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CXCL12 Retargeting of an Oncolytic Adenovirus Vector to the Chemokine CXCR4 and CXCR7 Receptors in Breast Cancer

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Abstract

Breast cancer is the most frequently diagnosed cancer in women under 60, and the second most diagnosed cancer in women over 60. While significant progress has been made in developing targeted therapies for breast cancer, advanced breast cancer continues to have high mortality, with poor 5-year survival rates. Thus, current therapies are insufficient in treating advanced stages of breast cancer; new treatments are sorely needed to address the complexity of advanced-stage breast cancer. Oncolytic virotherapy has been explored as a therapeutic approach capable of systemic administration, targeting cancer cells, and sparing normal tissue. In particular, oncolytic adenoviruses have been exploited as viral vectors due to their ease of manipulation, production, and demonstrated clinical safety profile. In this study, we engineered an oncolytic adenovirus to target the chemokine receptors CXCR4 and CXCR7. The overexpression of CXCR4 and CXCR7 is implicated in the initiation, survival, progress, and metastasis of breast cancer. Both receptors bind to the ligand, CXCL12 (SDF-1), which has been identified to play a crucial role in the metastasis of breast cancer cells. This study incorporated a T4 fibritin protein fused to CXCL12 into the tail domain of an adenovirus fiber to retarget the vector to the CXCR4 and CXCR7 chemokine receptors. We showed that the modified virus targets and infects CXCR4- and CXCR7-overexpressing breast cancer cells more efficiently than a wild-type control vector. In addition, the substitution of the wild-type fiber and knob with the modified chimeric fiber did not interfere with oncolytic capability. Overall, the results of this study demonstrate the feasibility of retargeting adenovirus vectors to chemokine receptor-positive tumors.

Keywords

Adenovirus; Breast Cancer; Cancer; Chemokine; CXCL12; CXCR4; CXCR7; Oncolytic; Preclinical; Receptor; Virotherapy; Virus

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Conflicts of Interest

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1. Introduction

Breast cancer remains the most commonly diagnosed cancer in women under 60 and second-most in women over 60 in the United States [1]. While significant progress has been made in developing targeted therapies for breast cancer, advanced breast cancer continues to have high mortality, with 5-year survival rates at 27% [1], thus highlighting the need for more efficient therapies. Specifically, new treatments are needed that can treat advanced and triple-negative breast cancer (TNBC). While a variety of targeted therapies are available for hormone receptor-positive breast cancers, especially at earlier stages, standard therapies such as chemotherapy and radiation remain the only choices for TNBC. Therefore, a more efficient therapy capable of targeting breast cancer cells while sparing normal tissue is sorely needed.

Adenoviruses have been investigated as therapeutic vectors in the context of breast cancer due to their natural ability to replicate in and lyse infected cells [2]. Adenoviruses are ideal therapeutic vectors due to being easy to manipulate, allowing for large transgene inserts and large-scale production. In addition, adenovirus vectors have been demonstrated to be clinically safe for local and systemic administration during oncolytic clinical trials [3] [4]. However, several characteristics of adenovirus vectors, including immunogenicity, liver sequestration, and low expression of the primary adenovirus receptor, CXADR or CAR, have limited their efficacy and have prevented their successful use in the clinic [5]. In recent years, the use of oncolytic adenovirus vectors in treating breast cancer has advanced to more sophisticated designs by incorporating tumor-specific promoters and transgene inserts [6] [7] [8] [9] [10], modifying fiber [11] and capsid proteins [12], and developing hybrid constructs [13] [14] [15] [16] [17] [18].

Adenovirus vectors have been extensively explored in breast cancer treatment utilizing various targeting approaches and induction of therapeutic transgenes. A predominant approach to engineering recombinant adenovirus vectors has been to target cancer cells directly. Due to the downregulation of the endogenous adenovirus 5 (Ad5) receptor, the Coxsackie, and Adenovirus receptor (CXADR or CAR), there has been a search for alternative receptors that would mediate efficient viral entry [19]. Several oncolytic vectors have been designed to target alternative entry pathways through fiber and knob modifications. For example, replacing the Ad5 knob with the Ad3 knob retargets an oncolytic adenovirus to the CD46 receptor [20] and has been utilized extensively to target breast cancer cells [13] [15] [16] [17] [21]. In addition, breast cancer cells overexpressing the receptors HER3 and HER4 have been targeted with a fiber-modified oncolytic adenovirus utilizing the epidermal growth factor-like domain of heregulin-α (HRG) inserted into the HI loop of the Ad5 fiber [14]. The insertion of RGD-4C into the HI loop also allowed retargeting of an adenovirus to integrin adhesion receptors on breast cancer cells [22]. An adenovirus modified to incorporate a polylysine motif within the fiber retargeted the adenovirus to heparan sulfate proteoglycans (HSPGs) in breast cancer cells *in vitro* and *in vivo* [23].

In this study, we proposed targeting CXCR4, a G-protein coupled receptor (GPCR), one of the most commonly overexpressed chemokine receptors in cancer, including breast cancer

[24]. CXCR4 functions as a mobilizer for hematopoietic stem cells and naïve lymphocytes and plays critical roles in the patterning of cell lineages during embryogenesis [25]. Currently, the only known ligand to CXCR4 is SDF-1, also known as CXCL12 [26]. In breast cancer cells, CXCR4 plays roles in the initiation, growth, progression, and, ultimately, the migration of tumor cells [27] and metastasis at distant sites [28] [29] [30]. In addition to CXCR4, a structurally related chemokine receptor, CXCR7, is capable of binding/sequestering SDF-1 [31]. CXCR7 is directly overexpressed in cancer cells [32], as well as in tumor-associated endothelial cells [33] and macrophages [34]. CXCR7 has also been implicated in driving tumor progression, e.g., tumor cell initiation, survival, progression, and metastasis [34] [35] [36]. Overall, the functional distribution of these receptors in breast tumors made them attractive targets for cancer therapy.

Previously, we successfully retargeted a replication-deficient Ad5 to CXCR4-overexpressing breast cancer cells via a bi-specific adaptor molecule, sCAR-CXCL12 [37]. The sCAR-CXCL12 adapter resulted in the efficient retargeting of the adenovirus to CXCR4-overexpressing cancer cells. In addition, in a human ex vivo liver slice model, the virus complexed with the adapter reduced liver tissue infection [37]. Furthermore, the adenovirus injection complexed with sCAR-CXCL12 into a SCID-bg mouse model further demonstrated liver-off and tumor-on biodistribution, supporting the safety rationale behind the targeting scheme [37]. Nonetheless, there are also significant disadvantages of using bispecific proteins in targeting oncolytic viruses [38].

Therefore, in the current study, we engineered a modified oncolytic adenovirus serotype 5 (Ad5) to express the human chemokine ligand CXCL12 in the context of a T4 fibritin-modified fiber for use as a single-component targeting moiety. We investigated the efficacy of infection of this new vector in a panel of breast cancer cell lines and determined its oncolytic capability.

2. Material and Methods

2.1. Cell Lines

The characteristics of the human breast cancer cell lines described by Smith *et al.* [39] and used for the cytotoxicity assays are shown in Table 1. The human embryonic kidney cell line HEK293 and breast cancer cell lines BT-20, MCF-7, MDA-MB-231, MDA-MB-436, and ZR-75-1 were obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS (Gemini, West Sacramento, CA), 1% penicillin/streptomycin (Thermo Fisher, Waltham, MA) and 1% NEAA (Thermo Fisher). The human mammary gland epithelial cell line, MCF-12A, was obtained from ATCC and maintained in DMEM/F12 containing 5% donor horse serum, 0.5 μg/mL hydrocortisone, 0.01 mg/mL bovine insulin, 100 ng/mL cholera toxin, and 20 ng/mL human EGF. The Chinese hamster ovary cell lines, CHO and CHO-CAR (stably expressing the CXADR or CAR cDNA) as characterized previously [40], were kindly provided by Rhonda Cardin (Louisiana State University School of Veterinary Medicine, Baton Rouge, LA) and maintained in RPMI 1640 (Genesee Scientific, San Diego, CA) containing 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 10 μg/mL thymidine (Sigma-Aldrich, St. Louis, MO), 10 μg/mL adenosine (Sigma-Aldrich) and 10 μg/mL 2-deoxyadenosine (Sigma-Aldrich). CHO-CAR

cells were also supplemented with 100 μg/mL Zeocin (InvivoGen, San Diego, CA). All cell lines were maintained at 37°C in a humidified 5% CO2 atmosphere.

2.2. CHO Cell Transfections

Plasmid and cell line transfections were performed via Lipofectamine 3000 (Invitrogen, Waltham, MA) according to the manufacturer's instructions. The CHO-CXCR4 cell line was established by transfection with a mammalian expression plasmid (pCMV6-AC-GFP; OriGene Technologies, Rockville, MD) containing the human CXCR4 transcript variant 1 cDNA sequence (NM_001008540) fused to a C-terminal tGFP tag. Similarly, the pCMV6-AC-GFP mammalian expression plasmid containing the human CXCR7 transcript cDNA sequence (NM_020311) fused to a C-terminal tGFP tag was used to establish the CHO-CXCR7 cell line. Transfected cells were incubated with transfection medium for 4 hours at 37°C, after which the media was exchanged with fresh media. Stable transfectant cells were selected by using G418 sulfate at 800 mg/ml. The CHO-CXCR4 and CHO-CXCR7 were maintained in the same medium as the parental CHO, with the addition of G418. All cell lines were maintained at 37°C in a humidified 5% CO2 atmosphere.

2.3. Adenovirus Vector Construction

We used a method described previously [41] [42] [43] to create a chimeric Ad5 fiber gene by genetic modification of the Ad fiber protein to contain a heterologous trimerization motif and a receptor-binding ligand. As shown in Table 2, sequences encoding the Ad5 fiber tail domain were fused to sequences encoding the bacteriophage T4 fibritin shaft and foldon domains, followed by sequences encoding the mature form of the CXCL12 protein. The Ad5 fiber, T4 fibritin, and CXCL12 sequences were separated by short peptide linkers; the total length of the construct was 1260 bp encoding 420 amino acids. In brief, a Srf I - Pac I Ad5 fragment containing the chimeric Ad5 fiber construct (5300 bp) was synthesized (GenScript, Piscataway, NJ) and used to replace the Srf I - Pac I fragment (5766 bp) of the wild-type Ad5 sequence (GenBank accession no. AY370909.2) within the pAdEasy-1 plasmid (Agilent Technologies, Santa Clara, CA). Ampicillin-resistant colonies were selected following ligation and transformation; DNA was extracted, and identities of positive clones were confirmed by restriction digestion and polymerase chain reaction (PCR).

2.4. Adenovirus Recombination

To produce the fiber-modified adenovirus construct, we used an established recombination technique [44]. Using this technique, a shuttle plasmid containing the wild-type E1a and E1b genes, and the red fluorescent protein sequence fused to the minor capsid protein IX gene was co-transformed into the electrocompetent E. coli strain, BJ5183 (Agilent Technologies, Santa Clara, CA), with an Ad5 viral backbone plasmid containing the fiber-fibritin-CXCL12 insert. BJ5183 is a recombination proficient bacterial strain necessary to efficiently execute a recombination event between a shuttle plasmid and an adenovirus backbone plasmid. After recombination, Ad5-pIX-RFP-FF/CXCL12 (Ad5-ffCXCL12) recombinant colonies were selected on 100 μg/mL kanamycin agar plates incubated overnight in 37°C for a maximum of 16 hours. After incubation, the smallest colonies were chosen for amplification as these were most likely to contain recombinants. Colony amplification was conducted in 5 mL LB broth with 100 μg/mL Kanamycin overnight in a shaking incubator for a maximum of 16

hours. Following amplification, plasmids were extracted via miniprep (Bio-Rad, Hercules, CA) and analyzed via Hind III restriction enzyme digestion (New England Biolabs, Ipswich, MA) to screen for positive recombinants. Upon positive recombinant clone verification, clones were then analyzed via PCR for the E1A, pIX, Ad5-E4, and Ad5-penton genes. A clone was chosen that was positive for all genes and subsequently transduced into XL10-Gold ultracompetent E. coli cells (Agilent Technology, Santa Clara, CA). Colonies were grown on 100 μg/mL kanamycin plates overnight at 37°C. The following day, a single colony was chosen and amplified further in XL10-Gold ultracompetent cells using 400 mL of LB broth with 100 μg/mL Kanamycin. After incubation overnight in a shaking incubator at 37°C, Ad5-ffCXCL12 was extracted using a maxiprep kit (Bio-Rad, Hercules, CA). The resulting DNA was then purified by phenol: $CHCl_3$ extraction and 70% ethanol precipitation overnight at −80°C. The next day the precipitated DNA was ultracentrifuged at 13,000 rpm for 30 minutes at 4°C. The resulting plasmid stock was resuspended in ultrapure H_2O and analyzed for concentration using a NanoDrop 8000 spectrophotometer (Thermo Fisher, Waltham, MA). The plasmid was confirmed to contain the E1a, pIX-RFP, CXCL12, and fiber-fibritin genes by PCR.

2.5. Adenovirus Rescue

To prepare the viral plasmid for transfection into the mammalian virus packaging cell line, HEK293-CXCR4, restriction enzyme digestion using Pac I (New England Biolabs) was used to linearize the plasmid. Following digestion, the plasmid was confirmed via gel-electrophoresis to confirm the presence of a small ligated portion of the viral plasmid, which confirms the linearization. The resulting linearized DNA was purified and concentrated via ethanol precipitation at −20°C. Purified viral DNA was then transfected using Lipofectamine 3000 reagent (Invitrogen, Waltham, MA) into HEK293-CXCR4 cells and incubated at 37°C for 7 to 14 days. Viral plasmid transfection was monitored for increasing fluorescence over the course of two weeks, after which potential virus was extracted in PBS from the cells via 3 freeze and thaw cycles using a dry ice bath. The resulting supernatant was then applied to a fresh dish of HEK293-CXCR4 cells at 60% confluency for viral rescue. The successful viral rescue was monitored via fluorescence microscopy until cells exhibited an extensive cytopathic effect and began to detach from the dish surface. Virus extraction was conducted via freeze/thaw cycles, and fresh HEK293-CXCR4 cells were infected for amplification. The virus was amplified to a maximum of sixty 150 mm dishes before harvesting for purification. The resulting virus supernatant (Ad5-ffCXCL12) was purified on a CsCl gradient, after which a titer was conducted via AdenoX Rapid Titer Kit (Clontech, Mountain View, CA). The control virus, Ad5-pIX-RFP-WT/Fiber (Ad5-wtFiber), was amplified in HEK293 cells and purified via CsCl gradient centrifugation, after which titer was determined in the same manner as Ad5-ffCXCL12.

2.6. PCR Analysis

The designated primers (Table 3) were used to test the viral clone and resulting viral DNA, post-production, for the presence of essential genes (Invitrogen, Waltham, MA). Polymerase chain reactions (PCR) reactions were performed for 36 cycles at 94°C for 1 minute, 56°C for 30 seconds, and 72°C for 2 minutes.

2.7. Immunoassays

For detecting CXCL12 (SDF-1) protein on the adenovirus virions, serial dilutions of Ad5-wtFiber and Ad5-ffCXCL12 were examined using an SDF-1 alpha/CXCL12A Human ELISA Kit (Thermo Fisher) according to the manufacturer's instructions. Absorbance was read at 450 nm using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA). SDF-1 (CXCL12) levels were calculated using a standard curve generated from the kit.

2.8. Western Blot Analysis

For the preparation of whole-cell lysates, 1.2 × 106 cells per sample were collected and harvested with reducing Laemmli buffer followed by 5 minutes of boiling. Samples were run on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour with 5% bovine serum albumin (BSA) and washed with TTBS (1% Tween-20 in Tris-buffered saline). Afterward, the membranes were incubated with mouse monoclonal antibodies overnight at 4°C. Membranes were analyzed for expression of CXADR (a.k.a. CAR or hCAR), CXCR4, and CXCR7, using primary antibodies PA5–31175, 35–8800, and PA5–28739, respectively (Thermo Fisher). Expression of β-actin was analyzed as a loading control using monoclonal antibody A1978 (Sigma-Aldrich). After three consecutive washes with TTBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology; Dallas, TX) for 1 hour and washed with TTBS. Finally, the membranes were developed using an enhanced chemiluminescent (ECL) reagent (GE Healthcare Biosciences, Pittsburgh, PA) for protein detection and visualized on a ChemiDoc imaging system (Bio-Rad).

2.9. Cell Receptor Analysis

The breast cancer cell lines were harvested with Versene and washed twice with cold PBS. Cells were incubated at 4°C for 30 minutes to 1 hour with PE-conjugated mouse anti-CXCR4 (FAB170P; R&D Biosystems, Minneapolis, MN) mouse anti-CXCR7 (FAB42271P; R&D Biosystems), or rabbit anti-CXADR (10799-R271-P; Sino Biological Inc, Chesterbrook, PA). PE-conjugated mouse IgG2A antibody (IC003P; R&D Biosystems) or monoclonal rabbit IgG (IC105P; R&D Biosystems) were used as isotype controls. After incubation, the cells were washed twice in cold PBS, then resuspended in 0.5 mL ice-cold PBS and placed on ice. Analysis of receptor expression was conducted via flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

2.10. Cell Lines

Cells were seeded overnight into 24-well tissue culture plates at 1 × 105 cells/well. The next day, cells were infected with 2% FBS DMEM media with Ad5-wtFiber or Ad5-ffCXCL12 at an increasing multiplicity of infection (MOI: 1, 10, 50, 100, 500, and 1000). After incubation for 4 hours at 37°C, virus-containing media was replaced with complete growth media and incubated at 37°C for 48 hours. At 48 hours, cells were harvested, washed with PBS, resuspended in 0.5 mL PBS, and analyzed by flow cytometry for the expression of the pIX-RFP protein.

2.11. Cell Viability Assay

Cells were plated at 1 × 104 cells per well into 96-well tissue culture plates. Cells were infected with the virus in 100 μL 2% FBS DMEM media. Infected cells were incubated at 37°C for 72 to 96 hours. Cell viability post-infection was measured using an XTT cell viability kit (Cell Signaling Technology, Danvers, MA) at 72 and 96 hours. Absorbance was read at 450 nm using a SpectraMax Plus microplate reader (Molecular De-vices).

2.12. siRNA Knockdown

CHO-CXCR4 cells were transfected with 45nM CXCR4 siRNA (Dharmacon, Lafayette, CO) at 37°C for 72 hours in transfection media according to the manufacturer's instructions. To confirm knockdown of CXCR4, the cells were harvested with Versene, imunostained using a PE-conjugated anti-CXCR4 antibody or a PE-conjugated isotype control antibody, and analyzed by flow cytometry. The cells were also analyzed for fluorescence detection of the CXCR4-tGFP fusion protein.

2.13. Virus Binding Assay

A qPCR-based method was used to assess the effect of CXCR4 knockdown on adenovirus virion binding to the cell surface. In this assay, untransfected CHO-CXCR4 cells or cells transfected with 45 nM CXCR4 siRNA were harvested with Versene, washed once with ice-cold PBS, and incubated with 100 MOI Ad5-wtFiber or Ad5-ffCXCL12 for 30 minutes on ice. Afterward, the cells were gently washed 3× with ice-cold PBS, pelleted by centrifugation, and resuspended in PBS. Total DNA was extracted from the cell samples using a QIAmp DNA mini kit (Qiagen; Germantown, MD) using the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher), and 25 ng of extracted DNA was used for real-time quantitative PCR (qPCR) to measure adenovirus E4 copy number. For qPCR of adenovirus E4 DNA, the following sets of primers and probes were synthesized (Thermo Fisher) and used: forward primer (5'-GGGTCGCCACTTAATCTACCT-3'); reverse primer (5'-dGCAAGGCGCTGTATCCAA-3'); and probe (5'-FAM-CGCTTGTGGTATGATGGCCACGT-TAMRA-3'). The following primers and probes were used to measure human GAPDH gene copy number: forward primer (5'-ACCAGGTGGTCTCCTCTGAC-3'); reverse primer (5'-TTGCTGTAGCCAAATTCGTT-3'); and probe (5'-FAM-TTTGTCAAGCTCATTTCCTGGTATGAC-TAMRA-3'). In this assay, the extracted DNA samples were amplified for 40 cycles at 94°C for 5 seconds and 60°C for 30 seconds, using a TaqMan Real-Time PCR Master Mix (Thermo Fisher Scientific) with the primers and probe on an Applied Biosystems 7900HT system (Thermo Fisher Scientific).

2.14. Statistics

Data are presenting as mean ± standard deviation (S.D.). Single comparative data were analyzed for significance by the Student's t-test using GraphPad Prism 8.0 software (GraphPad Software; La Jolla, CA). Two-way ANOVA followed by Bonferroni's multiple comparisons test was performed to compare the percent cell survival or percent infected parameters at different MOI using GraphPad Prism 8.0 software.

3. Results

3.1. Ad5-ffCXCL12 Characterization

After the shuttle and backbone plasmids were transduced into electrocompetent E. coli cells, the smallest colonies were selected for miniprep plasmid extraction. As shown in Figure 1(A), plasmid DNA samples isolated from 10 different clones digested with the restriction enzyme Hind III showed a characteristic pattern, which was diagnostic of adenovirus genomes successfully recombined. After recombinant clone selection, PCR was performed on the clones (5, 6, and 7) to further demonstrate the presence of key adenovirus genes: E1A, pIX, Ad5-E4, and Ad5-penton (Figure 1(B)). Based on these results, we identified clone 7 as a positive recombinant clone containing the screened genes. Clone 7 DNA was transduced into ultracompetent E. coli cells and amplified. After amplification, plasmid DNA was extracted, and PCR confirmed the presence of the modified fiber and CXCL12 insertion (Figure 1(C)). After transfection, production, and purification of the viral stocks for Ad5-wtFiber and Ad5-ffCXCL12, PCR on Ad5-ffCXCL12 viral stock confirmed the presence of the T4 fibritin and CXCL12 genes (Figure 1(D)). An ELISA for CXCL12 protein expression confirmed the CXCL12 ligand on the virus capsid (Figure 1(E)); the resulting design of the recombinant virus genome is depicted in Figure 1(F).

3.2. Breast Cancer Cell Lines Express Varying Levels of CXCR4, CXCR7, and CXADR

A panel of breast cancer cell lines (BT-20, MCF-7, MDA-MB-231, MDA-MB-436, and ZR-75-1) and the immortalized breast epithelial cell line, MCF-12A, were analyzed via flow cytometry for receptor expression. The data shown in Figure 2(A) indicate that the cell lines contain subpopulations expressing CXCR7 and CXADR at varying levels at the cell surface. CXCR4 expression was not detected in any of the cell lines via flow cytometry. However, western blot analysis of whole-cell lysate detected varying levels of CXCR4 expression (Figure 2(B)). The discrepancy between the two receptor identification methods may be due to endocytosis of the receptors upon antibody binding for flow cytometry or to the presence of intracellular CXCR4. CXCR7 was found in all cell lines as distinct populations of cells by flow cytometry, except in MDA-MB-436, where the cells express a more homogenous population (Figure 2(A)). Western blot confirmed the CXCR7 presence in most cell lines, with the highest expression observed in BT-20 cells (Figure 2(B)). Similarly, the endogenous Ad5 receptor, CXADR (a.k.a. CAR), was present at varying levels in all cell lines with high levels expressed on MCF-12A, MCF7, MDA-MB-231, and MDA-MB-436 (Figure 2(A)). Western blot of CXADR found similar receptor levels in most cell lines, with the highest expression detectable in MDA-MB-436 (Figure 2(B)). It is worth suggesting that the differences in expression levels between the two methods may lie in receptor detection capability by western blot (total expression) versus flow cytometry (cell surface expression).

3.3. Ad5-ffCXCL12 Efficiently Targets and Enhances Infection Efficacy in Breast Cancer Cells

As shown in Figure 3, breast cancer cell lines were infected for 72 hours with increasing MOI of either Ad5-wtFiber or Ad5-ffCXCL12. All breast cancer cell lines demonstrated a significantly greater infection with Ad5-ffCXCL12 than Ad5-wtFiber (Figures 3(A)–(F)). Even in the presence of high levels of CXADR, Ad5-ffCXCL12 demonstrated significantly

enhanced infectivity compared with Ad5-wtFiber, as observed in both the MDA-MB-231 (Figure 3(D)) and the MDA-MB-436 (Figure 3(E)) cell lines. While the MDA-MB-231 cell line analysis indicated low CXCR4 and CXCR7 expression (Figure 2), there was no correlation with Ad5-ffCXCL12 infection efficiency. As shown in Figure 3(D), infection efficiency was the highest of all cell lines tested, reaching 77.3% at 1000 MOI. This result suggests that minimal levels of the CXCR4 and CXCR7 receptors may be sufficient to mediate the efficient uptake of the modified virus. The overall infection efficiency of the non-tumorigenic breast epithelial cell line, MCF-12A (moderate internal CXCR4, low membrane CXCR7, high membrane CXADR), with Ad5-ffCXCL12 was lowest compared with the breast cancer cell lines (Figure 3(C)). Of note, even in the presence of high levels of CXADR, infection efficiency with Ad5-wtFiber did not correlate with the high CXADR expression suggesting that infection was not necessarily dependent on receptor abundance. For example, although we identified moderate levels of CXADR in MCF-7 cells (Figure 2), Ad5-wtFiber infection was largely ineffective (Figure 3(B)). Likewise, poor infection efficiency in MCF-12A by Ad5-wtFiber (Figure 3(C)) may be due to the low expression of integrin αvβ3 and αvβ5 co-receptor expression [37].

3.4. Ad5-wtFiber and Ad5-ffCXCL12 Exhibit Similar Oncolysis

After the infection, cell viability was assessed at increasing MOI of either Ad5-wtFiber or Ad5-ffCXCL12 using an MTT assay. In this analysis, two different cell lines, MCF-12A and MDA-MB-436, were compared at 72 hours and 96 hours post-infection. Both cell lines expressed variable levels of CXCR7 and CXADR (Figure 2(A)). We show that cell viability of cells infected with Ad5-ffCXCL12 was comparable to viability after Ad5-wtFiber infection (Figure 4), suggesting that oncolytic efficiency is similar between the two viruses; the incorporation of the ff-CXCL12 does not hinder oncolytic efficiency. Overall, a decrease of cell viability at MOI 100 and 1000 were statistically significant ($p < 0.001$) compared with that of uninfected cells. MCF-12A exhibits slighter decreases in cell survival between 72 and 96 hours (Figure 4(A) and Figure 4(B)); however, MDA-MB-436 cells seemed more susceptible to both viruses at higher doses (Figure (C) and Figure 4(D)) when compared with MCF-12A. Growth inhibition of cells at these MOIs could be attributed to the direct oncolysis mediated by either virus's cytotoxic and bystander effects.

3.5. Infection of Isogenic Cell Lines Engineered to Overexpress CXCR4, CXCR7, and CXADR

To further investigate the role of different receptors in Ad5-ffCXCL12 infection, the CHO Chinese hamster ovary cell line was used to overexpress the human receptors CXADR, CXCR4, and CXCR7. Overexpression of CXCR4 (Figure 5(A)) and CXCR7 (Figure 5(B)) were assessed by flow cytometry utilizing a tGFP reporter gene fused to the receptor gene sequences. Further flow analysis was conducted using antibodies targeting CXCR4 and CXCR7 (Figure 5(C) and Figure 5(D), magenta peaks). Of note, tGFP fusion protein expression was appeared higher than immune detection using anti-CXCR4 or anti-CXCR7 antibodies. This discrepancy could be attributed to the intracellular localization and trafficking of the CXCR4 and CXCR7 receptors from the cell surface. As expected, CHO parental cells were refractory to Ad5-wtFiber (Figure 5(E)) but were inherently susceptible to Ad5-ffCXCL12 infection (Figure 5(E)), with a statistically significant infection at 500

and 1000 MOI when compared with Ad5-wtFiber. This modest increase in infected cells may be attributed to unknown hamster protein interactions with the modified fiber and knob, as Ad5-wtFiber could not infect CHO cells. As shown in Figure 5(F), overexpression of human CXADR in CHO-CAR cells was accompanied by an increased infection with Ad5-wtFiber. In addition, CHO-CAR cells were also susceptible to Ad5-ffCXCL12 infection. In comparison, Ad5-ffCXCL12 infection was facilitated in CHO-CXCR4 (Figure 5(G)) and CHO-CXCR7 cells (Figure 5(H)). However, infection in both cell lines was lower than expected with Ad5-ffCXCL12. This result may suggest that additional factors may be necessary for efficient infection of adenovirus vectors in CHO cells.

3.6. siRNA Knockdown of CXCR4 Diminishes Ad5-ffCXCL12 Binding

CHO-CXCR4 cells were transfected with anti-CXCR4 siRNA. To determine the extent of CXCR4 expression, the cells were examined by two-parameter (dual color flow cytometry (Figure 6). In the CHO-CXCR4 cells, the CXCR4 cDNA was constructed to contain an N-terminal tGFP marker that allowed for fluorescence detection of total CXCR4 protein expression. The cells were also immunostained with a PE-labeled anti-CXCR4 antibody to assess cell surface expression of the CXCR4-tGFP fusion protein. As quantified in Table 4 and shown in Figure 6(A), flow cytometry analysis indicated approximately 14.1% of the untransfected CHO-CXCR4 cells were positive for tGFP-tagged CXCR4. Immunostaining with an anti-CXCR4 antibody revealed that approximately 10.1% of the GFP-positive cell population was positive for cell surface expression of CXCR4, while approximately 83.9% was negative (Table 4 and Figure 6(B)). These results are consistent with the process of internalization and recycling of GPCRs. Importantly, CXCR4 siRNA transfection of CHO-CXCR4 resulted in a decrease of tGFP-tagged CXCR4 (Table 4 and Figure 6(C)) to approximately 4% as determined at 72 hours post-siRNA transfection. Immunostaining with an anti-CXCR4 antibody showed that CXCR4 siRNA transfection resulted in similarly low levels of cell surface CXCR4 expression at 3.4% (Table 4 and Figure 6(D)). The effect of CXCR4 knockdown was next investigated on Ad5-ffCXCL12 using a virus binding assay. After 72 hours of siRNA transfection, the CHO-CXCR4 cells were infected with 100 MOI of either Ad5-wtFiber or Ad5-ffCXCL12 for 30 minutes. As shown in Figure 6(E), CXCR4 knockdown resulted in a decrease in Ad5-ffCXCL12 binding to 35% of control untransfected) cells. CXCR4 knockdown also decreased Ad5-wtFiber binding, although the effect was only at 65% of control (untransfected) cells.

4. Discussion

The wild-type receptor for Ad5, CXADR, is downregulated in the majority of breast tumors, limiting the therapeutic efficacy of wild-type fibered Ad5 vectors [45]. Thus, the present study sought to utilize this chemokine axis to retarget an oncolytic Ad5 to increase infection in breast cancer cells. The utility of retargeting Ad5 with fiber modifications has been shown to be a viable therapeutic approach [13] [14] [15] [16] [17] [21] [22] [23]. In this approach, the replacement of the Ad5 fiber-knob with T4 fibritin maintains the structural integrity of the Ad5 fiber and allows for larger ligand insertions into the knob domain [46] [47]. We previously utilized the T4 fibritin platform to successfully retarget an oncolytic adenovirus to the cMet receptor in hepatocellular carcinoma and breast cancer [48]. Ad5 binding is

mediated by the CXADR receptor, and entry is facilitated in a two-step uptake mechanism by the αvβ3 and αvβ5 integrins as well as other integrins that recognize Arg-Gly-Asp (RGD) motif that is expressed on the penton base protein [49]. However, in the absence of CXADR, Ad5 binding has been shown to be mediated by a host of cell surface receptors. Heparan sulfate glycosaminoglycans have been found to act as fiber-independent and fiber-dependent binding receptors for Ad5 [50] [51]. The αvβ5 integrin has also been shown to act as a primary receptor in the absence of CXADR [52]. Overall, Ad5-ffCXCL12 infection of MCF-12A cells was lower than breast cancer cell lines, possibly attributable to low levels of αvβ3 and αvβ5 at the cell surface [37].

The therapeutic importance of targeting the CXCR4-CXCR7-CXCL12 axis in breast cancer has been widely recognized, in part due to the overexpression of both receptors occurring at different stages and subtypes of breast cancer [53] [54] [55] [56] [57]. In this study, we evaluated the efficacy of incorporating the CXCL12 ligand into the C-terminus of a modified fiber gene containing the Ad5 tail domain and the T4 fibritin shaft and foldon domains. We previously demonstrated the efficacy of retargeting a replication-deficient adenovirus to CXCR4 and CXCR7 using a bi-specific adaptor molecule [37]. The replication-deficient Ad5 was successfully retargeted to cancer cells overexpressing CXCR4 and detargeted the liver uptake *in vivo* . However, due to the bi-specific nature of the adaptor and unknown safety and pharmacokinetics of the adapter-virus complex, we sought to incorporate the CXCL12 ligand directly into the adenovirus genome. We demonstrated the successful incorporation of the CXCL12 gene via PCR and confirmed CXCL12 protein expression using an ELISA binding assay (Figure 1(D)).

The infection efficiency of Ad5-ffCXCL12 was analyzed by treating the breast cancer cell lines at increasing MOI for 72 hours. In these studies, Ad5-ffCXCL12 consistently exhibited greater infection in breast cancer cells when compared with Ad5-wtFiber. Breast cancer cell lines MDA-MB-231 and MDA-MB-436 showed high levels of CXADR, and thus, Ad5-wtFiber infection was greater in these cell lines when compared with the other breast cancer cell lines. High levels of CXADR, however, were not sufficient for infectivity. Although the immortalized breast epithelial cell line (MCF-12A) exhibited high levels of CXADR, these cells were poorly infected with Ad5-wtFiber (Figure 3(A)), indicating that additional cellular components may be necessary to mediate wild-type adenovirus infection efficiently. In contrast, Ad5-ffCXCL12 infection was improved in MCF-12A cells, likely due to the expression of CXCR7 at the cell surface.

The CXCR4-CXCR7-CXCL12 axis has been implicated in the initiation, progression, and metastasis of cancer cells. CXCL12 has been known as a specific ligand to the chemokine receptor, CXCR4 [24], for over two decades. In 2005, CXCR7 was also dis-covered to have a high binding affinity to CXCL12 [35]. Since then, the involvement of the CXCR4-CXCR7-CXCL12 axis in breast cancer has been extensively explored. CXCR4 has been shown to be over-expressed in a variety of cancers, including breast cancer [58]. Specifically, CXCR4 has been implicated in tumor cell survival and progression, epithelial-mesenchymal transition (EMT), trafficking of cancer cells [24], and metastasis [24] [28] [29]. CXCR7 also is overexpressed in a variety of cancers, including breast cancer [36]. It has been primarily considered a scavenger receptor for CXCL12 to manage extracellular

CXCL12 levels and limit CXCL12-CXCR4 binding [59] [60]. It has also been implicated in the progression and metastasis of CXCR4-expressing tumor cells [60] [61]. In contrast, CXCR7 expression in breast cancer tumor vasculature has been shown to control and inhibit metastasis [33]. Despite the common consensus that CXCR7 acts as a sequestering receptor for CXCL12, recent evidence has been demonstrated that CXCL12-CXCR7 binding results in downstream signaling affecting cancer stem cell (CSC) survival and proliferation, as well as EMT [62] [63] and metastasis [36]. CXCR4 and CXCR7 have been found to be expressed either in separate populations of cells or co-expressed on the same cells within breast tumors [32] [36] [57] [64]. In addition to coexpression, both receptors have been observed to heterodimerize to mediate CXCR4 signaling [34] [65] [66].

5. Conclusions

In conclusion, this study showed an increased infection efficiency of a fiber modified adenovirus (Ad5-ffCXCL12) in breast cancer cells, primarily overexpressing CXCR7 and in HEK293 cells overexpressing CXCR4. In addition, Ad5-ffCXCL12 oncolytic efficiency was similar to the Ad5-wtFiber control, suggesting that the modifications did not impair the virus's ability to replicate within the cell. We demonstrated a degree of specificity to the CXCR4 and CXCR7 receptors in an isogenic hamster cell line. However, the major limitations we observed in controlling infection in breast cancer cells are likely due to additional proteins on the cell surface that mediate viral infection, independent of the fiber. Further studies will be needed to assess the ability to simultaneously target the adenovirus to a specific receptor and block binding of hexon to extracellular receptors, such as heparan sulfate glycosaminoglycans.

Overall, we have developed Ad5-ffCXCL12 as a therapeutic oncolytic adenovirus candidate for breast cancer therapy that warrants further investigation. Future *in vivo* studies will be needed to assess this virus a replication-permissive immunocompetent animal model to characterize its oncolytic ability in an *in vivo* setting adequately. The most common animal models to be utilized in preclinical oncolytic adenovirus studies have been murine xenograft models. However, since murine models are not permissive to human Ad5 replication due to species-specificity, preclinical assessment of Ad5 vectors has been limited. Thus far, Syrian hamster and porcine species have emerged as replication-competent animal models for investigating oncolytic adenoviruses [67] [68] [69]. Nonetheless, a suitable breast cancer cell line overexpressing CXCR4 or CXCR7 must also be developed to utilize these *in vivo* models.

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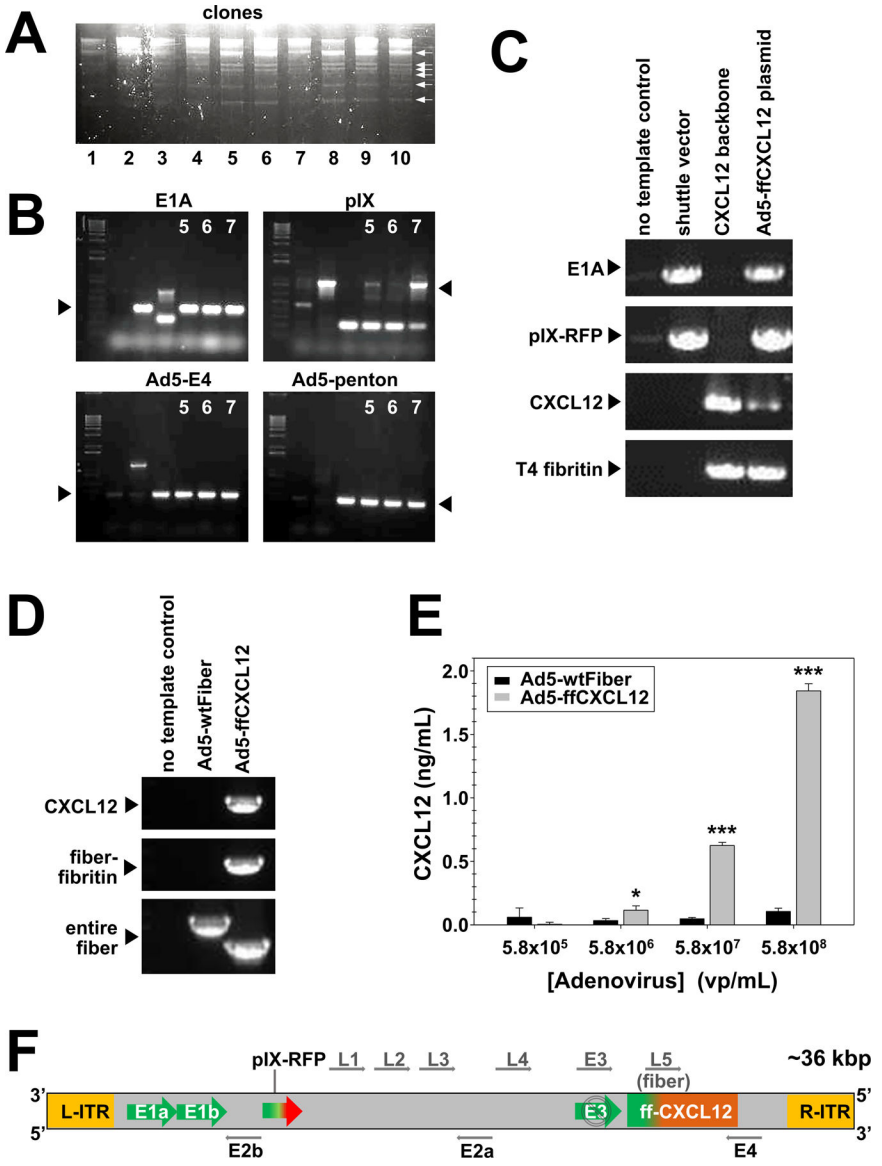

Figure 1.

Recombinant adenovirus clone confirmation. (A) DNA isolated from potential recombinant clones digested with Hind III. (B) Clones 5, 6, and 7 screened for E1A, pIX, Ad5-E4, and Ad5-penton by PCR. (C) PCR analysis of recombinant plasmid DNA from clone 7 by PCR. (D) PCR analysis of final virus stock after amplification in 293A-CXCR4 cell line and purification with CsCl gradient. (E) ELISA analysis of CXCL12 protein expression in purified viral stock. Bars are representative of mean values S.D. The differences between the two virus constructs at each adenovirus concentration were compared using Student's t-test and were considered statistically significant if $p < 0.05$. *$p < 0.05$, ***$p < 0.001$. (F) Schematic of Ad5-ffCXCL12 genome. Abbreviations: VP, viral particles; PCR, polymerase chain reaction; CsCl, cesium chloride; S.D., standard deviation.

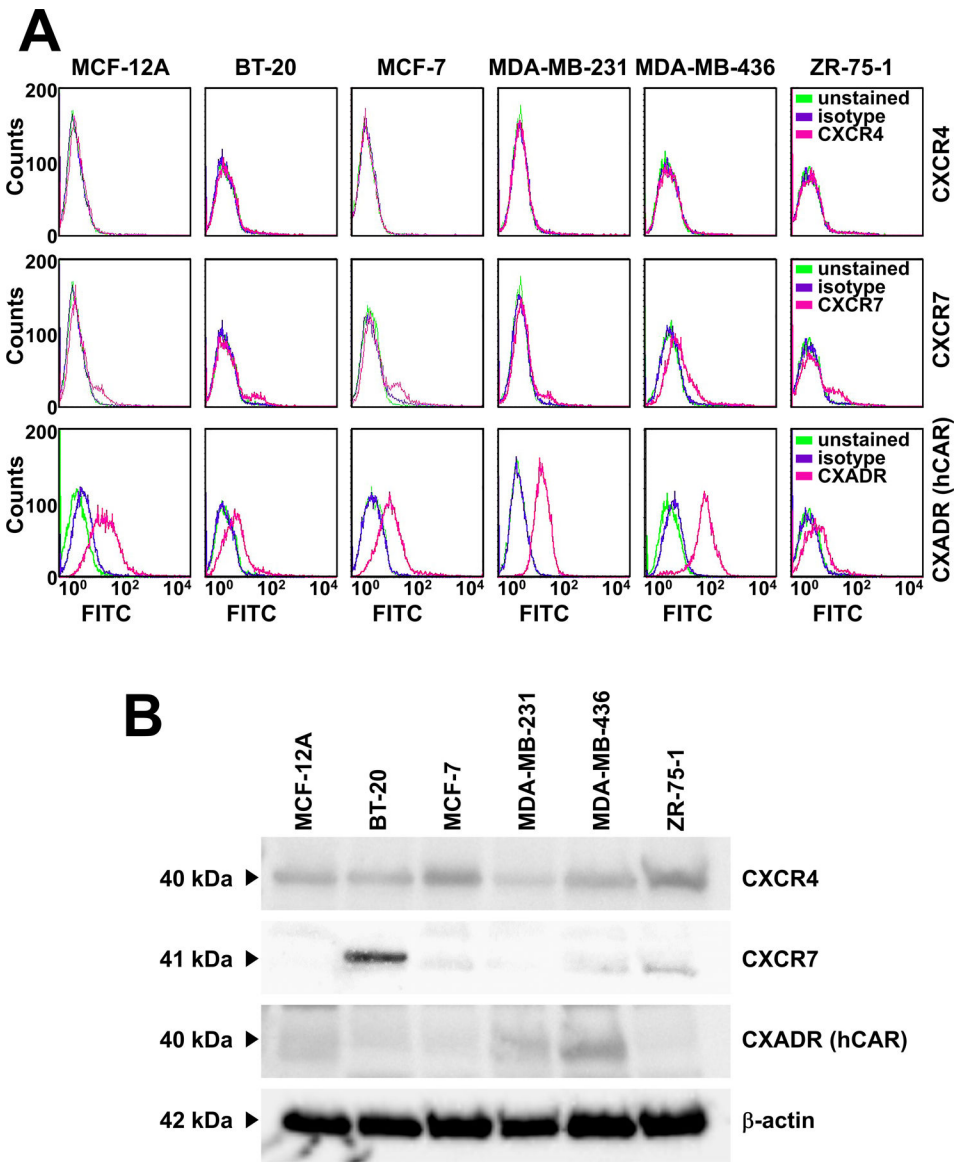

Figure 2.

Expression of CXCR4 and CXCR7 in breast cancer cell lines. (A) Cell receptor expression was determined by flow cytometry of breast cancer cell lines immunostained with FITC-conjugated antibodies specific for CXCR4, CXCR7, and CXADR (a.k.a. CAR). Fluorescence detection of unstained cells (green peaks) was compared with cells immunostained with an isotype IgG staining (purple peaks) or a receptor-specific antibody (magenta peaks). In each experiment, 10,000 cells were analyzed for each sample. Shown are representative results of three independent experiments. (B) Western blot analysis of breast cancer cell lines. Aliquots of whole-cell lysates from each cell line were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and probed with primary mouse anti-human antibodies specific for CXCR4 CXCR7, CXADR (a.k.a. CAR), or ?-actin. Membranes were subsequently incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody, developed using an ECL reagent, visualized by a Western blotting

imaging system. Shown are representative blots after visualization. Abbreviations: ECL, enhanced chemiluminescent; FITC, fluorescein isothiocyanate; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

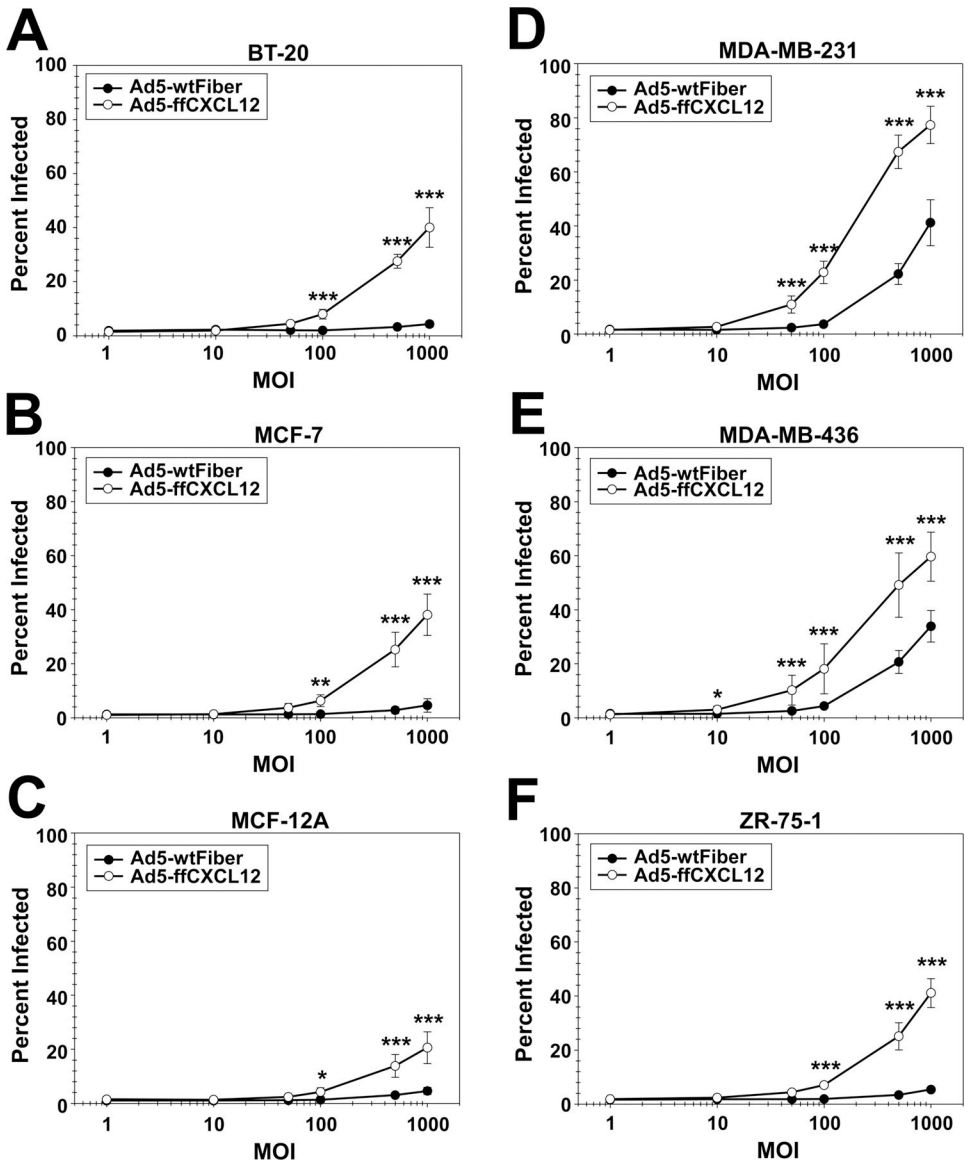

Figure 3.

Detection of adenovirus infection. Percent of (A) BT-20, (B) MCF-7, (C) MCF-12A, (D) MDA-MB-231, (E) MDA-MB-436, and (F) ZR-75-1 cell lines infected with Ad5-wtFiber (●) or Ad5-ffCXCL12 (○) after treatment for 72 hours with increasing MOI was determined by flow cytometry analysis of pIX-RFP expression. All data is representative of three replicate experiments. Points indicate the mean S.D. of percent RFP positive cells. Two-way ANOVA followed by Bonferroni's multiple comparisons test was performed to compare the percent infected cells at each MOI. The differences between the two virus treatments at each MOI were considered statistically significant if $p < 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: MOI, multiplicity of infection; S.D., standard deviation.

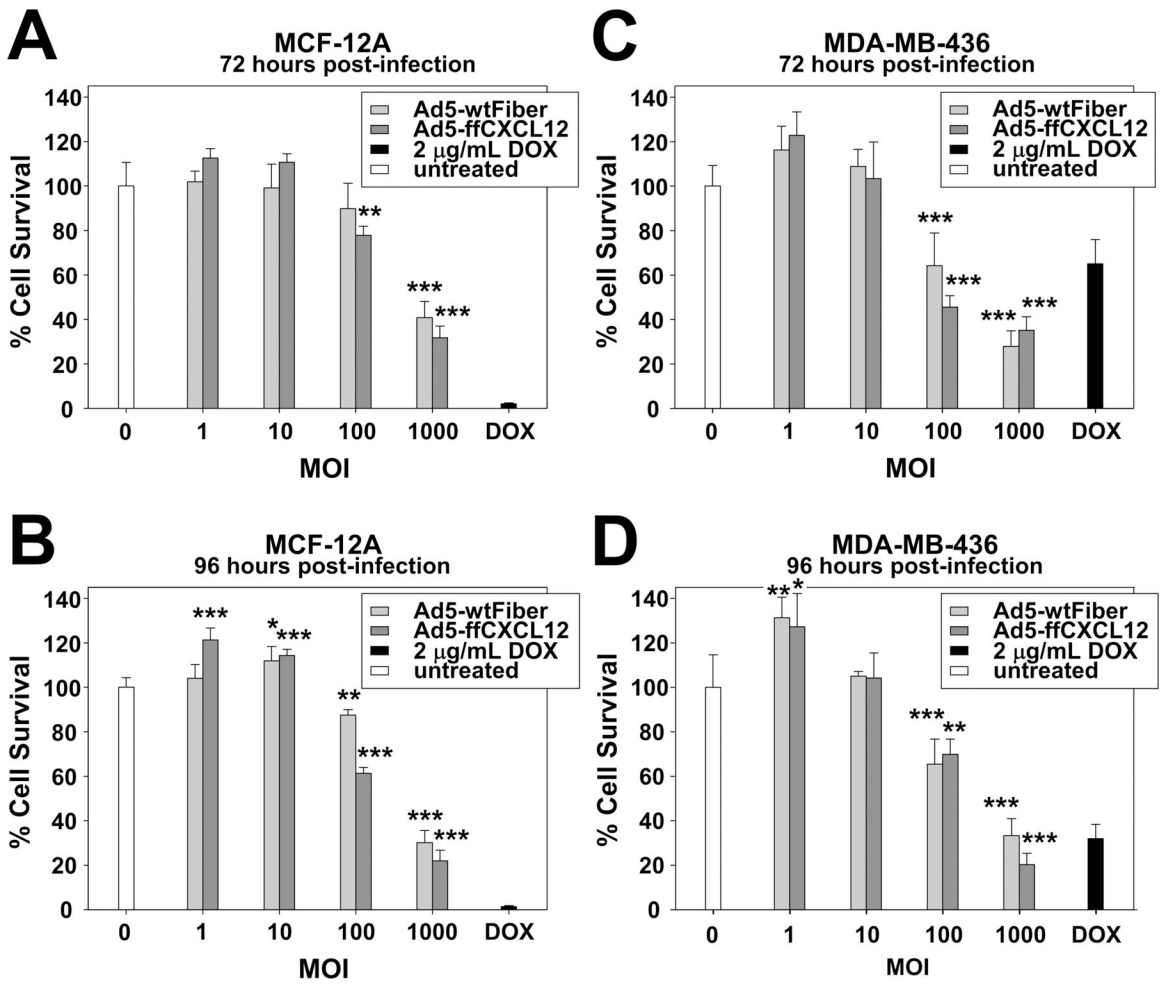

Figure 4.

Viability assays of MCF-12A and MDA-MB-436 after infection with increasing MOI of Ad5-wtFiber or Ad5-ffCXCL12. MCF-12A cells were infected with increasing MOI of Ad5-wtFiber or Ad5-ffCXCL12 for (A) 72 hours or (B) 96 hours before XTT assays. MDA-MB-436 cells were also infected with increasing MOI of Ad5-wtFiber or Ad5-ffCXCL12 for (C) 72 hours or (D) 96 hours before XTT assays. As a positive control, the cells were treated 2 ?g/mL DOX. All data are representative of five replicates normalized to untreated cells (100% cell viability). Bars indicate the mean S.D. Two-way ANOVA followed by Bonferroni's multiple comparisons test was performed to compare the percent cell survival at each MOI to uninfected cells (0 MOI). The differences between the virus treatment at each MOI and uninfected cells were considered statistically significant if $p < 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: DOX, doxorubicin; MOI, multiplicity of infection; S.D., standard deviation.

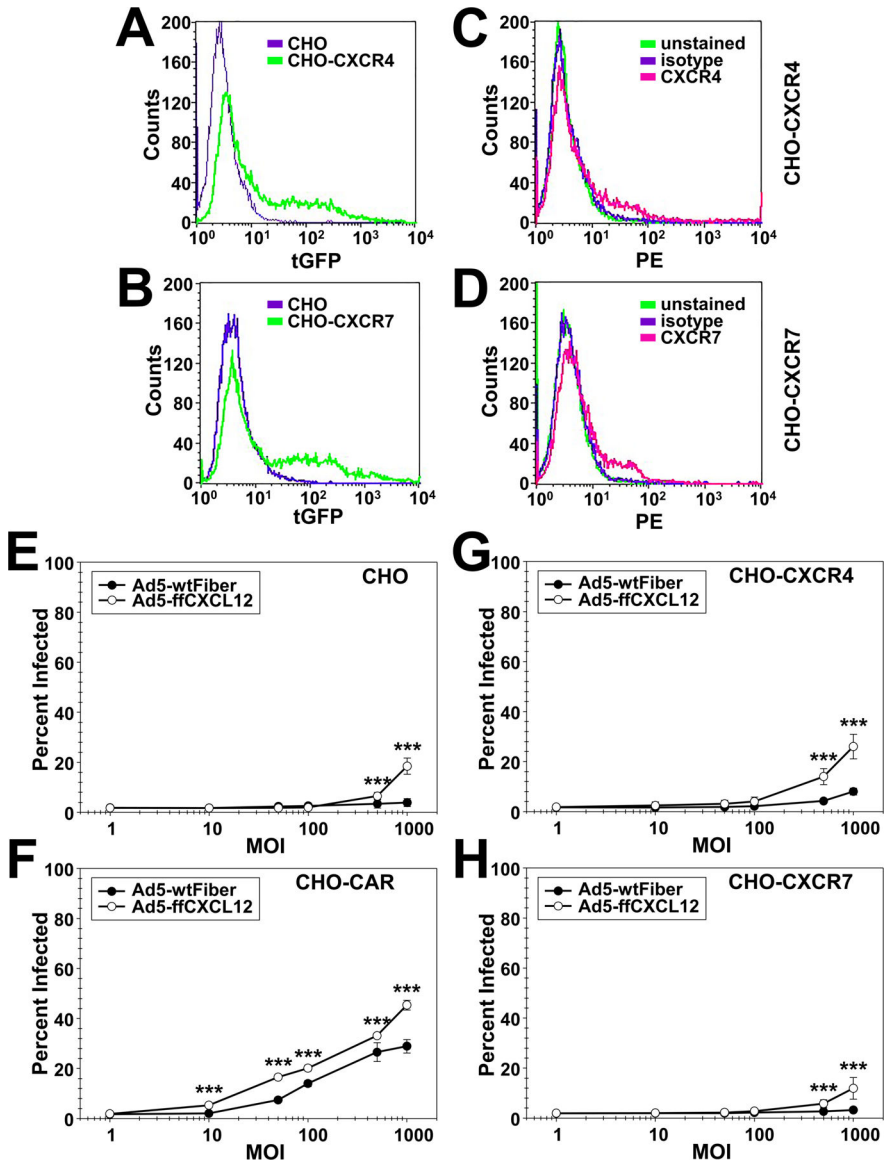

Figure 5.

Expression of CXCR4 and CXCR7 in stably transfected CHO cells. GFP-tagged receptor expression in (A) CHO-CXCR4 and (B) CHO-CXCR7 cells was compared to parental CHO cells by flow cytometry after stable transfection with mammalian expression plasmids. CHO-CXCR4 and CHO-CXCR7 cells were also examined by immunostaining for cell surface expression of (C) CXCR4 or (D) CXCR7 using PE-conjugated monoclonal antibodies specific for the human receptors. The cells were incubated with PBS alone (unstained), an isotype control antibody, or the receptor-specific antibody. Following incubation, the cells were washed, resuspended in 0.4 mL PBS, and analyzed by flow cytometry. The percent of (E) CHO, (F) CHO-CAR, (G) CHO-CXCR4, and (H) CHO-CXCR7 cell lines infected with Ad5-wtFiber (●) or Ad5-ffCXCL12 (○) for 72 hours at increasing MOI was determined by flow cytometry analysis of pIX-RFP expression. All data is representative of three replicate experiments. Points indicate the mean S.D. of percent

RFP positive cells. Two-way ANOVA followed by Bonferroni's multiple comparisons test was performed to compare the percent infected cells at each MOI. The differences between the two virus treatments at each MOI were considered statistically significant if $p < 0.05$; ***$p < 0.001$. Abbreviation: MOI, multiplicity of infection; PE, phycoerythrin; RFP, red fluorescent protein; S.D., standard deviation; tGFP, turbo green fluorescent protein.

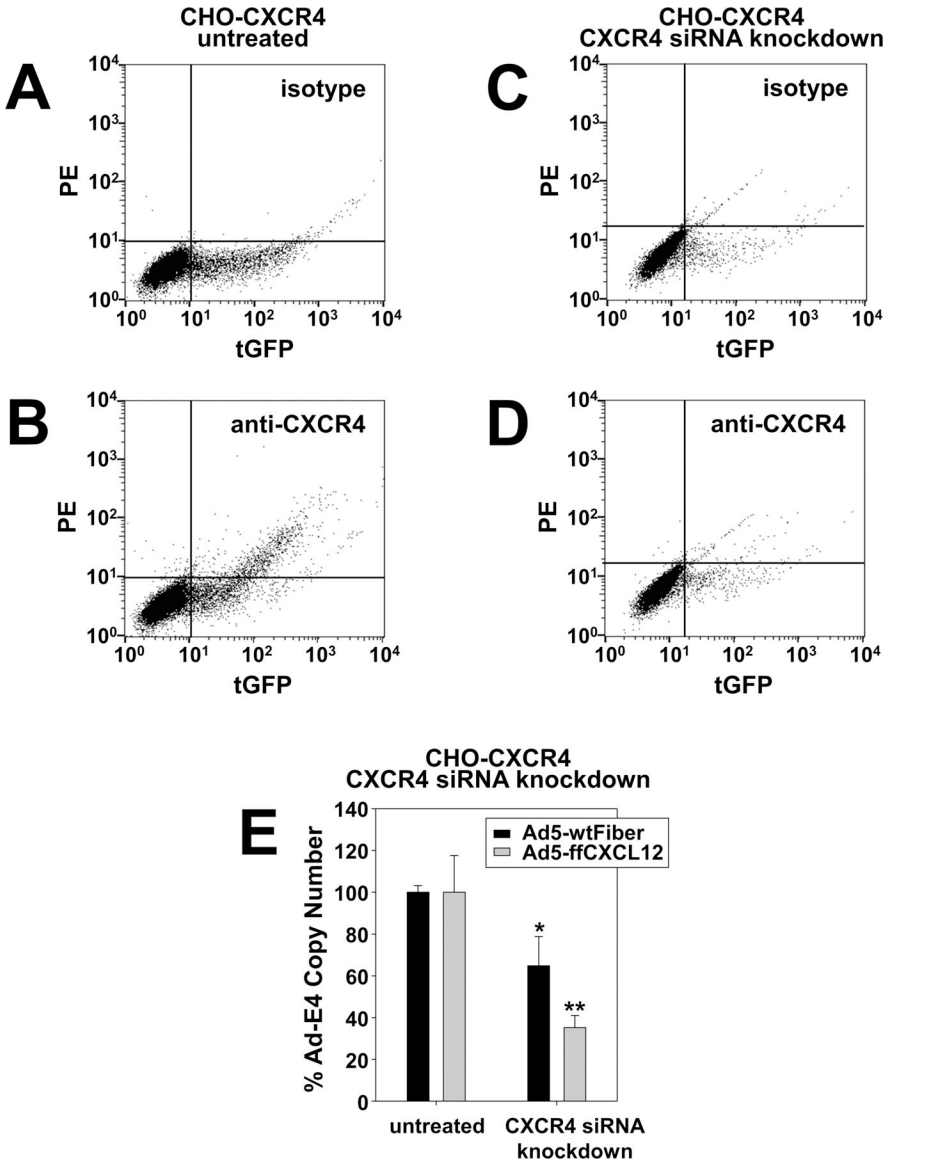

Figure 6.

Effect of CXCR4 knockdown on adenovirus binding. Parental CHO-CXCR4 cells and CHO-CXCR4 cells transfected with CXCR4 siRNA were characterized by two parameter (dual-color fluorescence) flow cytometry. Shown are representative fluorescence plots of tGFP-tagged receptor expression (x axis) and immunostaining of CXCR4 expression (y axis) in untransfected cells using an isotype control (A) or a PE-conjugated monoclonal antibody specific for the human receptor (B). CXCR4 expression was also determined in cells transfected for 72 hours with an anti-CXCR4 siRNA. Shown are representative fluorescence plots of tGFP-tagged receptor expression (x axis) and immunostaining of CXCR4 expression (y axis) in transfected cells using an isotype control (C) or a PE-conjugated monoclonal antibody specific for the human receptor (D). Binding of Ad5-wtFiber (black bars) or Ad5-ffCXCL12 (grey bars) at 100 MOI was determined by qPCR of DNA isolated from untransfected CHO-CXCR4 cells or CHO-CXCR4 cells transfected for 72 hours with

an anti-CXCR4 siRNA (E). All data are representative of five replicates normalized to untransfected cells (100% cell viability). Bars indicate the mean S.D. The differences between the untransfected and transfected groups were compared using Student's t-test and were considered statistically significant if $p < 0.05$; *$p < 0.05$, **$p < 0.01$. Abbreviations: MOI, multiplicity of infection; PE, phycoerythrin; qPCR, quantitative polymerase chain reaction; S.D., standard deviation; siRNA, small interfering RNA; tGFP, turbo green fluorescent protein.

Table 1.

Classification and basic expression profile of a panel of normal and breast cancer cell lines.

Cell Line	Tumor Type	Histological Classification	Molecular Classification	Receptor Status		
				ER	PR	HER2
MCF-12A	normal breast	spontaneous immortalization	normal epithelial	−	−	−
BT-20	primary breast	Basal A	TNBC	−	−	+
MCF-7	pleural effusion	Luminal A	ER+	+	+	−
MDA-MB-231	pleural effusion	Basal B	TNBC	−	−	−
MDA-MBA-436	pleural effusion	Basal B	TNBC	−	−	−
ZR-75–1	ductal carcinoma	Luminal A	ER+	+	+	+

Abbreviations: TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 2.

GenBank DNA and amino acid sequences associated with the chimeric Ad5 fiber construct.

fiber-fibritin-CXCL12 gene (ffCXCL12)	GenBank accession no.	Amino acid sequence	Nucleotide sequence (bp)
Ad5 fiber N-terminus tail domain	MF681662.1	amino acids 1 – 67	30997 – 31197 (201)
Linker sequence	JF745946.1	NGLSLDEAGNLT	31361 – 31396 (36)
T4 fibritin shaft and foldon domains	X12888.1	amino acids 231 – 487	688 – 1461 (774)
Linker sequence	LC498629.1	GGGGSGGGGSGGGGS	254 – 298 (45)
Human CXCL12, transcript variant 1	NM_199168.3	amino acids 21 – 89	156 – 359 (204)

Table 3.

Primers sets for PCR analysis.

Gene	Forward Primer	Reverse Primer
Ad5-E4	5'-TCATGATTGCAAAAATTCAGGTTCC-3'	5'-CTGTTGTAAGACAGGCTTCTAATGTTTA-3'
Ad5-penton	5'-CGCGGCGATGTATGAGGAAGGT-3'	5'-CCCGCGCCTTTAAACTTATTGG-3'
CXCL12	5'-CTGGCTTAAGAGCGAATG-3'	5'-TCCACTTTAGCTTCGGGT-3'
Ad5-E1A	5'-GCGGGAAAACTGAATAAGAG-3'	5'-AGGCTCAGGTTCAGACACAG-3'
Fibritin	5'-CGGCAACCTTACCCAGAA-3'	5'-CGCCATCTTTACGAACGT-3'
Ad5-Fiber	5'-ATGAAGCGCGCAAGACCGTC-3'	5'-AGCTATGTGGTGGTGGGGCT-3'
pIX-RFP	5'-GGAAGCATTGTGAGCTCATA-3'	5'-TCTTGACCTCAGCGTCGTAGTGGCC-3'

Abbreviations: Ad5, adenovirus serotype 5; CXCL12, C-X-C Motif Chemokine Ligand 12; PCR, polymerase chain reaction; pIX, adenovirus protein IX; RFP, red fluorescent protein.

Table 4.

Quadrant analysis of CXCR4-tGFP fusion detection and PE-conjugated anti-human CXCR4 immunostaining of CHO-CXCR4 cells by flow cytometry.

	untreated		CXCR4 siRNA knockdown	
quadrant	**isotype**	**anti-CXCR4**	**isotype**	**anti-CXCR4**
LL (tGFP− PE−)	85.0%	83.9%	95.4%	95.7%
UL (tGFP− PE+)	0.1%	0.4%	0.1%	0.1%
LR (tGFP+ PE−)	14.1%	10.1%	4.0%	3.4%
UR (tGFP+ PE+)	0.8%	5.6%	0.5%	0.8%

Abbreviations: LL, lower left; LR, lower right; UL, upper left; UR, upper right; PE, phycoerythrin tGFP, turbo green fluorescent protein.