Enhancing reovirus for use in oncolytic virotherapy

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Characterization of a replicating expanded-tropism oncolytic reovirus carrying the adenovirus E4orf4 gene

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ABSTRACT

While the mammalian orthoreovirus Type 3 Dearing (reovirus T3D) infects many different tumor cells, various cell lines resist the induction of reovirus-mediated cell death. In an effort to increase the oncolytic potency, we introduced transgenes into the S1 segment of reovirus T3D. The adenovirus E4orf4 gene was selected as transgene since the encoded E4orf4 protein induces cell death in transformed cells. The induction of cell death by E4orf4 depends in part on its binding to phosphatase 2A (PP2A). In addition to the S1-E4orf4 reovirus, two other reoviruses were employed in our studies. The reovirus rS1-RFA encodes an E4orf4 double-mutant protein that cannot interact with PP2A and the rS1-iLOV virus encoding the fluorescent marker iLOV as a reporter. The replacement of the codons for the junction adhesion molecule-A (JAM-A) binding head domain of the truncated spike protein blocks the entry of these recombinant viruses via the reovirus receptor JAM-A. Instead, these viruses rely on internalization via binding to sialic acids on the cell surface. This expands their tropism and allows infection of JAM-A deficient tumor cells. Here, we not only demonstrate the feasibility of this approach, but also established that the cytolytic activity of these recombinant viruses is largely transgene-independent.

INTRODUCTION

The lytic replication of mammalian orthoreovirus Type 3 Dearing (reovirus T3D) initiates preferentially cell death in transformed cells, but not in normal diploid cells. The cell's innate sensing of the virus, and more specifically the PKR-dependent inhibition of translation, has been demonstrated to underlie the difference in reovirus sensitivity between cancer cells and healthy cells. In many tumor cells, the pathways that control cell division and other regulatory processes are derailed. Mutations leading to constitutively active RAS signaling occur in approximately 30% of all human cancers and in some cancer types, for instance in pancreatic cancer, the incidence is even higher [1, 2]. The Ras signaling inhibits the PKR response and Ras-transformed cells are generally more sensitive to reovirus-induced apoptosis. This leads to enhanced virus release and spread from the infected cells [3, 4]. These observations led to the initiation of a series of clinical trials in which the wild-type reovirus T3D was administered to patients as viral anti-cancer agent [5]. Reovirus treatment in various cancer types proved to be well tolerated and safe for patients but when used as a monotherapy the anti-tumor efficacy was limited, warranting studies on combinatorial therapies. For such preclinical studies many rodent models are available. Studies in murine models are facilitated by the reovirus' capacity to replicate in human as well as in murine cells. This allows studies on the effect of immune modulation in immune-competent mouse tumor models [6].

Not all tumor cells are sensitive to reovirus infection and subsequent oncolysis. While some tumor cells resist infection by reoviruses due to the absence of the reovirus receptor junction adhesion molecule-A (JAM-A) at the cell surface, other cells resist reoviruses at a post-entry level. In several head and neck cancer cell lines, reovirus infection did not efficiently initiate cell death. In a panel of squamous cell carcinomas of the head and neck (SCCHN), the variation in sensitivity to reovirus infection was not linked to differences in EGFR/Ras/MAPK pathways [7]. In HT1080 fibrosarcoma cells, reovirus T3D exposure causes a persistent infection despite an activating N-Ras mutation. In the persistently reovirus-infected HTR1 cell line the apoptotic pathway is not completely abolished, since chemical-induced apoptosis and exposure to E1B-defective adenoviruses still result in apoptotic cell death [8].

To overcome the resistance to reovirus infection and oncolysis, we employed the plasmid-based reverse genetics system to generate replication-competent transgene-containing reoviruses. Recently, we and others demonstrated the feasibility of this approach by replacing the sequence coding for the JAM-binding head domain of the reovirus attachment protein σ1 by genes encoding the green fluorescent reporter proteins iLOV or UnaG [9, 10].

The location of the transgene in segment S1 of reovirus was based on our observation that the *jin* mutants obtained by bioselection of the reovirus T3D on JAM-A deficient U118-MG cells acquired the capacity to infect cells independent of the presence of the reovirus receptor JAM-A on the cell surface. One of the mutants, *jin*-3, harbors a single point mutation in the S1 segment, that leads to a Gly196Arg substitution in the tail region of the σ1 spike protein close to the region involved in sialic acid binding. This mutation allows the *jin* mutants to employ sialic acids on the cell surface as primary receptors and we demonstrated that the head-domain of the σ1 protein was not required for entry of the *jin* mutants [11]. Therefore, the codons in S1 that encode the head domain can be replaced by a heterologous transgene.

Several therapeutic proteins are candidates to be expressed by replicating reovirus vectors. One suitable candidate is the human adenovirus type 2 (HAdV-2) E4 open reading frame 4 (E4orf4) protein. This small 14 kDa protein is encoded by a fragment of only 345 nucleotides in length. The E4orf4 protein induces p53-independent apoptosis in transformed cells, but not in normal cells [12, 13]. This effect of E4orf4, however, is cell line dependent, since it can induce caspase-independent cell death in some other transformed cell lines [14]. Induction of cell death by E4orf4 is dependent on the association of E4orf4 with the Bα subunit of protein phosphatase 2A (PP2A). PP2A is an abundant cellular serine/threonine phosphatase that targets proteins implicated in many cell growth and signaling pathways [15]. Binding of E4orf4 to PP2A inhibits ATP-utilizing chromatin assembly and modifying factor (ACF) containing chromatin remodeling complexes, causing alterations in the cell's chromatin, leading to cell death [16]. Amino acid substitutions in the E4orf4 protein that inhibit its binding to PP2A prevent cell death induction in H1299 lung carcinoma cells. One such E4orf4 mutant harbors mutations that result in two amino acid substitutions: R81A and F84A (RFA for short) [12].

E4orf4 can also trigger a cytoplasmic-induced cell death, caused by interaction with Src family kinases (SFKs). The E4orf4-Src interaction is detected when E4orf4 is overexpressed alone, and outside the context of an adenovirus infection. In adenovirus-infected cells the E4orf4-Src association is rarely observed, since E4orf4 mainly resides in the nucleus and is therefore not available to Src [17, 18]. The changes in the RFA mutant do not affect the region involved in Src binding and therefore the RFA protein may still induce cytoplasmic programmed cell death.

The most studied reovirus-induced cell death mechanism is the apoptotic pathway [19-24]. As is mentioned before, induction of apoptosis is enhanced in reovirus-infected Ras-transformed cells and may stimulate virus release and spread of the virus to neighboring cells. The signaling events involved in the induction of reovirus-mediated cell death are both cell-type dependent and reovirus strain-specific [25, 26]. Necrosis, a caspase-independent cell death pathway, is controlled

by the sialic acid binding capacity of reovirus σ1 protein and requires the production of viral RNAs [27]. Our transgene-containing recombinant reoviruses rely on enhanced binding to sialylated glycans on the host cells, which could induce the caspase-independent necrosis type of cell death. This supported our hypothesis that combining reovirus infection of tumor cells with E4orf4 protein expression may increase the oncolytic potency of reovirus T3D for tumor types that resist wild-type reovirus T3D-mediated cell death.

Here, we generated reoviruses containing the wild-type E4orf4 gene, a virus carrying the gene encoding the double mutant of E4orf4 (RFA) and, as control, our previously generated reovirus containing the iLOV gene, and tested these in various cell types that resisted wild-type reovirus-induced cell death.

MATERIALS AND METHODS

Cell lines and viruses

The cell lines 911 [28], human glioblastoma cell line U118-MG, chicken hepatoma cell line LMH, p53-deleted human non-small-cell lung carcinoma cell line H1299, human bladder carcinoma cell line UMUC-3, and the normal human foreskin fibroblasts VH10 and VH25 [29] were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Life Technologies, Bleiswijk, the Netherlands), supplemented with 8% fetal bovine serum (FBS, Invitrogen) and with antibiotics penicillin and streptomycin (pen/strep). LMH cells were cultured on dishes coated with 0.1% gelatin (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). PC-3M-Pro4/Luc cells (Pro4-Luc cells in text) are highly metastatic human prostate cancer cells obtained from the Department of Urology of the Leiden University Medical Center [30]. Pro4-Luc cells are cultured in high glucose DMEM 8% FBS, pen/strep and 400 µg/ml G418 (Santa Cruz, Bio-Connect BV, Huissen, the Netherlands).

The 911-Flag.PP2A cells were generated by stable transfection of 911 cells with plasmid pcDNA3-FLAG.PP2A.Bα (kindly provided by Prof. P. Branton, Department of Biochemistry, McGill University, Montreal, Quebec, Canada [12]) and selected on medium containing 800 µg/ml G418. Cell line 911-Flag.PP2A was derived from one colony (#1) and further propagated after initial selection in high glucose DMEM 8% FBS, pen/strep and 400 µg/ml G418. Cell line 911scFvHis is a 911-cell derivative and expresses a single-chain antibody on its surface that is capable of binding His-tags. This cell line was used for the first propagation of recombinant reoviruses, and was cultured in high glucose DMEM 8% FBS, pen/strep and 400 µg/ml G418. The T7-RNA polymerase-expressing cell line BSR-T7 [31] was provided by Prof. K.K. Conzelmann (Ludwig-Maximilians-University Munich, München, Germany) and cultured in high glucose DMEM, 8% FBS, pen-strep and 400 µg/ml G418 [29]. All cells were cultured in an atmosphere of 5% CO_2 at 37°C. The identity

of the human cell lines used in this study was verified by short tandem repeat analyses and comparison with the STR databases at the Forensic Laboratory for DNA Research, Department of Human Genetics, Leiden University Medical Center. All cell lines used are regularly tested for mycoplasma with the MycoAlert mycoplasma detection kit (Lonza Benelux BV, Breda, the Netherlands).

The *wt*-T3D virus strain R124 was isolated from reovirus T3D stock VR-824 from the American Type Culture Collection by two rounds of plaque purification and propagated on 911 cells. In the text, R124 is referred to as *wt*-R124. *Jin-3* mutant reovirus was obtained by bioselection of *wt*-R124 on U118-MG cells as previously described [11] and further propagated on 911 cells. Recombinant reovirus rS1His-2A-iLOV (referred to as rS1-iLOV in this manuscript) was generated as described previously [9].

Plasmid constructs and recombinant reoviruses

PBacT7 constructs with S1His-2A-HA.E4orf4 and S1His-2A-HA.RFA

The S1His-2A-HA.E4orf4 segment was designed *in silico* and a DNA copy was synthesized by Eurofins Genomics (Ebersberg, Germany). The total length of this synthetic segment is 1419 bp (Figure 1). The segment sequence was assembled to contain the following features: 1) nt 1 to 768 from the S1 segment of reovirus mutant *jin-3*; this includes the 5'-untranslated region (UTR), entire σ1s ORF, and the first 252 amino acids of the *jin-3* σ1, including the codons for the G196R change near the sialic acid binding domain [11]; 2) the codons for a 6xHis-tag (18 bp) which was placed in frame with the σ1 open reading frame; 3) the codons for the porcine teschovirus-1 2A sequence (66 bp + 3 additional bp); 4) the HA-tagged E4orf4 encoding sequence (372 bp); 5) a stop codon, and 6) the 3'-UTR of the S1 segment from nt 1219 to 1416 of reovirus T3D, which includes the A-, B, and C-box elements implicated in encapsidation of the reovirus plus strand RNA in the viral capsid [32, 33].

The synthetic S1His-2A-HA.E4orf4 fusion construct was inserted in a pEX-A2 plasmid by Eurofins Genomics (to generate pEX-S1His-2A-HA.E4orf4). The S1His-2A-HA.E4orf4 part was further PCR amplified from this construct using forward primer T7_compl_S1For (5'-TAATACGACTCACTATAGCTATTGGTCGGATGGATCCTCGCCTACGT-3') and reverse primer S1EndR (5'-GATGAAATGCCCCAGTGC-3') with Pfu polymerase (Fermentas, Fisher Scientific, Landsmeer, the Netherlands). The underlined sequences are the parts complementary to the sequence in the pEX-S1His-2A-HA.E4orf4 construct. The PCR product was digested with restriction endonuclease *SacII* and purified with SureClean (Bioline; GC Biotech BV, Alphen aan den Rijn, the Netherlands) according to the manufacturer's protocol. The PCR product was inserted in the plasmid pBACT7 backbone of pBacT7-S1T3D [34]. Plasmid pBacT7-S1T3D was obtained at Addgene (plasmid 33282). The wt S1T3D was

removed by digestion with *Sma*I and *Sac*II and the pBACT7 backbone was isolated from a 1% agarose gel and purified by JetSorb (Genomed; ITK Diagnostics BV, Uithoorn, the Netherlands) according to the manufacturer's protocol. The *SacII*-digested S1His-2A-HA.E4orf4-containing PCR product was inserted in the *SmaI*- and *SacII*-digested pBACT7 DNA with T4 DNA ligase (Fisher Scientific, Landsmeer, the Netherlands), resulting in construct pBT7-S1His-2A-HA.E4orf4.

To obtain the RFA mutant of E4orf4 that no longer binds to PP2A as a result of amino acid substitutions R81A and F84A, plasmid pBT7-S1His-2A-HA.E4orf4 was used as input for site-directed ligase-independent mutagenesis (SLIM) PCR with forward primer RFAmutE4O4_For (5'-GATCTGTTTGTCACGCCGCCACCTGGGCTTGCTT CAGGAAATATGAC-3') and reverse primer RFAmutE4O4_Rev (5'-GTCATATTTCCTGAA GCAAGCCCAGGTGGCGGCGTGACAAACAGATC-3'). Underlined are the nucleotides encoding the substituted amino acids R81A and F84A in the mutated E4orf4 protein. The SLIM PCR was performed with the following components; 100 ng pBT7-S1His-2A-HA.E4orf4, 5 µl 10x KOD buffer, 1 mM MgSO4, 20 pmol of each primer (RFAmutE4O4_For and RFAmutE4O4_Rev), 250 µM dNTP's, 1U KOD polymerase (Novagen; Merck-Millipore, Amsterdam, the Netherlands) and molecular biology-grade water to a final volume of 50 µl. Cycling parameters: 2 min 94°C, 30 cycles 15 sec 94°C - 30 sec 58°C - 10 min 68°C and one final extension step of 10 min 68°C. The input plasmid was digested by adding 2 µl DpnI (Fermentas, Fisher Scientific, Landsmeer, the Netherlands) to the reaction and incubation for 2 hours at 37°C. An aliquot of 10 µl was used to transform chemically competent Top10 bacteria (Invitrogen, Fisher Scientific, Landsmeer, the Netherlands). DNA was extracted from colonies with GeneJet Plasmid Miniprep kit (Fisher Scientific, Landsmeer, the Netherlands) according to the manual and sent for sequencing to the Leiden Genome Technology Center (Leiden University Medical Center, Leiden, the Netherlands) to confirm the presence of the mutations, resulting in construct pBT7-S1His-2A-HA.RFA.

Reoviruses rS1-E4orf4 and rS1-RFA

Recombinant reoviruses were generated from both plasmids, pBT7-S1His-2A-HA.E4orf4 and pBT7-S1His-2A-HA.RFA as previously described [9]. In short, pBT7-S1His-2A-HA.E4orf4 or pBT7-S1His-2A-HA.RFA were transfected with TransIT-LT1 transfection reagent (Mirus, Sopachem BV, Ochten, the Netherlands) in BSR-T7 cells, together with four other plasmids containing the remaining reovirus segments (obtained through AddGene): pT7-L1T1L (plasmid 33286), pT7-L2-M3T3D (plasmid 33300), pT7-L3-M1T3D (plasmid 33301) and pT7-M2-S2-S3-S4T3D (plasmid 33302) [35]. Two days post transfection, the cells were harvested and lysed by three cycles of freeze-thawing and cell debris was cleared from supernatant by centrifugation.

Initial propagation was done in 911scFvHis cells until first signs of cytopathic effect (CPE) before further expanding the reoviruses on 911 cells. Official recombinant virus names rS1His-2A-HA.E4orf4 and rS1His-2A-HA.RFA are abbreviated to rS1-E4orf4 and rS1-RFA in this manuscript.

Accession number of the HAdV-2 E4orf4 protein: YP_001551773.

RNA isolation and S1 RT-PCR

Cells (911, $1x10^5$ per well) in 24-well plates were infected with rS1-E4orf4 or rS1-RFA. Since we had no indication of the titer of the early passaged batches, 1/20th part of the isolated reoviruses P1 was used for exposure to 911 cells. Total RNA was extracted, 24 hours post infection (hpi), with the Absolutely RNA miniprep kit (Stratagene, Agilent Technologies, Amstelveen, the Netherlands) from the infected cells according to the manual. cDNA was synthesized with the S1EndR primer (5'-GATGAAATGCCCCAGTGC-3') and SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Bleiswijk, the Netherlands). For the S1 PCR the following primers were used; S1For: 5'- GCTATTGGTCGGATGGATCCTCG-3' and S1EndR with GoTaq polymerase (Promega, Leiden, the Netherlands). The PCR products were purified with SureClean (Bioline, GC Biotech BV, Alphen aan den Rijn, the Netherlands) for the subsequent sequence reactions with primers S1For, S1EndR and S1Trunc_For: 5'-GACTGTGTTTGATTCTATCAACTC-3'. Sequence analysis was performed in the Leiden Genome Technology Center (Leiden University Medical Center, Leiden, the Netherlands).

Western blot analysis

Cell lysates were prepared in RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40) supplemented with protease inhibitors (complete mini tablets, Roche Diagnostics, Almere, the Netherlands). Protein concentrations in the lysates were measured by Bradford assay (Biorad, Veenendaal, the Netherlands).

For detection of the HA-tagged E4orf4 and RFA proteins in lysates, equal amounts of protein (30 µg) were loaded into the slots of a 15% polyacrylamide-SDS gel after addition of western sample buffer (final concentrations: 10% glycerol, 2% SDS, 50 mM Tris·HCl pH 6.8, 2.5% β-mercaptoethanol and 0.025% bromophenol blue). The proteins were transferred to Immobilon-PSQ (Merck-Millipore, Amsterdam, the Netherlands) and the blot was divided for staining with β-Actin antibody (ImmunO clone C4, MP Biomedicals, Eindhoven, the Netherlands) and HA.11 monoclonal antibody (Biolegend, ITK Diagnostics BV, Uithoorn, the Netherlands) for detection of the HA-tag. A Goat-anti-Mouse HRP-conjugated secondary antibody was used for detection and the signals were visualized by standard chemiluminescence techniques.

Reovirus proteins were detected by loading equal amounts of lysate (20 µg) into slots of a 12% polyacrylamide-SDS gel after addition of western sample buffer. The proteins were transferred to Immobilon-FL (Merck-Millipore, Amsterdam, the Netherlands) for detection with the Odyssey system (LI-COR Biotechnology, Westburg, Leusden, the Netherlands). The blot was divided for staining with mouse Vinculin antibody hVIN-1 (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and a combination of mouse anti-σ3 4F2 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA) and rabbit anti-P2A peptide serum ABS31 (Merck-Millipore, Amsterdam, the Netherlands). For the detection of the primary antibodies, the IRDye 800CW Donkey-anti-Rabbit IgG and IRDye 680RD Donkey-anti-Mouse IgG (LiCor, Westburg BV, Leusden, the Netherlands) were used, prior to analyzing the signals with the Odyssey.

Immunoprecipitation (IP) assay

Cell line 911-Flag.PP2A was infected with rS1-E4orf4 or rS1-iLOV at an MOI of 1 (in a 6-well plate). As controls, 911-Flag.PP2A cells were PEI-transfected with plasmid pEGFP-N2, plasmid pcDNA.HA.E4orf4, and plasmid pCDNA.HA.RFA (3 µg plasmid DNA per well). Lysates were made in Giordano buffer containing NP40 (50 mM Tris·HCl pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA and 0.5% NP40) at 24 hpi and 48 hours post transfection. A small amount of lysate was set aside for protein detection in whole cell extracts (WCE).

Remaining cell lysates were used in the IP procedure, with anti-flag M2 affinity gel beads (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) according to the manufacturer's protocol. In short, equilibrated anti-flag beads in Giordano buffer were added to the different lysates and tumbled for 2 hours at 4°C. The beads were washed 3x with Giordano buffer to remove unbound proteins, and subsequently 2x sample buffer without β-mercaptoethanol (125 mM Tris·HCl pH 6.8, 4% SDS, 20% glycerol and 0.01% bromophenol blue) was added. The samples were boiled for 5 minutes before loading on a 15% polyacrylamide-SDS gel. IP proteins in lysates were detected using anti-HA HA.11 (Biolegend, ITK Diagnostics BV, Uithoorn, the Netherlands). The blot with WCE was divided for staining with the same anti-HA antibody and monoclonal anti-flag M2 antibody (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). After the incubation with the anti-flag antibody, the membranes were re-stained with the anti-σ3 4F2 antibody. For the detection of the primary antibodies, IRDye 680RD Donkey-anti-Mouse IgG was used, prior to analyzing the signals with the Odyssey (infection panel) or Goat-anti-Mouse HRP-conjugated secondary antibody for detection with the standard chemiluminescence technique (transfection panel).

Transfections
Transfection controls in Western detection for HA.E4orf4 and HA.RFA proteins in 911 cells and in 911-flag.PP2A IP assay were generated using 25 kDa linear polyethyleneimine (PEI) at a concentration of 1 mg/ml (pH 7.4). For transfection of the 911 cell line, cells were grown in 24-well plates in 400 µl fresh DMEM containing 8% FBS/well and 1 µg DNA (either plasmid pcDNA.HA.E4orf4 or plasmid pCDNA.HA.RFA) mixed with 3 µg PEI in 100 µl Opti-MEM (Invitrogen, Life Technologies, Bleiswijk, the Netherlands). For the IP controls, 911-flag.PP2A cells in 1.5 ml DMEM, 8% FBS in 6 well plates were transfected using 3 µg DNA (pcDNA.HA.E4orf4, pCDNA.HA.RFA or pEGFP-N2 plasmids) and 9 µg PEI in 250 µl Opti-MEM. The next day the cells received fresh DMEM with 8% FBS and were allowed to recover for 48 hours before the cells were harvested and lyzed.

Viability (WST) assays
WST-1 reagent (Roche, Woerden, the Netherlands) was used to assay the viability of cells after reovirus infections. In a 96-well plate $5x10^3$ cells/well (in triplicate) were exposed to different reoviruses (rS1-E4orf4, -RFA, -iLOV, *jin-3* and wt-R124) at an MOI of 5 (Figure 4) or MOI of 30 (Figure 5B) in DMEM containing 2% FBS, or mock-infected. For the assay 5 µl of WST-1 reagent, which is half of the amount suggested by the manufacturer, was added to each well which already contained 100 µl medium, at the indicated days post infection. The percentage survival was calculated by dividing the OD_{450} values of the reovirus-exposed cells by the values of the mock-treated cells for each cell line, and multiplying this by 100%. Cultures with survival values below 1% were considered all dead and adjusted to 1% for plotting the data in log scale.

Caspase 3/7 assay
H1299 and 911 cells, grown in 96-well plates at $5x10^3$ cells/well were exposed to the reovirus variants wt-R124, *jin-3*, rS1-E4orf4, rS1-RFA or rS1-iLOV, each at an MOI of 30, or mock-infected. All conditions were tested in triplicate. Caspase activity was measured 24 hpi using the Caspase-Glo 3/7 assay (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. For the detection, a PerkinElmer's VictorX3 (PerkinElmer, Groningen, the Netherlands) multilabel plate reader was used.

Statistics
For the WST-1 assays the percentages survival were transformed to log kill with this formula: log kill=log(100/survival (%)). Subsequently multiple t tests were performed on the transformed data with corrections for multiple comparisons using the Holm-

Sidak method in GraphPad Prism version 7.0. The generated results (log kill and adjusted P values for comparisons) are summarized in separate tables.

RESULTS

Generation of recombinant reoviruses

To augment the oncolytic potency of reoviruses we have chosen to explore the possibility of incorporating a therapeutic transgene in their genome. Our previous results demonstrated that the S1 segment, encoding the reovirus attachment protein σ1, is a suitable location for inserting a gene encoding for a small fluorescent protein, called iLOV, without loss of virus replication [9]. The engineered recombinant reoviruses can no longer bind to the reovirus receptor junction adhesion molecule-A (JAM-A) but are thought to rely on enhanced binding to sialylated glycans on the cell surface. The removal of the codons for the JAM-A interacting head domain of the reovirus attachment protein σ1, allows for the insertion of small transgenes without exceeding the size of the wild-type T3D S1 segment. As a potentially clinically relevant protein in the context of anti-tumor features, we chose the HAdV-2 E4orf4. Protein E4orf4 induces p53-independent cell death in tumor cells and not in normal diploid cells. The size of the E4orf4 cDNA is 345 bp in length and therefore fits in the S1 segment to replace the sequence encoding the JAM-A binding domain. To detect E4orf4 in cells, the codons for an HA-tag were fused with the amino terminus of the E4orf4 gene, increasing the insert size with 27 nucleotides. The addition of the HA-tag to the amino terminus of HAdV-2 E4orf4 does not affect its function [12]. We employed the porcine teschovirus-1 2A element to allow separation of the truncated and C-terminal His-tagged σ1 from the HA-tagged E4orf4 protein, similar to the strategy previously used to generate the iLOV reporter virus (Figure 1). Similarly, we generated a derivative virus in which four point mutations in the E4orf4 gene substitute two amino acids at position 81 and 84 (R81A/F84A). This 'RFA' virus is unable to bind to PP2A.

To produce the recombinant reoviruses rS1-E4orf4 and rS1-RFA, the plasmids with the modified S1 segments together with four plasmids encoding the other nine genome segments of reovirus were transfected into BSR-T7 cells as previously described [9]. Newly assembled recombinant reoviruses from the transfected BSR-T7 cells were propagated on 911 cells containing an artificial receptor for the His-tag (911scFvHis), yielding passage 1 (P1) of the recombinant reovirus. For further propagation the 911 cell line was used.

The genetic integrity of the recombinant reoviruses was checked by sequencing the RT-PCR product of the isolated viral S1 RNA. The expected nucleotide changes in the RFA mutant are present (positions 1120-1121 CG in E4orf4 to GC in

RFA and at positions 1129-1130 TT in E4orf4 to GC in RFA) and no other mutations were found in either the S1-E4orf4 or in the S1-RFA segments (Figure 2A).

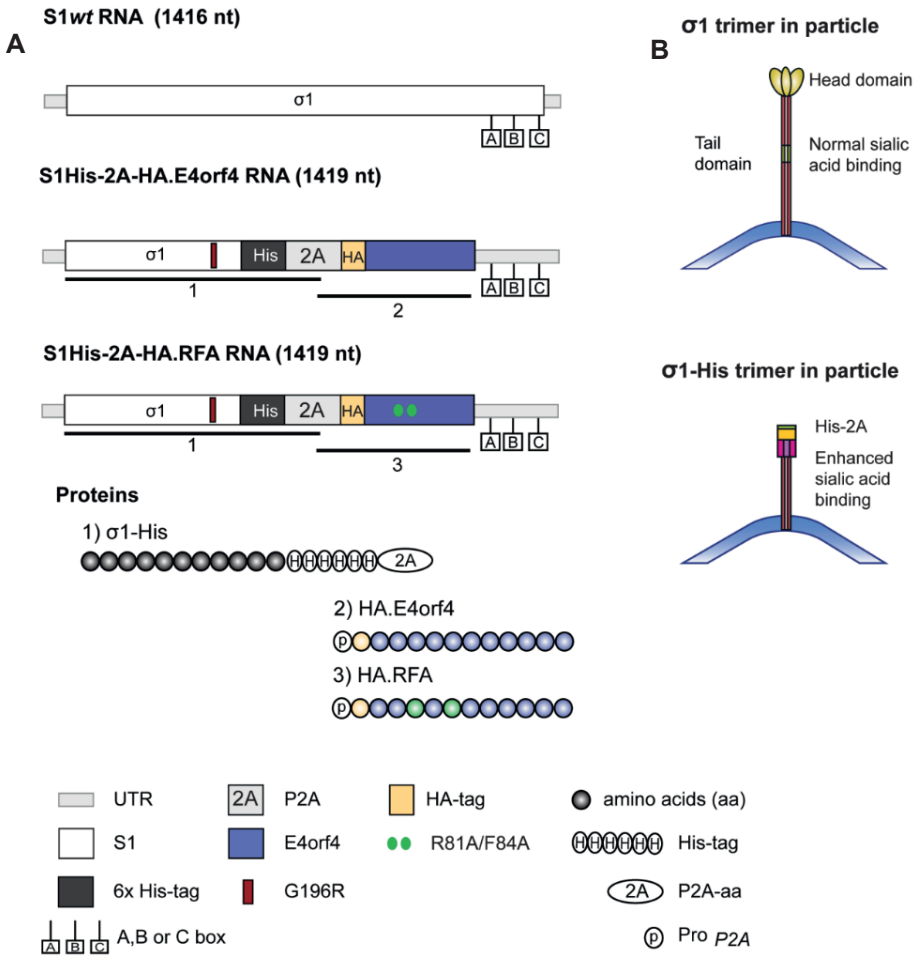

Figure 1. Schematic representation of the modified S1 gene segment. A) S1wt RNA compared with S1His-2A-HA.E4orf4 and the double mutant S1His-2A-HA.RFA RNA. Protein HA.RFA cannot bind to Protein Phosphatase 2A. The σ1-His protein is present in the particle, the HA-tagged proteins (E4orf4 or the double mutant RFA) only in infected cells. Modified reovirus S1 contains a point mutation in the sequence coding for the σ1 tail resulting in a G196R amino acid change. This change allows the virus to enter host cells by enhanced binding to sialic acids and compensates for the loss of the head domain. B) Simplified depiction of the wt σ1 trimer and the truncated σ1-His trimer in the capsid of a reovirus particle.

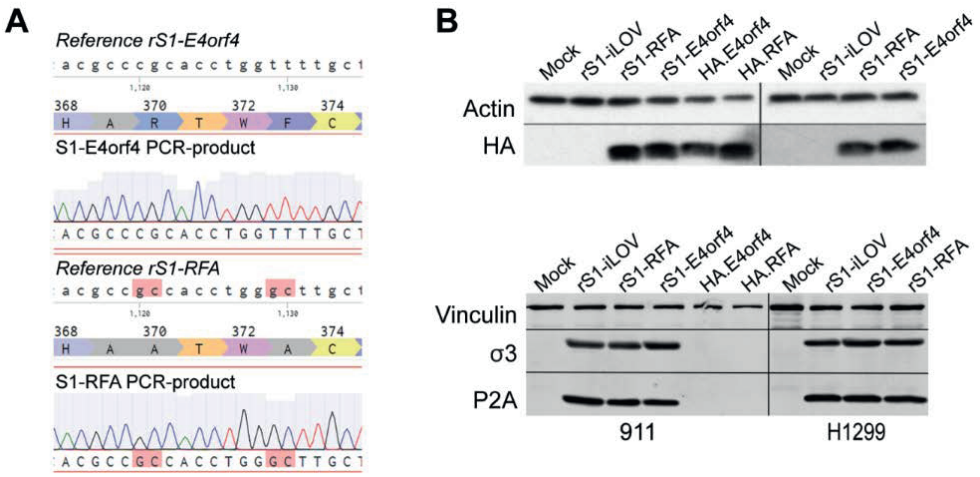

Figure 2. Confirmation of S1His-2A-HA.E4orf4 and -HA.RFA sequence and protein expression in cell lysates. A) Alignment of E4orf4 and RFA RT-PCR sequencing results with the reference sequences. The amino acid sequence is depicted in the reference panels. The nucleotide mutations in the S1His-2A-HA.RFA sequence responsible for the amino acid changes R to A and F to A are marked in red boxes. The amino acid numbering 368 to 374 is derived from the complete σ1-His-2A-HA.E4orf4/RFA protein. Alignments are generated with Benchling (Benchling Inc., San Francisco, USA). B) Protein detection by western blotting in 911 (left part) and H1299 (right part) cell lysates. 911 and H1299 cells were mock-infected or infected with recombinant reoviruses (at an MOI of 1) rS1-iLOV, rS1-E4orf4 or rS1-RFA. As positive controls for detection of HA-tagged proteins, 911 cells were transfected with pcDNA.HA.E4orf4 (HA.E4orf4) and pcDNA.HA.RFA (HA.RFA). RIPA lysates were generated 48 hours after infection/transfection. Upper panel: detection of HA-tagged E4orf4 or RFA proteins. Actin staining was included as loading control. Lower panel: detection of viral proteins σ3 (4F2 antibody) and σ1-His (P2A antibody) in lysates of infected cells. Loading control: vinculin.

To study the genetic stability of the recombinants, the viruses were serially passaged 10 times in 911 cells. In P10 of a rS1-E4orf4 reovirus batch we detected the presence of a minor population that contained a deletion in the S1-E4orf4 segment. Sequence analysis revealed the loss of 47 nucleotides at the 3' end of the E4orf4 sequence, resulting in a shift in location of the stop codon and a 6 nucleotide deletion in the A-box of S1 (Figure S1). In the E4orf4 protein this resulted in a loss of 5 amino acids at the C-terminus and two amino acid changes compared to the full-length E4orf4 protein. The C-terminus of E4orf4 in different adenovirus strains is the

least conserved region of the protein, in contrast to the highly conserved binding sites for PP2A and Src. In the deletion mutant found, the binding sites of PP2A and Src are not affected (Figure S2). The same deletion mutant emerged in high passage number batches of several independent transfection experiments to generate rS1-E4orf4 reovirus in BSR-T7 cells. Therefore, we decided to use for all experiments a low passage number of rS1-E4orf4 reovirus in which only trace amounts of the deletion mutant were detectable by RT-PCR of the S1-E4orf4 segment. Remarkably, no deletion mutants were detected in batches of rS1-RFA reoviruses.

To verify that the modified S1 segments were expressed and the E4orf4 proteins were produced in infected cells, 911 and H1299 cells were exposed to rS1-E4orf4 and rS1-RFA reoviruses at an MOI of 1. As positive controls, 911 cells transfected with plasmid pcDNA.HA.E4orf4 or plasmid pcDNA.HA.RFA were included (Figure 2B). HA-tagged proteins (14 kDa) were detected 48 hours post infection (hpi) in both the 911 and H1299 cell lines infected with rS1-E4orf4, rS1-RFA and in the plasmid-transfected 911 cells. No HA-tag could be detected in cells infected with the rS1-iLOV reovirus carrying the iLOV gene as reporter. On a separate blot, the same cell lysates were incubated with an antibody directed against reovirus σ3 (41 kDa) to show the presence of replicating reovirus and a P2A antibody to detect the recombinant truncated σ1 (30 kDa) proteins. P2A and σ3 were detected in the cell lysates of cells infected with the three recombinant reoviruses, but not in uninfected cells or transfected 911 cells.

Detection of σ3 and P2A in lysates of rS1-E4orf4 and rS1-RFA infected 911 and H1299 cells showed that the two recombinant reoviruses infect and replicate in both cell lines. In addition, detection of the HA-tagged proteins demonstrated the efficient expression of the E4orf4 and RFA transgenes in cells upon infection with the recombinant reoviruses.

E4orf4 binds to PP2A in context of reovirus infection

To confirm that the adenovirus E4orf4 protein expressed in the context of a reovirus infection can bind to the B55α subunit of cellular protein phosphatase 2A (PP2A), 911 cells expressing flag-tagged PP2A (911-Flag.PP2A) were infected with the rS1-E4orf4, rS1-RFA and rS1-iLOV reoviruses. In a co-immunoprecipitation assay, E4orf4 was found to interact with the flag-tagged PP2A in lysates of the rS1-E4orf4 infected cells (Figure 3). This interaction was also evident in 911-Flag.PP2A cells transfected with a plasmid encoding the HA-tagged E4orf4 protein. In contrast, in 911-Flag.PP2A cells infected with rS1-RFA or transfected with the double mutant HA.RFA plasmid, no association was visible on the Flag-IP blot. These results demonstrate the capacity of E4orf4 to interact with PP2A-B55α in the context of a reovirus infection.

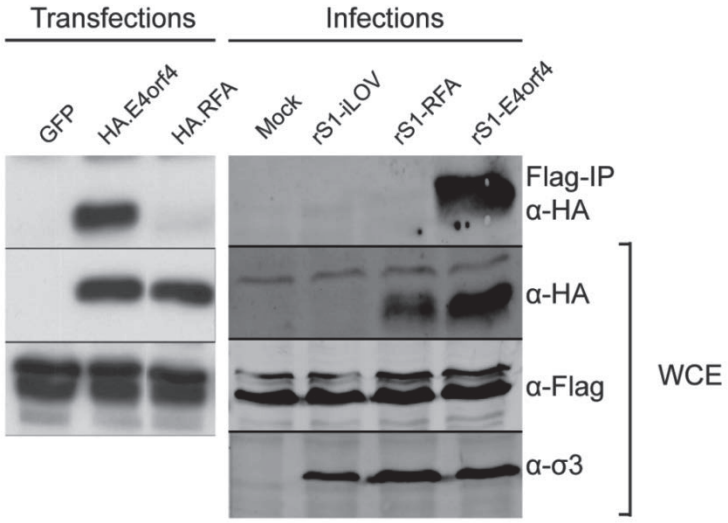

Figure 3. Detection of E4orf4 - PP2A interaction in reovirus rS1-E4orf4 infected 911-Flag.PP2A cells. HA.E4orf4 was co-immunoprecipitated using anti-flag antibodies to confirm the association of Flag.PP2A with E4orf4 in the context of reovirus rS1-E4orf4 infection. 911-Flag.PP2A cells were mock-infected or infected with rS1-iLOV, rS1-RFA and rS1-E4orf4 viruses at an MOI of 1. As controls 911-Flag.PP2A cells were transfected with pcDNA.HA.E4orf4 (HA.E4orf4) and pcDNA.HA.RFA (HA.RFA). As a negative control pEGFP-N2 (GFP) is included. Lysates were made 24 hours post infection (hpi) or 48 hours post transfection. Blots with whole cell extracts (WCE) were subjected to HA-antibody staining to show presence of HA-tagged E4orf4 and RFA proteins in lysates of infected and transfected cells. Expression of Flag-tagged PP2A in WCE was detected with anti-flag antibody and presence of reovirus protein in infected cells was detected using anti-σ3.

E4orf4 reoviruses induce cell death in most, but not all tumor cell lines tested

To test to what extent the recombinant reoviruses containing either iLOV, E4orf4 or RFA as transgenes are capable of inducing cell death in tumor cell lines, we used an assay based on cleavage of the tetrazolium salt WST-1 by living cells to quantify residual cell viability upon reovirus infection. Cells were exposed to the three recombinant reoviruses rS1-iLOV, rS1-E4orf4 and rS1-RFA, *wt*-R124, and *jin-3* mutant reovirus at an MOI of 5 and cell viability was measured 5 days post infection (dpi) (Figure 4A). In the permissive 911 cells all viruses induce cell death, with *jin-3* and *wt*-R124 reoviruses being more efficient than the recombinant viruses. The JAM-A negative glioblastoma cell line U118-MG could be very efficiently killed by *jin-3* but resisted *wt*-R124 infection. In addition, all three recombinant reoviruses induced cell

death in U118-MG cells to a similar extent. A comparable result was obtained for the chicken hepatoma cell line LMH. Chicken cells do not express a cellular receptor that can be used by mammalian reoviruses and therefore resist *wt*-R124 infection. The three recombinant reoviruses are comparably effective (10% cell survival or less) in induction of cell death in LMH cell cultures as *jin-3* (~20% cell survival). In the JAM-A negative human bladder cancer cell line UMUC-3 the recombinant reoviruses induce cell death but these cells are less sensitive to *jin-3* and fully resist *wt*-R124 infection. Of all cell lines tested here, only the JAM-A positive Pro4-Luc cells are not sensitive to infection by the recombinant reoviruses or *wt*-R124. However, these cells are efficiently infected and killed by *jin-3*.

An overview of the cell lines used with information on the JAM-A status and susceptibility to *wt*-R124 infection as well as a motivation for the cell lines used, is summarized in the supplementary data (Table S1). To confirm that reoviruses rS1-E4orf4 and rS1-RFA can enter the five cell lines, we exposed cells to rS1-RFA and rS1-E4orf4 at an MOI of 2 and checked for reovirus σ3 at 1 and 24 hpi in a western blot assay. In the cell lysates an increase in reovirus σ3 is detected 24 hpi compared to 1 hpi suggesting that indeed the viruses can enter and start to replicate in the cells. Even in the Pro4-Luc cells that resist oncolysis by both rS1-RFA and rS1-E4orf4 an increase in reovirus σ3 is evidently detected (Figure S3). In the cell lines LMH and UMUC-3 the amount of reovirus σ3 protein after 24 hours is lower than that in the other cell lines. It is possible that in LMH and UMUC-3 the replication process is slower, since 5 dpi the cells do respond to induction of cell death (Figure 4A).

Expression of the E4orf4 transgene after reovirus-mediated gene transfer into cells should not affect the viability of normal non-transformed cells. Cultures of the normal diploid skin fibroblast VH10, VH25 and control 911 cells were exposed to rS1-E4orf4, rS1-RFA, *jin-3* and *wt*-R124 at an MOI of 5. At 4 dpi, a WST-1-based cell viability assay was performed. In both fibroblast cell lines no reduction in cell viability was detected upon *jin-3*, *wt*-R124 and the two recombinant reoviruses containing E4orf4 or RFA (Figure 4B).

The results of the cell viability assays suggest that all three recombinant reoviruses tested are potent in inducing cell death in the tumor cell lines. A comparison of the mean log kill of rS1-E4orf4 to rS1-RFA or rS1-iLOV confirms that E4orf4 has no added effect on induction of cell death in the tested cell lines (Table 1). This suggests that the recombinant reoviruses with truncated σ1 protein and enhanced sialic acid binding capability are, by themselves, potent inducers of cell death in various tumor cells, while normal human diploid fibroblasts are not affected by these viruses.

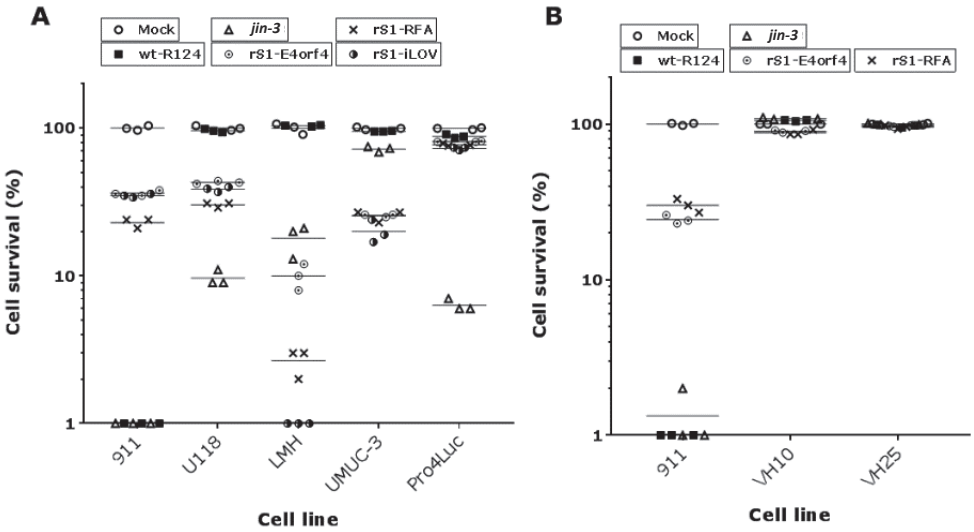

Figure 4. Cell viability assay in various tumor cell lines and normal fibroblasts after exposure to recombinant reoviruses, *jin-3*, or wt-R124. A) Cultures of various cell lines (*i.e.* 911, U118-MG, LMH, UMUC-3, and Pro4-Luc) are mock-treated or exposed in triplicate to one of the five reoviruses; wt-R124, *jin-3*, rS1-E4orf4, rS1-RFA or rS1-iLOV at an MOI of 5. WST-1 reagent was added 5 days post infection (dpi). B) Cells (911 and normal fibroblast cell lines VH10 and VH25) are mock-treated or exposed in triplicate to 4 reoviruses; wt-R124, *jin-3*, rS1-E4orf4 or rS1-RFA at an MOI of 5. WST-1 reagent was added 4 dpi. Cell survival (%) was calculated as the percentage of OD_{450} values of the infected cells over the mock-treated cells and plotted in a log scale on the Y-axis using GraphPad Prism 7.0d.

Cell line	Mean log kill			Mean log kill		
	S1-E4orf4	S1-RFA	Adjusted *P*	S1-E4orf4	S1-iLOV	Adjusted *P*
911	0.44	0.64	0.003	0.44	0.46	0.273
U118-MG	0.37	0.52	<0.001	0.37	0.41	0.047
LMH	1.01	1.58	0.005	1.01	2.00	<0.001
UMUC-3	0.59	0.59	0.964	0.59	0.70	0.124
Pro4Luc	0.09	0.11	0.028	0.09	0.14	0.007

Table 1. Mean log kill calculations belonging to Figure 4a.

Addition of E4orf4 in reovirus recombinants does not increase caspase 3/7 activity in both H1299 and 911 cells

Diverse mechanisms of cell death can be triggered by E4orf4 proteins. Several studies using the human non-small-cell lung carcinoma cell line H1299 show that transfection of a plasmid containing E4orf4 results in the induction of caspase 3/7 activity, but this activation is not required for the induction of cell death. In these cells the caspase inhibitor CrmA did not inhibit E4orf4-induced cell death [12, 14, 36]. We first evaluated the wt-R124 reovirus-induced cell death of H1299 cells, as there is a marked discrepancy in the effect of reovirus on these cells in the available literature data [26, 37].

The H1299 and 911 cells were exposed to the 5 different reovirus variants at an MOI of 30. At 5 dpi, the cell viability was measured (Figure 5A). The viability of H1299 cells exposed to wt-R124 was reduced to ~45% compared to uninfected cells. Recombinant reovirus rS1-E4orf4 and rS1-iLOV decreased viability of the exposed H1299 cells to ~60%, and rS1-RFA to ~40%. The reovirus mutant *jin-3* was the most potent inducer of cell death in H1299 cells (< 1%). In 911 cells all 5 reoviruses reduce the cell viability to ~10% or less. From these data we conclude that in H1299 cells the E4orf4 protein does not contribute to an increased oncolytic potency of the rS1-E4orf4 virus in comparison to wt-R124 reovirus. This is evident from the comparison of the mean log kill upon exposure of H1299 cells to the rS1-E4orf4 to rS1-RFA or rS1-iLOV viruses (Table 2).

In a subsequent experiment we measured the induction of caspase 3/7 activity in both H1299 and 911 cell lines upon infection with the different reoviruses (Figure 5B). In 911 cells, both wt-R124 and mutant *jin-3* strongly induced caspase 3/7 activity compared to mock-treated cells (a 12-fold and 14-fold increase, respectively). All three recombinant reoviruses (rS1-E4orf4, rS1-RFA, and rS1-iLOV) displayed only a 4-fold increase over mock-treated cells. In reovirus-infected H1299 cells induction of caspase 3/7 activity was for all five virus variants much lower; for wt-R124 there was only a very small increase compared to mock-treated cells, *jin-3* and the three recombinant reoviruses increased the caspase activity approximately 2-fold compared to mock-infected cells. Apparently, insertion of E4orf4 in reovirus did not lead to an additional increase in caspase 3/7 activity in H1299 cells. The 2-fold increase of caspase 3/7 activity in H1299 cells did not correlate to induction of cell death, since reovirus mutant *jin-3* effectively killed H1299 cells while the three recombinant reoviruses were as effective as wt-R124 in cell lysis.

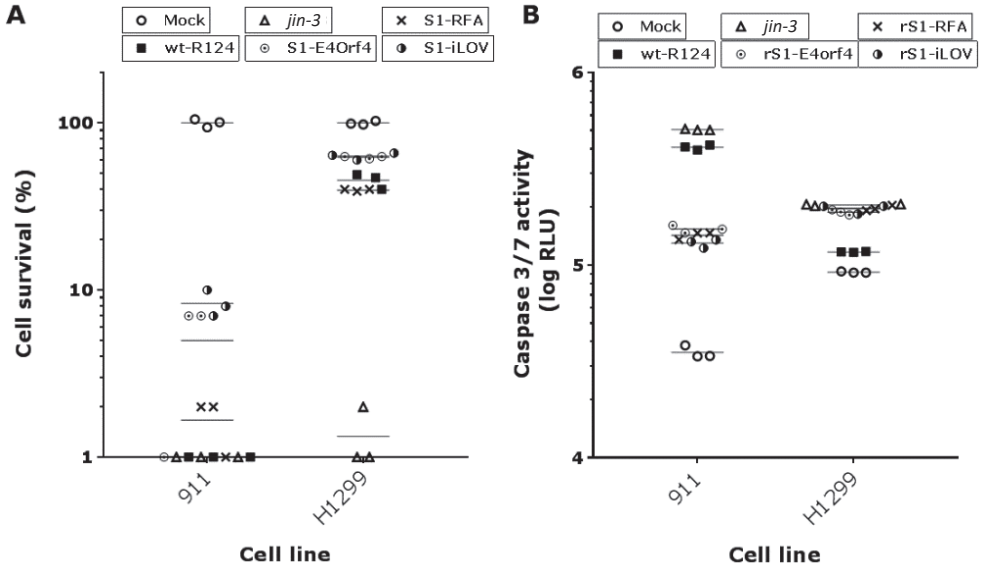

Figure 5. Caspase 3/7 activity and cell viability in H1299 and 911 cells after exposure to recombinant reoviruses, *jin-3*, or wt-R124. A) Cells (911 and H1299) were mock-treated or exposed in triplicate to five reoviruses; wt-R124, *jin-3*, rS1-E4orf4, rS1-RFA or rS1-iLOV at an MOI of 30. WST-1 reagent was added 5 days post infection. Cell survival (%) was calculated as the percentage of OD_{450} values of the infected cells over the mock-treated cells and plotted in a log scale on the Y-axis. B) Cells (911 and H1299) were mock-treated or exposed in triplicate to five reoviruses; wt-R124, *jin-3*, rS1-E4orf4, rS1-RFA or rS1-iLOV at an MOI of 30. Caspase 3/7 activity was measured 24 hours post infection. Caspase activity is plotted on the Y-axis in log relative light units (log RLU). Graphs are generated using GraphPad Prism 7.0d.

Cell line	Mean log kill			Mean log kill		
	S1-E4orf4	S1-RFA	Adjusted *P*	S1-E4orf4	S1-iLOV	Adjusted *P*
911	1.44	1.80	0.292	1.44	1.08	0.487
H1299	0.21	0.40	<0.001	0.21	0.20	0.639

Table 2. Mean log kill calculations belonging to Figure 5a.

DISCUSSION

Oncolytic virus therapy is a powerful approach for cancer treatment. Already a large number of clinical studies demonstrated the feasibility and safety of the approach [5, 38, 39]. Nevertheless, the anti-tumor response of oncolytic virus therapy needs improvement. Such enhancements may come from combining the administration of

the oncolytic viruses with immunomodulation or conventional anti-cancer treatments such as radiation or chemotherapy. In addition, the oncolytic virus may be modified to enhance tumor cell selectivity (tumor targeting), tumor cell infectivity (expanding the virus' tropism), or by including a transgene that may enhance anti-tumor efficacy. Such approaches have been extensively evaluated in a wide variety of preclinical and clinical studies involving adenoviruses [40, 41]. Here we demonstrated the feasibility of generating replication-competent, expanded-tropism reoviruses carrying a heterologous transgene for enhancing its cytolytic activity.

Previously, we demonstrated the feasibility of genetically retargeting reoviruses to an artificial receptor by inclusion of a receptor-binding ligand at the carboxyl terminus of the viral spike protein [42]. Furthermore, using bioselection we identified several *jin* mutants that were able to infect wt reovirus-resistant JAM-A deficient cells. The mutations in the *jin* viruses clustered in the region of the σ1-spike protein's shaft that is involved in sialic acid binding [11]. The enhanced-affinity sialic acid binding most probably underlies the viruses' capacity to infect cells independent of JAM-A expression.

This enhanced tropism for binding to sialic acids allows for the replacement of JAM-A interacting sequences in the head domain by heterologous sequences as we previously showed by insertion of a small reporter gene encoding the iLOV reporter that exhibits a green fluorescence protein [9]. In the study reported here we generated two new recombinant reoviruses that encode the HAdV-2 E4orf4 protein as well as the E4orf4 'RFA' mutant protein, which cannot interact with the Bα subunit of protein phosphatase 2A (PP2A). We showed that inclusion in the reovirus S1 segment of the codons for the HAdV-2 E4orf4 protein or its double mutant 'RFA', yields replication-competent recombinant reoviruses. In cell lysates of rS1-E4orf4 or rS1-RFA infected 911 and H1299 cells, HA.E4orf4 and HA.E4orf4.RFA could be detected (Figure 2B). Moreover, we confirmed that the wt E4orf4 protein can bind the Bα subunit of PP2A also in reovirus-infected cells.

The use of the rS1-RFA reovirus was based on loss of interaction between the RFA protein and PP2A. It should be noted that this is not an inert control as this protein retains the capacity to bind to Src family kinases [17]. Src kinases are tyrosine phosphatases that are involved in many cellular processes such as cell growth and differentiation. They interact with many cellular proteins including cell surface receptors [43]. Src kinase binds directly to the arginine-rich motif of E4orf4 and leads to tyrosine phosphorylation of E4orf4. This eventually results in caspase-independent induction of cytoplasmic cell death [44]. The E4orf4 mutant RFA can still bind Src kinase and cause cell death independent of binding to PP2A. Although the E4orf4 protein is mainly present in the nucleus during an adenovirus infection

and interaction with Src kinase is rarely observed, overexpression of E4orf4 in the cytoplasm, outside the context of an adenovirus infection, leads to binding of Src kinase and programmed cell death [15,16].

In rS1-E4orf4 virus infected cells, like in transfection experiments, E4orf4 is present in both the nucleus and cytoplasm (data not shown). Therefore, we also included the rS1-iLOV as an additional control in our studies. In our recombinant reoviruses only the N-terminal part of σ1 (*i.e.* the first 252 amino acids) is present. Our previous data with rS1-iLOV showed that this virus was able to induce cell death in several transformed cell lines that resisted *wt*-R124 cell killing. In another study an attenuated T3D reovirus mutant was found in persistently infected HT1080 cells, and this virus harbored a stop codon in S1 resulting in a truncated σ1 protein after the first 251 amino acids. This reovirus could still induce cell death in tumor cell lines, although with a slightly reduced efficiency. Furthermore, this reovirus was severely attenuated in SCID mice [45]. Our data demonstrate that our recombinant reoviruses with the truncated σ1 proteins are potent inducers of cell death in the tumor cell lines U118-MG, LMH and UMUC-3, but not in normal fibroblasts. The only cancer cell line of our panel that resists all three of our recombinant reoviruses is the Pro4-Luc cell line, but these cells are efficiently killed by *jin-3* (Figure 4).

In none of the cell lines tested, inclusion of transgenes encoding the E4orf4 protein or its RFA derivative did enhance the induction of cell death in infected cells compared to reovirus carrying the iLOV transgene. In many studies that evaluate the HAdV-2 E4orf4-induced cell death, H1299 cells were used as the model [12, 14, 36]. To explore if E4orf4 or RFA expression in the context of our recombinant reovirus infection could have an enhanced effect over *wt*-R124, *jin-3* or iLOV containing reovirus, we also used H1299 cells (Figure 5). However, infection with rS1-E4orf4 and rS1-RFA did not further decrease the viability of H1299 cells compared to rS1-iLOV. The most potent inducer of cell death in H1299 cells was *jin-3*. All five reovirus variants strongly reduce the viability of 911 cells after infection. We further checked whether caspase 3/7 activity was increased upon E4orf4 expression in H1299 cells. According to literature, caspase 3/7 activity was increased in H1299 cells in plasmid transfection experiments with E4orf4-containing plasmids, but addition of a caspase inhibitor did not inhibit the induction of cell death [14]. In cell lines 911 and H1299 a different caspase response was observed upon infection with the five reoviruses. Both *wt*-R124 and *jin-3* showed a great increase in caspase 3/7 activity in 911 cells but this effect is much less in H1299 cells. The three recombinant reoviruses, however, exhibit a moderate induction of caspase 3/7 in 911 cells (4-fold increase over mock). In H1299 cells the fold induction over mock is similar in cells exposed to *jin-3* and the recombinant reoviruses; approximately 2-fold. In the context of reovirus, E4orf4 does neither increase caspase 3/7 activity in infected H1299 cells

nor in 911 cells. The 2-fold induction of caspase 3/7 activity in H1299 cells did not correlate with the induction of cell death as observed in the WST-1 assay, since *jin-3,* which exerted a similar caspase induction, kills H1299 cells more effectively than the recombinant reoviruses.

In some cell types, binding of the reovirus σ1 protein to sialic acids induces a non-apoptotic cell death pathway, *i.e.* necrosis, much more efficiently than reoviruses lacking sialic acid binding capacity [27]. In other cell types, binding to sialic acids induces more potent apoptotic cell death [46]. Our finding that reoviruses with enhanced sialic acid binding capacity seem to induce pronounced cell death but with relatively limited caspase activity in 911 cells, are consistent with the results by Hiller *et al.* [27], who showed that reoviruses capable of binding to sialic acid induce a caspase-independent form of cell death. The presence of the JAM-A binding domain in σ1 of reovirus mutant *jin-3* could explain the enhanced induction of caspase 3/7 activity in 911 cells, and confirms the findings of Connolly *et al.* [46], that indicate that the JAM-A binding domain is involved in activation of NF-κB and subsequent induction of apoptosis.

For oncolytic viruses to be applicable in the clinic they need to be efficacious as well as genetically stable. While the viruses generated in this study are generally stable when propagated in the 911 production cell line, reovirus deletion mutants in batches of rS1-E4orf4 were occasionally detected. Most notably a 47-nucleotide deletion in rS1-E4orf4 batches that spans the last 24 nucleotides of the 3' part of the E4orf4 sequence, leads to relocation of the stop codon and disrupts the A-box in the S1 segment. The three boxes – A, B and C - (Figure 1) included in the transgene containing S1 sequence are thought to be important for packaging of the segment into the virions [32]. However, the described disruption of the A-box resulted in a replicating virus, indicating that the A-box is trivial for efficient viral genome packaging. The resulting E4orf4 protein in the deletion mutant is 5 amino acids shorter at the C-terminus and has two amino acid changes (valine and serine to tyrosine and glutamic acid, respectively) compared to the full-length E4orf4 protein (Figure S1). The C-terminus of E4orf4 proteins from different adenoviruses is quite variable, therefore we assume that our E4orf4 deletion mutant would not interfere with the interaction of the protein to its binding partners, PP2A and Src. In the E4orf4 proteins of the different adenovirus strains the binding domains of both PP2A and Src are highly conserved and these domains are unchanged in the deletion mutant of E4orf4 present in the rS1-E4orf4 batches (Figure S2). Remarkably, in batches with rS1-RFA reoviruses, deletion mutants were never observed upon serial propagation, even though the nucleotide substitutions are located at some distance (~ 70 nt) from the breakpoint.

In rS1-iLOV batches, also deletion mutants were detected in high passage number batches [9]. The mutations in these batches were larger and affected the function of the iLOV protein. They also located at the 3' end of the transgene sequence. In two different mutants found in rS1-iLOV batches, the nucleotides recognized as the A-box were completely deleted. In the E4orf4 deletion mutant reoviruses only 6 nucleotides of the A-box were deleted, further confirming that the A-box is dispensable for packaging of the S1 segment in reovirus particles.

In high passage number rS1-iLOV batches, no full-length S1-iLOV was detected, suggesting that the deletion mutants had a selective advantage over the iLOV-containing viruses. In the rS1-E4orf4 virus batches, even at a high passage number, the full-length S1-E4orf4 segment remained dominantly present.

It is most likely that the deletions occur at a viral RNA level and not by rearrangements of the plasmids used for reovirus generation. In an independent experiment where we used limiting dilutions after one single round of propagation on 911 cells we detected two different deletion mutants only upon continued passaging (data not shown), strongly suggesting that the deletions occurred during the replication of the reovirus RNA genomes during propagation.

No additional deletions or point mutations were found in the S1 sequence of the three recombinant reoviruses. This suggests that the point mutation underlying the *jin-3* phenotype is stable and that no additional mutations are required for effective replication of the recombinant reoviruses in the 911 helper cell line.

It remains to be established whether the recombinant reoviruses are effective *in vivo*. New variants that efficiently replicate, spread, and enhance anti-tumor immunity *in situ* may be required. It is encouraging that we can combine forward and reverse genetics approaches to generate such variants.

Since the recombinant reoviruses with truncated σ1 spikes are potent inducers of cell death by themselves, adding transgenes that encode proteins that amplify oncolysis may be obsolete. A better choice may be to use the available capacity for including foreign transgenes encoding immunostimulatory proteins or cancer vaccine peptides. This will combine the reovirus-induced cell death and possible release of tumor antigens in the tumor environment with the stimulation of an anti-cancer immune response.

SUPPLEMENTARY FIGURES

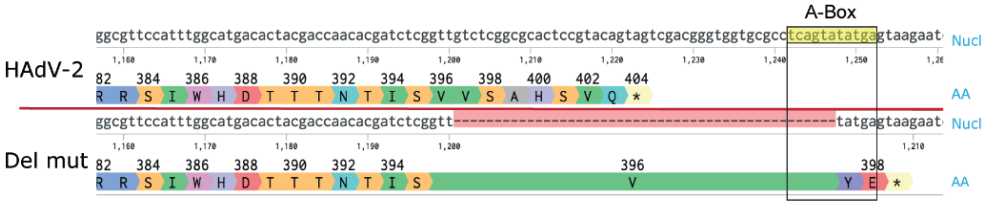

Figure S1. Partial alignment of the E4orf4 deletion mutant (Del mut) sequence with full-length E4orf4 (HAdV-2) sequence. The deletion is highlighted in pink-shaded boxes. The A-box is marked and spans nucleotides 1242-1252. The stop codons are marked with * in the yellow-shaded background. Abbreviations: Nucl - Nucleotide sequence; AA - amino acid translation. Alignments are generated with Benchling (Benchling Inc., San Francisco, USA).

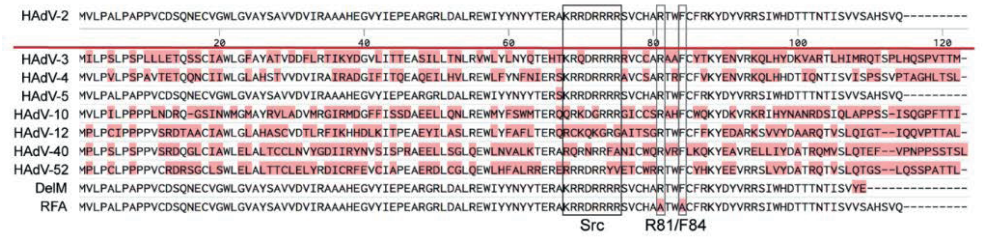

Figure S2. Alignment of E4orf4 proteins of various adenovirus types with deletion mutant E4orf4 (DelM) and the RFA protein. Mismatches are annotated with pink-shaded boxes. The conserved Src binding domain and conserved amino acids R81 and F84 are marked with black squares. Alignments are generated with Benchling (Benchling Inc., San Francisco, USA). Accession numbers used in the alignments of E4orf4 proteins are: HAdV-3 AET87291; HAdV-4 YP_068051; HAdV-5 AP_000229; HAdV-10 BAM66773; HAdV-12 CAB57853.1; HAdV-40 AMQ95251; HAdV-52 ABK35062. HAdV-2 E4orf4 is used in our recombinant reovirus and serves as the alignment template; Accession number YP_001551773.

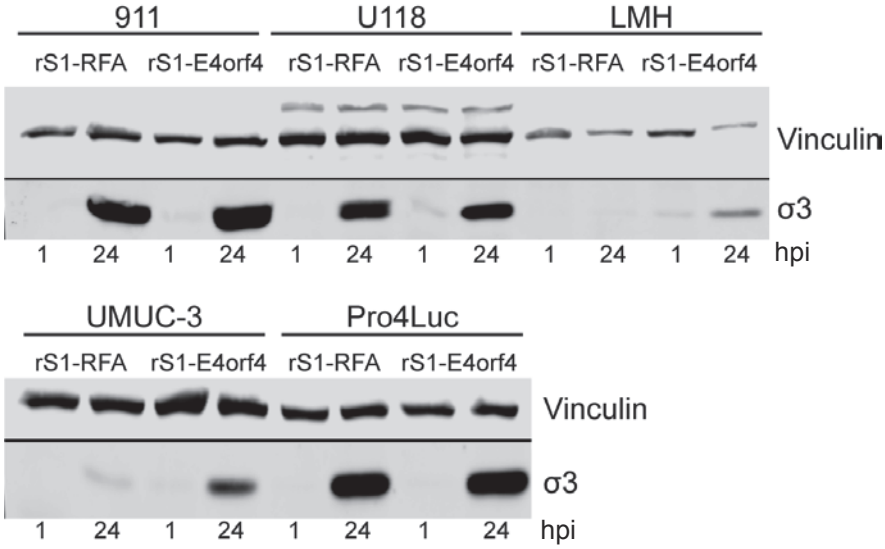

Figure S3. Indication of reovirus replication by comparing reovirus σ3 protein levels at 1 hour and 24 hours post infection (hpi). Cell lines 911, U118-MG (U118), LMH, UMUC-3, and Pro4-Luc were exposed to recombinant reoviruses rS1-RFA and rS1-E4orf4 at an MOI of 2 in 2 wells per virus for each cell line, in a 24-well plate. Giordano lysates were made 1 hour or 24 hours post infection. For the 24 hour timepoint, fresh medium was added to the cells in the well 1 hour post exposure. Equal volumes (1/4 of the total volume) of lysates were loaded into the slots of a 10% polyacrylamide-SDS gel. The proteins were transferred to Immobilon-FL. The blot was divided for staining with mouse Vinculin antibody (hVIN-1) and mouse anti-σ3 (4F2). For detection of the primary antibodies, IRDye 680RD Donkey-anti-Mouse IgG were used, prior to analyzing the signals with the Odyssey.

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