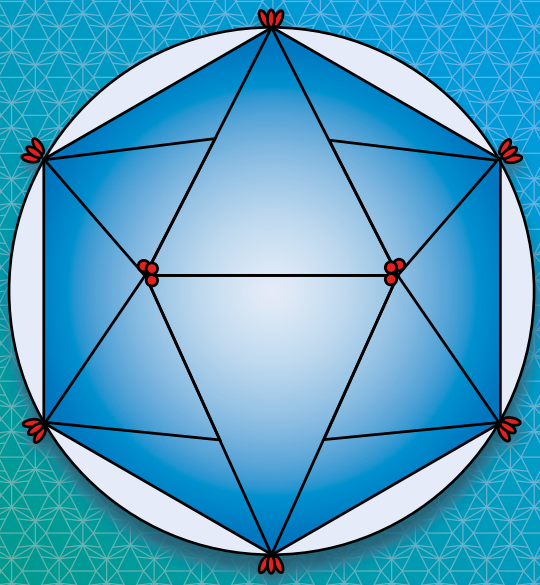

Tweaking Reovirus T3D to Boost the Oncolytic Potency

Diana van den Wollenberg

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General introduction

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1.1 General introduction

Halfway the twentieth century the first reports appeared on viruses isolated from the gastrointestinal tract of seemingly healthy persons. These viruses were grouped together under the name ECHO viruses. The term ECHO viruses being an acronym for enteric cytopathogenic human orphan viruses. Some of the ECHO viruses turned out to be less innocent and were ultimately linked to diseases. These viruses are now categorised as members of the Enterovirus genus (Picornaviridae) [1].

Other isolates in the ECHO virus group are now known as Mammalian orthoreoviruses (or shortly: reoviruses), based on their larger size. The ECHO virus type 10 is capable of inducing pathological effects in newborn mice and producing lesions in the brain, the myocardium and the liver. The Mammalian orthoreoviruses were categorised into three antigenically different serotypes by neutralisation and hemagglutinin-inhibition tests. The ECHO 10 virus became the prototype of reovirus serotype 1, "Lang" (T1L) [1, 2]. The prototype of serotype 2, "Jones" (T2J) strain was derived from a child with diarrhoea and was originally an unclassified D5 variant of echovirus type 10 [3]. The third prototype of serotype 3 was also isolated from a child with diarrhoea and was called the "Dearing" strain (T3D). This strain is commonly used in different laboratories around the world and in the work described in this thesis. In this context "wild-type" reovirus refers to the T3D reovirus strain.

In 1977 the group of Hashiro was one of the first to observe that for their replication reoviruses discriminate between tumour cells and normal diploid cells [4]. These first experiments were performed with T2J, but later it became clear that also T3D and T1L exhibit oncolytic properties. Of the three strains T3D is thought the most potent for use in anti-cancer therapies as will be explained in the next section. This section provides an introduction into the reovirus biology and its use as an oncolytic agent in the form of a review, published in the journal Expert Opinion on Biological Therapy in 2009. It explains two methods used in virology to isolate viruses with a different phenotype; viz. bioselection of viruses with desirable phenotypes or rational design of new genotypes by genetic modification. The final section of this chapter discusses the developments and insights in the reovirus field after 2009.

1.2 Modification of mammalian reoviruses for use as oncolytic agents

1.2.1 Abstract

The *Reoviridae* constitute a family of viruses with a non-enveloped icosahedral capsid and a segmented double-stranded RNA genome. Prototypes of the mammalian Orthoreoviruses have been isolated from the human respiratory and enteric tracts and are not associated with serious human disease. One of these, human reovirus type 3 Dearing (T3D), is studied frequently and usually serves as a model for the family. In the last decade the mammalian Orthoreoviruses, especially T3D, have been evaluated as an oncolytic agent in experimental cancer therapy. This is primarily based on the observation that reoviruses induce cell death and apoptosis in tumor cells, but not in healthy non-transformed cells. To-date, several clinical trials have been initiated in Canada, the United States, and the United Kingdom, to study the feasibility and safety of such an approach to cancer treatment. Due to the segmented structure of their double-stranded RNA genomes genetic modification of *Reoviridae* has been notoriously difficult. Several new techniques have been described recently that facilitate the genetic modification of reovirus genomes. The basis for reverse genetics of reovirus is the discovery in 1990 that reovirus RNA is infectious. Subsequently, it took ten years before a foreign gene was introduced into the reovirus genome. Here we will review the different methods for reovirus modification and their use for generating new reovirus-derived oncolytic agents.

1.2.2 Introduction

In the battle to fight cancer, a large variety of new therapeutic approaches are being explored. One such approach is the use of biological strategies. For this, viruses have been considered as oncolytic agents for more than a century. Clinical reports of cancer regressions that were coincidental with natural virus infections appeared throughout the first half of the twentieth century [5]. Based on these anecdotal observations, early clinical trials were performed with viral oncolytic agents [6, 7]. Often, the host immune response prevailed, but occasionally, the

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infection persisted and the cancer regressed. These studies provided proof of concept for the use of viruses as oncolytic agents [5].

Currently, many natural viral isolates, as well as their genetically-modified derivatives, are evaluated as oncolytic agents in clinical studies. These include adenovirus, herpes simplex virus, vaccinia virus, measles virus, Newcastle disease virus, and reovirus. Many of these viruses preferentially replicate in and kill tumor cell lines [5-8]. In the tumor the virus can trigger a systemic immune response against the tumor cells, potentially further boosting therapeutic efficacy [9-16]. The viruses' intrinsic selectivity for tumor cells can be further enhanced by genetic modification [17].

1.2.3 Human reoviruses

The family

The *Reoviridae* constitute a family of viruses with a non-enveloped icosahedral capsid and a segmented double-stranded RNA genome. The family name is an acronym of Respiratory Enteric Orphan viruses. The term "orphan virus" indicates that a particular virus is not associated with any known disease in humans. Prototypes of the mammalian Orthoreoviruses have been isolated from the human respiratory and enteric tracts and are not associated with serious human disease [18]. In contrast to the Orthoreoviruses rotaviruses are pathogenic and are the major etiologic agents of serious diarrhoeal illness in children throughout the world [19]. Despite the fact that some members of the *Reoviridae* family are associated with disease, the original name is still used [18]. There are three types of human Orthoreoviruses (called reoviruses here), which can be differentiated serologically. The three serotypes are each represented by prototype strains isolated from a human host: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Abney (T3A) and type 3 Dearing (T3D). Reoviruses have a wide geographic distribution, and virtually all mammals, including humans, can serve as hosts for infection [18]. While reoviruses are considered non-pathogenic in humans, newborn mice are very sensitive to reovirus infection and have been used to study reovirus pathogenesis. Following inoculation of newborn mice, both T1L and T3D invade the central nervous system, albeit with different routes and with distinct pathology. Whereas T1L spreads to the CNS hematogenously and infects ependymal cells, leading to hydrocephalus, T3D reaches the CNS by neural routes and causes lethal

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encephalitis. Studies using T1L x T3D reassortant viruses have shown that the pathways of viral spread and tropism for neural tissues segregate with the viral S1 gene, which encodes attachment protein σ1 [20].

Capsid structure

The reovirus' icosahedral capsid is composed of an outer and inner protein shell. Its genome contains 10 double-stranded RNA segments which are grouped into three categories corresponding to their size: L (large), M (medium) and S (small). Segments range from ~ 3.9 kbp – 1.2 kbp and each segment encodes one or two proteins. The viral capsid proteins are denoted by the Greek character corresponding to the segment it was translated from, the L segment codes for λ proteins, the M segment codes for μ proteins and the S segment codes for σ proteins, each protein numbered according to its relative mobility on SDS-PAGE. On the vertices, σ1 trimers of the protein form the spike. In the capsid the λ2 molecules reside as pentameric turrets that serve as the insertion site for the σ1spike [18]. A model of the reovirus capsid and its segments is provided in **Figure 1**.

Receptors

Wild-type reovirus T3D can use distinct receptors for binding to target cells. Sialic acids were the first molecules identified to bind reovirus T3D [21, 22]. Barton and co-workers described in 2001 that reovirus attachment to cells is a multi-step process [23]. In this process reovirus protein σ1 binds with the shaft domain to surface sialic acids with low affinity, before the head domain of σ1 connects to a cellular receptor with a higher affinity. The Junction Adhesion Molecule-A (JAM-A, also known as Junction Adhesion Molecule 1, or JAM-1) has been identified as the high-affinity receptor for reovirus T3D and can mediate virus attachment and infection [24, 25]. JAM-A is an integral tight junction protein of the immunoglobulin superfamily and contains two extracellular immunoglobulin-like domains and a short cytoplasmic tail. A region in the globular head of σ1 of reovirus T3D interacts with JAM-A [26].

Replication cycle

The start of a productive viral infection is docking to the target cell. The reovirus spike can establish a low-affinity interaction with sialic acids on the cell surface. Subsequently, the head domain of the spike binds JAM-A with high affinity. After engagement of JAM-A a secondary interaction is established between

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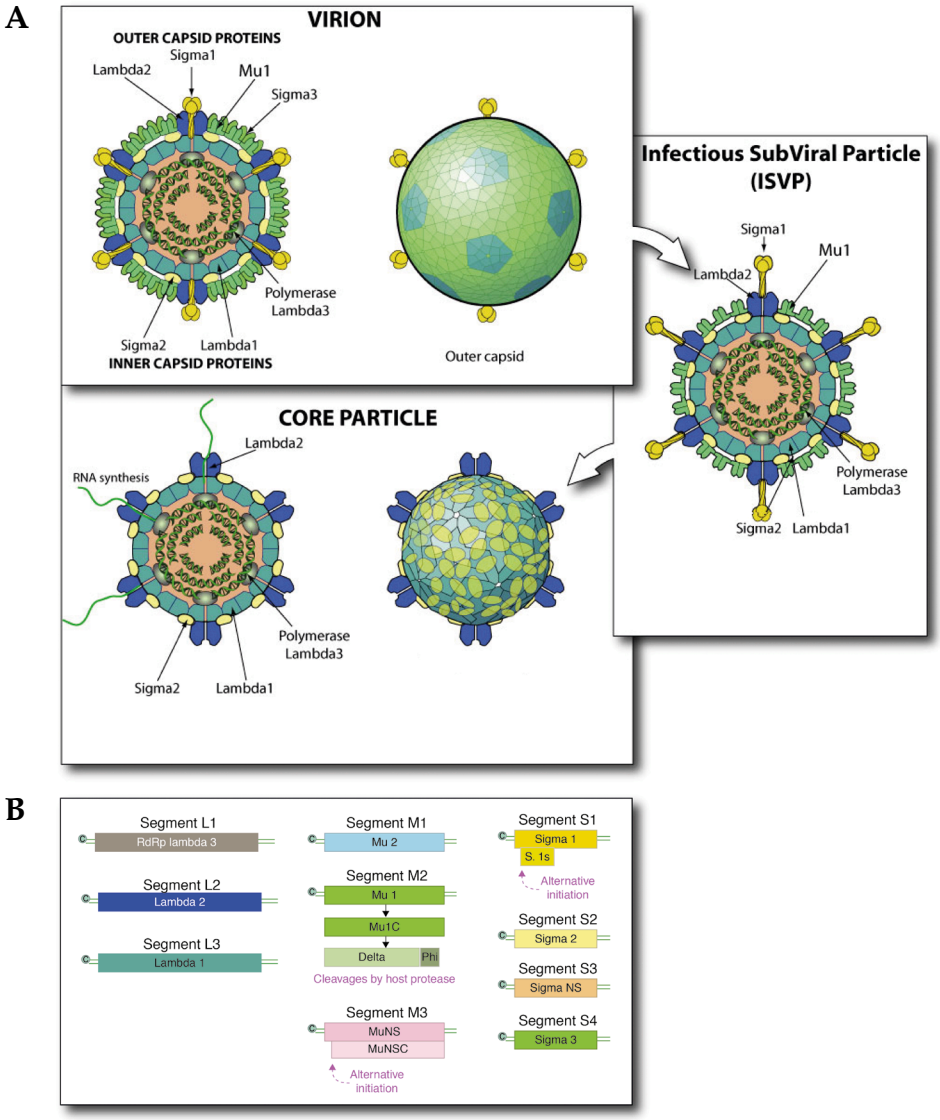

FIGURE 1.

A. Schematic representation of mammalian reovirus Type 3 Dearing. Depicted are the structure of the intact virion, the infectious sub-viral particle, and the core particle. The various capsid proteins are indicated.

B. Representation of the genomic segments of reovirus type 3 Dearing, and their encoded proteins. Pictures are courtesy of ViralZone. All pictures in ViralZone are copyright of the SIB Swiss Institute of Bioinformatics.
ViralZone www.expasy.ch/viralzone, Swiss Institute of Bioinformatics.

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cellular β1-integrins and the λ2 protein. This leads to endocytotic internalization of the particle [27]. Upon acidification of the endosome, the particles are partially uncoated, yielding so called infectious subviral particles (ISVP). In the ISVP the inner core protein μ1 is proteolytically cleaved resulting in formation of hydrophobic peptides which penetrate and locally disrupt the membrane [28]. In the cytoplasm, early transcription of the dsRNA genome by viral polymerase occurs. Full-length plus-strand transcripts from each of the dsRNA segments are synthesized, which are used as templates for translation. The translation products are concentrated in distinct cytoplasmic regions, the so called-viral factories (**Figure 2**), in which the transcripts are precisely assorted in newly formed subviral particles [22]. The packaging of the 10 genomic segments is well orchestrated since each viral core must contain a single copy of each of the plus-strand transcripts. Recent studies suggest that sequences contained within the 130 nucleotides (nt) at the 5′ terminus serve as identity label for each of the segments [29, 30]. The plus-strand RNAs contain the tetranucleotide sequence 'GCUA' at the 5′ end and the pentanucleotide sequence 'UCAUC' at the 3′ end, which may have a role in the encapsidation process [31]. In the newly formed core minus-strand synthesis ensues, producing double-stranded RNA genomes. Secondary transcription may amplify the genome copy number in the cell. The viral particles are matured and can be released after host cell membrane breakdown [32].

1.2.4 Reovirus and its relation with tumor cells

Preference for transformed cells

The human reovirus Type 3 Dearing is an interesting candidate because of its well documented inherent preference for replication in transformed cells. Reovirus type 3 was isolated from the intestinal tract from an apparently health individual. Serology demonstrates that most humans are exposed to human reoviruses before that age of 5, presumably with an asymptomatic infection [18, 33]. The impetus for the use of human reovirus T3D as an oncolytic agent has been the observation that although reovirus T3D is non-pathogenic, it preferentially lyses tumor cells, especially those with an activated Ras signaling pathway.

The human reovirus' preference for replication in transformed cells has been much studied [33]. A hallmark study was published by Patrick Lee's group which showed that activation of the Ras signaling pathway can sensitize cells to reovirus

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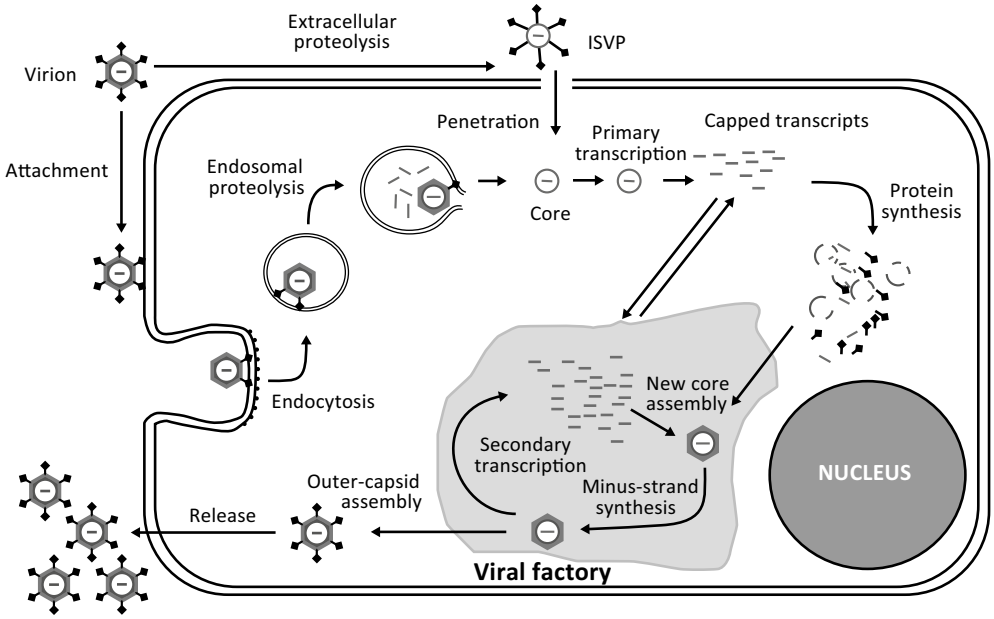

FIGURE 2.
Schematic representation of the reovirus life cycle. Both the endocytotic pathway of the intact virions, and the cytoplasmic membrane penetration routes of the infectious subviral particles are indicated (adapted from [22]).

replication [34]. This study revealed the essential role of the double-stranded-RNA-dependent protein kinase (PKR). In normal cells, the double-stranded reovirus RNA activates a PKR response and leads to PKR phosphorylation. This triggers phosphorylation of eukaryotic initiation factor 2α, which in turn, inhibits translation of mRNA. Strong and coworkers demonstrated that Ras signaling inhibits PKR phosphorylation, allowing translation of the reovirus plus-strand RNA's [34]. Further studies implicated the Ras/RalGEF/p38 signaling in the regulation of reovirus replication and cytolysis [35]. These data offered an explanation for the reovirus' preferential cytolytic effects on transformed cells. This renewed the interest in the use of reovirus as oncolytic agent. Already in 1978, Theiss, Stoner, and Kniazeff reported that infusion of reovirus type 3 into the lungs of mice reduced the progression of chemically induced pulmonary adenomas, and that multiple infusions enhanced the antitumor effect [36]. More elaborate studies confirmed the feasibility of reovirus-based cancer therapeutics

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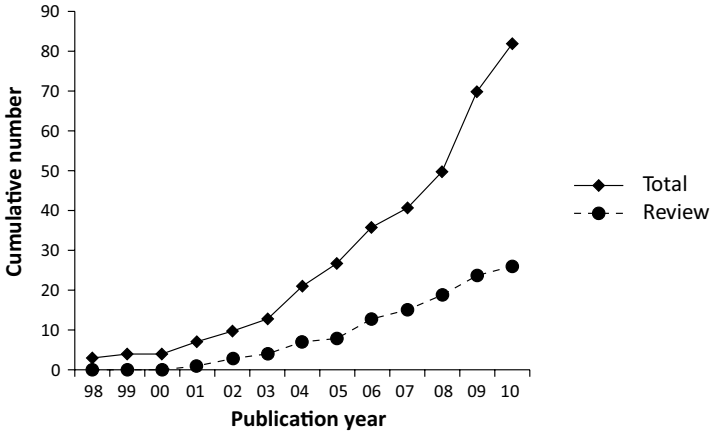

FIGURE 3.
Cumulative number of entries found in PubMed using (reovirus AND oncoly*) as search term. The Y axis represents the number of entries with a publication date before January 1st of the indicated year. The diamonds and the solid line represent the total number of publications and the circles and dashed line indicated the number of reviews on this topic. Note that approximately 30% of the publications represent review papers.
Data gathered on August 25, 2009.

[37, 38], and boosted the interest for developing reoviruses as an oncolytic agent [39], as is evident from a boost in the number of publications on this topic (**Figure 3**). In Canada, the company Oncolytics Biotech Inc. was founded, which is committed to explore the oncolytic capability of reovirus as oncolytic agent [40].

The relation between Ras signaling and reovirus replication has been much debated [41-43]. The PKR relation should not be taken too dogmatically as also other steps in the replication cycle may determine the cell's susceptibility to reovirus-mediated cytolysis. Kranenburg and collaborators demonstrated that activation of the Ras pathway sensitized the cells to reovirus-induced apoptosis, both in mouse [44] and in human tumor models [45]. However, virus yield was not affected by ras status. Reovirus T3D was strongly cytopathogenic in cells with activated Kras, but not in cells in which the activated Kras gene was inactivated. Similarly, viral uncoating is critical in determining the cells sensitivity to reovirus infection. Efficient uncoating is dependent on cellular proteases, such as cathepsin B and L. The expression of these proteases is often upregulated in tumor cells [46, 47]. Conversely, down-regulation of cathepsin L leads to reovirus resistance as

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this thwarts reovirus disassembly [48]. Also late events in the reovirus life cycle, such as the assembly of infectious viral particles, are correlated with Ras status [49]. It should be noted here, that it is probable that not the Ras-status itself, but rather the functional consequences of Ras signaling, are sensed by the reoviruses. Rubin and collaborators set up a gene-trap method to identify cellular genes involved in virus infection and cell killing. Intriguingly, the screen suggests that expression of the insulin-like growth factor II (IGF-II) gene could confer resistance to reovirus infection [50, 51]. Along similar lines the reovirus S4 gene may affect the efficiency of virus replication in Ras transformed cells [52].

Clinical studies: evidence for safety

The combination of the apathogenicity in humans and the preferential replication in, and cytolysis of, transformed cells lead to the initiation of at least 13 clinical trials in Canada, the US and the UK using human reovirus Type 3 Dearing [53-55]. So far the application of replicating wild-type reoviruses has been safe. In none of the studies reported on to date, dose-limiting toxicity has been encountered [55]. Recently, a series of reviews discussed the clinical results that have been obtained so far [53-55]. In the early-phase cancer gene therapy approaches clinical efficacy has not yet been demonstrated, although anecdotic evidence for antitumor efficacy exists. As with other oncolytic viruses several factors may limit effectiveness, including antiviral immunity, inefficient vector delivery to the tumor, limited vector spread within the tumor, and inefficient infection of tumor cells. Whereas some of these factors are inevitable, others may be circumvented by alterations to the therapeutic virus.

The reovirus receptor JAM-A is limiting transduction

While the reovirus receptor is widely expressed in several tissues, some tumor cells may have down regulated the receptors on their cell surface. For instance, Kranenburg and collaborators described that none of the 13 freshly isolated tumor fragments from patients with colorectal metastases were susceptible to reovirus T3D infection [56]. This is probably due to absence or inaccessibility of the reovirus receptor JAM-A on the tumor cells. Immunohistochemistry on tissue microarrays showed that JAM-A was expressed at very low levels in liver metastases and improperly localized in the cytoplasm of colorectal tumor cells. Remarkably tissue fragments and single-cell populations isolated from human colorectal tumor biopsies were susceptible to ISVPs, which have the capacity to directly

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penetrate the cell membrane independent of reovirus receptors [56]. Similarly, infectivity of the non-permissive human glioblastoma cell line U118MG could be restored by transfer and expression of a JAM-A cDNA clone [57]. This confirms that the receptor scarcity contribute to the resistance of tumor cells to reovirus T3D. This may be specific to particular tumor types. A recent study shows that reovirus T3D induces regression of human breast cancer primary tumor samples xenografted in immunodeficient mice. In addition, reoviruses not only eradicated the differentiated breast tumor cells, but also the cancer stem cells [58]. Interestingly, expression of the JAM-A molecule has suggested as a prognostic factor in breast cancer. JAM-A has been suggested to function as a key negative regulator of cell migration and invasion. While JAM-A is robustly expressed in normal human mammary epithelium, its expression is down-regulated in metastatic breast cancer tumors. MDA-MB-231 cells, which are highly migratory, expresses low amounts of JAM-A. Forced overexpression of JAM-A in MDA-MB-231 cells inhibited both migration and invasion through collagen gels, whereas knockdown of JAM-A using short interfering RNAs enhanced the invasiveness. These data suggest that JAM-A serves as a negative regulator of breast cancer cell invasion and possibly metastasis [59]. Similarly, recent data suggest a role for JAM1-A in progression of renal cell carcinoma [60] and endometrial carcinoma [61]. In contrast, based on patient survival studies and *in vitro* studies, McSherry and collaborators (2009) come to the opposite conclusion [62]. They suggest a correlation between increased JAM-A expression and reduced survival of breast cancer patients.

Taken together the data summarized above demonstrate that particular aspects of tumor biology, such as receptor availability, Ras signaling, cathepsin expression, antiviral immunity, etcetera, may affect the anti-tumor efficacy of reovirus-mediated oncolytic strategies. So how do we go from here, and how can we generate modified reoviruses with enhanced anti-tumor efficacy?

1.2.5 Generating improved oncolytic reoviruses

Bioselection

In virology two approaches are available to isolate viruses with distinct phenotypes. The classic approach involves bioselection for the desired phenotype, and subsequent characterization of the mutants, including the characterization of the

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genetic changes that underlie the new phenotype. In human reoviruses this method has been employed successfully, and has been used to select receptor mutants, interferon-resistant mutants, and temperature-sensitive mutants [63, 64]. Studies on such mutants provided insight in the biology of reoviruses. Starting with an isolate of T3D that could not be passaged in murine erythroleukemia (MEL) cells, Dermody and colleagues isolated mutants that were adapted to grow in MEL cells [64]. Reassortants were used to demonstrate that this trait is segregated with the S1 segment that encodes the σ1 protein. Sequence analysis mapped these mutations in part of the S1 segment that codes for amino acids 198-204 of σ1. The MEL-adapted mutants, but not the parental strain could hemagglutinate human and bovine erythrocytes. In addition, the binding of the mutants to MEL cells could be abolished by neuraminidase treatment. These data suggest that the MEL-adapted reoviruses employ sialic acid molecules as their receptor [64]. In addition, it demonstrates that receptor mutants of reoviruses can be selected for with relative ease. It should be noted however, that in these studies a phenotype was selected for that is present in many of the T3D isolates. Therefore it remains to be seen whether reoviruses with completely novel receptor specificities can be isolated with similar ease. Along similar lines Lemay and colleagues isolated T3D mutants with varying interferon sensitivity. One of the mutants had an interferon-hypersensitive phenotype. Interestingly this mutant was more dependent on Ras signaling than other viral isolates [65]. These data demonstrate that bioselection may be used to isolate reovirus mutants that are more efficient as oncolytic agent.

Rational design

In addition to the classical approach for isolating mutants, reverse genetics approaches have become feasible. For a long time the segmented dsRNA genomes of *Reoviridae* thwarted the use of genetic modification for constructing reovirus mutants. Pioneering experiments performed in the Joklik lab demonstrated that reovirus RNA is infectious. Transfection of T3D RNA (either plus strand or double-stranded RNA) into L929-cells yield T3D virus, but only if the cells were super-infected with a slow-plaqueing T2J virus as a helper [66]. This technique was used to create a T3D mutant with two temperature sensitivity mutations [67], and eventually the S2 segment was modified to carry the chloramphenicol acetyltransferase (CAT) gene [68]. This involved the *in vitro* synthesis of the heterologous S2, *in vitro* capping of this RNA, enzymatic degradation of the parental S2 segment, and co-transfection of S2-CAT RNA with single-stranded (plus-

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stranded) and/or double-stranded RNAs of the other nine segments. Although this experiment constituted a breakthrough and a single recombinant reovirus was generated, the method is inefficient and cumbersome.

Also in other members of the *Reoviridae* reverse genetics strategies have been developed. Roy and collaborators demonstrated that bluetongue virus RNA was infectious and infectious bluetongue virus (BTV) could be recovered from *in vitro*-synthesized RNA [69, 70]. BTV belongs to the genus *Orbivirus* and like mammalian reoviruses has a segmented genome consisting of 10 linear dsRNA molecules. This technique allowed replacement of individual segments with segments that were synthesized *in vitro* using synthetic T7-produced RNAs. A similar approach had been described for rotaviruses [71]. While the latter study described successful rescuing of a rotavirus with an artificial segment, the method is purportedly inefficient and needs to be improved.

An alternative approach has been used to generate genetically targeted T3D reoviruses [57]. The propagation of wild-type reoviruses on cells expressing a modified σ1-encoding segment embedded in a conventional RNA polymerase II transcript led to frequent substitution of the wild-type genome segment by the modified version. This technique allowed generating reoviruses that are genetically targeted to U118MG cells that express a single-chain Fv capable of binding a HIS-tag as an artificial receptor. The U118MG cells lack JAM-A and therefore resist infection by wild-type T3D. The targeted reoviruses were engineered to carry an HIS tag as ligand for this receptor fused at the C terminus of the σ1 spike protein. This demonstrates that the C terminus of the σ1 protein is a suitable locale for the insertion of oligopeptide ligands and that targeting of reoviruses is feasible. The genetically targeted viruses can be propagated using the receptor-modified U118MG cells as helper cells [57].

All methods described above require the use of helper viruses or viral core particles for generating infectious viruses. Recently, Kobayashi and collaborators described a new helper-free method for generating of recombinant reovirus T3D from cloned genome segments [72]. The method appears robust and productive viral infection could be established in approximately 1 in 10^5–10^6 cells transfected with plasmids encoding the ten reovirus gene segments. The method was used to engineer changes in S1 and S4. In addition, the enhanced green fluorescent (eGFP) gene was introduced into the S4 segment, demonstrating that the technology allowed the incorporation of heterologous transgenes in the reovirus genome.

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Since this virus lacks a normal S4 segment, the σ3 encoded by S4 needs to be provided *in trans*. Therefore the T3D/S4-GFP virus could only be propagated in cells genetically modified to produce the σ3 protein [72]. These data show that viruses with otherwise lethal mutations can be produced on helper cells that trans-complement the missing factor.

To summarize the data above, reoviruses are amendable both by classic genetic and by reverse-genetic approaches. With this a robust platform for generating specific cancer-targeted can be generated. This poses new questions. What characteristics of reoviruses should be modified to improve the safety and their efficacy of reovirus-derived oncolytic agents? And in what fields are new developments necessary to fulfil the promise of efficacious reovirus-derived oncolytic vectors?

Toward targeted reoviruses

In some tumors the reovirus receptor is not expressed or inaccessible. This may limit the efficacy of treatment. Amending reoviruses to use a receptor different than the canonical receptor JAM-A may enhance the efficacy. To this end both bioselection strategies, as well as genetic modification strategies may be employed. Similar to the studies with the MEL adapted virus mentioned above, reoviruses could be adapted to grow in tumor cells that lack JAM-A on their surface. Isolation of a series of mutants that were selected for replication in human glioblastoma cell lines has already been reported [73]. Sequence analysis demonstrated the presence of mutations in the S1 segment in all mutants, although the receptor used by these mutants is still enigmatic. Alternatively, the viruses may be genetically modified to encode new receptor binding ligands in one of their capsid proteins. In an elegant study, Rouault and Lemay used epitope tagging to identify regions in the σ3 protein that permit insertion of heterologous peptides [74]. The N-terminus of σ3 appears a promising location for such ligands. Similarly, the C-terminus of the σ1 was demonstrated as a feasible location, and peptide sequences inserted here could engage a single-chain Fv expressed on target cells as alternative receptor. These locations are as well attractive for insertion of small targeting peptides. Such peptides could be the integrin-binding 'RGD'-containing oligopeptides that have been inserted successfully in the fiber of the adenovirus and facilitate adenovirus-receptor independent infection of cells [75]. It remains to be determined, however, whether these sites tolerate inserting larger targeting peptides. If this proves feasible, then targeting ligands that have been successfully

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incorporated in the capsids of other non-enveloped oncolytic viruses may be incorporated in reoviruses. Such ligands could include single-chain Fv domains, single-chain T-Cell Receptors, or so-called affibodies [76].

Another class of modifications that are most useful in oncolytic reoviruses is modifications that reduce infection of non-target tissues. This could be accomplished by detargeting the virus, i.e. by preventing association with non-target cells. Such mutations should block the reovirus spike's capacity to bind JAM-A. The crystal structure of both the reovirus head domain of the σ1 spike and of the extracellular domain of the JAM-A have been reported [77, 78]. More recently also the σ1 spike in complex with JAM-A has been reported [79]. Based on this structure, amino acids in the spike that are critical for ligation to JAM-A were identified. The system of Kobayashi and collaborates [72] was used to generate mutant reoviruses harboring single amino acid changes in critical amino acids. Several of these mutants, *i.e.* T380A, G381A and D423A, exhibited strongly reduced binding to JAM-A in surface plasma resonance analyses, as well as to neuraminidase-treated HeLa cells [79]. Interestingly, these mutants were viable and could be propagated on L929 cells, infecting the cells presumably via direct association with sialic acid residues. These mutants can facilitate the development of oncolytic reoviruses with reduced infectivity of normal tissues. However, since no significant toxicity has been noted in the clinical studies reported on to date, it is not yet clear reoviruses de-targeting will be necessary.

1.2.6 Where do we go from here?

With these tools and techniques available and with evidence of excellent safety of the wild-type reoviruses in clinical studies we may further define key factors that may limit clinical efficacy. In what fields are new developments necessary to ful-fill the promise of efficacious cancer targeted reovirus vectors?

Cells for delivery

Many preclinical and clinical studies demonstrated that delivery of viral vectors to the tumor is often disappointingly inefficient. As outlined above, the expression of the reovirus receptor JAM-A may be low, or the receptor proteins are inaccessible to the virus particles. In addition, the high interstitial fluid pressure within most tumors causes a convective flow away from the tumor. This

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inhibits the passive diffusion of viral particles into the tumor. Moreover, the extracellular matrix may form physical barriers that prevent efficient spread of viral vectors [80, 81].

Given the inefficiency of virus delivery to tumor cells, new delivery methods are essential. An attractive option is to use cells with the capacity to migrate to umors as delivery vehicles of oncolytic viruses. In this strategy the tumor targeting cells are loaded with viruses and administered to the patient. After migration, the cells should hand off the cell-associated viruses to the tumor cells. Several cell types can migrate to tumors *in vivo*, including cytokine induced-killer cells, tumor-antigen specific T cells, macrophages, endothelial progenitor cells, and mesenchymal stem cells [82].

Cellular delivery may also circumvent the effects of neutralizing immunity [12, 83]. This is evidenced in a recent study that employed human reovirus T3D as oncolytic agent. In reovirus-naïve mice with B16tk lymph node melanoma metastases, free reovirus or reovirus delivered through mature Dendritic Cells (DC) or T cells was detected in the tumor-draining lymph nodes 3 days after treatment. Here, these viruses could eradicate local tumor cells. Only T cells carrying reovirus generated anti-tumor immune responses and long-term tumor clearance. In reovirus-immune mice, however, the results were different. Delivery of free reovirus was ineffective as a therapy, but if mature DC as well as T cells were used as carriers, reovirus was effectively delivered to melanoma *in vivo* [12]. These data show that whereas systemically administered reoviruses may not be suitable for therapy, DC may be an appropriate vehicle for delivery of significant amounts of reovirus to tumors. This and several other studies suggest that cellular delivery is feasible and may be applicable for delivery of viral oncolytic agents at the tumor sites. However, cellular delivery also adds a new level of complexity to the clinical studies, and are logistically challenging.

What cells to target?

With the aid of genetic modification and bioselection strategies new reovirus-derived cancer gene therapeutics can be developed that ensure efficient infection of cells within the tumor. It is known that tumors are usually markedly heterogeneous, consisting of complex mixtures of cancer cells with various grades of differentiation. A cell type that has attracted much attention in recent years is the tumor-initiating cell, or cancer stem cell. The tumor-initiating cell is a cell with the capacity to self-renew and differentiate into any of the lineages of cancer cells that

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comprise a tumor [84]. Presumably the tumor-initiating cells are derived from organ stem cells [85]. The latter are long-lived and change over the course of time by accumulating (epi)genetic alterations. In this model, the characteristics of cancer initiating cells and therefore of the resulting tumor, depend (in part) on these alterations. The cancer stem cell population in a tumor may not only govern tumor progression, but may also be responsible for the establishment of distant clones (metastases) [84, 86]. Therapeutic efficacy may therefore be directly related to the efficiency with which a particular treatment eradicates the cancer stem cell population. It may therefore be important to ensure that new reoviral oncolytic agents have the capacity to transduce and kill cancer stem cells [58]. It may even be desirable to generate reovirus variants that specifically target the cancer stem cell population, rather than the more differentiated tumor cells.

1.2.7 Conclusion

Recent years have witnessed a re-emergence of the oncolytic virus approach to cancer treatment. Initial clinical safety studies have demonstrated the validity of the concept, and feasibility of current approaches. The reovirus T3D used in the clinical studies reported on so far are well tolerated and safe. In the past years the knowledge on mammalian reoviruses, their biology and their interaction with the host have expanded rapidly. Whereas genetic modification of reoviruses' segmented double-stranded RNA genomes was notoriously difficult, new techniques facilitate the development of genetically modified variants. These techniques can be used to generate new tumor targeted oncolytic reoviruses that will hopefully be as safe as current ones, but more efficacious.

1.2.8 Expert opinion

Reovirus-based oncolytic virus therapies have been safe, but so far the efficacy is limited. As with other oncolytic viruses the efficacy may be enhanced by combining the oncolytic virus approach with more conventional therapeutic approaches such as radiation and chemotherapy [87, 88]. This concept is now being explored clinically [89].

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In parallel, the new techniques for reverse genetics together with more classical genetic approaches can be used to overcome some of the hurdles that thwart efficacious oncolytic virus therapy. Several factors limit the efficacy of treatment with oncolytic viruses. These include the inefficient delivery of oncolytic viruses to the tumors, the limited infectivity of the cancer cells within the tumors, pre-existing immunity in the patient population, and the marked heterogeneity of many tumor types. With the new (reverse-) genetics techniques, new tumor-targeted oncolytic reoviruses can be generated with improved efficacy and applicability. Hereby, the gene therapist can be guided by our knowledge in parallel fields. In this respect, the large body of knowledge on oncolytic adenoviruses may be extremely valuable. Despite the differences in their genomic constitution, adenoviruses and reoviruses have several aspects in common. Although reovirus T3D and human adenovirus-C (HAdV-C) recognize different receptors, there are overt structural similarities between the T3D spike and the adenovirus fiber [77, 90]. Hence for developing tumor-targeted reoviruses it may be useful to parallel the approaches that have been explored so successfully in the oncolytic adenovirus field [76, 91, 92].

Transient immune suppression may overcome immunological barriers that impede the effective reovirus delivery [93, 94], although it should be realized that this approach should be taken with caution. Reovirus infection can be associated with severely morbidity in immune compromised NOD/SCID mice [95]. Interestingly, it may be possible to select mutants with significantly reduced viral pathogenesis in immune compromised animals [96]. On the other hand, the immune system is an important mediator of anti-tumor activity, and the oncolytic virus infection may activate antitumor immune activity [15, 97].

The reverse genetic techniques can be used to generate reoviruses carrying heterologous genes [68, 72]. Transgenes may be included to arm the oncolytic viruses, for instance by including transgenes encoding cytokines for activating the immune system. This may increase the anti-tumor efficacy [17]. Also the replication of reoviruses may be reduced by insertion of microRNA target sequences, similar to the method used to prevent replication of adenoviruses in hepatocytes [98, 99]. Already, Dermody and colleagues demonstrated that RNA interference can be used to inhibit reovirus replication [100]. However, it remains to be established whether reoviruses carrying foreign sequences (i.e. transgenes, microRNA targets) are genetically sufficiently stable to allow production of clinical grade batches.

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In cases where the use of mutants may be ineffective, other changes to the virus particles could be used. One such hurdle is the occurrence of reovirus immunity in the human population. While it is unlikely that mutants can be isolated which are insensitive to preexisting immunity, polymer shielding may be used to mask the epitopes in the capsid. This approach has been found extremely effective to protect adenovirus vectors, and it seems reasonable to anticipate that this strategy will also be effective in reoviruses [101].

Despite the new opportunities there is one factor that may potentially limit use of genetically targeted reoviruses. Reoviruses display a high mutation rate compared to many DNA viruses. As a result, during prolonged passage reovirus mutants have a tendency to change or revert, forming so-called quasispecies. This genetic drift may be reduced by propagating the viruses under appropriate selective pressure. For large-scale production of reoviruses for clinical use, product consistency is important. Therefore, it is essential to design culture conditions that limit the expansion of mutants or revertants in the vector preparation. It remains to be seen whether that is feasible for novel tumor-targeted oncolytic reoviruses with new phenotypes. It is anticipated that the development of new production systems may be necessary, and should be developed in parallel with appropriate pre-clinical testing methods. This can warrant safe clinical application of new oncolytic reoviruses. With these we can keep the gene therapists' promise of providing new and effective treatment for malignant neoplastic disease.

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1.3 Update on reovirus modifications

Bioselection

The reovirus receptor JAM-A is thought to play an important role in the entry of reoviruses into tumour cells. However, a few years ago we discovered that this is mainly true for tumour cells in monolayer cultures (see **Chapter 3**). In a 3D spheroid model, the absence of JAM-A does not seem to influence the entry of wild-type reoviruses in cells that lack the receptor, which may have important consequences for the *in vivo* situation at a tumour site.

Although the JAM-A receptor may not be as important as expected, *in vitro* reovirus mutants have now been isolated that can enter independently of the canonical receptor in monolayers of cells that resist wild-type T3D reoviruses (see **Chapter 2**) These mutants are called Junction Adhesion Molecule-A Independent (*jin*) mutants.

Besides the entry route of the reovirus other factors involved in the replication cycle of reoviruses play a role in the sensitivity of cells to reovirus infection and cell death. Some tumour types are corrupted in their cell death pathways and are therefore difficult to defeat with chemotherapy. This may also have implications for the sensitivity of cells to reovirus cytotoxicity. Several cell lines derived from squamous carcinomas of the head and neck resist reovirus induced apoptosis mediated cell death [102]. The relationship between sensitivity to reovirus infection, replication, and induced cell death has not yet been fully resolved and seems quite complex. A tool that could assist in this quest is to use the ability of the reovirus to adapt to its environment. Upon forced entry and patience by the scientist mutants with more potent cytotoxicity can be selected from the wild-type reovirus-resistant cell types, as is done for the *jin* mutants. These viruses can provide new insights in the mechanisms involved in the different types of cell death (apoptosis, autophagy, necrosis; for a mini review on cell death, see [103]). They can also be evaluated for other tumour types with disrupted death pathways.

A different approach to select for reovirus variants was done in the lab of Patrick Lee [104]. They infected reovirus sensitive cells with all three reovirus serotypes (T1L, T2J and T3D) simultaneously and isolated two variants with larger plaques than T3D as a measure for improved infectivity. The two isolated reovirus variants turned out to be only of T3D origin, with additional mutations in the S1 (T3v2) or L1, L2 and L3 segments (T3v1). In the permissive cancer cells both variants produced larger plaques compared to the wild-type T3D. In the B16 melanoma mouse model the two variants show also superior oncologic activities over the wild-type T3D virus. The specificity for cancer cells *in vitro* remained and *in vivo* no host toxicity was observed.

Genetic modification

The availability of the plasmid-based reverse genetics system [105] inspires reovirus scientists to discover more on reovirus biology and to improve the reovirus efficacy in oncolytic cancer therapies. Most of the reports that appeared after the introduction of the system belong to the first category and resolve issues where reovirus segments are involved in apoptosis, assembly or disassembly, cell tropism and pathogenesis in mice [106-117].

Only a few reports deal with the addition of exogenous polypeptides added to gene segments. In 2011 Brochu-Lafontaine *et al*. [118] published about adding heterologous polypeptides to σ1, as we did with our first approach to modify σ1 with the addition of a His(6)-tag and the need to use a helper reovirus to generate the S1His reovirus (see **Chapter 4**). They showed that addition of a sequence of 750 nucleotides at the C-terminus of σ1 was not tolerated and managed to add a sequence of about 40 nucleotides to σ1.

Demidenko *et al.* added longer tandem repeats and used a tetra virus 2A element for exogenous polypeptide expression in three other segments (S3,M1 and L1). The total length of heterologous sequences they added in the reovirus genome was 1500 nucleotides divided over two segments (L1 and M2). This technique could be useful in the vaccine development [119], also regarding the simultaneous expression of peptides on different segments. They only showed that the modified reoviruses created were genetically stable for three passages in L929 cells. It remains to be seen what will happen if the viruses are propagated for additional rounds on L929 cells.

As explained in section 1.2 there are reports on the expression of exogenous proteins in reovirus segments, the techniques replaced the ORF of the segments and required that the viruses were propagated on cell lines that complement for the missing reovirus proteins [105, 120]. Slowly more becomes known about the different signals that are important for the replication of the segments and the virus. However, so far no one has found a location for inserting exogenous genes without losing the fitness of the virus. In **Chapter 5** we describe the expression of a small fluorescent protein at the location of the JAM-A binding region in σ1 as a proof for autonomously replicating genetic modified reoviruses. Based on this location it is possible to add small genes that express proteins with oncolytic properties or immune stimulating factors.

1.4 Aims and outline of this thesis

Aims

Exploring manipulation of reovirus T3D to enhance its oncolytic properties by:

1. *a bioselection procedure*. We aim at bypassing the Junction Adhesion Molecule-A (JAM-A) as a receptor for entry into JAM-A deficient glioblastoma cells. This would allow us to expand the tropism of reoviruses to cells that are difficult to infect with wild-type reovirus T3D due to a scarcity of JAM-A reovirus receptors.
2. *a genetic modification strategy*. We aim at evaluating the S1 segment as a location for inserting small foreign genes in the dsRNA genome of reovirus T3D.

Outline

Chapter 2 describes the isolation of reovirus mutants through bioselection in cells that lack the canonical reovirus receptor, Junction Adhesion Molecule-A (JAM-A). All three so-called *jin* mutants harbour mutations in the S1 segment resulting in amino acid alterations in the tail part of the S1-encoded attachment protein σ1. Besides the U118MG glioblastoma cells the *jin* mutants can infect other cells that resist *wt* reovirus T3D infection, including chicken and hamster cells, but not primary human fibroblasts. This expanded tropism makes the *jin* mutants useful as oncolytic agents in tumours that are difficult to infect by the *wt*T3D reovirus, as is shown with U2OS (osteosarcoma cell line) and STA-ET2.1 (Ewing sarcoma) cells.

JAM-A may not be the only important player in the context of tumours, as is shown in **Chapter 3**, where *wt*T3D reovirus is capable of infecting 3D-cultures of U118MG cells, independent of this receptor. Cells in spheroid cultures secrete factors (among others the extracellular proteases cathapsin B and L) that can convert reovirus particles into intermediate subviral particles (ISVPs). ISVPs are normally formed during the endocytosis process, but if generated outside cells (for example in the intestines by proteases present in the environment) ISVPs infect cells independently of the JAM-A receptor. In the microenvironment of tumours there is evidence for the presence of extracellular proteases and this should be taken into account when using reoviruses as oncolytic agents, since these proteases can enhance the oncolytic effectivity.

Genetic modification of reovirus T3D is the topic of **Chapters 4** and **5**. **Chapter 4** describes the first attempt to alter the S1-segment resulting in a σ1 protein with a His(6)-tag attached to the C-terminus. In this method, 911 cells transduced with the modified S1-His segment in the context of a RNA-polymerase II transcript and CMV-promoter are infected by wild-type reovirus. A subsequent selection step in

U118MG cells expressing an artificial receptor binding the His(6) tag eliminates the wild-type virus, which cannot enter the JAM-A negative U118MG cells from the modified reovirus population. A disadvantage of this system is the need for wild-type reovirus as a helper with the possibility to generate different mutants and losing the desired modified reovirus.

Another method for genetic modification of reovirus, omitting wild-type reovirus as a helper virus is described in **Chapter 5**. Based on the data with the *jin* mutants, the S1-sequence encoding the head domain of σ1 is replaced by a transgene encoding a small fluorescent protein, called iLOV. To employ the expanded tropism, the mutation found in S1 of *jin-3* is introduced into the sequence encoding the tail part of the σ1 spike. The truncated σ1 part is fused to a His(6)-tag and the iLOV protein is placed behind a porcine teschovirus-1 2A element to avoid incorporation of iLOV into the virus capsid. This recombinant S1His-2A-iLOV segment is placed in an expression plasmid under the control of a T7-promoter and a HDV ribozyme at the 3′ end to generate a genuine 3′-UTR. The modified reovirus plasmid is co-transfected with expression plasmids containing the other 9 reovirus segments into mammalian cells that stably express T7 RNA Polymerase. The rS1His-2A-iLOV virus proved viable and replication-competent despite the loss of its "head". Previously, foreign genes were inserted in the ORFs of one segment and this system requires helper cells for the trans-complementation of the deleted functions. Here, the S1 segment has been shown to be a feasible candidate location for the insertion of small genes. This strategy is currently being explored for the development of oncolytic reoviruses with improved antitumor activity.

A general discussion is provided in the final chapter, **Chapter 6**.

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Chapter 2

Isolation of reovirus T3D mutants capable of infecting human tumor cells independent of Junction Adhesion Molecule-A

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Contents chapter 2

2.1 Abstract

Mammalian Reovirus is a double-stranded RNA virus with a distinctive preference to replicate in and lyse transformed cells. On that account, Reovirus type 3 Dearing (T3D) is clinically evaluated as oncolytic agent. The therapeutic efficacy of this approach depends in part on the accessibility of the reovirus receptor Junction Adhesion Molecule-A (JAM-A) on the target cells. Here, we describe the isolation and characterization of reovirus T3D mutants that can infect human tumor cells independent of JAM-A. The JAM-A-independent (*jin*) mutants were isolated on human U118MG glioblastoma cells, which do not express JAM-A. All *jin* mutants harbour mutations in the S1 segments close to the region that encodes the sialic acid-binding pocket in the shaft of the spike protein. In addition, two of the *jin* mutants encode spike proteins with a Q336R substitution in their head domain. The *jin* mutants can productively infect a wide range of cell lines that resist *wt* reovirus T3D infection, including chicken LMH cells, hamster CHO cells, murine endothelioma cells, human U2OS and STA-ET2.1 cells, but not primary human fibroblasts. The *jin*-mutants rely on the presence of sialic-acid residues on the cell surface for productive infection, as is evident from wheat germ agglutinin (WGA) inhibition experiments, and from the *jin*-reovirus resistance of CHO-Lec2 cells, which have a deficiency of sialic-acids on their glycoproteins. The *jin* mutants may be useful as oncolytic agents for use in tumors in which JAM-A is absent or inaccessible.

2.2 Introduction

The *Reoviridae* constitute a family of viruses with a non-enveloped icosahedral capsid and a segmented double-stranded RNA genome. Prototypes of the mammalian Orthoreoviruses were isolated from the human respiratory and enteric tracts and have not been associated with serious human disease. The human reovirus type 3 Dearing (T3D) is frequently studied and often serves as a model for the family. The reoviruses have a lytic replication cycle and preferentially induce cell death and apoptosis in tumor cells but not in diploid, non-transformed cells [1-3]. In transformed cells reovirus uncoating and replication are stimulated [4-8]. In addition, Ras signalling sensitizes the cells to reovirus-induced apoptosis [9]. Based on these observations, reovirus T3D is a promising candidate for use as oncolytic agent, and is currently evaluated in a variety of clinical cancer therapy trials [10-13].

Reovirus attachment to cells is a multi-step process. The reovirus spike protein σ1 binds with a region of its shaft domain to cell surface-bound sialic acids with low-affinity, before the head domain of σ1 engages the high affinity receptor Junction Adhesion Molecule-A (JAM-A, also known as JAM-1) [14, 15]. Following receptor binding, virions become internalized by a mechanism involving the capsid protein λ2 binding to β1 integrins [16, 17]. An alternative entry pathway can be employed upon proteolytic removal of the reovirus outer capsid protein σ3 and cleavage of μ1/μ1C, yielding intermediate (or infectious) subviral particles (ISVPs). The ISVPs can directly penetrate the cellular membrane independent of the presence of JAM-A [18, 19]. The ISVPs are similar to the disassembly intermediates formed during cellular entry via the endocytosis pathway.

The reovirus receptor JAM-A is expressed in epithelial and endothelial cells of several tissues including lung, kidney, pancreas, heart, brain, intestine and lymph nodes [20] but some tumor cells have down-regulated the JAM-A receptors on their cell surface, thereby limiting the susceptibility to reovirus T3D. JAM-A expression was found significantly down-regulated in clear-cell renal carcinoma cells [21]. Also, cells grown from freshly isolated colorectal tumor metastases resist reovirus infection. Immunohistochemistry demonstrated that JAM-A is not accessible at the cell surface, although JAM-A is detectable intra-cellularly [22]. Furthermore, there is an inverse correlation of JAM-A expression in breast cancer cells and their ability to migrate. JAM-A is expressed in normal human mammary epithelial cells but in metastatic breast cancer tumors the expression is down-regulated [23].

Here we describe the isolation and characterization of reovirus T3D mutants that are adapted to propagation in JAM-A negative, reovirus-T3D resistant cell lines. The first was identified as a spontaneously occurring mutant in one of our batches genetically retargeted reovirus [24]. Subsequently two other mutants were isolated by selection on JAM-A negative human glioblastoma cells. We demonstrate that these JAM-A-independent (*jin*) mutants employ an as yet unidentified, but apparently ubiquitous receptor, which is present on a wide variety of cell types. Their potential use as novel oncolytic tools against tumor cells in which JAM-A is absent or inaccessible is discussed.

2.3 Results

Isolation of a JAM-A independent reovirus mutant

Previously we described a system for generating genetically modified reoviruses. The modification strategy relies on the exchange of a genome segment encoding the spike protein σ1 by a segment encoding a his-tagged spike. The

modified viruses can be selected and propagated on U118scFvHis cells. This cell line is a derivative of the JAM-A negative human glioblastoma cell line U118MG and expresses a single-chain Fv (scFv) on its surface that is capable of binding the His-tag. The scFv serves as an artificial receptor for the σ1-His containing viruses [24].

In one of the batches of σ1-His modified reoviruses, we noted that a cytopathic effect (CPE) was not only induced in the U118scFvHis cells, but also in the parental U118MG cells. This suggested that this batch contains viruses that are capable of infecting cells independent of the presence of JAM-A and independent of the artificial scFv-His receptor.

The first mutant virus isolated, which was called *jin-1* (JAM-A independent), was further propagated on U118MG cells. The *jin-1* mutant virus was compared to our lab reference *wt*T3D reovirus. In contrast to the *wt*T3D reovirus, the *jin-1* virus induces CPE in U118MG cells as is evident from a WST-1 cell viability assay (**Figure 1A**). Both viruses are equally cytolytic to 911 cells which do contain JAM-A [24]. Immunofluorescence assays using an antibody against the major capsid protein σ3 confirmed the presence of σ3 in U118MG cells infected with *jin-1*, but not with *wt*T3D (**Figure 1B**). To further verify that U118MG cells support the replication of the *jin-1* mutant, a metabolic labelling with [^{35}S]-Methionine was performed. As a positive control the U118-HAJAM cell line was included. This U118MG-derived cell line had been transduced with a lentivirus to overexpress an HA-tagged version of the JAM-A receptor. In U118-HAJAM and in 911 cells, exposure to the *wt*T3D as well as to *jin-1* reoviruses established infection as is evident from the synthesis of reovirus proteins. In contrast, in U118MG cells the reoviral protein synthesis was only detectable upon infection with the *jin-1* virus but not with our *wt*T3D (**Figure 1C**). These data demonstrate that the *jin-1* reovirus, in contrast to *wt*T3D, is capable of infecting and replicating in the JAM-A negative cell line U118MG.

Sequence analysis of the reovirus mutants

The *jin-1* mutant originated from the U118scFvHis cell line. Since this mutant can infect JAM-A negative U118MG cells, we speculated that the attachment protein σ1 was altered. After one round of plaque purification and further propagation for eleven passages on U118MG cells, the complete genome was sequenced. The primers used (for this) are listed in the supplementary table S1. The PCR products were purified and used for sequence analysis. In the S1 segment two mutations occurred. The mutation led to a threonine-to-methionine change at position 193 (T193M) and a glutamine-to-arginine change at position 336 of the protein (Q336R). Also in other segments mutations were found (**Table 1**).

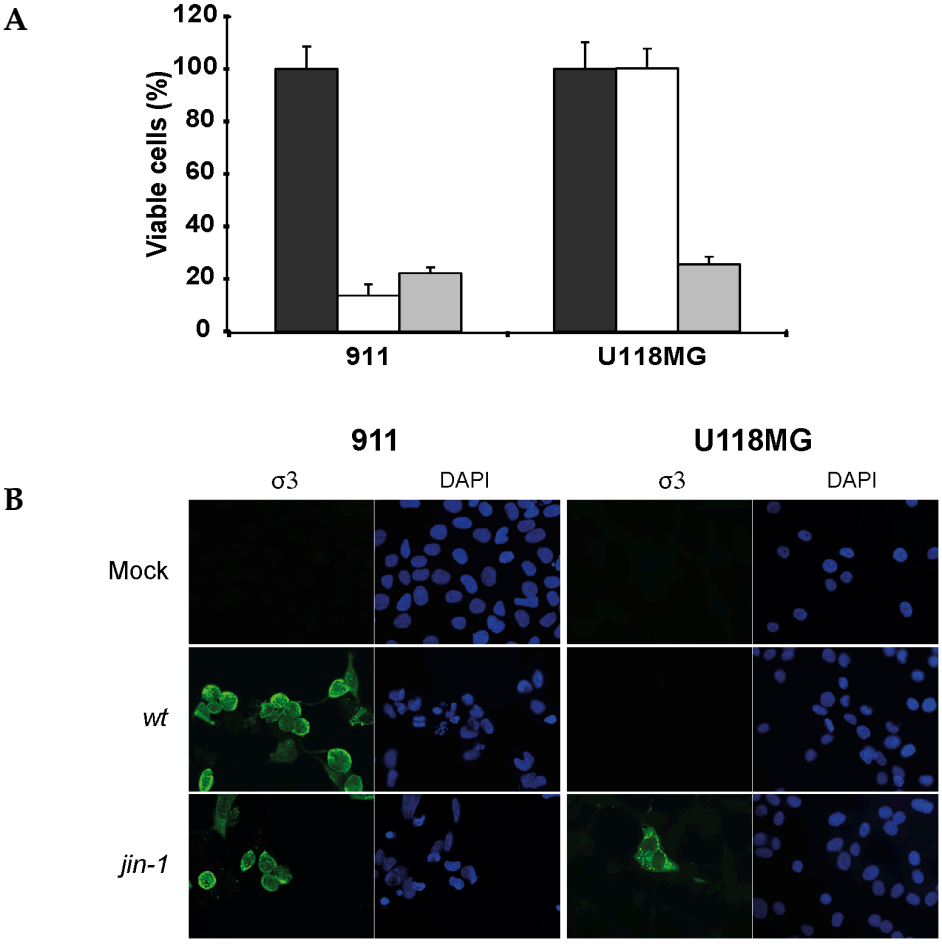


FIGURE 1.
Reovirus mutant *jin-1* is able to infect the JAM-A negative cell line U118MG.
A. Viability assay (WST-1) on 911 and U118MG cells. Cells were mock infected (black bar) or infected with *wt*T3D (white bar) or *jin-1* virus (grey bar) with an MOI of 10, six days post infection. Means (±standard deviation) from three wells.
B. Detection of outer capsid protein σ3 in 911 and U118MG cells after addition of *wt*T3D or *jin-1* virus with an MOI of 5. 40 hr post infection cells were stained with a monoclonal antibody directed against σ3 (4F2) and visualised with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse secondary antibody. The nuclei are visualised with 4′,6-diamidino-2-phenylindole (DAPI).

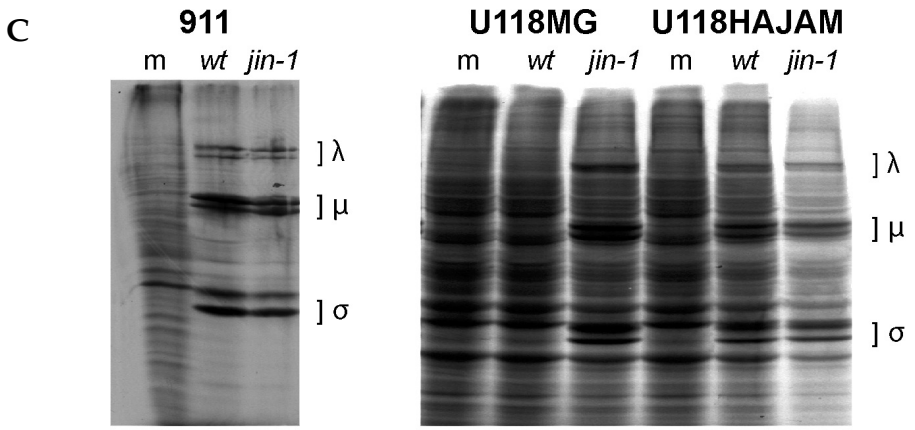
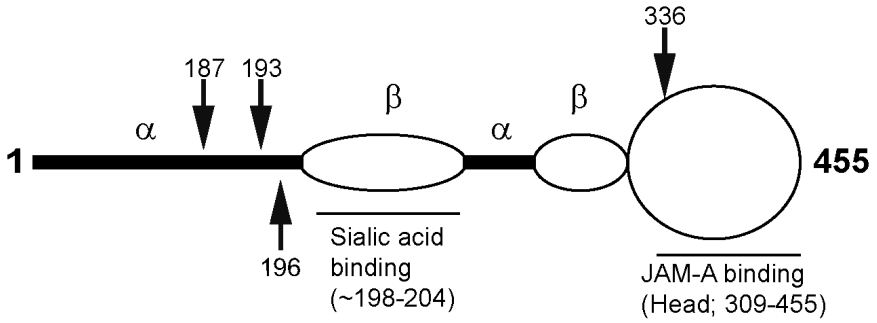


FIGURE 1.
Reovirus mutant *jin-1* is able to infect the JAM-A negative cell line U118MG.
C. Assessment of reoviral protein synthesis in *jin-1* or *wt*T3D infected cells. Indicated cells were infected with *wt*T3D or *jin-1* virus and labeled with [^{35}S]-methionine once CPE became apparent. 911 cells were infected with an MOI of 1 and U118MG or U118HAJAM cells with MOI of 5. Indicated are the positions of the reoviral λ, μ and σ proteins. m represents mock infected cells; *wt*: *wt*T3D infected cells and *jin-1*: *jin-1* infected cells.

TABLE 1.
Amino acid differences in reovirus proteins of the jin mutants and the *wt*T3D reovirus strain.

RNA segment (protein)	AA position	*wt*	*jin-1*	*jin-2*	*jin-3*
S1 (σ1)	187	Gly		Arg	
	193	Thr	Met		
	196	Gly			Arg
	336	Gln	Arg	Arg	
S2 (σ2)	254	Ser	Phe		
S3 (σNS)			No changes		
S4 (σ3)	177	Ser	Phe		
	198	Gly	Glu		
	357	Met	Thr		
M1 (μ2)			No changes		
M2 (μ1)	388	Lys	Arg		
	530	Thr	Ala		
M3 (μNS)	705	Ala	Val		
	706	Asp	Ala		
	708	Val	Ala		
L1 (λ3)	413	Ile	Ser		
L2 (λ2)	1101	Met	Ile		
L3 (λ1)	201	Thr	Ala		
	703	Arg			Gly
	1164	Ser			Phe



Reovirus	AA position	AA (wt-mutant)
jin-1	193	T-M
	336	Q-R
jin-1	187	G-R
	336	Q-R
jin-1	196	G-R

FIGURE 2.
Schematic representation of mutations in Sigma-1.
Model of the σ1 protein (adapted from Chappel et al, 2009). Arrows indicate the positions of the mutations.

To expand the pool of mutants, we repeated the procedure and exposed the U118scFvHis cells with our wild-type virus before further expansion on U118MG cells. After the first selection rounds in the U118scFvHis cells, we again found the Q336R mutation in the σ1 head domain. Upon prolonged propagation (10 passages) on U118MG cells an additional mutation was found in S1, resulting in a G187R change. This mutant strain, carrying mutations resulting in a Q336R and G187R change was named *jin-2*. Based on the findings in *jin-2* S1 in the earlier passage, we analyzed S1 of an earlier passage of *jin-1* as well (prior to plaque purification) and also in this S1 segment the only mutation present was the one resulting in the Q336R change in σ1.

Another mutant reovirus (*jin-3*) was obtained after direct exposure of U118MG cells at very high MOI to *wt*T3D reovirus. This virus was blindly passaged (i.e. the cells were lysed without signs of overt CPE at the time of virus harvest) for 6 rounds on U118MG cells. After 6 rounds in U118MG cells, CPE became apparent. After plaque purification on 911 cells and 10 additional passages on U118MG the complete genome of the *jin-3* mutant was sequenced. Only one mutation was found in the S1 segment, resulting in a G196R alteration. **Table 1** gives a summary

of all the amino acid changes found in the mutants, compared to our *wt*T3D. A schematic overview of the amino acid changes in σ1 is depicted in **Figure 2**. In all *jin* mutants the amino-acid alterations in the shaft of σ1 are located close to the sialic acid (SA) binding motif [6, 15, 25, 26].

Primary human fibroblasts (VH10 cells) do not support replication of the *jin* mutants or wtT3D

To study whether the *jin* mutants acquired the capacity to replicate in normal, non-transformed human cells, we exposed diploid human foreskin fibroblasts (VH10 cells) to *wt*T3D and to the *jin* mutants. The skin fibroblasts were chosen because primary human fibroblasts do not express JAM-A [27]. We studied the yields of the *jin* viruses and compared these with the yields of *wt*T3D on VH10 fibroblasts and on U118MG cells. As expected U118MG cells yielded high titers of the *jin* reoviruses, while *wt*T3D virus yields were below the amounts of virus added to the cells (**Figure 3A**). On VH10 fibroblasts, neither the *wt*T3D reovirus nor the three *jin*-mutants yielded significant titers (**Figure 3B**). Furthermore in the VH10 cell cultures no apparent signs of cell death were observed (data not shown). These data suggest that, like *wt*T3D our *jin* mutants do not replicate in normal non-transformed diploid fibroblasts.

During the plaque assays on 911 cells for the determination of viral yields, we noted that the plaques formed by the *jin-1* virus and *jin-3* virus were consistently smaller than those of the *wt*T3D virus; the plaque surface area of the initial *jin-1* virus and *jin-3* virus is approximately 10-fold lower (**Figure 3C**), suggesting reduced cell-to-cell spread of the mutants.

The *jin-2* virus also has a reduced plaque size compared with the *wt* virus, but the variation within the population is larger, which suggests heterogeneity in the population. Sequence analysis of the S1 segment of both the smaller and the larger *jin-2* plaques revealed that the smaller plaques contained the mutations for the G187R and Q336R change, while the larger plaques only contained the mutation that yield the Q336R alteration in σ1 (data not shown).

Protease inhibitor E64d blocks entry of *jin-1* virus

Reoviruses enter cells by receptor-mediated endocytosis after attachment of the 1 protein to the JAM-A receptor [16, 28]. Subsequently, the viral λ2 protein binds cellular integrins leading to endocytosis. In the endosomes the particle undergoes conformational changes by partial proteolysis, leading to intermediate subviral particles (ISVPs). The outer capsid proteins 3 and μ1/μ1C are cleaved by cellular proteases and 1 undergoes a conformational change. *In vitro*, this process can be mimicked by proteolytic treatment of complete virions. The generated

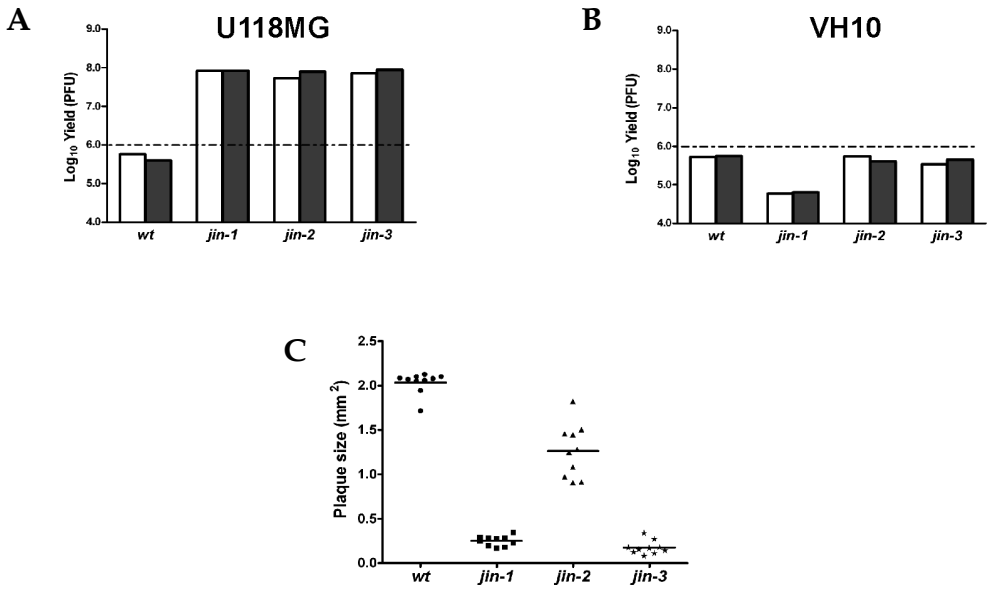


FIGURE 3.
Comparison of *wt*T3D with *jin*-viruses in terms of plaque size, yield in U118MG cells and primary human fibroblasts (VH10 cells).

A. Yield of *wt*T3D and *jin-1*, *jin-2* and *jin-3* mutants from U118MG cells infected with MOI 10 per virus. Yields were determined 72 hours post infection by plaque assays on 911 cells. The graph shows yields (Log_{10} PFU) of two independent U118MG cell infections: first one is shown as a white bar and second as a grey bar. The dashed line represents the input amount of the initial infection.

B. Yield of *wt*T3D and *jin-1*, *jin-2* and *jin-3* mutants from VH10 cells infected with MOI 10 per virus. Yields were determined 72 hours post infection by plaque assays on 911 cells. The graph shows yields (Log_{10} PFU) of two independent VH10 cell infections: first one is shown as a white bar and second as a grey bar. The dashed line represents the input amount of the initial infection.

C. Plaque sizes of *wt*T3D and *jin-1*, *jin-2* and *jin-3* mutants in 911 cells. Surface areas of 10 plaques per virus were measured four days post infection with Olympus DP-software.

ISVPs are capable to enter cells independent of the JAM-A receptor by penetration of the cytoplasmic membrane [18, 19, 29]. One possible explanation for the JAM-A independent entry of the *jin-1* virus could be a premature transition to ISVPs prior to entry into the cell. To test whether the *jin-1* virus is still dependent on cellular proteases the protease inhibitor E64d was used. If cells are exposed to E64d prior to infection, intact virions are trapped in the endosome while ISVPs can complete the replication cycle [30, 31]. To confirm that *wt* ISVPs are JAM-A independent we exposed the U118MG cells to *wt*T3D ISVPs and to intact T3D virions (**Figure 4A**). As expected, only in the cells exposed to the ISVPs, viral protein σ3 synthesis is

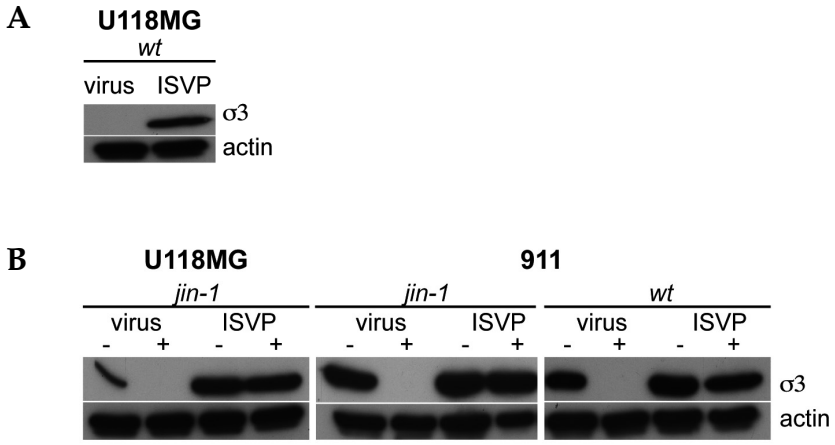


FIGURE 4.
Effect of the cysteine protease inhibitor E64d on *jin-1*, *wt*T3D virus, and ISVP entry into cells.

A. U118MG cells were exposed to purified *wt*T3D virus and *wt*T3D ISVP ($2*10^3$ particles per cell). Lysates were made 24 hours post-infection and analyzed by SDS-PAGE and western-blotting. The reovirus σ3 proteins were detected by the anti-reovirus σ3 antibody 4F2 and an anti-Actin serum was used to detect actin as a loading control.

B. Effect of 100 μM E64d on the entry of particles compared to entry of ISVPs. Cells, treated (+) or untreated (-) with 100 μM E64d, were exposed to *jin-1* (U118MG and 911 cells) or *wt*T3D (911 cells) virus or ISVPs ($2*10^3$ particles per cell). Lysates were made 24 hours post-infection. Equal amounts of protein were loaded on 10% SDS-polyacrylamide gel and detected with anti-reovirus σ3 antibody (4F2), and anti-Actin as a loading control.

detected, evidencing virus entry and viral protein synthesis. In 911 cells both the *jin-1* virus and *wt*T3D virus are inhibited by E64d (**Figure 4B**), while σ3 was detected in both the *jin-1* and *wt* ISVP infected cells. Also in the U118MG cells, the *jin-1* virus entry is blocked by the presence of E64d, but not the *jin-1* ISVP entry. These data demonstrate that like *wt*T3D reovirus, the reovirus mutant *jin-1* exploits the endocytotic pathway to enter U118MG cells.

jin-1 σ1 forms trimers

The change at amino-acid position 336 is located close to the domain that has been implicated in trimerization of σ1 [32]. Although the Q336R alteration is located at the outward surface-exposed side of every monomer in the trimeric conformation (**Figure 5A**, R336 is shown in red) it is essential to confirm that the Q336R alteration does not interfere with trimer formation. To this end an *in vitro* trimerization assay was performed as described by Leone at al. [33]. For this *wt* σ1, σ1-Q336R and σ1-Y313A proteins were synthesized *in vitro*. The Y313A change

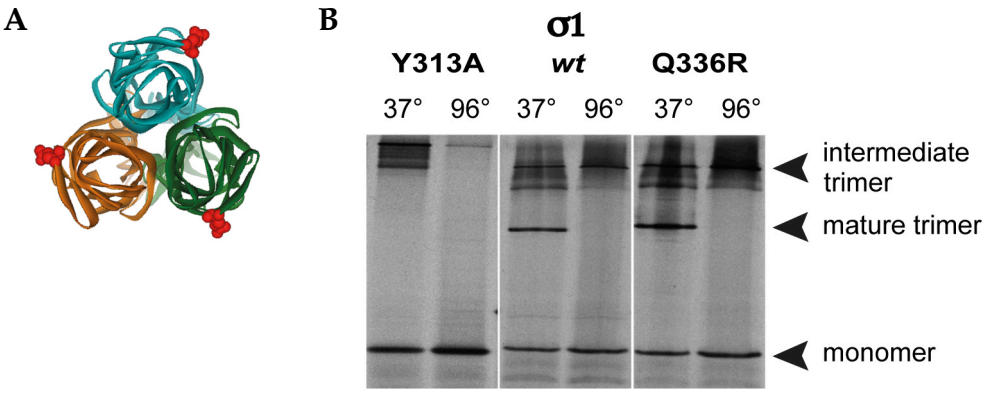


FIGURE 5.
Analysis of Sigma-1 trimers synthesized *in vitro*.
A. Top view of σ1-trimer, with colored monomer units (green, turquoise, orange). Position of the Q336R mutation in each monomer is indicated as red CPK symbol (Chappell *et al.*, 2002); PDB ID: 1KKE. The software used for the 3D graphs is Viewerlite 5.0 from Accelrys.
B. [^{35}S]-methionine labelled *in vitro* transcribed and translated products of plasmids pDGC-S1*wt* (S1wt), pDGC-S1Q336R (S1Q336R) and pDGC-S1Y313A (S1Y313A) were incubated for 30 minutes at 37°C (to stabilize the mature trimers) or boiled for 5 minutes (to disrupt the trimers), before loading on a 10% SDS-polyacrylamide gel at 4°C. The position of the three different conformations is indicated.

abolishes the capacity of σ1 to form trimers [32]. The σ1 products were analysed by mild PAGE at 4°C (**Figure 5B**). Whereas the σ1-Y313A protein does not form mature trimers, both the *wt*T3D σ1 and σ1-Q336R do. Intermediate trimers, which consist of σ1 molecules in which only the shaft is trimerized while the head domain is in a monomeric configuration, are detectable in all σ1 variants tested. Our data show that the Q336R alteration that occurs in the *jin-1* and *jin-2* viruses does not affect the formation of mature σ1-trimers *in vitro*.

jin-1 and *jin-2* have selective advantages over wild-type reovirus in U118MG cells

To confirm that *jin-1* and *jin-2* viruses have a selective advantage over *wt*T3D in U118MG cells, we mixed *jin-1* or *jin-2* with a 100-fold excess of *wt*T3D prior to infection of cells. Cultures of 911 cells were infected at an MOI 10 with the mixtures to allow reassortment of genome segments to take place. Two days post-infection, the virus was harvested by three freeze-thaw cycles and used to infect U118MG cells. While no CPE for *jin-1*/*wt* mixtures was observed at 7 days post-infection in the U118MG cells, the cells were freeze-thawed and the lysate

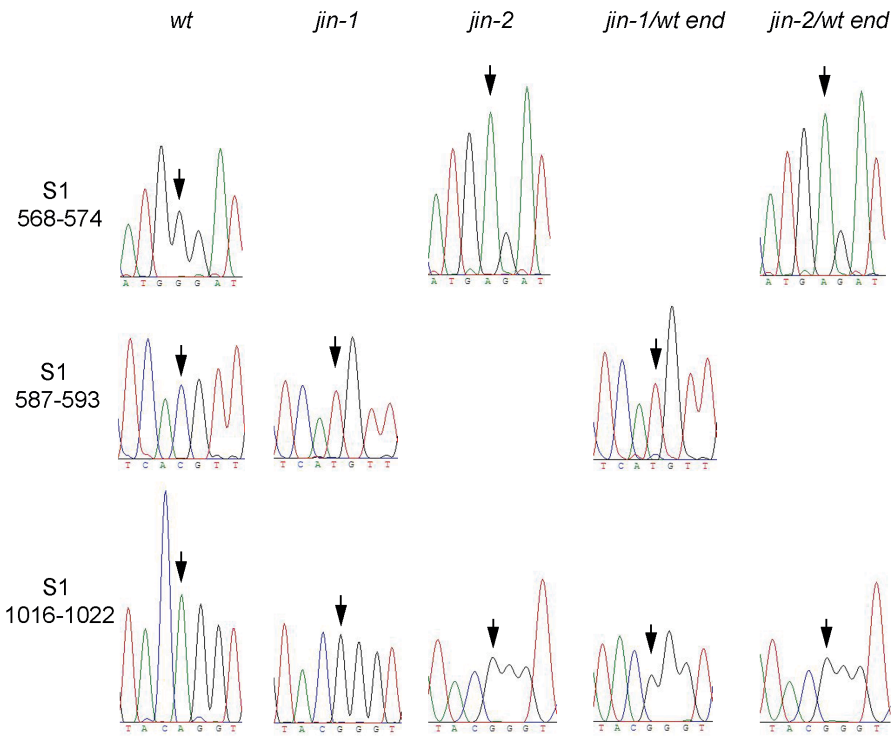


FIGURE 6.
S1-sequence analysis of the *jin-1*/*wt*T3D and *jin-2*/*wt*T3D selection assay on U118 MG cells. The *jin-1* or *jin-2* viruses were mixed with a 100-fold excess of *wt*T3D virus with regards to MOI. 911 cells were exposed to the mixtures first, before propagation on U118MG cells for three more passages. Reovirus RNA was isolated from the virus derived from the third passage on U118MG cells and subjected to RT-PCR to obtain the S1 products from the total population (*jin-1*/*wt* end or *jin-2*/*wt* end). Sequence histograms of the indicated regions were compared to the S1 sequences of the input reoviruses. Arrows indicate the nucleotide differences between the *wt*T3D and *jin-1* or *jin-2*. (S1 nucleotide positions 571, 590 and 1019).

was used to infect fresh U118MG cultures. In U118MG cells infected with *jin-2*/*wt*T3D mixture the virus was harvested after 4 days, with visible signs of CPE. This procedure was repeated for two more times. At passage 3 clear CPE was observed four days post infection in both *jin-1*/*wt* and *jin-2*/*wt* selections. Sequencing of PCR products after reverse transcription PCR of the S1 segment after the third selection on U118MG cells were compared with the S1 sequences of *wt*T3D, *jin-1* and *jin-2* (**Figure 6**). S1 of the *jin-1*/*wt* end population contains a T at nucleotide position 590 and a G at position 1019, identical to the *jin-1* S1 segment.

Sequence results for S1 of the *jin-2*/*wt* end population revealed an A at position 571 and a G at position 1019 and this is identical to *jin-2* S1 sequence. From these findings we conclude that in U118MG cells σ1 proteins from *jin-1* or *jin-2* provide a strong selective advantage over *wt*T3D σ1. These data provide evidence that the amino-acid alterations in σ1 provide the *jin* mutants with the capacity to infect and replicate in JAM-A-negative cells.

jin-1 reoviruses infect cells that are non-permissive for *wt*T3D

To study whether the *jin-1* mutant has expanded its tropism beyond the U118MG cell line, we evaluated whether this virus can replicate in a panel of cell lines that resists infection with *wt*T3D virus. These cell lines include chicken hepatoma cell line LMH [34], murine endothelioma cell line Eoma [35], human bone osteosarcoma cell line U2OS [36] and human Ewing sarcoma cell line STA-ET2.1 [37]. In parallel the cell lines 911 and U118-HAJAM were included as positive controls for infection. Each of the cell lines were exposed to *wt*T3D or *jin-1* viruses with an amount of virus corresponding to 8 PFU/cell as determined in 911 cells. While no major capsid protein 3 was detected in the *wt*T3D-resistant cell lines exposed to *wt*T3D, exposure of these cells to *jin-1* resulted in the detection of the 3 protein at 36 hr post infection (**Figure 7A**). In 911 and U118HA-JAM cells, the 3 protein is present in *wt*T3D infected cells as well as in the *jin-1* infected cells.

To verify that *wt*T3D resistant cells could support replication of the *jin-1* virus, the virus yields were determined in some of these cell lines (**Figure 7B**). In 911 cells both viruses give a similar yield, but in three other cell lines (U118MG, U2OS and LMH) more progeny virus was produced with the *jin-1* virus than with *wt* virus. The amount of *wt*T3D produced per cell did not rise above the amount added to the cells (MOI of 10, dashed lines). From these data we conclude that the *jin-1* virus is able to productively infect our panel of *wt*T3D resistant cells.

Cell entry of reovirus *jin-1* and *jin-3* relies on sialic acids

Apart from the Q336R mutation, the other mutations found in the S1 segments of the *jin* mutants are located close to the region involved in SA binding [6, 15]. Recently the crystal structure of the sialic acid – σ1 complex was elucidated [26]. There is a remarkable heterogeneity in the amino acid sequence of the SA-binding domains of different T3D and T1L strains. Some isolates cannot bind SA as determined on JAM-A negative murine erythroleukemia (MEL) cells. The forced selection of such strains yielded mutants that could infect MEL cells probably via the interaction with SA [38].

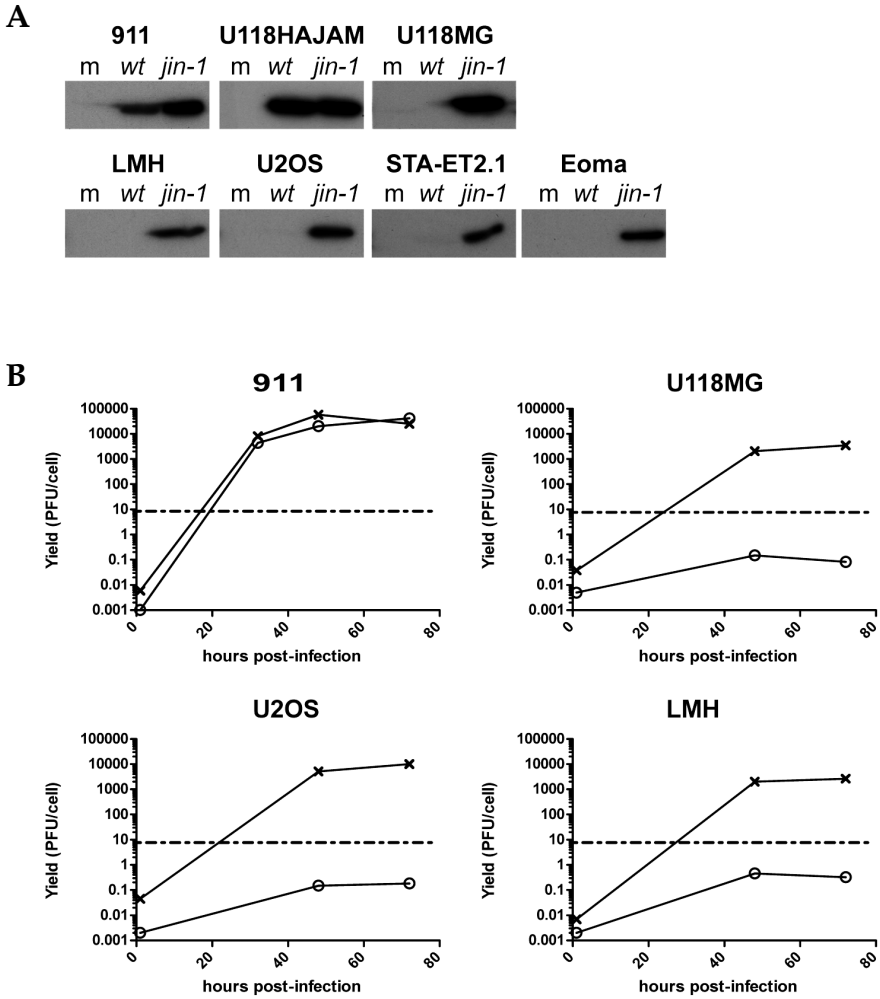


FIGURE 7.
Reovirus mutant *jin-1* can infect cell lines that resist *wt*T3D reovirus infection.

A. Several cell lines were infected with *wt*T3D or *jin-1* and 32 hr post-infection cells were lysed. Protein samples (30 μg) were analyzed by 10% SDS-polyacrylamide gel electrophoresis. For the immunodetection anti-reovirus σ3 (4F2) was used. The cell lines 911 and U118-HAJAM are included to serve as positive controls for the infectivity of *wt*T3D. The cells were mock-infected (m); *wt*T3D infected, or *jin-1* infected.

B. Virus production of *wt*T3D and *jin-1* in the different cell lines. Cells were exposed to virus at MOI of 10 for one hour, washed with PBS and immediately lysed (1 hour time point) or left for 48 or 72 hours. For 911 cells an additional harvest point at 32 hours post-infection was included. The viral titers in the samples were determined by plaque assays on 911 cells. The graph shows a representative example of the assay. Open circles: *wt*T3D(o), crosses: *jin-1*(x). The dashed line represents the input amount of the initial infection (10 PFU/cell).

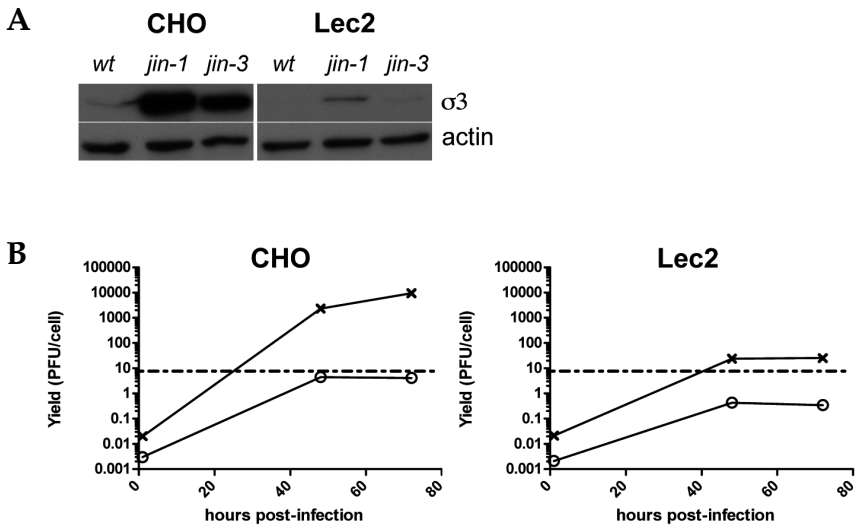


FIGURE 8.
Lec2 cells are poorly infected by reovirus mutants *jin-1* and *jin-3*.

A. CMP-sialic acid transporter defective Lec2 cells and parental cell line, CHO, were exposed to *wt*T3D, *jin-1*, and *jin-3* at MOI of 10. Protein lysates were made 32 hrs post-infection and analyzed by SDS-polyacrylamide gel electrophoresis. For the immunodetection of σ3 the anti-reovirus σ3 antserum 4F2 was used, and anti-actin (human) was used to detect actin as a loading control.

B. Virus production of *wt*T3D and *jin-1* in CHO and Lec2 cells. Cells were exposed for one hour to the viruses at MOI of 10, washed with PBS and immediately lyzed (1 hour time point), or incubated at 37°C for 48 hrs and 72 hrs. Virus yields were determined by plaque assays on 911 cells. The graph shows a representative example of the assay. Open circles: *wt*T3D(o), crosses: *jin-1*(x). The dashed line represents input amount of virus used at the initial infection (10 pfu/cell).

The *wt*T3D that was used in our studies has an amino-acid sequence of the sialic-acid binding pocket that is identical to strains capable of binding sialic acid. The S1 mutations found in the *jin* mutants are not located in the region coding for the SA-binding pocket of σ1 (viz. amino acids 198-204; ref [15, 26]), but are located in close proximity of this region. Nevertheless, it is conceivable that the amino acid alterations in the *jin* mutants affect the affinity or avidity of SA binding. To investigate the involvement of SA in binding of our mutant viruses we used Lec2 cells. Lec2 cells have a strongly reduced (by about 90%) amount of sialic acids on their cell surface [39, 40]. Lec2 cells are mutants derived from Chinese Hamster ovary (CHO) cells, which are poorly infected by *wt*T3D reovirus [41, 42]. In contrast, both the *jin-1* and *jin-3* mutants efficiently infect CHO cells (**Figure 8A**). The *wt*T3D nor *jin-1* and *jin-3* infect Lec2 cells, as is evidenced by lack of detectable σ3

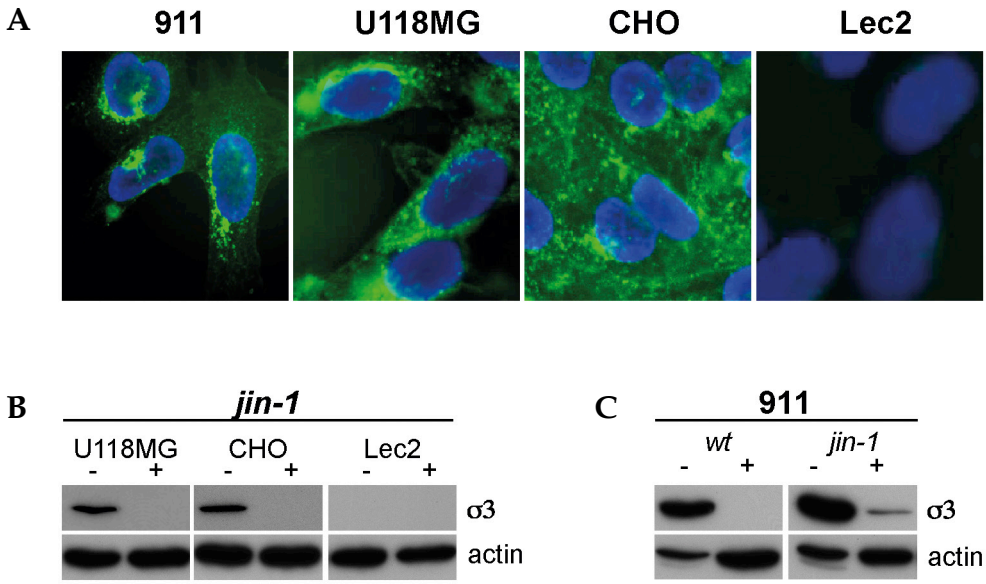


FIGURE 9.
WGA inhibits binding of reovirus to cells.
A. Detection of Sialic acids in cell lines (911, CHO, U118MG and Lec2) by FITC-labeled WGA immunofluorescence.
B. WGA inhibition of reovirus infection. Prior to exposure of reovirus *jin-1*, the cells (U118MG, CHO, and Lec2) were mock-treated (-) or treated with 100 μg/ml WGA for 1 hr. at 37°C (+). After exposure of the cells to the virus at 4°C the cells were washed with PBS and incubated for an additional 32 hours in a CO_2 incubator before protein lysates were made. For the immunodetection of the σ3 protein the anti-reovirus σ3 (4F2) was used and anti-actin was used as a loading control.
C. WGA inhibition of *wt*T3D and *jin-1* reovirus infection in 911 cells.

in the cells exposed to these viruses (**Figure 8A**). Also the replication of *jin-1* in the Lec2 cells is markedly reduced compared with the yields obtained in the parental CHO cells (**Figure 8B**). This suggests that the expanded tropism of *jin* is dependent on the presence of SA on the cell surface. To support the utilization of SA by the *jin-1* mutant, we shielded the SA on the surface of the cells by pre-incubating the cells with wheat germ agglutinin (WGA). WGA is a lectin with a strong affinity to a broad range of sialoconjugates. To confirm that WGA effectively binds to the cell lines 911, U118MG and CHO, but not to Lec2, we employed FITC-labeled WGA on fixed cells grown on cover slips (**Figure 9A**). For the competition experiments we blocked the sialic acids with WGA prior to the binding of *jin-1* to the cells. The addition of WGA to the cells inhibited entry of *jin-1* in U118MG and CHO cells (**Figure 9B**). Also in 911 cells, *jin-1* and *wt*T3D infection

are inhibited (**Figure 9C**). This confirms the dependency of *wt*T3D on SA for cell binding and entry. Our data demonstrate that also the *jin-1* mutant relies on SA binding for cellular entry.

2.4 Discussion

The use of tumor-selective oncolytic viruses for killing tumor cells that resist conventional therapeutic approaches is conceptually attractive. Human reoviruses are one of the promising candidates for use as replicating oncolytic agent [7, 43, 44]. Reoviruses preferentially induce cell death and apoptosis in tumor cells, but not in diploid, non-transformed cells [1-3]. However, in some tumor cells expression of the JAM-A receptors is down-regulated and absent on the cell surface, thereby limiting the susceptibility of the cells to reovirus T3D infection.

Here we report the isolation of JAM-A independent T3D reoviruses with an expanded tropism. These mutants, designated as *jin* mutants, may be considered as oncolytic agents in those tumor types that lack accessible JAM-A on their surface [21-23, 45]. Although we encountered the first *jin* mutant in a batch of S1-His modified reovirus after selection in the U118MG-scFvHis cell line [24], the *jin* mutants are not genetically modified viruses in the formal sense since they resulted from spontaneous mutations in T3D viruses.

In three independent virus batches we identified *jin* mutants. Two of these (*jin-1* and *jin-2*) carried an identical mutation in the head domain of the spike protein σ1. The mutation replaces the glutamine (Q) residue at position 336 by an arginine (R). The amino acid 336 is located at a surface exposed position, close to the region involved in the trimerization of the 1 spikes [32]. The alteration renders the area more positively charged. This could potentially result in conformational changes that may disturb the formation of 1-trimers. However, the results of a trimerization assay (depicted in **Figure 3**) revealed that altered σ1 of *jin-1* still forms mature trimers, showing that the Q336R alteration in S1 of the *jin-1* and *jin-2* viruses does not affect trimer formation of this domain.

While the *jin* mutants were isolated on human glioblastoma cell line U118MG, we found that the *jin-1* mutant efficiently infects a variety of reovirus T3D resistant cell lines, including the chicken hepatoma cell line LMH, but not non-transformed primary human skin fibroblasts (VH10) This expanded tropism, together with the small-plaque phenotype observed in the JAM-A positive cell line 911, is reminiscent of changes observed in other virus families. Adaptation to cell culture conditions can result in selection of viruses that acquired the capacity to bind heparan sulfates [46-48]. Variants of foot-and-mouth disease virus (FMDV) which

bind strongly to heparan sulfate *in vitro*, show small plaques on BHK cells and these variants are attenuated in cattle. Furthermore, they have a decreased ability to spread from the site of inoculation [48]. Alternatively, mutants of Sindbis Virus that exhibit a reduced binding to heparan sulfate give rise to larger plaques *in vitro* and are more virulent *in vivo* with slower clearance from the circulation [46]. However, the Q336R change in *jin-1* and *jin-2* does not yield a typical linear heparin-binding domain consensus -X-B-B-X-B-X- and -X-B-B-B-X-X-B-X- in which B is a basic residue (mainly K or R) and X a hydropathic residue [49-51]. It should be noted that the presence of a linear consensus sequence is not a strict prerequisite for glycosaminoglycan binding. Some of Venezuelan equine encephalitis virus (VEE) mutants bind heparan sulfates (HS) through a conformational domain and do not contain the linear HS-binding domain [52]. Our observation that reovirus *jin-1* entry into U118MG cells cannot be inhibited by incubation with heparin or heparan sulphate (data not shown) suggests that binding to glycosaminoglycans is not responsible for the broadened tropism of *jin-1* and *jin-2*. It therefore remains to be established if and how the Q336R change contributes to the expanded tropism. Also with respect to sialic acid binding, in some DNA viruses mutations in the SA-binding pocket resulted in changes in plaque morphology [53, 54].

Upon continued serial passaging of *jin* mutants in U118MG cells, additional amino acid alterations accumulated in spike protein σ1, T193M in *jin-1* and G187R in *jin-2*. For the *jin-3* mutant, a single mutation in S1 resulted in G196R substitution in σ1. Those changes are located in close proximity of the region implicated in sialic acid binding [6, 15, 25, 26]. This is in line with the previous observation that passaging of reoviruses incapable of binding SA on mouse erythroleukemia cells yielded mutants capable of binding sialic acids [38]. In co-crystalization experiments of σ1 in complex with SA it was shown that the changes were mapped in the σ1 region between amino acids 198 and 204 [26].

Also outer capsid protein σ3 plays an important role in the process of reoviral entry [16, 18, 55, 56]. A mutation found in so-called persistent-infection reoviruses leads to an amino acid change Y354H in the σ3 protein. This alteration has been linked to the reoviral resistance to the protease inhibitor E64d [57]. A mutation in *jin-1* results in M357T in σ3, which is in close proximity of position 354. However, no such mutations are found in *jin-2* and *jin-3*. Moreover, *jin-1* is sensitive to E64d, demonstrating that the *jin-1* virus still depends on cysteine proteases for uncoating and infection. This suggests that the *jin-1* virus enters JAM-A negative cells via the endocytic pathway, like *wt*T3D in JAM-A expressing cells.

So far, we have evaluated the *jin* mutants in *in vitro* studies only and it remains to be elucidated what the effect will be *in vivo*, both with regards to safety, as well as to their oncolytic efficacy.

While we cannot exclude the possibility of the recruitment of secondary receptors, our data suggest that the *jin* mutants rely on sialic acid binding for internalization. It is tempting to speculate that a changed affinity for sialic acids underlies the changed tropism of our *jin* viruses, since they show a decreased ability to spread in cultured cells, exhibit a small plaque phenotype, and shielding SA moieties with WGA prevents the *jin* viruses to enter cells. It remains to be established if the mutation in S1 affects reovirus pathology in mice. In this respect, it is noteworthy that the pathology of reoviruses is, in part, dependent on the σ1 protein [58].

2.5 Materials and methods

Cell lines

Cell lines 911 (generated previously in our lab, see reference [55]), U118MG (obtained from ATCC), U2OS (obtained from ATCC, see reference [29]), CHO (obtained from ATCC), Eoma (obtained from ATCC, see reference [28]) and VH10 (primary human foreskin fibroblasts, provided by B. Klein [59]) were cultured in Dulbeco's Modified Eagle Medium (DMEM) containing high glucose (Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin (pen-strep) and 8% fetal bovine serum (FBS) (Invitrogen, Breda, The Netherlands). The U118-HAJAM cells were cultured in DMEM plus 8% FBS and pen-strep, supplemented with 200 μg/ml G418. LMH cells (obtained from ATCC, see reference [27]) were grown on collagen I coated dishes (Rat Tail collagen, 2.5 $\mu g/cm^2$, Invitrogen, Breda, The Netherlands) in DMEM plus 8%FBS and pen-strep. STA-ET2.1 cells were grown on collagen I coated dishes (5.0 $\mu g/cm^2$) in RPMI 1640 medium (Invitrogen, Breda, The Netherlands), supplemented with pen-strep and 10% FBS. Lec2 cells (derived from ATCC, see reference [33]) were cultured in alpha-MEM (Invitrogen, Breda, The Netherlands) with 8% FBS. All cells are cultured in an atmosphere of 5% CO_2 at 37°C.

Reovirus propagation

The wild-type T3D virus strain R124 (see accession numbers) was isolated from a reovirus T3D stock obtained from the American Type Culture Collection (stock VR-824) by two rounds of plaque purification on 911 cells. The 911 cells were used to propagate R124 (referred to as *wt*T3D in the text) as described previously [60]. Briefly, cells were exposed to reovirus in DMEM plus 2% FBS for 2 hours at 37°C, 5% CO_2. Subsequently, the inoculum was replaced by DMEM containing 8% FBS. The virus was harvested 48 hours post infection by resuspend-

ing the cells in phosphate-buffered saline (PBS) with 2% FBS and subjecting the suspension to three cycles of freezing and thawing. The sample was cleared by centrifugation for 10 minutes at 800 g. For the mutant *jin* viruses, U118MG cells were used to propagate the viruses after one round of plaque purification on 911 cells. The *jin* viruses were routinely harvested from U118MG cells 72 hours post infection. The experiments were done with virus-containing freeze-thaw lysates, unless otherwise indicated. The infectious reovirus titers of the strains were determined by plaque assay on 911 cells.

Origin of the *jin*-mutants

jin-1 is derived from U118scFvHis cells during our experiments on genetically modifying reovirus [24]. The *jin-1* virus was first grown on U118scFvHis cells for two propagations, before three passages on U118MG cells (first point of S1-sequence analysis). The virus was subjected to one round of plaque purification on 911 cells to obtain a homogenous population. This was further propagated on U118MG cells for 11 rounds before analysis of the complete genome sequence (**Table 1**).

jin-2 was isolated from U118scFvHis cells infected with *wt*T3D reovirus and passaged twice in these cells. The S1 segment was sequenced from this passage. Subsequently it was passaged 10 times on U118MG cells. After plaque purification, the complete genome sequence was determined (**Table 1**).

jin-3 was isolated from U118MG cells exposed to *wt*T3D virus followed by blindly passaging the virus for 6 rounds. From the resulted preparation a virus was isolated by plaque purification and following by 10 additional passages on the U118MG cells. (**Table 1**).

Yield determinations

To determine the replication of *wt*T3D, *jin-1*, *jin-2* and *jin-3* in U118MG and VH10 cells (**Figure 3**), cells were seeded in 24-well plate with a cell density of $1*10^5$ cells/well. Viruses (in DMEM plus 2% FBS) were added to the cells with an MOI of 10, two wells per virus. After an exposure of one hour in incubator (37°C, 5% CO_2) the inoculum was removed and the cells were washed once with PBS and fed by fresh DMEM plus 2% FBS. Reoviruses were harvested from medium and cells by 3 cycles of freeze-thawing, 72 hours after infection. Yields were determined by plaque assays on 911 cells.

For the replication of *wt*T3D or *jin-1* in 911, U118MG, LMH, U2OS, CHO and Lec2 cells (**Figure 7B** and **8B**), cells were seeded in 6 well plates with a density of $1.5*10^6$ cells/well. Wild-type T3D or *jin-1* was added to 4 wells in case of 911 cells and 3 wells for the other cell lines with an MOI of 10 (in DMEM plus 2%FBS). After one hour of exposure in incubator, the viruses were washed from the cells

and medium was replaced. From one well, immediately after washing once with PBS, cells in medium were collected and subjected to freeze-thaw cycles (1 hour time point). 32 Hours (911 cells only), 48 and 72 hours after infection cells and medium were collected and subjected to freeze-thaw cycles. Viral yields were determined by plaque assays on 911 cells.

Cell viability assay

WST-1 reagent (Roche, Almere, The Netherlands) was used to assay the viability of cells after reovirus infections. U118MG and 911 cells in 96-well plate were mock-infected or infected with *wt*T3D or *jin-1* with an MOI of 10, in triplo. Six days post infection WST-1 reagent was added, according to the manufacturer's manual. The viability measurements in mock-infected cell cultures, were set to 100%.

[^{35}S]-Methionine labelling

Infected cells (911 cells infected at MOI = 1; U118MG and U118-HAJAM with MOI = 5) or mock-infected cells were incubated with TRAN^{35}S - LABEL™ (10mCi/ml; MP Biomedicals, Eindhoven, The Netherlands) for 4 h; one day (911 cells) or two days (U118MG and U118HA-JAM cells) post infection. Cells were washed once with phosphate-buffered saline and lysed in Giordano Lysis Buffer (50 mM Tris HCl pH 7.4, 250 mM NaCl, 0.1% Triton, 5 mM EDTA) containing protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). The labelling assays were performed in 24-well plates with 5 µl (50 µCi) TRAN^{35}S - LABEL™ per well. The cells were lysed with 100 µl lysis buffer. After addition of sample buffer, 50 microliters per lysate was loaded in the wells of a 10% SDS-polyacrylamide gel. Gels were dried and exposed to a radiographic film to visualize the labeled proteins.

Immunofluorescence assay

For immunofluorescence assays, U118MG and 911 cells were grown on glass coverslips in 24-well plates before infection with *wt*T3D or *jin-1* with an MOI of 5 or no virus. One day post infection the cells were fixed with cold methanol (15 minutes, 4°C), washed with PBS containing 0.05% Tween-20, and incubated with antibody 4F2 directed against reovirus σ3 (monoclonal antibody developed by T.S. Dermody [61]; obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242), diluted in PBS containing 3% BSA. After incubation at room temperature the cells were washed (PBS, 0.05% Tween-20) and incubated with secondary fluorescein isothiocyanate (FITC)-

conjugated goat anti-mouse serum for 30 minutes at room temperature. The mounting solution consisted of glycerol containing 0.02 M Tris HCl pH8.0, 2.3% 1,4-diazabicyclo-[2.2.2]-octane and 0,5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

RT-PCR and sequencing

Total RNA was isolated (Absolutely RNA miniprep kit; Stratagene, Agilent Technologies, Amstelveen, The Netherlands) from U118MG cells infected with the different reovirus mutants (*jin-1* or *jin-3*), one day post infection. For *wt*T3D total RNA was isolated from infected 911 cells. Primers used for the RT-PCR procedures are listed in Table S1. DNA synthesis of all the segments started with the unique endR primer designed for every segment, using SuperScript III (Invitrogen, Woerden, The Netherlands) for the reverse transcription process. For the PCR, PFU polymerase (Promega, Leiden, The Netherlands) was used with the primer combinations unique for every segment. PCR products were first cleaned with Sureclean (Bioline, London, UK), according to the manual, before direct analysis of the sequence. In some cases the resulting PCR products were cloned into plasmid pJet1 (GeneJet, PCR cloning kit; Stratagene, Agilent Technologies, Amstelveen, The Netherlands) and their DNA sequences were determined. All sequence data were generated by The Leiden Genome Center (LGTC, Leiden, The Netherlands).

In vitro transcription-translation and trimerization assay

All primer sequences can be found in table S1. With DualSHFor and DualSHRev primers, S1 PCR product was generated from plasmid pCDNART3S1 [24], according to manual of the pDual-GC vector (Stratagene, Agilent Technologies, Amstelveen, The Netherlands). The resulting construct (pDualS1His) contained no stop codon, meaning that the Myc-His tag was present behind S1. To introduce a stop codon behind the S1 ORF in pDualS1His, the DualS1st for and rev primers were used in a mutation PCR, with EXL polymerase (Stratagene, Agilent Technologies, Amstelveen, The Netherlands). This resulted in the plasmid pDGC-S1delTag, which was used for the trimerization assay and as start to generate the pDGC-S1QR and pDGC-S1Y313A (with S1-QRmut2Rev and For combi or S1-Y313AmRev and For combi, respectively) also with EXL polymerase. For the *in vitro* transcription-translation (ITT) part, TNT® T7 Quick Coupled Transcription/ Translation kit (Promega, Leiden, The Netherlands) was used. Input for the ITT assays were the plasmids pDGC-S1delTag, pDGC-S1QR and pDGC-S1Y313A. The total reaction volume was 15 μl, scaled according to the manual (in the presence of 6 μCi TRAN^{35}S - LABEL™; MP Biomedicals, Eindhoven, The Netherlands). For

the trimerization Assay, one fifth of every ITT reaction per construct was used and incubated with Sample buffer (final concentrations: 10% glycerol, 2% SDS, 60 mM Tris HCl pH 6.7, 2.5% β-mercaptoethanol and 2.5% bromophenol blue) for 30 minutes at 37°C to stabilize the trimers or boiled for 5 minutes 96°C to disrupt the trimers. After incubation the samples were loaded on a 10% SDS-polyacrylamide gel, which was kept at 4°C during the run. Gels were dried and exposed to a radiographic film to visualize the labeled proteins.

Plaque assay and size measurements

Plaque assays were performed in a standard assay as previous described for adenoviruses [62] with minor modifications. Briefly, virus stocks were serial diluted in DMEM containing 2% FBS. The dilutions were added to near-confluent 911 cells in six-well plates. Four hours after infection, medium was replaced with agar-medium. Agar-medium consists of (final concentrations) 0.5% agarose (Ultrapure™, Invitrogen, The Netherlands),1x minimal essential medium (MEM), 2% FBS, 12.5 mM $MgCl_2$, 2 mM GlutaMAX™ (Invitrogen, Almere, The Netherlands) and 1x pen-strep antibiotic mixture (Invitrogen, Almere,The Netherlands). Plaques are counted six days post infection. Plaques sizes were measured four days post infection. For the measurements a CKX41 Olympus microscope was used and the plaque area was measured with the software of Olympus: Olympus DP-soft.

Western analysis

Cell lysates were made in Giordano Lysis Buffer supplemented with protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). Total amount of protein in the lysates was measured (Bradford, Biorad, Veenendaal, The Netherlands) and the same amount of lysate (30 μg) was loaded into the wells of a 10% SDS-polyacrylamide 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-P (Millipore, Etten-Leur, The Netherlands) and visualized using standard protocols. Antibodies used in this study: 4F2 directed against reovirus σ3; β-Actin antibody: ImmunO anti-Actin clone C4 (MP Biomedicals, Eindhoven, The Netherlands).

E64d inhibition

E64d (Sigma Aldrich, Zwijndrecht The Netherlands) was dissolved in DMSO before use. U118MG and 911 cells were seeded in 24 well plates; half of the cells were exposed to 100 μM E64d at 37°C, 5%CO_2 for one hour. Purified *wt*T3D or

jin-1 virus and ISVPs (approximately $2*10^3$ particles per cell) were added to the cells and left for one hour at 4°C; cells were washed with PBS and transferred back to 37°C, 5%CO_2 in the absence or presence of E64d, for 36 hours. Lysates were made as described in Western analysis. For the immunodetection the anti-3 antibody (4F2) and β-Actin antibody were used.

Generation of ISVPs

*wt*T3D or *jin-1* virus ISVPs are freshly prepared by treating CsCl purified virions [60] with chymotrypsin. Purified viruses were diluted to a concentration of 10^{11} PFU/ml in Reovirus Storage Buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$) and treated with 200 μg/ml Chymotrypsin (TLCK treated, Sigma Aldrich, Zwijndrecht The Netherlands; C3142) at 37°C for 1 hour. Reaction was stopped by adding 5 mM phenylmethyl-sulfonyl fluoride (Sigma Aldrich, Zwijndrecht The Netherlands).

Wheat germ agglutinin (WGA) binding and competition experiment

For the detection of sialic acids in the different cell lines, FITC-labeled WGA (Sigma Aldrich, Zwijndrecht The Netherlands) was used. Cells (grown on round glass coverslips in 24-well plate) were fixed with ice-cold Methanol (15 minutes, 4°C) and washed with PBS containing 0.05% Tween-20. WGA-FITC was added to the cells at a concentration of 5 μg/ml in PBS containing 3% BSA and incubated for 1 hour at room temperature. Excess of unbound WGA-FITC was washed away with PBS containing 0.05% Tween-20. Nuclei were visualized by DAPI in the same mounting solution as is described for the immunofluorescence assay.

WGA competition experiment was done by exposing cells (in 24-well plate wells) to WGA at a concentration of 100 μg/ml in culture medium for one hour in CO_2 incubator at 37°C. The pre-incubation medium was removed and *wt*T3D or *jin-1* virus was added to the cells with an MOI of 10 in DMEM containing 2% FBS at 4°C for one hour. Cells were washed with ice-cold PBS and normal culture medium was added to the cells. Cells were left for 32 hr in CO_2 incubator (37°C) before lysates were made as described in Western analysis. For the immunodetection the anti-σ3 antibody (4F2) and β-Actin antibody were used.

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Accession numbers

GenBank ID's of *wt*T3D (R124) and *jin-1* segments:

R124 T3D-L1 GU991659; *R124* T3D-L2 GU991660; *R124* T3D-L3 GU991661; *R124* T3D-M1 GU991662; *R124* T3D-M2GU991663; *R124* T3D-M3 GU991664; *R124* T3D-S1 GU991665; *R124* T3D-S2 GU991666; *R124* T3D-S3 GU991667; *R124* T3D-S4 GU991668.

jin-1-L1 GU991669; *jin-1*-L2 GU991670; *jin-1*-L3 GU991671; *jin-1*-M1 GU991672; *jin-1*-M2GU991673; *jin-1*-M3 GU991674; *jin-1*-S1 GU991675; *jin-1*-S2 GU991676; *jin-1*S3 GU991677; *jin-1*-S4 GU991678.

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Chapter 3

Mammalian orthoreovirus T3D infects U-118 MG cell spheroids independent of Junction Adhesion Molecule-A

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Contents chapter 3

3.1 Abstract

In the canonical pathway, infection of cells by the wild-type mammalian orthoreovirus Type 3 Dearing (T3D) is dependent on the interaction of the viral spike protein σ1 with the high-affinity cellular receptor Junction Adhesion Molecule-A (JAM-A). We previously demonstrated that the human glioblastoma cell line U-118 MG does not express JAM-A and resists reovirus T3D infection in standard cell culture conditions (SCCC). Heterologous JAM-A expression sensitises U-118 MG cells to reovirus T3D. Here we studied reovirus infection in U-118 MG cells grown in spheroid cultures with the premise that cells in such cultures resemble cells in tumours more than those grown under standard adherent cell culture conditions on a plastic surface. Although the U-118 MG cells in spheroids do not express JAM-A, they are susceptible to reovirus T3D infection. We show that this can be attributed to factors secreted by cells in the spheroids. The concentration of active extracellular proteases cathepsin B and L in the medium of spheroid cultures was increased 19- and 24-fold, respectively, as compared with SCCC. These enzymes can convert the reovirus particles into a form that can infect the U-118 MG cells independent of JAM-A. Taken together, these data demonstrate that infection of tumour cells by wild-type reovirus T3D is not strictly dependent on the expression of JAM-A on the cell surface.

3.2 Introduction

The mammalian orthoreovirus Type 3 Dearing (T3D) is a promising oncolytic agent. Currently, its use as anti-cancer therapeutic, either in stand-alone treatment or in combination with existing medication, is explored in a variety of clinical trials. Reovirus T3D has been well tolerated and no dose-limiting toxicity has been reported so far. Anecdotal evidence of antitumour efficacy exists in stand-alone treatment and in combination therapies [1]. The reovirus T3D is a member of the *Reoviridae* family, which is characterized by a segmented double-stranded RNA genome. The non-enveloped virus contains two icosahedral protein shells. Reovirus has been long known to induce cell death preferentially in tumour cells, and much less in non-transformed diploid cells [2]. Various determinants underlie the virus' preferences for killing cancer cells of which active Ras signalling [3, 4], receptor availability [5], viral uncoating [6], and endosomal trafficking [7] appear the most important.

Reovirus attachment protein σ1 is considered as the main determinant of the virus' tropism. Attachment of the virus to the host cell is a three-step process. The

virus first scans the cell surface by high avidity binding to sialic acids with residues in the shaft domain of the spike protein [8]. This leads to the engagement of the high-affinity reovirus receptor Junction Adhesion Molecule-A (JAM-A, also named JAM-1) by a defined region in the head domain of σ1 [9]. JAM-A is a transmembrane protein with two extracellular immunoglobulin domains and a short cytoplasmic tail. The protein is concentrated at the apical region of intercellular tight junctions of epithelial and endothelial cells [10]. Following JAM-A binding, β1 integrins guide internalization of the virus, most likely by clathrin-mediated endocytosis [11]. In the endosome the virus is partially uncoated by proteolytic disassembly by the host proteases such as cathepsin B and L [12].This process is characterized by structural changes in σ1, removal of the outer-capsid protein σ3 and conformational rearrangements in outer-capsid protein μ1. In this stage the particles are designated as intermediate subviral particles (ISVPs) (reviewed by Danthi *et al.* [13]). ISVPs are able to escape the endosome, thereby delivering transcriptionally active cores into the cytoplasm [14]. In the intestines and the lungs, the initial sites of reovirus infection *in vivo*, disassembly of the virion by host proteases can take place extracellularly and the resulting ISVPs can enter cells independent of JAM-A directly at the cell membrane [15-18].

Under standard cell culture conditions, infection of glioma cells by reovirus T3D strictly depends on expression of JAM-A. We and others demonstrated that the reovirus-resistance of the glioblastoma cell line U-118 MG, upon its growth in standard cell culture conditions, can be overcome by heterologous expression of the JAM-A receptor or its extracellular domain coupled to a heterologous transmembrane domain [19, 20]. Remarkably, however, various JAM-A negative primary glioma cell cultures, that had been isolated from tumour resection material and were grown as spheroid cultures, were found to be sensitive to reovirus T3D infection [21]. These paradoxical results question the importance of JAM-A in reovirus infection. Therefore we decided to study the role of JAM-A in more detail in two *in vitro* cell culture models. These are the standard cell cultures condition (SCCC) cultures adherent to a polystyrene surface, and the three-dimensional tumour-cell spheroids.

The spheroid cell culture models are being used in cancer research since the 1970s [22] but gained renewed interest when the limitations of two-dimensional culture methods became apparent (reviewed by Achilli *et al.* [23]). Spheroids resemble the architecture of an *in vivo* tumour (including its micro environment) more closely than SCCC cultures. As an example, spheroids establish gradients of products that are secreted or taken up by cells. Also, cell-cell and cell-matrix interactions in spheroids better mimic tumours [24]. This also became evident from genomic profiles of adherent cell cultures and spheroids derived from resected

glioma material; spheroids more closely resemble the genetic characteristics of the parental tumour [25].

Here we grew U-118 MG glioblastoma cells as SCCC cultures and as multicellular spheroids to compare the susceptibility to reovirus in these two models. We show that, while U-118 MG cells grown in spheroid cultures are JAM-A deficient, they are highly susceptible to reovirus infection. This can be attributed to proteolytic activation of the reoviruses by proteases secreted by the cells in the spheroid cultures. This demonstrates that factors secreted by cells in the three-dimensional cultures can proteolytically activate the reoviruses which results in infection independent of JAM-A expression.

3.3 Results

Growth of U-118 MG cells in SCCC and spheroid cultures

To study the effects of the culture system on the growth rates of U-118 MG cells, the cell numbers were counted at various time points after initiation of the SCCC and spheroid cultures. Both cultures started with 5,000 cells per well and cell numbers were counted regularly during five days (**Figure 1A**). As expected, cells in SCCC cultures divide more frequently than in spheroid cultures. In five days after initiation, the cell numbers in adherent SCCC cultures increased almost ten-fold, whereas the cell number in the spheroid cultures increased only three-fold. The diameter of the spheroids increased linearly during time (**Figure 1B**). Ki-67 protein staining on microtome sections of spheroids revealed the presence of proliferating cells throughout the spheroid (**Figure 1C**). These data confirm the presence of viable cells within the spheroid. Analysis by Transmission Electron Microscopy (TEM) of a section of a spheroid (**Figure 1D**) revealed the presence of extracellular matrix (ECM). No evidence was obtained on the appearance of a necrotic centre, which is often observed in multicellular spheroids [24].

U-118 MG spheroids do not express Junction Adhesion Molecule-A

Cell-cell interactions of cells in spheroids inevitably differ from cells in SCCC cultures. U-118 MG cells in SCCC cultures do not express reovirus' primary cellular receptor, JAM-A [19]. To study whether JAM-A expression is induced by growth in spheroid cultures, we investigated the JAM-A expression status. As a positive control, U-118HA-JAM cells were used which were generated by transduction of U-118 MG cells with a lentiviral vector encoding JAM-A [19]. Flow-cytometry analyses showed that U-118 MG cells retrieved from spheroid cultures do not express JAM-A protein on their surface, whereas the U-118HA-JAM

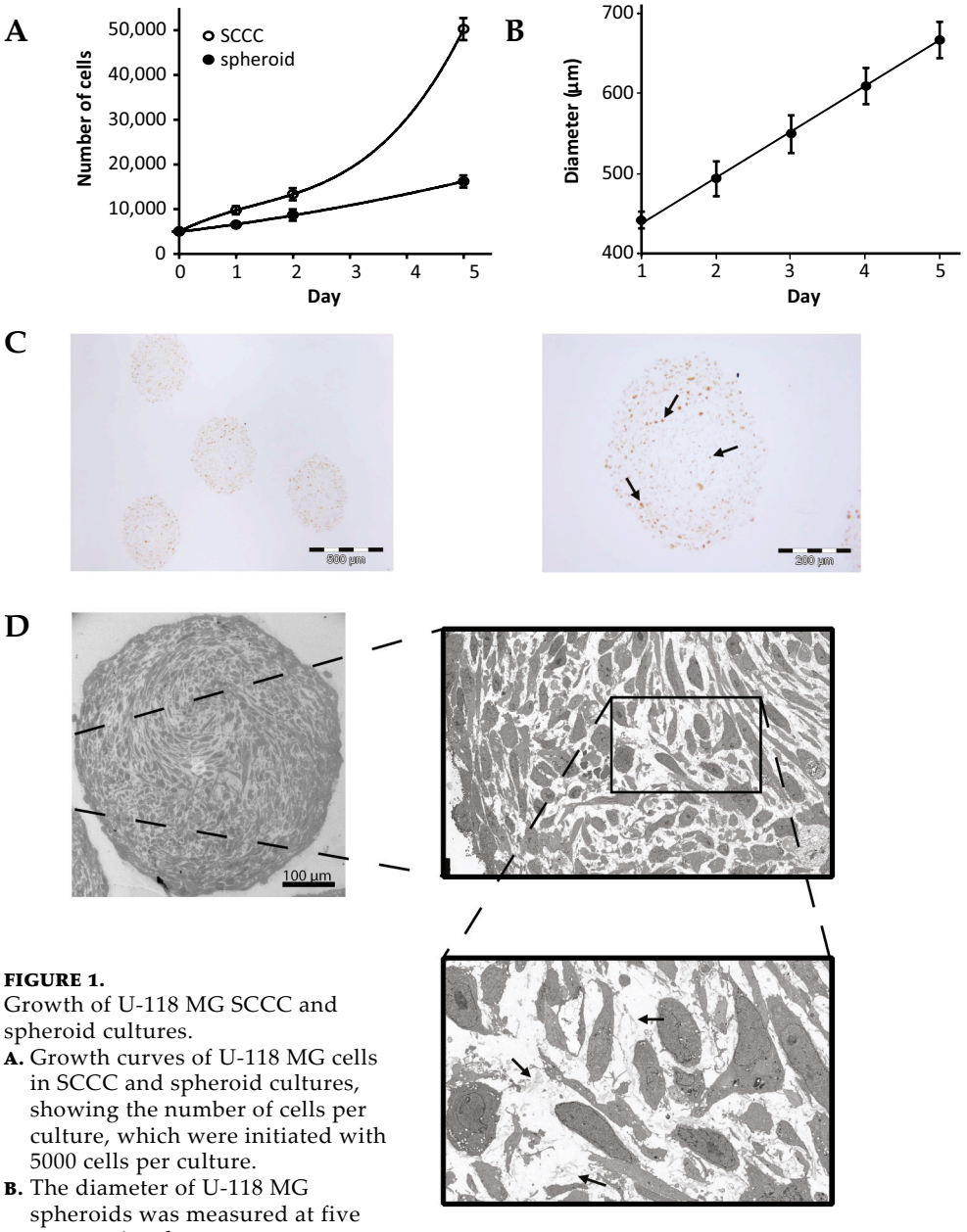

FIGURE 1.
Growth of U-118 MG SCCC and spheroid cultures.

A. Growth curves of U-118 MG cells in SCCC and spheroid cultures, showing the number of cells per culture, which were initiated with 5000 cells per culture.

B. The diameter of U-118 MG spheroids was measured at five consecutive days.

C. Viability assay by Ki-67 protein staining in cross-sections of U-118 MG spheroids, 2 days after preparation. The right panel is an enlargement of the lower right spheroid. Examples of Ki-67-positive cells are indicated by an arrow. To enhance the visibility of weak signals, no counterstain was applied in these sections.

D. Electron microscopy image of a U-118 MG spheroid, 2 days after preparation. The boxed sections are enlarged in the right, lower panel. The extracellular matrix (ECM) between cells is indicated by arrows.

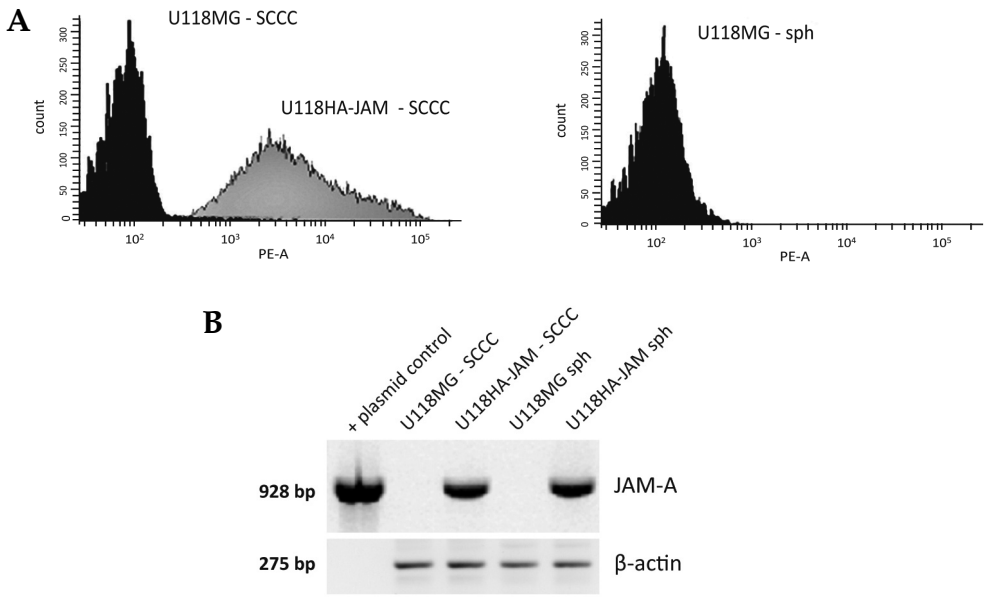

FIGURE 2.
JAM-A expression in U-118 MG cells.
A. Flow cytometry analysis of JAM-A expression on U-118 MG and U-118HA-JAM SCCC cells and U-118 MG spheroid cells using a JAM-A specific antibody.
B. RT–PCR assay on total cellular RNA of U-118 MG and U-118HA-JAM SCCC and spheroid cultures using JAM-A-specific primers. Beta-actin analysis was performed to confirm the integrity of the RNA.

spheroids were clearly JAM-A positive (**Figure 2A**). The absence of JAM-A expression in both U-118 MG SCCC cells and spheroids was confirmed by reverse transcription polymerase chain reaction (RT-PCR) on total cellular RNA (**Figure 2B**).

U-118 MG cells in spheroids are more susceptible to reovirus infection than U-118 MG SCCC cultures

Results from our previous study suggested that JAM-A expression is not a prime determinant of the sensitivity of human glioma cells to reovirus infection and that significant reovirus infection can occur in cells that have only very low amounts of JAM-A at their surface [21]. Therefore, we studied the difference in sensitivity to reovirus infection of U-118 MG cells grown as SCCC and as spheroid cultures. Spheroid cultures (consisting of 5000 cells) were prepared using methylcellulose-containing culture medium in non-adherent U-bottom plates [26]. As controls, SCCC cultures were prepared with the same number of cells. We exposed the cells in both cultures to wild-type reoviruses at varying multiplicities

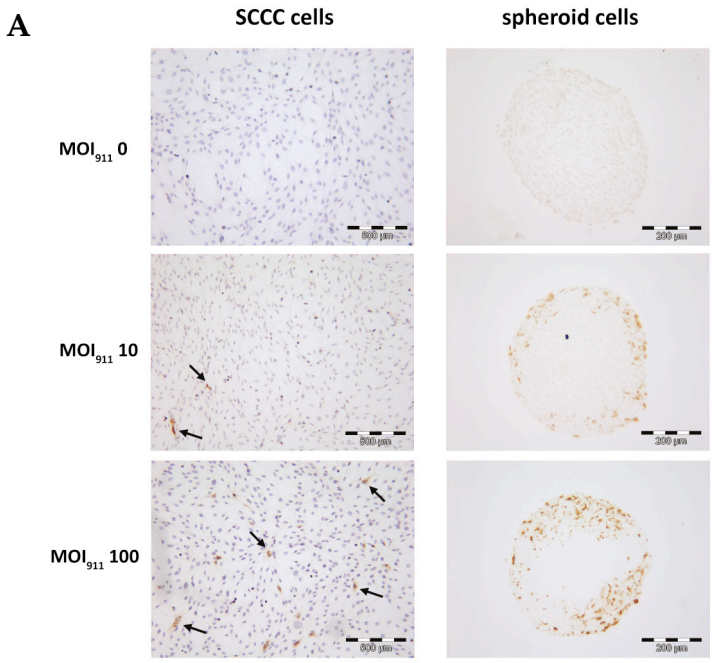

FIGURE 3.
Reovirus infection of U-118 MG SCCC and spheroid cultures.
A. Detection of reovirus protein σ3 by DAB staining in reovirus-infected U-118 MG SCCC and spheroid cultures, at 72 h post infection. The arrows indicate σ3-positive cells in the SCCC culture.

of infection (MOI_{911}), ranging from 0 to 100 PFU_{911} per cell. Three-days post infection, the presence of the reovirus protein σ3 was visualized by immunocytochemistry. This revealed only few positive cells at both MOI_{911} 10 and 100 in the SCCC cultures, while the outer rim of the spheroids was almost completely infected at MOI_{911} 10. Virus infection deep into the spheroid was clearly visible at MOI_{911} 100 (**Figure 3A**). The number of infected cells was quantified by flow cytometry by staining for the reovirus protein σ3 with a fluorochrome R-Phycoerythrin (PE)-coupled antibody. In SCCC cultures only 0.3 % and 2.4 % of the cells were positive for σ3 upon exposure to MOI_{911} of 10 and 100, respectively. The cells in the spheroids cultures were more efficiently infected, with 13.0 % and 39.0 % of the cells positive for σ3 expression upon exposure to MOI_{911} of 10 and 100, respectively (**Figure 3B**). Finally, the viability of SCCC cells and spheroids after reovirus infection was assessed eight days post infection (**Figure 3C**). While the viability of SCCC cultures only slightly decreased at increasing virus titer,

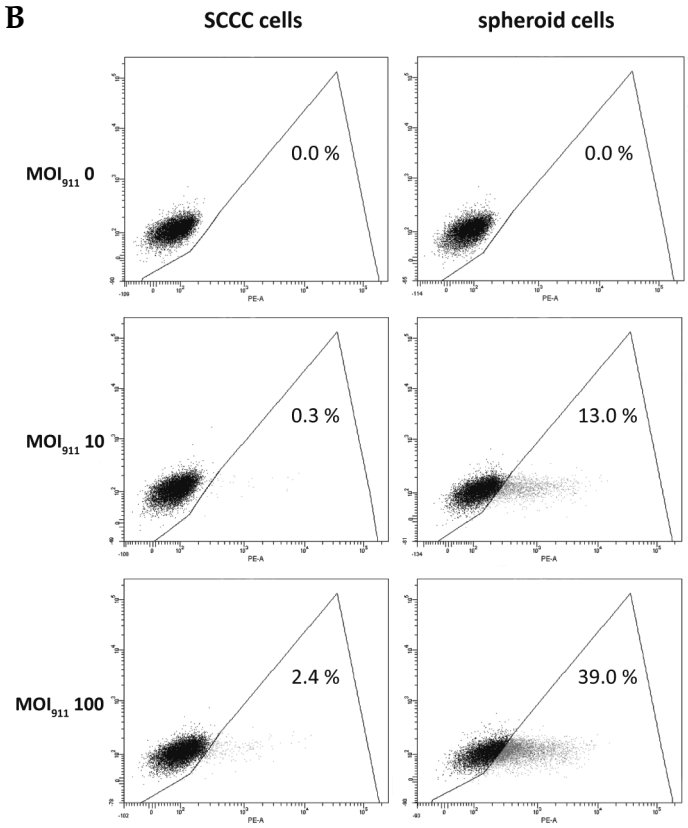

FIGURE 3.
Reovirus infection of U-118 MG SCCC and spheroid cultures.
B. Flow cytometry analysis of reovirus-infected U-118 MG SCCC and spheroid cells by staining for reovirus protein σ3 at 72 h post infection. The figure is representative for three independent samples.

spheroids showed a distinct decrease in viability especially at MOI_{911} 10 and 100. We considered the possibility that the methylcellulose used in formation of the cultures may increase the sensitivity of the cells to reovirus infection. To investigate this, we compared the viability of reovirus infected spheroids generated in culture medium containing methylcellulose or made on a solid agarose matrix. In both spheroids systems the cells were equally susceptible to reovirus (data not shown), demonstrating that the methylcellulose treatment does not increase the cells' susceptibility to reovirus infection.

As compared to cells in monolayer cultures, cells in a spheroid grow at higher concentrations. This allows the cells of a spheroid to affect their local environment

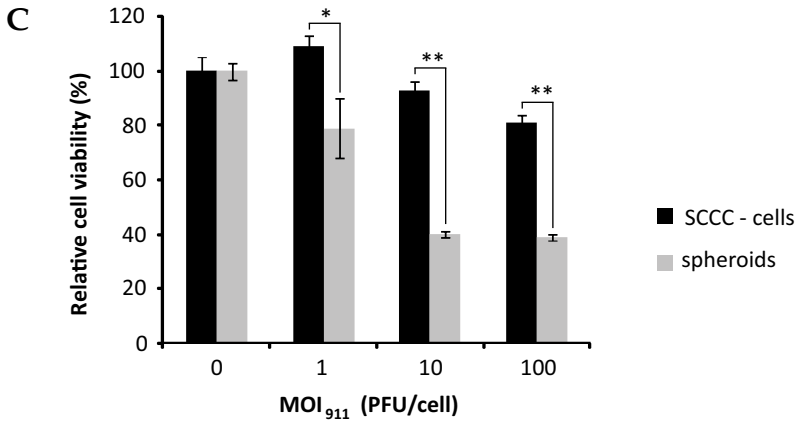

FIGURE 3.
Reovirus infection of U-118 MG SCCC and spheroid cultures.
C. Viability assay of reovirus-infected U-118 MG SCCC and spheroid cultures, at 8 days post infection. The viability of uninfected samples is set to 100%. The error bars represent the s.e.m. (n=12 samples), P-values: *P < 1.0E−2, **P < 5.5E−9.

to a greater extent than cells in monolayer cultures. To test whether a high local concentration of cells in SCCC cultures can mimic the spheroid environment and stimulated JAM-A-independent transduction, we generated SCCC cultures at low, normal, and high density and exposed them to reovirus at MOI_{911} 100. This assay revealed higher infection efficiency (as concluded from σ3 protein staining) for the high density cultures (**Figure 4A**). This was confirmed by flow cytometry to quantify the number of infected cells. Cell cultures with low and normal density exhibited only 0.9 % and 3.3 % reovirus-infected cells, respectively, while 20.8 % of cells in the high density culture were infected (**Figure 4B**). These results demonstrate that, as in spheroid cultures, JAM-A is dispensable for reovirus infection in dense SCCC cultures.

Secreted products enhance reovirus infection of JAM-A negative cells

Our data show that U-118 MG cells in spheroid cultures are more susceptible to reovirus infection than their counterparts in SCCC cultures, despite the absence of JAM-A. Since tumours are known to secrete proteases into their microenvironment [27], which may result in reovirus disassembly outside the cell, we speculated that a similar process may occur in the medium surrounding the U-118 MG cells in spheroid cultures. To test this hypothesis, we collected medium from U-118 MG cells in SCCC and in spheroid cultures five days after establishing

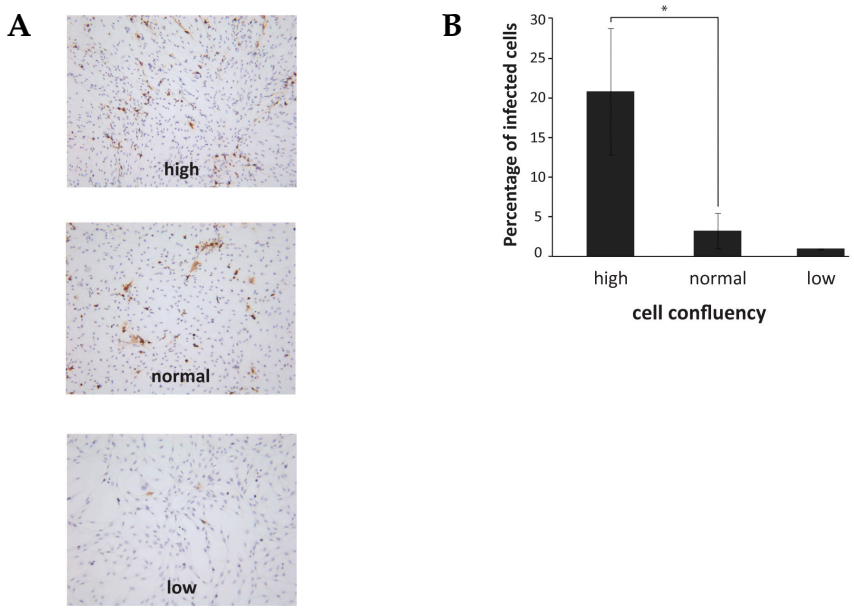

FIGURE 4.
Reovirus infection of U-118 MG SCCC cultures at different densities.
A. Detection of reovirus protein σ3 by DAB staining in SCCC cultures at different densities infected with reovirus at MOI_{911} 2.5 of 100, at 4 days post infection.
B. Flow cytometry analyses of reovirus-infected (MOI_{911} of 100) SCCC cultures at different densities. The error bars represent the s.d. (n=3), *P < 3.0E−2.

these cultures. Reoviruses were added to the collected media, which was subsequently exposed to semi-confluent U-118 MG SCCC cultures at MOI_{911} of 100. Three days post infection, the number of infected cells was analysed by flow cytometry. While U-118 MG cells infected with reovirus in presence of fresh medium or medium from adherent cells show only 2.8 % and 2.3 % infected cells, respectively, the spheroid medium with reovirus yielded an infection in 16.2 % of the cells (**Figure 5A**). This increase in infection was supported by a viability assay on semi-confluent U-118 MG cells. As semi-confluent U-118 MG cells do not readily exhibit a cytopathic effect upon reovirus infection, cells were replated in fresh medium three days post infection and allowed to attach and proliferate. The next day the cells' viability was assessed. While infection (at MOI_{911} of 10) in the context of fresh medium or SCCC-derived medium showed a marginal decrease in viability, the viability was reduced to 70% after infection in the context of spheroid-derived medium (**Figure 5B**). At MOI_{911} of 100, a more pronounced decrease in viability was observed for cells infected in presence of fresh medium

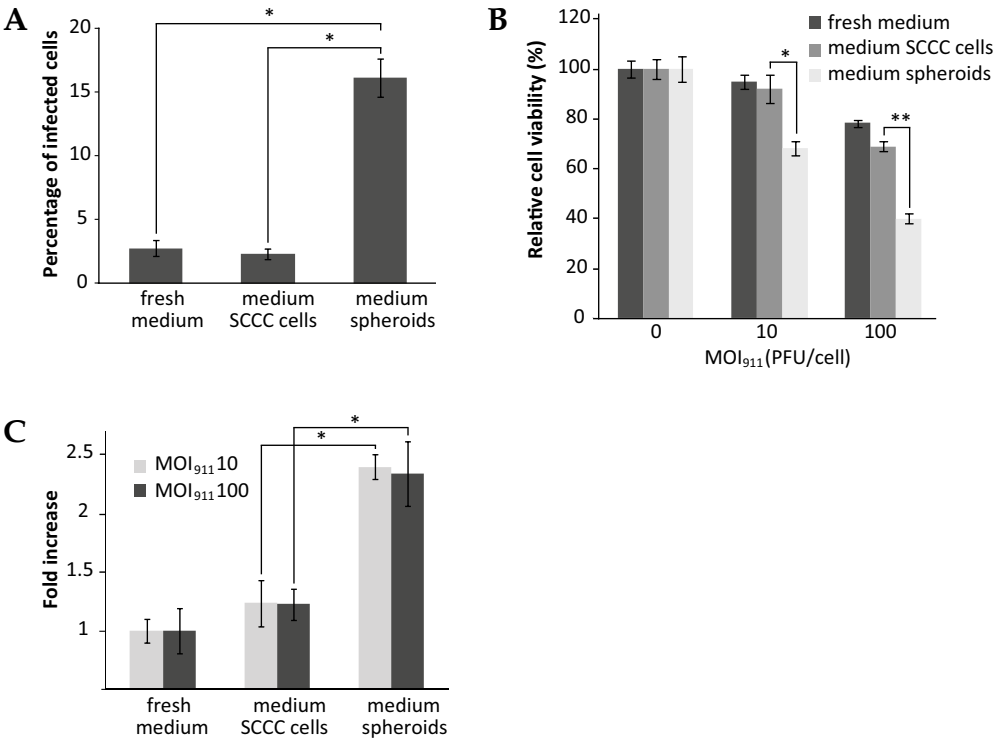

FIGURE 5.
Assessment of the effects of culture-derived factors on reovirus infection efficiency.
A. Flow cytometry assay on reovirus- infected U-118 MG cells (MOI_{911} of 100) in the presence of medium derived from U-118 MG SCCC cultures, U-118 MG spheroid cultures or fresh medium, at 3 days post infection. Error bars indicate the s.d. (n = 3), *P < 2.5E−4.
B. Viability analysis of U-118 MG cells exposed to reovirus at MOI_{911} of 100 in the presence of medium derived from U-118 MG SCCC cultures, spheroid cultures or fresh medium. Error bars represent the s.e.m. (n=12), *P < 3.6E−3, **P < 1.2E−6.
C. Flow cytometry analyses of U-118 MG cells infected with reovirus that were previously incubated with medium derived from U-118 MG SCCC cultures, spheroid cultures or fresh medium. The error bars indicate the s.d. (n=3), *P < 5.0E−3.

or SCCC cell medium. However, the viability of cells infected in spheroid-derived medium decreased even more, up to 40%.

We next investigated whether the medium derived from spheroid cultures indeed has a direct effect on the virions, thereby promoting the infection of JAM-A-deficient cells. We dissolved reovirus into medium from SCCC cells, medium from spheroids, and non-conditioned control medium, and subsequently purified the virus by CsCl banding. Infection of semi-confluent U-118 MG cells in monolayer cultures revealed that the virus particles incubated with SCCC-derived

medium do not enhance transduction of U-118 MG cells (**Figure 5C**). In contrast, infection with virus that had been pre-exposed with spheroid-derived medium was significantly increased. These data demonstrate that one or more factors secreted by the U-118 MG cells in spheroid cultures enhance the reovirus infection of JAM-A negative cells.

Cathepsin B and L secretion of spheroids is upregulated and enhances reovirus infection of cells

Many tumours produce high amounts of cathepsins that are secreted into the extracellular microenvironment [27, 28]. Cathepsin B and L are known to be involved in reovirus disassembly in the endosome [12]. To test whether cathepsin B and L are secreted by U-118 MG spheroids and could activate the reovirus entry, we examined the cathepsin B and L activity in cells and medium of U-118 MG SCCC and spheroid cultures. This demonstrated a two-fold increase in intracellular cathepsin B and L activity in the U-118 MG spheroid cultures compared to SCCC cultures (**Figure 6A**). In the media, this difference was even more pronounced, with a 19 and 24-fold increase, respectively. To investigate whether cathepsin B and L can modify reovirus particles and enhance infection of semi-confluent SCCC cultures of U-118 MG cells, we incubated reovirus stocks with either cathepsin B or L. After virus purification, semi-confluent SCCC cultures of U-118 MG cells were exposed to these particles and analysed three-days post infection by flow cytometry. This revealed a five-fold increase in the percentage of infected cells incubated with cathepsin B-treated virus, as compared to virus incubated in the cleavage buffer alone. Cathepsin L treatment rendered the virus two-fold more infectious in U-118 MG cells (**Figure 6B**). These data demonstrate that cathepsins secreted by U-118 MG cells grown in spheroid cultures can modify reovirus particles allowing them to infect JAM-A deficient U-118 MG glioma cells.

3.4 Discussion

Based on *in vitro* studies, JAM-A has originally been characterized as the major cellular receptor of reovirus [9]. The function of JAM-A in reovirus T3D infection *in vivo* became evident by studies using JAM-A-deficient animals [29]. While the virus' distribution towards the central nervous system, which is the major tropism in mice, can occur via both neural- and bloodstream routing, only the bloodstream routing appears to be JAM-A dependent [29]. Also, JAM-A is not required for the initial *in vivo* infection with reovirus T3D in the intestines [15, 16] and in the lungs

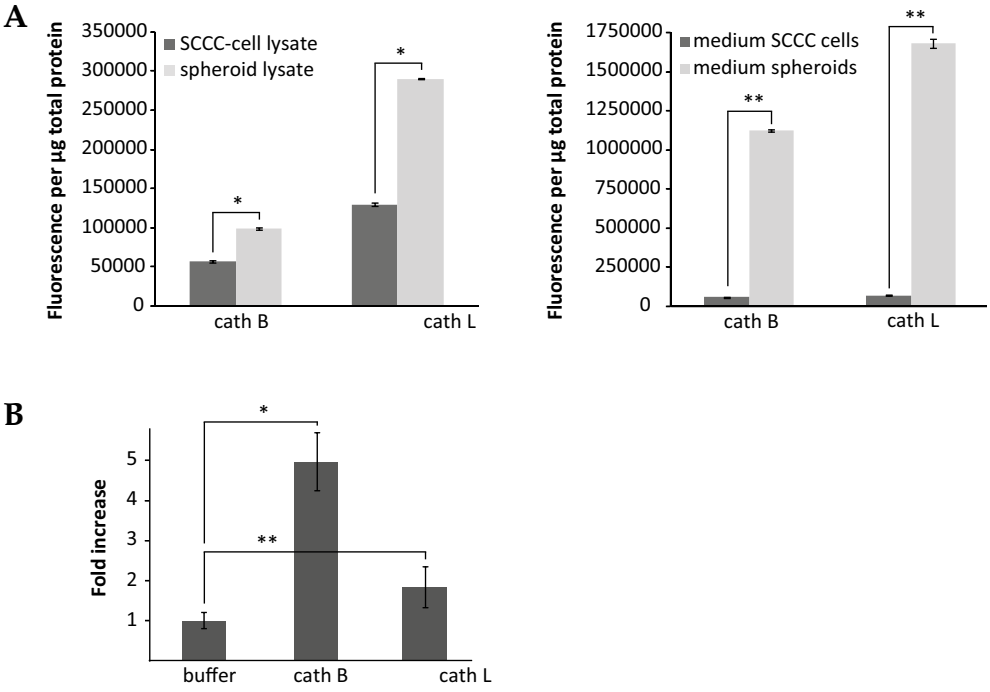

FIGURE 6.
Cathepsin B and L activity in U-118 MG cultures and culture medium, and its effect on reovirus infection.

A. Cathepsin B and L activity in the cells and conditioned media of U-118 MG SCCC cultures and spheroids. Error bars indicate the s.d. (n = 3), *P < 2.0E−6 **P < 9.0E−10.

B. Increase in reovirus infectivity in semi-confluent SCCC cultures of U-118 MG cells upon exposure of the virus to Cathepsin B, Cathepsin L or buffer. After treatment, the virus preparations were purified by CsCl centrifugation before the infectivity assay. The number of infected cells was assayed by flow cytometry at 3 days post infection. The graph shows the fold increase in infectivity over buffer-treated virus. The error bars indicate the s.d. (n = 3), *P < 5.0E−3; **P < 5.0E−2.

[17]. Here the reovirus can infect as ISVPs upon proteolytic conversion by extracellular proteases.

For reovirus' use as oncolytic agent, the importance of JAM-A is unclear. Whereas reovirus is able to induce apoptosis after JAM-A independent cell entry [30], the presence of JAM-A on the cell membrane of tumour cell lines has been reported to be important for the cells' susceptibility to reovirus infection *in vitro* [31, 32]. JAM-A positive tumours *in vivo* are generally well infected by reovirus [32, 33] and resistance to reovirus is observed when JAM-A is absent, or not located on the tumour cell surface [5, 32]. Remarkably, some cell lines that are

JAM-A negative *in vitro*, can form reovirus susceptible tumours upon implantation as xenografts in mice. Alain *et al.* demonstrated that U-118 MG tumours in mice are susceptible to reovirus infection, in marked contrast to the reovirus resistance of U-118 MG cells grown in adherent cell monolayer cultures [6]. Here we demonstrated that also *in vitro* JAM-A can be dispensable for reovirus infection. Despite high resistance to reovirus of U-118 MG cells in monolayer cultures, U-118 MG spheroids can be readily infected and killed. Similar observations have been made in glioblastoma stem-like cell (GSC) cultures, isolated from tumour resections of glioma patients [21].

We demonstrate that the increased sensitivity to reovirus infection of U-118 MG cells in spheroid cultures is mediated by components secreted into the medium. Infection of JAM-A deficient adherent cells in presence of conditioned medium from spheroid cultures significantly enhances the capacity of the reovirus to infect these cells. In many tumour types, such as mammary-carcinoma, prostate cancer and glioma, the secretion of proteases is upregulated [27, 28]. In a tumour and its microenvironment these proteases are part of complex, multidirectional pathways interacting to modulate key processes as angiogenesis, metastasis and ECM composition, ultimately promoting tumour progression [27, 34]. The main players in this network are cathepsins (predominantly cathepsin B), metalloproteinases (MMPs) and urokinase plasminogen activator [27, 28, 35]. For the reovirus infectious pathway, cathepsins B, L and S are important in the disassembly process of the virion [12, 36, 37], creating ISVPs that are able to escape the endosome. In the acidic environment of tumours and spheroids [24, 38, 39], reovirus ISVPs may be generated extracellularly. This could provide the reoviruses with a mechanism that bypasses JAM-A for cell entry [14]. There is evidence that cathepsin B expression is upregulated in U251N glioma spheroids compared to monolayer cells [40] and that cathepsin B and L expression is higher in reovirus sensitive U-118 MG tumours in mice than in resistant adherent cells [6]. We extend these observations and show that cathepsin B and L expression and secretion is strongly enhanced in U-118 MG cells grown in spheroids compared to cells in adherent monolayer cultures. Approximately 20-25 fold enhanced secretion of active cathepsins B and L were found in the medium of spheroids, reminiscent of the enhanced secretion of cathepsins by tumours *in vivo*. The cathepsins may be involved in the deposition and organisation of ECM components, such as fibronectin and proteoglycans, as observed in U-118 MG and other glioma spheroids [41]. The depositions of ECM in our spheroid cultures, as readily observed by EM analysis, may have been the result of increased cathepsin secretion.

We also demonstrated that the infectivity of reovirus particles in U-118 MG cells can be affected by components in the spheroid-conditioned medium. The

amount of activating proteases is limiting activation, as is evident from the observation that five- and 25-fold dilutions of the conditioned media in fresh medium were incapable of generating reoviruses capable of infecting U118MG cell in SCCC cultures (data not shown). It should be noted however, that the effective concentrations of activating proteases at the cell surface may be higher than the amounts that are present in the conditioned media. Purified cathepsins could convert reoviruses to particles that are infections for U118 cells in SCCC cultures. Moreover, we demonstrated that JAM-A independent infection by reoviruses could be enhanced when the U-118 MG cells were grown at very high densities (**Figure 4A**). These conditions may mimic the high cell density seen in spheroid cultures.

There may be other cellular processes or components that enhance reovirus infection in our spheroid model. Our data do not exclude the potential involvement of other secreted proteases such as MMPs. MMP expression is upregulated in many gliomas [27] and MMPs have been shown to enhance the intratumoral spread of herpes simplex viruses and adenoviruses by remodelling the ECM [42, 43]. A similar mechanism might also augment reovirus spread in spheroids and tumours. It may therefore be useful to study the production of proteases by cellular explants from solid tumors that will subjected to oncolytic reoviruses therapy.

By showing that spheroids can upregulate the secretion of proteases, and consequently display an enhanced susceptibility to reovirus, we demonstrated that spheroids are a valuable *in vitro* model, better resembling the *in vivo* situation than SCCC cultures. The spheroid model should therefore be considered as alternative for monolayer tumour cell cultures. It could be implemented in studies for new therapeutics prior to experiments in tumour-bearing animals. It could provide information about the anticancer agent's efficacy that may be more faithfully mimicking the *in vivo* effects than adherent cell monolayer cultures.

3.5 Materials and methods

Cell lines

The human cell lines U-118 MG (glioblastoma) and 911 (adenovirus type 5 early region 1 transformed human embryonic retinoblasts [44]) were maintained at 37°C in a humidified atmosphere of 5% CO_2, in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL) and penicillin-streptomycin (penstrep). The generation of U-118HA-JAM cells by transducing U-118 MG cells with a lentiviral vector encoding hemagglutinin (HA)-tagged JAM-A, was described

previously [19]. These cells were cultured in DMEM high glucose, supplemented with 8% FBS, penicillin, streptomycin, and 200 μg/ml G418 (Life Technologies, Carlsbad, CA, USA).

Reovirus production and purification

The wild-type T3D reovirus strain R124 (here referred to as reovirus) was isolated previously from a reovirus T3D stock (stock VR-824) obtained from the American Type Culture Collection (ATCC) by two rounds of plaque purification using 911 cells. Large-scale virus production was performed by infecting 911 cells with a multiplicity of infection (MOI) of 1 to 3 plaque forming units (PFU_{911}), followed by medium replacement at three hours post infection, and harvesting of cells and medium at 72 hours post infection. Cells and medium were separated by centrifugation for 15 minutes at 3000 x g. The pellet was resuspended in 2 ml of the medium fraction and subjected to three cycles of freezing and thawing. The sample was cleared by centrifugation for 10 minutes at 3000 x g and the supernatant was mixed with the medium fraction. From this suspension, the virus was further purified by a double discontinuous caesium chloride (CsCl) gradient protocol. Hereby, the virus suspension was layered onto a double-layered (1.45 and 1.20 g/cm^3) CsCl cushion in phosphate buffered saline (PBS) and centrifuged in a SW28 rotor at 95,000 x g for 3 hours at 16°C. After the first centrifugation step the lowest of the two virus bands (containing the infectious virions) was isolated with a syringe, and loaded onto a new CsCl gradient and centrifuged as before. After isolation the virus particles were desalted in an Amicon Ultra-15 Centrifugal Filter Device (molecular weight limit of 100 kDa, Millipore, Billerica, MA, USA). The virus was stored in reovirus storage buffer (10 mM Tris pH 7.5, 150 mM NaCl, 10mM $MgCl_2 \cdot 6\ H_2O$, 5% sucrose) at -80°C. The infectious titer of the virus was determined by plaque assay on 911 cells.

Preparation and infection of SCCC cultures and spheroid cultures

To grow U-118 MG and U-118HA-JAM cells in standard cell cultures conditions (SCCC) (adherent to a plastic surface) and as spheroid cultures, the cells were harvested from semi-confluent monolayers by trypsin treatment, counted and resuspended in medium containing 2.4 mg/ml methylcellulose (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) at the concentration of 50,000 cells/ml (described in [26]). Of these suspensions, 100 μl was added into each well of a flat-bottom (for SCCC cultures) or U-bottom (for spheroids) 96-well-plate. Cells were re-inserted in the incubator for 24 hours to allow their adherence or establishment as spheroid (one spheroid per well (5,000 cells/well)). Methylcellulose was removed by repeated washes with medium. Reovirus infection was done

with an MOI_{911} ranging from 0 to 100 PFU per cell. Unless stated otherwise, the virus concentrations (PFU/µl) were kept equal for SCCC cultures and spheroids. As the number of cells per well differed between the two cell culture methods, the culture medium volume in the wells was adjusted to obtain the desired titer in PFU per cell. To count the number of cells per well, cells were dissociated using TrypLe Select (Gibco-BRL) and counted using a hemocytometer. The diameter of the spheroids was measured on an Olympus CK40 microscope, using an Olympus Camedia Digital Camera C-3030 and Olympus software; Olympus DP-soft.

Immunocytochemistry analysis

SCCC cultures

For immunocytochemistry analysis, SCCC cultures were grown on glass coverslips, fixed in 4% formaldehyde for 15 minutes at room temperature (RT), washed with PBS and further permeabilized in PBS containing 0.1% triton for 15 minutes. After exposure to blocking solution (10% goat serum in PBS) for 1 hour at RT, cells were incubated for 3 hours at RT with antibody 4F2 directed against reovirus σ3 protein (monoclonal antibody [45], obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242), 1:200 diluted in blocking solution. After washing in PBS, the samples were incubated with polyclonal goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) (P0447, Dako, Glostrup, Denmark), 1:50 in blocking solution, for 30 minutes at RT. After extensive washing, the cells were stained with a filtered 3,3'-diaminobenzidine (DAB) solution (10mg DAB dissolved in 10 ml PBS and mixed with 10 ml H_2O and 10 µl 30% H_2O_2) under the microscope and immersed in H_2O after appearance of the brown colour. After dehydration by incubation in increasing concentrations of ethanol (50-100%), the samples were briefly exposed to xylene and mounted in Pertex (Pertex Mounting Medium, Leica Biosystems, Wetzlar, Germany).

Spheroids

Spheroids were fixed overnight in 4% formaldehyde at 4°C, dehydrated and stained in an eosin solution (1% eosin in ethanol) for 10 minutes. After removing the excess of eosin by washing with 100% ethanol, spheroids were incubated twice in xylene for 15 minutes and embedded in paraffin. The paraffin blocks were sectioned in 6 µm thick slices on a microtome and after paraffin stretching in a 40°C water bath, transferred to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and allowed to dry. The slides were deparaffinised in xylene, before rehydration of the samples by immersion in decreasing concentrations of ethanol (100-50%). The antigens were retrieved by heating the slides in 10

mM sodium citrate buffer (0.19 g citric acid monohydrate and 1.2 g trisodium citrate dehydrate in 500 ml H_2O) and maintaining the temperature just below the boiling point for 6 minutes. Samples were allowed to cool, washed twice in H_2O and incubated in 0.3% H_2O_2 for 10 minutes. After a brief wash in H_2O and PBS, samples were blocked and further processed as described above for the SCCC samples. Ki-67 protein staining was performed to detect proliferating cells. For this, uninfected spheroids were treated as outlined above, though incubated with a primary purified mouse antibody against human Ki-67 (BD Biosciences), 1:100 diluted in blocking solution.

Electron microscopy analysis of the spheroids

Spheroids were fixed in 1.5% glutaraldehyde, postfixed in 1% osmium tetroxide and subsequently en-block stained in 1% aqueous uranyl acetate. After dehydration in ethanol, the samples were embedded in Epoxy resin LX-112 (Ladd Research). Ultrathin 100-nm sections were prepared and post-stained with uranyl acetate and lead citrate for ultrastructural analysis. Electron microscopy images were obtained in an FEI Tecnai 12 BioTwin operated at 120 kV and equipped with an Eagle cooled slow-scan charge-couple device (CCD) camera (FEI Company, USA). A total of 3545 overlapping images were collected at a magnification of x 6800 and binning 2 (pixel size of 3.2 nm at the specimen level) and stitched together into a single virtual slide as previously described [46].

Flow cytometry analysis

Anti-JAM immunostaining

Adherent cell cultures of U-118 MG and U-118HA-JAM cells and spheroids of U-118 MG cells were prepared as described before. Three days post preparation of spheroids or SCCC cultures, cells were harvested by dissociating the cells using TrypLe Select. Cells originating from 24 wells were pooled and fixed by incubation in 4% formaldehyde for 15 minutes at RT. After washing cells in staining buffer (1% FBS, 0.09% w/v sodium azide in PBS, pH 7.5, filtered), cells were incubated for 1 hour at RT with a 1:200 dilution of mouse monoclonal anti-JAM-A antibody (clone M.Ab.F11, Abcam, Cambridge, UK) in staining buffer. After washing, the cells were exposed to R-phycoerythrin (PE) fluorochrome-conjugated rat anti-mouse immunoglobulins (IgG_1, BD Biosciences) for 30 minutes at RT in the dark, 1:100 diluted in staining buffer. Subsequently, the cells were washed extensively, resuspended in FACS buffer (0.5% BSA, 2mM EDTA in PBS) and assayed on a BD LSRII flow cytometer, 10,000 events per sample. Data were analyzed with FACSDiva software (BD Biosciences).

Anti-reovirus immunostaining

To analyze the susceptibility to a reovirus infection, SCCC cultures and spheroids of U-118 MG cells were prepared and infected with MOI_{911} of 10 or 100 PFU per cell. Three days post infection cells were dissociated, pooled and fixed as above, washed in staining buffer. After permeabilizing the samples in Perm/Wash Buffer (BD Biosciences, San Jose, CA, USA) for 15 minutes at RT, they were incubated with primary antibody against reovirus σ3 protein, 1:200 diluted in Perm/Wash Buffer for 1 hour at RT. Subsequently cells were washed and exposed to PE fluorochrome-conjugated rat anti-mouse antibody ($IgG2_{a+b}$, BD Biosciences) for 30 minutes at RT in the dark, 1:100 diluted in Perm/Wash buffer. After extensive washing, the samples were resuspended in FACS buffer and analyzed on the flow cytometer as described above.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from SCCC cultures or spheroids of U-118 MG and U-118HA-JAM cells was extracted using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol, upon sonification at 4°C of the spheroids. First-strand cDNA was generated with 100 ng total RNA using SuperScript II reverse transcriptase (Life Technologies - Invitrogen, Carlsbad, CA, USA) and oligonucleotide hJAM_RT Rev (5'-CACCAGGAATGACGAGGTC-3'). The resulting cDNA was amplified in 30 cycli using oligonucleotide hJAM_RT Rev and hJAM For (5'-ATGGGGACAAAGGCGCAAGTC-3') employing PFU DNA polymerase (Thermo Fisher Scientific – Fermentas) and visualized with gel electrophoresis on a 1% agarose matrix together with plasmid pcDNA-HA-JAM as a positive control. As a loading control, 25 ng of total RNA was used to generate cDNA with oligonucleotide Hum_β-actin Rev (5'-TCCTTCTGCATCCTGTCGGGCA-3'). Amplification was performed using oligonucleotides Hum_β-actin For (5'-CAAGAGATGGCCACGGCTGCT-3') and Hum_β-actin Rev.

Cell viability assay

Eight days post infection of SCCC cultures or spheroids the cell viability was assessed by replacing the culture medium by fresh medium containing 10% WST-1 reagent (Roche, Penzburg, Germany) in 12 wells per condition. After 1 hour (for SCCC cultures) or overnight (for spheroids) the absorbance was measured in a microplate reader (Bio-Rad model 550, Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm. The viability measurements were normalized to the viability of uninfected cultures.

Analyzing high and low confluent adherent cells cultures

MG cells in low (split ratio 1 to 25), normal (1 to 10) or high (1 to 2.5) confluence in 6 well (high and normal confluence) or 24 well (low confluence) plates. Two days post preparation the cells were counted and infected as described before Four days post infection the cells were harvested, fixed and assayed for reovirus expression on the flow cytometer or stained for immunocytochemistry analysis as described before.

Analyzing secretion products of spheroids

Semi confluent U-118 MG monolayers were grown in 6 well plates (flowcytometry assay) or 96 well plates (viability assay) and infected with reovirus with an MOI ranging from 0 – 100 PFU per cell in fresh culture medium or culture-derived medium (isolated from SCCC cultures or spheroid cultures after 5 days of incubation). The percentage of reovirus infected cells was analyzed on the flow cytometer. For viability analysis, cells were dissociated, at four days post infection, using TrypLe Select and seeded in 48 well plates using fresh medium. The next day, the viability of the cells was examined by WST-1 assay.

Reovirus incubation with conditioned medium or cathepsins

The effect of secretion products of spheroids on the virus was studied by incubating 10^9 PFU reovirus with 1.5 ml fresh medium, or 5-day-old conditioned medium from adherent cells or spheroids at 37°C. At 24 and 48 hours post incubation, 1 ml fresh or conditioned medium was added to the incubation, to ensure the presence of active secretion products. For determining the effect of cathepsins on the virus, 10^9 PFU reovirus was incubated with 12.5 μg cathepsin B or L in cathepsin cleavage buffer (100 mM NaCl, 15 mM $MgCl_2$, 50 mM sodiumacetate (pH 5.0) [47]. After 72 hours incubation, the solutions were loaded on a CsCl gradient (1.55, 1.45 and 1.20 g/cm^3 in PBS) and centrifuged as described before. The virus band was isolated and the CsCl was removed by Amicon filter purification. In the purification the volumes and conditions were standardized for all samples. The purified virus was added to semi-confluent cultures of U-118 MG cells at concentrations of 10 and 100 PFU/cell. Three days post-infection the percentage of reovirus-infected cells was analyzed by flow-cytometetry.

Measurement of cathepsin L and B activity

Enzymatic cathepsin L and B activity was examined in conditioned medium and cell lysates of SCCC and spheroid cultures with a Cathepsin B or Cathepsin L Activity Assay Kit (Abcam). After five days of culturing, cells and medium were

separated, medium was stored at 4°C and cells were counted. Cells were lysed in the kit's lysis buffer and the lysate from two 96 well plates was pooled, centrifuged and protein content was measured using a BCA protein assay (Pierce, Etten-Leur, The Netherlands). 50 µl of cell lysate or medium was assayed for cathepsin B or L activity according to the manufacturer's protocol.

Statistical analysis

The statistical significance of key data was assessed using GraphPad Prism V6 by performing a the Student's t Test. P-values below 0.05 were considered significant.

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Chapter 4

A strategy for genetic modification of the spike-encoding segment of human reovirus T3D for reovirus targeting

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Contents chapter 4

4.1 Abstract

Human Orthoreovirus Type 3 Dearing is not pathogenic to humans and has been evaluated clinically as oncolytic agent. Its transduction efficiency and the tumor cell selectivity may be enhanced by incorporating ligands for alternative receptors. However, genetic modification of reoviruses has been difficult and genetic targeting of reoviruses has not been reported so far. Here we describe a technique for generating genetically targeted reoviruses. Propagation of wild-type reoviruses on cells expressing a modified σ1-encoding segment embedded in a conventional RNA-polymerase-II transcript leads to substitution of the wild-type genome segment by the modified version. This technique was used for generating reoviruses which are genetically targeted to an artificial receptor expressed on U118MG cells. These cells lack the Junction Adhesion Molecule-1 and therefore resist infection by wild-type reoviruses. The targeted reoviruses were engineered to carry the ligand for this receptor at the C-terminus of the σ1 spike protein. This demonstrates that the C-terminus of the σ1 protein is a suitable locale for the insertion of oligopeptides ligands and that targeting of reoviruses is feasible. The genetically targeted viruses can be propagated using the modified U118MG cells as helper cells. This technique may be applicable for the improvement of human reoviruses as oncolytic agent.

4.2 Introduction

Orthoreovirus Type 3 Dearing (T3D) has been evaluated clinically as oncolytic agent [1, 2]. The impetus for these studies has been the observation that reovirus T3D preferentially lyses tumor cells, especially those with an activated Ras-signaling pathway [3-7]. The *Reoviridae* (Respiratory Enteric Orphan viruses) constitute a family of non-enveloped viruses with segmented double-strand (ds)RNA genomes. The three types of human reoviruses have 10 genome segments and are classified in the genus Orthoreovirus. These viruses are not associated with disease in humans. In newborn mice, however, these viruses can cause lethal infections [8]. The Orthoreovirus Type 3 Dearing (T3D) is often studied and serves as a model for the family. Reovirus T3D enters the host cell through the interaction of the spike protein 1 with its cognate receptor, the Junction Adhesion Molecule (JAM-1). In addition, sialic-acid groups at the cell surface can act as receptors [9-13]. The JAM-1-binding amino acids are located in the C-terminal head domain of σ1 [11, 14-16]. After cell attachment, integrin-binding motifs in the capsid protein λ2, which forms the structural base for σ1, bind β1 integrins and mediate

endocytosis [17]. Following endosomal escape viral replication ensues. Capped plus-strand RNA molecules are formed by transcription of the genome segments by the viral RNA-dependent RNA-polymerase. Plus-strand RNA molecules are used for translation and also associates with viral core proteins in special virus-induced cellular compartments, the so-called "viral factories" [18]. The packaging of the 10 genomic segments must be well orchestrated since each viral core must contain a single copy of each of the plus-strand transcripts. Recent studies suggest that sequences contained within the 130 nucleotides (nt) at the 5′ terminus serve as identity label for each of the segments [19]. Negative-strand synthesis takes place within the newly-formed core, yielding dsRNA segments. These can be further transcribed, or included in maturing virions to be released by the infected cell [20-22]. The plus-strand RNAs contain the tetranucleotide sequence 'GCUA' at the 5′ end and the pentanucleotide sequence 'UCAUC' at the 3′ end, which may have a role in the encapsidation process [23].

The efficiency of reovirus-based oncolytic therapies is likely compromised by the scarcity of reovirus receptors on the surface of tumor cells. Freshly isolated colorectal tumor cells resist T3D infection, probably due a lack of JAM-1 on their surface [24]. The transduction efficiency and the tumor cell selectivity of oncolytic viruses, such as adenoviruses, has been enhanced by genetically incorporating ligands for alternative receptors [25]. However, genetic modification of reoviruses has been notoriously difficult. A few reports have been published on the genetic modification of member of the *Reoviridae* family [21, 26]. However, these systems are arduous and rather inefficient. Recently, a reverse genetics method has been described that relies on transfection of 10 different expression plasmids encoding all of the viral segments [27]. With this method a heterologous transgene was engineered into one of the genome segments. However, the genetic modification of capsid components to amend viral tropism was not reported.

Here we describe a new technique for genetically modifying reoviruses. The technique was used for generating targeted T3D variants carrying $(His)_6$ tags at exposed positions in the head domain of the σ1 spike. Reoviruses carrying the 1-$(His)_6$ spikes, but not wild-type T3D, can infect genetically engineered U118MG glioblastoma cells displaying a single-chain antibody fragment (scFv-His) recognizing the $(His)_6$ tag as an artificial receptor on their surface. The $(His)_6$-tagged reoviruses, in combination with the scFv-His-expressing U118MG cells, can serve as a basis for developing tumor-targeted reoviruses.

4.3 Results

The efficacy and specificity of oncolytic-virus approaches can be enhanced by targeting infection to tumor cells. The development of targeted reoviruses has been hampered by the difficulties in applying reverse-genetics procedures to the segmented dsRNA genomes of these viruses. Therefore we set out to develop a novel technique for generating such genetically targeted reoviruses (**Figure 1**). A cell line was selected that supports reovirus replication but resists infection due to

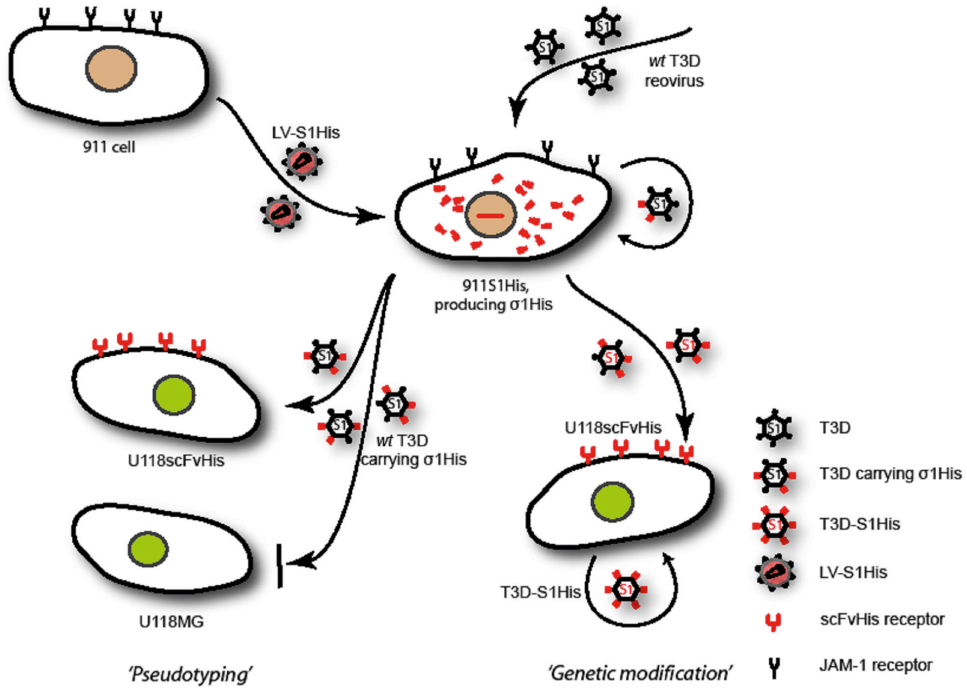


FIGURE 1.
Schematic representation of the selection system for a targeted reovirus.
The lentivirus vector LV-S1His was used to transfer a reovirus S1-expression cassette into 911 cells. The resulting 911S1His cells produce the σ1 protein carrying the $(His)_6$ tag at its C-terminus. Upon infection of the 911S1His cells with wild-type T3D, the progeny viruses may carry a mixture of σ1-His and σ1 spike proteins in their capsid.
As a consequence, these viruses can infect the U118scFvHis cells that display an anti-His-tag single chain-antibody on their cell surface which can serve as an artificial receptor. Unmodified U118MG cells resist reovirus infection. Sequential passaging of virus in U118scFvHis cells selects for viruses in which the wild-type S1 segment has been replaced by the heterologous S1His segment during replication in the 911S1His cells.

the absence of reovirus receptors. This cell line was endowed with a cell-surface protein that can function as an artificial receptor. The codons for the peptide ligand were engineered in a suitable position in one of the reovirus capsid proteins. Finally, the genomic segment encoding this modified capsid protein was introduced in the reovirus genome.

JAM-1 deficiency underlies U118MG resistance to reovirus infection

A cell line lacking reovirus receptors and, as a result resistant to infection, is crucial in the development of genetically retargeted reoviruses. Based on published data [28], the human glioblastoma cell line U118MG was selected as a candidate. To confirm its resistance to reovirus infection, cultures of U118MG cells were exposed to T3D and assayed for cell viability (**Figure 2A**). The viability of U118MG cells was not affected by T3D, up to a multiplicity of infection (MOI) of 100. In contrast, survival of the control cell line 911 declined significantly after infection at a MOI of 0.001. The viability of the cells as measured by WST-1 assay strongly correlated with the induction of CPE (data not shown).

To determine whether the resistance of U118MG cells to T3D is due to the absence of the reovirus receptor JAM-1 [9, 29, 30], RNA isolated from U118MG cells and 911 cells was assayed for the presence of JAM-1 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) using a JAM-1-specific primer set. The RNA from U118MG cells yielded no detectable PCR product (**Figure 2B**), confirming the absence of JAM-1. This was further corroborated by immunofluorescence microscopy with JAM-1-specific antisera (data not shown). In contrast, JAM-1 mRNA was readily detectable in 911 cell-derived RNA (**Figure 2B**).

To study whether the absence of JAM-1 is the sole factor contributing to the resistance of U118MG cells to T3D infection, U118MG cells were transduced with a lentiviral vector encoding Hemagglutinin (HA)-tagged JAM-1 (**Figure 3**). In another vector (LV-JAM-ECD) the extracellular domain (ECD) of JAM-1 was linked to a heterologous transmembrane domain, to formally rule out JAM-1-mediated signaling (**Figure 2C**). Parental U118MG cells and HA-JAM- or JAM-ECD-expressing U118MG cells were exposed to T3D and metabolically labeled with [^{35}S]-Methionine to assess reovirus-protein synthesis, as an indicator for viral replication. No synthesis of reovirus proteins was observed up to 7 days post infection in U118MG. In contrast, synthesis of the reovirus proteins was detectable in the HA-JAM- and JAM-ECD-expressing U118MG derivatives (**Figure 2D**). Note that host-protein synthesis is not fully shut-off by reovirus in U118MG cells. Taken together, these data show that the absence of JAM-1 on the surface of U118MG is the only reason for its resistance to reovirus T3D.

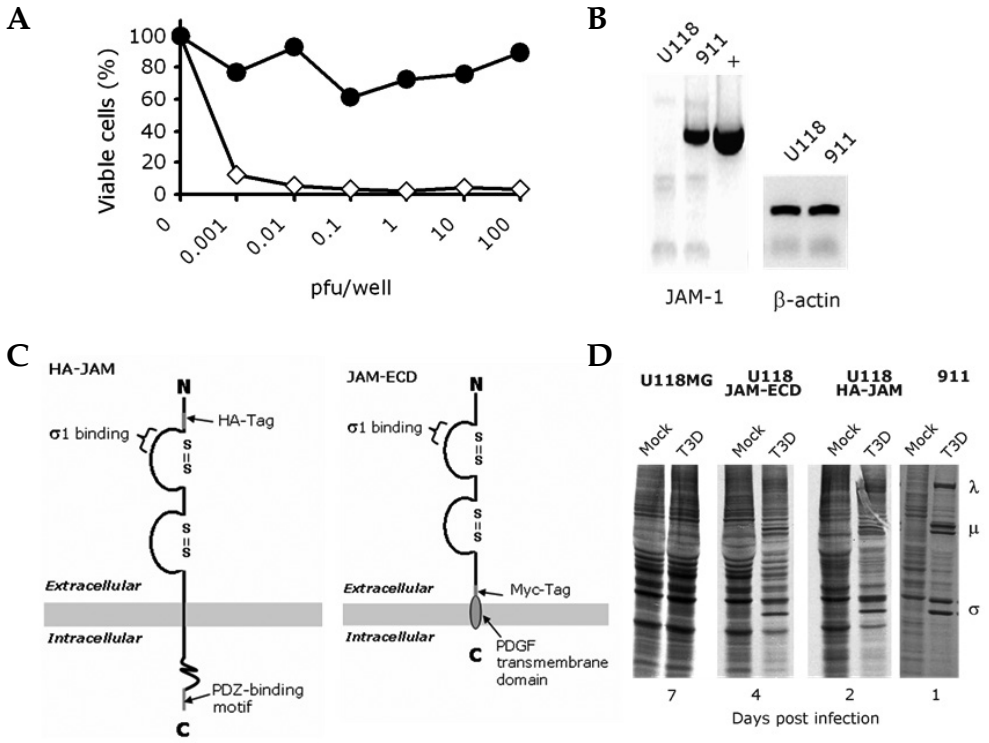


FIGURE 2.
U118MG cells resist reovirus infection due to a lack of JAM-1 expression.

A. U118MG cells survive reovirus infection. The viability of cultures of U118MG cells (●) and cultures of 911 cells (◇) infected with increasing amounts of reovirus T3D was measured two days post infection by WST-1 assay.

B. hJAM-1 RNA is absent in U118MG cells. RNA from U118MG cells (U118) and 911 cells was isolated and used for cDNA synthesis with primer hJAM_RT rev (See **Table 1**). As a positive control (+), a small amount of the pCDNA-HA-JAM plasmid was included in a PCR reaction (product length see **Table 1**). A β-actin-specific RT-PCR assay was performed to confirm the integrity of the RNA.

C. Schematic representation of the HA-JAM and JAM-ECD proteins displayed at the cell membrane.

D. Display of JAM-1 or its extracellular domain sensitized U118MG cells to reovirus T3D. Reovirus T3D-infected cells and mock-infected cells were labeled with [^{35}S]-methionine once CPE became apparent, and protein samples were analyzed by SDS-PAGE. The positions of the reoviral σ, μ, and λ proteins are indicated. JAM-1, junction adhesion molecule-1; RT-PCR, reverse transcription-PCR.

Expression of an artificial receptor on the surface of U118MG cells

As an artificial receptor that could be utilized by targeted reoviruses, we exploited a single-chain antibody fragment specific for stretch of 6 C-terminal Histidine residues (scFv-His) [31] as an artificial receptor for the reovirus T3D.

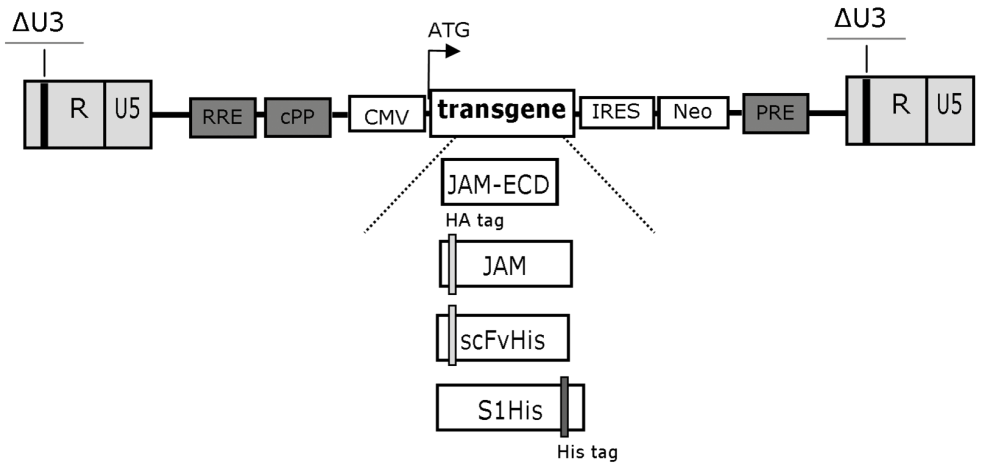


FIGURE 3.
Lentiviral vectors used in this study.
The scheme represents the integrated lentiviral vectors. The vectors are derived from a self-inactivating, third-generation HIV-1 vector. Upon integration, the vector loses its capacity to produce mRNA other than the mRNA derived from the transgene-expression cassette. The positions of the Rev-responsive Element (RRE), the central polypurine tract (cPPT), and the posttranscriptional regulatory element (PRE) are indicated. The transgenes were inserted upstream of the internal ribosome entry site (IRES) and the neomycin phosphotransferase (Neo) selection marker. JAM is the cDNA encoding human Junction Adhesion Molecule. JAM-ECD encodes the extracellular domain of JAM-1 fused to the PDGF transmembrane domain. scFvHis represents the single-chain antibody directed against the $(His)_6$ tag. S1His is a modified cDNA of the reovirus T3D S1 segment in which the $(His)_6$ tag is fused with σ1.

This scFv is expressed on the surface of mammalian cells if linked to a transmembrane domain and has been previously used as an artificial receptor for propagation of recombinant adenoviruses [32]. The codons for scFv-His, linked to the transmembrane domain of the human PDGF receptor and an HA tag, were inserted in the vector pLV-CMV-x-IRES-Neo (**Figure 3**) and the resulting vector was used to transduce U118MG cells. Immunochemical staining confirmed homogeneous expression of the artificial receptor in the transduced U118MG cell population (data not shown).

Modification of σ1 by adding a $(His)_6$-tag to its C terminus

To produce σ1-variants, we opted to using a *trans*-complementation approach in which reovirus was propagated on a cell line producing one of the capsid components. The virus may incorporate the modified component in its capsid during virus generation. To generate reoviruses with modified σ1-variants in its capsid,

TABLE 1.
Primers used in this study.
Abbreviations: JAM-1, junction adhesion molecule-1; RT-PCR, reverse transcription-PCR.
The primers used in this study and fragment lengths of the PCR products expected with the designated primer combination are shown. The restriction sites (primers 4 & 5) and the codons for the $(His)_6$ tag (primer 6) are underlined.

Method	Primer name	Primer number	Sequence	Primer combination	Fragment length (bp)
Jam Detection	hJAM For	1	5′-ATGGGGACAAAGGCGCAAGTC-3′		
	hJAM_RT Rev	2	5′-CACCAGGAATGACGAGGTC-3′	1+2	928
S1 Cloning	ReoS1 For	3	5′-CCAAGCTTGCTATTGGTCGGATG-GATCCTCG-3′		
	ReoS1Rev	4	5′-ATTGCGGCCGCGAT-GAAATGCCCCAGTGCCG-3′	3+4	1435
His-tag addition	HisReoS1 Rev	5	5′-GCAGGGTGGTCTGATCCTCAGT-GATGGTGATGGTGATGCGTGAAAC-TACGCGGGTA-3′	3+5	1423
	SigmaEnd Rev	6	5′-GATGAAATGCCCCAGTGCCGCG-GGGTGGTCTGATCCTCA-3′	3+6	1442
S1His RT-PCR	S1endR	7	5′-GATGAAATGCCCCAGTGC-3′	3+7	1442
	His-Rev	8	5′-GTGATGGTGATGGTGATG-3′	3+8	1403
β-actin RT-PCR	Hum_ β-actin For	9	5′-CAAGAGATGGCCACGGCTGCT-3′		
	Hum_ β-actin Rev	10	5′-TCCTTCTGCATCCTGTCGGGCA-3′	9+10	275
JAM-ECD cloning	JamDP For	11	5′-TGTACTGCAGTGCACTCTTCT-GAACCTGAAGT-3′		
	JamDP Rev	12	5′-TATGCTGCAGGACCCCCACATTC-CGCT-3′	11+12	755

911 cells were generated that stably express a modified S1 gene segments. The S1 genome segment was cloned from wild-type reovirus T3D-infected 911 cells, using primers chosen on the basis of the published S1 sequence [33]. The deduced σ1 amino-acid sequence of the S1 genome segment cloned from the ATCC VR-824 T3D reovirus batch was found to differ from the published S1 sequence at two positions, Ile^{246} to Thr and Thr^{249} to Ile. These mutations are known to abolish a trypsin-sensitive site in the σ1 shaft [34].

The codons for $(His)_6$ tag were inserted by mutation PCR downstream of the triplet encoding the C-terminal Thr of σ1. The resulting recombinant S1-genome segment encoding $(His)_6$-tagged σ1 (S1His) was inserted into a lentiviral vector (**Figure 3**). This vector was used to generate 911 cells which stably express S1His.

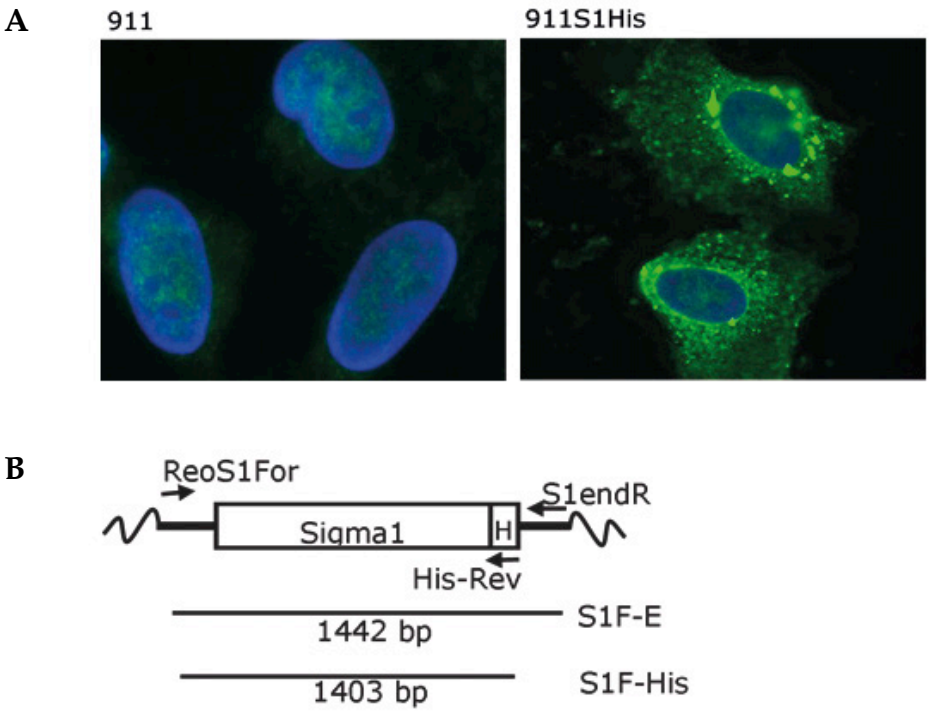


FIGURE 4.
Characterization of the 911S1His cell line.

A. Immunofluorescence assay demonstrating the presence of the -His protein in 911S1His cells. The mouse -His antibody and FITC-coupled Goat-anti-Mouse sera (green) were used to detect the 1-His protein in the LV-S1His-transduced 911 cells. The nucleus is stained with DAPI (Blue).

B. Schematic representation of S1His-expression cassette after lentivirus-mediated gene transfer. The region coding for 1 is indicated by the open box. The primers used in the PCR assay and the expected PCR products are indicted. H represents the position of the $(His)_6$ tag (see **Table 1** for the sequence of the primers).

The synthesis of the $(His)_6$-tagged σ1 (σ1-His) in the polyclonal 911S1His cell lines was verified by immunofluorescence (**Figure 4A**), RT-PCR (**Figure 4B-C**, and **Table 1**), and Western-blot analysis (**Figure 4D**). The staining pattern of σ1-His (**Figure 4A**) is reminiscent of the morphology of the viral factory [8, 35]. These data led us to conclude that σ1-His protein was produced in the transduced cells.

Reovirus pseudotyping by modification of σ-1

To evaluate whether the σ1-His protein was incorporated in the capsid, the 911S1His cells were infected with wild-type T3D, and the virus was passaged three times on the 911S1His cells. If σ1-His is incorporated it could amend the

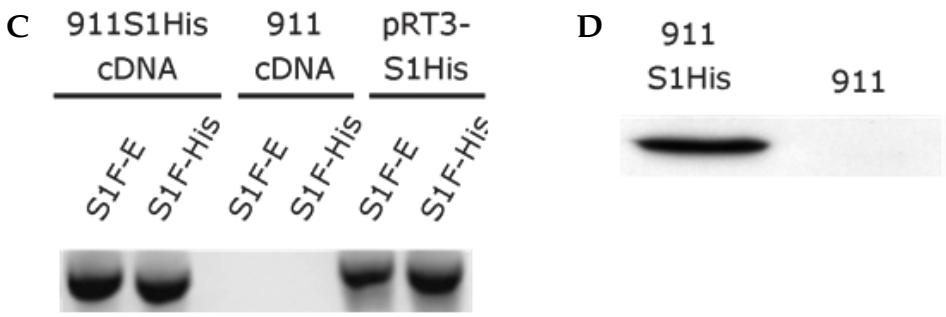


FIGURE 4.
Characterization of the 911S1His cell line.
C. RT-PCR to detect S1 RNA in 911S1His cells. Reverse transcription of 911S1His and 911-cell RNA was performed using primer S1endR. The plasmid pRT3S1His was used as a positive control for the PCR analysis which used the primers indicated in (**B**). Positions of the primers in the S1 segment as well as the expected PCR products S1F-E and S1F-His are indicated in (**B**).
D. Western analysis demonstrating the presence of the 1-His protein in 911S1His cell lysates. 1-His protein was detected with the Penta-His antibody. DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; RT-PCR, reverse transcription-PCR.

tropism. To test the tropism U118MG, U118HA-JAM and U118scFvHis cells were infected with either wild-type T3D or with T3D from the 3rd passage on 911S1His cells. T3D harvested from 911S1His cells, but not wild-type T3D virus, could infect and lyse U118scFvHis cells (**Figure 5A**). The cell-viability assay confirmed that U118MG cells resist the σ1-His containing T3D, and showed survival of 10% of the U118HA-JAM cells at 3 days after exposure to the 911S1His-cell derived T3D. U118scFvHis cells exposed to the σ1-pseudotyped T3D showed a drop of 55% in viability (**Figure 5B**). These data demonstrate that the 911S1His-derived reovirus can infect U118MG cells that express the scFvHis as an artificial receptor. From these results, we conclude that σ1-His is incorporated in the reovirus capsid, that the $(His)_6$ tag is accessible to the artificial receptor, and that interaction of this tag with its receptor leads to productive infection.

Selecting a genetically modified reovirus

Recent data suggest that the sequence motifs that facilitate assortment and packaging of the reovirus segments are contained within a 130 nt region at the 5′ end of reoviral RNAs [19]. This region is also present in the heterologous transcripts produced in 911S1His cells. If the reoviral signals in the LV-S1His-derived σ1-His-encoding transcripts are functional, these transcripts should associate with newly formed cores and replace the wild-type S1 segment in the progeny virus.

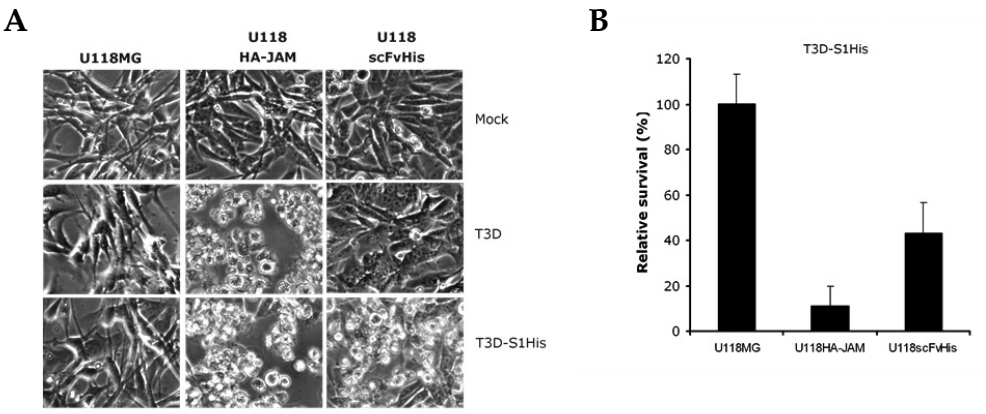


FIGURE 5.
Pseudotyping of T3D reovirus after propagation in 911S1His cells.
A. CPE induction in cultures of U118scFvHis and U118HA-JAM cells, but not U118MG cells, infected with reovirus T3D harvested from 911S1His cells. Photos of CPE were taken three days post infection.
B. Cell viability of U118MG, U118HA-JAM and U118scFvHis cells, as measured by WST-1 assay, three days after infection with reovirus T3D harvested from 911S1His cells. The relative survival is depicted, normalized to mock-infected U118MG cells (mean of three measurements).

To test this hypothesis, we harvested the virus progeny from the U118scFvHis cells that were infected with 1-pseudotyped T3D. If the particles contain the S1His genome segment, it should be possible to propagate them on U118scFvHis cells. Indeed, a metabolic labeling experiment performed at three days post infection showed that the reovirus particles produced in U118scFvHis cells could again infect U118scFvHis, in contrast to unmodified T3D (**Figure 6A**). The propagated virus, which was named T3D-S1His, was passaged 11 times and maintained the 1-His protein as assayed by Western blotting (**Figure 6B**). The presence of the $(His)_6$-tag was also confirmed by sequence analysis of cloned RT-PCR products (**Figure 7**). These data suggest that S1His segment is incorporated as a genome segment in T3D, and expanded its tropism.

Selection for the $(His)_6$-tag co-selects other alterations in genome segment S1

So far we demonstrated that the $(His)_6$ tag of the S1 segment is incorporated in the reovirus. To test whether selection for the presence of the $(His)_6$ tag could be used for selecting other mutations in the same segment, we repeated the selection experiment with the T3D laboratory strain R124. Because the nucleotide sequence

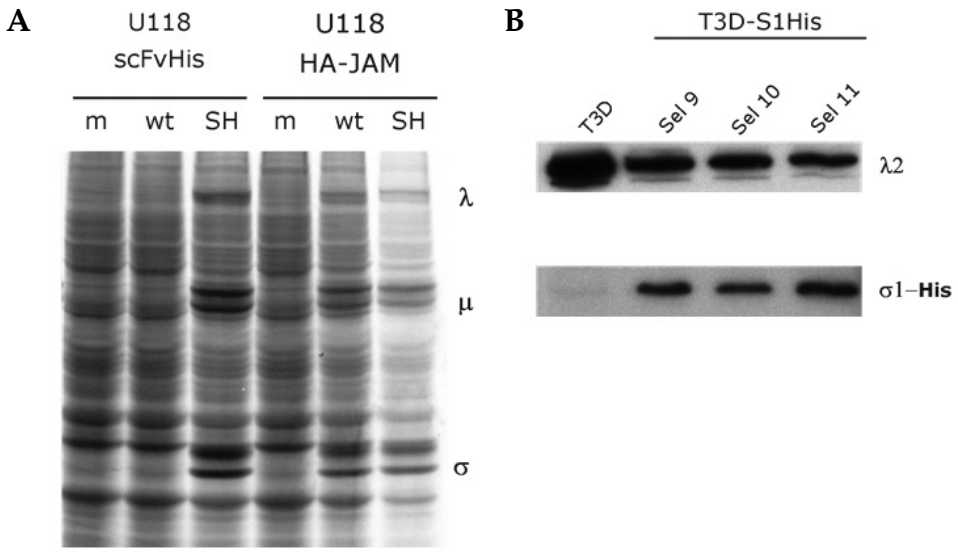


FIGURE 6.
Selection of genetically stable T3D-S1His reovirus stock on U118scFvHis cells.
A. U118scFvHis cells synthesize reovirus proteins upon infection with T3D-S1His. [^{35}S]-Methionine-incorporation assay were performed three-days post infection on U118scFvHis and U118HA-JAM cells infected with wild-type reovirus T3D (*wt*), T3D-S1His 740 (SH) reoviruses, or mock-infected (m), respectively.
B. 1-His is present in S1His reovirus batches as evidenced by Western analysis of reovirus-containing lysates. Western analyses were performed on different viral lysates for the presence of the $(His)_6$-modified 1 using the anti His-tag serum. T3D depicts a lysate of the unmodified reovirus T3D. Sel9 -11 represents T3D reovirus lysates propagated for 9 to 11 passages on U118scFvHis cells. Loading controls using the antibody 7F4 against reovirus λ2 are depicted in the upper panel. $(His)_6$-tagged σ1 detected with Penta-His antibody is shown in the lower panel.

of S1 from R124 differs in two nucleotides from that used to construct LV-S1His (**Figure 7**). This allows distinguishing the origin of the S1 sequences in the resulting T3D-S1His independent of the His tag sequences. After three passages of R124 on 911S1His cells, the virus was harvested and used to infect U118MG, U118HA-JAM, and U118scFvHis cells. CPE was observed in U118HA-JAM and U118scFvHis cell cultures but not in the infected U118MG cultures. In contrast, non-pseudo-typed R124 only induced cell death in U118HA-JAM cells (data not shown).

The virus produced in the U118scFvHis cells was passaged for 6 times on this cell line. The resulting virus, named R124-S1His, was used again to infect U118scFvHis cells. After confirming the presence of the $(His)_6$-tagged σ1 (**Figure**

8A), RNA was isolated from infected cells and the S1 segments were cloned after RT-PCR amplification. Sequence analysis confirmed the presence of the $(His)_6$-tag encoding sequence. All clones have a sequence identical to the cloned S1His, rather than the R124-S1, at the positions which differ between isolates (**Figure 8B** and **C**). These data suggest that selection for the presence of the $(His)_6$ tag leads to incorporation of the entire S1 segment into reovirus particles. A few additional changes were found in the 1 encoded by the S1 of R124-S1His (**Figure 7**). This can be attributed to the RNA-dependent RNA polymerase's relative lack of fidelity [36]. Taken together, our data are consistent with a mechanism in which the entire S1 genome segment is replaced by the S1His segment.

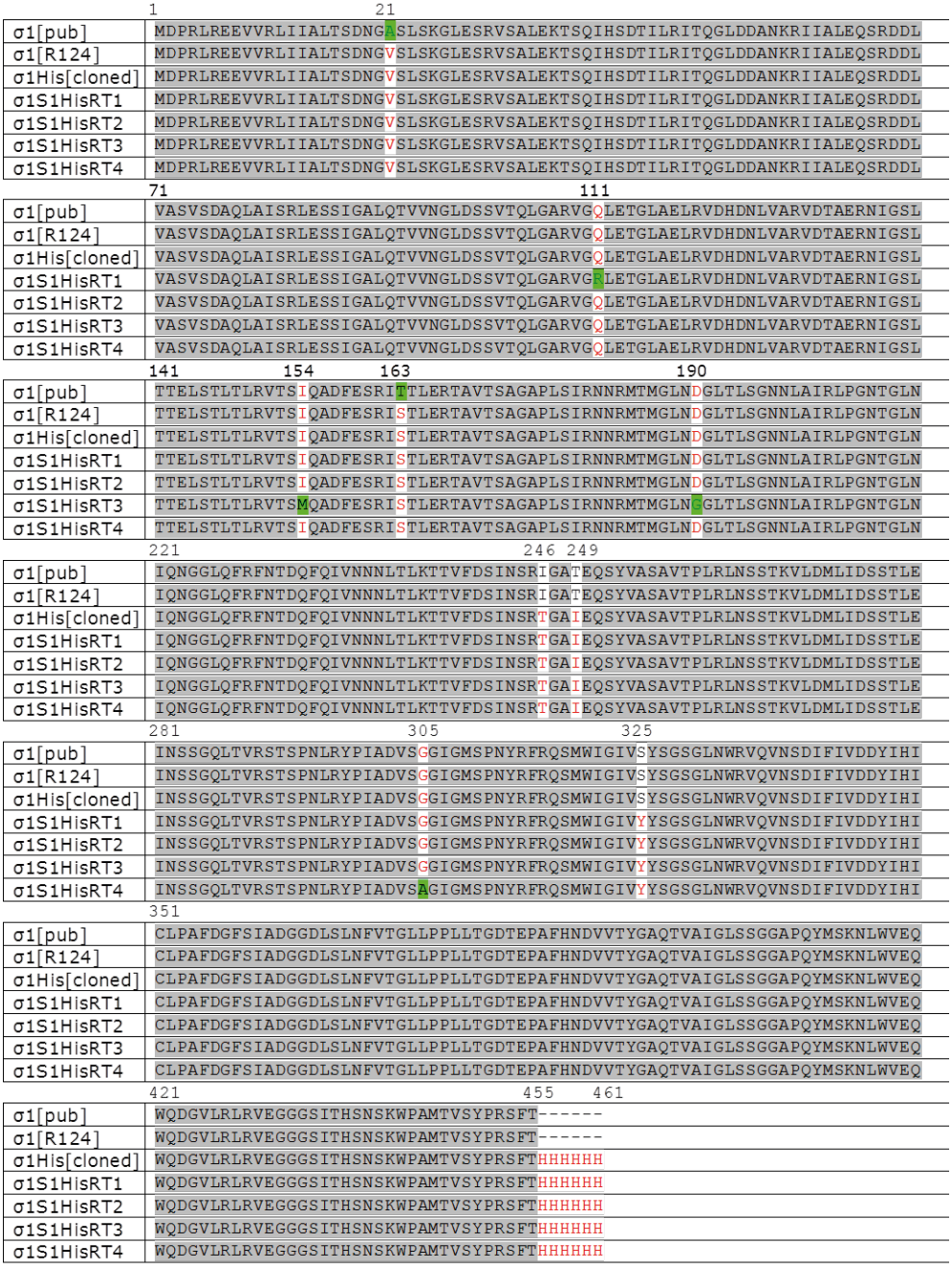
Selective infection of cells in a mixed population by a targeted reovirus

To verify that genetically targeted reoviruses could be used to selectively eradicate specific cells in a mixed population, U118scFvHis cells were plated with U118-eGFP cells to represent target and non-target cells, respectively. A number of these mixed cultures were exposed to the R124-S1His virus at an MOI of 1.5 at various time points after infection the relative number of eGFP-positive cells was determined by flow-cytometry (**Figure 8D**). The relative number of eGFP-positive cells increased more than 5.4-fold in comparison with mock-infected cultures at 96 hrs post infection. These data demonstrate selective eradication of sensitized cells by a genetically-targeted reovirus.

4.4 Discussion

Reverse genetics in RNA viruses with segmented genomes has been technically challenging. For *Reoviridae*, this is due, at least in part, to the difficulties in achieving sufficient quantities of non-poly-adenylated plus-strand reovirus RNA's in the cytoplasm of mammalian cells. The previously described methods to

FIGURE 7.
Amino-acid sequence of different σ1 and σ1-His proteins.
The amino-acid sequence alignment of σ1 (455 aa) and σ1His (461 aa) is shown. The upper sequence (1[pub]) is the published sequence of σ1 (accession number gi|333742|gb|M10262.1). R124 is our lab strain of reovirus T3D. The isolate was purified from the VR-824 stock obtained from the ATCC by two rounds of plaque purification on 911 cells. σ1His (cloned) is the sequence in pRT3S1His. σ1His RT1 and 2 are two RT-PCR amplified clones from the ATCC-T3D-derived T3D-S1His viruses. σ1His RT3 and 4 are amplified clones from the R124-S1His virus. The amino-acid changes are colour-coded. RT-PCR, reverse transcription-PCR.


1 21
σ1[pub] MDPRLREEVVRLIIALTSDNGASLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1[R124] MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1His[cloned] MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1S1HisRT1 MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1S1HisRT2 MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1S1HisRT3 MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
σ1S1HisRT4 MDPRLREEVVRLIIALTSDNGVSLSKGLESRVSALEKTSQIHSDTILRITQGLDDANKRIIALEQSRDDL
71 111
σ1[pub] VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
σ1[R124] VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
σ1His[cloned] VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
σ1S1HisRT1 VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGRLETGLAELRVDHDNLVARVDTAERNIGSL
σ1S1HisRT2 VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
σ1S1HisRT3 VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
σ1S1HisRT4 VASVSDAQLAISRLESSIGALQTVVNGLDSSVTQLGARVGQLETGLAELRVDHDNLVARVDTAERNIGSL
141 154 163 190
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σ1[R124] TTELSTLTLRVTSIQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNDGLTLSGNNLAIRLPGNTGLN
σ1His[cloned] TTELSTLTLRVTSIQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNDGLTLSGNNLAIRLPGNTGLN
σ1S1HisRT1 TTELSTLTLRVTSIQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNDGLTLSGNNLAIRLPGNTGLN
σ1S1HisRT2 TTELSTLTLRVTSIQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNDGLTLSGNNLAIRLPGNTGLN
σ1S1HisRT3 TTELSTLTLRVTSMQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNGGLTLSGNNLAIRLPGNTGLN
σ1S1HisRT4 TTELSTLTLRVTSIQADFESRISTLERTAVTSAGAPLSIRNNRMTMGLNDGLTLSGNNLAIRLPGNTGLN
221 246 249
σ1[pub] IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRIGATEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1[R124] IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRIGATEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1His[cloned] IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRTGAIEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1S1HisRT1 IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRTGAIEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1S1HisRT2 IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRTGAIEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1S1HisRT3 IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRTGAIEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
σ1S1HisRT4 IQNGGLQFRFNTDQFQIVNNNLTLKTTVFDSINSRTGAIEQSYVASAVTPLRLNSSTKVLDMLIDSSTLE
281 305 325
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σ1[R124] INSSGQLTVRSTSPNLRYPIADVSGGIGMSPNYRFRQSMWIGIVSYSGSGLNWRVQVNSDIFIVDDYIHI
σ1His[cloned] INSSGQLTVRSTSPNLRYPIADVSGGIGMSPNYRFRQSMWIGIVSYSGSGLNWRVQVNSDIFIVDDYIHI
σ1S1HisRT1 INSSGQLTVRSTSPNLRYPIADVSGGIGMSPNYRFRQSMWIGIVYYSGSGLNWRVQVNSDIFIVDDYIHI
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σ1S1HisRT3 INSSGQLTVRSTSPNLRYPIADVSGGIGMSPNYRFRQSMWIGIVYYSGSGLNWRVQVNSDIFIVDDYIHI
σ1S1HisRT4 INSSGQLTVRSTSPNLRYPIADVSAGIGMSPNYRFRQSMWIGIVYYSGSGLNWRVQVNSDIFIVDDYIHI
351
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σ1His[cloned] CLPAFDGFSIADGGDLSLNFVTGLLPPLLTGDTEPAFHNDVVTYGAQTVAIGLSSGGAPQYMSKNLWVEQ
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σ1S1HisRT3 CLPAFDGFSIADGGDLSLNFVTGLLPPLLTGDTEPAFHNDVVTYGAQTVAIGLSSGGAPQYMSKNLWVEQ
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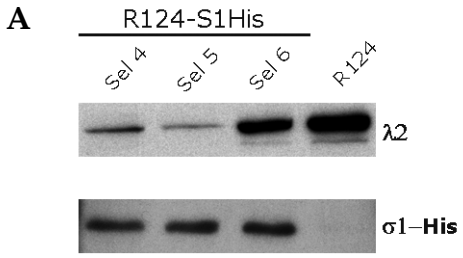
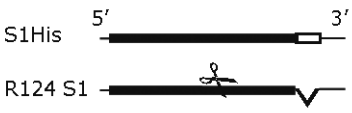
421 455 461
σ1[pub] WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFT------
σ1[R124] WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFT------
σ1His[cloned] WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFTHHHHHH
σ1S1HisRT1 WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFTHHHHHH
σ1S1HisRT2 WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFTHHHHHH
σ1S1HisRT3 WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFTHHHHHH
σ1S1HisRT4 WQDGVLRLRVEGGGSITHSNSKWPAMTVSYPRSFTHHHHHH





	Nucleotide	Position
	749	758
S1His	C	T
R124 S1	T	C

FIGURE 8. Selection of R124-S1His reoviruses on U118scFvHis cells.
A. The 1-His protein is present in lysates of R124-S1His reoviruses serially passaged on U118scFvHis cells. R124 is a lysate of the plaque-purified T3D reovirus. Sel 4-6 depict R124-S1His reovirus lysates isolated after the indicated number of passages on U118scFvHis cells. Upper panel: Western blot analysis for 2. The presence of λ2 was confirmed by western blotting using the 7F4 specific for λ2. Lower panel: Western blotting demonstrates the presence of $(His)_6$-tagged σ1 with the Penta-His antibody in the R124-S1His virus.
B. Nucleotide differences distinguish the cloned S1His sequence and the R124 S1 sequence. The amino acids coded by codons at positions 749 and 758 of the R124-S1 create a trypsin-cleavable site in σ-1 (✂).

manipulate reovirus genomes either involve transfection of RNA synthesized *in vitro*, or employ T7 polymerase-driven expression cassettes [21, 26, 27]. In these techniques, ribozymes generate transcripts with 3′ ends that are identical to normal segment termini. In general, such transcripts are unstable since they lack a poly-A tract [21, 26, 27]. Here we show that also polyadenylated transcripts generated with a conventional RNA polymerase II expression cassette can be used to replace a genome segment. A transcript containing a modified copy of the reovirus T3D S1 segment, encoding a $(His)_6$-tagged version of the spike protein σ1, was stably expressed. This protein was capable of binding a single-chain antibody fragment for oligo (His) tags. Propagation of wild-type T3D on cells producing 1-His led to its incorporation into reovirus capsids. A similar strategy has been previously used for other capsid proteins of reoviruses [37] and other non-enveloped viruses such as adenoviruses [38, 39]. However, if the genetic sequences encoding the modified capsid protein are not encapsidated, the altered targeting specificity would be lost after a single round of replication.

The transcripts encoded by the lentivirus vector LV-S1HIS contain all genetic information and most likely also the structural information contained within the S1 segment, e.g. the motifs regulating plus- and minus-strand synthesis. We hypothesized that these transcripts would be targeted to the "viral factories" and here associate with newly-formed viral cores. Furthermore, if the signals for

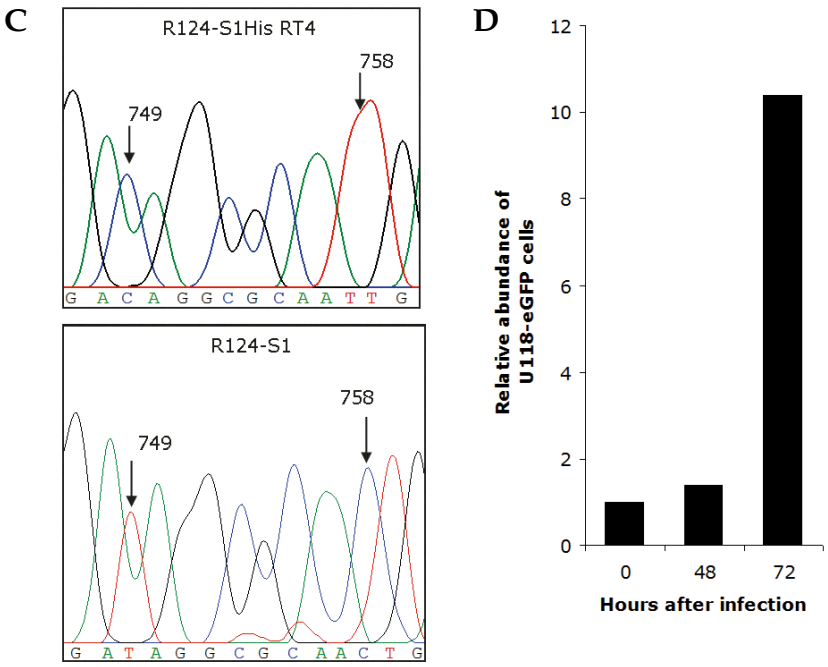


FIGURE 8. Selection of R124-S1His reoviruses on U118scFvHis cells.
C. Sequence analyses depict the differences at nucleotides 747-760 of RT-PCR clone R124-S1His RT4 and R124. Arrows represent the nucleotide differences at position 749 and 758.
D. The R124-S1His reovirus specifically eradicates the U118scFvHis cells from a mixed population. A mixture of U118scFvHis and U118-eGFP cells were infected with R124-S1His reovirus at an MOI of 2, and samples were taken for flow-cytometric analyses at the indicated times after the infection. Represented is relative frequency of GFP-positive cells in the virus-infected cultures relative to mock-infected cultures. These data show that the relative frequency of the R124-S1His-resitant eGFP-positive cells increased more than 10-fold upon selective eradication of the cells which are sensitive to the targeted reovirus. GFP, green fluorescent protein; MOI, multiplicity of infection; RT-PCR, reverse transcription-PCR.

minus-strand synthesis [22, 40] are functional, the S1 part of the lentiviral transcript should be converted in dsRNA. Secondary transcription of this dsRNA would lead to S1 segments with authentic termini. Our results propagating the T3D-S1His variant support these hypotheses.

Based on our hypothesis, we anticipated that the entire LV-S1His-derived genome segment, rather than only the region encoding the $(His)_6$ tag, would become incorporated into the virion. This was confirmed using the R124 strain in the selection. The reovirus selected after infection of the U118scFvHis with the R124 strain propagated in 911S1His cells contained the sequence of the $(His)_6$-

tagged-1 encoding segment instead of the S1 from R124 at the isolate-specific positions. These findings are consistent with the model that the entire S1His segment is derived from the transcript specified by LV-S1His.

This approach may prove useful for improving reoviruses as oncolytic agents. To date, only wild-type T3D has been used in clinical trials [1, 6, 41]. The results of this approach are encouraging and the procedure is well tolerated. However, the scarcity or inaccessibility of reovirus receptors on the surface of tumor cells may limit the efficacy of wild-type reoviruses as oncolytic agents. We recently reported that cultures of colorectal tumor cells isolated from resected tumor material resist reovirus infection, despite the presence of JAM-A [24]. This is likely attributable to the insufficient expression of reovirus receptors at their plasma membranes since T3D-derived infectious sub-viral particles, which are known to enter cells independent of JAM-1 and sialic acid, efficiently infect and lyse these cells. Further development of the approach presented here should allow modification of reoviruses to enhance tumor cell transduction and reduce the infection of non-target cells. This will increased the safety and efficacy of anticancer strategies using reoviruses as oncolytic agents.

We have developed a simple technique to genetically-modify reoviruses, and identified the C-terminus of σ1 as a good location for insertion of a targeting oligopeptides. Moreover, the $(His)_6$ tag at the C-terminus of σ1, in combination with the U118scFvHis cells allowed the construction of reoviruses that do not depend on JAM-1 for attachment and cell entry. Furthermore the development of tumor-targeted reoviruses can benefit from the experience with targeted adenoviruses, especially since the spatial structure of the reovirus spike is remarkably similar to the adenovirus fiber [42].

This reovirus genetic-modification technique developed in this study may be widely applicable. It may be adapted for other reovirus genome segments [43]. This would allow generation of defined mutants for gaining insight in the intricate interactions between the reoviruses and their hosts, and to address questions about virus structure, function, and pathogenesis. The strategy may be amendable for use in other *Reoviridae*. Although from the family name one may get the impression that all its members are "orphan" viruses, some of them cause severe diseases and have considerable economic impact. Members of its genera Orbivirus and Coltivirus cause diseases of humans, e.g. Colorado tick fever, and of domestic livestock, e.g. African horse sickness, and bluetongue disease [44]. Moreover, members of the genus Rotaviruses are the major etiologic agents of serious diarrhea in children under 2 years of age [45]. In these genera, too, this reverse genetics technique may be very useful.

4.5 Materials and methods

Cell lines

911 cells are adenovirus type 5 early region 1-transformed human embryonic retinoblasts [46]. U118MG human glioblastoma cells were obtained from Dr. B. de Leeuw (Erasmus Medical Center, Rotterdam, The Netherlands). All cell lines were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Breda, The Netherlands) supplemented with penicillin, streptomycin, glucose, and 8% Fetal Bovine Serum (FBS; Invitrogen), unless otherwise specified. Cells were cultured in a 5% CO_2 atmosphere at 37°C.

Reovirus propagation

911 cells were used to propagate wild-type T3D (American Type Culture Collection, VR-824) as described previously [47]. Briefly, cells were exposed to reovirus in DMEM/2% FBS for 2 hours at 37C, 5% CO_2. Subsequently, the inoculum was replaced by DMEM containing 8% FBS. The virus was harvested 48 hours post infection by resuspending the cells in phosphate-buffered saline (PBS) with 2% FBS and subjecting the suspension to three cycles of freezing and thawing. The sample was cleared by centrifugation for 10 minutes at 800x g. Where indicated, the virus was further purified by CsCl equilibrium centrifugation essentially as described [46]. In some experiments the R124 clonal isolate was used. This isolate was purified from the VR-824 stock obtained from the ATCC by two rounds of plaque purification on 911 cells. The infectious reovirus titer was determined by plaque assay on 911 cells.

Cell viability assay

WST-1 reagent (Roche, Woerden, The Netherlands) was used to assay the viability of cells after reovirus infections. Cells were infected with different amounts of reovirus in 96-well plates and WST-1 reagent was added, according to the manufacturer's instructions, at various time points post infection.

Cloning of T3D S1 from infected 911 cells

911 cultures were infected with to wild-type reovirus T3D at an MOI of ~2 plaque-forming units (PFU)/cell. Total cellular RNA was extracted 24 hours post-infection using the Absolutely RNA miniprep kit (Stratagene, Huissen, The Netherlands). First-strand cDNA synthesis employed the ReoS1 Rev primer, using SuperScript II reverse transcriptase (Invitrogen). PFU polymerase (Promega, Leiden, The Netherlands) was used for template amplification. See **Table 1** for

details about the primers and the PCR fragments. The PCR product was purified from a 1% agarose gel using the JetSorb kit (Genomed, ITK Diagnostics, Uithoorn, The Netherlands). The product was digested using Hind III and Not I, and cloned into the plasmid pCDNA3.1+ (Invitrogen) digested with the same enzymes, yielding plasmid pRT3S1. The sequence of the insert was verified at the Leiden Genome Technology Center.

To insert the codons for the $(His)_6$-tag at the C-terminus of σ1, the σ1-coding region was PCR amplified with primers HisReoS1_Rev and ReoS1_For (**Table 1**). The PCR product was digested with HindIII and ligated to HindIII-and EcoRV-digested pCDNA3.1+. The resulting plasmid was used as template for PCR amplification with primers SigmaEnd_Rev and ReoS1_For and, after digestion with HindIII, ligated into Hind III and EcoRV-digested plasmid pCDNA3.1+, generating plasmid pRT3S1His.

The sequences of all the constructs described in this study are available on request.

RT-PCR

Primers used for the RT-PCR procedures are listed in **Table 1**. For the detection of human JAM-1 RNA , 911 cells and U118MG cells were seeded on 5 cm dishes and total cellular RNA was isolated using the Absolutely RNA miniprep kit (Stratagene). In all cases, SuperScript II was used to generate first-strand cDNA. For the characterization of the 911S1His cell line, total cellular RNA was extracted from confluent cultures of 911 and 911S1His cells, as described above. For the detection of S1His in reovirus batches, infections of 911 cells were performed. RNA was isolated 24 hours after infection and used for RT-PCR amplification. The resulting PCR products were cloned into plasmid pTOPO-TA (Invitrogen) and their DNA sequences were determined.

Production of lentiviral vectors

All lentiviral constructs used in this study were based on the pLV-CMV-x-IRES-Neo vector [48] (see **Figure 3**). The S1His region of plasmid pRT3S1His was released by *Eco*105I and *Xba*I digestion and inserted in the same restriction sites of pLV-CMV-x-IRES-Neo. To generate the HA-JAM lentiviral expression vector, plasmid pCDNA-HA-JAM [49] (kindly provided by Dr. U.P Naik, Delaware, Newark) was digested with *Eco*105I and *Xba*I and inserted in pLV-CMV-BC-Neo. Plasmid pLV-JAM-ECD-IRES-Neo was made by first inserting the codons for the extracellular domain of JAM into pDisplay (Invitrogen) and cloning the *Eco*105I - *Xho*I fragment into the same sites of the pLV vector. pCDNA-HA-JAM was used

as template to amplify the JAM-ECD (see **Table 1**). To generate pLV-scFvHis-IRES-Neo, encoding the anti-His tag single-chain antibody fragment, pHissFv.rec [32] (a kind gift from Dr. D.T. Curiel, Univ. of Alabama) was digested with *Eco*105I and *Xho*I and inserted in the pLV vector. Production of the lentiviral vectors and transduction of the cells were performed as described previously [48, 50]. The LV-S1His vector was used for transducing cultures of 911 cells at an estimated MOI of 0.5. The polyclonal, G418-restistant cell population, referred to as 911S1His cells were used for further studies.

Generation of modified reoviruses carrying the S1His segment

Wild-type reoviruses were used to infect 911S1His cells according to routine procedures. After three rounds of propagation, the resulting σ1-His containing viruses were harvested by freeze thawing and used to infect U118scFvHis cells. The virus produced in U118scFvHis cells was harvested at the first signs of CPE, and serially passaged several times in U118scFvHis cells.

[^{35}S]-Methionine labeling

Infected or mock-infected cells were incubated with Redivue [^{35}S]-Methionine Pro-mix (200 μCi/ml; Amersham, Roosendaal, the Netherlands) for 4 hours at various time points post infection. Cells were washed once with PBS and lysed in Giordano Lysis Buffer (50 mM Tris-Cl [pH=7.4], 250 mM NaCl, 0.1% Triton, 5 mM EDTA) containing protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). All labeling assays were performed in 24-wells plates with 5 μl Pro-mix per well. The cells were lysed with 100 μl lysis buffer per well. Fifty μl of the lysates was loaded on a 10% SDS-Polyacrylamide gel after addition of sample buffer. Gels were dried and exposed to a radiographic film to visualize the labeled proteins.

Immunofluorescence assay

For immunofluorescence assays, cells were grown on round glass cover slips in 24-well plates, fixed with methanol, washed with PBS containing 0.05% Tween-20, and incubated with a primary antibody against the oligo (His) tag primary antibody (Sigma Aldrich, Zwijndrecht, The Netherlands). The coverslips were washed and incubated with secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse serum for 30 minutes at room temperature. The mounting solution consisted of glycerol containing 0.02 M Tris-HCl [pH=8.0], 2.3% 1,4-diazabicyclo-[2.2.2]-octane and 0,5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

Western blot analysis

Cell lysates were made in Giordano lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.1% Triton, 5 mM EDTA) supplemented with protease inhibitors. Reovirus lysates were prepared by adding 15 l of cleared reovirus to 5 l of Western sample buffer (final concentrations: 10% glycerol, 2% SDS, 60 mM Tris-Cl [pH 6.7], 2.5% -mercaptoethanol, and 2.5% bromophenol blue). After incubation for 3 min at 100C, the samples were analyzed on SDS 10% polyacrylamide gels. The proteins were transferred to Immobilon-P (Millipore, Etten-Leur, The Netherlands) and visualized using standard protocols. Primary antibodies used were the Penta-His antibody (Qiagen, The Netherlands) for detection of the $(His)_6$ tag, 7F4 directed against reovirus protein λ2 [51] (kindly provide by Dr. K. Tyler, University of Colorado Health Science Center, Denver, Colorado). The secondary antibody used was HRP-conjugated Goat anti-Mouse IgG (Santa Cruz Biotechnology, Santa Cruz, USA).

Selective eradication assay

U118MG cells were transduced with a lentiviral vector encoding the eGFP gene [50]. The resulting U118eGFP cells were mixed with U118scFvHis cells and seeded in 6-well plates. The mixed cultures were either mock infected, or infected with R124-S1His (MOI of ~ 1 PFU/cell). At the indicated time points after the infection, the co-cultures were trypsinized and the cells were collected in PBS. These cell suspensions were analyzed for eGFP activity using a BD LSRII flow cytometer and BD FACSDiva software (BD Bioscience, Breda, The Netherlands).

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Chapter 5

Replicating reoviruses with a transgene replacing the codons for the head domain of the viral spike

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Contents chapter 5

5.1 Abstract

The capacity to modify the reovirus genome facilitates generation of new therapeutic reoviruses. We describe a method for generating replication-competent reoviruses carrying an heterologous transgene. The strategy is based on the expanded-tropism reovirus mutant *jin-3*, which can infect cells independent of the reovirus receptor JAM-A. *Jin-3* harbors a mutation in the S1 segment resulting in a G196R substitution in the tail of the spike protein σ1. The use of the *jin-3* tail-encoding S1 segment allows replacing the codons for the JAM-A-binding head domain by up to 522 nt of foreign sequences, without exceeding the size of the wild-type S1 segment. We inserted the codons for the porcine teschovirus-1 2A element fused with those encoding the fluorescent protein iLOV. Replicating rS1His-2A-iLOV reoviruses were generated by co-transfection of expression plasmids for all reovirus segments. These reoviruses contain the S1His-2A-iLOV segment in absence of the wild-type S1 segment. Density-gradient centrifugation confirmed the association of the σ1-tail fragment with the capsid. Both JAM-A positive and JAM-A negative cells exposed to the rS1His-2A-iLOV reoviruses exhibited iLOV fluorescence, confirming the *jin-3* derived expanded-tropism phenotype. These data demonstrated the feasibility of generating decapitated replication-competent T3D reoviruses carrying a heterologous transgene.

5.2 Introduction

Mammalian orthoreoviruses are non-enveloped viruses with a segmented double-stranded RNA (dsRNA) genome. The name Reovirus is an acronym of Respiratory and Enteric Orphan virus. So far the virus has not been associated with severe pathology in humans, hence its designation as an orphan virus. In cell culture reovirus infection initiates a lytic replication cycle. The reovirus preferentially infects and lyses transformed cells. This has been attributed to the stimulating effects of the active RAS-signaling on virus uncoating, replication, egress, and the induction of apoptosis [1-4]. The reovirus' preference for transformed cells and the absence of pathology in humans have led to the development of reovirus Type 3 Dearing as a viral oncolytic agent for clinical use in a number of cancer types [5, 6]. The results of the clinical trials published so far demonstrate that the reovirus-administrations are well tolerated. Moreover, evidence of anti-tumor efficacy has been acquired. Nonetheless, the antitumor efficacy could be improved. This may be accomplished by increasing the infection efficiency in tumor cells and by increasing the oncolytic potency of the virus. Here we describe an approach to generate replication-competent expanded-tropism reoviruses that harbor a heterologous transgene.

The mammalian reovirus genome consists of ten double stranded RNA segments, making genetic modification of the reovirus genome especially difficult. The development of plasmid-based reverse genetic systems for mammalian reoviruses facilitated the development of reverse genetics approaches [7]. However, there is limited information available regarding locations in the genome that could accommodate the insertion of new protein coding genes without compromising the virus' capacity for autonomous replication. So far, small exogenous sequences have been successfully added to the genome segments S1, S3, M1 and L1 [8-10]. Insertion in these locations increases the size of the genome segments and this may compromise the genomic stability of the modified reoviruses. Others report the replacement of the open reading frame (ORF) from some of the segments by heterologous genes, but this yields replication-incompetent reoviruses that require trans-complementation of the deleted functions for propagation in helper cells [7, 11]. Here, we present the generation of replication-competent reoviruses carrying the sequence encoding the fluorescent protein iLOV within the S1 segment.

In our approach we employed a feature of the *jin* mutants of the Type 3 Dearing reovirus. The *jin* mutants were obtained upon serial passaging of the T3D reovirus on glioblastoma cells that lack expression of the high-affinity reovirus receptor Junction-Adhesion Molecule A (JAM-A) [12]. The absence of JAM-A expression makes these cells non-permissive for *wt* reovirus infection in monolayer cultures [13]. The *jin* mutants could productively infect a variety of JAM-A negative cells. All *jin* mutants harbor mutations in the S1 segment close to the region previously identified as the sialic acid-binding domain in the tail of the trimeric spike protein σ1 [14, 15]. This suggests that the *jin* mutants do not require the presence of the head domain for cell entry.

We used the S1 segment of reovirus *jin-3*, which harbors only one point mutation that results in a G196R change in the tail of the σ1-spike. To reduce the size of the S1 segment, the σ1 protein was truncated after amino acid 252. This allows inserting transgenes up to a length of 470 nt without exceeding the length of the wild-type S1 segment. The ORF of the nonstructural protein σ1s, which entirely overlaps the σ1 ORF, but is read from another reading frame, is left intact. Although in different field isolates of T3 reoviruses the amino acid sequence of the σ1s is more heterogeneous than that of σ1, some regions are conserved [16]. The role of σ1s in the reovirus replication cycle has not yet been fully elucidated, but evidence suggests that it is involved in virulence and apoptosis [17] and is required for induction of G2/M cell cycle arrest in some cell lines [18, 19].

In our study we have used the small fluorescent protein iLOV as the reporter [20]. The 13 kDa iLOV protein is evolved from the Light, Oxygen or Voltage

sensing domain of the blue-light receptor phototropin 2 of Arabidopsis thaliana. The protein contains the flavin mononucleotide (FMN) as chromophore. The small size (~330 nt) of the synthetic codon-optimized gene encoding the iLOV fluorescent protein makes it a suitable reporter for our approach.

Here we show how a synthetic transgene-containing segment can be incorporated in the genome of a mammalian reovirus without compromising the replicative capacity of the resulting recombinant virus. Furthermore we demonstrate that the codons of the head domain of the spike protein σ1 can be replaced by a small transgene, and that the transgene is expressed. The reoviruses carrying the σ1-tail of *jin-3* and the transgene on the S1 segment retained their capacity to infect JAM-A deficient cells. This technique may facilitate the development of new replication competent-recombinant reoviruses armed with a therapeutic transgene for oncolytic virus therapy. In addition this approach may be exploited for the generation of new reovirus-based life-virus vaccines.

5.3 Results

Generation of the rS1His-2A-iLOV reoviruses

The *jin* reoviruses infect cells through an interaction between the tail domain of the reovirus spike protein and sialic-acid residues at the cell surface. This observation, together with the notion that the head domain is not essential for trimerization of σ1 molecules [21], led us to postulate that in *jin* viruses the head domain of the spike protein could be replaced by transgene sequences. To test this hypothesis, the codons for the head domain of σ1 were replaced by the iLOV-coding region in the S1 segment of *jin-3*. The trypsin cleavage site, which is present in the tail domain of the σ1 protein of the *wt*T3D reovirus was retained in our modified variant (**Figure 1**). Trypsin cleavage occurs after the arginine residue at position 245 of the T3D-σ1 protein [22]. This cleavage is important in the formation of Infectious Subvirion Particles (ISVP's) and can be of importance during the proteolytic disassembly [23].

The modified S1 segment encodes only the tail of σ1 (amino acids 1 - 252), followed by a 6xHis tag, the porcine teschovirus-1 2A sequence (2A), and the iLOV coding sequence. The ORF is 1176 nt long (ORF of S1-*wt* is 1377 nt) and is followed by 198 nt of the 3' region of S1. The latter region contains sequences reported to be involved in encapsidation [24]. Translation of the modified S1 ORF generates two distinct proteins that are separated upon the ribosome skipping induced by the 2A peptide: the σ1-His protein and the iLOV fluorescent reporter protein (**Figure 1**).

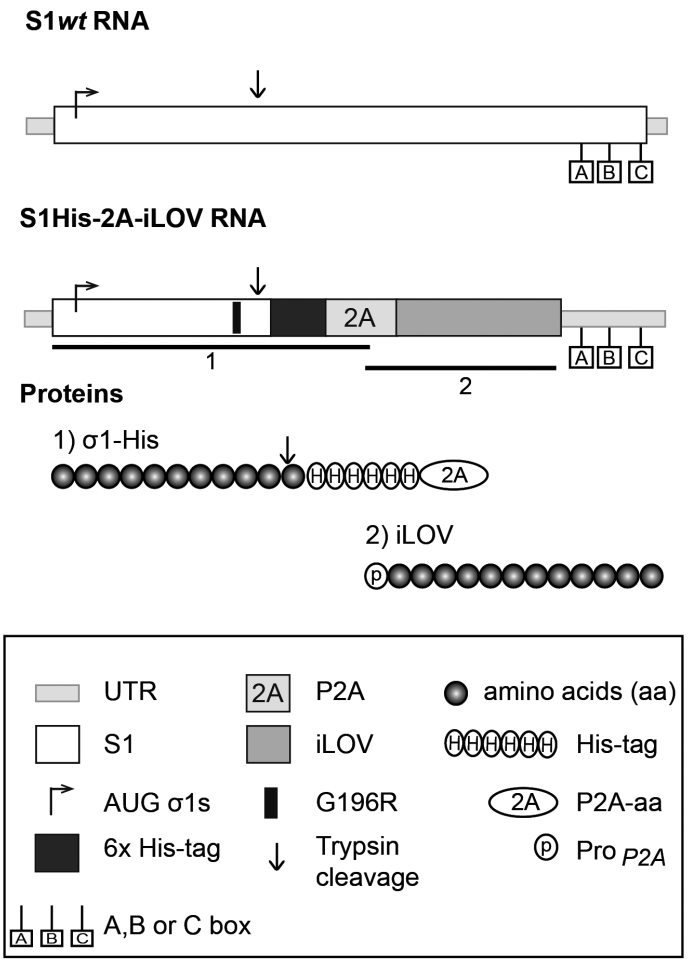

FIGURE 1.
Schematic representation of S1-*wt* and S1His-2A-iLOV RNA. The length of the S1His-2A-iLOV and the *wt*-S1 segment is 1386 nucleotides and 1416 nucleotides, respectively. Two proteins are synthesized from the S1His-2A-iLOV ORF: 1) the σ1-His that contains some additional amino acids derived from the 2A peptide, and 2) the iLOV protein that contains an additional proline at the N-terminus as result of processing of the 2A sequence. (The drawing is not depicted in scale with regard to sizes of the molecules).

To generate the recombinant reovirus, plasmid pBT7-S1His-2A-iLOV encompassing the modified S1 segment, preceded by a T7 RNA polymerase dependent promoter and tailed by the hepatitis delta virus ribozyme, was transfected into BSR-T7 cells together with four plasmids encoding the other nine segments, essentially as described [25]. Virus was harvested at 48 hours post-transfection (P0).

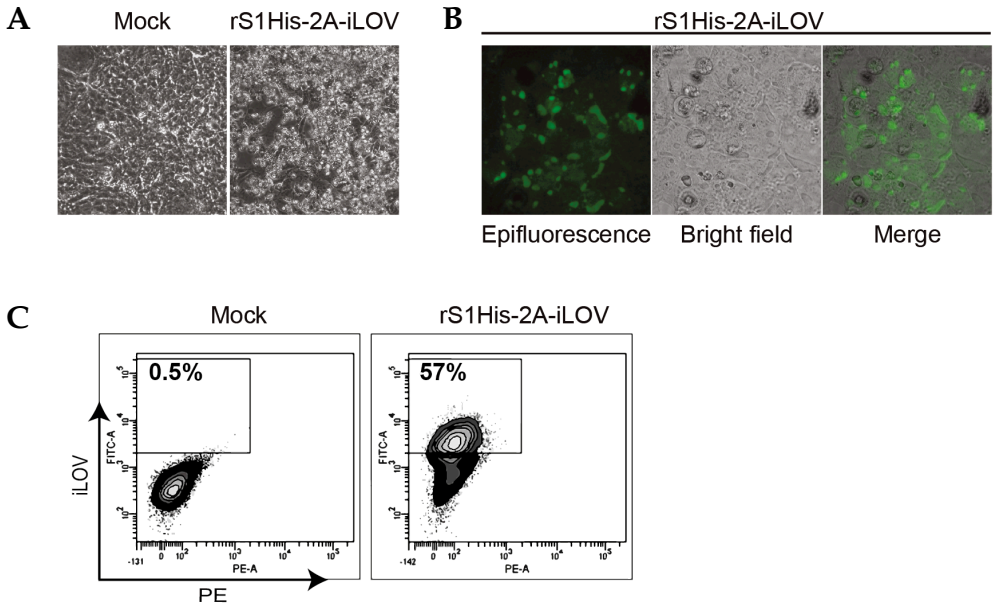

FIGURE 2.
The recombinant S1His-2A-iLOV reovirus replicates in cells and is infectious.
A. Morphological appearance of mock infected 911scFvHis cells and 911scFvHis cells infected with an early passage of rS1His-2A-iLOV.
B. Detection of iLOV positive 911scFvHis cells infected with an early passage rS1His-2A-iLOV virus (MOI_{911} ~ 0.3). Bright-field and fluorescence photographs were taken using an Olympus CKX41 microscope.
C. Detection of iLOV-positive 911scFvHis cells infected with rS1His-2A-iLOV by flow cytometry at 72 hours post infection. 911scFvHis cells were either mock infected or infected with an early passage of rS1His-2A-iLOV (MOI_{911} ~ 0.4). The iLOV signal is plotted on the Y-axis (FITC-A) against the PE-A signal on the X-axis.

Part of the yield was used to infect 911scFvHis cells (P1). The presence of the 6xHis tag in the capsid allows the modified virus to use the membrane-bound single-chain Fv recognising the His tag (scFVHis) as an artificial receptor [8]. Clear cytopathic effects (CPE) became apparent at 10-days post infection, suggesting the presence of infectious viruses (**Figure 2A**). Upon harvesting, the virus was used to infect cultures of 911 cells in a plaque assay experiment. The plaques formed by rS1His-2A-iLOV viruses were smaller than those of *wt* reoviruses, and the borders are not clearly defined. The plaque morphology was similar to those of the *jin-3* viruses (data not shown). To determine the titers of the freeze-thaw batches of rS1His-2A-iLOV viruses we used the TCID50 method on 911 cells. On average the yields are lower compared to the *wt*T3D reovirus and around 4*106 Infection Units per ml (IU_{911}/ml). For the rS1His-2A-iLOV viruses the yields per cell in three

independent CsCl-density gradient purified virus batches ranged between 5.2 x 103 and 1.0 x 104 physical particles per cell, which is 10 – 30 fold lower than the yields obtained for *wt* and *jin-1* viruses. The iLOV-derived fluorescent signal was detectable by fluorescence microscopy (**Figure 2B**). The fluorescence was quantified by flow cytometry. For this, 911scFvHis cells were either mock infected or infected with an early passage (P2) rS1His-2A-iLOV at a multiplicity of infection (MOI_{911}) of 0.4 IU_{911}/cell. Fluorescence was analysed by flow cytometry at 72 hours post-infection (**Figure 2C**). In the rS1His-2A-iLOV infected cultures, 57% of the cells exhibited the iLOV fluorescence. These data demonstrate that the replacement of the codons for the head domain of the reovirus spike protein σ1 by an iLOV coding fragment yields infectious and replicating reoviruses.

S1His-2A-iLOV RNA is present in the recombinant reoviruses

To confirm the presence of the modified S1His-2A-iLOV segment in the recombinant reoviruses, RT-PCR was performed using total RNA isolated from rS1His-2A-iLOV-infected 911scFvHis cells at 24 and 48 hours post-infection. A schematic overview of the primers and their binding sites is represented in **Figure 3A**. The S1 primer pair amplified the *wt* S1 segment as well as the iLOV-containing segment. The length of the PCR product of the iLOV-containing S1 segment almost equals the length of the *wt*T3D-S1 segment (**Figure 3B**) and the segment is present in both isolations (24 and 48 hour). The iLOV-specific PCR product is detectable in both isolates but not in the *wt*T3D reovirus sample. As expected the PCR primers used to detect the region encoding the head domain of σ1 only amplified a fragment in the RNA isolated from *wt* reovirus infected cells but not in the samples from rS1His-2A-iLOV virus infected cells. Sequence analysis of the amplified S1His-2A-iLOV PCR product showed no additional mutations within the segment (data not shown).

To further confirm the presence of replicating reoviruses, total RNA was isolated from cells infected with either *wt*T3D reovirus or with the rS1His-2A-iLOV reovirus, and used for poly-acrylamide gel electrophoresis (**Figure 3C**). The ten individual segments could be detected, further confirming the presence of replicating reoviruses. The modified S1 segment is slightly smaller than the wild-type S1 segment, resulting in a marginally increased electrophoretic mobility. From these data we conclude that the S1His-2A-iLOV segment is packaged in the reovirus particles and that the *wt* S1 segment is absent from these reovirus preparations. These data demonstrate that the rS1His-2A-iLOV reoviruses replicate independent of viral helper functions.

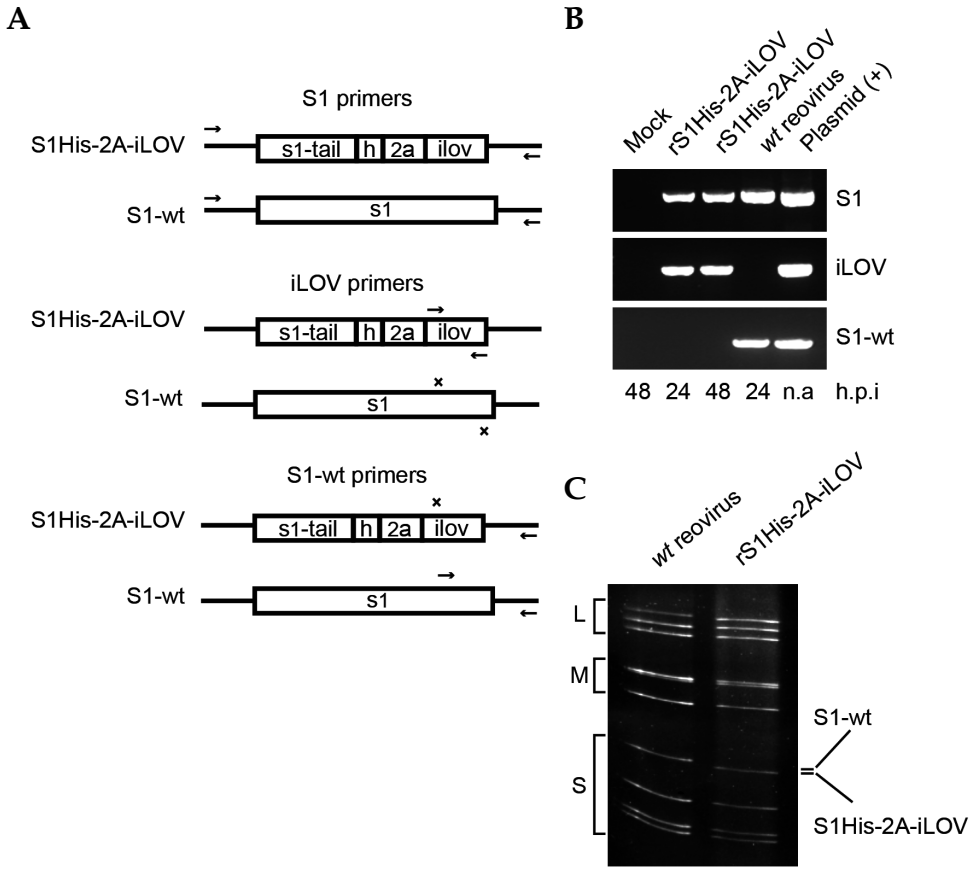

FIGURE 3.
Detection of S1His-2A-iLOV RNA in infected 911scFvHis cells.

A. Schematic representation of binding sites for the primers used in the RT-PCR procedure. The primer-binding sites are indicated by arrows. The crosses denote non-binding sites. PCR products formed with S1 primers (S1For and S1endR) are 1416 bp (S1 derived from *wt* Reovirus) or 1386 bp (S1 derived from rS1His-2A-iLOV) in length. The PCR product generated with iLOV primers (iLOVFor and iLOVRev) is 303 bp and with S1-*wt* primers (S1testFor and S1endR) is 354 bp.

B. RT-PCR to detect S1 RNA, iLOV or absence of the S1 head domain in 911scFvHis cells infected with passage 1 of the rS1His-2A-iLOV reovirus, at 24 and 48 hours post infection (h.p.i). Controls are RNA isolated from either uninfected (mock; negative) or *wt* reovirus infected 911scFvHis cells, resp. 48 or 24 hours post infection. The positive DNA controls are plasmids pBACT7S1His-2A-iLOV in case of S1 and iLOV PCR and pBACT7S1T3D for the S1-*wt* PCR. Time post infection is not applicable (n.a.) for the PCR controls.

C. RNA electrophoresis of *wt* reovirus and rS1His-2A-iLOV virus. RNA was isolated from infected 911scFvHis cells, analysed by 12% PAGE-SDS and detected with Sybr-Gold. L, M and S and specifically S1 RNA segments are indicated.

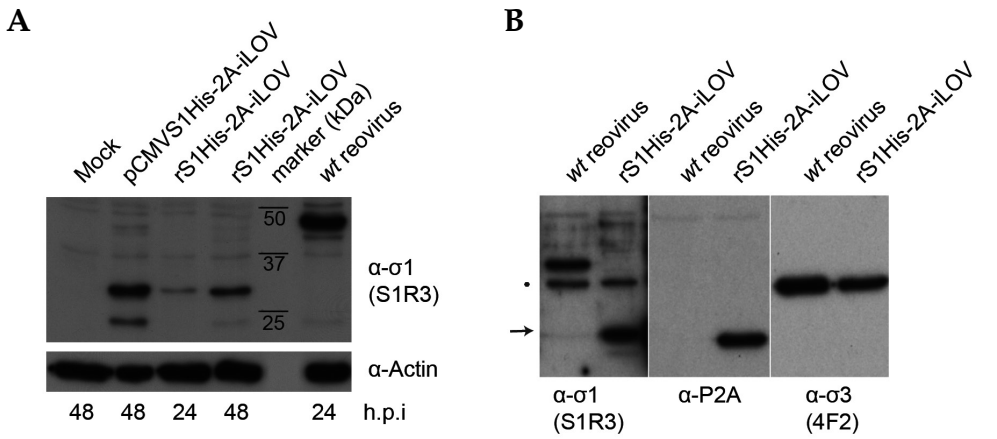

FIGURE 4.
Detection of σ1-His in infected 911scFvHis cells and CsCl purified particles.

A. 911scFvHis cells were infected with early passage rS1His-2A-iLOV. Lysates of infected cells were made 24 hr or 48 hr post infection (h.p.i) and analysed by Western blotting. As a positive control for σ1His protein, cells transfected with pCMVS1His-2A-iLOV are used and lysates were made 48 hr post transfection. Cells infected with *wt*-reovirus (24 h.p.i) are taken along to visualize the difference in size; predicted size of *wt* σ1 is about 49 kDa and σ1-His after P2A processing is about 30 kDa. σ1 is detected with S1R3, polyclonal rabbit antibody directed against N-term of σ1. As a loading control a β-actin antibody is used.

B. Approximately $5*10^9$ particles of CsCl purified *wt*-reovirus or σ1-His containing rS1His-2A-iLOV reovirus was loaded onto 12% SDS-PAGE gels. S1R3 polyclonal antibody against N-term of σ1 was used for detection of both, the *wt*-σ1 and the σ1-His protein (arrow). The smaller σ1-His protein is also detected with an antibody directed against the P2A peptide. The dot indicates a background band, probably cross reactivity with σ3. As an indication of the amount of particles that are loaded on gel, σ3 is detected with monoclonal antibody 4F2.

The σ1-His protein is detectable in 911scFvHis cells infected with the recombinant reovirus and is incorporated in the rS1His-2A-iLOV particles

To verify that the inclusion of the porcine teschovirus-1 2A sequence yields a truncated σ1 protein in the rS1His-2A-iLOV infected cells, 911scFvHis cells were exposed to rS1His-2A-iLOV virus at an MOI_{911} of 2. At 24 and 48 hours post infection the cells were harvested and protein lysates of the infected cells were analysed by western-blotting. The presence of truncated σ1 was detected by immunoblotting using the polyclonal rabbit antibody S1R3 directed against a σ1-derived synthetic peptide. The σ1-His protein is detectable already 24 hours after infection. A specific band of approximately 30 kDa is seen only in lysates prepared from the rS1His-2A-iLOV infected cells. This is in agreement with the calculated molecular mass of the truncated σ1-His protein. In contrast, in the

lysates from the cells infected with the *wt* reovirus show a band that corresponds to the calculated molecular mass of *wt*-σ1 (approximately 49 kDa) (**Figure 4A**).

To confirm the presence of the truncated σ1-His protein in the virus capsid, rS1His-2A-iLOV particles were produced and purified by CsCl density gradient centrifugation. Protein lysates from these purified particles were used for western blot analysis. As a control, purified *wt*T3D reovirus was included. Again a band was detected migrating at an apparent molecular mass of 30 kDa only in the rS1His-2A-iLOV particles (**Figure 4B**) and this band was also detected with the antibody directed against the 2A peptide. The σ3 is used as a loading control. Taken together, our data demonstrate that the 2A sequence yields separate σ1 and iLOV proteins. In addition, our data show that at least part of the truncated σ1 protein is associated with the reovirus capsid.

rS1His-2A-iLOV virus can infect cells independently of the artificial scFvHis receptor and the natural JAM-A receptor

So far, the rS1His-2A-iLOV reovirus was propagated in cells that contain the scFvHis as an artificial receptor for the modified virus. To test whether the rS1His-2A-iLOV virus can infect cells independently of this scFvHis receptor, cultures of 911 cells were exposed to the rS1His-2A-iLOV virus at an MOI_{911} of 0.4 and analyzed by flow cytometry for iLOV expression at 72 hours post infection (**Figure 5A**). Approximately 46% of the 911 cells exhibited iLOV fluorescence. This demonstrates that the rS1His-2A-iLOV recombinant reovirus can also infect cells in absence of the artificial scFvHis receptor. To further test if the infection is independent of the reovirus receptor JAM-A, cultures of the JAM-A-deficient U118MG cells were exposed to rS1His-2A-iLOV at an MOI_{911} of 0.4 (**Figure 5A**). Approximately 21% of the U118MG cells exhibit iLOV fluorescence. This demonstrates that the rS1His-2A-iLOV virus does require neither expression of scFvHis nor JAM A as receptor for entry into cells.

To test the cytolytic activity of the rS1His-2A-iLOV reovirus, monolayer cultures of several cell lines that resist *wt*T3D reovirus infection were exposed to rS1His-2A-iLOV, or *wt*T3D reovirus at an MOI_{911} of 1, or mock infected. Four days post infection, cells were inspected visually for signs of CPE and micrographs were taken with an original magnification of 200x (**Figure 5B**). Subsequently the viability of the cultures was established on the same day (**Figure 5C**). In parallel, cultures of 911 cells were included to demonstrate that both *wt*T3D reovirus (*wt*-Reo) and the modified rS1His-2A-iLOV virus induce cell death in JAM-A positive, permissive cells (**Figures 5B** and **5C**). In monolayer cultures of JAM-A deficient U-118MG cells, only the rS1His-2A-iLOV reoviruses, but not *wt*T3D reoviruses, induce CPE, resulting in a reduced viability (**Figures 5B** and **C**). The U251

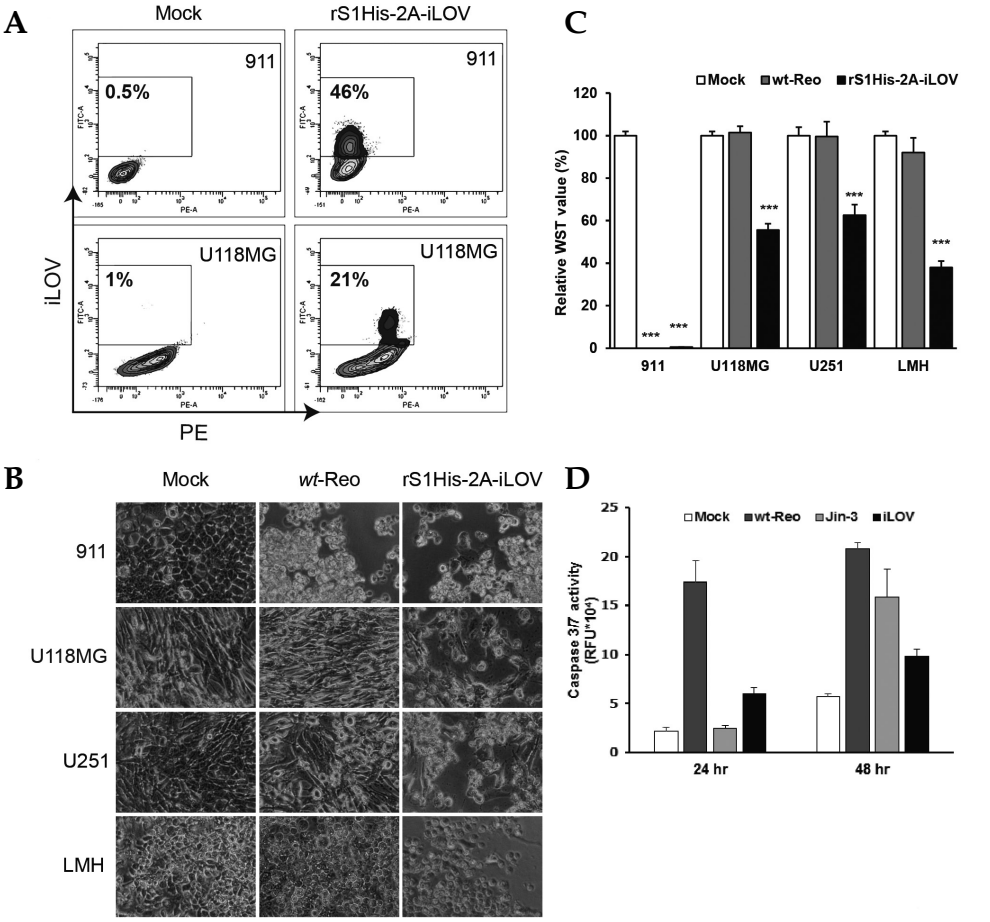

FIGURE 5.
rS1His-2A-iLOV reovirus infection is independent of the scFvHis receptor and Junction Adhesion Molecule-A.

A. 911 cells and U118MG cells were mock infected or infected with an early passage rS1His-2A-iLOV reovirus (MOI_{911} ~ 0.4). Cells were analysed by flow cytometry 72 hours post infection. iLOV signal is plotted on the Y-axis (FITC-A) against the PE-A signal on the X-axis.

B. CPE induction in the cell lines upon mock infection or upon infection with *wt*T3D reovirus or rS1His-2A-iLOV (MOI_{911} ~1). Micrographs were taken 4 days post infection, prior to WST-1 assay.

C. Cell viability, as measured by WST-1 assay, of the various cell lines upon mock infection or infection with *wt*T3D reovirus or rS1His-2A-iLOV (MOI_{911} ~1). Relative WST values are calculated as percentages of OD_{450} values of the infected cells over the mock treated cells. The error bars represent the SD (n=4). *** P<0.0001 and * P<0.01.

D. Capsase-3/7 activity in mock-infected culture or cell cultures (911) infected with *wt*T3D reovirus, *jin-3*, and rS1His-2A-iLOV at an MOI_{911} ~ 5. Caspase 3/7 activity was measured 24 and 48 hours post infection. The error bars represent the SD (n=4). OD, optical density.

cell line has a low JAM-A expression (data not shown) and as a result is weakly permissive to *wt*T3D reovirus. In contrast to the *wt*T3D reoviruses the rS1His-2A-iLOV reovirus induces marked cell death in the U251 cells. Finally, the chicken hepatoma cell line LMH does not express a functional JAM-A receptor and therefore resists *wt*T3D reovirus infection. It is however sensitive to rS1His-2A-iLOV (**Figure 5C**).

The *wt*T3D reovirus induces apoptosis in many transformed cells ([3]). The viral spike protein σ1 has been implicated in the induction of the programmed cell death ([26]). To study whether the rS1His-2A-iLOV viruses, which lack the head domain of the σ1 spike protein, induce apoptosis we measured the activity of the caspases 3/7 upon reoviruses exposure in 911 cells. The cells were exposed to rS1His-2A-iLOV and, as controls, to *wt*T3D and *jin-3* reoviruses at an MOI=1. Caspase-3/7 activity was measured 24 or 48 hours post infection (**Figure 5D**). In the 911 cells infected with rS1His-2A-iLOV the induction of caspase-3/7 activity is evident at 24 hours and 48 hours post infection, although in a lesser extent than upon *wt*T3D reovirus infection. Also in cell exposed to *jin-3* caspase-3/7 activity is induced albeit somewhat delayed.

Taken together, these results demonstrate that the rS1His-2A-iLOV reovirus is cytotoxic in JAM-A deficient as well as in JAM-A-expressing cell lines. In 911 cells an increase in caspase-3/7 activity is detected upon rS1His-2A-iLOV infection, suggesting that these viruses can exert their cytolytic activity by the induction of classic apoptosis.

Lec2 cells are less sensitive for rS1His-2A-iLOV infection than the parental CHO cells

The JAM-A independent cell entry of rS1His-2A-iLOV is possible by the interaction of the *jin-3* derived sialic-acid binding region in the tail region of σ1. To confirm that sialic-acids are involved in binding of rS1His-2A-iLOV to cells, we exposed Lec2 cells to this virus. Lec2 cells are Chinese Hamster Ovary mutant cells that have a defect in the translocation of CMP-sialic-acid into the Golgi compartment causing a 90% reduction of sialic acids on their cell surface [27]. To verify this phenotype in Lec2 cells we exposed Lec2 and normal CHO cells to FITC-A-conjugated Wheat Germ Agglutinin (WGA-FITC) and measure the binding of WGA-FITC to the cells by flow cytometry (**Figure 6A**). The FITC signal of WGA-FITC exposed CHO cells is stronger than in Lec2 cells (Median FITC-A value in WGA-FITC CHO cells is 18349 and for WGA-FITC-exposed Lec2 cells 2012), confirming the sialic acids-deficient phenotype of the Lec2 cells.

Next we assessed the sensitivity of Lec2 cells to infection with reovirus rS1His-2A-iLOV. Lec2, CHO and 911 cells were mock infected, infected with

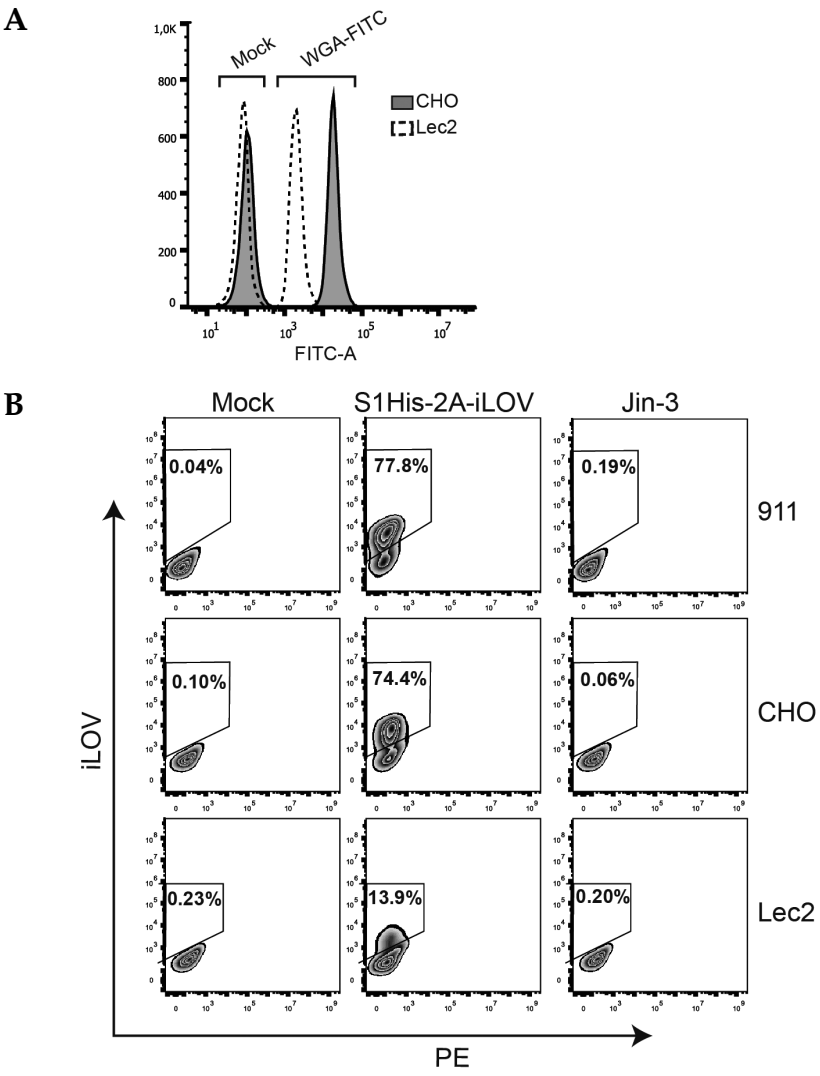

FIGURE 6.
The fraction of iLOV-positive cells upon reovirus rS1His-2A-iLOV exposure of Lec2 and CHO cells.

A. Lec2 cells and CHO cells were exposed to WGA-FITC and WGA-binding was evaluated by flow cytometry analysis. Lec2 cells have a ~90% reduction in WGA-FITC binding.

B. Flow cytometry analysis of 911, CHO, and Lec2 cells following infection with rS1His-2A-iLOV, *jin-3* (MOI_{911}~0.5) or mock infected cells, 48 hours post infection. iLOV signal is plotted on the Y-axis against the PE-A signal on the X-axis. Gates for iLOV positive cells are set per cell line.

rS1His-2A-iLOV, or with *jin-3* at an MOI_{911} of 0.5 and 48 hours post infection the iLOV-positive cells were detected by flow cytometry (**Figure 6B**). As expected exposure to *jin-3* did not result in detectable green fluorescence. This confirms the absence of autofluorescence upon infection with reovirus. In Lec2 cells infected with rS1His-2A-iLOV only 14% of the cells are iLOV positive, while the CHO and 911 cells infected with rS1His-2A-iLOV contain over 70% of iLOV positive cells. These data demonstrate that the reduction of the sialic-acids concentration on the surface of the Lec2 cells correlates with a decreased sensitivity to rS1His-2A-iLOV virus infection.

rS1His-2A-iLOV reoviruses retained the preferential cytolytic activity in tumor cells

To verify that the preferential cytolysis of transformed cells is maintained for rS1His-2A-iLOV, cultures of diploid human skin fibroblasts (VH10 cells) were used as a model. VH10 cells were exposed to rS1His-2A-iLOV, and *jin-3* reoviruses or mock infected. Compared to the 911-control infection (same as in **Figure 6B**) only a few iLOV positive cells (3.19%) were detected in the VH10-infected cells (**Figure 7A**). To further demonstrate that the VH10 cells resist rS1His-2A-iLOV-induced cell death, cells were exposed to rS1His-2A-iLOV and *jin-3* reoviruses at an MOI_{911}~5, or mock infected. Viability was measured four days post infection. As a positive control, 911 cells were included in the assay (**Figure 7B**). Whereas in 911 cells both reoviruses induce significant cell death in the monolayer cultures, the viability of the VH10 cell cultures is not affected. These results confirm that similar to the *jin-3* and *wt*T3D reoviruses, the rS1His-2A-iLOV viruses do not infect and lyse normal diploid skin fibroblasts.

Characterization of two deletion mutants that lost the iLOV gene

In two batches of the rS1His-2A-iLOV reoviruses, derived from independent transfection experiments, we noted the loss of transgene expression and the appearance of deletion mutants upon prolonged passaging. The viruses had been propagated for 10 rounds on 911 cells (Experiment 1) and 911scFvHis cells (Experiment 2). RT-PCR analysis revealed the presence of deletion mutants in the rS1His-2A-iLOV stocks (data not shown). This was confirmed by the gel electrophoresis of RNA isolated from infected cells (**Figure 8A**). Already at passage 3 of the reovirus on 911 cells, the deleted segment could be seen as an aberrant RNA segment migrating faster than the smallest S segment. On gel, both the full-length S1His-2A-iLOV segment and the S1 deletion segment are visible, suggesting a heterogeneous population of viruses. In RNA isolated from passage 10 only the S1 deletion segment could be detected, suggesting that this deletion mutant overgrew the population.

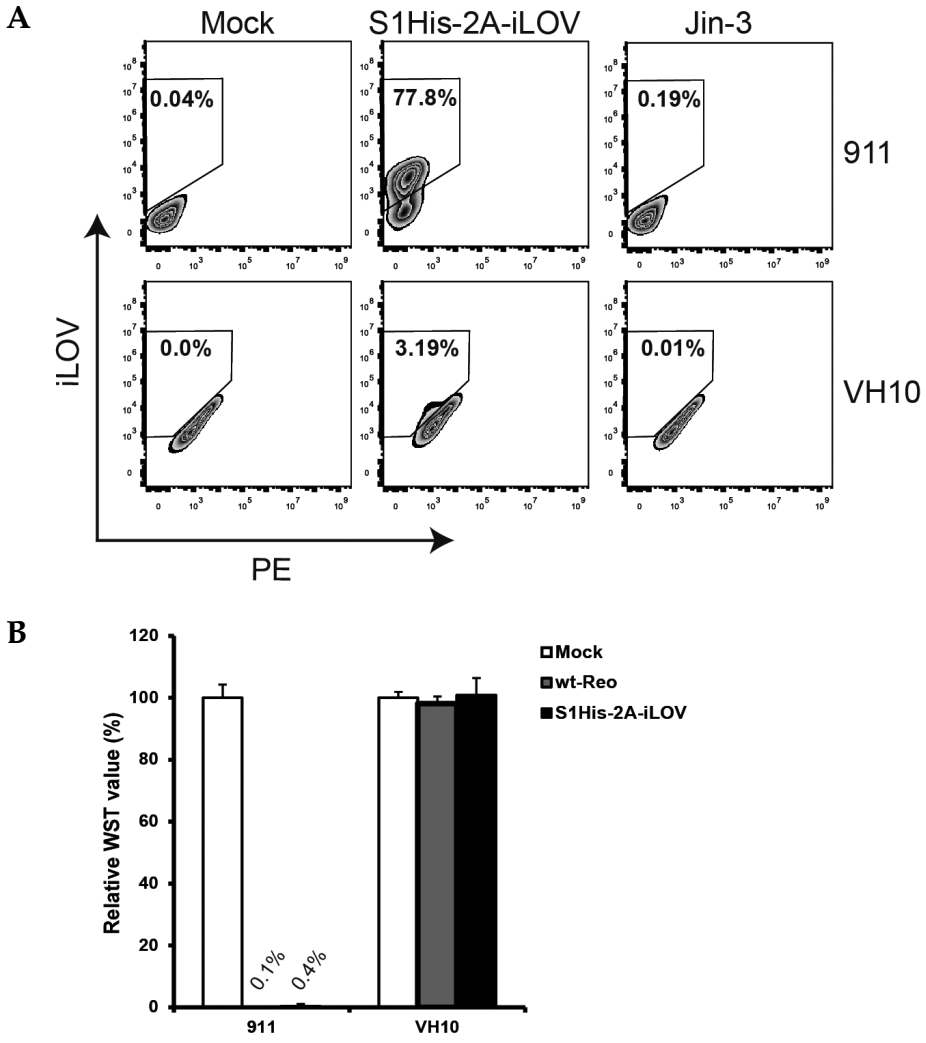

FIGURE 7.
Detection of iLOV positive cells and viability of VH10 cells exposed to rS1His-2A-iLOV.

A. Flow cytometry analysis of 911 and VH10 cells exposed to rS1His-2A-iLOV, *jin-3* (MOI_{911}~0.5) or mock treated cells, 48 hours post infection. The panel of 911 cells is copied from fig 6 b (VH10 samples were included in the same FACS experiment). iLOV signal is plotted on the Y axis against the PE-A signal on the X-axis. Gates for iLOV positive cells are set per cell line.

B. Cell viability, as measured by the WST-1 assay, four days post infection with rS1His-2A-iLOV, *jin-3* (MOI_{911}~1) or mock infected. Relative WST values are calculated as percentages of OD_{450} values of the infected cells over the mock treated cells. The error bars represent the SD (n=4). OD, optical density.

In a batch of rS1His-2A-iLOV reovirus propagated on 911scFvHis cells a deletion mutant was observed after 10 passages with an even smaller S1 segment. Sequence analyses of PCR products of the deletion mutants revealed that in both mutants the iLOV encoding region had been deleted resulting in S1 segments of 1001 nt (dl1, S1His-2A-iLOVΔnt 877-1261) and 922 nt (dl2, S1His-2A-iLOVΔnt 751-1214) in length, respectively (**Figure 8B**). In dl2, the 6xHis-tag and the 2A sequence had also been deleted. Interestingly, in dl2 the A-box, and in dl1 the A-box and the B-box, which both have been implicated in encapsidation of reovirus RNA into the viral capsid [24], were deleted, while the C-box element had been retained in both mutants. This suggests that the A and B boxes are dispensable for packaging the S1 segment RNA into the viral capsid.

The results from the RNA analyses were further verified at the protein level in 911scFvHis-cell lysates from cells infected with the reovirus batches containing the deletion mutants (**Figure 8C**). In passage 2 of both batches and passage 10 of the first batch, the σ1 protein contains the 2A element, whereas 2A is no longer detectable in passage 10 of batch 2. As expected the truncated σ1 protein migrates at an apparent molecular mass of 26 kDa instead of 30 kDa.

Taken together our data indicate that it is possible to insert the iLOV coding region in the S1 segment. However care should be taken to test the integrity of the transgene containing segment if the recombinant viruses are passaged to high passage number. Notably, apart from these deletions no other mutations were found in the S1 segment. This suggests that the sequence encoding the N-terminal 252 amino acids of the *jin-3* σ1 is sufficient for cell entry.

5.4 Discussion

Our data suggests that the S1 segment encoding σ1 is suitable for insertion of a transgene encoding a heterologous protein. We demonstrate that the S1 segment can harbor heterologous sequences that encode the fluorescent protein iLOV. In this construct, the iLOV-encoding sequence was preceded by the codons for the porcine teschovirus-1 2A peptide. Upon translation this yields separate proteins from the fused open reading frame. To reduce the size of the transgene-containing S1 segment, the sequences encoding the head-domain of σ1 were deleted. The head domain harbors the amino acids that interact with the JAM-A receptor. The head-domain could be removed by employing the σ1 sequences from the *jin-3* mutant [12]. This mutant is one of a series of reovirus T3D mutants that can infect cells in a JAM-A receptor-independent fashion. The mutations in these *jin*-mutants lead to amino-acid alterations close to the sialic-acid binding site in the σ1 protein, which is located in the tail domain of the spike [14, 15, 26].

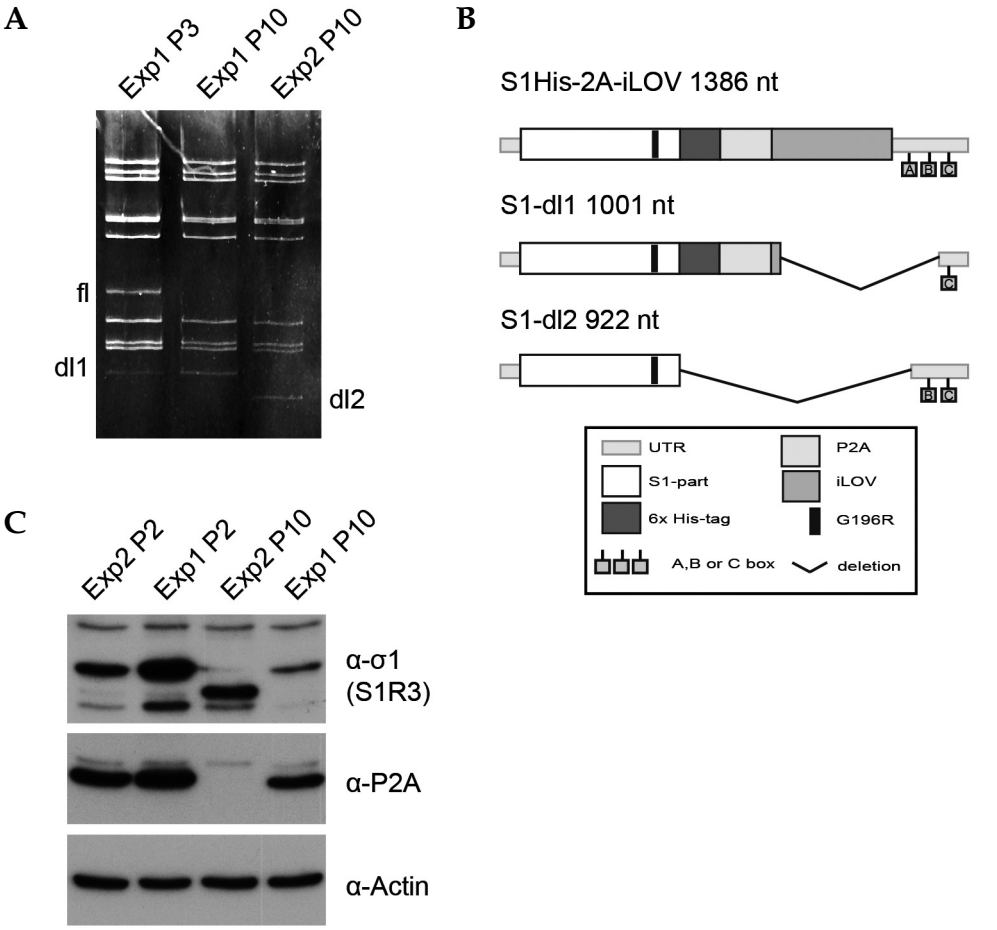

FIGURE 8.
Deletion mutants are detected in two batches of the rS1His-2A-iLOV virus.

A. RNA electrophoresis of 3 rS1His-2A-iLOV isolates from 2 independent experiments. In P3 of experiment 1 (Exp1) the deletion in the S1 segment (dl1) is already present next to the full-length (fl) S1His-2A-iLOV segment, while in P10 only S1-dl1 is present. Size of S1-dl1 is 1001 bp. Size of S1-dl2, derived from second experiment (Exp2) after 10 passages in 911scFvHis cells is 922 bp.

B. Schematic overview of the two S1-deletion mutants compared to the full length S1His-2A-iLOV.

C. Detection of σ1 protein in infected 911scFvHis cells in two independent batches of rS1His-2A-iLOV reoviruses containing deletion mutants. Lysates were made 48 hours after infection and equal amounts of protein were loaded on 12% SDS-PAGE gels and detected with antibodies directed against σ1 (S1R3), P2A (ABS31) or actin. The predicted size of σ1 in the deletion mutant (S1-dl2) is about 26 kD.

To release the S1-His protein from the iLOV protein, the porcine teschovirus-1 2A element was introduced. The length of the fragment encoding the 2A peptide is only 66 nt, and it also includes the codons for a GSG-linker to improve the efficiency [28, 29]. The 2A mediated separation is highly efficient and results in reovirus particles that do not contain the iLOV protein.

The S1His-2A-iLOV RNA was found to be packaged in the reovirus particles in 911 and 911scFvHis cells and transferred to target cells as was evident from RT-PCR analyses. The truncated σ1-His protein fragment was detected in infected cells and in CsCl-gradient purified reovirus particles.

The utility of the σ1 head domain-coding region as a locale for the transgene was further supported by the observation that an attenuated T3D reovirus derived from persistently infected HT1080 cells carries a mutation in S1 which resulted in a stop codon near the codons encoding the trypsin-sensitive site at amino acids 245 of the spike protein. This mutation results in truncation of the σ1 protein leading to attenuation of the virus upon inoculation of immune-deficient mice [30]. While the size of the S1 segment in this isolate was not altered, the σ1 protein lacks the head domain. This mutant reovirus replicated efficiently in L929 cells. As in our experiment the truncated σ1 protein was detected by immunoblots in purified particles produced on of L929 cells. The truncated protein consisted of the first 251 amino acids of σ1, and still contained the sialic-acid binding domain and the open reading frame encoding the σ1S protein [15, 31, 32].

In our modified S1 segment, the last 195 nucleotides of wild-type S1 segment were left intact. This region has been predicted to contain three cis-acting sequences required for packaging of the reovirus plus-strand RNA in the core; A, B and C-boxes [24]. In a deletion mutant characterized in our study we found that the A and the B-box were deleted. This suggests that the A and B box are dispensable, reducing the length of the region required for packaging to only 36 nt. The 5'-end of the S1 segment has been left intact. In our initial experiments, we tried to include the ORF encoding the Discosoma sp. red fluorescent protein (DsRed). This ORF is 678 nt in length, and was placed in-frame, downstream of the 6xHis-tag and the porcine teschovirus-1 2A element. Although we noticed the formation of replicating reoviruses, none of these transferred an intact copy of the DsRed transgene, as was evident from the complete absence of red fluorescence in the infected cells, despite the presence of marked cytopathicity. In this construct the total length of the modified S1 segment was 1722 nt, and we speculated that our inability to generate the recombinant dsRed-gene containing reoviruses was due to the larger size of the S1-dsRed segment. So far, limited data is available on the factors that restricts the packaging capacity of foreign sequences into the reovirus genome. In a recent study the insertion of long unmodified Simian Immuno-

deficiency Virus derived gag sequences in the reovirus M1 and L1 segments were not compatible with replication [9]. The failure to recover the recombinant reoviruses with the longer gag inserts was explained by the formation and stability of the RNA secondary structures of the inserted gag sequence. Upon reduction of the stability of the predicted RNA structure by wobble-mutagenesis, infectious recombinant reoviruses with the longer gag inserts could be obtained.

To circumvent the problem of possible size constraints, we evaluated the use of the smaller reporter iLOV. The coding region of this reporter is only 333 nucleotides in length. With the S1His-2A-iLOV construct we could generate replicating reoviruses harboring the iLOV ORF as a reporter gene. The iLOV signal could be detected in infected cells by fluorescence microscopy, but the signal was often weak and bleached rapidly. Nevertheless, the signal was detectable by flow cytometry. The iLOV sequence was based on a codon-optimized version of the iLOV protein sequence described in 2008 [20]. A codon-optimized iLOV has been used to generate an infectious recombinant foot-and-mouth disease virus [33]. Here too, the fluorescent signal of iLOV was reported to be relatively weak. Recently, more stable iLOV variants were generated that provided a stronger signal in bacterial and mammalian cells [34].

We have used a similar strategy for the expression of a small viral protein fused to the 2A sequence. Viable recombinant viruses could be generated and our preliminary results demonstrate the absence of deletions in the virus preparation upon prolonged passaging (up to 7 passages) (data not shown). These data further suggest that our head-replacement approach for expressing heterologous transgenes is feasible. It may be warranted that the integrity of the viruses should be tested regularly to avoid the presence of deletion mutants lacking the transgene product.

Based on the identification of the two viable S1-deletion mutants in our batches of rS1His-2A-iLOV reoviruses, the truncated S1 RNAs are effectively packaged into the reovirus cores. Smaller M1 deletion fragments were detected upon serial passages of reovirus Type 1 x Type 3 reassortants [35]. The smallest deletion mutant of M1 that was assembled into progeny viruses was approximately 350 nucleotides in length. The M1-deletion mutants are defective and rely on the presence of helper viruses that contain the full-length M1 to complement the deficiency. Based upon the analyses of different deletion mutants the authors concluded that the deletions do not occur randomly, instead they depend on the presence of particular sequence elements. In avian reoviruses, which are more distant members of the orthoreovirus genus [36], high MOI passaging yielded defective interfering (DI) particles containing S1-deletion mutants in which the residual S1 segment was approximately 400 nucleotides in length [37]. The

increase in the presence of the S1-deletion segment coincided with a decrease in full-length S1 RNA in purified avian virus particles. In infected chick embryo fibroblasts (CEF), the presence of the DI particles decreases the viral yields. As with the mammalian M1-deletion mutants, the replication of these DI mutants in cells relies on the presence of viruses with the full-length S1. Our S1-deletion mutants are distinct from these DI particles by virtue of their capacity to replicate autonomously and hence independently of wild-type helper viruses. Therefore such mutants may pave the way for generating recombinant reoviruses with even larger replacements of the S1 segment by heterologous sequences. The replacement of the head domain allows the insertion transgenes up to 522 nucleotides in length without exceeding the size of the *wt* S1 segment. It remains to be established whether the occurrence of deletion mutants in reoviral vectors constitutes a limitation for their use as oncolytic agents in clinical applications. Whereas deletion mutants are detected, in our hands the frequency of their occurrence is low enough to prevent them from impeding the preclinical experiments with these transgene containing viruses. Stocks containing deletion mutants can just be discarded. In both cases the deletion mutants could be traced back to low passage number stocks, suggesting that they may have been produced already during the generation of the recombinant viruses. The methodology to generate these viruses involves the generation of non-polyadenylated RNA molecules in the cells using T7 polymerase and ribozymes and such transcripts are known to be instable. This is supported by the observation that the rS1His-2A-iLOV virus could be stably propagated for 10 passages after expansion of a clonal isolate (data not shown).

So far, the yields of the head-domain replacement reovirus vectors is 10 - 30-fold lower than those of the *wt*T3D reoviruses on a per-cell basis. Several mechanisms could contribute to the reduced yields. The initial infections of the producer cells may be more asynchronous than with head-domain containing viruses, leading to reduced yields in single-step growth cultures. However, delaying the harvest did not compensate for this and did not normalize the yield (data not shown). Alternatively, the region of the *wt*T3D S1 segment that was deleted in the rS1His-2A-iLOV virus may contain cis-acting elements that negatively affect the virus yields when deleted. So far we have found no evidence for such mechanism. Also it could be conceivable that the iLOV transgene contains sequences that inhibit efficient production of particles. This seems unlikely, since the mutants from which the iLOV transgene had been deleted did not yield higher titers. Also, reoviruses with other small transgenes did yield similar titers (data not shown). Prolonged passaging of the rS1His-2A-iLOV virus did not yield other mutations in S1 than the deletion mutants mentioned above. This suggests that the *jin-3* mutation leading to the G196R substitution is stable and does not evolve further to further

enhance reovirus yields. Our current efforts to improve the yields employ reoviruses carrying therapeutic transgenes and involve bioselections.

The virus retained the capacity of the parental *jin-3* mutant to infect cells in the absence of the JAM-A receptor on the target cells, which is evident from the virus' capacity to infect JAM-A negative U118MG cells and chicken LMH cells in monolayer cultures The virus rS1His-2A-iLOV induces caspase 3/7 activity in 911 cells. This demonstrates that the induction of apoptosis by reovirus is independent of the virus' capacity to bind JAM-A. This is consistent with roles of either sialic-acid binding by the reovirus spike protein, or the σ1S protein in this process [18, 26], as both of these functions are retained in the reovirus vectors described in our study.

The modification of the S1 segment in the rS1His-2A-iLOV does not result in a loss of preference for transformed cells. Neither rS1His-2A-iLOV virus nor the *jin-3* virus induce CPE or apoptosis in human diploid skin fibroblasts. This, together with the virus' capacity to induce cytopathic cell death makes the modification strategy particularly suited for arming oncolytic reoviruses with transgenes that encode immune stimulatory molecules, antigenic proteins, or anti-tumor polypeptides. This strategy may be suitable for generating replication-competent reoviruses carrying a transgene for use as a vaccine vector or for boosting the anti-tumor-efficacy of oncolytic reoviruses.

5.5 Materials and methods

Cell lines and viruses

The cell lines U118MG, U251, LMH, CHO, Lec2 (all obtained from ATCC) and 911 were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Life Technologies, Bleiswijk, The Netherlands), supplemented with 8% fetal bovine serum (FBS; Invitrogen) and with penicillin and streptomycin (pen-strep) as described [13, 38]. LMH cells were cultured on dishes coated with 0.1% gelatin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Lec2 cells were cultured in alpha-MEM, supplemented with 8% FBS and pen-strep. The 911scFvHis cells were generated by transduction of 911 cells with the lentivirus vector pLV-scFvHis-IRES-Neo as described before [8]. The resulting polyclonal cell line was cultured in DMEM containing 8% FBS, pen-strep and 200 μg/ml G418. The T7-RNA polymerase expressing cell line BSR-T7 [39] was provided by K. Conzelman and cultured in DMEM, 8%FBS, pen-strep and 400 μg/ml G418. Normal foreskin fibroblasts (VH10), were provided by B. Klein [40] and cultured in high-glucose DMEM supplemented with 8% FBS and pen-strep. All cells are cultured in an atmosphere of 5% CO_2 at 37°C.

The wild-type T3D virus strain R124 was isolated from reovirus T3D stock VR-824 from the American Type Culture Collection (stock VR-824) by two rounds of plaque purification and propagated on 911 cells. In text this virus is referred to as *wt*T3D reovirus. The viruses were quantitated by plaque assay on 911 cells as described and virus concentrations and multiplicities of infection were defined on the bases of these titers, e.g. MOI_{911}.

Cloning of S1His-2A-iLOV in pBacT7 backbone

The S1His-2A-iLOV segment was designed in silico and a DNA copy was synthesized by Eurofins MWG Operon (Ebersberg, Germany). The total length of this synthetic segment is 1386 bp (**Figure 1**). The segment sequence is assembled to contain the following features: 1) nt 1 to 768 from the S1 segment of reovirus mutant *jin-3*; this includes the 5′ 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 [12]; 2) the codons for a 6xHis-tag (18 bp) which is placed in frame with the σ1 part; 3) the codons for the porcine teschovirus-1 2A sequence (66 bp + 3 additional bp); 4) the iLOV-encoding sequence (333 bp) which was reverse translated and human-codon optimized from the amino acid sequence published by Chapman *et al*. [20] using the web-based Encor Biotechnology Inc. (http://www.encorbio.com/protocols/Codon.htm); 5) the stop codon and 3′ end of S1 segment from nt 1219 to 1416 of reovirus T3D, which include A-, B-, and C-box elements predicted for encapsidation of the reovirus ssRNA in the capsid [24, 41], as well as the 3′ UTR. The resulting sequence was manually edited to remove or include specific restriction enzyme recognition sites in the designer DNA fragment.

The synthetic S1His-2A-iLOV fusion construct was inserted in a pBluescript plasmid by Eurofins MWG Operon (to generate pBSmwg-S1His-iLOV). The S1His-2A-iLOV part was further PCR amplified from this construct using forward primer T7S1For (5′-TAATACGACTCACTATAGCTATTGGTCGGATGGATCC-TCGCCTACGT-3′) and reverse primer S1iLOVendR (5′-ACGTGATATCCCTCG-CGATGAAAT-3′) with PFU polymerase (Fermentas, FisherScientific, Landsmeer, The Netherlands). The underlined sequences are the parts complementary to the sequence in the pBSmwg-S1His-2A-iLOV construct. The PCR product was digested with SacII and purified with SureClean (Bioline; GC Biotech BV, Alphen aan den Rijn, The Netherlands) according to the manual. The PCR product was inserted in the pBACT7 backbone of pBacT7-S1T3D [7]. Plasmid pBacT7-S1T3D was purchased at Addgene (plasmid 33282, www.addgene.org). The *wt* S1T3D was removed by digestion with SmaI and SacII and the pBACT7 backbone was isolated from a 1% agarose gel and purified by JetSorb (Genomed; ITK Diagnos-

tics BV, Uithoorn, Netherlands) according to the manufacturer's manual. The SacII-digested S1His-2A-iLOV-containing PCR product was inserted in the SmaI and SacII digested pBACT7 DNA with T4 DNA ligase (Fermentas, FisherScientific, Landsmeer, The Netherlands), resulting in construct pBT7-S1His-2A-iLOV.

Generation of reovirus rS1His-2A-iLOV

For generation of recombinant reoviruses we used the procedure described by Boehme *et al*. [42] with some modifications. Whereas the original article employs 10 plasmids for providing the full complement of reovirus segments [7], our system uses five plasmids. In addition to pBT7-S1His-2A-iLOV, we used the following plasmids which were all obtained from Addgene: pT7-L1T1L (plasmid 33286), pT7-L2-M3T3D (plasmid 33300), pT7-L3-M1T3D (plasmid 33301) and pT7-M2-S2-S3-S4T3D (plasmid 33302). For generating recombinant reoviruses the five plasmids were transfected into BSR-T7 cells using the TransIT-LT1 transfection reagent (Mirus; Sopachem BV, Ochten, Netherlands), according to the manufacturer's manual. Two μg of each of the plasmids was mixed and added to the cells with 30 μl TransIT reagent. Two-day post-transfection the cells were harvested and lysed by three cycles of freeze-thawing. After pelleting the cell debris, the cleared supernatant containing the recombinant reoviruses was added to 911scFvHis cells in a 6-well plate. Upon the first appearance of cytopathic effects (CPE) the cells were harvested and the rS1His-2A-iLOV reovirus was released from the cells by three cycles of freeze-thawing. The cell debris was removed from the reovirus-containing lysate by centrifugation (10' at 3000xg), and stored at -20°C until further use. The virus was routinely passaged by exposing fresh semi-confluent cultures to dilutions of the cleared lysates.

A medium-sized batch of cesium chloride-purified rS1His-2A-iLOV was prepared by loading the cleared freeze-thaw lysates onto a discontinuous CsCl gradient (1.45 g/cm^3 and 1.2 g/cm^3) in PBS. After centrifugation in a SW28 rotor at 95000xg for 4 hours at 16°C, the lower band, containing the infectious particles, was harvested and desalted in an Amicon® Ultra 100K device according to manufacturer's manual (Millipore, Merck Chemicals BV, Amsterdam, Netherlands). The CsCl-purified reovirus was recovered in reovirus storage buffer (10 mM Tris. HCl pH 7.5, 150 mM NaCl, 10mM $MgCl_2$.6 H_2O, 5% sucrose) was aliquoted and stored at -80°C until use. The amount of particles was calculated based on the OD_{260} values [43].

Reverse transcriptase PCR and sequence analysis

Cells (911scFvHis, 1*10^5) in 24 well plates were infected with rS1His-2A-iLOV. Since we had no indication of the titer of the early passaged batch (P1 of rS1His-

2A-iLOV) obtained in experiment 1, 1/20th part of the isolated reovirus was used for exposure to the cells. Total RNA was extracted with the Absolutely RNA miniprep kit (Stratagene, Agilent Technologies, Amstelveen, The Netherlands) from the infected cells according to the manual. cDNA was synthesised with the S1endR primer (5′-GATGAAATGCCCCAGTGC-3′) and SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Bleiswijk, The Netherlands). In the PCR the following primers were used to detect 1) S1 (S1For: 5′-GCTATTGGTCGGATGGATCCTCG-3′ and S1endR); 2) iLOV (forward primer: 5′-ATGATCGAGAAGAACTTCGTGATC-3′ and reverse primer: 5′-CACGTGGTCGCTGCCGTCCAGCTG-3′) and 3) S1Head part (S1testFor: 5′-GAGCATGTGGATAGGAATTG-3′ and S1endR) with GoTaq polymerase (Promega, Leiden, The Netherlands). The positions of the primer binding sites are shown in **Figure 3B**.

For sequence analysis PFU was used as the polymerase and the PCR products of the S1 segments (full-length in early passages and S1-dl1 and –dl2) were sent to our LGTC department (LUMC, Leiden, the Netherlands). Primers used in the sequence reactions: S1endR and S1For.

Western blot analysis

Cell lysates were prepared in Giordano Lysis Buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.1% Triton, 5 mM EDTA) supplemented with protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). Total amount of protein in the lysates was measured by Bradford assay (Biorad, Veenendaal, The Netherlands). Equal amounts of lysate (30 μg) were loaded into the wells of a 12% 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).

For electrophoresis of CsCl purified reoviruses ~$5*10^9$ particles were mixed with the above-mentioned western sample buffer and added to a 12% polyacrylamide SDS gel. The proteins were transferred to Immobilon-P (Millipore, Etten-Leur, The Netherlands) and visualized using standard protocols. For every antibody a different polyacrylamide-SDS gel was used unless otherwise stated.

The antibodies used in this study were S1R3, a monospecific polyclonal rabbit serum directed against the synthetic peptide glaelrvdhdnlvarv, representing amino acids 115-130 of the σ1-tail (generated by A. D. Lipińska, University of Gdańsk, Gdańsk, Poland). The peptide was glutaraldehyde-conjugated to glutathione S-transferase purified from the pGEX system, according to the manufacturer instructions (Amersham) and used to immunize rabbits. Mouse monoclonal antibody 4F2, directed against reovirus σ3 obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and

maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242 [44]. Antibody (mouse) directed against β-Actin: ImmunO anti-Actin clone C4 (MP Biomedicals, Eindhoven, The Netherlands). And rabbit anti-P2A peptide serum ABS31 (Merck-Millipore, Amsterdam, The Netherlands).

Flow cytometry analysis

Cells (911scFvHis, 911 or U118-MG) were infected with rS1His-2A-iLOV (MOI_{911}'s are indicated in the text). Cells were detached from the 24-well plates with trypsin and fixed in 4% paraformaldehyde at 72 hours post infection. After three washes in FACS buffer (PBS with 0.5% BSA and 2 mM EDTA) cells were assayed in FACS buffer for iLOV presence on a BD LSRII flow cytometer. ILOV signal was measured by selecting the blue laser (excitation at 488 nm) with the green fluorescence channel (FITC A in the plots) and the yellow fluorescence channel for PE (negative) signal. For the detection of iLOV positive cells in relation to sialic-acid binding or normal diploid cells 911, CHO, Lec2 or VH10 cells were mock infected, infected with rS1His-2A-iLOV or *jin-3* as an additional control. Both viruses were added to cells with the same MOI_{911} (see text) and 48 hours post infection prepared for FACS analysis as described above. In both experiments, gates for iLOV positive cells were set per cell line, depending on the population of the negative mock infected cells. Data were analysed with FACS-Diva software (BD Bioscience, Breda, The Netherlands).

Detection of WGA-FITC binding

CHO and Lec2 cells were transferred by trypsin treatment to tubes. Trypsin was inhibited by adding the cell suspension to DMEM containing 2% FBS. After one centrifugation step for 3 min. at 350xg, cells were resuspended in PBS (mock) or WGA-FITC (1 μg/ml in PBS) to a final concentration of ~$7.5*10^5$ cells per 500 μl. Cells were placed at room temperature for 15 min. to initiate binding of WGA-FITC, followed by three washes with FACS buffer (3 min. at 350xg) to remove excess of unattached WGA-FITC. Detection of FITC-positive cells was done in 500 μl FACS buffer on a BD LSRII flow cytometer. Data were analysed with FACS-Diva software (BD Bioscience, Breda, The Netherlands).

Reovirus RNA electrophoresis

For reovirus RNA analyses the method used is adapted from a rotavirus protocol described by Navarro *et al.* [45]. Cells in 6-well plates were infected with *wt*T3D or rS1His-2A-iLOV reoviruses. Upon the first appearance of CPE, the cells were harvested from the medium and subjected to three cycles of freeze-thawing

in 100 µl PBS containing 2% FBS. For the dsRNA isolation, SDS was added to the samples to a final concentration of 1%, boiled for 3 minutes, and placed on ice for at least 1 minute before 1 µl RNAse free DNAse (Fermentas, FisherScientific, Landsmeer, The Netherlands) was added. The samples were then incubated for 30 minutes at 37°C. Subsequently Proteinase K (Fermentas, FisherScientific, Landsmeer, The Netherlands) was added to a final concentration of 2 mg/ml and left at 37° C for 2 hours. RNA was extracted by 1 volume of Trizol reagent (Ambion, Life Technolgies, Bleiswijk, The Netherlands) followed by isopropanol precipitation. RNA was taken up in 25 µl RNase free water. To separate the RNA segments 10 µl was used and loaded on a 12% polyacrylamide-SDS gel after addition of 2x RNA loading dye (Fermentas, FisherScientific, Landsmeer, The Netherlands). Electrophoresis was carried out with 20 mA for ~12 hours, before staining with SYBR gold (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) and photographed on a BioRad Gel Doc™ XR+ System (BioRad, Veenendaal, The Netherlands).

Viability assay

WST-1 reagent (Roche, Woerden, The Netherlands) was used to assay the viability of cells after reovirus infections. Cells were grown in 96-well plates and mock infected or infected with *wt*T3D reovirus or early passage (P2) rS1His-2A-iLOV reovirus with an MOI_{911} of 1, in quadruple. Four days post infection the WST-1 reagent was added to the wells according to the instructions. The mock infected cells were set to 100% viability. The statistical significance was determined with GraphPad Prism v6 using the 2-way ANOVA with Dunnett's multiple comparison test. The results are considered significant for $P<0.05$.

For the viability assay with the VH10 cells and 911 cells as a control, cells were infected with rS1His-iLOV, *jin-3* (MOI_{911}~1) or uninfected in quadruplicate and four days post infection the viability of the cultures was determined by WST-assay.

Caspase-3/7 activity assay

For measuring caspase activity, the 911 cell line was cultured in 96-well plates ($1.5*10^4$ cells per well in quadruplicate) and mock infected, infected with reovirus containing lysates of *wt*T3D reovirus, rS1His-2A-iLOV, or *jin-3* mutant reovirus at an MOI_{911}~5. Twenty four hours and 48 hours post infection caspase-3/7 activity was measured by the Caspase-Glo 3/7 detection kit (Promega, Leiden, The Netherlands).

Determination of rS1His-2A-iLOV by TCID50 (50% tissue culture infective dose) assay

One day prior to the assay, 911 cells were plated in 96-well plates. The next day cells were infected with 10-fold serially dilutions of rS1His-2A-iLOV virus, starting with a 100-fold dilution up to 10^{-9} in DMEM with 2% FBS. Of each dilution 50 µl/well was added to 10 wells containing 911 cells in 50 µl DMEM plus 2% FBS per well. The last 2 wells of each row were used as uninfected controls. After ~7 days, CPE was scored under a light microscope and titer was determined using the Reed-Müench method [46] and expressed as Infectious Units (IU_{911})/ml.

Nucleotide sequences

The sequence of the S1His-2A-iLOV segment is deposited in Genbank under accession number KJ806995. The GenBank ID's of our *wt*T3D (R124) segments are: R124 L1 GU991659; R124 L2 GU991660; R124 L3 GU991661; R124 M1 GU991662; R124 M2 GU991663; R124 M3 GU991664; R124 S1 GU991665; R124 S2 GU991666; R124 S3 GU991667; R124 S4 GU991668.

Genbank ID of *jin-3* S1segment is: KJ806994.

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Chapter 6

General discussion

Contents chapter 6

6.1 Overall conclusions

The clinical evaluation of reovirus T3D as an anti-cancer agent for the treatment of cancers revealed several obstacles. The antiviral immune response and the inefficient delivery of reovirus to the tumour site are identified as the main hurdles that limit efficacy [1]. The work described in this thesis is focused on this last barrier; the resistance of cancer cells to reovirus induced cell death [2, 3] and enhancing the oncolytic properties of reovirus T3D. The studies follow two basic approaches: 1) bioselection and 2) genetic modification.

The results of the work led us to the following conclusions:

1. The high mutation rate of the dsRNA of reoviruses is applicable for generating viruses that are capable of infecting cells that resist infection with wild-type reovirus due to the lack of the JAM-A receptor. The new viruses have expanded their tropism.
2. In more complex culture conditions such as spheroid cultures, reovirus T3D is not dependent on the interaction with the JAM-A receptor for cell entry.
3. The spike protein σ1 tolerates the insertion of small heterologous proteins including a teschovirus-1 2A element and permits the removal of the JAM-A binding domain of σ1.
4. It is possible to insert a small heterologous transgene into the S1 segment of reovirus T3D without the need to use helper cell lines or wild-type reoviruses to rescue the recombinant reovirus.

6.2 Reovirus in relation to sialylated glycans

Reovirus T3D has been applied in several clinical trials and has been well tolerated and safe in humans (e.g. see website of Oncolytics Biotech for the latest developments and trials; http://www.oncolyticsbiotech.com). So far, the bioselected *jin* reoviruses described in **Chapter 2** of this thesis and the rS1His-2A-iLOV recombinant reovirus (**Chapter 5**) have only been studied in tissue culture conditions. The next step would be to test them in an experimental animal cancer model. A problem is that for neonatal mice [4] and severe immunodeficient mice [5], reoviruses can be fatal. Most of the animal experiments in reovirus research are therefore performed in adult nude mice [6, 7].

One of the important determinants for reovirus pathogenesis in the central nervous system of newborn mice is the binding of σ1 to sialic acids. In contrast to our *jin* mutants with an enhanced binding to sialic acids an amino acid substitution of R202W in σ1 is no longer capable of binding to sialic acids [8]. The T3D-σ1R202W

virus was less neurovirulent in 2-day-old mice. In the spinal cords of mice infected with T3D-σ1R202W less apoptotic cells were detected than in the spinal cords of mice infected with the wild-type T3D virus. In this respect it is interesting to see what happens with our *jin* mutants or the head-less rS1His-2A-iLOV virus in newborn mice.

In 911 cells, the caspase 3/7 activity induced after infection of the recombinant rS1His-2A-iLOV virus is less than in the wild-type reovirus infected 911 cells. In *jin-3* infected 911 cells, the induction of caspase 3/7 is delayed, but at 48 hr post infection the activity is higher than in the recombinant virus infected cells (**Chapter 5**, **Figure 5C**). The paper by Danthi *et al.* [9] mentions that reovirus strains containing both JAM-A and sialic acid binding domains are the most potent inducers of apoptosis. This can explain our observations of a reduced caspase 3/7 activity in 911 cells with the headless rS1His-2A-iLOV virus.

Binding of σ1 to sialic acids (α2,3; α2,6 or α2,8-linked sialylated glycans) is specific for reovirus strain T3D. It was found that strain T1L utilizes ganglioside GM2 as a coreceptor [10]. While the glycan binding domain of T3D-σ1 is located in the tail part of the protein, in T1L-σ1 the glycan binding domain is located in the head domain, near the JAM-A binding site. Noteworthy in this respect is the location of the amino acid change Q336R in σ1 of *jin-1* and *jin-2* (**Chapter 2**, **Table 1**). The alignment of the glycan binding domain of T1Lσ1 with T3Dσ1 and *jin-1*σ1 is depicted in **Figure 1**. It is possible that the alteration of Q336R in the two *jin*-mutants renders a conformation that allows binding to a glycan at this position. Generating the T3D-σ1Q336R protein would allow interaction studies with different glycans.

6.3 Reovirus and cell entry

Since the discovery that the three reovirus strains (T1L, T2J and T3D) attach to JAM-A it is generally accepted that JAM-A is the high affinity receptor, at least in cells cultured *in vitro*. The situation *in vivo* is more complex, as is explored in JAM-A$^{-/-}$ mice infected with reovirus T1L, T3D and a strain defective of binding to sialic acids (T3SA^{-}) [11]. In this report it is concluded that JAM-A is not involved in reovirus replication in the intestine and the brain of mice infected with the T3SA^{-} strain. Based on findings that specific regions in the brains of both WT and JAM-A$^{-/-}$ mice are infected with reovirus T3D and T3SA^{-} it is hypothesized that in neurons a different receptor is involved in reovirus T3D uptake. In 2014 this receptor was identified as the Nogo receptor, NgR1; a leucine-rich repeat protein expressed on the surface of neurons [12]. Adult mice, however, are protected from

T1Lσ1	(350-380)	WR	ANVT	LNLMKVDDWLVLSF	SQMT	TNSIMAD
T3Dσ1	(333-363)	WR	VQVN	SDIFIVDDYIHICL	PAFD	GFSIADG
jin-1σ1	(333-363)	WR	VRVN	SDIFIVDDYIHICL	PAFD	GFSIADG

FIGURE 1. Amino Acid alignment of the glycan binding domain of T1L-σ1 (350-380) with the corresponding region of T3D-σ1 and *jin-1*-σ1 (333-360). The glycan- binding sites in T1L-σ1 are highlighted by yellow boxes. The altered amino acid of *jin-1* (R336) is displayed in red. Adapted from [10].

reovirus-induced CNS disease. This can be explained by the preference for reovirus T3D to infect only the unmyelinated CNS of newborn mice in which the NgR1 receptor is not yet fully associated to myelin and therefore available for reovirus binding. This age-dependent pathogenesis had already been reported previously [13]. More research is needed to identify the reovirus protein that is involved in binding to NgR1. It seems that σ3 or part of σ1 that stays associated with virions, but not with the conformationally changed σ1 in ISVPs, binds NgR1 [12].

In the intestine where many proteases are present, reovirus particles may be extracellular digested to ISVPs and the ISVPs no longer depend on JAM-A for cell entry [14-16]. Also in U118MG cells grown in 3D-cultures we found that JAM-A is dispensable for cell entry. The sensitivity for reovirus acquired by the cells when grown in spheroids, is dependent on cathapsins secreted by the U118MG spheroids (**Chapter 3**). In the microenvironment of tumours many proteases are present, including different cathapsins, that can promote tumour progression (for a review see Alain *et al.* [17]) and may help reovirus to enter the tumour cells independent of the JAM-A receptor.

JAM-A and CAR (coxsackievirus and adenovirus receptor) are both membrane proteins involved in adhesive junctions (AJs) on epithelial and endothelial cells. Many viruses utilize different families of the membrane proteins in AJs as receptor to enter cells. Adenoviruses use CAR for cell entry in a similar fashion as reovirus exploits JAM-A [18, 19]. In 2002 a report appeared with evidence that adenoviruses not only use CAR for cell entry but also for their escape [20]. Cells infected with adenovirus produce an excess of fibers (the spike protein of adenovirus that attaches to the CAR receptor) that are not all incorporated in the nascent virions. The produced fibers may bind CAR, thereby competing with the CAR-CAR interactions that mediate cell-cell adhesion. This may allow the adenoviruses to escape and cross tissue barriers. Based on the similarity in structure of the adenovirus fiber and the reovirus spike it is possible that also reoviruses may use secretion of excess amounts of spike protein to facilitate viral escape into the

bloodstream by disrupting JAM-A homodimer interactions [21]. The data summarized here indicates that reoviruses can use several strategies and receptors for their replication in different circumstances.

6.4 Reovirus and host immune response

Administration of oncolytic viruses to patients with an intact immune system has been a challenge. The viruses have to overcome many barriers before reaching their potential target, the tumour site [22]. In the early days of oncolytic virus delivery, the focus was on direct intratumoral delivery. This, of course, is only possible if the tumour site is easily accessible for direct injection of the oncolytic agent. For a more general solution, including reaching metastasis, the preferred method is by systemic delivery. In the oncolytic viral field, many strategies are being developed to deal with acquired immune responses against the virus [23-25].

One of the obstacles is the pre-existing immunity against reovirus in the majority (± 90%) of the human population. In most of the patients enrolled in clinical trials neutralizing antibodies (NABs) are detected prior to administration of the reovirus [26-28]. Experiments in reovirus-immune tumour bearing mice revealed that intravenous injected reovirus, antitumour therapy was completely ineffective [29]. The same research shows that delivery of the reovirus on carrier cells (mature Dendritic Cells (mDC) and T-cells) protects the virus against the circulating NABs resulting in clearance of the cancer cells. Furthermore, when reoviruses were delivered by T-cells an anti-tumour immune response was triggered resulting in long-term tumour clearance. These findings imply that besides the negative effect of the immune response towards the reovirus, there is also a beneficial effect of the immune system on the reovirus mediated anti-tumour effect. This observation is not new to the field of oncolytic virus therapy. It is shown that uninfected tumour cells in tumours treated with vesicular stomatitis virus (VSV) were also killed despite the initial poor delivery of the VSV to the tumours in a mouse model [30]. Over the years, more is published on the topic of oncolytic viruses and the complex interaction with the host immune system and the anti-tumour response [22, 25, 31].

In the human situation the circumstances for reovirus are different compared to mouse models. Despite the presence of NABs, reovirus could be detected in tumour biopsies of patients after intravenous administration [27, 32, 33]. One hypothesis is that reoviruses in the bloodstream bind to and/or infect blood cells (mononuclear cells, granulocytes and platelets) and are transported to the tumour

sites, shielded from the circulating NABs. To further test this, a study was performed that includes stimulation of the potential carrier cells prior to systemic reovirus delivery [28]. They confirmed that cytokine stimulation of the recipient cells (monocytes/macrophages) *in vivo* enhanced the viral delivery to the tumour cells. This cytokine conditioning therapy was even more effective in reovirus-immune mice with pre-existing NABs. More research is needed to fully understand the complex interaction of the immune system with oncolytic viruses and the anti-tumour effect. For reovirus therapy it is at least promising that also in the presence of pre-existing NABs the viruses can find their way to the tumours.

6.5 Reovirus and its next generation

A proof of concept for adding a therapeutic transgene is described in this thesis (**Chapter 5**). Here we describe the introduction of a gene encoding a fluorescent reporter protein in the segment encoding the reovirus spike protein. Based on the observations that it may be useful to enhance the immune response to tumours in combination with reoviruses, next generation reoviruses would carry immune-stimulating molecules. This would give the virus a dual-acting function for inducing tumor cell death.

Already GM-CSF is used in the context of an oncolytic herpes simplex virus for the treatment of melanoma [34]. The study shows that intratumoral administration of the so called Oncovex$^{GM\text{-}CSF}$ virus induced an immune response in metastatic melanoma lesions. This could be used in the field of anti-cancer immunotherapy, which is developing towards personalized peptide vaccination as a novel treatment [35].

Another, related, strategy based on our recombinant reoviruses is to enhance the oncolytic potency by adding anti-tumor proteins [36]. There are many candidates, among others the viral proteins apoptin [37], the adenovirus-derived E4Orf4 [38], or even non-viral anticancer peptides [39, 40]. One of those anticancer peptides, LyP-1 is already exploited in combination with baculovirus [41].

In our initial design of the rS1His-2A-ilOV virus we found several viable deletion mutants. In those deletion mutants, sequences in the untranslated region (A and B-boxes) were deleted, suggesting that these two conserved elements are not necessary for incorporation of the recombinant S1 segment. This provides another 52 nucleotides for adding foreign sequences, for example codons for a tumour-homing sequence, like LyP-1 (27 nucleotides long). For a targeting strategy, the codons could be directly fused with the S1His codons to ensure incorporation of the targeting peptide into the viral capsid.

6.6 References chapter 6

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Addendum

Contents Addendum

Summary

In the war against cancer many viruses have been tried as bullets. Even viruses that cause diseases in humans have been adapted to destroy cancer cells. Such viruses were rendered less pathogenic and thereby reduced their fitness and reproductive capacity. Some viruses discriminate by default between normal cells and transformed cells: such viruses are named 'oncolytic viruses'. They preferentially infect, multiply and destroy transformed cells for their release and dispersion to the surrounding cells. One of these viruses is the mammalian orthoreovirus T3D, or reovirus, in short. **Chapter 1** gives an introduction on the characteristics of reoviruses and their potential use as oncolytic agents.

Cancer cells have developed various strategies to escape control of normal regulators in tissue. This may have consequences for oncolytic viruses if their strategy involves evading cell death pathways. For their anti-tumour effect oncolytic viruses need to attach to receptors on the surface of the cell before their entry into the cell. In some tumour cells the canonical reovirus receptors may not always available. Reovirus cell entry is a multiple step process. The first step involves attaching to sialic-acid residues on the cell surface. It is followed by a higher affinity binding to Junction Adhesion Molecule-A (JAM-A). **Chapter 2** describes the isolation of reovirus mutants that are able to infect cells that lack JAM-A, the JAM-A independent (in short: *jin*) reovirus mutants. The *jin* mutants harbour mutations in the S1 segment resulting in amino acid changes in the tail of the spike protein σ1, and in two mutants (*jin-1* and *jin-2*), also in the head domain region. The changes in the three *jin* mutants in the tail of σ1 are located in close proximity of the region that is implicated in sialic-acid binding. Our hypothesis is that the changed affinity for sialic-acids underlies the expanded tropism of the *jin* viruses. The question remains how important this is in tumours *in vivo*.

The tumour microenvironment *in vivo* differs strongly from the conditions in cell culture. This is noticeable when cells are grown as spheroids. **Chapter 3** reveals that JAM-A is dispensable for reovirus infection when cells are grown in a 3D cultures. The U118MG cells that are reovirus resistant under normal cell culture conditions, due to the absence of JAM-A, are susceptible to reovirus infection when grown in spheroid cultures. The increased infection is mediated by the presence of extracellular cathepsins in the U118MG spheroids. Cathepsins are proteases that can convert the reovirus particles to so-called intermediate subviral particles (ISVPs). ISVPs have been described to be capable of infecting cells independent of the JAM-A receptor.

The tumour microenvironment contains many proteases secreted by the tumour cells. These proteases promote tumour progression by modulation of key

processes such as angiogenesis, metastasis, and composing the extra-cellular matrix (ECM). The presence of proteases in tumour could be enhance reovirus infection and replication.

Genetic modification of the segmented dsRNA genome of reoviruses is more complicated than modification of dsDNA viruses such as Herpes Simplex virus or Adenovirus. Based on previous research in the group on Adenovirus and modifications to its capsid proteins to expand the tropism, **Chapter 4** describes the modification of the reovirus S1 segment that encodes for the spike protein σ1 in the viral capsid. This approach requires the presence of wild-type reovirus to rescue the genetically modified virus. The only segment that was changed is S1 and included the codons for a 6xHis-tag at the 3'end of the open reading frame for the σ1 protein. Our results demonstrate that the C-terminus of σ1 is a suitable location for adding heterologous peptides and that the S1His segment stably delivered with a lentivirus into 911 cells is encapsulated in the reovirus particles. A drawback of this system is the presence of wild-type reovirus necessary to provide the other reovirus segments. Although a selection system is used that consists of cells that are resistant to wild-type reovirus and are only sensitive to the His-tagged reovirus, the mutation rate of this dsRNA viruses interferes with the reliability of the system and the chance that revertant viruses are isolated is relatively high (see **Chapter 2**).

In 2007 a plasmid-based reverse genetics system for mammalian reoviruses was described which allowed generation of recombinant reoviruses without the requirements of wild-type reovirus as a helper. In **Chapter 5**, this system was used for generating a recombinant reovirus that carries a gene that encodes for a small fluorescent protein (called iLOV) and replaces the head domain of the σ1 protein. The choice for this location was based on the knowledge that the *jin-3* virus, with only one point mutation in the tail of σ1, efficiently infects cells independent of the JAM-A receptor. The S1His-2A-iLOV segment encodes the first 252 amino acids of σ1, including the point mutation in the tail of *jin-3* σ1 which lead to the G196R substitution fused to the His-tag and a teschovirus-1 2A peptide that ensures the synthesis of a separated iLOV protein in infected cells. The data presented in this chapter demonstrate for the first time that σ1 tolerates replacement of the head domain of the spike protein by a small heterologous proteins. With this system, next generation reoviruses could be generated that are armed with therapeutic transgenes to enhance the oncolytic efficacy of these viruses.

Finally, **Chapter 6** provides a general discussion on some of the aspects involved in reovirus entry of cells and the immune response in men and mice.

Nederlandse samenvatting

'Sleutelen aan Reovirus T3D om de Oncolytische Effectiviteit te Verhogen'

Om de strijd te winnen in de oorlog tegen kanker worden zelfs virussen gebruikt als kogels. Ook virussen die ziekten kunnen veroorzaken bij de mens: zij worden zodanig aangepast dat ze kankercellen vernietigen en minder ziekteverwekkend zijn. Dit heeft soms tot gevolg dat deze virussen minder levensvatbaar zijn en zich slechter kunnen vermenigvuldigen. Een aantal virussen is van nature in staat om te discrimineren tussen gezonde cellen en kankercellen: de zogenoemde oncolytische virussen. Deze virussen vermenigvuldigen zich voornamelijk in de kankercellen en bij de ontsnapping uit de cellen waarbij deze cellen worden vernietigd. Eén van die virussen is "Mammalian Orthoreovirus T3D", hier verder reovirus genoemd. **Hoofdstuk 1** geeft een inleiding in de biologie van reovirussen en hoe ze worden ingezet bij behandeling van kanker. Kankercellen hebben strategieën ontwikkeld om aan de normale regulatoren van celdeling te ontsnappen. Dit heeft ook gevolgen voor de oncolytische virussen als kankercellen niet meer reageren op prikkels voor celdood, waar virussen op rekenen in hun strategie om te ontsnappen uit de cellen.

Voordat de oncolytische virussen een anti-tumor effect kunnen veroorzaken zullen de virussen eerst de cellen binnen moeten komen. Dit doen virussen door te hechten aan bepaalde receptoren die aanwezig zijn op de oppervlakten van de cellen. Helaas zijn niet bij alle typenkanker deze receptoren aanwezig. Het reovirus komt de cellen binnen met een proces dat meerdere stappen kent. Als eerste bindt het reovirus aan siaalzuren die op de oppervlakte van cellen aanwezig zijn om vervolgens aan de "echte" receptor te binden, genaamd JAM-A (Engelse afkorting voor Junction Adhesion-Molecule A). In **hoofdstuk 2** wordt beschreven hoe reovirus mutanten werden geïsoleerd die in staat zijn om cellen te infecteren die geen JAM-A receptor bevatten; deze aangepaste reovirussen worden *jin*-mutanten genoemd. *Jin* staat voor JAM-A onafhankelijk (JAM-A independent). *Jin* reovirussen bevatten puntmutaties in het S1 genoom. Deze leiden tot aminozuur veranderingen in het σ1 eiwit, verantwoordelijk voor hechting van de reovirussen aan de cellen. De drie geïsoleerde *jin* reovirussen hebben veranderingen in de staart van het σ1 eiwit en twee van de drie (*jin-1* en *jin-2*) ook nog in het kopdomein. De veranderingen in de staart van σ1 liggen allemaal in de buurt van de regio die verantwoordelijk wordt gehouden voor de binding aan siaalzuren op het celoppervlake. Onze hypothese is dat de *jin* reovirussen zich sterker binden aan siaalzuren door deze veranderingen en daardoor meer verschillende gastheer-

cellen kunnen infecteren, ook aan de cellen die geen JAM-A receptor hebben. De vraag blijft of dit in tumoren van het menselijk lichaam ook geldt.

Het micromilieu in tumoren is anders dan van cellen die in het laboratorium op plastic worden gekweekt. Er is al een verschil waarneembaar als cellen in 3D klompjes (spheroids) worden gegroeid. **Hoofdstuk 3** geeft aan dat JAM-A niet nodig is voor het infecteren van U118MG spheroids door reovirus. Onder normale celkweek omstandigheden kan reovirus, door het ontbreken van JAM-A, de U118MG cellen niet infecteren, maar in de 3D spheroids zijn deze cellen ineens wel gevoelig voor reovirusinfectie. Een verklaring voor de verhoogde gevoeligheid is de aanwezigheid van bepaalde enzymen die in staat zijn om andere eiwitten af te breken, cathepsins. Cathepsins behoren tot een groep enzymen die in staat zijn om reovirussen af te breken tot zogenoemde ISVPs (Intermediate Subviral Particles). Een eigenschap van deze ISVPs is dat het binnendringen van cellen onafhankelijk is van de aanwezigheid van JAM-A op het celoppervlak. In het micromilieu van tumoren is bekend dat veel van dit soort enzymen (proteases in dit geval) aanwezig zijn. De proteases zijn mede verantwoordelijk voor de ontwikkeling van de tumor naar een agressievere vorm door bijvoorbeeld het aanleggen van bloedvaten (angiogenese), en hebben een rol bij het vermogen om te metastaseren en ze zijn belangrijk voor het remodeleren van de extracellulaire matrix van de tumor. De aanwezigheid van deze proteasen kunnen reovirussen helpen om de tumorcellen binnen te komen.

Genetische modificatie van het reovirus genoom is door de aanwezigheid van meerdere dsRNA segmenten een stuk moeilijker dan bij dsDNA virussen, zoals bijvoorbeeld herpes simplex virus en adenovirus. Gebaseerd eerder ervaringen in ons lab met het modificeren van adenoviruscapside eiwitten om het tropisme te veranderen wordt in **hoofdstuk 4** beschreven hoe het S1 segment van reovirus genetisch wordt veranderd. S1 codeert voor het capside eiwit σ1, dat de spike vormt waarmee het virus de JAM-A receptor bindt. Om het veranderde reovirus te vermenigvuldigen is er echter wild-type reovirus nodig. Het enige segment dat wordt veranderd is S1. Hier wordt de sequentie coderend voor een 6xHis-tag geplaatst dat er zo voor te zorgen dat aan de C-terminus van het σ1 eiwit een His-tag vast zit. De experimenten tonen aan dat deze locatie geschikt is voor het aanbrengen van heterologe peptiden en dat het S1His genoom dat wordt aangeleverd door een 911 cellijn die was getransduceerd met een lentivirus, wordt ingepakt in de nieuwe reovirussen. Een nadeel van het systeem is dat er wild-type reovirus nodig is om de andere negen segmenten te leveren. Ondanks het gebruik van een selectiesysteem tegen het wild-type reovirus is de hoge mutatiesnelheid van reovirus een bedreiging voor de betrouwbaarheid van dit systeem, waardoor de kans op het ontstaan van mutanten toeneemt (zie ook **hoofdstuk 2**).

Het beschikbaar komen van een plasmide systeem voor het modificeren van reovirus (in 2007) heeft ertoe geleid dat er geen wild-type reovirus meer nodig is. In **hoofdstuk 5** is dit systeem gebruikt voor het maken van een recombinant reovirus met een S1 segment waarin het coderende gedeelte voor het kopdomein van σ1 wordt vervangen door de codons van een klein fluorescerend eiwit (iLOV). De keuze van deze locatie kwam voort uit de kennis dat *jin-3*, met maar één aminozuursubstitutie in de staart van σ1, niet langer JAM-A nodig heeft om cellen binnen te komen. Het nieuwe S1His-2A-iLOV segment codeert de sequentie voor de eerste 252 aminozuren van σ1, met de puntmutatie resulterend in de G196R verandering, gefuseerd aan de codons voor een His-tag en die voor het teschovirus-2A peptide dat ervoor zorgt dat het iLOV eiwit wordt gescheiden van het σ1-His eiwit. Uit de gegevens van dit hoofdstuk blijkt voor het eerst dat σ1 de toevoeging van een klein heteroloog eiwit tolereert en daarbij het hoofddomein niet nodig heeft. Met deze kennis is het mogelijk om nieuwe generaties reovirussen te maken die gewapend zijn met therapeutische genen die de oncolytische effectiviteit kunnen verhogen.

Het laatste hoofdstuk, **hoofdstuk 6**, vat de algemene conclusies samen en bespreekt een aantal onderdelen die van belang zijn bij het binnenkomen van de reovirussen in cellen en de reactie van het immuunsysteem op de aanwezigheid van reovirus in mensen en muizen.

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Curriculum vitae

Diana van den Wollenberg is geboren op 3 maart 1969 in Tilburg. Na het doorlopen van de Beatrix MAVO in Tilburg werd in 1985 begonnen aan de HLO opleiding Medische Biochemie aan de Hogeschool West-Brabant (Dr. Struycken Instituut) in Etten-Leur, waarvan het eerste jaar in een speciale schakelklas MLO-HLO. De opleiding werd afgesloten in 1990 met een stage bij het Nederlands Kanker Instituut, Sectie Celbiologie, onder begeleiding van Dr. J. Collard.

In juli 1990 werd ze aangenomen als analist bij de sectie Moleculaire Carcinogenese van de Universiteit Leiden. Zij ging werken in de groep van Prof. Dr. A.J. van der Eb en werd ingezet bij het promotieonderzoek van R.C Hoeben (inmiddels Prof.) naar gentherapie voor hemofilie A. In de loop van de jaren werd de nadruk van het onderzoek steeds meer gelegd op de ontwikkeling van nieuwe virale vectoren voor gebruik in gentherapie en in oncolytische behandelingen. Inmiddels was de sectie Moleculaire Carcinogenese opgegaan in de afdeling Moleculaire Celbiologie onder leiding van Prof. Dr. H.J. Tanke en werd de Medische faculteit ondergebracht bij het Leids Universitair Medisch Centrum. Eind jaren 90 heeft ze meegewerkt aan de ontwikkeling van de helpercellijn PER.C6 (Crucell) om adenovirus vectoren op te groeien. In 2005 werden de reovirussen geïntroduceerd binnen de Virus- en Stamcelbiologiegroep.

Naast het onderzoek is ze, sinds 2006, actief binnen het LUMC in de medezeggenschap, eerst als lid van de onderdeelcommissie divisie 5 (OC5), later als duo-voorzitter van OC5 en momenteel als duo-voorzitter van de onderdeelcommissie behorende bij divisie 4 (OC4).

Vanaf 2008 heeft ze het promotieonderzoek verricht dat is beschreven in dit proefschrift.