Forward and reverse genetics strategies for improving oncolytic reoviruses

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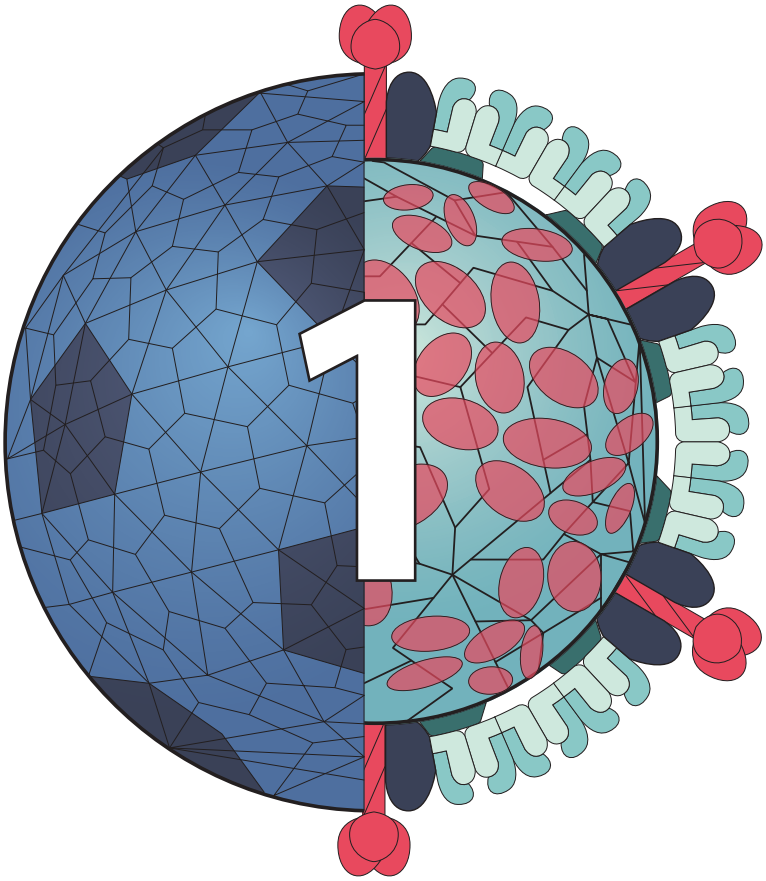

INTRODUCTION

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PART I

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Worldwide, almost 40% of people will be diagnosed with cancer at some point during life[1]. Although the five-year survival rate for the most common cancer types has been improving since 1975[1], cancer is the second leading cause of death globally[2]. Taken together, the disease causes a massive physical and mental burden for the patients and their relatives and has a significant societal impact[2]. These facts necessitate improved and new strategies to combat cancer.

Oncolytic viruses are among the most promising emerging anti-cancer agents. A variety of wild-type or genetically-modified viruses has been tested in the clinic[3,4] and in 2015 the first oncolytic virus was approved in the US, Europe and Australia for treatment of advanced stage melanoma, thereby establishing oncolytic viruses as a new category of cancer therapeutics[5].

While the approval of an oncolytic virus as an anti-cancer drug has been a recent development, the concept of exploiting viruses as ammunition in the battle against cancer has intrigued scientists for more than a century[6]. Already in the nineteenth century it was observed that cancer patients can go into (partial) remission when suffering from an infectious disease[7,8]. The uncovering of the particular nature of viruses and their visualisation by electron microscopy in the first half of the twentieth century encouraged scientists to study viruses, including mumps[9], adenovirus[10], West Nile[11], and hepatitis B[12], as anti-cancer agents in clinical trials[6]. Although complete tumour regression was observed occasionally[9], generally these virotherapies lacked efficacy. Moreover, regulatory barriers were tightened due to severe side effects and combined with the emerging success of chemotherapy, this resulted in a drastic decrease of the number of clinical trials involving oncolytic viruses in the 1970 and 1980s[6].

During this bleak era in oncolytic virotherapy, another virus was discovered to cause lysis in tumour cells; the mammalian orthoreovirus. While the virus generally does not lead to significant disease in humans, it was observed that transformed cell lines exhibit sensitivity to mammalian orthoreovirus type 2 Jones (T2J) and 3 Dearing (T3D), while normal diploid cells appeared resistant[13,14]. In later studies, predominantly reovirus T3D was used as oncolytic agent and this virus appeared highly potent against cancer cells, making it the archetype oncolytic reovirus[15,16].

Oncolytic virus therapy reappeared on the therapeutic horizon with the advent of recombinant DNA technology, and the prospects of using genetic modification to improve safety by attenuating the candidate viruses and by increasing tumour cell specificity. In addition, viruses could be made more powerful e.g. by inserting a transgene with therapeutic value[17]. This culminated into the first clinical trials with a

genetically modified adenovirus in 1996 and also reaffirmed the interest in the wild-type, unmodified oncolytic reovirus[15]. To date the wild-type reovirus T3D has been examined as single agent or in combination with chemo- or radiotherapy in more than 30 clinical trials against various cancer types[18]. These cancer types include glioblastoma, head- and neck cancer, melanoma, lung cancer and prostate cancer[19]. In these studies reovirus demonstrated to be safe for the patient and in some patients anti-tumour efficacy has been observed.

With increased understanding on mechanisms responsible for virus-mediated killing of cancer cells, also several barriers were identified that hamper the anti-tumour efficacy of reovirus and oncolytic viruses in general[3,19]. Anti-viral immune responses, the scarcity or inaccessibility of the virus receptor on the tumour cells, and physical barriers formed by the stromal component of tumours are examples of obstacles that an oncolytic virus must overcome before being able to infect and eliminate a tumour[20].

To overcome these hurdles, modifications can be introduced to the viral genome. In general two strategies are employed to improve the therapeutic potential of oncolytic viruses: (I) *Forward-genetics*, in which desired/aberrant phenotypes are isolated prior to identification of the alterations in the genome responsible for the phenotype, and (II) *Reverse genetics* or *rational design of modifications* to the virus genome. Both approaches are used for the research described in this thesis. A forward-genetics strategy was employed (called bioselection or natural selection) to select for a mutant reovirus with improved cancer-killing capacity (chapter 3). Due to the absence of proofreading activity in the reovirus' RNA-dependent RNA polymerase (RdRp), the intrinsic mutation rate in reoviruses is high, leading to a rapid adaptation of the virus to host cells and a natural selection for virus mutants that are favourably adapted to the host cell. Reverse genetics strategies are described in chapter 2 and chapter 5, which involve rational modifications on respectively adenovirus, to target the virus to cancer-specific surface antigens, and reovirus, to introduce heterologous transgenes in the genome that could facilitate viral imaging or improve therapeutic efficacy. As a result of reovirus' complex genomic architecture, which consists of a ten-segmented double-stranded RNA genome, the use of reverse genetics on reovirus is highly challenging. Our successful incorporation of a transgene in the reovirus genome (chapter 5)[21] provides novel opportunities and demonstrates conceptual proof on reverse genetic approaches for reovirus modification.

The work described in this thesis mainly focuses on the intracellular delivery of reovirus in tumour cells and three-dimensional tumour spheroids (chapter 4) and the improvement hereof using both bioselection and rational design strategies (chapter 3,

5 and 6). Other chapters describe the improved transduction of tumour cells using a genetically modified human adenovirus type 5 (chapter 2) and the role of the cellular autophagy machinery on reovirus infection (chapter 7).

1.1.1 Outline of the thesis

Chapter 1 provides a brief introduction on oncolytic viruses, with a particular focus on reoviruses (which are the protagonist entities in this thesis). Besides basic information on reovirus' structure and biology, also genetic modification strategies and mechanisms of viral oncolysis, with the activated Ras status of transformed cells as key player, are described.

Chapter 2 describes the improved transduction of tumour cells by human adenovirus type 5. Similar to reovirus, adenovirus has great potential as oncolytic agent, which is illustrated by the approval of the genetically modified adenovirus type H101 for the treatment of nasopharyneal carcinoma in combination with chemotherapy by the China's State Food and Drug Administration in 2005. This made adenovirus the first oncolytic virus to be approved by a regulatory agency[4]. Adenovirus was first isolated in the 1950s from adenoid tissue and currently more than 50 human serotypes of adenovirus are identified[22]. In oncolytic virotherapy predominantly adenovirus type 5 of subgroup species C is used. Adenovirus has a linear dsDNA genome of 30-40 kb, packaged into a icosahedral shaped protein capsid of 60-90 nm in diameter. Because of the genomic stability of the virus, the relative non-pathogenic behaviour in immunocompetent adults and the ability to infect a wide range of cells, adenovirus is not only an attractive oncolytic agent, but has also proven to be a suitable vehicle for gene delivery. Adenovirus vectors in which adenoviral genes are replaced by therapeutic transgenes are currently in development to ameliorate various illnesses. Being mostly replication-deficient, these adenoviruses e.g. encode lethal genes for cell killing in tumours, display pathogenic antigens as viral vaccine, or have the purpose to repair cellular functions by gene correction[22,23]. Adenoviruses are the most widely studied viruses for gene delivery and oncolytic virotherapy.

As for most oncolytic viruses, the poor transduction of tumour cells can be a bottleneck that hampers the anti-tumour efficacy of adenoviruses. This can be attributed, in part, to the low or heterogeneous expression of the primary receptor for adenovirus type 5, the coxsackie and adenovirus receptor (CAR). As adenovirus is relatively easy to genetically engineer, the transduction of tumour cells by the virus can be improved by retargeting of the virus. In this strategy a tumour-targeting ligand is introduced in one of the capsid proteins of adenovirus, thereby creating adenoviruses with a higher specificity for tumour-cells. **Chapter 2** describes the generation and evaluation of a

tumour-targeted adenovirus vector, which has a tumour-targeting ligand (HER2/neu-binding ZH *affibody* molecule) fused to the minor capsid protein IX. The high-affinity binding between the protein IX-coupled ZH targeting ligand and its HER2/neu receptor may result in inadequate release of the virus from the targeted receptor in the endosome. To allow for intracellular release of the viral particle from the HER2/neu molecule, a cathepsin-cleavage site was introduced between the protein IX anchor and the ZH targeting ligand. This resulted in significantly enhanced transduction of tumour cells in two- and three-dimensional cell culture models as well as in a chorioallantoic membrane tumour model, as compared to the control adenovirus.

Chapter 3 describes the isolation of reoviruses with an expanded tropism after bioselection. The mutant viruses were generated by propagation of reovirus on a glioblastoma cell line that is negative for the cellular receptor for reovirus, the Junction Adhesion Molecule-A (JAM-A). The isolated mutants appeared capable of infecting various cancer cell types independent of JAM-A, whilst primary human fibroblasts remained insensitive. These so-called *jin* mutants hold mutations in the S1 segment of the virus which encodes for the virus attachment protein σ1.

In **Chapter 4** it is demonstrated that infection of tumour cells by reovirus is not strictly dependent on the presence of JAM-A on the cell surface. While JAM-A-negative glioblastoma cells were resistant to reovirus under standard two-dimensional cell culture conditions, they became sensitive to reovirus infection when cells were grown as three-dimensional spheroids. This could be attributed to the enhanced secretion of cathepsin proteases by the three-dimensional cell cultures. It was hypothesized that these extracellular proteases convert intact reovirus virions into intermediate sub-viral particles (ISVPs), which are able to infect the spheroid cells independent of JAM-A. For tumours, extensive secretion of proteases into the tumour micro-environment has been described[25,26], as well as the entry of reoviruses into JAM-A negative glioblastoma tumours in mice[27]. Accordingly, the tumour spheroid model appears to be a highly valuable *in vitro* model, as its protease secretion characteristics mimic the actual *in vivo* situation more faithfully than adherent two-dimensional cell cultures.

Chapter 5 describes the use of forward-genetics to generate a modified reovirus. The *jin-3* mutant, generated in chapter 3, harbours an amino acid substitution in the tail region of the σ1 protein, enabling the virus to infect cells independent of JAM-A. This allows the replacement of the JAM-A-binding head domain of σ1 by foreign sequences without exceeding the size of the S1 segment or compromising the transduction capacity of the virus. For that reason, the JAM-A-binding domain of the S1 segment was replaced with the coding sequence for the fluorescent protein iLOV. Correct production of the (truncated) σ1 and iLOV proteins from the S1-iLOV sequence was established

by introducing a 'self-cleavage' element (porcine teschovirus-1 (P2A)) in between. The resulting virus remained replication competent, retained its oncolytic capacity and displayed iLOV fluorescence in both JAM-A positive and JAM-A negative cells.

Chapter 6 demonstrates proof-of-concept on using a non-oncolytic viral vector (baculovirus) as a tool for improved anti-tumour performance of oncolytic reovirus. The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), was previously shown to possess remarkable characteristics with potential for tumour therapy, that is, the ability to transduce a broad panel of mammalian cells and to penetrate through several cell layers into spheroids and tumours[28]. To employ these characteristics of baculovirus in oncolytic virotherapy, we equipped baculovirus vectors with full-length JAM-A receptors to allow for the binding ('piggybacking') of reovirus particles. Hereby, biviral complexes were formed in which baculovirus and reovirus particles were coupled. In this biviral context, reovirus was able to infect, replicate in and kill glioblastoma cells, which were resistant to standard treatment with (single-particle format) reovirus. Moreover, in the presence of the baculovirus, reovirus showed deeper penetration and spread into glioblastoma tumour spheroids and increased spheroid cell death.

Chapter 7 describes a novel mechanism associated with reovirus infection; induction of macroautophagy. Autophagy is a highly conserved homeostatic process to degrade and recycle unnecessary cellular components. Autophagy can either combat or, on the contrary, facilitate viral infections. Many autophagy-related gene (Atg) products play a role in the autophagy process. We revealed, using mouse embryonic fibroblasts (MEFs) and glioblastoma cell lines, that reovirus induces autophagy, e.g. demonstrated by the appearance of double-membraned vesicles, an increase in acidic vesicles, conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3), p62 degradation and the formation of GFP-LC3 puncta in infected cells. Furthermore, the obtained results demonstrated that a productive reovirus infection is not strictly necessary, but strongly stimulates the induction of autophagy.

Chapter 8 provides a general discussion on the results described in this thesis and on the future perspectives of using reovirus as an oncolytic agent.

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PART II

GENETIC MODIFICATION IN MAMMALIAN ORTHOREOVIRUSES

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1.2.1 Introduction

Mammalian reoviruses are members of the *orthoreovirus* genus of the family of *Reoviridae.* They were first isolated from the gastrointestinal and respiratory tracts of healthy and sick humans in the early 1950s[1]. The name reovirus is an acronym of *R*espiratory and *E*nteric *O*rphan virus. The term 'orphan' is used to indicate that no serious disease is linked to this virus in humans, while a large proportion of the human population has been exposed to the virus and built neutralising immunity to reoviruses[2,3].The *orthoreoviruses* have a wide geographic distribution and are isolated from a broad range of mammals, birds and reptiles. Although in humans the mammalian *orthoreoviruses* are non-pathogenic, in newborn mice they can cause severe disease[4].
The capsid of the *orthoreovirus* is a non-enveloped icosahedral structure composed of outer and inner protein shell. In 1963, it was discovered that the genome consists of double-stranded RNA (dsRNA)[5], and soon after it was found that the genome of mammalian *orthoreoviruses* consist of 10 distinct genome segments[6].

1.2.1.1 Taxonomy

To date, three *orthoreovirus* species groups are recognised. In this chapter we will focus on the mammalian *orthoreoviruses*. In this species group three serotype strains of mammalian *orthoreoviruses* have been identified, with the type 1 Lang (T1L), type 2 Jones (T2J), type 3 Abney (T3A) and type 3 Dearing (T3D) as the prototypical representatives. The serotypes are classified according to their neutralisation by specific antibodies and by the classical hemagglutination inhibition assay[7,8]. Genomic variation between the serotypes is found for all segments. The S1 segments, coding for the σ1 attachment protein, has the largest sequence divergence. The sequence variations can have biological consequences[4]. It was observed by reassortment studies between T1L and T3D that the S1 segments define the pathology. While the serotypes T1L and T3D both infect the central nervous system (CNS) in newborn mice, their routes of infection differ. T1L causes hydrocephalus by spreading hematogenously in the CNS by infecting ependymal cells. In contrast, T3D causes viral encephalitis by spreading via the neural routes in the CNS[9].

Table 1.1 Overview of the genome segments, proteins and prototypes of the various *ts* mutants

Segment			Protein			*ts* mutant				
	Total size (nt)	ORF (nt)		Size (aa)	Location	Group	Protoype	ssRNA	Phenotype Protein	dsRNA
L1	3854	19-3819	λ3	1267	Core	D	*tsD357*	+/−	+	−
L2	3916	14-3880	λ2	1289	Core	B	*tsB352*	+	+	+
L3	2901	14-3838	λ1	1275	Core	I	*tsI138*	?	+	−
M1	2304	14-221	μ2	736	Core	H	*tsH11.2*	++	−	−
M2	2203	30-2153	μ1	708	Outer capsid	A	*tsA201*	++	++	++
M3	2241	19-2181	μNS μNSC	721 681	NS NS	F	*tsF556*[1]	++	++	++
S1	1516	13-1377; 71-430	σ1 σ1s	455 120	Outer capsid NS	J	*tsJ128*	?	?	?
S2	1331	19-1272	σ2	418	Core	C	*tsC447*	?	?	−
S3	1198	28-1125	σNS	366	NS	E	*tsE320*	+/−	+	−
S4	1196	33-1127	σ3	365	Outer capsid	G	*tsG453*	+	+	+

Note: bp - base-pairs; aa - amino acids; [1] Tentative, leaky mutant; ++ (almost) as wild type; + less than 50% of wild type; +/− less than 10% of wild type; − less than 1% of wild type.

1.2.1.2 Genome segments and their proteins

Structural proteins. The genome of the reovirus consists of 10 dsRNA segments with a total size of 23.5 kb. The segments are named according to their sizes[6]. There are three large segments (L1, L2 and L3) ranging in size from 3854 to 3916 nt, three medium segments (M1, M2 and M3) with sizes between 2206 to 2304 nt, and four small segments (S1, S2, S3 and S4) varying from 1196 to 1416 nt[4,10]. Each segment encodes a single protein, with as a sole exception the S1 segment which encodes two proteins[11,12]. The proteins are named and numbered according to their apparent molecular weight on SDS-PAGE. The names are derived from the Greek character of the segment it was translated from, the L segments code for the λ proteins, the M segments code for the μ proteins, and the S segments encode the σ proteins. The numbering of the proteins does not always correspond to the genome size of the segment from which they are transcribed[4]. An overview of the segments, and the proteins that they code for is provided in Table 1.1.

The non-enveloped icosahedral outer capsid is about 70 nm in diameter. The inner capsid or core structure is about 52 nm in diameter. Together, these capsids are composed of eight structural proteins. The five structural proteins forming the inner core structure are λ1, λ2, λ3, μ2 and σ2. The λ1 protein is encoded by L3 segment and is a major structural protein of the inner capsid. This protein is involved in transcription. The λ2 protein is translated from L2 RNA and forms the pentameric turrets of the core structure. During primary transcription this protein adds a methylated cap structure to nascent plus-strand RNA (see below). The λ3 protein is encoded by L1 segment and functions as the RNA-dependent RNA polymerase (RdRp) and it transcribes the plus and the minus strands[13]. The exact function and location of the μ2 protein which is encoded by the M1 segment in the core are still unknown. However, it has been suggested as a co-factor or second subunit of RdRp. The σ2 protein, encoded by the S2 segment, is involved in the assembly of the core particles. This protein decorates the λ1 shell (reviewed by[4,10]).

The outer capsid is composed of three structural proteins. The two major outer capsid constituents are σ3 and μ1 proteins. The third minor outer-capsid protein σ1 forms the spikes at the vertices of the icosahedron and functions as the attachment protein. The σ3 protein is encoded by the S4 segment and provides stability to the capsid and functions as a shield for μ1. Removal of σ3 protein occurs through proteolytic cleavage upon entrance. The μ1 protein is encoded by the M2 segment and provides stability to the outer capsid. This protein is degenerated by proteolysis during viral entry to yield the μ1N and μ1C proteins. This is an important process during replication. Removal of σ3 and cleavage of μ1 yield a partially uncoated particle, the so-called intermediate

subviral particles (ISVP). These ISVPs have the capacity to disrupt cellular membranes and may function in endosomal escape. Moreover, ISVPs generated *in vitro* by treatment of purified reovirus particles with chymotrypsin or *in-vivo* in the lumen or small intestines by proteases, yields particles that retain their infectivity, and can enter the cell independent of the presence of the canonical reovirus receptor Junction Adhesion Molecule A (JAM-A) (reviewed by[4,10]).

Trimers of σ1 proteins encoded by S1 segment form the spikes on the vertices of the icosahedral capsid (see representative figure[14]). The spike is anchored in the λ2 protein turret. Through removal of σ1 protein during virus entry, the λ2 turrets undergo a conformational change and become active. The spikes contain a tail and a head domain. The protein spike interacts with cellular receptors. Initially a low-affinity interaction is established with sialic acid residues on the cell surface via interactions with the tail of the spike. Subsequently, high-affinity interactions can establish between the head domain of the spike and JAM-A. Subsequently, Arg-Gly-Asp (RGD) domains in the λ2 protein bind β1-integrins on the cell surface and mediate particle entry by endocytosis[15,16].

Non-structural proteins. At least three non-structural proteins are generated during infection. The σ1NS is translated from the second open reading frame of the S1 mRNA. This is a non-essential protein which causes cell cycle arrest during infection[11,12]. The second non-structural protein, σNS, is encoded by the S3 segment. This protein has a strong affinity with ssRNA[17]. It is suggested that it plays a role in the replication and the assembly of core particles[18]. Furthermore it associates with μNS and μ2 to form inclusion domains or viral factories.

The μNS protein is encoded by the M3 segment. It is the third non-structural protein and associates with the viral mRNA shortly after transcription, and to viral cores, probably to anchor viral components needed for assembly or replication[19-22]. In addition, by proteolytic cleavage of μNS μNSC is generated. The precise function of this protein is unknown[4].

1.2.1.3 Replication

Entry and uncoating. As for most RNA viruses, genome replication of reoviruses takes place in the cytoplasm, schematically represented in Plate 5b. Reoviruses are internalised into the endosomes. In the endosomes proteolysis facilitates the partial removal of the outer capsid. The uncoating process includes additional cleavage and removal of μ1 and σ3. Subsequently, σ1 molecules are detached from the particles. The activated particles escape from the endosomes by penetrating the endosomal membrane. Through removal of μ1 and σ1 the λ3 RNA-dependent RNA polymerase

(RdRp) is activated and initiates transcription, yielding full-length plus-strand RNA molecules. The conformational change of λ2 pentamers, through removal of σ1, turns them into channels for the release of plus-strand mRNA into the cytosol. In addition, the λ2 turrets add a methylated cap structure to the 5' end of the plus-strand RNAs[23-25]. On these transcripts the 3' end remains non-poly-adenylated. The methylated cap structure (7-methyl guanosine triphosphate (m7G(5')ppp)) is identical to the cap structures of cellular mRNAs and is therefore recognised by the host ribosomes and translated. Initially the four segments L1, M3, S3 and S4 are transcribed and translated which stimulates subsequent transcription and translation of all segments[9,26-28]. At this stage in the replication cycle, translation of viral and host proteins occurs simultaneously.

The assembly of new particles occurs in newly formed non-membranous structures in the cytoplasm, the so-called viral factories. These may aid in shielding the viral processes from the host cell's components of the innate immune system. The plus-strand transcripts are incorporated into these new cores. Within the core-particles the minus-strand synthesis takes place to yield dsRNA[18,29]. In the newly formed core particles, a secondary round of plus-strand synthesis initiates, yielding uncapped viral transcripts. The uncapped viral transcripts are translated very efficiently since the reoviruses employ a mechanism to modify the ribosomes in such a way that non-capped transcripts are preferentially translated. At this point the host cell protein synthesis is shut down. This mechanism ensures that the host cell's protein synthesising machinery is used for synthesis of viral proteins rather than for cellular proteins (reviewed by[10]).

Lytic infection. Reoviruses normally cause a lytic infection in permissive host cells. The reovirus-induced cell death is independent of productive infection. Exposure of cells to UV-inactivated virus particles can still induce apoptosis. The primary determinants of reovirus induced-apoptosis are associated with the outer capsid protein μ1, although attachment of σ1 to the cell surface strongly enhances the apoptotic signal. The cleavage of the μ1 protein during internalisation is essential for the induction of apoptosis. If the disassembly of the viral particles is blocked by monoclonal antibodies against σ3 or μ1, apoptosis is not initiated. Also monoclonal antibodies against σ1 inhibit apoptosis; however the mechanism here is prevention of binding to the cells surface. Pro-apoptotic signals are generated by binding of the attachment protein to sialic acid and JAM-A. Reovirus mutants lacking the sialic acid binding capacity are still able to induce apoptosis albeit to a lesser extent[9,30-32].

1.2.1.4 Cis-acting sequences

Knowledge of the replicative cycle of reoviruses has been essential for the development of a reverse genetics system. The essential *cis*-acting sequences that reside in the genome segments need to be identified. Such sequences include the elements required for the initiation of plus and minus-strand synthesis, the sequences in the plus-strand RNA that function as segment-identity labels, and the sequences that are required for incorporating the plus strand RNA's into the newly formed cores. Also it is crucial to identify other constraints that may limit the incorporation of heterologous sequences in reovirus genome segments. In the following paragraphs we will describe the functional elements identified in reovirus genome segments.

Non-translated regions. The particle to infectious unit ratio of reoviruses is low[33], indicating that the assembly of new particles is effective and precise and the infection process is highly efficient. The encapsidation involves assembly of 8 structural proteins with one copy of each of the 10 segments. The precise assorting mechanism which directs one copy of each segment into the particle is not yet well understood. Sequences contained within the 130 nt at the 5′ terminus serve as identity label for each of the segments[34,35]. The termini of all segments contain relatively short (15–33 nt) non-translated region (NTR) with short (4–5 nt) identical sequences at the extreme ends. The 5′ end of the plus-strand RNA starts with the tetranucleotide sequence GCUA- and the sequence at the 3′ end is a pentanucleotide –UCAUC. No other sequence homology exists in the NTR regions of the segment termini[29,36,37].

Assortment of the segments. The first indications that the 5′ and 3′ ends of the segments are important for replication and assembly stem from work by Schlesinger *et al*.[38], and later by Zou *et al*.[39]. In these studies deletion mutants were used. These deletion mutants were formed spontaneously when T3D was passaged at a high multiplicity of infection and contained deletions in the middle of the segments. The deleted segments were replicated and encapsidated in new particles although the presence of helper virus was required. It led to the hypothesis that sequences near the 3′ end are necessary to function as promoter for the RdRp and that the termini are needed for the assembly of new particles[38,39].

By employing a reverse genetics system for inserting a chloramphenicol acetyl transferase (CAT) reporter gene in different genome segments (discussed below) more information on the required *cis*-acting sequences at the 5′ and 3′ ends was obtained[40]. From three segments, for example, S2, M1 and L1, the regions which are important for assembly have been determined. The lengths of these regions were identified through varying the length of the 5′ and 3′ ends and measuring the CAT activity of the progeny virus. The lengths of the elements required for encapsidation correlated

with the lengths of the genome segments. L1 required the longest 5′ and 3′ regions (i.e. respectively 129 nt and 139 nt) while S2 needed the shortest regions, at the 5′ end 96 nt and at the 3′ end 98 nt. The M1 segment required at the 5′ end 124 nt and 172 nt at the 3′ end[34,35]. These results indicate that the *cis*-acting sequences do not only encompass the non-translated regions, but extend into the coding regions of the segments. Furthermore, Roner *et al.*, in 2004 and 2006, demonstrated that the signals at the 5′ end act independently from the signal at the 3′ end. In the process the signals at the 5′ end function as the segment-identity labels. This became apparent from the observation that chimeric segments, containing the 5′ end of S2 or M1 and the 3′ end of L1 are incorporated as S2 or M1 segments, not as a L1 segment[29,41].

1.2.1.5 Genome-size constraints

The genome of the mammalian reoviruses is packaged within the 52 nm inner core. In comparison to other RNA viruses, reoviruses have one of the most densely packaged genomes[35]. Experiments of the group of Roner and colleagues indicate that the maximum amount of RNA that can be included into the core is almost reached. They showed with their reverse genetics system that upon increasing the length of L1 with 726 nt this segment was still incorporated. However, when creating chimeric segments with the 5′ end of M1 or S2 and the 3′ end of L1, and increasing the size of the resulting segments by 2307 nt or 2500 nt respectively, full incorporation of these segments was inhibited. The modified 5′- L1.CAT.3′-S2, 5′-M1.CAT.3′-S2 and 5′-S2.CAT.3′-L1 segments, in which the sizes were decreased by approximately 980 bp, were still packaged[35]. These data suggest that there are limitations to the packaging capacity of reoviruses. Such limitations are important if one considers developing heterologous transgene-containing reoviruses.

1.2.2 Forward-genetics in orthoreoviruses

In forward-genetics studies aberrant phenotypes are isolated followed by the identification of the mutation responsible for this phenotype. Much of today's knowledge on reovirus genes and genome segments has been obtained from studies with this forward-genetics approach. One of the strategies used most commonly was the selection of temperature-sensitive mutants after chemical mutagenesis. This method was often used in combination with the use of reassortants. A third strategy discussed in this chapter is the selection of natural mutants.

1.2.2.1 Temperature-sensitive mutants

The initial isolation of many reovirus temperature sensitive (*ts*) mutants was after chemical mutagenesis of reovirus stocks. The mutated reoviruses were plated and propagated at a low permissive temperature (usually 32 °C), yielding virus which could efficiently replicate at this temperature. Subsequently, clones were isolated that exhibited an impaired growth at a higher temperature (often 39 °C was used).

With this strategy large series of mutants with aberrant phenotypes have been generated, and designated as *ts* mutants. These *ts* mutants were classified in complementation groups on the basis of the absence of complementation between pairs of *ts* mutants. Subsequently these complementation groups could be assigned to the different genome segments of the reovirus (reviewed by[10]). A summary of the different groups and the segments to which they have been mapped is provided in Table 1.1.

The phenotypes of these mutants have been instrumental in defining the roles of the individual viral proteins in the infectious pathways of the human reoviruses[10].

1.2.2.2 Reassortants

In addition to *ts* mutants, reassortment strategies were applied. The reassortants can arise during co-infections of cells with two different *ts* mutants or between different *orthoreovirus* serotypes. Co-infection can lead to exchange of entire genome segments between the viruses. During co-infection, a reassorted genome can be detected in up to 15% of the progeny viruses[42].

1.2.2.3 Natural selection/bioselection

A third forward-genetics strategy is the isolation of spontaneous mutants and naturally occurring reoviruses[43,44]. The absence of proofreading in the reoviral RNA-dependent RNA polymerase (RdRp) leads to a high mutation rate[45]. This leads to rapid adaptation of the reoviruses and the selective outgrowth of mutants that have favorably adapted to the host[46,47].

1.2.3 Reovirus/cell interactions

1.2.3.1 A key role of σ1 in cell binding

Since the early 1960s it has been well established that Reovirus T3D, but not T1L, could haemagglutinate bovine erythrocytes[48]. Reassortment studies identified σ1 protein as the haemagglutinin. In these studies T1L x T3D reassortants were generated and by using serotype-specific σ1 anti-sera it was demonstrated that the capacity to haemagglutinate the bovine erythrocytes was strictly correlated with the presence

of the σ1 protein of T3D[7,8,49,50]. Further studies using these reassortants and serotype specific sera pinpointed σ1 also as the protein responsible for infection of permissive nucleated cells[51].

1.2.3.2 Sialic acids

Attachment studies of the T3D virus to different cell types (erythrocytes, L cells, lymphocytes and murine erythroleukaemia (MEL) cells) made clear that haemagglutination (HA) in erythrocytes is caused by binding to terminal sialic acids of glycoproteins on the cell surface[48,52-55]. Sialic acid is a generic term for the *N*- or *O*-substituted derivatives of neuraminic acid and also the name of the most common neuraminic acid, the terminal N-acetylneuraminic acid (NeuAc). With S1 reassorted viruses of T3D and T1L (reassortants T1L+S1-T3D (1HA3) and T3D+S1-T1L (3HA1)) it was demonstrated that reovirus attachment to sialylated oligosaccharides on glycoproteins was solely mediated by the σ1 attachment protein[56].

The σ1 protein is present as a homo-trimer[57,58] at the vertices of the viral particle. It has a distinct 'head' and 'tail' region[59,60]. The domains that interact with cellular receptors have been identified. The knowledge of the interaction of the σ1 protein with cellular sialic acids was derived from studies employing reovirus T3D field isolates that differ in their capacity to agglutinate human and bovine erythrocytes and to bind sialic acids. Sequence analyses of the S1 genes of the different virus isolates revealed single point mutations that only cause weak haemagglutination and which are unable to bind sialic acids. These mutations all cluster in one region in the σ1 tail, residue 198-204, which demonstrated that not only the head but also the tail region of the spike is exposed on the capsid in such a way that it can interact with cellular receptors[61].

The phenomenon that only T3D and 1HA3 and not T1L viruses could infect murine erythroleukemia (MEL) cells was exploited in studies that mapped the sialic-acid binding-domain of σ1. The capacity of the viruses to infect MEL cells is strictly correlated with the viruses' capacity to haemagglutinate erythrocytes. This suggested sialylated proteins to be involved in MEL cell binding[62]. To further identify the receptor binding region of the σ1 tail, reovirus type 3 field isolates which were unable to bind sialic acids were adapted by serial passaging to grow in MEL cells. Sequence analyses of these MEL adapted (MA) viruses revealed point mutations that were clustered near the residues 198-204 that had previously been identified as the sialic acid binding region of the σ1 tail. These data demonstrated that this part of the tail of the spike is involved in sialic-acids binding[43].

Similarly, T3D reoviruses were selected for growth in the presence of a monoclonal antibody that inhibited hemagglutination. This selection yielded mutants that exhibit a strongly reduced neurovirulence upon inoculation of newborn mice[44]. These data demonstrate the relative ease with which reovirus mutants can be obtained by conventional forward genetics strategies.

1.2.3.3 Junction Adhesion Molecule-A

Evidence that not only the fibrous tail of the σ1 spike but also a domain in the head is involved in receptor binding has accumulated over the years[43,62-67]. In 2001, Barton and colleagues identified Junction Adhesion Molecule (JAM) as the cellular receptor to which the σ1 protein's head domain can attach. Additional binding-inhibition experiments with antibodies show that JAM directly binds to σ1. Transient expression of JAM renders reovirus-resistant cells sensitive to infection[68]. Furthermore, Campbell and colleagues defined that only JAM-A (also known as JAM-1) and not JAM-B or JAM-C can serve as a receptor for reovirus type 1, 2 and 3[69].

JAM is a 25kDa type I transmembrane protein with two extracellular Ig domains (D1 and D2) and a short cytoplasmic tail. The membrane distal extracellular domain (D1) forms a homodimer[70]. The protein is concentrated at the apical region of intercellular tight junctions of epithelial and endothelial cells (for reviews, see[70-72]).

Structure-guided mutational analysis revealed three amino acids located in the D1 domain of JAM-A which are individually required to bind σ1. The amino acids Glu[61] and Lys[63] participate in salt bridges with opposing amino acids and thereby have a role in stabilisation of the D1 dimer. The amino acid Leu[72] is part of a hydrophobic interaction with a residue of the opposing dimer[15]. Since all amino acids required for binding σ1 were located in the dimeric interface of the D1 domain, it was reasoned that σ1 first disrupts the JAM-A dimer and then binds to the monomeric form of the D1 domain. Binding studies indeed showed that the binding affinity between σ1 and the monomeric form of the D1 domain is higher than between the JAM-A homodimers. In addition, using cryo-crystallography it was clearly demonstrated that only monomers of JAM-A were bound by σ1[73]. Insight in the amino acid residues important for binding JAM-A was obtained by using the helper-free reverse-genetics system (see below)[74]. The engineered reoviruses with mutant forms of σ1 revealed that the JAM-A binding domain is located at the lower part of the head domain. Moreover, one reovirus σ1 molecule can bind three JAM-A monomers[73]. (Picture of JAM in[15] and JAM-σ1 interaction in[73]).

The knowledge of reovirus T3D binding to its cellular receptors culminated in a multi-step binding model in which attachment protein σ1 first engages sialic acids on the cell surface in a low-affinity interaction and subsequently binds JAM-A with high affinity[74,75]. The virion is internalised via clathrin-mediated endocytosis upon interaction between β1 integrins on the cell surface with the capsid protein λ2[16]. This has been suggested since λ2 contains the integrin binding sequences Arg-Gly-Asp (RGD)[16]. Furthermore, β1 integrins contain a cytoplasmic domain containing two Asn-Pro-any amino acid-Tyr (NPXY) motifs which are required for functional reovirus entry[76].

1.2.3.4 Persistent infection

Rather than causing a lytic infection, the T3D reovirus can establish a persistent infection. The initial observations stem from experimental infections of cultured human embryonic cells[77]. Infectious virus could be recovered from the cultures after 9–12 passages without appearance of overt cytopathic effects.

Taber and colleagues (1976) isolated a culture of persistently infected CHO cells. In these cultures a large number of cells were infected, and the cultures produced reoviruses that were cytopathic for the parental CHO cell line[78]. This suggests that the persistently infected cell lines adapted to resist the reovirus-induced cytopathic effects. Ahmed and co-workers (1981) demonstrated in an L-cell system that the persistently infected cells as well as the reoviruses propagated in the persistently infected cultures acquire changes and thus eventually differ from the parental virus and host cells. The L cells cured from the persistent infection had an increased resistance to *wild-type* T3D viruses, suggesting that the cells adapted by genetic or epigenetic mechanisms, leading to increased virus resistance[79]. More recently it was demonstrated that persistently infected cells express reduced amounts of the cathepsins B and L, which are known to be involve in reovirus uncoating[80-82]. These data suggest a co-evolution of host and virus to eventually reach a state of a stable but dynamic equilibrium.

Persistently infected L-cell cultures obtained after co-infections with T2J, which does not result in persistent infections, together with T3D, which is able to give persistent infections, yielded various hybrid recombinant mutants containing all the S4 segment of T3D. This suggested that sequences in this segment underlie the capacity of the virus to establish a latent infection[83]. Via a similar approach the capacity of human reoviruses to inhibit host cell RNA and protein synthesis was also been mapped to the S4 segment[84]. Taken together, these data imply that persistent infection can only exist if the host cells retain the capacity to synthesise proteins. Strong inhibition, as is the case upon infection with T2J, would be incompatible with such persistence. Furthermore, sequence analyses of the persistent infections demonstrated acquisition

of additional mutations in the S4 segment and an increased resistance to ammonium chloride, a weak base that inhibits the pH decrease in endosomes and lysosomes. This again suggests that the viruses adapted to selective pressure operating at the level of cell entry.

In addition to alterations in S4, sequence analyses of the viruses causing persistent infection also revealed mutations in the S1 segment, encoding the spike protein[83,85-87]. The σ1 protein is known to be involved in the induction of apoptosis in reovirus infected cells[9]. Isolation of an S1 attenuated reovirus mutant form from persistently infected cells, revealed that although virus replication was maintained the apoptotic potential was reduced. Sequence analysis revealed that the most significant mutation was a nonsense mutation which truncated the σ1 protein, however it could not be ruled out that other mutations in S1 and S4 too are involved in this phenomenon[85]. It would therefore be interesting to test whether the mutations found in the S1 segments of the persistently infected viruses affect the capacity of σ1 to bind sialylated proteins and to induce apoptosis[4,88].

In the preceding sections the conventional forward-genetics strategies have been discussed as well as some of their applications. Although they have been instrumental for determining the functions of the different segments, these techniques have their limitations. No directed mutations can be introduced. Therefore, reverse genetics systems have been developed, facilitating not only new studies into the functions of individual reovirus proteins, as well as the development of new reoviruses for use as oncolytic agents.

1.2.4 Reverse-genetics in orthoreoviruses

So far development of reverse genetic systems for reoviruses has been notoriously difficult. Although in 1982 the cDNAs of all the genome segments had been cloned for the purpose of sequencing[89], the first reverse genetics system was only described in 1990[90]. Genetically modified particles were generated with the aid of helper reoviruses. In 2007 the first helper-free system was described[74]. To date, three different systems have been developed which have all their merits and weaknesses.

1.2.4.1 The infectious-RNA system

The first method for reverse genetics in reovirus T3D employs RNA transfections of all the 10 genome segments, a cell-free translation system, and helper reoviruses[90].

Active core structures, generated by *in vitro* disassembly of reoviral particles, were used for transcription of plus-strand RNAs *in vitro*. The RNA transcripts were translated using a rabbit reticulocyte lysates (RRL) system. While this step was not essential, it increased

the efficiency by 2–3 orders of magnitude. The newly translated viral proteins, together with dsRNA, and ssRNA, were introduced into mouse L fibroblasts by lipofection. Usage of only ssRNA or dsRNA in this mixture is possible but less efficient. A few hours later the helper virus, which can be either serotype 1 or serotype 2, is added to the cultures. Between 24 to 48 hours post infection, virus can be harvested. Either plaque purification, or the use of serotype-specific neutralising anti-sera, was required to eliminate the helper-viruses[90,91].

This technique was used to generate a compound *ts* mutant. Two *ts* mutants were chosen both of which contain a *ts* mutation albeit on different genome segments (genome segment M2 coding for μ1 and segment S2 encoding σ2). These segments were jointly incorporated in progeny virions generating the compound double *ts* mutant. Creating these double *ts* mutant required removal of the corresponding *wild-type* segments from transfected RNA pool. This can be accomplished by sequence-specific degradation of the *wild-type* segments through addition of complementary oligonucleotides and RNase H treatment. This enzyme degrades the RNA-strand in complementary DNA/RNA duplexes. Analysis of the double mutant showed that the *ts* phenotype was enhanced compared to the parental single *ts* mutants. Furthermore, exposure of mice revealed that the double *ts* mutant was less pathogenic than the parental viruses, while protective neutralising immunity was still induced[92].

Transgene-containing reoviruses. The first example of the introduction of a heterologous transgene gene in the reovirus genome was described by Roner and Joklik in 2001. To this end, the infectious RNA-system was used. In this experiment the S2 segment was replaced by an S2 segment modified to include the chloramphenicol acetyl transferase (CAT) gene, as a reporter. The coding sequence of the CAT gene is smaller than the coding sequence of σ2 (753 nt versus 1331 nt, respectively), and its activity can be easily monitored in cell lysates[40].

The CAT gene was placed in-frame in the S2 open reading frame. The total lengths of the S2 sequences flanking the CAT gene were 198 nt at the 5′ end and 284 nt at the 3′ end. The modification inactivated the σ2 open reading frame. Therefore all experiments were performed in helper cells that expressed an intact copy of S2 to trans-complement the missing σ2 protein. To generate infectious virus with the modified S2 gene, the wild-type S2 was removed from viral RNA preparation by addition of an oligodeoxyribonucleotide complementary to the S2-transcript and RNase-H treatment. To provide the modified S2 transcripts, the capped S2-CAT RNA was generated by transcribing a cloned version of this S2-CAT segment by T7 RNA polymerase. After

removal of the wild-type S2 RNA and addition of capped S2-CAT RNA the mixture was lipofected into helper cells, yielding reoviruses carrying the CAT gene in their S2 segment.

This system was used to identify the regions that are essential for replication and packaging of the segments. These regions were mapped at the segment termini. In an experiment in which the CAT gene was inserted between the 5' terminal end of the L1 segment and 3' terminal of the S2 segment, the CAT containing segment was found to replace the L1 segment, but not the S2 segment. Similarly, a chimeric 5'-S2.CAT.L1-3' segment and a 5'-M1.CAT.L1-3' replace the S2 and M1 segments, respectively. These data indicate that the 5' terminus determines the segment identity in the segment assortment. Moreover the 5' and 3' termini act independently[35].

In similar studies, the size constraints were established for packaging and replication as discussed in genome-size constraints. These data show that although the size of the segments can be increased by inserting heterologous sequences, the capacity is limited[35].

The infectious-RNA method has been effectively used to engineer alterations in the reovirus genome. However, the method is technically demanding. The RNase H procedure is not fully efficient, resulting in the presence of residual RNA of the targeted segment, and appearance of viruses with wild-type segments. In addition, the requirement of helper viruses during the generation of the modified viruses is undesirable. The helper virus may yield the formation of reassortants between the 'helper' viruses and the generated virus, although the use of T2J as helper reduces the magnitude of the problem[91]. Despite these weaknesses, the results obtained with this system have been extremely informative.

1.2.4.2 The segment-replacement technique

Van den Wollenberg *et al.* recently described an alternative approach for generating genetically modified reoviruses. In an effort to modify the σ1 spike, an S1 segment was generated encoding a σ1 that harbours a C-terminal histidine-tag. The presence of this tag would allow particles to infect JAM-A-negative cells that express on their surface a single-chain antibody recognising the His-tag, as an artificial receptor[93].

This strategy was based on experiments by Rouault and Lemay[94]. These authors expressed modified versions σ1, μ1, σ3 and used these proteins to recoat reovirus ISVPs and cores *in vitro*[95-97] to study reovirus capsid protein structures and functions. In a proof of principle study a foreign epitope, viz. a hexahistine tag, was added to the amino terminus of σ3[94]. For recoating, purified wild-type virions were treated with chymotrypsin to generate ISVPs. The ISVPs were incubated with cellular extracts that

contain mutant σ3 proteins. Free σ3 proteins were removed from the recoated ISVPs and by immunoblotting procedure the modified σ3 on the recoated ISVPs could be detected. While this strategy can be used to introduce modified proteins in the capsid, the modification will not persist upon replication since the viral genome is not modified. Van den Wollenberg *et al*. produced particles with modified proteins incorporated in their capsid by propagating reoviruses on helper cell lines that synthesise modified reoviral proteins. These modified proteins would be incorporated in the capsid during replication of the reovirus. In a first series of experiments reoviruses were propagated on modified cells that express a S1 segment that included a His-tag at the carboxyl terminus of the σ1-encoding open reading frame. It was anticipated that the viruses harvested from these cells would carry the σ1-His in their capsid, but would not carry the modified S1-His segment. However, the authors noted that propagation of wild-type reoviruses on cells expressing the σ1 His-encoding segment as a conventional RNA polymerase II transcript led to frequent replacement of the wild-type genome segment with the modified version. The resulting viruses could be serially passaged on JAM-A-deficient U118MG cells that were modified to express the single-chain Fv capable of binding a His-tag. Hence, this technique allowed the generation of reoviruses that are genetically retargeted. It also demonstrated that the C terminus of the σ1 protein is a suitable location for the insertion of oligopeptide ligands and shows that it is possible to use genetic modification to retarget the infection of reoviruses[93].

The precise mechanism by which the modified segment is incorporated is still unclear. Two mechanisms could be envisaged. In a first mechanism, the RNA-polymerase II transcript that contains the modified S1 segment, associates with newly formed core particle despite the presence of the long 3' extension and a poly-A tail. In the core particle the minus-strand synthesis would start even with the 3' extension and poly-A tail. This would yield a partially double-stranded RNA copy of the polymerase II transcript. If this dsRNA copy serves as template for secondary plus-strand synthesis, new plus strands would be generated that harbour the modified σ1 open reading frame, but are otherwise identical to the wild-type S1 segment. Alternatively, one could anticipate a mechanism that would involve RNA recombination or a template switch during replication of the reovirus genomes. Future studies will aim at resolving the mechanism for the replacement of genetic information.

The segment-replacement system is relatively straightforward, as it is based on the selective advantage for the modified σ1 over the wild-type σ1. The selection is essential for selectively expanding the viruses that have the wild-type genome segment replaced by the modified version. Unfortunately, this method is thwarted by the fact that, with a low frequency, mutants arise in these cultures that have the capacity to infect the

U118MG cells independent of JAM-A and the His-tag-specific scFV. This leads to the occurrence of replicating reoviruses that do not carry the desired mutation. Therefore, plaque purification and screening are important to characterise and purify the desired mutants.

1.2.4.3 A helper-free reverse-genetics systems

A fully helper-free system for reverse genetics was described by Kobayashi and colleagues. It employs 10 different plasmids, each containing a single cloned genome segment. The method starts with infection of susceptible cells with an attenuated vaccinia virus expressing the T7 RNA polymerase, and is followed by naked-DNA transfection of the 10 plasmids encoding each of the genome segments. The genome segments are inserted in the plasmid as full-length cDNA clones derived from the wild-type T3D. These cloned segments are inserted downstream of a bacteriophage T7 promoter. The 3' terminus of each cloned segment was fused to the hepatitis delta virus (HDV) ribozyme, which generates native 3' ends without poly-A tail by self-cleavage of the RNA transcript. The attenuated vaccinia virus is replication-deficient and serves to express the T7 RNA polymerase at high level. The supernatants of the transfected cultures were plaque assayed on L-cells and replication-competent viruses could be isolated. The method is robust and productive, viral infection could be established in approximately 1 in 10^5–10^6 cells transfected. This system was validated to confirm that a single amino acid change in σ1 conferred resistance of σ1 to trypsin cleavage, and that a single amino acid change in σ3 accelerates proteolytic disassembly of the reovirus. Furthermore, with this system the functional domains in μ1 responsible for the induction of reovirus apoptosis in host cells could be mapped[31,98]. Introduction of the enhanced green fluorescent protein (eGFP) gene into the S4 segment demonstrated that the technology allowed the incorporation of heterologous transgenes into the reovirus genome. 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[74].

Taken together, these data show that this helper-free reverse genetics method can be used to generate viruses with single amino-acid changes as well as for generating viruses carrying heterologous transgenes.

The advantage in comparison of the two other systems is that there is no requirement for helper viruses. The system is easily amendable for the introduction of various kinds of mutations. However, the efficiency may need further improvement since it is estimated that about 1 in 10^5–10^6 transfected cells can establish viral progeny. As a first step in improvement, instead of inserting the genome segments in 10 separated

plasmids, the system has been amended to four plasmids including all the 10 segments. The genome segments are still independently flanked by the T7 promoter and the HDV ribozyme. Furthermore, instead of using the attenuated vaccinia virus, BHK cells stably expressing the T7 polymerase under control of cytomegalovirus promoter can be used, further simplifying the technology[99]. With these improvements, the reverse genetics system is more effective in generating progeny virus.

1.2.4.4 Rotavirus

Rotaviruses are classified in a separate genus of the *Reoviridae*. Unlike the mammalian *orthoreoviruses*, the human rotaviruses are very pathogenic, causing severe diarrhoea, especially in young children[100]. It is a major health problem, particularly in developing countries[101]. At this moment, attenuated live virus is used as vaccine[102]. The development of a reverse genetics system would allow us to gain more knowledge about the biology of rotaviruses.

Rotaviruses are very similar to the mammalian o*rthoreoviruses*. They contain 11 dsRNA genome segments encoding 13 proteins, and they have a non-enveloped three-layer icosahedral capsid structure[103]. Although they resemble reovirus, the development of a reverse genetics system is even more difficult.

So far, attempts to generate a plasmid-based reverse genetics system have been unsuccessful. A first reverse-genetics system was described in 2006 by Komoto *et al*.[104] These authors managed to replace the spike-encoding segment of a human rotavirus by that of a simian homologue. The system employed resembles *the segment-replacement technique* described above, except the cDNA of the simian spike-encoding segment was flanked by a T7 promoter and the HDV ribozyme. Infection of a vaccinia virus vector expressing T7 polymerase was required to provide the T7 polymerase. Selection of the modified virus employed neutralising antibodies against the human spike-protein, selectively enriching the virus population for the viruses containing the simian spike-protein[104]. So far this method relied on a high selective pressure for the modified rotavirus which limits the application of the technique.

More recently, Troupin *et al.* describe a modified version of the Komoto method to generate a recombinant rotavirus carrying an artificially rearranged rotavirus segment 7, coding for non-structural protein 3 (NSP3)[105]. Their method is based on the observation that rearranged segments 7 and 11 are preferentially packaged[106]. The usefulness of this system is under debate[107], since it requires extended passaging at a high multiplicity of infection to recover the recombinant virus.

The latest development in rotavirus reverse genetics is based on the method of Komoto *et al.* together with a dual selection mechanism[107]. Trask *et al.* combined a *ts* mutation of non-structural protein 2 (NSP2), with RNAi-mediated degradation of NSP2 transcripts to select for a recombinant rotavirus evading both mechanisms, *ts* and RNAi degradation. Therefore, the recombinant segment 8 cDNA, coding for NSP2, was modified to contain silent mutations in the region targeted by the RNAi to avoid degradation. The combination of the *ts* helper virus with the RNAi-mediated selection significantly improved the recovery of the recombinant rotavirus. An analysis demonstrated that eight of eight rotavirus isolates contained the recombinant segment 8.
This 'two-hit' method may enhance the efficiency of recombinant virus recovery. One limitation of this system is the requirement for gene-specific RNAi, which may not be equally effective for all the 11 segments of the rotavirus.
So far, reverse genetic systems for the introduction of a foreign gene in rotaviruses have not been reported.

1.2.5 Reovirus as an oncolytic agent

The observation that reoviruses preferentially lyse transformed cells was first made in 1977 by Hashiro *et al*.[108]. One year later Theiss and colleagues described the suppressing effects of reovirus infection on lung tumours in mice[109]. There is a strong indication that the preferential lysis of transformed cells depends on the RAS status of these cells. In non-transformed cells, dsRNA activates the cell's defence mechanism by phosphorylation of the double-stranded RNA-dependent protein kinase (PKR). This inhibits translation of viral transcripts. If the RAS pathway is constitutively active, phosphorylation of PKR is inhibited, facilitating reovirus replication[58,110]. Although that activated RAS status is important for oncolysis, it is not the sole determinant of cellular sensitivity to reovirus. Kranenburg and colleagues showed that the reovirus propagation and yields are not dependent on the RAS status, although the active RAS stimulated cell lysis[111,112]. In other studies, cellular resistances to reovirus infection were correlated with cathepsin B and L activity. These enzymes were identified as important factors for the reovirus proteolytic disassembly[80,113,114].
The preference for transformed cells plus the absence of significant pathology in humans made reoviruses excellent candidates for oncolytic virus therapies. The Canadian company Oncolytics Biotech initiated several clinical trials with their lead product, Reolysin, which was based on the wild-type T3D strain. At this moment at least 24 clinical trials are active or completed in the UK, US, Canada, and Belgium. The indications comprise a range of cancer types, including prostate cancer, malignant gliomas, pancreatic cancer, lung cancer, and head and neck cancer[115]. Initially, the virus

was administered as mono-therapy but in more recent clinical trials reoviruses are combined with more conventional treatment modalities. In these studies no dose-limiting toxicity was reached, underscoring the safety of reovirus-based oncolytic virus therapy[116]. Despite anecdotal evidence of anti-tumour activity, the efficacy has been limited. Currently, several factors such as insufficient tumour penetration of the therapeutic virus, limited spread of the virus within the tumour cell mass, insufficient expression of the reovirus receptor JAM-A on tumour cells, and pre-existing humoural immunity, have been proffered to explain the limited tumour–cell transduction[117]. It is anticipated that technology to modify reovirus genomes by reverse genetics may aid the generation of new mutants that overcome these current limitations.

1.2.5.1 Optimising reovirus therapy

To overcome some of the bottlenecks, different options are being explored. An interesting approach to improve the delivery of reoviruses to tumours may involve the use of tumour-seeking cells as delivery vehicles. Several cell types (e.g. T-cells, dendritic cells, macrophages, mesenchymal stem cells) have the ability to migrate to tumours. If these cells are loaded with oncolytic viruses, they may release the virus in the tumour mass after migration. In addition, this approach may shield the virus from the neutralising immunity immune system[118].

The scarcity of the reovirus receptor JAM-A on tumour cells may be overcome by modification of the attachment protein σ1. Either bioselection of spontaneous mutants or the use of reverse genetics strategies can be employed. In the latter approach the lessons learned from generating Adenovirus-C mutants that by-pass the dependency of the adenovirus receptor, can be very helpful. Adenovirus-C is widely studied as an oncolytic virus. The attachment protein of adenovirus-C, fiber, recognises the Coxsackie and Adenovirus Receptor (CAR) receptor. Although the adenovirus fiber and the reovirus spike have no sequence similarity, their 3D structures are remarkably similar[69,119]. In the adenovirus fiber protein, a wide variety of ligands have been inserted. Such ligands include single-chain Fv domains, single-chain T-cell receptors, so-called *affibody*™ molecules, and integrin-binding RGD motifs. This successfully changed the cellular tropism of the adenoviruses[120-122]. It remains to be determined whether the same approach is equally effective in modifying the cellular tropism of reoviruses.

Not only the infection efficiency, but also the tumour -cell specificity of infection can be enhanced. To avoid the infection of non-target cells expressing JAM-A, the σ1 protein can be modified to ablate its association with JAM-A. Viruses with reduced JAM-A binding have been isolated[73,85], these mutants were still viable and infected cells presumably via binding to sialic acid residues[73]. The mutant with the truncated σ1, isolated by Kim

and colleagues, still preferentially targeted tumour cells, however, it showed a reduced toxicity *in vivo*. Also exposure of naïve mice to this virus still resulted in the induction of neutralising immune response. Kobayashi and co-workers used their helper-free reverse-genetics system to generate a mutant which harbours a single amino acid change in the σ1 protein which ablates the capacity to bind JAM-A[74]. Such viruses may facilitate the development of more efficacious tumour-cell selective reoviruses for application as oncolytic agent.

1.2.6 Conclusion

To date, several reverse-genetics systems have been described for manipulation of mammalian *orthoreovirus* genomes. These systems have already proved their effectiveness and were used to reveal new viral functions and facilitated the introduction of modified genome segments. Nevertheless, the robustness of these techniques should be further improved to make them more widely applicable. Some of these systems require selection methods to enrich the mutant viruses. The developments of genetically modified variants should be accompanied by the parallel development of procedures to make the mutant reoviruses a safe pharmaceutical product. This requires manufacturing processes that prevent reversion of the mutants to *wild-type* viruses. To ensure that the mutations are retained during the prolonged passaging that is required for production of large clinical-grade batches, the production systems should be developed in parallel. Such systems must provide a continued positive-selection pressure for the presence of mutations or modifications. Only if the development of reverse genetics systems goes hand in hand with the development of dedicated production systems, can the new technology be employed to generate improved mammalian orthoreoviruses for clinical application.

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