Realia et naturalia

DISSERTATIONES
TECHNOLOGIAE
UNIVERSITATIS
TARTUENSIS
20

KALLE PÄRN

Studies on inducible alphavirus-based antitumour strategy mediated by site-specific delivery with activatable cell-penetrating peptides

DISSERTATIONES TECHNOLOGIAE UNIVERSATIS TARTUENSIS
20

DISSERTATIONES TECHNOLOGIAE UNIVERSATIS TARTUENSIS
20

KALLE PÄRN

Studies on inducible alphavirus-based antitumour strategy mediated by site-specific delivery with activatable cell-penetrating peptides

UNIVERSITY OF TARTU
PRESS

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

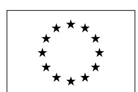
Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on June 29, 2015 by the Council of Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

Supervisors: Prof. Andres Merits, Ph.D. Institute of Technology, University of Tartu, Estonia
Prof. Ülo Langel, Ph.D. Institute of Technology, University of Tartu, Estonia
Chairman, Department of Neurochemistry, Stockholm University, Stockholm, Sweden

Opponent: Kenneth Lundström, Ph.D. PanTherapeutics, Rue des Remparts 4, Lutry, Switzerland

Commencement: Auditorium 121, Nooruse 1, Tartu at 12.15 on August 14th, 2015

Publication of this thesis is granted by the Institute of Technology, Faculty of Science and Biotechnology, University of Tartu and by the Graduate School of Biomedicine and Biotechnology created Under the auspices of European Social Fund.



ISSN 2228-0855
ISBN 978-9949-32-889-5 (print)
ISBN 978-9949-32-890-1 (pdf)

Copyright: Kalle Pärn, 2015

University of Tartu Press
www.tyk.ee

Contents

LIST OF ORIGINAL PUBLICATIONS 6
LIST OF ABBREVIATIONS 7
INTRODUCTION 8
REVIEW OF LITERATURE 9
Alphaviruses 9
Alphavirus virion and genome organization 12
Alphavirus infection-replication cycle 16
Alphavirus based expression and gene therapy vectors 20
Examples of practical applications of alphavirus vectors 27
Non-viral transfection methods 32
Cell-penetrating peptides (CPPs) 34
Internalisation mechanisms of CPPs 36
Tissue-specific delivery of CPPs 38
RESULTS AND DISCUSSION 42
Aims of the present study 42
A method for controlling the rescue of infectious full-length RNAs from DNA/RNA layered alphavirus-based expression vectors (I) 42
Studies on the properties and virus interactions of the CPP-based transfection reagent PepFect6 (II) 48
The development of a tumour-specific nucleic acid delivery strategy for *in vivo* applications (III) 53
Future perspectives 56
CONCLUSION 58
SUMMARY IN ESTONIAN 60
REFERENCES 62
AKNOWLEDGEMENTS 80
PUBLICATIONS 81
CURRICULUM VITAE 137
ELULOOKIRJELDUS 138

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by the following Roman numerals:

I. Viru, L., G. Heller, T. Lehto, **K. Pärn,** S. El Andaloussi, Ü. Langel and A. Merits (2011). “Novel viral vectors utilizing intron splice-switching to activate genome rescue, expression and replication in targeted cells.” Virol J 8: 243.

II. **Pärn, K.,** L. Viru, T. Lehto, N. Oskolkov, Ü. Langel and A. Merits (2013). “Transfection of infectious RNA and DNA/RNA layered vectors of semliki forest virus by the cell-penetrating peptide based reagent PepFect6.” PLoS One **8**(7): e69659.

III. Veiman, K. L., K. Künnapuu, T. Lehto, K. Kiisholts, **K. Pärn,** Ü. Langel and K. Kurrikoff (2015). “PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo.” J Control Release 209: 238–247.

Articles are reprinted with the permission of copyright owners.

Author’s contribution:

I. Participated in the construction of the DNA/RNA-layered vectors, took part in performing of the experiments with these vectors and provided minor contributions to the writing of the article.

II. Designed the experiments, performed all of the the experiments and wrote the article.

III. Produced the plasmid vector used in the experiments and provided insight about the behaviour and characteristics of the plasmid vector.

Other publications:

IV. Vasconcelos, L., **K. Pärn** and Ü. Langel (2013). “Therapeutic potential of cell-penetrating peptides.” Ther Deliv 4(5): 573–591.

V. **Pärn, K.,** Eriste, E. and Langel, Ü. (2015) Antiviral and antimicrobial applications of cell penetrating peptides. In: Cell-penetrating peptides. Methods and protocols, Second Edition. Űlo Langel, Editor, Methods in Molecular Biology. pp. (in print).

LIST OF ABBREVIATIONS

cDNA	complementary DNA
CHIKV	Chikungunya virus
CMV	human Cytomegalovirus
CPP	cell-penetrating peptide
DREP	DNA/RNA-layered replicon
EGFP	enhanced green fluorescent protein
GMP	good manufacturing practice
GOI	gene of interest
IRES	internal ribosome entry site
LTR1	long terminal repeat
MMP	matrix metalloprotease
mRNA	messenger RNA
NLS	nuclear localisation signal
nsP	non-structural protein
ORF	open reading frame
PEG	polyethylene glycol
SCO	Splice-correcting oligonucleotide
SFV	Semliki Forest virus
SIN	Sindbis virus
UTR	untranslated region
VEEV	Venezuelan Equine Encephalitis virus
VRP	virus replicon particle

INTRODUCTION

Alphaviruses are a group of RNA viruses with a single-stranded positive-sense genome. Best studied members of the genus alphavirus (family Togaviridae) include Semliki Forest virus (SFV), Sindbis virus (SIN) and lately also the Chinkungunya virus (CHIKV). While the first two viruses have been widely investigated mainly because of their responsiveness and ease of handling, then the latter has emerged as a prominent pathogen with multiple outbreaks during the last years, causing the researchers to increase the effort in understanding the virus and in developing a treatment or a vaccine.

Since the construction of the first alphavirus-based vectors in 1980's, numerous practical applications have been demonstrated for them, both for laboratory and for medical purposes. Alphavirus vectors can be characterised by their small size, allowing for fast and simple propagation, and high levels of introduced transgene expression in addition to the numerous copies of the genome which can be created in a small time frame. One of the interesting properties of alphaviruses is the destruction of their host cells through guiding them to apoptosis. Although this feature does not seem perspective for any long-term applications, it has, instead, been utilised for the treatment of tumours, allowing the destruction of malignant growths together with the release of previously synthesised beneficial proteins upon the destruction of the cell. These proteins are encoded by the vector itself as corresponding sequence(s) can be inserted into the genome of the vector.

The work at hand tries to investigate the possibility of the creation of a tumour-specific alphavirus-based therapeutic system through the co-application of cell-penetrating peptides (CPPs) as delivery mediators. For this, an inducible virus vector system is studied, determining the suitability of defective introns for the inhibition of virus replication and the efficiency of splice correction oligonucleotides (SCOs) in reversing the suppressive effect of these insertions in order to fully restore the functionality of the vectors. Next, the suitability of PepFect6, a peptide-based transfection system was assessed in combination with an assortment of DNA and RNA-based virus vectors to ensure the suitability of such delivery system for virus-related applications; more specifically, we were interested in its efficacy and in the prevention of the unexpected emergence of an inhibitory effect, often derived from the use of different types of delivery systems or methods. Finally, a possible approach for the deactivation of a previously efficient cell-penetrating peptide was studied with a goal of creating a tumour-selective inducible peptide through the introduction of a protection group, cleavable by the enzymes present in the tumour microenvironment.

REVIEW OF LITERATURE

Alphaviruses

Alphaviruses are small viruses, with positive-sense RNA genomes, belonging to the *Togaviridae* family. This family consists of two genera: Alphavirus and Rubivirus. While the Rubivirus genus contains only one species, the Rubella virus, the 30 known members of the *Alphavirus* genus include virus species with varying degrees of pathogenicity. Alphaviruses, infecting terrestrial vertebrates can be further classified as Old World and New World alphaviruses, depending on their native geographical habitat (Alam et al. 2014, Hyde et al. 2015). Alphavirus infections *in vivo* usually last around 5 to 7 days and are primarily controlled by IFNα/β response (Ryman and Klimstra 2008). There have been various contradictory reports about the role of T- and NK cells during the infection process, making their role in the regulation of infection still unclear (Peck et al. 1979, Singh et al. 1987, Alsharifi et al. 2006, Chu et al. 2013, Teo et al. 2013). Antibodies are known to neutralize infectious alphaviruses and play role in the clearance and prevention of alphavirus infection (Griffin 2010, Kam et al. 2012, Lum et al. 2013)

Infection with Old World alphaviruses is often associated with several clinical symptoms such as rheumatic disease, primarily polyarthralgia and polyarthritis (Suhrbier et al. 2012). Neurotropic alphaviruses (more common in group of New World alphaviruses) are reported to replicate to a high titer in the central nervous system cells (Steele and Twenhafel 2010). For Western-, Eastern- and Venezuelan Equine Encephalitis viruses (VEEV), which are neurotropic and cause severe inflammation of central nervous system, there have been reports of mortality rates reaching up to 70% with survivors having long-term side effects (Delekta et al. 2014).

Although the infection with “milder” arthritogenic members of the genus from Old World, such as the SFV or SIN, tends to cause only minor side effects like headaches and muscle pains, the infection with CHIKV virus can lead to much more severe consequences including high fever, painful swelling of joints and, on the rare occasions, the emergence of large blisters (Enserink 2007). There have also been reports of changes in bone structure in relation to arthritogenic alphavirus infections (Chen et al. 2015). Such side effects can be accounted to a persistent post-viraemia infection which is common for many alphaviruses and is believed to be the cause of persistent symptoms such as chronic arthritis (Suhrbier and Mahalingam 2009).

The earlier research on alphaviruses was conducted mainly on SFV and SIN models due to their ability to replicate to a high titer in a cell culture environment and availability of convenient mice models. However, lately the focus of research has shifted more towards to the work with CHIKV as this virus had demonstrated itself to be a high-profile human pathogen with several massive outbreaks during this century (Leung et al. 2011). The basic features of alphavirus genomes have been researched quite thoroughly. There is not much more

to learn about their genome organisation; in contrast even the basics of their replication cycles are not so well understood and lot of contradictory data exists. Even less is known about alphavirus-host interactions and mechanisms, how and why alphaviruses cause diseases in their vertebrate hosts. Correspondingly, there are numerous laboratories dedicated to the research of various processes and interactions taking place during the infection cycle of alphavirus in order to gain insight into specific molecular mechanisms that play an important role for these viruses. Such research might eventually lead to the development of efficient anti-alphavirus vaccines or therapies (Kaur and Chu 2013, Khan et al. 2014). Less pathogenic alphaviruses, such as SFV and SIN, have also been widely applied in an attempt to further understand viral neuropathogenicity (though both of these viruses are from Old World alphavirus group, they do cause encephalitis in mouse model) and to conduct research aiming to understand several aspects of neurodegenerative diseases. This is achieved through the application of neuropathogenic strains of these viruses, such as the L10, SFV4 and SFV6 strains, all of which are capable of replicating to a high titer in the central nervous system cells (Rheme et al. 2005, Ferguson et al. 2015).

As stated above, the genomes of different alphaviruses have a similar organization. This has led to attempts to transfer the knowledge obtained about one type of virus to the other members of the genus. In a certain degree, it has been successful, especially concerning the basic enzymatic functions of closely related viruses. In the case of more distantly related alphaviruses such generalizations are often misleading. Furthermore, some of the viruses have specific properties that are often confined within the limits of one specific strain. Such differences might either be innate or caused by mutations during the replication by viral polymerases with high error rates. Many of the single-nucleotide mutations will not have any effect on the structure of the virus proteins but there is always a chance to have a mutation that results in a change in the encoded amino acid. Most of such cases result in a defective virus that is either unable to replicate or does not form virions correctly, but the occurrence of such mutations can also lead to increased infectivity, resulting in a chance that a more viable virus will emerge (Jones et al. 2008). This is probably best illustrated by the example of CHIKV adaptation to alternative mosquito vector, *Aedes albopictus* (Halstead 2015, Higgs and Vanlandingham 2015, Lee and Chu 2015, Vega-Rua et al. 2015).

While the specific symptoms of alphavirus infection might vary, the host range and transmission routes are conserved in most cases (Hyde et al. 2015). Large majority of alphaviruses infect vertebrate hosts and are transmitted between hosts by mosquitoes. However, few exceptions, such as the Eilat virus, which has been found to infect mosquitos but not vertebrates (Nasar et al. 2012), and salmonide alphavirus, clearly lacking insect vector, are known. Thus, in general, alphaviruses are capable of infecting vertebrate hosts such as primates, small rodents or birds and they do require mosquitos (or other blood-feeding arthropods) as their intermediate vectors (Hyde et al. 2015). The alphaviruses replicate in their vectors and should reach to a high titer before being

transferred to a new host. There have been no reports about a vertical transmission in mosquitos for some alphaviruses, such as SIN, which gives reason to believe that this step is not crucial for their infection cycle. In case of better studied (in this regard) CHIKV, the results are contradictory: most researchers have documented the presence of viral RNA in the mosquito larvae but have, with one exception, failed to isolate an infectious virus (Mourya 1987, Vazeille et al. 2009, Zayed et al. 2012, Agarwal et al. 2014). What makes the infection of insect vectors especially interesting is the fact that such replication in the arthropod host does not affect considerably the viability of insect vectors (Ciano et al. 2014).

The emergence of alphaviruses, similar to other arthropod-borne viruses, is closely related to various environmental changes which allow their vectors to occupy new habitats, resulting in wider spread of the viruses (Figueiredo and Figueiredo 2014). This has become especially problematic in various urban areas that facilitate the expansion and reproduction of mosquito populations (Barraza et al. 2015). This, in turn, has led to various measures, such as the reduction of the number of mosquitoes by the introduction of infertile males to the population, in order to control the spread of the more severe viruses such as CHIKV (Enserink 2007).

Semliki Forest virus.

SFV was one of the first alphaviruses ever to be described after its isolation from mosquitoes collected from the Semliki forest in Uganda in 1944 (Ketola et al. 2008). Its natural habitat is the sub-Saharan area and the spread of the virus is mostly restricted to densely forested areas. Due to this fact, its natural host is still unknown. Nonetheless, it is one of the most thoroughly researched alphaviruses, probably second only to SIN. Like other arboviruses in the alphavirus genus, SFV spreads with the help of mosquitoes, which enable the transmission to the vertebrate host or hosts. Although SFV is capable of infecting humans, it does not cause any severe side effects apart from a mild headache and muscle pain (Ketola et al. 2008), and even these are not clearly documented. In rodents, however, SFV is highly pathogenic and has therefore become a model system in viral encephalitis research (Atkins et al. 1985, Atkins et al. 1990). The capability of the virus to replicate to a high titer in rodents supports idea that the natural vertebrate host for SFV might indeed be small rodents. SFV wild-type isolates, such as the L10 isolate, cause fatal encephalitis in laboratory mice. However, there are also multiple other laboratory strains of SFV in existence, the most common examples include A7, A7(74) and M9. These viruses originate from a different isolation of SFV and are generally non-lethal for laboratory mice. Curiously, the lack of neurovirulence is not unconditional: for example the SFV A7(74) strain has been known to cause lethality in mice which were younger than 11 days during the moment of infection (Fragkoudis et al. 2009). The age-dependent virulence is actually common for many, if not all,

alphaviruses: they cause disease (often encephalitis, even if the virus originates from the Old World alphaviruses) in neonatal mice but often fail to do this in older mice (Couderc et al. 2008, Ziegler et al. 2008). The main difference between the more virulent strains of SFV and the non-virulent ones is the ability of the first group to induce severe neuronal damage while the latter are incapable of causing any substantial damage to their hosts, with the exception of central nervous system demyelination, which is the result from SFV's capability to infect oligodendrocytes in addition to neuronal cells (Mokhtarian et al. 2003). However, at such cases, the host is usually capable of clearing the virus from its organism within approximately one week (Atkins et al. 1990). Clearance is assisted by the adaptive immune response as in its absence the virus can persist in central nervous system for a long period of time without producing any disease (Metcalf and Griffin 2011, Priya et al. 2014). This property has led to attempts to develop alphavirus vectors for long-term expression of target genes in neuronal cells (Lundström 2005, Ehrengruber and Goldin 2007, Lundström 2012, Lundström 2015).

Alphavirus virion and genome organization

The virion of alphaviruses is 70 nm in diameter (Figueiredo and Figueiredo 2014) and is regular and spherical in shape. 240 subunits of the capsid protein C form a shell with a T=4 icosahedral symmetry which is responsible for the encapsulation of viral RNA. This protein shell is further surrounded by a lipid bilayer originating from the cell membrane of its host (Rheme et al. 2005, Fragkoudis et al. 2009). On the surface of the enveloped viral capsid, exactly 80 glycoprotein spikes are displayed, representing trimers of the E1-E2 heterodimeric glycoproteins of the virus. E1 and E2 are transmembrane type I triplicate membrane-anchored proteins; similarly to the capsid protein they are also arranged in a T=4 icosahedral symmetry and correspond to the orientation of C protein (Strauss and Strauss 1994, Smith et al. 1995, Ferlenghi et al. 1998, Mukhopadhyay et al. 2006). The glycoproteins are responsible for recognising the host cell receptors and facilitate the entry process by membrane fusion (Strauss and Strauss 1994). In addition, they are both antigenic, allowing the infected host to produce antibodies to combat the infection. The antigenicity of E2 and E1 proteins could be exploited for the development of effective vaccines against alphaviruses (Khan et al. 2014).

The alphavirus ssRNA genome is non-segmented and 11–12kb in length. The 5' end of the genome has a type 0 cap structure and there is a polyA sequence present at the 3' end of genome. These features make the cellular factors recognise the viral genome as a messenger RNA, resulting in immediate translation after the entry to cytoplasm has taken place (Strauss and Strauss 1994). Cap 0 structure makes the RNA unrecognisable for the cellular RIG-I receptors while specific secondary structures of 5' region of genome prevent its recognition by IFIT1 system (Iwasaki 2012, Reynaud et al. 2015). The genomic

RNA encodes nine or ten proteins in total and can be divided into two coding regions. First open reading frame (ORF), corresponding to the first two thirds of genome, encodes the non-structural (ns) proteins 1–4 that are required for the replication of the genome. The latter one third of genome contains the ORF encoding for structural proteins, which are needed for the formation and the exit of the virions (Hyde et al. 2015). Both ns- and structural proteins are expressed in the form of polyprotein precursors; former directly from the RNA genome and the latter from the subgenomic RNA synthesised in infected cells. There are also untranslated regions (UTR-s) present on the both ends of the genome which contain conserved regions acting as parts of the promoters and are recognized by alphavirus replication complex (Frolov et al. 2001, Kulasegaran-Shylini et al. 2009, Arias-Goeta et al. 2014, Figueiredo and Figueiredo 2014). The 5' UTR generally consists of slightly less than 100 nucleotides while the length of the 3' UTR is more variable and is generally between 200 and 700 nucleotides. The 5' UTR together with the various non-structural proteins and the E2 glycoprotein can be regarded as the source for determining the virulency of the alphaviruses. Additionally, there is a short non-translated region (around 50 nucleotides in length) present between the two ORF-s (Figueiredo and Figueiredo 2014).

The non-structural protein 1 (nsP1) is an anchoring molecule for viral replicase complexes as, in contrast to other nsPs, it is capable of attaching itself to cellular membranes (Peränen et al. 1995). It also plays an important role in the synthesis of minus-stand RNA (Wang et al. 1991) and possesses guanine-7-methyltransferase and guanylyl transferase activities that facilitate the capping of the genomic and subgenomic RNA molecules (Mi and Stollar 1991, Ahola and Kääriäinen 1995).

The C-terminal region (approximately half of the protein) of nsP2 is a papain-like cystein protease that is required for the processing of the P1234 polyprotein into intermediate and final components of viral replicase. The N-terminal part of nsP2 possesses basal NTPase and RNA triphosphatase activities (Hardy and Strauss 1988, Rikkonen et al. 1994, Gomez de Cedron et al. 1999, Vasiljeva et al. 2000). At least the NTPase activity of nsP2 is greatly activated by the presence of protease domain, conversely, some of the protease activity requires the presence of the N-terminal domain (Lulla et al. 2012, Das et al. 2014). RNA helicase activity of nsP2 strictly requires the presence of all parts of the protein as an individual N-terminal region completely lacks this activity. Finally, the full-length nsP2 also has RNA winding activity (Das et al. 2014). In infected cells, nearly 50% of the protein ends up inside the nucleus (Peranen et al. 1990), where it is involved in the suppression of type-I interferon signalling (Breakwell et al. 2007, Fros et al. 2010) and in the suppression of cellular transcription by the induction of host RNA polymerase II degradation (Akhrymuk et al. 2012). This localization pattern has led to the hypothesis that the nsP2 may have nuclear localization signal (NLS). Indeed, a SV40-like putative NLS signal has been found in nsP2; its disruption leads to a reduced replication efficiency and cytotoxicity, in addition to the generation of a avirulent phenotype of

virus (Kaariainen and Ahola 2002, Tamm et al. 2008). However, none of NLS-es predicted for the nsP2 of SIN have any role for nuclear transport of the protein (Frolov et al. 2009). Furthermore, the nsP2 of CHIKV lacks the putative NLS and insertions in region, corresponding to the NLS of SFV, have no effect on nuclear localization of nsP2 of CHIKV (Utt et al. 2015). Thus, the functional significance of nuclear localization of nsP2 is clear but the role of classical NLS'es in this process is unclear.

The specific functions of nsP3 are harder to determine as the protein lacks enzymatic functions, except the ADP-ribose 1-phosphate phosphatase activity (Malet et al. 2009) which is difficult to link to alphavirus replication. There is, however, increasing evidence that nsP3 represents a platform through which the RNA is able to bind to the replication complex (Malet et al. 2009). Coherently, it is also a required component for the minus-strand and subgenomic RNA synthesis (Lemm et al. 1994). Another studies have determined the nsP3 to be an important component for pathogenicity, especially in mice where the mutations in this protein changed the profile of virus behaviour (Tuittila and Hinkkanen 2003, Park and Griffin 2009). For some reason, a significant part of the total nsP3 protein has been found to be separate from the replication complexes and localised onto the nuclear envelope (Gorchakov et al. 2008). This may reflect another important property of nsP3 – the protein possesses hypervariable and intrinsically disordered C-terminal region, which has been shown to be crucial for numerous virus-cell interactions (Neuvonen et al. 2011, Foy et al. 2013, Panas et al. 2014) and believed to be involved in interactions with many more cellular proteins and pathways (unpublished data from our laboratory).

The nsP4 is a RNA dependent RNA polymerase of alphaviruses (Rubach et al. 2009). It also has adenylyl transferase activity which is required for the synthesis of polyA tails in template-independent fashion (Tomar et al. 2006). Compared to other ns-proteins, the amount of nsP4 in the infected cell is much lower. This is because, in some alphaviruses (but not in SFV4 or SFVL10), the coding region of nsP4 is preceded by a leaking opal codon system which allows to synthesise a polyprotein containing nsP4 with a relatively low efficiency (Li and Rice 1993). The second reason for the low amounts of nsP4 is its rapid degradation by proteasomes according to the N-end rule (de Groot et al. 1991). NsP4 becomes stable only when it interacts with other ns-proteins. Thus, it has been hypothesized that the nsP4 protein acts as an integral part of the replication complex as it provides a base onto which the other non-structural proteins can attach through their N-terminal regions. The N-terminus of the nsP2 can also be used for interactions with the host proteins (Shirako et al. 2000).

The structural polyprotein is divided into five separate proteins – C, E3, E2, 6K and E1. The capsid protein, as the name indicates, is used for capsid formation during the late stages of the infection and contains a RNA-binding signal through its Arg, Pro and Lys residues, located in the N-terminus, which are used to initiate the RNA packaging (Owen and Kuhn 1996). This region interacts with the packaging signal present in the viral genomic RNA (but not in the

subgenomic RNA). The formation of packaging complexes is possibly mediated by a leucine zipper structure, which drives the aggregation of capsid proteins to form dimeric complexes (Perera et al. 2003). The C-terminal region of capsid protein is a serine protease which cleaves itself off from the rest of the polyprotein. The C-terminal end of the capsid protein allows the binding of viral glycoproteins through the hydrophobic pocket located near the substrate binding site (Leung et al. 2011).

The functionality of the E3 protein has been reported to vary between alphaviruses. Although the specific properties are poorly understood, it is believed that the E3 protein is somehow related to virion formation while not being present in a fully formed virion of SIN (Leung et al. 2011). At the same time, in the case of some alphaviruses, such as SFV, the mature E3 protein is present in virons as abundantly as the E1 and E2 proteins (240 copies) (Riezebos-Brilman et al. 2006).

The structure of the E2 protein contains three domains which resemble immunoglobulin. When in a complex, the E2 protein forms a roughly 2500 $Å^2$ contact surface with the E1 protein and is located on the outer surface of the particle which results in the heightened exposure to the surrounding solvents when compared to E1 (Xiong et al. 1989, Berglund et al. 1993, Rausalu et al. 2009, Voss et al. 2010). It has been established that alphaviruses are capable of infecting a wide range of hosts, possibly due to the characteristic of the E2 glycoprotein to interact with a large number of cellular receptors. This is possibly the reason why the E2 protein is considered to be a determining factor for neurovirulence (Davis et al. 1986, Tucker et al. 1997, Ferguson et al. 2015). Although beneficial for entering the cell, this property also results in heightened levels of host antibody response towards the E2 protein, reducing the chances of the virus particles to carry out a successful infection in an immunocompetent host (Atkins et al. 1990). Thus, the E2 protein is a viral antireceptor and a major antigen.

The role of the 6K protein is to facilitate the transport of various components to the sites in which the virion particles are to be assembled. The part played by this protein during the replication is not vital, but still of great importance, as the lack of this protein does not block the release of infectious viruses (Liljestrom et al. 1991, Loewy et al. 1995). After fulfilling the essential role of transport arrangement, the palmitoylated protein then becomes a part of the virion in small parts (Gaedigk-Nitschko and Schlesinger 1990). Although the specific characteristics for this protein are also yet to be fully determined, it has been demonstrated that the 6K protein is capable of influencing the transportation of the glycoproteins and affecting the assembly process of the virion itself (Loewy et al. 1995). Being a part of virion, the protein is believed to intervene with the membrane integrity of bacterial and mammalian cells, in addition to its capability to form cation-selective ion channels (Melton et al. 2002, Sanz et al. 2003). The region, encoding 6K, encodes also for another protein via ribosomal frame-shift. As the protein is encoded by different reading frames it was named as TF, short for trans-frame. It was discovered relatively recently (Firth et al.

2008) and has been shown to be essential (though not crucial) for alphavirus infection (Snyder et al. 2013). It is possible that some of the functions, previously attributed to 6K protein, are actually the functions of TF.

The E1 protein is a fusion protein of alphavirus virions. As described above it is incorporated into the E2-E1 spike structures. Upon the dissociation of spikes at low pH, the E1 forms homotrimers. E1 facilitates various interactions between the virus particle and the cell. Monomers of the E1 protein are usually located at the base of the surface glycoprotein spikes and they form a uniform coating on the surface of the virion (Mukhopadhyay et al. 2006). In its trimeric form, the E1 protein is responsible for the fusion of the viral envelope with the target cell upon entry and facilitates the transition of the surface proteins into ion-permeable pores (Wengler et al. 2003). Like the E2 protein, the E1 has a transmembrane helix which is used to anchor the protein to the lipid bilayer (Schmidt et al. 1979).

Alphavirus infection-replication cycle

Alphavirus replication is a term often used to designate two different processes: the replication of virus RNA and the process of virus multiplication. Furthermore, the replication taking place in the cells of a vertebrate host and the replication inside the cells of its arthropod vector are rather different. In the case of infection taking place in a vertebrate host, alphaviruses are able to enter the cell through receptor-mediated endocytosis facilitated by the interactions between the host cell and the exposed E2-E1 spikes (Figueiredo and Figueiredo 2014). A number of proteins can be bound by E2, including the major histocompatibility complex I, high-affinity laminin receptors (Rheme et al. 2005) and many others. This route is preferred even on the occasions upon which direct access to cytoplasm is available to the virion (Mercer et al. 2010). Once inside the cell, alphaviruses use the endocytotic vesicles as a means of transportation to reach the desired sites and to maximise the potential of replication – in addition to the correct placement of complexes, this strategy allows the prevention of the early immune response signals, which are triggered when the virus moves freely in the cytoplasm of the cell (Marsh and Helenius 2006, Mercer et al. 2010). In order to exit the vesicular compartments, a membrane fusion between the virus envelope and the vesicle wall takes place. This process is common to a wide range of viruses that use endocytosis as the preferred method of entry. The fusion of the vesicle and viral envelope is facilitated by the low pH levels (5 or lower) inside the vesicle which allows the E1 to form fusogenic homotrimers (Tuittila et al. 2000, Wu et al. 2007). These complexes, in turn, allow the merging of the virion membrane with that of endosome (Zhong et al. 2008, Kielian et al. 2010, Zeng et al. 2014).

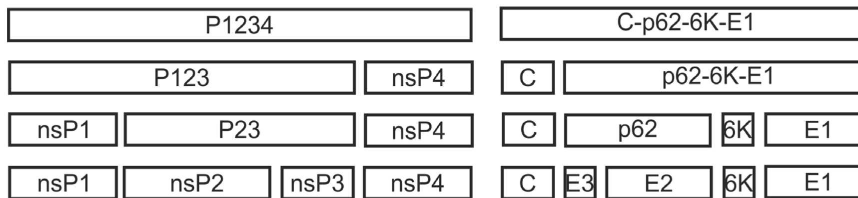


Figure 1. The cleavage process of alphavirus non-structural (P1234) and structural (C-p62-6K-E1) polyproteins. The non-structural polyprotein is first cleaved between the nsP3 and nsP4, allowing the formation of P123+nsp4 (early replication complex) which is able to produce negative-strand RNA. The P123 polyprotein is then further cleaved from the cleavage site after nsP1, after which the site between nsP2 and nsP3 is also cleaved. The process is tightly regulated and allows the conversion of the early replication complex to late replication complex capable of synthesizing genomic and subgenomic RNAs.

Through its autoproteolytic activity the C protein is co-translationally released from the rest of structural polyprotein. The remaining polyprotein is further cleaved in ER by cellular factors into p62, 6K and E1 proteins, of which the p62 is further cleaved into E2 and E3 proteins during the later stages of the structural protein maturation.

After the escape of the virus capsid from endosome has taken place, the capsid itself is dissolved through the interactions with the ribosomes, allowing the release of virus genome. The replication and protein production (Figure 1) takes place in cytoplasm where the virus replication organelles are formed. These organelles are called cytoplasmic vacuoles type I, which represent modified endosomes and lysosomes. (Froshauer et al. 1988). From the first ORF, a non-structural polyprotein P1234 and, in case of viruses having an in-fame opal codon, also P123, are translated. The efficiency of read-through is roughly 10–20% (Takkinen 1986, Li and Rice 1993). Polyproteins are then processed with the help of a protease domain located in nsP2 region. First, P1234 is cleaved to form P123 and a separate nsP4 protein. These proteins interact with each other, cellular plasma membrane and viral RNA genome and form an early replication complex that allows the synthesis of the negative-polarity RNA, using genomic RNA as a template. Next, the P123 polyprotein is further cleaved in an organized manner into nsP1 and P23. After the cleavage of the latter, all 4 non-structural proteins (nsP1, nsP2, nsP3 and nsP4) are formed. These proteins form a different replication complex, which cannot perform the synthesis of the negative-sense RNA (Strauss and Strauss 1994, Kim et al. 2004). Instead, the complex, termed as late (or positive-strand) replicase, is used for the transcription and replication of viral RNA (Figueiredo and Figueiredo 2014). To do this, the negative-sense RNA (almost certainly in the form of double-stranded replication intermediate) is used as a template for the synthesis of numerous new positive-sense RNA-s, which can be divided as genomic (42S for SFV) and subgenomic (26S) RNAs. The synthesis process for these RNAs is very active, resulting in hundreds of thousands of RNA molecules with the genomic to subgenomic RNA ratio of 1:2-3. The 26S subgenomic mRNA acts as a template for

the translation of the structural polyprotein. The processing of the structural polyprotein C-p62-6K-E1 starts with the co-translational removal of capsid protein by its own serine protease activity. This re-directs the translation to cellular membranes and, concurrently with the synthesis process, the rest of the polyprotein is transported to the lumen of endoplasmatic reticulum. In this compartment, its co- and post-translational processing occurs; first resulting in three surface proteins p62 (a precursor for E2 and E3), 6K and E1). At the final stage of the processing, p62 protein is cleaved to E3 and E2; this process occurs during the transport of glycoprotein complexes in the trans-Golgi compartment. Thus, the processing results in the five (or 6) structural proteins – capsid, E3, E2, 6K (TF) and E1 (Suomalainen et al. 1992, Khan et al. 2002, Garcia-Moreno et al. 2015). The processing is well regulated, for example, the p62 first (co-translationally, concurrently with the process of protein folding in ER) forms a dimer with the E1 protein to ensure the proper folding (Atasheva et al. 2010). As the E1 protein plays an important role in the exit process of the virion (Kim et al. 2000), it is important for the p62 to protect it from the low-pH conditions in order to retain the full functionality of the protein. This stage is also believed to be the responsible for the initial creation of the single trimeric viral spikes (Garmashova et al. 2007). Cleavage of E1-p62 by furin allows the formation of mature trimers of E2-E1 heterodimers (Zhang et al. 2003, Rheme et al. 2005, Fields and Kielian 2013).

At the late stages of infection, the formation of new virus particles takes place. In order for this to happen, the virus RNA is packed densely inside the virus capsid. For SIN (and most of alphaviruses), the packaging signal, required for this process, is located inside the region of viral RNA encoding for nsP1 (Strauss and Strauss 1994); for SFV, however, the packaging signal is located in nsP2 and has different structure (Kim et al. 2011). In this step, only the complete virus genome will be packaged into the capsid, as neither the negative-strand RNA nor the subgenomic RNA possess the signal required to initiate the packaging process. At the same time, the mature glycoproteins accumulate to the cell plasma membrane and once the virus genome has been packed inside the capsid protein, an interaction between the cytoplasmic tail of E2 glycoprotein and the RNA-containing capsid takes place, resulting in the budding of mature virions (Barry et al. 2010).

The infection with alphaviruses induces various changes, both functional and structural, inside the host cell. These changes can best be observed in a cell culture environment. From the structural side, infected cells become more round in appearance and have a bloated look. From the molecular perspective, there are high levels of virus RNA replication taking place. In turn, the synthesis of host RNA and proteins becomes inhibited. In part, it occurs due to the cytotoxic properties of nsP2, however the effects are further amplified by the endoplasmatic reticulum stress caused by the synthesis of virus membrane proteins (Barry et al. 2010). Although all the underlying mechanisms of this inhibition are not yet fully understood, it has become clear that, at the cases of Old- and New World alphaviruses, it is caused through the action of different viral pro-

teins. Indeed, in case of Old World alphaviruses the nsP2 has major role (Garmashova et al. 2007, Akhrymuk et al. 2012); however, the effect is enhanced by the action of capsid protein and the RNA replication process. In the case of New World alphaviruses the capsid protein has major role (Atasheva et al. 2010). Regardless of the exact mechanism(s), the shutdown of the host cell allows the virus to divert resources from cellular pathways (which may also be anti-viral) into those more beneficial for the virus. As a result, such reassigning of cellular systems, through the inhibition of host transcription, translation and the transportation of various molecular building blocks between the nucleus and cytoplasm, leads to the death of the infected cells which is reported to occur by apoptosis (Glasgow et al. 1998). Furthermore, cells respond to virus replication by activating innate immune responses. Apparently, these processes are aiming to block the virus infection; however they too can be diverted by virus to fulfil its goals. Thus, the replication of virulent strains of SFV in cells of central nervous system triggers the production of type-I interferons and inflammatory cytokines; in contrast no such immune response has been registered during the infection with non-virulent strains (Tuittila et al. 2000). The molecular bases of this phenomenon remained unknown for long time; only recently it has been discovered that the ability to induce cytokine production is not related to viral RNA replication; instead it is a novel property of replicase complex of virulent, but not avirulent, strains of SFV (Utt et al. 2015), unpublished data from our lab).

At first, the infection in invertebrate hosts follows the similar route as in infected vertebrate cells. Again, high-level synthesis of viral RNA and protein takes place and new generation of virions are released (though this occurs via exocytosis as virions bud from intracellular membranes). For some reason, however, these processes are far less damaging to the host cell when compared with a similar infection in vertebrate hosts, enabling the infected cell to continue its normal functions and, in some cases, even to replicate. In most cases the molecular bases of these differences are unknown. However, it has been demonstrated that the nsP2 of Old World alphaviruses does not cause the degradation of cellular RNA polymerase II in insect cells (Akhrymuk et al. 2012).

Roughly 24 hours post-infection, rapid and effective suppression of virus production occurs and cell culture becomes persistently infected. Virus production is not completely eliminated, probably because the replication still occurs in small fraction of cells. Although the full set of mechanisms involved in the emergence of this persistent infection have not yet been determined, it has been suggested that inhibitory molecules are synthesised by the host cells. Multiple studies have revealed that siRNAs and RNAi play the most important role in controlling the alphavirus infection in insect cells (Cirimotich et al. 2009, Nogueira et al. 2011, Siu et al. 2011, Ratnik et al. 2013). Other mechanisms, like piRNAs, pro-phenol-oxydase system and mosquito antiviral signalling have also shown to play a role (Fragkoudis et al. 2008, Rodriguez-Andres et al. 2012, Schnettler et al. 2013). Interestingly, these mechanisms are not really needed to protect the hosts from the harmful effects associated with the replication of the

virus: in their absence, the replication is enhanced and a persistent infection may not occur, nonetheless, the cells experience minimal damage form a high-level long-lasting virus infection. In part, it may be due to the fact that in insect cells the virus affects the vital biosynthesis processes only in a minor way, allowing most of the infected insect cells to remain viable, although there is always the presence of some levels of cell death, not as a result of apoptosis, but rather because of cell necrosis (Fragkoudis et al. 2009).

Alphavirus based expression and gene therapy vectors

Since the construction of the first alphavirus vectors based on SIN and VEEV in 1989 (Davis et al. 1989, Xiong et al. 1989) and on SFV in 1991 (Liljestrom et al. 1991), alphaviruses are becoming an increasingly important part of the field of genetic engineering and gene delivery vectors. Alphaviruses, such as SFV and SIN, are able to replicate in a wide selection of cells and are also capable of high levels of protein expression in a short period of time: these properties are among the reasons why they are considered to be promising vector systems. Alphaviruses can be used to deliver protein-coding sequences into cells which are either non-transfectable under normal conditions or into non-dividing cells, which are resistant to infection with other virus-based vector systems. The second advantage of alphavirus-based vectors is the size of their genome, which is rather small when compared with many other viruses. This feature allows the researchers to reconstruct, manipulate and apply the vectors in a laboratory setting with a relative ease; during the recent years the methods of synthetic biology (gene synthesis) are frequently used for this purpose. However, such manipulations do not come without its consequences – the insertion of a foreign sequence into the viral genome leads to an increase in its length, resulting in problems like the decrease of the replication rates, changes in the regulation of gene expression and possible difficulties relating to genetic instability when packaging the lengthened genome into the capsid (Rausalu et al. 2009). The third advantage of using alphaviruses as vectors is that their RNA is active when transfected into cell, therefore being capable of establishing a viral infection, resulting in the production and release of new generations of virus particles. In addition to these general advantages there are also numerous more specific (sometimes restricted to specific virus) but no less attractive properties including natural tropism to cancer cells of SIN (Suzme et al. 2012), ability of SFV to break immunotolerance to cancer cells (Riezebos-Brilman et al. 2005) and ability of SFV to prime immune response possibly due to its superior ability to trigger production of cytokines (Naslund et al. 2011, Nikonov et al. 2013).

The ability of alphaviruses to successfully and rapidly carry out their infection-replication process in a wide selection of cells is also one of their downsides. As the life cycle of the virus is relatively short and results in the death of the host cell through the apoptotic pathways (Barry et al. 2010), un-modified alphaviruses are not ideal for the generation of long-lasting expression systems.

Another inhibitory property for these vectors is the possibility to insert only relatively short foreign sequence as the complete genomes with inserts significantly longer than 2kb will have problems completely fitting into the virus capsid during the packaging process. Finally, a set of limitations originates from the basic properties of alphaviruses. As their replication depends on the interactions with host components (proteins, membranes) these cannot be eliminated. As RNA viruses, alphaviruses replicate with high error rate and are prone for recombination – both of these factors reduce stability of inserted sequences.

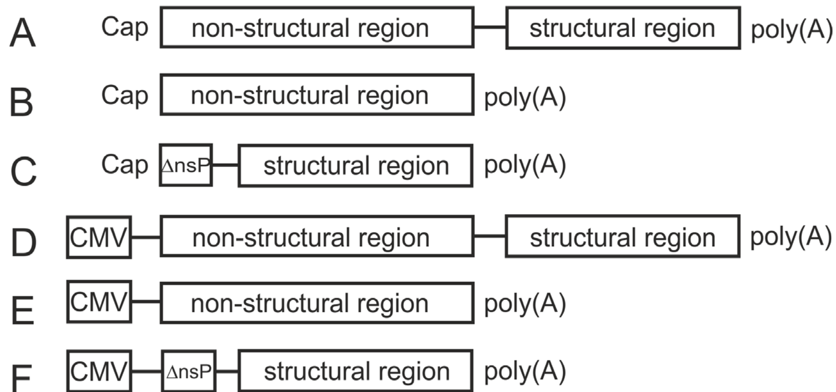


Figure 2. Common vector types based on alphavirus genomes.

A. Full-length genomic RNA vector, essentially identical to the wild-type virus (can contain point mutations and/or small mutations altering its properties).
B. RNA-based replicon vector that has its structural region removed and therefore is not able to form virus particles for exiting the cell.
C. Helper vector for RNA-based replicon vector, which contains a coding region for structural proteins required for virion formation.
D. Full-length DNA/RNA-layered vector, structurally similar to the wild-type virus but in a DNA plasmid form and containing a CMV promoter for the start of cDNA transcription and the initiation of the replication process.
E. DNA/RNA-layered replicon vector, similar to the RNA-based replicon vector but in the form of a DNA plasmid (same principle as for E).
F. Helper vector for DNA/RNA-layered replicon vector.

At cases A, B, D and E genes of interest (GOI) can be inserted either into existing reading frame(s), under duplicated subgenomic promoter or inserted IRES element. At cases B and E the duplication of subgenomic promoter (and/or IRES insertion) is not strictly required as GOI can be inserted under control of existing subgenomic promoter.

As alphavirus vectors have been in use for more than 25 years, very large varieties of different designs have been tested and used; the set is too extensive and diverse to be reviewed comprehensively. Hence, a somewhat simplified classification of vectors has been provided below. Although the various types of

alphavirus-based vectors (Figure 2) can be classified according to numerous criteria, such as the expressed marker of interest or their pathogenicity, in this work, the vectors will be classified according to their genomic structure. Following this strategy, four distinct main types of vectors can be identified – the RNA based full-length genomic vector, the DNA based full-length DNA-RNA layered vector and the replicon vectors, in the form of either DNA or RNA, which are devoid of their structural ORF region and therefore are not capable for virion formation and release.

Full length genomic alphavirus RNA vectors became possible after the successful transition of the RNA based virus genome into a cDNA molecule and its insertion into a plasmid, downstream of a promoter from DNA bacteriophages (such as SP6 or T7). This approach would allow the *in vitro* transcription of infectious RNA transcripts using the corresponding RNA polymerase. The main advantage of this approach is its ability to enable the insertion of mutations or the introduction of foreign sequences into the virus genome, effectively turning them into gene delivery vectors. Since then, these vectors have been widely used and their main advantage is their tendency to behave in a similar fashion to the unmodified virus from which they were derived (Rice 1992) and the high infectivity of *in vitro* synthesised RNAs allowing the construction of representative libraries and/or the insertion of mutations compromising virus replication. This is due to the fact that these vectors have no protein-encoding sequences of their original genome removed; instead they often contain an inserted gene of interest (GOI) or a regulatory sequence. This allows the vectors to replicate and to form new virions which contain the same genome which was originally delivered to the cell, allowing the formation of novel generations of genetically engineered virus genomes. The inserted sequences can commonly be found either in the non-structural region of the alphavirus genome (in most cases inside the nsP3 protein coding sequence), in their structural region (for example between the C/E3 protein sequences) or placed under a separate subgenomic promoter which is often located at the end of the genome between the region encoding the E1 protein and 3' UTR; alternatively the GOI can be inserted under the control of the native subgenomic promoter and the duplicated promoter is used for the synthesis of subgenomic RNA for structural proteins.

The similarities between the full-length vector and the wild-type virus allow the vector to express proteins in a highly efficient manner; furthermore, in theory, such vectors could repeatedly be amplified in susceptible cells without the need to repeat the virus rescue process. However, the similarities to the wild type virus can also be a source for problems, especially when it comes to the practical applications of the virus-based vectors. By far the most prominent of these problems is the stability of recombinant genomes; this is followed by concern over safety of such vectors (Vähä-Koskela et al. 2003). The issues relating the safety are, unfortunately, not completely avoidable for a full-length genomic vector as it is capable of producing infectious virions as a result of its infection-replication cycle. However, numerous approaches can be used to alleviate the safety concerns including miRNA based regulation of vectors (Bhomia et al.

2013, Ratnik et al. 2013), engineering of conditional lethality of alphavirus vectors (Sawicki and Sawicki 1993) and introduction of mutations reducing pathogenicity of virus (Tamm et al. 2008). The stability issues, which are mostly reflected by the poor expression of the foreign transgene and low viability of the correct constructs, originate from the basics of alphavirus molecular biology and are therefore impossible to address completely. No matter what, alphavirus replication will always be carried out by the error-prone replicase (even if the recently discovered high-fidelity mutations are used (Coffey et al. 2011, Rozen-Gagnon et al. 2014)), which is capable for a copy-choice recombination between two different RNA molecules or between the different regions of one and the same molecule. Similarly, the spherical capsid will always limit the maximal size of the genome which can be packed into the virions. Nevertheless, some aspects of the stability problem can be solved using a few specific strategies. This includes the introduction of GOIs only into the few pre-determined positions, which will result in a minimal effect on the virus. As obtained results have demonstrated, viruses are able to express the insert as a fusion protein together with its own native ns-proteins or as an individual protein, if protease sites are included into design (Tamberg et al. 2007). The regulation of GOI expression can also be achieved: it occurs early, but at low level, if the insertion is made into the ns-region or it can occur later, but at a higher level, if the insertion is made into the structural region. Alternatively, a relatively stable and efficient expression of the foreign sequence can be achieved by the insertion of the GOI under the control of a subgenomic promoter positioned in the viral genome as described above. In addition to the consistent expression of the GOI, vectors with such design can also be used in research to determine the exact function and efficiency of subgenomic promoters and have even been reported to be used in determining the localisation and transportation pathways of the genomic material of the virus inside the infected cell (Cook and Griffin 2003, Rausalu et al. 2009). It should be noted, however, that alphaviruses are highly individual and an approach, which leads to a stable vector for one virus, will result in unstable vectors in the case of another. Thus, SIN with EGFP inserted between C and E3 was reported to be stable (Thomas et al. 2003) while SFV of similar design was unstable with compromised replication (Fragkoudis et al. 2009). For SFV, the most stable vectors contain duplicated subgenomic promoter downstream of the structural region (Rausalu et al. 2009) while in the case of CHIKV more stable vectors are obtained by inserting duplicated promoter upstream of structural region (Vanlandingham et al. 2005). There is no theory capable of explaining these inconsistencies, thus the selection of optimal design is largely based on a trial and error approach.

A structurally similar construct to the RNA-based genomic vector is the full-length DNA/RNA layered vector. While having a similar genome organisation as the corresponding RNA vector, these DNA based vectors are somewhat easier to manipulate and do not require transcription prior to the transfection of susceptible cells. As it can be seen from the name, the main difference between these two types of vectors is that one of them, like the virus itself, is based on

the infectious RNA molecule while the other is delivered to the cells in the form of DNA. As the *in vitro* transcription is be quite costly and RNA is difficult to handle (prone to degrade easily) molecule, the use of DNA instead significantly decreases the workload. In addition, DNA is much easier to produce in large amounts and with GMP (good manufacture practice) quality, which is the pre-requirement of any use on human subjects. However, DNA/RNA layered vectors require a more complex activation system from the cell: just the delivery of DNA to the cytoplasm of cells is insufficient; instead the molecule must first be transported into the nucleus of the cell where it can be used as a template to synthesise a RNA molecule with the help of a specific promoter placed up-stream of the virus cDNA sequence, which is recognised by the transcription factors in the eukaryotic cell. The first alphavirus-based layered DNA-RNA vectors, which were based on SIN, took advantage of either Rous sarcoma virus LTR or a CMV promoter, but most of the following alphavirus constructs have utilised the CMV promoter (Berglund et al. 1998). However, not all transfection methods and reagents allow efficient nuclear entry of the DNA. The CMV promoter is a strong and immediate early promoter of virus, the RNA synthesis process starts relatively fast and occurs using cellular RNA polymerase II. The primary transcript must be terminated correctly (therefore the DNA/RNA layered vectors also contain an eukaryotic termination signal), spliced (if introns are inserted to the construct, see paper I for the details of such technology) and transported to the cytoplasm where it will behave in a similar way as a normal RNA-based genomic vector (Dubensky et al. 1996, Berglund et al. 1998, Rheme et al. 2005). All this is somewhat time-consuming (in a range of an hour or so) and different hurdles reduce efficiency of virus rescue (which is considerably, often 10-fold or more, lower than in case of *in vitro* RNA transcripts). In the context of full-length constructs, it is generally not regarded as a problem as the rescued recombinant viruses can subsequently be propagated (in theory as many times as needed).

One approach to combat the previously mentioned safety and stability issues is to use a viral replicon vector. In case of alphaviruses, replicon is a vector which does not contain any region encoding for structural proteins, making it unable to exit the cell, which means that the infection will not be able to spread inside of a cell culture or in a living organism, effectively reducing the biological hazard originating from the virus-based construct. Replicons are derived from the full-length vectors and can also be based either on DNA (layered vector) or RNA (Figure 2). The first of such alphavirus vectors was created in 1989 by Xiong through the modification of SIN genome, during which the region coding for the structural proteins was replaced with a gene encoding chloramphenicol acetyltransferase (Xiong et al. 1989). Since then the replicon versions of various viruses such as SFV, VEEV and CHIKV have been created, all of which follow a similar design and therefore possess similar properties (Berglund et al. 1993). In these vectors the non-structural part of the viral genome is left intact (though it may contain mutation or mutations aiming to improve efficiency of replicon) to allow the construct to replicate to a high copy

number through the first phases of the virus life cycle. As in the case of native virus, only the non-structural proteins (replicase) are translated directly from replicon RNA (unless it contains an IRES element upstream of the GOI). In transfected cells the replicase synthesises the negative strand and, after the conversion into its late form, multiple new copies of replicon genome as well as an extreme abundance of subgenomic RNAs (in general, they are more abundant than the subgenomic RNAs of the full virus, often outnumbering the replicon genomes by the value of 10 or more). The reduced size of the replicon genome and the limited rounds of replication also reduce the issues related to stability of these vectors – genomes which have a smaller size also have less problems with packaging. The single-round nature of replicons eliminates the main mechanism behind of instability of full-length genomes – outcompeting of original vector genomes by more rapidly replicating mutant ones which have growth advantage due the loss of GOI and/or inserted control sequences (Rausalu et al. 2009, Ratnik et al. 2013). The inability to produce new generations of the virions can be very helpful when developing virus-based vaccines. Such vectors do not express the major viral antigens and thus the immune response against vector is weak or non-existent, allowing the patient to receive multiple doses of the same vector (Berglund et al. 1998, Rodriguez-Madoz et al. 2007).

Although the results obtained with replicon vectors can be very promising, there are several downsides. If used in the basic studies, one needs to remember that as the replicon vectors do not mimic the whole alphavirus life cycle, the results obtained from such vectors might not be reflective of processes taking place during the real infection (Delekta et al. 2014). Furthermore, these vectors are not able to infect the cells by themselves, making them dependant on various transfection methods, which might possess adverse effects on cellular viability and seriously limit their *in vivo* applicability. One of the methods designed to overcome this problem is known as the construction of virus replicon particles (VRPs). At this case, the region, encoding for the structural proteins of the virus, is placed between the 5’ and 3’ UTRs (and most typically also under the control of a subgenomic promoter; Figure 2) and co-delivered to susceptible cells together with the replicon. In these cells the replication of both the replicon RNA and the helper vector RNA is facilitated by the virus replicase complex, which is able to recognise the replication signals on both sequences. Subgenomic RNA (if the helper vector has a subgenomic promoter) is produced and the structural proteins will be expressed. Such co-incubation allows the formation of VRPs which, however, contain only the replicon RNA as the helper is not packed due to the absence of a packaging signal. Another approach for the construction of VRPs requires the creation of a stable packaging cell line stably producing low-level helper-RNA from the integrated DNA copy in the cell genome. Structural proteins, which require the synthesis of subgenomic RNA, are not produced in such cells until the cells are transfected by replicon RNA. This approach removes the need to transfect cell with two separate molecules – every cell already contains the sequence of the helper vector in its genome. Furthermore, in the culture of such packaging cells the replicon vectors are no longer

restricted to initially transfected cells: released VRPs can infect neighbouring cells (or fresh culture of packaging cells) and produce new VRPs. This property compensates for the fact that the VRP titers, obtained through the use of this method, do not reach the levels obtained by the co-transfection of cells by the replicon and helper RNAs. This effect is possibly resulting from the lower gene expression efficiency of the integrated sequence when compared with a separate molecule which is able to replicate to a high copy number (Polo et al. 1999).

When a normal cell is infected with a VRP particle, the first stages of the infection take place in a similar fashion as during a full-length virus infection. The only difference is that no secondary infectious virus particles will be produced, rendering the construct as harmless as a normal (non-packed) replicon vector (Delekta et al. 2014). However, if VRPs are based on the Old World alphaviruses, the replication of such vectors still results in cell death as the vital mechanisms, through which these viruses supress cellular metabolism and induce cell death, are intact. Due to this property, VRPs are also known as the suicide vectors: they kill the cell and die with it without being able to continue their existence through progeny (Smerdou and Liljestrom 1999). Such approach might also be used to research the various aspects of viral entry without the need of a higher-risk fully functioning virus (especially important for the studies on alphaviruses which are pathogenic for humans such as CHIKV). The problem with this method is the risk of recombination which can happen during the period where the replicon and its helper are co-incubated inside the cell. Such copy-choice recombination results in a wild type virus genomes or, in full-length genomic virus vectors, depending on the presence of any artificially inserted sequences (Weiss and Schlesinger 1991). In order to decrease the chances of unwanted recombination taking place, further modifications of this system have been developed – for example, it is possible to create conditional helper systems, which are dependent on some external stimulus in order to be active or to split sequence encoding for structural protein between two separate helper vectors, which would ensure additional safety, as the reversion back to the wild type genome would require the recombination to take place both between the replicon and its two helper vectors (Berglund et al. 1993, Frolov et al. 1997). However, this split-helper system might not always be very efficient as there have been reports on some types of replicon vectors which have been unable to transcribe helper-RNA in a correct fashion, resulting in an extremely low yield of replicon particles from the process (Tamm et al. 2008). Therefore, alternative approaches to reduce recombination, such as the development of promoterless helper vectors (Kamrud et al. 2010) or the elimination of the viability of recombinant full-length genomes by inserting target sequences for cellular miRNAs (Ratnik et al. 2013), have been developed.

Examples of practical applications of alphavirus vectors

The most common application of alphavirus vectors is to use them for basic research, often to study the corresponding alphavirus. Replicons are used to increase the safety of such studies and are also useful if the events of an infection in a single cell are analysed (thus, in the conditions where the release of infectious progeny is unwanted). However, the field that has gained much more attention nowadays is the use of alphaviruses for a more patient-related (directly or indirectly) purposes. Thus, alphavirus vectors have emerged as efficient tools with potential for the treatment and prevention of medical conditions. As an example of indirect usage, alphavirus vectors can be used for recombinant protein production. Several alphavirus-based genetic vectors have even entered the first phases of clinical trials during the last 10 years and many more have been used in preclinical studies. Lately, however, the focus has shifted towards using alphaviruses in antitumor treatment and they significant advantages have been made in the field. Additionally, the vectors have been applied to target central nervous system which is extremely difficult to reach by other means (Atkins et al. 2008).

The paths leading to the immunisation against a disease through the use of alphavirus-based vectors can broadly be divided into two distinct categories. The first approach resembles the most common vaccination strategies and its purpose is to stimulate the immune system of the host with a foreign antigen in order to achieve a heightened immune response and an increase in the number of respective antigens. The second approach is the fortification of the immune system by the expression of such proteins which would help to protect the cell against the pathogen of interest. The common feature for both of these strategies is that the antigen-coding sequences are introduced inside the vector sequence in order to achieve high-level expression once the vector has been delivered to the target organism. As described above, the construction and application of alphavirus vectors can result in many different forms through which they can be used, this is also the case in vaccine development. The vectors used for such applications can be based on either full length RNA or DNA/RNA layered systems or they can be built upon replicon vector technologies (Berglund et al. 1998, Fleeton et al. 2001, Ljungberg et al. 2007). Though promising in cell culture, most of these approaches have, however, yielded low efficiency when applied in an *in vivo* condition. The only approaches that have shown real promise are the use of VRPs, through which the immunity against many different diseases has been achieved in model test systems (Berglund et al. 1998, Atkins et al. 2008) and the use of DREPs (DNA/RNA layered replicons), which are much more immunogenic than the standard DNA based vaccines (Naslund et al. 2011).

The main focus for the virus-based vaccine development has been to combat such widespread viruses like HIV. At this case, it is not correct to say that alphavirus systems have performed worse than the alternatives – no anti-HIV vaccine has ever produced good results (and some, such as the vaccines based

on adenovirus vectors, have produced results opposite to the desired ones). In fact, several *in vivo* experiments, where alphavirus VRPs have been applied in order to prevent the infection by HIV itself or its primate-based model system, SIV, have shown promise. Thus, the repeated inoculations of primates with virus-based vectors (multiple vaccine carriers are often used, as the first inoculation might result in an immune response towards the virus upon which the first construct was based) have been successful in reducing the initial spread of the virus, or in producing high immune responses. However, the vaccines were not able to completely prevent the infection of the host (Nilsson et al. 2001, Xu et al. 2006). In mice, the obtained results were even more promising: upon the immunisation of mice with the vectors there was a significant increase in observed anti-HIV T-cell responses and even a protective response was achieved on the mucosal surfaces, which efficiently prevented the entry of the virus into the immunised host (Vajdy et al. 2001, Sundback et al. 2005). However, it is still unclear how it would translate to applicable results in primates and humans. As mentioned above, the lack of clear success in case of HIV is more likely due to the difficult nature of the target rather than the shortcomings of any vector system (including the ones based on alphaviruses).

Another vaccine-like application for alphavirus-based vectors is the development of antiviral molecules which could then be used to combat virus infections. For this purpose, replicon vectors have been exploited to determine the specific effect of various substances in relation to the replication of the virus. Once such molecules have been isolated, it is possible to incorporate them into vaccine shots, providing that they possess no significantly adverse effects on living organisms, which would render the application of these molecules impossible. One of such examples is the use of WEEV replicons for the discovery of viral RNA replication inhibitors (Peng et al. 2009, Delekta et al. 2014).

It has been theorised that due to the ability of alphaviruses to infect neuronal cells, the vectors based on these viruses could be used to deliver foreign genes into the central nervous system. This capability has been demonstrated to be rather efficient in tissue culture but the first *in vivo* assays have been conducted using a non-virulent SFV-based vector in mice. This test resulted in the successful initial expression of EGFP in glial cells, however the vector failed to infect neuronal cells as efficiently (Ehrengruber et al. 2003). The direct introduction of a virus construct to the central nervous system cells has also been attempted but it has resulted in cytotoxic side effects in the infected area, making this approach non-efficient for any clinical purpose. Thus, alphaviruses can still be considered as an efficient means for applications which do not require an extended survivability period such as the labelling of certain brain regions (Furuta et al. 2001, Graham et al. 2006). There have been reports of using non-neuropathogenic alphavirus-based vectors for the attempts of treatment of multiple sclerosis. The results obtained from mouse models have been quite promising. The used vector, based on an avirulent SFV A7(74) virus, where a sequence coding for a component of immune system, able to reduce the progress of the disease and to inhibit the clinical symptoms, was inserted. This

vector was then introduced through the olfactory bulb (approach allowing to bypass the blood-brain barrier). To note, this route of infection would result in lethal consequences if the neurovirulent SFV4 or SFV6 virus would have been used as bases of the vector. As A7(74) is significantly less pathogenic, it was able to reach directly the central nervous system without causing lethal disease (Jerusalmi et al. 2003, Vähä-Koskela et al. 2007).

The application of alphaviruses to combat various types of tumours, including glioma, papillomavirus-associated carcinoma, prostate cancer, melanoma and breast cancer has received increasing amounts of attention as the insight into the genetic background and the treatment of tumours is considered to be a high-priority field in medical research. This has resulted in two distinct, but in some ways interconnected, approaches for anti-tumour applications of these vectors. One of the approaches is somewhat similar to vaccinations – the main purpose of the alphavirus vector is to increase the potential immune response to tumours by introducing sequences coding for various antigens and/or immunity-boosting molecules which would prevent the emergence of tumour cells altogether. The common problem with alphavirus-based expression systems, however, is their low duration of expression, especially when working in *in vivo* systems, which makes any application, that requires a consistent expression of transgene, highly dependent on the reintroduction of the virus to the system. This, however, is not a limitation in anti-cancer therapy. Thus, the second approach does not attempt to express something for extended periods. For anti-cancer treatment to be effective it is more efficient to have the construct replicate in a way which would allow the creation of high amounts of virus particles and once the infection has reached the final stages, the cells become apoptotic (Atkins et al. 2008, Quetglas et al. 2010).

The idea to use alphaviruses as anti-cancer vaccines first surfaced in the 1970's when it was observed that once SFV-infected tumour cells were introduced to mice, they were able to prevent the emergence of the same tumour type (Griffith et al. 1975). This property of the alphaviruses has, since then, been studied further. It has been discovered that the use of alphavirus vectors to combat tumour is a much more efficient method when compared to the use of a DNA plasmid-based transgene vectors for the same purpose; furthermore, in the case of using alphavirus VRPs there is no risk of genome integration (Leitner et al. 2000). An additional benefit of applying alphaviruses for this purpose is their tendency not to affect the general immune responses, a feature which allows the expressed protein to be fully efficient (Rayner et al. 2002). Although the expression levels of the foreign protein do not differ that much between the standard and alphavirus-based vectors, the ability of alphaviruses to induce the synthesis of different cytokines and ultimately apoptotic cell death is where the difference is most prominent. As the idea of such vaccination is to target the immune system against tumours and the destruction of the tumour cells with the help of the virus vector is not a goal by itself, it is interesting to find out that the induced apoptosis still plays an important role in enhancing the efficiency of produced antigens. It is proposed that once the cell is destroyed, various antigen-interact-

ing cells of the immune system are able to recognise remains of the apoptotic cell, and through this, also come in contact with the antigens or cytokines produced by the vector. This interaction results in greatly increased immune response when compared to the production of antigens inside the healthy cells. In addition to the produced molecules, which will be exposed during the process, a significant amount of genomic material originating from the alphavirus vectors is also released, resulting in a general state of alarm in the host and further activating the internal defence mechanisms (Ying et al. 1999, Leitner et al. 2006). Indeed, it was found that the anti-tumour effect of such vaccine candidates depends on the ability of vector to induce apoptosis. When the apoptosis rates of the alphavirus-infected cells were decreased, the resulting immunogenicity against tumours was decreased by a significant rate, clearly indicating the important role of cell death induced by alphavirus vectors (Leitner et al. 2004). Another proposed mechanism for efficient vaccine-like activity is the ability of alphaviruses to directly infect the dendritic cells (major antigen presenting cells). This can lead to a direct antigen presentation without the need of the cell to enter apoptosis (Quetglas et al. 2010).

As already indicated above, not all alphavirus vectors, expressing a sequence of interest, are aimed to function as anti-cancer vaccines. It is also possible to use these vectors in a context where the purpose of the transgene is to limit the growth of tumours; for this, vectors, expressing various inhibitory molecules, have been constructed. The usefulness of such approach is further emphasized by the ability of some alphaviruses to preferentially infect tumour tissue. For example, SIN has been reported naturally to accumulate in tumours in an experimental setup designed to inhibit their growth by systemic injection of the virus (Tseng et al. 2002, Tseng et al. 2004). This selectivity is assumed to be the result of an altered signalling profile present on tumorous cells but can also result from the over-expression of receptors with a high affinity towards SIN on tumour cells and/or from leaky tumour vasculature. At the cases of alphaviruses lacking natural affinity to tumours, this property can be engineered by modifying the E2 envelope glycoprotein and changing the ability of the virus to be recognised by receptors (Iijima et al. 1999, Tseng et al. 2007). Another approach is to modify the vector so it would be able to initiate replication only in tumour cells – a tumour-specific promoter can be included to a DNA/RNA layered vector instead of a CMV or some other similar promoter, making the vector viable only in cells which would be able to recognise the sequence (Guan et al. 2006). The obvious limitation of this approach is that only the virus rescue, but not its subsequent spread, can be controlled using this approach. The fact that tumour cells are highly dependent on blood supply and that growing tumours induce the development of novel blood vessels from the pre-existing ones, in order to continue growing and to receive valuable substances (a process known as angiogenesis), can also be used to develop anti-tumour alphavirus-based vectors. Thus, if a virus construct expresses an antiangiogenic protein, the resulting release of the molecules could inhibit, and possibly block, the progression of tumour tissue. Such experiments have been indeed conducted by the use

of vectors expressing various angiogenesis-inhibiting molecules such as endostatin, a fragment of collagen-18α which has displayed antiangiogenic properties and angiostatin, a fragment of plasminogen. As a result, various degrees of tumour reduction and increase of mouse survivability were observed (Yamanaka et al. 2001, Lee et al. 2006). Fusing the tumour cells together would also be beneficial in reducing the tumour size as the cells, which have been affected by the fusion process, will enter the apoptosis. The fusion process can be induced by expressing the hyperfusogenic version of the gibbon ape leukemia virus envelope glycoprotein or hemaglutinin-neuraminidase and a fusion protein from Newcastle disease virus, expression of which is shown to result in the aggregation of the target cells (Galanis et al. 2001, Zeng et al. 2004). The coding sequences for such proteins have been introduced to the alphavirus vectors but so far most of these approaches have been tested only in cell culture and the number of corresponding *in vivo* studies is still rather low (Quetglas et al. 2010).

It has also been determined that, if delivered intratumourally, alphavirus vectors with no transgene expression, or expressing only a marker protein, are also capable of reducing tumour growth, most probably through the extensive apoptotic effect induced by the vectors on tumour tissues (Vähä-Koskela et al. 2006). The tumour reduction has been observed both in a cell culture environment and in *in vivo* nude mice models. As was to be expected, the ability of the vector to reduce the growth of malignant tissue was somewhat reduced in a living animal (Murphy et al. 2000, Murphy et al. 2001). The efficiency of this approach can be further increased through the co-application of apoptosis-inducing alphavirus vectors and cancer vaccines which are injected directly into tumour (Smyth et al. 2005). The use of full length vectors, capable of inducing the generation of novel virions, however, is not always the best choice as, in some cases, they might cause lethal side effects. To overcome this problem, the application of VRPs containing replicon vectors has been proposed (Atkins et al. 2008). This does, however, result in reduced anti-tumour activity. As an alternative, an interesting approach, also capable of magnifying the apoptotic effect of alphavirus vectors in tumour tissues, has been developed. It is based on the inclusion of a gene, the product of which would be able to induce cell death under certain conditions, to the alphavirus genome, thus creating a double-lethal system. This has been achieved through the use of herpes simplex virus type-1 thymidine kinase, which is not apoptosis-inducing by itself but rather transforms the cell to be susceptible to nucleoside analogues (ganciclovir and similar anti-herpesvirus drugs) which, when modified, are capable of inducing cytotoxicity (Tseng et al. 2006). In addition to the increased efficacy, such vectors also have increased safety, as any cell, infected by a runaway virus, would be killed by the drug, preventing any further amplification and the spread of the virus. Similarly, the number of target cells, available for therapeutic virus, can be narrowed down by introducing miRNA target sites into the virus genome. The selection of targets is based on these miRNAs, under-expressed in targeted tumours but present in high levels in normal cells or, at least in the cells, the

infection of which is associated with viral disease (such as neurons in case of SFV and SIN). Several viruses of this type have been tested; results have shown that while it is rather difficult to keep the virus restricted to cancer cells (Ratnik et al. 2013), it is relatively easy to keep it away from its normal target cells (Ylösmaki et al. 2013).

Fortunately, the application of alphaviruses as anti-tumour agents has not remained only a laboratory perspective. Although numerous candidates have been suggested as perspective drugs for clinical trials, not all of them have made it so far. Nevertheless, there are some promising vaccine-type drugs based on such vectors which have passed the preclinical phase and entered the first phases of clinical trials. The safety and possible anti-drug immune response of virus particles based on VEEV, which expressed carcinoembryonic antigen CEA(6D), also known as AVX701, was evaluated in a small number of advanced-stage cancer patients in a phase I/II trial by Alphavax (Morse et al. 2010). The aim of the treatment was to induce host immune response against the tumour-associated antigens. The results obtained from the trial revealed that no significant harm was caused by the recombinant virus, indicating its possible usefulness in anti-cancer treatment. It was also observed that the drug induced heightened levels of antibodies respective to the expressed foreign antigen. However, as the goal of the test was to determine the presence of possible immune responses in patients that had already developed malignant growths, it is not possible to say to what extent the drug is effective against the emergence of novel tumours and how it might behave when applied to broader test groups. Answers to these questions can possibly be achieved through the ongoing trial expected to end in 2017. Another upcoming phase I test of an alphavirus-based drug AVX901, to be performed by the same company, is targeting breast cancer and the study is designed to observe both the efficiency and safety of this drug. This drug is also based on VEEV which has been modified to be unable to cause active infection, instead it expresses human epidermal growth factor receptor 2, which should boost the immune response towards the cancer cells expressing the same protein. The basic idea behind this vaccine was demonstrated to be viable already in 2005, where it was shown that replicon particles containing this transgene were successful in the inhibition of the growth of the tumour model (Wang et al. 2005). VEEV VRPs, expressing membrane antigens characteristic of prostate tumour cells, have also been applied in combating prostate cancer in a phase I trial. Again, no adverse cytotoxic effects were observed; however there was also no significant improvement of the general condition of the treated patients (Slovin et al. 2013).

Non-viral transfection methods

Gene delivery systems can broadly be classified as viral- and non-viral vectors. Although the virus-based systems often provide high replication rates and are not easily supressed by the host's immune system, there is a downside to their

application – with virus-based delivery systems, there is an increased risk of an viral infection taking place in the target organism and the personnel handling these vectors need to be previously trained in the virus-related biosafety in order to ensure the proper exploitation of the system. Non-viral vectors, on the other hand, can be applied with relative ease as they are not based on a pathogen and possess no risk of spreading uncontrollably into the healthy tissues of the patient or between individuals. This fact has greatly boosted the research in the field of the vectors to create efficient tools for either laboratory or therapeutic use.

Non-viral delivery systems can be further characterised as physical and chemical transfection methods (Kaestner et al. 2015). This classification reflects on the underlying mechanisms which are in use while transporting the genetic material into cells with either of the approaches. While the physical methods entail the use of some type of a physical force, either a physical contact with an injector needle or an electric field, which is applied to the cells, then the chemical approaches can be summarised as methods which require the presence of a transporting molecule which facilitates the creation of a biological interaction between the transfection complex and the host cells, resulting in a successful delivery across the plasma membrane.

One example of physical methods is microinjection, which consists of fixing the cell between microscopic tweezers which hold the cell in place while the injection is carried out (Zhang and Yu 2008). Although this method guarantees the successful delivery of genomic material of interest into the target cell, the process itself can be rather time consuming, especially as the injection takes place one cell at a time, making this method more useful in approaches, where there is no need for the transfection of whole cell populations, such as the *in vitro* fertilisation. Another example in this field is electroporation – an approach, which is widely used both in laboratory settings and even on living subjects. This method entails the creation of an electrical field for a short period of time. Such field creates disruptions in the molecular connections of the cell membrane, allowing the genetic material to be internalised by the cell (Gehl 2003). In contrast to microinjection, this approach allows the transfection of larger cell populations in a short time period but at the cost of cell viability – usually a significant number of cells does not survive the procedure, resulting in the reduction of the number of cells possessed by the end of the experiment. Another approach for electroporation is the transfection of living tissue with the help of electrostatic field. This procedure is often carried out in the outer layers of the organism, such as skin and requires the target area to contain the genetic material and to be placed between the electrodes, which are then used to create an electric pulse (Matsuda and Cepko 2007).

Chemical transfection methods can vary greatly from the more robust approaches such as the calcium phosphate method to the more refined ones such as the cationic liposomes or cell-penetrating peptides. The main problem here is that negatively charged genomic material must be transported across the exterior membrane of the cell, which is composed of phospholipids and also has a

negative charge (Vasconcelos et al. 2013). Therefore the molecule facilitating this process must possess either charge-shielding properties or have a positive charge on its own, to balance out the charge originating from the cargo molecule. Perhaps one of the best known chemical approaches to transfection is the use of cationic liposomes, namely the Lipofectamine series, which has proved itself to be rather reliable all-round transfection reagent, working in nearly every possible *in vitro* scenario possible. Such broad applicability has lead this reagent to become a kind of a positive control or a benchmark in many laboratories, conducting research in the field of transfection, against which to compare the efficiency of other chemical transfection approaches.

Various cationic lipid based reagents are often mixtures of various liposomal components, among which some carry out the interaction with the cargo, while others act as helper lipids and help to further stabilise the nanocomplexes and facilitate the interaction between the transfection complex and the cell. As a result, positively charged liposomal compartments are formed, with a diameter of roughly 100 nm, which interact with the genomic material and allow it to pass through the phospholipid bilayer while the negative charges are being shielded from the cell (Dalby et al. 2004). However, this method is not without its downsides – although the transfection in cell culture is a rather reliable process, the lipid-based reagents are not very suitable for situations where one requires the conduction of transfections with a large amount of genomic material or to carry out multiple transfections on the same cell population, as large doses of the liposomes result in a decrease in cell viability, making many liposome-based reagents, like Lipofectamine 2000, unusable in live animal models (Zhong et al. 2008). The reagent has also been demonstrated to inhibit the capability of the virus to infect the cell and to begin its replication cycle, leading to unreliable data in some virus-related experiments which require the prior transfection of the cell (Pärn et al. 2013).

Cell-penetrating peptides (CPPs)

Cell-penetrating peptides are small molecules, roughly 5–40 amino acids in length, which are capable of translocation across the cell plasma membrane and, in doing so, enabling the internalisation of genetic material. The emergence of CPPs first started in the end on 1980's when it was discovered that the HIV-1 trans-activating transcriptional activator (Tat) protein was capable of entering eukaryotic cells without any additional help (Frankel and Pabo 1988). Further research narrowed the focus down to a single region of the protein, now known as the Tat-peptide (Vives et al. 1997). During the same period, it was discovered that the third helix of Antennapedia protein homeodomain was also capable of translocation across the plasma membrane, leading to the emergence of the penetratin peptide (Joliot et al. 1991, Derossi et al. 1994). Since then, the field of CPPs has received a great amount of attention, resulting in the advent of

numerous new CPPs and delivery approaches both for *in vitro* and *in vivo* settings.

The main advantage of using CPP-based transfection systems over other transfection methods is the relatively low cytotoxicity of such peptides. Unless used at an extremely high concentration, CPPs have displayed almost negligible adverse side effects when used in a cell culture environment. Similar lack of cytotoxicity can also be observed in *in vivo* animal models, however this does not apply to every type of CPP, as some of them might be more harmful than others, depending on their molecular characteristics (Bechara and Sagan 2013). Another positive feature is the suitability of CPPs for the use with different kinds of genomic cargo. The main limiting factor with many chemical transfection approaches is the observance that although the transporter molecules seem to be efficient with some type of cargo, they might be completely inefficient when transporting something else. This does not seem to be an issue with CPPs as they are capable of transporting both smaller molecules, such as siRNAs and SCOs while also being extremely efficient in delivering larger DNA or RNA based constructs, such as plasmid-based expression systems (Pärn et al. 2013, Trabulo et al. 2013).

CPPs can be classified on the basis of many different parameters: one of these is the classification according to the origin of the CPP. The first group is known as natural CPPs. These peptides are derived directly from a natural source, such as a parent protein, and do not contain any modifications in their amino acid sequence. Examples of this group are the Tat and penetratin peptides (Vives et al. 1997, Derossi et al. 1998). The second group, called chimeric peptides, is based on the combination of sequence parts originating from different parent molecules. This approach allows the combination of positive properties from mixed backgrounds and makes it possible to discard such parts of the sequence, which might not play a very important role in the transfection process. An example of this group is the peptide transportan, in which the researchers have combined the peptide sequences from the neuropeptide galanin and wasp venom mastorpan in order to achieve a potent transfection vector (Pooga et al. 1998). The third group is known as synthetic peptides. This group consists of peptides which possess significant sequence modifications compared to their peptides of origin or are completely synthetic, without the use of any molecule as a template, often using repeats of similar amino acids (Wang et al. 2015).

Another approach to CPP classification is to differentiate them according to the type of the interaction taking place between the peptide and its cargo. Such interactions can be classified into two distinct subgroups: covalent and non-covalent. The covalent approach is used rather widely and while it ensures that each CPP molecule transports an additional cargo molecule across the plasma membrane, the use of such interactions results in a rather significant increase in the workload related to the transfection process as the cargo molecule must be conjugated to the peptide during the synthesis and for every different type of cargo, a whole new molecule must be created. Non-covalent strategy, on the other hand, allows for an increased flexibility as the formation of complexes

takes place once the peptide and transported genetic material have been mixed together and incubated for a short period of time. In addition to being suitable for various cargo molecules without additional synthesis, this approach allows to adjust the ratio of peptide and cargo molecules, to ensure optimal transfection parameters in a specific setting (Deshayes et al. 2008, Vasconcelos et al. 2013).

One of the classifications takes the molecular characteristics of the peptides into account. Chemically, a distinction can be made between various CPP-s which are classified as either cationic, hydrophobic or amphipathic peptides (Vasconcelos et al. 2013). The cationic peptides can be identified with ease as they feature a number of positively charged amino acids in their sequence, such as lysine, arginine and histidine, resulting in a positive net charge of the peptide, hydrophobic and amphipathic CPP-s, on the other hand, are recognised by the presence of hydrophobic amino acids and a low net charge or by the presence of lipophilic and hydrophilic blocks respectively (Madani et al. 2011).

Internalisation mechanisms of CPPs

The pathways which are utilised by CPPs in order to cross the plasma membrane of a eukaryotic cell are still subject to a considerable debate and controversy. Even if some reports try to establish a commonly used pathway for CPP entry, there is always a contradicting paper present, further contributing to the uncertainty. Due to these issues, a definite final viewpoint cannot be established in the work at hand, but in order to shed some light into this issue, a few of the viewpoints, which have gathered significant support from the research community, will hereby be explained (Figure 3).

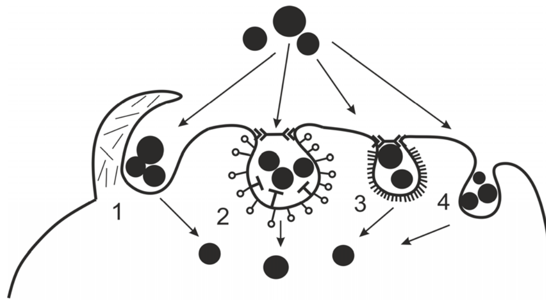


Figure 3. The demonstrated endosomal internalisation routes for the peptide nanocomplexes. Four proposed routes of entry among the numerous strategies suggested for the entry of peptide transfection complexes.

1. Macropinocytosis
2. Clathrin-dependent endocytosis
3. Caveolae-mediated endocytosis
4. Caveolae/clathrin-independent endocytosis

In some cases the internalisation of CPPs is regarded to take place via the direct translocation across the cell membrane. This approach is considered to be more "physical" in nature as the translocation occurs using the energy-independent routes. Some groups have suggested that this pathway does not affect cell membrane in any way and the peptide molecule together with its cargo flips across the membrane from the extracellular environment into the cytoplasm of the cell. Other papers claim that the peptides create miniature disruptions in the lipid bilayer which allow the passing of extracellular molecules such as the peptide and the genomic material, resulting in a successfully transfected cell. This final viewpoint is supported by numerous cytotoxicity experiments which determine the viability of cell populations through membrane leakage, demonstrating that, in many cases, membrane leakage can be observed during the earlier points of measurement and cannot be observed later during the experiments (Jobin et al. 2013, Vasconcelos et al. 2014).

A more common suggestion claims that the entry of CPPs is an energy-dependent process instead. Supporters of this concept believe that the main route which leads the peptide inside the cell takes place through the endocytosis-related pathways. Most authors supporting this viewpoint agree that the interaction with cell membrane and its receptors is crucial for the internalisation but their opinions differ on the specific endocytosis route utilised by the CPP. Such pathways include, but are not limited to: clathrin-mediated endocytosis, macropinocytosis, and caveolae/lipid-raft-mediated endocytosis (Figure 3). Once inside the cell via the endocytosis uptake route, the complexes must find a strategy to escape from the endosomes in order to be able to release their cargo into the cytoplasm. The escape is usually facilitated by the acidic conditions present inside the endosomes, however other strategies, such as the addition of some special molecules to the peptide sequence in order to facilitate endosomal exit or membrane disruption, have been suggested (Koren and Torchilin 2012, Wang et al. 2014).

Finally there are some, who believe that the internalisation of the peptide molecule can be a result of both of the abovementioned strategies. The defining viewpoint in this case is that the specific route of entry is dependent on the type of the peptide itself and the cargo it is transporting. But once again conflicting opinions can be found – while some authors claim that when transporting a smaller cargo, CPPs enter the cell through direct translocation and the complex formation with a larger payload forces the peptide to use endocytotic pathways, there are authors who believe the exact opposite, claiming that endocytosis facilitates the entry in the case of smaller complexes and larger ones pass directly through the cell wall (Jiao et al. 2009, Vasconcelos et al. 2013).

Regardless of the specific strategy used to cross the plasma membrane, there is a final step which is common for all the peptides – the release of the cargo molecule. In many cases the complexes just dissociate when exposed to the intracellular environment, this is prevalent when using non-covalent binding strategy but when using a covalent bond, the molecule often stays in one piece as there is no differentiation between the peptide motif and its cargo. In some

cases, the peptide can contain a linker sequence which can be cleaved by various enzymes (such as the MM2 linker described in the following chapter) or some features of the molecule can be attached through a disulfide bridge which can be cleaved upon entering the cell (Mäger et al. 2012).

While the characterisation of the overall transfection efficiency might be sufficient for the *in vitro* cell culture systems, using an *in vivo* animal model requires much more thorough development and analysis of the peptide. The main difference between the two systems is that while the cells on a cell culture dish all originate from the same source, and are genetically identical, then a living organism is essentially a collection of different interconnected types of tissues that have varying molecular characteristics and also display an organ-specific affinity for the uptake of transfection complexes. *In vivo* biodistribution can vary greatly between the peptides originating from a different background, also there have been different reports on the preferred organs for the CPP class as a whole, but peptides generally tend to accumulate in the metabolism-related organs such as liver, lungs, kidney and spleen, which can be characterised as organs, which are either functioning to clean up the body from various foreign substances or possess high levels of blood supply, leading to an increased chance of interaction with the peptide (Hayashi et al. 2011, Kim et al. 2015). There are also some organs which are not as easy to reach when compared to the previously mentioned ones, such as the brain and heart. The delivery to these organs has been unsuccessful or with a low efficiency for a long time however, during the last few years there has been an increasing number of reports describing successful delivery into these tissue types (Bechara and Sagan 2013). Rat cardiomyocytes (cardiac muscles) have been successfully targeted by the cell-penetrating peptides, resulting in a future perspective for heart failure treatment and the exon-skipping therapy required for the treatment of Duchenne muscular dystrophy (Betts et al. 2012, Chien et al. 2014, Lehto et al. 2014). Delivery to brain tissue is considered a demanding task as the peptide complexes must first cross the blood-brain barrier. Although there are examples of intranasal introduction or direct injection of the delivery systems, allowing to bypass the barrier (Kanazawa et al. 2011, Kizil et al. 2015), there have also been reports of peptide complexes capable of enhancing the delivery of cargo molecules across the blood-brain barrier (Liu et al. 2014, Yao et al. 2015).

Tissue-specific delivery of CPPs

The lack of tissue-specificity and the common tendency of CPPs to accumulate either in the major organs which require constant and high levels of blood supply, such as the lung or liver, or in the tissues located in the region of the place of injection, has made the use of CPPs somewhat problematic when trying to apply them for the treatment of specific tissue-specific genetic diseases. To overcome this problem, modifications have been introduced into the structures and functionalities of CPPs in an attempt to achieve higher tissue-selectivity. In

this section a short overview of such approaches will be given in order to demonstrate the diversity of this field.

One of the possibilities for achieving high transfection rates into tumour tissues would be to use a targeting sequence. For this, a specific amino acid motif is integrated into the sequence of the CPPs, often located in the terminus, as such location has minimal chances of disturbing the secondary structure of the peptide, which often plays an important part in ensuring the successful entry of the peptide into a cell. Such sequences are usually recognised by the specific molecules overexpressed on the surface of the tumour cells, such as the epidermal growth factor receptor, which is a common tumour-associated target for many strategies involving the reduction of tumour growth. It has been even suggested that the inhibition of this gene will result in positive outcomes in relation to tumour progression. A CPP has been fused with an EGFR-interacting domain derived from vaccinia virus growth factor, enabling significant increase in uptake levels of the fusion peptide when compared to the unmodified peptide (Nguyen et al. 2015). Another example of a targeting sequence is the addition of a CREKA sequence to a peptide. CREKA was identified by the screening of peptide libraries in order to find sequences capable of binding to tumour receptors (Zanuy et al. 2008, Karmali et al. 2009). Since then, it has been applied in combination with a peptide pVEC, resulting in improvement in cargo translocation rates into tumour cells (Regberg et al. 2012). Not all peptides require the addition of the specific targeting region, some of them already contain it in their sequence. Such CPPs might reveal their affinity towards tumour tissues in general or some certain types of tumours once a screening process has been applied, allowing to filter out peptides binding to some specific extracellular marker present in tumour tissues (Higa et al. 2015). Another interesting finding is a peptide with its sequence derived from the oligomerisation domain of Hsp70, a membrane-bound protein which is expressed on the cell surface of tumour cells, which enables the targeting of the tumour cells expressing Hsp70 in their membrane. The potential of this system lies in the fact that this protein is expressed only on the surface of tumour cells, while their healthy counterparts possess no such binding site (Gehrmann et al. 2014). Although the ability of this sequence to facilitate binding to a nucleic acid cargo and to ensure its internalisation to cell has not been demonstrated, the sequence could prove to be, not unlike the CREKA sequence, a potential component of a tumour-targeting chimeric delivery peptide.

An approach somewhat similar to the addition of a targeting sequence is the attachment of a targeting molecule to the formed transfection complex to ensure the correct delivery of the peptide and its cargo. Such molecules must display similar characteristics to other targeting mechanisms and be able to conduct specific binding to the unique cell surface profile of the tumours. One elimination strategy of tumours is to target the cancer-associated fibroplasts, which provide a supporting scaffold to promote tumour growth and development (Stylianopoulos et al. 2012). For this purpose, mouse monoclonal antibody molecules capable of binding to human fibroblast activation protein-α have

been bound to the surface of the delivery complexes through electrostatic binding, resulting in the successful interactions with the tissue and in the reduction of tumour sizes in mice through the anti-tumour drug, doxorubicin, included in the complex as a cargo molecule (Ji et al. 2015). A similar route was taken in another experiment, where the peptide-toxin complex intended for the neutralisation of tumour cells was targeted to the specific tissue by mixing an antibody for the anti-carcinoembryonic antigen and heparin conjugate with the transfection complexes, also resulting in electrostatic interactions between the two components. This allowed to achieve specific binding to the carcinoembryonic antigen over-expressing tumour cells in a mouse model system, resulting in the significant inhibition of tumour growth without a noticeable systemic toxicity (Shin et al. 2014).

An efficient strategy to keep the CPPs from targeting unwanted tissue types is the construction of such peptides, which would be activated at the location of interest through an external stimulus. Such activation requires the peptide to be in conjugation with molecules which are capable of inhibiting the interactions between the peptide complex and the target cell. These molecules are receptive to a specific signal, which can be in the form of pH, temperature or light, upon receiving which they will detach themselves from the peptide, allowing it to carry out its internalisation process as intended. Such approach has been utilised by Yang et al, who used the triggered caged peptide as a guidance system for their lipid-based delivery mechanism (Yang et al. 2015). Their system involved activating the peptide using a near-infrared light, which they considered to be able to ensure site-specific activation, which could be harder to accomplish through the other approaches aiming to change the tumour microenvironment, and to penetrate deeper compared with other types of light impulses, in addition to possessing no considerable side effects, which might be present for UV activation. Another approach for site-specific delivery through activation is to use a strategy, which would allow the interaction of inactive transfection complexes with the tumour-specific components and, through such interactions, to restore the full activity of the peptide. A system consisting of a CPP and an inhibitory peptide has been devised, using an MMP (matrix metalloprotease)-specific substrate to bind the two sequences together. As growing tumours require the remodelling of the extracellular matrix, achieved through the product of MMP, this gene is upregulated in tumour cells, allowing its product to interact with the substrate introduced in the peptide complex and to cleave the inhibitory peptide attached to the delivery system (Olson et al. 2009). Lately, the use of such cleavage system has received growing attention, having been applied on multiple occasions (Gao et al. 2014, Mei et al. 2014). A similar tumour-dependent de-shielding has also been utilised in our work, which involves the shielding of the peptide complex through the use of polyethylene glycol (PEG) particles (Veiman et al. 2015). Although the *in vivo* results are rather promising, there are some minor concerns about the tumour-specificity of this transportation system, as there have been reports of a premature activation taking place in the vasculature system instead of the tumour tissue (van Duijnhoven et al. 2011, van

Duijnhoven et al. 2015). Regardless of this unwanted de-shielding taking place outside the tumour tissue, the practical evidence indicates the ability of these particles to be internalised by tumours to a great extent and therefore assures the potential of this method. However, further specificity might be achieved through the selection of a similar, but more tumour-specific enzyme which would be able to ensure the cleavage of the shield when located in the correct tissue (van Duijnhoven et al. 2015).

RESULTS AND DISCUSSION

Aims of the present study

The thesis at hand presents three different but yet interconnected studies with a general goal of developing the tools for efficient approach for combating tumours. The specific goals related to each of the separate studies were the following:

(I) To develop an original inducible alphavirus-based DNA/RNA layered vector system where the rescue of infectious virus genomes could be controlled by the combination of introduction of aberrantly spliced introns and application of specific molecules, such as SCOs, capable of correcting the introduced defects

(II) To test the capabilities of a CPP-based transfection system to deliver larger nucleic acid constructs, such as the alphavirus DNA/RNA layered vectors and infectious *in vitro* transcripts into eukaryotic cells. To estimate the efficiency of this delivery and to compare it with the delivery achieved by the use of cationic lipid based reagents. To observe the potential adverse effects which might inhibit the replication of rescued viruses. Based on the obtained data to estimate the applicability of CPP based transfection regents for the transfection of cells with alphavirus vectors and for the studies of alphavirus biology;

(III) To develop a peptide-based transfection system which would be able to facilitate preferential delivery into tumour cells through the application of tumour-specific de-shielding of the transport molecules

A method for controlling the rescue of infectious full-length RNAs from DNA/RNA layered alphavirus-based expression vectors (I)

The efficiency of virus-based approaches as tools for the treatment and prevention of various diseases, such as tumours, has previously been extensively demonstrated (Barber 2004, Atkins et al. 2008, Harrington et al. 2010, Kanai et al. 2010, Rommelaere et al. 2010, Toth et al. 2010). However, such application of viruses does not come without its cost as the vectors contain an inherent risk for damaging their host cells and spreading the infection (Ehrengruber and Lundström 2007). A possibility to overcome the occurrence of a widespread unwanted infection is to use truncated versions of the viruses, such as the alphavirus-based replicon vectors (Liljeström and Garoff 1991). Such vectors are incapable of virion production but are still able to facilitate high levels of transgene expression and replication inside the cell and to terminate it by inducing apoptotic death (Urban et al. 2008). Though such vectors are safe and useful for applications where apoptosis is a desired effect (such as the termination of the diseased tissue and/or to produce immune response-inducing molecules),

they may be not efficient enough to combat aggressive (and metastatic) forms of the cancer. Another shortcoming of these vectors is that due to their non-propagative nature, they must be produced and applied in large amounts and the production of VRPs, RNAs or even GMP quality plasmid DNA is rather expensive (Steel et al. 2011). Finally, such vectors have limited specificity – either natural specificity intrinsic for the original virus or artificial specificity created by researchers. Given the difficulties to regulate the replication of RNA viruses, it may be not sufficient to ensure the replication of virus genomes in cancer cells and to leave the other types of cells unharmed (Piver et al. 2005).

Why are RNA viruses difficult to regulate? The problem is that the huge arsenal of regulatory elements, known for scientists, is almost exclusively designed (or found in nature) for the regulation of gene expression starting from the transcription of mRNA from a DNA template. Alphaviruses do not use this process at all and hence all promoters, transcription factors, enhancers, terminators, etc., have absolutely no meaning for them (Strauss and Strauss 1994). Only the elements functioning on the level of RNA can be used directly in their genomes and there are not so many of such elements. MiRNAs and their targets are relatively new thing – when this study was first designed back in 2009, we did not know if miRNAs could be used (in fact, our lab initiated a specific study to investigate this topic, the results are published by Ratnik (Ratnik et al. 2013)) in alphavirus vectors. There are riboswitches and aptamers – however the previous studies in our lab had already shown that these elements are not effective in an alphavirus context. There are specific RNA secondary structures which can be used to supress the gene expression, but it is not trivial (though not likely completely impossible) to make them work in some specific cells and, at the same time, not in the other cell types. In short, the tools for generating cell specificity directly at the RNA level seemed to be (with exception of miRNAs) rather inadequate.

To overcome these difficulties, a novel type of a control mechanism was required. Ideally, is should result in the virus remaining unable to successfully replicate unless it is activated through a specific external stimulus. As indicated above it is hard to achieve on the level of virus itself as the suitable tools do not exist (now there is evidence that miRNA targeting can be used but not without limitations). Thus, it leaves the option to regulate the release of the virus from a layered DNA/RNA vector. It is not the same thing – once released, the virus cannot be controlled by the engineered mechanisms – but at least this approach allows to achieve virus rescue in the specific, targeted sites (for anti-cancer therapy that would be the cancer cell).

One approach for the regulation of gene expression in biotechnology is to use aberrant introns, which are not spliced according to the correct splicing pattern under the normal conditions (Sazani and Kole 2003). While the non-defective introns are used as regulatory mechanisms which may inhibit or enhance the expression of genes, depending on the specific situation, then the introns with an aberrant splice site block the gene expression as the splicing activity is diverted away from the original location and the resulting mRNA

encodes for a protein which will be distorted and will most likely contain incorrect amino acid sequence after the splice site due to a shift in the translational reading frame (Ladomery 2013). The introduction of introns directly to a positive-stand RNA virus, such as SFV, makes no sense: these viruses are strictly cytoplasmic. The elements can, however, be inserted into the DNA/RNA layered vectors (Johansen 1996, Yang et al. 1998). One of these vectors, the pCMV-SFV4, previously constructed by our workgroup, which already contains an intron (normal intron from the rabbit beta-globin gene) in the region encoding for capsid protein (Ülper et al. 2008), was therefore taken for the bases of the new vector construction. Suitable aberrantly spliced intron sequences used for this purpose originated from humans suffering from β-thalassemia, a disorder related to haemoglobin production. In these people, the expression of β-globin is altered by the presence of various mutations, two of which, the point mutations located at positions 654 and 705 of the second intron of the gene, were used as examples of naturally occurring splice-altering mutations (Busslinger et al. 1981, Finotti et al. 2015). Third defective intron, used in the study, represented an artificial construct encompassing both of the mutated sites; the idea was to gain more efficient suppression of correct splicing. In order to allow the release of the replication competent RNA from the vectors to resume, a strategy which would allow to deactivate the negative effect of the defective introns must be present. It is possible to revert effect on aberrantly spliced introns through the intracellular delivery of splice-correcting oligonucleotides (SCOs) which are capable of binding to the mutated site in the intron sequence and, in doing so, they are able to shield the location from being spliced, resulting in the production of correct mRNA and, consequently, replication-competent virus vector (Zeng et al. 1999, El-Beshlawy et al. 2008).

The approach to use defective introns to affect the replicative capabilities of viruses was first shown to be efficient on adenovirus-based vector systems (Figure 2, paper I). This was an expected result, however, the demonstration of the application and potency of such mechanism in relation to alphavirus constructs was more challenging. Namely, as only the rescue of the virus is controlled, the control must be as efficient as possible – a single successful rescue event can lead to a spread of replicating alphavirus vector in cell culture. To exclude the possibility of the spread of infection, DNA/RNA layered replicon vectors were first constructed and analysed. In order to fully evaluate the potential of the selected and constructed introns, a set of alphavirus replicon vectors was constructed, containing either a wild-type intron, intron 654, intron 705 or the artificially constructed double-mutant intron 654+705. As the specific location of these introns is also of utmost importance, they were to be placed in a region which plays an important role in the replication process of the virus, otherwise it would have been impossible to observe the effect they play on the rescue of the replicon or the virus. Keeping this in mind, two locations were selected as suitable targets for the intron placement, both located in the non-structural protein encoding region. Within this region, it does not matter where exactly the introns are placed – any mistake leading to the disruption of the

reading frame is fatal. Furthermore, the introns can also be inserted into the sequences of reporter genes inserted in the frame of non-structural proteins to achieve the same effect. The latter property allowed us to use firefly luciferase reporter gene, located in the nsP3 region of the base genome, as one site for intron insertion, while an additional site, inside the nsP4 region, was used as the second position for intron insertion (Figure. 1, Table 1, paper I). To note, this selection had more to do with convenience because, as indicted above, it does not matter where exactly these elements are located. The resulting vector system would allow to analyse two key issues which are of interest in relation to the designed system. Firstly, the insertions would allow to assess the overall functionality of the defective introns as aberrant splicing in either or both of the locations would result in the inability of the virus systems to produce functional nsP4, which is required for the genome replication. To measure if the construct is capable of replication, the EGFP sequence was placed under the subgenomic promoter. Thus, the expression of EGFP marker is strictly dependent on the replication of vector. The problem with this approach was, however, that it is not very well quantifiable: it will produce either a yes (virus replicates and EGFP is produced) or no (replication does not occur and EGFP is not produced) as the answer. As, in some cases, this is a limitation, a second method, which includes the more specific determination of the intron mediated suppression and SCO mediated activation efficiency, was used. It takes advantage of the presence of Luc-marker. In constructs, where the defective intron is placed only in the nsP4 region, Luc is expressed both from the replicating and non-replicating transcripts. In contrast, if the intron is located in the Luc sequence itself, only the transcripts, which are correctly spliced, express luciferase and can become replicating replicon-vector RNAs. To note, the situation is actually somewhat more complicated: it is unlikely that the incorrect splicing or its correction by SCOs can occur at 100% efficiency in any cell. It is more likely that each cell has pool of correct and incorrect transcripts and the presence of SCOs shifts their balance in the favour of correct ones. Given the ability of alphavirus replicase to replicate the templates in *trans* (replicase made on the basis of correct RNA can bind and replicate the incorrect ones as well), it can blur the results; therefore both activities (the production of EGFP and Luc activity) were always monitored.

At first, the efficiencies of the introns were evaluated separately at both positions. Although the effect obtained from different introns varied, it was clear that a single intron in either location was not sufficient to inhibit the replication process to a great extent; even the strongest (double-mutant) intron allowed a quite efficient replicon rescue. This contrasted to the data obtained for the adenovirus vector and most likely reflected the fact that production of very small amounts (possibly a single copy per cell) of correctly spliced RNA was sufficient to initiate the replication. This data led to the introduction of two introns in different positions of the DNA/RNA layered vector. This resulted in drastic (nearly tenfold) improvement in the reduction of infectivity levels when compared to the approach utilising a single defective intron. This indicated that,

in the large majority of transfected cells, no RNA transcripts, capable of initiating replicase synthesis and entering into the replication cycle, were produced.

Another approach, enabling to determine the efficiency of the intron-based inhibition strategy, is to look into the reduction of the capability of the vectors to spread the infection across the cells. This is even more demanding as infectious RNA rescue must be supressed not only in an individual cell but in all transfected cells of the transfected culture – any rescued virus will sooner or later infect all the cells. As this is something which is not obtainable through the previously used replicon vectors, a set of full-length vectors, containing introns in the same positions as their replicon counterparts, had to be constructed. To quantify the results, an infectious center assay, which enables the determination of the number of cells where rescue of infectious virus occurs (rescued viruses are then capable of infecting cells adjacent to them and form plaques), was performed. As expected, the results obtained were consistent with those predicted based on the observations made from the replicon experiment. Again, the introduction of a single intron did not influence the emergence of virions to a great extent, however the presence of two introns produced a significant inhibition. The inhibition of the rescue was greatest for the vector containing two double-defective introns; the infectivity of such construct was close to background in HeLa cells (not reliably detected due small values (Figure 4, paper I)) and reduced nearly 50-fold in more sensitive BHK-21 cells. Similar reduction was also observed for Luc expression, and as this is much more convenient to quantify (compared with the performance of ICA), subsequent analysis were based on use of this approach. It should be mentioned, however, that complete inhibition (at least in BHK-21 cells) was still not achieved. It could be speculated that the insertion of additional aberrantly spliced introns would have been needed to achieve this goal. Nevertheless, based on the obtained data, the method for controlling the infectious replicon/virus rescue (exploitation of two aberrantly spliced introns, both of which preferably containing two aberrantly spliced sites) was quite efficient and provides the proof-of-principle of applicability of this approach.

Thus far, however, we have provided an elegant method to inactivate DNA/RNA layered vector. In order for this system to have any practical value for research or in developing medical tools, a method, allowing vectors to regain their original infectivity and replication capabilities, is required. This means that such vectors must respond positively to the SCOs introduced to the system. Such SCOs have been designed to reverse the effect of the defective introns by blocking either the 654 or the 705 aberrant splicing site and forcing the use of correct one at the end of intron. It was soon determined that the two SCOs (SCO705 and SCO654) were not equal in their functionality; the SCO654 showed significantly greater activation levels and was, to the extent capable also to rescue the constructs containing intron with mutation in position 705. This effect, most probably, originated from the splice-enhancing activity of SCO654 – a property which has not previously been noted for this specific molecule, but has been known to be present in a small number of other SCOs

(Resina et al. 2007, Svasti et al. 2009) In a similar fashion to the two different splice-switching mutations, the two SCOs also acted in synergy, resulting in increased efficiency when both of them were applied. The ability of the nucleotide sequences to rescue the release of replication-competent RNA from alphavirus constructs containing aberrantly spliced introns was observed for replicon and full length vectors alike, demonstrating the broad applicability of this system. As previously stated, the inhibition levels obtained from the combination of different introns varied between the different types of vectors, allowing for speculation in regards to the possible variations in the activation efficiencies for these constructs. To our relief, the introduction of the mixture containing both SCOs returned the infectivity all of the intron-containing vectors to levels comparable to vectors without a splice-deficient intron. The effect was especially prominent for the vector containing two double defective introns as the activation levels reached more than one hundred fold, proving that this system is viable regardless of the levels of previous inhibition. Though it was not experimentally tested, it could be speculated that the presence of larger number of aberrantly spliced introns could also be compensated by the use of mixture of corresponding SCOs.

Judging by the results, it can be concluded that the construction of the controllable release systems based on the DNA/RNA layered vectors of alphaviruses was achieved and it leads the way to various potential applications where a selective expression is required. However the system is not without its setbacks as it only provides a strategy for influencing the first step in the introduction of a layered vector, but once activated, the further control will be lost. Nevertheless, this method provides an excellent approach for applications where the beginning of the infection must be contained to a specific type of tissue for the replication competent vectors and when using replicon vectors, the infection will be localised only to the SCO-containing cells. Also, as this general system has been successfully transferred from adenoviruses to alphaviruses, it is only logical that the specific and highly efficient activation system described here can also be applied to other types of viruses, especially to those based on DNA, allowing an increase in the control period which is not obtainable for viruses with a RNA genome.

Another problem which must be solved, for this method to be truly efficient, is the method of delivery. First of all, the intron-containing vectors are in the form of DNA-RNA layered vectors (in practical terms they are plasmids), which indicates that the constructs are not capable of entering the cells through the pathways normally used by the virus. Instead, they must rely on the external factors and be transported with the help of an efficient transfection method. Same problem can be applied to the delivery of SCOs, which must also be able to reach the same cells occupied by the vector. While a much easier task to accomplish in a cell culture environment, the real problem arises in an *in vivo* model system, where the delivery needs to be much more efficient and much more specific. The main problem encountered here is that the method of delivery must not have any harmful effects on the cells – at least not any effects

which would prevent virus rescue from the vector and its replication. Additional important aspect to consider is the site-specific delivery of SCOs as their role is to activate the constructs. While the correct delivery of vectors themselves is important as well, it does not, at least, pose a significant threat as, in the absence of SCOs, the virus is not rescued and even if this occurs at very low level it is unlikely sufficient enough to start an infection (though debated, the infectious dose of human-infecting alphavirus in around 100 plaque forming units if delivered by mosquito and higher than that if delivered by injection). Though this approach increases the safety of the system, it also emphasizes that the focused delivery of SCOs is very important. First, the activation of the virus in the target tissue must be efficient. Second, the activation of the vectors outside the target tissue would abolish or reduce the usefulness of this system: incorrect rescue can activate unwanted immune response. Finally, if SCOs and alphavirus vectors never meet at the correct location, no activation can take place.

With the goal of finding a suitable transfection method for the purpose of practical application of the controllable vectors, study II was undertaken. Its aim was to determine if a widely published class of transfection molecules, CPPs, would be able to facilitate the entry of virus genomes and smaller molecules (siRNAs, comparable with size of SCOs) into eukaryotic cells without harming the viability of target cells and negatively affecting infection capabilities of the virus constructs and infectious virions.

Studies on the properties and virus interactions of the CPP-based transfection reagent PepFect6 (II)

Due to the inability of DNA and RNA to efficiently cross the cell plasma membrane, a method of transfection must be used for a successful delivery of nucleic acids into cells. Keeping the practical application of therapeutic drug development and *in vivo* applications in mind, most of the common transfection approaches become obsolete as they are either too damaging for the recipient cell and exert general toxicity on the organism or do not allow to achieve high transfection rates in a reasonable time scale to be efficient enough for the treatment of medical conditions. This is the reason why, in most cases, physical transfection methods have been filtered out for animal studies as well as many chemical approaches, such as the widely used Lipofectamine series, which is based on cationic liposomes and is commercially available throughout the world. Although efficient and rather consistent in its transfection profile, it has displayed inherent toxicity, as a result of which the viability of the cell is decreased in a cell culture setting. Due to these effects, multiple transfections on the same cell population cannot be conducted by the same lipid-based approach, not to mention any possible application in a live animal model system, where the resulting adverse effects usually result in fatal consequences.

In an attempt to find a solution to this problem, the attention has been shifted towards another class of chemical reagents which have been previously demon-

strated to be without any cytotoxic side effects when used for transfection either in the cell culture or for molecule delivery in an *in vivo* setting. These reagents are CPPs, short peptide sequences which are efficient in nearly every possible setting and which have demonstrated such proficiency in delivering its cargo without causing harm, that many of the therapeutic systems based on this approach have entered clinical trials. Another positive aspect of these peptides is that many of the impressive traits related to them can be extrapolated to the whole molecule group: many characteristics observed on one occasion can usually be seen in other CPPs designed in a similar fashion, and in many cases, in all of the peptides with cargo delivery capability. Based on this knowledge, a CPP, PepFect6, which is an improvement derived from a chimeric transfection system, transportan 10 (Andaloussi et al. 2011), was selected in order to assess its delivery capabilities and any possible side effects related to the use of this substance.

As the PepFect6 was developed about the time when the work, described in paper II, was initiated, not much was known about its specific properties. Therefore it was essential to determine the exact transfection parameters which could be used for the delivery of virus-based vector plasmids and *in vitro* generated RNA transcripts. Although the peptide had been used before on multiple occasions, it had always been applied (and has still been applied in every other publication to date) for the delivery of smaller nucleic acid based cargo molecules: siRNAs and, on some occasions, smaller reporter plasmids (unpublished data). However, the length of the alphavirus genomes (ca 12 kb RNA) and corresponding DNA/RNA layered vectors (roughly 16 kbp for a full-length DNA/RNA layered vector containing an integrated sequence for expression of fluorescent marker) are significantly longer than the length of siRNAs or even that of the simple reporter plasmids. This raised the question if the peptide would still be able to efficiently deliver such large cargo molecules into cell. Indeed, other reagents meant for siRNA delivery (such as the commercial Lipofectamine RNAiMax reagent), do not generally display such universal adaptive properties. What is even more interesting is that, up to this date, it is hard to find publications on the topic of the delivery of larger plasmid sequences, especially those which are related to viruses, with the help of CPPs, making the insights gained from this specific study rather unique.

At first, it was important to determine the exact amount of the reagent to be used for the transfection and the time required for the successful delivery of cargo molecules. The results obtained from this step formed a basis for the conduction of the following experiments, for which the selected optimal conditions could then be applied. The realisation that the amount of PepFect6, required for the successful transfection, was nearly the same in all cases (charge ratio of 1:5, with one notable exception of 1:3 for RNA transfection into BHK-21 cells, Figure 2, paper II), was quite interesting, meaning that the target cell line does not influence the uptake to a great extent. A far more surprising result was obtained in regards to the incubation period required for a successful delivery. It was found that even upon the immediate removal of the media containing the trans-

fection mixture, still a large percentage of the total population of the cells was transfected and the transfection efficiency peaked as early as at 20 minutes of incubation (Figure 3, paper II). These results leave some room for speculation: would the peak levels have appeared sooner if there had been intermediate time points included in the experimental setup, perhaps it would be enough to incubate the cells only for 5 or 10 minutes, which would noticeably reduce the time required for any experimental setup. An additional concern is that although the cells were transfected at the maximum level possible, it is highly unlikely that the complexes were able to enter the cell during such a short period. This is especially relevant for the first experimental time point, therefore it could be deducted that one of the properties of peptide-based transfection complexes is their ability to attach themselves the outer membrane of the cell in such a fashion that they will not be removed by the external stimuli which are accompanied with the changing of the culture media.

Previous observations had demonstrated the presence of a certain delay in the emergence of the florescent signal when applying the two reagents under the similar conditions during the transfection of DNA/RNA layered vectors but not for the RNA transcripts. This occurrence was verified through the measurement of the increase in the luciferase levels in time; this experiment confirmed that there was nearly 2-hour delay in marker protein accumulation in the case of using the PepFect6 reagent for the delivery of DNA/RNA layered vector compared to delivery of RNA transcripts (Figure 4, paper II). Further insight about the intracellular fate of the delivered plasmids was gained through the use of actinomycin D, an inhibitor supressing synthesis of infectious RNA transcripts from a plasmid but not the replication of the already released viral (vector) RNA genomes. A corresponding experiment allowed to confirm the previously raised hypothesis that the difference in the accumulation of the marker protein was the result of a delay in the release of the cargo molecule delivered by the peptide-based transfection reagent (Figure 4, paper II). Thus, inside the cell, DNA molecules are released and transported into the nucleus at a relatively slow manner. This results in a delay of approximately 1.5 hours, during which time the replicating vector RNAs appear in cytoplasm and their replication becomes insensitive to the actinomycin D treatment. Although this delay is apparently not harmful for the virus life cycle, it is certainly something that should be taken into account when designing experimental or treatment procedures containing DNA-based sequences; among other things this allows to avoid false interpretation of the data related to the ability of different (RNA versus DNA) vectors to enter into their productive phase.

For a system likely to be successfully applied in a more practical setting, it is vital to ensure that it would not cause adverse effects on the transfected cells. Although the class of CPPs is regarded to be safe for a number of applications, there was not any information available about the specific context in which these reagents were used in this study. The problem is that alphavirus infection is, by definition, damaging for the cells and virus-induced damage may be affected (increased, decreased or modified) by the effects caused by transfection

reagent. Typically, when a reagent starts to affect the integrity of the transfected cell, some specific visual changes can be observed which indicate a deviation of cells from their normal conditions. However, it is not always enough to rely only on the visual signs of cytotoxicity as they might not always be recognised by the observing party and, if virus vectors are involved, the cytotoxic effects of reagent might overlap with the changes induced by the replication of the virus. In addition, some cytotoxic effects might not be visually detectable. Thus, to ensure the safety of the peptide-based delivery system, the effect of the transfection complexes on the cell viability had to be measured. The corresponding experiment resulted in a more specific determination of cellular viability under the optimal and near-optimal transfection conditions; to eliminate the effects caused by virus infection, constructs, which are unable to release replication competent viral vectors, were used. Such experiments demonstrated that, under the applied conditions, there was no inhibitory effect of transfection reagent/procedure on the viability of the cell populations and neither did the transfection affect the replicative capabilities of the cells (Figure 2, paper II).

The final issue which needed clarification was to determine if and to what extent does the transfection reagent affect the properties of the virus, its replication and capability to infect cells. If a virus-based controllable vector described in the study I were to be used for medical applications, such as the inhibition of the growth of tumours, it must be ensured that using a CPP-based delivery agent does not undo the potential benefits obtained by this proposed therapeutic system. Furthermore, the lack of effect on the part of transfection reagent is essential for studies of the viruses as well. If the infection proceeds differently in reagent treated cells (compared to non-treated cells) the reagent/procedure is of a limited value for such studies. To note, the lipid based reagents often have such problems, not only because of the cytotoxic effects, but also because the virus replicase complexes are formed and are anchored to lipid membranes. To reveal such properties (or their absence) of PepFect6 reagent, the morphology of the transfected cells and the localisation of viral replication organelles inside the cells was observed after the transfection of the viral RNA or corresponding DNA/RNA layered vectors. This experiment did not reveal any deviations, attributable to transfection reagent/procedure, from the results obtained by previous studies on alphaviruses and their effects on the cellular structure (Figure 5, paper II). In another setup PepFect6 was used to transfect cells with siRNAs and then transfected cells were infected with SFV. It was found that the infection profile of the cells transfected with a peptide-based reagent remained close to that in non-treated cells; importantly the spatial arrangement of the replication organelles also did not deviate from the expected pattern. In contrast, when the cells were previously transfected with a liposomal reagent, the transfection procedure affected subsequent virus infection, if the two events (transfection and infection) took place in a short time frame. It should be noted, however, that 24 h post-transfection neither reagent (PepFect6 or Lipofectamine 2000) had any detectable effect on SFV infection in transfected cells (Figure 6, paper II). Finally, it was observed that while PepFect6 was certainly able to deliver both

SFV RNA and corresponding DNA/RNA layered vector to transfected cells, the transfection occurred at moderate efficiency which was generally lower than in case of use of Lipofectamine 2000 reagent (Figure 3, paper II).

Taken together, this study revealed that there are certain aspects which must be considered when applying PepFect6 to deliver a DNA-based cargo, which must reach nucleus (moderate efficiency, delay in infectious genome rescue). The positive effects, observed in relation to this reagent, were mostly associated with the lack of transfection induced cell damage and the lack of effect on virus replication process. These observations encourage the further applications of peptide-based reagents in the systems related to alphavirus (and likely other viruses) based vectors. An especially useful feature of the CPP was its lack of interference with the infection-replication cycle of the virus itself, which was something that could have been expected, as the entry mechanisms of the viruses and the proposed route of entry for the peptide complexes overlap, creating a possibility for the inhibition of virus entry or incorrect placement of replication complexes. These results give reason to believe that this transfection approach might not affect the functionality of other virus-based systems. This may extend to the potentially efficient use of a controllable system based on an intron-containing DNA virus vectors (paper I) as CPPs could be used to deliver both vectors and SCOs to targeted cells. In these cells, viruses can be produced, released and then spread further; in case these vectors are based on DNA viruses, they will be able to successfully replicate only in the specific cells where the correcting molecule has been transported. To achieve tumour inhibition without the threat of a systematic infection for the RNA viruses, only DNA-RNA layered replicon vectors can be used at this point, as the application of a full-length RNA virus would result in a new generation of virus particles which lack the engineered control mechanisms.

To conclude this part of study, nearly all CPP-s have displayed the capabilities of transporting smaller cargo molecules such as siRNAs and SCOs. In contrast, transfection of large plasmid molecules and long RNA transcripts has not been so widely assessed. Results obtained in this study give reason to believe that CPPs are capable of the delivery of such molecules. In turn, it means that if CPPs can be used for site-specific delivery of such molecules, the same system would probably have no restrictions regarding the transport of smaller cargo molecules as well. One of such tumour-targeting delivery systems is assessed in study III, where a tumour-dependent de-shielding mechanism was applied in order to prevent the transfection complexes becoming active prior to reaching the tissue of interest.

The development of a tumour-specific nucleic acid delivery strategy for *in vivo* applications (III)

The ultimate goal for the development of various delivery mechanisms is to find an efficient system which could be used for the purposes related to the treatment and inhibition of severe medical conditions which are not responsive to common treatment procedures or persistently tend to reoccur after a successful procedure. This is the reason why it is important to develop delivery vectors which would not display any toxic properties on the patients, even on the occasions when repeated administration is required to reach the full potential of the treatment. One of the possibilities for achieving this is to use targeting mechanisms, which would enable the carrier system to differentiate between the healthy and the diseased tissue and only interact with the latter. Such systems are especially potent when a clear difference can be made between the tissues, either through the secreted molecules present in the microenvironment or through the altered receptor profile. A good example of such tissues are the growths formed by the abnormal cells which make up the tumour tissue, a common medical problem for which an efficient and selective treatment procedure has still not been established. In this study, a potential delivery system is proposed and investigated, which could be used for the delivery of treatment-related molecules into tumour cells. Although these molecules can include such members which induce a specific effect immediately after the administration, the focus of the current research project is the delivery of plasmid-based sequences inside the tumour cells in order to create an intracellular expression strategy.

Keeping the general purpose of virus-based tumour delivery in mind, it was important to formulate a tumour-specific delivery which would be efficient in mediating the transportation of larger plasmids across the cell plasma membrane. Unfortunately, using a virus-based expression system at this point would have proven to be a challenge, both in the regards of biosafety and the measurable result, as a virus-based replicon vector would have resulted in cell apoptosis – a desirable effect for the purpose of tumour treatment but an obstacle for the determination of transfection system capabilities. This is one of the reasons why it was beneficial to include a plasmid expression vector with a noticeably longer (roughly 10kbp) nucleotide base pair sequence than the sequences of common reporter systems, allowing the experiments to closely mimic the delivery of alphavirus-based replicon DNA-RNA layered vectors and still enable the quantification of the results through transgene expression.

The CPP chosen for this delivery purpose was PepFect14, a transportan10 analogue, not unlike the PepFect6 transfection system used in the previous study assessing the suitability of CPPs for virology-related applications (Ezzat et al. 2011). Based on its promising performance facilitating *in vitro* plasmid delivery, it was believed that similar characteristics would also be present when applying the peptide as the basis for the development of an *in vivo* tissue specific delivery system. First, it was necessary to determine the behaviour of the peptide in a live animal setting, as the biological distribution and the specific

effects on the animals cannot be predicted solely based on the results obtained from the cell culture. As a result of the transfection, greatest accumulation of the peptide and its cargo was seen in lungs but minor increases in luciferase activities were also recorded in other tissues indicating a broad delivery spectrum for this reagent (Figure 1, paper III). The most efficient charge ratio between the peptide and its cargo appeared to be 1:4 while transporting 20 μg of genomic material, however the introduction of the complexes to the circulatory system resulted in acute toxic effects observed in some of the mice and, in some more severe cases, even leading to the death of the subject.

After confirming the delivery capabilities of the unmodified peptide, it was important to develop an approach for the inhibition of the activity to keep the complexes from interacting with possible targets throughout the host organism. While a selection of inhibitory strategies can be applied, the use of PEG molecules to shield the peptides was considered to be the best solution in this situation as it has been known to prevent the complexes from coming into contact with cells and, hopefully, prevent the DNA from being degraded by intracellular enzymes encountered in blood. As the behaviour of PEG-coupled peptides was previously unknown, three different types of shielding molecules (PEG600, PEG1000 and PEG2000) were used to determine if the CPP retained its ability to form complexes with its cargo and if the shielding strategy would be able to efficiently inhibit the activity of the particles. While the exact behaviour of specific peptides was shown to differ in the amount of the peptide required for complex formation, it was clear that all of the constructed molecules possessed molecular characteristics similar to PepFect14 and were able to interact with their cargo and bond with it (Figure 2, paper III), being incorporated either partly in the particle core (smaller PEG molecules) or forming a stable outer layer (PEG 2000). Similar characteristics could also be observed when complexes were tested for resistance to degradation, leading to the theory that the resistance to enzymes is can mostly be accounted to the peptide itself and the presence of PEG does not have a positive effect on the stability of the complexes. Interestingly, the ability of some constructs to interact with the core of the formed nanoparticle greatly enhanced the stability and binding strength of the complexes when compared to the larger molecules which were not able to do so. Nevertheless, the presence of any length of PEG used in the experimental setup was enough to cause a general weakening of the complex structures in comparison with the unmodified parent peptide, a problem which could be solved by the introduction of the same peptide into the reagent mixture, resulting in a cocktail of peptides to be used for particle formation.

Functional *in vitro* testing of the constructs indicated a significant inhibition of internalisation of the transportation complexes, however upon applying a mixture of modified and unmodified peptides to the plasmid, a concentration dependent increase in cell penetration activity could be observed, displaying the suitability of PEG as an inhibitor of molecular interactions which, in addition to efficient inhibition, allows for the adjustment of transfection rates through a simple mixing procedure. The results obtained from *in vivo* displayed a similar

efficiency when compared to the results obtained from cultured cells (Figure 4, paper III), indicating a decrease in the uptake levels and a longer blood circulation time for the molecules, allowing for a reduction in lung accumulation for the peptide-DNA nanocomplexes, as a result of which, a significant increase in passive accumulation to other tissue types was observed, however the molecules which were not internalised soon after the introduction were, with high probability, excreted from the organism as no delayed induction of reporter activity could be observed. The uptake profiles of the mixtures of protected and unprotected peptides also followed the previously obtained results rather closely, as the internalisation process seemed to be dependent on the peptide rations included in the complexes. One of the main differences between the original and the modified CPP was that through the reduction of general transfection efficiency through the application of an activity inhibitor, the viability of test subjects increased significantly as the shielding effect decreased the mortality rates to none.

Although the shielding strategy can be considered as a success, it is not enough just to shield the molecule and prevent it from interacting with the cell to create a potential medically efficient treatment system. Although there is a tendency for the complexes to accumulate in various types of tissue, including tumours, the accumulation rates are not sufficient for achieving any noticeable affinity over the other possible targets and even if such preference existed, the presence of the shield would negate all possible entry. To access the full potential of particle shielding, it must somehow be possible to neutralise the inhibitory effect achieved through the introduction of the PEG group. One approach for making the shield conditional is to introduce a linker which would allow the shield to detach at a suitable moment. Such linkers can be activated by an array of methods, varying from the presence of specific physical conditions to the more specific reactions with enzymes. An especially beneficial possibility would be to utilise a linker which would allow the detachment of shielding group directly at the site of tumour tissue, therefore enabling a type of site-specific delivery to be achieved. One of such molecules is a MMP2-cleavable linker, which interacts with the matrix metalloprotease enzyme present in many of the tumour tissues but not to a great extent in normal cells. One of the obstacles with similar approaches has always been the possibility of inefficient cleavage, leading to the reduced internalisation levels when compared with unmodified transporter molecule. Fortunately, this is not the case for this experimental strategy as the preliminary tests indicate a near-complete activation of the previously inactive peptide by the MMP2 enzyme, which was further confirmed by *in vitro* assays, through which the most efficient ratio of cleavable and parent peptide was found to be 50%. As the particle including the smallest of the tested PEGs proved itself to be the most efficient, it was selected to undergo *in vivo* testing to further explore the functionality of the delivery mechanism when other possible target organs are also available. While the previously efficient ratio of 50% was able to facilitate significant tumour delivery, minor levels of reporter activity were still present in other tissues where the peptide

complexes typically tend to accumulate. However, once the ratio of protected peptide was increased to 70%, a nearly complete tumour-specific delivery efficiency could be observed, indicating the success of this approach (Figure 5, paper III). While the application of peptide constructs containing larger PEG molecules also resulted in increased reporter levels measured from tumour tissues, their capability to inhibit the overall interaction with tissues was not as complete as the one observed by the PEG 600-containing PepFect144 and therefore can be considered of a little practical value in the practical medicine-related situations where minimal side effects are desired.

Based on the conducted experiments, it was demonstrated that the conversion of an efficient delivery vector into one with the properties of a tissue-specific activation was possible through the introduction of an interaction-inhibiting shield attached to the complexes by a tumour-cleavable linker. What makes this achievement more special is the fact that the molecules delivered into the tumour cells were not limited to small siRNAs or SCOs with a fluorescent tag, but rather longer than usual plasmid-based expression systems which, as a result of the delivery, managed to reach the nucleus of the cell and retain their functionality of expressing a measurable marker. This knowledge provides grounds for the expectations that plasmid vectors encoding virus replicon genomes, such as the ones described in studies I and II, could also be delivered specifically to tumour tissues with high efficiency without the threat of affecting the surrounding tissues or other organs present in the host. The development of this strategy also opens up countless possibilities for further practical applications regarding the treatment of these types of tumours which are capable of expressing MMP2, such as the high-level development of chemotherapy complexes to be used on patients with malignant growths or even for more basic research on tumour-inhibitory strategies. A possible development on the basis of this work is to apply a similar approach of tissue-selective activation to other CPPs in order to achieve even greater delivery efficiency, also it would theoretically be possible to use a similar system for the targeting other tissues, provided that an enzyme is identified which is characteristic only of the desired target.

Future perspectives

All of the systems, described in the studies listed here, have their own applications and possess numerous potential uses on their own or when combined with each other and/or with other strategies not described in the work at hand. However, the combination and the practical application of the knowledge obtained through this work would be of great benefit and therefore also a logical direction to take as a next step. As the reason for undertaking this project was to offer a mechanism for tumour treatment through an approach which had not yet been described, it is important to continue with the practical demonstration of the co-application of these systems which would allow the researchers to move

a step closer to an efficient tumour-treatment strategy through the virus-induced apoptosis, which can also be combined with the production of therapeutic molecules, such as tumour inhibitors or anti-cancer vaccines designed to prevent the further growth of malignancies already present and the emergence of novel tumours. Although the perspective of clinical trials is still out of reach, it is still the envisioned goal and therefore something that should be kept in mind when designing the experiments and conducting the work with the aim of perfecting the components included in the proposed system. As mentioned before, the virus activation system achieved through the aberrant splicing of introns could, in theory, be transferred to other types of viruses and possibly even more efficiently to those having DNA genomes. This would allow the application of vectors based on full-length viruses, capable of leaving their host cells and re-infecting others, in the service of tumour treatment; when coupled with efficient and target tissue specific delivery of SCOs, it should enable the virus replication only in the tissues of interest. Also the CPPs, used for the delivery, must not always remain to be the same. As the field of drug delivery is changing with an immense speed, it is only logical to assume that within a relatively small time period, novel peptide builds will be proposed, which offer even further possibilities for delivery of the components of the developed systems and which then could be used as a basis for the therapeutic system proposed in this thesis.

CONCLUSION

The current state of tumour treatment is not perfect. The existing treatment procedures rely heavily on surgery, as a result of which the malignant mass is removed but still has a risk of recurrence. In addition, radiation or chemotherapy approaches can be applied, however both of these approaches often result in unwanted side effects in the patient without ensuring the complete neutralisation of the tumour cells. This is the reason why it is important to continue with the development of novel anti-tumour approaches which would enable to offer the patients fresh solutions to their health problems, allowing for higher treatment rate and the possibility of reduction in unwanted symptoms.

The work at hand has approached the issue of tumour therapy by exploring three systems which have been linked with each other through the potential practical application which involves the creation of an efficient and safe tumour inhibition system with a perspective for future clinical applications. In the first study, a control mechanism was proposed for alphavirus-based DNA/RNA-layered vectors, which would allow the system to be controlled via the introduction of one or more defective intron sequences placed in region corresponding to the non-structural ORF of virus genome. Based on the obtained results, it can be concluded that the rescue of infectious RNAs from these constructs was successfully inhibited to a great extent with the possibility to reverse the situation by the introduction of SCOs, which would allow to correct the aberrant splicing profile. As the intron-containing sequence must be based on a DNA/RNA-layered vector, rather than on RNA genomes, packed into a virus particle, an efficient method for transfection using these constructs is required. This issue was addressed in a study, where a promising class of delivery agents, CPPs (exemplified by the novel PepFect6 reagent), was evaluated for such application. The main goal for this research was to determine the properties of the peptide-based delivery system when in interaction with virus-based sequences. It was demonstrated that the application of CPPs for the delivery of virus-based constructs has significant potential, as the reagent managed to induce high levels of intracellular delivery together with the lack of detectable interference with the infection process or without the suppression of replication capabilities of the virus vectors and virus particles. Based on this knowledge, a CPP-based tumour-specific delivery strategy was devised for large plasmid delivery into malignant tissues. This approach entailed constructing a peptide which would be completely inactivated by a shielding molecule PEG, connected to the CPP through a MMP2-cleavable linker. This allows the transfection complexes to lay dormant upon entering the blood circulation and only be activated once encountering the specific protease, MMP2, which is greatly upregulated in tumour cells, effectively creating a tissue-specific delivery system.

Based on this research, a safe and tumour-selective approach for chemotherapy can be proposed, consisting of a controllable DNA virus and/or DNA/RNA-layered replicon vector, expressing a therapeutic transgene, which would be selectively delivered into tumour cells by the tumour-targeting CPP

complexes together with the SCOs required for the activation of the vectors. Such system would enable the tissue-specific induction of cell apoptosis; in addition, enhanced resistance or inhibitory effect towards the tumour tissues can be exerted by the expression of therapeutic proteins, which will be released to organism after the termination of the transfected/infected tumour cells.

SUMMARY IN ESTONIAN

Uurimus indutseeritava alfaviirusel põhineva kasvajavastase strateegia kohta, mis on vahendatud läbi aktiveeritavate rakku sisenevate peptiidide poolt võimaldatava kohtspetsiifilise kohaletoimetamise.

Alfaviirused on grupp lühikesi positiivse polaarsusega üheahelalisel RNA-l põhinevaid viiruseid, mille enimtuntud liikmete hulgas on Semliki Forest viirus, Sindbis viirus ning viimasel ajal ka Chikungunya viirus. Esimest kahte on tänu nende kiirele paljunemisele ja väiksele patogeensusele kasutatud juba aastaid, kuid tänu hiljutistele Chikungunya viiruse epideemiatele on intensiivsem uurimistöö laienenud ka selle viiruse valdkonda.

Alates esimeste alfaviirustel põhinevate vektorite loomisest on neid rakendatud mitmete praktiliste ülesannete jaoks nii laboratoorseks uurimistööks kui ka meditsiinilistel eesmärkidel. Kuna alfaviiruste üheks omaduseks on oma peremeesrakk viiruse paljunemise tagajärjel apoptoosi suunata, on see omadus ravimitööstuses laialdast rakendust leidnud. Kuigi see omadus tundub esmapilgul kahjulikuna, saab seda rakendada kasvajate vastases ravis, lastes viirusel tuumorirakke hävitada ning samas väljutada nendest terapeutilisi molekule, mis on sünteesitud viiruse genoomi integreeritud transgeense järjestuse baasil.

Käesoleva töö eesmärgiks ongi tegeleda kasvajate ravimise valdkonnaga, mis on praegu kaugel ideaalsest. Hetkel olemasolevad raviprotseduurid põhinevad peamiselt kirurgial, mille tagajärjel eemaldatakse küll kahjulik kude, kuid jääb püsima kasvaja uuesti tekkimise oht. Selle lisaks saab rakendada ka kiiritus- või kemoteraapiat, kuid mõlemad lähenemised võivad endaga kaasa tuua soovimatuid kõrvalnähte, mistõttu on oluline jätkata uuringutega uute kasvajavastaste lähenemiste välja arendamiseks, mis pakuksid patsientidele uusi võimalusi oma tervise taastamiseks ning lubaksid saavutada suurema raviefektiivsuse ja soovimatute kõrvalnähtude vähenemise.

Antud töös uuritakse lähemalt kolme süsteemi, mis on üksteisega ühendatud läbi potentsiaalse praktilise rakenduse, mis kujutab endast efektiivse tuumoriteraapia süsteemi, millel on perspektiivi jõuda ka kliinilise rakenduseni. Esimeses uurimustöös pakuti välja kontrollmehhanism alfaviirustel põhinevate DNA/RNA-kihtvektorite jaoks, mis lubaks süsteemi kontrollida läbi ühe või enama defektse introni sisestamise regiooni, mis vastab viiruse genoomi mittestruktuursele lugemisraamile. Nakatamisvõimeliste RNA-de avaldumine nendelt konstruktidelt suruti edukalt maha, samas oli võimalik seda protsessi taastada läbi splaissingut korrigeerivate oligonukleotiidide rakendamise, mis võimaldavad ebakorrektse splaissingumustri korrigeerimist. Kuna intronit sisaldavad järjestused peavad põhinema DNA/RNA-kihtvektoril, mitte tavapärasel RNA genoomil, mis on viiruseosakesse pakitud, esineb nende konstruktide rakendamiseks suur vajadus efektiivse transfektsioonisüsteemi järele. Sellele probleemile keskenduti järgmises töös, milles hinnati paljulubava transfektsioonisüsteemi klassi, rakku sisenevate peptiidide, esindaja, PepFect6-e, sobivust

sellisteks rakendusteks. Uurimuse peamiseks eesmärgiks oli peptiidipõhise ülekandesüsteemi omaduste hindamine kokkupuutel viirusel põhinevate järjestustega. Näidati, et rakku sisenevate peptiidide rakendamine viirusel põhinevate konstruktide rakku toimetamiseks omab märgatavat potentsiaali, kuna antud molekul suutis saavutada kõrgetasemelist rakkudesse toimetamise efektiivsust, omamata samal ajal mingit mõju viiruse nakatamisprotsessile ning mõjutamata viirusosakeste ning viirusel põhinevate vektorite paljunemise võimekust. Põhinedes sellel teadmisel, loodi rakku sisenevatel peptiididel põhinev tuumorispetsiifiline transpordistrateegia, mis oleks suuteline toimetama suuri plasmiide kasvajakudedesse. Selleks konstrueeriti peptiid, mis inaktiveeriti täielikult varjestusmolekuli PEG poolt, mis on rakku siseneva peptiidiga ühendatud läbi MMP2 geeniprodukti poolt lõigatava motiivi. Kuna MMP2 on geen, mis on kasvajarakkudes üleekspresseeritud, võimaldab see transfektsioonikompleksidel jääda inaktiivseks kehasse sisenemisel ning ainult muutuda aktiivseks juhul, kui nad puutuvad kokku spetsiiflise proteaasiga, mida leidub kasvajarakkudes, luues sedaviisi koespetsiifilise transpordisüsteemi.

Põhinedes antud uurimistööle, saab välja pakkuda ohutu ning kasvajaspetsiifilise lähenemise kemoteraapiaks, mis koosneb kontrollitavast DNA viirusest ja/või DNA/RNA-kihtvektorist, mis ekspresseerib terapeutilist valku, mida oleks võimalik koos neid aktiveerivate splaissingut korrigeerivate oligonukleotiididega, tuumorispetsiifilise rakku siseneva peptiidi abil, selektiivselt tuumorirakkudesse toimetada. Selline süsteem võimaldaks koespetsiifilist rakkude apoptoosi viimist, mille lisandub ekspresseeritavatest terapeutilistest valkudest, mis vabanevad eelnevalt transfekteeritud/nakatunud rakkude hukkumise tagajärjel peremeesorganismi, tulenev resistentsus või kasvaja arengut pidurdav efekt.

REFERENCES

Agarwal, A., P. K. Dash, A. K. Singh, S. Sharma, N. Gopalan, P. V. Rao, M. M. Parida and P. Reiter (2014). "Evidence of experimental vertical transmission of emerging novel ECSA genotype of Chikungunya Virus in Aedes aegypti." PLoS Negl Trop Dis **8**(7): e2990.

Ahola, T. and L. Kääriäinen (1995). "Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP." Proc Natl Acad Sci U S A **92**(2): 507–511.

Akhrymuk, I., S. V. Kulemzin and E. I. Frolova (2012). "Evasion of the innate immune response: the Old World alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of RNA polymerase II." J Virol **86**(13): 7180–7191.

Alam, C. M., A. K. Singh, C. Sharfuddin and S. Ali (2014). "In- silico exploration of thirty alphavirus genomes for analysis of the simple sequence repeats." Meta Gene **2**: 694–705.

Alsharifi, M., M. Lobigs, M. M. Simon, A. Kersten, K. Muller, A. Koskinen, E. Lee and A. Mullbacher (2006). "NK cell-mediated immunopathology during an acute viral infection of the CNS." Eur J Immunol **36**(4): 887–896.

Andaloussi, S. E., T. Lehto, I. Mäger, K. Rosenthal-Aizman, Oprea, II, O. E. Simonson, H. Sork, K. Ezzat, D. M. Copolovici, K. Kurrikoff, J. R. Viola, E. M. Zaghloul, R. Sillard, H. J. Johansson, F. Said Hassane, P. Guterstam, J. Suhorutsenko, P. M. Moreno, N. Oskolkov, J. Halldin, U. Tedebark, A. Metspalu, B. Lebleu, J. Lehtio, C. I. Smith and Ü. Langel (2011). "Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo." Nucleic Acids Res **39**(9): 3972–3987.

Arias-Goeta, C., S. Moutailler, L. Mousson, K. Zouache, J. M. Thiberge, V. Caro, F. Rougeon and A. B. Failloux (2014). "Chikungunya virus adaptation to a mosquito vector correlates with only few point mutations in the viral envelope glycoprotein." Infect Genet Evol **24**: 116–126.

Atasheva, S., A. Fish, M. Fornerod and E. I. Frolova (2010). "Venezuelan equine Encephalitis virus capsid protein forms a tetrameric complex with CRM1 and importin alpha/beta that obstructs nuclear pore complex function." J Virol **84**(9): 4158–4171.

Atkins, G. J., M. N. Fleeton and B. J. Sheahan (2008). "Therapeutic and prophylactic applications of alphavirus vectors." Expert Rev Mol Med **10**: e33.

Atkins, G. J., B. J. Sheahan and N. J. Dimmock (1985). "Semliki Forest virus infection of mice: a model for genetic and molecular analysis of viral pathogenicity." J Gen Virol **66 (Pt 3)**: 395–408.

Atkins, G. J., B. J. Sheahan and D. A. Mooney (1990). "Pathogenicity of Semliki Forest virus for the rat central nervous system and primary rat neural cell cultures: possible implications for the pathogenesis of multiple sclerosis." Neuropathol Appl Neurobiol **16**(1): 57–68.

Barber, G. N. (2004). "Vesicular stomatitis virus as an oncolytic vector." Viral Immunol **17**(4): 516–527.

Barraza, S. J., P. C. Delekta, J. A. Sindac, C. J. Dobry, J. Xiang, R. F. Keep, D. J. Miller and S. D. Larsen (2015). "Discovery of anthranilamides as a novel class of inhibitors of neurotropic alphavirus replication." Bioorg Med Chem **23**(7): 1569–1587.

Barry, G., R. Fragkoudis, M. C. Ferguson, A. Lulla, A. Merits, A. Kohl and J. K. Fazakerley (2010). “Semliki forest virus-induced endoplasmic reticulum stress accelerates apoptotic death of mammalian cells.” J Virol **84**(14): 7369–7377.

Bechara, C. and S. Sagan (2013). “Cell-penetrating peptides: 20 years later, where do we stand?” FEBS Lett **587**(12): 1693–1702.

Berglund, P., M. Sjoberg, H. Garoff, G. J. Atkins, B. J. Sheahan and P. Liljestrom (1993). “Semliki Forest virus expression system: production of conditionally infectious recombinant particles.” Biotechnology (N Y) **11**(8): 916–920.

Berglund, P., C. Smerdou, M. N. Fleeton, I. Tubulekas and P. Liljestrom (1998). “Enhancing immune responses using suicidal DNA vaccines.” Nat Biotechnol **16**(6): 562–565.

Betts, C., A. F. Saleh, A. A. Arzumanov, S. M. Hammond, C. Godfrey, T. Coursindel, M. J. Gait and M. J. Wood (2012). “Pip6-PMO, A New Generation of Peptide-oligonucleotide Conjugates With Improved Cardiac Exon Skipping Activity for DMD Treatment.” Mol Ther Nucleic Acids **1**: e38.

Bhomia, M., A. Sharma, M. Gayen, P. Gupta and R. K. Maheshwari (2013). “Artificial microRNAs can effectively inhibit replication of Venezuelan equine encephalitis virus.” Antiviral Res **100**(2): 429–434.

Breakwell, L., P. Dosenovic, G. B. Karlsson Hedestam, M. D'Amato, P. Liljestrom, J. Fazakerley and G. M. McInerney (2007). “Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response.” J Virol **81**(16): 8677–8684.

Busslinger, M., N. Moschonas and R. A. Flavell (1981). “Beta + thalassemia: aberrant splicing results from a single point mutation in an intron.” Cell **27**(2 Pt 1): 289–298.

Chen, W., S. S. Foo, N. A. Sims, L. J. Herrero, N. C. Walsh and S. Mahalingam (2015). “Arthritogenic alphaviruses: new insights into arthritis and bone pathology.” Trends Microbiol **23**(1): 35–43.

Chien, W. M., Y. Liu and M. T. Chin (2014). “Genomic DNA recombination with cell-penetrating peptide-tagged cre protein in mouse skeletal and cardiac muscle.” Genesis **52**(7): 695–701.

Chu, H., S. C. Das, J. F. Fuchs, M. Suresh, S. C. Weaver, D. T. Stinchcomb, C. D. Partidos and J. E. Osorio (2013). “Deciphering the protective role of adaptive immunity to CHIKV/IRES a novel candidate vaccine against Chikungunya in the A129 mouse model.” Vaccine **31**(33): 3353–3360.

Ciano, K. A., J. J. Saredy and D. F. Bowers (2014). “Heparan sulfate proteoglycan: an arbovirus attachment factor integral to mosquito salivary gland ducts.” Viruses **6**(12): 5182–5197.

Cirimotich, C. M., J. C. Scott, A. T. Phillips, B. J. Geiss and K. E. Olson (2009). “Suppression of RNA interference increases alphavirus replication and virus-associated mortality in Aedes aegypti mosquitoes.” BMC Microbiol **9**: 49.

Coffey, L. L., Y. Beeharry, A. V. Borderia, H. Blanc and M. Vignuzzi (2011). “Arbovirus high fidelity variant loses fitness in mosquitoes and mice.” Proc Natl Acad Sci U S A **108**(38): 16038–16043.

Cook, S. H. and D. E. Griffin (2003). “Luciferase imaging of a neurotropic viral infection in intact animals.” J Virol **77**(9): 5333–5338.

Couderc, T., F. Chretien, C. Schilte, O. Disson, M. Brigitte, F. Guivel-Benhassine, Y. Touret, G. Barau, N. Cayet, I. Schuffenecker, P. Despres, F. Arenzana-Seisdedos, A. Michault, M. L. Albert and M. Lecuit (2008). “A mouse model for Chikungunya:

young age and inefficient type-I interferon signaling are risk factors for severe disease." PLoS Pathog **4**(2): e29.

Dalby, B., S. Cates, A. Harris, E. C. Ohki, M. L. Tilkins, P. J. Price and V. C. Ciccarone (2004). "Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications." Methods **33**(2): 95–103.

Das, P. K., A. Merits and A. Lulla (2014). "Functional cross-talk between distant domains of chikungunya virus non-structural protein 2 is decisive for its RNA-modulating activity." J Biol Chem **289**(9): 5635–5653.

Davis, N. L., F. J. Fuller, W. G. Dougherty, R. A. Olmsted and R. E. Johnston (1986). "A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice." Proc Natl Acad Sci U S A **83**(18): 6771–6775.

Davis, N. L., L. V. Willis, J. F. Smith and R. E. Johnston (1989). "In vitro synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant." Virology **171**(1): 189–204.

de Groot, R. J., T. Rumenapf, R. J. Kuhn, E. G. Strauss and J. H. Strauss (1991). "Sindbis virus RNA polymerase is degraded by the N-end rule pathway." Proc Natl Acad Sci U S A **88**(20): 8967–8971.

Delekta, P. C., C. J. Dobry, J. A. Sindac, S. J. Barraza, P. K. Blakely, J. Xiang, P. D. Kirchhoff, R. F. Keep, D. N. Irani, S. D. Larsen and D. J. Miller (2014). "Novel indole-2-carboxamide compounds are potent broad-spectrum antivirals active against western equine encephalitis virus in vivo." J Virol **88**(19): 11199–11214.

Delekta, P. C., A. Raveh, M. J. Larsen, P. J. Schultz, G. Tamayo-Castillo, D. H. Sherman and D. J. Miller (2014). "The Combined Use of Alphavirus Replicons and Pseudoinfectious Particles for the Discovery of Antivirals Derived from Natural Products." J Biomol Screen.

Derossi, D., G. Chassaing and A. Prochiantz (1998). "Trojan peptides: the penetratin system for intracellular delivery." Trends Cell Biol **8**(2): 84–87.

Derossi, D., A. H. Joliot, G. Chassaing and A. Prochiantz (1994). "The third helix of the Antennapedia homeodomain translocates through biological membranes." J Biol Chem **269**(14): 10444–10450.

Deshayes, S., M. Morris, F. Heitz and G. Divita (2008). "Delivery of proteins and nucleic acids using a non-covalent peptide-based strategy." Adv Drug Deliv Rev **60**(4–5): 537–547.

Dubensky, T. W., Jr., D. A. Driver, J. M. Polo, B. A. Belli, E. M. Latham, C. E. Ibanez, S. Chada, D. Brumm, T. A. Banks, S. J. Mento, D. J. Jolly and S. M. Chang (1996). "Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer." J Virol **70**(1): 508–519.

Ehrengruber, M. U. and A. L. Goldin (2007). "Semliki Forest virus vectors with mutations in the nonstructural protein 2 gene permit extended superinfection of neuronal and non-neuronal cells." J Neurovirol **13**(4): 353–363.

Ehrengruber, M. U. and K. Lundström (2007). "Alphaviruses: Semliki Forest virus and Sindbis virus vectors for gene transfer into neurons." Curr Protoc Neurosci **Chapter 4**: Unit 4 22.

Ehrengruber, M. U., M. Renggli, O. Raineteau, S. Hennou, M. J. Vähä-Koskelä, A. E. Hinkkanen and K. Lundström (2003). "Semliki Forest virus A7(74) transduces hippocampal neurons and glial cells in a temperature-dependent dual manner." J Neurovirol **9**(1): 16–28.

El-Beshlawy, A., A. Mostafa, I. Youssry, H. Gabr, I. M. Mansour, M. El-Tablawy, M. Aziz and I. R. Hussein (2008). "Correction of aberrant pre-mRNA splicing by antisense oligonucleotides in beta-thalassemia Egyptian patients with IVSI-110 mutation." J Pediatr Hematol Oncol **30**(4): 281–284.

Enserink, M. (2007). "Infectious diseases. Chikungunya: no longer a third world disease." Science **318**(5858): 1860–1861.

Ezzat, K., S. E. Andaloussi, E. M. Zaghloul, T. Lehto, S. Lindberg, P. M. Moreno, J. R. Viola, T. Magdy, R. Abdo, P. Guterstam, R. Sillard, S. M. Hammond, M. J. Wood, A. A. Arzumanov, M. J. Gait, C. I. Smith, M. Hällbrink and Ü. Langel (2011). "PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation." Nucleic Acids Res **39**(12): 5284–5298.

Ferguson, M. C., S. Saul, R. Fragkoudis, S. Weisheit, J. Cox, A. Patabendige, K. Sherwood, M. Watson, A. Merits and J. K. Fazakerley (2015). "The ability of the encephalitic arbovirus Semliki Forest virus to cross the blood brain barrier is determined by the charge of the E2 glycoprotein." J Virol.

Ferlenghi, I., B. Gowen, F. de Haas, E. J. Mancini, H. Garoff, M. Sjoberg and S. D. Fuller (1998). "The first step: activation of the Semliki Forest virus spike protein precursor causes a localized conformational change in the trimeric spike." J Mol Biol **283**(1): 71–81.

Fields, W. and M. Kielian (2013). "A key interaction between the alphavirus envelope proteins responsible for initial dimer dissociation during fusion." J Virol **87**(7): 3774–3781.

Figueiredo, M. L. and L. T. Figueiredo (2014). "Emerging alphaviruses in the Americas: Chikungunya and Mayaro." Rev Soc Bras Med Trop **47**(6): 677–683.

Finotti, A., L. Breda, C. W. Lederer, N. Bianchi, C. Zuccato, M. Kleanthous, S. Rivella and R. Gambari (2015). "Recent trends in the gene therapy of beta-thalassemia." J Blood Med **6**: 69–85.

Firth, A. E., B. Y. Chung, M. N. Fleeton and J. F. Atkins (2008). "Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma." Virol J **5**: 108.

Fleeton, M. N., M. Chen, P. Berglund, G. Rhodes, S. E. Parker, M. Murphy, G. J. Atkins and P. Liljestrom (2001). "Self-replicative RNA vaccines elicit protection against influenza A virus, respiratory syncytial virus, and a tickborne encephalitis virus." J Infect Dis **183**(9): 1395–1398.

Foy, N. J., M. Akhrymuk, A. V. Shustov, E. I. Frolova and I. Frolov (2013). "Hypervariable domain of nonstructural protein nsP3 of Venezuelan equine encephalitis virus determines cell-specific mode of virus replication." J Virol **87**(13): 7569–7584.

Fragkoudis, R., Y. Chi, R. W. Siu, G. Barry, G. Attarzadeh-Yazdi, A. Merits, A. A. Nash, J. K. Fazakerley and A. Kohl (2008). "Semliki Forest virus strongly reduces mosquito host defence signaling." Insect Mol Biol **17**(6): 647–656.

Fragkoudis, R., N. Tamberg, R. Siu, K. Kiiver, A. Kohl, A. Merits and J. K. Fazakerley (2009). "Neurons and oligodendrocytes in the mouse brain differ in their ability to replicate Semliki Forest virus." J Neurovirol **15**(1): 57–70.

Frankel, A. D. and C. O. Pabo (1988). "Cellular uptake of the tat protein from human immunodeficiency virus." Cell **55**(6): 1189–1193.

Frolov, I., E. Frolova and S. Schlesinger (1997). "Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA." J Virol **71**(4): 2819–2829.

Frolov, I., N. Garmashova, S. Atasheva and E. I. Frolova (2009). "Random insertion mutagenesis of sindbis virus nonstructural protein 2 and selection of variants incapable of downregulating cellular transcription." J Virol **83**(18): 9031–9044.

Frolov, I., R. Hardy and C. M. Rice (2001). "Cis-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis." RNA **7**(11): 1638–1651.

Fros, J. J., W. J. Liu, N. A. Prow, C. Geertsema, M. Ligtenberg, D. L. Vanlandingham, E. Schnettler, J. M. Vlak, A. Suhrbier, A. A. Khromykh and G. P. Pijlman (2010). "Chikungunya virus nonstructural protein 2 inhibits type I/II interferon-stimulated JAK-STAT signaling." J Virol **84**(20): 10877–10887.

Froshauer, S., J. Kartenbeck and A. Helenius (1988). "Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes." J Cell Biol **107**(6 Pt 1): 2075–2086.

Furuta, T., R. Tomioka, K. Taki, K. Nakamura, N. Tamamaki and T. Kaneko (2001). "In vivo transduction of central neurons using recombinant Sindbis virus: Golgi-like labeling of dendrites and axons with membrane-targeted fluorescent proteins." J Histochem Cytochem **49**(12): 1497–1508.

Gaedigk-Nitschko, K. and M. J. Schlesinger (1990). "The Sindbis virus 6K protein can be detected in virions and is acylated with fatty acids." Virology **175**(1): 274–281.

Galanis, E., A. Bateman, K. Johnson, R. M. Diaz, C. D. James, R. Vile and S. J. Russell (2001). "Use of viral fusogenic membrane glycoproteins as novel therapeutic transgenes in gliomas." Hum Gene Ther **12**(7): 811–821.

Gao, H., S. Zhang, S. Cao, Z. Yang, Z. Pang and X. Jiang (2014). "Angiopep-2 and activatable cell-penetrating peptide dual-functionalized nanoparticles for systemic glioma-targeting delivery." Mol Pharm **11**(8): 2755–2763.

Garcia-Moreno, M., M. A. Sanz and L. Carrasco (2015). "Initiation codon selection is accomplished by a scanning mechanism without crucial initiation factors in Sindbis virus subgenomic mRNA." RNA **21**(1): 93–112.

Garmashova, N., R. Gorchakov, E. Volkova, S. Paessler, E. Frolova and I. Frolov (2007). "The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff." J Virol **81**(5): 2472–2484.

Gehl, J. (2003). "Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research." Acta Physiol Scand **177**(4): 437–447.

Gehrmann, M., S. Stangl, G. A. Foulds, R. Oellinger, S. Breuninger, R. Rad, A. G. Pockley and G. Multhoff (2014). "Tumor imaging and targeting potential of an Hsp70-derived 14-mer peptide." PLoS One **9**(8): e105344.

Glasgow, G. M., M. M. McGee, C. J. Tarbatt, D. A. Mooney, B. J. Sheahan and G. J. Atkins (1998). "The Semliki Forest virus vector induces p53-independent apoptosis." J Gen Virol **79 (Pt 10)**: 2405–2410.

Gomez de Cedron, M., N. Ehsani, M. L. Mikkola, J. A. Garcia and L. Kääriäinen (1999). "RNA helicase activity of Semliki Forest virus replicase protein NSP2." FEBS Lett **448**(1): 19–22.

Gorchakov, R., N. Garmashova, E. Frolova and I. Frolov (2008). "Different types of nsP3-containing protein complexes in Sindbis virus-infected cells." J Virol **82**(20): 10088–10101.

Graham, A., R. Walker, P. Baird, C. N. Hahn and J. K. Fazakerley (2006). "CNS gene therapy applications of the Semliki Forest virus 1 vector are limited by neurotoxicity." Mol Ther **13**(3): 631–635.

Griffin, D. E. (2010). “Recovery from viral encephalomyelitis: immune-mediated noncytolytic virus clearance from neurons.” Immunol Res **47**(1–3): 123–133.
Griffith, I. P., N. E. Crook and D. O. White (1975). “Protection of mice against cancer by immunization with membranes but not purified virions from virus infected cancer cells.” Br J Cancer **31**(6): 603–613.
Guan, M., J. R. Rodriguez-Madoz, P. Alzuguren, C. Gomar, M. G. Kramer, S. Kochanek, J. Prieto, C. Smerdou and C. Qian (2006). “Increased efficacy and safety in the treatment of experimental liver cancer with a novel adenovirus-alphavirus hybrid vector.” Cancer Res **66**(3): 1620–1629.
Halstead, S. B. (2015). “Reappearance of chikungunya, formerly called dengue, in the Americas.” Emerg Infect Dis **21**(4): 557–561.
Hardy, W. R. and J. H. Strauss (1988). “Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo by using monospecific antibodies.” J Virol **62**(3): 998–1007.
Harrington, K. J., E. M. Karapanagiotou, V. Roulstone, K. R. Twigger, C. L. White, L. Vidal, D. Beirne, R. Prestwich, K. Newbold, M. Ahmed, K. Thway, C. M. Nutting, M. Coffey, D. Harris, R. G. Vile, H. S. Pandha, J. S. Debono and A. A. Melcher (2010). “Two-stage phase I dose-escalation study of intratumoral reovirus type 3 dearing and palliative radiotherapy in patients with advanced cancers.” Clin Cancer Res **16**(11): 3067–3077.
Hayashi, Y., J. Yamauchi, I. A. Khalil, K. Kajimoto, H. Akita and H. Harashima (2011). “Cell penetrating peptide-mediated systemic siRNA delivery to the liver.” Int J Pharm **419**(1–2): 308–313.
Higa, M., C. Katagiri, C. Shimizu-Okabe, T. Tsumuraya, M. Sunagawa, M. Nakamura, S. Ishiuchi, C. Takayama, E. Kondo and M. Matsushita (2015). “Identification of a novel cell-penetrating peptide targeting human glioblastoma cell lines as a cancer-homing transporter.” Biochem Biophys Res Commun **457**(2): 206–212.
Higgs, S. and D. Vanlandingham (2015). “Chikungunya virus and its mosquito vectors.” Vector Borne Zoonotic Dis **15**(4): 231–240.
Hyde, J. L., R. Chen, D. W. Trobaugh, M. S. Diamond, S. C. Weaver, W. B. Klimstra and J. Wilusz (2015). “The 5' and 3' ends of alphavirus RNAs – Non-coding is not non-functional.” Virus Res.
Iijima, Y., K. Ohno, H. Ikeda, K. Sawai, B. Levin and D. Meruelo (1999). “Cell-specific targeting of a thymidine kinase/ganciclovir gene therapy system using a recombinant Sindbis virus vector.” Int J Cancer **80**(1): 110–118.
Iwasaki, A. (2012). “A virological view of innate immune recognition.” Annu Rev Microbiol **66**: 177–196.
Jerusalmi, A., M. M. Morris-Downes, B. J. Sheahan and G. J. Atkins (2003). “Effect of intranasal administration of Semliki Forest virus recombinant particles expressing reporter and cytokine genes on the progression of experimental autoimmune encephalomyelitis.” Mol Ther **8**(6): 886–894.
Ji, T., Y. Ding, Y. Zhao, J. Wang, H. Qin, X. Liu, J. Lang, R. Zhao, Y. Zhang, J. Shi, N. Tao, Z. Qin and G. Nie (2015). “Peptide assembly integration of fibroblast-targeting and cell-penetration features for enhanced antitumor drug delivery.” Adv Mater **27**(11): 1865–1873.
Jiao, C. Y., D. Delaroche, F. Burlina, I. D. Alves, G. Chassaing and S. Sagan (2009). “Translocation and endocytosis for cell-penetrating peptide internalization.” J Biol Chem **284**(49): 33957–33965.

Jobin, M. L., P. Bonnafous, H. Temsamani, F. Dole, A. Grelard, E. J. Dufourc and I. D. Alves (2013). "The enhanced membrane interaction and perturbation of a cell penetrating peptide in the presence of anionic lipids: toward an understanding of its selectivity for cancer cells." Biochim Biophys Acta **1828**(6): 1457–1470.

Johansen, I. E. (1996). "Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo." Proc Natl Acad Sci U S A **93**(22): 12400–12405.

Joliot, A., C. Pernelle, H. Deagostini-Bazin and A. Prochiantz (1991). "Antennapedia homeobox peptide regulates neural morphogenesis." Proc Natl Acad Sci U S A **88**(5): 1864–1868.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman and P. Daszak (2008). "Global trends in emerging infectious diseases." Nature **451**(7181): 990–993.

Kaariainen, L. and T. Ahola (2002). "Functions of alphavirus nonstructural proteins in RNA replication." Prog Nucleic Acid Res Mol Biol **71**: 187–222.

Kaestner, L., A. Scholz and P. Lipp (2015). "Conceptual and technical aspects of transfection and gene delivery." Bioorg Med Chem Lett **25**(6): 1171–1176.

Kam, Y. W., D. Simarmata, A. Chow, Z. Her, T. S. Teng, E. K. Ong, L. Renia, Y. S. Leo and L. F. Ng (2012). "Early appearance of neutralizing immunoglobulin G3 antibodies is associated with chikungunya virus clearance and long-term clinical protection." J Infect Dis **205**(7): 1147–1154.

Kamrud, K. I., K. Alterson, M. Custer, J. Dudek, C. Goodman, G. Owens and J. F. Smith (2010). "Development and characterization of promoterless helper RNAs for the production of alphavirus replicon particle." J Gen Virol **91**(Pt 7): 1723–1727.

Kanai, R., H. Wakimoto, T. Cheema and S. D. Rabkin (2010). "Oncolytic herpes simplex virus vectors and chemotherapy: are combinatorial strategies more effective for cancer?" Future Oncol **6**(4): 619–634.

Kanazawa, T., H. Taki, K. Tanaka, Y. Takashima and H. Okada (2011). "Cell-penetrating peptide-modified block copolymer micelles promote direct brain delivery via intranasal administration." Pharm Res **28**(9): 2130–2139.

Karmali, P. P., V. R. Kotamraju, M. Kastantin, M. Black, D. Missirlis, M. Tirrell and E. Ruoslahti (2009). "Targeting of albumin-embedded paclitaxel nanoparticles to tumors." Nanomedicine **5**(1): 73–82.

Kaur, P. and J. J. Chu (2013). "Chikungunya virus: an update on antiviral development and challenges." Drug Discov Today **18**(19–20): 969–983.

Ketola, A., A. Hinkkanen, F. Yongabi, P. Furu, A. M. Maatta, T. Liimatainen, R. Pirinen, M. Bjorn, T. Hakkarainen, K. Makinen, J. Wahlfors and R. Pellinen (2008). "Oncolytic Semliki forest virus vector as a novel candidate against unresectable osteosarcoma." Cancer Res **68**(20): 8342–8350.

Khan, A. H., K. Morita, C. Parquet Md Mdel, F. Hasebe, E. G. Mathenge and A. Igarashi (2002). "Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site." J Gen Virol **83**(Pt 12): 3075–3084.

Khan, M., R. Dhanwani, J. S. Kumar, P. V. Rao and M. Parida (2014). "Comparative evaluation of the diagnostic potential of recombinant envelope proteins and native cell culture purified viral antigens of Chikungunya virus." J Med Virol **86**(7): 1169–1175.

Kielian, M., C. Chanel-Vos and M. Liao (2010). "Alphavirus Entry and Membrane Fusion." Viruses **2**(4): 796–825.

Kim, D. Y., A. E. Firth, S. Atasheva, E. I. Frolova and I. Frolov (2011). "Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution." J Virol **85**(16): 8022–8036.

Kim, H., S. Moodley and M. Liu (2015). "TAT cell-penetrating peptide modulates inflammatory response and apoptosis in human lung epithelial cells." Drug Deliv Transl Res **5**(3): 275–278.

Kim, K. H., T. Rumenapf, E. G. Strauss and J. H. Strauss (2004). "Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts." Virology **323**(1): 153–163.

Kim, K. H., E. G. Strauss and J. H. Strauss (2000). "Adaptive mutations in Sindbis virus E2 and Ross River virus E1 that allow efficient budding of chimeric viruses." J Virol **74**(6): 2663–2670.

Kizil, C., A. Iltzsche, A. K. Thomas, P. Bhattarai, Y. Zhang and M. Brand (2015). "Efficient Cargo Delivery into Adult Brain Tissue Using Short Cell-Penetrating Peptides." PLoS One **10**(4): e0124073.

Koren, E. and V. P. Torchilin (2012). "Cell-penetrating peptides: breaking through to the other side." Trends Mol Med **18**(7): 385–393.

Kulasegaran-Shylini, R., S. Atasheva, D. G. Gorenstein and I. Frolov (2009). "Structural and functional elements of the promoter encoded by the 5' untranslated region of the Venezuelan equine encephalitis virus genome." J Virol **83**(17): 8327–8339.

Ladomery, M. (2013). "Aberrant alternative splicing is another hallmark of cancer." Int J Cell Biol **2013**: 463786.

Lee, J. S., J. H. Lee, H. Poo, M. S. Kim, S. H. Lee, M. H. Sung and C. J. Kim (2006). "Growth inhibitory effect of triple anti-tumor gene transfer using Semliki Forest virus vector in glioblastoma cells." Int J Oncol **28**(3): 649–654.

Lee, R. C. and J. J. Chu (2015). "Proteomics profiling of chikungunya-infected Aedes albopictus C6/36 cells reveal important mosquito cell factors in virus replication." PLoS Negl Trop Dis **9**(3): e0003544.

Lehto, T., A. Castillo Alvarez, S. Gauck, M. J. Gait, T. Coursindel, M. J. Wood, B. Lebleu and P. Boisguerin (2014). "Cellular trafficking determines the exon skipping activity of Pip6a-PMO in mdx skeletal and cardiac muscle cells." Nucleic Acids Res **42**(5): 3207–3217.

Leitner, W. W., E. S. Bergmann-Leitner, L. N. Hwang and N. P. Restifo (2006). "Type I Interferons are essential for the efficacy of replicase-based DNA vaccines." Vaccine **24**(24): 5110–5118.

Leitner, W. W., L. N. Hwang, E. S. Bergmann-Leitner, S. E. Finkelstein, S. Frank and N. P. Restifo (2004). "Apoptosis is essential for the increased efficacy of alphaviral replicase-based DNA vaccines." Vaccine **22**(11–12): 1537–1544.

Leitner, W. W., H. Ying, D. A. Driver, T. W. Dubensky and N. P. Restifo (2000). "Enhancement of tumor-specific immune response with plasmid DNA replicon vectors." Cancer Res **60**(1): 51–55.

Lemm, J. A., T. Rumenapf, E. G. Strauss, J. H. Strauss and C. M. Rice (1994). "Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis." EMBO J **13**(12): 2925–2934.

Leung, J. Y., M. M. Ng and J. J. Chu (2011). "Replication of alphaviruses: a review on the entry process of alphaviruses into cells." Adv Virol **2011**: 249640.

Li, G. and C. M. Rice (1993). "The signal for translational readthrough of a UGA codon in Sindbis virus RNA involves a single cytidine residue immediately downstream of the termination codon." J Virol **67**(8): 5062–5067.
Liljestrom, P., S. Lusa, D. Huylebroeck and H. Garoff (1991). "In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release." J Virol **65**(8): 4107–4113.
Liljeström, P. and H. Garoff (1991). "A new generation of animal cell expression vectors based on the Semliki Forest virus replicon." Biotechnology (N Y) **9**(12): 1356–1361.
Liu, H., W. Zhang, L. Ma, L. Fan, F. Gao, J. Ni and R. Wang (2014). "The improved blood-brain barrier permeability of endomorphin-1 using the cell-penetrating peptide synB3 with three different linkages." Int J Pharm **476**(1–2): 1–8.
Ljungberg, K., A. C. Whitmore, M. E. Fluet, T. P. Moran, R. S. Shabman, M. L. Collier, A. A. Kraus, J. M. Thompson, D. C. Montefiori, C. Beard and R. E. Johnston (2007). "Increased immunogenicity of a DNA-launched Venezuelan equine encephalitis virus-based replicon DNA vaccine." J Virol **81**(24): 13412–13423.
Loewy, A., J. Smyth, C. H. von Bonsdorff, P. Liljestrom and M. J. Schlesinger (1995). "The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process." J Virol **69**(1): 469–475.
Lulla, A., V. Lulla and A. Merits (2012). "Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein." J Virol **86**(1): 553–565.
Lum, F. M., T. H. Teo, W. W. Lee, Y. W. Kam, L. Renia and L. F. Ng (2013). "An essential role of antibodies in the control of Chikungunya virus infection." J Immunol **190**(12): 6295–6302.
Lundström, K. (2005). "Biology and application of alphaviruses in gene therapy." Gene Ther **12 Suppl 1**: S92–97.
Lundström, K. (2012). "In vivo administration of recombinant alphavirus into rodents." Cold Spring Harb Protoc **2012**(8).
Lundström, K. (2015). "Semliki Forest Virus-Based Expression of Recombinant GPCRs." Methods Enzymol **556**: 331–350.
Madani, F., S. Lindberg, Ü. Langel, S. Futaki and A. Graslund (2011). "Mechanisms of cellular uptake of cell-penetrating peptides." J Biophys **2011**: 414729.
Malet, H., B. Coutard, S. Jamal, H. Dutartre, N. Papageorgiou, M. Neuvonen, T. Ahola, N. Forrester, E. A. Gould, D. Lafitte, F. Ferron, J. Lescar, A. E. Gorbalenya, X. de Lamballerie and B. Canard (2009). "The crystal structures of Chikungunya and Venezuelan equine encephalitis virus nsP3 macro domains define a conserved adenosine binding pocket." J Virol **83**(13): 6534–6545.
Marsh, M. and A. Helenius (2006). "Virus entry: open sesame." Cell **124**(4): 729–740.
Matsuda, T. and C. L. Cepko (2007). "Controlled expression of transgenes introduced by in vivo electroporation." Proc Natl Acad Sci U S A **104**(3): 1027–1032.
Mei, L., Q. Zhang, Y. Yang, Q. He and H. Gao (2014). "Angiopep-2 and activatable cell penetrating peptide dual modified nanoparticles for enhanced tumor targeting and penetrating." Int J Pharm **474**(1–2): 95–102.
Melton, J. V., G. D. Ewart, R. C. Weir, P. G. Board, E. Lee and P. W. Gage (2002). "Alphavirus 6K proteins form ion channels." J Biol Chem **277**(49): 46923–46931.
Mercer, J., M. Schelhaas and A. Helenius (2010). "Virus entry by endocytosis." Annu Rev Biochem **79**: 803–833.

Metcalf, T. U. and D. E. Griffin (2011). "Alphavirus-induced encephalomyelitis: antibody-secreting cells and viral clearance from the nervous system." J Virol **85**(21): 11490–11501.
Mi, S. and V. Stollar (1991). "Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli." Virology **184**(1): 423–427.
Mokhtarian, F., C. M. Huan, C. Roman and C. S. Raine (2003). "Semliki Forest virus-induced demyelination and remyelination – involvement of B cells and anti-myelin antibodies." J Neuroimmunol **137**(1–2): 19–31.
Morse, M. A., A. C. Hobeika, T. Osada, P. Berglund, B. Hubby, S. Negri, D. Niedzwiecki, G. R. Devi, B. K. Burnett, T. M. Clay, J. Smith and H. K. Lyerly (2010). "An alphavirus vector overcomes the presence of neutralizing antibodies and elevated numbers of Tregs to induce immune responses in humans with advanced cancer." J Clin Invest **120**(9): 3234–3241.
Mourya, D. T. (1987). "Absence of transovarial transmission of Chikungunya virus in Aedes aegypti & Ae. albopictus mosquitoes." Indian J Med Res **85**: 593–595.
Mukhopadhyay, S., W. Zhang, S. Gabler, P. R. Chipman, E. G. Strauss, J. H. Strauss, T. S. Baker, R. J. Kuhn and M. G. Rossmann (2006). "Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses." Structure **14**(1): 63–73.
Murphy, A. M., M. M. Morris-Downes, B. J. Sheahan and G. J. Atkins (2000). "Inhibition of human lung carcinoma cell growth by apoptosis induction using Semliki Forest virus recombinant particles." Gene Ther **7**(17): 1477–1482.
Murphy, A. M., B. J. Sheahan and G. J. Atkins (2001). "Induction of apoptosis in BCL-2-expressing rat prostate cancer cells using the Semliki Forest virus vector." Int J Cancer **94**(4): 572–578.
Mäger, I., K. Langel, T. Lehto, E. Eiriksdottir and Ü. Langel (2012). "The role of endocytosis on the uptake kinetics of luciferin-conjugated cell-penetrating peptides." Biochim Biophys Acta **1818**(3): 502–511.
Nasar, F., G. Palacios, R. V. Gorchakov, H. Guzman, A. P. Da Rosa, N. Savji, V. L. Popov, M. B. Sherman, W. I. Lipkin, R. B. Tesh and S. C. Weaver (2012). "Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication." Proc Natl Acad Sci U S A **109**(36): 14622–14627.
Naslund, T. I., L. Kostic, E. K. Nordstrom, M. Chen and P. Liljestrom (2011). "Role of innate signalling pathways in the immunogenicity of alphaviral replicon-based vaccines." Virol J **8**: 36.
Neuvonen, M., A. Kazlauskas, M. Martikainen, A. Hinkkanen, T. Ahola and K. Saksela (2011). "SH3 domain-mediated recruitment of host cell amphiphysins by alphavirus nsP3 promotes viral RNA replication." PLoS Pathog **7**(11): e1002383.
Nguyen, L. T., X. Z. Yang, X. Du, J. W. Wang, R. Zhang, J. Zhao, F. J. Wang, Y. Dong and P. F. Li (2015). "Enhancing tumor-specific intracellular delivering efficiency of cell-penetrating peptide by fusion with a peptide targeting to EGFR." Amino Acids **47**(5): 997–1006.
Nikonov, A., T. Molder, R. Sikut, K. Kiiver, A. Mannik, U. Toots, A. Lulla, V. Lulla, A. Utt, A. Merits and M. Ustav (2013). "RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication." PLoS Pathog **9**(9): e1003610.
Nilsson, C., B. Makitalo, P. Berglund, F. Bex, P. Liljestrom, G. Sutter, V. Erfle, P. ten Haaft, J. Heeney, G. Biberfeld and R. Thorstensson (2001). "Enhanced simian immunodeficiency virus-specific immune responses in macaques induced by

priming with recombinant Semliki Forest virus and boosting with modified vaccinia virus Ankara." Vaccine **19**(25–26): 3526–3536.
Nogueira, M. L., M. C. Nogueira and C. Pacca (2011). "Arboviral encephalitis and RNAi treatment." Cent Nerv Syst Agents Med Chem **11**(4): 296–304.
Olson, E. S., T. A. Aguilera, T. Jiang, L. G. Ellies, Q. T. Nguyen, E. H. Wong, L. A. Gross and R. Y. Tsien (2009). "In vivo characterization of activatable cell penetrating peptides for targeting protease activity in cancer." Integr Biol (Camb) **1**(5–6): 382–393.
Owen, K. E. and R. J. Kuhn (1996). "Identification of a region in the Sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation." J Virol **70**(5): 2757–2763.
Panas, M. D., T. Ahola and G. M. McInerney (2014). "The C-terminal repeat domains of nsP3 from the Old World alphaviruses bind directly to G3BP." J Virol **88**(10): 5888–5893.
Park, E. and D. E. Griffin (2009). "The nsP3 macro domain is important for Sindbis virus replication in neurons and neurovirulence in mice." Virology **388**(2): 305–314.
Peck, R., A. Brown and C. J. Wust (1979). "In vitro heterologous cytotoxicity by T effector cells from mice immunized with Sindbis virus." J Immunol **123**(4): 1763–1766.
Peng, W., D. C. Peltier, M. J. Larsen, P. D. Kirchhoff, S. D. Larsen, R. R. Neubig and D. J. Miller (2009). "Identification of thieno[3,2-b]pyrrole derivatives as novel small molecule inhibitors of neurotropic alphaviruses." J Infect Dis **199**(7): 950–957.
Peranen, J., M. Rikkonen, P. Liljestrom and L. Kaariainen (1990). "Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2." J Virol **64**(5): 1888–1896.
Perera, R., C. Navaratnarajah and R. J. Kuhn (2003). "A heterologous coiled coil can substitute for helix I of the Sindbis virus capsid protein." J Virol **77**(15): 8345–8353.
Peränen, J., P. Laakkonen, M. Hyvonen and L. Kääriäinen (1995). "The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles." Virology **208**(2): 610–620.
Piver, E., C. Collin, A. Diatta, P. Vaudin and J. C. Pages (2005). "Cellular factors influencing Semliki Forest Virus vector biology." Gene Ther **12 Suppl 1**: S111–117.
Polo, J. M., B. A. Belli, D. A. Driver, I. Frolov, S. Sherrill, M. J. Hariharan, K. Townsend, S. Perri, S. J. Mento, D. J. Jolly, S. M. Chang, S. Schlesinger and T. W. Dubensky, Jr. (1999). "Stable alphavirus packaging cell lines for Sindbis virus and Semliki Forest virus-derived vectors." Proc Natl Acad Sci U S A **96**(8): 4598–4603.
Pooga, M., M. Hällbrink, M. Zorko and Ü. Langel (1998). "Cell penetration by transportan." FASEB J **12**(1): 67–77.
Priya, R., I. K. Patro and M. M. Parida (2014). "TLR3 mediated innate immune response in mice brain following infection with Chikungunya virus." Virus Res **189**: 194–205.
Pärn, K., L. Viru, T. Lehto, N. Oskolkov, Ü. Langel and A. Merits (2013). "Transfection of infectious RNA and DNA/RNA layered vectors of semliki forest virus by the cell-penetrating peptide based reagent PepFect6." PLoS One **8**(7): e69659.
Quetglas, J. I., M. Ruiz-Guillen, A. Aranda, E. Casales, J. Bezunartea and C. Smerdou (2010). "Alphavirus vectors for cancer therapy." Virus Res **153**(2): 179–196.

Ratnik, K., L. Viru and A. Merits (2013). "Control of the rescue and replication of Semliki Forest virus recombinants by the insertion of miRNA target sequences." PLoS One **8**(9): e75802.

Rausalu, K., A. Iofik, L. Ulper, L. Karo-Astover, V. Lulla and A. Merits (2009). "Properties and use of novel replication-competent vectors based on Semliki Forest virus." Virol J **6**: 33.

Rayner, J. O., S. A. Dryga and K. I. Kamrud (2002). "Alphavirus vectors and vaccination." Rev Med Virol **12**(5): 279–296.

Regberg, J., A. Srimanee and Ü. Langel (2012). "Applications of cell-penetrating peptides for tumor targeting and future cancer therapies." Pharmaceuticals (Basel) **5**(9): 991–1007.

Resina, S., R. Kole, A. Travo, B. Lebleu and A. R. Thierry (2007). "Switching on transgene expression by correcting aberrant splicing using multi-targeting steric-blocking oligonucleotides." J Gene Med **9**(6): 498–510.

Reynaud, J. M., D. Y. Kim, S. Atasheva, A. Rasalouskaya, J. P. White, M. S. Diamond, S. C. Weaver, E. I. Frolova and I. Frolov (2015). "IFIT1 Differentially Interferes with Translation and Replication of Alphavirus Genomes and Promotes Induction of Type I Interferon." PLoS Pathog **11**(4): e1004863.

Rheme, C., M. U. Ehrengruber and D. Grandgirard (2005). "Alphaviral cytotoxicity and its implication in vector development." Exp Physiol **90**(1): 45–52.

Rice, C. M. (1992). "Examples of expression systems based on animal RNA viruses: alphaviruses and influenza virus." Curr Opin Biotechnol **3**(5): 523–532.

Riezebos-Brilman, A., A. de Mare, L. Bungener, A. Huckriede, J. Wilschut and T. Daemen (2006). "Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy." J Clin Virol **35**(3): 233–243.

Riezebos-Brilman, A., J. Regts, E. J. Freyschmidt, B. Dontje, J. Wilschut and T. Daemen (2005). "Induction of human papilloma virus E6/E7-specific cytotoxic T-lymphocyte activity in immune-tolerant, E6/E7-transgenic mice." Gene Ther **12**(18): 1410–1414.

Rikkonen, M., J. Peranen and L. Kääriäinen (1994). "ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2." J Virol **68**(9): 5804–5810.

Rodriguez-Andres, J., S. Rani, M. Varjak, M. E. Chase-Topping, M. H. Beck, M. C. Ferguson, E. Schnettler, R. Fragkoudis, G. Barry, A. Merits, J. K. Fazakerley, M. R. Strand and A. Kohl (2012). "Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest virus." PLoS Pathog **8**(11): e1002977.

Rodriguez-Madoz, J. R., J. Prieto and C. Smerdou (2007). "Biodistribution and tumor infectivity of semliki forest virus vectors in mice: effects of re-administration." Mol Ther **15**(12): 2164–2171.

Rommelaere, J., K. Geletneky, A. L. Angelova, L. Daeffler, C. Dinsart, I. Kiprianova, J. R. Schlehofer and Z. Raykov (2010). "Oncolytic parvoviruses as cancer therapeutics." Cytokine Growth Factor Rev **21**(2–3): 185–195.

Rozen-Gagnon, K., K. A. Stapleford, V. Mongelli, H. Blanc, A. B. Failloux, M. C. Saleh and M. Vignuzzi (2014). "Alphavirus mutator variants present host-specific defects and attenuation in mammalian and insect models." PLoS Pathog **10**(1): e1003877.

Rubach, J. K., B. R. Wasik, J. C. Rupp, R. J. Kuhn, R. W. Hardy and J. L. Smith (2009). "Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro." Virology **384**(1): 201–208.

Ryman, K. D. and W. B. Klimstra (2008). "Host responses to alphavirus infection." Immunol Rev **225**: 27–45.

Sanz, M. A., V. Madan, L. Carrasco and J. L. Nieva (2003). "Interfacial domains in Sindbis virus 6K protein. Detection and functional characterization." J Biol Chem **278**(3): 2051–2057.

Sazani, P. and R. Kole (2003). "Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing." J Clin Invest **112**(4): 481–486.

Sawicki, D. L. and S. G. Sawicki (1993). "A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis." J Virol **67**(6): 3605–3610.

Schmidt, M. F., M. Bracha and M. J. Schlesinger (1979). "Evidence for covalent attachment of fatty acids to Sindbis virus glycoproteins." Proc Natl Acad Sci U S A **76**(4): 1687–1691.

Schnettler, E., C. L. Donald, S. Human, M. Watson, R. W. Siu, M. McFarlane, J. K. Fazakerley, A. Kohl and R. Fragkoudis (2013). "Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells." J Gen Virol **94**(Pt 7): 1680–1689.

Shin, M. C., J. Zhang, K. A. Min, K. Lee, C. Moon, J. P. Balthasar and V. C. Yang (2014). "Combination of antibody targeting and PTD-mediated intracellular toxin delivery for colorectal cancer therapy." J Control Release **194**: 197–210.

Shirako, Y., E. G. Strauss and J. H. Strauss (2000). "Suppressor mutations that allow sindbis virus RNA polymerase to function with nonaromatic amino acids at the N-terminus: evidence for interaction between nsP1 and nsP4 in minus-strand RNA synthesis." Virology **276**(1): 148–160.

Singh, V. K., G. P. t. Damewood, R. M. Friedman and R. K. Maheshwari (1987). "Tunicamycin enhances virus replication and inhibits antiviral activity of interferon in mice: correlation with natural killer cells." J Exp Pathol **3**(1): 19–33.

Siu, R. W., R. Fragkoudis, P. Simmonds, C. L. Donald, M. E. Chase-Topping, G. Barry, G. Attarzadeh-Yazdi, J. Rodriguez-Andres, A. A. Nash, A. Merits, J. K. Fazakerley and A. Kohl (2011). "Antiviral RNA interference responses induced by Semliki Forest virus infection of mosquito cells: characterization, origin, and frequency-dependent functions of virus-derived small interfering RNAs." J Virol **85**(6): 2907–2917.

Slovin, S. F., M. Kehoe, R. Durso, C. Fernandez, W. Olson, J. P. Gao, R. Israel, H. I. Scher and S. Morris (2013). "A phase I dose escalation trial of vaccine replicon particles (VRP) expressing prostate-specific membrane antigen (PSMA) in subjects with prostate cancer." Vaccine **31**(6): 943–949.

Smerdou, C. and P. Liljestrom (1999). "Two-helper RNA system for production of recombinant Semliki forest virus particles." J Virol **73**(2): 1092–1098.

Smith, T. J., R. H. Cheng, N. H. Olson, P. Peterson, E. Chase, R. J. Kuhn and T. S. Baker (1995). "Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy." Proc Natl Acad Sci U S A **92**(23): 10648–10652.

Smyth, J. W., M. N. Fleeton, B. J. Sheahan and G. J. Atkins (2005). "Treatment of rapidly growing K-BALB and CT26 mouse tumours using Semliki Forest virus and its derived vector." Gene Ther **12**(2): 147–159.

Snyder, J. E., K. A. Kulcsar, K. L. Schultz, C. P. Riley, J. T. Neary, S. Marr, J. Jose, D. E. Griffin and R. J. Kuhn (2013). “Functional characterization of the alphavirus TF protein.” J Virol **87**(15): 8511–8523.

Steel, J. J., B. R. Henderson, S. B. Lama, K. E. Olson and B. J. Geiss (2011). “Infectious alphavirus production from a simple plasmid transfection+." Virol J **8**: 356.

Steele, K. E. and N. A. Twenhafel (2010). “REVIEW PAPER: pathology of animal models of alphavirus encephalitis.” Vet Pathol **47**(5): 790–805.

Strauss, J. H. and E. G. Strauss (1994). “The alphaviruses: gene expression, replication, and evolution.” Microbiol Rev **58**(3): 491–562.

Stylianopoulos, T., J. D. Martin, V. P. Chauhan, S. R. Jain, B. Diop-Frimpong, N. Bardeesy, B. L. Smith, C. R. Ferrone, F. J. Hornicek, Y. Boucher, L. L. Munn and R. K. Jain (2012). “Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors.” Proc Natl Acad Sci U S A **109**(38): 15101–15108.

Suhrbier, A., M. C. Jaffar-Bandjee and P. Gasque (2012). “Arthritogenic alphaviruses – an overview.” Nat Rev Rheumatol **8**(7): 420–429.

Suhrbier, A. and S. Mahalingam (2009). “The immunobiology of viral arthritides.” Pharmacol Ther **124**(3): 301–308.

Sundback, M., I. Douagi, C. Dayaraj, M. N. Forsell, E. K. Nordstrom, G. M. McInerney, K. Spangberg, L. Tjader, E. Bonin, M. Sundstrom, P. Liljestrom and G. B. Karlsson Hedestam (2005). “Efficient expansion of HIV-1-specific T cell responses by homologous immunization with recombinant Semliki Forest virus particles.” Virology **341**(2): 190–202.

Suomalainen, M., P. Liljestrom and H. Garoff (1992). “Spike protein-nucleocapsid interactions drive the budding of alphaviruses.” J Virol **66**(8): 4737–4747.

Suzme, R., J. C. Tseng, B. Levin, S. Ibrahim, D. Meruelo and A. Pellicer (2012). “Sindbis viral vectors target hematopoietic malignant cells.” Cancer Gene Ther **19**(11): 757–766.

Svasti, S., T. Suwanmanee, S. Fucharoen, H. M. Moulton, M. H. Nelson, N. Maeda, O. Smithies and R. Kole (2009). “RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice.” Proc Natl Acad Sci U S A **106**(4): 1205–1210.

Zanuy, D., A. Flores-Ortega, J. Casanovas, D. Curco, R. Nussinov and C. Aleman (2008). “The energy landscape of a selective tumor-homing pentapeptide.” J Phys Chem B **112**(29): 8692–8700.

Zayed, A., A. A. Awash, M. A. Esmail, H. A. Al-Mohamadi, M. Al-Salwai, A. Al-Jasari, I. Medhat, M. E. Morales-Betoulle and A. Mnzava (2012). “Detection of Chikungunya virus in Aedes aegypti during 2011 outbreak in Al Hodayda, Yemen.” Acta Trop **123**(1): 62–66.

Zeng, J., P. Fournier and V. Schirrmacher (2004). “High cell surface expression of Newcastle disease virus proteins via replicon vectors demonstrates syncytia forming activity of F and fusion promotion activity of HN molecules.” Int J Oncol **25**(2): 293–302.

Zeng, X., S. Mukhopadhyay and C. L. Brooks, 3rd (2014). “Residue-level resolution of alphavirus envelope protein interactions in pH-dependent fusion.” Proc Natl Acad Sci U S A **112**(7): 2034–2039.

Zeng, Y., X. Gu, Y. Chen, L. Gong, Z. Ren and S. Huang (1999). “Reversal of aberrant splicing of beta-thalassemia allele by antisense RNA in vitro and in vivo.” Chin Med J (Engl) **112**(2): 107–111.

Zhang, X., M. Fugere, R. Day and M. Kielian (2003). "Furin processing and proteolytic activation of Semliki Forest virus." J Virol **77**(5): 2981–2989.
Zhang, Y. and L. C. Yu (2008). "Single-cell microinjection technology in cell biology." Bioessays **30**(6): 606–610.
Zhong, Y. Q., J. Wei, Y. R. Fu, J. Shao, Y. W. Liang, Y. H. Lin, J. Liu and Z. H. Zhu (2008). "[Toxicity of cationic liposome Lipofectamine 2000 in human pancreatic cancer Capan-2 cells]." Nan Fang Yi Ke Da Xue Xue Bao **28**(11): 1981–1984.
Ziegler, S. A., L. Lu, A. P. da Rosa, S. Y. Xiao and R. B. Tesh (2008). "An animal model for studying the pathogenesis of chikungunya virus infection." Am J Trop Med Hyg **79**(1): 133–139.
Takkinen, K. (1986). "Complete nucleotide sequence of the nonstructural protein genes of Semliki Forest virus." Nucleic Acids Res **14**(14): 5667–5682.
Tamberg, N., V. Lulla, R. Fragkoudis, A. Lulla, J. K. Fazakerley and A. Merits (2007). "Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus." J Gen Virol **88**(Pt 4): 1225–1230.
Tamm, K., A. Merits and I. Sarand (2008). "Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus." J Gen Virol **89**(Pt 3): 676–686.
Teo, T. H., F. M. Lum, C. Claser, V. Lulla, A. Lulla, A. Merits, L. Renia and L. F. Ng (2013). "A pathogenic role for CD4+ T cells during Chikungunya virus infection in mice." J Immunol **190**(1): 259–269.
Thomas, J. M., W. B. Klimstra, K. D. Ryman and H. W. Heidner (2003). "Sindbis virus vectors designed to express a foreign protein as a cleavable component of the viral structural polyprotein." J Virol **77**(10): 5598–5606.
Tomar, S., R. W. Hardy, J. L. Smith and R. J. Kuhn (2006). "Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity." J Virol **80**(20): 9962–9969.
Toth, K., D. Dhar and W. S. Wold (2010). "Oncolytic (replication-competent) adenoviruses as anticancer agents." Expert Opin Biol Ther **10**(3): 353–368.
Trabulo, S., A. L. Cardoso, A. M. Cardoso, C. M. Morais, A. S. Jurado and M. C. Pedroso de Lima (2013). "Cell-penetrating peptides as nucleic acid delivery systems: from biophysics to biological applications." Curr Pharm Des **19**(16): 2895–2923.
Tseng, J. C., B. Levin, T. Hirano, H. Yee, C. Pampeno and D. Meruelo (2002). "In vivo antitumor activity of Sindbis viral vectors." J Natl Cancer Inst **94**(23): 1790–1802.
Tseng, J. C., B. Levin, A. Hurtado, H. Yee, I. Perez de Castro, M. Jimenez, P. Shamamian, R. Jin, R. P. Novick, A. Pellicer and D. Meruelo (2004). "Systemic tumor targeting and killing by Sindbis viral vectors." Nat Biotechnol **22**(1): 70–77.
Tseng, J. C., P. B. Zanzonico, B. Levin, R. Finn, S. M. Larson and D. Meruelo (2006). "Tumor-specific in vivo transfection with HSV-1 thymidine kinase gene using a Sindbis viral vector as a basis for prodrug ganciclovir activation and PET." J Nucl Med **47**(7): 1136–1143.
Tseng, J. C., Y. Zheng, H. Yee, D. E. Levy and D. Meruelo (2007). "Restricted tissue tropism and acquired resistance to Sindbis viral vector expression in the absence of innate and adaptive immunity." Gene Ther **14**(15): 1166–1174.
Tucker, P. C., S. H. Lee, N. Bui, D. Martinie and D. E. Griffin (1997). "Amino acid changes in the Sindbis virus E2 glycoprotein that increase neurovirulence improve entry into neuroblastoma cells." J Virol **71**(8): 6106–6112.

Tuittila, M. and A. E. Hinkkanen (2003). “Amino acid mutations in the replicase protein nsP3 of Semliki Forest virus cumulatively affect neurovirulence.” J Gen Virol **84**(Pt 6): 1525–1533.

Tuittila, M. T., M. G. Santagati, M. Roytta, J. A. Maatta and A. E. Hinkkanen (2000). “Replicase complex genes of Semliki Forest virus confer lethal neurovirulence.” J Virol **74**(10): 4579–4589.

Urban, C., C. Rheme, S. Maerz, B. Berg, R. Pick, R. Nitschke and C. Borner (2008). “Apoptosis induced by Semliki Forest virus is RNA replication dependent and mediated via Bak.” Cell Death Differ **15**(9): 1396–1407.

Utt, A., P. K. Das, M. Varjak, V. Lulla, A. Lulla and A. Merits (2015). “Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2.” J Virol **89**(6): 3145–3162.

Vajdy, M., J. Gardner, J. Neidleman, L. Cuadra, C. Greer, S. Perri, D. O'Hagan and J. M. Polo (2001). “Human immunodeficiency virus type 1 Gag-specific vaginal immunity and protection after local immunizations with sindbis virus-based replicon particles.” J Infect Dis **184**(12): 1613–1616.

van Duijnhoven, S. M., M. S. Robillard, K. Nicolay and H. Grull (2011). “Tumor targeting of MMP-2/9 activatable cell-penetrating imaging probes is caused by tumor-independent activation.” J Nucl Med **52**(2): 279–286.

van Duijnhoven, S. M., M. S. Robillard, K. Nicolay and H. Grull (2015). “In vivo biodistribution of radiolabeled MMP-2/9 activatable cell-penetrating peptide probes in tumor-bearing mice.” Contrast Media Mol Imaging **10**(1): 59–66.

Wang, F., Y. Wang, X. Zhang, W. Zhang, S. Guo and F. Jin (2014). “Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery.” J Control Release **174**: 126–136.

Wang, W., S. Abbad, Z. Zhang, S. Wang, J. Zhou and H. Lv (2015). “Cell-penetrating Peptides for Cancer-targeting Therapy and Imaging.” Curr Cancer Drug Targets.

Wang, X., J. P. Wang, M. F. Maughan and L. B. Lachman (2005). “Alphavirus replicon particles containing the gene for HER2/neu inhibit breast cancer growth and tumorigenesis.” Breast Cancer Res **7**(1): R145–155.

Wang, Y. F., S. G. Sawicki and D. L. Sawicki (1991). “Sindbis virus nsP1 functions in negative-strand RNA synthesis.” J Virol **65**(2): 985–988.

Vanlandingham, D. L., K. Tsetsarkin, C. Hong, K. Klingler, K. L. McElroy, M. J. Lehane and S. Higgs (2005). “Development and characterization of a double subgenomic chikungunya virus infectious clone to express heterologous genes in Aedes aegypti mosquitoes.” Insect Biochem Mol Biol **35**(10): 1162–1170.

Vasconcelos, L., F. Madani, P. Arukuusk, L. Pärnaste, A. Graslund and Ü. Langel (2014). “Effects of cargo molecules on membrane perturbation caused by transportan10 based cell-penetrating peptides.” Biochim Biophys Acta **1838**(12): 3118–3129.

Vasconcelos, L., K. Pärn and Ü. Langel (2013). “Therapeutic potential of cell-penetrating peptides.” Ther Deliv **4**(5): 573–591.

Vasiljeva, L., A. Merits, P. Auvinen and L. Kääriäinen (2000). “Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2.” J Biol Chem **275**(23): 17281–17287.

Vazeille, M., L. Mousson and A. B. Failloux (2009). “Failure to demonstrate experimental vertical transmission of the epidemic strain of Chikungunya virus in Aedes albopictus from La Reunion Island, Indian Ocean.” Mem Inst Oswaldo Cruz **104**(4): 632–635.

Vega-Rua, A., R. Lourenco-de-Oliveira, L. Mousson, M. Vazeille, S. Fuchs, A. Yebakima, J. Gustave, R. Girod, I. Dusfour, I. Leparc-Goffart, D. L. Vanlandingham, Y. J. Huang, L. P. Lounibos, S. Mohamed Ali, A. Nougairede, X. de Lamballerie and A. B. Failloux (2015). "Chikungunya virus transmission potential by local aedes mosquitoes in the americas and europe." PLoS Negl Trop Dis **9**(5): e0003780.

Veiman, K. L., K. Künnapuu, T. Lehto, K. Kiisholts, K. Pärn, Ü. Langel and K. Kurrikoff (2015). "PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo." J Control Release **209**: 238–247.

Weiss, B. G. and S. Schlesinger (1991). "Recombination between Sindbis virus RNAs." J Virol **65**(8): 4017–4025.

Wengler, G., A. Koschinski, G. Wengler and F. Dreyer (2003). "Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents." J Gen Virol **84**(Pt 1): 173–181.

Vives, E., P. Brodin and B. Lebleu (1997). "A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus." J Biol Chem **272**(25): 16010–16017.

Voss, J. E., M. C. Vaney, S. Duquerroy, C. Vonrhein, C. Girard-Blanc, E. Crublet, A. Thompson, G. Bricogne and F. A. Rey (2010). "Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography." Nature **468**(7324): 709–712.

Wu, S. R., L. Haag, L. Hammar, B. Wu, H. Garoff, L. Xing, K. Murata and R. H. Cheng (2007). "The dynamic envelope of a fusion class II virus. Prefusion stages of semliki forest virus revealed by electron cryomicroscopy." J Biol Chem **282**(9): 6752–6762.

Vähä-Koskela, M. J., J. P. Kallio, L. C. Jansson, J. E. Heikkila, V. A. Zakhartchenko, M. A. Kallajoki, V. M. Kahari and A. E. Hinkkanen (2006). "Oncolytic capacity of attenuated replicative semliki forest virus in human melanoma xenografts in severe combined immunodeficient mice." Cancer Res **66**(14): 7185–7194.

Vähä-Koskela, M. J., T. I. Kuusinen, J. C. Holmlund-Hampf, P. T. Furu, J. E. Heikkila and A. E. Hinkkanen (2007). "Semliki Forest virus vectors expressing transforming growth factor beta inhibit experimental autoimmune encephalomyelitis in Balb/c mice." Biochem Biophys Res Commun **355**(3): 776–781.

Vähä-Koskela, M. J., M. T. Tuittila, P. T. Nygardas, J. K. Nyman, M. U. Ehrengruber, M. Renggli and A. E. Hinkkanen (2003). "A novel neurotropic expression vector based on the avirulent A7(74) strain of Semliki Forest virus." J Neurovirol **9**(1): 1–15.

Ülper, L., I. Sarand, K. Rausalu and A. Merits (2008). "Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron." J Virol Methods **148**(1–2): 265–270.

Xiong, C., R. Levis, P. Shen, S. Schlesinger, C. M. Rice and H. V. Huang (1989). "Sindbis virus: an efficient, broad host range vector for gene expression in animal cells." Science **243**(4895): 1188–1191.

Xu, R., I. K. Srivastava, C. E. Greer, I. Zarkikh, Z. Kraft, L. Kuller, J. M. Polo, S. W. Barnett and L. Stamatatos (2006). "Characterization of immune responses elicited in macaques immunized sequentially with chimeric VEE/SIN alphavirus replicon particles expressing SIVGag and/or HIVEnv and with recombinant HIVgp140Env protein." AIDS Res Hum Retroviruses **22**(10): 1022–1030.

Yamanaka, R., S. A. Zullo, J. Ramsey, M. Onodera, R. Tanaka, M. Blaese and K. G. Xanthopoulos (2001). "Induction of therapeutic antitumor antiangiogenesis by intratumoral injection of genetically engineered endostatin-producing Semliki Forest virus." Cancer Gene Ther **8**(10): 796–802.

Yang, S. J., F. Revers, S. Souche, H. Lot, O. Le Gall, T. Candresse and J. Dunez (1998). "Construction of full-length cDNA clones of lettuce mosaic virus (LMV) and the effects of intron-insertion on their viability in Escherichia coli and on their infectivity to plants." Arch Virol **143**(12): 2443–2451.

Yang, Y., Y. Yang, X. Xie, X. Cai, Z. Wang, W. Gong, H. Zhang, Y. Li and X. Mei (2015). "A near-infrared two-photon-sensitive peptide-mediated liposomal delivery system." Colloids Surf B Biointerfaces **128**: 427–438.

Yao, H., K. Wang, Y. Wang, S. Wang, J. Li, J. Lou, L. Ye, X. Yan, W. Lu and R. Huang (2015). "Enhanced blood-brain barrier penetration and glioma therapy mediated by a new peptide modified gene delivery system." Biomaterials **37**: 345–352.

Ying, H., T. Z. Zaks, R. F. Wang, K. R. Irvine, U. S. Kammula, F. M. Marincola, W. W. Leitner and N. P. Restifo (1999). "Cancer therapy using a self-replicating RNA vaccine." Nat Med **5**(7): 823–827.

Ylösmaki, E., M. Martikainen, A. Hinkkanen and K. Saksela (2013). "Attenuation of Semliki Forest virus neurovirulence by microRNA-mediated detargeting." J Virol **87**(1): 335–344.

AKNOWLEDGEMENTS

I would like to extend my gratitude to all the people who have made this thesis possible either through good advice or practical help. This includes the work groups of Professor Andres Merits and Professor Ülo Langel, all the members of which have my sincere appreciation.

Although the members of both of the work groups have helped me in one way or another, I would like to name a few special people who have played an important role in my journey towards a scientific degree. From the alphavirus group, I would like to thank my first supervisor, Liane Viru, who has offered me a lot of knowledge and wisdom about molecular biology and life in general, I would also like to thank Eva Žusinaite and Margus Varjak, who have helped me with their expertise on the occasions where my own knowledge was not sufficient, also I am immensely grateful for all the help, guidance and suggestions received from Andres Merits. From the Peptide group I would like to thank Ülo Langel, who has accepted me as a Ph.D. student in his workgroup and has offered me the chance to author various publications and Kaido Kurrikoff, who has coordinated my activities during that period.

Last but not least, it gives me great pleasure to express my gratitude towards Professor Andres Metspalu from the Estonian Genome Center, who has sparked my interest in human genomics and motivated me to complete my thesis at a fast pace.

CURRICULUM VITAE

Name: Kalle Pärn
Date of Birth: 21.09.1988
Citizenship: Estonian
E-mail: kalle.parn@ut.ee

Education

2004–2005 Pärnu Sütevaka Secondary School of Humanities
2005–2007 Tartu Miina Härma Secondary School
2007–2010 University of Tartu, BSc (Gene Techonology)
2010–2012 University of Tartu, MSc (Biomedicine)
2012– University of Tartu, Institute of Technology, doctoral studies (Engineering and Technology)

Professional employment

2012–2014 University of Tartu, Institute of Technology, Specialist
2014– University of Tartu, Institute of Technology, Junior Scientist

Publications:

1. Viru, L., G. Heller, T. Lehto, **K. Pärn,** S. El Andaloussi, Ü. Langel and A. Merits (2011). "Novel viral vectors utilizing intron splice-switching to activate genome rescue, expression and replication in targeted cells." Virol J 8: 243.
2. Vasconcelos, L., **K. Pärn** and Ü. Langel (2013). "Therapeutic potential of cell-penetrating peptides." Ther Deliv 4(5): 573–591.
3. **Pärn, K.,** L. Viru, T. Lehto, N. Oskolkov, Ü. Langel and A. Merits (2013). "Transfection of infectious RNA and DNA/RNA layered vectors of semliki forest virus by the cell-penetrating peptide based reagent PepFect6." PLoS One **8**(7): e69659.
4. Veiman, K. L., K. Künnapuu, T. Lehto, K. Kiisholts, **K. Pärn,** Ü. Langel and K. Kurrikoff (2015). "PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo." J Control Release 209: 238–247.
5. **Pärn, K.,** Eriste, E. and Langel, Ü. (2015) Antiviral and antimicrobial applications of cell penetrating peptides. In: Cell-penetrating peptides. Methods and protocols, Second Edition. Űlo Langel, Editor, Methods in Molecular Biology. pp. (in print).

ELULOOKIRJELDUS

Nimi: Kalle Pärn
Sünniaeg: 21.09.1988
Kodakondsus: Estonian
E-post: kalle.parn@ut.ee

Haridus

2004–2005 Pärnu Sütevaka humanitaargümnaasium
2005–2007 Tartu Miina Härma gümnaasium
2007–2010 Tartu Ülikool, BSc (Geenitehnoloogia)
2010–2012 Tartu Ülikool, MSc (Biomeditsiin)
2012– Tartu Ülikool, tehnoloogiainstituut, doktorantuur (tehnika ja tehnoloogia)

Teenistuskäik

2012–2014 Tartu Ülikool, tehnoloogiainstituut, spetsialist
2014– Tartu Ülikool, tehnoloogiainstituut, nooremteadur

Publikatsioonid:

1. Viru, L., G. Heller, T. Lehto, **K. Pärn,** S. El Andaloussi, Ü. Langel and A. Merits (2011). "Novel viral vectors utilizing intron splice-switching to activate genome rescue, expression and replication in targeted cells." Virol J 8: 243.
2. Vasconcelos, L., **K. Pärn** and Ü. Langel (2013). "Therapeutic potential of cell-penetrating peptides." Ther Deliv 4(5): 573–591.
3. **Pärn, K.,** L. Viru, T. Lehto, N. Oskolkov, Ü. Langel and A. Merits (2013). "Transfection of infectious RNA and DNA/RNA layered vectors of semliki forest virus by the cell-penetrating peptide based reagent PepFect6." PLoS One **8**(7): e69659.
4. Veiman, K. L., K. Künnapuu, T. Lehto, K. Kiisholts, **K. Pärn,** Ü. Langel and K. Kurrikoff (2015). "PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo." J Control Release 209: 238–247.
5. **Pärn, K.,** Eriste, E. and Langel, Ü. (2015) Antiviral and antimicrobial applications of cell penetrating peptides. In: Cell-penetrating peptides. Methods and protocols, Second Edition. Űlo Langel, Editor, Methods in Molecular Biology. pp. (avaldamisel).

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto.** Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.
3. **Hannes Luidalepp**. Studies on the antibiotic susceptibility of *Escherichia coli*. Tartu 2012, 111 p.
4. **Vahur Zadin**. Modelling the 3D-microbattery. Tartu 2012, 149 p.
5. **Janno Torop**. Carbide-derived carbon-based electromechanical actuators. Tartu 2012, 113 p.
6. **Julia Suhorutšenko.** Cell-penetrating peptides: cytotoxicity, immunogenicity and application for tumor targeting. Tartu 2012, 139 p.
7. **Viktoryia Shyp.** G nucleotide regulation of translational GTPases and the stringent response factor RelA. Tartu 2012, 105 p.
8. **Mardo Kõivomägi.** Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in *Saccharomyces cerevisiae*. Tartu, 2013, 157 p.
9. **Liis Karo-Astover.** Studies on the Semliki Forest virus replicase protein nsP1. Tartu, 2013, 113 p.
10. **Piret Arukuusk**. NickFects–novel cell-penetrating peptides. Design and uptake mechanism. Tartu, 2013, 124 p.
11. **Piret Villo.** Synthesis of acetogenin analogues. Asymmetric transfer hydrogenation coupled with dynamic kinetic resolution of α-amido-β-keto esters. Tartu, 2013, 151 p.
12. **Villu Kasari.** Bacterial toxin-antitoxin systems: transcriptional cross-activation and characterization of a novel *mqsRA* system. Tartu, 2013, 108 p.
13. **Margus Varjak.** Functional analysis of viral and host components of alphavirus replicase complexes. Tartu, 2013, 151 p.
14. **Liane Viru.** Development and analysis of novel alphavirus-based multifunctional gene therapy and expression systems. Tartu, 2013, 113 p.
15. **Kent Langel.** Cell-penetrating peptide mechanism studies: from peptides to cargo delivery. Tartu, 2014, 115 p.
16. **Rauno Temmer**. Electrochemistry and novel applications of chemically synthesized conductive polymer electrodes. Tartu, 2014, 206 p.
17. **Indrek Must.** Ionic and capacitive electroactive laminates with carbonaceous electrodes as sensors and energy harvesters. Tartu, 2014, 133 p.
18. **Veiko Voolaid.** Aquatic environment: primary reservoir, link, or sink of antibiotic resistance? Tartu, 2014, 79 p.
19. **Kristiina Laanemets.** The role of SLAC1 anion channel and its upstream regulators in stomatal opening and closure of *Arabidopsis thaliana.* Tartu, 2015, 115 p.