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Alphaviral vectors as gene delivery tools for cancer therapy

DOCTORAL THESIS

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Abstract

Application of recombinant viral vectors has become one of the most explored strategies for cancer gene therapy. This is due to the unique ability of viruses, given the right conditions, to very specifically target, infect and kill cancer cells. Among therapeutic viruses, due to high-level transgenic expression and induction of p53-independent apoptosis in infected cells, alphaviral vectors are thought to harbor high potential.

The aim of this research was to optimize the efficient gene delivery strategy by investigating different mouse tumor models in conjunction with recombinant alphavirus vectors. Special attention was given to the application of recombinant particles and viral RNA and to the characterization of alphavirus oncotropism in different tumor models *in vitro* and *in vivo*. In parallel, we have detected intracellular mechanisms that could affect vector transduction and replication in cancer cells.

We have compared the biodistribution of recombinant SFV particles (recSFV) and naked viral RNA replicon (recRNA) in tumor-free, as well as 4T1 mammary tumor and B16 melanoma tumor-bearing mice, as a consequence of different vector administration strategies. In both tumor models, we could show for the first time the predominant tumor targeting-capabilities of recSFV at a reduced viral dose upon systemic virus inoculation.

The high potential of SFV recRNA as a biosafe approach for the development of therapeutic treatment was demonstrated. Intravenous inoculation of recRNA provided primary brain targeting, whereas intratumoral inoculation triggered high and localized transgene expression levels in tumors. To prolong transgene expression, we have tested several re-inoculation strategies of virus-based and RNA-based vectors. To enhance vector therapeutic potential, recSFV was applied in combination with different dosages of chemotherapeutic drug 5-fluorouracil to mammary tumor-bearing mice. This adjunct treatment facilitated intratumoral spread of recSFV and increased transgene synthesis resulting in significant reduction of tumor volume. This synergetic therapeutic effect was achieved due to enhanced vector permeability and inhibition of interferon (IFN) response.

Investigating alphavirus infectivity, we found that alphaviruses efficiently infected B16 mouse melanoma tumors *in vivo* via predominant tumor targeting. In contrast their transduction efficiency in B16 cells cultivated *in vitro* was blocked. Therefore, we hypothesize that the tumor microenvironment alters gene expression in B16 cells, leading to up-regulation of virus-binding receptors or factors associated with virus entry and replication. Subsequent proteome analysis comparing B16 mouse melanoma cells cultured *in vitro* versus B16 cells isolated from tumors, unrevealed 277 differently regulated proteins. Annotating these proteins with their biological and molecular functions, we could identify a complex of genes associated with antiviral responses and cytoskeleton organization, respectively that could determine alphavirus activity. Importantly, we observed a decrease in expression of interferon alpha (IFN-alpha) in tumor-isolated cells that resulted in the suppression of several IFN-regulated genes and pathways. We therefore speculate that these specific mechanisms may critically abrogate host cells' antiviral defense in tumors. Additionally, differences in the expression of genes that regulate cytoskeletal organization caused significant alterations in cell membrane elasticity. As a consequence, for the first time, the physical state of cell membranes could be associated with conditions favorable to alphavirus infection and replication.

Taken together, our findings extend our knowledge about alphaviruses and their therapeutic properties with respect to tumor targeting and organ-directed gene delivery. The studies also provide a foundation regarding their application in future, more clinically oriented, studies.

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Abbreviations

Gene abbreviations

Actc1	Actin
Adh7	Alcohol dehydrogenase class 4 mu/sigma chain
Aif1l	Allograft inflammatory factor 1-like
Aspm	Abnormal spindle-like microcephaly-associated protein homolog
Bcl-2	Apoptosis regulator Bcl-2
Cald1	Non-muscle caldesmon
Capg	Macrophage-capping protein
Cdk2	cyclin dependent kinase 2
Cd97	CD97 antigen
Cfl1	Cofilin-1
Crem	Four and a half LIM domains protein 5
Cryab	Alpha-crystallin B chain
Csf1	Macrophage colony-stimulating factor 1
Csnk2b	Casein kinase II subunit beta
Ctla-4	Cytotoxic T-lymphocyte protein 4
DC-SIGN	dendritic cell-specific HIV-1-binding protein
Dst	Dystonin
Ds-Red	Discosoma sp. red fluorescent protein
Dync1h1	Cytoplasmic dynein 1 heavy chain 1
Dynll2	Dynein light chain 2
Dynlt1	Dynein light chain Tctex-type 1
EGFP	Enhanced Green Fluorescent Protein
Etv6	Transcription factor ETV6
Flnc	Filamin-C
Gfap	Glial fibrillary acidic protein
GITR	Glucocorticoid-Induced TNFR family Relatedgene
GM-CSF	Granulocyte-macrophage colony-stimulating factor

Gpx1	Glutathione peroxidase 1
Gsto1	Glutathione S-transferase omega-1
Gstz1	Maleylacetoacetateisomerase
Her2/neu	Human epidermal growth factor receptor-2
Hist1h1a	Histone H1.1
Hist1h1t	Histone H1t
Hspb1	Heat shock protein beta-1
Hsp90aa1	Heat shock protein HSP 90-alpha
Ifi35	Interferon-induced 35 kDa protein homolog
Il-4	Interleukin 4
Il-12	Interleukin 12
Il-15	Interleukin 15
Il-18	Interleukin 18
IFN-alpha	Interferon alpha
IFN-beta	Interferon beta
Kif1a	Kinesin-like protein Kif1a
LacZ	β-galactosidase,
Lamp-1	lysosome-associated membrane protein 1
Lmna	Prelamin-A/C
L-SIGN	C-type lectin
Luc	Firefly luciferase
MAGE	Melanoma-associated antigen
Map7	Ensconsin
MART-1/Melan-A	Melanocytic proteinMelan
Mlph	Melanophilin
MUC18	Cell surface glycoprotein MUC18
Myo5a	Unconventional myosin-Va
Myo18a	Unconventional myosin-XVIIIa
Nfe2l2	Nuclear factor erythroid 2-related factor 2

Opa1	Dynamin-like 120 kDa protein
Pdcd4	Programmed cell death protein 4
Pmel17/gp100	Melanocyte protein pmel
Psca	Prostate stem cell antigen
Psma	Prostate-specific membrane antigen
Pycard	Apoptosis-associated speck-like protein containing a CARD
Rps21	40S ribosomal protein S21
Scrib	Protein scribble homolog
Sgtb	Small glutamine-rich tetratricopeptide repeat-containing protein beta
Ss18	Protein SSXT
Stat1	Signal transducer and activator of transcription 1
Steap	Metalloreductase
S100a11	Protein S100-A11
S100b	Protein S100-B
Timm10b	Mitochondrial import inner membrane translocase subunit Tim10 B
Tmod1	Tropomodulin-1
Trp-1	Tyrosinase-related protein-1
Trp-2	Tyrosinase-related protein-2
Tubb6	Tubulin beta-6 chain
Txnl1	Thioredoxin-like protein 1
VEGFR2	Vascular endothelial growth factor receptor 2

Other abbreviations

AAV	Adeno-associated virus
AFM	Atomic force microscopy
APCs	Antigen-presenting cells
CAN	Acetonitrile
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen

CRT	Calreticulin
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EMA	European Medicine's Agency
EPR	Enhanced permeability and retention
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration (USA)
FDR	False discovery rate
GO	Gene Ontology
Gln	Glutamine
Glu	Glutamic acid
HCC	Hepatocellular carcinoma
HDACi	Histone deacetylase inhibitors
HIV	Human immunodeficiencyvirus
HPV	Human papilloma virus
HSV	Herpes virus
H&E	Hematoxylin and eosin staining
i.p.	Intraperitoneal injection
i.t.	Intratumoral injection
i.u.	Infection units
i.v.	Intravenous injection
LC-MS	Liquid chromatography-mass spectrometry
Lys-C	Lysylendopeptidase
mAbs	Monoclonal antibodies

MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MLN	Mediastinal lymph nodes
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTT	MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole)
PANTHER	Protein ANalysisTHrough Evolutionary Relationships
PBS	Phosphate-buffered saline
PI	Propidium iodide
PKR	Protein kinase R
p53	Cellular tumor antigen p53
RC	Replication competent
RD	Replication deficient
recSFV	Recombinant Semliki Forest virus
recSIN	Recombinant Sindbis virus
RLUs	Relative light units
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous injection
siRNA	Small interfering RNA
ssDNA	Single-stranded DNA
TAA	Tumor-associated antigens
TFA	Trifluoroacetic acid
tRNA	Transfer RNA
VEE	Venezuelan Equine Encephalitis virus
VLP	Virus-like particles
v.p.	Viral particles
β-hCG	Human chorionic gonadotropin

5-FU	5-fluorouracil

Introduction

Oncolytic alphaviral systems were successfully used in various mouse tumor models. In clinics however, alphavirus-based cancer virotherapy is not yet considered a mature field. The major limitations for broad application of alphavirus are the lack of detail characterization of these viruses and understanding optimal treatment strategy. There is insufficient information about virus infectivity of different tumor models, distribution, transgene expression time, effect of vector re-administration and interrelationships between alphavirus infectivity and tumor microenvironment. Moreover, alphavirus-activity in combination with chemotherapeutic drugs is poorly explored.

Aim of the study

Our research focuses on characterization of alphavirus oncotropism and its oncolytic properties in cancer models *in vitro* and *in vivo.* The major aim of the study was the development of an optimal strategy for efficient alphaviral vector delivery and transgene expression in mouse tumors. To achieve a synergic therapeutic effect, application of recombinant alphavirus vectors in combination with classical chemotherapeutic drugs was tested. Special attention was given to the investigation of favorable intracellular conditions that promote alphavirus transduction and replication.

Objectives

This study has five major objectives which are also outlined in **Figure 1**

Objective 1: Generation of recombinant SFV and SIN viruses, their characterization as gene transfer vectors and evaluation of their utility in comparison with one and other in context with different cancer cell lines. On this basis, by analyzing susceptibility to viral infection, cytotoxicity and inhibition of cell proliferation, identify the most effective therapeutic vector for specific tumor cell lines

Objective 2: Investigation of recSFV virus-based and RNA-based vector biodistribution and infection ability in 4T1 mouse mammary tumor model.

Task 1: to investigate, by applying several vector inoculation modes, recSFV biodistribution and transgene expression level in tumor-free and 4T1 tumor-bearing mouse models.

Task 2: to investigate naked recSFV RNA biodistribution and transgene expression level in tumor-free and 4T1 tumor-bearing mice.

Task 3: to optimize recSFV and recSFV RNA vectors re-administration strategies in order to prolong transgene expression in tumor-free and 4T1 tumor-bearing mouse models.

Objective 3: Development of an optimal cancer treatment strategy using recombinant recSFV virus vector and chemotherapeutic drug 5-FU in 4T1 mouse mammary tumor models.

Task 1: to examine potential synergistic effects of recSFV and 5-FU on 4T1 cells *in vitro*.

Task 2: to explore whether combining recSFV vector and 5-FU treatment in 4T1 tumor-bearing mice would potentiate tumor clearance.

Task 3: to elucidate factors which underlie potential synergistic therapeutic effects of recSFV and 5-FU drug administration in 4T1 tumors *in vivo*.

Objective 4: Investigation of recSFV vectors' infection ability and biodistribution in a B16-F10 mouse melanoma tumor model.

Task 1: to assess recSFV biodistribution and transgene expression level in B16-F10 tumor-bearing mouse models in dependence with several vector inoculation modes.

Task 2: to compare recSFV infectivity of B16 melanoma cells at different conditions (*in vitro* and *ex vivo*)

Objective 5: By profiling B16 melanoma cells susceptible or non-susceptible for alphavirus infection, identify candidate genes that are associated with establishing favorable conditions for recSFV infection and replication.

Task 1: to compare protein profiles form B16 cells cultivated *in vitro* versus B16 cells isolated from tumor (*ex vivo*).

Task 2: to analyze in-depth these proteins to understand biological mechanisms that govern alphavirus infectivity

Task 3: to compare the physical features of cell membranes from B16 cultured *in vitro* and tumor isolated B16 cells (*ex vivo*) using atomic force microscopy (AFM).

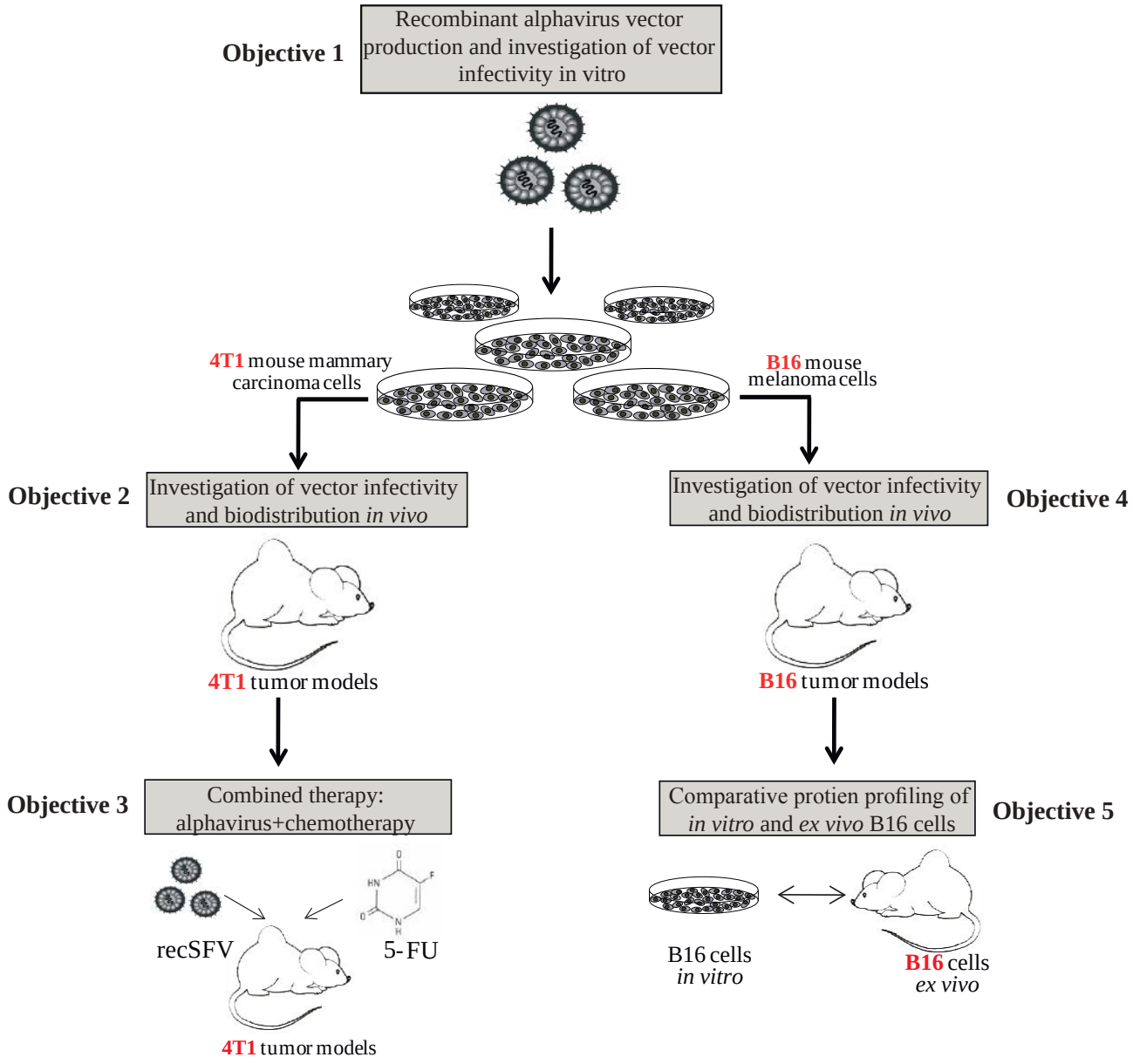

Figure 1: Schematic representation of the objectives. Objective 1 includes alphavirus infection of different cancer cell lines *in vitro*, where 2 cell lines, namely 4T1 and B16 cells, were selected for further experiments. Objective 2 and 3 involve *in vitro* and *in vivo* experiments using 4T1 cells. Objective 4 and 5 contain *in vitro*, *in vivo* and *ex vivo* experiments using the B16-F10 cell line.

List of papers

Original paper I: Vasilevska J, Skrastina D, Spunde K, Garoff H, Kozlovska T, Zajakina A. Semliki Forest virus biodistribution in tumor-free and 4T1 mammary tumor-bearing mice: a comparison of transgene delivery by recombinant virus particles and naked RNA replicon. *Cancer Gene Therapy J* 2012, 19(8): 579-587.

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Original paper III: Vasilevska J, De Souza GA, Stensland M, Skrastina D, Zhulenkovs D, Paplausks R, Kurena B, Kozlovska T, Zajakina A. Comparative protein profiling of B16 mouse melanoma cells susceptible and non-susceptible to alphavirus infection: effect of the tumor microenvironment. *Cancer Biology&Therapy* 2016, 11: 1-16.

Review paper I: Zajakina A, Vasilevska J, Kozlovska T, Lundstrom K. Alphavirus vectors for cancer treatment. *Viral Nanotechnology, 1st Edition* 2015. Boca Raton: CRC Press, Taylor & Francis Group, 467-485.

(* equal contribution)

Conferences

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Review of the literature

1.1 Cancer gene therapy and viral vectors; introduction

Gene therapy is a relatively new paradigm in medicine with enormous therapeutic potential. Actually, almost 2000 clinical trials using gene therapy were approved worldwide in period from 1989 to 2016 (Gene Therapy Clinical Trials Worldwide). Initially, therapeutic transgenes were delivered via non-specific vectors that induced toxicity to the cancer cells but to normal cells as well. Thus, viruses represent an attractive vehicle for cancer gene therapy due to their capacity to effectively deliver genes to their specific targets. The idea of using viral replication triggering subsequent cell destruction for treatment of malignant diseases emerged for the first time more than a century ago. A report by Dock in 1904 documented dramatic remission of leukemia in a patient who suffered from influenza infection (Dock, 1904). In nowadays, viruses are being investigated in three main gene therapy treatment approaches: immunotherapy, gene transfer and oncolytic virotherapy. In virus-based cancer immunotherapy genetically modified cells and viral particles stimulate the immune system to destroy cancer cells. For gene transfer, that is a more recent treatment modality, transgenes are directly delivered into cancerous cells. In comparison, oncolytic virotherapy uses viral particles that replicate within the cancer cell subsequently causing cell death.

Recent reviews have summarized therapeutic achievements of viral vector-based cancer treatment in completed and ongoing clinical trials (Donnelly et al., 2012, Fukuhara H et al., 2016; Gong et al., 2016; Russell et al., 2012) as follows: as in preclinical studies, they support the key concepts of efficient therapy: i) successful viral vectors should have predominant tumor tropism to be able to target metastatic cancer after systemic vector infusion, ii) physical barriers that limit vector distribution within the tumors can be overcome by additional chemical adjuvants, and iii) immune cells and tumor microenvironment can either be synergic and inhibiting factors for virus vector activity.

Despite several types of viruses, including retrovirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus, have been modified to enhance their efficacy in gene therapy applications; all these vector systems have unique advantages and limitations. For instance, retroviral vectors can permanently integrate into the host's genome, but require mitotic cell division for transduction. Adenoviral vectors can efficiently deliver genes to a wide variety of dividing and non-dividing cell types, but immune elimination of infected cells often limits gene expression *in vivo.* AAV also infects many non-dividing and dividing cell types, but has a limited DNA capacity. Herpes simplex virus can deliver large amounts of exogenous DNA; however, cytotoxicity and

maintenance of transgene expression remain as obstacles. Since 1950's, safety and efficacy of several viral vectors including adenovirus, retrovirus, AAV virus (Chira et al., 2015) as well as Semliki Forest virus, Newcastle virus, Sendai virus, rabies virus, measles virus, mumps virus, influenza virus and others have been reported in several anecdotal and formal clinical trials (Chiocca et al., 2002). Nevertheless, only in 2015 the U.S. Food and Drug Administration (FDA) and the European Medicine's Agency (*EMA*) approved the first virus-based advanced therapy "OncoVEX$^{GM\text{-}CSF}$" for melanoma treatment. OncoVEX$^{GM\text{-}CSF}$ is a genetically modified herpes simplex virus type 1 designed to replicate within tumors and produce an immunostimulatory protein called granulocyte-macrophage colony-stimulating factor (GM-CSF). The latter molecule induces cell death and promotes an anti-tumor immune response (Pol et al., 2015). Additionally, according to BioCentury, many clinical trials with several viral vectors are currently ongoing. Among these two viral vectors are tested in phase III trials, nine in phase II and at least eight in phase I. Probably these numbers will increase further (Cuickner-Meisne, 2016), what supports the positive dynamics of virotherapy in cancer. Taken together this clearly emphasizes the necessity of additional preclinical studies aiming at exploiting viruses for designing novel strategies for cancer treatment.

1.2 Alphavirus

1.2.1 Alphavirus structure and replication

Alphaviruses are enveloped viruses that belong to the *Togaviridae* family which are predominantly transmitted to vertebrates by mosquitoes.The classical vectors for the expression of heterologous genes were developed based on Semliki Forest virus (SFV) (Liljestrom and Garoff, 1991), Sindbis virus (SIN) (Xiong et al., 1989) and Venezuelan equine encephalitis virus replicons (VEE) (Davis et al., 1989). Alphaviruses contain approximately 11.5 kb long capped and polyadenylated positive-strand RNA genome that is functionally divided in two parts (two open reading frames ORF) coding the nonstructural and structural proteins correspondingly (**Figure 2A**).Upon delivery into the cytoplasm, from the first ORF the genomic RNA is processed into four viral nonstructural proteins nsP1-4 making the replication complex. The role of each nsP protein is different. Briefly, nsP1 protein initiates the negative strand RNA synthesis and capping of virus RNAs. NsP2 is the protease responsible for processing of the viral replication proteins (Strauss and Strauss, 1994). No enzymatic function was shown for the nsP3, however, it is essential for the synthesis of negative strand and SG RNAs (LaStarza et al., 1994). NsP4 acts as catalytic subunit of the RNA polymerase in the alphavirus replication complex (Strauss and Strauss, 1994). Together, this complex mediates replication of the plus strand (42S) genome into full-length minus strands that is later transcribed

into new 42S genomic RNA and 26S subgenomic (SG) RNA. The SG RNA internally initiated at the 26S promoter on the 42S minus RNA strands (**Figure 2B**). The second ORF codes for the structural proteins which also are synthesized as a large polyprotein to produce capsid protein (C) and the three envelope proteins (p62, which is the precursor of E2, 6K, and E1) (Hahn and Strauss 1990; Schlesinger and Schlesinger, 2001). After synthesis, the C proteins complex with genomic RNA into nucleocapsid structures in the cell cytoplasm. Only the genomic RNA could be packaged because of the encapsidation signal that is located within the nsP1 gene for SIN and in the nsP2 gene for SFV (Frolova et al., 1997). Cleavage of the intermediates of the other structural proteins is required for the encapsidation and budding of infectious viral particles (Garoff et al., 2004).

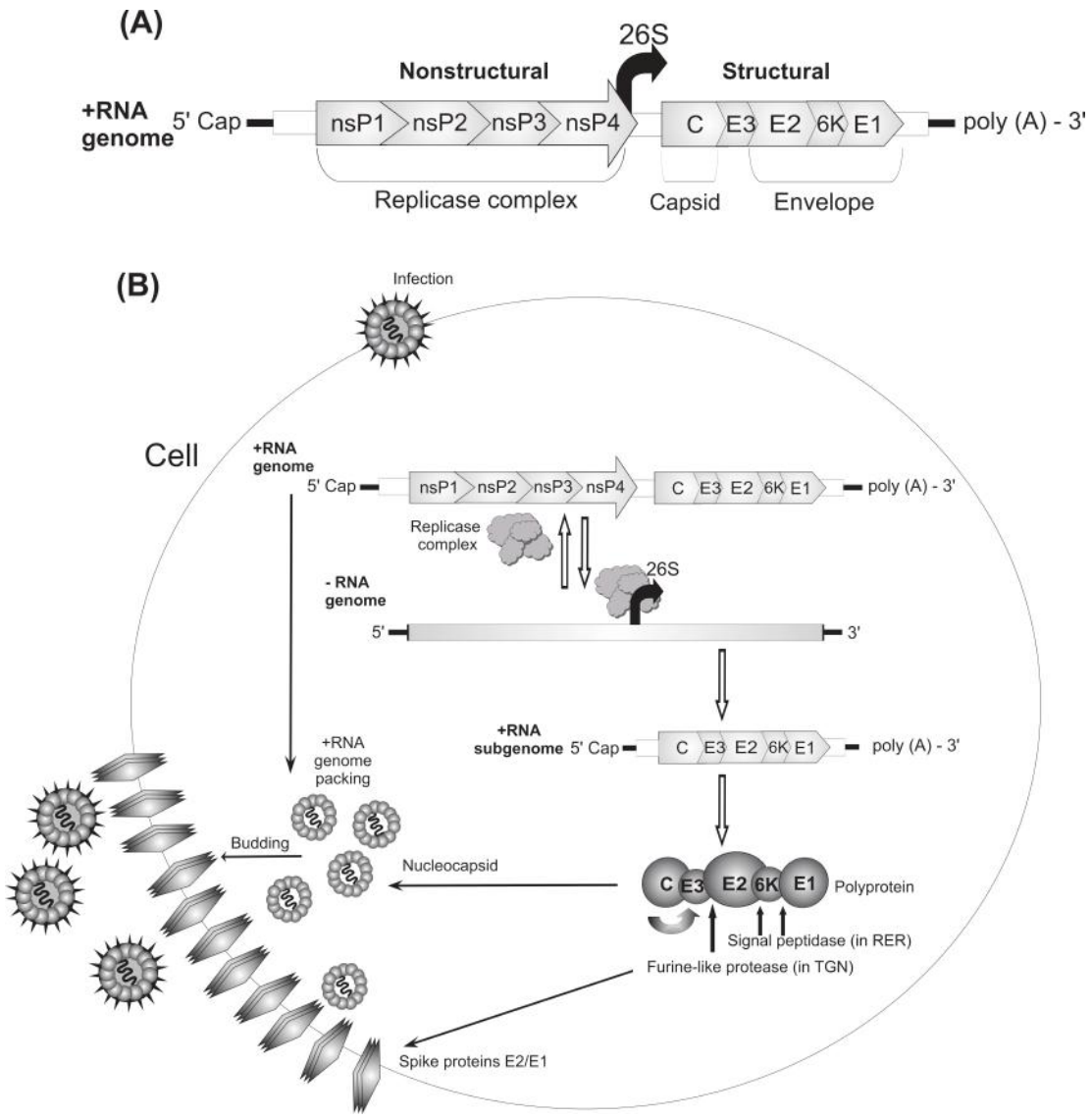

Figure 2: Genome structure (A) and replication cycle (B) of alphaviruses. RER – rough endoplasmic reticulum; TGN – trans Golgi network; NC – nucleocapsid.

In most cases replication of alphavirus RNA drives cell death in infected cells. Alphaviruses may

have developed multiple strategies to induce programmed cell death via the mitochondria. One of the main players in apoptosis induction by alphavirus infection is the host protein kinase R (PKR) (Balachandran et al., 2000; Gorchakov et al., 2004; Venticinque et al., 2010). PKR activation results in significant changes to the cell, which manifest as both cellular stress and translational inhibition through eIF2a phosphorylation (Williams, 2001; Williams, 1999). Another death mechanism is triggered by activation of proapoptotic*bcl-2* family members *Bax* and *Bak* leading to the release of cytochrome c and other apoptotic factors (caspase 9, caspase 3 and caspase 7) (Urban et al., 2008; Scallan et al., 1997).

A key step in virus infection and replication is the entry of the viral particle into the host cell. Unfortunately, little is known about the viral determinants and cell surface receptors that modulate entry and replication. Various studies have reported that alphaviruses enter the cell via receptor-mediated endocytosis, involving multiple proteins implicated in virus absorption/transduction, such as heparan sulfate, laminin receptor, the major histocompatibility complex (MHC), DC-SIGN, L-SIGN, heat shock 70 protein, and α1β1 integrin, among others (Smit et al., 2002; Wang et all., 1992; Helenius et al., 1978; Klimstra et al., 2003; Ryan et al., 1992; La Linn et al., 2005). However, it is most likely that alphaviruses may utilize multiple surface proteins as receptors or alternative entry pathways in different cells.

1.2.2. Recombinant alphavirus vectors

Semliki Forest virus (SFV) and Sindbis virus (SIN) are the best-studied alphaviruses which have been used for the development of different gene expression systems. Three types of vectors have been engineered, namely: replication-deficient vectors, replication-competent vectors and layered DNA vectors (**Figure 3**).

Replication-deficient vectors or one-step infection RNA vectors.

For this application, vectors were generated in such a way where the alphavirus nonstructural and structural genes have been split on separate plasmid vectors. The expression vector plasmid contain only the nonstructural coding region, which is required for the production of the nsP1–4 replicase complex, 26S subgenomic promoter, and a multiple cloning site with several unique restriction sites for the foreign gene insertion under the control of SP6 RNA promoter for in vitro transcription of RNA. In the same manner, the structural genes are transcribed from helper vectors. For the production of the infectious particles, in vitro-transcribed recombinant and helper RNAs are cotransfected into packaging mammalian cells (usually BHK-21 cells) by either electroporation or applying transfection/lipofection reagents, leading to generation of replication-deficient particles.

The helper construct provides the structural proteins to assemble new virus particles, while the recombinant construct provides the nonstructural proteins for RNA replication of both recombinant and helper RNAs. The system is self-limiting because helper-RNAs are not encapsidated into new generated viral particles due to absence of encapsulation signal. The produced recombinant virus contains only recombinant RNA genome without structural genes encoded, thereby is able to infect animal cells and express the transgenes providing a one-step infection without propagation of new virus particles.

However, the production of replication-deficient alphavirus vectors has been complicated by the emergence of propagation-competent vector during the vector production. There have been several attempts to eradicate this problem, including construction of split helper systems for Sindbis, SFV and VEE vector production (Frolov et al., 1997; Pushko et al., 1997; Smerdou and, Liljestrom, 1999). Frolov *et al.* constructed chimeric split helper RNA for the production of alphavirus vectors. In this approach, the helper RNA was divided into two parts, one coding for capsid and the other coding for envelope glycoproteins (**Figure 3;** helper part).

Replication-competent RNA vectors

Replication-competent alphavirus vectors contain full virus genome encoding non-structural genes as well as structural genes. These vectors may contain two 26S promoters providing synthesis of two SG mRNAs: the first controls expression of the heterologous product, and the second controls production of virus structural proteins (**Figure 3**, see the structure of the replication-competent vector). For the expression of gene of interest, these vectors may synthesize the transgene as cleavable part of both structural and non-structural polyprotein (Thomas et al., 2003; Tamberg et al., 2007). In other cases, the insert is located in non-structural polyprotein genes and produced as a fusion protein (Atasheva et al., 2007). In contrast to one step infection vectors, replication-competent vectors have self-replicating RNA genome. They are able to produce infectious virus particles in host cell after infection and to spread from cell to cell. Despite replication-competent vectors could provide enhanced distribution and expression in infected tissue, they have been less frequently used for *in vitro* and *in vivo* application than replication-deficient vectors.

DNA-based vectors

DNA-based alphavirus vectors have been generated by replacement the SP6 RNA polymerase promoter with DNA promoter (e.g., CMV IE, RSV LTR) that makes it possible to express the transgenes directly from alphavirus plasmid DNA (**Figure 3**, see alphavirus DNA vector). The introduction of this DNA-based vector into host cells provides transient expression of the transgene, however, the efficacy of gene delivery in this case fully relies on the efficacy of transfection

technique. To produce recombinant viral particles, the DNA-based expression vector could be cotransfected with DNA-based helper vector, however, using RNA-based vectors the number of the obtained virus particles could be 10^2-10^3 higher (Diciommo and Bremner, 1998).

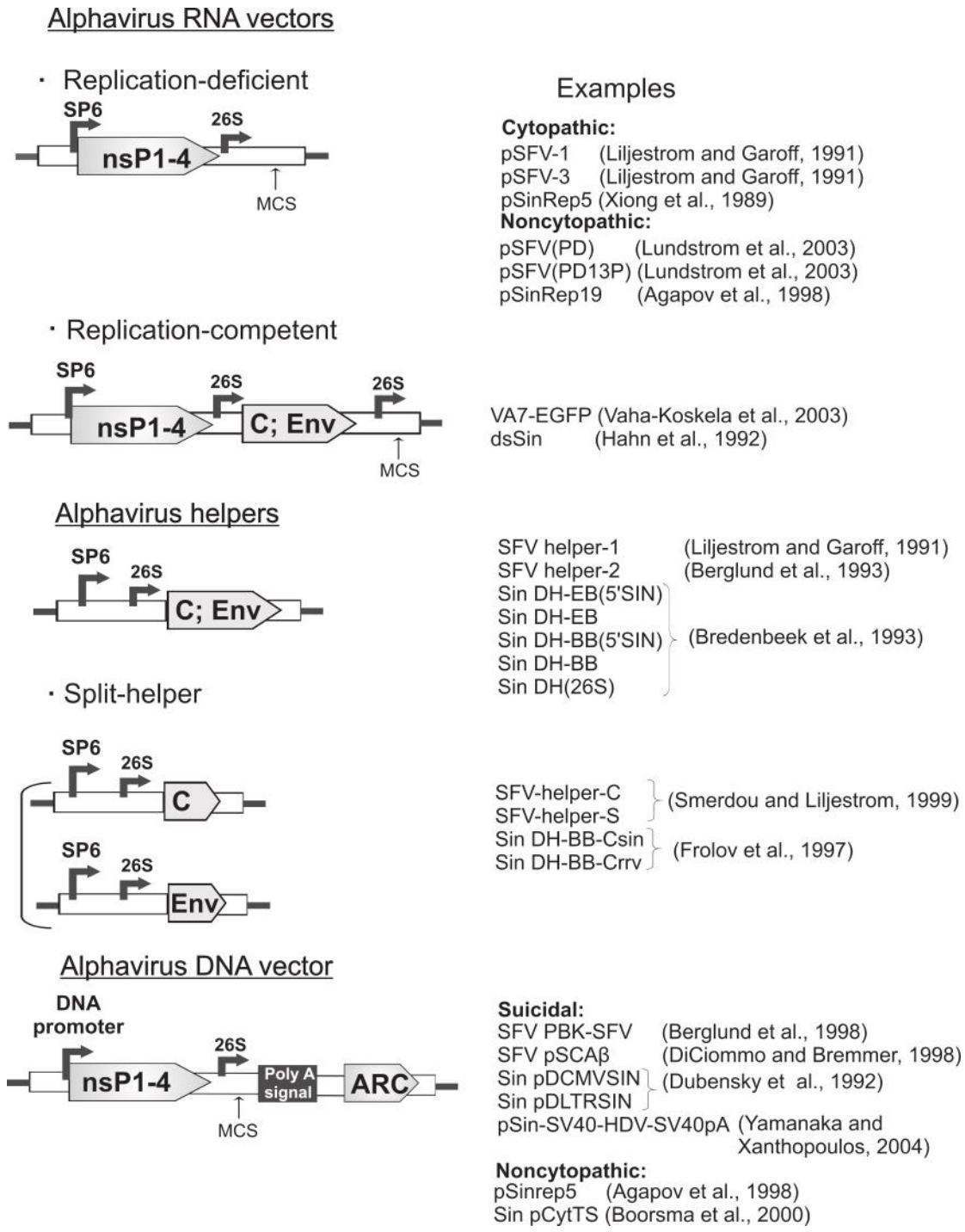

Figure 3: Schematic representation of recombinant alphavirus-based constructs. The major vector types are demonstrated: replication-deficient RNA vectors, replication competent RNA vectors, alphavirus helpers and DNA layered vectors. The examples of each type of vector are shown for Semliki Forest (SFV) and Sindbis (SIN) viruses. MCS – multiple cloning site; ARC – antibiotic resistance cassette.

1.2.3. Alphavirus vectors as gene delivery tools in cancer therapy

The application range of the virus-based expression systems in gene therapy depends on several properties of the vectors, including packaging capacity, replication sufficiency, host range, tissue specific targeting, duration of transgene expression, and complexity and efficacy of the vector production. Alphavirus vectors have a lot of natural features that make them attractive for application in cancer gene therapy. The major advantages of the alphaviruses include (i) an ability to infect a broad host range of mammalian cells, (ii) induction of strong induction of p53-independent apoptosis and (iii) an ability to efficiently overcome immunological tolerance by the activation of innate antiviral pathways (Lundstrom, 2009; Quetglas et al., 2010; Osada et al., 2012). Replication of alphavirus-based systems is extremely efficient producing around 10^5 new virions per host cell (Berglund et al., 1993; Schlesinger, 1995). Vectors have an inserted gene size capacity of at least 5 kb and the efficacy of transgene production under favorable intracellular conditions reaches approximately 20% of the total cell protein (Pushko et al., 1997). Low specific immune response against the vector itself and the absence of vector pre-immunity allow application of alphaviruses in broad spectrum of gene therapy strategies.

The history of using the alphaviruses for cancer treatment started in 1975, when Griffith *et al.* has shown that pre-immunization with extract of fibrosarcoma cells infected with SFV has induced antitumoral protection against malignant tumor in treated mice (Griffith et al., 1975). In nowadays, cancer virotherapy is based not only on stimulation of anti-tumor immune response, but also on the ability of the viruses to preferentially infect and kill cancer cells. The possibility to apply systemic administration modes of alphaviruses for predominant infection of cancer cells would open a new door to widespread application of the vectors. Systemic administration may be the most effective method to reach various metastatic sites and is much more amenable than a surgical procedure from a translational perspective. Thus far, only SIN has been considered to be capable of targeting tumors upon systemic injection of the vector in mouse models (Meruelo, 2004; Tseng et al., 2004; Unno et al., 2005; Tseng et al 2010; Suzme et al 2012). In contrast, specific tumor targeting by SFV vectors has not been confirmed *in vivo* until 2012 and vectors were administrated by direct intratumoral route in most studies (Murphy et al., 2000; Rodriguez-Madoz et al., 2007; Maatta et al., 2007; Quetglas et al., 2012b)**.** Only recently, we have demonstrated that SFV has a predominant tumor tropism upon systemic injection with a reduced viral dose, proving that tumor-targeted delivery by the vector could be possible under certain conditions (Vasilevska et al., 2012, **Paper I**).

Alphaviruses have been tested as therapeutic approaches in different ways to establish the best strategy for efficient cancer therapy. Positive therapeutic effect has been achieved in several mouse

tumor models after alphavirus inoculation due to natural oncolytic properties of the vector. Rapid induction of apoptosis in the infected tumor cells affected the tumor growth, however, this strategy did not result in complete tumor regression (Asselin-Paturel et al., 1999; Chikkanna-Gowda et al., 2005; Smyth et al., 2005). In contrast, treatment based on high level expression of immunomodulator genes, such as cytokines or growth factors by the vectors resulted in significant inhibition of tumor development or complete regression in animal models (Asselin-Paturel et al., 1999; Rodriguez-Madoz et al., 2005; Chikkanna-Gowda et al., 2005; Lyons et al., 2007). Despite recombinant virus particles are the most frequently applied vectors in cancer gene therapy, viral RNA (naked or encapsulated into transfection vesicles) also can be used as an alternative gene delivery vector. *In vitro* transcribed alphaviral RNA is capable to replicate itself providing equal efficiency of transgene expression and induction of cell apoptosis. However, in comparison with enveloped viral particles, administration of self-replicating RNA does not induce antivector immunity and is safe for *in vivo* application (Vignuzzi et al., 2001; Cheng et al., 2001a). So far, inoculation of SFV RNA was applied only as a technique to stimulate or enhance the immune system's responses against cancer. Ying et al., have demonstrated that intramuscular pre-vaccination with small dose of naked SFV RNA (1 μg) was enough to trigger complete protection from tumor development extending the survival of treated mice (Ying et al., 1999).

1.3 Efficacy of therapeutic and prophylactic anti-cancer vaccines based on alphaviral vectors

Several treatment strategies using replication-deficient and replication-competent alphaviruses such as SFV, SIN and VEE were found effective in different cancer models due to targeted immunogene delivery and oncolytic properties of the vectors. It was shown, that therapeutic efficacy of alphavirus-based vaccines depends on vaccination settings (or administration mode): prophylactic vaccine (immunization was applied prior cancer cells challenge) or therapeutic vaccine (immunization was applied in tumor-established models). Here the brief overview of alphavirus application in melanoma and breast cancer treatment is provided. The therapeutic results of alphavirus-based vaccines in other cancer types are shown in supplementary **Review paper 1**.

1.3.1 Melanoma

Melanoma is one of the most aggressive forms of skin cancer. According to Cancer Research statistics, mortality rates induced by malignant melanoma have increased by 156% since the early 1970s (CancerStatistics.org). Melanoma tumors arise from melanocytes and contain specific tumor-associated antigens (TAA), which can be categorized as differentiation antigens such as

Pmel17/gp100, p75/tyrosinase-related protein TRP-1, MART-1/Melan-A and the retained intron in tyrosinase-related protein (TRP-2-INT2), as well as tumor-associated antigens, like MAGE, or a melanoma cell adhesion molecule (MUC18) (Pleshkan et al., 2011). These antigens have been used as main targets for alphavirus-based prophylactic and therapeutic immunotherapy of melanoma.

The tyrosinaseis an enzyme that plays a key role in melanogenesis (Kumar et al., 2011). Successful stimulation of immune response against melanoma by alphavirus-based vaccines expressing either mouse or human tyrosinase-related protein 1 (TRP-1) has been demonstrated in several studies. For the first time, in 2003, Wolfgang W. Leitner *et al*., have evidenced that prophylactic intramuscular pre-immunization with Sindbis DNA-based pSin-mTRP-1 and pSin-hTRP-1 tumor vaccines was capable to break immunological tolerance and activate anti-cancer immune system in B16 mouse melanoma models (Leitner et al., 2003). Two years later, these results were re-proved by Stacie M. Goldberg *et al*., who demonstrated that prophylactic inoculation of VEE virus-like particles encoding mouse or human TRP-1 has stimulated high immune responses and caused a significant delay of tumor development in immunocompetent melanoma tumor-bearing mice (Goldberg et al., 2005).

In 2010, Avogadri *et al*., has examined the potential of therapeutic application of VEE-TRP-2 VLP vaccines in controlling of melanoma tumor growth. In their study, administration of VEE-TRP-2 VLP started as late as 5 days after tumor inoculation has triggered time-dependent anti-tumor protection (Avogadri et al., 2010). Interestingly, the applied therapeutic treatment was significantly more efficient in contrast to immunization with combination of VEE-gp100 and VEE-tyrosinase vectors. Moreover, vaccination with all three VEE vectors resulted in similar therapeutic efficacy as VEE-TRP-2 alone. Continuing the set up of the more potent treatment based on VEE-TRP-2 vector, 4 years later, the same group demonstrated that efficacy of VEE-TRP-2 vaccine can be increased in combination with either antagonist anti-CTLA-4 or agonist anti-GITR immunomodulatory monoclonal antibodies (mAbs). This combined strategy induced complete B16 tumor regression in 90% and 50% of mice respectively and augmented ~4- and 2-fold the TRP2-specific CD8+ T-cell response and circulating Abs, compared to the vaccine alone (Avogardi et al., 2014).

Murine melanoma cell adhesion molecule (MCAM/MUC18) seems to be another attractive target for therapy, because it is expressed in late primary and metastatic melanoma and almost is not expressed by healthy melanocytes. Although, immunization with Sindbis DNA vector (SINCp) expressing MCAM/MUC18 has not revealed any therapeutic effects against parental B16-F10 cells. Apparently, the negative data were obtained due to extremely low expression of this antigen in B16-F10 cells (Leslie et al., 2007).

In another study devoted to alphavirus DNA-based vaccines, the combination of SFV DNA expressing 1–4 domains of murine VEGFR2 and IL12 together with additional SFV DNA vaccine targeting survivin and β-hCG was proposed as a novel synergic strategy that targeted tumor cells and angiogenesis simultaneously. The combined vaccines inhibited the tumor growth and improved the survival rate in B16 melanoma mouse models. Furthermore, the treatment elicited efficient humoral and cellular immune responses against survivin, β-hCG and VEGFR2 and led to decrease of microvessel density in the tumors (Yin et al., 2015).

Several studies have demonstrated that delivery and expression of cytokine genes in melanoma cells can drive dramatic inhibition of tumor development and strong induction of specific anti-tumor immunity. In preclinical models of cancer, gene therapy with interleukin 12 has reached unprecedented levels of success, especially if it was combined with immunotherapy. In alphavirus context, Asselin-Paturel with co-authors in 1999 have demonstrated that single intratumoral injection of recombinant SFV-IL-12 VLP induced dramatic tumor necrosis in all treated mice allografts leading to 70–90% of tumor growth inhibition. However, the completed tumor regression was not achieved in this study (Asselin-Paturel et al., 1999). To improve the outcomes of the treatment strategy, in another research, the inoculation of SFV-IL-12 was applied in combination with co-stimulation with agonist anti-CD137 monoclonal antibodies, that revealed powerful synergic therapeutic effect (Quetglas et al., 2012a). In more details, stimulation of immune system with agonist agents acting on CD137 expressed on primed T cells promoted an increase of tumor-eradicating cytotoxic T-cell responses. Thus, combined treatment with both SFV-IL-12 and CD137 mAb extremely enhanced the therapeutic efficacy causing50% and 75% of complete tumor remissions (Melero et al., 1997).

In battle with melanoma progression, oncolytic properties of the replication-competent alphavirus vectors were also explored. In that context, avirulent SFV strain A7 was used as a therapeutic vaccine upon systemic and direct inoculation routesin human melanoma-bearing SCID xenografts (Vaha-Koskela et al., 2006). The tested treatment strategy has indicated promising dynamic independently on vector injection mode, showing that significant tumor regression could be achieved by a single administration of VA7. The neurotropism of SFV did not restrict its ability to target tumors, as within 3 weeks VA7 had caused regression of tumors to far below the starting volume. Nevertheless, in depth analysis identified integration of small groups of dividing tumor cells in strands of connective tissue, indicating the potential tumor remission in the future.

1.3.2 Breast cancer

Breast cancer is the most common cancer in women worldwide and the second most common cancer overall. About 30-40% of breast cancers overexpress the growth-promoting protein HER2/neu that correlates with tumor aggressiveness and poor prognosis, making it one of the major target for immunotherapy (Banin Hirata et al., 2014). Beneficial therapeutic data were achieved after intramuscular vaccination with Sindbis VLP DNA-based expression system (ELVIS-*neu*) expressing *neu* gene 14 days before injection of cancer cells overexpressing *neu.* A strong protection of mice against tumor development was observed. Pre-immunization with ELVIS-*neu* resulted in decrease of lung metastasis' incidence from mammary fat pad tumors, as well as caused a reduction of the number of lung metastases developed from intravenous injection of *neu* overexpressing cells. It should be noted, that intradermal vaccination was also efficient, despite required 80 % less plasmid for a similar level of protection (Lachman et al., 2001). In similar experiment, Wang *et al*., has demonstrated that treatment efficacy of pSINCP/neu vector application is depended on vaccination type: therapeutic or prophylactic. They showed that the vaccine could be effective only if administrated before tumor challenge (Wang et al., 2005).

The effect of immunotherapy stimulated by pSINCP/neu DNA vaccine and VEE/neu VLP could be favored by co-treatment with chemotherapy. Application of chemical anticancer agent doxorubicin at concentration 5 mg/kg prior alphavirus-based vaccination significantly delayed tumor progression. Interesting, but mice treated only with chemotherapy alone did not show regression of tumor growth, despite doxorubicin is established as standard adjuvant in breast cancer therapy. It should be emphasized that alphavirus-based vaccines have synergic therapeutic effect only with particular chemical agents. In contrast, combination of the paclitaxel drug at concentration 25 mg/kg and pSINCP/neu vector was ineffective (Eralp et al., 2004).

Important, but in the case of the breast cancer therapy, the therapeutic alphavirus-based vaccines are not effective against established tumor. Only 36% of rat breast tumors were affected by VEE-neu VLP vaccination, when it was subcutaneously administrated 2 days after the cancer cells (Laust et al., 2007). However, different modulations in treatment strategies can significantly improve therapeutic outcomes. For instance, immunization of transgenic human breast tumor-bearing mice with dendritic cells infected with VEE VLP expressing the extracellular-transmembrane domains of rat *neu* oncoprotein has revealed an activation of both cellular and humoral immune response against *neu* and caused inhibition of tumor development (Moran et al., 2007).

Replication-enhanced alphavirus vectors expressing cytokines such us IL-12 have been generated as

a cancer therapy agents. Chikkanna-Gowda *et al*., has applied enhanced Semliki Forest virus vector (SFV10-E) that expresses foreign genes (in this case IL-12) at levels up to 10 times higher than the original SFV10 vector. Direct intratumoral inoculation of this vector at high titre led to complete tumor regression in four out of six mice and decreased the numbers of lung metastases (Chikkanna-Gowda et al., 2005). The efficacy of the same improved SFV10-E VLP vector was re-proved in another study, where therapeutic inoculation of SFV10-E encoding VEGFR-2 and IL4 reduced significantly inhibited tumor growth and pulmonary metastatic spread in treated mice (Lyons et al., 2007).

Aiming to develop an effective therapy against metastatic breast cancer and to enhance the efficacy of alphavirus vectors, the novel treatment strategy based on the combined intratumoral administration of SFV-IL-12 and attenuated Salmonella LVR01 followed by surgical removal of the treated primary 4T1 tumors was tested. This neoadjuvant combined therapy was markedly synergistic compared to each treatment alone resulting in 90% long-term survival (Kramer et al., 2015).

1.4 **Clinical trials**

Despite a lot of beneficial results of cancer treatment have been shown in preclinical studies, only some of therapeutic strategies were translated in clinical trials for human treatment. In alphavirus context, the first clinical data were published in 2003 by Ren *et al*., where first phase I/II clinical study using Semliki forest virus vector expressing the human interleukin 12 gene and encapsulated in cationic liposomes (LSFV–IL12) was described. To evaluate the biosafety and optimal dosage of the vector, LSFV–IL12 was intravenously administrated in cancer patients with stage III or IV metastasizing melanoma or renal cell carcinoma every 3 days for 4 weeks in two different concentrations. No toxic effect has been determined after the therapy was as well as the functionality of the internal organs has not been affected, however, the treatment resulted in 10-fold increase of IL-12 concentration in the peripheral blood of the treated patients (Ren et al., 2003).

Another phase I/II study was based on alphavirus ability to infect dendritic cells with high efficacy. Patients with advanced or metastatic cases of lung, colon, breast, appendix or pancreatic cancers were pretreated with multiple courses of chemotherapy and then got injected up to four times with VEE VLP expressing the carcinoembryonic antigen (CEA). Despite the majority of patients had dramatically low rate of clinical response after the therapy, the CEA-specific T cell and antibody response was found to be clinically relevant. The regression of liver metastasis has been observed in patient with pancreatic cancer. Additionally, two patients with no evidence of disease remained in

remission and two patients were able to maintain stable disease (Morse et al., 2010).

Recently, in 2012, phase I clinical trials have been performed accessing the therapeutic efficacy of VEE VLP expressing prostate specific membrane antigen (PSMA) in patients with castration resistant metastatic prostate cancer. Patients were vaccinated with different vector dosages by maximally 5 subcutaneous administration routes in the deltoid region. However, the therapeutic effect was very low. The treatment was generally well-tolerated and did not demonstrate any toxic side effects, but only a small number of patients demonstrated a humoral response to PSMA. No cellular immune response to PSMA has been observed (Slovin et al., 2013).

2 A short description of the methods

2.1 Cell lines

BHK-21 cells (baby hamster kidney cells), H2-35 cells (mouse hepatocytes transformed with a temperature-sensitive strain of simian virus 40) and the tumor cell lines 4T1 (mouse mammary carcinoma), Huh-7 (human hepatocarcinoma), PA1 (human ovarian teratocarcinoma), C-33A (human cervical carcinoma), B16-F10 (mouse melanoma) and MCF-7 (human breast adenocarcinoma) were obtained from the American Type Culture Collection (ATCC/LGC Prochem).

BHK-21 cells were cultured in BHK - Glasgow MEM (GIBCO/Invitrogen) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 2 mM L-glutamine, 20mM HEPES and antibiotics (100 µg ml^{-1} streptomycin and 100 U ml^{-1} penicillin). The 4T1 and MCF-7 cell lines were cultured in Dulbecco's Minimal Essential Medium (D-MEM, GIBCO/Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine. The Huh-7, PA1 and B16-F10 cell lines were cultured in D-MEM-GlutaMax (GIBCO/Invitrogen, UK) supplemented with 10% FBS and 40 µg ml^{-1} gentamicin. H2-35 cells were cultured in D-MEM-GlutaMax (GIBCO/Invitrogen) supplemented with 5% FBS without antibiotics. C-33 A cells were cultured in 40% D-MEM (GIBCO/Invitrogen) and 45% RPMI 1640 (GIBCO/Invitrogen) supplemented with 15% FBS, 10 mM HEPES, 2 mM L-glutamine and antibiotics (40 µg ml^{-1} gentamicin).

2.2 Generation of the plasmids

pSFV/EGFP and pSINRep5/EGFP

The pSFV1 (Liljestrom and Garoff, 1991) and pSinRep5 (Bredenbeek et al., 1993) vectors were used in this study. The enhanced green fluorescent protein (*EGFP*) gene was introduced into both vectors under the 26S subgenomic promoter. The *EGFP* gene was cut out of the pEGFP-C1 plasmid (Clontech) with *Nhe*I and *Hpa*I restriction endonucleases, treated with T4 DNA polymerase (Thermo Scientific) to blunt the DNA ends and ligated with the pSFV1 and pSinRep5 vectors, which were cleaved with *Sma*I and *Pml*I, respectively. Additionally, a pSFV1/DS-Red construct carrying the red fluorescent protein gene (*DS-Red*) (Matz et al., 1999) was generated.

recSFV/DS-Red

The *DS-Red* gene was amplified by PCR (primers: 5'-ATTAGGATCCACCGGTCGCCACCATG-3' and 5'-TATCCCGGGCTACAGGAACAGGTGGTG - 3') using the pDsRed-Monomer-C1 plasmid as a template (Clontech). The PCR fragment was cleaved with *Bam*HI and *Sma*I and

ligated into a pSFV1 vector hydrolyzed with the same enzymes.

recSFV/EnhLuc

The pSFV1/Enh vector was generated based on the pSFV1 vector by insertion of the 50 region of SFV capsid gene sequence (36 codons), acting as a translational enhancer for efficient heterologous gene expression, under the control of the 26S subgenomic promoter. This element is followed by in-frame insertion of foot-and-mouth-disease virus 2A autoprotease sequence (Ryan and Drew, 1994). Located downstream of this cassette, the polylinker allows for the cloning of a gene of interest. The firefly luciferase gene (*Luc*) was amplified by PCR using the pGL3-Basic (Promega) plasmid as a template. The primers for amplification were as follows: 5'-GACCCGGGAAGGATCTCTCGAGGATGGAAGAC-3' (forward) and 5'-CCGTTTAAACTATCCTAGAATTACACGGCGATCTTTCCGCCCTTC-3' (reverse). The *Luc* PCR fragment was cut using *SmaI* and *PmeI* restriction enzymes and ligated into the pSFV1/Enh vector, which had also been cleaved with the same enzymes. The resulting pSFV1/Enh.Luc vector carries the *Luc* gene fused with the SFV core enhancer and 2A autoprotease-encoding sequence at the 5' end. The presence of the SFV enhancer provides an approximate 10-fold increase in the transgene expression in comparison with the classical SFV1 vector (Chikkanna-Gowda et al., 2005). The foreign N terminal 36 aa, which may affect the luciferase enzymatic activity, are removed by the activity of 2A autoprotease.

2.3 *In vitro* RNA transcription

The resulting plasmids were used to produce recRNA and recombinant virus particles. pSFV-Helper (Liljestrom and Garoff, 1991) and pSIN-DH-EB helper (Bredenbeek et al., 1993) were used to produce the SFV and SIN particles, respectively. pSFV/EGFP, pSFV/DS-Red, pSFV1/Enh.Luc and pSFV-Helper plasmids were linearized using the *Spe*I restriction enzyme (Thermo Scientific). pSinRep5 and pSIN-DH-EB helper were linearized using *Xho*I respectively. *In vitro* RNA transcription was performed using 3 µg of linearized DNA and 40 U of SP6 RNA polymerase (Thermo Scientific) in a 50 µl reaction mixture. The RNA transcripts were capped during the transcription reaction by adding 1mM of the 5' (ppp)5'G cap-analogue (New England Biolabs). The DNA template was removed by digestion using RNase-free DNase (Thermo Scientific).

2.4 Production of recSFV and recSIN virus particles

For the packaging of SFV/SIN RNAs into SFV/SIN particles, 20 µg of *in vitro* transcribed RNAs (recSFV RNA + SFV-helper RNA; recSIN RNA +SIN-DH-EB) were co-electroporated into 1×10^7 BHK cells (850 V, 25 mF, 2 pulses) using a Bio-Rad Gene Pulser apparatus (Bio-Rad) without the

pulse controller unit. The electroporated cells were resuspended in 15 ml of complete BHK medium containing 1% FBS, transferred into tissue culture flasks (75cm^2) and incubated at 37ºC (5% CO2). After a 24-h incubation, recSFV/rec SIN-containing medium was harvested, rapidly frozen and subsequently used as a virus stock. The virus stocks did not contain the replication proficient wild-type virus (confirmed by cell reinfection). To concentrate recSFV and recSIN particles, 25ml of the virus-containing medium was ultracentrifuged through a double sucrose cushion with a top layer comprising 3ml of 20% sucrose and a bottom layer comprising 3ml of 50% sucrose (Beckman SW32 rotor; 160 000 g for 1.5h at 41C; Beckman Coulter). After ultracentrifugation, the medium and 1ml of the upper sucrose cushion were removed and the next 4ml, representing the recSFV/recSIN particle-containing fraction, was collected and dialyzed in phosphatebuffered saline (PBS) overnight at 4ºC, dialysis membrane: CelluSep H1, 25 kDa cutoff (Orange Scientific). Next, the concentrated recSFV stocks were aliquoted and rapidly frozen. The concentration led to an approximate fivefold increase in the recombinant particle titer.

The viral titers (infectious units per ml, iu ml^{-1}) were quantified by infecting BHK-21 cells with serial dilutions of viral stock and analyzing *EGFP* or *DS-Red* expression via fluorescence microscopy on a Leica DM IL microscope (Leica Microsystems). For recSFV/Enh.Lucthe viral titer was quantified by Real-time PCR as described below.

2.5 Real-time PCR for recSFV/Enh.Luc virus quantification

The method is described in ***Paper I***.

Briefly, the viral RNA was isolated from recSFV/Enh.Luc virus particles (volume 500 µl) using TRI Reagent BD (Sigma) following the manufacturer's protocol. The RNA was reverse transcribed using the First-Strand cDNA Synthesis Kit (USB-Affymetrix) applying the random hexamer oligonucleotide strategy. A 183-bp DNA fragment was amplified by Real-Time PCR reaction using *Luc* gene-specific primers. To make a quantification of virus titre, the fivefold serial dilutions of the plasmid DNA (pSFV1/Enh.Luc) were used to establish a standard curve. The viral samples, negative controls (that is, isolated viral RNA without complementary DNA synthesis) and serially diluted standards were synchronously processed. The results were analyzed using OpticonMonitor3 software (Bio-Rad) and expressed as the virus replicon genome copy number ml^{-1} (virus replicon particles, v.p.).

2.6 Infection of cell lines with recombinant virus particles

Cells were cultivated in 24-well plates at a density of 2×10^5 cells per well in a humidified 5%

CO_2incubator at 37°C. For transduction, the cells were washed twice with PBS containing Mg^{2+} and Ca^{2+}(Invitrogen). Next, 0.3 ml of the solution containing the virus particles was added. The recSFV/Enh.Luc, recSFV/EGFP, recSFV/DS-Red and recSIN/EGFP virus particles were diluted in PBS (containing Mg^{2+} and Ca^{2+}) to achieve a multiplicity of infection (MOI) of 10. The cells were incubated for 1 h in a humidified 5% CO_2incubator at 37°C. The control cells (uninfected) were incubated with PBS (containing Mg^{2+} and Ca^{2+}). After incubation, the solution containing the virus was replaced with 0.5 ml of growth medium. The cells were gently washed with PBS and transferred to fresh medium every day.

2.7 MTT cell proliferation assay

The cell proliferation was quantified using the MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based cell viability assay. Cells were infected in 24-well plates as described above, and proliferation was analyzed 0, 1, 2, 3, 4 and 5 days after infection. The medium was replaced with 0.3 ml of solution containing 0.5 mg ml^{-1} MTT (Affymetrix) dissolved in D-MEM without phenol red (GIBCO/Invitrogen) supplemented with 5% FBS. The cells were incubated for 2 h in a humidified 5% CO_2incubator at 37°C. After incubation, the formazan crystals were dissolved by adding 0.3 ml of MTT solubilization solution consisting of 10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol. The absorbance was measured using a microplate spectrophotometer (BioTek Instruments) at a test wavelength of 570nm and a reference wavelength of 620nm. Cell viability (%) was obtained using the following equation: Percent cell viability = (test 570 nm – 620 nm)/(control 570 nm – 620 nm) × 100, where the control is the value obtained from uninfected cells.

2.8 Fluorescence-activated cell sorting (FACS) analysis

Cells were infected on 6-well plates with recSFV/EGFP and recSIN/EGFP virus particles at an MOI of 10 as described above (1 ml of virus-containing solution was used for the infection). The infected cells were harvested 24 h after infection. Detached cells were harvested from the cell medium by centrifugation, and attached cells were trypsinized. The collected cells (approximately 10^6) were washed with PBS and resuspended in 1 ml of PBS. For propidium iodide (PI) staining, the cells were incubated with 10 µl of 50 µg ml^{-1} PI solution (Becton Dickinson Biosciences) and immediately processed for FACS analysis. *EGFP* and PI fluorescence was measured using a FACS Aria II (Becton Dickinson Biosciences). The FACS data were analyzed by BD FACS Diva 6.1.2 software. Uninfected cells were used as a negative control for both the PI and *EGFP* FACS analysis and contained approximately 1-2% PI-positive cells in 4T1 culture.

2.9 Induction of 4T1 and B16 tumor nodules and recSFV virus or recRNA administration

The methods are described in ***Papers I, II and III***

Briefly, 4T1 or B16-F10 tumor cells were trypsinized, washed with PBS and resuspended in PBS at a final concentration of 2.5×10^6 cells ml^{-1} and 3×10^5 cells ml^{-1} respectively. Two hundred microliters of the corresponding cell suspension were subcutaneously injected above the right shoulder blade of the mice. After 8-12 days, the tumor diameters were measured using linear callipers, and the tumor volume in mm^3 was calculated using the following formula: volume = shorter measurement^2×longer measurement/2 ($V=(width)^2\times length/2$) (Tomayko and Reynolds, 1989). When tumor volumes reached 1000 mm^3, 4T1 tumor-bearing mice (n = 5 or more) and tumor-free mice were inoculated either i.v. or i.p. of recSFV particle-containing stocks. Two viral titers were used: 6×10^9 v.p. (concentrated and dialyzed as described above) and 2×10^8 v.p. (unconcentrated recSFV particle-containing medium). B16 tumor-bearing mice (n=3) were i.t. inoculated with 10^8 v.p. or i.p. inoculated with 2×10^8 v.p. of recSFV/Enh.Luc particle-containing stocks.

For *in vivo* application, recRNA was synthesized as described above. For i.t. administration, 50 µl of the transcription mix containing 130 µg of recRNA was diluted in sterile diethylpyrocarbonate-treated water to a final volume of 250 µl with 5% glucose (w/v) and total volume (250 µl) was divided into five portions, and recRNA was injected into five different places in the 4T1 or B16 tumor.

RecSFV virus/recRNA expression and biodistribution in mice organs and tumors were analyzed 24h after inoculation.

2.10 Analysis of *Luc* gene expression in mice organs

The method is described in ***Paper I***.

Briefly, *Luc* gene expression analysis was performed by measuring the luciferase enzymatic activity in tissue homogenates at 24h after vector inoculation (48 h; 72 h or 168 h depending on experiment set up). The organs were manually homogenized in a 2× concentration of ice-cold lysis buffer (Cell Culture Lysis buffer, Promega) containing a protease inhibitor cocktail (10 µl per 1ml of lysis buffer) (Sigma) and the protein concentration was determined in tissue lysates using the RC DC Protein Assay (DC Protein Assay, Bio-Rad). The luciferase activity was measured by adding 100 µl of freshly reconstituted luciferase assay buffer to 20 µl of the tissue homogenate (Luciferase Assay

System, Promega). *Luc* gene expression was then quantified as relative light units (RLUs) using a conventional luminometer (Luminoskan Ascent, Thermo Scientific). The RLU values were expressed per 1mg of protein (RLU/mg^{-1}) or total protein content in each organ (RLU per mg total).

2.11 **4T1 cell and tumor treatment with 5-FU**

The methods are described in ***Paper II***.

Briefly, 4T1 cells were seeded in a 24-well plate (2×10^5 cells per well) and on the next day, the cells were treated with medium containing 5-FU (Sigma) at 13, 26, 65 or 130 μg ml^{-1}. Every day for 5 days, the cells were gently washed with PBS to remove dead and detached cells, and fresh medium containing 5-FU was added. The control cells were not treated with 5-FU. The cell proliferation was analyzed by MTT cell proliferation assay at 0, 1, 2, 3, 4 and 5 days after the start of 5-FU treatment.

To access the therapeutic efficacy of 5-FU treatment *in vivo*, 4T1 tumor-bearing mice ($n \geq 5$) were treated with 5-FU at different doses (40, 150 or 400 mg kg^{-1}) via peroral administration 4 times over a period of 8 days (every other day). For the recSFV and 5-FU combined treatment experiments, One hour after the last 5-FU treatment, the mice were inoculated either i.t. (intratumoral) or i.p. (intraperitoneal) with 200 μl (4 injections of approximately 50 μl each) or 300 μl of recSFV/Enh.Luc particle-containing stocks (6×10^9v.p. ml^{-1}), respectively.

2.12 **Analysis of FITC-dextran accumulation in 4T1 tumors**

The method is described in ***Paper II***.

Briefly, two groups of mice (treated with 150 mg kg^{-1} 5-FU group (n = 3) and untreated control group (n = 3)) were inoculated i.v. with 120 μl of FITC-dextran 2000 kDa solution (40 mg/ml in PBS) (Sigma) 24h after the last 5-FU treatment. Two hours later tumors were collected and incubated overnight in 4% paraformaldehyde and frozen OCT compound (Sigma). Cryosections (10 μm) were prepared and the intensity of FITC-dextran leakage was visualized by fluorescent microscopy. Pixels of images were measured by ImageJ software.

2.13 **Analysis of IFN-alpha in 4T1 tumor lysates**

The method is described in ***Paper II***.

Briefly, 4T1 tumor-bearing mice were either treated or non-treated with 150 mg kg^{-1} 5-FU and inoculated i.t. with 200 μl (4 injections of approximately 50 μl each) of recSFV/EnhLuc particle-containing stocks (6×10^9 v.p. ml^{-1}) as described above. 18 hours after the virus administration, 4T1 tumors were isolated and frozen in liquid nitrogen. Then frozen tumors were manually

homogenized in ice cold PB. Tissue lysates were prepared and IFN-alpha expression was analyzed according to the manufacturer's protocol of ELISA Kit for Interferon Alpha (Uscn Life Science Inc.). The obtained data (pg/ml) were expressed in % relative to lysates non-treated with both the 5-FU and the virus.

2.14 **Isolation and cultivation of *ex vivo* B16 cells**

The method is described in ***Paper III***.

Briefly, freshly isolated B16-F10 tumors were manually homogenized in PBS and filtered through 40-µm diameter filters. The obtained cells were seeded in 24-well plates at a density of approximately 5×10^5 cells per well in Dulbecco's GlutaMAX medium (GIBCO/Invitrogen) supplemented with 10% FBS, 100 mg ml^{-1} streptomycin, and 40 µg ml^{-1} gentamicin and cultivated or 5-7 days until the cell monolayer reached 80% confluence. Then, 1st passage of *ex vivo* cells was trypsinized and plated in 24-well plates at a low density of approximately 4×10^3 cells per well (for infection or melanin staining) and in 10-cm Petri dishes at a density of 2×10^4 cells for further *ex vivo* B16 cell passaging (2nd passage, 3rd passage, etc.).

2.15 **Label-free LC-MS analysis of *ex vivo* and *in vitro* B16 cells**

The method is described in ***Paper III***.

Briefly, for the samples preparation, *ex vivo* B16 cells were isolated from three B16-F10 tumor-bearing mice and cultivated for 5-7 days until the monolayer of the first passage reached 80% confluence (see above). The control *in vitro* B16-F10 cells were cultivated as described above until the monolayer reached 80% confluence. Both *in vitro* and *ex vivo* B16 cells were lysed with 0.1% ProteaseMax in 50 mM ammonium bicarbonate buffer (Promega) and sonicated for 15 min at 35 kHz. The lysate protein concentration was measured using Direct Detect® Assay-free Cards (Merck Millipore). For LC-MS analysis, samples were prepared using the FASP protocol (Wiśniewski et al., 2012).

For LC-MS analysis, all experiments were performed on an Easy nLC1000 nano-LC system connected to a quadrupole – Orbitrap (QExactive) mass spectrometer (ThermoElectron) equipped with a nanoelectrospray ion source (EasySpray/Thermo). For liquid chromatography separation, we used an EasySpray column (C18, 2-µm beads, 100 Å, 75-µm inner diameter) (Thermo) capillary with a 25-cm bed length. The flow rate was 300 nl/min, and the solvent gradient was 2% B to 5% B in 10 min followed by 5% to 26% B in 230 min, and then 90% B wash in 20 min. Solvent A was aqueous 0.1% formic acid, whereas solvent B was 100% acetonitrile in 0.1% formic acid. The

column temperature was kept at 60°C.

The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 400 to 1.200) were acquired in the Orbitrap with resolution R = 70.000 at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadruple). The method used allowed for the sequential isolation of the most intense multiply charged ions, consisting of up to ten depending on the signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or a maximum acquisition time of 100 ms. MS/MS scans were collected at a resolution of 17.500 at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were as follows: electrospray voltage of 2.1 kV, no sheath and auxiliary gas flow, heated capillary temperature of 250°C, and normalized HCD collision energy of 25%. The ion selection threshold was set to 1e4 counts. An isolation width of 3.0 Da was used.

For the protein identification, MS raw files were submitted to MaxQuant software version 1.4.0.5 for protein identification (Cox et al., 2008). Parameters were set as follows: protein N-acetylation, methionine oxidation and pyroglutamate conversion of Glu and Gln as variable modifications. First, we used a search error window of 20 ppm and a main search error of 6 ppm. The Lys-C or Trypsin enzyme option, both without proline restriction, was used depending on the sample, with two allowed miscleavages. Minimal unique peptides were set to 1, and the FDR allowed was 0.01 (1%) for peptide and protein identification. Label-free quantitation was set with a retention time alignment window of 3 min. The UniProt Reference Proteome mouse database was used (download from April 2014). Generation of reversed sequences was selected to assign FDR rates. All quantitative analyses were performed using the Perseus suit from MaxQuant. Briefly, MaxLFQ values were loaded and log-transformed, and 0 values were replaced by noise detection values using an imputation approach based on the normal distribution of the whole data. Differential proteins were assigned by a t-test analysis using S0 = 0.5, a p-value threshold of 0.01 and a permutation-based FDR correction.

2.16 **Bioinformatics analysis**

A total of 277 differentially expressed genes in *ex vivo* and *in vitro* B16 cells were selected by filtering with confidence at $p<0.01$ from a total of 4980 proteins with a difference in expression of at least 1.4-fold. The biological classification of associated genes in terms of their biologic processes and molecular functions was obtained by Gene Ontology (GO) analysis using the Protein

Analysis THrough Evolutionary Relationships (PANTHER) classification system (version: PANTHER 9.0;http://www.pantherdb.org) (Thomas et al., 2003; Mi et al., 2013).Analysis of the cellular localization of selected genes was conducted using the UniProt Knowledgebase (http://www.uniprot.org/).

2.17 **Analysis of IFN-alpha and IFN-beta in *ex vivo* and *in vitro* B16 cells**

The method is described in ***Paper III***.

Briefly, control *in vitro* B16 cells and freshly isolated *ex vivo* B16 cells were seeded in 24-well plates and cultivated until the cell monolayer reached 80% confluence as described above. Expression levels of the IFN-alpha and IFN-beta were determined in *in vitro* and first-passage *ex vivo* B16 cell lysates before SFV infection (0 h) and at 1 h, 3 h, 6 h, 9 h and 18 h after infection with SFV/Ds-Red at an MOI of 10. The cells were trypsinized, washed with PBS and resuspended in 100 µl of PBS. The cell lysates were prepared according to recommended technique described in manufacturer's protocols. The protein concentration was equalized in all samples using the BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific). The expression levels of IFN-alpha and IFN-beta in the cell lysates were determined using the Interferon Alpha ELISA Kit (Uscn Life Science Inc.) and Interferon Beta ELISA Kit (Cusabio Biotech) according to the provided protocols.

2.18 **Analysis of cell membrane elasticity by AFM**

The method is described in ***Paper III***.

Briefly, control *in vitro* B16 cells and freshly isolated *ex vivo* B16 cells were seeded in 8-well plastic chambers and cultivated until the cell monolayer reached 80% confluence as described above. To measure cell membrane hardness, an uncoated atomic force microscope cantilever (Olympus AC240TS) with spring constant C=2 N/m and resonant frequency F=70 kHz was used. Cantilever calibration was conducted by standard operations using an MFP-3D atomic force microscope (Asylum Research) and Igor Pro 6.34A software. The AFM tip with a radius of 10 nm was manually positioned at the middle point between the cell nucleus and the elongated cell body using an OLYMPUS IX71 inverted optical microscope. After positioning, the tip was engaged without scanning the surface of the cell to maintain viability, and single force curves were acquired. The force curve data were exported to Microsoft Excel and analyzed as described in **Paper III.**

2.19 **Statistical analysis**

Statistical analyses of the results were performed using Microsoft Excel and Statistica7 (StatSoft, Tulsa). All error terms presented in the results are representative of minimum 2 or more independent experiments or at least 3 different samples. Statistically significant differences were determined using Student's t-test ($p<0.05$).

3 Results

The results are presented here as original publications.

The author's contribution to the enclosed original publications:

Original paper I: **Vasilevska J**, Skrastina D, Spunde K, Garoff H, Kozlovska T, Zajakina A. Semliki Forest virus biodistribution in tumor-free and 4T1 mammary tumor-bearing mice: a comparison of transgene delivery by recombinant virus particles and naked RNA replicon. *Cancer Gene Therapy J* 2012 Aug;19(8):579-87. Doi: 10.1038/cgt.2012.37.

(Performed: alphavirus production, concentration and titration; participated in design and analysis of the *in vivo* experiments; prepared the figures and wrote the manuscript)

Original paper II: Zajakina A, **Vasilevska J***, Zhulenkov D, Skrastina D, Spaks A, Plotniece A, Kozlovska T. High efficiency of alphaviral gene transfer in combination with 5-fluorouracil in mouse mammary tumor model. *BMC Cancer* 2014 Jun 20;14:460. Doi: 10.1186/1471-2407-14-460

(Performed: alphavirus production and titration; all *in vitro* experiments with alphavirus infections, treatment with 5-FU and cell proliferation analysis; participated in design and performance of *in vivo* experiments; prepared the figures for the manuscript)

Original paper III: **Vasilevska J**, De Souza GA, Stensland M, Skrastina D, Zhulenkovs D, Paplausks R, Kurena B, Kozlovska T, Zajakina A. Comparative protein profiling of B16 mouse melanoma cells susceptible and non-susceptible to alphavirus infection: effect of the tumor microenvironment. *Cancer Biology&Therapy* 2016 Aug 11:1-16. Doi: 10.1080/15384047.2016.1219813

(Performed: alphavirus production and titration; all *in vitro* experiments with *in vitro* and *ex vivo* B16 cells isolation, cultivation and infection; analysis of protein profilingby LC-MS, precipitated in *in vivo* experiments and AFM analysis; prepared the figures and wrote the manuscript)

Review paper I: Zajakina A, **Vasilevska J**, Kozlovska T, Lundstrom K. Alphavirus vectors for cancer treatment. Book chapter in "Viral Nanotechnology" 2015. Doi: 10.1201/b18596-31

(Designed the figures; collected the literature and partially wrote the manuscript)

3.1 **Introduction in the results.**

Transduction efficiency and cytotoxicity of alphaviral vectors *in vitro*

In order to determine the most potent vector and the most optimal cancer cell line for further preclinical studies *in vivo*, we have investigated transduction efficiency and oncolytic properties of recombinant SFV and SIN vectors in various mouse and human cancer cell lines. Series of experiments have been set up where seven cell lines were infected with equal amount of recombinant particles (MOI = 20) encoding *EGFP* gene. FACS analysis of *EGFP*-positive cells was performed at 24 h postinfection.

Despite both recSFV and recSIN viruses have similar structures and biology, our results indicated differences in the infectivity and cytotoxic properties of the vectors in cancer cell lines (**Figure 4**). Four cell lines (Huh-7, PA1, 4T1 and H2-35) could be considered as good targets for both recSFV and recSIN vectors. Interestingly, the *human C33-A* cervical carcinoma was the only cell culture that showed exceptional transduction specificity with recSIN vector. Two cell lines (B16-F10 and MCF-7) were not susceptible to transduction with applied vectors.

To evaluate the immediate cytotoxic effect of alphaviral infection (24 h), cells were stained with propidium iodide (PI). Percentage of PI-positive cells (dead cells) was measured by FACS and presented versus *EGFP*-positive cells (**Figure 4**, FACS results). Uninfected cells were used as a control for both PI and *EGFP* FACS analysis and showed 1-4 % of PI-positive cells in population for uninfected cell cultures (not shown).The strongest cytopathic effect was detected in PA1 cell line for both recSFV and recSIN vectors (36 % and 41 % of dead cells correspondingly), these cells were almost completely detached after 24 h postinfection. In contrast to recSFV vector, recSIN vector infection was extremely cytotoxic to H2-35 cell line. The *EGFP*-positive cells could be considered as conditionally dead cells, because these cells will die due to active alphaviral replication. Therefore among highly susceptible cell lines almost 50% of the cells in population were affected by recombinant virus (PI+*EGFP*-positive cells). Remarkably, two cell lines, which did not show the *EGFP* expression (B16-F10, MCF-7), displayed the increased percentage of dead cells in population upon alphaviral infection comparing to uninfected control cells.

In order to compare the ability of various cancer cell cultures to survive alphaviral infection, we evaluated the cell proliferation during 5 days postinfection.For this purpose MTT assay, providing the quantitative measurement of viable cells based on the action of mitochondrial dehydrogenase enzymes in living cells, was applied. As shown in **Figure 4**, recSFV vector displayed stronger inhibition of 4T1 cell proliferation, whereas, C33-A and H2-35 were more affected by recSIN

vector. Proliferation of two cell lines Huh-7 and PA1 was equally inhibited by both recSFV and recSIN vectors leading to complete cell death at 4^{th}-5^{th} day.The unsusceptible cell lines were also affected by both vectors.

Summarizing the data, we have showed a significant variation of alphavirus infection ability of different cancer cell lines *in vitro* that let us separate tested cell lines into two groups: i) infection susceptible and ii) unsusceptible cell lines. For the further experiments we have separated our research into two PARTS, selecting one cell line from infection susceptible or unsusceptible group for each part. Thus, as the most susceptible cell line to recSFV vector infection, 4T1 mouse mammary carcinoma (60% of positive cells) was selected for PART 1 of the study to investigate and to optimize different gene delivery strategies by recSFV vector. In contrast, B16-F10 mouse melanoma was chosen for PART 2 as uninfectable cell culture (1% of positive cells) to explore intracellular conditions that can facilitate or block alphavirus transduction and replication. The recSFV vector has been selected for further studies.

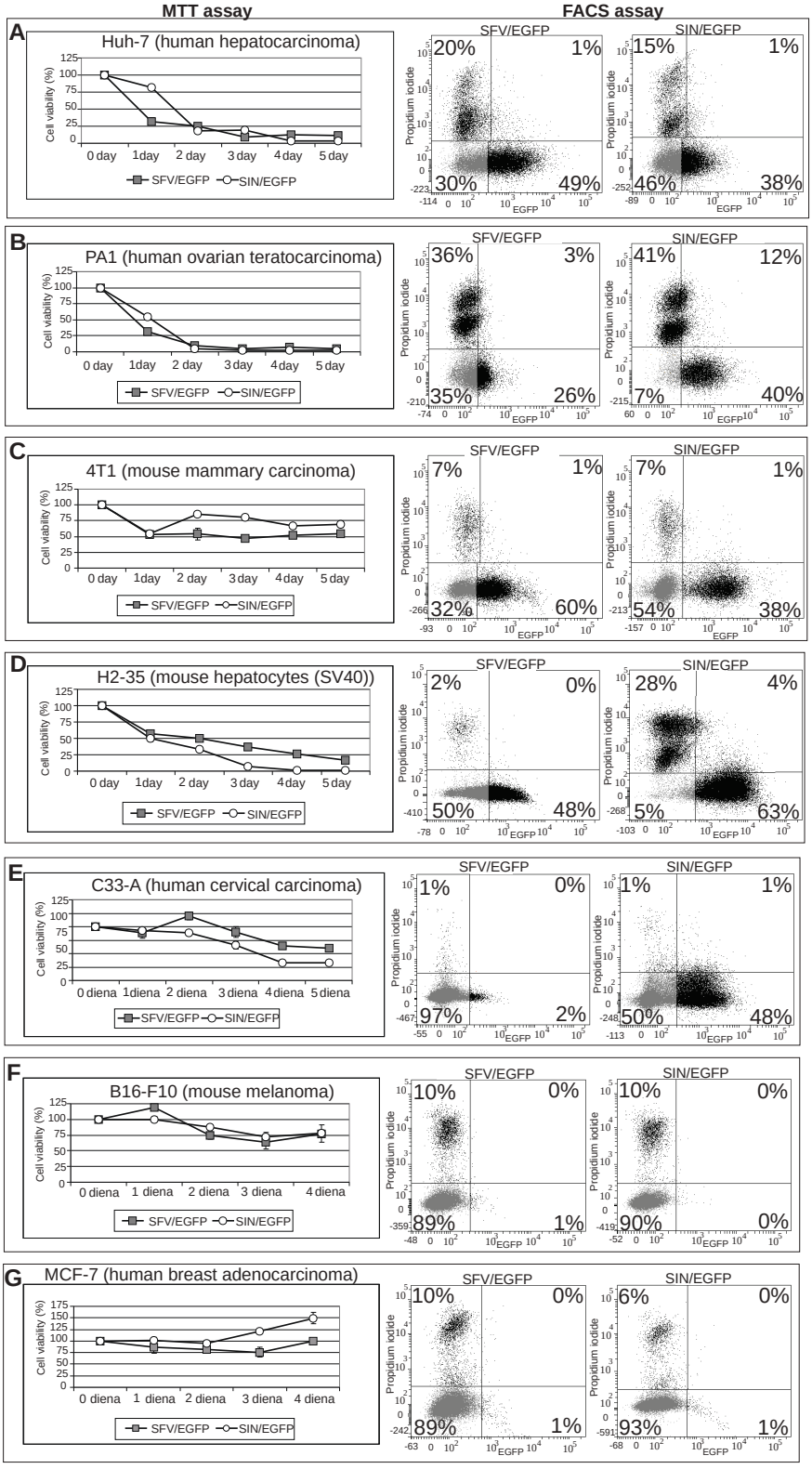

Figure 4: Infection efficacy of alphaviral vectors in different cancer cell lines. Cells were infected with recSFV and recSIN encoding EGFP. At 24 h post-infection cells were analyzed by FACS: the x-axis and the y-axis represent EGFP and propidium iodide fluorescence, respectively. The percentage of living/dead cells and EGFP positive/negative cells is indicated on the scatter plot. The diagrams on the left show the cytotoxic effect of recSFV and recSIN infection in corresponding cell line. MTT cell viability assay was conducted during 4-5 days post-infection and the results are presented as percentage of viable cells relative to control (uninfected cells). Error bars show the standard deviation of three experiments.

Paper I

Semliki Forest virus biodistribution in tumor-free and 4T1 mammary tumor-bearing mice: a comparison of transgene delivery by recombinant virus particles and naked RNA replicon.

(2012)

Cancer Gene Ther 8: 579-87

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ORIGINAL ARTICLE

Semliki Forest virus biodistribution in tumor-free and 4T1 mammary tumor-bearing mice: a comparison of transgene delivery by recombinant virus particles and naked RNA replicon

J Vasilevska[1], D Skrastina[1], K Spunde[1], H Garoff[2], T Kozlovska[1] and A Zajakina[1]

Semliki Forest virus (SFV) vectors are promising tools for cancer gene therapy because they ensure a high level of transgene expression and a rapid and strong cytopathic effect. However, broad tissue tropism and transient expression make it more difficult to develop an optimal cancer treatment strategy. In this study, we have compared the distribution of recombinant SFV particles (recSFV) and naked viral RNA replicon (recRNA) in tumor-free and 4T1 mammary tumor-bearing mice as a consequence of different vector administration strategies. The high potential of SFV recRNA as a biosafe approach for the development of therapeutic treatment was demonstrated. Intravenous (i.v.) inoculation of recRNA provided primary brain targeting in both tumor-free and 4T1 tumor mouse models, but local intratumoral inoculation revealed a high expression level in tumors. Moreover, we observed the predominant tumor targeting of recSFV at a reduced viral dose on i.v. and intraperitoneal (i.p.) virus inoculation, whereas the dose increase led to a broad virus distribution in mice. To prolong transgene expression, we have tested several i.v. and i.p. reinoculation strategies. A detailed evaluation of vector distribution and readministration properties could have an impact on cancer gene therapy clinical trial safety and efficacy.

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Keywords: RNA inoculation; SFV distribution; cytopathic effect

INTRODUCTION

The latest virus-based technologies have significantly contributed to the progress of cancer therapy.[1,2] Among other recombinant viruses, alphaviral vectors are efficient mammalian expression systems, allowing transient high-level transgene expression and rapid high-titer recombinant particle production.[3,4] These vectors are promising in the field of cancer gene therapy because of their strong cytopathic effects through the induction of p53-independent apoptosis,[5,6] their ability to efficiently overcome immunological tolerance by the activation of innate antiviral pathways[7,8] and the subsequent triggering of the cytotoxic T-lymphocyte response against tumors.[9,10] The advantages also include a low specific immune response against the vector itself, the absence of vector pre-immunity and a high level of biosafety.[11,12]

Alphaviruses are enveloped viruses containing a positive-strand RNA genome and belonging to the *Togaviridae* family.[13] The classical Semliki Forest virus (SFV) replicon vector is generated in such a way that the heterologous insert replaces the structural genes under the control of the 26S viral subgenomic promoter.[14] The vector RNA can be packaged into recombinant viral particles by co-transfection into cells together with a helper RNA that encodes structural genes (capsid and envelope). On infection, the SFV RNA can replicate and lead to high expression of the heterologous gene; however, the vector cannot propagate because it lacks genes coding for viral structural proteins.

SFV can efficiently infect and induce apoptosis in many types of cancer cells *in vitro*.[15,16] However, in contrast to the closely related Sindbis virus, specific tumor targeting by SFV vectors has not been confirmed *in vivo*.[17] Therefore, the most commonly used approach has been intratumoral (i.t.) injection of SFV vectors carrying a reporter and/or a therapeutic gene. Therapeutic efficacy by direct, repeated i.t. injections of recombinant SFV (recSFV) has been shown in several mouse tumor models, demonstrating tumor growth inhibition or complete regression.[18–22] Despite the promising treatment results, local i.t. vector administration has some disadvantages. For example, many tumors are not readily accessible for i.t. administration. Furthermore, this inoculation type is not appropriate for metastasis treatment, and i.t. injection doses are limited. Systemic administration may be the most effective method to reach various metastatic sites and is much more amenable than a surgical procedure from a translational perspective. However, the systemic application of oncolytic vectors is limited by their toxicity; therefore, the virus distribution has to be carefully investigated in tumor-free and tumor-bearing animal models.

A broad SFV dissemination by systemic administration has been shown in mouse models.[17,23] The intravenous (i.v.) and intraperitoneal (i.p.) injection of recSFV particles resulted in a similar recombinant virus distribution with high infectivity detected in the heart and lung and moderate infectivity detected in other organs. To prolong the transgene expression that significantly drops on

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just the third day after inoculation, systemic vector reinjection attempts within a 20-day interval were performed.[17] These reinjection results showed that the vector expression was only slightly above background in all of the organs tested, indicating recombinant virus activity inhibition by the immune system. Nevertheless, the vector injection method (i.v., i.p. and i.t.), dose, and reinjection strategy may significantly contribute to the virus targeting and transgene expression efficacy, which has not been evaluated in detail thus far.

Taking into consideration that the SFV replicon vector is based on *in vitro* transcribed RNA, the corresponding RNA (recRNA) (naked or encapsulated into transfection vesicles) can be used as an alternative vector for cancer therapy. Self-replicating RNA can provide the same efficient cytoplasmic transgene expression and induction of apoptosis in the host cells, and it is safe for *in vivo* application and does not induce antivector immunity.[24–26] The high potential of recSFV RNA as a tumor vaccine was shown in one study where intramuscular injection of as little as 1 μg of naked SFV RNA provided complete tumor protection and extended the survival of treated mice when tumor cells were injected 2 days before immunization.[27]

To develop optimal treatment strategies regarding the viral dose, the administration method and the regimen, we performed a detailed study of SFV biodistribution in tumor-free, immunocompetent Balb/c mice and 4T1 mammary tumor-bearing mice. 4T1 is a mouse transplantable tumor cell line that is highly tumorigenic and invasive.[28] In contrast to previous studies, we have demonstrated that reduced dosage of recSFV provides preferential SFV infection in 4T1 tumor nodules, whereas the dosage increase contributes to broad dissemination of infection in the organism. Moreover, for the first time, the biodistribution of naked SFV recRNA in tumor-free and tumor-bearing mice was investigated on i.v., i.p. and i.t. RNA administration. Finally, we have tested several readministration strategies using recSFV particles and recRNA. This study emphasizes the importance of the vector inoculation method and highlights the relevance of systemic SFV injection (viral particles and naked RNA) in the context of transgene distribution and tumor delivery.

MATERIALS AND METHODS

Cell lines and animals

The BHK-21 cells (baby hamster kidney cells) and the tumor cell line 4T1 (metastasizing mammary carcinoma from BALB/c mouse) were obtained from the American Type Culture Collection (ATCC/LGC Prochem, Boras, Sweden). BHK-21 cells were propagated in BHK—Glasgow MEM (GIBCO/Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 2 mM L-glutamine, 20 mM HEPES and antibiotics (streptomycin 100 μg ml^{-1} and penicillin 100 U ml^{-1}). The 4T1 cell line was cultured in Dulbecco's minimal essential medium (GIBCO/Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics.

Specific pathogen-free 4- to 6-week-old female BALB/c mice were obtained from Latvian Experimental Animal Laboratory of Riga Stradins University and maintained under pathogen-free conditions in accordance with the principles of the Latvian and European Community laws. All experiments were approved by the local Animal Protection Ethical Committee and the Latvian Food and Veterinary Service (permission for animal experiments no. 32/23.12.2010).

Construction of the pSFV1/Enh.Luc expression vector

The pSFV1/Enh vector was generated based on the pSFV1 vector[14] by insertion of the 5′ region of SFV capsid gene sequence (36 codons), acting as a translational enhancer for efficient heterologous gene expression, under the control of the 26S subgenomic promoter. This element is followed by in-frame insertion of foot-and-mouth-disease virus 2A autoprotease sequence.[29] Located downstream of this cassette, the polylinker allows for the cloning of a gene of interest. The firefly luciferase gene (*Luc*) was amplified by PCR using the pGL3-Basic (Promega, Madison, WI, USA) plasmid as a template. The primers for amplification were as follows: 5′-GACCCGGGAAGGATCTCTCGAGGATGGAAGAC-3′ (forward) and 5′-CCGTTTAAACTATCCTAGAATTACACGGCGATCTTTCCGCCCTTC-3′ (reverse). The *Luc* PCR fragment was cut using *Sma*I and *Pme*I restriction enzymes and ligated into the pSFV1/Enh vector, which had also been cleaved with the same enzymes. The resulting pSFV1/Enh.Luc vector carries the *Luc* gene fused with the SFV core enhancer and 2A autoprotease-encoding sequence at the 5′ end. The presence of the SFV enhancer provides an approximate 10-fold increase in the transgene expression in comparison with the classical SFV1 vector.[18] The foreign N-terminal 36 aa, which may affect the luciferase enzymatic activity, are removed by the activity of 2A autoprotease.

In vitro RNA transcription and recSFV virus production

The synthesis of recSFV RNA and the generation of infectious vector particles have been previously described.[30] Briefly, pSFV1/Enh.Luc and pSFV-helper[14] plasmids were linearized using the *Spe*I restriction enzyme (Fermentas, Vilnius, Lithuania). *In vitro* RNA transcription was performed using 3 μg of linearized DNA and 40 U of SP6 RNA polymerase (Fermentas) in a 50 μl reaction mixture. The RNA transcripts were capped during the transcription reaction by adding 1 mM of the 5′(ppp)5′G cap-analogue (New England Biolabs, Hitchin, UK). The DNA template was removed by digestion using RNase-free DNase (Fermentas).

For the packaging of pSFV1/Enh.Luc RNA (recRNA) into SFV particles (recSFV), *in vitro* transcribed RNAs (recRNA and SFV-helper RNA, 20 μg each) were co-electroporated into 1×10^7 BHK cells (850 V, 25 μF, 2 pulses) using a Bio-Rad Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) without the pulse controller unit. The electroporated cells were resuspended in 15 ml of complete BHK medium containing 1% FBS, transferred into tissue culture flasks (75 cm^2) and incubated at 37 °C (5% CO_2). After a 24-h incubation, recSFV-containing medium was harvested, rapidly frozen and subsequently used as a virus stock. The virus stocks did not contain the replication proficient wild-type virus (confirmed by cell reinfection).

To concentrate recSFV particles, 25 ml of the virus-containing medium was ultracentrifuged through a double sucrose cushion with a top layer comprising 3 ml of 20% sucrose and a bottom layer comprising 3 ml of 50% sucrose (Beckman SW32 rotor; 160 000 *g* for 1.5 h at 4 °C; Beckman Coulter, Fullerton, CA, USA). After ultracentrifugation, the medium and 1 ml of the upper sucrose cushion were removed and the next 4 ml, representing the recSFV particle-containing fraction, was collected and dialyzed in phosphate-buffered saline (PBS) overnight at 4 °C, dialysis membrane: CelluSep H1, 25 kDa cutoff (Orange Scientific, Braine-l'Alleud, Belgium). Next, the concentrated recSFV stocks were aliquoted and rapidly frozen. The concentration led to an approximate fivefold increase in the recombinant particle titer.

Real-time PCR recSFV virus quantification

To determine the genome copy number (that is, virus titer) of recSFV, viral RNA was isolated from the recSFV particle-containing aliquots (500 μl) using TRI Reagent BD (Sigma, St Louis, MO, USA) and dissolved in 30 μl of diethylpyrocarbonate-treated water. In all, 11 μl of RNA was reverse-transcribed using the First-Strand cDNA Synthesis Kit (USB-Affymetrix, Santa Clara, CA, USA) applying the random hexamer oligonucleotide strategy. The following *Luc* gene-specific primers were generated using Beacon Designer 7 software (Premier Biosoft, Palo Alto, CA, USA): 5′-ACGGATTACCAGGGATTTC-3′ (forward) and 5′-GCAGGCAGTTCTATGAGG-3′ (reverse). The PCR produced a 183-bp DNA fragment using the MiniOpticon system (Bio-Rad). The reaction mixture contained the VeriQuest SYBR Green qPCR Master Mix (USB-Affymetrix), 600 nmol of each primer and 0.5 μl of complementary DNA in a 25 μl reaction volume under the following conditions: pre-denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Fivefold serial dilutions of the plasmid DNA (pSFV1/Enh.Luc) were used to establish a standard curve. A linear range was observed when 10^2–10^7 copies of plasmid DNA were used as a template in the amplification reaction. The assay detection limit was 20 plasmid DNA copies per reaction, and the amplification efficiency was 80–90%. Triplicate PCR reactions were performed for all standards and samples. The viral

samples, negative controls (that is, isolated viral RNA without complementary DNA synthesis) and serially diluted standards were synchronously processed. The amplification product was confirmed by melting curve analysis (product melting temperature 78.2–79.2 °C). The results were analyzed using OpticonMonitor3 software (Bio-Rad) and expressed as the virus replicon genome copy number ml^{-1} (virus replicon particles, v.p.).

Induction of tumor nodules and recSFV virus or recRNA administration

4T1 tumor cells were trypsinized, washed with PBS and resuspended in PBS at a final concentration of $2.5x10^6$ cells ml^{-1}. Two hundred microliters of the 4T1 cell suspension were subcutaneously injected above the right shoulder blade of the mice. After 10 days, the tumor diameters were measured using linear callipers, and the tumor volume in mm^3 was calculated using the following formula: volume = shorter measurement^2 × longer measurement/2 (V = (width)^2 × length/2).[31] All obtained tumor volumes reached at least 1000 mm^3.

Tumor-free and 4T1 tumor-bearing mice ($n = 5$ or more) were inoculated either i.v. or i.p. with 1 ml of recSFV particle-containing stocks. Two viral titers were used: 6×10^9 per ml (concentrated and dialyzed as described above) and 2×10^8 per ml (unconcentrated recSFV particle-containing medium).

For *in vivo* application, recRNA was synthesized as described above. For i.v. and i.p. administration, 50 μl of the transcription mix containing 130 μg of recRNA was diluted in sterile diethylpyrocarbonate-treated water to a final volume of 250 μl with 5% glucose (w/v). The same amount of recRNA was applied for i.t. injection; however, the total volume (250 μl) was divided into five portions, and recRNA was injected into five different places in the tumor.

Analysis of *Luc* gene expression in mice organs

Luc gene expression analysis was performed by measuring the luciferase enzymatic activity in tissue homogenates at 24 (first day), 48 (second day) and 72 h (third day) or 7 days after recSFV virus administration and on the first and second day after recRNA administration. The organs were excised and manually homogenized in a 2 × concentration of ice-cold lysis buffer (Cell Culture Lysis buffer, Promega) containing a protease inhibitor cocktail (10 μl per 1 ml of lysis buffer) (Sigma). The volume of lysis buffer used for each organ (or pairs of bilateral organs) is indicated in Table 1. After homogenization, the samples were centrifuged for 10 min at 9000 *g*, and the protein concentration was determined in tissue lysates using the RC DC Protein Assay (DC Protein Assay, Bio-Rad). The luciferase activity was measured by adding 100 μl of freshly reconstituted luciferase assay buffer to 20 μl of the tissue homogenate (Luciferase Assay System, Promega). *Luc* gene expression was then quantified as relative light units (RLUs) using a conventional luminometer (Luminoskan Ascent, Thermo Scientific, Welwyn Garden City, UK). As a result of the different 4T1 tumor sizes and the high tissue density, tumors were weighed and homogenized in lysis buffer using a ratio of 200 μl of lysis buffer per 100 mg of tumor tissue (2:1).

To evaluate the vector distribution in the entire organism and to determine the viral dose per organ in general, rather than the viral dose per 1 mg of protein (RLU mg^{-1}) as presented in other studies,[17,32] the RLU values were expressed per total protein content in each organ (RLU per mg total). Nevertheless, using the data in Table 1, where the total mg of proteins for each organ are indicated, the 'RLU per mg total' can be easily transformed into 'RLU mg^{-1}' by dividing the RLU value presented in each diagram (see figures) by the total protein amount in the organs. RLUs were calculated as an average mean from the mice in a group ($n = 5$ or more). As a negative control, mice were inoculated with PBS, and the maximal negative values in mice group $n = 7$ (Table 1) were subtracted in the presented diagrams. Each experiment was repeated at least twice.

Statistical analysis

The RLU results are presented as the mean ± s.e. The data were transformed to the logarithmic scale. Statistical analyses of the results were performed using Microsoft Excel and Statistica7 (StatSoft, Tulsa, OK, USA). Statistically significant differences were determined using Student's *t*-test ($P < 0.05$).

Table 1. Protein concentration and negative values of luciferase detection system in organ homogenates

Organ	*Protein concentration (mg ml^{-1})*[a]	*Lysis buffer volume (μl)*	*Total protein amount in organ (mg)*[a]	*Luciferase-negative values per organ (RLU per mg total)*[b]
Heart	11.97 ± 0.04	200	2.39 ± 0.009	255
Lung	9.82 ± 0.05	200	1.96 ± 0.01	143
Brain	12.00 ± 0.08	400	4.80 ± 0.03	121
Kidney	22.23 ± 0.05	300	6.67 ± 0.01	215
Liver	36.97 ± 0.06	1000	36.97 ± 0.06	700
Ovary	1.04 ± 0.05	50	0.05 ± 0.03	373
Tumor	4.69–9.58	400–1600	2.14–8.61	1315

Abbreviation: RLU, relative light unit.
[a]Mean ± s.e.
[b]In mice group, the negative values varied from zero to indicated maximal value.
The organs of Balb/c mice were manually homogenized in 2 × lysis buffer. The insoluble fraction was separated by centrifugation, and the protein concentration was measured using the RC DC Protein Assay (Bio-Rad, Hercules, CA, USA). The minimal and maximal values for the tumors are indicated. The background of luciferase detection system was measured in tissue homogenates on the first day after mice inoculation with PBS (mock). The maximal RLU values per mg total in mice group ($n = 7$) are indicated.

RESULTS

Comparison of the recSFV virus biodistribution in tumor-free and 4T1 tumor-bearing mice

To investigate the biodistribution of recSFV *in vivo*, immunocompetent, tumor-free mice were i.v. or i.p. inoculated with 2×10^8 v.p. recSFV/Enh.Luc (recSFV) particles. Luciferase expression analysis in the organ homogenates was performed on the first (24 h), second (48 h) or seventh day after virus inoculation (Figures 1a and b). The results are presented as the amount of RLUs per total protein (mg) in each organ homogenate, allowing for the evaluation of the recombinant virus distribution and *Luc* expression level in organs in general (see Materials and methods section).

On the first day, i.v. recombinant virus injection resulted in the highest level of transgene expression in the heart, moderate expression in the brain and lung, and low expression in the liver, kidney and ovary (Figure 1a). On the second day, a rapid drop in the *Luc* expression was observed in all of the examined organs, with a further decrease on the seventh day, confirming the transient expression of luciferase by recSFV *in vivo*. In contrast to the results obtained after i.v. injection of the virus, i.p. administration revealed high levels of transgene expression on the first and second days (Figure 1b). Nevertheless, starting from the third day, we observed a significant ($P < 0.05$) decrease in the expression of the transgene in all organs (data not shown). Comparing the level of the *Luc* gene expression in all organs on i.v. and i.p. administration, the i.v. method provided a considerably higher level of *Luc* expression in the heart and lungs (that is, active blood circulation), whereas i.p. administration provided higher expression in the ovary, liver and kidney (more accessible organs by i.p. injection), and the *Luc* expression level in the brain was similar for both types of injections. In addition, the overall remaining level of *Luc* expression on the seventh day was noticeably higher after i.p. virus administration compared with i.v. injection (Figures 1a and b), demonstrating the prolonged expression in the case of i.p. administration.

To evaluate the recSFV distribution in 4T1 tumor-bearing mice, animals were i.v. and i.p. inoculated using the same 2×10^8 amount of recSFV v.p., and *Luc* gene expression was analyzed in organ and tumor homogenates on the first day after injection. Surprisingly, the most predominant tumor targeting (the highest RLU value in tumor) was detected after i.v. and i.p. recSFV

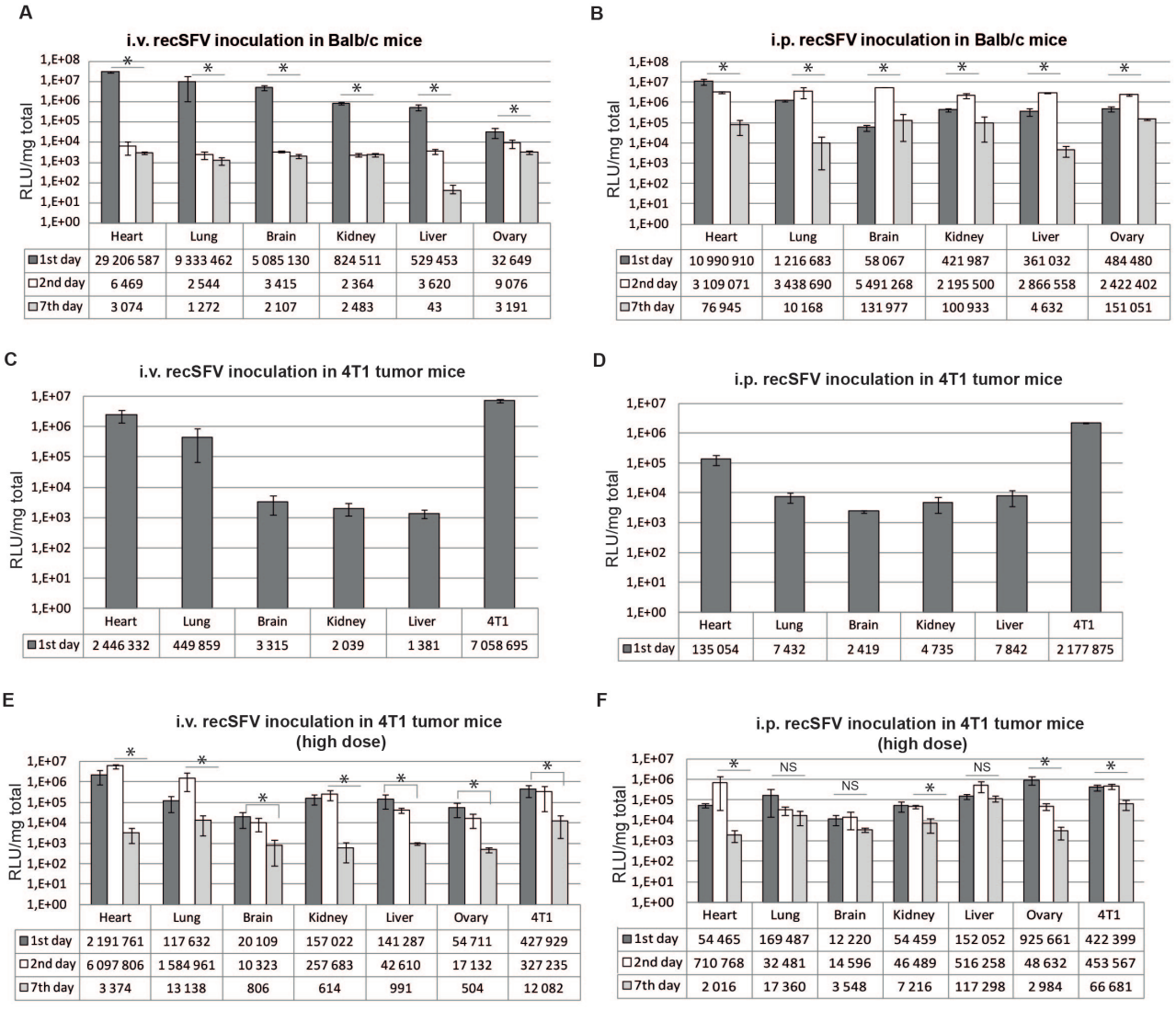

Figure 1. *Luc* gene expression in tumor-free Balb/c and 4T1 tumor-bearing mice inoculated intravenously (i.v.) and intraperitoneally (i.p.) with recSFV/Enh.Luc virus particles (recSFV). The luciferase activity was measured in organ homogenates from tumor-free mice ($n = 5$) on the first (24 h), second (48 h) and seventh days after i.v. (**a**) and i.p. (**b**) inoculation with 2×10^8 recSFV virus replicon particles (v.p.). Luciferase expression in 4T1 tumor-bearing mouse organs ($n = 5$) was analyzed on the first day after i.v. (**c**) and i.p. (**d**) injections of 2×10^8 recSFV v.p. and on the first, second and seventh days after i.v. (**e**) and i.p. (**f**) inoculation with high viral dose (6×10^9 recSFV v.p.). The graphs present the RLUs per total mg protein in each organ or tumor (see Materials and methods section). The results are presented as the mean ± s.e. The average RLU values are indicated in the tables. *Significant differences vs the seventh day ($P < 0.05$). NS, nonsignificant differences; RLU, relative light unit.

administration (Figures 1c and d), which provided a high level of transgene expression in tumors compared with the heart and lungs. As the absolute RLU values in the organs of tumor-bearing mice were significantly lower than the values observed in tumor-free mice (resembling some organs 7-day after inoculation), we did not analyze the subsequent days (that is, days second, third and seventh) after 2×10^8 recSFV particles inoculation. Therefore, to evaluate the recSFV distribution in 4T1 tumor-bearing mice, the viral load was increased, and the results of i.v. and i.p. inoculations were investigated on the first, second and seventh days.

As shown in Figures 1e and f, no preferential *Luc* gene tumor expression was observed at high doses of recSFV (that is, 6×10^9 v.p.), which were applied via i.v. and i.p. injection, as all organs received a high dose of the virus. Interestingly, we did not detect a strong decrease in *Luc* expression on the second day after i.v. administration (Figure 1e), as was shown in the tumor-free animals with the low virus dose, and the expression indexes in all organs widely fluctuated. The ovary and liver can be efficiently targeted by i.p. administration (Figure 1f). Remarkably, the transgene expression level in subcutaneous 4T1 tumors was not affected by the method of virus administration, demonstrating a similar *Luc* expression on i.v. and i.p. injections. However, i.p. administration provided a higher level of the *Luc* expression on the seventh day after inoculation in almost all organs and tumors. These data confirm that i.p. inoculation is able to provide prolonged transgene expression in tumor-free and tumor-bearing mice. Finally, despite the high virus dose, the overall expression level in tumor-bearing mice was significantly lower than that observed in tumor-free animals using a lower virus dose, whereas in tumor-free mice the same high virus dose provided the expected increase in *Luc* expression in all organs (data not shown).

Comparison of recRNA biodistribution in tumor-free and 4T1 tumor-bearing mice

In addition to recombinant virus particle (recSFV) application, the unpacked self-replicating SFV RNA may be considered an

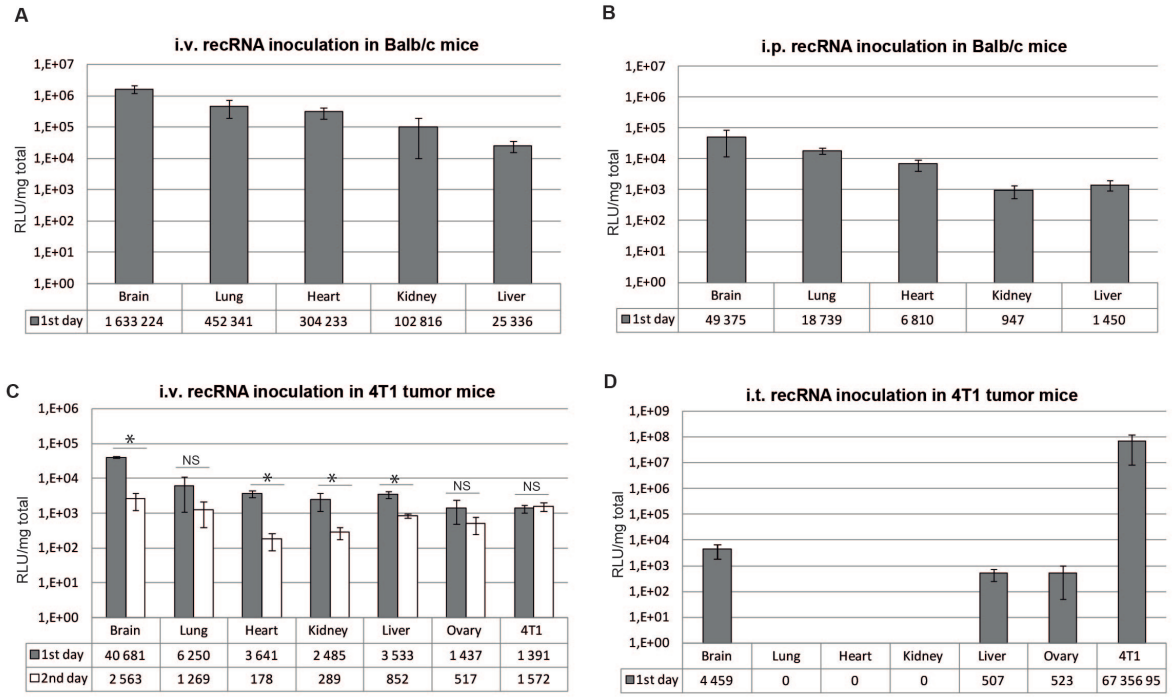

Figure 2. recSFV/Enh.Luc RNA (recRNA) biodistribution in tumor-free Balb/c and 4T1 tumor-bearing mice. The luciferase activity was measured in organ homogenates from tumor-free mice (**a**, **b**) and 4T1 tumor-bearing mice (**c**, **d**) on the first or first and second day after recRNA intravenous (i.v.) (**a**, **c**), intraperitoneal (i.p.) (**b**) and intratumoral (i.t.) (**d**) ($n = 5$) inoculations with 130 μg of Semliki Forest virus (SFV) recRNA expressing firefly luciferase. In the case of i.t. inoculation, recRNA was sequentially injected for two days (that is, 130 μg each day), and *Luc* gene expression was analyzed in tumor and organ homogenates one day after the last injection (first day). The graphs present the RLUs per total mg protein in each organ or tumor (see Materials and methods section). The results are presented as the mean ± s.e. The average RLU values are indicated in the tables. *Significant differences ($P < 0.05$). NS, nonsignificant differences; RLU, relative light unit.

alternative gene transfer vector for cancer gene therapy. Based on this assumption, we investigated the biodistribution of recSFV/Enh.Luc RNA (recRNA) by determining *Luc* gene expression in selected organs of tumor-free and 4T1 tumor-bearing mice. For this purpose, 130 μg of *in vitro* prepared recRNA was used for i.v. and i.p. mouse injections. The luciferase activity was measured in organ homogenates on the first (24 h) and second (48 h) days after RNA inoculations.

The luciferase production in mouse organs after systemic recRNA administration is shown in Figures 2a and b. Surprisingly, in contrast to the virus distribution, where the maximal expression was detected in the heart, the highest recRNA expression was observed in the brain as a consequence of both i.v. and i.p. recRNA administrations. Other organs demonstrated a much lower transgene expression level compared with that of virus inoculation. Moreover, the injection type significantly influences the level of recRNA activity: only i.v. inoculation provides a relatively high level of *Luc* gene expression.

The recRNA distribution in 4T1 tumor-bearing mice was investigated only for i.v. inoculation (Figure 2c), as the i.p. route did not demonstrate a high expression level. The results showed the highest recRNA expression in the brain, as was also detected in tumor-free animals. We observed low *Luc* gene expression in other examined organs and tumors on i.v. injection. Moreover, a rapid decrease in transgene expression in all examined organs, excluding the tumors, was observed on the second day after recRNA inoculation. Interestingly, the *Luc* gene expression in tumor-bearing mice was significantly lower compared with the tumor-free animals, and the same was observed for the recombinant virus vector expression.

In addition, the recRNA expression after i.t. administration was examined (Figure 2d). For this purpose, i.t. injections consisting of 130 μg of recRNA were performed each day for 2 days, and *Luc* gene expression was analyzed in tumor and organ homogenates 1 day after the second injection (first day). Our results showed extremely high *Luc* gene expression in 4T1 tumors (Figure 2d). Low vector activity in other organs showed poor RNA distribution in mice on i.t. administration. However, obvious transgene expression was found in the brain even after i.t. RNA vector inoculation.

recSFV virus and recRNA reinjection in Balb/c mice

The transient mode of SFV-driven gene expression *in vivo* is demonstrated by the strong transgene expression decrease on the third day after inoculation. Virus readministration usually does not provide a significant enhancement or prolongation of transgene expression in mice.[17,33] It is possible that the viral vector provoking the immune response is the reason for readministration failure. To develop an optimal strategy to increase and prolong transgene expression, several vector reinjection schemes using recSFV virus and recRNA combinations and different injection methods were evaluated (Figures 3a and b).

Initially, (Figure 3a), Balb/c mice were i.v. inoculated with 2×10^8 recSFV virus, and 7 days later, one group ($n = 5$) was i.p. readministered with the same amount of recSFV virus, the second group ($n = 5$) was i.v. readministered with 130 μg of recRNA. Luciferase expression analysis was performed in tissue homogenates 24 h after recSFV virus or recRNA reinjection. To observe the increase in the transgene expression level, the results were compared with the values of the remaining *Luc* expression on the seventh day after the first recSFV virus inoculation. In both reinjection combinations (that is, recSFV virus and recRNA), we succeeded in significantly increasing the *Luc* expression in all

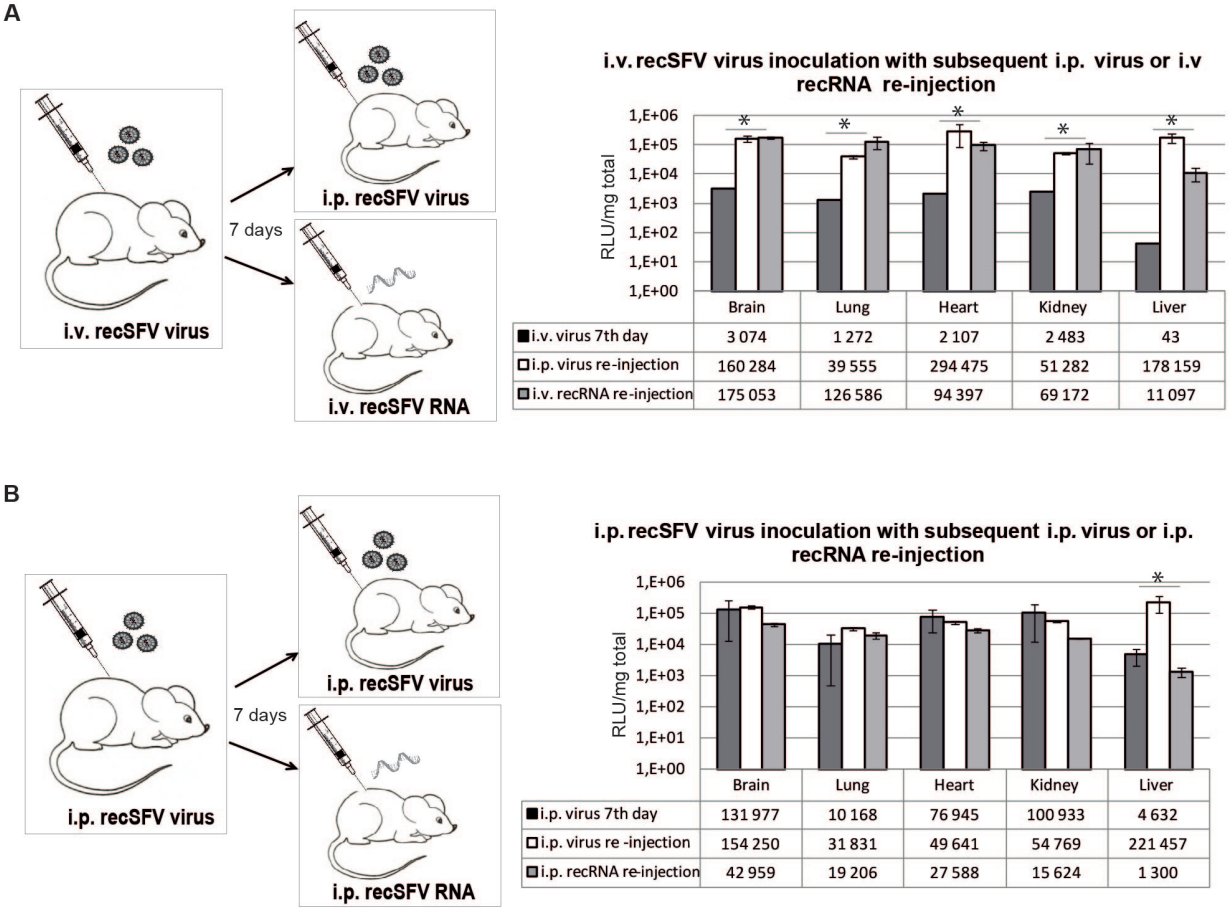

Figure 3. Semliki Forest virus (SFV) vector administration strategies in Balb/c mice using SFV/Enh.Luc virus particles (recSFV) and SFV/Enh.Luc RNA (recRNA) reinjection combinations. (**a**) Balb/c mice were intravenously (i.v.) inoculated with 2×10^8 virus replicon particles (v.p.) of the recSFV. After 7 days, one group of mice ($n = 5$) was intraperitoneally (i.p.) reinoculated with 2×10^8 v.p. of the recSFV, and another group ($n = 5$) was i.v. reinoculated with 130 μg recRNA. (**b**) Balb/c mice were i.p. inoculated with 2×10^8 v.p. of the recSFV. After 7 days, one group of mice ($n = 5$) was i.p. reinoculated with 2×10^8 v.p. of the recSFV, and another group ($n = 5$) was i.p. reinoculated with 130 μg of recRNA. The *Luc* gene expression was analyzed in tissue homogenates on the seventh day after the first recSFV inoculation and on the first day after the recSFV or recRNA reinoculations. The graphs demonstrate RLUs per total mg protein in each organ (see Materials and methods section). The results are presented as the mean ± s.e. The average RLU values are indicated in the tables. *Significant differences vs the seventh day ($P < 0.05$). RLU, relative light unit.

examined tissues; however, this level did not reach the values of the primary i.p. recSFV virus or i.v. recRNA inoculations on the first day (Figures 1b and 2a). Nevertheless, the recRNA i.v. readministration values were comparable with the recSFV virus i.p. reinjection results, demonstrating the high potential of recRNA application, particularly if the brain is the targeted organ.

According to another combination, the effect of recSFV virus/recRNA reinjections using the i.p. method was investigated (Figure 3b). For this purpose, Balb/c mice were i.p. inoculated with 2×10^8 recSFV virus, and 7 days later, one group ($n = 5$) received a readministration of the same amount of recSFV virus i.p., and the second group ($n = 5$) received a readministration of 130 μg of recRNA i.p. Luciferase expression was measured in the organ homogenates that were collected on the first day (24 h) after reinoculation. Compared with the values on the seventh day, the repeated i.p. recSFV virus injection ensured a significant increase in the transgene expression only in the liver. In contrast with the i.v. recSFV virus readministration (Figure 3a), the repeated i.p. method is much less effective for increasing the transgene expression at least with 7-day time interval. The recRNA i.p. reinjection also did not provide a remarkable increase in the transgene expression. As shown in Figures 1a and b (seventh day), i.p. virus inoculation, in contrast to i.v. inoculation, provided prolonged *Luc* gene expression in all organs. Therefore, the relatively high values remaining on the seventh day after i.p. inoculation were not significantly increased by the recSFV virus and recRNA reinjections.

recSFV virus and recRNA reinjection in 4T1 tumor-bearing mice

As the recRNA i.v. reinoculation in tumor-free mice demonstrated the significant increase in transgene expression that resembled recSFV reinoculation (Figure 3a), we were interested in evaluating the recRNA readministration properties and transgene expression biodistribution in tumor-bearing mice that were primary i.v. injected with a high (6×10^9) virus dose. For this purpose, 4T1 tumor-bearing mice were i.v. inoculated with 6×10^9 recSFV virus, and after 7 days, the mice were i.v. inoculated with 130 μg of recRNA (Figure 4a). *Luc* gene expression was measured in tissue homogenates 24 h after recRNA inoculation and compared with the *Luc* values on the seventh day after the first recSFV virus inoculation (that is, the remaining values). Using i.v. recRNA readministration, a significant increase in transgene expression was observed in the brain and heart. All other organs and tumors demonstrated statistically insignificant *Luc* expression differences.

Moreover, to investigate the ability of recRNA to prolong *Luc* gene expression, we evaluated repeated i.v. recRNA administration (Figure 4b). For this purpose, 4T1 tumor-bearing mice were i.v.

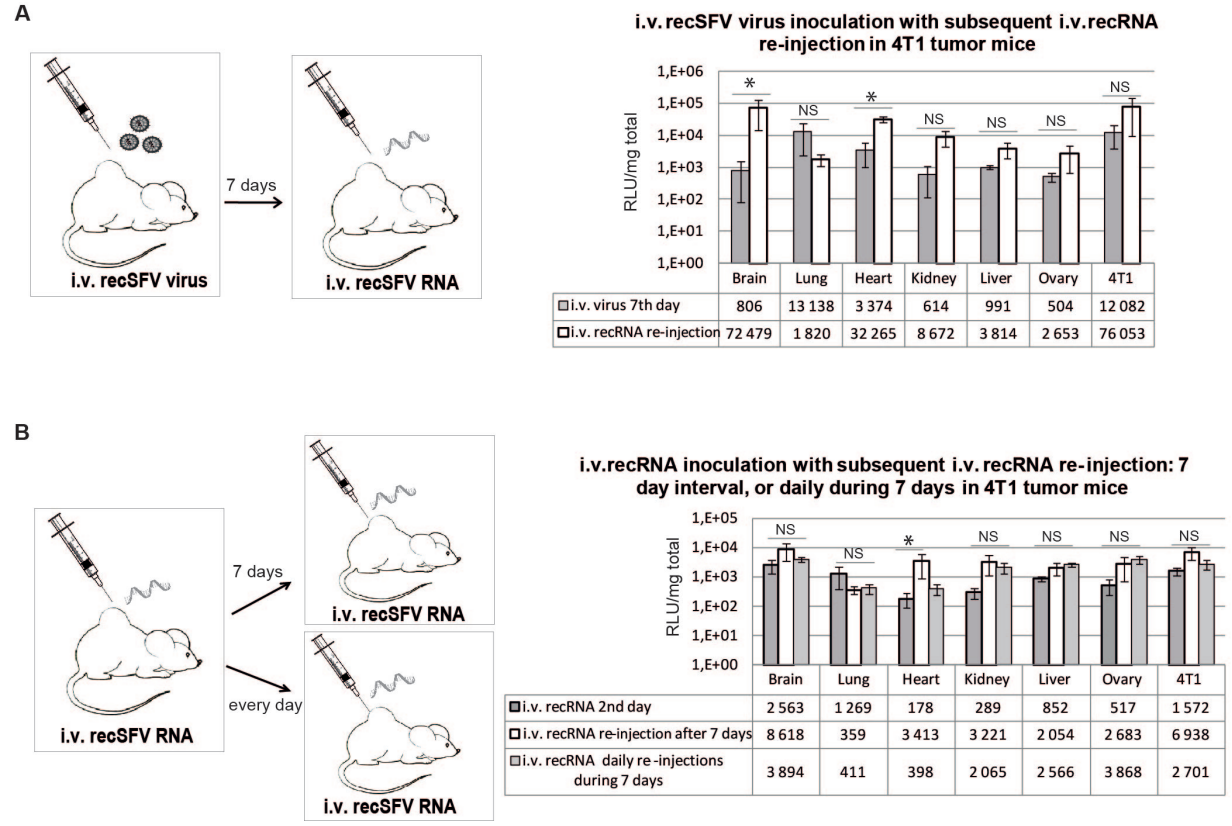

Figure 4. Semliki Forest virus (SFV) vector administration strategies in 4T1 tumor-bearing mice using SFV/Enh.Luc virus particles (recSFV) and SFV/Enh.Luc RNA (recRNA) reinjection combinations. (**a**) 4T1 tumor-bearing mice were intravenously (i.v.) inoculated with 6×10^9 virus replicon particles (v.p.) recSFV. After 7 days, one group of mice ($n = 5$) was i.v. inoculated with 130 μg recRNA. Luciferase expression was analyzed in tissue homogenates on the seventh day after the first recSFV virus inoculation and on the first day after the recRNA inoculation. (**b**) 4T1 tumor mice were i.v. inoculated with 130 μg of recRNA. After 7 days, one group of mice ($n = 5$) was i.v. reinoculated with 130 μg of recRNA. Another group of mice ($n = 5$) was daily i.v. inoculated with 130 μg of recRNA for 7 days. Luciferase expression was analyzed in tissue homogenates on the 2nd day after the first recRNA inoculation and on the first day after the last recRNA reinoculation. The graphs demonstrate RLUs per total mg protein in each organ or tumor (see Materials and methods section). The results are presented as the mean ± s.e. The average RLU values are indicated in the tables. *Significant differences ($P < 0.05$), NS, nonsignificant differences; RLU, relative light unit.

inoculated with 130 μg of recRNA and 7 days later the second 130 μg recRNA dose was i.v. reinjected. In addition, another group of mice was used to examine the prolongation of transgene expression by daily i.v. injections with 130 μg recRNA for 7 days (Figure 4b). *Luc* expression was measured in tissue homogenates 24 h after the last recRNA injection.

The recRNA reinjection with 7-day interval provided the same level (that is, statistically insignificant differences) of *Luc* expression in all organs and tumors as was observed on the second day after the first recRNA inoculation. This observation indicates that recRNA is able to prolong transgene expression on systemic reinjection during a 7-day interval, since without reinjection the luciferase level dropped to the background (not shown). Remarkably, the daily reinjection of 130 μg recRNA did not enhance the transgene expression, demonstrating generally the same result as reinjection using 7-day interval (Figure 4b).

Despite the fact that recRNA administration did not show high values of transgene expression, repeated i.v. recRNA injection resulted in the same level of expression in tumors (or even higher) as was detected after the first (primary) recRNA inoculation. Therefore, repeated recRNA injection could provide prolonged expression in contrast with repeated administration of recSFV, which does not result in the same level of transgene expression as was achieved after the first (primary) virus inoculation.

DISCUSSION

Biodistribution studies are important for the validation of viral vector tumor uptake and the assessment of their potential toxicity to normal tissues. In this study, we were interested in comparing the recSFV vector biodistribution in tumor-free and 4T1 tumor-bearing mice after i.v. and i.p. administration of recombinant viral particles. Our results confirmed the broad *in vivo* recSFV dissemination that has been described by other authors,[17,23] showing the highest SFV-driven transgene expression in organs with active blood circulation, including the heart and lung. Nevertheless, the virus dose may affect the vector distribution, toxicity and therapeutic effect.[34]

In this study, we observed a significant correlation between recSFV dose and biodistribution in a 4T1 tumor model: the lower virus dose provided predominant tumor recSFV targeting as a consequence of both types (that is, i.v. and i.p.) of recombinant virus administration (Figures 1c and d). Moreover, the increase in the recSFV dose changed not only the biodistribution pattern of the vector (the main expression switches to the heart), but also led to a decrease in transgene expression in tumors, whereas other organs demonstrated a high level of expression. Therefore, we conclude that the increase in recSFV titer does not provide predominant virus tumor targeting and enhancement of the i.t. transgene expression level in the 4T1 tumor model. In contrast, the abundant virus circulation more efficiently promotes the

infection of the organs but not the tumors. A possible explanation for this difference could be the hypothesis that after virus injection, the majority of the virus particles circulating in blood infect cancer cells, which are more accessible for infection, and the rest of the virus disseminates to other organs, primarily leading to infection in the heart (and other organs depending on the method of administration).

The serum is known to inhibit alphavirus infectivity *in vitro*. One of the reasons is that components in serum (complement, immunoglobulins, and so on) interfere with the virus adsorption. In our experiments, the low recSFV dose represents the virus preparation containing cell culture medium with 1% FBS, in contrast to the high dose recSFV, which was purified by sucrose gradient and dialyzed in PBS. These two virus preparations (low dose and high dose) did not demonstrate the significant differences in recSFV distribution in tumor-free mice (not shown), only the level of *Luc* expression was higher using high virus dose, as was expected. We assume that the infectivity also is not affected significantly by FBS presence (10 μl per 1 ml dose) in low virus dose preparation, because the native mouse serum components already present in blood and equally interfere with the virus infection. Nevertheless, we do not exclude the influence of FBS and other factors of cell culture medium on immunity and transgene expression properties that have to be evaluated in longer period of time after recSFV inoculation (>7 days).

In all experiments, we verified the higher transgene expression level in tumor-free animals as compared with 4T1 tumor-bearing mice. This was observed for the i.v. and i.p. methods and for the recSFV and recRNA inoculation experiments. The low level of viral expression in tumor-bearing mice may be affected by the upregulation of tumor stress factors and immune-related genes that suppress the virus entry and replication. We may also assume that vector uptake by tumor nodules in tumor-bearing mice leads to a decrease in the viral dose that is available for other organs, therefore reducing the total transgene expression level in tumor-bearing mice.

The virus injection method (i.v. and i.p.) may significantly contribute to the virus allocation and affect the duration of transgene expression in an organism. Although recSFV did not demonstrate the most predominant tumor targeting at high doses, the ability of the vector to disseminate throughout the body may be important for gene delivery to a specific organ where metastases may be expected. With regard to our results, the liver, ovary and kidney were more efficiently targeted by i.p. recSFV administration, whereas the i.v. method targeted the other organs.

Moreover, in contrast to i.v. administration, i.p. recSFV inoculation revealed a higher *Luc* gene expression level on the seventh day, ensuring prolonged transgene synthesis. To solve the problem of transgene expression prolongation, other authors have attempted repeated vector administration.[33,35,36] It is likely that the repeated virus inoculation is suppressed by induction of antivector immune response. To avoid this, the recSFV particles were incapsulated into cationic liposomes.[36] Possibly, the liposome protects the incapsulated SFV particle from inactivation by immune system.

On the other hand, a naked recRNA vector could be used as an alternative strategy for repeated transgene delivery. In this study, we investigated the recRNA biodistribution on i.p., i.v. and i.t. administration. The use of naked RNA represents a safe, promising transgene delivery option,[37] but suffers from poor stability and lack of efficient and clinically feasible delivery technologies. In contrast, alphavirus replicon RNA probably has an increased stability because of its secondary structure, which may protect it from degradation.[38] In addition to high expression levels of inserted gene provided by self-replicating RNA, the various RNA-species produced by the replicon amplification initiates antiviral program resulting in type I interferon production and induction of apoptosis.[6,39] Therefore, the RNA-based vectors are potent immunostimulatory ligands providing excellent adjuvant properties and inducing strong cytopathic effect. As shown in previous studies, recRNA vectors have been used successfully for vaccine development through intramuscular injections.[26,27,40]

Here, we demonstrated for the first time that i.t. administration of self-replicating recRNA provides efficient transgene expression in tumors without significant vector dissemination to organs (Figure 2d), in contrast to recSFV particles i.t. inoculation.[32] Therefore, recRNA is a safe vector for i.t. application. As the i.t. recSFV administration was applied in numerous studies, we did not investigate this type of vector inoculation. However, the transgene expression efficiency of recSFV and recRNA vectors has to be compared in future.

Surprisingly, systemic recRNA inoculation revealed predominant transgene expression in brains of tumor-free and 4T1 tumor-bearing mice (Figures 2a and c). It is tempting to speculate that in contrast to the recSFV particles, recRNA molecules may cross the hematoencephalic barrier more efficiently. This recRNA property may potentially be used for brain-targeted gene delivery. However, the mechanisms of naked RNA delivery into target cells *in vivo* remain to be explored.

In this study, we showed that recRNA i.v. readministration can be successfully applied to prolong transgene expression without the risk of deterioration of viral vectors associated with the immune response. Nevertheless, for unknown reasons, neither recRNA reinjections nor recSFV particles reinjections allow for increasing the transgene expression up to values obtained after the primary vector inoculation (first day).

Here, we describe a comprehensive study of SFV vector distribution with regard to the vector injection type, viral dose, expression time and vector readministration. These results may be analyzed from a different point of view, depending on the priority of the vector application. In conclusion, we would like to emphasize the observed recSFV tropism in 4T1 tumors at certain doses of systemic viral inoculation. Thus far, only the Sindbis virus of the alphavirus genus has been considered to have tumor-targeting properties;[41–44] however, a correlation with the virus dose has not been investigated in that case. Another important observation indicates a high potential for the self-replicating recRNA application for gene delivery demonstrated in this paper. The main advantages of recRNA vectors over the recSFV particles application include safety, reinoculation efficiency, reduced injection volume and simple preparation (that is, *in vitro* RNA synthesis over virus production). Our findings make SFV vectors attractive for future studies with respect to tumor targeting and gene delivery into organs that may be applied in combination with the recSFV virus and recRNA administration.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paper II

High efficiency of alphaviral gene transfer in combination with 5-fluorouracil in mouse mammary tumor model

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High efficiency of alphaviral gene transfer in combination with 5-fluorouracil in a mouse mammary tumor model

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Abstract

Background: The combination of virotherapy and chemotherapy may enable efficient tumor regression that would be unachievable using either therapy alone. In this study, we investigated the efficiency of transgene delivery and the cytotoxic effects of alphaviral vector in combination with 5-fluorouracil (5-FU) in a mouse mammary tumor model (4 T1).

Methods: Replication-deficient Semliki Forest virus (SFV) vectors carrying genes encoding fluorescent proteins were used to infect 4 T1 cell cultures treated with different doses of 5-FU. The efficiency of infection was monitored via fluorescence microscopy and quantified by fluorometry. The cytotoxicity of the combined treatment with 5-FU and alphaviral vector was measured using an MTT-based cell viability assay. *In vivo* experiments were performed in a subcutaneous 4 T1 mouse mammary tumor model with different 5-FU doses and an SFV vector encoding firefly luciferase.

Results: Infection of 4 T1 cells with SFV prior to 5-FU treatment did not produce a synergistic anti-proliferative effect. An alternative treatment strategy, in which 5-FU was used prior to virus infection, strongly inhibited SFV expression. Nevertheless, *in vivo* experiments showed a significant enhancement in SFV-driven transgene (luciferase) expression upon intratumoral and intraperitoneal vector administration in 4 T1 tumor-bearing mice pretreated with 5-FU: here, we observed a positive correlation between 5-FU dose and the level of luciferase expression.

Conclusions: Although 5-FU inhibited SFV-mediated transgene expression in 4 T1 cells *in vitro*, application of the drug in a mouse model revealed a significant enhancement of intratumoral transgene synthesis compared with 5-FU untreated mice. These results may have implications for efficient transgene delivery and the development of potent cancer treatment strategies using alphaviral vectors and 5-FU.

Keywords: Semliki Forest virus, Cytotoxic effect, 5-fluorouracil, Combined cancer treatment, 4 T1 tumor

Background

Several preclinical studies in recent years have demonstrated therapeutic synergy between viral vectors and chemotherapy [1,2]. As reported previously, chemical compounds might be acting as adjuvants for the applied genetic vaccines [3] and/or could enhance the infectivity and gene transfer efficiency of the viral vector [4]. Among the potential therapeutic viruses, alphaviral vectors are good candidates for cancer therapy because of the high level of transgene expression and their ability to mediate strong cytotoxic effects through the induction of p53-independent apoptosis [5,6]. The advantages of alphaviral vectors also include a low specific immune response against the vector itself, the absence of vector pre-immunity and a high level of biosafety [7,8].

Alphaviruses are enveloped viruses that belong to the *Togaviridae* family and contain a positive-strand RNA genome. The classic vectors for the expression of heterologous genes were developed primarily based on Semliki

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Forest virus (SFV) and Sindbis virus (SIN) replicons. In these vectors, a heterologous insert replaces the structural genes under the control of the 26S viral subgenomic promoter [9,10]. The vector RNA can be packaged into recombinant alphaviral particles in cells via co-transfection with a helper RNA encoding structural genes (capsid and envelope). Upon infection, the vector RNA replicates and generates a high level of expression of the heterologous gene. The vector cannot propagate because it lacks the genes encoding the required viral structural proteins. Replication of the recombinant alphaviral genome, which occurs on the cytoplasmic membrane, causes cellular apoptosis, even in the absence of viral structural gene expression [11].

Due to the rapid induction of apoptosis in infected cells, treatment with natural oncolytic alphaviral vectors results in tumor regression [12-15]. Administration of replication-deficient vectors encoding reporter or immunomodulator genes, such as cytokines or growth factors, has also been demonstrated. This leads to successful tumor inhibition or complete regression in animal models [16-19]. Nevertheless, the application of alphaviral immunogene therapy in a clinical study using Venezuelan equine encephalitis (VEE) virus (VEE/CEA) in phase I/II demonstrated insufficient anti-tumor efficacy in patients, most likely due to the inefficient induction of anti-tumor immune responses in patients with end-stage disease [20]. Moreover, the alphaviral vectors were administered to patients after standard treatment (usually chemotherapy), which may significantly reduce the efficiency of alphavirus infection and transgene expression. Remarkably, the majority of the successful preclinical studies using alphaviral vectors were performed in animal cancer models that did not involve pretreatment with chemical drugs. Therefore, the effect of combined chemotherapy and alphaviral therapy has not been comprehensively studied.

The efficacy of virotherapy depends on specific tumor targeting and the level of viral replication [21]. It has been reported that the application of classical chemical drugs, e.g., 5-fluorouracil (5-FU) and gemcitabine, in combination with oncolytic herpes or adenoviral vectors make cancer cells more prone to virus infection and replication [4,22], thereby enhancing the therapeutic effects of the viral vector. Alternatively, the viruses may improve the chemotherapy outcomes. For example, Newcastle disease virus has been shown to assist in overcoming cisplatin resistance in a lung cancer mouse model [23]. Moreover, the use of herpes simplex virus following doxorubicin treatment was demonstrated to eradicate chemoresistant cancer stem cells in a murine breast cancer model [24]. Also co-administration of reovirus with docetaxel synergistically enhanced chemotherapy in a human prostate cancer model [25], allowing reduced doses of chemotherapeutics to be used. Furthermore, the combination of an asymptomatic low dose of 5-FU with recombinant adenoviruses produces a synergistic effect in various cell lines and *in vivo* tumor models [26-30]. Although the detailed molecular mechanism underlying the therapeutic benefits of the combined treatment remains unknown, such a treatment has already demonstrated promising results in a clinical setting [31,32].

Whether the synergistic anti-tumor effect can be achieved using a drug combination that includes alphaviral vectors has been poorly investigated. One study showed that application of a Sindbis vector with oncolytic properties in combination with the topoisomerase inhibitor irinotecan in SCID mice bearing human ovarian cancer resulted in prolonged animal survival [33]. The authors highlight the role of natural killer cells in the induction of the anti-cancer effect by the combined treatment. Targeting of different anti-cancer mechanisms involving immune cell activation could lead to effective combinatorial therapies, though these would have to be evaluated in immunocompetent tumor models.

Using a 4 T1 mouse mammary tumor model, we investigated the efficiency of combined 5-FU and SFV vector treatment. We focused on the inhibition of cell proliferation and efficiency of transgene delivery under combined treatment *in vitro* and *in vivo*.

Methods

Cell lines and animals

BHK-21 (baby hamster kidney cells) and 4 T1 cells (metastasizing mammary carcinoma from BALB/c mice) were obtained from the American Type Culture Collection (ATCC/LGC Prochem, Boras, Sweden). BHK-21 cells were propagated in BHK - Glasgow MEM (GIBCO/Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 2 mM L-glutamine, 20 mM HEPES, streptomycin 100 mg ml^{-1} and penicillin 100 U ml^{-1}. The 4 T1 cell line was cultured in Dulbecco's minimal essential medium (GIBCO/Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, streptomycin 100 mg ml^{-1} and penicillin 100 U ml^{-1}. Specific pathogen-free 4- to 6-week-old female BALB/c mice were obtained from Latvian Experimental Animal Laboratory of Riga Stradin's University and maintained under pathogen-free conditions in accordance with the principles and guidelines of the Latvian and European Community laws. All experiments were approved by the local Animal Protection Ethical Committee of the Latvian Food and Veterinary Service (permission for animal experiments no. 32/23.12.2010).

Production of SFV (SFV/EGFP, SFV/DS-Red, SFV/EnhLuc) and SIN (SIN/EGFP) recombinant virus particles

The pSFV1 [9] and pSinRep5 [10] vectors were used in this study. The enhanced green fluorescent protein (EGFP)

gene was introduced into both vectors under the 26S subgenomic promoter. The EGFP gene was cut out of the pEGFP-C1 plasmid (Clontech, CA, USA) with *Nhe*I and *Hpa*I restriction endonucleases, treated with T4 DNA polymerase (Thermo Scientific, Lithuania) to blunt the DNA ends and ligated with the pSFV1 and pSinRep5 vectors, which were cleaved with *Sma*I and *Pml*I, respectively. Additionally, a pSFV1/DS-Red construct carrying the red fluorescent protein gene (DS-Red) [34] was generated. The DS-Red gene was amplified by PCR (primers: 5′-ATTAGGATCCACCGGTCGCCAC CATG-3′ and 5′-TATCCCGGGCTACAGGAACAGG TGGTG-3′) using the pDsRed-Monomer-C1 plasmid as a template (Clontech, CA, USA). The PCR fragment was cleaved with *Bam*HI and *Sma*I and ligated into a pSFV1 vector cleaved with the same enzymes. An SFV vector carrying the firefly luciferase gene was used for the *in vivo* experiments [35].

The resulting plasmids were used to produce recombinant virus particles as previously described [35]. pSFV-Helper [9] and pSIN-DH-EB helper [10] were used to produce the SFV and SIN particles, respectively. The DNA template was removed by digestion with RNase-free DNase (Fermentas, Lithuania). The viral titers (infectious units per ml, iu ml^{-1}) were quantified by infecting BHK-21 cells with serial dilutions of viral stock and analyzing EGFP or DS-Red expression via fluorescence microscopy on a Leica DM IL microscope (Leica Microsystems Wetzlar GmbH, Germany). For the *in vivo* application, SFV/EnhLuc viral particles (v.p.) were concentrated, and the viral titer was quantified by Real-time PCR as previously described [35].

Infection of cell lines with recombinant virus particles

Cells were cultivated in 24-well plates at a density of 2×10^5 cells per well in a humidified 5% CO_2 incubator at 37°C. For transduction, the cells were washed twice with PBS containing Mg^{2+} and Ca^{2+} (Invitrogen, UK). Next, 0.3 ml of the solution containing the virus particles was added. The SFV/EGFP, SFV/DS-Red and SIN/EGFP virus particles were diluted in PBS (containing Mg^{2+} and Ca^{2+}) to achieve a multiplicity of infection (MOI) of 10. The cells were incubated for 1 h in a humidified 5% CO_2 incubator at 37°C. The control cells (uninfected) were incubated with PBS (containing Mg^{2+} and Ca^{2+}). After incubation, the solution containing the virus was replaced with 0.5 ml of growth medium. The cells were gently washed with PBS and transferred to fresh medium every day.

MTT cell proliferation assay

The cytotoxicity was quantified using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based cell viability assay. Cells were infected in 24-well plates as described above, and proliferation was analyzed 0, 1, 2, 3, 4 and 5 days after infection. The medium was replaced with 0.3 ml of solution containing 0.5 mg ml^{-1} MTT (Affymetrix, Cleveland, USA) dissolved in D-MEM without phenol red (GIBCO/Invitrogen, UK) supplemented with 5% FBS. The cells were incubated for 2 h in a humidified 5% CO_2 incubator at 37°C. After incubation, the formazan crystals were dissolved by adding 0.3 ml of MTT solubilization solution consisting of 10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol. The absorbance was measured using a microplate spectrophotometer (BioTek Instruments, Winooski, USA) at a test wavelength of 570 nm and a reference wavelength of 620 nm. Cell viability (%) was obtained using the following equation: Percent cell viability = (test 570 nm − 620 nm)/(control 570 nm − 620 nm) × 100, where the control is the value obtained from uninfected cells (the standard error of the control was less than 3% for days 0–3 and less than 6% for days 4–5 in three independent experiments).

Fluorescence-activated cell sorting (FACS) analysis

Cells were infected on 6-well plates with SFV/EGFP and SIN/EGFP virus particles at an MOI of 10 as described above (1 ml of virus-containing solution was used for the infection). The infected cells were harvested 24 h after infection. Detached cells were harvested from the cell medium by centrifugation, and attached cells were trypsinized. The collected cells (approximately 10^6) were washed with PBS and resuspended in 1 ml of PBS. For propidium iodide (PI) staining, the cells were incubated with 10 μl of 50 μg ml^{-1} PI solution (Becton Dickinson Biosciences, San Jose, California, USA) and immediately processed for FACS analysis. EGFP and PI fluorescence was measured using a FACSAria II (Becton Dickinson Biosciences, San Jose, California, USA). The FACS data were analyzed by BD FACSDiva 6.1.2 software. Uninfected cells were used as a negative control for both the PI and EGFP FACS analysis and contained approximately 1-2% PI-positive cells in 4 T1 culture.

Fluorometry of infected/reinfected cells

Cells were seeded on 24-well plates and infected with SFV/EGFP as described above. After 24, 48 and 72 h, the infected cells were reinfected with the SFV/DS-Red virus. DS-Red fluorescence was measured 24 h after each reinfection using a fluorometric plate reader (Tecan Infinite M 200, Austria) with an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The fluorometry data were expressed as the percentage of the reinfected cell fluorescence units relative to the fluorescence units obtained from the control cells infected with SFV/DS-Red alone (positive control, 100%). The experiments were performed in triplicate.

Treatment of cells with 5-FU

5-FU powder (Sigma, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 70 mg ml^{-1} and further diluted in filtered water to 7 mg ml^{-1}. 4 T1 cells were seeded in a 24-well plate (2×10^5 cells per well). The next day, the cells were treated with medium containing 5-FU at 13, 26, 65 or 130 μg ml^{-1}. Every day for 5 days, the cells were gently washed with PBS to remove dead and detached cells, and fresh medium containing 5-FU was added. The control cells were not treated with 5-FU. The MTT cell proliferation assay was performed 0, 1, 2, 3, 4 and 5 days after the start of 5-FU treatment. The presence of DMSO traces did not affect 4 T1 cell proliferation.

Induction of tumor nodules

A 4 T1 mouse mammary tumor model was established as previously described [35]. Briefly, 4 T1 tumor cells were resuspended in PBS at a final concentration of 2.5×10^6 cells ml^{-1}. Two hundred microliters of the 4 T1 cell suspension were subcutaneously injected above the right shoulder blade of the mice. After 10 days, the obtained tumor volumes reached at least 1000 mm^3.

5-FU treatment and SFV/EnhLuc injection *in vivo*

5-FU powder (Sigma, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 300 mg ml^{-1} and then diluted in filtered water to 30 mg ml^{-1}. 4 T1 tumor-bearing mice ($n \geq 5$) were treated with 5-FU at different doses (40, 150 or 400 mg kg^{-1}) via peroral administration 4 times over a period of 8 days (every other day). One hour after the last 5-FU treatment, the mice were inoculated either i.t. (intratumoral) or i.p. (intraperitoneal) with 200 μl (4 injections of approximately 50 μl each) or 300 μl of SFV1/EnhLuc particle-containing stocks (6×10^9 v.p. ml^{-1}), respectively. As a control, 4 T1 tumor-bearing mice not treated with 5-FU were i.t or i.p. inoculated with the same dose and volume of SFV1/EnhLuc.

Analysis of luciferase gene expression in mouse organs and tumors

The Luc gene expression level was estimated by measuring luciferase enzymatic activity in tissue homogenates 24 h after SFV/EnhLuc virus administration. The tumors and organs were excised and manually homogenized in a 1x concentration of ice-cold lysis buffer (Cell Culture Lysis buffer, Promega) containing a protease inhibitor cocktail (10 μl per 1 ml of lysis buffer) (Sigma, St. Louis, MO, USA). After homogenization, the samples were centrifuged for 10 min at $9000 \times g$, and the protein concentration was determined in tissue lysates using the BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, UK). Luciferase activity was measured by adding 100 μl of freshly reconstituted luciferase assay buffer to 20 μl of the tissue homogenate (Luciferase Assay System, Promega, USA) and then was quantified as relative light units (RLUs) using a luminometer (Luminoskan Ascent, Thermo Scientific, UK). The RLU values were expressed per mg of protein in the lysates. As a negative control, 4 T1 tumor-bearing mice were inoculated with PBS, and the maximal negative values were subtracted from the presented results.

The efficacy index of the 5-FU and SFV combined treatment was calculated using the formula (RLU in 5-FU treated mice/RLU in 5-FU non-treated mice)/(tumor weight in 5-FU treated mice/tumor weight in 5-FU non-treated mice). For example: the efficacy index = (3497925.0/1397062.5)/(681.3/690.9) = 2.5. The efficacy index thus reflects the level of SFV expression (increase in RLU) and the effect of the 5-FU treatment (reduction in tumor weight).

Analysis of FITC-dextran accumulation

The first group of 4 T1 tumor-bearing mice (n = 3) was treated with 150 mg kg^{-1} 5-FU as described above and the second group (n = 3) was untreated with 5-FU. Next day after the last 5-FU treatment the mice from both groups were inoculated i.v. with 120 μl of FITC-dextran 2000 kDa solution (40 mg ml^{-1} in PBS) (Sigma). Two hours later tumors were collected and incubated overnight in 4% paraformaldehyde. After cryoprotection in 20% sucrose tumors were frozen in OCT compound (Sigma). Cryosections (10 μm) were prepared and the intensity of FITC-dextran leakage was visualized by fluorescent microscopy. Pixels of images were measured by ImageJ software.

Analysis of IFN-alpha in tumor lysates

Two groups of 4 T1 tumor-bearing mice (n = 6 each) were either treated or non-treated with 150 mg kg^{-1} 5-FU as described above. One hour after the last 5-FU treatment, three mice from each group (n = 3) were inoculated i.t. with 200 μl (4 injections of approximately 50 μl each) of SFV1/EnhLuc particle-containing stocks (6×10^9 v.p. ml^{-1}). 18 hours after the virus administration, 4 T1 tumors were isolated and frozen in liquid nitrogen. Frozen tumors were manually homogenized with homogenization hammer and tissue powders were resuspended in 500 μl of PBS. To provide better tumors homogenization, two freeze-thaw cycles were performed. After homogenization, samples were centrifuged for 10 min at $5000 \times g$ and the protein concentration was equalized in all tissue lysates using the BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, UK). Expression of IFN-alpha in 4 T1 lisates was determined using ELISA Kit for Interferon Alpha (Uscn Life Science Inc., China), according provided protocol. The obtained data (pg/ml)

were expressed in % relative to lysates non-treated with both the 5-FU and the virus.

Statistical analysis

The cell viability and RLU results are presented as the means ± standard error of 3 independent experiments. The statistical analysis of the results was performed using Microsoft Excel and Statistica7 (StatSoft, Tulsa, OK, USA). Statistically significant differences were determined using Student's t-test ($P < 0.05$).

Results

Transduction efficiency and cytotoxicity of alphaviral vectors in 4 T1 cells

To select the most efficient cytotoxic alphaviral vector for 4 T1 mouse mammary carcinoma cells, we compared cell survival and transduction efficiency for two commonly used vectors based on SFV and SIN replicons. 4 T1 cells were infected with equal amounts of recombinant particles (multiplicity of infection, MOI = 10) encoding the EGFP gene. FACS analysis of EGFP-positive cells was performed at 24 h post-infection. As shown in Figure 1a (FACS assay), the SFV vector yielded a higher proportion of EGFP-positive cells (60%) compared with the SIN vector (38%).

The percentage of EGFP-positive cells measured via FACS indicates the transduction efficiency and the ability of the vector to express the gene of interest. However, alphaviral vectors may provoke cytopathic effects without generating observable transgene expression. This discrepancy is due to the strong induction of rapid apoptosis, which prevents the accumulation of the recombinant product within the cell. To evaluate the immediate (24 h after infection) cytotoxic effects of alphaviral infection, 4 T1 cells were stained with propidium iodide (PI), a membrane-impermeable fluorescent dye that is generally excluded from viable cells. The percentage of PI-positive (dead) cells measured by FACS was similar for the SFV and SIN vectors (7%) (Figure 1a). Nevertheless, the SFV vector provoked a stronger inhibition of cell proliferation than the SIN vector in 4 T1 cells, as demonstrated by the MTT cell viability assays performed over the 5 days following infection (Figure 1a). Despite the strong cytotoxic effect of the SFV vector, the 4 T1 cell culture (in contrast with other highly infectable cancer cell lines, e.g., Huh-7, PA1, H2-35, not shown) survived infection at present conditions, and cell proliferation was completely restored within 8–10 days.

Repeated infections were next tested as a means of enhancing the infectivity and cytotoxicity of the alphavirus. Remarkably, repeated infection of surviving cell culture with the same or a different alphaviral vector (SFV or SIN, respectively) did not produce a significant enhancement of transgene production or prolongation of cytotoxicity. As shown in Figure 1b, the 4 T1 cell culture infected with SFV/EGFP were less susceptible to repeated infection with SFV/DS-Red particles encoding the DS-Red fluorescent protein [34]. Only a very small number of EGFP-negative cells (which did not express the transgene after the first infection) were able to express the DS-Red gene, indicating that the cells could not be doubly infected by both alphaviruses. Similar results were obtained with the SIN vector and with other combinations of SFV/SIN and SIN/SFV reinfection (not shown). Moreover, an MTT cell viability analysis did not reveal a difference in the cell proliferation patterns of singly and doubly-infected cells (not shown). We conclude that the repeated application of alphaviral vectors is not an efficient strategy to achieve complete inhibition of cancer cell proliferation. This effect may be attributable to the overall cellular protein synthesis down regulation [11] and strong induction of an anti-viral response [36,37] that makes the repeated application of the vector inefficient.

The SFV vector was selected for further cytotoxicity analysis in combination with 5-FU.

Combined treatment of 4 T1 cells with SFV and 5-FU

The low efficiency of oncolytic virotherapy in preclinical studies might be associated with anti-vector immunity or the resistance of tumors to repeated infections. Recently, multiple strategies involving the combination of oncolytic vectors with classic cytotoxic drugs have proven to be advantageous for certain types of cancer (for review, see Wennier et al. 2012) [1]. Here, we analyzed whether the combination of the SFV alphaviral vector and 5-FU exerts a synergetic effect on cancer cell proliferation.

To analyze the cytotoxic effect of 5-FU on 4 T1 cells, cell monolayers were exposed to different concentrations of 5-FU for 5 days (Figure 2a). After 5 days of incubation, high concentrations of 5-FU (65 and 130 μg ml^{-1}) resulted in complete inhibition of cell proliferation on days 5 and 4, respectively. Cells incubated with a low concentration of 5-FU (13 μg ml^{-1}) displayed approximately 25% viability on day 5, but further incubation did not lead to complete cell death under these conditions. For the combined treatment, the highest (130 μg ml^{-1}) and the lowest (13 μg ml^{-1}) 5-FU doses were tested.

The notion that recombinant alphaviruses expressing, e.g., anti-tumor genes and/or inducing anti-tumor immune responses must be applied prior to chemical drug treatment is rational. Therefore, we first tested whether 5-FU could inhibit the proliferation of cells previously infected with SFV. As shown in Figure 2b, 4 T1 cells were infected with SFV/EGFP 2 days prior to treatment with 5-FU. The kinetics of 4 T1 cell proliferation in the

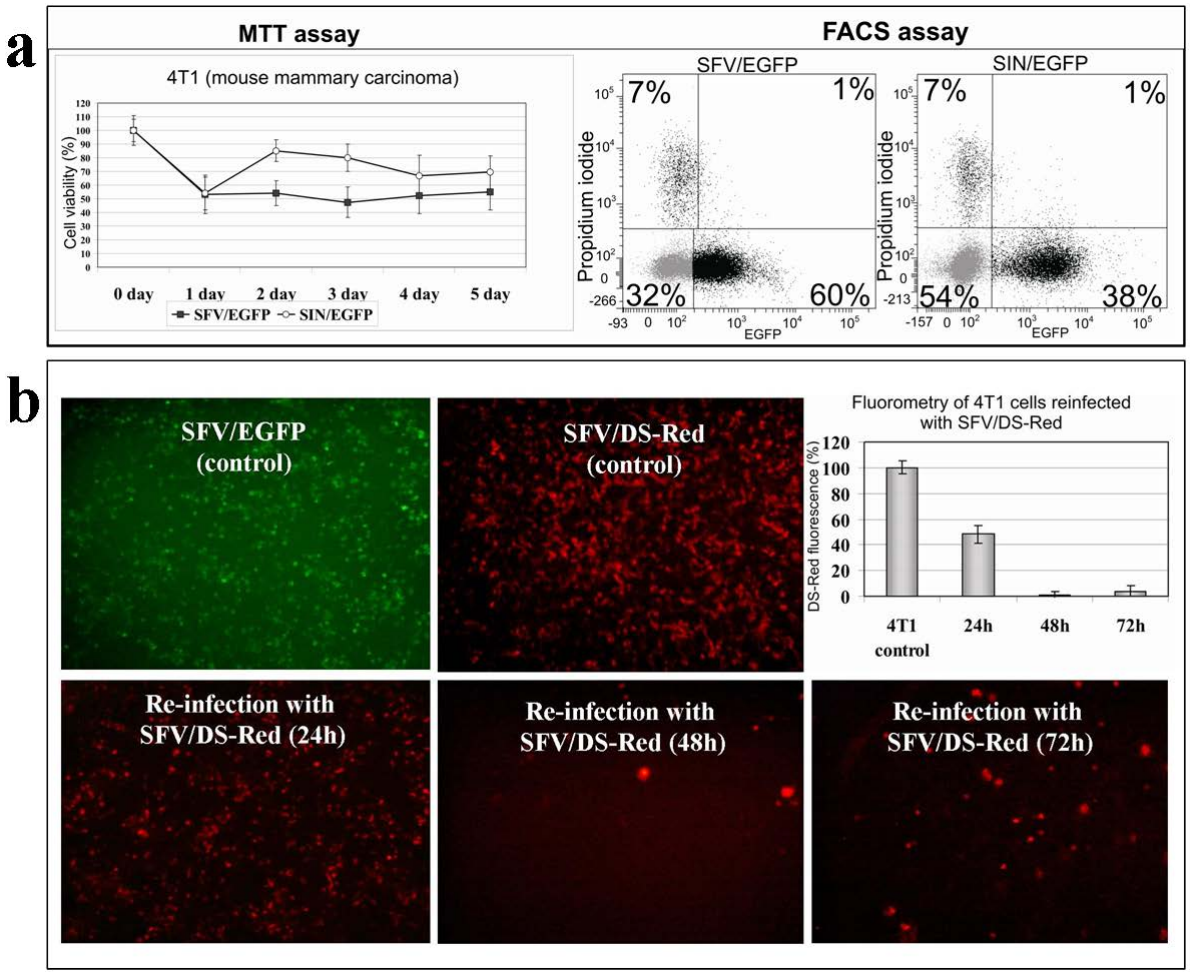

Figure 1 Transduction efficiency and cytotoxicity of SFV and SIN alphaviral vectors in 4 T1 cells. (a) 4 T1 cells were infected with SFV and SIN particles encoding EGFP. At 24 h post-infection, the cells were harvested, stained with PI and subjected to dual FACS analysis. The x-axis and the y-axis represent E*GFP* and *PI* fluorescence, respectively. The percentage of living/dead cells and EGFP-positive/negative cells is indicated on the plot. The FACS data shown are from representative experiments (n = 3). The diagram on the left (MTT assay) demonstrates the cytotoxic effects of SFV and SIN infection. An MTT cell viability assay was performed every day for 5 days post-infection. The results are presented as the percentage of viable cells relative to the control (uninfected cells). The error bars indicate the standard error of 3 independent experiments. **(b)** Repeated infection of 4 T1 cells. The cells were infected with SFV expressing EGFP (pictures show green fluorescence) and then re-infected 24, 48 and 72 h later with SFV expressing DS-Red (pictures show red fluorescence). Fluorometry of DS-Red fluorescence was performed 1 day after each re-infection. The diagrams represent the percentage of fluorescence units in re-infected cells relative to control cells (100%), which were primarily infected with only SFV/DS-Red. The error bars indicate the standard error of three experiments.

combined treatment approach (SFV plus 5-FU) was similar to those of infected 4 T1 cells. The SFV infection of 4 T1 cells alone resulted in 55% of cell viability on day 5 after infection (Figure 1a, MTT-test, SFV). In the case of combined treatment, the cell viability was not significantly changed and resulted in 50% and 40% viability after treatment with 13 μg and 130 μg of 5-FU on day 5, respectively (Figure 2b). Therefore, the application of 5-FU after SFV did not significantly influence the survival of the 4 T1 cell culture, even at the high drug dose (130 μg ml^{-1}), providing the evidence for infected cell culture resistance to further treatment with cytotoxic agent.

Short pretreatment of cancer cells with 5-FU has recently been shown to significantly enhance the infectivity of adenoviruses [30,38]. To investigate the effect of 5-FU on alphavirus infection, 4 T1 cells were pretreated with high (130 μg ml^{-1}) and low (13 μg ml^{-1}) concentrations of 5-FU for 2 days and then infected with SFV/DS-Red. As shown in Figure 3, preincubation of cells with 5-FU almost completely inhibited alphaviral infection. Moreover,

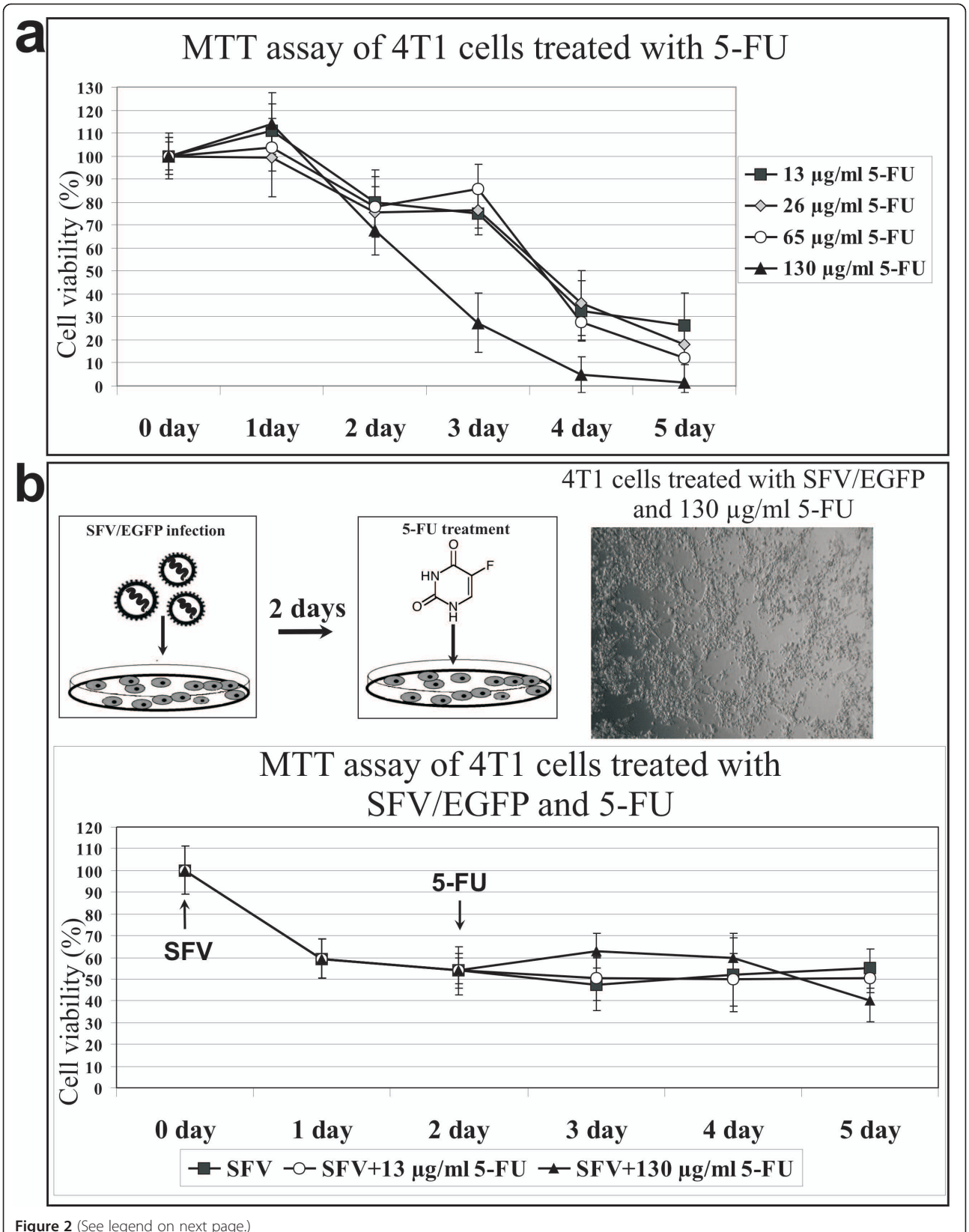

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Figure 2 Evaluation of 4 T1 cell proliferation after 5-FU treatment and in combination with SFV infection. (a) 5-FU treatment. 4 T1 cells were grown in cell culture medium (24-well plates) containing the indicated concentrations of 5-FU. The MTT cell viability assay was performed every day for 5 days. The diagram shows the cytotoxic effect of 5-FU on 4 T1 cells as the percentage of viable cells relative to the control (untreated cells). **(b)** Schematic representation of the combined treatment with SFV and 5-FU. The cells were infected with SFV/EGFP particles, and the medium was replaced 2 days later with medium containing 5-FU. The MTT cell viability assay was performed every day for 5 days. The arrows designate the day of infection (SFV) and the beginning of the drug treatment (5-FU). The diagram shows the cytotoxic effect of 5-FU following SFV infection as the percentage of viable cells relative to the control (untreated cells). The error bars indicate the standard error of 3 independent experiments. The microscopy image shows a 4 T1 cell monolayer at day 5 after treatment with SFV and the highest concentration of 5-FU.

in contrast to the adenoviral vector, a short (2 h) pretreatment of 4 T1 cells with a low dose of 5-FU (13 μg ml^{-1}) slightly inhibited alphaviral infection, with a total decrease in fluorescence of approximately 10-15% compared with infected cells not treated with 5-FU (not shown). Lower 5-FU concentrations (below 13 μg ml^{-1}) had no significant effect on alphaviral infectivity in 4 T1 cells (not shown).

To measure the inhibition of cell proliferation produced by the combined treatment, 5-FU-pretreated 4 T1 cells were infected with SFV and subjected to cell viability analysis over a period of 5 days (Figure 4). Pretreatment of 4 T1 cells for 2 h with a high dose of 5-FU (130 μg ml^{-1}) followed by infection with SFV did not significantly impair cell proliferation compared with 4 T1 cells that were only infected with SFV (Figure 4b). On day 5, the cell viability was approximately 52%. In a similar way, application of a low dose of 5-FU (13 μg ml^{-1}) for 2 h did not provoke a significant enhancement of cytotoxic effect of SFV (70% on day 5) compared to the SFV infection alone (60% on day 5), indicating the absence of synergy between 5-FU and SFV. Furthermore, prolonged incubation with 5-FU (for 2 days) also did not produce a significant difference in infected cell proliferation at either dose tested, comparing to uninfected cells under similar conditions (Figure 4c). The cells that were pretreated with a low dose of 5-FU began to resume cell division (49% cell viability) by day 5, whereas the cells treated with a high dose reached 24% cell viability, similar to the controls: cells that were treated with 5-FU but not infected with SFV (64% and 23%, respectively). Therefore, the treatment strategy, in which 5-FU was used prior to virus infection, strongly inhibited SFV expression and did not produce synergistic cytotoxic effect in 4 T1 cells.

The effect of 5-FU treatment on SFV expression in 4 T1 tumor-bearing mice

To investigate the efficiency of SFV-driven transgene expression after 5-FU chemotherapy, 4 T1 tumor-bearing mice were perorally (p.o.) treated with 5-FU and then inoculated with SFV/EnhLuc by intratumoral (i.t.) injection of 3×10^8 virus particles encoding firefly luciferase. The mice were treated with different doses of 5-FU 4 times, every other day (Figure 5a). The lower dose (40 mg kg^{-1}) resulted in no visible toxic effects or any significant tumor inhibition; this dose is therefore considered asymptomatic. The medium dose (150 mg kg^{-1}) produced a minimal tumor size reduction and medium toxicity (loss of appetite). The high dose (400 mg kg^{-1}), by contrast, yielded significant tumor inhibition and strong side effects (watery diarrhea, weight loss, hunched posture). After the last 5-FU treatment (1 h later), the mice were i.t. inoculated with SFV/EnhLuc virus particles, and Luc gene expression was measured 24 h later via luminometry on tumor lysates. The highest luciferase activity was detected in the tumors of mice treated with the highest dose of 5-FU (400 mg kg^{-1}) (Figure 5b), with increases in transgene production of approximately 50-fold compared with mice not treated with 5-FU and approximately 14-fold compared with the low dose treatment (40 mg kg^{-1}). Remarkably, this asymptomatic low dose also produced a statistically significant 3.6-fold increase in luciferase activity ($p < 0.05$).

Because the low dose improved transgene expression and had no signs of toxicity, this dose was used to evaluate the tumor targeting and biodistribution of SFV particles upon intraperitoneal (i.p. 1.8×10^9 v.p.) administration in combination with 5-FU. As presented in Figure 5c, the highest levels of Luc gene expression were detected in the tumors and hearts of mice treated with 40 mg kg^{-1} 5-FU. Although significantly lower total Luc expression was observed with i.p. inoculation compared with the i.t. route, the Luc level in the tumors was still 2.1-fold higher ($p < 0.05$) in i.p. inoculated mice relative to 5-FU untreated mice. Among the other organs, only the heart showed an increase in Luc expression after 5-FU treatment (1.4-fold; not significant). Remarkably, there were no significant changes in vector biodistribution observed in the case of i.t. administration (not shown). The i.t. inoculation provided no further distribution of the vector to organs in both 5-FU treated and untreated mice, confirming therefore the enhancement of vector expression specifically in tumor of 5-FU treated animals.

Discussion

One strategy to enhance cancer virotherapy is to apply viral vectors in combination with standard and

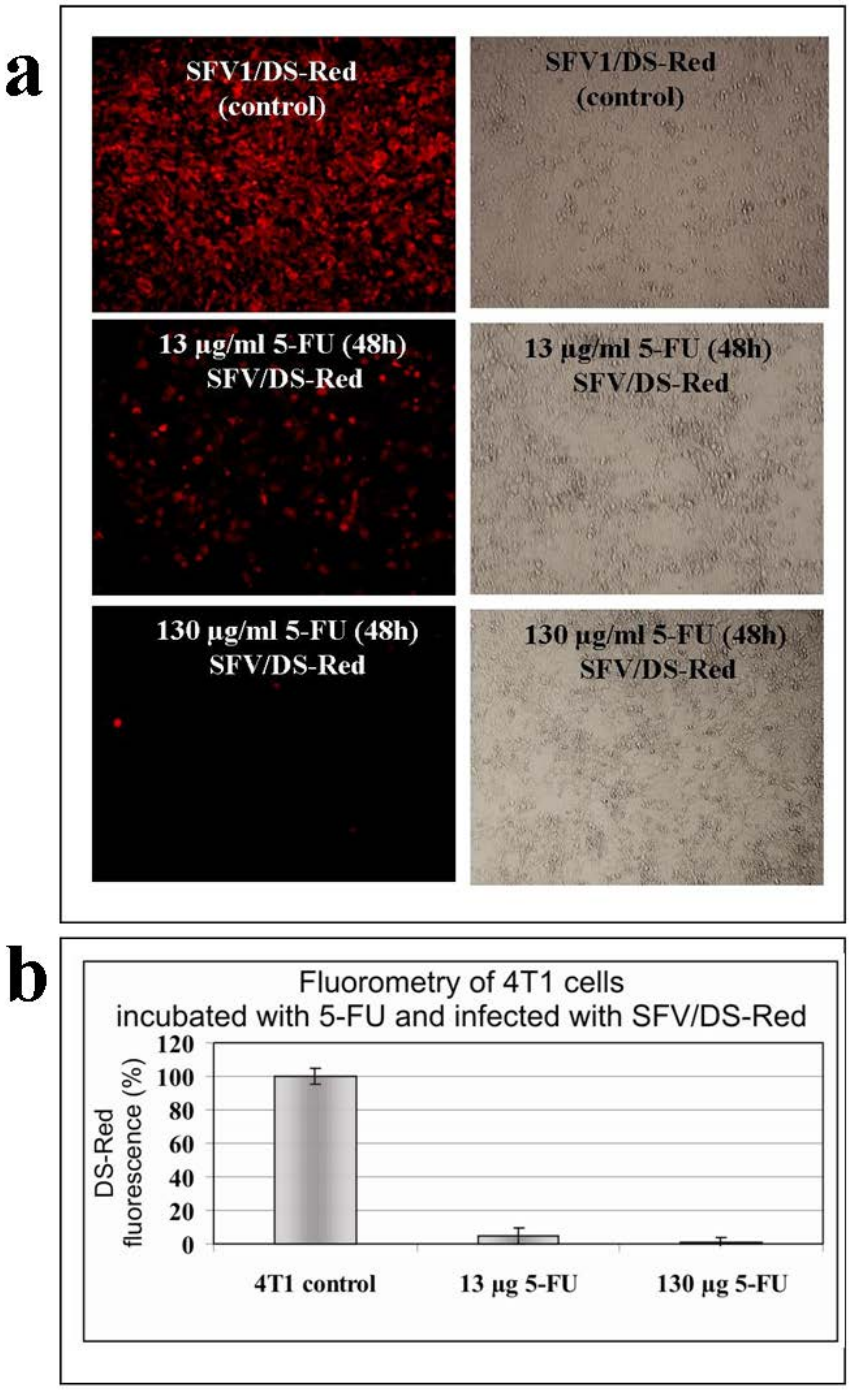

Figure 3 Inhibition of S**FV/DS-Red infection in 4 T1 cells pretreated with 5-FU.** 4 T1 cells were treated with high (130 µg ml^{-1}) or low (13 µg ml^{-1}) concentrations of 5-FU for 2 days, then infected with SFV/DS-Red particles. **(a)** Fluorescence and phase contrast microscopy pictures. **(b)** Fluorometric measurement of DS-Red fluorescence in infected cells at 24 h post-infection. The diagram shows the percentage of fluorescence units measured in the cells pretreated with 5-FU (13 µg ml^{-1} or 130 µg ml^{-1}) and then infected with SFV/DS-Red relative to 4 T1 control cells (100%) that were only infected with SFV/DS-Red. The error bars indicate the standard error of three independent experiments.

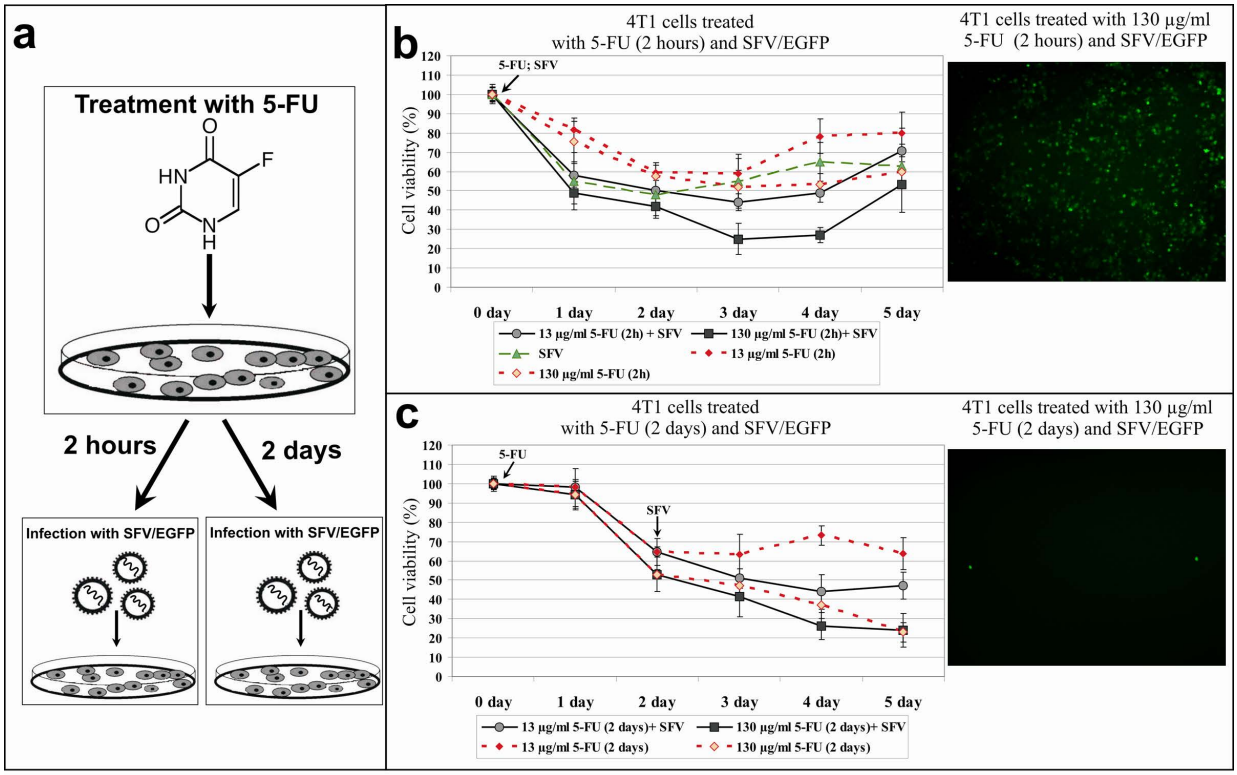

Figure 4 Evaluation of cytotoxicity in 4 T1 cells treated with 5-FU and then infected with SFV. (a) Schematic representation of the experiment. 4 T1 cells were pretreated with high (130 µg ml^{-1}) or low (13 µg ml^{-1}) doses of 5-FU for 2 h or 2 days and then infected with SFV/EGFP particles. **(b)** 4 T1 cells treated with 5-FU for 2 h and infected with SFV/EGFP particles (solid lines). The dotted lines (red) show the controls: cells treated with 5-FU for 2 h and then incubated in complete medium for 5 days. The dashed line (green) shows the cells infected with SFV/EGFP particles. **(c)** 4 T1 cells treated with 5-FU for 2 days and infected with SFV/EGFP particles (solid lines). The dotted lines (red) show the controls: cells treated with 5-FU for 2 days (day 0–2) and then incubated in complete medium for further three days. An MTT cell viability assay was performed every day for 5 days. The diagrams show the cytotoxic effects of 5-FU and SFV/EGFP, which are expressed as the percentage of viable cells relative to the untreated cells. Arrows indicate the beginning of drug treatment (5-FU) and the day of infection (SFV). Error bars show the standard error of three experiments. Fluorescent images demonstrate the efficiency of SFV/EGFP expression on the day after infection of 5-FU pretreated 4 T1 cells.

well-studied chemical drugs to promote synergistic actions and potentially lead to effective therapy outcomes. Classic alphaviral vectors based on SFV and SIN replicons have been used for *in vitro* and *in vivo* cancer gene therapy experiments and have shown promising results in different cancer models [39,40]. Nevertheless, the problems of tumor recovery and the inefficiency of repeated vector administration remain to be solved. In this study, we explored the efficiency of SFV-mediated gene transfer in combination with 5-FU and the possibility of a synergistic cytotoxic effect of the combined treatment in the highly proliferative 4 T1 mouse breast cancer model.

5-FU is an antitumor drug typically included in breast carcinoma chemotherapeutic regimens [41,42]. The cytotoxic effect of 5-FU occurs through the inhibition of the synthesis and functioning of DNA and RNA. Although the general mechanism of 5-FU action as an anti-metabolite has been investigated [43], little is known about the intracellular molecular changes that lead to apoptosis in the presence of 5-FU. Protein kinase R (PKR) has been shown to be a molecular target of 5-FU-induced apoptosis [44], suggesting that 5-FU might induce apoptosis via a mechanism similar to that of alphaviruses: the double-stranded RNA intermediates made during alphavirus genome/subgenome replication also activate PKR, which contributes to the inhibition of protein synthesis [45]. PKR has also been shown to play an important role in the induction of apoptosis by other drugs, such as doxorubicin and etoposide [46,47], which have been successfully used in combination with other viruses [48,49]. Therefore, the combined treatment with alphavirus and 5-FU presented herein could potentially produce a synergistic effect due to the targeting of similar pathways that may work together to enhance cytotoxicity in cancer cells. Nevertheless, this combined treatment showed poor efficiency in

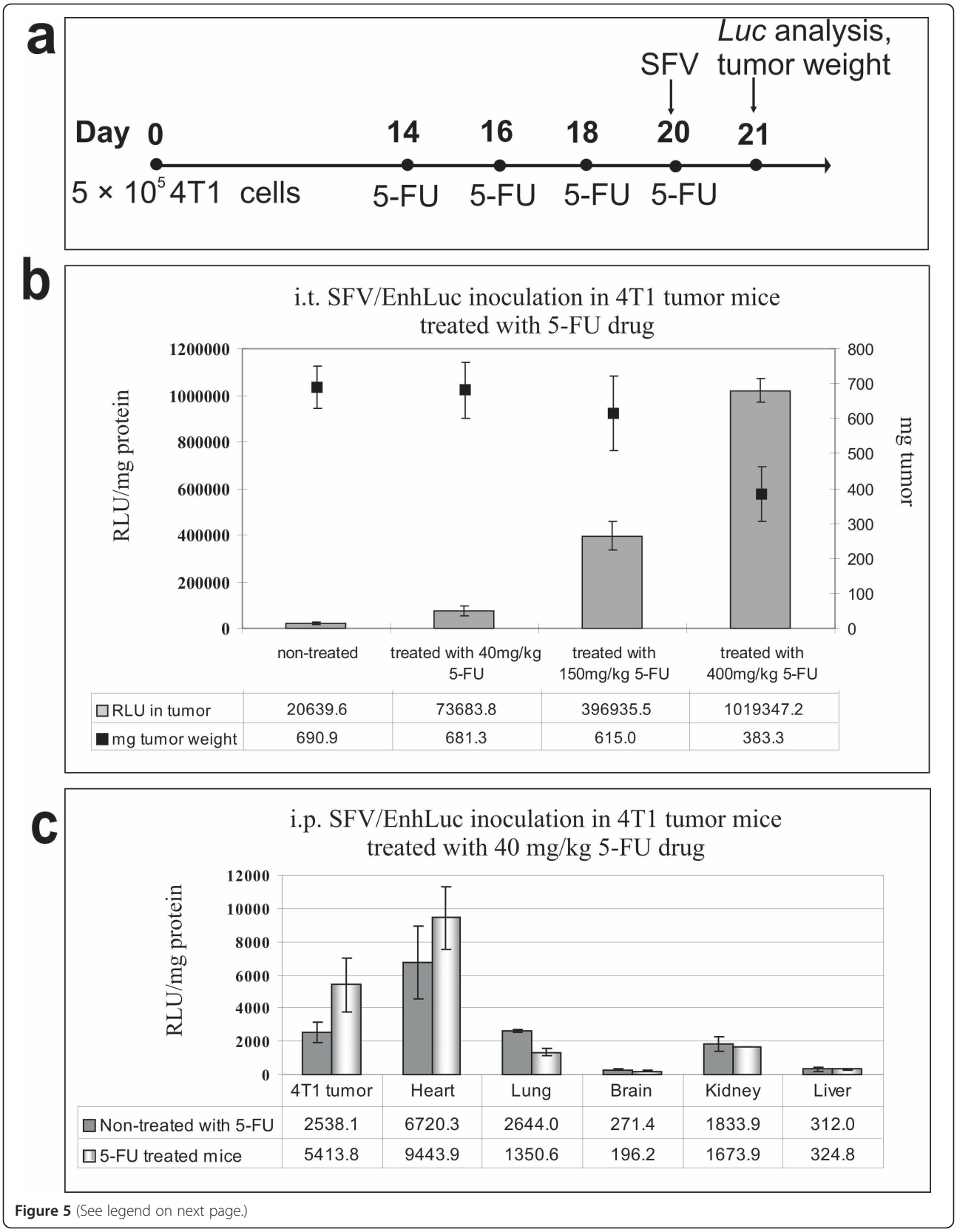

Figure 5 (See legend on next page.)

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Figure 5 SFV expression in 4 T1 tumor-bearing mice treated with 5-FU. (a) Experiment design: Balb/c mice (n = 5 in each group) were subcutaneously inoculated with 4 T1 cells; beginning on day 14, the mice were treated four times with 5-FU, every other day (40 mg kg^{-1}, 150 mg kg^{-1} or 400 mg kg^{-1}). On day 20, after the last 5-FU administration, the mice were i.t. or i.p. inoculated with SFV/EnhLuc virus particles. Tumor weight and Luc gene expression were measured 24 h after viral inoculation. **(b)** Intratumoral Luc gene expression after i.t injection of SFV/EnhLuc virus particles in 5-FU-treated mice. Luciferase activity was measured in tumor homogenates 24 h after virus inoculation. Tumor weights were measured prior to homogenization (scale on the right). **(c)** SFV/EnhLuc virus biodistribution in 4 T1 tumor-bearing mice treated with 40 mg kg^{-1} 5-FU. Luciferase activity was measured in tumor and organ homogenates 24 h after i.p. virus inoculation. The graphs present the RLUs per mg protein in each organ or tumor (see Methods section). The results are presented as the means ± s.e. The average RLU values and tumor weights are indicated in the tables. RLU, relative light unit.

4 T1 cells *in vitro*. Neither SFV infection with subsequent 5-FU treatment (Figure 2b) nor the opposite strategy of pretreatment with 5-FU and later infection with SFV (Figure 4) produced a more efficient inhibition of cell proliferation compared with SFV or 5-FU alone (Figures 1a and 2a). Moreover, pretreatment of cells with 5-FU significantly inhibited SFV infection and transgene expression (Figure 3).

The basis for the resistance of the surviving cell population to high 5-FU doses and SFV infection in the combined treatment remains unclear. Cabrele et al. [4] and others demonstrated stimulation of adenoviral vector infection via 2 h of low-dose pretreatment with 5-FU in human colon carcinoma cell lines. In contrast to adenoviruses, RNA containing alphaviruses replicate their genome in the cytoplasm. The extremely efficient alphaviral RNA replication is regulated by the virus-encoded replicase complex and the specific secondary structure of the RNA genome [50]. As previously described, incorporation of 5-FU metabolites into RNA may change RNA structure and/or affect tRNA and rRNA function [43]. It is thus possible that a similar incorporation of 5-FU metabolites into alphaviral genomic and subgenomic RNAs may likewise alter RNA secondary structure and inhibit its replication and translation. The presence of 5-FU and its metabolites could also inhibit the viral replicase in a similar manner to that observed in the inhibition of the active center of thymidylate synthetase by 5-fluorodeoxyuridine monophosphate [51]. We conclude that this combined treatment produces no synergy in the induction of apoptosis but rather inhibits alphaviral replication and transgene production.

Several oncolytic viruses have been applied in combined treatments in mouse tumor models [1]. However, less is known about the efficiency of infection or the kinetics of virus persistence under combined treatment in mice because most studies focused on the significant therapeutic effects and tumor growth inhibition. The fact that multiple different combinations of viruses (enveloped, unenveloped, dsDNA, RNA, ssDNA) and cytotoxic chemical drugs (antimetabolites, antibiotics) all produce synergistic therapeutic effects implies a common non-specific mechanism underlying such a benefit. Here, we observed a significant enhancement of intratumoral SFV-mediated transgene expression in mice treated with 5-FU (Figure 5). The low dose (suboptimal) of 5-FU provoked a 3.6-fold increase in Luc gene expression, whereas the high dose (400 mg kg^{-1}, which is close to the maximum-tolerated dose of chemotherapy regimens) yielded a 50-fold increase. This positive correlation between 5-FU dose and the level of Luc expression contradicts the *in vitro* results; however, this correlation is in line with the promising results obtained using other viruses in combination with 5-FU in mouse models [52-54].

5-FU is widely distributed to all tissues, including sites of active cell proliferation [55]. In addition to the tumor, the primary target cells are endothelial cells in blood vessels. Therefore, 5-FU treatment leads to massive cell death in places with high vascularization (including the tumor), which may increase tissue permeability to macromolecules and viruses in particular. The high level of SFV expression observed following 5-FU treatment might be explained by the enhanced permeability and retention (EPR) effect [56,57], which leads to passive and preferential accumulation and more efficient intratumoral distribution of the virus at sites of enhanced vascular permeability. To compare tumor vascular leakage in mice treated and untreatedwith 5-FU, we used fluorescein isothiocianate-conjugated dextran 2000 kDa (FITC-dextran 2000), a polysaccharide with a high molecular mass that is used as a model of permeability and retention for macromolecular structures such as nanoparticles, liposomes and viruses [58]. FITC-dextran 2000 was injected via the tail vein in 4 T1 tumor bearing mice and 2 h later the tumor cryosections were subjected to fluorescence analysis. As shown in Figure 6a and b, the distribution intensity of FITC-dextran 2000 within the tumor was significantly higher in 5-FU treated mice (150 mg kg^{-1}) comparing to the untreated control. This observation supports the idea that 5-FU treatment elevates tumor vascular permeability of macromolecular structures that might lead to enhanced virus distribution and high level of transgene production in 5-FU treated animals. The concept of enhanced virus intratumoral distribution after drug treatment is also supported by the

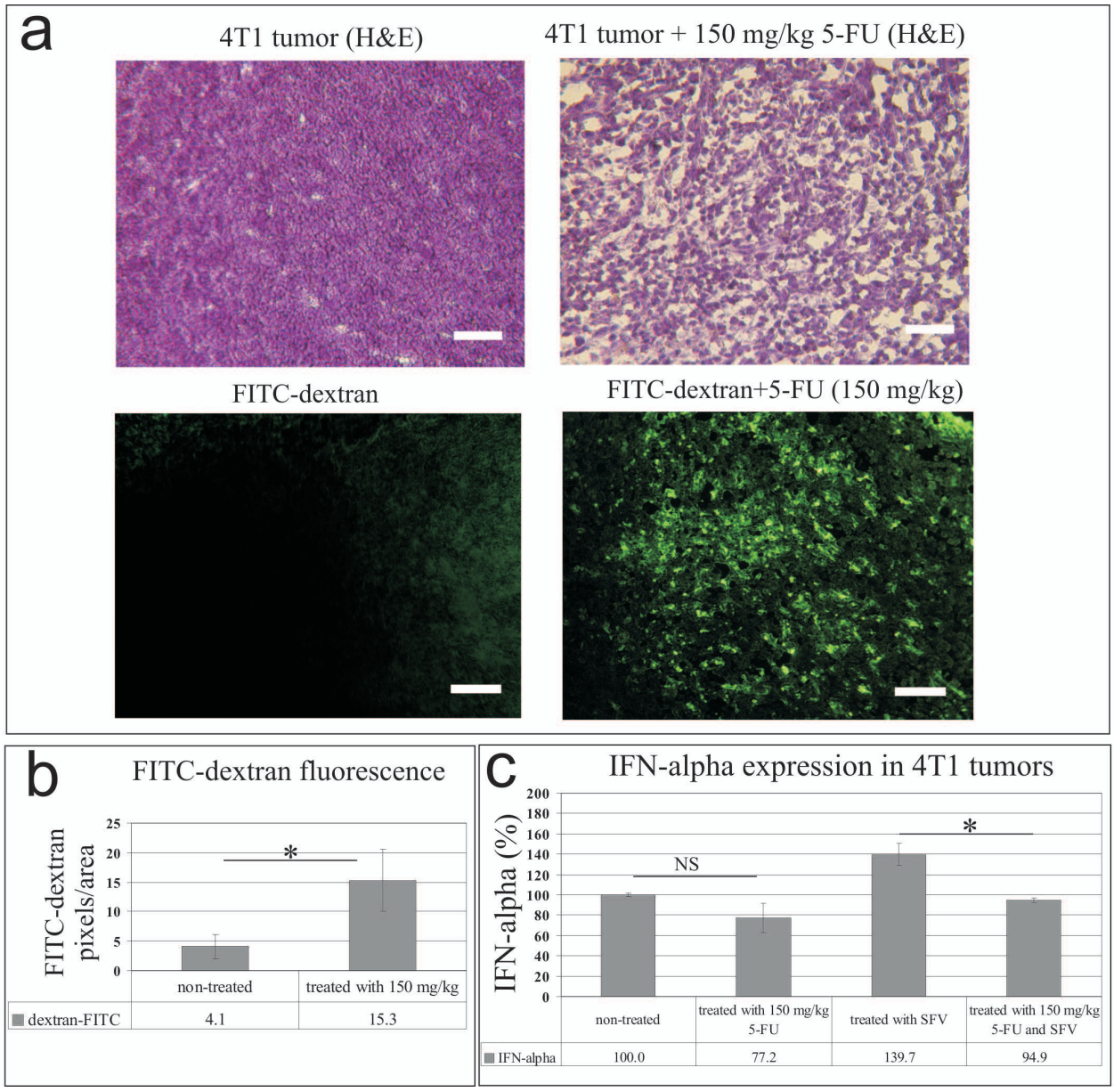

Figure 6 Evaluation of vascular permeability and intratumoral level of IFN-alpha in 4 T1 tumor-bearing mice treated with 5-FU. **(a)** Fluorescent microscopy of 4 T1 tumor cryosections demonstrating a FITC-dextran 2000 kDa accumulation in untreated (left) and 5-FU treated (right) tumors. FITC-dextran 2000 kDa (green) was intravenously-administrated and 2 h later tumors were processed as described in methods. FITC, fluorescein isothiocyanate. H&E, hematoxylin and eosin staining of cryosections. The 5-FU exhibited morphological changes in tumor sections, microvessel density was significantly lower than that in the untreated control, large patchy necrosis was visualized. **(b)** The diagram shows the data from three tumors, evaluating ten sections per tumor. **(c)** IFN-alpha response to intratumoral SFV/EnhLuc injections in tumor-bearing mice treated and untreated with 5-FU. The IFN-alpha level is expressed in % relative to tumors non-treated with 5-FU and virus. Two groups of mice (n = 6) were treated and non-treated with 5-FU, respectively, then a half of each group of mice was subjected to intratumoral injections of SFV/EnhLuc. The IFN-alpha response was measured in tumor homogenates after 18 h of virus injection. NS – non-significant differences; * - significant differences ($p < 0.05$), mean ± SD; bar 200 μm.

results of Tseng et al. [59], who demonstrated a significant enhancement in tumor vascular permeability and oncolytic Sindbis vector targeting following chemotherapy. Notably, those authors also did not observe a positive effect of the drug treatment (paclitaxel) on virus infection and replication *in vitro*.

Besides the changes in tumor vascular permeability mediated by 5-FU treatment, an antiviral immune response

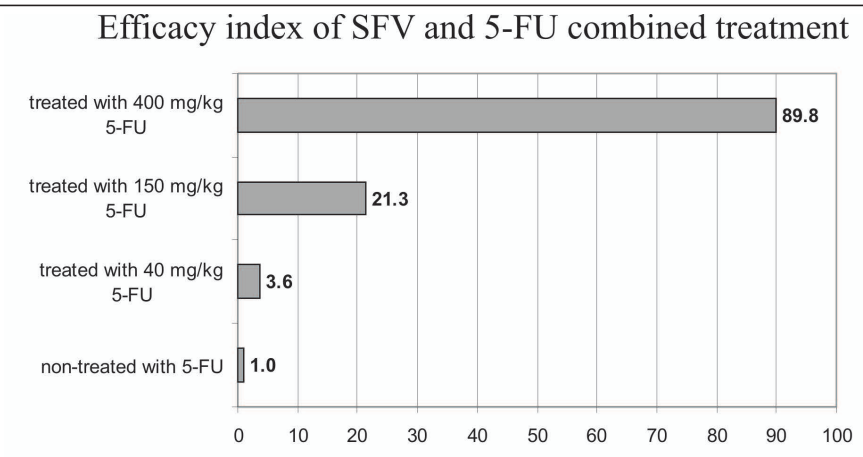

Figure 7 The efficacy index of SFV and 5-FU combined treatment of 4 T1 tumor-bearing mice. The mice were treated with different doses of 5-FU and then i.t. inoculated with SFV/EnhLuc virus particles. The efficacy index reflects the correlation of tumor growth inhibition with the level of SFV expression in 5-FU treated mice. The calculations are described in the Methods section.

has to be considered as a factor which affects the infection. At the early step of infection alphaviruses are sensitive to type I IFN production [60,61]. We have examined the intratumoral level of IFN-alpha in 5-FU treated and untreated tumor bearing mice as a response to i.t. administrated SFV (Figure 6c). The results indicate a significant inhibition of IFN-alpha antiviral response in 5-FU treated tumors, evidencing the innate immunity inhibition by 5-FU that at the same time might lead to enhanced virus replication.

Therefore, we propose that pretreatment with a cytotoxic drug may enhance the efficiency of alphaviral-mediated transgene delivery through the EPR effect and the inhibition of antiviral IFN-alpha response. Here we have demonstrated a significant 3.6-50.0 fold increase in Luc transgene expression that can be regulated by 5-FU dose. Although we did not observe any differences in tumor growth and survival rates (not shown) between the groups of animals treated with 5-FU and treated with combination of 5-FU and SFV/EnhLuc, the observed enhancement of intratumoral virus expression mediated by 5-FU pretreatment has a potential to advance the alphavirus-driven transgene delivery field. The insertion of proinflammatory transgenes into the vector instead of reporter *luc* gene could be promising for further optimization of SFV-based virotherapy of cancer to enhance the effect of chemotherapy and to prevent tumor recurrence and metastasis.

Conclusions

In this study, we describe the enhanced intratumoral expression of a replication-deficient SFV vector following 5-FU treatment in the 4 T1 mouse mammary tumor model. To illustrate the efficacy of the combined treatment, we introduced "the efficacy index", which revealed a decrease in tumor weight upon 5-FU treatment that was correlated with an increase in SFV expression. As presented in Figure 7, the highest efficacy index (89.8) was observed with the high 400 mg kg^{-1} 5-FU treatment, which provoked a significant inhibition of tumor growth and the most efficient intratumoral SFV expression. The application of a subtherapeutic dose (40 mg kg^{-1}) of 5-FU also led to a 3.6-fold enhancement of SFV expression upon i.t. vector administration. Moreover, 5-FU treatment did not change the distribution of SFV upon i.p. inoculation, allowing preferential vector expression in the tumor and heart and leading to a 2.1-fold increase in intratumoral SFV expression.

Although the combined treatment did not show a synergistic anti-proliferative effect *in vitro* due to the strong inhibition of SFV replication by 5-FU, the significant increase observed in intratumoral SFV expression (even at a low drug dose) might enhance the transgene delivery of alphaviral vectors and their general therapeutic potential.

Competing interest

The authors declare no conflict of interest.

Authors' contributions

AZ conducted the study, participated in its design and data interpretation and wrote the manuscript; JV carried out the *in vitro* and *in vivo* studies, performed the calculations, produce the figures; DZ carried out the fluorometric assay; DS carried out experiments with animals; AS performed FACS analysis; AP helped with 5-FU application; TK participated in coordination of the study and data analysis. All authors read and approved the final manuscript.

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Paper III

Comparative protein profiling of B16 mouse melanoma cells susceptible and non-susceptible to alphavirus infection: effect of the tumor microenvironment

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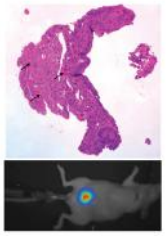

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RESEARCH PAPER

Comparative protein profiling of B16 mouse melanoma cells susceptible and non-susceptible to alphavirus infection: Effect of the tumor microenvironment

Jelena Vasilevska[a], Gustavo Antonio De Souza[b], Maria Stensland[b], Dace Skrastina[a], Dmitry Zhulenvovs[a], Raimonds Paplausks[c], Baiba Kurena[a], Tatjana Kozlovska[a], and Anna Zajakina[a]

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ABSTRACT

Alphavirus vectors are promising tools for cancer treatment. However, relevant entry mechanisms and interactions with host cells are still not clearly understood. The first step toward a more effective therapy is the identification of novel intracellular alterations that could be associated with cancer aggressiveness and could affect the therapeutic potential of these vectors. In this study, we observed that alphaviruses efficiently infected B16 mouse melanoma tumors/tumor cells *in vivo*, whereas their transduction efficiency in B16 cells under *in vitro* conditions was blocked. Therefore, we further aimed to understand the mechanisms pertaining to the differential transduction efficacy of alphaviruses in B16 tumor cells under varying growth conditions. We hypothesized that the tumor microenvironment might alter gene expression in B16 cells, leading to an up-regulation of the expression of virus-binding receptors or factors associated with virus entry and replication. To test our hypothesis, we performed a proteomics analysis of B16 cells cultured *in vitro* and of B16 cells isolated from tumors, and we identified 277 differentially regulated proteins. A further in-depth analysis to identify the biological and molecular functions of the detected proteins revealed a set of candidate genes that could affect virus infectivity. Importantly, we observed a decrease in the expression of interferon α (IFN-α) in tumor-isolated cells that resulted in the suppression of several IFN-regulated genes, thereby abrogating host cell antiviral defense. Additionally, differences in the expression of genes that regulate cytoskeletal organization caused significant alterations in cell membrane elasticity. Taken together, our findings demonstrated favorable intracellular conditions for alphavirus transduction/replication that occurred during tumor transformation. These results pave the way for optimizing the development of strategies for the application of alphaviral vectors as a potent cancer therapy.

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Alphavirus; B16 melanoma; cancer; intracellular alterations; tumor microenvironment

Introduction

The application of recombinant viral vectors has become one of the most intensively developed strategies in cancer gene therapy. Such therapy is based on the ability of the viruses to preferentially infect and kill cancer cells. Alphaviral vectors function as efficient mammalian expression systems because of their high-level transgenic expression and induction of p53-independent apoptosis in infected cells.[1,2] These vectors also have a broad range of hosts and induce a weak immune response against the vector.[3] Alphaviruses are small, enveloped, positive-stranded RNA viruses that belong to the *Togaviridae* family. The classical Semliki Forest virus (SFV) replicon vector is generated by replacing the structural genes under the control of the 26S viral subgenomic promoter with a heterologous insert of interest.[4] The vector RNA can be packaged into recombinant viral particles during co-transfection of the host cells with a helper RNA that encodes structural genes, i.e., capsid and envelope proteins. SFV RNA replicates actively during infection, and the heterologous gene is expressed at a high level. However, the vector cannot propagate because it lacks genes encoding the viral structural proteins.

The expression efficacy of all viral vectors relies on the virus transduction, replication and distribution ability. Alphaviruses are able to infect a broad range of cancer cell lines with widely divergent biochemical and genetic environments both *in vitro* and *in vivo*.[5-7] However, the tumor microenvironments are capable of forming a barrier that is highly impermeable to the virus.[8] Viral penetration, persistence and spreading may be impeded alone or in combination, thus causing a wide variation in viral transduction/replication capacity even within a single cell line under *in vitro* and *in vivo* conditions. We and other authors have found that alphaviruses can efficiently infect B16 mouse melanoma tumors *in vivo*, whereas the infection *in vitro* is blocked for unknown reasons.[6] This observation has encouraged us to perform an in-depth analysis of intracellular factors that could vary in the same cells before and after administration in mice. Melanoma is a complex multistep heterogeneous disease in which most of the steps in the tumor transformation process, such as proliferation, invasion, angiogenesis and metastasis, are modulated by microenvironmental factors such as growth factors and proteolytic enzymes produced by stromal cells.[9] However, the ability of these factors to affect viral

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infectivity has yet to be explored. Taking into consideration that the tumor microenvironment is able to influence gene expression in cancer cells, we hypothesized that it might also play a role in the upregulation of virus-binding receptors or other factors, which in turn affect viral entry and replication. To date, only Sindbis virus has demonstrated tumor tropism *in vivo*.[10-11] We recently demonstrated that SFV is capable of predominant tumor infection upon systemic vector administration at an optimized dose.[12] However, the interaction/entry mechanisms have not been investigated in detail.

Various studies have reported that alphaviruses enter the cell via receptor-mediated endocytosis, involving multiple proteins implicated in virus absorption/transduction, such as heparan sulfate, laminin receptor, the major histocompatibility complex (MHC), DC-SIGN, L-SIGN, heat shock 70 protein, and $\alpha 1\beta 1$ integrin, among others.[13-18] However, it is most likely that alphaviruses may utilize multiple surface proteins as receptors or alternative entry pathways in different cells. In addition to receptors, cytoskeletal organization plays an essential role in the interaction of viruses with the host cell, affecting penetration through the membrane and further development of infection and viral expression.[19,20] The microtubule network regulates several processes, including intracellular transport, transcription, replication and secretion of progeny virions, as well as assembly and cell-to-cell spread.[21] Recent studies indicate that the cell membrane interacts with the attached cytoskeleton/associated motor proteins, thus controlling endocytosis/exocytosis and modulating physical features of the cell such as its shape, motility and membrane elasticity.[21] All these processes could potentially affect viral activity;[22] however, the mechanism(s) associated with the alphaviruses remain to be elucidated.

Efficient viral replication is one the most important prerequisites for successful gene therapy. Alphaviral replication is extremely high under favorable intracellular conditions, resulting in expression levels of nearly 20% of the total cell protein.[23] However, infection triggers cellular defenses induced by a combination of different factors. Thus, the up-regulation of several immune system proteins or stress factors, such as interferon-stimulated genes (ISGs),[24] zinc-finger antiviral proteins[25] or eukaryotic translation initiation factors,[26] can significantly suppress alphavirus-driven gene expression. Interferons were shown to be the major players in the modulation of the pervasive antiviral environment, leading to the prevention of viral spread and replication. Several studies have shown that enhanced alphavirus infectivity is mediated by a decrease in the intracellular level of type I IFNs, which is true both *in vitro* and *in vivo*.[27,28] Interesting results, which are consistent with the current research, have been shown for Vesicular Stomatitis Indiana virus (VSV). Ovarian and breast cancer cells were highly susceptible to VSV infection *in vitro*, but the infection of the developed tumors *in vivo* was inefficient due to activation of the JAK/STAT pathways and overexpression of interferon-stimulated genes induced by tumor-infiltrating macrophages.[29] This study confirms our hypothesis that the tumor microenvironment is able to induce intracellular changes in cancer cells, thus leading to variation in viral activity *in vivo*.

In this current study, we performed a proteomics analysis of B16 cells cultivated *in vitro* and of tumor-isolated B16 cells and the results were compared. Our goal was to identify genes that are differentially expressed in mouse melanoma cells before and after their inoculation in mice. Based on a quantitative analysis of the detected proteins, we report a list of gene candidates (*S100b, Pycard, CD97, Pdcd4, Gpx1, Csnk2b, Gstz1, Gsto1, Scrib, Hsp90aa1, Hspb1, Cryab, Csf1, Adh7, Sgtb, Aif1l, Crem, Etv6, Nfe2l2, Stat1, IFI35, S100a11, Txnl1*) that may be involved in the antiviral response. We also identified cytoskeletal organization gene candidates (*Tmod, Dst, Dynll2, Opa1, Mlph, Actc1, Myo18a, Dynlt1, Myo5a, Map7, Csnk2b, Lmna, Dync1h1, Timm10b, Kif1a, Gfap, Capg, Flnc, Aspm, Hist1h1a, Ss18, Hist1h1t, Cfl1, Cald1, Rps21, Tubb6*), which might provide optimal intracellular conditions for the infection and further expression of transgenes. Our findings could extend what is currently known about the alterations in the melanoma microenvironment during tumor development. In turn, this information could provide a significant contribution toward the modulation of more efficient strategies for cancer gene therapy.

Results

Comparison of SFV infectivity in B16 cells and B16 tumors

To compare the efficiency of SFV-driven transgene expression in mouse melanoma cells *in vitro* and *in vivo*, cultured B16 cells were infected (*in vitro*) with the SFV/Enh.Luc vector at a multiplicity of infection (MOI) of 10. To examine SFV infectivity *in vivo*, immunocompetent B16 tumor-bearing mice were inoculated intratumorally (i.t.) with 10^8 SFV/Enh.Luc particles. Luciferase expression was analyzed in the cell lysates and tumor homogenates at 24 h post-infection.

In vitro, very low expression of the transgene was detected in B16 cells post-infection, revealing the transduction/replication inefficiency of the SFV vector under such conditions. By contrast, intratumoral administration of the same recombinant virus resulted in high expression of the transgene in B16 melanoma tumors (Fig. 1A). These data indicate that there is variation in the genetic background between *in vitro*-cultivated B16 cells and B16 cells in tumor-bearing mice, which facilitates transduction or replication of the SFV vector.

To determine the vector distribution within a subcutaneous melanoma tumor nodule, 2 SFV vectors expressing green fluorescence protein (SFV/EGFP) and red fluorescence protein (SFV/DS-Red) were inoculated into different points of a B16 tumor nodule (Fig. 1B). The analysis of tumor cryosections revealed only local expression of the corresponding fluorescent protein at the location of the intratumoral injections, with no broad intratumoral dissemination of the virus due to the absence of SFV/EGFP expression at locations of SFV/DS-Red injection and vice versa. Therefore, it is possible to conclude that although the SFV vector is capable of a high level of expression of the transgenes in B16 tumors, the efficacy of the alphavirus-based therapy could be increased by enhancing the tumor permeability, thus promoting the wide intratumoral spread of the vector.

Comparison of SFV infectivity of in vitro *B16 cells and* ex vivo *B16 cells*

To investigate whether B16 cells were modified by the tumor microenvironment, rendering them susceptible to SFV vector

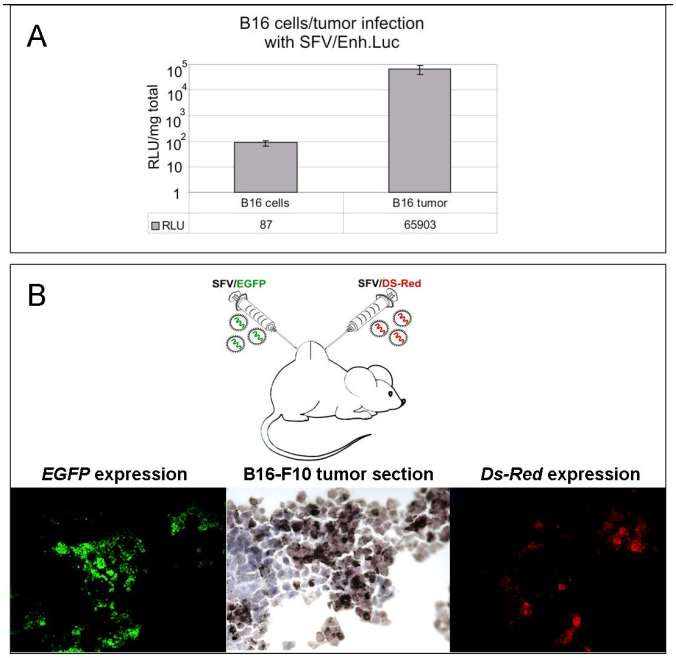

Figure 1. SFV expression and intratumoral spread in a melanoma mouse model. (A) Infection of B16 melanoma cells *in vitro* and B16 tumor cells *in vivo* with SFV/Enh.Luc vector. The B16 cells were infected with SFV at an MOI of 10 *in vitro*. For the *in vivo* experiment, B16 tumor-bearing mice were i.t. inoculated with 10^8 SFV v.p. The luciferase expression analysis in cell lysates and tumor homogenates was performed 24 h post-infection by luminometry. The bar graph presents the RLUs per 1 mg protein in the cell lysate/tumor homogenate. The results represent the mean ± s.e. RLU - relative light unit. (B) Administration strategy of SFV vectors and fluorescence microscopy of B16 tumor cryosections, demonstrating SFV/FGFP and SFV/Ds-Red virus spread in the tumor. A total of 10^6 v.p. of SFV/EGFP and SFV/Ds-Red were injected in different tumor sides by direct intratumoral injections. The tumors were cryosectioned and analyzed 24 h after SFV vector administration.

infection, we conducted a series of SFV transduction experiments using 2 types of melanoma cells: i) B16 *in vitro* cells – a standard B16-F10 cell line cultured under ordinary cell culturing conditions *in vitro*, and ii) B16 *ex vivo* cells - melanoma cells isolated from B16-F10 tumors. The susceptibility of B16 *ex vivo* cells to SFV infection was analyzed over time at different cell passages. *In vitro* and *ex vivo* B16 cells were infected with SFV/Ds-Red at an MOI of 10. Ds-Red gene expression was analyzed 24 h post-infection by fluorescence microscopy.

Our results demonstrated extremely high SFV-mediated transgene expression during the first passage of B16 *ex vivo* cells in contrast to control B16 cells *in vitro* (Fig. 2). Surprisingly, further cultivation of the *ex vivo* cells led to a dramatic inhibition of SFV vector infection. We hypothesize that the tumor microenvironment can induce alterations at the level of gene expression, which affect the morphology and physical parameters of cells, such as cell shape, motility and membrane elasticity. In turn, these changes provide conditions favorable for SFV vector transduction/replication. Notably, although the *ex vivo* B16 cells displayed these features for a short period, these cells subsequently began to lose susceptibility to SFV infection by the second passage upon splitting. Remarkably, the replacement of fetal bovine serum to freshly prepared autologous mouse serum for cultivation of *ex vivo* and *in vitro* B16 cells had no effect on SFV infectivity. We did not observe any significant increase in SFV/Ds-Red activity in B16 cells cultivated in cell medium supplemented with mouse serum (not shown).

To demonstrate that the *ex vivo*-isolated cells were B16 melanoma cells, we performed melanin staining using the classical Fontana-Manson method (Fig. 2B). The results revealed melanin production in the isolated cells. Moreover, in contrast to *in vitro* B16 cells, the level of expression of melanin in *ex vivo* cells was significantly increased. Further cultivation of *ex vivo* cells led to a decrease in melanin synthesis (data not shown).

Protein profile analysis of ex vivo *and* in vitro *B16 cells*

To determine the intracellular conditions that could enhance SFV infection, we performed a comparative proteomics analysis of *ex vivo* B16 (first passage) and *in vitro* B16 cells using liquid chromatography-mass spectrometry (LC-MS). To characterize the *in vitro* and *ex vivo* B16 cells, we identified individual proteomes of 3 *in vitro* B16 cell samples and 3 *ex vivo* B16 samples (isolated from 3 different mice). A total of 4980 proteins were identified using the UniProt database, among which 277 proteins were differentially regulated (165 up-regulated and 112 down-regulated), with a fold change >1.4 in *ex vivo* B16 cells in comparison to the *in vitro* B16 cells ($p < 0.01$; Table S1 and Table S2). All these proteins were identified in triplicate for each sample. The quantified proteins were functionally annotated using the PANTHER bioinformatics resource (version PANTHER 9.0; http://www.pantherdb.org/) and further classified according to their functions in biological processes (Fig. 3A-B) and molecular mechanisms (Fig. 3C-D).[30] The cellular localization of the detected proteins were determined by manual classification using the UniProt database (http://www.uniprot.org/) (Fig. 3E-F).

We identified 165 proteins that were upregulated in the *ex vivo* B16 cells during tumor development in comparison to the *in vitro* B16 cells. According to the analysis of the biological functions, the majority of the identified proteins belonged to different metabolic processes (37.8%). Smaller groups comprised cellular process proteins (19.3%), cell component organization and biogenesis proteins (8.2%), biological regulation proteins (7.7%), developmental process proteins (7.7%) and localization proteins (7.3%). Several other identified proteins were classified as multicellular organismal process (4.7%), response to stimulus (3%), immune system process (2.1%), reproduction (1.3%) and apoptotic process (0.9%) proteins (Fig. 3A).

Regarding molecular functions, more that 70% of all up-regulated proteins were grouped into 2 leading categories: catalytic activity proteins (47%) and binding proteins (25.5%). The other overexpressed gene products were functionally distributed as structural molecule activity proteins (12.1%), enzyme regulator activity proteins (7.4%), receptor activity (2%), translation regulators (2%), nucleic acid binding/transcription factor activity (2.7%), transporters (0.7%) and antioxidant activity (0.7%) proteins (Fig. 3C).

Proteins perform their functions in specific cellular locations. Careful analysis of their subcellular localization revealed that the majority of the upregulated proteins were localized in the mitochondria (27%) and the cytoplasm (21%) (Fig. 3E). Importantly, a large portion of the mitochondrial proteins were NADH dehydrogenases, which are fundamentally crucial for growth signaling and transcription in a broad array of

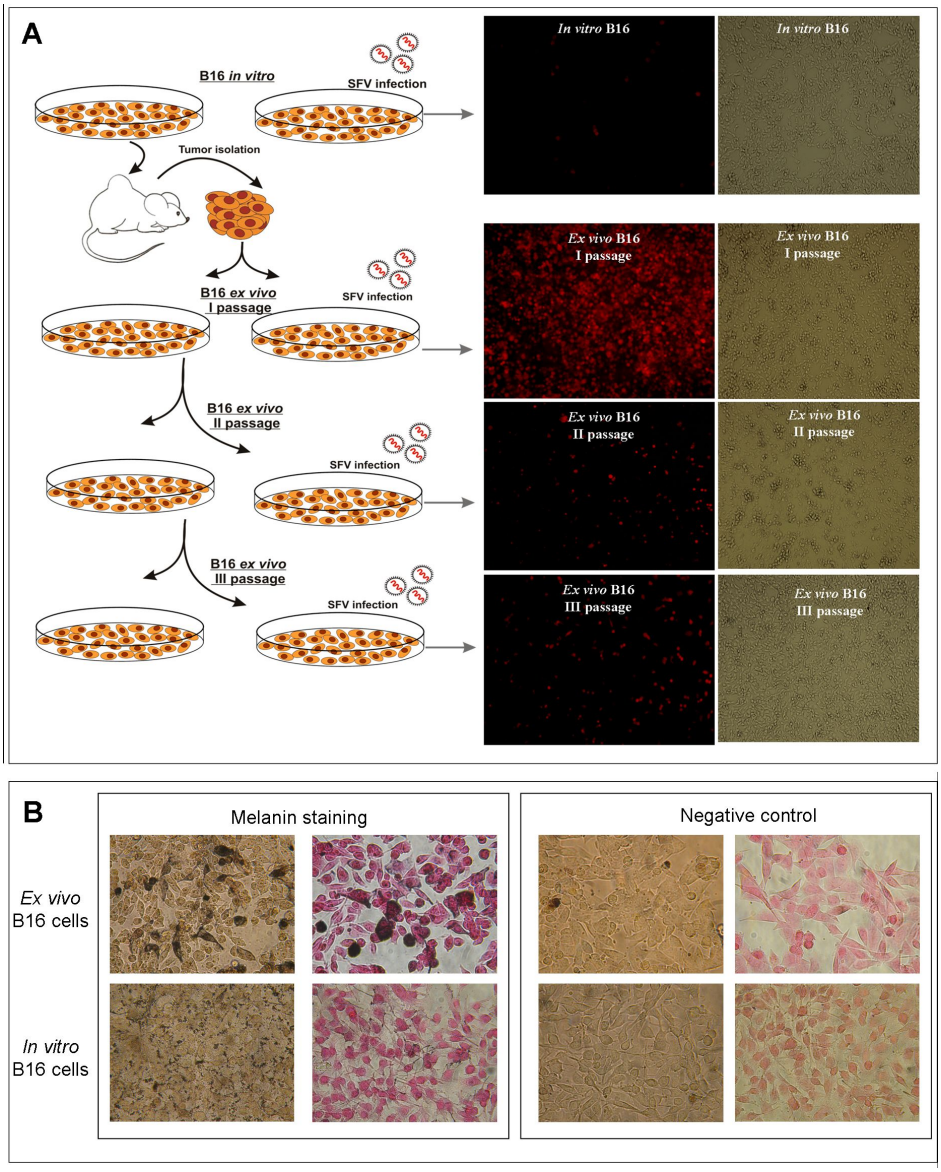

Figure 2. Comparison of SFV infection susceptibility and melanin expression of *in vitro* and *ex vivo* B16 cells. (A) Schematic illustration of the isolation, propagation and infection of *ex vivo* B16 cells with SFV vector compared with the control *in vitro* B16 cells. The control *in vitro* B16 cultured cells were subcutaneously injected into immunocompetent C57BL/6 mice. Ten days after cell inoculation, B16 tumor nodules were isolated, homogenized and plated as *ex vivo* B16 cells in 2 plates as the first cell passage. When the cell monolayer reached 80% confluency, the 1st plate of cells was split for further cultivation (second cell passage), whereas the 2nd plate of cells was infected with SFV/Ds-Red vector for transgene expression analysis, which is presented as fluorescence and phase contrast microscopy images. (B) Melanin staining of *in vitro* and *ex vivo* B16 cells. The cells were treated with Fontana-Masson silver stain to detect melanin (dark brown, black color). Negative control – non-stained cells. The nuclei were counterstained with nuclear fast red.

melanoma tumors,[31] indicating enhanced aggressiveness of the *ex vivo* B16 cells in contrast to the control *in vitro* cells. The intracellular distribution of the other proteins was predicted to be localized in the nucleus (13%), cellular components (12%), endoplasmic reticulum (7%), cell membrane (5%), Golgi apparatus (3%) and both lysosomal and secreted (2%). More stringent analysis was performed for the proteins localized in melanosomes (4%), which are unique organelles in melanoma cells. The *ex vivo* B16 cells overexpressed genes such us *Typr1, Tyr, Dct, Mlph* and *Myo5a*, which are involved in melanin synthesis, explaining the enhanced pigmentation of the *ex vivo* cells, which resulted in the histological difference between the *ex vivo* and *in vitro* B16 cells (Fig. 2B).

LC-MS analysis identified 112 downregulated proteins in the *ex vivo* B16 cells. Regarding their involvement in biological processes, the majority of the down-regulated proteins were also categorized into different metabolic and cellular processes (32% and 20.3%, respectively) in a similar manner as the upregulated proteins (see above). The other proteins were involved in biological regulation (10.5%), developmental process (9.8%), response to stimulus (7.8%), biogenesis (6.5%), immune system process (5.2%), multicellular organismal process (3.9%), localization (3.3%), biological adhesion (1%) and apoptotic process (0.7%) (Fig. 3B).

Biological classification of the downregulated proteins in terms of their molecular mechanisms revealed that most of them had binding (44.3%) and catalytic activity (22.7%) functions. A significantly smaller number of proteins were related to nucleic acid binding transcription factor activity (13.6%) and structural molecule activity (11.4%) proteins, respectively. The smallest number of proteins were categorized as enzyme regulator (3.4%), protein binding transcription factor (2.3%), and receptor and transporter (1.1%) activity proteins (Fig. 3D).

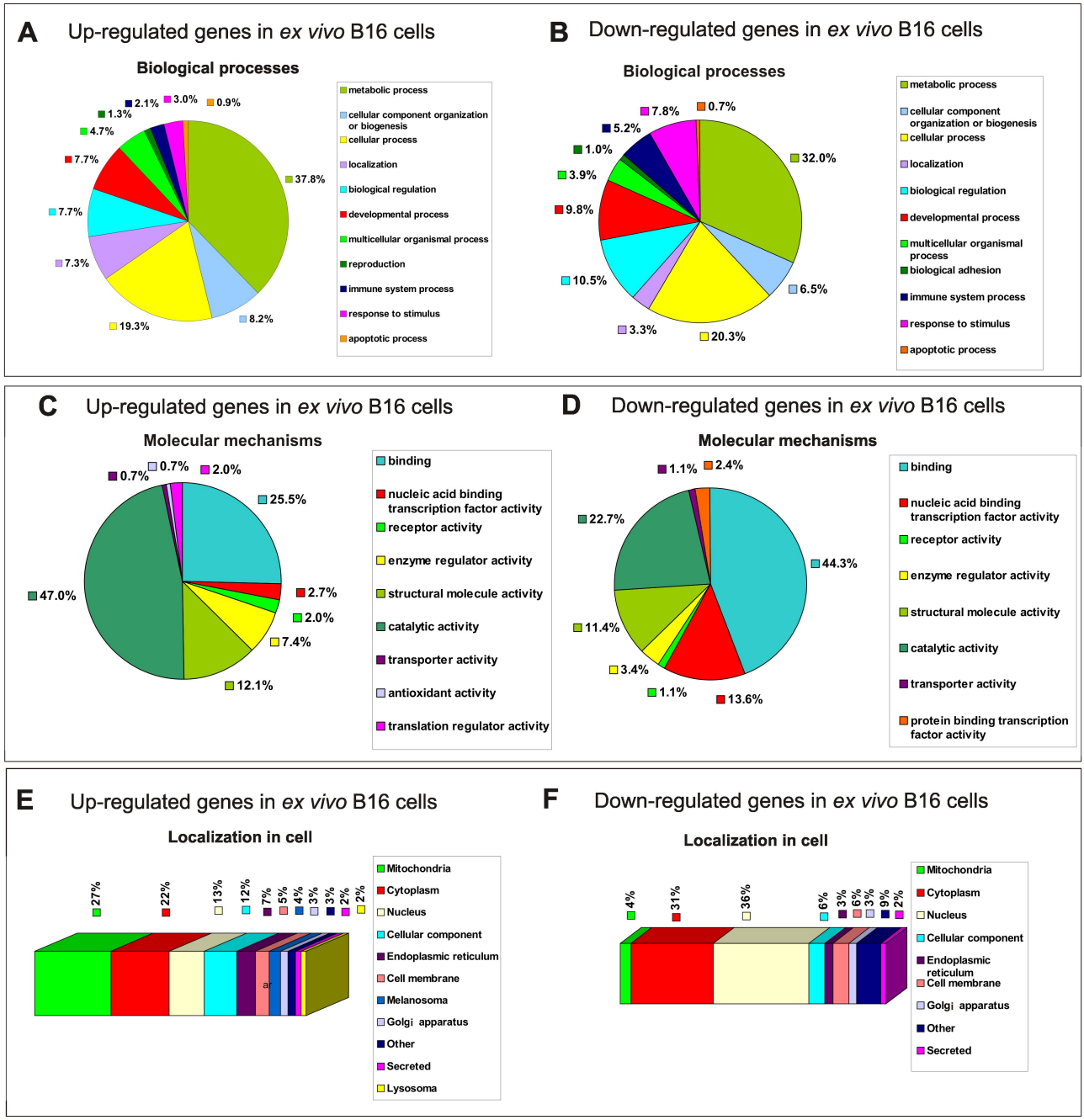

Figure 3. Functional classification of 277 differentially regulated proteins in *ex vivo* B16 cells identified in total protein extracts by LC-MS. Protein profile analysis of both *in vitro* and *ex vivo* B16 cells was performed by LC-MS, and *in vitro* B16 cells were used as reference cells. The pie charts demonstrate the distribution of 165 upregulated (left panel) and 112 downregulated (right panel) proteins in *ex vivo* B16 cells according to their biological processes (A-B) and molecular mechanisms (C-D). Categorizations were based on information provided by the PANTHER classification system (see methods). The subcellular localization prediction of up-regulated (E) and down-regulated (F) proteins in B16 *ex vivo* cells was annotated manually using the UniProt database. The percentages shown in the pie and bar charts represent the percentage of genes belonging to each group.

Most of the down-regulated proteins were predicted to be localized in the nucleus (36%) and the cytoplasm (31%) (Fig. 3F). A total of 6% were classified as cell membrane and cellular component proteins. Interestingly, only 4% of the downregulated proteins exhibited mitochondrial localization, which is in contrast to 27% of the upregulated proteins (see above), indicating decreased mitochondrial activity in the *in vitro* B16 cells. A total of 3% of the proteins were localized in the endoplasmic reticulum and Golgi apparatus, and 2% of the proteins were identified as secreted proteins.

In summary, a functional analysis of the biological processes of differentially regulated proteins revealed a similar distribution, in which more than 50% of all up/down-regulated proteins participated in different metabolic processes (mostly in primary metabolic processes such as protein, nucleobase-containing compound, lipid, carbohydrate and tricarboxylic acid cycle metabolic processes) and cellular processes, which included cell communication, cell cycle, movement of cellular components, chromosome segregation and cytokinesis (PANTHER annotation). According to the molecular mechanisms, most of the detected up-regulated proteins were related to

catalytic activity proteins, indicating enzyme regulator, hydrolase, isomerase, ligase, oxidoreductase and transferase activity. By contrast, the majority of the downregulated proteins were responsible for binding calcium ions, calcium-dependent phospholipids, chromatin, nucleic acids and other proteins. The difference in molecular functions between the up- and downregulated proteins was the reason for the different predominant intracellular localization of these proteins.

Classical alphavirus-recognizing receptors, such as membrane heparan sulfate, laminin receptor, major histocompatibility complex (MHC), DC-sign, L-sign, heat shock 70 protein, and $\alpha 1\beta 1$ integrin,[13-18] which are implicated in virus absorption/transduction and are described in several studies, were not identified by LC-MS analysis in our experiment or were not found to be significantly up/downregulated. In addition, real-time PCR analysis did not demonstrate any significant differences in the gene expression of some of these candidates (data not shown).

Antiviral response genes

The main biological processes that initiate intracellular responses to viral infection and could potentially block replication primarily comprise the response to a stimulus, immune system processes and apoptosis.[32] The category of responses to environmental stimuli (such as stress, endogenous or external stimuli) displays considerable overlap with the immune response category, usually regulating the expression of the same genes. The induction of apoptosis is often a consequence of such processes because all these features largely overlap. Some of the genes could be classified into 2 or 3 biological processes by the PANTHER classification system. Based on our proteomics classification results (as mentioned above), we performed a detailed analysis of the up/down-regulated genes that participated in 3 biological processes, response to stimulus, immune system response and apoptosis, summarizing these genes in one "antiviral response genes" category (Fig. 4A). In total, we identified 10 upregulated antiviral response genes in the *ex vivo* B16 cells: *S100b* (6.2-fold increase), *Pycard* (4.9-fold increase), *CD97* (4-fold increase), *Pdcd4* (3.1-fold increase), *Gpx1* (2.3-fold increase), *Csnk2b* (2.2-fold increase), *Gstz1* (2.2-fold increase), *Gsto1* (1.9-fold increase), *Scrib* (1.7-fold increase) and *Hsp90aa1* (1.6-fold increase).

Overexpression of casein kinase II *Csnk2b*, scribble protein *Scrib* and heat shock protein *Hsp90aa1* has been reported to potentially improve alphavirus-host cell binding and transduction (see Discussion). Additionally, all these proteins are correlated with high tumor aggressiveness and poor prognosis.

Overexpression of S100b (*S100b*) in the *ex vivo* B16 cells could be explained by active B16 tumor development because S100b is generally synthesized only under pathological circumstances and not under normal physiological conditions.[33]

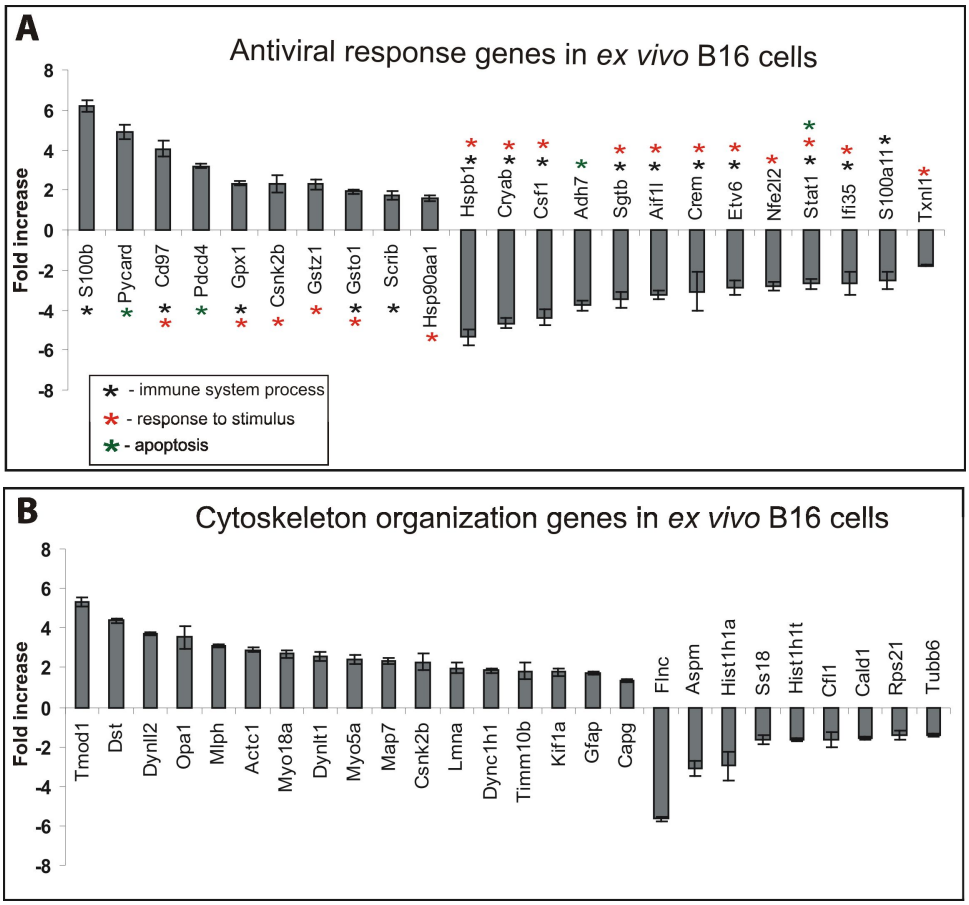

Figure 4. Up/downregulated genes in *ex vivo* B16 cells classified as antiviral response genes and cytoskeletal organization genes. (A) Based on the LC-MS data classification by PANTHER, genes that participated in apoptosis, response to stimulus and immune system processes were summarized in the antiviral response genes group. (B) Genes responsible for cellular component organization. The graph shows the fold-change in the expression of corresponding genes in *ex vivo* B16 cells compared with *in vitro* B16 cells. The results represent the mean ± s.e.

Moreover, a high level of the S100B protein serves as a reliable prognostic biomarker in patients with malignant melanoma.[34]

The overexpressed glutathione peroxidase 1 Gpx1, maleylacetoacetate isomerase Gstz1 and glutathione S-transferase omega-1 Gsto were sub-classified as toxic substance responsible genes (PANTHER classification). Because there is limited information available regarding these multifunctional enzyme activities in cancer models, the upregulation of the genes could be explained as a consequence of a stress response caused by B16 cells upon transfer from the mice (in vivo conditions) to the plates (ex vivo conditions).

Although the up-regulation of CD97 antigen in melanoma cells has not been previously described, it was overexpressed in advanced stages of different cancers. The protein is mostly located in invasive tumors with higher cell motility than the cells in solid tumors.[35-37]

The up-regulated apoptotic genes, *Pycard* (apoptosis-associated speck-like protein containing a CARD) and *Pdcd4* (programmed cell death protein 4), in the *ex vivo* B16 cells could act as key mediators in apoptosis and inflammation; however, no studies have described their roles in melanoma or their impacts on viral infectivity and replication.

In addition to the 10 upregulated genes described above, 13 downregulated genes involved in apoptosis, response to stimuli and immune system processes have been identified: *Hspb1* (5.3-fold decrease), *Cryab* (4.6-fold decrease), *Csf1* (4.3-fold decrease), *Adh7* (3.7-fold decrease), *Sgtb* (3.4-fold decrease), *Aif1l* (3.2-fold decrease), *Crem* (3-fold decrease), *Etv6* (2.9-fold decrease), *Nfe2l2* (2.8-fold decrease), *Stat1* (2.6-fold decrease), *IFI35* (2.6-fold decrease), *S100a11* (2.5-fold decrease) and *Txnl1* (1.7-fold decrease) (Fig. 4A).

The roles of genes such as alcohol dehydrogenase 7 (*Adh7*, also known as *Adh3*) and small glutamine-rich tetratricopeptide repeat-containing protein *Sgtb* have not been described for alphavirus infection/replication. However, reduced *Adh7* expression has been implicated in oncogenesis and might influence viral life cycles through increased S-nitrosylation and formaldehyde-induced changes in cellular redox.[38,39] By contrast, the down-regulation of SGT proteins remarkably enhances the activity of HIV-1 virus.[40]

An in-depth analysis of other down-regulated genes showed that most of them participated in 3 IFN-regulated pathways: JAK-STAT, p38 MAPK and PI3K. Specifically, those genes were the heat shock protein *Hspb1*, α-B crystallin *Cryab*, macrophage colony-stimulating factor 1 *Csf1*, cAMP-responsive element modulator *Crem*, allograft inflammatory factor 1 *Aif1l*, transcription factor *Etv6*, nuclear factor erythroid 2-related factor 2 *Nfe2l2*, signal transducer and activator of transcription 1 *Stat1*, protein S100-A11 *S100a11* and thioredoxin-like protein 1 *Txnl1* (Table S3). These findings indicated that the described signaling pathways were suppressed in the *ex vivo* B16 cells. The expression of interferon-inducible 35 kDa protein *(IFI35)* was also significantly inhibited. These results present a network of downregulated genes modulated by IFN that could cooperatively provide favorable conditions for SFV transduction/replication.

Cytoskeletal organization genes

The cytoskeleton plays an important role in the life cycle of every virus during attachment, internalization, endocytosis, nuclear targeting, transcription, replication, transport of progeny subviral particles, assembly, exocytosis or cell to-cell spread.[21] To investigate the genes regulating cytoskeletal organization that could facilitate alphavirus activity in *ex vivo* B16 cells, we performed a detailed analysis of the up/down-regulated proteins that participated in cellular component organization (or biogenesis) categorized by the PANTHER classification system (Fig. 4B). We identified 17 up-regulated genes: *Tmod* (5.3-fold increase), *Dst* (4.3-fold increase), *Dynll2* (3.7-fold increase), *Opa1* (3.5-fold increase), *Mlph* (3.1-fold increase), *Actc1* (2.9-fold increase), *Myo18a* (2.7-fold increase), *Dynlt1* (2.5-fold increase), *Myo5a* (2.4-fold increase), *Map7* (2.3-fold increase), *Csnk2b* (2.2-fold increase), *Lmna* (1.9-fold increase), *Dync1h1* (1.8-fold increase), *Timm10b* (1.8-fold increase), *Kif1a* (1.7-fold increase), *Gfap* (1.7-fold increase), and *Capg* (1.7-fold increase).

The cytoskeleton has 3 major types of filaments: actin, intermediate filaments and microtubules. Each type of filament has its own specific stabilization and motor proteins. In this experiment, we identified the upregulation of the filamentous protein α actin, *Actc1*. Moreover, both tropomodulin 1 (*Tmod1*) and macrophage-capping protein (*Capg*), which serve as actin filament stabilization proteins,[41,42] were also found to be overexpressed in B16 *ex vivo* cells. The up-regulation of the intermediate filament protein Laminin (*Lmna*) was shown to increase the stiffness matrix, to confer nuclear mechanical properties, and to influence the differentiation of mesenchymal stem cells.[43] Conversely, tubulin, which is the main component of microtubules, was found to be 1.4-fold downregulated in the *ex vivo* B16 cells (see below). However, the microtubule-stabilizing protein, *Map7*, which might play an important role during the reorganization of microtubules, was found to be 2.3-fold overexpressed.

Interestingly, in the ex vivo B16 cells, all the major motor proteins involved in movement on cytoskeletal filaments were upregulated. The myosins, *Myo18a* and *Myo5a*, are the only motor proteins that are able to bind to actin. Myosin acts upon actin filaments to generate cell surface contractions and other morphological changes, including vesicle motility and cytoplasmic streaming. Additionally, *Myo5a* has been considered to regulate melanosome transport in cooperation with the up-regulated *Mlph*.[44] Both the upregulated kinesin, *Kif1a*, and the dyneins, *Dynll2, Dynlt1*, and *Dync1h1*, act as the main microtubule motor proteins, providing the intracellular retrograde motility of vesicles and organelles along microtubules.[45] There are no known motor proteins for intermediate filaments.

Up to 4.3-fold upregulation of dystonin (*Dst*), which acts as an integrator of intermediate filaments and actin and microtubule cytoskeleton networks, facilitates intracellular transport by regulating organelle organization.[46] The overexpression of another cytoskeletal organization factor, glial fibrillary acidic protein (*Gfap*), could be associated with cell mechanical strength and shape, but its exact function remains poorly understood.[47]

Casein kinase II subunit β (*Csnk2b*) is the only common protein that was detected in both categories of antiviral response genes and cellular component organization genes (Fig. 3A-B). Several studies have shown that casein kinase II is able to phosphorylate cytoskeletal proteins including tubulin[48] or microtubule-associated protein 1B,[49] thus contributing to morphological changes that occur during mitosis and cytokinesis in differentiating cells. Additionally, we identified the up-regulation of 2 mitochondrial membrane organization proteins, *Opa1* and *Timm10b*, which are responsible for multi-pass transmembrane protein transport.[50,51]

Nine down-regulated genes responsible for cytoskeletal organization have been identified in the *ex vivo* B16 cells: *Flnc* (5.6-fold decrease), *Aspm* (3.1-fold decrease), *Hist1h1a* (2.9-fold decrease), *Ss18* (1.6-fold decrease), *Hist1h1t* (1.6-fold decrease), *Cfl1* (1.6-fold decrease), *Cald1* (1.5-fold decrease), *Rps21* (1.4-fold decrease) and *Tubb6* (1.4-fold decrease). In this gene category, the muscle-specific filamin-C (*Flnc*) was detected as one of the most downregulated genes in B16 *ex vivo* cells that could potentially affect melanoma cell structure. The reduced expression of *Flnc* in myoblast cells has been shown to lead to defects in cell differentiation and fusion ability. These cells form multinucleated "myoballs" rather than maintaining an elongated morphology.[52] Interestingly, the elongation of B16 *ex vivo* cells could also be impeded by a low level of the protein SSXT (*Ss18*), which has been described as a cytoskeletal phenotype-associated protein that plays a role in the elongation of the cell body via the induction of detyrosinated Glu tubulin.[53] Our results demonstrate the downregulation of abnormal spindle-like microcephaly-associated (*Aspm*) protein in B16 *ex vivo* cells. Some studies have suggested that mutations or deregulation of *Aspm* could cause microcephaly due to the dysregulation of mitotic spindle activity, which increases the probability of asymmetric cell division.[54]

Caldesmon (*Cald1*) and cofilin (*Cfl1*), which are important for cytoskeletal organization and dynamics, were also found to be down-regulated up to 1.5- and 1.6-fold, respectively. Caldesmon is a multifunctional ubiquitous regulator of the actin cytoskeleton that when expressed at low levels, determines the bipolar shape and linear migration of cells.[55] By contrast, defects in cofilin expression alter the morphology of actin networks *in vivo* and reduce the rate of actin flux through actin networks. The consequences of decreasing actin flux are manifested by decreased, but not blocked, endocytic internalization at the plasma membrane.[56] Additionally, inhibition of the production of the 40S ribosomal protein S21 (*Rps21*) was also detected in B16 *ex vivo* cells.

Finally, suppression of H1 histone expression was detected in B16 *ex vivo* cells (2.9-fold decrease in *Hist* and 1.6-fold decrease in *Hist1h1t*), which could significantly impair the cell differentiation capacity. H1 histones have been shown to contribute to efficient repression of the expression of pluripotency factors and to participate in the establishment and maintenance of the epigenetic marks necessary for silencing pluripotency genes during embryogenesis and stem cell differentiation.[57]

Comparison of membrane elasticity between in vitro *and* ex vivo *B16 cells*

The transition of a tumor cell from fluid to adhesive conditions involves an early polarization event and major rearrangements of the submembrane cytoskeleton. This process can change the mechanical properties of the membrane, for example by increasing its elastic properties, which could affect cell endocytosis,[58] virus-cell interactions and transduction.[22] In our study, we used atomic force microscopy (AFM) to compare the cell membrane elasticity (or hardness)[59] of the *in vitro* B16 cells and the first passage of the *ex vivo* B16 cells.

Several AFM force measurements were performed in which equal contact points of the AFM tip and cell surface were selected (the middle point between the cell nucleus and the elongated cell body) (Fig. 5A). After tip-cell contact, the

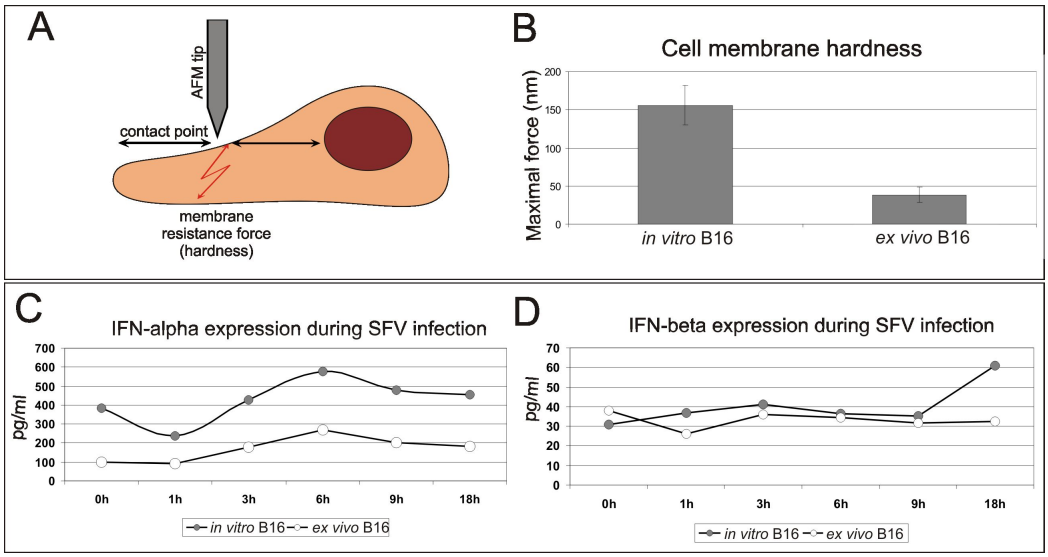

Figure 5. Comparison of cell membrane elasticity and IFN-α/β expression between *in vitro* and first-passage *ex vivo* B16 cells. The principle of AFM measurement is schematically demonstrated in panel A. The middle point between the cell nucleus and the elongated cell body was selected as the contact point of the AFM tip and the cell surface. The pushing force was increased slowly until the membrane was punctured. The acquired maximal forces essential for membrane puncturing of *in vitro* and *ex vivo* B16 cells are shown in panel B. The membrane resistance force indicates the elasticity of the sample surface; higher maximal force indicates higher membrane hardness. The results represent the mean ± s.e. Expression levels of IFN-α (C) and IFN-β (D) were determined in *in vitro* and first-passage *ex vivo* B16 cell lysates before SFV infection (0 h) and at 1 h, 3 h, 6 h, 9 h and 18 h post-infection. The results represent the concentration of IFN protein per 1 ml of cell lysate (pg/ml).

pushing force was slowly increased until the membrane was punctured. The elasticity (or hardness) defined by the resistance of the cell to the changes was measured. The greater the resistance, the greater was the elasticity of the material and the faster it regained its original shape or configuration upon withdrawal of the deforming force.

In this experiment, the membrane resistance force indicated the elasticity of the sample surface. A higher maximal force indicated a greater membrane hardness. The AFM data demonstrated that the *ex vivo* B16 cells had significantly lower cell membrane elasticity (Fig. 5B), with an average maximal force of 38 nN compared with the control cells with maximal forces within the range of 120–180 nN. The low level of membrane hardness in the case of the *ex vivo* B16 cells could be one of the reasons for the improved alphavirus-cell interaction and transduction by endocytosis.

IFN-α/β analysis

Type I interferon (IFN) is an extremely powerful antiviral response factor that is capable of affecting alphavirus infections.[60] We compared the expression levels of IFN-α and IFN-β in the *in vitro* B16 cells and the first passage of the *ex vivo* B16 cells before infection (0 h) and at different time points during infection and replication (1 h, 3 h, 6 h, 9 h, and 18 h) (Fig. 5C-D). Our data demonstrated a significant down-regulation of IFN-α expression (4-fold decrease) in the *ex vivo* B16 cells before infection compared with the control *in vitro* cells. Melanoma cells do not secrete IFN under normal conditions, which results in the accumulation of IFN within the cells. In our experiment, we observed an equal tendency of IFN-α accumulation in both *in vitro* and *ex vivo* B16 cells starting from 1 h after infection (Fig. 5C). However, the expression level of IFN-α in *in vitro* cells was remarkably higher 1 h post-infection and increased over time to reach a maximum of 576 pg/ml at 6 h post-infection. By contrast, the maximum expression in *ex vivo* cells reached only 269 pg/ml at the same time point. The inhibited expression of IFN-α could be the main cause of the enhanced alphavirus replication in the *ex vivo* melanoma cells.

There were no significant differences in the expression and accumulation of IFN-β in either the *in vitro* or the *ex vivo* B16 cells, thus providing evidence that IFN-β might play a less important role in host cell protection against alphaviral infection.

Discussion

Although alphaviruses are successfully used as tumor-targeting agents in cancer gene therapy, the key factors providing intracellular conditions that are favorable for more efficient viral activity as well as the virus-host interaction dynamics are not well understood. The tumor microenvironment and innate immune responses could cause a wide range of variations in viral activity, blocking or facilitating the replication and spread of the viral particles. We obtained interesting results demonstrating an extremely high infectiousness of alphaviruses in B16 melanoma tumors *in vivo*, whereas delivery of the marker transgenes was inefficient for the same cells *in vitro* (Fig. 1A). These data corresponded well with the results of other authors.[6] We hypothesized that this difference resulted from the influence of the tumor microenvironment on the total cellular proteome.

Alphavirus infectivity and spread in vivo

Although a high level of alphavirus-driven transgene expression was determined, the SFV vector displayed a limited distribution in B16 tumors, demonstrating patchy expression of the virus only in local injection areas (Fig. 1B). Several factors affect the capacity of the virus to spread within the tumor, such as virus neutralization by blood components or rapid generation of an antiviral immune response; however, the main factor is the intratumoral stromal barriers. For alphaviruses, it is possible to increase the tissue permeability and promote their intratumoral distribution, simultaneously using synergic chemotherapeutic drugs.[27,61] However, the slow kinetics of viral spread after intratumoral injections is a common disadvantage that has also been reported for other vectors, e.g., adenoviruses[62] and retroviruses.[63] The oncolytic properties of alphaviruses make them a promising tool with the potential to significantly improve the field of cancer treatment. Thus, it is clearly important to investigate the details of the processes that determine the efficiency of virus spread and infection within tumor microenvironments.

Alphavirus infectivity in vitro

The *ex vivo* B16 tumor cells clearly demonstrated a high efficacy of SFV infection in the first passage after isolation, whereas the control B16 cells cultivated *in vitro* did not support the viral activity. However, the *ex vivo* cells were not able to retain these properties for a long time during the cultivation *in vitro* (Fig. 2A). This observation indicated that the B16 tumor microenvironment was able to provide favorable conditions for alphaviral transduction and replication that were not present in the *in vitro* culture.

The ability to support virus entry and subsequent expression of the marker transgene is most likely determined by differences between the protein profile of the cells residing in the tumor microenvironment and the cells cultivated in a monolayer. The alterations of cellular protein levels might be reflected in numerous ways, from changes in metabolic activity to the physical parameters of the cell membranes, which facilitate endocytosis. Moreover, it is likely that a combination of several appropriate properties is required simultaneously, which is why we focused our efforts on the systematic top-down analysis of the proteomic differences between *ex vivo* and *in vitro* cells.

Role of antiviral response genes in viral activity

The comparative analysis of the protein profile of B16 *ex vivo* cells yielded the list of up/downregulated gene candidates, which were classified into several groups based on their functions and roles in biological processes and molecular mechanisms (Fig. 3A-D). The statistical analysis showed that none of the designated subgroups representing specific biological processes and molecular mechanisms was significantly up- or down-regulated in the *ex vivo* cells in general. This result was expected because although the *ex vivo* and *in vivo* cells

demonstrate altered properties, they still exhibit similar phenotypes and thus similar expression patterns.

Because the main biological processes capable of dramatically affecting viral activity are the response to stimuli, immune system processes and apoptosis,[32] it is very likely that alterations of the expression and translation levels of these specific proteins could ensure favorable conditions for viral activity (Fig. 4A). The most interesting candidate genes that were found to be up-regulated were *Csnk2b* ((CK2) family member), *Scrib* and *Hsp90aa1*. Overexpression of all these genes not only serves as an indicator of the increased aggressiveness of the *ex vivo* B16 cells by facilitating malignant transformation[64,65] but also potentially facilitates viral vector activity. Recent studies have shown that CK2 is essential for the infectious cycle of alphaviruses and other different DNA/RNA viruses.[66,67] The inhibition of *Hsp90* reduces chikungunya virus infection and inflammation *in vivo*.[68] *Scrib* acts as a binding protein and plays a role in virus-host cell interactions in the case of several viral infections.[69] Moreover, *Scrib* is important for cell polarity, acting as a crucial factor in cell membrane architecture and physiology.[70,71] Therefore, upregulation of *Scrib* could play a significant role in the increased membrane elasticity of *ex vivo* cells, which could in turn impact the process of viral entry.

Among the downregulated proteins, one of the most attractive candidates was IFN-α because it is one of the main negative regulators of tumor growth. Inhibition of IFN-α expression has been shown to cause transcriptional downregulation of several anti-proliferative IFN-α-inducible signaling pathways, namely, PI3K, p38 MAPK and JAK-STAT,[72,73] which was correlated with extremely high alphavirus activity.[74,75] A detailed analysis of down-regulated antiviral response genes in B16 *ex vivo* cells showed that most of them participate in the PI3K, p38 MAPK and JAK-STAT pathways (Table S3), but the expression of genes such as *Hspb1 (Hsp27), Cryab, Csf1, Stat1* and *S100a11* is mediated by all 3 pathways. The PI3K pathway has been shown to be essential for the replication of different viruses. However, inhibition of the activity of PI3K in B16 *ex vivo* cells apparently did not function as a key factor affecting SFV vector-driven expression. Alphaviruses are able to auto-activate and auto-regulate PI3K signaling during infection, providing essential conditions for cap-dependent translation of viral proteins.[76] By contrast, down-regulation of p38 MAPK and JAK-STAT signaling has a remarkable effect on the virulence potential of alphaviruses. Both of these pathways can promote apoptosis, thereby enhancing the cytopathic properties of the virus during infection.[74,75] The inhibited activity of both the p38 MAPK and JAK-STAT pathways in B16 *ex vivo* cells facilitated alphavirus replication and transgene expression, possibly by delaying the onset of apoptosis during infection. The downregulation of several genes coordinated by the IFN-inducible pathways shown in Table S3 such us *Nfe2l*,[77-79] *Ifi35*,[80] *S100A11*[81] has been shown to promote different levels of virus infectivity.

Role of genes regulating cytoskeletal organization during virus entry

An additional group, which might be responsible for the improved viral activity, comprises the cytoskeleton-related proteins. Genes regulating cytoskeletal organization are essential during the virus life cycle. Up-regulation of several cytoskeleton motor proteins such us dynein and kinesin in *ex vivo* B16 cells facilitates the activity of various virus vectors. Generally, invading viruses use dynein to reach the nucleus for replication. In turn, kinesin is used to reach the cell membrane where viral budding and exit occur.[82] Dynein has been reported to interact with purified adenovirus,[83] adeno-associated virus,[84] parvovirus,[85] herpes virus[86] and HIV-1.[87] Kinesin appears to have several important roles during the replication cycle of vaccinia virus[88] and to provide cellular transport for herpes virus (HSV)[89] along microtubules.[90] Interestingly, the release of enveloped HSV virions at the plasma membrane was promoted by Myosin 5A (*Myo5a*), which is another overexpressed gene candidate detected in *ex vivo* B16 cells.[91] Additionally, a delay of HSV replication and inhibition of capsid movement in the cytoplasm during egress was observed under the condition of dystonin (*Dst*) depletion, suggesting that this non-motor protein is an important part of the virus transport machinery.[92] Furthermore, changes in the expression of cellular component organization genes could also affect virus entry by altering the physical features of the cell membrane, such as the elasticity and the rigidness. During the endocytosis process, the cell membrane must deform and accommodate a high degree of curvature that requires low elastic features. We presume that the reduced elasticity of the *ex vivo* B16 cells observed by AFM analysis (Fig. 5B) was responsible for the improved alphavirus transduction, whereas the re-organized cytoskeleton facilitated its replication and spread.

In summary, we presented candidate genes (Fig. 4) that provided favorable conditions in combination for increased alphavirus infectivity, thereby providing new possibilities for the enhanced efficacy of alphavirus-based cancer gene therapy. Based on the results of this study, we emphasize the role of combined alterations in gene expression. Individual functional experiments of the identified genes were not a focus of this study because such experiments fail to reproduce the full network of different factors that occur during malignant tumor development and affect alphavirus infection. However, continuous progress in these investigations in the future might impact our understanding of the alphavirus transduction/replication process.

Materials and methods

Cell lines and animals

The BHK-21 (baby hamster kidney cells) and B16-F10 (metastatic mouse melanoma) cells were obtained from the American Type Culture Collection (ATCC/LGC Prochem). The BHK-21 cells were propagated in BHK - Glasgow MEM (GIBCO) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, 2 mM L-glutamine, 20 mM HEPES and antibiotics (streptomycin 100 mg ml^{-1} and penicillin 100 U ml^{-1}). The B16-F10 cell line was cultured in Dulbecco's GlutaMAX medium (GIBCO) supplemented with 10% FBS and antibiotics streptomycin 100 mg ml^{-1} and 40 μg ml^{-1} gentamicin. Specific pathogen-free 4- to 6-week-old female C57BL/6 mice were obtained from Latvian Experimental Animal Laboratory of Riga Stradins University and maintained under pathogen-free conditions in the accordance with the principles of the Latvian

and European Community laws. All experiments were approved by the local Animal Protection Ethical Committee and the Latvian Food and Veterinary Service (permission for animal experiments no. 32/23.12.2010).

Production of SFV (SFV/EGFP, SFV/DS-Red and SFV/Enh.Luc) recombinant virus particles

The pSFV1[93] vector was used in this study. The pSFV/EGFP, pSFV/Ds-Red and pSFV/Enh.Luc vectors were generated as described previously.[12,27] The resulting plasmids were used to produce SFV/EGFP, SFV/DS-Red and SFV/Enh.Luc virus particles. Briefly, the pSFV/EGFP, pSFV/DS-Red, pSFV/Enh.Luc and pSFV-Helper[93] plasmids were linearized using the *Spe*I restriction enzyme. *In vitro* RNA transcription was performed using 1–2 μg of linearized DNA and 40 U of SP6 RNA polymerase (Thermo Scientific) in a 50 μl reaction mixture, as described by the manufacturer. The RNA transcripts were capped during the transcription reaction by adding 1 mM of the 5′(ppp)5′G cap-analog (New England Biolabs). The DNA template was removed by digestion with RNase-free DNase (Thermo Scientific).

For packaging, the corresponding *in vitro* transcribed recombinant RNA (20 μg each) were co-electroporated with the helper RNA into 1×10^7 BHK-21 cells (850 V, 25 μF, 2 pulses) using a Bio-Rad Gene Pulser apparatus without the pulse controller unit. The electroporated cells were resuspended in 15 ml of complete BHK medium, transferred into tissue culture flasks (75 cm^2) and incubated at 37°C (5% CO_2). After 24 h, the recombinant SFV particle-containing medium was harvested, rapidly frozen and subsequently used as the virus stock for cell culture infection.

The virus titer (infectious units per ml, iu ml^{-1}) was quantified by infection of BHK-21 cells with serial dilutions of viral stock and analysis of *EGFP* or *DS-Red* expression by fluorescence microscopy on a Leica DM IL microscope (Leica Microsystems Wetzlar GmbH). SFV/Enh.Luc virus titer was quantified by Real-time PCR as previously described.[12]

Infection of cell lines with recombinant virus particles

The BHK-21 and B16-F10 cells were cultivated in 24-well plates at a density of 2×10^5 cells per well in a humidified 5% CO_2 incubator at 37°C. For transduction, the cells were washed twice with PBS containing Mg^{2+} and Ca^{2+} (Invitrogen). Then, 0.3 ml of the medium containing the virus particles was added. The SFV/DS-Red and SFV/Enh.Luc virus particles were diluted in PBS (containing Mg^{2+} and Ca^{2+}) to achieve a multiplicity of infection (MOI) of 10. The cells were incubated for 1 h in a humidified 5% CO_2 incubator at 37°C. The control cells (uninfected) were incubated with PBS (containing Mg^{2+} and Ca^{2+}). After incubation, the solution containing virus was replaced with 0.5 ml of growth medium.

Induction of tumor nodules

The B16-F10 cells were trypsinized, washed with PBS and resuspended in PBS at a final concentration of 3×10^5 cells ml^{-1}. Two hundred microliters of the B16-F10 cell suspension was subcutaneously injected above the right shoulder blade of the mice. After 8–12 days, when tumor volumes reached 1000 mm^3, the B16 nodules were i.t. injected with SFV particles or isolated for further tumor cell cultivation.

SFV/Enh.Luc administration and analysis of luciferase gene expression in tumors

B16 tumor-bearing mice (n = 3) were i.t. inoculated with 0.25 ml of SFV/Enh.Luc particle-containing stocks (4×10^8 i.u. per ml). The *Luc* gene expression level was estimated by measuring luciferase enzymatic activity in tumor homogenates 24h after SFV/EnhLuc virus administration. The tumors were excised and manually homogenized in a 1x concentration of ice-cold lysis buffer (Cell Culture Lysis buffer, Promega) containing a protease inhibitor cocktail (10 μl per 1 ml of lysis buffer) (Sigma). After homogenization, the samples were centrifuged for 10 min at 9000 × g, and the protein concentration was determined in tissue lysates using the BCA Protein Assay Kit (Thermo Scientific). Luciferase activity was measured by adding 100 μl of freshly reconstituted luciferase assay buffer to 20 μl of the tissue homogenate (Luciferase Assay System, Promega) and then was quantified as relative light units (RLUs) using a luminometer (Luminoskan Ascent, Thermo Scientific). The RLU values were expressed per mg of protein in the lysates. As a negative control, B16 tumor-bearing mice were inoculated with PBS, and the maximal negative value was subtracted from the presented results.

SFV/EGFP, SFV/Ds-Red administration and analysis of vector intratumoral spread

B16 tumor-bearing mice were i.t. inoculated with 10^6 i.u. of SFV/EGFP and SFV/Ds-Red in different tumor sides (SFV/EGFP was injected into right side of tumor, where SFV/Ds-Red was injected into tumor left side). 24h after vectors administrations the tumors were isolated and frozen in OCT compound (Sigma). The cryosections (5–10 μm) were prepared and *EGFP* and *Ds-Red* expression was visualized by fluorescent microscopy.

Isolation and cultivation of ex vivo B16 cells

Freshly isolated B16-F10 tumors were manually homogenized in PBS and filtered through 40-μm diameter filters. The obtained cells were washed twice with PBS and seeded in 24-well plates at a density of approximately 5×10^5 cells per well in Dulbecco's GlutaMAX medium (GIBCO/Invitrogen) supplemented with 10% FBS, 100 mg ml^{-1} streptomycin, and 40 μg ml^{-1} gentamicin. Two days later, the cells were washed with PBS to remove unattached cells and cultivated in Dulbecco's GlutaMAX medium containing 10% FBS, 100 mg ml^{-1} streptomycin, and 40 μg ml^{-1} gentamicin for 5–7 d until the cell monolayer reached 80% confluency. The medium was replaced every 2 d during cultivation. Next, the 80% monolayer 1st passage of *ex vivo* cells was trypsinized and plated in 24-well plates at a low density of approximately 4×10^3 cells per well (for infection or melanin staining) and in 10-cm Petri dishes at a

density of 2×10^4 cells for further *ex vivo* B16 cell passaging (2nd passage, 3rd passage, etc.).

Melanin staining of B16 cells was performed using a Fontana-Masson Stain kit according to the protocol provided by the manufacturer (Abcam). Briefly, 80–100% monolayered cells were washed with PBS and fixed with 3% paraformaldehyde for 10 min. The cells were then washed with PBS and treated according to the Fontana-Masson staining protocol. The cell nuclei were counterstained with nuclear fast red.

Sample preparation for label-free LC-MS analysis

Ex vivo B16 cells were isolated from 3 B16-F10 tumor-bearing mice and cultivated for 5–7 d until the monolayer of the first passage reached 80% confluency (see above). The control *in vitro* B16-F10 cells were cultivated as described above until the monolayer reached 80% confluency. Both *in vitro* and *ex vivo* B16 cells were lysed with 0.1% ProteaseMax in 50 mM ammonium bicarbonate buffer (Promega) and sonicated for 15 min at 35 kHz. The lysate protein concentration was measured using Direct Detect® Assay-free Cards (Merck Millipore). For LC-MS analysis, samples were prepared using the FASP protocol.[94] Briefly, 80 μg of proteins from each sample were mixed with DTT at a final concentration of 10 mM and incubated for 15 min at 56°C. The cell lysate was then incubated with 6 M urea and filtered through a YM-30 spin column (Millipore). The sample-containing columns were washed twice with 100 mM Tris pH 8.5 and then digested by addition of lysyl endopeptidase (Lys-C) (Wako) solution or trypsin to the column overnight at 37°C. The resulting Lys-C and trypsin fractions were eluted with H_2O by centrifugation and mixed with 0.1% trifluoroacetic acid (TFA). All samples were purified using microcolumns prepared by placing a C18 Empore Extraction Disk (Varian, St. Paul, MN) into 200-μl pipet tips. Peptides were eluted by applying 80 μl of 80% acetonitrile (ACN) and 0.1% formic acid in water. ACN was evaporated in a vacuum drier, and the samples were diluted in 0.1% formic acid in water.

LC-MS

All experiments were performed on an Easy nLC1000 nano-LC system connected to a quadrupole – Orbitrap (QExactive) mass spectrometer (ThermoElectron) equipped with a nanoelectrospray ion source (EasySpray/Thermo). For liquid chromatography separation, we used an EasySpray column (C18, 2-μm beads, 100 Å, 75-μm inner diameter) (Thermo) capillary with a 25-cm bed length. The flow rate was 300 nl/min, and the solvent gradient was 2% B to 5% B in 10 min followed by 5% to 26% B in 230 min, and then 90% B wash in 20 min. Solvent A was aqueous 0.1% formic acid, whereas solvent B was 100% acetonitrile in 0.1% formic acid. The column temperature was kept at 60°C.

The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 400 to 1,200) were acquired in the Orbitrap with resolution R = 70,000 at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadruple). The method used allowed for the sequential isolation of the most intense multiply charged ions, consisting of up to 10 depending on the signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or a maximum acquisition time of 100 ms. MS/MS scans were collected at a resolution of 17,500 at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were as follows: electrospray voltage of 2.1 kV, no sheath and auxiliary gas flow, heated capillary temperature of 250°C, and normalized HCD collision energy of 25%. The ion selection threshold was set to 1e4 counts. An isolation width of 3.0 Da was used.

Protein identification and label-free quantitation

MS raw files were submitted to MaxQuant software version 1.4.0.5 for protein identification.[95] Parameters were set as follows: protein N-acetylation, methionine oxidation and pyroglutamate conversion of Glu and Gln as variable modifications. First, we used a search error window of 20 ppm and a main search error of 6 ppm. The Lys-C or Trypsin enzyme option, both without proline restriction, was used depending on the sample, with 2 allowed miscleavages. Minimal unique peptides were set to 1, and the FDR allowed was 0.01 (1%) for peptide and protein identification. Label-free quantitation was set with a retention time alignment window of 3 min. The UniProt Reference Proteome mouse database was used (download from April 2014). Generation of reversed sequences was selected to assign FDR rates. All quantitative analyses were performed using the Perseus suit from MaxQuant. Briefly, MaxLFQ values were loaded and log-transformed, and 0 values were replaced by noise detection values using an imputation approach based on the normal distribution of the whole data. Differential proteins were assigned by a t-test analysis using S0 = 0.5, a p-value threshold of 0.01 and a permutation-based FDR correction.

Bioinformatics analysis

A total of 277 differentially expressed genes in *ex vivo* and *in vitro* B16 cells were selected by filtering with confidence at $p < 0.01$ from a total of 4980 proteins with a difference in expression of at least 1.4-fold. The biological classification of associated genes in terms of their biologic processes and molecular functions was obtained by Gene Ontology (GO) analysis using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (version: PANTHER 9.0; http://www.pantherdb.org).[30,96] Analysis of the cellular localization of selected genes was conducted using the UniProt Knowledgebase (http://www.uniprot.org/).

Analysis of IFN-α and IFN-β in ex vivo and in vitro B16 cells

Control *in vitro* B16 cells and freshly isolated *ex vivo* B16 cells were seeded in 24-well plates and cultivated until the cell monolayer reached 80% confluency as described above. Expression levels of the IFN-α and IFN-β were determined in *in vitro* and first-passage *ex vivo* B16 cell lysates before SFV infection (0 h) and at 1 h, 3 h, 6 h, 9 h and 18 h after infection with SFV/Ds-Red at an MOI of 10. The cells were trypsinized, washed with PBS and resuspended in 100 μl of PBS. For the lysates, 3 freeze-thaw cycles of the cell suspensions were performed. The

cell lysates were centrifuged for 10 min at 5000 × g, and the protein concentration was equalized in all samples using the BCA Protein Assay Kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific). The expression levels of IFN-α and IFN-β in the cell lysates were determined using the Interferon Alpha ELISA Kit (Uscn Life Science Inc.) and Interferon Beta ELISA Kit (Cusabio Biotech) according to the manufacturers' protocols.

Analysis of cell membrane elasticity by AFM

Control *in vitro* B16 cells and freshly isolated *ex vivo* B16 cells were seeded in 8-well plastic chambers and cultivated until the cell monolayer reached 80% confluency as described above. To measure cell membrane hardness, an uncoated atomic force microscope cantilever (Olympus AC240TS) with spring constant C = 2 N/m and resonant frequency F = 70 kHz was used. Cantilever calibration was conducted by standard operations using an MFP-3D atomic force microscope (Asylum Research) and Igor Pro 6.34A software. The AFM tip with a radius of 10 nm was manually positioned at the middle point between the cell nucleus and the elongated cell body (Fig. 5A) using an OLYMPUS IX71 inverted optical microscope. After positioning, the tip was engaged without scanning the surface of the cell to maintain viability, and single force curves were acquired. Single force curve data were exported to Microsoft Excel. For each cell, a particular force curve was acquired using only trace data, where x was the distance to the cell and y was the applied force. The maximum force that could be endured by a cell was calculated by identifying the intersection point of 2 linear trendlines: one describing the tip approach region and the second describing the cell perforation region. The trendlines fit the experimental data with a coefficient of determination of no less than 95%.

Statistical analysis

The RLU results of the *in vivo* and *in vitro* experiments presented in Fig. 1A are presented as the mean ± s.e. of replicate analyses and are representative of 2 independent experiments. The data were transformed to the logarithmic scale. All error terms shown in Figs. 4 and 5B are expressed as the standard error of the mean from at least 3 different samples. Statistical analyses of the results were performed using Microsoft Excel and Statistica7 (StatSoft, Tulsa). Statistically significant differences were determined using Student's t-test ($p < 0.05$).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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4. Discussion

Efficient gene delivery and high transgene expression via alphavirus vectors make them an excellent platform for the development of anticancer vaccines. However, these vectors need to be well characterized and the conditions that provide selective infection of cancerous tissues without causing harm to normal tissues have to be determined. Classic alphaviral vectors, based on SFV and SIN replicons, have been used for cancer gene therapy experiments *in vitro* and *in vivo* and have shown promising results in different cancer models (for reference see **Review paper I** in attachment). Nevertheless, the problems of targeted gene delivery, concerning inefficiency of repeated vector administration and tumor recovery, remain to be solved. Moreover, relevant entry mechanisms of alphaviruses and their interactions with host cells remain unclear.

In the studies presented in this thesis we delineate the potential of alphavirus for targeted cancer gene therapy. In depth analysis of these vectors' infectivity in several cancer cell lines *in vitro,* SFV vector distribution *in vivo* with regard to the vector injection mode, viral dose, expression time and vector re-administration is provided. To develop more effective therapeutic strategy for cancer treatment, we also have explored the efficiency of SFV-mediated gene transfer alone in combination with 5-FU with respect to possible synergistic cytotoxic effects. Additionally, we identified and characterized intracellular mechanisms that occur during the tumor development and may contribute to the capacity of alphaviruses to target tumor cells more specifically.

4.1 Alphavirus distribution and biosafaty

Investigation of virus biodistribution is a key step in understanding virus-host associations and development of novel therapies for clinical application. The most important technical challenges, continuing to attract the attention of the virotherapy research community, are the optimization and enhancement of systemic virus delivery, degree of intratumoral virus spread and extent of transgene production. In **Paper I**, we have evaluated recSFV vector propagation in tumor-free and 4T1 tumor-bearing mice after i.v. and i.p. administration of recombinant viral particles. As an additional tumor model, B16 melanoma tumor-bearing mice were used to examine dissemination of recSFV vectors after direct (i.t) and systemic (i.p.) inoculation (**Supplementary Poster 1**). Despite we could confirm the data published by others (Rodriguez-Madoz et al., 2007; Colmenero et al., 2001), demonstrating a broad spread of recSFV *in vivo,* we would like to emphasize our observations regarding recSFV predominantly targeting 4T1 and B16 tumors. It should also be noted that the highest transgene expression in both tumor models was achieved at specific doses of systemic viral

inoculation, indicating a dose-depended tumor tropism of recSFV. Thus far, only the Sindbis virus of the alphavirus genus has been considered to have tumor targeting properties (Meruelo, 2004; Tseng et al., 2004b; Tseng et al., 2010; Unno et al., 2005). However, this study did not address a potential dependence of distribution patterns with the dose virus inoculated. Despite several ongoing trials underline the advantages of intratumoral delivery, systemic delivery and tumor-specificity of viral vectors is extremely important; both for clinical trials in general and for treatment of metastatic cancer in particular.

The continued innovation of new therapeutic modalities based on gene therapy holds promise for improving treatment of cancer patients. Here we show the high potential of self-replicating recRNA vector for gene delivery. The main advantages of recRNA vectors over recSFV particles include safety, reinoculation efficiency, reduced injection volume and the more simple preparation of RNA synthesis *in vitro* compared to virus production. Before, self-replicating alphavirus RNA was used as a vaccine in order to induce specific immune response. As shown by Cheng *et al.*, immunization with Sindbis virus RNA replicon vectors fused with herpes simplex virus type 1 protein VP22 and human papillomavirus type 16 E7, resulted in significant E7-specific T-cell-mediated immune responses. In this study the authors noted, that due to the inability of RNA vectors to spread *in vivo*, they facilitate the vectors' capability to distribute via linkage of VP22 (Cheng et al., 2001). However, investigation of alphavirus RNA dissemination and its expression abilities *in vivo* without any help of additional transport proteins was not performed. In **Paper I** we show how systemic i.p., i.v. versus direct i.t. routes impacted luciferase expression as an index of alphavirus RNA vector biodistribution in the whole animal. Significant recRNA-driven *Luc* expression was detected in all organs analyzed, indicating a broad dissemination and significant expression potency of self-replicating RNA vector. Moreover, we therein present for the first time recRNA as a promising vector for targeted gene delivery to the brain. Because administration of recSFV particles did not lead to predominant expression in the brain, we speculate that recRNA molecules may cross the hematoencephalic barrier more efficiently than viral vector.

Relevant in this context, in later studies aiming target glioblastoma cells in the brain, multivalent naked RNA nanoparticles based on pRNA 3-way-junction from bacteriophage phi29 were generated. However, in this research the authors decided to increase the efficacy of the specific vector targeting by linkage of folate ligand. Thus, confirming our hypothesis, systemic inoculation of this vector successfully targeted and delivered siRNA into malignant brain cells of mice (Lee et al., 2015).

Delivery of recombinant genes into the brain is becoming an increasingly important; this not only

for brain cancer therapy, but also as a strategy for answering questions about the molecular mechanisms underlying brain function. Thus we believe that further optimization of brain-targeted recRNA-based gene delivery is warranted.

Despite having demonstrated broad dissemination and intensive expression of both recSFV and recRNA in mice, we would like emphasize the high level of biosafety of these vectors. No toxic side effects, inflammation or organs disfunction were observed in infected mice during our experiments. As a confirmation of alphavirus's biosafety, thus far no patient has developed any serious side effects during clinical studies relevant there (for references see Review of the literature). According to these reports, alphavirus-based treatment was well tolerated by all patients.

Although other viral vectors have been used with greater success in clinical trials (for the review see Kotterman et al., 2015), some clinical cases demonstrated that biosafety of gene delivery vectors require more attention and development. In 1999, in clinical trials aimed to treat ornithine transcarbamylase deficiency and following adenoviral vector administration, a young patient died due to systemic inflammation and multiorgan failure. Of note, this patient had a higher level of pre-existing immunity to the vector. Based on this fact, this patient's immune memory to the vector was put forward as one possible reason for this fatality. Another hypothesis focused on the possibility that certain proteins of the vector capsid inadvertently induced excessive release of inflammatory cytokines by antigen presenting cells (Wilson 2009). In this case, the absence of alphavirus pre-immunity should be considered as a major advantage. Moreover, alphaviruses elicit only a low grade of specific immune responses against the vector itself.

Another example underlining the requirement of applying only highly biosafe viral vectors was described in 2000. During the X-SCID trial, where the γ-retrovirus MLV was applied, 5 of 20 patients developed clonal T-cell leukemia (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2003). This outcome was caused by the vector's efficient integration into the host's genome, preferentially near the transcriptional start site of genes and within hot spots enriched for proto-oncogenes and genes controlling cell-growth (Cattaglio et al., 2007). In view of these issues, replication of RNA replicon–based vectors such as alphaviruses takes place in the cytoplasm and, as a consequence, integration into the host genome does not occur. Thus from our point of view, there is no current need to significantly improve clinical safety the case of alphaviruses.

In summary, alphavirus vectors have been shown to display a strong safety profile and induce clinically relevant gene expression with subsequent protein activity. However, extremely efficient therapeutic outcomes were not yet achieved (see references in the Review of the literature). Main reasons underlying this lack of efficiency are thought to be poor characterization of these vectors

and the lack of innovative treatment strategies such as combined therapies. The latter are discussed below.

4.2 Perspectives of cancer gene therapy based on combination of alphavirus vectors with other adjuvants in clinic

By demonstrating numerous synergistic interactions that improve vector intratumoral spread and expression, thus far preclinical studies provide a strong basis for virotherapy (see review paper I in supplementary). In **Paper II**, enhanced intratumoral expression of replication-deficient SFV vectors upon direct and systemic inoculation was achieved by combination of the viral vector with 5-FU. Using a comparable approach, such combined therapy was applied for treatment of recurrent head and neck cancer patients in II phase of clinical trial. Administration of adenovirus vectors (ONYX-015) together with cisplatin and 5-FU resulted in more than 50% reduction in tumor volume in 19 out of 30 patients, including 8 complete responses and 11 partial responses (Khuri et al., 2000). In this case, adenovirus vectors were engineered to selectively replicate in p53-deficient cancer cells and subsequently lyse these cells. As alphaviruses naturally induce apoptosis in a p53-independet fashion, conducting similar trials are an intriguing proposition. Hence, we also believe that identification of additional drugs that positively interact with alphaviruses is of high priority.

Investigating the reasons for high recSFV activity in mouse melanoma tumors *in vivo* compared to the same cells *in vitro*, we have concluded that alphaviruses likely benefit from or even require specific alterations to host cell pathways which are induced by tumor microenvironment. The identification of individual molecular markers determining a vector's activity and specific antitumor properties may indeed significantly aid in predicting gene therapy outcomes. As shown in **Paper III**, high alphavirus infectivity was promoted by inhibition of IFN-alpha and down-regulation of IFN-regulated pathways (PI3K, p38 MAPK and JAK-STAT). These findings allow designating our vectors as interferon (IFN)-sensitive. One interesting strategy for cancer therapy in this context would be exploring the effects of alphavirus-based therapy with drugs specifically suppressing innate immune responses of virus infected cells. For example combination of alphaviruses and IFN inhibitors such as Ruxolitinib or histone deacetylase inhibitors (HDACi) could be explored. Advantageous in this respect if the fact that individually these drugs are already being evaluated for the treatment of cancer (Hurwitz et al., 2015; Kummar et al., 2007) and are known to prevent the transcriptional activation of antiviral genes after virus infection (Chang et al., 2004). However, additional preclinical studies to test the efficacy of these combinations are required as such intervention may in some cases interfere with the host's own anti-tumor response.

Tumor cell respond to the tumor microenvironment on a molecular level that may have as a consequence alterations of the biomechanical properties of these cells and tissues. Understanding such processes may be instrumental in increasing our understanding of cellular behavior and cellular manifestations in cancer tissues. In **Paper III**, delineating the effects of alphaviruses in a mouse model of melanoma, we identified 26 genes, all associated with cytoskeleton organization, that were differently expressed depending on the method of culture (cells cultivated *in vitro* or *in vivo* during tumor development). Such alterations may not only affect virus traffic and secretion, but, by altering the physical features of cell membrane, also impact virus entry. During endocytosis, the cell membrane must deform and accommodate a high degree of curvature. In the case of HIV it was shown that elastic deformations of the cell membrane affect the required energy for HIV-host cell interaction (Sun et al., 2006). We would speculate that changes in cell membrane elasticity may also affect alphavirus infectivity.

The possibility to measure elasticity in a cellular network has led to the development of drug molecules for cancer therapeutics attractive to the pharmaceutical industry (Pillet et al., 2013). One example is cytoskeleton-destabilizing agent (Cyt-B) that is able to attenuate the average elasticity of cells by disrupting actin filaments (Cai et al., 2010). It was shown that cisplatin-resistant ovarian cancer cells respond to a combination of cisplatin and Cyt-B treatment by reducing the elastic features of cell membranes (Sharma et al., 2012). Considering our results regarding membrane elasticity, it would be interesting to explore therapeutic efficacy of cytoskeleton-reorganization agents in combination with alphavirus vectors.

4.3 Lessons from unexpected and negative results

Twice during our studies *in vitro* experiments yielded results contrary towhen investigating the same question using *in vivo* model systems. First, in contrast to 4T1 allograft, combined treatment of 4T1 cells with recSFV and 5-FU *in vitro* did not cause synergic effects with respect to inhibition of cell proliferation and vector expression. In another experiment, whereas B16 mouse melanoma cells culturing *in vitro* ere insensitive to recSFV infection, the same cells were rendered susceptible to recSFV infection and triggered extremely high transgene expression after exposing them to *in vivo* conditions. These disparities have been incredibly informative and as described above in **Paper II** and **III** have fronted new concepts in the field. These include the need for a better understanding of the tumor microenvironment, challenges associated with a tumor tissue's heterogeneity as well as drugs such as 5-FU in promotion modulating alphavirus vector activity. Based on our experience, we conclude that solely relying on results form *in vitro* experiments can

be misleading and must be validated further during the development phases of new cancer therapeutics. Correspondingly at the price of time and funds, results generated using *in vivo* model systems reflect complexities such as a tumor's microenvironment what might be crucial under some circumstances while not in others. In this context, any approach and type of sample studied, form cell culture systems to animal models and clinical specimens, have their own sets of strength and weaknesses. Cross-validation and exploitation of different strategies to answer a specific objective are therefore key to provide a sound basis for proposing novel concepts for virus-based cancer therapy. We would like to emphasize the importance of investigation unexpected, incoherent and negative results, because they might hold the key to true understanding of mechanisms underlying infection quality and quantity as well as explain variability observed for host responses.

By revealing alphaviruses as potent vectors for targeted gene delivery, this work contributes to providing novel and attractive options for designing innovative virus-based cancer therapy.

5 Conclusions

1. Alphavirusescan infect a wide range of cancer cell lines *in vitro*. Despite recSFV and recSIN being closely related viruses, the transduction ability and oncolytic properties of these vectors differ significantly. In addition, infectivity and cytotoxic properties of the same vector varies noticeably depending on cancer cells types.
2. Despite recSFV vectors having a broad dissemination in tumor-bearing mice upon systemic vector administration, predominant tumor targeting could be attained via dose adaptation in mouse models for mammary carcinoma and melanoma. However, the best results for recSFV-mediated transgene expression were achieved when choosing the i.t. as the route of administration.
3. Unpacked self-replicating recRNA can be used successfully as an alternative and safe methodology for gene delivery. I.v. inoculation of recRNA provided primary brain targeting in both tumor-free and mammary carcinoma bearing mice, whereas intratumoral inoculation revealed localized high expression levels in tumors.
4. Although 5-FU inhibited SFV-mediated transgene expression in metastatic mammary carcinoma (4T1) cells *in vitro*, delivery of 5-FU and recSFV as a combined treatment option *in vivo* revealed synergic therapeutic effects. These include significant enhancement of intratumoral transgene synthesis and tumor cleavage compared with mice only treated with 5-FU.
5. Comparative protein profiling of B16 mouse melanoma cells susceptible and non-susceptible to alphavirus infection revealed differences in the expression of genes that regulate antiviral responses and cytoskeletal organization processes. Indeed, inhibition of IFN-alpha led to suppression of signaling cascades such as the PI3K, p38 MAPK and JAK-STAT pathways and could represent a major key to modulate recSFV activity. The alterations observed in proteins related with cytoskeleton organization in B16 cells when isolated form tumors had as a consequence a significant modification of cell membrane's elasticity. Taken together these intracellular and physical alterations may promote alphavirus entry and replication.

6 Theses for defense

1. Recombinant Semliki Forest virus is an efficient vector for targeted gene delivery to tumors upon systemic administration.

2. Self-replicating alphavirus RNA may be used as alternative vector for efficient gene delivery and expression.

3. The therapeutic efficacy of the recombinant Semliki Forest virus may be enhanced by combination with chemotherapeutic drugs in cancer gene therapy.

4. A tumor's microenvironment may affect recSFV infectivity. Alphavirus transduction and replication thereby depends on changes in the host cell which are in turn triggered by the tumor's microenvironment.

5. In virology and when evaluating new approaches to cancer gene therapy, unexpected, incoherent and conflicting results obtained via different methodologies and model systems are common. Thus successful development of such therapies, depends on understanding the strength and limitations of each approach and system.

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Supplementary 1

Review paper I

Alphavirus vectors for cancer treatment

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28 Alphaviral Vectors for Cancer Treatment

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CONTENTS

28.1 INTRODUCTION

28.1.1 Overview of Alphavirus Replication and Assembly

Alphaviruses represent a large class of arthropod borne viruses (Arboviruses) belonging to the Togaviridae family of viruses, which cause transient febrile illness or more severe diseases such as encephalitis. The most commonly used vectors were generated on the basis of three alphaviruses: Semliki Forest virus (SFV) (Liljestrom and Garoff 1991), Sindbis virus (SIN) (Xiong et al. 1989), and Venezuelan equine encephalitis virus (VEE) (Davis et al. 1989).

The biology of these alphaviruses is similar. Their genome comprises a 5′ end capped and 3′ end polyadenylated RNA molecule of approximately 12 kb in length. Since the RNA has a positive polarity, it is infectious, capable of initiation of replication and translation when introduced into the cytoplasm of host cells. Functionally, the genome is divided into two parts coding for the nonstructural and structural proteins, respectively (Figure 28.1). Two-thirds of the 5′ end of the RNA genome encodes a polyprotein that is processed into four viral nonstructural proteins responsible for the replication of the plus strand (42S) genome into full-length minus strands. These molecules then serve as templates for the production of new 42S genomic RNAs and subgenomic 26S RNAs. The latter is an approximately 4000 nucleotide long subgenomic RNA and is collinear with the last third of the genome. Its synthesis is internally initiated at the 26S promoter on the 42S minus RNA strand.

The subgenomic RNA codes for the structural proteins of the virus, which are also synthesized as a polyprotein precursor in the order C-E3-E2-6K-E1. Once the capsid (C) protein has been synthesized, it acts as an autoprotease, cleaving itself off the nascent chain (Hahn and Strauss 1990; Schlesinger and Schlesinger 2001). At the endoplasmic reticulum (ER) membrane, the nascent chain is cotranslationally translocated and cleaved further by a signal peptidase to the three structural membrane proteins, p62 (precursor of E3-E2), 6K, and E1. After synthesis, the C protein complexes with genomic RNA into nucleocapsid structures in the cell cytoplasm. Usually, only the genomic RNA is packaged due to the presence of the encapsidation signal within the nsP1 and nsp2 genes for SIN and SFV, respectively (Frolova et al. 1997).

The membrane proteins undergo extensive posttranslational modifications within the biosynthetic transport pathway of the cell. The precursor protein p62 is proteolytically cleaved during the transport to the cell surface to form the mature envelope glycoprotein E2 (Lobigs and Garoff 1990). The p62 forms a heterodimer with E1 in the ER (Barth et al. 1995). This dimer is transported to the plasma

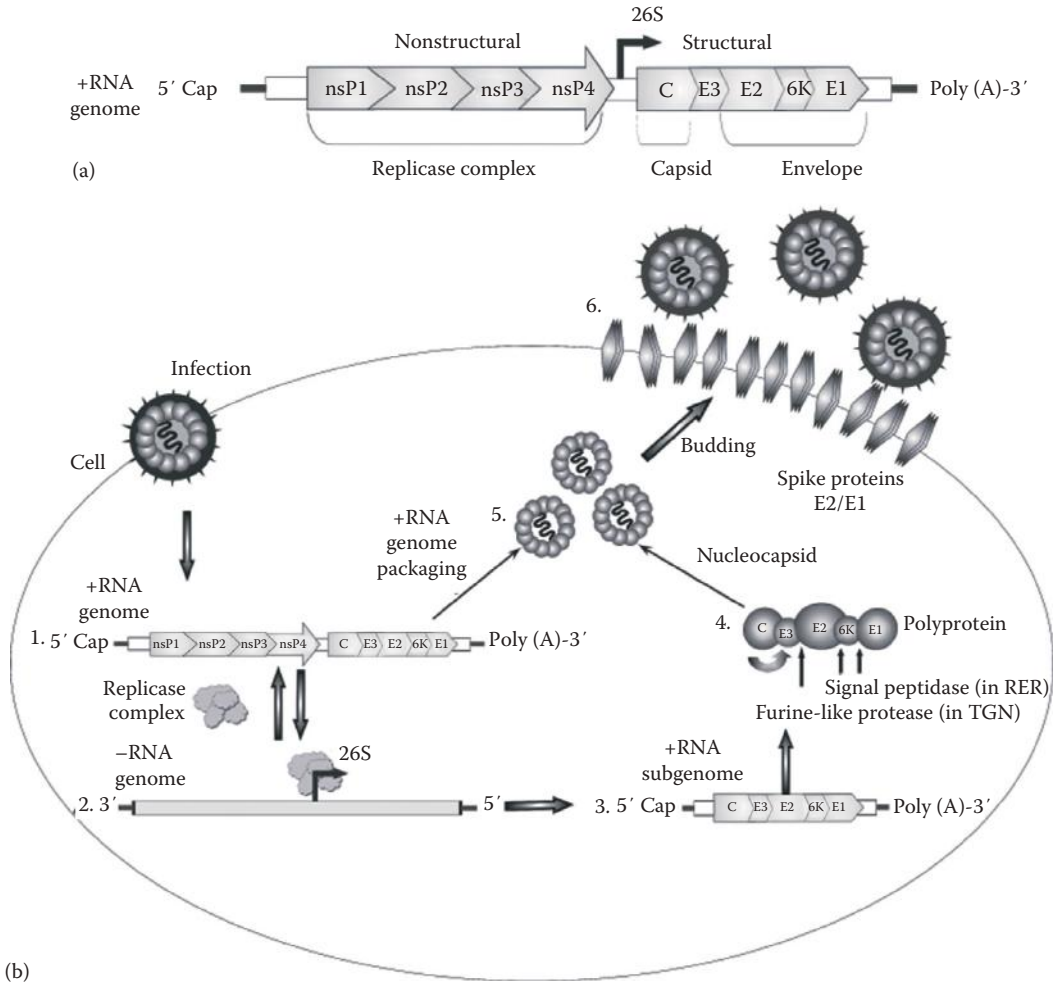

FIGURE 28.1 (a) Schematic structure of the alphaviral genome. The genome is a positive-strand RNA molecule that is capped and polyadenylated. It encodes two polyproteins indicated as nonstructural and structural parts of the genome. (b) Replication cycle of alphaviruses. After virus particle penetration and uncoating, the viral genomic RNA is released into the cytoplasm. The replication is initiated by translation of the nonstructural proteins (nsP1–4) and formation of the replicase complex (1). The positive strand genomic RNA serves as a template for a full-length complementary negative strand (2). The negative strand in turn serves as a template for the synthesis of subgenomic RNA expressed from 26S subgenomic minus RNA promoter (3). The structural proteins are translated from the subgenomic RNA and are processed posttranslationally into the individual proteins (4). The positive RNA genome is encapsidated by the capsid protein (5). Virus budding occurs on the cytoplasmic membrane via spike proteins and nucleocapsid interactions (6). RER, rough endoplasmic reticulum; TGN, trans-Golgi network; NC, nucleocapsid.

membrane, where virus budding occurs via spike nucleocapsid interactions (for review, see Jose et al. 2009). At a very late (post-Golgi) stage of transport, the p62 protein is cleaved to E3 and E2 by host furin-like proteases. This cleavage activates the host cell-binding function of the virion as well as the membrane fusion potential of E1. In the absence of p62 cleavage, virus particles are noninfectious. This feature was used for the construction of conditionally infectious particles (Salminen et al. 1992). In SFV, E3 remains part of the mature virion (Garoff et al. 1990), whereas it is shed from the spike in SIN (Welch and Sefton 1979).

Active alphaviral RNA replication triggers cell death in infected cells. The nonstructural region of the genome has been shown to be sufficient for the induction of apoptosis, while the structural region can be replaced by a gene of interest (Urban et al. 2008). Although the precise mechanisms of cell death and virus persistence remain unclear, activation of double-stranded RNA-activated protein kinase R (PKR) has been proposed to contribute to blocking protein synthesis and induction of apoptosis in infected cells (Balachandran et al. 2000; Gorchakov et al. 2004; Venticinque and Meruelo 2010). Other factors, such as caspase cleavage (Nava et al. 1998), reduction in intracellular superoxide levels (Lin et al. 1999), bcl-2 downregulation (Scallan et al. 1997), and cyclin-dependent kinase 2 (CDK2) activation (Hu et al. 2009), may participate in alphavirus-mediated induction of apoptosis, suggesting that several pathways are involved in this potentially cell-specific process.

28.1.2 Structure of Alphavirus Vectors

The essential elements of the expression plasmids are shown in Figure 28.2. The expression system is based on the full-length cDNA clone of the corresponding alphavirus. The classical vectors were generated in such a way that the heterologous insert replaces the structural genes downstream of the 26S subgenomic promoter. Therefore, the vectors contain only the nonstructural coding region, which is required for the production of the nsP1–4 replicase complex, the 26S subgenomic promoter, and a multiple cloning site with several unique restriction sites for the foreign gene insertion. Because the RNA replication is dependent on short sequence elements located at the 5′- and 3′-ends of the genomic RNA (Kuhn et al. 1990), these regions are also included in the vector construct.

To generate infectious particles, the genes encoding structural proteins can be provided in trans. This is a central part of the alphavirus expression technology representing the packaging of recombinant RNAs into infectious particles using a helper construct encoding the viral structural genes. In this procedure, in vitro-made recombinant and helper RNAs are cotransfected into animal host cells (Figure 28.3). The recombinant RNA codes for the RNA replicase needed for the amplification of both incoming RNA species

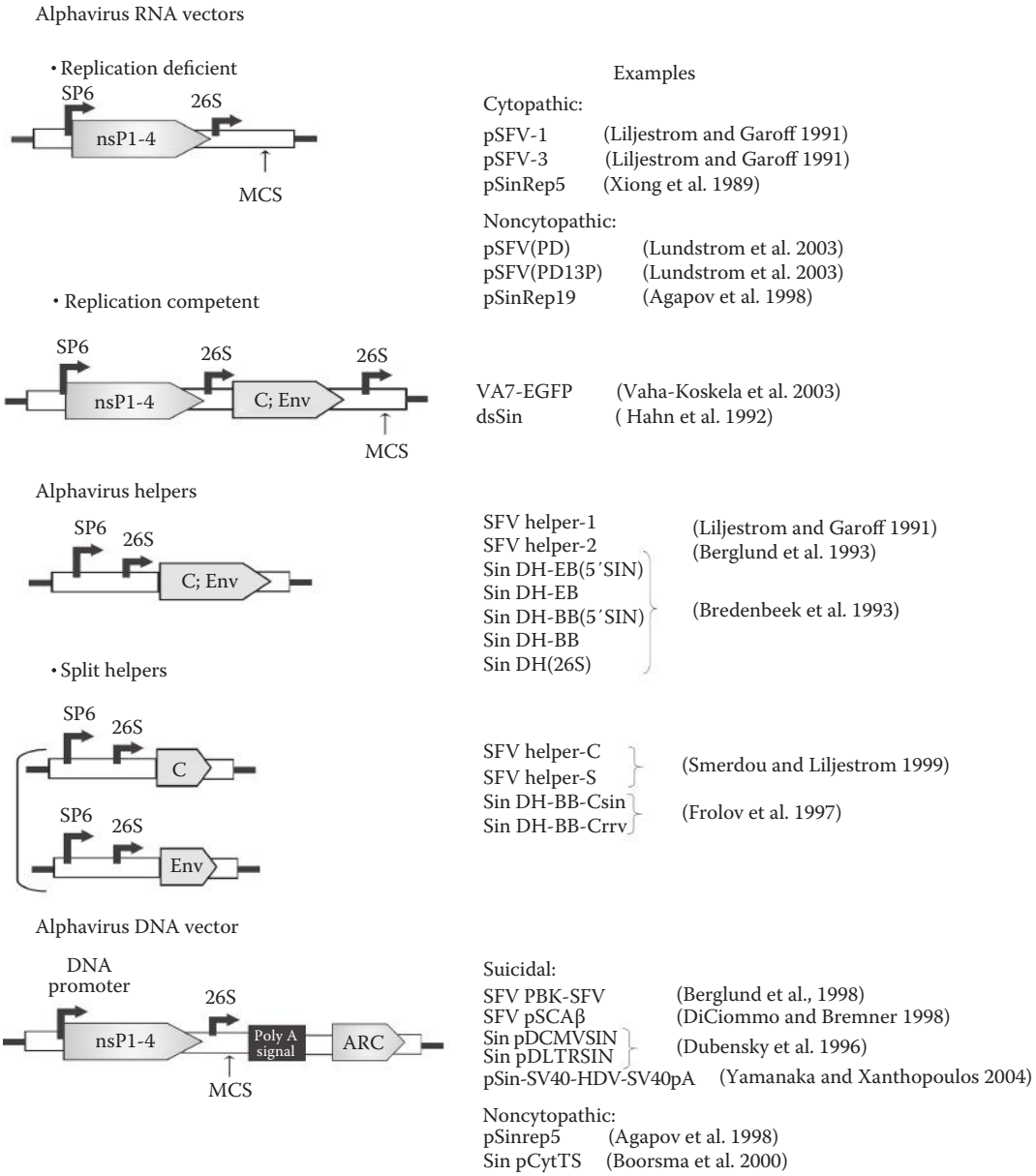

FIGURE 28.2 Schematic representation of recombinant constructs based on alphaviruses. The main classes of vectors are indicated: RNA vectors, including replication-deficient (with helper systems) and replication-competent vectors, and DNA/RNA layered vectors. Examples of each type of vector are indicated for Semliki Forest (SFV) and Sindbis (SIN) viruses. MCS, multiple cloning site; ARC, antibiotic resistance cassette.

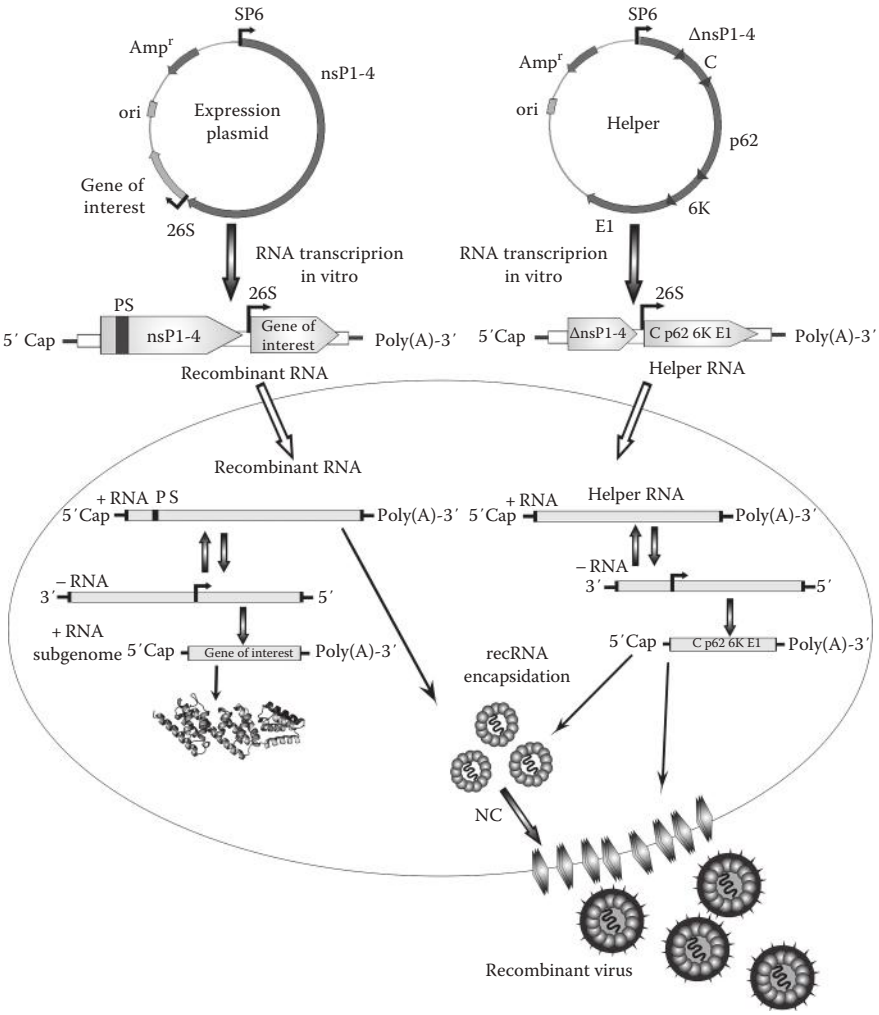

FIGURE 28.3 Recombinant alphavirus production. Two types of in vitro synthesized RNAs (recombinant RNA and helper RNA) are cotransfected into cells. Both RNAs are replicated by the alphavirus replicase complex (nsP1–4). The helper RNA provides the alphavirus structural proteins, which form recombinant virus particles with encapsidated (packaged) recombinant RNA. PC, packaging signal; NC, nucleocapsid.

and the gene of interest, whereas the helper RNA encodes the structural proteins for the assembly of new virus particles. The helper vector is constructed by deleting a large portion of the nonstructural genes, retaining the 5′ and 3′ end signals needed for RNA replication. Since almost the complete nsP region of the helper is deleted, RNA produced from this construct will not replicate in the cell, due to the lack of a functional replicase complex. When helper RNA is cotransfected with recombinant RNA, the helper construct provides the structural proteins in trans to assemble new virus particles, while the recombinant construct provides the nonstructural proteins for RNA replication of both recombinant and helper RNAs The goal for this trans-complementation process is selective packaging of only recombinant RNAs into virus particles, because the helper vector lacks RNA packaging sequence signals recognized by the capsid protein. This packaging signal is located on the replicase-coding sequence. The produced recombinant virus stock, therefore, contains only recombinant genomes, and when such virus particles are used to infect animal host cells, no helper proteins are expressed, providing a one-step virus infection.

In order to reduce the chance of recombination and generation of replication-competent virus, a packaging system in which capsid and envelope genes are produced from separate vectors has been developed (Smerdou and Liljeström 1999). Since the capsid gene contains a translational enhancer (Sjoberg et al. 1994), this sequence was inserted in front of the spike sequence p62-6K-E1. On the other hand, to provide cotranslational removal of the enhancer sequence and normal biosynthesis of the spike complex, a sequence coding for the foot-and-mouth disease virus 2A autoprotease was inserted in frame between the capsid translational enhancer and the spike genes. Cotransfection of cells with both helper RNAs (SFV-helper-C and SFV-helper-S) and the SFV vector replicon carrying a foreign gene led to the production of recombinant particles with high titers (up to 8×10^8 particles per 10^6 cells). An empirical frequency of recombination and replication-competent virus appearance in this system would be very low (10^{-13}),

emphasizing the high biosafety of the system based on two-helper RNAs. A similar strategy of the two-helper RNA system was developed on the basis of the Sindbis virus replicon (Frolov et al. 1997). In this system, the Sindbis spike genes were fused to the capsid gene of Ross River virus containing deletions in the RNA-binding domain, which maintained both the translation-enhancing and the self-cleaving activities. The same bipartite helper packaging system has also been described for VEE (Pushko et al. 1997), but in this case, the spike proteins were expressed without the capsid translation enhancer, which apparently is not needed in the VEE context.

In contrast to replication-deficient particles providing one round of infection, production of replication-competent virus can be achieved by cell transfection with a single construct (Hahn et al. 1992; Vaha-Koskela et al. 2003). These vectors may contain two 26S promoters, leading to the synthesis of two subgenomic mRNAs: one is responsible for the expression of the heterologous product, and the other for the synthesis of virus structural proteins (Figure 28.2, replication-competent vectors). Alternatively, the gene of interest can be inserted into nonstructural polyprotein genes and expressed as a fusion protein (Atasheva et al. 2007), or it can be expressed as a cleavable part of a structural or nonstructural polyprotein (Thomas et al. 2003; Tamberg et al. 2007). These vectors are self-replicating, produce infectious virus particles, and can spread from cell to cell in a manner similar to that of the parental virus. The obvious advantage of replication-competent vectors is the increased efficacy of in vivo gene delivery, which should allow for spreading in infected tissue and, therefore, enhancing the therapeutic efficacy. However, the disadvantage is the safety concern related to potential uncontrolled spread of infectious particles.

Another type of alphaviral vectors commonly used in vaccine development is based on DNA vectors. To allow direct application of plasmid DNA, the SP6 RNA polymerase promoter has been replaced by a DNA promoter (e.g., CMV IE, RSV LTR). In this case, transient transfection of plasmid DNA will result in expression of the gene of interest. DNA vectors could also contain a selection marker for stable transfection. Moreover, it is possible to cotransfect the DNA vector with a DNA-based helper vector, and to obtain recombinant particles or to use helper-producing cell lines. However, the virus titer has generally been significantly lower in this system than in the case of RNA-based particle production (Diciommo and Bremner 1998).

28.1.3 Gene Delivery by Alphavirus Vectors

Among recombinant viruses, alphaviral vectors are good candidates for cancer gene therapy due to their ability to mediate strong cytotoxic effects through the induction of p53-independent apoptosis, their ability to efficiently overcome immunological tolerance by the activation of innate antiviral pathways, and the subsequent triggering of cytotoxic T-lymphocyte responses against tumors (Lundstrom 2009; Quetglas et al. 2010; Osada et al. 2012). The advantages of alphaviral vectors also include a low specific immune response against the vector itself and the absence of vector preimmunity in the major part of the population.

Alphaviruses have been widely accepted to display a broad tissue tropism and can efficiently infect and induce apoptosis in many types of cancer cells (Wahlfors et al. 2000; Rheme et al. 2005). In practice, the infectivity and cytotoxic properties of alphaviruses can vary significantly between different types of cancer cells. A low level of IFN-β production in some tumor cells has been shown to determine the susceptibility and oncolysis of tumors to SIN virus (Huang et al. 2012). Whether this correlation exists for other alphaviruses remains unknown. Although SFV, SIN, and VEE are closely related, substantial differences may exist in their tissue tropism, vector infectivity, and cytotoxicity (Wahlfors et al. 2000).

The mechanism of virus infection and possible regulation of entry, which is important for tumor targeting, is currently controversial. Different proteins have been proposed as candidate receptors for alphavirus infection (for review, see Kononchik et al. 2011; Leung et al. 2011). However, because alphaviruses infect genetically divergent cells, they probably utilize multiple proteins as receptors or alternative entry pathways in different cells. Thus far, only SIN has been considered to be capable of targeting tumors upon systemic injection into mouse models (Meruelo 2004; Tseng et al. 2004b, 2010; Unno et al. 2005). The mediastinal lymph nodes (MLNs) were shown as a site of early transient heterologous protein expression after intraperitoneal injection of SIN vectors, providing the generation of effector and memory $CD8^+$ T cells against expressed tumor-associated antigen (Granot et al. 2014). In contrast, SFV vectors have been applied in most studies through intratumoral administration (Murphy et al. 2000; Maatta et al. 2007; Rodriguez-Madoz et al. 2007; Quetglas et al. 2012b). We have recently observed that SFV can target tumors upon systemic injection at a reduced viral dose, demonstrating that tumor-targeted delivery of the vector may be possible under certain conditions (Vasilevska et al. 2012).

Despite rapid induction of apoptosis in infected cells, treatment with natural oncolytic alphaviral vectors did not result in complete tumor regression (Chikkanna-Gowda et al. 2005; Smyth et al. 2005). The administration of immunomodulator genes, such as cytokines or growth factors, was more efficient and led to successful tumor inhibition or complete regression in animal models (Asselin-Paturel et al. 1999; Rodriguez-Madoz et al. 2005; Lyons et al. 2007). The use of replication-competent viruses is limited by safety restrictions, and most studies in recent years have focused on the use of suicidal replicons in the form of recombinant particles and DNA- or RNA-based vectors.

A promising new approach in vaccine development has been the use of plasmid DNA for immunization. In difference to conventional plasmid DNA, protein expression directed by alphaviral DNA vectors has a suicidal effect due to their strong cytopathic effect. Therefore, the use of these vectors eliminates the undesirable consequences of DNA integration into the host genome. Taking into consideration that the SFV replicon vector is based on in vitro transcribed RNA, the

corresponding RNA (naked or encapsulated into transfection vesicles) can also be used as an alternative vector for cancer therapy. Self-replicating RNA can provide the same efficient cytoplasmic transgene expression and induction of apoptosis in host cells. It is safe for in vivo applications and does not induce antivector immunity (Cheng et al. 2001; Vignuzzi et al. 2001; Saxena et al. 2009). Intramuscular injection of as little as 1 μg of naked SFV RNA provided complete tumor protection and extended the survival of treated mice when tumor cells were injected 2 days before immunization (Ying et al. 1999).

The issue of transgene distribution and persistence in vivo is of special importance when applications in gene and cancer therapy are considered. Therefore, in order to evaluate persistence and distribution of recombinant SFV particles, SFV-replicon-based DNA plasmid and conventional DNA plasmid were compared after intramuscular injection mouse and chicken model (Morris-Downes et al. 2001). The presence of transgene was detected by RT-PCR. Recombinant SFV particles persisted for 7 days at the injection site, while SFV replicon-based plasmid and conventional DNA plasmid could be detected up to 93 and 246 days, respectively, at the injection site. In chickens, transgene could be detected up to 1 day at the injection site in case of recombinant SFV particles and up to 17 days for the SFV-based plasmid and 25 days for the conventional DNA plasmid. In mice lymph nodes, the recombinant SFV particles were detectable for 1 day, and both plasmids for 3 months. Similarly, the two plasmids were present up to 3 months in tissues distal from the site of injection, indicating dissemination.

Localization and persistence of replicon RNA is also dependent on the route of administration (Colmenero et al. 2001). Intravenous administration resulted in a systemic distribution, and the reporter gene was detectable in spleen and lymph nodes as well as in nonlymphoid tissues. Subcutaneous injection leads to a local distribution in the draining lymph nodes and skin surrounding the injection site, while intramuscular injection resulted in expression in local lymph nodes and at the injection site. This study confirmed the transient nature of SFV particles in vivo, since the reporter gene was almost undetectable by day 6 after injection by all examined administration routes. Intratumoral injection of SFV leads to localization of SFV-RNA in tumor cells and draining lymph node only (Colmenero et al. 2002). The short persistence renders the recombinant replicon particles as a safe vaccine tool, but not relevant for applications where prolonged gene expression in vivo is desired.

28.2 EFFICACY OF THERAPEUTIC AND PROPHYLACTIC ANTICANCER VACCINES BASED ON ALPHAVIRUS VECTORS

Mainly SFV, SIN, and VEE vectors have been tested for cancer treatment in animal models. Here we provide a detailed overview on different strategies of cancer treatment using immunogene delivery as naked RNA, plasmid DNA, and virus particles by replication-deficient vectors. Moreover, the possibility to employ oncolytic replication-competent alphaviruses is discussed.

28.2.1 Melanoma

Melanoma is one of the most aggressive forms of skin cancer. Melanoma tumors arise from melanocytes and contain specific tumor-associated antigens (TAAs), which can be categorized as differentiation antigens such as Pmel17/gp100, p75/tyrosinase-related protein TRP-1, MART-1/Melan-A and the retained intron in tyrosinase-related protein (TRP-2-INT2) as well as TAAs like MAGE or melanoma cell adhesion molecule (MUC18) (Pleshkan et al. 2011). These antigens act as ideal targets for melanoma immunotherapy because of their preferential expression in melanocytes and melanoma cells. Alphavirus vectors have demonstrated advances in targeted prophylactic and therapeutic immunotherapy of melanoma in several preclinical studies.

Tyrosinase is an essential enzyme involved in the initial stages of melanin biosynthesis in melanocytes and melanoma cells (Kumar et al. 2011). The ability to stimulate or augment an immune response against melanoma by alphaviral antitumor vaccines expressing either murine or human tyrosinase-related protein 1 (TRP-1) has been shown in two different studies. The efficacy of DNA-based SIN tumor vaccines pSIN-mTRP-1 and SIN-hTRP-1 was evaluated in a B16 mouse melanoma model (Leitner et al. 2003). It was one of the first demonstrations of prophylactic immunization with an alphavirus DNA vector where intramuscular injection was able to break immunological tolerance and provide immunity against melanoma, when inoculated 5 days prior to cancer cells challenge. Similarly, the high prophylactic potential of alphavirus-based vaccines was confirmed by demonstrating the ability of VEE virus-like particle (VLP) vectors encoding murine or human TRP-1 to induce strong immune responses and to provide a significant tumor growth delay in immunocompetent melanoma tumor–bearing mice (Goldberg et al. 2005).

In another study, the transmembrane melanosomal glycoprotein TRP-2 was applied as a therapeutic gene in controlling of melanoma growth (Avogadri et al. 2010). It has been shown that VEE-TRP-2 VLPs induced time-dependent tumor protection when vaccination was started as late as 5 days after tumor inoculation. Importantly, vaccination with VEE-TRP-2 was more effective than combination of VEE-gp100 with VEE-tyrosinase vectors. Moreover, the efficacy of the combination of all three VEE vectors was not significantly better than VEE-TRP-2 alone.

The SIN DNA vector (SINCp) was used to express the murine melanoma cell adhesion molecule (MCAM/MUC18) for vaccination against murine melanoma (Leslie et al. 2007). MUC18 is expressed in late primary and metastatic melanoma, but hardly at all in healthy melanocytes. Immunization with this vector showed no antitumor effect against parental B16F10 cells, probably because of extremely low expression of murine MUC18 in those melanoma cells. To increase this antigen expression in tumor cells, new B16F10 cells transduced with MUC18 were obtained. Vaccination against MUC18 resulted in the induction of humoral and $CD8^+$ T-cell immune responses against melanoma. In order to investigate the efficacy of recombinant alphavirus-based vaccines for the

stimulation of human immune responses, SFV VLPs encoding MAGE-3 were administered in humanized BALB/c mice (Trimera murine model) (Ni et al. 2005). The results showed that the SFV vector elicited human MAGE–specific antibody and CTL responses in Trimera mice.

Several studies in melanoma therapy development have shown that expression of cytokine genes in tumor cells generally resulted in dramatic alteration of tumor cell growth and induction of tumor specific immunity. For instance, a single intratumoral injection of recombinant SFV particles expressing IL-12 caused significant inhibition of melanoma growth in tumor-bearing mice (Asselin-Paturel et al. 1999). Intratumoral administration of SFV-IL12 led to dramatic tumor necrosis in all treated mice, resulting in 70%–90% tumor growth inhibition. However, complete tumor regression was not achieved in this study. To improve the efficacy of antimelanoma therapy, a combined strategy including administration of SFV-IL-12 VLPs and systemic costimulation with agonist anti-CD137 monoclonal antibodies has been explored (Quetglas et al. 2012a), showing the powerful synergistic effects. Briefly, immune system stimulation with agonist agents acting on CD137 expressed on primed T cells resulted in the enhancement of tumor-eradicating cytotoxic T-cell responses (Melero et al. 1997). In contrast to suboptimal therapeutic effect provided by intratumoral injection of SFV-IL-12 VLPs alone, combined administration of both SFV-IL-12 and CD137 mAb dramatically increased the therapeutic efficacy, inducing 50% and 75% of complete tumor remission, respectively.

The oncolytic potential of a replication-competent alphavirus vector was also investigated for the treatment of melanoma. In this context, the avirulent SFV strain A7 expressing EGFP was applied intravenously, intraperitoneally, and intratumorally as a therapeutic vaccine in human melanoma-bearing SCID xenografts (Vaha-Koskela et al. 2006). A single inoculation of the VA7 vector resulted in significant tumor regression, irrespective of the route of administration. The neurotropism of SFV did not restrict its ability to target tumors, as within 3 weeks, VA7 had caused regression of tumors to far below the starting volume. Despite the positive treatment dynamic, small isolated groups of dividing tumor cells were detected within strands of connective tissue, indicating the potential tumor remission in the future.

28.2.2 Breast Cancer

Worldwide, breast cancer is the most common cancer among women. The HER2/neu is a member of the tyrosine kinase receptor family overexpressed in 30%–40% of breast cancers, correlating with increased metastasis and poor prognosis, due to increase of mitotic activity, mutation of the p53 gene, negative estrogen receptor status, and absence of bcl-2 (Banin Hirata et al. 2014). The HER2/neu has a high potential as tumor antigen in breast cancer therapy. A DNA-based SIN vector ELVIS-*neu* expressing the *neu* gene was used for intramuscular vaccination 14 days before injection of cancer cells overexpressing *neu* (Lachman et al. 2001). The results showed a strong protection of mice against tumor development. Vaccination led to reduction of the incidence of lung metastasis from mammary fat pad tumors and also reduced the number of lung metastases resulting from intravenous injection of *neu*-overexpressing cells. Interestingly, intradermal vaccination also provided protection and required 80% less plasmid for a similar level of protection. The beneficial results of cancer vaccines based on the SIN *neu* vector to treat pre-existing tumors were also confirmed (Wang et al. 2005). It was shown that therapeutic efficacy of the pSINCP/neu vaccine depended on the order of vector and cancer cell injection, indicating that the prophylactic vaccine was effective only when administered before tumor challenge.

The therapeutic potential of immunotherapy with the pSINCP/neu DNA vaccine and the VEE/neu VLPs was enhanced by combination with the chemical anticancer agents doxorubicin and paclitaxel (Eralp et al. 2004). Administration of 5 mg/kg doxorubicin prior to pSINCP/neu DNA and VEE/neu VLPs vaccination led to a significant delay in tumor progression. Despite doxorubicin being established as a standard adjuvant therapy for breast cancer, mice receiving chemotherapy alone did not demonstrate reduced tumor growth. Interestingly, but in contrast to the previous results, combined treatment with paclitaxel (25 mg/kg) increased the effectiveness of only the VEE/*neu* VLP vaccine.

In another study, 36% of rat breast tumors were eliminated when a VEE-neu VLP vaccine was subcutaneously administered to animals with aggressive preexisting mammary tumors (Laust et al. 2007). When DC-based cancer immunotherapy was combined with VEE-neu VLP administration, induction of both cellular and humoral immunity against *neu* was observed in transgenic human breast tumor–bearing mice (Moran et al. 2007). Furthermore, the combination treatment led to significant inhibition of tumor growth. Not only tumor antigens have been used for immune system stimulation. Cytokines such as IL-12 have potential as cancer therapy agents because of their antitumor and antimetastatic activities. For instance, enhanced IL-12 expression has been established from SFV10-E VLPs as a potential treatment for breast cancer (Chikkanna-Gowda et al. 2005). The enhanced SFV10-E vector has shown up to 10 times higher expression levels of foreign genes as compared to the original SFV10 vector. Intratumoral administration of high titer SFV-E-IL-12 VLPs caused complete tumor regression in four out of six mice and noticeably reduced the amount of lung metastases.

The vascular endothelial growth factor receptor-2 (VEGFR-2) serves also as an attractive therapeutic target, because it is required for neovascularization within tumors and has been shown to be important for tumor growth, invasion, and metastasis. The requirements for therapeutic efficacy of VEGFR-2-expressing vectors are associated with the induction of an antibody response against VEGFR-2. It was demonstrated that both tumor growth and pulmonary metastatic spread were significantly inhibited in mice with preexisting tumors when subjected to five immunizations with SFV10-E VLP expressing VEGFR-2 (Lyons et al. 2007). Moreover, a significant tumor regression was observed after coimmunization of mice with SFV particles encoding VEGFR-2 and IL-4.

28.2.3 Lung Cancer

Lung cancer is the leading cause of cancer death in the industrialized world. The majority of patients are diagnosed at a locally advanced or metastatic stage, making systemic therapies the mainstay for treatment (Blanchon et al. 2006). In case of lung cancer, promising results were obtained only using direct intratumoral alphaviral vector administration. The first preclinical study using alphaviral vectors in lung cancer treatment was performed by direct intratumoral injections of SFV VLPs expressing EGFP into human non–small lung cancer xenografts (Murphy et al. 2000). The outcome was tumor growth inhibition, and in some cases, complete tumor regression was achieved. The effect was mediated by p53-independent apoptosis and necrosis, but required repeated intratumoral administration (three to six injections) and very high doses of the vector (1×10^{10} IU/mL).

In another lung tumor study, the synergistic effect of combined therapy with SFV-IL-12 VLP and anti-CD137 monoclonal antibodies was demonstrated (Quetglas et al. 2012a). Similar to B16 mouse melanoma, syngeneic TC-1 lung carcinoma was inhibited by a clinically feasible therapeutic combination involving intratumoral treatment with SFV-IL-12 and systemic costimulation with anti-CD137 monoclonal antibodies.

Oncolytic virotherapy with the attenuated replication-competent SFV vector VA7-EGFP showed a good safety profile and resulted in almost complete inhibition of tumor growth in human lung adenocarcinoma NMRI nu/nu mouse models upon intratumoral administration (Maatta et al. 2007). In contrast, systemic administration resulted in only delayed tumor growth (intravenous injection) or total absence of response (intraperitoneal injection).

28.2.4 Colon Cancer

Colon cancer is the second most common cancer in the European Union, and at least 50% of patients develop recurrences or metastases during their illness due to aggressive behavior of this cancer type (Kuipers et al. 2013). Several preclinical studies have demonstrated high potential of alphavirus vectors in the development of colorectal cancer therapy. For instance, an alphaviral DNA vector encoding LacZ (pSIN1.5-β-gal) was compared to a conventional CMV promoter-based β-gal plasmid in CT26.CL25 tumors (Leitner et al. 2000). It was shown that intramuscular immunization with plasmid DNA replicons of mice with preexisting tumors elicited immune responses at doses 100–1000-fold lower than when performed with conventional DNA plasmids. Mice bearing experimental tumors expressing the β-gal reporter antigen were effectively treated and resulted in significant prolongation of survival rates.

Several alphavirus vectors have been engineered to express different cytokines to enhance antitumoral immune responses against colon cancer. A single intratumoral injection of 10^7 and 10^8 VLP of SFV-IL-12 resulted in a complete tumor regression in 36% and 80%, respectively, in a mouse colon adenocarcinoma model (Rodriguez-Madoz et al. 2005). Moreover, application of the modified SFV vectors with a natural capsid translation enhancer significantly increased IL-12 expression and tumor regression in treated mice. Six doses of high titer SFV10-E VLPs expressing IL-12 induced complete regression in all colon carcinoma tumor–bearing mice (CT26 model). During the treatment stage, tumor swelling occurred in relation to intratumoral necrosis and inflammation (Chikkanna-Gowda et al. 2005). In a similar study, colon carcinoma tumor–bearing mice were treated with six inoculations of high titer SFV10-E VLP expressing the murine IL-18 gene along with an Ig-kappa leader sequence (Chikkanna-Gowda et al. 2006). Although the growth of treated tumors was delayed, complete tumor regression was achieved only in 33% of treated mice, where the induction of avascular and suppurative necrosis was observed.

In order to investigate the potential of angiogenesis inhibition in primary colon carcinoma, mice were immunized with SFV10-E VLP expressing VEGFR-2 10 days prior to tumor cell injection (Lyons et al. 2007). Similar to 4T1 mouse breast carcinoma, the growth of CT26 colon carcinoma was inhibited in vaccinated mice. Microvessel density analysis showed that immunization with SFV-VEGFR-2 VLPs led to a significant inhibition of tumor angiogenesis. Moreover, coimmunization of mice with SFV VLPs encoding VEGFR-2 and IL-4 led to enhancement of mice survival and production of high titers of anti-VEGFR-2 antibodies in contrast to coimmunization with VEGFR-2 and IL-12, or VEGFR-2 alone.

The therapeutic potential of alphaviruses as oncolytic agents has also been studied. The virulent SFV4 strain and its derivative recombinant SFV-p62-6k vector, containing deletions of the capsid and E1 genes, were used to stimulate immunity in a CT26 model (Smyth et al. 2005). Direct intratumoral injection of replication-deficient VLPs or virulent SFV4 resulted in an immediate and intense inflammatory reaction and significant effect on survival. No differences were observed in inhibition of tumor growth between VLP- and SFV4-treated animals. However, the antitumor effect could be enhanced by preimmunization of animals with the VLP vector.

28.2.5 Ovarian Cancer

Ovarian cancer is the sixth most common malignancy in women. In advanced ovarian cancers, tumors spread throughout the peritoneal cavity and induce the production of ascites. Therefore, the high potential of systemic tumor targeting by SIN vectors is an important factor in treatment of this type of cancer. Efficacy of SIN vector application in ovarian cancer treatment has been evaluated using several strategies, such as immunotherapy, oncolytic virotherapy, and combined therapy with chemical agents. It has been shown that SIN VLP vectors have the ability to systemically and specifically target metastasized tumors within the peritoneal cavity, leading to significant suppression of tumor

growth in ovarian tumor–bearing xenograft models (Tseng et al. 2004b). However, incorporation of antitumor cytokine genes such as IL-12 and IL-15 genes significantly enhanced the efficacy of the vector (Tseng et al. 2004a; Granot et al. 2011). Additionally, using C.B-17-SCID beige mice with selective impairment of natural killer (NK) cell functions, and C.B-17-SCID ovarian tumor–bearing mice, it was demonstrated that anticancer efficacy of SIN vectors is largely NK cell dependent and depletion of these cells caused a significant decrease in the therapeutic potential (Granot et al. 2011; Granot and Meruelo 2012). Because of low efficacy of penetration in tumor vascular structures, the SIN vectors were not able to reach and kill all tumor cells to ensure complete tumor regression. To solve this problem, a chemotherapeutic drug was used. Due to the ability of paclitaxel to inhibit tumor angiogenesis at low concentration and increase blood vessel permeability, the combined treatment with paclitaxel (taxol) at a concentration of 16 mg/kg and intraperitoneally inoculated SIN-LacZ VLPs dramatically enhanced therapeutic effects (Tseng et al. 2010).

SFV replicon vectors were also utilized for ovarian cancer immunotherapy with granulocyte-macrophage colony-stimulating factor (GM-CSF), which is an important hematopoietic growth factor and immune modulator (Klimp et al. 2001). It was shown that intraperitoneal injection of SFV-GM-CSF VLPs in murine ovarian tumor models provided rise in the number of macrophages and neutrophils. It resulted in a modest tumor growth inhibition, but with no survival benefit.

The potential of the oncolytic SIN AR339 strain was evaluated for ovarian cancer treatment (Unno et al. 2005). Although the SIN AR339 is a replication-competent vector, this strain has not been reported to cause any serious human disease. Intraperitoneal vector administration in the human ovarian xenograft mouse model provided significant suppression of ascite formation, an important therapeutic outcome in the treatment of ovarian cancer. In another study, mouse ovarian carcinoma xenografts were treated with an oncolytic SFV vector, using a readministration strategy with the same or another (vaccinia) viral vector (Zhang et al. 2010). In contrast to reinoculations of the same virus, the heterologous vector administration led to remarkably increased oncolysis and generation of antitumor immunity that significantly prolonged the survival.

28.2.6 Cervical Cancer

Human papilloma virus (HPV) E6 and E7 oncogenes are promising targets for cervical cancer vaccine development. SFV VLPs expressing the HPV16 E6 and E7 as separate proteins were applied as prophylactic vaccines in a mouse model for cervical cancer based on TC-1 cells expressing HPV16 E6E7 (Daemen et al. 2000). Preimmunization with three injections of 10^4 pSFV-E6E7 VLPs induced HPV-specific CTL response in 50% of the mice, whereas three inoculation with an increased virus dose of 10^6 resulted in CTL response in all treated mice. Furthermore, immunization with the highest dose of 5×10^6 of SFV-E6E7 VLPs protected 40% of the mice from tumor challenges. To enhance oncogene production and improve the cellular immune responses against E6 and E7, a new vector encoding a fusion protein of E6 and E7 together with the SFV core translational enhancer (pSFV3enh-E6,7) was generated (Daemen et al. 2002). Immunizations with 5×10^6 SFV3-enhE6,7 VLPs protected four out of five mice from tumor development, and a second tumor challenge in tumor-free animals revealed complete long-term protection against tumor occurrence. In a further study, authors confirmed the high potential of the proposed SFV-enhE6,7 VLP vaccine by intravenous and intramuscular administrations (Daemen et al. 2004).

In addition to the promising prophylactic properties of the SFV3-enhE6,7 VLP vector, its therapeutic potential in tumor-bearing mice was also investigated (Daemen et al. 2003). Subcutaneous injections of the vector at the 2nd, 7th, and 14th day after TC-1 cell challenge resulted in rapid CTL response induction and efficient protection against fast-growing tumors (90%–100% of treated mice were protected even after a second tumor challenge). Importantly, the efficacy of using adenovirus Ad-E6,7 VLPs was dramatically lower (20%–40% of treated mice were protected) (Riezebos-Brilman et al. 2007). To improve the therapeutic efficacy, coadministration of SFV3-enhE6,7 VLPs and different doses of SFV-IL-12 VLPs was examined (Riezebos-Brilman et al. 2009). The results of coinoculation of both vectors depended significantly on the viral dose and injection schedule. Synergistic antitumor activity was observed only at a low dose of SFV-IL12. Furthermore, heterologous prime-boost immunization strategy was shown to provide advantages over single immunization (Walczak et al. 2011). Heterologous prime boost with SFV3-enhE6,7 VLPs and virosomes containing the E7 protein resulted in higher numbers of antigen-specific CTL in mice than applying homologous protocols. Nevertheless, the high number of CTL initially primed by the heterologous protocols did not correlate with enhanced antitumor responses in vivo.

SIN self-replicating RNA vectors were developed to induce E7-specific immunity in a TC-1 mice model (Cheng et al. 2001). Intramuscular inoculation of RNA, encoding HPV E7 oncogene alone (SINrep5-E7), induced poor humoral and cellular immune responses and provided no protection against tumor challenge. However, another construct expressing E7 as a fusion with secretory Sig protein and lysosome-associated membrane protein-1 (LAMP-1) in the SINrep5-Sig/E7/LAMP-1 vector demonstrated E7-specific $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cell activity and increased in vivo antitumor effect. Addition of the LAMP-1 endosomal/lysosomal sorting signal to the E7 protein significantly enhanced the oncogene processing and presentation in vivo in the case of uptake of apoptotic cells by APCs at sites of vector RNA inoculation.

The efficacy of prophylactic and therapeutic vaccines based on SIN VLPs expressing both E7 and calreticulin (CRT), an ER Ca^{2+}-binding transporter participating in antigen processing and presentation with major histocompatibility complex (MHC) class I, was tested (Cheng et al. 2006).

The developed SINrep5-CRT/E7 VLP vector was able to generate antigen-specific immune responses, antiangiogenic effect, and a strong antitumor activity. Intramuscular vaccination with SINrep5-CRT/E7 VLPs 1 week prior to the challenge with TC-1 cells provided excellent protection of all treated animals. To determine the therapeutic potential of the vector in established tumors, both immunocompetent and nude mice were intramuscularly inoculated with SINrep5-CRT/E7 VLPs 2 days after tumor cell injection. Although this strategy did not provide complete tumor elimination as a prophylactic approach, it resulted in a significantly lower number of pulmonary tumor nodules in both mice groups.

Similar to other alphaviral vectors, VEE VLP-based vectors expressing E7 provided satisfactory results when used as a prophylactic vaccine. It was shown that two subcutaneous preimmunizations with VEE-E7 VLPs 2 weeks prior to cancer cell injection prevented tumor formation in mice (Velders et al. 2001). Moreover, mice challenged 3 months after immunization with cancer cells did not develop tumors, indicating induction of long-term memory responses by the vector. In contrast, the therapeutic approach was efficient only in 67% of treated tumor-bearing mice. In other studies, the efficacy of the VEE vector was increased by expression of both E6 and E7 oncogenes from the same vector (Eiben et al. 2002; Cassetti et al. 2004). To test the HLA-restricted capabilities of the vaccine, an HPV tumor model was established on the basis of HLA-A*0201 transgenic mice. In this case, preimmunization with VEE-E6E7 VLPs protected 100% of immune-competent and HLA-A*0201 transgenic mice from tumor development and induced specific T cell immune response against HLA-A*0201-restricted HPV16 epitopes. As a therapeutic vaccine, VEE VLPs were inoculated after a tumor challenge. Although the therapy did not completely eradicate tumors, approximately 90% of immune competent and transgenic mice demonstrated elimination of established tumors.

The anticancer potential of the oncolytic replication-competent SIN virus AR339 strain was also explored using different human cervical cancer xenografts in mice (C33A) (Unno et al. 2005). Therapeutic treatment by intratumoral or intravenous injection of the vector resulted in remarkable regression of tumor growth through induction of necrosis.

28.2.7 Prostate Cancer

Alphavirus-based gene therapy represents an attractive strategy for noninvasive treatment of prostate cancer, where current clinical interventions show limited efficacy. The first promising results in preclinical trials were obtained using apoptosis-resistant tumor models. Immunodeficient mouse models with established rat prostate tumors, overexpressing the *Bcl-2* oncogene, were treated by intratumoral injections of SFV VLPs encoding the proapoptotic gene *Bax*, which plays a key role in programmed cell death (Murphy et al. 2001). Expression of the *Bax* gene by the SFV1 vector enhanced its cytopathic potential and led to a remarkable 47% reduction in tumor volume compared to the control. However, complete regression was not achieved in this study.

One alternative strategy for prostate cancer treatment is immune system stimulation against specific prostate cancer antigens, like the prostate-specific membrane antigen (PSMA), the six transmembrane epithelial antigen of the prostate (STEAP), and the prostate stem cell antigen (PSCA) (Naz and Shiley 2012). PSMA is a highly restricted prostate cell surface antigen. The VEE VLP vector, producing human PSMA, has demonstrated strong cellular and humoral immunities in mice upon subcutaneous inoculation (Durso et al. 2007). Although additional preclinical studies were not conducted, due to the absence of relevant PSMA tumor challenge models, the efficacy of VEE-PSMA VLP was studied in clinical trials (see Section 28.3).

The STEAP antigen is also an attractive target for immunotherapy, because it is predominantly expressed in prostate tissue, and is found to be upregulated in multiple cancer cell lines (Gomes et al. 2012). The potential of VEE VLPs expressing mouse STEAP was assessed in the context of prophylactic and therapeutic approaches (Garcia-Hernandez et al. 2007). Mice preimmunized with VEE-STEAP VLPs showed a specific induced immune response and significantly prolonged overall survival of TRAMPC-2 prostate tumor–bearing mice. The therapeutic effect of the VEE vector was tested by coadministration with the STEAP plasmid DNA vaccine, demonstrating short, but statistically significant delay in tumor growth. More beneficial results were obtained using a PSCA as a target, which *is* upregulated in a large proportion of localized and metastatic prostate cancers. Prophylactic vaccination of transgenic (TRAMP) mice with the PSCA-cDNA plasmid followed by VEE-PSCA VLP inoculation generated a specific immune response and antitumor protection in 90% of TRAMP mice.

28.2.8 Brain Cancer

In preclinical studies, intracranial injection of B16 mouse melanoma cells was applied to generate a mouse brain tumor model. This model was used to investigate the therapeutic potential of an SFV VLP vaccine–encoding mouse endostatin—a protein, possessing antiangiogenic properties (Yamanaka et al. 2001a). A significant reduction of intratumoral vascularization was observed in tumor sections after intratumoral injection of SFV-endostatin VLPs.

Another strategy of brain tumor therapy includes the immunization of mice with DCs isolated from bone marrow and transduced with SFV VLPs expressing cytokines or specific cDNAs from melanoma or glioma cells (Yamanaka et al. 2001b). It was shown that prevaccination with DCs transduced by the same type of cDNA as the tumor (SFV-mediated B16 complementary cDNA or SFV-mediated 203 glioma cDNA vectors for B16 and 203 glioma tumors, respectively) provided protection from tumor challenge. Moreover, therapeutic vaccination of brain tumor–bearing mice prolonged the overall survival.

Therapeutic immunization with DCs that have been pulsed with SFV IL-12 also significantly prolonged survival of B16 brain tumor–bearing mice (Yamanaka et al. 2002). A similar survival rate has been detected after stimulation of the immune system with DCs transduced with SFV-IL-12 VLPs in combination with systemically administrated IL-18 (Yamanaka et al. 2003). Interestingly, that combination of DCs pulsed with SFV-IL-12 and systemic inoculation of IL-18 has increased the survival rate.

Human melanoma–associated antigen gp100 is a melanocyte differentiation antigen, which has been also detected in multiple glioma cancer cell lines (Liu et al. 2004). It was shown that vaccination with a plasmid DNA-based SIN vector expressing human gp100 and murine IL-18 induced specific antitumor CTL immune responses and provided antitumor protection (Yamanaka and Xanthopoulos 2005). Three prophylactic immunizations with both pSIN-hgp100 and pSIN-IL-18 DNA resulted in prevention of the formation of B16-hgp100-transfected tumors. Therapeutic vaccination of mice with established B16-hgp100 tumors showed significant survival prolongation (90 days) with both vectors, where median mice survival treated with either pSIN-hgp100 or pSIN-IL-18 DNA was 24–28 days.

The antitumor capacity of the oncolytic replication-competent SFV VA7 vector was investigated in immunocompetent rat glioma-tumor models (Maatta et al. 2007). Neither intravenous nor intraperitoneal administration provided any positive therapeutic efficacy in glioma-bearing rats. In contrast, direct intratumoral injections of SFV VA7 led to a significant reduction of tumor growth. However, these beneficial results were followed by accelerated increase in tumor mass, leading to eventual death of the animals. Despite the promising results of oncolytic virotherapy in other cancer types described earlier, the SFV VA7 vector demonstrated insufficient efficacy in brain tumors in immunocompetent mice. Nevertheless, systemic inoculation of the SFV VA7 vector in nude mice caused complete subcutaneous brain tumor eradication while leaving healthy brain tissue unharmed (Heikkila et al. 2010). Furthermore, improved long-term survival was observed in 16 of a total of 17 animals.

28.2.9 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a liver cancer that has limited therapeutic options. In preclinical studies, woodchucks chronically infected with woodchuck hepatitis virus serve as a model for liver cancer therapy development. The therapeutic potential of SFV-E-IL12 VLPs was evaluated in woodchucks with hepatic tumors (Rodriguez-Madoz et al. 2009). The results indicated that a single intratumoral injection of vector provided partial, dose-dependent tumor regression in 58% of treated animals, leading to reduction in tumor volume of up to 70% 4 weeks after treatment. The promising therapeutic results were associated with a general activation of cellular immune responses against HCC. Nevertheless, tumor growth was restored thereafter. In a recent study, an L-PK/c-myc transgenic mice model was applied, providing spontaneous appearance of hepatic tumors with latency, histopathology, and genetic characteristics similar to human HCCs (Rodriguez-Madoz et al. 2014). Intratumoral inoculation of SFV-IL-12 induced growth arrest in most tumors, providing 100% survival rate.

28.2.10 Osteosarcoma

Osteosarcoma is the most common primary malignant bone tumor, which typically metastasizes into bones, lungs, and other soft tissues. The oncolytic SFV vector VA7 was tested as a virotherapy candidate against unresectable osteosarcoma. Subcutaneous human osteosarcoma nude mice xenografts were treated by three intratumoral injections of SFV VA7-EGFP (Ketola et al. 2008). Treatment with the oncolytic SFV was highly efficient, showing significant reduction of tumor size in comparison with the oncolytic adenoviral Ad5Δ24 vector. Additionally, a highly aggressive orthotopic osteosarcoma nude mouse model characterized by invasion to surrounding tissues, and emergence of hematogenous pulmonary metastases, was treated with VA7-EGFP. Intratumoral inoculations of oncolytic SFV significantly enhanced the survival rate in the orthotopic osteosarcoma model. However, none of the mice were eventually cured.

28.3 CLINICAL TRIALS

Following beneficial results of cancer treatment in preclinical trials, some therapeutic strategies have been evaluated in humans. The first phase I/II clinical study was performed using the SFV vector expressing the human IL-12 gene and encapsulated in cationic liposomes (LSFV–IL12) (Ren et al. 2003). To assess the biosafety and optimal dosage of the vector, LSFV–IL12 was intravenously administrated in cancer patients with stage III or IV metastasizing melanoma or renal cell carcinoma every third day for 4 weeks in two different concentrations. The therapy demonstrated no toxicity or any significant changes in the function of internal organs. However, therapeutic potential was indicated by a 10-fold increase in IL-12 concentration in the peripheral blood of treated patients. In another phase I/II study, repeated inoculations of VEE VLPs expressing the carcinoembryonic antigen (CEA) induced clinically relevant CEA-specific T cell and antibody responses due to the ability of alphaviruses to infect DCs (Morse et al. 2010). The study which included patients with advanced or metastatic cases of lung, colon, breast, appendix, or pancreatic cancers were pretreated with multiple courses of chemotherapy and received up to four injections of VEE-CEA VLPs. The majority of patients showed a dramatically low rate of clinical responses after the therapy. Regression of liver metastasis in one patient with pancreatic cancer was detected. Moreover, two patients with no evidence of disease remained in remission and two patients were able to maintain stable disease. One of the most recent alphavirus-based clinical trials targeted prostate cancer

TABLE 28.1
Prophylactic Anticancer Vaccines Based on Recombinant Alphavirus Vectors

Vector	Expressed Transgene	Tumor Model	Vaccination and Vector Administration Types	Vaccination Efficacy	References
SIN DNA/RNA layered vectors					
SIN DNA	TRP-1	Mouse melanoma B16	Prophylactic (i.m.)	Immunity Tumor prevention (60%–70%)	Leitner et al. (2003)
SIN DNA	MUC18	Mouse melanoma B16	Prophylactic (s.c.)	Ineffective	MC Leslie et al. (2007)
		Mouse melanoma B16 transduced with MUC18	Prophylactic (s.c.)	Immunity Tumor prevention (50%)	
SIN DNA	HER/neu	Mouse breast cancer A2L2	Prophylactic (i.m.), (i.d.)	Tumor prevention (80%) Reduction of metastasis Increase of survival	Lachman et al. (2001)
SIN DNA	HER/neu	Mouse breast cancer A2L2	Prophylactic (i.m.)	Tumor prevention (50%) Partial tumor reduction Reduction of metastasis	Wang et al. (2005)
SIN DNA	LacZ	Mouse colon cancer CT26.CL25, β-gal-expressing clone	Prophylactic (i.m.)	Complete tumor prevention (100%)	Leitner et al. (2000)
SIN DNA + SIN DNA	Gp100	Mouse brain cancer model B16	Prophylactic (i.m.)	Tumor prevention (40%)	Yamanaka et al. (2005)
	IL-18			Increase of survival	
SIN DNA	P1A	Mouse mastocytoma P815	Prophylactic (i.m.)	Tumor prevention (60%–70%)	Ni et al. (2004)
SIN RNA	Sig/E7/ LAMP-1	Mouse cervical cancer model TC1	Prophylactic (i.m.)	Immunity Reduction of metastasis	Cheng et al. (2001)
SIN viral particles vectors					
SIN VLP	CRT/E7	Mouse cervical cancer model TC1	Prophylactic (i.m.)	Complete tumor prevention (100%) Immunity	Cheng et al. (2006)
SIN VLP	P1A	Mouse mastocytoma P815	Prophylactic (i.p.)	Tumor prevention (80%)	Ni et al. (2004)
SFV and SFV-E viral particles vectors					
SFV VLP	HPV16 E6, E7	Mouse cervical cancer model TC1	Prophylactic (s.c.), (i.p.)	Tumor prevention (40%)	Daemen et al. (2000)
SFV-E VLP	HPV16 E6, E7	Mouse cervical cancer model TC1	Prophylactic (s.c.), (i.p.)	Complete tumor prevention (100%)	Daemen et al. (2002)
SFV10-E VLP	VEGFR-2	Mouse breast cancer 4T1	Prophylactic (s.c.)	Partial tumor reduction Reduction of metastasis	Lyons et al. (2007)
SFV-E VLP	VEGFR-2	Mouse colon cancer CT26	Prophylactic (s.c.)	Partial tumor reduction Increase of survival Angiogenesis inhibition	Lyons et al. (2007)
SFV-E VLP + IL-12	VEGFR-2	Mouse colon cancer CT26	Prophylactic (s.c.)	Inefficient	Lyons et al. (2007)
SFV-E VLP + IL-4	VEGFR-2	Mouse colon cancer CT26	Prophylactic (s.c.)	Immunity Increase of survival	Lyons et al. (2007)
SFV-E VLP	P1A	Mouse mastocytoma P815	Prophylactic (i.v.)	Immunity Tumor prevention (90%)	Colmenero et al. (1999)
SFV VLP + Ad VLP	P1A	Mouse mastocytoma P1.HTR3	Prophylactic (i.v.)	Increase of survival	Näslund et al. (2007)
VEE viral particles vectors					
VEE VLP	TRP-1	Mouse melanoma B16	Prophylactic (s.c.)	Immunity Partial tumor reduction	Goldberg et al. (2005)
VEE VLP	Gp100	Mouse melanoma B16	Prophylactic (s.c.)	Ineffective	Avogadri, et al. (2010)
VEE VLP	Tyr	Mouse melanoma B16	Prophylactic (s.c.)	Ineffective	Avogadri et al. (2010)
VEE VLP	TPR-2	Mouse melanoma B16	Prophylactic (s.c.)	Partial tumor reduction	Avogadri et al. (2010)
VEE VLP	HER/neu	Mouse breast cancer A2L2	Prophylactic (s.c.)	Complete tumor prevention (100%) Complete metastasis prevention (100%)	Wang et al. (2005)

(*Continued*)

TABLE 28.1 (*CONTINUED*)
Prophylactic Anticancer Vaccines Based on Recombinant Alphavirus Vectors

Vector	Expressed Transgene	Tumor Model	Vaccination and Vector Administration Types	Vaccination Efficacy	References
VEE VLP	E7	Mouse cervical cancer C3	Prophylactic (s.c.)	Complete tumor prevention (100%)	Velders et al. (2001)
VEE VLP	HPV16 E6, E7	Cervical cancer model HLF16	Prophylactic (s.c.)	Complete tumor prevention (100%)	Eiben et al. (2002)
VEE VLP	HPV16 E6, E7	Mouse cervical cancer C3, TC1	Prophylactic (s.c.)	Complete tumor prevention (100%)	Cassetti et al. (2004)
VEE VLP	STEAP	Mouse prostate cancer TRAMPC-2	Prophylactic (s.c.)	Immunity Increase of survival	Garcia-Hernandez et al. (2007)
VEE VLP + cDNA PSCA	PSCA	Mouse prostate cancer TRAMPC-2	Prophylactic (s.c.)	Tumor prevention (76%)	Garcia-Hernandez et al. (2008)
DCs + VEE VLP	HER/neu	Human breast cancer NT2	Prophylactic (s.c.)	Immunity Partial tumor reduction	Moran et al. (2007)

(Slovin et al. 2013). The immunotherapeutic efficacy of VEE VLPs carrying PSMA was evaluated for patients with castration resistant metastatic prostate cancer. The patients received a maximum of five subcutaneous injections in the deltoid region at two different vector concentrations. The vaccination did not cause adverse systemic or local toxicity and was generally well tolerated. However, the therapeutic effect of both immunization strategies was very low. No cellular immune response to PSMA was observed, and only a small number of patients demonstrated a humoral response to PSMA.

28.4 CONCLUDING REMARKS

The most interesting and promising applications of alphaviruses for cancer treatment are summarized in Tables 28.1 through 28.3. Alphavirus-based delivery platforms have numerous advantages, which render them attractive tools for immunotherapeutic vaccine and cancer therapy.

Safety: When the suicidal replication-deficient alphavirus particles are used, viral structural genes are not present and the infectious virus capable of infecting new target cells cannot generate virus progeny in immunized host cells. Particularly, the use of the second-generation helper vector (Berglund et al. 1993) and the split helper system (Smerdou and Liljeström 1999) prevents homologous recombination events and generation of replication-competent virus progeny. Moreover, RNA replicon–based vaccines are not prone to random integration into the host genome, thus avoiding the risk of cell transformation and development of tolerance or anti-DNA antibodies due to persistence, which present a limitation for the conventional DNA vaccines. Furthermore, the viral RNA is degraded within 5–7 days. Additionally, apoptosis induced by alphaviruses in transfected cells is another safety feature of vaccines based on suicidal alphavirus vectors.

No preexisting immunity: Alphaviruses generally possess no widespread immunity in the human and animal populations although some epidemics related to SFV, SIN, and VEE has been documented. This limitation observed for other viral expression systems does not prevent the use of alphaviruses for in vivo expression of heterologous genes.

Repeated administration: The viral structural genes are not intracellularly expressed. Therefore, alphavirus replicons can be repeatedly administered, since the host immune response to the vector itself does not cause rejection when subjected to booster immunizations, which has presented some serious limitations for other vector systems.

Stimulation of immune responses: Due to induction of apoptosis, gene expression is transient and lytic. The induced apoptosis assists in the uptake of transfected cells by DCs and, subsequently, facilitates activation and stimulation of these cells. Additionally, the viral double-stranded RNA molecules generated during alphavirus RNA replication provide an immunostimulatory effect on DCs and on innate immunity.

Application of alphavirus replicon systems induces broad and robust humoral and cellular immune responses to a wide array of tumor antigens and confers protection against tumor challenges as has been demonstrated in numerous studies employing several animal models. The demonstrated ability of alphavirus-based vaccines to break immunological tolerance to self-antigens is crucial for cancer therapy. Indeed, these vaccines have been found effective in cancer therapy models both in prophylactic and therapeutic settings. Plethora of successful preclinical studies already performed and future vector developments and improvements in vector delivery and targeting will contribute to widening the range of alphavirus vector applications and potentially paving the way for extremely versatile tools for future immunotherapy and cancer gene therapy.

TABLE 28.2
Therapeutic Anticancer Vaccines Based on Recombinant Alphaviral Vectors

Vector	Expressed Transgene	Tumor Model	Vaccination and Vector Administration Types	Vaccination Efficacy	References
SIN DNA/RNA layered vectors					
SIN DNA	HER/neu	Mouse breast cancer A2L2	Therapeutic (into the foot pad)	Ineffective	Eralp et al. (2004)
SIN DNA + DOX	HER/neu	Mouse breast cancer A2L2	Therapeutic (into the foot pad)	Partial tumor reduction	Eralp et al. (2004)
SIN DNA + PTX	HER/neu	Mouse breast cancer A2L2	Therapeutic (into the foot pad)	Ineffective	Eralp et al. (2004)
SIN DNA	HER/neu	Mouse breast cancer A2L2	Therapeutic (i.m.)	Ineffective	Wang et al. (2005)
SIN DNA + Ad vector	HER/neu	Mouse breast cancer A2L2	Therapeutic (i.m.)	Increase of survival	Wang et al. (2005)
SIN DNA	LacZ	Mouse colon cancer CT26.CL25	Therapeutic (i.m.)	Increase of survival	Leitner et al. (2000)
SIN DNA + SIN DNA	Gp100 IL-12	Mouse brain cancer model B16	Therapeutic (i.m.)	Increase of survival	Yamanaka et al. (2005)
SIN DNA	P1A	Mouse mastocytoma P815	Therapeutic (i.m.)	Tumor prevention (40%)	Ni et al. (2004)
SIN viral particles vectors					
SIN VLP	P1A	Mouse mastocytoma P815	Therapeutic (i.p.)	Tumor prevention (50%)	Ni et al. (2004)
SIN VLP	LacZ IL-12	Human ovarian cancer ES-2	Therapeutic (i.p.)	Partial tumor reduction	Tseng et al. (2004)
SIN VLP	IL-12 IL-15	Human ovarian cancer ES-2	Therapeutic (i.p.)	Partial tumor reduction	Tseng et al. (2004)
SIN VLP + paclitaxel	LacZ	Human ovarian cancer ES-2	Therapeutic (i.p)	Partial tumor reduction Increase of survival	Tseng et al. (2010)
SIN VLP	CRT/E7	Mouse cervical cancer model TC1	Therapeutic (i.m.)	Reduction of pulmonary nodules	Cheng et al. (2006)
SFV and SFV-E viral particles vectors					
SFV VLP	IL-12	Mouse melanoma B16	Therapeutic (i.t.)	Partial tumor reduction (70%–90%)	Asselin-Paturel et al. (1998)
SFV-VLP + CD137 mAb	IL-12	Mouse melanoma B16	Therapeutic (i.t.)	Complete tumor reduction (50%, 75%) Immunity	Quetglas et al. (2012)
SFV-IL-with CD137 mAb	IL-12	Mouse lung cancer TC1	Therapeutic (i.t.)	Partial tumor reduction	Quetglas et al. (2012)
SFV VLP	EGFP	Human lung cancer H358a	Therapeutic (i.t.)	Partial tumor reduction Complete tumor reduction (40%)	Murphy et al. (2000)
SFV VLP	GM-CSF	Mouse ovarian cancer MOT	Therapeutic (i.p.)	Partial tumor reduction Immunity	Klimp et al. (2001)
SFV VLP	BAX	Rat prostate cancer AT3	Therapeutic (i.t.)	Partial tumor reduction	Murphy et al. (2001)
SFV VLP	Endostatin	Mouse brain cancer model B16	Therapeutic (i.t.)	Partial tumor reduction Increase of survival	Yamanaka et al. (2001)
SFV-E VLP	IL-12	Woodchucks liver cancer WCH17	Therapeutic (i.t.)	Partial tumor reduction	Rodriguez-Madoz et al. (2009)
SFV-E VLP	P1A	Mouse mastocytoma P815	Therapeutic (i.t.), (peritumoral)	Partial tumor reduction Complete tumor reduction (46%)	Colmenero et al. (2002)
SFV VLP	IL-12	Mouse mastocytoma P815	Therapeutic (i.t.), (peritumoral)	Partial tumor reduction Complete tumor reduction (53%)	Colmenero et al. (2002)
SFV VLP	IL-12	Mouse colon cancer MC38	Therapeutic (i.t.)	Complete tumor reduction (80%)	Rodriguez-Madoz et al. (2005)
SFV-E VLP	IL-12	Mouse colon cancer MC38	Therapeutic (i.t.)	Complete tumor reduction (92%)	Rodriguez-Madoz et al. (2005)

(*Continued*)

TABLE 28.2 (*CONTINUED*)
Therapeutic Anticancer Vaccines Based on Recombinant Alphaviral Vectors

Vector	Expressed Transgene	Tumor Model	Vaccination and Vector Administration Types	Vaccination Efficacy	References
SFV-E VLP	IL-18	Mouse colon cancer CT26	Therapeutic (i.t.)	Partial tumor reduction Complete tumor reduction (33%)	Chikkanna-Gowda et al. (2006)
SFV10-E VLP	VEGFR-2	Mouse breast cancer 4T1	Therapeutic (i.t.)	Partial tumor reduction Reduction of metastasis	Lyons et al. (2007)
SFV-E VLP	HPV16 E6, E7	Mouse cervical cancer model TC1	Therapeutic (s.c.)	Complete tumor reduction (100%	Daemen et al. (2003)
SFV-E VLP + SFV VLP	HPV16 E6, E7 IL-21	Mouse cervical cancer model TC1	Therapeutic (s.c.)	Complete tumor reduction (28%)	Riezebos-Brilman et al. (2009)
VEE viral particles vectors					
VEE VLP	TPR-2	Mouse melanoma B16	Therapeutic (s.c.)	Immunity Reduction of metastasis	Avogadri et al. (2010)
VEE VLP	HER/neu	Rat breast cancer 13762 MAT B III	Therapeutic (s.c.), (i.m.)	Immunity Complete tumor reduction (50%)	Nelson et al. (2003)
VEE VLP	HER/neu	Rat breast cancer 13762 MAT B III	Therapeutic (s.c.)	Partial tumor reduction	Laust et al. (2007)
VEE VLP	E7	Mouse cervical cancer C3	Therapeutic (s.c.)	Partial tumor reduction	Velders et al. (2001)
VEE VLP	HPV16 E6, E7	Cervical cancer model HLF16	Therapeutic (s.c.)	Partial tumor reduction	Eiben et al. (2002)
VEE VLP	HPV16 E6, E7	Mouse cervical cancer C3 cell line	Therapeutic (s.c.)	Partial tumor reduction	Cassetti et al. (2004)
		HLF16 cell line	Therapeutic (s.c.)	Complete tumor reduction (90%–100%)	
VEE VLP + STEAP cDNA	STEAP	Mouse prostate cancer TRAMPC-2	Therapeutic (s.c.)	Partial tumor reduction	Garcia-Hernandez et al. (2007)

TABLE 28.3
Oncolytic Anticancer Vaccines Based on Replication-Competent Alphavirus

Vector	Expressed Transgene	Tumor Model	Vaccination and Vector Administration Type	Vaccination Efficacy	References
Sindbis virus					
SIN AR339	EGPF	Human ovarian cancer OMC-3	Therapeutic (i.p.)	Suppression of ascites formation	Unno et al. (2005)
SIN AR339	Nonspecified	Human cervical cancer HeLaS3 and C33A	Therapeutic (i.t.)	Partial tumor reduction Increase of survival	Unno (2005)
SIN AR339	Nonspecified	Human cervical cancer C33A cell line	Therapeutic (i.v.)	Partial tumor reduction	Unno et al. (2005)
SFV virus					
SFV VA7	EGFP	Human melanoma A2058	Therapeutic (i.v.), (i.p.), (i.t.)	Partial tumor reduction	Vähä-Koskela et al. (2006)
SFV VA7	EGFP	Human lung cancer A549	Therapeutic (i.t.) Therapeutic (i.v.) Therapeutic (i.p.)	Almost complete tumor reduction Partial tumor reduction Ineffective	Määttä et al. (2008)
SFV VA7	EGFP	Rat brain cancer BT4C	Therapeutic (i.v.), (i.p.) Therapeutic (i.t.)	Ineffective Partial tumor reduction	Maatta et al. (2007)
SFV VA7	EGFP	Human brain cancer U87	Therapeutic (i.v.)	Complete tumor reduction (95%)	Heikkilä et al. (2010)
SFV VA7	EGFP	Human osteosarcoma Saos2LM7	Therapeutic (i.t.)	Partial tumor reduction	Ketola et al. (2008)
SFV + VV	Non-specified	Mouse ovarian cancer MOSEC	Therapeutic (i.p.)	Increase of survival Immunity	Zhang et al. (2010)
SFV wild type	Non specified	Mouse fibrosarcoma WEHI-11	Prophylactic (i.p.)	Complete tumor reduction (80%)	Griffith et al. (1975)

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Supplementary 2

Poster I

Intratumoral delivery and biodistribution of oncolytic alphaviral vectors in mouse melanoma model.

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Intratumoral delivery and biodistribution of oncolytic alphaviral vectors in mouse melanoma model

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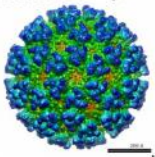
Recombinant vectors based on alphaviral replicon are promising tools in the field of oncolytic cancer gene therapy because of their strong cytopathic effect through the induction of p53-independent apoptosis.

Nevertheless, broad tissue tropism and transient expression make it more difficult to develop an optimal cancer treatment strategy. In this study we have investigated the intratumoral delivery and distribution of replication deficient **Semliki Forest virus** (recSFV) in B16 **melanoma-bearing mice** as a consequence of intraperitoneal (**i.p.**) and itratumoral (**i.t.**) vector administration strategies. For this purpose a group subcutaneous B16 tumor-bearing mice were i.p. or i.t. inoculated with 10^8 recSFV particles encoding **firefly luciferase**. The tissues homogenates (**heart, brain, liver, kidney, lung**) and **subcutaneous tumor** nodules were subjected for luciferase expression analysis at 24 h postinoculation. We observed a broad recSFV distribution in mice upon i.p. vector administration and the predominant tumor targeting of recSFV upon i.t. inoculation.

In order to investigate the vector distribution within subcutaneous tumor nodule, two vectors expressing green fluorescence protein (**EGFP**) and red fluorescence protein (**DS-Red**) were inoculated into different points of tumor nodule. The analysis of **tumor cryosections revealed only local expression** of the corresponding fluorescence protein without virus intratumoral dissemination. Our results show that the i.t. vector inoculation is **biosafe** approach for the development of therapeutic treatment. Nevertheless the additional strategies to enhance the vector dissemination within the tumor have to be developed in future. A detailed evaluation of vector distribution properties could have an impact on cancer therapy **clinical trial safety and efficacy**.

Semliki Forest virus (SFV)

The Semliki Forest virus was first isolated in the Semkili Forest (Central Africa) in 1942. SFV is transmitted to humans by mosquitoes and is able to cause a lethal encephalitis in rodents, but only one lethal human infection has been reported.



Mancini *et al*, 2000

SFV vector advantages

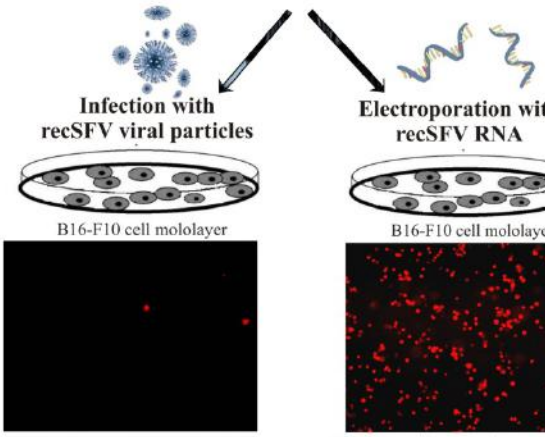
- High transgene production
- High vector capacity
- Biosafety
- Induction of 53-independet apoptosis

SFV application:

- Recombinant protein production
- Gene therapy vector
- Oncolytic cancer treatment
- Vaccine development

Virus classification: (+) ssRNA
Family: Togaviridae
Genus: Alphavirus

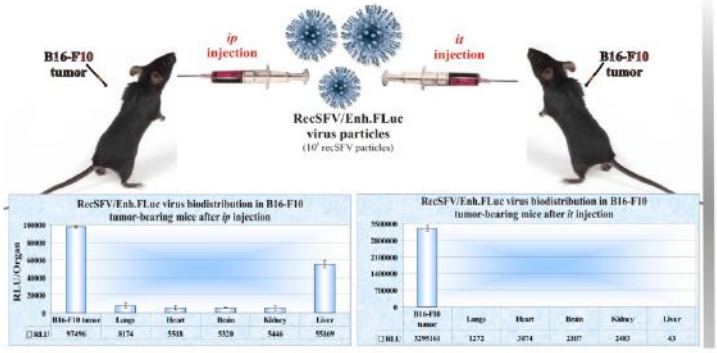
B16-F10 mouse melanoma treatment with recSFV vector *in vitro*



B16-F10 cells were infected with 10^8 recSFV/Ds-Red virus. Infection time 1h. Positive cells were visualized by fluorescent microscopy 24h post infection.

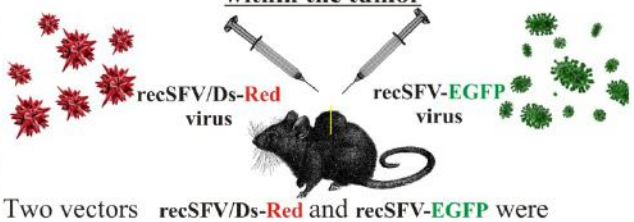
B16-F10 cells were electroporated with 50μg recSFV RNA (electroporation parameters: 850V; 25μF). Positive cells were visualized by fluorescent microscopy 24h post infection.

RecSFV virus biodistribution in melanoma B16-F10 tumor-bearing mice

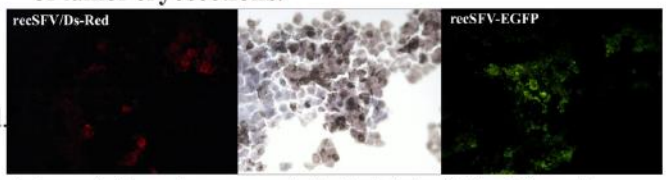


Luciferase transgene expression was analysed 24h after mice inoculation. The diagrams present the RLUs per total mg protein in each organ or tumor. RLU - relative light units.

RecSFV virus distribution inB16-F10 within the tumor



Two vectors **recSFV/Ds-Red** and **recSFV-EGFP** were inoculated into different points of tumor nodule on the left and right side correspondingly. The vector distribution was analyzed by fluorescence microscopy of tumor **cryosections**.



Conclusions:

1. Only local thransgene expression without intratumoral vector dissemination upon i.t. SFV inoculation was observed.
2. The high potential of naked SFV recRNA as a biosafe approach for the development of therapeutic treatment was demonstrated.

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