

Novel developments for applications of alphavirus vectors in gene therapy

Review Article

Kenneth Lundstrom

F. Hoffmann-La Roche, Research Laboratories, CH-4070 Basel, Switzerland

Correspondence: Kenneth Lundstrom, Ph.D. Tel: 41-61-687 8653, Fax: 41-61-688 4575, E-mail: Kenneth.Lundstrom@Roche.com

Abbreviations: SFV, Semliki Forest virus; CTL, cytotoxic T-cell; GFP, green fluorescence protein; GPCRs, G-protein coupled receptors

Key words: Semliki Forest virus, gene therapy, vaccine, LacZ, cell targeting, gene expression

Received: 19 January 2001; **accepted:** 23 January 2001

Summary

As a prerequisite for gene therapy applications, alphavirus-mediated delivery of reporter genes to different brain regions in rodents has resulted in local, high-level expression of a transient nature. Infection of human prostate tumor cell lines with recombinant Semliki Forest virus (SFV)-LacZ particles demonstrated a strong induction of apoptosis that led to premature cell death. Injection of self-replicative SFV-LacZ RNA showed in prophylactic and therapeutic effects in animals. Furthermore, injection of SFV vectors expressing interleukin-12 resulted in tumor regression in a mouse B16 melanoma tumor model. Similarly, injection of SFV vectors expressing GFP, -galactosidase or even empty SFV vectors led to p53-independent induction of apoptosis in nude mice with implanted human lung carcinomas. Repeated injections showed improved anti-tumor responses without any visible immune reaction against injected SFV particles. The envelope structure of alphaviruses has been modified to allow cell/tissue specific targeting. Moreover, SFV vectors have been used for the production of retrovirus-like particles. Extensive development on alphavirus vectors has resulted in novel non-cytopathogenic and replication-persistent forms. Overall, alphavirus vectors can be considered highly attractive for future gene therapy applications.

I. Introduction

Viral vectors have proven to be powerful for efficient gene delivery both *in vitro* and *in vivo*. At present there are many efficient viral vector systems described including retroviruses, adenoviruses, AAV (adeno-associated virus), herpes simplex virus and lentivirus. One group of viruses that recently has received increased attention is alphaviruses. The three most common alphaviruses, of which expression vectors have been developed are Semliki Forest virus (SFV) (Liljeström and Garoff, 1991), Sindbis virus (Xiong et al., 1989) and Venezuelan Equine Encephalitis virus (VEE) Davis et al., 1989). The expression vector systems are generally based on three modifications of the alphavirus genome (**Fig. 1**). I. Replication-competent vectors, where in addition to the full-length genome a second subgenomic promoter is engineered to express the foreign gene of interest. II. Replication-deficient vectors, where a helper vector is required for the expression of the viral structural proteins.

III. Layered DNA vectors, where an RNA polymerase II expression cassette drives the transcription of a self-replicative RNA vector (replicon) (Berglund et al., 1996, Dubensky et al., 1996). All three vector systems have their special features and their advantages as well as their disadvantages for different applications. The replication-competent vectors have the greatest potential of infecting a large population of cells in a tissue/organism due to their capability to produce progeny virus. However, these generated particles can pose a safety risk when used *in vivo* if efficient cell/tissue-specific targeting is not developed. The replication-deficient vectors represent a higher safety level because no further virus production occurs from them. However, the gene delivery efficiency is impaired especially in larger tissue sections. On the other hand, the expression levels obtained are extremely high, which can contribute to better by-stander effects seen in neighboring non-infected cells. Finally, the layered DNA vectors are the safest gene delivery vehicles since no viral particles are present at any stage. Obviously, their

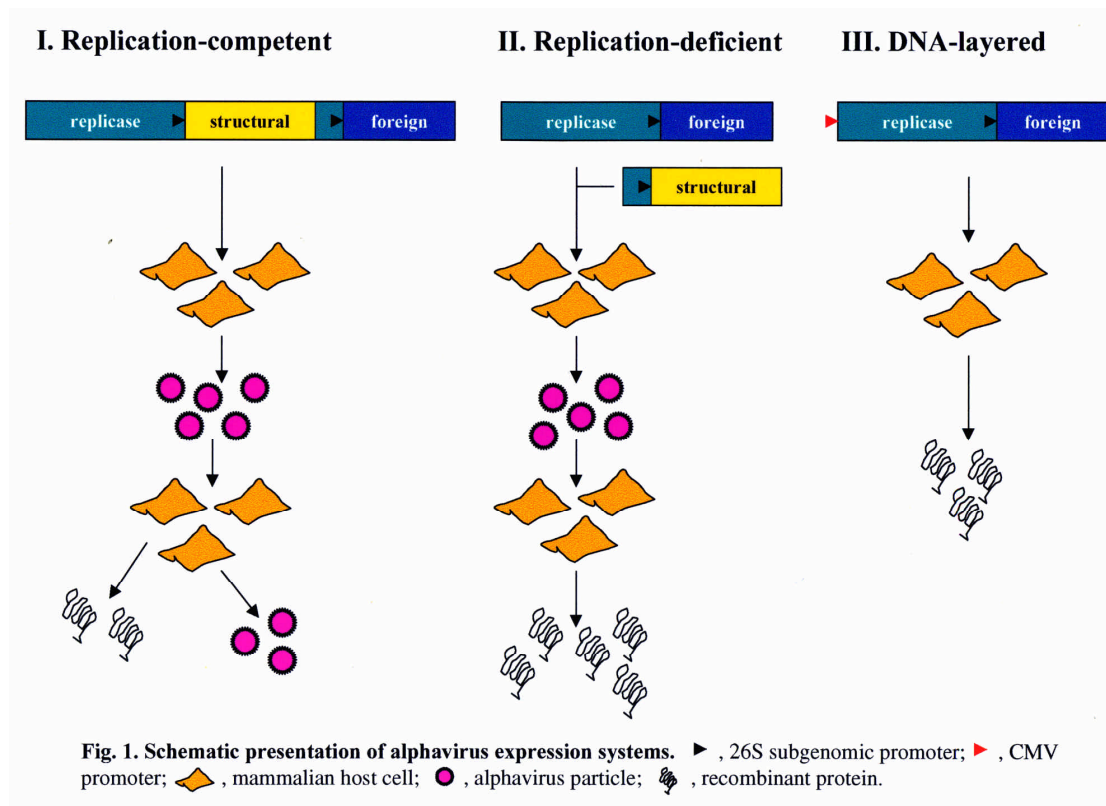
main disadvantage is the poor gene delivery efficiency common to all plasmid vectors.

In this review are highlighted the use of alphavirus vectors for expression of recombinant proteins as well as *in vivo* expression studies in rodents. The efficiency of gene delivery to tumor cells and the efficacy of intratumoral injections into animals with implanted tumors are described. Furthermore, alphavirus vectors can be employed for the production of retrovirus-like particles in a simple and efficient way. Alphavirus particles, self-replicating RNA molecules as well as layered DNA vectors have been used for vaccine approaches that can be applied to both prophylactic and therapeutic cancer gene therapy. Emphasis is also put on the development of novel alphavirus vectors for cell/tissue-specific targeting, for lower toxic effects on host cells and prolonged expression time.

II. Gene expression *in vitro*

Topologically different proteins (nuclear, cytoplasmic, membrane and secreted proteins) have been expressed at high levels from SFV vectors in a variety of cell lines and primary cell cultures (Lundstrom, 1999). Particularly G protein-coupled receptors (GPCRs) and ligand-gated ion channels have been expressed at high densities in mammalian host cells. Saturation binding

studies indicated that more than 100 pmol receptor per mg protein and receptor densities of more than 6 million receptors per cell were achieved. Functional coupling of GPCRs to G-proteins could be demonstrated by measuring intracellular Ca^{2+} -release, inositol phosphate accumulation, cAMP stimulation and GTP S binding. However, there are some potential disadvantages of using SFV vectors for functional studies. SFV infection inhibits the host cell protein synthesis, which will deprive the cells from endogenous G-proteins and therefore result in less efficient coupling. The other issue is the extreme receptor levels obtained, which in itself will lead to an inappropriate receptor / G-protein ratio for detection of strong functional responses. To overcome these problems, multiple infections with SFV vectors expressing both GPCRs (α_1 -adrenergic receptor) and G-proteins (G_q , G_{12} and G_{13}) were carried out in the same cells resulting in substantially increased functional responses in COS7 cells (Scheer et al., 1999). Establishment of large-scale SFV technology in suspension cultures of mammalian cells led to the production of high receptor yields (1-5 mg / l), which has allowed efficient receptor purification for structural studies (Hovius et al., 1998). This efficient gene expression and the possibility of simultaneous expression of several proteins in the same host cell should be a good basis for using alphavirus vectors for *in vivo* applications.



III. Gene expression *in vivo*

Both Sindbis and SFV vectors have been used for efficient gene delivery to rodent brain *in vivo*. Stereotactic injections of replication-deficient Sindbis virus carrying the LacZ gene into mouse *nucleus caudatus/putamen* and *nucleus accumbens septi* resulted in high transient β -galactosidase expression (Altman-Hamandzic et al., 1997). Similar observations were made after injection of SFV-LacZ particles into the *striatum* and *amygdala* of male Wistar rats (Lundstrom et al., 1999a). The injected animals were monitored for general health (food intake, body weight, body temperature), sensorimotor function, muscle strength and exploratory behavior and compared to control animals injected with cell culture medium. No significant differences were found between the two groups. High, local expression of β -galactosidase was observed at 1-2 days post-injection. The transient nature of expression from replication-deficient SFV vectors became evident from time-course studies that showed a decrease in β -galactosidase levels at 4 days post-injection. Some staining was still detectable at 28 days post-injection, most likely due to the high stability of β -galactosidase. *In situ* hybridization data also confirmed the transient nature of expression. The SFV-mediated transgene expression *in vivo* was remarkably neuron-specific. Similar observations were obtained from primary rat hippocampal neurons cultured on a feeder layer of astrocytes, where SFV-mediated GFP expression was mainly detected in neurons

(> 90%) and in very few glial cells (< 5%) (**Fig. 2**). Likewise, injection of SFV-GFP vectors into organotypic hippocampal slices demonstrated that more than 90% of the GFP-positive cells were of neuronal origin (Ehrengruber et al., 1999).

IV. Alphavirus vectors in tumor cells and animal models

It is well documented that SFV vectors causes a strong induction of apoptosis after infection (Lundstrom et al., 1997). This feature could therefore make SFV vectors attractive for cancer gene therapy applications. It has been shown that SFV vectors can efficiently infect human prostate tumor cell lines and prostate duct epithelial cells *ex vivo* (Hardy et al., 2000). Furthermore, strong apoptosis was detected in cells infected with SFV-LacZ virus and led to premature cell death. To achieve even stronger responses in tumor cells, cytokine genes have been introduced into the SFV vector. Intratumoral injections of SFV-IL12 virus particles, expressing the p40 and p35 subunits of IL-12 from the same SFV vector, showed significant tumor regression and inhibition of tumor blood vessel formation in a mouse B16 melanoma model when monitored by Doppler ultrasonography (Asselin-Paturel et al., 1999). Moreover, multiple injections resulted in increased anti-tumor response, but interestingly no anti-viral immune reaction could be detected.

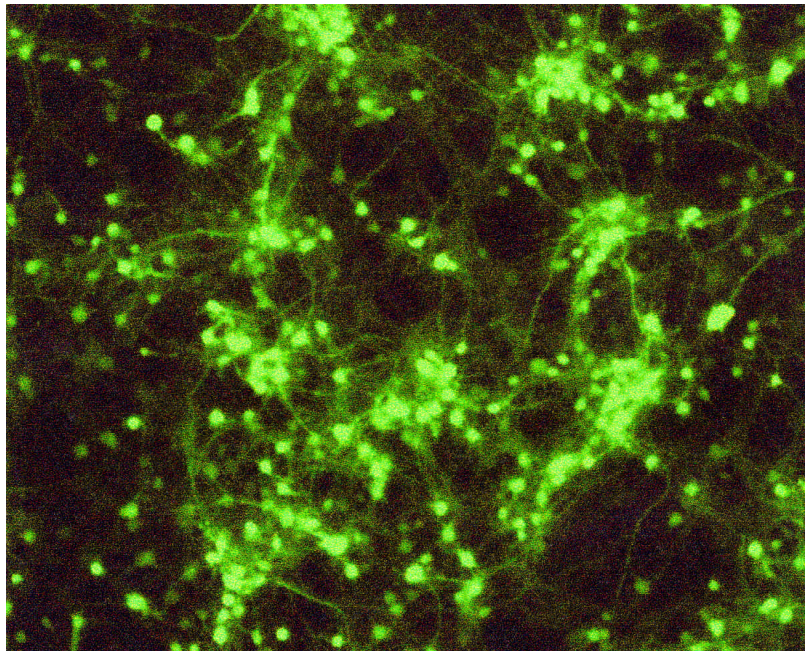


Figure 2. Expression of GFP in primary rat hippocampal neurons. Primary hippocampal neurons were infected with SFV-GFP at a multiplicity of infection (MOI) of 10 and visualized by fluorescence microscopy at 2 days post-infection.

In another study, nude mice with implanted human lung carcinomas were injected with various SFV vectors (Murphy et al., 2000). It could be concluded that intratumoral injections with SFV-LacZ, SFV-GFP or even empty SFV particles (containing only the SFV replicase genes) resulted in induction of p53-independent apoptosis and in significant tumor shrinkage. Again, repeated injections according to a scheme of 3 injections on consecutive days followed by another series of 3 injections one week later turned out to be beneficial. Most encouragingly, these repeated injections resulted in no antiviral responses in the treated animals.

V. Cell/tissue-specific targeting

To further increase the safety of using alphavirus vectors for gene therapy applications it would be of great advantage to be able to specifically target virus infections to specific cells / tissue. Sindbis virus (SIN) vectors with 10^5 -fold reduction in infection rates of normal host cells were obtained by introduction of IgG-binding domains of protein A into the E2 membrane protein (Ohno et al., 1997). Protein A-mediated infection occurred through specific monoclonal antibodies reacting with cell surface proteins. In another approach - and -hCG gene sequences were introduced into the Sindbis envelope, which led to no infection of BHK cells nor human cancer cells lacking LH/CG receptors (Sawai and Meruelo, 1998). In contrast, choriocarcinoma cells showed high infection rates. Targeting of SFV vectors has also been initiated by the generation of chimeric SFV particles with protein A domains in various regions of SFV E1 and E2 membrane proteins. Most of these chimeric SFV vectors were not capable of producing virus progeny due to incorrect folding of envelope structures (Lundstrom, unpublished results). However, EM studies confirmed that vectors with inserts close to the N-terminus of the E2 protein generated viable SFV particles. The infection rate of BHK cell was dramatically reduced and studies on infection through the protein A domains are now in progress. The advantage of using SFV compared to SIN vectors is the possibility to generate conditionally infectious particles with the second-generation pSFV-Helper2 vector (Berglund et al., 1993). These SFV particles are almost non-infectious (1 particle out of 10^5 are infectious) without -chymotrypsin treatment, which means that chimeric SFV particles will have a further 10^5 -fold down-regulation of infectivity.

VI. Production of retrovirus-like particles

The efficient recombinant protein expression from alphavirus vectors has encouraged to produce retrovirus-like particles by co-transfection of SFV vectors carrying the *gag-pol*, *env* and LTR-⁺-*neo*-LTR constructs from

Moloney murine leukemia virus (MMLV) into BHK cells (Li and Garoff, 1996). The advantage of this approach is that helper-free high-titer retrovirus-like particles possessing reverse-transcriptase activity could be rapidly produced, even when constructs contain intron sequences (Li and Garoff, 1998). In another approach, retrovirus virion RNA was cloned into the SFV expression vector. Introduction of *in vitro* transcribed full-length chimeric SFV-retrovirus RNA into retrovirus packaging cell lines by electroporation or SFV infection resulted in retrovirus-like particles that were capable of transducing target cells, showed reverse transcriptase activity and could integrate into the host cell genome (Wahlfors et al., 1997). Finally, in a hybrid application of virus targeting and retrovirus production, the SFV envelope protein genes were replaced by the *env* gene from murine leukemia virus (MuLV), which resulted in packaging of minimal virus particles with strong affinity to host cells carrying MuLV receptors (Lebedeva et al, 1997).

VII. Vaccine strategies

Traditionally application of vaccine strategies has been to use alphavirus particles, self-replicative naked RNA or layered DNA vectors for immunization of animals to obtain cytotoxic T cell (CTL) responses and protection against lethal challenges with virus (Lundstrom, 2001). In this sense, VEE has turned out to be a particularly efficient vector. Immunization with VEE particles expressing influenza HA resulted in complete protection against intranasal challenge with influenza virus in BALB/c mice (Caley et al., 1997). Likewise, macaques vaccinated with VEE vectors expressing the GP and NP structural proteins of Marburg virus remained aviremic and were completely protected from the disease (Hevey et al., 1998). The use of naked RNA or layered DNA vectors for vaccination has become attractive because of the simple and safe administration. The self-replicative alphavirus vectors have gained much popularity mainly because antigen-specific immune responses could be obtained at concentrations 1,000-fold lower than those for conventional plasmids (Hariharan et al., 1998; Berglund et al., 1998).

More closely related to gene therapy applications, vaccine strategies have also been initiated to induce tumor immunity. Expression of the P1A gene from recombinant SFV vectors resulted in induction of P185 tumor immunity (Colmenero et al., 1999). Injection of self-replicating SFV-LacZ RNA intramuscularly protected mice from tumor challenge and prolonged the survival time of mice with established tumors (Ying et al., 1999). Furthermore, immunization of mice with 5×10^6 SFV particles expressing the human papillomavirus early genes E6 and E7 protected 40% of the animals from cervical cancer challenge (Daemen et al., 2000).

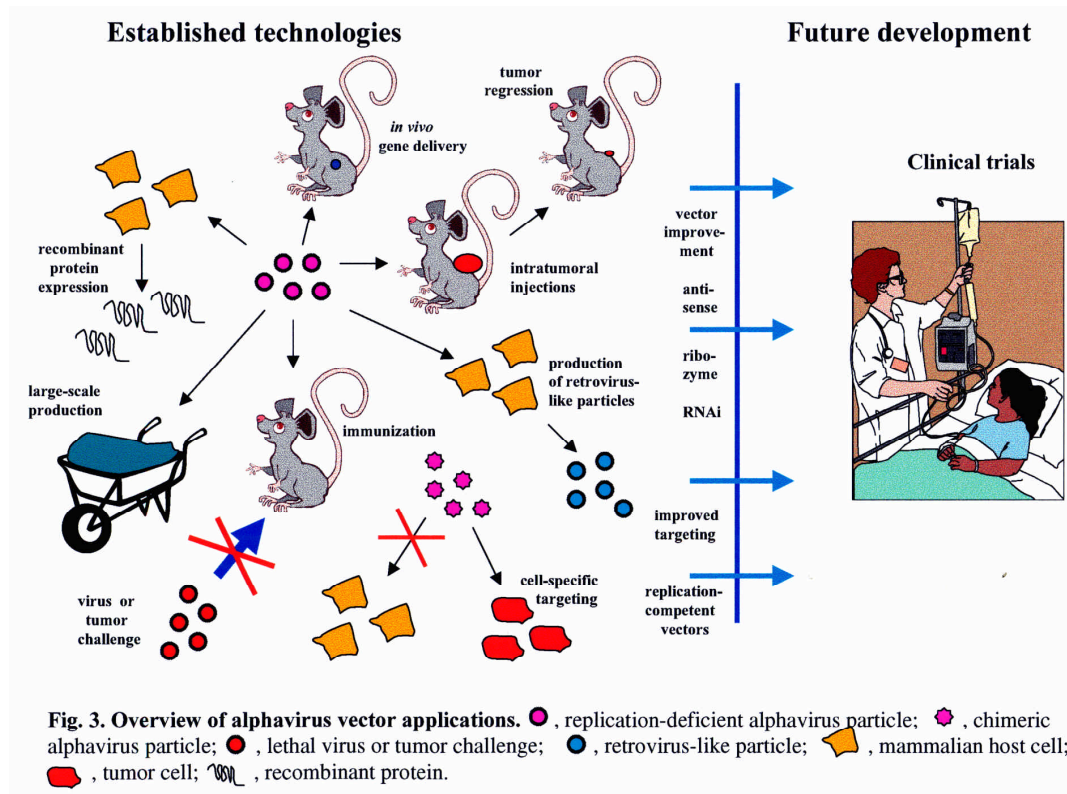
VIII. Vector development

Although the alphavirus vectors are rather efficient in relation to gene delivery and transgene expression efficiency, there are some disadvantages that need to be addressed. The vectors are highly toxic to the host cells and typically induce apoptosis. Additionally, shortly after alphavirus infection there is generally a dramatic shut down of endogenous gene expression, which will certainly contribute to the premature cell death. In the case of cancer gene therapy, this is necessarily not a negative feature, but for other applications it might be favorable to have a prolonged survival of the host cells. For this reason, novel non-cytopathogenic vectors have been developed for both Sindbis (Agapov et al., 1998) and SFV (Lundstrom et al., 1999b). In both cases it turned out that point mutations in the nonstructural gene nsP2 resulted in the non-cytopathogenic phenotype. The non-cytopathogenic Sindbis vector showed change in phenotype only in a limited number of host cells (BHK and Vero cells) and a significantly reduced RNA replication, whereas the SFV vector showed a substantially reduced cytotoxicity in all host cells tested (BHK, CHO, HEK293, HeLa cells) including primary neurons in culture. Moreover, the recombinant protein expression from the mutant SFV vector was increased by 5- to 10-fold.

Another drawback with applying alphavirus vectors has been their transient nature of gene expression. This has been partly due to the high cytotoxicity subjected to the host cells, but also the novel non-cytopathogenic alphavirus vectors clearly show only short-term expression mainly due to RNA degradation and termination of RNA replication. Recently, it was demonstrated that some point mutations or deletions in the nsP2 gene of both Sindbis and SFV generated vectors with persistent RNA replication that allowed a substantially prolonged transgene expression in host cell lines (Perri et al., 2000). Development of these novel vectors for long-term gene therapy applications could be very attractive.

IX. Conclusions and future prospects

As described in this review alphavirus vectors can be used as versatile tools for *in vitro* and *in vivo* gene expression studies (Fig 3). The rapid high-titer virus production, broad host range, cytoplasmic RNA replication and extreme overexpression of recombinant proteins are features that have made alphaviruses attractive. Today, a multitude of recombinant proteins have been successfully expressed from SFV vectors, not the least the highly important family of GPCRs. Moreover, techniques for *in vivo* gene delivery are well established.



Preliminary studies on tumor models in animal have resulted in promising regression in tumor size and the absence of immune responses against the virus after repeated injections have been most encouraging. The vaccine approach for cancer therapy with both therapeutic and prophylactic efficacy obtained is also very exciting. The proof of principle demonstrated for cell/tissue specific targeting, should also encourage further development. Novel non-cytopathogenic and replication persistent vectors will certainly increase the application possibilities of alphavirus vectors and should allow the use of antisense, ribozyme and RNA interference technologies as modes of cancer gene therapy. In general, it can be concluded that the wide range of applications of alphavirus vectors today should with further development make them highly attractive for clinical trials and gene therapy applications in the future.

References

- Agapov, E.V., Frolov, I., Lindenbach, B.D., Pragai, B.M., Schlesinger, S. and Rice, C.M. (1998) Noncytopathogenic Sindbis RNA vectors for heterologous gene expression. **Proc Natl Acad Sci USA** 95: 12989-12994.
- Altman-Hamandzic, S., Groceclose, C., Ma, J.-X., Hamandzic, D., Vrindavanam, N.S., Middaugh, L.D., Paratto, N.P. and Sallee, F.R. (1997) Expression of β -galactosidase in mouse brain: Utilization of a nonreplicative Sindbis virus vector as a neuronal gene delivery system. **Gene Ther** 4: 815-822.
- Asselin-Paturel, C., Lassau, N., Guinebreteire, J.-M., Zhang, J., Gay, F., Bex, F., Hallez, S., Leclere, J., Peronneau, P., Mami-Chouaib, F. and Chouaib, S. (1999) Transfer of the murine interleukin-12 gene in vivo by Semliki Forest virus vector induces B16 tumor regression through inhibition of tumor blood vessel formation monitored by Doppler ultrasonography. **Gene Ther** 6: 606-615.
- Berglund, P., Sjöberg, M., Garoff, H., Atkins, G.J., Sheahan, B.J. and Liljeström P. (1993) Semliki Forest virus expression system: production of conditionally infectious recombinant particles. **Bio/Technology** 11: 916-920.
- Berglund, P., Smerdou, C., Fleeton, M.N., Tubulekas, I. and Liljeström, P. (1998) Enhancing immune response using suicidal vaccines. **Nat Biotechnol** 16: 562-565.
- Berglund, P., Tubulekas, I. and Liljeström, P. (1996) Alphaviruses as vectors for gene delivery. **Trends Biotechnol** 14: 130-134.
- Caley, I.J., Betts, M.R., Irlbeck, D.M., Davis, N.L., Swannstrom, R., Frelinger, J.A. and Johnston, R.E. (1997) Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. **J Virol** 71: 3031-3038.
- Colmenero, P., Liljeström, P. and Jondal, M. (1999) Induction of P185 tumor immunity by recombinant Semliki Forest virus expressing the P1A gene. **Gene Ther** 6: 1728-1733.
- Daemen, T., Pries, F., Bungener, L., Kraak, M., Regts, J. and Wilschut, J. (2000) Genetic immunization against cervical carcinoma: induction of cytotoxic T lymphocyte activity with a recombinant alphavirus vector expressing human papillomavirus type 16 E6 and E7. **Gene Ther** 7: 1859-1866.
- Dubensky, T.W. Jr., Driver, D.A., Polo, J.M., Belli, B.A., Latham, E.M., Ibanez, C.E., Chada, S., Brumm, D., Banks, T.A., Mento, S.J., Jolly, D.J., Chang, S.M.W. (1996) Sindbis virus DNA-based expression vectors: Utility for in vitro and in vivo gene transfer. **J Virol** 70: 508-519.
- Ehrensgruber, M.U., Lundstrom, K., Schweitzer, C., Heuss, C., Schlesinger, S. and Gähwiler, B.H. (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. **Proc Natl Acad Sci USA** 96: 7041-7046.
- Hariharan, M.J., Driver, D.A., Townsend, K., Brumm, D., Polo, J.M., Belli, B.A., Catton, D.J., Hsu, D., Mittelstaedt, D., McCormack, J.E., Karavodin, L., Dubensky, T.W. Jr., Chang, S.M.W. and Banks, T.A. (1998) DNA immunization against herpes simplex virus: enhanced efficacy using Sindbis virus-based vector. **J Virol** 72: 950-958.
- Hardy, P.A., Mazzini, M.J., Schweitzer, C., Lundstrom, K. and Glode, L.M. (2000) Recombinant Semliki Forest virus infects and kills human prostate cancer cell lines and prostatic duct epithelial cells *ex vivo*. **Int J Mol Med** 5: 241-245.
- Hevey, M., Negley, D., Pushko, P., Smith, J. and Schmaljohn, A. (1998) Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. **Virology** 251: 28-37.
- Hovius, R., Tairi, A.-P., Blasey, H., Bernard, A., Lundstrom, K. and Vogel, H. (1998) Characterization of a mouse 5-HT₃ receptor purified from mammalian cells. **J Neurochem** 70: 824-834.
- Lebedeva, I., Fujita, K., Nihrane, A. and Silver, J. (1997) Infectious particles derived from Semliki Forest virus vectors encoding murine leukemia virus envelope. **J Virol** 71: 7061-7067.
- Li, K.-J. and Garoff, H. (1996) Production of infectious recombinant Moloney murine leukemia virus particles in BHK cells using Semliki Forest virus-derived RNA expression vectors. **Proc Natl Acad Sci USA** 93: 11658-11663.
- Li, K.-J. and Garoff, H. (1998) Packaging of intron-containing genes into retrovirus vectors by alphavirus vectors. **Proc Natl Acad Sci USA** 95: 3650-3654.
- Liljeström, P. and Garoff, H. (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. **Bio/Technology** 9: 1356-1360.
- Lundstrom, K. (1999) Alphaviruses as tools in neurobiology and gene therapy. **J Receptor & Signal Transd Res** 19: 673-686.
- Lundstrom, K. (2001) Alphavirus vectors: applications for DNA vaccine production and gene expression. **Intervirology**, in press.
- Lundstrom, K., Pralong, W. and Martinou, J.-C. (1997) Anti-apoptotic effect of Bcl-2 overexpression in RIN cells infected with Semliki Forest virus. **Apoptosis** 2: 189-191.
- Lundstrom, K., Richards, J.G., Pink, J.R. and Jenck, F. (1999a) Efficient *in vivo* expression of a reporter gene in rat brain

- after injection of recombinant replication deficient Semliki Forest virus. **Gene Ther Mol Biol** 3: 15-23.
- Lundstrom, K., Schweitzer, C., Richards, J.G., Ehrenguber, M.U., Jenck, F. and Mülhardt, C. (1999b) Semliki Forest virus vectors for in vitro and in vivo applications. **Gene Ther Mol Biol** 4: 23-31.
- Murphy, A.-M., Morris-Downes, M.M., Sheahan, B.J. and Atkins, G.J. (2000) Inhibition of human lung carcinoma cell growth by apoptosis induction using Semliki Forest virus recombinant particles. **Gene Ther** 7: 1477-1482.
- Ohno, K., Sawai, K., Iijima, Y., Levin, B. and Meruelo, D. (1997) Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. **Nat Biotechnol** 15: 763-767.
- Perri, S., Driver, D.A., Gardner, J.P., Sherrill, S., Belli, B.A., Dubensky, T.W. Jr. and Polo, J.M. (2000) Replicon vectors derived from Sindbis virus and Semliki Forest virus that established persistent replication in host cells. **J Virol** 74: 9802-9807.
- Sawai, K. and Meruelo, D. (1998) Cells-specific transfection of choriocarcinoma cells by using Sindbis virus hCG expressing chimeric vector. **Biochem Biophys Res Comm** 248: 315-323.
- Scheer, A., Björklöf, K., Cotecchia, S. and Lundstrom, K. (1999) Expression of the α_1 -adrenergic receptor and G protein subunits in mammalian cell lines using the Semliki Forest virus expression system. **J Receptor & Signal Transd Res** 19: 369-378.
- Wahlfors, J., Xanthopoulos, K.G. and Morgan, R.A. (1997) Semliki Forest virus-mediated production of retroviral vector RNA in retroviral packaging cells. **Hum Gene Ther** 8: 2031-2041.
- Ying, H., Zaks, T.Z., Wang, R.-F., Irvine, K.R., Kammula, U.S., Marincola, F.M., Leitner, W.W. and Restifo, N.P. (1999) Cancer therapy using self-replicating RNA vaccine. **Nat Medic** 5: 823-827.