Dmitry Sergeevich Kravchenko et al /J. Pharm. Sci. & Res. Vol. 9(12), 2017, 2544-2548

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Lentiviral system for T-lymphocyte-based apoptin expression and internalization

Dmitry Sergeevich Kravchenko, Yuriy Nikolaevich Lezhnin,
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences
117997, Russian Federation, Moscow, GSP-7, Ulitsa Miklukho-Maklaya, 16/10
Engelhardt Institute of Molecular Biology, Russian Academy of Sciences 119991 Moscow, Russia

Anna Yuryevna Hristichenko, Anna Evgenyevna Ivanova, Stepan Petrovich Chumakov,
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences
117997, Russian Federation, Moscow, GSP-7, Ulitsa Miklukho-Maklaya, 16/10

Abstract

Apoptin, a small proline-rich protein represents a potential future anti-cancer therapeutic, due to its ability to induce tumor cell death without affecting normal primary cells. The presented study was aimed at the development of a T-lymphocyte-based lentiviral system of apoptin expression and internalization. Here we tried to select the most effective signal peptide, providing a high level of apoptin expression, and a transport peptide, which ensures efficient penetration of the oncotoxic protein into tumor cells. As a result, we developed an effective therapeutic approach, which leads to intracellular apoptin accumulation and selective elimination of cancer cells.

Keywords: Apoptin, Immunotherapy, Oncotoxic proteins, Cancer treatment, T-lymphocytes, Lentiviral expression system, HMMsp38, Gaussia luciferase signal sequence

INTRODUCTION

Traditional approaches for cancer treatment, including surgery, chemotherapy and radiotherapy have demonstrated very limited efficacy for patients with late-stage and refractory forms of cancer [1, 2]. An innovative method which has shown great promise in treatment of tumors least sensitive to standard medications, is immunotherapy, particularly adoptive cell transfer of cytotoxic T-lymphocytes (CTLs) [3, 4]. This technique relies on transplantation of immune cells capable of recognizing and selectively eliminating tumor cells. The specificity of adoptive cell transfer can be increased by genetic modification of infused cells, for example, by improvement of signal regions of chimeric antigen receptors [5-7]. Such genetic manipulations, however, often result in decrease of the cytotoxic potential of immune cells [8]. This could be circumvented by developing genetically modified cells which express proteins or peptides with anti-tumor activity.

One of the most promising oncotoxic proteins used in cancer treatment, is apoptin [9]. Apoptin is a protein encoded by an avian virus, which induces p53-dependent apoptosis in various tumor and transformed cell lines without affecting normal cells [10, 11]. The aim of our research was to construct an effective system for apoptin expression in cytotoxic T-lymphocytes with its subsequent internalization by tumor cells. That novel therapeutic approach, combining specificity of cancer cell detection by CTLs with high oncotoxicity of apoptin, may lead to efficient elimination of cancer cells regardless of their phenotypic profile.

METHODS

CD8+ T-cells isolation

Peripheral blood samples were obtained from healthy adult donors. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque (Sigma Aldrich, St Louis, MO, USA) density gradient centrifugation. CD8+ T-cell fraction was isolated using EasySep Human CD8+ Positive Selection Kit, according to the manufacturer's instructions.

Cell culture

CD8+ T-cells were cultured at 37°C and 5% CO2 in AIM-V serum-free medium, supplemented with IL-2 (5 ng/ml) и IL-7 ng/ml.

Adherently growing human embryonic kidney (HEK) cell lines HEK 293T and HEK 293 were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose (4.5 g/L) with 2 mM L-glutamine) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) (Thermo scientific, USA) and 1% (v/v) of a 10.000 U/mL penicillin and 10 mg/mL streptomycin stock solution (Paneco, Russia) at 37°C, 5% CO2.

RNA isolation and cDNA preparation

Total RNA was extracted from cells using TRIzol isolation reagent (Invitrogen, USA). The RNA was reverse transcribed using oligo-$(dT)^{20}$ by incubation with 200 U of SuperScript III reverse transcriptase at 50°C for 50 min in the presence of 25 mM MgCl2, 10x RT Buffer, 10 mM dithiothreitol (DTT), 40U of RNase OUT (RNase inhibitor) and 10 mM dNTP Mix.

Table 1. Restriction sites and primer sequences, used for vector construction

Gene name	Restriction sites	Primer sequences
Gaussia luciferase signal sequence combined with TAT-peptide sequence	*EcoRI - XbaI*	5'ATATATGAATTCATGGGCGTGAAGGTGCTGTTCGCCCTGATCTGCAT CGCCGTCGCCGAGGCGGAGTACGGCCGCAAGAAACGCCGCCAGCGCC GCCGCTCTAGAAGAGA 3' 5'ATATATCTAGAGCGGCGGCGCTGGCGGCGTTTCTTGCGGCCGTAGAT GCAGATCAGGGCGAACAGCACCTTCACGCCCAT 3'
HMMsp38 signal sequence combined with TAT-peptide sequence	*EcoRI - XbaI*	5'AATTCATGTGGTGGCGCCTGTGGTGGCTGCTGCTGCTGCT3' 5'CACACCATGGGCCACAGCAGCAGCAGCAGCAGCAGCAGCCACCAC AGGCGCCACCACATG3' 5'GCTGCTGCTGTGGCCCATGGTGTGGGCCCTCGAGTACGGC3' 5'CTAGAGCGGCGGCGCTGGCGGCGTTTCTTGCGGCCGTACTCGAGGG CC3' 5'CGCAAGAAACGCCGCCAGCGCCGCCGCTCTAGACATCTGCTGTTTCC T3'
Apoptin/VP3	*Xba - BamHI*	5'ATATATCTAGAAAGATGAACGCTCTCCAAGAAGAT3' 5'ATATAGAGGATCCTCATTAGTGATGGTGATGAT GATGCAGTCTTATACGCCTTTTTG 3'
EF1alpha promoter	*ClaI - XbaI*	5'ATATAGAGGATCCTCATTAGTGATGGTGATGAT GATGCAGTCTTATACGCCTTTTTG 3' 5'ATATAGAGGATCCTCATTAGTGATGGTGATGAT GATGCAGTCTTATACGCCTTTTTG 3'

Construction recombinant pL-CMV-Gluc-TAT-Apoptin-wpre vector

The lentiviral vector pL-CMV-MCS-wpre containing multiple cloning site was used as a basis for cloning of studied functional elements. The CMV promoter was replaced by EF1alpha, which was amplified from cDNA of HEK 293T cells by Tersus HS DNA Polymerase (Evrogen, Russia). HMMsp38 signal sequence, TAT-peptide sequence and Gaussia luciferase signal sequence were synthesized by Evrogen (Russia), and then were cloned into pL-CMV-MCS-wpre vector. Restriction sites and primers used for cloning and amplification are listed in the table 1 for all the sequences.

Lentiviral packaging and virus collection

Twenty-four hours before transfection HEK 293T cells growing at 70% confluency, were trypsinized and cell density was adjusted to 1.0×10^6 cells/mL with complete growth medium. Recombinant viral vector pL-CMV-Gluc-TAT-Apoptin-wpre and three packaging plasmids: pRSV-REV, pCMV-VSV-G and pCMV-GAG were co-transfected into HEK 293T cells using TurboFect reagent (Thermo Scientific, USA) according to the manufacturer's instructions. After 8 hours of incubation, cell culture medium was replaced with fresh DMEM medium supplemented with 2% FBS; 48 hours later the culture medium was collected and filtered through a 0.45-um membrane to remove any cellular debris. Then PEG 8000 was added and the supernatant was incubated on ice for 24 h. To obtain high virus titer, supernatant was centrifuged at 4000×g at 4 °C for 30 min and white precipitate was resuspended in 1 ml PBS.

Lentiviral transduction of human T-lymphocytes

T-cells were collected by centrifugation at 1200 rpm for 10 min at 25°C, 4 hours before the procedure. The cell pellet was resuspended in AIM-V serum-free medium and then placed in 6 cm Petri dishes at a ratio of $2x10^6$/ml. Lentiviral particles, carrying a sequence of ontotoxic apoptin protein fused with secretion and transduction signal sequences, were added to T-cells at a ratio of 10:1 respectively. After 4 h of transduction, the cells were collected by centrifugation at 1200 rpm for 10 min at 25°C and then were resuspended in AIM-V serum-free medium supplemented with IL-2 (5 ng/ml) и IL-7 ng/ml in 10 cm Petri dishes. After 96 hours of incubation the expression of the transgene sequence was analyzed by immunoblotting and RT-PCR.

SDS-PAGE electrophoresis and Immunoblot analysis

1 µg/ml Protease Inhibitor Cocktail Set III (Calbiochem, Billerica, MA, USA) was added to cell lysates. Protein concentrations were determined using Bradford protein assay (Fermentas, Thermo Scientific, Waltham, MA, USA). 20 ug of total protein per sample was subject to 10% or 6% SDS-PAGE. Separated proteins were transferred to PVDF (Polyvinylidene fluoride) membrane (Hybond GE Healthcare, USA). PVDF was blocked with PBS containing 3% BSA and incubated with primary monoclonal mouse antibodies for 1 h at room temperature, followed by three steps of washing by PBS containing 0.1 % Tween 20 and incubated with a peroxidase-labelled secondary antibodies. Peroxidase activity was visualized using femtoLUCENT luminescent substrate (GBiosciences, USA).

Immunofluorescence

Cells were split into six-well plates containing sterile coverslips. After 24 h, media was aspirated from the plates and cells were washed three times with PBS. Cells were fixed using 1 ml of 4% paraformaldehyde for 30 min with gentle shaking. After fixing, cells were washed three times with PBS and permeabilized with 1 ml 0.1% Triton X-100 for 10min. Cells were then washed three times with PBS and blocked with 1% BSA for 30 min. Coverslips were incubated with 100 µl 1 : 100 primary antibody (ab1936120) overnight at 4 °C. The following day, coverslips were washed three times with PBS and incubated

with 100 µl of secondary antibodies (DyLight 488 Donkey anti-rabbit IgG) for 45min in the dark. After incubation, coverslips were washed three times with PBS and stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) for 10 min. Slides were labelled and the coverslips were mounted and sealed with transparent nail varnish. Slides were analysed using ZOE Fluorescent Cell Imager (BioRad) microscope in the GFP detection channel.

Statistical analysis

Data were analyzed using the unpaired Student t test with the help of GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Selection of Signal Peptide

Lentiviral transduction of T-lymphocytes is the most effective method of genetic modification of human T-cells [12]. This approach provides successful insertion of a specific sequence into cell genome, stable level of gene expression over a long period of time, and a significantly higher efficiency of cell transformation relatively to methods of chemical transfection [13]. To compare efficiencies of different signal peptides, we constructed lentiviral vectors, containing sequences of two secretion signals, known for their efficacy: HMMsp38 - an artificial secretory peptide, characterized by a high efficacy index (D = 0.932) and the GLuc peptide - *Gaussia princeps* luciferase secretion signal, which has the highest efficiency among natural signals (D = 0.88) [14-16]. Both of constructed vectors: pL-CMV-HMMsp38-Apoptin and pL-CMV-Gaussia-Apoptin (shown in Figure 1) contained sequences of chosen signal peptides fused to the apoptin sequence.

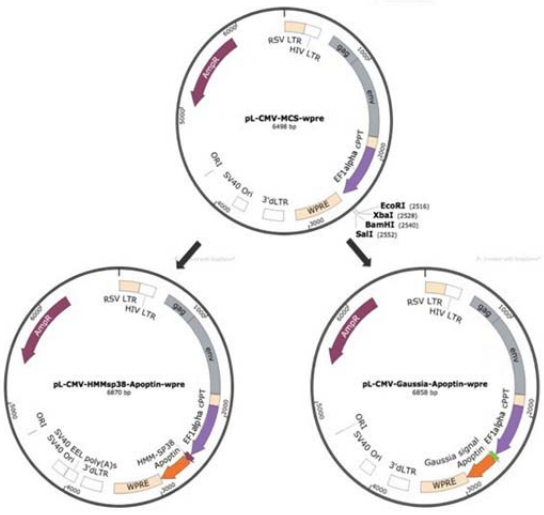

Figure 1. Construction of lentiviral vectors encoding sequences of extracellular localization signals Gaussia and HMMsp38

Created genetic constructs were successfully introduced in a population of T-cells with the help of lentiviral transduction. The efficiency of each studied secretion signal was compared by measuring the amount of the target protein in culture medium 36 hours after the transduction. Presence of the target protein was determined by ELISA (Figure 2A), PAGE-electrophoresis and Western blot analysis (Figure 2B).

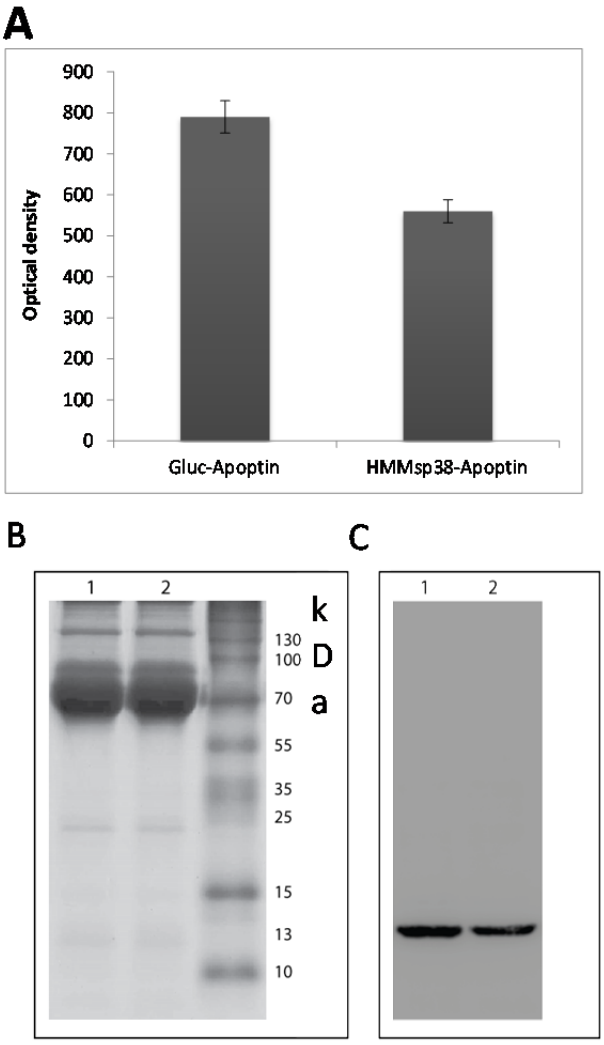

Figure 2. Expression and secretion analysis of recombinant forms of apoptin oncoprotein: HMMsp38-apoptin and GLuc-apoptin; A - the enzyme-linked immunosorbent analysis (ELISA) of T-cells culture medium; B - SDS-PAGE electrophoresis of the T-cells culture medium with a total protein concentration of 100 µg (lane 1 corresponds to the GLuc apoptin sample, lane 2 to HMMsp38 apoptin); C - Western blot analysis of media samples hybridization with anti-apoptin antibodies (lanes 1-2 correspond to the tracks in Figure B)

The results, shown in Figure 2, support the fact, that the *Gaussia princeps* luciferase secretion signal provides a higher level of apoptin expression compared to HMMsp38. This data determined the choice of the GLuc sequence for further use during engineering of the apoptin expression system.

Testing of transport peptide

The next step in creating the system for apoptin secretion and internalization was selection of the most efficient transport peptide. In most cases, these peptides are unique and are responsible for the transport of specific proteins [17]. However, there are also non-specific types of traffic signals, best known of them is the transducing transcription factor of the human immunodeficiency virus (TAT-HIV1). This non-specific transport peptide is used in

most experimental works, related to the transport of various protein structures to target cells [18, 19]. It is worth noting that TAT signaling factor was also used in the research of Guelen (2004) and Lee (2012), who described the possibility of using recombinant apoptin for clinical practice [20, 21]. Thereby, this transport peptide seemed to be an effective choice for the apoptin internalization system, and the sequence of TAT-HIV1 was cloned at the N-terminus of apoptin sequence after GLuc secretion signal (Figure 3),

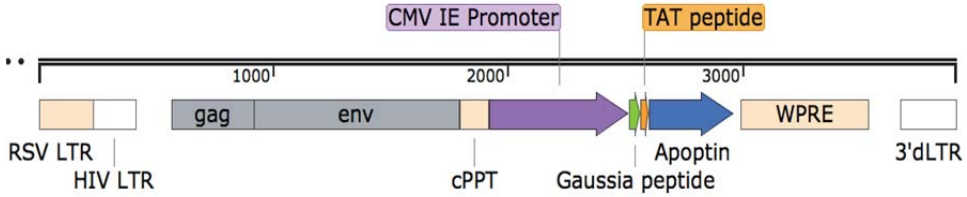

Figure 3. Schematic representation of the pL-CMV-Gluc-TAT-Apoptin-WPRE expression vector

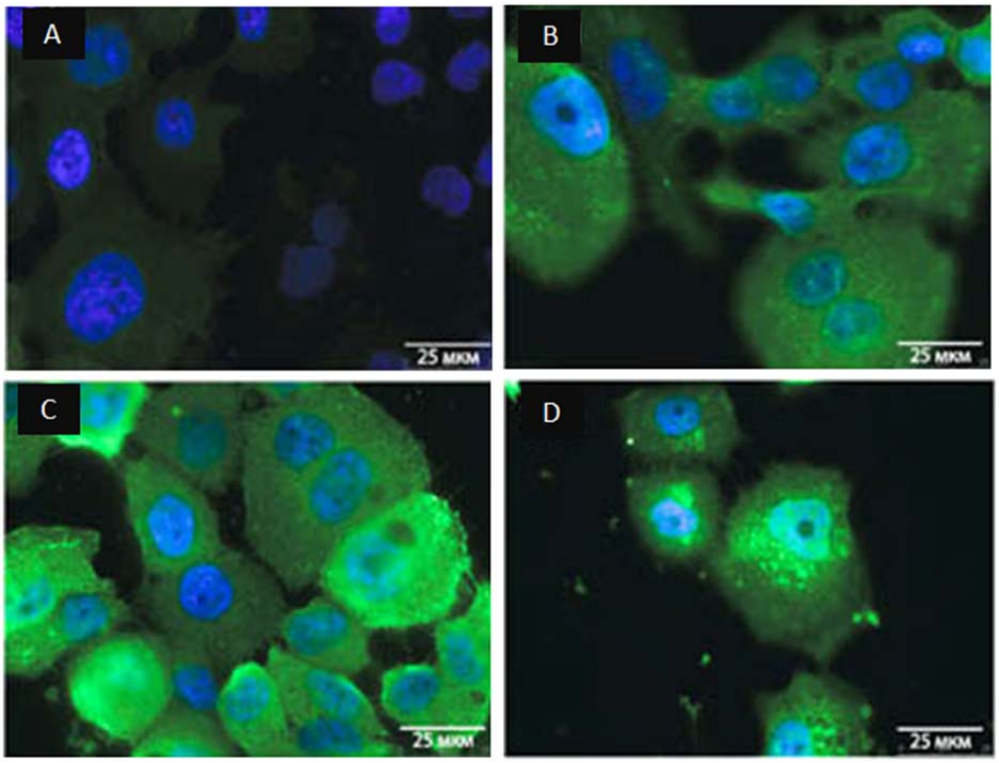

Figure 3. Immunofluorescence analysis of apoptin accumulation in MCF7 cells, treated with the Gluc-TAT-Apoptin-containing medium; photos were made after 12 (A), 24 (B), 48 (C) and 72 (D) hours of incubation.

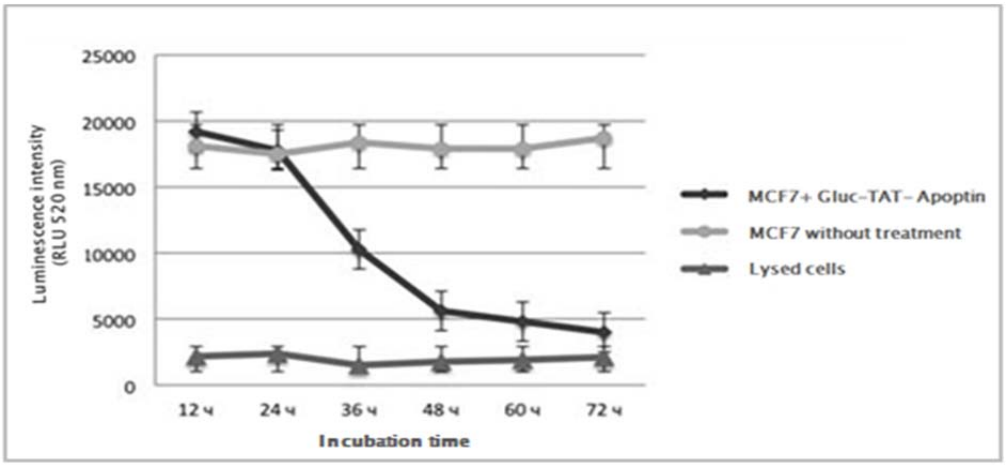

Figure 5. Analysis of MCF7 viability after treatment with the Gluc-TAT-Apoptin-containing medium; untreated cells were used as a positive control; negative control was treated with camptothecin. All measurements were carried out using a CellTiter Glo 2.0 reagent (Promega) and a microplate reader (BioRad).

In vitro analysis of cytotoxic potential of the apoptin intercellular delivery system

Constructed pL-CMV-Gluc-TAT-Apoptin-WPRE expression vector was used for lentiviral transduction of human T-lymphocytes. To estimate efficiency of the apoptin intercellular transport system, we studied an accumulation of the recombinant protein in MCF7 cell line. Twelve hours after transfection of the T-cells, the culture medium was collected and transferred to MCF7 cell culture; changing of culture medium was carried out 2 times per day. Every day after first medium replacement, one of the samples of MCF7 cells was stained and then analyzed by fluorescence microscopy (Figure 4).

According to the presented micrographs, the fluorescence intensity of MCF7 cells increases proportionally to incubation time, which indicates that the intercellular transport system based on Gluc and TAT signal peptides provides an efficient transport of recombinant apoptin from the producer cell line into target cancer cells.

To study the oncotoxic proporties of the apoptin internalization system, we performed an analysis of MCF7 viability after treatment with Gluc-TAT-Apoptin-containing medium; viability was measured with CellTiter Glo 2.0 kit every 12 hours after the beginning of cell treatment (Figure 5).

Results indicate that after 24 hours of incubation, amount of apoptin in treated cells reached a critical level, which leads to a sharp increase in apoptosis intensity. After 72 hours, luminescence level detected for treated cells was comparable to the signal for camptothecin-treated cells used as a negative control, which indicates high cytotoxic properties of apoptin.

CONCLUSION

Summarizing obtained results, we can conclude that developed system of apoptin expression and internalization provides an efficient elimination of cancer cells, which allows us to expect its further application in cancer therapy.

ACKNOWLEDGEMENTS

The work was funded by MESR, contract No. 14.607.21.0140 (RFMEFI60715X0140).

REFERENCES

[1] Ashdown M.L., Robinson A.P., Yatomi-Clarke S.L., Allison A., Abbott D., Markovic S.N., Coventry B.J. 2015. Chemotherapy for Late-Stage Cancer Patients: Meta-Analysis of Complete Response Rates. *F1000Res*. **4,** 232.

[2] Owonikoko T.K., Behera M., Chen Z., Bhimani C., Curran W.J., Khuri F.R., Ramalingam S.S. 2012. A systematic analysis of efficacy of second-line chemotherapy in sensitive and refractory small-cell lung cancer. *J Thorac Oncol*. **7,** 866-872.

[3] Wang M., Yin B., Wang H.Y., Wang R.F. 2014. Current advances in T-cell-based cancer immunotherapy. *Immunotherapy*. **6,** 1265-1278.

[4] Rosenberg S.A., Restifo N.P., Yang J.C., Morgan R.A., Dudley M.E. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer*. **8,** 299-308.

[5] Essand M.,Loskog A.S. 2013. Genetically engineered T cells for the treatment of cancer. *J Intern Med*. **273,** 166-181.

[6] Varela-Rohena A., Carpenito C., Perez E.E., Richardson M., Parry R.V., Milone M., Scholler J., Hao X., Mexas A., Carroll R.G., June C.H., Riley J.L. 2008. Genetic engineering of T cells for adoptive immunotherapy. *Immunol Res*. **42,** 166-181.

[7] Barrett D.M., Grupp S.A., June C.H. 2015. Chimeric Antigen Receptor- and TCR-Modified T Cells Enter Main Street and Wall Street. *J Immunol*. **195,** 755-761.

[8] Sharpe M.,Mount N. 2015. Genetically modified T cells in cancer therapy: opportunities and challenges. *Dis Model Mech*. **8,** 337-350.

[9] Backendorf C.,Noteborn M.H. 2014. Apoptin towards safe and efficient anticancer therapies. *Adv Exp Med Biol*. **818,** 39-59.

[10] Noteborn M.H., Zhang Y.H., van der Eb A.J. 1998. Apoptin specifically causes apoptosis in tumor cells and after UV-treatment in untransformed cells from cancer-prone individuals: a review. *Mutat Res*. **400,** 447-455.

[11] Los M., Panigrahi S., Rashedi I., Mandal S., Stetefeld J., Essmann F., Schulze-Osthoff K. 2009. Apoptin, a tumor-selective killer. *Biochim Biophys Acta*. **1793,** 1335-1342.

[12] Bilal M.Y., Vacaflores A., Houtman J.C. 2015. Optimization of methods for the genetic modification of human T cells. *Immunol Cell Biol*. **93,** 896-908.

[13] Frecha C., Levy C., Cosset F.L., Verhoeyen E. 2010. Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Mol Ther*. **18,** 1748-1757.

[14] Luft C., Freeman J., Elliott D., Al-Tamimi N., Kriston-Vizi J., Heintze J., Lindenschmidt I., Seed B., Ketteler R. 2014. Application of Gaussia luciferase in bicistronic and non-conventional secretion reporter constructs. *BMC Biochem*. **15,** 14.

[15] Stern B., Optun A., Liesenfeld M., Gey C., Grafe M., Pryme I.F. 2011. Enhanced protein synthesis and secretion using a rational signal-peptide library approach as a tailored tool. *BMC Proc*. **5 Suppl 8,** O13.

[16] Clerico E.M., Maki J.L., Gierasch L.M. 2008. Use of synthetic signal sequences to explore the protein export machinery. *Biopolymers*. **90,** 307-319.

[17] Yang N.J.,Hinner M.J. 2015. Getting across the cell membrane: an overview for small molecules, peptides, and proteins. *Methods Mol Biol*. **1266,** 29-53.

[18] Rizzuti M., Nizzardo M., Zanetta C., Ramirez A., Corti S. 2015. Therapeutic applications of the cell-penetrating HIV-1 Tat peptide. *Drug Discov Today*. **20,** 76-85.

[19] Vives E., Richard J.P., Rispal C., Lebleu B. 2003. TAT peptide internalization: seeking the mechanism of entry. *Curr Protein Pept Sci*. **4,** 125-132.

[20] Guelen L., Paterson H., Gaken J., Meyers M., Farzaneh F., Tavassoli M. 2004. TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene*. **23,** 1153-1165.

[21] Lee M.S., Sun F.C., Huang C.H., Lien Y.Y., Feng S.H., Lai G.H., Chao J., Chen H.J., Tzen J.T., Cheng H.Y. 2012. Efficient production of an engineered apoptin from chicken anemia virus in a recombinant E. coli for tumor therapeutic applications. *BMC Biotechnol*. **12,** 27.