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Research Article

Construction and Characterization of Recombinant HEK Cell Over Expressing α_4 Integrin

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Abstract

Purpose: Integrins are heterodimeric membrane proteins, which are exposed to post translational modifications in eukaryotic cells in contrast to prokaryotic cells. These modifications provide advantages for production of proper nanobody, mono and polyclonal antibody against this surface protein and also in aptamer selection process. Since the majority of diagnostic and therapeutic antibodies, target the surface epitopes, eukaryotic membrane proteins provide an appropriate model for further investigation on therapeutic agents.

Methods: Escherichia coli strain top 10, was used as host for ITGA-4 expression vector encoding the human integrin α_4. The plasmid was extracted and consequently, ITGA-4 vector was digested to make a linear plasmid. Human Embryonic Kidney-293 (HEK-293) cell transfected with linear plasmid and subsequently screened for stable ITGA-4 expressing Cells. Three separated clones were isolated twenty one days after transfection. Chromosomal DNA was extracted from ITGA-4-transfected cells. The presence of ITGA-4 gene in HEK-293 genome was confirmed by PCR. The expression level of ITGA-4 on HEK-293 cells was also analyzed by Flow cytometry.

Results: Flow cytometric analysis showed that HEK-293 cells have no expression of integrin α_4 on their surface while 95% of transfected HEK-293 cells with ITGA4, expressed different levels of integrin α_4 on their surfaces which correlates well with genomic DNA PCR amplification results.

Conclusions: The results suggest that we have successfully constructed the integrin α_4 expressing HEK293 cell, which will facilitate further research into the production of antibody, nanobody and aptamer against α_4 integrin.

Introduction

Integrins are heterodimeric membrane proteins, which play a crucial role in cell adhesion and signal transduction.[1] Integrins consist of an α and a β subunit in humans. 18 alpha and 8 beta subunits have been identified that form at least 24 different heterodimers through different combination of α and β subunits.[2]

Integrins mediate cell-cell and cell-Extra Cellular Matrix (ECM) adhesion, providing adhesion for adherent cells, drive many signalling pathways that regulate diverse processes including proliferation, migration, cell survival, differentiation, tumor invasion and metastasis.[3,4]

$\alpha_4\beta_1$ is a receptor for the immunoglobulin adhesion ligand, Vascular Cell Adhesion Molecule 1 (VCAM-1), and fibronectin which are expressed on endothelial cell and ECM respectively. $\alpha_4\beta_1$ integrin is expressed at moderate-to-high levels on almost all lymphocytes, monocytes and eosinophils.[5]

$\alpha_4\beta_1$ integrin-mediated cell-ECM adhesion, seems important for neural crest cell migration, cardiac development and blood vessel remodelling.[6-8]

$\alpha_4\beta_1$ integrin-mediated cell-cell adhesions, plays a key role in tumor angiogenesis through homing of both endothelial progenitor cells and monocytes to neovascular tissue. Moreover $\alpha_4\beta_1$ integrin is essential for inflammation process, through leukocytes attachment to vascular endothelial cells during extravasation.[9,10]

Binding of $\alpha_4\beta_1$ to VCAM-1, provides tumor angiogenesis and homing of hematopoietic stem and progenitor cells.[11]

Interaction of circulating leukocytes, with endothelium of the blood–brain barrier (BBB) and intestine is a critical step in pathogenesis of inflammatory diseases of the Central Nervous System (CNS) and Crohn's disease (CD). Previous studies, demonstrated T lymphocyte interaction, with the vascular endothelial cells of brain and intestine through α_4 integrin binding to VCAM-1 and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) respectively.[12,13]

α_4 integrin-mediated trafficking of pathogenic effector T cells to the brain and intestine has been considered as a validated therapeutic target for the treatment of Multiple Sclerosis (MS) and CD.[14,15] Furthermore, regarding to α_4

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integrin involvement in tumor angiogenesis, it also could be authorised as a target for cancer therapy.[16]

Tysabri (natalizumab), an antibody which blocks α_4 integrin and inhibits the α_4-mediated adhesion of leukocytes to their counter receptors, has been used for treatment of patients with relapsing remitting multiple sclerosis (RRMS) and CD.[17] As monoclonal antibodies (mAbs) have high immunogenicity and molecular weight and low heat stability, are not in favour of treatment approaches. Thus research on other agents lacking the above disadvantages seems crucial for mAbs replacement.

Aptamers are new tools with low immunogenicity and molecular weight and high heat stability which make them appropriate candidates for mAbs substitution.

Aptamers are selected by a process known as Systemic Evolution of Ligands by Exponential Enrichment (SELEX). One of the most common SELEX methods is cell SELEX. In this method, those aptamers would be selected that bind to protein targets expressed on the cell surface with high specificity and affinity. Hence, type of cells used in this selection process is a critical parameter in cell SELEX. The selected aptamers will be useful for detection and blockade of specific surface markers.[18]

In the present study, we intended to construct an over-expressing full length of human ITGA-4 recombinant HEK cell (Hek293/ITGA-4). This construct could further be employed in SELEX process to select a specific aptamer or immunization of camel to generate nanobody against alpha4 integrin that could inhibit alpha4 integrin as a mediator of adhesion and migration. This aptamer could be utilized in preclinical studies for treatment of MS and Crohn's disease in future.

Materials and Methods

Transformation

Escherichia coli strain top 10 (Pasture, Tehran), was used as host for Z2827-M67-(ITGA4) expression vector (GeneCopoeia, MD, USA) encoding the human integrin α_4. E.coli top 10 was grown in Luria–Bertani (LB) (Sigma-Aldrich, St. Louis, MO, USA) broth medium, containing tetracycline (50 µg/ml), under aerobic condition and shaking at 250 rpm at 37°C. E.coli top 10, was then transformed with Z2827-M67-(ITGA4) expression vector in order to plasmid amplification using the $CaCl_2$ procedure.[19] The transformed cells were spread on LB agar plates containing 100 µg/ml ampicillin and incubated at 37°C overnight. Isolated single colonies were inoculated into LB broth with ampicillin (100 µg/ml) and grown at 37°C for 24 hours with shaking at 250 rpm for the purpose of plasmid extraction. The plasmids were extracted using commercial plasmid extraction kits (Feldan, Canada). Consequently, Z2827-M67-(ITGA4) vector was digested with Eco31I (Thermo Scientific, Waltham, MA, USA) enzyme according to manufacturer's instruction to make a linear plasmid. Digested plasmid was evaluated by agarose gel electrophoresis (Figure 1).

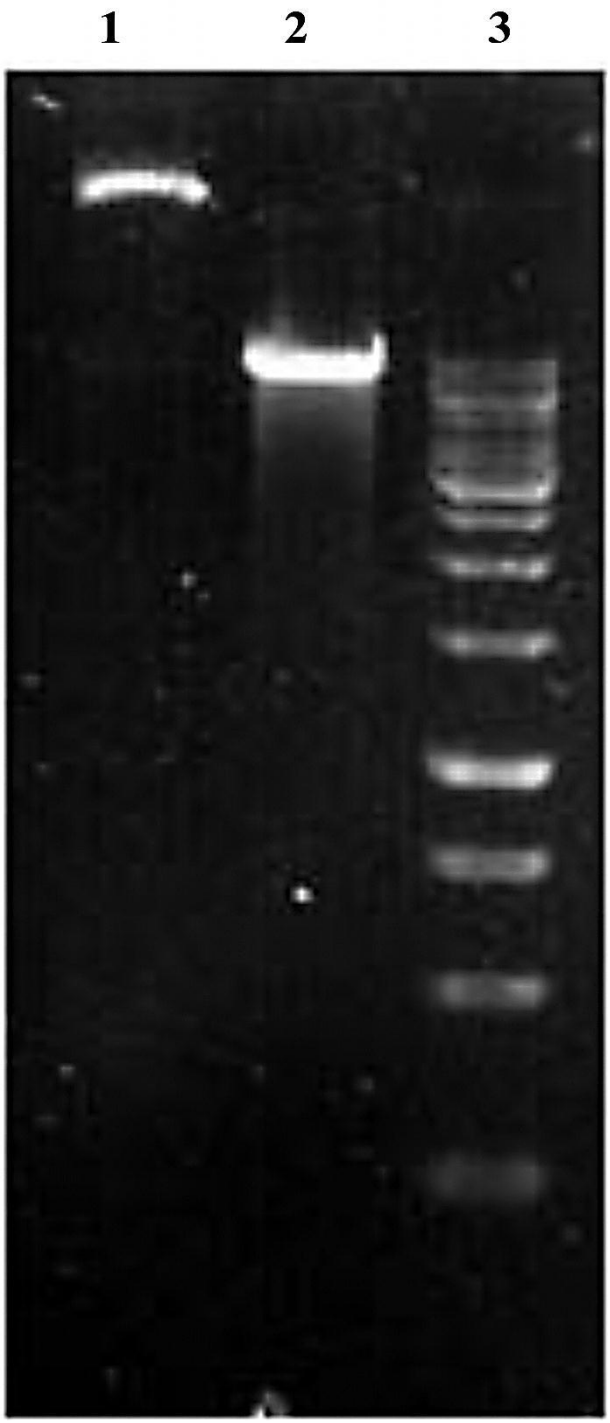

Figure 1. Agarose gel electrophoresis image. Lane 1=Undigested plasmid pEZ-M67- (ITGA4), Lane 2= digested plasmid pEZ-M67- (ITGA4) with enzyme Eco31i, lane 3= 1Kb ladder.

Cells culture

All cell culture reagents were purchased from Gibco-life technologies (CA, USA). Human Embryonic kidney (HEK-293) cell line (Pasteur institute, Tehran, Iran) were grown in Dulbecco's Modified Eagle Media (DMEM) culture medium supplemented with 10% Fetal Calf Serum (FBS), 100 U/ml Penicillin and 100µg streptomycin at 37°C in a 5% CO_2 atmosphere.

Transfection of HEK-293 Cell and Screening for Stable α_4 integrin expressing Cells

HEK-293 Cells in exponential growth phase were seeded (5 × 10^6cells) into 10-cm plate the day before transfection. Culture medium was changed with fresh medium two hours before transfection. Distilled water was added to the mixture of 21µgr of linearized plasmid up to 365 µl. 13.2 µl TE 1X, 42 µl CaCl2 2.5M and 420 µl of Hepes Buffered Saline (HBS) 2X were added drop wise and slowly under agitation by vortexing . In this step additional severe vortexing for 1 min was done and the tube incubated for 15 min at 37°C. The resulted DNA complex was added on cell culture drop wise and the plate was swirled. The cells were exposed to the precipitate for 2–6 h at 37°C. Z2827-M67-(ITGA-4) linearized plasmid contained hygromycin B phosphotransferase gene (hph) as a selective marker. Transfected cells were grown in non-selective medium for 2 days. Forty eight hours after transfection, cells were exposed to 200µg/ml hygromycin B (Roche, IN, USA). After 21 days of selection, hygromycin B -resistant clones were randomly picked up, and cultured in the medium containing 200 µg/ml hygromycin B in separated flasks. After more than 14 days cells which grow under hygromycin B treatment likely, has some copy of hygromycine B resistant gene in their genome and usually are named as stable transfected cell lines. HEK-293 cells that were not transfected were used as negative control.

DNA extraction and Polymerase Chain Reaction (PCR) on genomic DNA

Chromosomal DNA was extracted from ITGA4-transfected cells using commercial extraction kit (BIONEER, Korea) according to manufacturer's instructions. The presence of ITGA-4 gene in HEK-293 genome was assayed by PCR. Amplification of extracted genomic DNA was performed using specific primers designed for partial human ITGA-4 cDNA (forward 5′-cgggatccatgtggagctggaag-3′ and reverse 5′-cgggatcctcagcggcgtttgagt-3′).

All PCR reagents were purchased from Thermo Scientific (Waltham, MA, USA). The reactions performed using Biorad thermal cycler (Bio-Rad Laboratory, USA). PCR conditions were optimized as follows: a total reaction mix of 50 µl contained 250ng of genomic DNA as template, 100 pmol of each primer, 1 U of Taq DNA polymerase, 1.5mM MgCl2, 200 mmol of each dNTP, and the volume was adjusted to 50 µl with deionised water. PCR program was 94° C for 3 min and 30 cycles of 94° C for 30 sec, 58° C for 30 sec, and 72° C for 2 min and 30 sec followed by a final extension step of 72°C for 10 min. Gel electrophoresis was performed after PCR amplification on 1% agarose gel (Figure 2).

Flow cytometric analysis

100 µl of Hek293/ITGA-4 and HEK-293 cell suspension staining were done with 5 µl anti-human CD49d antibody (Biolegend, CA, USA) conjugated with phycoerythrin (PE) for 30 min at 4°C. Isotope control was used to enable correct compensation and to confirm antibody specificity. Flow cytometric analysis was performed on FACS Callibur Flow cytometer (BD bioscience, NJ, USA) by accumulating up to 10,000 events per tube.

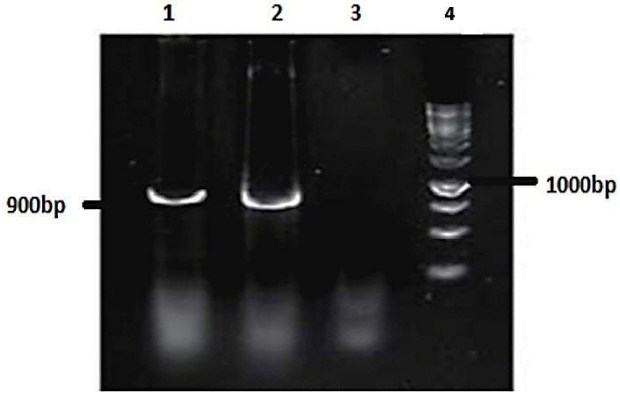

Figure 2. Agarose gel electrophoresis image showing PCR product of ITGA4 gene. Lane 1= amplification product of 900bp portion of ITGA4 gene produced by targeting extracted genomic DNA from ITGA4-transfected HEK cells. Lane 2=amplification product of 900bp portion of ITGA4 gene produced by targeting plasmid pEZ-M67- (ITGA4). Lane 3=Negative control. Lane 4=1Kb ladder.

Results and Discussion

Twenty one days after transfection, there were three separated clones in 100 mm plate.

Chromosomal DNA extraction and amplification

Genomic DNA from transfected cells was extracted. Primers were chosen to target the 900bp portion of the ITGA-4 cDNA. The PCR product illustrated amplification of a 900bp DNA band on agarose gel (Figure 2).

Flow cytometric analysis

As shown in Figure 3, HEK-293 cells have no expression of α_4 integrin on their surface while 95% of transfected HEK-293 with ITGA4 were expressed α_4 integrin which correlates well with genomic DNA PCR amplification results.

Different eukaryotic proteins have been used in immunization of mouse and camel to produce nanobody, mono, and polyclonal antibody and in SELEX process to select specific aptamers. Eukaryotic proteins usually have post translational modifications e.g. glycosylation, phosphorylation and fatty acid addition. The majority of diagnostic and therapeutic antibodies, target the special epitopes which contain post translational modification residues. Eukaryotic proteins could be expressed in prokaryotic expression systems in high yield and low cost however; these proteins lack post translational modifications and even proper folding. On the other hand, although protein expression in eukaryotic expression systems like mammalian cell lines has low yield and the resulted proteins are expensive, the resulted

protein has an accurate folding and appropriate post translational modifications. Displaying eukaryotic membrane or even secreting and intracellular proteins on surface of mammalian cell lines e.g. HEK, is an alternative method to overcome the above cited obstacles.

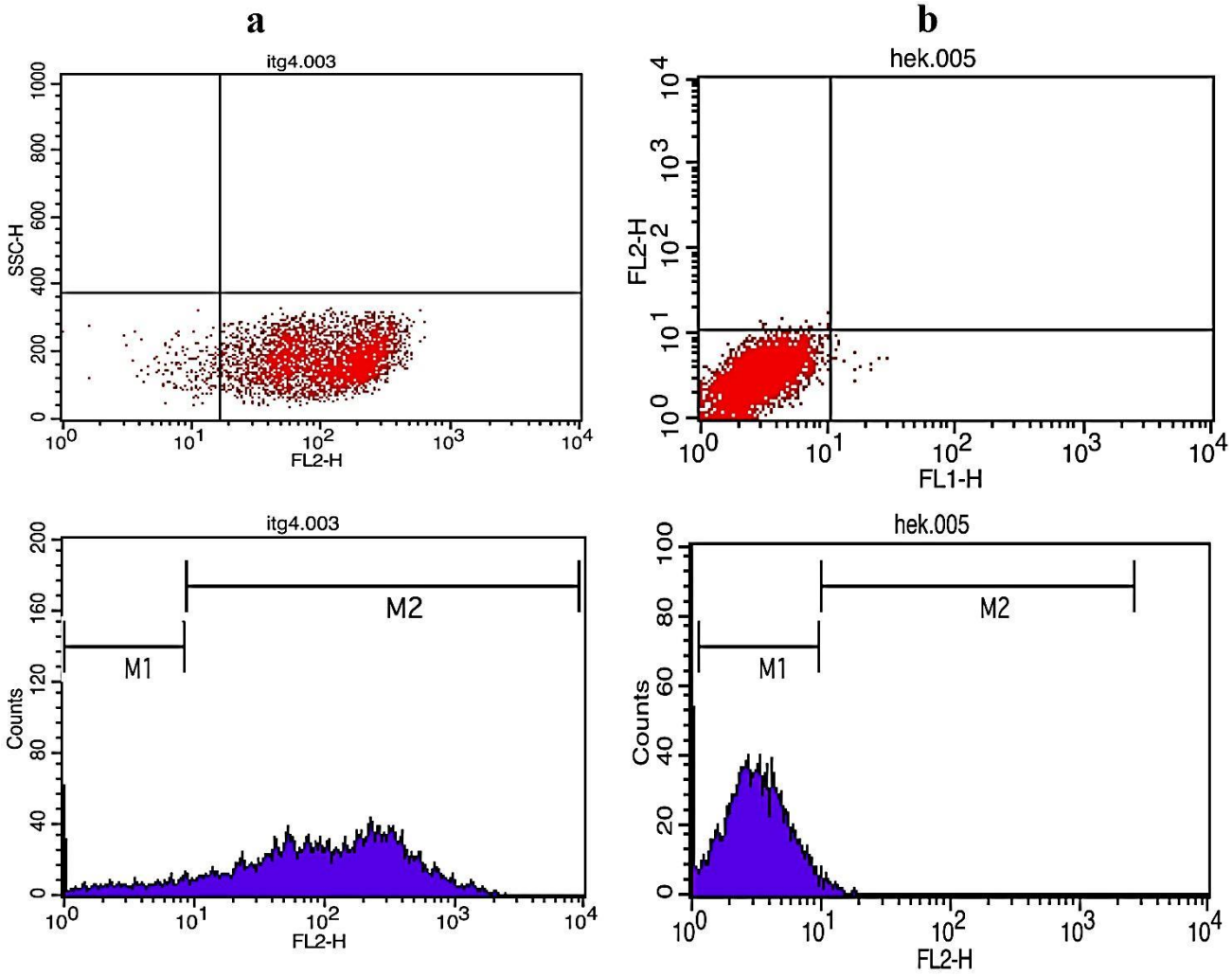

Figure 3. Flow cytometric results of α_4 integrin expression on transfected (a) and un-transfected (b) HEK cells.

The HEK-293 overexpressing ITGA-4 may provide an appropriate model for further investigation on diverse therapeutic agents. The existence of protein as target, on cell surface has advantages that stand it out comparing to other methods for biomolecule selection. Live cells with overexpression of a certain molecule, are required for production and selection process of biomolecules e.g. mono and polyclonal antibodies, nanobodies and aptamers. Most antibody targets are membrane proteins with post translation modifications, which are difficult to express and purify as recombinant proteins. The availability of such proteins in sufficient quantities for immunization and selection is a hurdle in all antibody technology platforms, thus using model cells overexpressing the desired protein could resolve this problem. The present model provides an approach for antibody production through minimizing impasses. A set of related molecules with antibodise are nanobodise founded in some animals e.g., camelides. Nanobodies are unique class of antibodies lacking L chains, thus, form a homodimeric H2‑type (vHH) antibodies.

In case of nanobody production, immunization of camel with peptides (even coupled to larger protein carriers) is not recommended as the majority of nanobodies distinguish conformational epitopes and a poor vHH response would rise subsequent to peptide injection. DNA immunization, cell immunization and DNA prime/cell boost are potential alternatives to protein immunization, thus presence of such a model seems crucial for nanobody production.[20]

Another group of high specificity biomolecules are aptamers. Aptamers have been compared to antibodies on the basis of their target-recognition capability, selective binding and high affinity, however aptamers have advantages over antibodies including low blood residence time, low toxicity or immunogenicity, long shelf life, high affinity and specificity, thermal stability, low cost, unlimited applications and chemically synthesis possibility.[21]

Aptamers usually are selected by screening nucleic acid libraries through SELEX process.

Purified recombinant proteins are traditional targets for aptamer SELEX, although these proteins might have an impaired three dimensional structure and/ or post translational modification. Aptamers required native state of target molecule with their natural folding and modifications for a better isolation in SELEX process. The present HEK-293 cell over expressing α_4 integrin provides the native state of α_4 integrin with appropriate post translational modification on the cell surface, thus offers an appropriate tool for specific aptamer selection against α_4 integrin.[22]

Conclusion

In this paper, we constructed a recombinant cell line (Hek293/ITGA4) over-expressing α_4 integrin. The expression of α_4 chain on cell surface was characterized by flow- cytometric staining. The results indicated that the ITGA-4 expression vector was successfully transfected into target cell, HEK-293. A highly and stably expressed α_4 integrin cell line obtained with hygromycin B antibiotic selection which could be applied in future investigations.

Acknowledgments

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

References

1. Davenport RJ, Munday JR. Alpha4-integrin antagonism--an effective approach for the treatment of inflammatory diseases? *Drug Discov Today* 2007;12(13-14):569-76. doi: 10.1016/j.drudis.2007.05.001
2. Huhtala M, Heino J, Casciari D, de Luise A, Johnson MS. Integrin evolution: Insights from ascidian and teleost fish genomes. *Matrix Biol* 2005;24(2):83-95. doi: 10.1016/j.matbio.2005.01.003
3. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010;339(1):269-80. doi: 10.1007/s00441-009-0834-6
4. Serini G, Valdembri D, Bussolino F. Integrins and angiogenesis: A sticky business. *Exp Cell Res* 2006;312(5):651-8. doi: 10.1016/j.yexcr.2005.10.020
5. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci* 2006;119(Pt 19):3901-3. doi: 10.1242/jcs.03098
6. Strachan LR, Condic ML. Neural crest motility and integrin regulation are distinct in cranial and trunk populations. *Dev Biol* 2003;259(2):288-302. doi: 10.1016/S0012-1606(03)00187-8
7. Sengbusch JK, He W, Pinco KA, Yang JT. Dual functions of [alpha]4[beta]1 integrin in epicardial development: Initial migration and long-term attachment. *J Cell Biol* 2002;157(5):873-82. doi: 10.1083/jcb.200203075
8. Grazioli A, Alves CS, Konstantopoulos K, Yang JT. Defective blood vessel development and pericyte/pvsmc distribution in alpha 4 integrin-deficient mouse embryos. *Dev Biol* 2006;293(1):165-77. doi: 10.1016/j.ydbio.2006.01.026
9. Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J. Integrin alpha4beta1-vcam-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. *J Clin Invest* 2005;115(6):1542-51. doi: 10.1172/JCI23445
10. Engelhardt B. Immune cell entry into the central nervous system: Involvement of adhesion molecules and chemokines. *J Neurol Sci* 2008;274(1-2):23-6. doi: 10.1016/j.jns.2008.05.019
11. Qian H, Georges-Labouesse E, Nystrom A, Domogatskaya A, Tryggvason K, Jacobsen SE, et al. Distinct roles of integrins alpha6 and alpha4 in homing of fetal liver hematopoietic stem and progenitor cells. *Blood* 2007;110(7):2399-407. doi: 10.1182/blood-2006-10-051276
12. Engelhardt B. Molecular mechanisms involved in t cell migration across the blood-brain barrier. *J Neural Transm* 2006;113(4):477-85. doi: 10.1007/s00702-005-0409-y
13. Lazebnik LB, Boldyreva ON, Parfenov AI, Trubitsyna IE, Shcherbakov PL, Khomeriki SG, et al. [cell adhesion molecules in evaluation of crohn's disease therapy]. *Eksp Klin Gastroenterol* 2013(3):31-8.
14. Kawamoto E, Nakahashi S, Okamoto T, Imai H, Shimaoka M. Anti-integrin therapy for multiple sclerosis. *Autoimmune Dis* 2012;2012:357101. doi: 10.1155/2012/357101
15. Bamias G, Clark DJ, Rivera-Nieves J. Leukocyte traffic blockade as a therapeutic strategy in inflammatory bowel disease. *Curr Drug Targets* 2013;14(12):1490-500. doi: 10.2174/13894501113149990158
16. Lu X, Lu D, Scully M, Kakkar V. The role of integrins in cancer and the development of anti-integrin therapeutic agents for cancer therapy. *Perspect Medicin Chem* 2008;2:57-73.
17. O'Connor P. Natalizumab and the role of alpha 4-integrin antagonism in the treatment of multiple sclerosis. *Expert Opin Biol Ther* 2007;7(1):123-36. doi: 10.1517/14712598.7.1.123
18. Banerjee J, Nilsen-Hamilton M. Aptamers: Multifunctional molecules for biomedical research. *J Mol Med (Berl)* 2013;91(12):1333-42. doi: 10.1007/s00109-013-1085-2
19. Sambrook j, W.russell d. Molecular cloning: A Laboratory Manual. 3rd ed. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor; 2001.

20. Hassanzadeh-Ghassabeh G, Devoogdt N, De Pauw P, Vincke C, Muyldermans S. Nanobodies and their potential applications. *Nanomed* 2013;8(6):1013-26. doi: 10.2217/nnm.13.86
21. Mairal T, Ozalp VC, Lozano Sanchez P, Mir M, Katakis I, O'Sullivan CK. Aptamers: Molecular tools for analytical applications. *Anal Bioanal Chem* 2008;390(4):989-1007. doi: 10.1007/s00216-007-1346-4
22. Sefah K, Shangguan D, Xiong X, O'Donoghue MB, Tan W. Development of DNA aptamers using cell-selex. *Nat Protoc* 2010;5(6):1169-85. doi: 10.1038/nprot.2010.66