Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Fusion of EGFP and porcine α 1,3GT genes decrease GFP expression

Yongxiang Zhao[1,2,3,a], Jing Tang[3,a], Qin Yao[3,a], Yuan Zhou[4,a], Huange Zhao[1], Xiaoyun Zeng[5], Jiaqi Shi[6], Guorong Luo[5], Xiaoxun Xie[5], Sufang Zhou[5], Zuguo Liu[7], Xiaoling Lu[5*], Donghai Lin[8*], Jianming Liu[9*]

[1]*Hainan Medical University, Haikou, Hainan, 571101, P.R. China*
[2]*The First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi 530021, P. R. China*
[3]*Zhongshan Hospital, Xiaman University, Xiamen, Fujian 361004, P. R. China*
[4]*Department of Pharmacology, Medical College, Hunan Normal University, Changsha, Hunan 41013, P. R. China*
[5]*Preclinical Medical College, Guangxi Medical University, Nanning, Guangxi 530021, P. R. China*
[6]*Department of Pathology, Arizona Cancer Center, University of Arizona, Tucson, AZ 85724, U.S.A.*
[7]*Ophthalmology Institute, Xiaman University, Xiamen, Fujian 361005, P. R. China*
[8]*The Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engneering, Xiamen 361005, P. R. China*
[9]*The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, P. R. China*

ARTICLE INFO

Article history:
Received 26 September 2010
Received in revised form 27 October 2010
Accepted 25 November 2010
Available online 20 December 2010

Keywords:
Enhanced green fluorescent protein
Porcine α 1, 3 galactosyltransferase
Fusion gene
Fluorescence intensity

ABSTRACT

Objective: To investigate the effect of fusion proteins expressed by the fused gene of porcine α 1,3 galactosyltransferase (α 1,3 GT) and enhanced green fluorescent protein (EGFP) on the green fluorescence intensity of EGFP. **Methods:** The fragment containing α 1,3GT was firstly recovered after the pcDNA3.1- α 1,3GT recombinant vector were digested with *Bam*HI and *Eco*RI, and then, the resultant fragment was ligated to the pEGFP-N1 vector which was also digested with the same enzymes. The new recombinant eukaryotic expression pEGFP/ α 1,3GT vector was obtained and sequenced. The pEGFP/ α 1,3GT was used to transfect human lung carcinoma cells A549 and HEKC 293FT, and the expression of EGFP was quantitatively analyzed by fluorescent microscope and flow cytometry. **Results:** The positive percentage of A549 was 80.5%, and that of 293 FT was 86.5% 48 hours after the two cell lines both were transfected by pEGFP-N1. The positive percentage of A549 was 75.8%, and that of 293 FT was 81.2% 48 hours after the two cell lines were transfected by pEGFP/ α 1,3GT. The mean fluorescence intensities of A549 transfected with pEGFP-N1 and pEGFP/ α 1,3GT were 1.21 and 0.956, respectively when compared with that of A549 without transfection. Meanwhile, the those of the 293FT that were transfected with pEGFP-N1 and pEGFP/ α 1,3GT were 7.66 and 1.00, respectively when compared with that of 293FT cells without transfection. **Conclusions:** These results suggested that the expression of EGFP gene fused with porcine α 1,3GT gene was partly inhibited.

1. Introduction

The green fluorescent protein (GFP) is a kind of monomer photoprotein that is composed of 238 amino acids and has molecular weight of 27-30 Kd[1-4]. It can undergo autocatalysis to form chromophore without any cofactors, showing green fluorescence once it is excited by near-ultraviolet or blue light. The enhanced green fluorescent protein (EGFP) has been produced by replacing Thr and Leu with Ser65 and Phe64, respectively, and it shows enhanced fluorescence strength by around 35 times when compared with GFP, and also exhibits improved sensitivity for reporter gene[5]. Therefore, EGFP has been widely used in the biomedical field as a multi-functional tool for some qualitative or quantitative investigations such as protein interaction, enzymatic activities, drug monitor, gene expression, protein localization, evaluation on the therapeutic effects of tumor vaccines, fluorescence labeling of targeted proteins and so on[6-17]. But it is still unclear whether its fluorescence intensity would be changed or not when it is fused with some exogenous genes.

*Corresponding author: Xiaoling Lu, Preclinical Medical College, Guangxi Medical University, Nanning, Guangxi 530021, P. R. China.
Donghai Lin, The Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engneering, Xiamen 361005, P. R. China.
Jianming Liu, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, P. R. China.
Tel.: +86 771 5350 964
E-mail: luwuliu@163.com(X. Lu), dhlin@xmu.edu.cn (D.Lin), liujianming@126.com(J. Liu)
Foundation Project: This work was supported in part by National Natural Scientific Foundation of China(81072148, 30760248, 30660185, 30000203).
[a] These authors contributed equally to this work.

Gal α 1-3Gal β 1-(3)4GlcNAc-R, *i.e.*, α Gal antigen, is a glycoprotein that is catalyzed and synthesized by α 1,3GT on the cell surface of all non-primate mammals except for the old century primates and human. Gal α (1,3)Gal antigen is not expressed in old century primates and human since it is depleted or replaced by a pseudogene during the evolution of α 1,3GT gene[18,19]. However, old century primates and human can produce the natural antibody to Gal epitope after their birth as a result of the immune responses to gastrointestinal bacterial flora and other pathogens that carry the Gal α (1,3)Gal saccharide group structures[20]. During xeno-transplantation, such as the transplantation of organs from pigs into human bodies, the anti-Gal antibody can bind to the α Gal antigen on the surface of vascular endothelial cells of pigs and thus induce the hyperacute rejection, and finally lead to the deactivation and cellular necrosis of the grafts[21-24]. On the basis of the hyperacute rejection mechanism, we attempted to express the porcine α Gal antigen in human tumor cells or/and tumor vascular endothelial cells by the transfection of these cells with the fusion gene of porcine α 1,3GT and EGFP, and thus the anti-tumor effects can be realized by the hyperacute rejection that was induced by the α Gal antigen binding to the natural anti-Gal antibody in the human body, with an objective to observe the production process of the fusion protein under the fluorescent microscope and understand the *in vivo* expression of the target gene in the near future, and finally realize the efficient monitoring and analysis on the effects of targeted killing of tumor cells by using the target gene α 1,3GT.

This investigation tried to understand the effect of fusion of porcine α 1,3GT and EGFP genes on the expression of the green fluorescent protein in order to establish the basis for the evaluations on the therapeutic effects of anti-tumor vaccine by using porcine α 1,3GT gene.

2. Materials and methods

2.1. Plasmids and strains

Porcine gene α 1,3GT and pCDNA3.1/ α 1,3GT gene were constructed previously in our laboratory. pEGFP-N1 (CLONTECH Laboratories, Inc. Mountain View, CA, U.S.A.) contains CMV promoter and EGFP gene. DH5 α strain was purchased from Invitrogen (Carlsbad, CA, U.S.A.).

2.2. Cell lines

A549 (American Type Tissue Culture Collection, Manassas, VA, U.S.A.) and 293FT (Invitrogen Life Technologies, CA, U.S.A.) were maintained in DMEM media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, USA) and antibiotics.

2.3. Reagents

Lipofectimine 2000 transfection reagents were purchased from Invitrogen (Carlsbad, CA, USA). Plasmid miniprep kit, ligation kit, restriction enzymes *Bam*HI and *Eco*RI, recovery and purification reagents for target gene were purchased from Takara Company (Osaka, Japan).

2.4. Construction of pEGFP/ α 1,3GT eukaryotic expression vector

pEGFP-N1 and pCDNA3.1/ α 1,3GT both were digested with *Bam*HI and *Eco*RI, and 1050 bp target fragment of α 1,3GT was recovered. The fragment was then ligated to the pEGFP-N1 vector at 16 ℃ for 3 h. Ten μL ligation product was used to transform DH5 α, subsequently the recombinant plasmid was extracted and subjected to enzymatic digestion identification, and finally, the eukaryotic expression vector containing EGFP was constructed (Figure 1). The recombinant plasmid was verified with DNA sequencing.

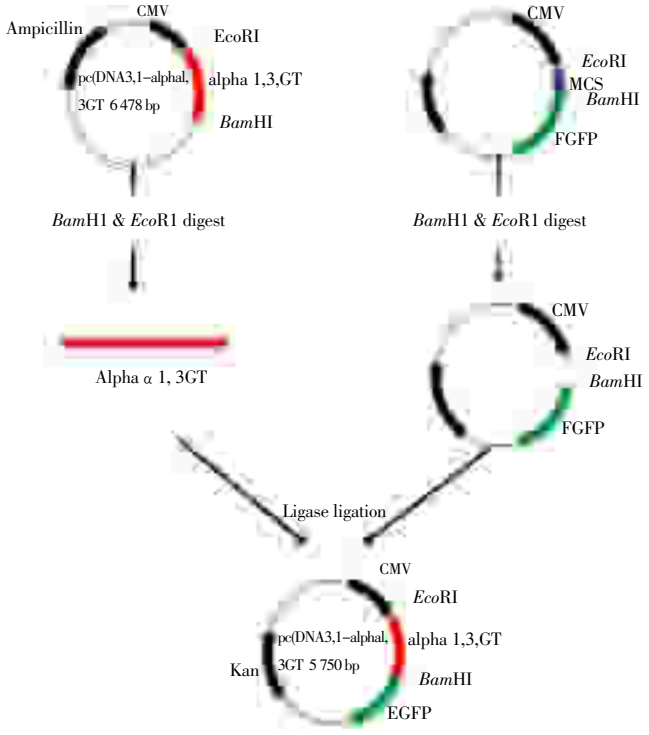

Figure 1. The vector for fusion of EGFP and porcine α 1,3GT genes.

2.5. Transfection of A549 and 293FT cells

A549 and 293FT cells were inoculated in two six-well plates and cultured overnight in order to reach a cell-fusion rate between 80%-90%. The blank control groups (which were not transfected), the experimental control groups (which were transfected with pEGFP-N1) and the experimental groups (which were transfected with pEGFP/ α 1,3GT) were established, and the cells were transfected by using the liposome method in which about 4.0 μg plasmid and 10 μL liposome were added into the six-well plates.

2.6.Detection of EGFP expression by the fluorescent microscope

The expression of EGFP was observed by using the

fluorescent microscope after the transfection of the A549 and 293FT cells for 24, 48 and 72 h, respectively. To quantify the expression of EGFP, the number of positive cells was counted in 10 random fields per well.

2.7. Detection of EGFP expression by the flow cytometry

A549 and 293FT cells were rinsed twice with PBS for 48 h after the transfection, subsequently they were digested with trypsin and prepared into cell suspension with PBS and then filtered with a copper screen of 200 meshes. The samples were analyzed by FACScalibur (Becton Dickinson, Heidelberg, Germany).

3. Results

3.1. Verification of pEGFP/α 1,3GT recombinant plasmid

The recombinant plasmid was verified with restriction enzyme analysis and DNA sequencing. The plasmid was digested by *Bam*HI and *Eco*RI, digests were analyzed with 1% agarose gel electrophoresis (data not shown). Both the digested fragments and DNA sequence analysis (data not shown) indicated that the recombinant plasmid was constructed correctly.

3.2.The expression and localization of pEGFP-N1 and pEGFP/α 1,3GT

The positive rates for the transfection of A549 and 293FT cells with pEGFP-N1 vector after 48 h were 80.5% and 86.5%, respectively, and the transfection efficiency of A549 and 293FT cells with pEGFP/α 1,3GT after 48 h were 75.8% and 81.2%, respectively. The number of fluorescent cells and the fluorescence intensities of pEGFP/α 1,3GT transfection groups were significantly lower than those of pEGFP-N1 groups, while no fluorescence was found in the blank control groups (Figure 2). The localization of the fusion protein was observed under laser scanning confocal microscope 48 h after the transfection (Figure 3).

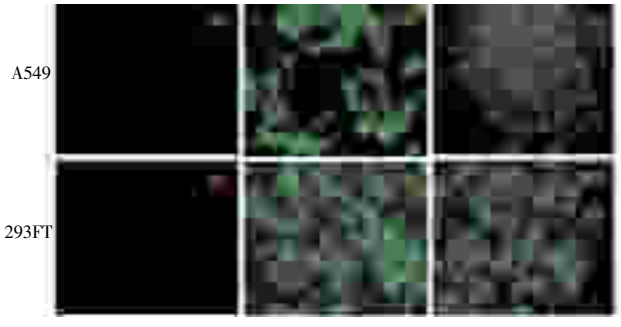

Figure 2. Expression and localization of pEGFP-N1 and pEGFP/α 1,3GT. A: A549 cell lines. B: 293FT cell lines. A1(×200) and B1(×200) are the blank controls. A2 (×200) and B2 (×200) are transfected by pEGFP-N1. A3 (×200) and B3 (×200) are transfected by pEGFP/α 1,3GT. Arrows indicated the signals for the expression of EGFP.

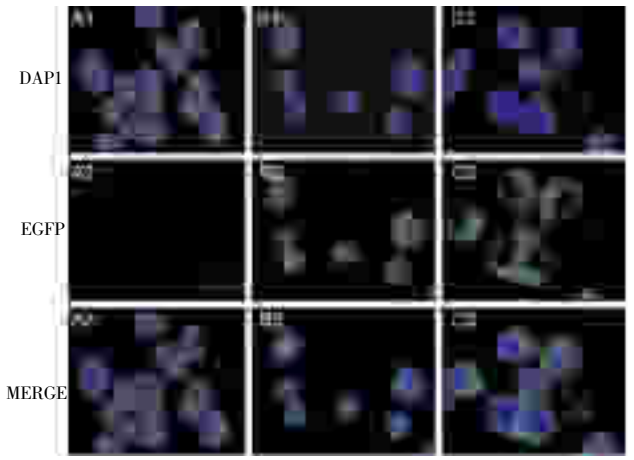

Figure 3. The transfection of A549 cell lines.
A: the blank control. B: pEGFP-N1 transfection. C: pEGFP-α 1,3GT transfection. Arrows indicated the localization for the expression of EGFP. All of the figures were captured under the laser scanning confocal microscope (×1600).

3.3. The flow cytometry analysis of the fluorescence intensity

As shown in Figure 4, the mean fluorescence intensity of A549 cells that were transfected by pEGFP-N1 and pEGFP/α 1,3GT was 1.21 and 0.956, respectively, and the mean fluorescence intensity of 293FT cells that were transfected with pEGFP-N1 and pEGFP/α 1,3GT was 7.66 and 1.00, respectively. The fluorescence expression intensities of pEGFP-N1 and pEGFP/α 1,3GT groups were significantly higher than those of the blank control groups, whereas the fluorescence expression intensities of the pEGFP/α 1,3GT groups were significantly lower than those of the pEGFP-N1 groups (Figure 4).

4. Discussion

Reporter genes have been used in the investigations on gene therapy[25,26]. The advantages of EGFP are as follows: high fluorescence intensity, easily observation under common fluorescent microscopes, no requirements for substrates and cofactors, liable fusion by using relatively small molecules and monomer protein, and no effects on activities of normal cells. However, the study for its fluorescent intensity stabilization has not been reported so far after EGFP gene is fused with exogenous genes.

The present study found fluorescence intensity of pEGFP/α 1,3GT was significantly lower than that of the pEGFP-N1 in the transfection of A549 or 293FT cells that were transfected with recombinant plasmid pEGFP/α 1,3GT. The reasons may be that the α 1,3GT gene was inserted into the C-terminus of pEGFP-N1 and the molecular weight of fusion protein was heavier than that of a single EGFP protein, which may affect the formation of green fluorescence chromophore in the spatial structure of protein conformation. On the other hand, fluorescence intensity was also related to the transfection conditions of cell lines. This investigation also found that the fluorescence intensity was different although the cell lines

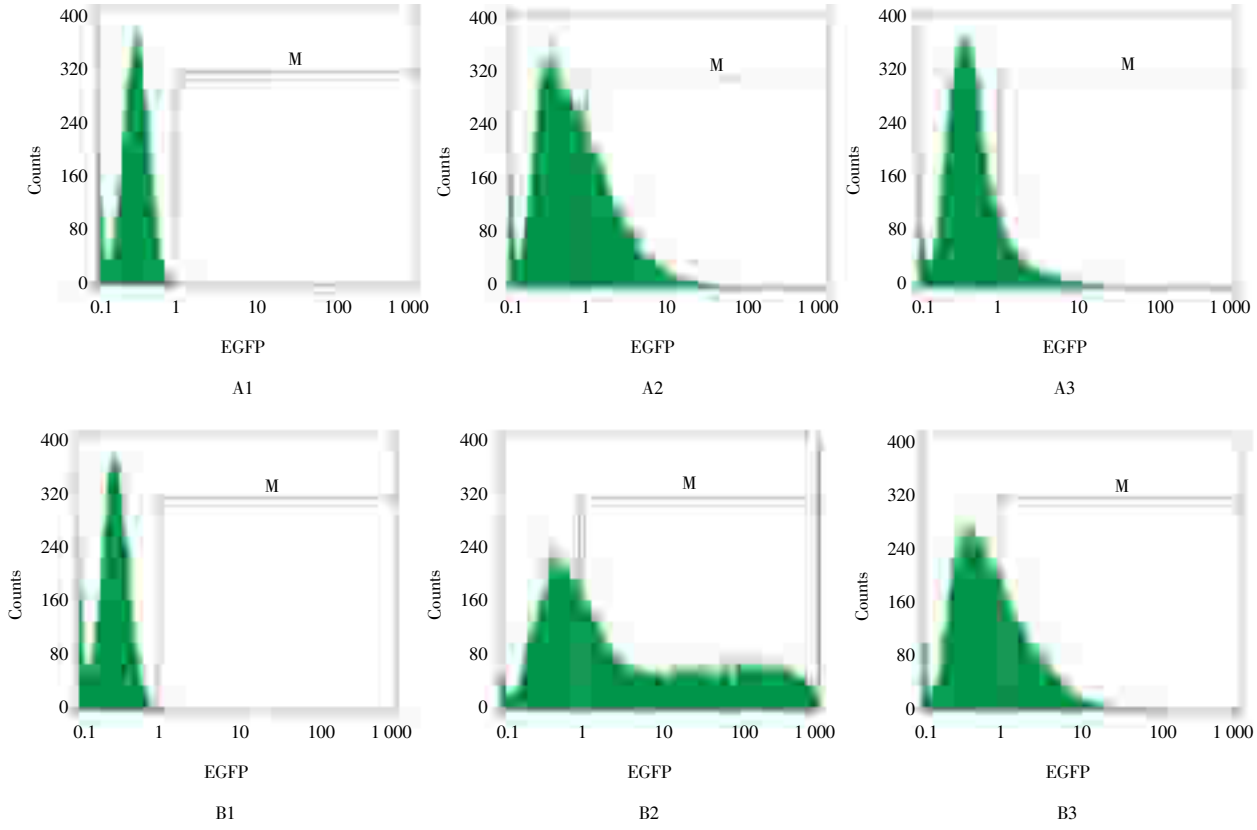

Figure 4. Flow cytometry analysis of the fluorescence intensity.
Group A: A549 cell lines, Group B: 293FT cell lines. A1 and B1 were the blank controls and the mean fluorescence intensity of them was 0.577 and 0.516, respectively. The mean fluorescence intensities of A2 and B2 transfected with pEGFP-N1 were 1.21 and 7.66, respectively. The mean fluorescence intensities of A3 and B3 transfected with pEGFP- α 1,3GT were 0.956 and 1.000, respectively.

A549 and 293FT were transfected with the same plasmid, but the expression intensity of pEGFP-N1 before the fusion was significantly higher than that of the pEGFP/ α 1,3GT after the recombination. These results reveal that further reformation need performing on EGFP in order to improve the expression level of the recombinant green fluorescent protein, while the method for the fusion protein synthesis by genetic engineering and stability for the fusion protein expression still need to be further improved. Therefore, the following points should be minded when green fluorescent fusion proteins containing target genes are used for the evaluations on the therapeutic effects of antitumor tracing. The first one is that the green fluorescence intensity may be reduced after exogenous genes are introduced into target cells, which may lead to certain influence on the expression and localization of target genes. The second one is whether the expression of green fluorescent fusion protein will affect the functional expression of target proteins. Investigations from Huang *et al* on ZZ-EGFP fusion protein indicated that the expression of fusion protein did not significantly affect the expression of target gene and the ZZ-EGFP fusion protein can retain all expected characteristics (extremely high solubility and relatively high yield)[27], which was in accordance to the results in the present study (the experimental data will be published in another manuscript). The third one is whether EGFP shows toxicity to cells. The poor status of cells may directly affect the transfection efficiency if EGFP shows toxicity to cells, which may inevitably lead to the decrease in the expression level of green fluorescent fusion protein. Alexander *et al* cloned the target gene fragment into pEGFP-C1 vector and constructed a recombinant plasmid, and they discovered that EGFP did not show toxicity to cells[28, 29], which was similar to our current results. However, Liu *et al* found that NIH/3T3 and BHK-21 cell lines underwent apoptosis shortly after the transfection with EGFP, which may be related to the different tolerance of cells[30].

The treatment of tumors with target genes is a kind of theoretically effective method[31], but substantial improvements are still needed for practical operation process. With the developments in investigations on the mechanisms of fusion protein formation, variations and mutations during the synthesis process of fusion genes and the introduction of cofactors, it is believed that breakthroughs in the gene therapy on tumors are inevitably achieved.

Role of funding source

Program for New Century Excellent Talents in University of China(NCET-06-0761), International Cooperation Project of The Ministry of Science and Technology of the People's Republic of China (2008DFA31380), Project of Scentific Activity of Ministry of Personnel for Returnee (2007-170), Program of Science and Technology of Hunan

Province(06SK4060), Program of Science and Technology of Hainan Province (070210), Program of Science and Technology of Fujian Province (2008-59-08), Project of Medical Innovation of Fujian Province (2007CX18) and Program of Science and Technology of Xiamen (3502Z20084012).

Conflict of interest statement

The authors have no financial conflict of interest.

References

[1] Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Sci* 1996; **273**: 1392-5.

[2] Yang F, Moss LG. The molecular structure of green fluorescent protein. *Nat Biotechnol* 1996; **14**: 1246-51.

[3] Henderson JN, Gepshtein R, Heenan JR, Kallio K, Huppert D, Remington SJ. Structure and mechanism of the photoactivatable green fluorescent protein. *J Am Chem Soc* 2009; **131**(12): 4176-7.

[4] Prasher DC, Eckenrode VK, Ward WW. Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* 1992; **111**: 229-33.

[5] Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 1996; **173**: 33-8.

[6] Butera D, Piazza RMF, Lane MAM. Molecular engineering of an EGFP/ disintegrin- based integrin marker. *Toxicon* 2005; **46**: 178-84.

[7] Ueta Y, Fujihara H, Serino R. Transgenic expression of enhanced green fluorescent protein enables direct visualization for physiological studies of vasopressin neurons and isolated nerve terminals of the rat. *Endocrinol* 2005; **146**: 406-13.

[8] Warmann SW, Fuchs J, Seitz G, et al. New trends in tumor biology transfection of a human hepatoblastoma cell line with green fluorescent protein. *J Pediatric Surg* 2005; **40**: 653-7.

[9] Fujio T, Fujihara H, Shibata M, Yamada S, Onaka T, Tanaka K, et al. Exaggerated response of arginine vasopressin enhanced green fluorescent protein fusion gene to salt loading without disturbance of body fluid homeostasis in rats. *J Neuroendocrinol* 2006; **18**: 776-85.

[10]Peckham GD, Bugos RC, Su WW. Purification of GFP fusion proteins from transgenic plant cell cultures. *Prot Expr & Pur* 2006; **49**: 183-9.

[11]Takanishi CL, Bykova EA, Cheng W. GFP-based FRET analysis in live cells. *Brain Res* 2006; **1091**: 132-9.

[12]Magliery V, Regan L. Reassembled GFP: detecting protein-protein interactions and protein expression patterns. *Meth Biochem Analysis*. 2006; **47**: 391-405.

[13]Meima ME, Weening KE, Schaap P. Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium. *Prot Expr & Pur* 2007; **53**: 283-8.

[14]Shibata M, Fujihara H, Suzuki H, Ozawa H, Kawata M, Dayanithi G, et al. Physiological studies of stress responses in the hypothalamus of vasopressin enhanced green fluorescent protein transgenic rat. *J Neuroendocrinol* 2007; **19**: 285-92.

[15]Zhuang R, Zhang Y, Zhang R, Song CJ, Song CH, Yang K, Yang A, et al. Purification of GFP fusion proteins with high purity and yield by monoclonal antibody-coupled affinity column chromatography. *Prot Expr & Pur* 2008; **59**: 138-43.

[16]Suzuki H, Kawasaki M, Ohnishi H, Nakamura T, Ueta Y. Regulatory mechanism of the arginine vasopressin-enhanced green fluorescent protein fusion gene expression in acute and chronic stress. *Peptides* 2009; **30**: 1763-70.

[17]Suzuki H, Onaka T, Kasai M, Kawasaki M, Ohnishi H, Otsubo H, et al. Response of arginine vasopressin enhanced green fluorescent protein fusion gene in the hypothalamus of adjuvant-induced arthritic rats. *J Neuroendocrinol* 2009; **21**:183-90.

[18]Yoshimura N, Sawada T, Furusawa M, Fuchinoue S. Expression of xenoantigen transformed human cancer cells to be susceptible to antibody-mediated cell killing. *Cancer Lett* 2001; **164**(2): 155-60.

[19]Yuriev E, Agostino M, Farrugia W, Christiansen D, Sandrin MS, Ramsland PA. Structural biology of carbohydrate xenoantigens. *Expert Opinion Biol Ther* 2009; **9**(8): 1017-29.

[20]Shohet SB, Griffiss JM, Galili U, Mandrell RE, Hamadeh RM. Interaction between human natural anti- α -galactosyl immunoglobulin G and bacteria of the human flora. *Inf & Imm* 1988; **56**(7): 1730-7.

[21]Griesemer AD, Hirakata A, Shimizu A, Moran S, Tena A, Iwaki H, et al. Results of gal-knockout porcine thymokidney xenografts. *Am J Transplantation* 2009; **9**(12): 2669-78.

[22]Lin CC, Cooper DK, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. *Transplant Immunol* 2009; **21**(2): 75-80.

[23]Ekser B, Rigotti P, Gridelli B, Cooper DK. Xenotransplantation of solid organs in the pig-to-primate model. *Transplant Immunol* 2009; **21**(2): 87-92.

[24]Hisashi Y, Yamada K, Kuwaki K, Tseng YL, Dor FJ, Houser SL, et al. Rejection of cardiac xenografts transplanted from alpha1,3-galactosyltransferase gene-knockout (GalT-KO) pigs to baboons. *Am J Transplantation* 2008; **8**(12): 2516-26.

[25]Lee YL, Lee YJ, Ahn SJ, Choi TH, Moon BS, Cheon GJ, et al. Combined radionuclide-chemotherapy and *in vivo* imaging of hepatocellular carcinoma cells after transfection of a triple-gene construct, NIS, HSV1-sr39tk, and EGFP. *Cancer Lett* 2010; **290**(1): 129-38.

[26]Buchwald M, Krämer OH, Heinzel T. HDACi--targets beyond chromatin. *Cancer Lett* 2009; **280**(2):160-7.

[27]Huang QL, Chen C, Chen YZ, Gong CG, Cao L, Wang J, et al. Application to immunoassays of the fusion protein between protein ZZ and enhanced green fluorescent protein. *J Immunological Meth* 2006; **309**: 130-8.

[28]Alexander L, Lee H, Rosenzweig M, Jung JU, Desrosiers RC. EGFP-containing vector system that facilitates stable and transient expression assays. *Biotechniques* 1997; **23**: 64-6.

[29]Gotoh H, Matsumoto Y. Cell-surface streptavidin fusion protein for rapid selection of transfected mammalian cells. *Gene* 2007; **389**: 146-53.

[30]Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells. *Biochem Biophys Res Comm* 1999; **260**: 712-7.

[31]Vähä-Koskela MJ, Heikkilä JE, Hinkkanen AE. Oncolytic viruses in cancer therapy. *Cancer Lett* 2007; **254**(2): 178-216.