GSF-Forschungszentrum für Umwelt und Gesundheit
in der Helmholtz-Gemeinschaft
Institut für Pathologie

LacZ reporter fusion assay for rapid and easy identification of highly efficient siRNA and its delivery by Lentivirus into suspension cell lines

Nataša Anastasov, Margit Klier, Daniela Angermeier and Leticia Quintanilla-Martinez

Institute of Pathology, GSF National Research Center for Environment and Health, Germany

Introduction

Malignat lymphoma is a relatively frequent disorder and the third most common cancer of childhood. There are more than 30 subtypes of lymphoma including Hodgkin's lymphoma (HL), and B- and T-cell non-Hodgkin lymphomas (NHL). For this study, we have chosen mantle cell lymphoma (MCL), as example of a B-cell NHL, and anaplastic large cell lymphoma (ALCL), as example of a T-cell NHL. MCL is a lymphoproliferative disorder characterized by the t(11;14) translocation, which results in the deregulation and overexpression of cyclin D1. The inappropriate expression of cyclin D1 in MCL, which is not normally expressed in lymphocytes or myeloid cells, has been proposed to deregulate the normal control of cell cycle and growth and to contribute to lymphomagenesis in MCL. ALCL, on the other hand, consists of a proliferation of predominantly large lymphoid T-cells with strong expression of the cytokine receptor CD30, and the chromosomal translocations t(2;5), which results in the expression of a chimeric protein known as NPM-ALK (nucleophosmin- anaplastic lymphoma kinase).

The new emerging technologies, such as RNA interference, give us the unique opportunity to analyze and understand the consequences of these molecular alterations in NHL. However, lymphoid cells show very low transfection efficiency by standard methods, and virus-based constructs are needed to achive a high transfection efficency. Among these, lentiviruses have the distinguishing property of being able to infect both the dividing and non-dividing cells.

The aim of the present study is 1) to design the cyclin D1 and Stat3 siRNA with the highest efficiency, 2) to establish the efficient transduction of lymphoma cell lines by lentiviral pFUGW vector containing the specific siRNA sequence for downregulation, 3) to analyze the effect of cyclin D1 and Stat3 knock-down in the expression of different proteins in lymphoma cell lines, and 4) to identify critical genes that ultimately could be used for targeted gene therapy.

Materials and Methods

To study the regulation of Stat3 expression in ALCL and cyclin D1 expression in MCL cells, we used specific genetic approach for targeted gene silencing by specific siRNA. Human embryonic kidney cell line (293T), MCL cell line (Granta 519) and ALCL cell line (Karpas 299) were used for analysis. 293T adherent cell line, which has high lipofectamine 2000 transfection efficiency, was used to prove the cyclin D1 and Stat3 knockdown by the β-galactosidase RNAi Screening System (BLOCK-iT, Invitrogen) (Figure 1). The different specifically designed siRNA sequences for knockdown effect were cloned into pSuper expression vector. To precisely quantify the shRNA effect on Stat3 expression, specific fusion protein LacZ-Stat3 was constructed (named pLS3) (Figure 2). For quantification of shRNA effect on cyclin D1 expression specific fusion protein LacZ-cyclin D1 was constructed (named pLC1) (Figure 3). The shRNAs induce RNA interference in the cell causing lacZ fusion mRNA degradation and downregulation of synthesis of the specific fusion protein. The reduction of β - galactosidase reporter expression activity is precisely measured by a Tecan Fluorescence plate reader. To confirm the absence of cyclin D1 and Stat3, Western blot analysis was performed using a total of 30 µg of protein extracts, separated by a 12% SDS-polyacrylamide gel electrophoresis. The antibody used was polyclonal anti-cyclin D1 (Santa Cruz, Biotechnology) and monoclonal anti-Stat3 (Transduction Laboratories). Specific sequence for downregulation of cyclin D1 and Stat3 was recloned into pFUGW vector containing GFP, designed for production of lentivirus by cotransfection with suitable packaging plasmids (Figure 4). The GFP expression, of infected cells, was detected by flow cytometry (FACS) (Figure 5 and 7) and Laser Scan Microscopy (LSM) (Figure 6).

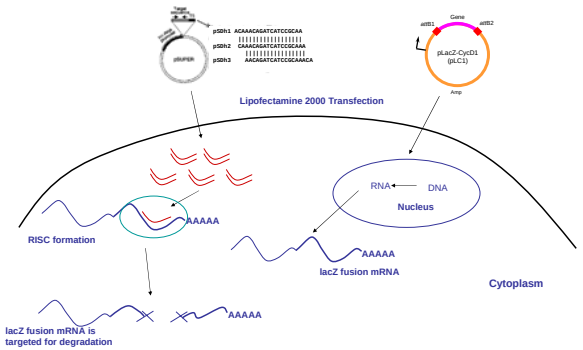


Figure 1. β-galactosidase RNAi Screening System for precise quantification of knockdown effect

Figure 2. Efficiency of shRNA transcribed from pSuper vector

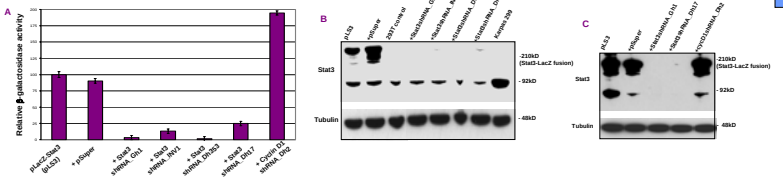


Figure 3. Sensitivity and reproducibility of β-galactosidase assay

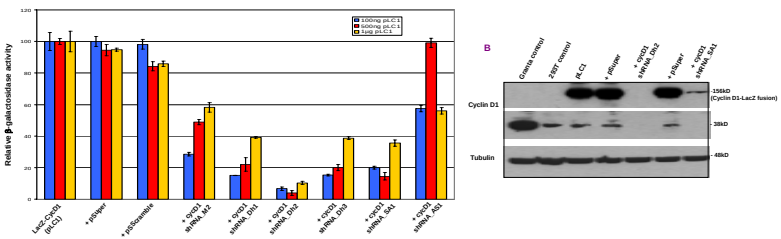


Figure 4. Lentiviral Production

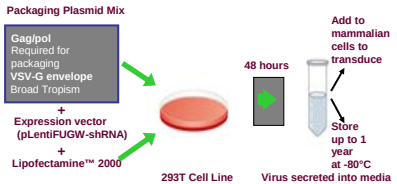
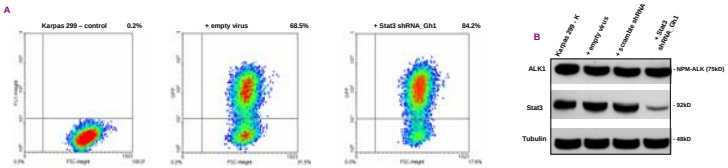


Figure 5. Infection of T-cell lymphoma cells (Karpas 299) with Lentivirus



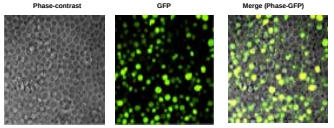
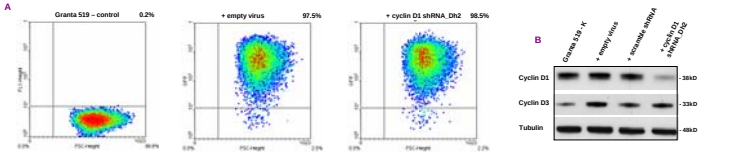


Figure 6. Karpas 299 infected with shRNA virus. GFP expression from viral infections was detected by Laser Scan Microscopy (LSM).

Figure 7. Infection of B-cell lymphoma cells (Granta 519) with Lentivirus



Results

shRNAs against Stat3 are able to specifically silence lacZ-Stat3 reporter expression and endogenously expressed Stat3 in a 293T cotransfection cell model. (Figure 2) **A)** Simultaneous delivery of different shRNAs for Stat3 to the cells with lacZ-Stat3 (pLS3) fusion plasmid induce reduction of β-galactosidase reporter expression activity. The β-galactosidase activity is normalized to 100% in 293T cells transfected only with specific LacZ fusion. pSuper represent empty vector control. Cyclin D1 shRNA_Dh2 was used as independent control for LacZ-Stat3 fusion expression. Cells were analyzed 48h after transfection. **B)** Western blot analysis showed knockdown effect with the specific Stat3 shRNAs on the 210kD fusion protein. Cell extracts were analysed 48h after cotransfection of shRNA expression plasmids with fusion plasmid (pLS3). However, the endogenously expressed Stat3 protein (92kD) was not downregulated. **C)** Since the half-life of Stat3 is approximately 72 hours, we additionally analyzed the cell extracts of 293T cell line 72h after co-transfection with Gh1-shRNA construct, demonstrating the knockdown for both, the fusion (210kD) protein and the endogenously expressed Stat3 protein.

High Sensitivity and reproducibility of β-galactosiadse assay. (Figure 3) **A)** The β - galactosidase activity is normalized to 100% in cell extracts from 293T cells transfected only with lacZ-cyclinD1 fusion (pLC1). We cotransfected 6 different shRNA constructs for cyclin D1, whereas only three show more than 80% reduction in β - galactosidase activity. Cyclin D1 shRNA _Dh1, Dh3 and SA1 were characterized as moderately active molecules with efficiency of 80% and construct pSDh2 was characterized as highly active shRNA with 95% efficiency. The ratio between different shRNA plasmids and reporter fusion (pLC1) in cotransfection varied. Concentration of shRNA plasmids for cotransfection was constant (5µg) and pLC1 was 100ng (blue boxes), 500ng (red boxes) and 1µg (yelow boxes). Cells were analyzed 48h after transfection. **B)** The level of expression of endogenous cyclin D1 from cell extracts prepared from control cell lines Granta and 293T was analyzed with anti-Cyclin D1 antibody. Also the level of expression of reporter fusion from the cells transfected either with lacZ-cyclin D1 fusion (pLC1) or cotransfected with specific shRNA constructs (pSDh2 and pSSA1) was analyzed 48h after transfection. The same membrane was probed with anti-tubulin. The knockdown effect is very high for cyclinD1 shRNA_Dh2, which correlates with the β-galactosidase assay.

Successful Infection of T-cell lymphoma cells (Karpas 299) with Lentivirus (Figure 5-6) **A)** The efficiency of viral infection was analysed detecting GFP expression by FACS. Karpas 299 control cells and infected cells were 90% viable three days after infection and 70-80% infected. **B)** The level of knockdown effect for Stat3 was analysed by Western blot from cell extracts prepared three days after infection. The specific downregulation of Stat3 protein was above 80% in the infected cells and no side effects were seen in the controls.

Successful Infection of B-cell lymphoma cells (Granta 519) with Lentivirus. (Figure 7) **A)** The efficiency of viral infection was analysed detecting GFP expression by FACS. Granta 519 control cells and infected cells were 65% viable three days after infection and 98% infected. **B)** Specific knockdown effect for cyclin D1 was shown by Western blot from cell extracts prepared three days after infection. The specific downregulation of cyclin D1 protein was above 80% in the infected cells and no side effects were seen in the controls.

Conclusions

- The LacZ reporter fusion assay is an excellent method to precisely quantify knockdown efficiency of siRNA-sequences and can be corroborated by Western blot analysis.
- Efficient production of Lentiviruses and high transduction efficiency (up to 98%) were achieved on lymphoid B- and T-cells.
- The pFUGW lentiviral vector with GFP was very useful to quantitate transduction efficiency by flow cyctometry (FACS).
- Successful knockdown effect with shRNA for Stat3 and cyclin D1 was observed in Granta 519 and Karpas 299 cells three days after lentiviral infection.

Further prospects are to analyze the effect of specific protein knockdown on the expression of different cell cycle proteins in lymphoma cell lines by qRT-PCR and Western blot. Also knockdown effect is going to be analyzed for G1/S phase cell cycle progression and apoptosis by flow cytometry (FACS).