Analysis of IL-4 Dependent Gene Expression in Namalwa Cells by siRNA Transfection: An Example of Pathway Analysis Using the Gene Pulser MXcell™ Electroporation System

Claudia M. Litterst,[1] Tobias Münz,[2] Edith Pfitzner,[2] and Luis Ugozzoli[1]
[1]Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA; [2]Department of Biochemistry and Biophysics, Friedrich-Schiller-Universität Jena, Center for Molecular Biomedicine, Hans-Knöll-Straße 2, D-07745 Jena, Germany

Introduction

siRNA-mediated knockdown of gene expression is a powerful functional genomics tool to interrogate signaling pathways in basic research and drug discovery. However, such studies can be technically challenging since they require efficient siRNA delivery to achieve significant reduction of protein levels. In addition, the siRNA transfection procedure must not perturb the cell physiology and alter the signaling pathway under investigation.

Figure 1 shows a simplified overview of the IL-4 signaling pathway. Binding of IL-4 to the IL-4 receptor leads to dimerization of the signal transducer and activator of transcription 6 (STAT6). The activated STAT6 dimer translocates to the nucleus and drives the expression of its target genes, for example, thymus and activation-regulated chemokine (TARC/CCL17, hereafter referred to as CCL17) (Wirnsberger et al. 2006). CCL17 is a high-affinity ligand for the CC chemokine receptor 4 (CCR4 receptor) and acts as a selective chemoattractant for T cells expressing this receptor (Imai et al. 1997, Imai et al. 1999). It has been suggested that CCL17 produced by B cells plays an important role in the pathogenesis of allergic disease (Lin et al. 2003).

The difficult-to-transfect Burkitt lymphoma cell line Namalwa, is a recognized model system to study the IL-4 signaling pathway (Rousset et al. 1988). In this article we describe the development of an optimized electroporation protocol for siRNA transfection of Namalwa cells using the Gene Pulser MXcell electroporation system along with Gene Pulser® electroporation buffer for siRNA transfection, and the application of siRNA-mediated gene silencing in a functional study on IL-4 dependent gene expression.

Methods

Namalwa cells (American Type Culture Collection, ATCC #CRL-1432) were grown in GIBCO RPMI Media 1640 (Invitrogen Corporation) with 7.5% FBS and 1% pyruvate.

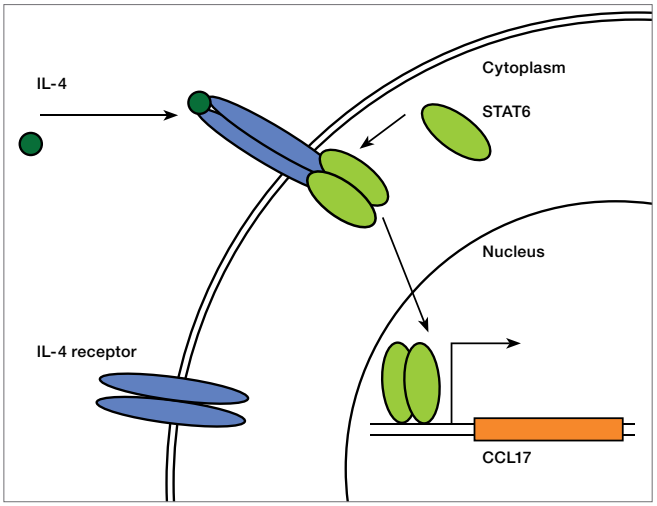

Fig. 1. Overview of the IL-4 signaling pathway. Binding of IL-4 to its receptor leads to dimerization and activation of STAT6. Activated STAT6 translocates to the nucleus where it binds to the promoter region of its target genes, for example, CCL17, and activates transcription.

Recombinant human IL-4 was purchased from R&D Systems, Inc. and was used at 10 ng/ml final concentration. STAT6 siRNAs were purchased from Integrated DNA Technologies, Inc. (IDT); all other siRNAs were from Bio-Rad.

For electroporation, cells were washed in PBS and resuspended in Gene Pulser electroporation buffer (Bio-Rad Laboratories, Inc.) at a density of 5 x 10^6 cells/ml. siRNAs were added at 100 nM final concentration. The cell suspension was mixed and 150 µl suspension/well was transferred to the appropriate wells of a 96-well electroporation plate (Bio-Rad). Electroporations were carried out on the Gene Pulser MXcell electroporation system (Bio-Rad). After electroporation, cell suspensions were immediately transferred to growth media (7.5 x 10^5 cells/ml final concentration) and incubated for 24 hr.

LDH levels in cell culture supernatants were determined using the CytoScan LDH Cytotoxicity Assay (G Biosciences). Relative LDH levels were calculated in reference to untreated lysed cells according to the manufacturer's recommendations. RNA was

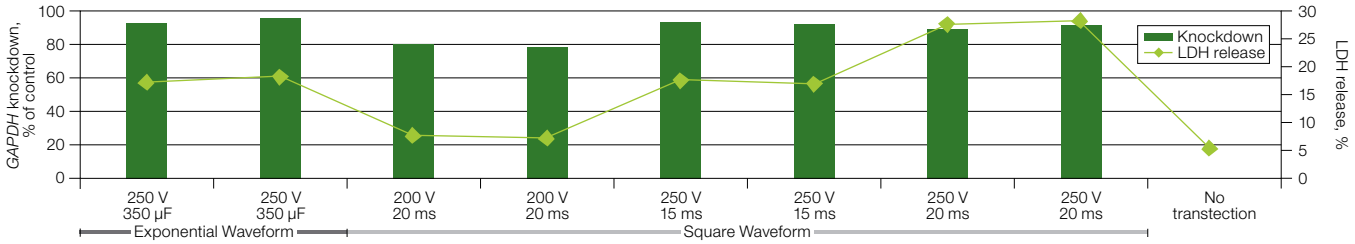

Fig. 2. Optimization of electroporation conditions. Namalwa cells were electroporated with nonsilencing control siRNA and *GAPDH* siRNA using the Gene Pulser MXcell system with the settings at 1000 Ω. Capacitance was set to 1000 µF for square-wave conditions. Twenty-four hours after transfection, cell supernatants were collected and subjected to LDH release assay. Total RNA was prepared from cells and relative levels of *GAPDH* mRNA were determined by RT-qPCR using the CFX384 real-time PCR detection system. Relative *GAPDH* mRNA levels were determined with respect to the levels measured in cells transfected with the nonsilencing control siRNA for each condition.

purified using the Aurum™ total RNA 96 kit, then cDNA was prepared with the iScript™ cDNA synthesis kit (both kits from Bio-Rad). Real-time PCR reactions were performed using iQ™ supermix on the CFX384™ real-time PCR detection system, and data was analyzed using CFX Manager™ software (all from Bio-Rad). Primers for amplification of the actin gene, *GAPDH*, and the CCL17 gene were purchased from IDT. Primer sequences for CCL17 were described previously (Radstake et al. 2005). Primer sequences for *GAPDH* and the actin gene can be obtained upon request. Actin was used as a reference gene for the analysis shown in Figure 2, and *GAPDH* was the reference gene for the experiment shown in Figure 3A.

For western blotting, cells were lysed in lysis buffer (1% Triton, 20 mM Tris-HCl, pH 8, 0.1% SDS, 150 mM NaCl) containing Protease Inhibitor Cocktail Set III (Calbiochem). Protein concentrations were determined using the *DC* protein assay (Bio-Rad). Total protein (50 µg/sample) was separated on 10% Ready Gel® Tris-HCl gels (Bio-Rad) and transferred to PVDF membranes using the Mini-PROTEAN® Tetra system (Bio-Rad). Membranes were probed with BD Transduction Laboratories STAT6 (BD Biosciences) and alpha-tubulin (Sigma-Aldrich, Inc.) antibodies. Western blots were developed using HRP-conjugated secondary antibodies according to standard procedures.

Results and Discussion

Preliminary experiments were first performed to identify optimal electroporation conditions for siRNA delivery into Namalwa cells. A range of settings (shown in Figure 2), that had been previously optimized for electroporation of plasmid DNA in Namalwa cells (Litterst and Ugozzoli 2009), was screened. Namalwa cells were electroporated with *GAPDH* and a nonsilencing control siRNA for each condition. Twenty-four hours after transfection, cytotoxicity and knockdown of *GAPDH* expression were analyzed.

As shown in Figure 2, strong *GAPDH* knockdown (>90%) was obtained with both exponential-decay and square-wave conditions (250 V/350 µF and 250 V/15 ms, respectively). LDH levels (a measure of cell lysis) were comparable for both of these conditions (approximately 17% relative LDH release), indicating that both settings result in efficient siRNA transfection of Namalwa cells with minimal effect on cell viability. Other square-wave conditions were either less efficient with respect to knockdown (200 V/20 ms) or resulted in higher cytotoxicity (250 V/20 ms) and are therefore not recommended for siRNA transfection of Namalwa cells. The exponential-decay condition (250 V/350 µF) was also the optimal setting for DNA transfection (Litterst and Ugozzoli 2009), indicating that once conditions are established for DNA delivery, they can be used for efficient siRNA transfection as well. This condition was subsequently used for our IL-4 dependent gene expression analysis.

Namalwa cells were transfected with GFP siRNA and two STAT6 siRNAs as indicated in Figure 3A. Twenty-four hours after transfection, one set of replicates was treated with recombinant IL-4 and levels of CCL17 mRNA were determined after 24 hr by reverse transcription quantitative PCR (RT-qPCR). As shown in Figure 3A, control samples transfected with GFP siRNA showed similar induction of CCL17 expression (8-fold induction) upon IL-4 treatment as nontransfected cells (11-fold induction), indicating that the electroporation procedure does not significantly interfere with the IL-4 pathway and the cells retain their ability to upregulate CCL17 after siRNA transfection. IL-4 treatment of samples transfected with STAT6 siRNAs induced expression of CCL17 mRNA only 2-fold. To confirm that the reduced response of transfected cells to IL-4 was due to knockdown of STAT6, STAT6 protein levels were analyzed in transfected cells by western blotting. siRNA transfection of STAT6 resulted in almost complete depletion of the STAT6 protein (Figure 3B, lanes 2 and 3) compared to the cells transfected with control siRNA (Figure 3B, lanes 1 and 4) and nontransfected cells (Figure 3B, lane 5), while tubulin protein levels were similar in all samples. These results are in agreement with previous studies showing that IL-4 induced expression of CCL17 is STAT6 dependent (Wirnsberger et al. 2006). Control samples transfected with *GAPDH* siRNA showed an up to 86% reduction of *GAPDH* mRNA after 48 hr (data not shown), indicating that the knockdown was still effective at the time expression of CCL17 was measured.

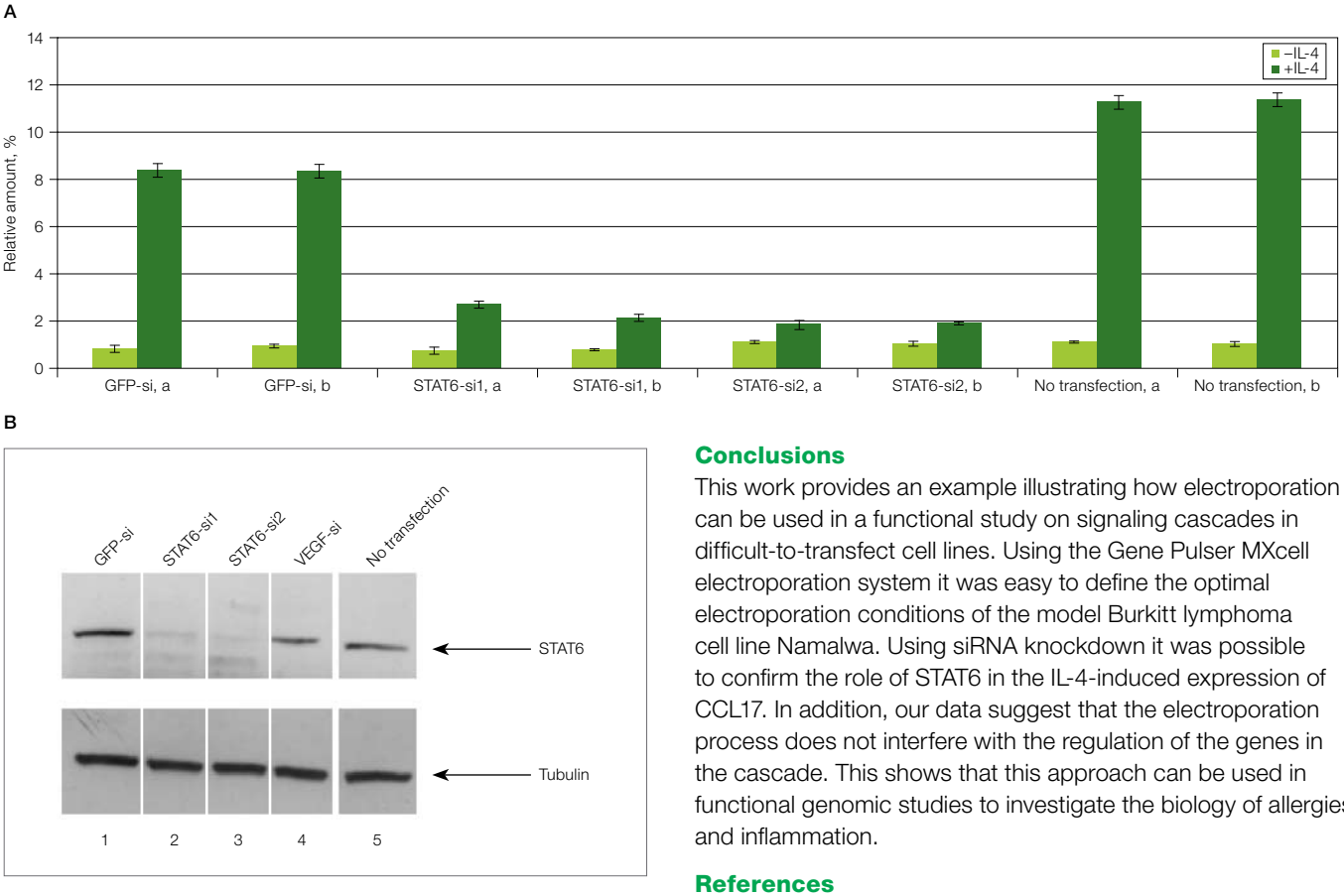

Fig. 3. STAT6 is required for IL-4-induced CCL17 expression. A, cells were transfected with control siRNAs (GFP-si), STAT6 siRNA (STAT6-si1, STAT6-si2), or not transfected as indicated. Each transfection was performed in four replicates. Twenty-four hours after transfection, two samples per set of replicates were treated with IL-4 (■) and two samples were left untreated (■). Forty-eight hours after transfection, RNA was prepared from cells and CCL17 mRNA levels were determined by RT-qPCR. Relative levels of CCL17 expression were calculated with respect to the GFP siRNA-transfected sample without IL-4; **B**, cells transfected with STAT6 (STAT6-si1, STAT6-si2) and control siRNAs (GFP-si, VEGF-si) as indicated in 3A were lysed 48 hr after transfection. VEGF-siRNA was used as an additional unrelated control to monitor variations in protein levels. Total protein extract (50 µg) was resolved by SDS-PAGE. Protein levels of STAT6 and tubulin were analyzed by western blotting using specific antibodies.

Conclusions

This work provides an example illustrating how electroporation can be used in a functional study on signaling cascades in difficult-to-transfect cell lines. Using the Gene Pulser MXcell electroporation system it was easy to define the optimal electroporation conditions of the model Burkitt lymphoma cell line Namalwa. Using siRNA knockdown it was possible to confirm the role of STAT6 in the IL-4-induced expression of CCL17. In addition, our data suggest that the electroporation process does not interfere with the regulation of the genes in the cascade. This shows that this approach can be used in functional genomic studies to investigate the biology of allergies and inflammation.

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