

Knockdown of chimeric glucocerebrosidase by green fluorescent protein-directed small interfering RNA

Tessa N. Campbell and Francis Y.M. Choy

Centre for Biomedical Research Department of Biology,
University of Victoria, Victoria, BC, Canada
Corresponding author: T.N. Campbell
E-mail: tessac@uvic.ca

Genet. Mol. Res. 3 (2): 282-287 (2004)

Received March 10, 2004

Accepted May 3, 2004

Published June 14, 2004

ABSTRACT. Gaucher disease, the most common type of lysosomal storage disorder, is characterized by an inherited deficiency of the membrane-associated hydrolase, glucocerebrosidase. Glucocerebrosidase catalyzes the hydrolysis of glucocerebroside to ceramide and glucose, a crucial step in the recycling of membrane sphingolipids. The exorbitant cost of the current treatment standard for Gaucher disease, enzyme replacement therapy, prevents many from receiving treatment. This limitation has led to a wide-spread search for more efficient and cost-effective methods of protein production and alternate therapies, resulting in a closer examination of glucocerebrosidase biosynthesis and current treatment techniques. The use of specific small interfering RNAs (siRNAs) to knock down target genes is an attractive option for studying such processes, though a glucocerebrosidase-specific siRNA has yet to be reported. We note, however, that green fluorescent protein (GFP)-directed siRNAs can not only provide a positive control to test siRNA delivery and system integrity, but also serve as a means to knock down a fusion partner without having to design siRNAs specific to the partner. After effectively co-transfecting COS-1 cells with enhanced GFP (EGFP)-tagged glucocerebrosidase constructs and GFP-directed siRNAs,

we report successful knockdown of all EGFP-containing constructs at both the RNA and protein levels. This provides a method of examining enzyme biosynthesis and treatment options. Furthermore, this technique is applicable to other systems, since we have demonstrated the usefulness of GFP as a siRNA target in mammalian cells when fused to another gene of interest.

Key words: Green fluorescent protein, Glucocerebrosidase, siRNA, RNAi

INTRODUCTION

Gaucher disease is an autosomal recessive disorder characterized by a heterogeneous set of signs and symptoms caused by the defective lysosomal hydrolysis of glucocerebroside and related glucosphingolipids. This defective hydrolysis results from dysfunction of the enzyme glucocerebrosidase (GBA; glucosylceramidase, acid β -glucosidase, EC 3.2.1.45) which acts as the penultimate step in conversion of glucocerebroside to glucose and ceramide (Beutler and Grabowski, 2001). Accumulated glucocerebroside is taken-up by macrophages and subsequently deposited in the spleen, liver, and bone marrow. Histological examination of affected tissues reveals lipid engorged macrophages (Gaucher cells) which lead to the disruption of normal organ function. The resultant hepatosplenomegaly, bone crises, and pancytopenia are the characteristic symptoms of the disease. Gaucher disease is currently the most common lysosomal storage disorder and the first to be successfully treated by enzyme replacement therapy (de Fost et al., 2003). However, the exorbitant cost of such therapy prevents many from receiving treatment (Grabowski et al., 1998). This limitation has led to a wide-spread search for more efficient and cost-effective methods of protein production and alternate therapies, resulting in a closer examination of GBA biosynthesis and current therapeutic techniques.

The discovery and manipulation of RNA-mediated interference (RNAi), a term coined by Fire et al. (1998), has presented the scientific community with new ways to examine old mechanisms. RNAi is a multi-step process involving the generation of small interfering RNAs (siRNAs) *in vivo* through the action of the RNase III endonuclease Dicer. The resulting 21-23 nucleotide siRNAs mediate degradation of their complementary RNA (Hannon, 2002; Shi, 2003). One successful siRNA target of interest to researchers from many different backgrounds is the *Aequorea victoria* green fluorescent protein (GFP). GFP has been fused to numerous other proteins and peptides, thus serving as a visible molecular reporter (Tsien, 1998). Since GFP has been effectively knocked down by specific siRNAs, it could also potentially serve as the target segment in a chimera, permitting knockdown of the partner-of-interest without having to design specific siRNAs for the partner. Because the prediction and selection of successful siRNAs for any target gene is still not fully developed, many time-consuming trial-and-error experiments are often required to eventually knock down the gene of interest. Hence, targeting GFP in a chimera instead of the partner of interest could save both time and resources. Therefore, we examined the use of enhanced GFP (EGFP) as a siRNA target for the knockdown of fused GBA as a method to examine enzyme biosynthesis and treatment options for Gaucher disease.

MATERIAL AND METHODS

To create EGFP-tagged GBA constructs beginning at either the first (GBA1) or the second (GBA2) ATG initiation site, template DNA was PCR-amplified with primers A and B for GBA1 and C and B for GBA2 (Table 1). PCR amplification was performed with an initial denaturation at 94°C for 2.5 min, followed by 28 cycles of 94°C for 1 min, 58°C for 1.5 min, and 72°C for 1.5 min. Reactions were carried out with 1 µl GBA cDNA template, 1 µl *Pfu* polymerase, 5 µl 10X PCR buffer, 5 µl 25 mM MgCl₂, 5 µl 2.5 mM dNTPs and 4 µl forward and reverse primers in a total volume of 50 µl. These amplified cDNAs were digested with *Eco*RI and inserted in-frame upstream of EGFP in the vector pEGFP-N1 (Clontech, Palo Alto, CA, USA). Transformants were selected by resistance to 30 µg/ml kanamycin on Luria Bertani plates. Clones were confirmed by PCR amplification and sequenced to ensure the fidelity of the PCR and cloning steps.

Table 1. Primers used in the construction and screening of glucocerebrosidase (GBA) and enhanced green fluorescent protein (EGFP) expression plasmids in COS-1 cells.

Primer	DNA	Sequence (5' to 3')	Location in cDNA ¹	Orientation
A	GBA	ACTCGAATTCTTCATCTAAGGACCCTGAGG	minus 32-minus 3	sense
B	GBA	ATCGAATTCGTCCTGGCGATGCCAC	1621-1596	antisense
C	GBA	CCTGTGAGGCTGCCAGCCATGAATTCGGTA	51-80	sense
D	EGFP	ATGGTGAGCAAGGGCGAGGAGCTGT	679-703	sense
E	EGFP	TACAGCTCGTCCATGCCGAGAGTGATCC	1391-1364	antisense

¹The first base of the GBA upstream initiator codon is designated #1 (Sorge et al., 1987). Numbers for EGFP correspond to those in Clontech manuals (Palo Alto, CA, USA).

True positive pGBA1-EGFP and pGBA2-EGFP clones were isolated and purified prior to co-transfection with either control *Thermotoga merittimia* siRNA (section 21 to 136 of the complete genome; sense:UUCUCCGAACGUGUCACGUTT, antisense: ACGUGACACGUU CGGAGAATT; QIAGEN, Valencia, CA, USA) or *Aequorea victoria* GFP siRNA (base pairs 122-143; sense:GCAAGCUGACCCUGAAGUUCAU, antisense: GAACUUCAGGGUCAG CUUGCCG; QIAGEN; Caplen et al., 2001). Transfection of 80% confluent COS-1 cells in 12-well plates with TransMessenger™ (QIAGEN) was performed according to the manufacturer's modified instructions for DNA/RNA co-transfections.

RNA was isolated at 48 h post-transfection with the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's directions. RT-PCR was performed using the SuperScript™ system (Invitrogen, Carlsbad, CA, USA) with EGFP primers D and E (Table 1). RNA was also analyzed by Northern blotting using a full-length EGFP cDNA probe. Protein expression was visualized every 24 h with both a Zeiss LSM 410 confocal microscope and an epifluorescence microscope.

RESULTS

RT-PCR results indicated transcript presence for all pEGFP-N1, pGBA1-EGFP, and pGBA2-EGFP co-transfected cultures. Negative controls showed no amplification (Figure 1). Northern blotting revealed bands of cross-reactivity for all expected cultures when co-transfected with control siRNA, but decreased band intensity for pEGFP-N1 when co-transfected

with GFP siRNA. No bands were visible for either glucocerebrosidase-containing construct when co-transfected with GFP siRNA. Green fluorescence was regularly observed in all pEGFP-N1-expressing cultures at each time point. Co-transfection of pEGFP-N1 with GFP siRNA resulted in markedly decreased fluorescence (Figure 2). No fluorescence was observed at any time in mock-transfected COS-1 cells. With respect to pGBA1-EGFP and pGBA2-EGFP, green fluorescence was visualized at 48-72 h post-transfection with decreased intensity and numbers of fluorescing cells noted when co-transfected with GFP siRNA (Figure 2).

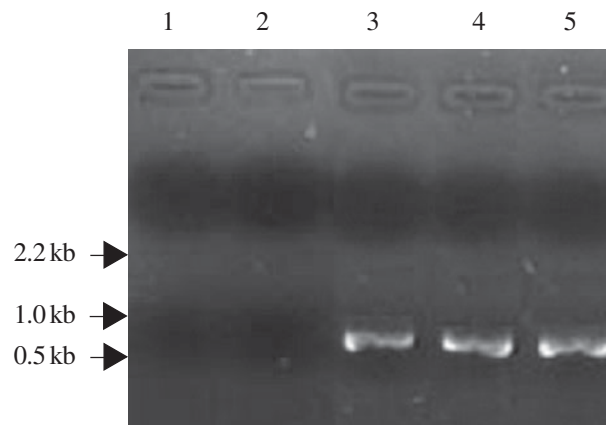


Figure 1. Glucocerebrosidase (GBA) and enhanced green fluorescent protein (EGFP) RNA isolated from COS-1 cells at 48 h post-transfection, subjected to RT-PCR and electrophoresed in a 0.7% agarose gel. From left to right (lanes 1-5): negative control (no reverse transcriptase added), COS-1 cells, pEGFP-N1, pEGFP•GBA1, pEGFP•GBA2.

DISCUSSION

Current standard treatment for Gaucher disease, the most common lysosomal storage disorder, is based upon enzyme replacement. Unfortunately, this treatment is exorbitantly expensive, thus preventing many patients from receiving such therapy. This limitation has led to a wide-spread search for more efficient and cost-effective methods of protein production and alternate therapies, resulting in a closer examination of glucocerebrosidase biosynthesis and current treatment techniques. The use of specific siRNAs to knock down target genes is an attractive option for studying such processes, though a GBA-specific siRNA has yet to be reported. One successful siRNA target of interest, however, is the *Aequorea victoria* GFP. GFP has been fused to numerous other proteins and peptides, thus serving as a visible molecular reporter (Tsien, 1998). Our research indicates that GFP-directed siRNAs not only provide a positive control to test siRNA delivery and system integrity, but also serve as a means to knock down a fusion partner without having to design siRNAs specific to the partner. After effectively co-transfecting COS-1 cells with EGFP-tagged GBA constructs and GFP-directed siRNAs, we report successful knockdown of all EGFP-containing constructs at both the RNA and protein levels. In the case of Gaucher disease, this method will prove useful when examining the synthesis/trafficking of normal and mutant versions of GBA in Gaucher fibroblasts, as well as the impact and localization of introduced recombinant enzyme in transgenic knockout models. In other systems whereby no siRNA has been specifically designed for a desired gene with little or no endogenous expression, a chimeric GFP fusion gene can be utilized, providing a target for temporal or localized knockdown.

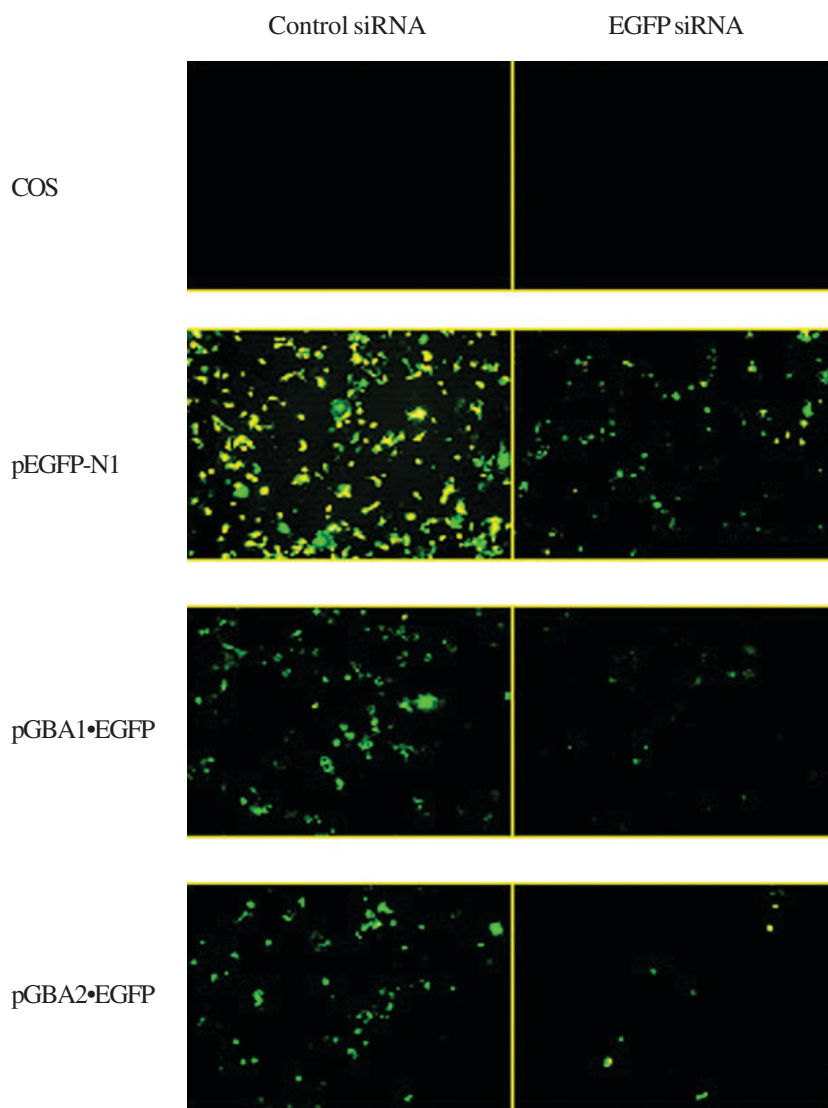


Figure 2. Glucocerebrosidase (GBA) and enhanced green fluorescent protein (EGFP) expression visualized with a Zeiss epifluorescence microscope at 20X magnification in COS-1 cells 72 h following co-transfection with small interfering RNAs (siRNAs).

ACKNOWLEDGMENTS

Research supported by Natural Sciences and Engineering Research Council grant #138216-01 to Francis Choy and a Scottish Rite Charitable Foundation of Canada award to Tessa Campbell.

REFERENCES

Beutler, E. and Grabowski, G.A. (2001). Gaucher disease. In: *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds.). McGraw-Hill, New York,

- NY, USA, pp. 3635-3668.
- Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A.** (2001). Specific inhibition of gene expression by small double stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* 98: 9742-9747.
- de Fost, M., Aerts, J.M.F.G. and Hollak, C.E.M.** (2003). Gaucher disease: from fundamental research to effective therapeutic interventions. *Neth. J. Med.* 61: 3-8.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C.** (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806-811.
- Grabowski, G.A., Leslie, N. and Wenstrup, R.** (1998). Enzyme therapy for Gaucher disease: the first 5 years. *Blood Rev.* 12: 115-133.
- Hannon, G.J.** (2002). RNA interference. *Nature* 418: 244-251.
- Shi, Y.** (2003). Mammalian RNAi for the masses. *Trends Genet.* 19: 9-12.
- Sorge, J.A., West, C., Kuhl, W., Treger, L. and Beutler, E.** (1987). The human glucocerebrosidase gene has two functional ATG initiator codons. *Am. J. Hum. Genet.* 41: 1016-1024.
- Tsien, R.Y.** (1998). The green fluorescent protein. *Annu. Rev. Biochem.* 67: 509-544.