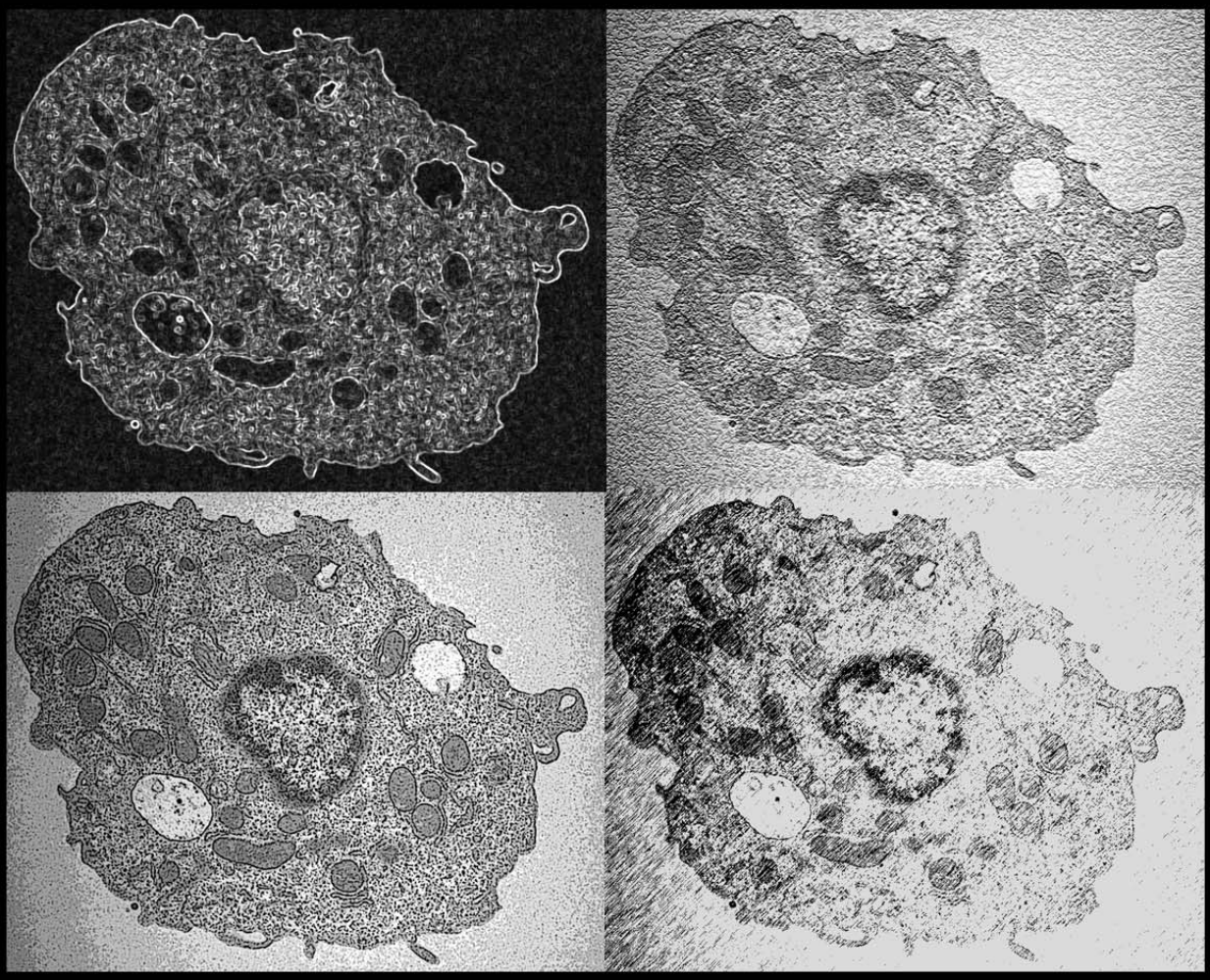
Caracterització de mutacions causants de la malaltia de Gaucher. Aproximació a una teràpia gènica.



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Resultats

CAPíTOL 4

UTILITZACIÓ DE TÈCNIQUES D'INTERFERÈNCIA D'RNA COM A POSSIBLE TERÀPIA DE LA MALALTIA DE GAUCHER

4.1. Inhibició de la Glucosilceramida Sintasa (GCS) mitjançant la utilització de siRNAs

Una de les teràpies que actualment s'està utlitzant en la malaltia de Gaucher és la teràpia de reducció de substrat com a complement o alternativa a la teràpia de reemplaçament enzimàtic. La teràpia de reducció de substrat es basa en la inhibició parcial de la glucosilceramida sintasa (GCS), enzim encarregat de sintetitzar glucosilceramida a partir de ceramida i glucosa. D'aquesta manera s'evita l'acúmul de glucosilceramida que provoca la malaltia.

En els darrers anys, els siRNAs (small interference RNAs) s'han utilitzat en diferents tipus d'experiments per inhibir l'expressió gènica. L'objectiu d'aquest treball és la inhibició de la expressió del gen de la *GCS* mitjançant siRNAs com una possible alternativa terapèutica per a la malaltia de Gaucher.

Vam dissenyar quatre siRNAs per a inhibir el gen *GCS* humà i els vam transfectar en cèl·lules HeLa. Amb dos d'aquests siRNAs vam aconseguir reduir la expressió de *GCS* a nivell de RNA. També vam comprovar la reducció tant a nivell d'activitat enzimàtica com de formació de glucosilceramida.

Després d'aconseguir silenciar el gen de la *GCS* utilitzant siRNAs, vam decidir probar-ho utilitzant shRNAs (short-hairpin RNAs). Els shRNAs són siRNAs generats per un plasmidi on se li ha insertat la seqüència que s'ha de transcriure per formar aquest shRNA. Vam generar dos shRNAs diferents aconseguint inhibir també l'expressió de *GCS*.

Pensant en una futura aplicació en animals models vam voler posar a punt la inhibició del gen Ugcg (homòleg de GCS en ratolí) en cèl·lules de ratolí. Utilitzant siRNAs vam aconseguir també inhibir la expressió d'aquest gen.

Treball sotmès a publicació:

RNAi-mediated inhibition of the glucosylceramide synthase (*GCS*) gene: a possible therapeutic strategy for Gaucher disease.

RNAi-mediated inhibition of the glucosylceramide synthase (*GCS*) gene: a possible therapeutic strategy for Gaucher disease

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Running title: RNAi-mediated inhibition of the GCS gene

ABSTRACT

In the last few years, small interference RNAs (siRNAs) have been used in a number of different experimental settings to silence gene expression. In some of them, chemically synthesized or *in vitro* transcribed siRNAs have been transfected into cells. In others, siRNAs have been expressed endogenously from siRNA expression vectors. Enzyme replacement and substrate deprivation therapies are the current approaches to treat Gaucher disease. Although good results have been reported there are several limitations and side effects that make interesting to search for new alternatives. We present here a new approach based on the inhibition of the *GCS* gene using siRNAs as a possible future therapeutic strategy for Gaucher disease. We have designed four siRNAs for the human *GCS* gene and transfected them into HeLa cells. With two of them, a clear reduction of *GCS* RNA levels and enzyme activity was obtained. Consistently, a reduction in glucosylceramide synthesis was observed. Similar results were obtained when plasmids expressing shRNAs (targeting the same sequences) were transfected into the cells. The inhibition of the mouse homolog *Ugcg* gene was also achieved, using a siRNA that targeted both human and mouse sequences. In summary, we have shown that siRNAs are a good tool to silence *GCS* gene expression and, thus, to reduce glucosylceramide formation. This success at the cellular level, including human and mouse cells, should be followed by experiments in animal models to develop a new therapeutic strategy for Gaucher disease.

OVERVIEW SUMMARY

In the last few years, small interference RNAs (siRNAs) have been used in a number of different experimental settings to silence gene expression. Enzyme replacement and substrate deprivation therapies are the current approaches to treat Gaucher disease. Although good results have been reported there are several limitations and side effects that make interesting to search for new alternatives. We present here a new approach based on the inhibition of the *GCS* gene using siRNAs as a possible future therapeutic strategy for Gaucher disease. A clear reduction in *GCS* RNA levels, enzyme activity and glucosylceramide synthesis was achieved. Similar results were obtained when using plasmids expressing shRNAs. Also, siRNAs to inhibit the mouse homolog *Ugcg* gene were successfully assayed.

INTRODUCTION

The glycosphingolipid (GSL) lysosomal storage disorders are a group of inherited genetic diseases caused by mutations in the genes coding for enzymes involved in GSL catabolism. These diseases are characterised by the accumulation of the GSL substrates within lysosomes of cells, which results in cellular dysfunction and damage. The most common of the glycosphingolipidoses is Gaucher disease (GD), an autosomal recessive trait due to the accumulation of glucosylceramide caused by deficient activity of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45). The main clinical symptoms are progressive anaemia, thrombocytopenia, hepatosplenomegaly and skeletal disease in the presence (types II and III) or absence (type I) of central nervous system involvement.

Several disease-specific therapies have been developed. These therapies include bone marrow transplantation, enzyme replacement therapy (ERT) or gene therapy, all three focused on the partial recovery of the enzyme activity. ERT has proved successful for the treatment of most type I GD patients (Beutler *et al.*, 1991). However, some limitations and disadvantages of ERT, such as the lack of effect on neurological symptoms, the high cost or the lifelong dependence on frequent infusions have prompted the search for new therapeutic strategies. In the last years a new treatment for Gaucher disease, substrate reduction therapy (SRT), proved to be efficient (Cox *et al.*, 2000) and was approved for particular cases. It is based on the partial inhibition of the amount of synthesis of the substrate (glucosylceramide) by *N*-alkylated iminosugar analogues to a level that can be effectively cleared by the affected enzyme with residual hydrolytic activity. These compounds act on glucosylceramide synthase (GCS) or UDP-glucose ceramide glucosyltransferase (UGCG), EC 2.4.1.80, which catalyses the first step in GSL synthesis. In particular, the iminosugar *N*-butyldeoxynojirimycin (*N*B-DNJ; OGT 918, referred to as miglustat) has been through clinical trials (Cox *et al.*, 2000) and is the one approved for treatment (Weinreb *et al.*, 2005). However, some annoying side effects have also been reported.

Other experimental approaches have been tested. On one hand, some inhibitors as those mentioned above seem to act as chaperons and, thus, to assist protein folding and stability of mutant GBA enzymes (Sawkar *et al.*, 2002). On the other hand, gene therapy attempts, reached the clinical trials with disappointing results (Becker, 2005). However, research in this field is still active to find methodological improvements that might make gene therapy feasible (Cheng and Smith, 2003). Other approaches, such as the use of chimeraplasts for gene repair, have been shown to be unsuccessful to correct GD mutations (Diaz-Font *et al.*, 2003).

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs), a type of posttranscriptional gene silencing, has been used to mediate the down-regulation of gene expression. This methodology has a wide range of possible applications including those with a potential therapeutic effect. In this respect, research projects focused on the treatment of viral, oncological, or neurological diseases have been reported (for a review see Uprichard, 2005).

In this study, this technology is applied to inhibit *GCS* gene expression. We analyzed the effect of different siRNAs and, with some of them, a clear reduction in GCS mRNA level was achieved. A decrease in the activity of the enzyme and in the GlcCer formation was also observed.

MATERIALS AND METHODS

Design and synthesis of siRNAs

Four siRNAs (siRNA11, siRNA34, siRNA48 and siRNA68) designed to inhibit the human *GCS* mRNA (GenBank accession no. NM_003358) were selected according to previously described guidelines (Elbashir *et al.*, 2002; Ambion Target finder and Design Tool): a sequence of the type AA(N19)UU, with a G/C content between 30-50%, and not found in other genes or with the shortest homology region with other genes searched in Blast (http://www.ncbi.nlm.nih.gov/BLAST/). The situation of the siRNAs on the predicted secondary structure of the GCS mRNA was determined using Michael Zucker Mfold program (http://bioinfo.rpi.edu/applications/mfold/old/rna). The 21-nucleotide double stranded RNAs were synthesized by *in vitro* transcription (*Silencer siRNA Construction Kit*; Ambion). The oligonucleotides used are listed in Table 1. The siRNAs for human *GAPDH*, used as a control, were synthesized using primers provided in the *Silencer siRNA Construction Kit* (Ambion). siRNAs

were quantified measuring by absorbance at 260 nm and their double-stranded nature was tested in 1% agarose gel by ethidium bromide staining.

shRNA expression vectors

Short hairpin RNA (shRNA) expression vectors were constructed using the oligonucleotides GCS11_F, GCS11_R, GCS68_F and GCS68_R described in Table 1. They were annealed to form double-stranded DNA fragments and inserted into the *Acc65*I and *Hind*III sites of the psiRNA-hH1-Zeo plasmid (Invivogen San Diego, CA, USA), containing a GFP:Zeo fusion gene which allows GFP detection and Zeocin™ (Invitrogen) selection.

Cell culture and transfection

HeLa, WEHI-3B and RAW264.7 (the two latter were a gift from Dr. Celada) cells were cultured in DMEM medium (Gibco-BRL) containing 100 U/ml penicillin/streptomycin (Gibco-BRL), and 10% fetal calf serum (FCS) at 37ºC and 5% CO_2. Cells were seeded in 6-well culture plates at a density of 1.3 x 10^5 cells per well (30% confluency) 18 hours prior to transfection. The reagent used to transfect was Lipofectamine (Invitrogen), according to the manufacturer's instructions. For stable transfection, HeLa cells were selected with 400 µg/ml of Zeocin™ (Invitrogen) during 3 weeks.

For fluorescence microscope examination of the stable transfection, cells were washed twice in PBS and fixed for 5 min in 4% paraformaldehyde at RT, and the nucleus was stained with DAPI (Sigma) 1:200 in PBS for 15 min at RT. The coverslips were mounted in Vectashield® mounting medium H-1000 (Vector) for fluorescence.

RNA isolation and Reverse Transcription

Total RNA from HeLa cells was isolated using the *QIAshredder™ homogenizer (*Qiagen*)* and the *RNeasy Mini Kit (*Qiagen*)*. Reverse transcription was performed using 200 U *M-MLV Reverse Transcriptase, Rnase H Minus, Point Mutant (Promega)*, 1 μg of total RNA, and 1 μg of oligo-dT for 1h at 42ºC and 10 min at 70ºC.

Real Time PCR

The quantification of human *GCS* mRNA and murine *Ugcg* mRNA was performed by Real Time PCR. The following Assays-on-Demand™ Gene Expression Products (TaqMan® MGB probes, Applied Biosystems) were used: for human GCS, assay ID Hs00234293_m1; for human beta actin, assay ID Hs99999903_m1; for murine Ugcg, assay ID Mm00495925_m1; and for murine beta-2-microglobulin, assay ID Mm00437762_m1. The analysis was performed in the ABI PRISM®7700 Sequence Detection System. Results are presented as ratios of *GCS* mRNA to beta-actin mRNA levels.

Radio-labelled RT-PCR

The *GCS* mRNA was analyzed by semiquantitative RT-PCR. PCR was performed using 1 U Taq DNA Polymerase (Promega), 5 pmols of each *GCS* primer and 2.5 pmols of each *GAPDH* primers used as an endogenous control (Table 1), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.1 μl ^{32} -P dATP (10μCi/μl) and 5-10 ng of HeLa cells cDNA in the recommended buffer (final volume 25 μl). PCR conditions were as follows: initial denaturation at 94ºC for 2 min, 25 cycles of denaturation at 94ºC for 40s, annealing for 30s at 56ºC and extension for 30s at 72ºC. Samples were analysed in a 5% polyacrilamide gel electrophoresis (PAGE), gel were dried and visualised in a Molecular Imager FX (BioRad). Quantification was performed using the *Quantity One* ® (BioRad) software.

GCS activity assay

HeLa cells were cultured in 60 mm plates, harvested at 72 h and resuspended in phosphate-buffered saline (PBSx1). Cell free extracts were prepared by sonication and total protein quantified by the Bradford assay (Bradford, 1976). The assay for GlcCer synthase activity was performed according to the methods of Shukla and Radin (Shukla and Radin, 1990) and Egido-Gabás *et al.* (2005) with modifications. Cell-free extracts (500 μg of protein) were incubated with 50 μM [^{14}C]UDP-glucose (20 nCi), 100 μM of N-Octanoyl-D-sphingosine (C8-ceramide, Sigma), 100 μM BSA, 16 mM NAD^+, in a total volume of 200 μl at 37°C (40 min). For activity assays in the presence of inhibitors, cell-free extracts were incubated with CBE 1,25 mM or *N*B-DNJ 125 μM. Every sample was also assayed without ceramide as a negative control.

Lipids were extracted by adding 500 µl of chloroform-methanol (2:1, v/v). The mixture was centrifuged for 3 min at 10,000 rpm. The lower organic phase was washed with 500 µl chloroform-methanol-0.1M KCl (3:47:48, v/v), and the [^{14}C]UDP-glucose incorporation was determined by liquid scintillation spectrometry using a Wallac 1410 (Pharmacia) liquid scintillation counter.

Thin Layer Chromatography (TLC)

Lipid extraction was performed as described above and, afterwards, samples were resuspended in chloroform. Samples (normalized for protein content) were spotted onto thin-layer chromatography silica plates (Merck) and resolved in chloroform-methanol-water (65:25:4, v/v). To localize glucosylceramide, galactosylceramide was added in every sample as a marker and non-radioactively detected by Molish spraying. Plates were air dried and visualised in a *Molecular Imager FX* (BioRad). Quantification was performed using the *Quantity One* ® (BioRad) software.

RESULTS

Downregulation of GCS mRNA level by siRNAs

One of the critical steps for siRNA-mediated inhibition is the efficient transfection of siRNA duplexes. Different reagents were tested, such as siPORT *Amine* and siPORT *Lipid* (AMBION), -data not shown- and the best results were obtained with Lipofectamine (Invitrogene).

HeLa cells were transfected with 40 nM of the four different GCS-siRNAs. After 24 h, total RNA was extracted and cDNA was synthesized by reverse transcription. Quantification of *GCS* mRNA by Real Time PCR (Fig. 1A) revealed that 2 of the 4 siRNAs tested, siRNA-11 and siRNA-68, were able to considerably reduce the expression of *GCS* in HeLa cells (3 and 4-fold, respectively). On the contrary, siRNA-34 and siRNA-48 showed no effect. The situation of the target sequences of these siRNAs on the predicted secondary structure of the *GCS* mRNA are depicted in Figure 1B.

To analyze the dose-dependent effect on the reduction produced by siRNA-11 and siRNA-68, different amounts of these siRNAs were transfected (Fig. 2A). Real Time PCR results showed that siRNA-11 significantly reduces *GCS* mRNA levels at 8 nM and reaches the maximum inhibition at 16 nM while siRNA-68 needs 32 nM or 40 nM to exert a similar effect. Values are means of four independent experiments.

The long-lasting effect of siRNA-dependent inhibition of *GCS* mRNA levels was addressed by performing the analysis at different times. A similar reduction of *GCS* expression was observed at 24h, 48h and 72h (data not shown). At longer times no clear reduction was observed.

The inhibitory effect of the siRNAs was also shown by semiquantitive radio-labelled RT-PCR experiments. Figure 2B shows one of these experiments for siRNA-11 and siRNA-68, using *GAPDH* mRNA as an endogenous control. Levels of *GCS* mRNAs were quantified. The values, expressed as percentage of the mean of the two control samples, were for siRNA-11: 8 nM, 24.12%; 16 nM, 21.62%; 24 nM, 18.22%; 32 nM, 17.19%; 40 nM, 22.46%; and for siRNA-68, 32 nM 31.05%; 40 nM, 11.45%.

As a control experiment to confirm the specificity of siRNAs, GAPDH-siRNA was transfected in HeLa cells. *GCS* mRNA level was quantified in these transfected cells both by Real Time PCR (using beta-actin as endogenous controls) and by radio-labelled RT-PCR. Results of both Real Time PCR (not shown) and RT-PCR (Fig. 2C) showed that the GAPDH-siRNA was unable to reduce *GCS* expression.

Reduction of GCS enzyme activity and Glc-Cer formation

To analyze the siRNA-dependent reduction of GCS enzyme activity, HeLa cells were harvested 72 h after siRNA transfection, total protein was extracted, and the GCS activity

assayed. *N*B-DNJ, a GCS inhibitor, was used on untransfected cells, as a control and for comparison.

Figure 3A shows GCS activity from HeLa cells transfected with different amounts of siRNA-11 (8-80 nM final concentration). Any amount of siRNA-11 used is able to reduce GCS activity. The transfection of 8 or 16 nM of siRNA reduces control activity in approximately 1.7-fold (56% ± 0.9 and 60% ± 7.5 respectively), while transfections of 24 or 32 nM produce a 3.2-fold reduction of GCS activity (32% ± 1.7 and 31% ± 4.0, respectively). These samples present a reduction of the activity similar to that obtained with *N*B-DNJ treatment. The activity shown by cells transfected with 40, 56 or 80 nM of siRNA is negligible.

To detect the glucosylceramide (Glc-Cer) synthesized in the activity assays, a thin layer chromatography (TLC) was performed (Fig. 3B). HeLa cells transfected with 40 nM of siRNA-11 showed a clear reduction of Glc-Cer formation compared with control HeLa cells. Cells without ceramide were used as negative control. Quantification of the TLC plate shows that the siRNA-transfected cells produced approximately 20-fold less Glc-Cer than untransfected cells.

Downregulation of GCS expression by shRNAs

The reduction of gene expression by RNA interference can be achieved using synthetic siRNAs, as described above, or by shRNAs coded in a DNA expression vector. The advantage of the shRNAs is that stable transfected cells with a permanent reduction of the expression of the target gene can be selected.

Based on siRNAs results, two different shRNAs expression vectors were constructed (shRNA-11 and shRNA-68), using the psiRNA-hH1 vector. The *GFP* gene included in the plasmid allowed the detection of the selected cells. As observed in Figure 4, all selected cells show expression of the GFP as expected.

To determine the effect of shRNAs at the mRNA level, radio-labelled RT-PCR was performed using GAPDH as endogenous control (Fig. 5A). Both, shRNA-11 and shRNA-68 moderately inhibited *GCS* expression. While, shRNA-11 produced a 49% reduction, the reduction obtained with shRNA-68 was about 65%.

Similar levels of reduction were obtained when GCS activity was measured (Fig. 5B). Cells transfected with shRNA11 showed a 58% of GCS activity compared to that of wild-type, while those transfected with shRNA-68 had a GCS activity of 50% of wild-type. Cells transfected with the vector alone (psiRNA) showed an activity of 75% of wild-type. The inhibition was confirmed measuring the levels of glucosylceramide formation by TLC (Fig. 5C), in which the values for the different transfections were: psiRNA (vector without insert) 81% of wild-type, shRNA-11 74% and shRNA-68 53%.

Downregulation of Ugcg expression in mouse cell line

The mouse *Ugcg* gene is the orthologue of the human *GCS* gene. Using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/) the four siRNAs previously designed for human studies were localized on the *Ugcg* sequence. Only the target sequence of the siRNA-68 completely matched the mouse sequence.

We decided to test this siRNA in mouse macrophage cell lines (RAW264.7 and WEHI-3B). The lack of consistent results with the WEHI-3B cells in the preliminary experiments prompted us to give up using these cells for the rest of the work.

siRNA-34 (which has 16 out of 20 nucleotides homologous to the mouse sequence) was used as a negative control. This siRNA was unable to reduce *Ugcg* expression in RAW264.7 cells (data not shown) as expected.

RAW264.7 were transfected with different amounts of siRNA-68 and *Ugcg* RNA levels were quantified by Real Time PCR, 24 hours after transfection (Fig. 6). A reduction of about 60-70% was obtained for all siRNA concentrations. Similar levels of inhibition were still observed after 48 hours (data not shown).

DISCUSSION

RNA interference (RNAi) is a natural occurring post-transcriptional gene silencing process, mediated by dsRNAs. It was discovered in *Caenorhabditis elegans* by Fire *et al.* (1998) and showed to be present in Protozoa and almost all eukaryotes tested (Dorsett and Tuschl, 2004). In the last years, it has been adopted as a powerful tool to reduce gene expression both for basic studies and to develop novel therapeutic strategies (Uprichard, 2005). For the application in mammals, since long exogenous dsRNAs cause apoptotic cell death by an interferon response (Lee *et al.*, 1997; Stark *et al.*, 1998), the introduction of small interfering effector molecules, siRNAs, 21-23 bp long, is necessary (Elbashir *et al.*, 2001). Alternatively, and in an attempt to overcome one of the main limitations of the use of siRNAs, i.e. the transient nature of siRNAs transfection, different groups have developed plasmids in which siRNAs are expressed as fold-back stem-loop structures (shRNAs) that give rise to siRNAs after intracellular processing (Tuschl, 2002 and references therein).

The first step to perform an RNAi approach to silence gene expression is to design siRNAs for specific target sequences. The siRNAs used in our work were designed according to guidelines previously reported (Elbashir *et al.*, 2002; Ambion web site). The superior efficiency of siRNA-11 over the other siRNAs tested may reflect its ability to target longer regions of stable internal loops (see Fig. 1B), a result that is consistent with the recent analysis by Schubert *et al.* (2005) on siRNA efficacy.

Previous studies demonstrated that off-target gene regulation was also detectable at high concentration of siRNAs (Jackson *et al.*, 2003). SiRNA concentration of 100 nM causes a nonspecific induction of a larger number of common genes, including some apoptosis-related and stress-response genes (Semizarov *et al.*, 2003). For this reason, although at 40 nM siRNA-68 seems to be slightly more efficient than siRNA-11 (Fig. 1A), we decided that siRNA-11 was better since it is efficient at lower concentrations (Fig. 2A).

GCS activity assay was performed to confirm inhibition at the protein level, due to the lack of GCS antibodies. The reduction obtained with 24 and 32 nM of siRNA-11 was similar to the reduction caused by 125 M *N*B-DNJ, (about 30%), a well-known inhibitor of the GCS enzyme. A higher decrease of GCS activity was obtained with the siRNA-11 at concentrations of 40 nM or higher, showing less than 4 % of control activity. As mentioned above, a high concentration (100 nM) of siRNA causes nonspecific induction of genes (Semizarov *et al.*, 2003). In this case, a reduction similar to that of *N*B-DNJ was obtained with a concentration 3 or 4-fold lower than that reported to exert a nonspecific effect. This reduction in GCS activity correlates with a reduction in GlcCer formation, as shown by TLC results.

Once the efficacy of siRNAs to inhibit human *GCS* expression was proved, we decided to repeat the experiment to knock down the mouse orthologous gene, *Ugcg*. The fact that the target sequence of siRNA-68 is identical in mouse and human, made us use this siRNA on mouse

cells. The results showed that the siRNA-68 was able to reduce the RNA levels of the mouse gene and open the possibility to perform analysis in a mouse model of the disease.

If siRNAs are going to be used as therapeutic tools, either they should be continuously provided to the cells or the cells should be forced to produce the siRNA on their own. This can be achieved by the transfection of DNA vectors bearing shRNAs. Our results with shRNAs were positive since they reduced GCS RNA levels. However, their efficacy was lower than that obtained with synthetic siRNAs. The efficacy of these two systems has been recently reviewed (Bantounas *et al.*, 2004). In this regard, these authors showed that in HeLa cells transient siRNAs reduced the expression of the target gene more efficiently than adenovirally delivered shRNAs. However, in T3 cells, the result is the opposite. They got to the conclusion that the effect is cell-type dependent.

New vectors have been recently developed (for example, see Amarzguioui *et al.* 2005) both, viral-derived or non-viral (Rossi, 2005) and shRNA-libraries have been generated (Silva *et al.*, 2005) which seem to overcome some of the problems of the first generation shRNAs products.

In the lysosomal disease field, RNAi was used to inhibt the *GBA*, *GCS* and *PSAP* genes, using a vector-encoded dsRNA 800-bp long (Diallo *et al.* 2003). They obtained a complete knockout of these genes in different cell types. These results, appropriate to study gene function, are also suitable for a therapeutic strategy in which a harmful protein should be eliminated. However, the complete shutdown of *GCS* expression is not the aim of our work, since some GCS activity should remain for the correct function of the organism (Yamashita *et al*., 1999).

The therapeutical apllications of siRNAs have been extensively discussed in recent publications (for example Uprichart, 2005; Thakker *et al.*, 2005). Most of the work has focused on cancer (reviewed in Izquierdo, 2005) and viral diseases (reviewed in Colbere-Garapin *et al.*, 2005). However, several positive results were also obtained in animal models of inherited diseases such as Huntington disease (Harper *et al.* 2005, Wang *et al.* 2005), non-syndromic hearing loss (Maeda *et al.* 2005), or amyotrophic lateral sclerosis (Ralph *et al.* 2005). Recently, two phase-I clinical trials for age-related macular dystrophy (AMD) have been reported (Check, 2005; Whelan, 2005). In one of them, the siRNA sirna-027 is used to target the VEGF-R gene while in the other trial, the siRNA Cand5, is used to target the VEGF gene. For the latter, successful results have been announced and a Phase-II study is underway.

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FIGURE LEGENDS

FIG. 1. Effect (**A**) and design (**B**) of four siRNAs targeted to the human *GCS* gene. (**A**) Real Time PCR quantification of *GCS* RNA levels in HeLa cells transfected with 40 nM of the indicated siRNAs expressed as percentage of control, untransfected, HeLa cells. Each value is the mean of two independent experiments, each of which was replicated three times. (**B**) Predicted secondary structure of *GCS* RNA. Insets correspond to enlarged parts of the RNA that include the targeting locations of the designed siRNAs.

FIG. 2. Effect of different concentrations of siRNA-11 and siRNA-68 on *GCS* mRNA levels. (**A**) Real Time PCR quantification of *GCS* mRNA levels in HeLa cells transfected with the indicated concentration of siRNA-11 (●) and siRNA-68 (■), expressed as percentage of control, untransfected, HeLa cells. (**B**) Semiquantitative radio-labelled RT-PCR of *GCS* mRNA from HeLa cells transfected with the indicated concentration of siRNA-11 and siRNA-68. *GAPDH* RNA was used as control. Quantification of the bands is shown as a histogram below. (**C**) Control experiment in which a siRNA targeting the *GAPDH* gene was assayed. Conditions as those used in (**B**).

FIG. 3. Effect of siRNA-11 on GCS activity and glucosylceramide formation. (**A**) GCS activity from HeLa cells transfected with the indicated concentration of siRNA-11. The effect of 125 M of *N*B-DNJ on untransfected HeLa cells is shown for comparison. Each value is the mean of three independent experiments, each of which was replicated twice. (**B**) TLC analysis of GlcCer synthesis in HeLa cells transfected with 40 nM of siRNA-11. C+: untransfected HeLa cells. C-: untransfected HeLa cells grown in the absence of ceramide. Quantification of the bands is shown as a histogram below.

FIG. 4. Fluorescence microscopy of untransfected HeLa cells (control), cells transfected with the GFP-bearing psiRNA-hH1 vector (psiRNA), or with the vector carrying either shRNA-68 or shRNA-11. DAPI staining was used for the identification of nuclei.

FIG. 5. Effect of shRNAs on *GCS* expression. HeLa cells were transfected with the psiRNA-hH1 vector (psiRNA), or with the vector carrying either shRNA-68 or shRNA-11. (**A**) Quantification of radio-labelled RT-PCR of *GCS* mRNA. (**B**) Quantification of GCS activity. (**C**) Quantification of TLC analysis of GlcCer formation.

FIG. 6. Real Time PCR quantification of *Ugcg* mRNA levels in RAW264.7 cells transfected with the indicated concentration of siRNA-68, expressed as percentage of control, untransfected cells.

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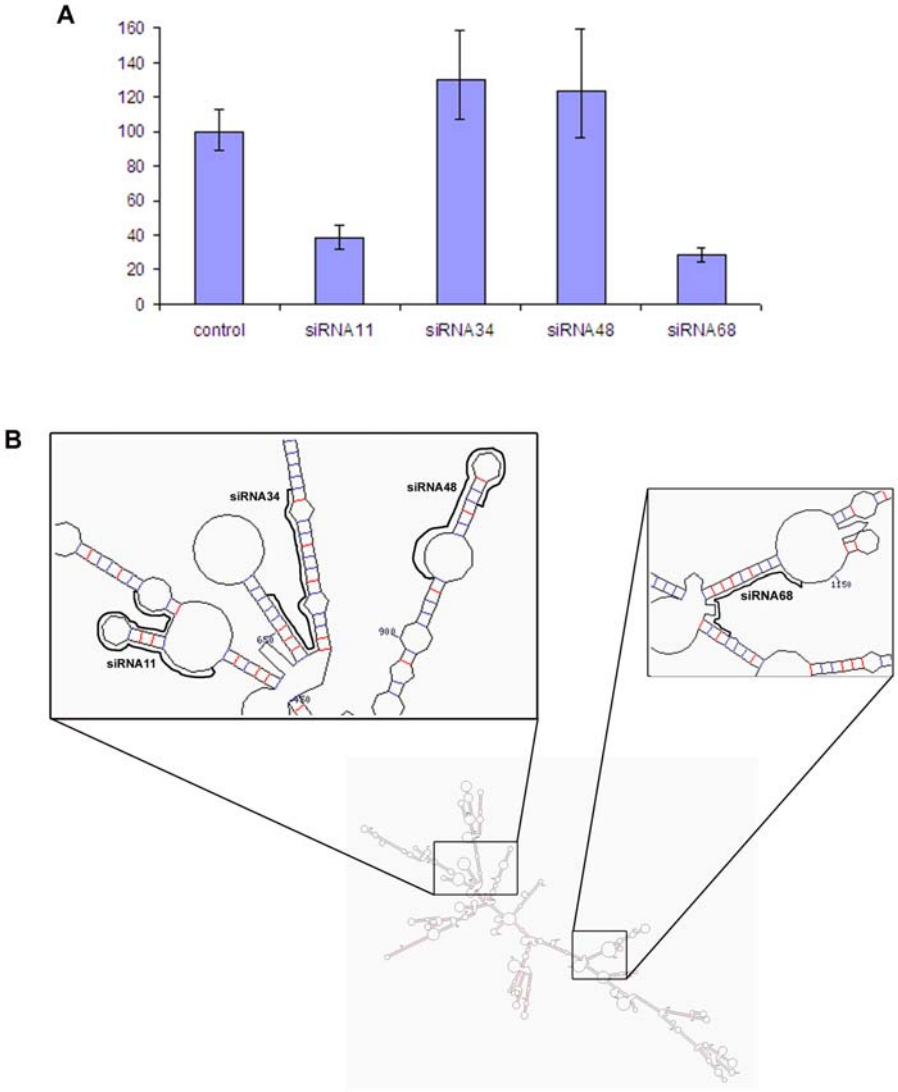
TABLE 1. OLIGONUCLEOTIDES USE IN THIS WORK

si/shRNA name	Oligonucleotide name	Oligonucleotide sequence (*target sequence*)	Position in cDNA	Exon	G/C content
siRNA11	AsiRNA11	5'- ***AAAGGGGTAGATCCTAACTTA***CCTGTCTC -3'	468	2	38.1%
	SsiRNA11	5'- AATAAGTTAGGATCTACCCCTCCTGTCTC -3'			
siRNA34	AsiRNA34	5'- ***AATGCCAGGATATGAAGTTGC***CCTGTCTC -3'	674	4	42.9%
	SsiRNA34	5'- AAGCAACTTCATATCCTGGCACCTGTCTC -3'			
siRNA48	AsiRNA48	5'- ***AAATGTGTGACAGGAATGTCT***CCTGTCTC -3'	906	6	38.1%
	SsiRNA48	5'- AAAGACATTCCTGTCACACATCCTGTCTC -3'			
siRNA68	AsiRNA68	5'- ***AATTTGTGAGCCAATTTCAGA***CCTGTCTC -3'	1154	8	33.3%
	SsiRNA68	5'- AATCTGAAATTGGCTCACAAACCTGTCTC -3'			
shRNA11	GCS11_F	5'- GTACCTC***AAAGGGGTAGATCCTAACTTA***TCAAG AGTAAGTTAGGATCTACCCCTTTTTTTTGGAAA -3'	468	2	
	GCS11_R	5'- AGCTTTTCCAAAAAAAAGGGGTAGATCCTAAC TTACTCTTGATAAGTTAGGATCTACCCCTTTGAG -3'			
shRNA68	GCS68_F	5'- GTACCTC***AATTTGTGAGCCAATTTCAGA***TCAAG AGTCTGAAATTGGCTCACAAATTTTTTTGGAAA -3'	1154	8	
	GCS68_R	5'- AGCTTTTCCAAAAAAATTTGTGAGCCAATTTCA GACTCTTGATCTGAAATTGGCTCACAAATTGAG -3'			
	GCS_exon5F	5'- CTTTGCTGCCACCTTAGAGC -3'			
	GCS_3'UTR	5'- GCAAGTGCCATGCAAAAATA -3'			
	GAPDH_F	5'- GTCAGTGGTGGACCTGACCT -3'			
	GAPDH_R	5'- AGGGGTCTACATGGCAACTG -3'			

FIGURE 1


A
160
140
120
100
80
60
40
20
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control
siRNA11
siRNA34
siRNA48
siRNA68
B
siRNA34
siRNA48
siRNA11
siRNA68

FIGURE 2

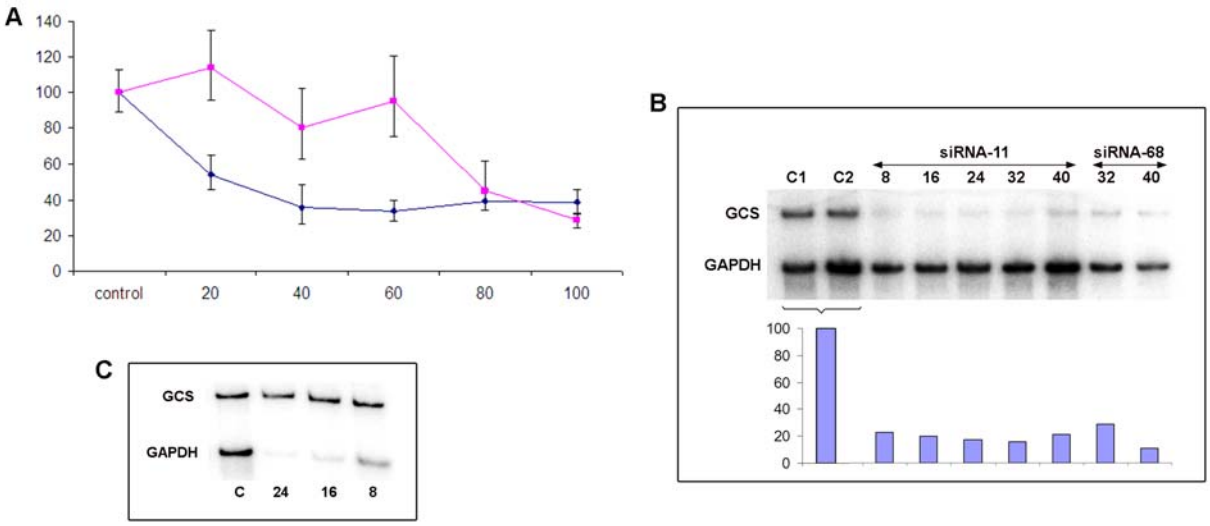


FIGURE 3

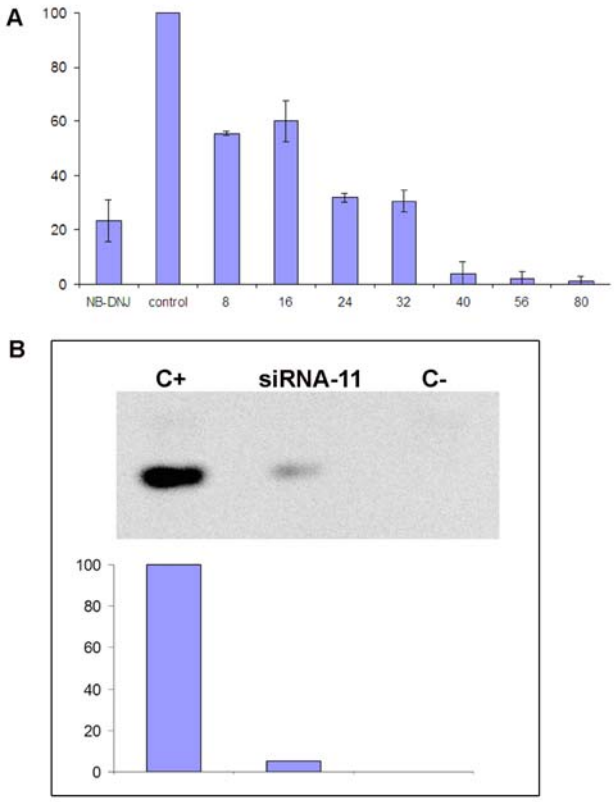


FIGURE 4

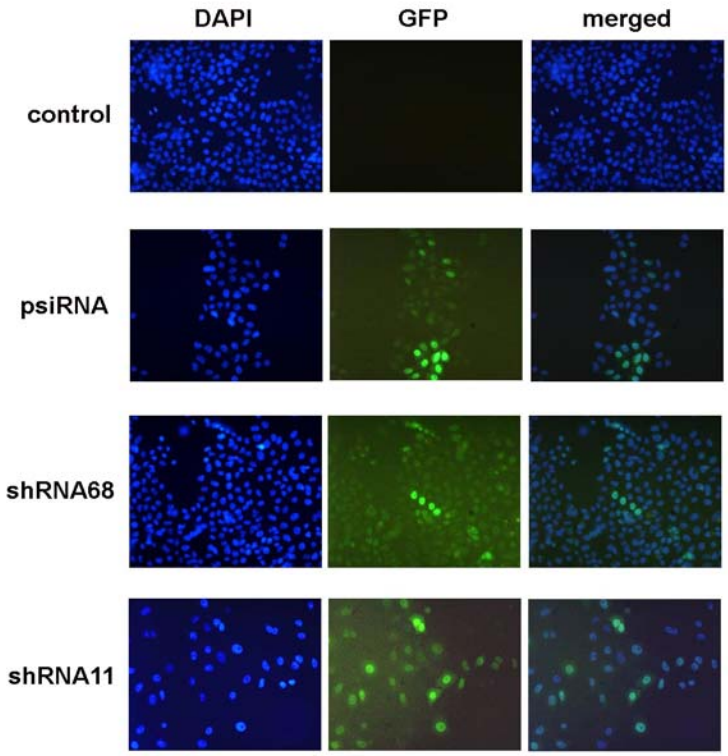


FIGURE 5

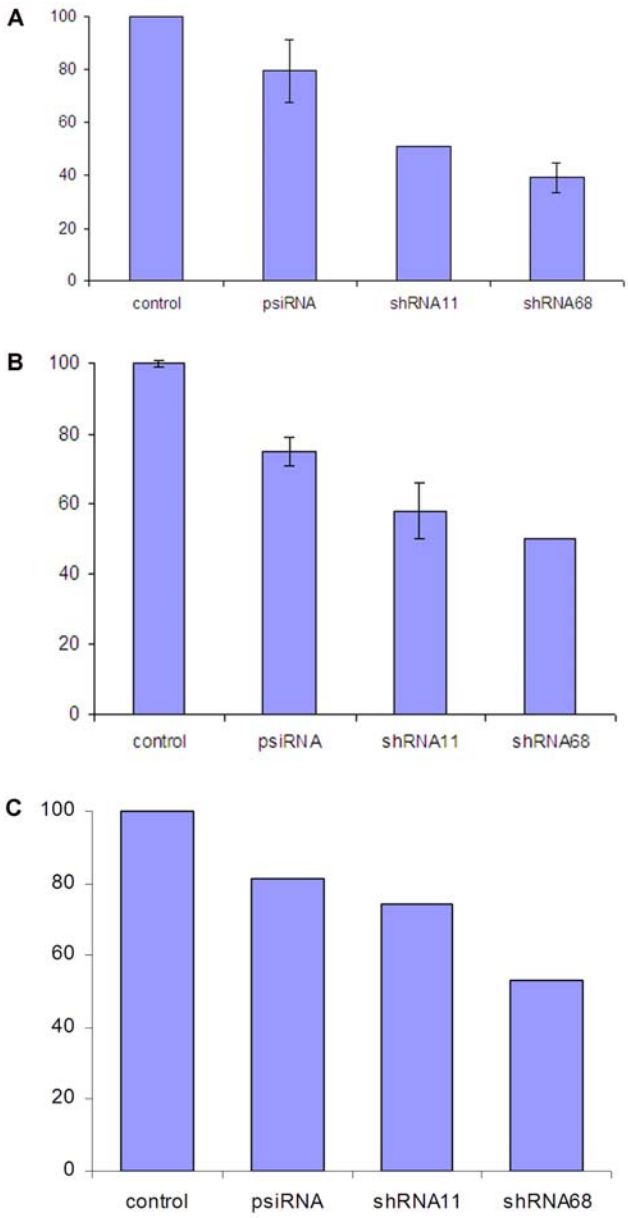
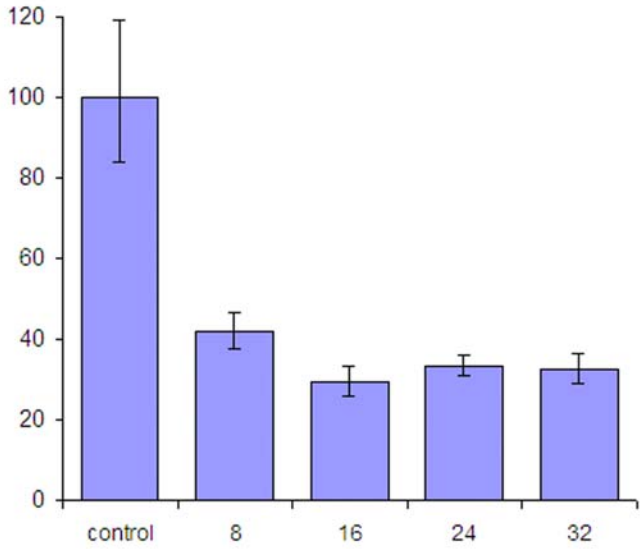

A
100
80
60
40
20
0
control
psiRNA
shRNA11
shRNA68
B
100
80
60
40
20
0
control
psiRNA
shRNA11
shRNA68
C
100
80
60
40
20
0
control
psiRNA
shRNA11
shRNA68

FIGURE 6



***Dades suplementàries*:**

4.2. Detecció de l'acúmul de glucosilceramida en macròfags de ratolí amb microscopia electrònica

El silenciament de la GCS utilitzant tècniques de RNAi es basa en la mateixa idea utilitzada en la teràpia de reducció de substrat, és a dir, inhibir o reduir la síntesi de glucosilceramida per compensar la poca o nul·la degradació d'aquest esfingolípid. En la teràpia de reducció de substrat s'utilitzen iminosucres, com el *N*B-DNJ, per inhibir la GCS.

En l'anàlisi de l'acció d'aquests inhibidors s'havia descrit que es podia veure el seu efecte en l'acúmul de GlcCer en lisosomes de macròfags de ratolí utilitzant tècniques de microscopia electrònica (Platt i col., 1994). Nosaltres vam pensar d'utilitzar la mateixa estratègia per saber si la inhibició per RNAi podia revertir l'acúmul de GlcCer en els macròfags de ratolí i si es podia detectar amb el microscopi electrònic.

4.2.1. Materials i mètodes

Cèl·lules: Es van utilitzar macròfags de ratolí RAW264.7 cultivats i transfectats amb el shRNA68 com es descriu en el capítol 4.1.

Tractament amb CBE: Per generar l'acúmul de glucosilceramida les cèl·lules es van tractar amb un inhibidor de la glucocerebrosidasa, mimetitzant l'efecte de la malaltia de Gaucher. Aquest inhibidor era el epòxid-β-conduritol (CBE). Les cèl·lules es van incubar amb 50, 100 o 200 μM de CBE durant 7, 10 o 15 dies.

Microscopia electrònica: Un cop les cèl·lules havien estat incubades amb el CBE, es van preparar per observar-les en el microscopi electrònic. La preparació de les cèl·lules (fixació, osmificació, deshidratació, inclusió en la reïna i ultramicrotomia) es van realitzar en el Serveis Científico-Tècnics, en el departament de Reconeixement molecular *in situ*, segons el protocol descrit en Platt i col. (1994).

4.2.2. Resultats

Inhibició de Ugcg *mitjançant shRNAs*

Es van transfectar les cèl·lules RAW264.7 amb el shRNA68, que ens permetia mantenir la inhibició del gen *Ugcg* durant més temps que utilitzant el siRNA68. Per comprovar que el shRNA68 era capaç d'inhibir el gen, es va quantificar l'expressió a nivell de mRNA amb la Real Time PCR. En la gràfica de la figura 1 es mostra l'expressió del gen Ugcg en cèl·lules control (sense transfectar), transfectades amb el plàsmid psiRNA i amb les transfectades amb el

shRNA68. Com es pot observar en la figura amb el shRNA68 obtenim una inhibició de la expressió del gen *Ugcg* del 50%.

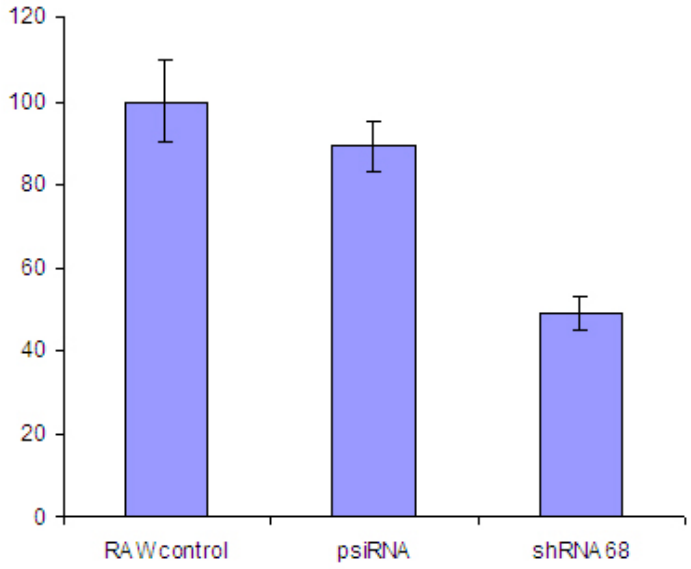


***Figura 1**. Expressió del gen Ugcg en cèl·lules RAW control, cèl·lules transfectades amb el vector psiRNA i cèl·lules transfectades amb shRNA68.*

Detecció de l'acúmul de glucosilceramida en macròfags de ratolí

Un cop haviem comprovat que podiem inhibir la expressió del gen *Ugcg* (que codifica la glucosilceramida sintasa en ratolí) amb siRNAs i shRNAs, ens vam plantejar generar els macròfags "Gaucher" cultivant-los amb CBE en el medi. Estava descrit que amb 14 dies d'incubació amb 50μM de CBE els macròfags acumulaven suficient quantitat de lípids per detectar-los en el microscopi electrònic (Platt i col., 1994).

En el nostre cas, tot i que vam utilitzar diferents concentracions de CBE i vam incubar les cèl·lules a diferents temps, no vam obtenir resultats quantificables clars. Els resultats que es mostren són els obtinguts en utilitzar 200 μM de CBE i incubant les cèl·lules durant 15 dies. Amb aquest tractament vam aconseguir detectar lisosomes que acumulaven lípids, però aquests resultats no ens permetien distingir clarament entre les cèl·lules control i les tractades amb CBE, és a dir, les que acumulaven glucosilceramida i les normals. Tot i que es podien detectar alguns lisosomes carregats de lípids en els macròfags tractats i no en els sense tractar amb CBE, el mètode no era útil per quantificar aquest acúmul i veure després si es reduia per efecte dels siRNAs.

En la figura 2 es mostren alguns exemples dels macròfags no tractats. Com es pot observar en la figura 2A, 2B, 2C i 2D, en els macròfags controls no observem lisosomes carregats de lípids (de color negre), i els veiem grisos.

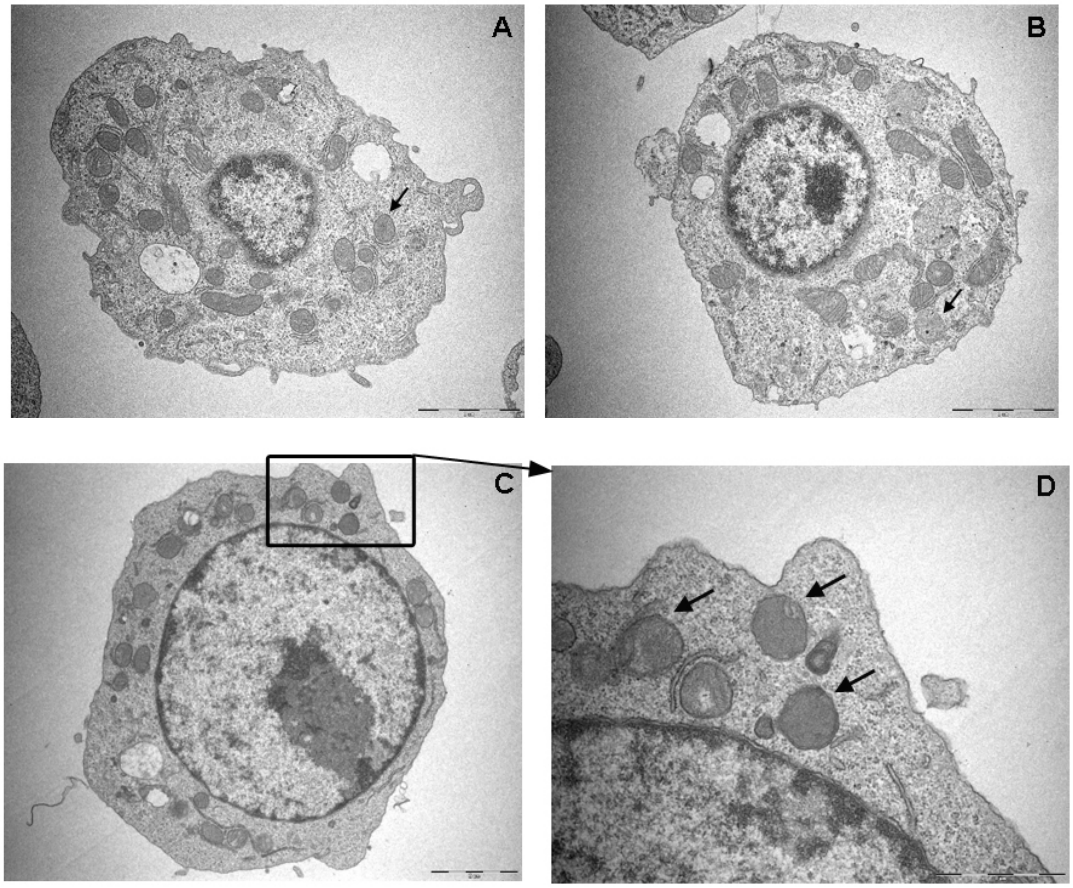


***Figura 2**. Macròfags RAW264.7 control (sense tractar). En la figura es pot veure com en aquestes cèl·lules els lisosomes no es veuen carregats de lípids. Podem observar 3 cèl·lules diferents (A, B i C) i l'amplificació d'una regió de la C (D). La barra de l'escala equival a 2 μm en A, B i C i a 1 μm en la D. Les fletxes negres (➔) senyalen els lisosomes.*

En canvi, en els macròfags tractats, podem veure lisosomes amb lípids acumulas (color negre). En la figura 3 podem observar dos camps (A i B) de cèl·lules tractades amb CBE, on apareixen lisosomes carregats de lípids que es poden apreciar millor en les imatges amplificades. Tot i que sí que es poden detectar alguns d'aquests lisosomes, no tots els lisosomes de la cèl·lula presenten aquest aspecte ni es detecten en totes les cèl·lules. Això fa que no sigui una bona eina per quantificar l'acúmul de glucosilceramida o la disminució d'aquest compost.

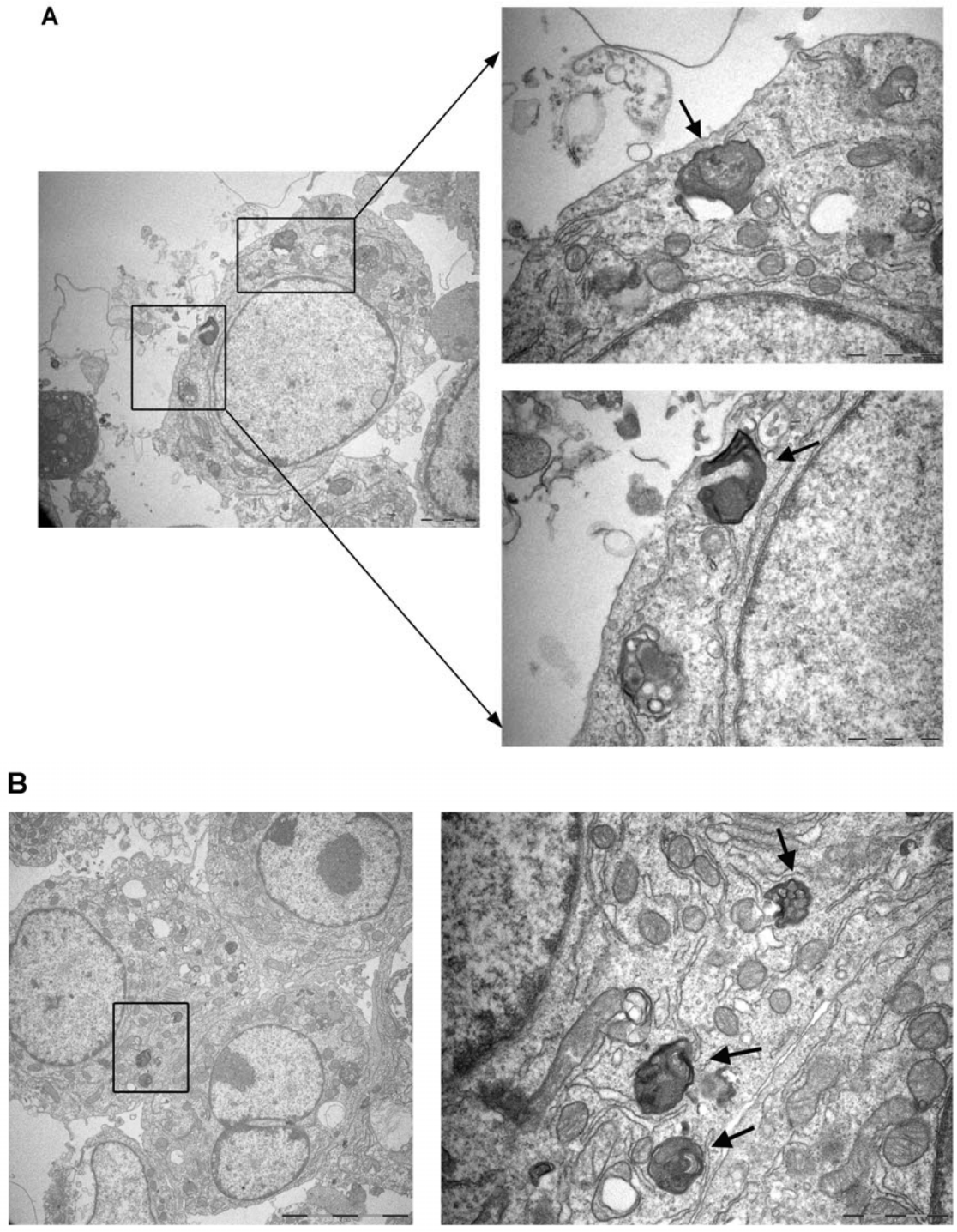


***Figura 3**. En la figura A i B veiem dos camps de cèl·lules diferents tractades amb CBE. Els quadres negres assenyalen els camps que s'amplifiquen al costat. En la figura A la barra de l'escala són 2 μm i 1 μm en les ampliacions, i en la figura B de 5 μm i 1 μm en l'ampliació. Les fletxes negres (➔) senyalen els lisosomes.*

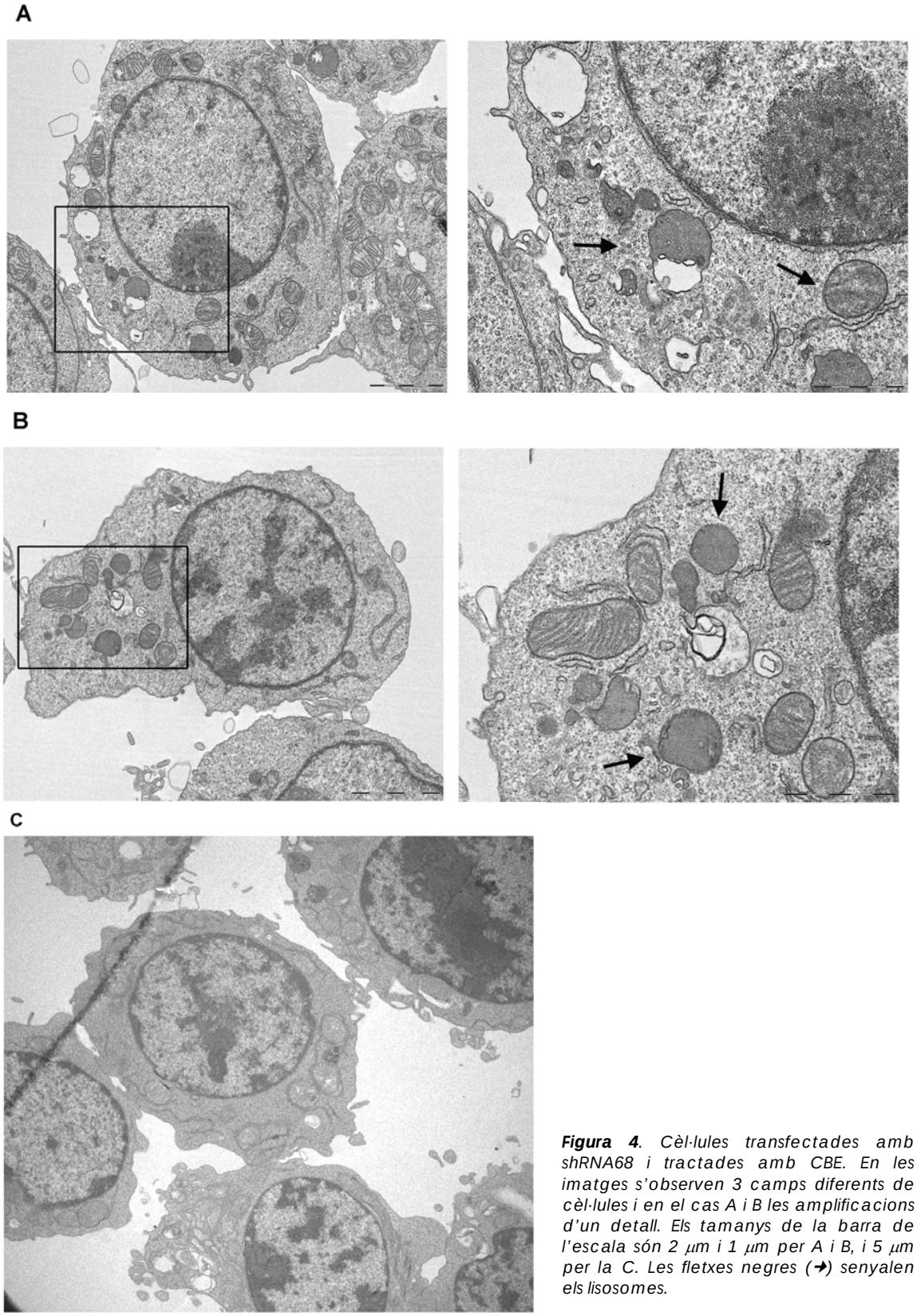


Figura 4. *Cèl·lules transfectades amb shRNA68 i tractades amb CBE. En les imatges s'observen 3 camps diferents de cèl·lules i en el cas A i B les amplificacions d'un detall. Els tamanys de la barra de l'escala són 2 μm i 1 μm per A i B, i 5 μm per la C. Les fletxes negres (➔) senyalen els lisosomes.*

En la figura 4 veiem macròfags de ratolí RAW264.7 tractats amb CBE i transfectats amb shRNA68. Aquestes cèl·lules es van seleccionar prèviament amb l'antibiòtic Zeocina, per obtenir les que havien incorporat la construcció amb el shRNA68. Es va comprovar també que la expressió de *Ugcg* estaba reduïda, indicant que el shRNA68 havia funcionat. Després de 15 dies de tractament amb CBE, tot i que no es detectaven lisosomes carregats de lípids, o s'en detectaven menys que en les cèl·lules que no expressen shRNA68, no es podia quantificar l'efecte de l'inhibició de *Ugcg* mitjançant shRNAs.