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Simultaneous siRNA-mediated silencing of pairs of genes coding for enzymes involved in glycosaminoglycan synthesis

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It has been demonstrated recently that it is possible to decrease expression of genes coding for enzymes involved in synthesis of glycosaminoglycans (GAGs) by using specific siRNAs which interfere with stability of particular mRNAs. This procedure has been proposed as a potential treatment for patients suffering from mucopolysaccharidoses, a group of inherited metabolic diseases caused by dysfunction of enzymes required for GAG degradation, and resultant storage of these compounds in cells of affected persons. Here, we asked if the simultaneous use two species of specific siRNAs aimed at silencing two genes involved in particular steps of GAG synthesis may be more effective than the use of single siRNA. We found that inhibition of GAG synthesis in cells treated with two siRNAs is generally more effective than using single siRNAs. However, the differences were not statistically significant, therefore the potential benefit from the use of two siRNAs over the use of a single siRNA is doubtful in the light of the cost-benefit ratio and possibly stronger side-effects of the putative therapy.

Key words: GAGs, MPSs

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INTRODUCTION

Mucopolysaccharidoses (MPSs) are a group of devastating disorders that belong to lysosomal storage diseases, each of which is produced by an inherited deficiency of an enzyme involved in the degradation of glycosaminoglycans (GAGs) (Neufeld & Muenzer, 2001). Storage of GAGs in cells results in a progressive damage of the affected tissues. Organs may also be enlarged, specifically the liver and spleen (Neufeld & Muenzer, 2001). Eleven distinct clinical types and subtypes of mucopolysaccharidoses, depending on the deficient enzyme, have been identified. Each type of MPS differs in clinical presentation and has varying degrees of severity. Decreased intellectual capacity, hyperactivity, and aggressive behavior are often clinical signs of MPS II and MPS III (Neufeld & Muenzer, 2001).

Enzyme replacement in treatment of human MPS was first reported in MPS type I in 2001 (Kakkis *et al*., 2001). Currently, enzyme replacement therapy (ERT) is available for treatment of only three of MPSs: MPS I, MPS II and MPS VI (Kakkis *et al*., 2001; Harmatz *et al*., 2006; Muenzer *et al*., 2006). This therapy has proven useful in reducing non-neurological symptoms and pain. ERT does not relieve the problems that may exist in the central nervous system because an active, recombinant form of a deficient enzyme does not cross the blood-brain barrier. Neurological symptoms are present in some types of MPS and may vary in severity. They occur in some MPS I patients (primarily subtype MPS IH), most of MPS II and MPS VII patients, and all MPS III patients (Neufeld & Muenzer, 2001), where they are especially severe.

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a broad-spectrum protein tyrosine kinase inhibitor which acts on several different growth factor receptors (Akiyama *et al*., 1987). Recent work has shown that genistein can reduce GAG synthesis in *in vitro* cultures of patients' cells from several MPS subtypes and there is evidence to suggest that it may be an effective substrate reduction therapy agent for MPS (Piotrowska *et al*., 2006; Jakóbkiewicz-Banecka *et al*., 2009). Free genistein has been found in the brain of rats consuming dietary genistein, suggesting that this compound is capable of crossing the blood-brain barrier (Chang *et al*., 2000). Therefore, impairment of substrate production may be an effective therapeutic option for patients suffering from neuronopathic forms of lysosomal storage diseases (Jakóbkiewicz-Banecka *et al*., 2007). Substrate deprivation therapy (SDT), based on the use of general inhibitors of GAG synthesis, improved neurological functions, but it was not specific to any MPS type. Recently, methods based on the phenomenon of RNA interference (RNAi) for specific inhibition of GAG synthesis have been reported, which could potentially be used in treatment of MPS III patients (Dziedzic *et al*., 2010; Kaidonis *et al*., 2010). Those studies indicated that deprivation through siRNA or shRNA-mediated RNA interference phenomenon may be considered as a potential therapy for MPS III and perhaps also for other diseases from this group (Dziedzic *et al*., 2010; Kaidonis *et al*., 2010).

RNA interference is a form of post-transcriptional gene regulation in which non-translated double-stranded RNA molecules called small interfering RNA (siRNA) mediate sequence-specific degradation of target messen-

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Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; ERT, enzyme replacement therapy; FBS, fetal bovine serum; GAG, glycosaminoglycan; GALT, galactosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GlcNAc, *N*-acetylglucosamine; GlcAT, glucuronosyltransferase; HS, heparin sulfate; MPS, mucopolysaccharidosis; RNAi, RNA interference; SDT, Substrate deprivation therapy; siRNA, small interfering RNA; XT, xylosyltransferase

ger RNA (mRNA). Researchers have discovered ways to control RNAi in order to regulate gene expression in a variety of biological schemes (Stevenson, 2004). This system could be used to interrupt disease processes such as those caused by human immunodeficiency virus type 1 (HIV-1) (Crowe, 2003), hepatitis viruses (Kusov *et al*., 2006), and influenza virus (McSwiggen & Seth, 2008). Potential treatments for neurodegenerative diseases have also been proposed (Raoul *et al*., 2006). RNA interference is also often seen as a promising way to treat cancer by silencing genes differentially upregulated in tumor cells or genes involved in cell division (Devi, 2006). Despite a decade of intensive research by leading pharmaceutical companies, only a limited number of clinical trials of synthetic siRNAs are currently under way (Kubiak and Nawrot, 2009). A major challenge with the use of siRNAs in mammals is their intracellular delivery to specific tissues and organs that express the target gene. Davis *et al*. (2010) have recently demonstrated for the first time in humans that siRNA molecules can be systemically delivered using nanoparticles to a solid tumor.

Glycosaminoglycan biosynthesis is initiated by the formation of the linkage tetrasaccharide, GlcAβ1,3Galβ1,3Galβ1,4Xylβ-*O*Ser (Esko & Lindahl, 2001). The first four steps of the synthesis of GAG chains linked to core proteins, chondroitin sulfate/dermatan sulfate (CS/DS), and heparin sulfate (HS) share a common pathway involving xylosyltransferases — XT-I and XT-II (encoded by the *XylT-1* and *XylT-2* genes) (Götting *et al*., 2000), β4-galactoslytransferase — GALT-I (encoded by the *B4GalT7* gene) (Almeida *et al*., 1999; Okajima *et al*., 1999), β3-galactosyltransferase — GALT-II (encoded by the *B3GalT6* gene) (Bai *et al*., 2001), and β3-glucuronosyltransferase — GlcAT-I (encoded by the *B3Gat3* gene) (Kitagawa *et al*., 1998; Tone *et al*., 1999). Thereafter, the glycosaminoglycan chains are elongated by alternating disaccharides, which are then partly modified by sulfation, epimerization or deacetylation (Esko & Lindahl, 2001).

The addition of the first GlcNAc residue to HS chain is catalyzed by EXTL3 glycosyltransferase, followed by the chain extension steps catalyzed by EXT1 and EXT2 transferases (Busse *et al*., 2007). As the chain is elongated, sulfate groups are introduced at various positions by enzymes from at least four families of sulfotransferases and some of the glucuronic acid residues are converted into iduronic acid by C5-epimerase (Li, 2010; Shworak *et al*., 1999).

Biosynthesis of the CS/DS linkage region and chain extension needs additional enzymatic activities, β4-GalNAc transferase 1 and 2 (exhibiting activities of GalNAct-I), and bifunctional enzyme activity - chondroitin synthase (Chsy1, Chsy2 and Chsy3/CSS3), which has both β1-3 glucuronosyltransferase and β1-4 N-acetylgalactosaminyltransferase (GlcAT-II and GalNAcT-II, respectively) activities required to form the repeating disaccharide (Kitagawa *et al*., 2001; Uyama *et al*., 2003). During assembly, the chain is modified by sulfation in various positions by a group of specific sulfotransferases (Okuda *et al*., 2000). The creation of IdoUA units occurs by epimerization of a portion of the GlcA residues, previously incorporated into the polysaccharide chain by two DS-epimerases (DS-epi 1 and DS-epi 2) and generating a highly modified dermatan sulfate chain (Pacheco *et al*., 2009).

UDP-xylosyltransferase (UDP-D-xylose:proteoglycan core protein beta-D-xylosyltransferase, EC 2.4.2.26) initiates the assembly of glycosaminoglycan chains to the core proteins of proteoglycans (Wilson, 2004) and has been shown to play an important role in proteoglycan homeostasis (Götting *et al*., 2007). In mammals, two highly homologous active isoenzymes named XT-I and XT-II (Götting *et al*., 2000; Pönighaus *et al*., 2007; Voglmeir *et al*., 2007) are encoded by *XylT-1* and *XylT-2* genes, respectively. The xylosyltransferase (XT) isoforms initiate the glycosaminoglycan synthesis, specifically catalyze the transfer of D-xylose from UDP-D-xylose to specific serine residues in the core protein. XT-I and XT-II are highly similar xylosyltransferases with tissue-specific expression, in particular, XT-II is highly expressed in the liver (Voglmeir *et al*., 2007; Pönighaus *et al*., 2007). Both xylosyltransferases I and II have stem regions like other glycosyltransferases (Kleene & Berger, 1993) that are susceptible to proteolytic cleavage allowing the enzymes to exit the cell's Golgi apparatus and ultimately to enter the circulation (Götting *et al*., 1999). It is uncertain which isoenzyme(s) is responsible for the steady-state level of xylosyltransferase activity in normal serum and which is responsible for the increased serum activities in various disease states (Condac *et al*., 2009). Therefore, which isoenzyme predominates in the serum of healthy individuals and patients and from which tissue it arises is unclear.

The key function of chondroitin sulfate N-acetylgalactosaminyltransferase (CSGalNAcT) is the transfer of 1,4-*N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to glucuronic acid (GlcUA) in chondroitin sulfate chains. CSGalNAcT-1 and -2 have one glycosyltransferase domain and exhibit GalNAcT activity in the initiation and elongation processes, indicating that CSGalNAcT-1 and -2 have both GalNAcT-I and -II activities (Uyama *et al*., 2003). The amino acid sequence of human CSGalNAcT-2 displays 60% identity to that of CSGalNAcT-1. CSGalNAcT-2 shows much stronger N-acetylgalactosaminyltransferase activity toward glucuronic acid of chondroitin poly- and oligosaccharides, and chondroitin sulfate poly- and oligosaccharides with a β1–4 linkage, i.e. elongation activity for chondroitin and chondroitin sulfate, but shows much weaker activity toward the tetrasaccharide of the glycosaminoglycan linkage structure (GlcA-Gal-Gal-Xyl-*O*-methoxyphenyl), i.e. initiation activity, than CSGalNAcT-1 (Sato *et al*., 2003).

Since we have demonstrated previously that GAG synthesis can be slowed down by silencing of a single gene encoding only one of an isoenzyme pair (Dziedzic *et al*., 2010), the aim of this study was to test if a simultaneous use of pairs of specific siRNAs devoted to silencing a both genes involved in a particular step of GAG synthesis may be more effective than the use of single siRNA.

MATERIALS AND METHODS

Cell cultures of MPS fibroblasts. MPS human skin fibroblasts obtained from The Children's Memorial Health Institute (Warsaw, Poland) were established from primary skin biopsies from MPS patients. The MPS I cultured skin fibroblast cell lines were deficient in α-L-iduronidase activity. They derived from patients with a clinically severe phenotype (MPS IH — Hurler disease) and having dermatan sulfate- and heparan sulfate-uria. The MPS III culture skin fibroblast cell lines were confirmed to have deficient sulfamidase activities (MPS IIIA — Sanfilippo A disease) and derived from patients with accumulation of undegraded heparan sulfate in urine. The MPS IH and MPS IIIA skin fibroblasts were transfected with siRNA against *CSGalNAcT* and *XylT* genes,

respectively. All cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS and antibiotic antimycotic solution (AAS) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2.

siRNA oligonucleotides. Pre-designed siRNAs for human *XylT-1* and *XylT-2* genes were as described previously (Dziedzic *et al*., 2010). siRNAs for the *CSGalNAcT-1* gene (*Silencer*® Select Pre-Designed siRNA #s31518 5'-GGG ACA UUG UAU GAG CUC Att-3' (sense) and 5'-UGA GCU CAU ACA AUG UCC Ctt-3' (antisense), *Silencer*® Select Pre-Designed siRNA #s31519 5'-GCA CCU UUA UCG CAA GUA Utt-3' (sense) and 5'-AUA CUU GCG AUA AAG GUG Cac-3' (antisense)) and the *CSGalNAcT-2* gene (*Silencer*® Select Pre-Designed siRNA #s30880 5'CUA GUG AUC UUU UAG AGU Utt-3' (sense) and 5'-AAC UCU AAA AGA UCA CUA Ggt-3' (antisense), *Silencer*® Select Pre-Designed siRNA #s 30881 5'-GGA CCU CUC AUG AAA GUG Att-3' (sense) and 5'-UCA CUU UCA UGA GAG GUC Caa-3' (antisense)) were purchased from Applied Biosystems/Ambion (Austin, TX, USA). Unrelated nonspecific siRNAs (AllStars Negative Control siRNA, Qiagen GmbH, Hilden, Germany) were used as controls.

RNA interference. Cells were transfected either with control siRNA, or with Mock without siRNA or with siRNA against a single gene coding for GAG synthetase as described previously (Dziedzic *et al*., 2010). In parallel, but only for GAG synthesis assays, cells were co-transfected with siRNAs targeting both sibling genes in combination: *Xylt-1* and *Xylt-2*, both at 25 nM, or *CSGalNAcT-1* at 50 nM and *CSGalNAcT-2* at 25 nM. At 48 h post-transfection, the procedure was repeated and cells were cultured for next 24 h before the analysis. After treatment of the cells for 72 h in total, the cells were harvested and real-time PCR and transmission electron microscopy were performed to determine mRNA expression and the contents of GAG.

Quantitative real-time PCR. Isolation of RNA, cDNA preparation and quantification of mRNA expression were performed as described previously. *Xylt-1* and *Xylt-2* cDNAs were amplified using the primers described earlier (Dziedzic *et al*., 2010). In this work, appropriate primers for *CSGalNAcT-1* and *CSGalNAcT-2* were chosen (Gene-Specific RT2 qPCR Primer Assay, SuperArray Bioscience Corporation, Frederick, MD, USA). Quantification was performed with the use of LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN, USA). The parameters of real-time PCR amplification were as follows: 10 min at 95°C, 45 cycles for 10 s at 95°C, 10 s at 55°C, and 20 s at 72°C. Gene expression was quantified by the 2-ct method, normalizing cT-values to a housekeeping gene (*GAPDH*) and calculating the relative expression values (Livak & Schmittgen, 2001).

GAG synthesis assay. GAG synthesis was monitored by measuring incorporation of ^{35}S (from radioactive sodium sulfate) into proteoglycans, according to the method described previously (Murata *et al*., 2003) and modified (Jakóbkiewicz-Banecka *et al*., 2009). In all experiments, ^{35}S counts were expressed per nanogram of DNA following quantitation using PicoGreen dsDNA Quantitation (Molecular Probes-Invitrogen, Carlsbad CA, USA) according to the manufacturer's protocol (Invitrogen).

Transmission electron microscopy. In order to determine the effect of downregulation of GAG synthetase genes transmission electron microscopy analysis was done essentially as described by Watkins and Cullen (Watkins & Cullen, 1987). Briefly, MPS fibroblasts were plated in 12-well plates and allowed to attach overnight. The cells were then transfected either with control siRNA, or with Mock without siRNA or with siRNAs against genes coding for GAG synthetases. Following treatment for specified time periods, transmission electron microscopy studies were performed as described previously (Herman-Antosiewicz *et al*., 2006), using a Philips CM100 (Philips Electron Optics, The Netherlands) microscope.

Statistical Analysis. Continuous variables were compared using one-way analysis of variance (ANOVA). Tukey's multiple comparisions test was used as a post-hoc comparator. Data are presented as mean ± standard deviation. All statistical assessments were two-sided and evaluated at the 0.05 significance level. All tests were performed using Statistica 8.0 (StatSoft, Poland) software with significance indicated where p values were less than 0.05.

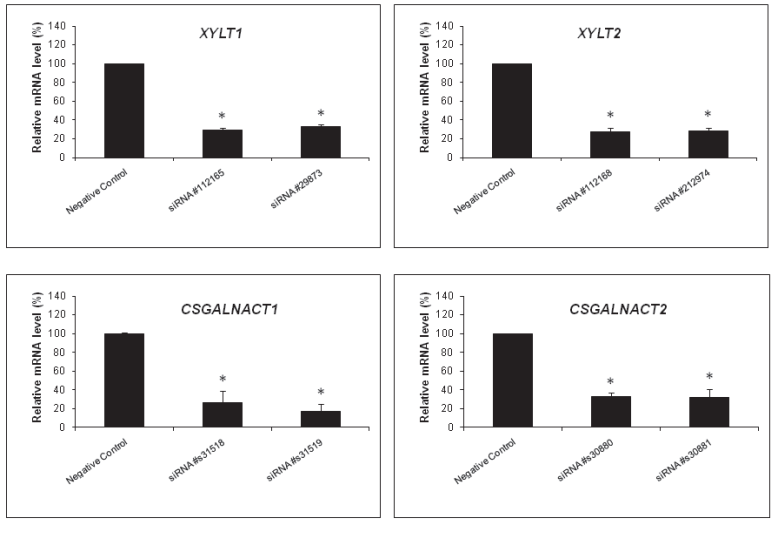

Figure 1. Effects of siRNAs on target gene down-regulation.
siRNA-mediated silencing of *XylT-1, XylT-2, CSGalNAcT-1* and *CSGalNAcT-2* genes was performed as described in Experimental Procedures. Gene expression remaining is expressed as the percentage of the amount of the examined gene's mRNA in cultures transfected with *XylT-1, XylT-2, CSGalNAcT-1* or *CSGalNAcT-2* siRNAs vs cells transfected with nontargeting control siRNA (AllStars Negative Control siRNA). The expression levels of the silenced genes were analyzed relative to *GAPDH* expression level, set as 100%. The effects of siRNAs were determined in three independent transfections, and quantitative RT-PCR reactions were performed in duplicate. The data are shown as mean ±S.D. *indicates $p<0.0001$ compared with Negative Control siRNA.

RESULTS AND DISCUSSION

We have demonstrated previously that it is possible to decrease expression of genes coding for enzymes involved in GAG synthesis by using specific siRNAs which interfere with stability of mRNAs of *XylT-1, XylT-2, GalTI* (*B4GalT7*) and *GalTII* (*B3GalT6*) genes (Dziedzic *et al*., 2010). This silencing of certain genes resulted in impairment of GAG production in cells derived from MPS III patients. Those results suggested that treatment with siRNA could be considered as a potential therapy for mucopolysaccharidoses. However, in the course of our work on improving

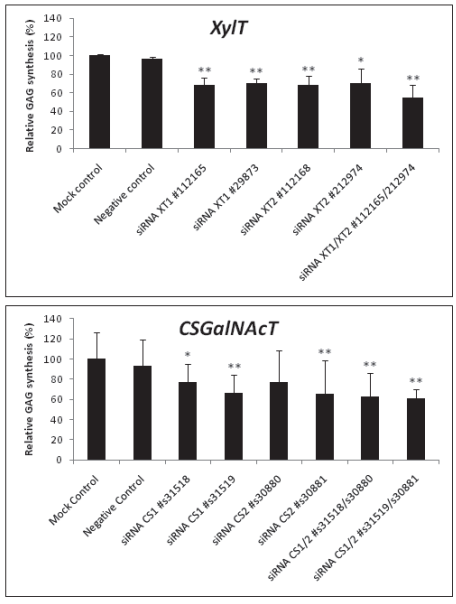

Figure 2. GAG synthesis in fibroblasts transfected with indicated siRNAs.
Effect of silencing of particular genes on the synthesis of sulfated glycosaminoglycan (GAG) was measured as [^{35}S]sulfate uptake into GAG in fibroblasts. Fibroblasts were transfected with indicated siRNAs, and following 48 h cultivation the transfection was repeated and cultivation was prolonged for another 24 h. The presented values are means of three independent experiments with corresponding standard deviations. 'Mock control' indicates conditions under which only buffer was used in transfection-like experiments (without any siRNA), and 'Negative control' represents results with AllStars Negative Control siRNA. *indicates $p<0.05$, and **indicates $p<0.01$ compared with the Mock control. No statistical significance was found when effects of addition of two siRNAs to one cell culture were compared to effects of addition of any siRNA alone.

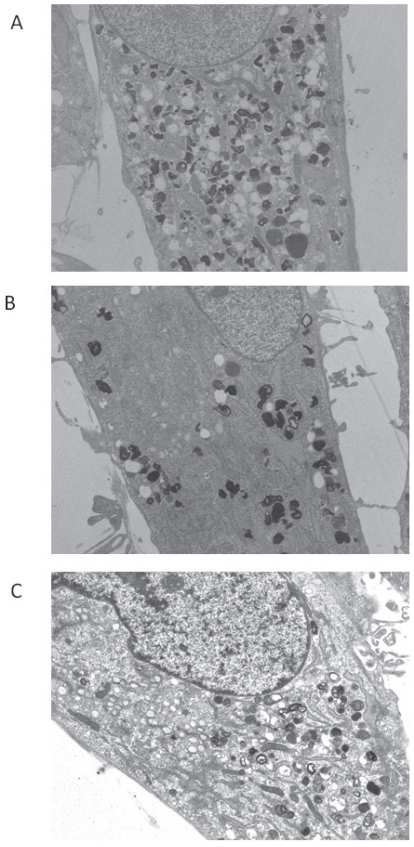

Figure 3. Ultrastructure of MPS III fibroblasts transfected with indicated siRNAs.
Note overcrowded lysosomes and peroxisomes in untreated cells **(A)** and healthy-looking cells transfected with siRNA against *XylT-2* gene (siRNA #s212974) **(B)**, and *CSGalNAcT-1* gene (siRNA #s31518) **(C)**. Representative electron micrographs are shown magnification 1650 ×.

the GAG synthesis inhibition, we asked if it was possible to increase the efficiency of this inhibition by using a combination of different siRNAs to affect expression of pairs of genes whose isoemzyme products are involved at a particular stage of GAG production.

Effects of siRNAs on levels of mRNAs for *XylT-1, XylT-2, CSGalNAcT-1* and *CSGalNAcT-2* were tested. Two kinds of siRNA were used for each gene in individual assays. We found that by using these siRNAs it was possible to silence expression of corresponding genes significantly (Fig. 1). In fact, we were able to decrease the levels of particular mRNAs to 20–30% of those in control samples.

Although a significant decrease in the efficiency of expression of genes coding for enzymes involved in GAG synthesis was achieved (Fig. 1), the major issue in treatment of MPS is actual reduction of GAG production, which may depend on activities of such enzymes to various extent, depending on the limiting steps of the process. Therefore, we determined the kinetics of GAG synthesis by measuring the efficiency of incorporation of inorganic ^{35}S. A statistically significant decrease in the level of GAG synthesis was observed in experiments with all siRNAs tested except for oligonucleotide no. 30880 (for the *CSGalNAcT-2* gene) (Fig. 2). Interestingly, this oligonucleotide caused an about 3-fold reduction in the level of corresponding mRNA (Fig. 1). These results confirmed that a significant decrease in the level of a particular mRNA does not necessarily mean a decrease in the efficiency of the biochemical process one of whose steps is catalyzed by the product of the silenced gene.

When effects of combinations of various siRNAs devoted to silencing the expression of pairs of genes coding for isoenzyme pairs (either *XylT-1 and XylT-2* or *CSGalNAcT-1* and *CSGalNAcT-2*) were tested, we found that GAG synthesis was decreased somewhat more efficiently than when cells were treated with siRNAs silencing of single genes (Fig. 2). Nevertheless, the differences between the treatment with single siRNA and combinations of siRNAs did not reach statistical significance. Definitely, the effects of simultaneous silencing of two genes were not additive.

To assess the effects of siRNAs on lysosomal storage, we analyzed treated and non-treated MPS IH and MPS IIIA fibroblasts by electron microscopy. We found that when those cells were transfected with siRNA the intracellular deposits (presumably GAGs) accumulated to lower levels than in untreated cells (Fig. 3 and data not shown). However, the differences between cells treated with single siRNAs and combinations of siRNAs were small if any.

The use of combinations of siRNAs in potential therapeutic procedures has been assessed to date in the development of anti-cancer and anti-viral approaches rather than in approaches devoted treating metabolic disorders (Kang *et al.*, 2007; Chang *et al.*, 2009; Kim *et al.*, 2009; Mcintyre *et al.*, 2009; Lambeth *et al.*, 2010). In anti-cancer

and anti-viral therapies, combinations of siRNAs have been proposed to prevent survival of mutants resistant to a single siRNA. However, in the studies mentioned above, the final biological effects of combined siRNAs were either not compared with or only slightly different from the efficiency of single siRNAs. The lack of a significantly improved impact on biological functions of a treatment with two siRNAs relative to a single siRNA (Chang *et al*., 2009; Kim *et al*., 2009) is analogous to the results described in this report. However, although prevention of survival of putative mutants resistant to one type of siRNA is important in potential anti-cancer and anti-viral therapies, the strategy to inhibit any mutation-caused escape from the inhibition of a particular biochemical process is not crucial in metabolic diseases, as one should not expect that such an escape could result in extensive propagation of mutant cells.

In conclusion, the results of our studies confirmed that treatment with siRNA may be an effective method to decrease GAG synthesis is cells from patients suffering from MPS. However, they also indicated that although treatment with a pair of siRNAs silencing expression of genes coding for both isoenzymes involved in particular steps of GAG production may cause a somewhat more efficient inhibition of GAG synthesis than the use of single siRNAs, the differences were not statistically significant. Therefore, the potential benefit from the use of a set of siRNAs (over the use of a single siRNA) is doubtful if we consider the cost-benefit ratio in the putative therapy.

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