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RNA interference against aldehyde dehydrogenase-2: development of tools for alcohol research

Gabriel Cortínez[a,*], Amalia Sapag[a], Yedy Israel[a,b,c]

[a]*Laboratory of Gene Therapy, Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, and*
[b]*Department of Molecular and Clinical Pharmacology, Institute of Biomedical Sciences (ICBM), Universidad de Chile, Olivos 1007, Independencia, Santiago, 8380492, Chile*
[c]*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA*

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Abstract

Liver alcohol dehydrogenase oxidizes ethanol to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenase-2 (ALDH2*1). Individuals who carry a low-activity ALDH2 (ALDH2*2) display high blood acetaldehyde levels after ethanol consumption, which leads to dysphoric effects, such as facial flushing, nausea, dizziness, and headache (''Asian alcohol phenotype''), which result in an aversion to alcohol and protection against alcohol abuse and alcoholism. Mimicking this phenotype may reduce alcohol consumption in alcoholics. RNA interference (RNAi) is a cell process in which a short interfering RNA (siRNA) of 21–25 bp guides the degradation of a complementary target mRNA. Thus, siRNAs may be useful in mimicking the Asian phenotype by inhibiting ALDH2 gene expression. We determined the inhibitory effect of three chemically synthesized siRNAs targeted against rat ALDH2 mRNA in human embryonic kidney cells (HEK-293 cell lines) transfected with a plasmid carrying the rat ALDH2 cDNA. Two of the three siRNAs were active, yielding a 65–75% reduction of ALDH2 activity. Based on the most promising siRNA sequence, three short hairpin RNA (shRNA) genes driven by the human U6 RNA promoter were designed and cloned in a plasmid. After transfection of HEK-293 cells, one of the genes was shown to be active, yielding a 50% reduction of ALDH2 activity. This effect is consistent with a 50% reduction in ALDH2 mRNA, whereas neither β-actin mRNA nor the interferon-inducible transmembrane protein-1 mRNA levels were affected. This study describes chemically synthesized siRNAs and an endogenously synthesized shRNA, which reduce ALDH2 activity and constitute tools that should be of value for further alcohol research.

Keywords: Acetaldehyde; siRNA; shRNA; Interference; Gene therapy

Introduction

Liver alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde, which is further oxidized to acetate by aldehyde dehydrogenase-2 (ALDH2). Some individuals in the East Asian population carry a dominant negative ALDH2 allele (*ALDH2*2*), which codes for a less active ALDH2 (Yoshida and Davé, 1985). After alcohol consumption, these individuals display high blood acetaldehyde levels (Wall et al., 1997), which are responsible for a number of effects, such as facial flushing, nty 7ausea, dizziness and headache (''Asian alcohol phenotype''). Individuals carrying one or two copies of *ALDH2*2* drink moderately or are virtually abstemious respectively, and are protected against alcohol abuse and alcoholism by 60–99% (Chen et al., 1999; Harada et al., 1982b; Higuchi, 1994; Luczak et al., 2006; Thomasson et al., 1991; Tu and Israel, 1995; Zintzaras et al., 2006).

Disulfiram has been used in the treatment of alcoholism for many decades (Hald and Jacobsen, 1948). A disulfiram metabolite binds to the sulfhydryl groups of ALDH2 (Jin et al., 1994; Rossi et al., 2006), blocking the oxidation of acetaldehyde to acetate and increasing the levels of blood acetaldehyde after alcohol consumption (Jones and Teng, 1983). Patients treated with disulfiram experience the equivalent of the Asian phenotype (Harada et al., 1982a), developing an aversion to alcohol. However, disulfiram has several side effects (Bessero et al., 2006; Kalant and Khanna, 1998; Orakzai et al., 2007; Rossi et al., 2006) because of its nonspecific interaction with sulfhydryl groups of other proteins. An alternative to disulfiram to reduce the activity of ALDH2 would be to inhibit *ALDH2* gene expression by the process of RNA interference (RNAi).

* Corresponding author. Tel.: +56-2-978-2934; fax: +56-2-737-7291.
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E-mail address: gcortine@ciq.uchile.cl (G. Cortínez).

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RNAi occurs naturally in several species from plants to animals, including mammals (reviewed by McManus and Sharp, 2002; Fire, 1999; Hannon, 2002). This process requires double-stranded RNA molecules to direct the degradation of a complementary target mRNA, which results in the reduced synthesis of the encoded protein. In the process of RNAi, the antisense strand of the siRNA, usually ranging from 21 to 25 bp (Bernstein et al., 2001; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000), is incorporated into the RNAi silencing complex (RISC), a cytosolic protein complex that guides the cleavage of the complementary mRNA. This degradation is highly specific, discriminating between messengers differing in a single nucleotide (Miller et al., 2004; Schwarz et al., 2006). Nevertheless, not all siRNAs are active. Although some rules have been established to guide their design (Elbashir et al., 2002; Reynolds et al., 2004), they do not take into account the fact that the secondary structure of the target mRNA may hinder the RNAi process (Ameres et al., 2007).

Marked inhibition of the expression of several target genes has been achieved by transfecting cells in culture with siRNAs (see Akhtar and Benter, 2007). For in vivo studies, genes encoding short hairpin RNAs (shRNAs), which are endogenously generated in the cell and further hydrolyzed to siRNA, allow longer acting effects than siRNA (McAnuff et al., 2007). The shRNA genes can be transcribed by RNA polymerase III, which typically synthesizes small RNAs lacking both a Cap structure and a poly A tail (Geiduschek and Tocchini-Valentini, 1988). An RNA pol III promoter that has been widely used to drive the synthesis of therapeutic shRNAs is that of the U6 RNA gene (Brummelkamp et al., 2002; Paul et al., 2002). Once formed, shRNA molecules are processed by Dicer, a type III RNAse (Bernstein et al., 2001), releasing a small double-stranded RNA, which may be recognized and loaded onto RISC. The aim of this study was to design and test synthetic siRNAs and shRNA-encoding gene constructs to reduce ALDH2 activity.

Materials and methods

Cell culture

The HEK-293 cells were obtained from the American Type Culture Collection (ATCC catalog no. CRL-1573) and were grown in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L of glucose (Gibco-Invitrogen, Grand Island, NY) and 1.5 g/L of sodium bicarbonate, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in 5% CO_2 at 37°C.

Short interfering RNA formation

Short RNA hybrids (siRNAs) of 19 bp were formed by annealing two 21-mer oligoribonucleotides (Eurogentec, Belgium), each having two thymidines at their 3′ end (Fig. 1). The sense and antisense oligonucleotides were incubated together (1.5 nmol each) in 75 μL of 50 mM Tris (pH 7.5) and 100 mM NaCl for 2 min at 94°C, 5 min at 78°C and 5 min at 65°C. Finally, the annealed siRNAs were cooled down to 20°C, aliquoted, and stored at −80°C. All the cooling down transitions were carried out at a rate of 2°C/min.

Cell type model

It is well known that primary hepatocytes cannot be readily transfected with lipofectamine, the standard reagent in the field (Holmen et al., 1995). Moreover, the half-life of ALDH2 in primary hepatocytes (Garver et al., 2001) does not allow a rapid estimation of the inhibitory effects of siRNA or shRNA. To circumvent these problems, we lipofected HEK-293 cells simultaneously with both a plasmid coding for the rat ALDH2 enzyme and with the siRNA molecules or a siRNA precursor (shRNA gene). The HEK-293 cells are readily lipofected, and, in addition, do not express ALDH2 activity (0.16 ± 0.14 nmol of NADH/mg of protein/min; not significantly different from zero). The ALDH2 activity of HEK-293 cells transfected with the plasmid encoding the rat ALDH2 ranges from 5 to 10 nmol NADH/mg of protein/min. In vivo, liver ALDH2 activity is reported as 12 nmol NADH/mg of protein/min (Ocaranza et al., 2008), whereas that in rat hepatoma cells (H4-II-E-C3) is 3 nmol NADH/mg of protein/min (Karahanian et al., 2005). H4-II-E-C3 cells are not readily lipofected (only 4% in our hands; data not shown).

Lipofection of HEK-293 cells with short interfering RNA and plasmid

The HEK-293 cells were plated in 1 mL of medium at 1×10^6 to 1.2×10^6 cells per 35-mm culture plate. The

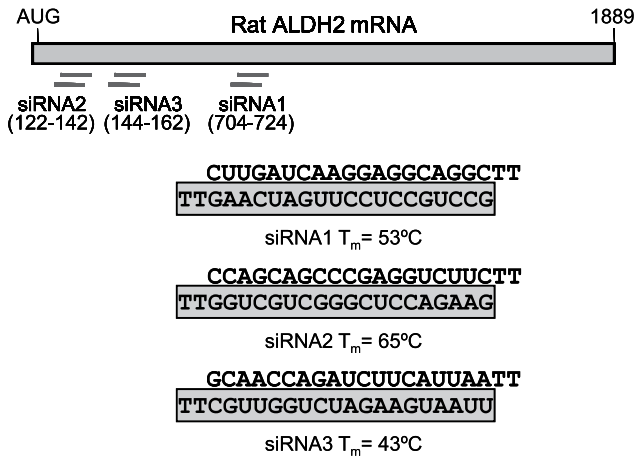

Fig. 1. Targets for short interfering RNA (siRNA) on rat aldehyde dehydrogenase-2 (ALDH2) mRNA. The oligoribonucleotides have two thymidines at the 3′ end that improve resistance to nuclease degradation. The antisense oligoribonucleotides are highlighted in *gray boxes*. The antisense strand of siRNA2 is also complementary to the human ALDH2 mRNA. The T_m was calculated according to Xia et al. (1998).

medium was replaced 24 h later with 1 mL of DMEM without serum, and the cells were lipofected with a complex of cationic liposomes and DNA containing 5 μL of lipofectamine 2000 (Invitrogen), 50 nmol of either the siRNA duplex or the sense or antisense oligonucleotide and 2 μg of plasmid pALDH2 encoding the rat ALDH2 in a gene having a CMV promoter and an SV40 polyadenylation signal. pALDH2 was generated by Eduardo Karahanian in our laboratory; the rat ALDH2 cDNA was excised from p19NSALDH2 (Jeng and Weiner, 1991), a gift from Henry Weiner, and cloned in plasmid pACCMV-pLpLARS (+) (a derivative of pACCMV-pLpA, Gómez-Foix et al., 1992), a gift from Hernán Grenett. After 5 h of lipofection, the medium was replaced with 2 mL of culture medium with serum. Cells were lysed 68 h later for ALDH2 activity measurements. Alternatively, the HEK-293 cells were transfected with a lipofectamine 2000-DNA complex formed by 10 μL of liposomes and 2 μg of each pALDH2 and a plasmid carrying the shRNA gene.

Construction of short hairpin RNA genes

The human U6 RNA promoter was amplified by polymerase chain reaction (PCR) from gDNA (HepG2 cells) with primers TG-205 (AAGGTCGGGCAGGAAGAGG) and TG-228 (CCAAAAACCCAA TGGTAGTATATGTGC TGCCAGAG); the latter has an *Xcm* I restriction site sequence (underlined) and a transcriptional termination signal recognized by RNA pol III. Amplification was carried out with *Taq* DNA polymerase for 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 10 s and elongation at 72°C for 60 s. The amplicon was ligated to pGEM-T Easy (Promega Corporation, Madison, WI), and the clone (pControl) was sequenced to confirm the promoter sequence (GenBank X07425).

The shRNA-coding regions were generated by ligating two previously phosphorylated and annealed oligonucleotide pairs between them and simultaneously to pControl digested with *Xcm* I. It is noted that the ligation of the two separately annealed oligonucleotide pairs (one for the sense and one for the antisense shRNA-coding regions) reduces the probability of self-hybridization (a first-order reaction) of two long complementary single-stranded oligonucleotides. The double-stranded fragments can only be cloned together and in one direction in the *Xcm* I restriction site. Clones were identified by PCR with a primer annealing to the promoter and a primer annealing to the shRNA-coding region and were designated as pshRNA a, b, and c, which varied in the size of the hairpin loop and its orientation.

Determination of aldehyde dehydrogenase-2 activity

Cells were lysed with 0.5 mL of 1% triton X-100 and 1 mM ethylenediaminetetraacetic acid (EDTA) and stored at −80°C. Cell debris was removed by centrifugation, and the supernatant was used to assay ALDH2 activity. The reaction mixture contained 34 mM phosphate buffer (pH 8.75), 4 mM dithiothreitol, 0.8 mM β-NAD^+, 5 mM $MgCl_2$, and 1.4 mM 4-methyl pyrazole. Between 200 and 400 μg of total protein were assayed; the reaction was carried out at 35°C and initiated by the addition of 14 μM propionaldehyde. Production of NADH was measured at 340 nm every 30 s for 10 min. The activity was expressed as nmol NADH/min/mg of total protein.

Calculation of aldehyde dehydrogenase-2 activity reduction

The HEK-293 cells transfected with a plasmid encoding the rat ALDH2 displayed activity of this enzyme with minor variation among culture plates in the same transfection series; however, a larger difference was obtained among different transfection series. To help in the calculation of enzyme activities, we incorporated an oligonucleotide control group in every transfection series: HEK-293 cells were transfected with the plasmid encoding the rat ALDH2 and with an oligonucleotide whose sequence is not related to that of the ALDH2 mRNA, such that all plates (test and control) were transfected with the same amount of nucleic acid. The ALDH2 activity measured in the control group was normalized to total protein and set as 100% ALDH2 activity (range: 5–10 nmol/mg protein/min). The activities in any one lipofection series were expressed as percentages of the activity in the control group of the same series.

Northern blot analysis

RNA from HEK-293 cells lipofected with pshRNAc was prepared from two 35-mm culture plates using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was incubated at −20°C for 16 h before precipitation. The RNA pellets were resuspended in 50 μL of RNAse-free water and quantified spectrophotometrically at 260 nm. Total RNA (20 μg) was subjected to electrophoresis in denaturating 20% polyacrylamide gels prepared in 7 M urea and TBE buffer (90 mM Tris, 90 mM borid acid, 2 mM EDTA; pH 8.4) and run at 15 W in TBE buffer. RNAs were transferred to a Hybond-XL nylon membrane (Amersham Biosciences, UK) in a Trans-Blot (Bio-Rad) electrophoretic transfer chamber at 100 V for 1 h. Membranes were preincubated for 1 h at 65°C in a solution containing 6 × SSC (900 mM sodium chloride, 123 mM sodium citrate; pH 7.0), 5 × Denhardt's solution, 0.5% Derhardts' solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/mL of salmon sperm DNA. RNA hybridizations were carried out with 6 pmol of an oligonucleotide DNA probe (CAG CAGCCCGAGGTCTTC) labeled on the 5′ end with ^{32}P. Membranes were washed three times with 10 mL of a solution containing 2 × SSC and 0.2% SDS, once at 65°C and twice at 55°C. The hybridization signal was collected on BioMax XAR film (Kodak, Rochester, NY).

Results

Three siRNAs were designed against the rat ALDH2 mRNA, two of these (siRNA1 and siRNA2) according to Elbashir et al. (2002) and one (siRNA3) according to Reynolds' rules (Reynolds et al., 2004). Two of these siRNAs are specific for rat ALDH2 mRNA (siRNA1 and siRNA3), whereas siRNA2 targets a sequence shared by both rat and human ALDH2 mRNAs. To test these siRNAs, HEK-293 cells were lipofected simultaneously with a plasmid encoding the rat ALDH2 (pALDH2) and with each of the synthetic siRNAs. Figure 2 shows that siRNA2 and siRNA3 afforded a reduction in ALDH2 activity of 65–75%, whereas siRNA1 was fully inactive. In all cases, the sense and antisense oligoribonucleotide moieties of the active siRNAs, tested separately, had no significant effect on ALDH2 activity, indicating that the siRNAs acted by means of a true RNAi process rather than by an antisense mechanism.

Short interfering RNA2 was chosen as the most promising sequence to develop an interference gene, because it targets both the rat and human ALDH2 mRNAs. To obtain shRNAs, we designed plasmids carrying the human U6 RNA promoter followed by the sequence encoding the hairpin. The 5′ end of the shRNA transcript included the first 27 nucleotides of the human U6 RNA, because an shRNA carrying this sequence on its 5′ end was shown, for another gene, to be more efficient in the silencing process than the shRNA sequence alone (Paul et al., 2002), probably because of an increased stability of the RNA provided by the initial 27 bases. The "UUCG" sequence was initially chosen for the hinge region connecting the sense and antisense strands of the RNA, because it forms a very stable turn or loop (Cheong et al., 1990; Varani et al., 1991) that may allow the folding of the RNA into a hairpin structure that may be recognized and cleaved by Dicer. This initial connector sequence did not result in active shRNAs anti-ALDH2 independently of its relative orientation in the hairpin (Fig. 3A—shRNAa and shRNAb; Fig. 3B—shRNAa, $P < 0.9$ and shRNAb, $P < 0.09$). In contrast, a larger connecting hinge having the sequence "UUCAAGAGA," which has been used in other shRNA constructs (Takigawa et al., 2004; Zhou et al., 2005), allowed a plasmid encoding this shRNA (shRNAc) to diminish the ALDH2 activity by 50% ($P < .05$) (Fig. 3B, shRNAc, $P < .05$). From previous work (Garver et al., 2001; Ocaranza et al., 2008), it is known that reductions in ALDH2 activity of the order of 40% result in marked inhibition of alcohol consumption in rats.

To determine if the reduction in ALDH2 activity obtained with shRNAc was because of lower mRNA levels, *Aldh2* transcripts from HEK-293 cells simultaneously lipofected with pALDH2 and the plasmid coding for shRNAc (or a control plasmid) were quantitated by reverse transcription followed by PCR. Data in Figure 4, showing a 58.4% reduction of *Aldh2* mRNA by shRNAc, are consistent with the reduction of ALDH2 activity in cells lipofected with pshRNAc. Transcripts of the β-actin gene showed no changes (4% reduction in mRNA). Additionally, the level of interferon-inducible transmembrane protein-1 (IFITM-1) mRNA, which increases when the interferon pathway is

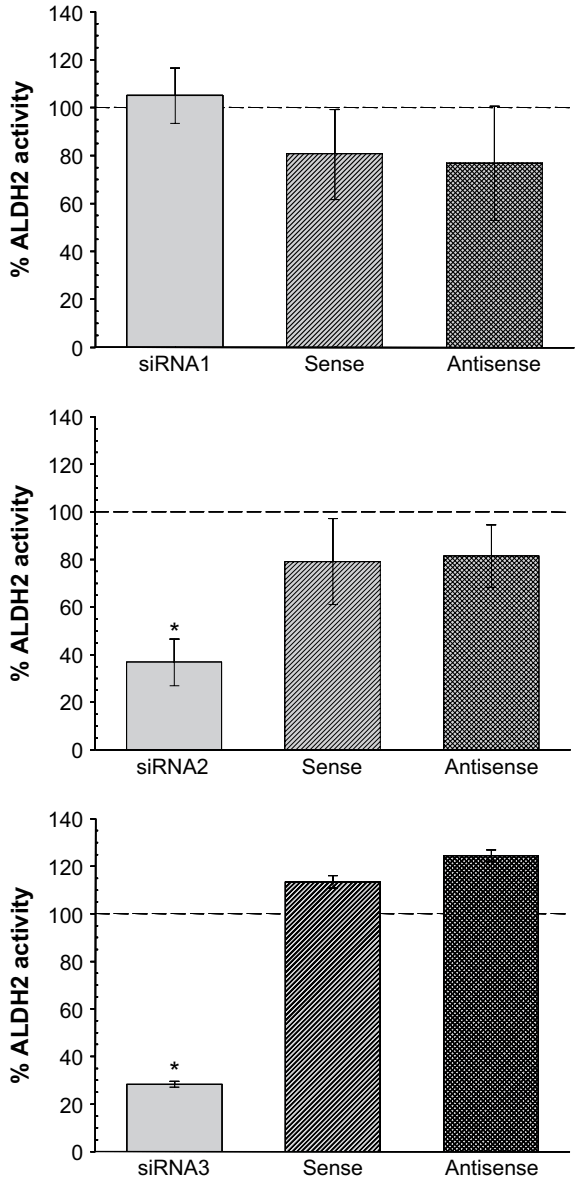

Fig. 2. Aldehyde dehydrogenase-2 (ALDH2) activity of human embryonic kidney cells (HEK-293) lipofected with siRNAs. ALDH2 activity of HEK-293 cells lipofected with plasmid encoding ALDH2 (pALDH2), a plasmid-encoding rat ALDH2, and either short interfering RNA (siRNA)1, 2, 3 or their respective sense and antisense oligonucleotides. Activities were measured 72 h after lipofection, corrected for protein, and normalized according to the activity present in cells lipofected with a non-related oligonucleotide within the same lipofection session (control: *dashed line*). Short interfering RNAs 2 and 3 result in significant reductions ($P < .05$) of ALDH2 activity, compared to the respective sense or antisense moieties. Reductions of ALDH2 activity with siRNAs 2 and 3 are equivalent. Results are from three independent experiments performed in triplicate.

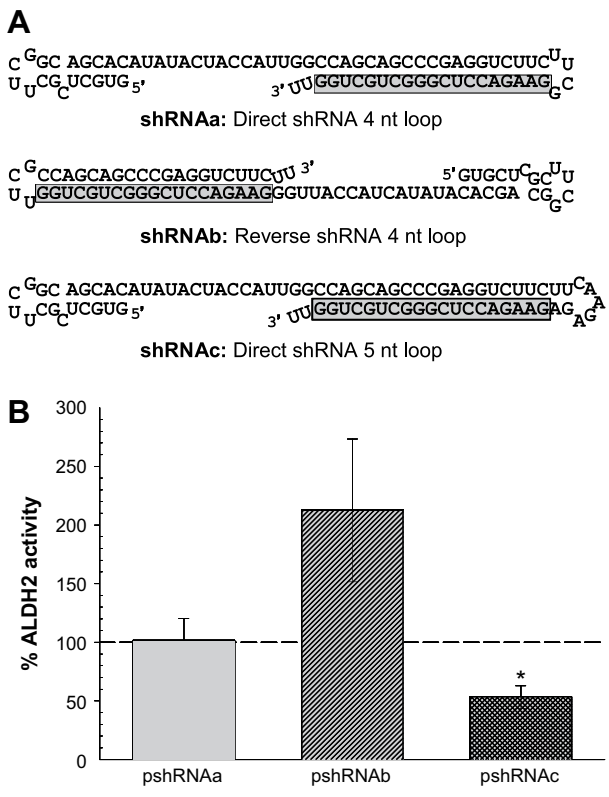

Fig. 3. Folding and inhibitory activity of short hairpin RNAs (shRNAs) obtained by transcription from genes controlled by the human U6 RNA promoter. (A) The diagram shows the folding of each shRNA predicted by the MFOLD program (Zuker, 1989). All three shRNAs have the first 27 nucleotides of the human U6 RNA, as this sequence improves silencing. The hairpins were designed to be processed by Dicer and release short interfering RNA2 (siRNA2). Sequences complementary to the aldehyde dehydrogenase-2 (ALDH2) mRNA are highlighted in *gray boxes*. Short hairpin RNAa and shRNAb are hairpins having a 4-nucleotide hinge region upstream and downstream of the antisense sequence, respectively. Short hairpin RNAc is a hairpin RNA having a 9-nucleotide hinge region upstream of the antisense sequence. (B) ALDH2 activity of HEK-293 cells 72 h postlipofection with plasmids coding for rat ALDH2 and shRNAs a, b, or c. The activity of ALDH2 was reduced 50% by pshRNAc ($P < .003$). The construct yielding siRNAs was inactive, whereas the increase in ALDH2 activity seen with shRNAb was not statistically significant ($P < .09$). The ALDH2 activity of human embryonic kidney (HEK 293) cells lipofected with pControl was defined as 100% (*dashed line*). Results are from three (pshRNAa and pshRNAb) or four (pshRNAc) independent experiments performed in triplicate.

activated (Yang et al., 2007), was not augmented by the shRNAc gene (3.5% mRNA reduction), indicating that the toxic interferon system was not activated (Fig. 4). Northern blot analysis of RNA extracted from HEK-293 cells transfected with a plasmid-encoding shRNAc demonstrated that the shRNAc gene is transcribed and that shRNAC is cleaved to generate siRNA (Fig. 5).

Discussion

The main contributions of this communication are: (1) the design of two siRNAs directed against ALDH2 mRNA that markedly reduce ALDH2 activity; (2) the generation of

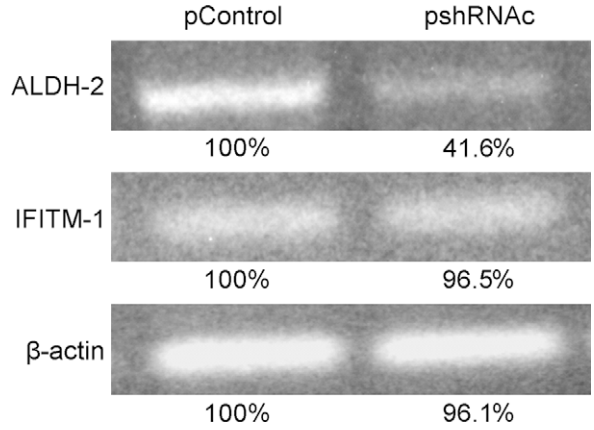

Fig. 4. Specific reduction of rat aldehyde dehydrogenase-2 (ALDH2) mRNA by short hairpin RNA (shRNA) without activation of the interferon system. Reverse transcription followed by polymerase chain reaction of ALDH2 interferon-inducible transmembrane protein-1 (IFITM-1), and β-actin mRNA of human embryonic kidney (HEK-293) cells lipofected with pALDH2 and pshRNAc or pControl. The β-actin mRNA levels were constant. The IFITM-1 mRNA levels, which rise when the toxic interferon system is activated, remained unchanged. Relative quantification is shown (Scion program).

a gene construct driven by an RNA polymerase III promoter that yields an active shRNA; and (3) the confirmation that not all siRNAs display interference effects and not all hairpin hinges result in active constructs. These molecules should be of value for further studies aimed at developing medications as adjuncts for the treatment of alcoholism or in basic alcohol research.

Active short interfering RNA constructs

Notably, one of the two siRNAs tested initially (siRNA2) was active, whereas the other one (siRNA1), designed applying the same criteria (Elbashir et al., 2002), was fully inactive. An additional consideration for the design of siRNA2 was the fact that its antisense strand hybridizes to both the rat and human ALDH2 mRNAs. The availability of subsequent rules for the design of siRNAs (Reynolds et al., 2004) allowed the synthesis of a third siRNA, was also active. Using Reynolds' rules, candidate siRNAs can be ranked according to a score based on their primary structure, with a higher score predicting a greater inhibition. A cutoff score of 6 predicts siRNAs that are likely to achieve or exceed a 50% inhibition of gene expression; siRNA3, having a score of 9, showed 70% inhibition of *Aldh2* gene expression. Nevertheless, an siRNA can be active despite having a low score; siRNA2, with a score of 2, afforded 65% reduction of ALDH2 activity. This may indicate that other factors, such as the accessibility of the target sequence, are relevant and should be incorporated into the algorithm.

Short hairpin RNA-generating genes

To obtain a drug that exerts its effects for prolonged periods, a desirable condition is the continuous generation

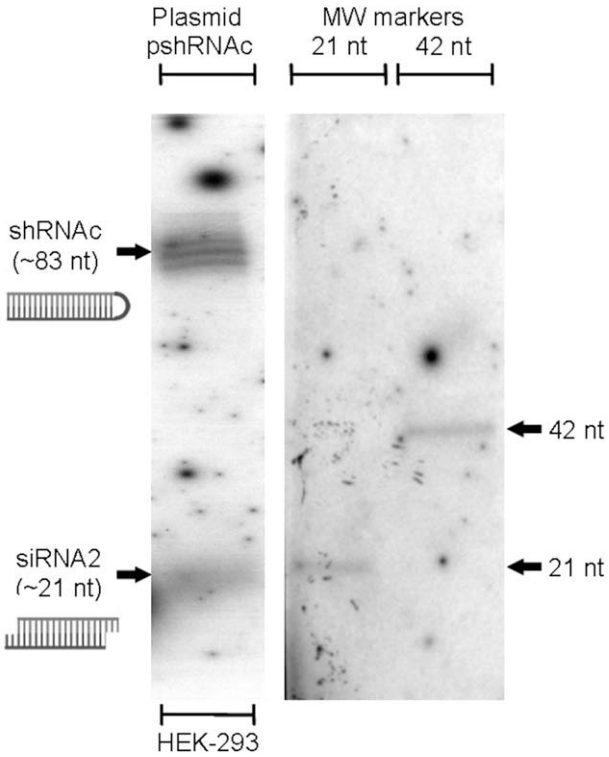

Fig. 5. Northern blot analysis of RNA from HEK-293 cells lipofected with pshRNAc. Detection of short hairpin RNAc (shRNAc) and short interfering RNA2 (siRNA2) in human embryonic kidney (HEK-293) cells lipofected with pshRNAc. Total RNA (10 μg) was electrophoresed and hybridized with a radioactive probe against shRNA and siRNA. Detection of shRNAc (83 nucleotides; see Fig. 3) indicates that the shRNA gene was transcribed, and detection of siRNA2 indicates that shRNAc was cleaved to yield siRNA2.

of the active siRNA in the cell itself, such as the generation of shRNAs, which are processed by Dicer to siRNAs. In the present study, three gene constructs having the U6 RNA pol III promoter and coding for shRNAs (a, b, and c), based on the siRNA2 sequence were generated. One of these gene constructs reduced ALDH2 activity by 50% ($P < .003$), a slightly lower effect than that of siRNA2, probably because the gene must first reach the nucleus to be transcribed, and the resulting shRNA must be subsequently exported to the cytoplasm to be processed by Dicer to exert its intended biological function. In contrast, synthetic siRNAs reach the cytosol more readily because, when delivered by lipofection, the siRNA is released from endosomes directly into the cytoplasm where both mRNA recognition and cleavage occur. Unexpectedly, shRNAb provided an increase in ALDH2 activity, although with marginal significance ($P < 0.09$), an effect that was not investigated further.

Relevance of the hinge region of short hairpin RNA

We show that the sequence of the hinge region connecting the arms of the hairpin is important to allow RNAi activity. With the 4-nucleotide connector "UUCG," no reduction in ALDH2 activity was obtained, independently of whether the hinge was placed upstream (shRNAa) or downstream (shRNAb) of the antisense strand (Fig. 3), whereas the 9-nucleotide hinge "UUCAAGAGA" placed upstream of the antisense strand (shRNAc) resulted in 50% inhibition of ALDH2 activity. Because all hairpins were designed to release the same siRNA (siRNA2), the differences are likely to be in the efficiency of cleavage by Dicer. It should be noted that the 9-nucleotide connector sequence of the active shRNAc has the potential of extending the stem by 2 bp, adding almost one-sixth of a turn (60°) to the helix (Tang and Draper, 1994) and providing a 5-nucleotide loop, both of which might facilitate the cleavage of the target by Dicer.

Feasibility of a gene therapy against alcoholism

Studies by Helander and Carlsson (1990) showed that alcoholics receiving therapeutic doses of disulfiram display 40–60% inhibition of ALDH2 activity (determined in leukocytes). Garver et al. (2001) showed that a 40% reduction of liver ALDH2 activity by an antisense phosphorothioate oligodeoxynucleotide administered in vivo results in 65% reduction of voluntary alcohol consumption in rats, demonstrating that a gene-based therapy for alcoholism is feasible. However, phosphorothioate oligonucleotides have a half-life of 48 h (Zhang et al., 1996), requiring the continuous administration of the antisense drug. Our cell culture systems do not allow the determination of long-term effects, given that once they reach confluence, 72–96 h post-transfection, cells tend to detach from the plate and show erratic ALDH2 activities.

Although the antisense RNA generated in the studies of Ocaranza et al. (2008) is likely capable of inhibiting the expression of several *Aldh* genes, the siRNA and shRNA molecules of the present study are only complementary to the mRNA of ALDH2. The siRNA technology constitutes a promising tool to inhibit the expression of a gene in a specific fashion as it can discriminate between alleles differing in a single nucleotide (Schwarz et al., 2006). This specificity may also reduce the side effects that normally arise by the use of chemical inhibitors (Bessero et al., 2006; Kalant and Khanna, 1998; Orakzai et al., 2007; Rossi et al., 2006).

To determine whether there were toxic secondary effects of shRNAc because of activation of the interferon system, we measured the IFITM-1 mRNA level (a marker of activation of the interferon pathway). We did not observe increases in IFITM-1 mRNA levels, indicating that the interferon pathway was not activated. Grimm et al. (2006) reported that shRNAs with a short stem length of 19 bp, similar to our active shRNAc, were nontoxic and showed silencing effects that lasted over 400 days.

Overall, the present study describes the generation of gene tools to reduce ALDH2 gene expression based on RNAi. Synthetic siRNA- and shRNA-coding genes aimed

at reducing ALDH2 activity should be of value for subsequent studies in the field of alcoholism.

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