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Research Paper

Gene Silencing with siRNA Duplexes Composed of Target-mRNA-Complementary and Partially Palindromic or Partially Complementary Single-Stranded siRNAs

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NOTE

Supplemental information can be found at www.landesbioscience.com/journals/rnabiology/supplement/Hossbach_Suppl_Table1.xls

ABSTRACT

Synthetic small interfering RNA (siRNA) duplexes are widely used to transiently and sequence-specifically disrupt gene expression in mammalian cultured cells. The efficiency and specificity of mRNA cleavage is partly affected by the presence of the nontargeting "passenger" or "sense" siRNA strand, which is required for presentation of the target-complementary or guide siRNA strand to the double-strand-specific RNA silencing protein machinery. We show that siRNA duplexes can be designed that are solely composed of two fully target-complementary guide strands that are sufficiently complementary to each other to form stable duplexes with characteristic 3′ overhanging ends. The general feasibility of this approach is documented by transient knockdown of lamin A/C and emerin in HeLa cells. The silencing efficiencies of guide-only siRNA duplexes are comparable to prototypical fully paired passenger/guide duplex siRNAs, even though guide-only siRNA duplexes may contain a significant number of nonWatson-Crick and G/U wobble base pairs. Such siRNA duplexes may offer advantages regarding production costs and specificity of gene silencing.

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INTRODUCTION

Transient gene silencing using small interfering RNAs (siRNAs) is a powerful method for loss-of-function studies in cultured mammalian cells (reviewed in ref. 1). RNA interference (RNAi) functions by incorporation of one of the strands of the duplex siRNAs into a mRNA-targeting effector complex, known as the RNA-induced silencing complex (RISC).[2,3] In mammalian systems, RISC is formed after loading of one of the duplex-constituting siRNA strands into the Argonaute 2 (Ago2) endonuclease protein.[4-7] The presence of the target-mRNA-complementary strand in RISC is a prerequisite for effective gene silencing.

siRNA-mediated gene knockdown has not only been used in cultured cells but it has also been accomplished in animals under certain conditions. Genes expressed in the nervous system of mice and rats have been specifically targeted by nuclease-stabilized as well as chemically unmodified siRNAs by local delivery and slow, continuous release of the siRNAs over one or two week periods.[8,9] Systemic injection of relatively high doses of cholesterol-conjugated siRNAs (dose of 50 mg/kg) was demonstrated to effectively repress genes expressed in the liver and jejunum.[10] More recently, systemic injection of non-conjugated siRNAs formulated in liposomes yielded effective and long-lasting repression in monkeys at a dose as low as 2.5 mg/kg.[11] These early findings indicate that siRNAs can be developed to silence genes involved in human disease.

Besides siRNA delivery, which still represents the major obstacle to in vivo application, the identification of highly effective and specific siRNAs that enable durable and gene-specific silencing represents the other hurdle. The latter is generally overcome by screening a fairly large number of possible target-specific siRNAs. Various factors seem to be responsible for causing variations in silencing efficiency: (1) Asymmetry of assembly of the RNA-induced silencing complex (RISC) causing the sense or passenger siRNA strand to enter more effectively into RISC than the target-complementary guide siRNA;[12-15] (2) Inaccessibility of the targeted segment on the mRNA might reduce accessibility to RISC;[16] (3) A high degree of off-target activity by a given siRNA may reduce its on-target activity, assuming that the amount of RISC formed in a cell is constant;[17,18] (4) Natural Dicer RNase III processing products vary in size between 21- and 23-nt with a 2- or 3-nt 3' overhang[19] and similar sequence-dependent variations for processing have been observed for

mature miRNAs.[20,21] It can be speculated that conventional 21-nt siRNAs with 2-nt 3' overhangs are suboptimal for certain sequences with respect to reentry into the RNAi pathway. This might explain why longer blunt-ended duplex or hairpin RNAs (of up to 29 bp), which are still substrates for Dicer processing, sometimes appear more effective than conventional siRNA duplexes;[16,22-24] (5) Finally, the balance of structural and kinetic effects described above may be important and its impact on siRNA silencing efficiency has been discussed in various contexts.[25-29]

It is apparent that one can increase the likelihood of identifying functional siRNAs by statistical and bioinformatic assisted methods for selecting siRNA sequences to a target mRNA sequence (for a recent review see ref. 30). Aside from the fact that existing bioinformatics tools increase, but not guarantee selection of efficient siRNA target sites, specific association of siRNA and/or target mRNA with RNA-binding proteins cell-type or tissue-specific changes in the composition of such proteins are factors that one might consider if one is trying to seek explanations.

We examined if a less conventional approach to the design of siRNAs could be taken and developed a simple software tool to identify siRNA duplexes that are only composed of sequences complementary to the target mRNAs. We selected the siRNA strands based on their property to be either partially self-palindromic or partially complementary to each other, while at the same time being fully complementary to the target sequence. This strategy eliminates the need for the presence of a nontargeting passenger siRNA strand. Many of the selected and tested siRNA sequences were effectively silencing the targeted gene. We also show that the targeting of two genes with different sequences by one single siRNA duplexes is possible.

MATERIALS AND METHODS

siRNA sequence selection and siRNA synthesis. To identify targeting mRNA segments that were partially palindromic or partially self-complementary and for siRNA design, we wrote a Perl script that we made accessible as web-tool for guide-only siRNA selection (http://www.mpibpc.mpg.de/groups/luehrmann/siRNA). The program accepts one or two cDNA or mRNA sequences in raw format and computes the degree of complementarity for all possible base-paired combinations of 19-nt complementary sequence segments of the query sequences. The paired sequences are analyzed for their content of G/U wobble and other nonWatson-Crick base pairs. For selection of guide-only siRNAs the content of G/U wobble and other nonWatson-Crick base pairs within the 19-nt paired regions is defined by an adjustable filter function, where we did not permit more than seven nonWatson-Crick base pairs including G/U wobbles. The sequence of the 2-nt 3' overhang was chosen as dTdT and was not required to match the target mRNA(s). The program output provides graphic and text output for the positions of the candidate oligos relative to the target mRNAs, and illustration, description and sequence for the siRNA duplexes and their Watson-Crick and non-Watson-Crick base pair composition. Text output is comma-delimited for easy import into spreadsheet programs. The conventional perfectly paired siRNA duplexes that we used for comparison were specific to the target mRNA according to BLAST searches against the human genome sequence (NCBI UniGene database).

To estimate the probability for identifying suitable guide-only siRNA duplexes, we selected 100 different mRNA sequences from GenBank in a range of 400 to 9000 nt (GenBank accession numbers are listed in Supplementary Table 1), and counted all possible guide-only siRNA duplexes for each mRNA sequence using our siRNA selection software. We only counted duplexes that had no more than 7 nonWatson-Crick base pairs, whereby the number of nonG/U wobble base pairs was limited to 5, but the number of G/U wobble base pairs could reach up to 7. Sequences with runs of 4 or more nucleotides were also excluded. For the counting of dual-targeting siRNA combinations, NM_012084 (2348 nt encoding GLUD2) was selected as the invariant first target mRNA, and scanned against the set of 100 target mRNAs described above. Statistical tools were used from Microsoft Excel for scatter plot data presentation, regression analysis with linear and potential trendline, and for the calculation of average and standard deviation.

21-nt siRNAs were purchased 5'-phosphorylated from Dharmacon, Lafayette, CA. Duplex siRNAs were formed by incubating 20 μM single-stranded siRNAs or 40 μM palindromic siRNA in 100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES/KOH (pH 7.4) for one minute at 90°C followed by incubation for one hour at 37°C. Annealing was examined by running aliquots on 4% NuSieve agarose gels.[31]

Cell culture and transfection of siRNA. The human HeLa SS6 cell line was grown in DMEM containing 10% fetal calf serum, penicillin and streptomycin at 37°C with 5% CO_2. Transfection with siRNA and Oligofectamine (Invitrogen) was performed as previously described.[31]

Antibodies and indirect immunofluorescence microscopy. The monoclonal lamin A/C antibody clone 636.23 (mouse IgG),[32] the vimentin antibody clone V9 (mouse IgG)[33] and the monoclonal emerin antibody (mouse IgG) (Novocastra, Germany) were used as primary antibodies. Secondary fluorescently labelled goat anti-mouse antibodies were purchased from Dianova (Hamburg, FRG). Cells were grown on glass coverslips, washed with PBS and fixed at -20°C with methanol for 10 min. Fixed cells were washed briefly with PBS. Primary antibodies were then added and incubated for one hour at 37°C in a humid chamber. Coverslips were washed three times with PBS and incubated with the labelled secondary antibodies for one hour at 37°C. Cells were again washed three times in PBS and stained with 1 μM Hoechst 33342 (Sigma) to visualize the DNA. The coverslips were subsequently mounted on glass slides in Mowiol (Hoechst, Frankfurt, FRG).

Western blotting. SDS gel electrophoresis was performed according to standard protocols. Proteins were separated by standard SDS gel electrophoresis and then transferred onto nitrocellulose membrane using the semi-dry transfer procedure.[34] Membranes were blocked in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.4) containing 5% skim milk powder. Antibodies against lamin A/C, emerin, or vimentin were diluted in the same buffer but with 2.5% skim milk powder and incubated with the membrane for 1 h at room temperature. Vimentin protein levels were measured to control equal protein loading of the wells in Western blot analysis. Membranes were washed twice with TBST and once with TBST containing 0.5% Triton X-100. Affinity purified horseradish-conjugated swine anti-mouse immunoglobulins were from Dako (Copenhagen, Denmark. They were diluted 1:10,000 in the blocking buffer with 2.5% skim milk powder and applied for two hours at room temperature. The bands were detected using the ECL kit (Amersham Biosciences) and quantified on a LumiImager (Boehringer/Roche, Germany).

bDNA assay. The branched DNA (bDNA) assay (QuantiGene Discovery System, Genospectra, Fremont, CA) was used for

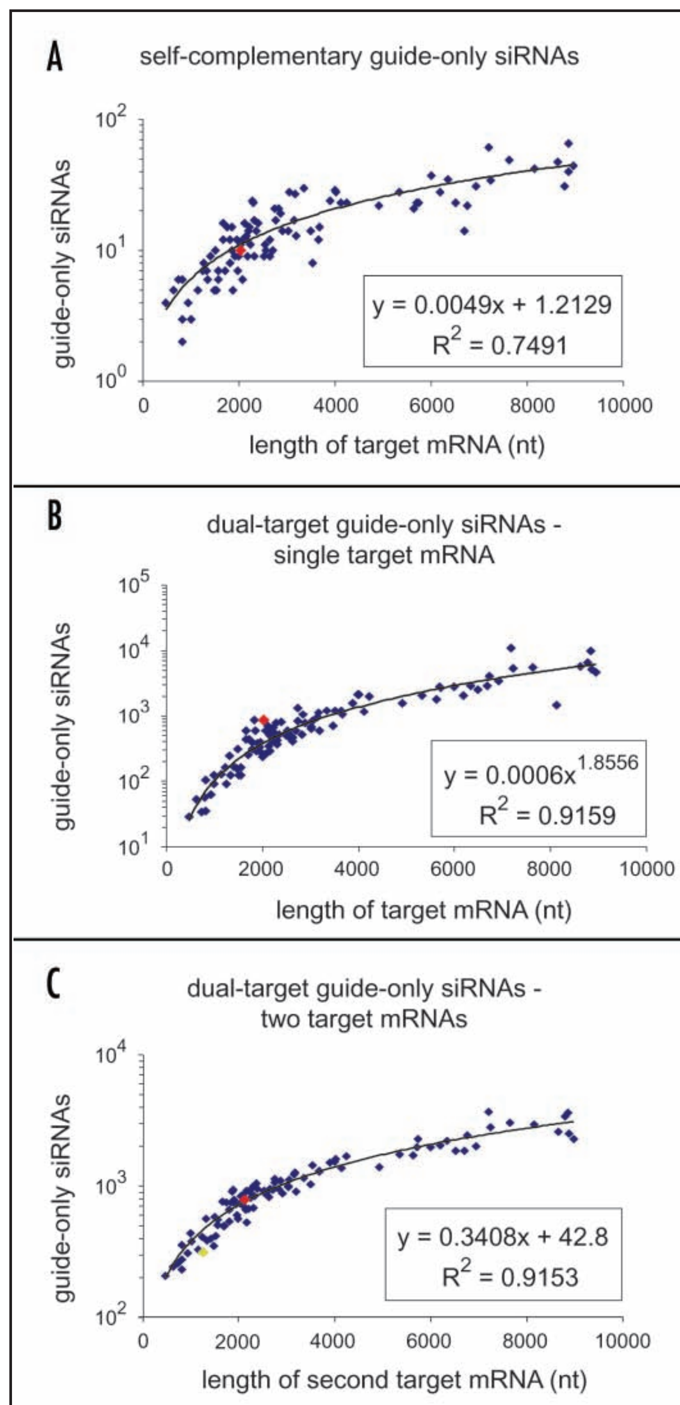

Figure 1. Analysis of the frequency of finding guide-only siRNA target sites in a set of 100 different mRNAs. The graphs display the number of siRNA duplexes as function of the length of the selected mRNAs. The data points were fitted to linear or exponential equations and the coefficients of determination (R^2) for the plots are listed. The required stringency of base pairing is described in the Methods. Red dots indicate datapoints for lamin A/C mRNA. Green dot indicates datapoint for emerin mRNA. (C) The first mRNA sequence (GenBank identifier NM_012084) with a length of 2348 bp was kept constant while for the second mRNA sequence the set of 100 different mRNAs was used.

quantification of mRNA levels in cellular extracts as previously described[35,36] and also following the manufacturer's protocol. The assay is based on specific cohybridization of the target mRNA with oligodeoxynucleotide probes provided by Genospectra. A probe set consists of three types of oligonucleotides: (1) the capture extenders (CEs), which are complementary to the target mRNA and to nucleotides, which are immobilized in the capture plate; (2) the label extenders (LEs), which bind the bDNA amplifier and also the target mRNA; (3) the blocking oligonucleotides (BLs) required for subsequent enzymatic development specific chemiluminescence. CEs, LEs and BLs are designed to hybridize to a 300 to 500 nucleotide region along the target mRNA forming a DNA/RNA duplex. HeLa SS6 cells were grown in 96-well plates and transfected as described.[37,38] Cells were disrupted by application of lysis buffer containing CEs, LEs and BLs. 50 μl of lysis buffer and 100 μl medium were applied and cells were incubated for one hour at 37°C. Cell lysates where transferred to the capture plates, which were sealed and incubated at 52°C overnight. Capture plates were washed and incubated with labelling/amplifier solution for one hour at 37°C. Subsequently plates were developed for 30 min at 52°C with substrate solution. Emitted luminescence was measured with a luminometer (VICTOR Light, PerkinElmer, Wellesley, MA).

bDNA reagents specific for lamin A/C, emerin and β-actin mRNA were used. Cell lysates were prepared 44 h post siRNA transfection and the mRNA levels were determined using the appropriate probes, but similar mRNA levels were measured when cell lysates were prepared between 18 to 96 h post transfection. Lamin A/C and emerin mRNA levels were expressed as ratios relative to the non-targeted β-actin mRNA control. The mRNA knockdown (in %) was expressed as the ratio of specific siRNA treated cells versus control- GL2-siRNA transfected cells.

RESULTS

siRNA design and feasibility analysis. Analysis of the base-pairing patterns in pre-miRNA RNase III processing products (miRNA/miRNA* duplexes) revealed that multiple mismatches are tolerated in these dsRNA processing intermediates. This suggests significant flexibility for the sequence selection of a passenger siRNA strand in an RNAi experiment and that deviations from the prototypical fully complementary strand should be allowed. We first evaluated computationally if it was practical to identify sufficiently large numbers of stable siRNA duplexes in which the guide and passenger strand siRNA were both, complementary to target mRNA segments, and hybridized stably to each other. Three types of sequence selections meet this requirement: (1) The guide siRNA is selected to be partially self-complementary (or palindromic). It is obvious though important to note that a partially self-complementary siRNA strand is always in equilibrium with a short fold-back hairpin structure and the equilibrium between duplex and fold-back structure depends on the nature of the mismatches and the strand concentration. This is also illustrated in the literature in an example where it was attempted to crystallize a stable hairpin RNA, yet the final obtained structure revealed a duplex formed by two strands of the partially single strand so that the segment of the centered hairpin loop was paired in a nonWatson-Crick though stable manner flanked by perfectly paired stems.[39] (2) Because palindromic sequence duplexes compete in structure with the fold-back hairpin structure of the single strand, we thought to avoid this potential problem by selecting siRNA duplexes that are composed of nonpalindromic strands fully complementary to two distinct segments of the target mRNA, yet at the same time being partially complementary to itself. (3) The two strands composing a nonpalindromic siRNA duplex are selected to

be complementary to two different target mRNAs, allowing simultaneous silencing of two genes with a single siRNA duplex.

To assess the frequency for finding siRNA duplexes that meet our design criteria, we examined a dataset of 100 different mRNAs covering a range of 400 to 9000 nt (Supplementary Table 1). Partially self-complementary guide-only siRNAs occurred with an average frequency of 5 ± 2 siRNAs per 1000 nt of target mRNA, and dual-target guide-only siRNAs were found with 598 ± 443 (one target sequence) and 358 ± 58 (two target sequences, one of them constant) siRNAs per 1000 nt of target mRNA (Fig. 1). These numbers indicate that this approach is feasible and that many more siRNA candidates can be identified then one normally would be able to test. For our experimental analysis, we selected lamin A/C and emerin as targets because we had previously targeted these genes using numerous prototypical siRNAs.[16,31,37,38]

Silencing of lamin A/C using partially palindromic siRNA sequences. We first identified partially self-complementary (or palindromic) 18- or 19-nt segments within the human lamin A/C mRNA using a modified local version of our software accessible as web-tool under http://www.mpibpc.mpg.de/groups/luehrmann/siRNA. The program examined all 18- or 19-nt antisense segments derived from the input target mRNA sequences for being partially self-complementary while applying a filter function to control for the stability of the self-complementarity duplex. Though the stability of duplexes containing certain nonWatson-Crick pairs has been evaluated within certain sequence contexts,[40] the rules and parameters are incomplete to reliably predict the stability of mismatched duplexes and experimental approaches were included.

We synthesized the partially palindromic guide siRNA strands as well as the complementary perfectly paired passenger siRNA strand cognate to positions 16, 94, 447 and 608 relative to the first nucleotide of the start codon of lamin A/C mRNA. The predicted structures of the paired self-complementary sequences are shown in (Fig. 2A). We also synthesized packaging strands fully complementary to the partially palindromic sequences and prepared prototypical siRNA duplexes. All siRNAs were synthesized 5'-phosphorylated. The prototypical passenger/guide siRNA duplex and the guide-only palindrome were subsequently transfected into human HeLa SS6 cells. A noncognate conventional siRNA duplex directed against GL2 firefly luciferase was used as control. The amount of lamin A/C protein knockdown was assessed 44 h after transfection using indirect immunofluorescence and the lamin A/C specific antibody. All of the prototypical passenger/guide siRNAs and three out of the four guide-only siRNAs were silencing the lamin A/C gene (Fig. 2B). The passenger/guide or

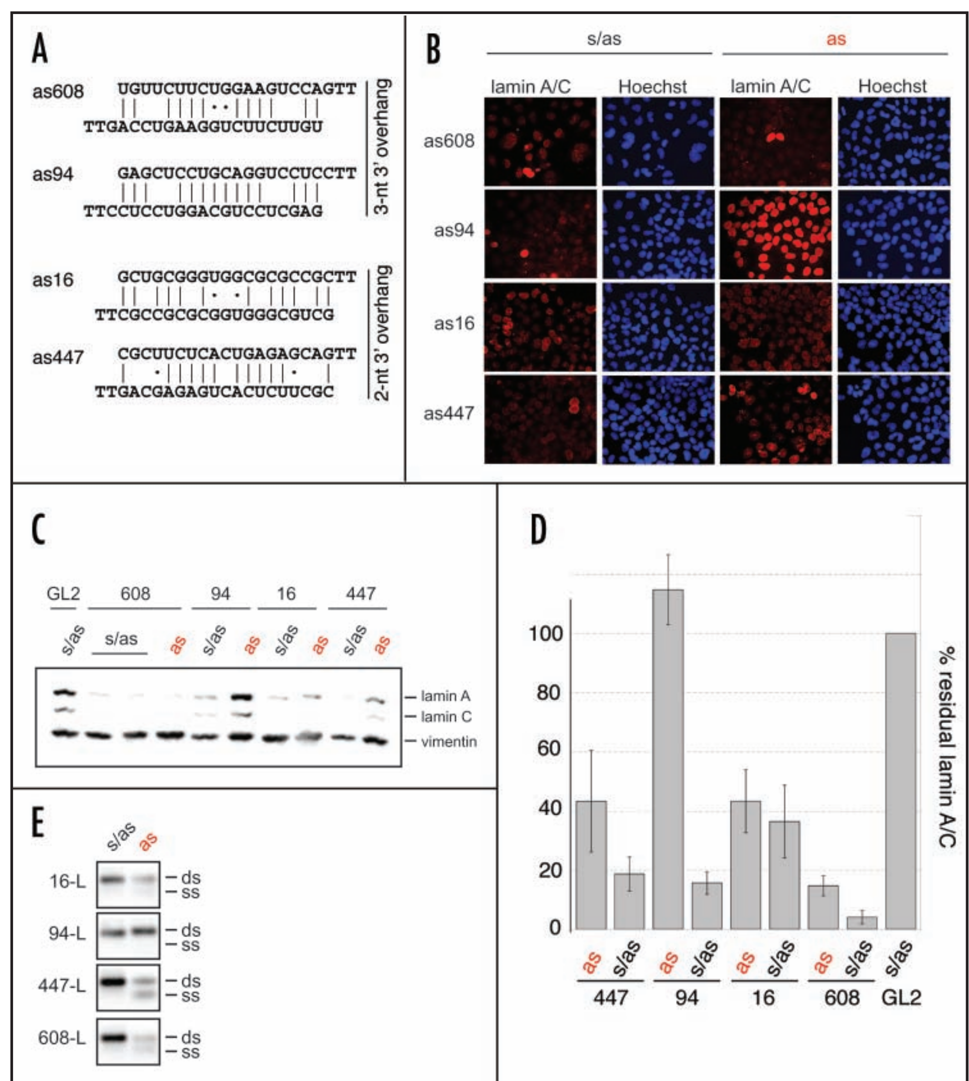

Figure 2. Silencing of lamin A/C using partially palindromic siRNAs. (A) Sequence and structure of the predicted palindromic siRNA duplexes. The GenBank sequence identifier for lamin A/C was NM_005572. (B) Indirect immunofluorescence micrographs of HeLa cells after transfection of prototypical (s/as) and palindromic duplex (as) siRNAs. Cells were stained with the lamin A/C antibody 44 h after transfection of siRNAs. DNA was visualized with Hoechst 33342. Positions of siRNA on the lamin A/C mRNA are given on the left side. The left panel shows cells transfected with prototypical siRNAs (s/as), the right panel shows cells transfected with the corresponding guide-only (as) siRNAs. Cells are shown at 400-fold magnification. (C) Western blot analysis of HeLa cells after transfection with palindromic siRNAs. Cell extracts were harvested and blotted with antibodies specific for lamin A/C and vimentin 44 h after siRNA transfection. The unspecific GL2 siRNA served as a negative control and the nonphosphorylated lamin A/C siRNA as a positive control. The GenBank sequence identifier for GL2 luciferase was X65324. Reduction of lamin A/C was observed for transfection of both prototypical and guide-only siRNAs targeting the regions 608, 16 and 447. (D) Quantitative western blot analysis of HeLa cell extracts after transfection of prototypical and guide-only siRNAs. The blots were probed as in (C) and examined by a luminescence reader. Values shown are standardized with vimentin and normalized with the GL2 negative control set to 100%. (E) Analysis of siRNA duplex formation by native gel electrophoresis. Guide-only siRNAs (as), and annealed passenger/guide (s/as) siRNAs were separated on 4% NuSieve agarose gels. The mobility of the duplex (dsRNA) and the single-stranded or fold-back siRNA (ssRNA) are indicated. The bands were visualized by UV light after ethidium bromide staining and digitally inverted.

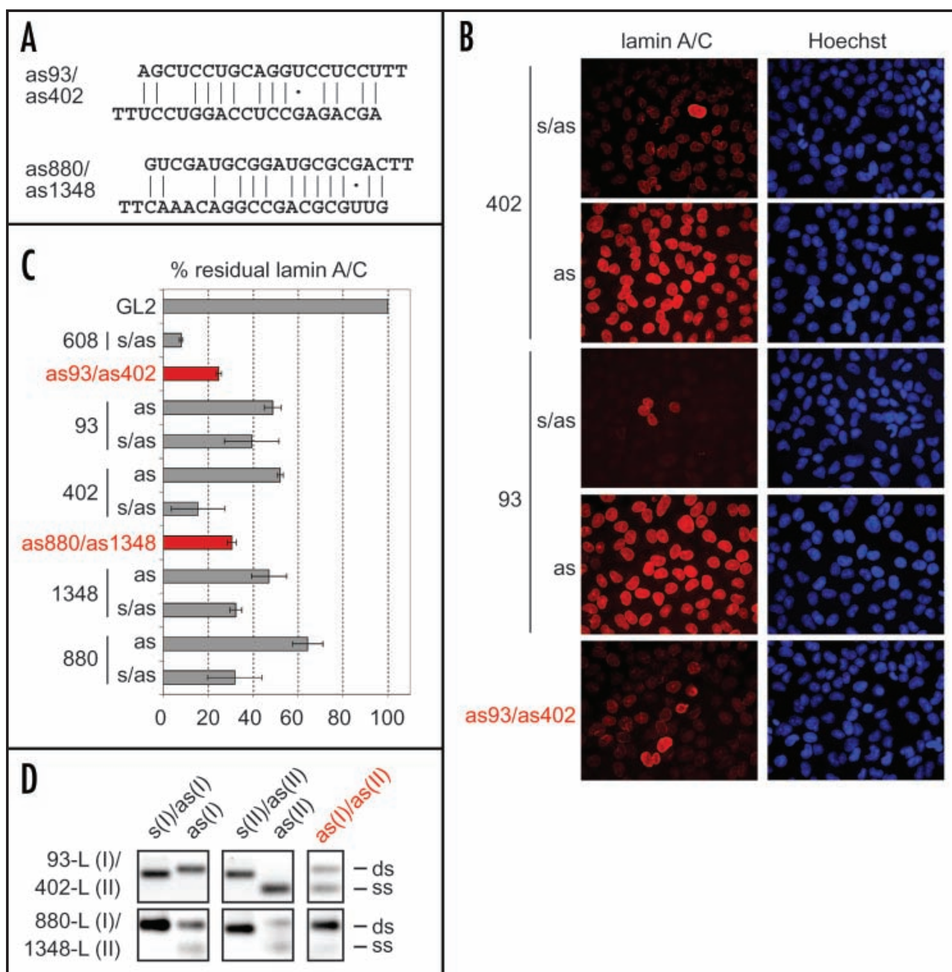

Figure 3. Silencing of lamin A/C by siRNA duplexes composed of two distinct, partially complementary antisense siRNA sequences. (A) Sequence and structure of the partially complementary siRNA duplexes. (B) Indirect immunofluorescence micrographs were taken 44 h after siRNA transfection. DNA was visualized with Hoechst 33342. (C) Quantitative Western blot analysis with antibodies 44 h after transfection. The bar graph displays relative values of residual lamin A/C as standardized with vimentin normalized to the GL2 control siRNA treated cells. (D) Analysis of single-stranded sense (s), antisense (as) or guide siRNA, and duplex siRNAs by native gel electrophoresis (see also Fig. 2E).

guide-only siRNAs corresponding to positions 608, 16 or 447 were comparably effective. The palindromic siRNA targeting position 94 was completely inactive, while the passenger/ guide siRNA showed significant protein reduction similar to the most potent passenger/ guide or guide-only siRNAs against position 608 (Fig. 3B).

The observations from indirect immunofluorescence microscopy were confirmed in a more quantitative manner by Western blotting using extracts from transfected HeLa cells (Fig. 3C and D). Cell extracts were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane and developed with antibodies recognizing lamin A/C and vimentin. The vimentin antibody allowed confirmation of equal protein loading and was also used to normalize the data obtained in quantitative Western blots. The Western blot signal from ECL development was recorded using a luminescence reader. The target positions 608, 16 and 447 displayed comparable knockdown levels for both passenger/guide and guide-only siRNAs. The guide-only siRNA targeting position 94 was ineffective though the prototypical siRNA showed moderately effective silencing. Together, these are surprising findings that indicate gene silencing can be accomplished in an effective and general manner using a guide-only siRNA composed of two identical single-stranded RNA molecules paired to each other.

We then examined the siRNAs by native agarose gel electrophoresis. All of the partially palindromic siRNAs showed a band comparable in electrophoretic mobility with the perfectly paired prototypical siRNA duplex, though two of the four partially palindromic siRNAs also showed a faster mobility second band presumably corresponding to the approximately 10 bp competing fold-back structure (Fig. 2E). There was no apparent correlation between homogeneity of structure and activity.

Silencing of lamin A/C using siRNA duplexes composed of two distinct, partially complementary guide siRNA sequences. We used our software tool to select siRNA duplexes that were composed of nonpalindromic strands fully complementary to two distinct segments of the target mRNA and that were partially complementary to itself. We then synthesized and tested as93/as402 and as880/ as1348 siRNAs and compared the silencing efficiencies to canonical siRNA duplexes pairing each guide siRNA to a fully complementary passenger siRNA or by examining the silencing potential of the unpaired single-stranded guide siRNAs. The selected guide-only siRNA duplexes both contained six nonWatson-Crick base pairs including one G/U wobble (Fig. 3A). The formation of duplex siRNA after siRNA strand annealing was confirmed using native agarose gel electrophoreses as described previously.[31] Gene silencing was monitored by immunofluorescence analysis (Fig. 3B) and confirmed quantitatively using Western blotting as described above (Fig. 3C). Native gel electrophoresis confirmed the presence of duplex siRNA after annealing (Fig. 3D). Both guide-only siRNA duplexes specifically reduced lamin A/C expression by 70 to 80% (comparable to the canonical passenger/ guide siRNAs), while the single-stranded guide siRNAs were less effective with lamin A/C reduction of only 50% or less. These data confirm the feasibility of the new siRNA selection approach.

Simultaneous silencing of two distinct genes using siRNA duplexes composed of partially complementary guide siRNA sequences. We next examined if it was possible to target two genes simultaneously by a single siRNA duplex composed of strands complementary to two distinct genes and also partially complementary to each other. We adjusted the software tool to allow for input of two mRNA target sequences and chose to target lamin A/C and the lamin A interacting protein emerin. Both genes are nonessential when silenced in HeLa cells.[37,38] A duplex was selected composed of lamin-specific as778-L and emerin-specific as569-E siRNAs that was predicted to contain seven mismatches including two G/U wobbles (Fig. 4A). The silencing efficiencies of the prototypical

siRNA duplexes as well as those of the single-stranded siRNAs alone were determined using immunofluorescence (Fig. 4B) as well as qualitative Western blotting analysis (Fig. 4C). Silencing of lamin A/C by the prototypical passenger/guide position 778-L siRNA duplex was effective and associated with the known mislocalization of emerin to the cytoplasm from previous siRNA knockdown studies.[38] Similarly, emerin was specifically knocked down using the prototypical passenger/guide position 569-E siRNA duplex. The individual guide siRNAs showed no silencing when transfected. The dual-targeting as778-L/as569-E-siRNA silenced as effectively as the individual prototypical siRNA duplexes. In an effort to obtain a more quantitative and rapid method to assess the effectiveness of dual-targeting siRNAs we also established and applied branched-DNA assays for measuring residual mRNA levels after siRNA delivery (Fig. 4D). Again, native gel electrophoresis confirmed the presence of some duplex siRNA after annealing (Fig. 4E). We tested two additional dual-targeting siRNA duplexes using this assay and showed that as78-L/as627-E silenced emerin by 50% and lamin A/C by 90%, as574-L/as495-E only reduced lamin A/C by 25% (data not shown). These findings indicate that it is possible to obtain potent and specific siRNA duplexes of dual targeting function.

DISCUSSION

We have shown that siRNA duplexes can be designed that are devoid of non-targeting passenger (sense) strands and are composed of two fully target-complementary strands that are sufficiently complementary to each other to form stable duplexes with characteristic 3' overhanging ends. The silencing efficiencies of guide-only siRNAs are comparable to prototypical passenger/guide or sense/antisense siRNAs, even though guide-only siRNA duplexes may contain a significant number of non-Watson-Crick and G/U wobble base pairs. Imperfect base-pairing is also frequently encountered within the 25- to 30-bp precursors of microRNAs, which similar to siRNAs, enter the RNAi pathway by incorporation of the mature miRNA strand into Argonaute proteins.[4,5,26,41,42] The distribution of the number of mispairs, including G/U wobbles, in miRNA precursors centers between 5 and 6, and was used as a threshold for the selection of

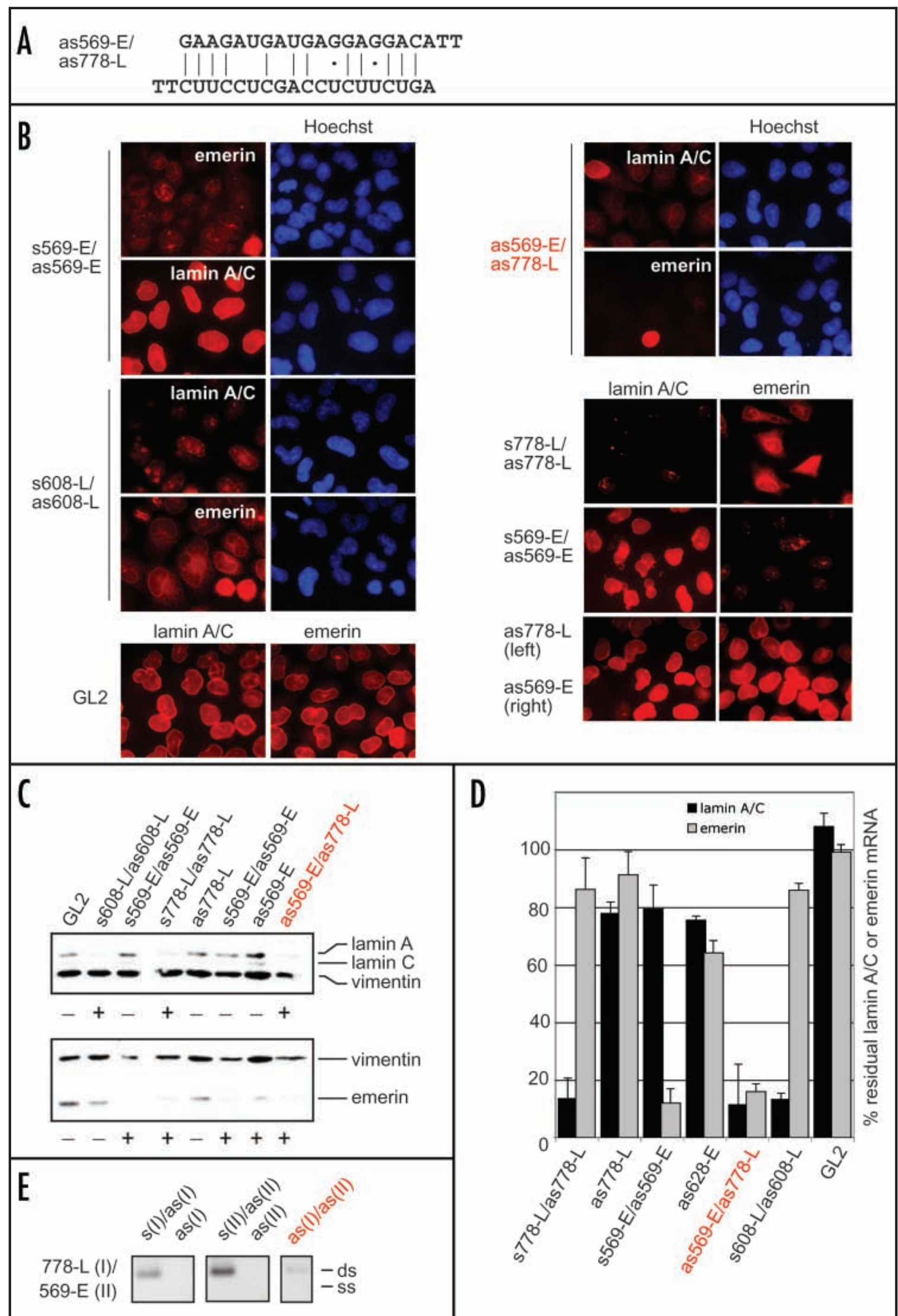

Figure 4. Simultaneous silencing of lamin A/C and emerin by dual-targeting siRNA duplex. (A) Sequence and structure of the dual-targeting siRNA duplexes. The GenBank sequence identifier for emerin was NM_000117. (B) Indirect immunofluorescence micrographs of cells stained with emerin and lamin A/C antibodies. (C) Western blot analysis of siRNA transfected cell extracts. (D) mRNA levels of both lamin A/C and emerin siRNA-treated cells were examined after transfection of the dual targeting siRNAs in quantitative analysis. siRNAs were transiently transfected into Hela SS6 cells, and mRNA levels of emerin and lamin A/C was analyzed after 48 h by the branched DNA assay. Values shown are standardized with actin mRNA isolated from siRNA-transfected cells. Data were normalized to mRNA levels observed in GL2 control-siRNA treated cells. (E) Analysis of single-stranded sense (s), antisense (as) or guide siRNA, and duplex siRNAs by native gel electrophoresis (see also Fig. 2E).

partially complementary or palindromic siRNA strands.

Though duplex siRNAs have overall structural dyad symmetry, the different sequence of the passenger and guide siRNA strands generally introduces thermodynamic and kinetic asymmetry that affects the association of various dsRNA binding proteins. These differences can determine which of the two duplex-constituting strands preferentially enters RISC and certain rules have been proposed for increasing the identification of effective siRNA duplexes.[12-14] It was also shown at the same time that mismatches introduced by sequence changes of the nontargeting siRNA strand rescued silencing of ineffective siRNA duplexes to a certain extent. Partially palindromic siRNAs, which are formed by intermolecular annealing of the same guide siRNA strand, have full dyad symmetry so that both strands enter with equal probability. However, because of the equilibrium between inter- and intramolecular paired structure, it also remains a possibility that such siRNAs (or a subset of such siRNAs) bypass the regular duplex-initiated assembly and that the guide siRNA engages as a single-strand directly with the Argonaute proteins. The assembly of RISC using single-stranded siRNAs has been demonstrated previously and cell-based RNA silencing by guide siRNAs unlikely to form palindromic structures has also been reported, though it is generally less effective or undetectable for most single-stranded siRNAs.[16,43,44]

The design of siRNA duplexes composed of two distinct, partially complementary guide siRNA sequences introduces significant asymmetry, but because both strands are complementary to its target mRNA, it is only the silencing efficiency of the least potent guide siRNA strand that possibly limits the silencing efficiency. The dual-targeting siRNAs directed against lamin A/C in this study were either as effective as the most effective prototypical siRNAs or in-between the activities of the prototypical siRNAs. Therefore, the breaks in dyad symmetry within our partially paired siRNA duplexes bear no consequence and dual-targeting siRNAs provide an alternative to prototypical siRNAs based on asymmetry rules. When dual-targeting siRNAs are designed that aim at two distinct genes, asymmetry of RISC assembly can become problematic and two out of the three functional duplexes targeting lamin A/C and emerin showed such problems. Nevertheless, only a very small number of duplexes were tested for identifying a very effective siRNA duplex silencing both targets simultaneously.

Dual-targeting siRNAs might provide an elegant solution to address the problem of rapidly emerging resistance in siRNA-mediated antiviral approaches.[45,46] The delivery of two targeting siRNA strands requires that two mutations must be simultaneously selected by the virus to escape siRNA targeting. Dual-targeting siRNAs are likely to also show increased specificity compared to prototypical siRNA duplexes. Off-target activities in siRNA silencing experiments are due to undesired silencing activity of the passenger and the guide strand of partially complementary, noncognate mRNAs.[17,47] Because of the absence of a nontarget-complementary passenger siRNA, the only possible off-target activities are associated with the guide strands. Off targeting activities are generally low and become undetectable when combining several protoyipical siRNA duplexes, each having its distinct off target but identical on-target activity. Similarly, a dual-targeting siRNA dilutes the off-target activities associated with the two antisense strands two-fold while retaining the on-target activity. With respect to siRNAs as potential drugs, manufacturing partially palindromic siRNA would only require the synthesis of a single siRNA strand and therefore could substantially reduce large-scale production costs.

The only conceivable disadvantages of our new approach are the limited number of siRNAs that may be identified by imposing partial sequence complementarity between target-complementary strands. This may limit the choice of designing cross-species targeting siRNA duplexes or highly target-specific siRNA duplexes that are selected to contain unique sequences within a genome.

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