Expanding the action of duplex RNAs into the nucleus: redirecting alternative splicing

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ABSTRACT

Double-stranded RNAs are powerful agents for silencing gene expression in the cytoplasm of mammalian cells. The potential for duplex RNAs to control expression in the nucleus has received less attention. Here, we investigate the ability of small RNAs to redirect splicing. We identify RNAs targeting an aberrant splice site that restore splicing and production of functional protein. RNAs can target sequences within exons or introns and affect the inclusion of exons within SMN2 and dystrophin, genes responsible for spinal muscular atrophy and Duchenne muscular dystrophy, respectively. Duplex RNAs recruit argonaute 2 (AGO2) to pre-mRNA transcripts and altered splicing requires AGO2 expression. AGO2 promotes transcript cleavage in the cytoplasm, but recruitment of AGO2 to pre-mRNAs does not reduce transcript levels, exposing a difference between cytoplasmic and nuclear pathways. Involvement of AGO2 in splicing, a classical nuclear process, reinforces the conclusion from studies of RNA-mediated transcriptional silencing that RNAi pathways can be adapted to function in the mammalian nucleus. These data provide a new strategy for controlling splicing and expand the reach of small RNAs within the nucleus of mammalian cells.

INTRODUCTION

Newly synthesized transcripts (pre-mRNAs) contain intervening sequences (introns). These introns must be excised from the pre-mRNA by the spliceosome, a ribonucleoprotein complex. The remaining portions of the pre-mRNA (exons) are then spliced to form the mature mRNA that codes for proteins. Splicing occurs in the nucleus and spliced transcripts are exported into the cytoplasm. Splicing usually does not produce a single mRNA species for each gene. Instead, pre-mRNAs are spliced in alternate ways, leading to production of different proteins. This phenomenon is known as alternative splicing and is observed in >90% of all human genes (1).

Approximately 60% of disease-causing point mutations are related to defective splicing (2) and chemical agents that redirect splicing may promote production of protein isoforms to compensate for genetic defects (3–5). For example, Duchenne muscular dystrophy is an incurable disease caused by mutations in the *DMD* gene-encoding dystrophin protein (6). Agents that promote alternative splicing can yield a truncated version of dystrophin that is naturally found in patients suffering from a more mild disease, Becker's muscular dystrophy. Induction of truncated dystrophin might convert a fatal genetic disease into a condition where patients experience a normal lifespan and a good quality of life.

Efforts to develop chemical agents to redirect splicing have focused on single-stranded oligomers including PNAs (7), LNAs (8), morpholino oligomers (9) and 2′-modified oligonucleotides (10). Phase I and Phase I-2a clinical trials have shown promising results. Both intramuscular (11) and systemic administration (12) have partially restored dystrophin expression and systemic administration also yielded significant improvements in muscle function. While this progress is promising, the challenges facing drug development are high and there remains an urgent need to explore multiple strategies.

Short-interfering RNAs (siRNAs) offer another strategy for recognizing mRNA. siRNAs are powerful tools for gene silencing in the laboratory and are being tested in several clinical trials (13). Typically, siRNAs bind argonaute 2 (AGO2) (14), recognize mRNA in the cytoplasm, guide cleavage of the RNA target by AGO2 and inhibit gene expression. While cleavage of an mRNA target is desirable for inhibiting gene expression,

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destruction of mRNA would be incompatible with redirecting splicing. It may be possible, however, to separate the recognition and cleavage functions of AGO2. We previously observed that small duplex RNAs can target non-coding transcripts and trigger transcriptional silencing or activation of adjacent genes (15,16). This gene modulation requires AGO2 protein and recognition occurs without causing cleavage of the targeted non-coding transcripts.

This observation led us to hypothesize that small RNAs might have the ability to alter another nuclear event splicing. To test this hypothesis, we have conducted a detailed analysis of duplex RNAs complementarity to key sequences within introns and exons. For three different genes, we find that duplex RNAs promote intron/exon exclusion. The mechanism involves AGO2 protein, recognition of pre-mRNA and does not involve changes in chromatin. These data provide an unexpected mechanism for RNA-mediated alteration of splicing and further demonstrate the reach of RNAi pathways into the nuclei of mammalian cells. The ability of small RNAs to alter splicing provides another option for developing therapeutic nucleic acids.

MATERIALS AND METHODS

General

Unless otherwise noted, duplex RNAs were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Duplex RNAs complementary to AGO1 or AGO2 mRNA were provided by Dharmacon (16). 2′-O-methyl RNA was obtained from Sigma. Trichostatin A (TSA) and 5-aza-2′-deoxycytidine (5-Aza-dC) were obtained from Sigma. All conclusions derive from multiple independent experiments.

Cell culture

HeLa pLuc/705 cells were provided from Dr Ryszard Kole (University of North Carolina) and cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, D5796) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 1% Sodium Pyruvate (Sigma) and 0.5% MEM non-essential amino acids (Sigma). Patient-derived SMA type I homozygous fibroblast cell (GM03813; Coriell Cell Repositories, Camden, NJ, USA) were cultured in Minimum Essential Medium Eagle (MEM; Sigma, M4655) supplemented with 10% (v/v) fetal bovine serum and 1% MEM non-essential amino acids (Sigma, M7145). Patient-derived Duchenne Muscular Dystrophy fibroblast cell (GM03429; Coriell Cell Repositories) were cultured in Minimum Essential Medium Eagle (MEM; Sigma, M4655) supplemented with 15% (v/v) fetal bovine serum and 1% MEM non-essential amino acids (Sigma, M7145).

Transfections

HeLa pLuc/705 cells were plated 24 h in advance so that their density on the day of transfection was ~90%. For GM03813 fibroblast transfection, cells were plated at a density of ~$1.2(10)^5$ per well of a 6-well plate 24 h before transfection. Cells were transfected with siRNAs using RNAiMAX (Invitrogen) (15,16). Unless indicated otherwise, total RNA was isolated 24 h after transfection with Trizol (Invitrogen) for RT–PCR.

Luciferase assay

Twenty-four hours after transfection, cells were washed twice with 1XPBS and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). Protein concentrations were determined by the bicinchoninic acid (BCA assay). Luciferase activity was measured on a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) by mixing 50 μl of cell lysate with 100 μl of Luciferase Assay substrate (Promega). Luciferase expression was calculated as relative luminescence units (RLU) per microgram protein and shown as fold increase in luminescence compared with negative control. All experiments were performed in multiple independent transfections. Error bars are standard deviation.

RT–PCR

To generate cDNA, total RNA was extracted and treated with DNase I (Worthington Biochemical) at 25 μM for 10 min. Reverse transcription was performed using high-capacity reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Generally, 2.0 μg of total RNA was used per 20 μl of reaction mixture. PCR was performed on a 7500 real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix (BioRad) using the following primers for HeLa cell: Luci forward primer 5′-TTGATA TGTGGATTTCGAGTCGTC-3′ and Luci reverse primer 5′-TGT CAATCAGAGTGCTTTTGGCG-3′. PCR for SMA cell used forward primer: 5′-CCTCCCATATGTC CAGATTCTCTTGATG-3′ and reverse primer: 5′-CAG ATGGTTTTTCAAAATAGAGTCC-3′. For DMD, primary PCR was carried out with outer primer set (44 FP: 5′-GATATAAAGATATTTAATCAGTGGCTAA; 83 RP: 5′-CCGTAATGATTGTTCTAGCCTCTTGAT TGC-3′) for 28 cycles. Then, nested PCR was performed with 1 μl of primary PCR product as template using an inner primer set (61 FP: 5′-TCAGTGGCTAACAGAAG CTGAACAGTTT-3′ and 83RP) for 30 cycles.

All the PCR products were separated on a 2% agarose gel and visualized on an AlphaImager. The bands were quantified using ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2007). Splicing correction on HeLa pLuc705 model was calculated as a percentage of the total amount of spliced mRNA: correct mRNA × 100/(correct mRNA + aberrant mRNA). For SMA, inclusion of exon 7 was calculated as a percentage of the total amount of spliced mRNA, i.e. included mRNA × 100/(included mRNA + skipped mRNA). The increment of aberrant mRNA was calculated as a fold mRNA level above a control sample.

RNA immunoprecipitation

HeLa Luc/705 cells were grown in $150 \, cm^2$ dishes and transfected with duplex RNAs. Cells ($\sim 4 \times 10^7$) were harvested 24 h after transfection and nuclear fraction was isolated (15–17). A nuclear lysis buffer [150 mM KCl, 20 mM Tris–HCl 7.4, 3 mM $MgCl_2$, 0.5% NP-40, $1 \times$ Roche protease inhibitors cocktail, RNAse in (50 U/ml final)] was added to the nuclei (Note: no formaldehyde cross-linking is used in this protocol) (16,17). The mixture was left on ice for 10 min. After vigorous vortexing and pipetting, nuclei were freeze-thawed three times in liquid nitrogen and a $22°C$ water bath. Insoluble material was removed by centrifugation at maximum speed for 15 min at $4°C$. Nuclear extracts were quickly frozen in liquid nitrogen and stored at $-80°C$. Sixty microliters of protein A/G agarose Plus was washed with phosphate-buffered saline ($1 \times PBS$, pH 7.4) and incubated with 5 µg of anti-AGO1(4B8, gift from from Dr Gunter Meister, Universität Regensburg) and anti-AGO2 (4G8, 011-22033, Wako) antibody in 0.5 ml at $4°C$ with gentle agitation for 2 h. After one wash with $1 \times PBS$ and one wash with nuclear lysis buffer, beads were incubated with nuclear cell lysate under constant rotation for 3 h at $4°C$. After washing with nuclear lysis buffer (three times), the beads were then treated with elution buffer (1% SDS, 0.1M $NaHCO_3$ and RNase inhibitor). Following proteinase K treatment, RNA extraction and precipitation, samples were treated with recombinant DNase I followed by reverse transcription. Corresponding cDNA was amplified using reverse primer complementary to Luc/705 pre-mRNA (5′-AAAACGAT CCTGAGACTTCCACACTGATG-3′) with Luci forward primer (Supplementary Figure S1i) and GAPDH mRNA (Applied Biosystems).

Results were normalized by measuring two parameters simultaneously: (i) binding of Luc/705 pre-mRNA to IgG, to anti-Ago1 and to anti-Ago2 antibodies as an indicator of fold enrichment of Luc/705 pre-mRNA in Ago1 or Ago2 IP relative to IgG IP; (ii) binding of GAPDH mRNA (in both Ago1 or Ago2 IP and IgG IP) to the above antibodies as an indicator of a housekeeping control for background binding.

Chromatin immunoprecipitation assay

ChIP assays were performed as described (16). Anti-trimethyl-histone H3 (Lys27) and anti-dimethyl-histone H3 (Lys9) antibodies were supplied by Millipore.

RESULTS

Duplex RNAs promote exon exclusion

To examine the effect of duplex RNAs on splicing, we used an engineered HeLa cell line (HeLa pLuc/705) that expresses a chromosomally encoded luciferase gene interrupted by intron 2 from the β-globin gene (18) (Figure 1a). The β-globin intron has been mutated to introduce a new splice site that causes retention of a fragment of the intron and production of inactive luciferase protein. Antisense oligonucleotides complementary to the introduced splice site redirect splicing toward normal length luciferase protein. HeLa pLuc/705 is an excellent model for splicing studies because splicing can be monitored at the RNA level by semi-quantitative PCR or at the protein level by luciferase activity.

We designed several duplex RNAs that were fully complementary to sequences near the introduced splice site within the β-globin-derived intron (Supplementary Table S1). We observed that duplex 709 was a potent activator of luciferase expression while duplex RNAs targeting nearby sites were less active (Figure 1b and c; Supplementary Figure S1a). Half-maximal activity was achieved by adding 10 nM duplex while previously characterized (18) antisense oligomer 705OMe (a single-stranded oligomer composed of 2′-O-methyl RNA bases) required >200 nM (Figure 1d and e; Supplementary Figure S1b and c). These experiments established that fully complementary duplex RNAs were capable of entering the nucleus and shifting splicing to efficiently exclude an intron.

In the experiments, the component single strands that make up the RNA duplexes were annealed at equimolar ratios to minimize the presence of single-stranded RNA. The extent of duplex formation was confirmed by monitoring thermal denaturing/renaturing and by determining melting temperatures. We have previously observed that single-stranded RNA is much less stable than duplex RNA. In cell culture medium, single-stranded RNA has a half-life of <30 s, while duplex RNAs are stable for up to 72 h (19). Chemical modifications can increase the stability of RNA, but the RNA used in this study was unmodified and the single strands would not be expected to survive initial contact with cell culture media. Consistent with this assumption, we have found that single-stranded RNAs are inactive when added to cells (Hu, J. and Corey, D.R., unpublished data).

RNA-mediated alternative splicing tolerates non-seed sequence mismatches

Some sequences that affect splicing are located within exons. Duplex RNAs that target exons would have the potential to act like standard siRNAs and cause cleavage of mature mRNA in the cytoplasm. Such cleavage would be undesirable if preservation of the exon-included spliceform was necessary. It is possible to disrupt cleavage by introducing mismatched bases located within the central region (bases 10 or 11) (20,21) of the duplex RNA. The presence of centrally located mismatches should, therefore, avoid destruction of mature mRNA and increase the options for obtaining different ratios of the desired spliceforms.

To test if mismatch-containing RNAs could be active splice modulators and expand the versatility of our approach, we tested mismatched duplexes targeted to sequences near the aberrant splice site for their ability to redirect splicing. Similar to our results with fully complementary duplex RNA (709), only duplex 709 mm was an efficient agent for redirecting splicing (Figure 1f; Supplementary Figure S1d and e). When introduced into
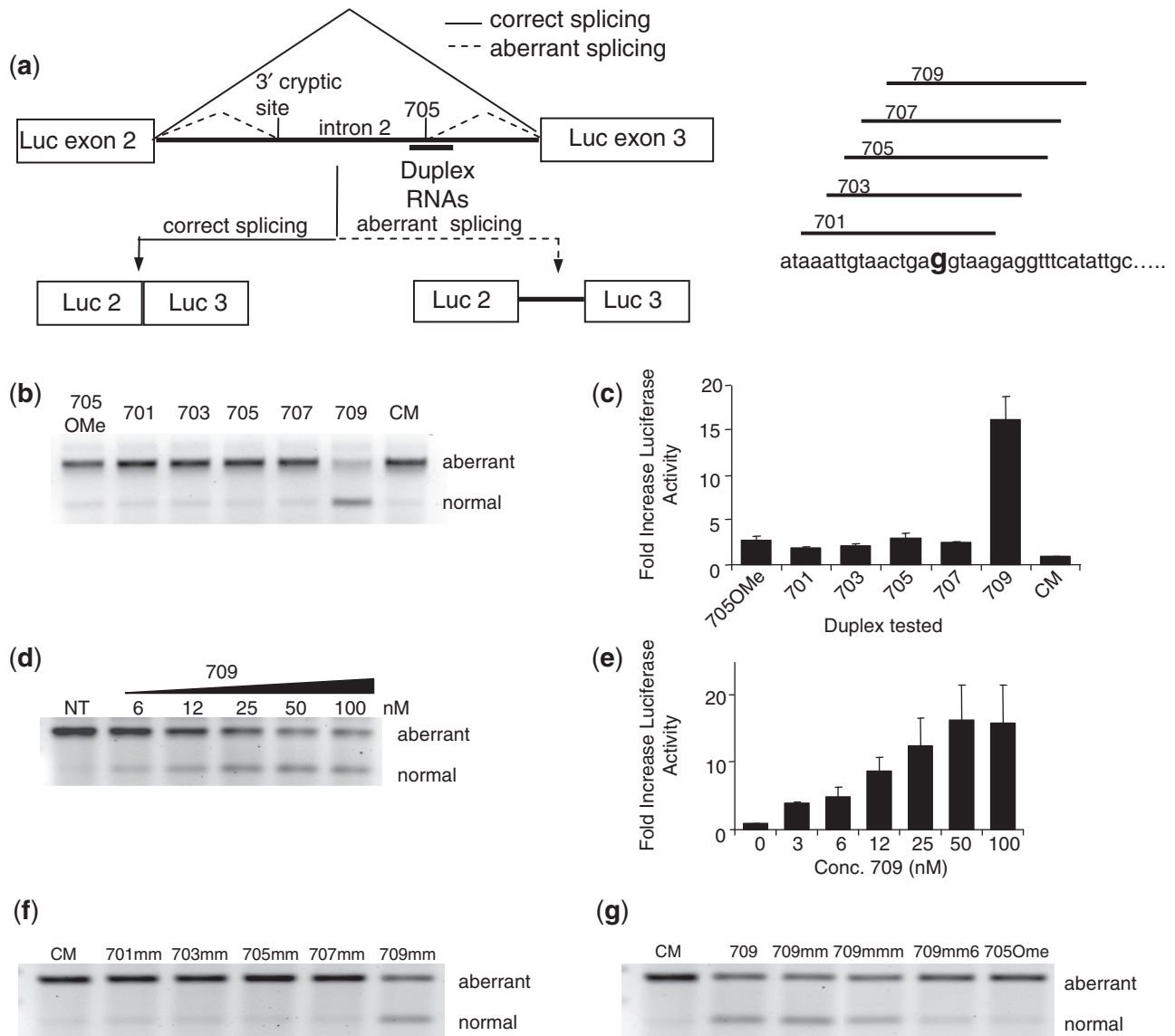
Figure 1. Duplex RNAs alter splicing of luciferase pre-mRNA in HeLa Luc/705 cells. (**a**) Left: schematic showing splicing of pre-mRNA. Boxes represent sequences derived from luciferase exons. The intervening region is derived from human β-globin inton 2 with T/G mutation at nucleotide 705. The 3′ cryptic site and target site for duplex 705 is noted. Right: target sites for duplex RNAs within the β-globin inton 2. The mutation site is in bold. (**b**) PCR amplification followed by gel electrophoresis to separate aberrant and correct splice products upon the addition of duplex RNAs. (**c**) Increase in luciferase activity upon the addition of duplex RNAs. (**d** and **e**) Effect on splicing and luciferase activity from increasing concentrations of duplex RNA 709. (**f**) PCR amplification followed by gel electrophoresis to separate aberrant and correct splice products after adding mismatch-containing RNA duplexes. (**g**) Effect on splicing of multiple or seed sequence mismatches. Duplex RNAs were transfected into HeLa-derived pLUC/705 cells at 50 nM unless otherwise noted. CM: non-complementary control duplex. Error bars represent standard deviation (SD). Experiments were performed in triplicate or quadruplicate.

cells at varying concentrations, 709 mm achieved half maximal activity at 10 nM (Supplementary Figure S1f).

After observing that one centrally located mismatch was tolerated, we tested whether multiply mismatched duplexes or duplexes were capable of redirecting splicing. We tested a duplex with three centrally located mismatches within a duplex RNA (709mmm) and observed altered splicing (Figure 1g; Supplementary Figure S1g and h). Duplex RNAs containing multiple mismatches are similar in structure to miRNAs and their ability to alter splicing suggests that some

miRNAs might be capable of changing the ratio of spliceforms.

Recognition of RNA substrates is highly dependent on binding of the seed sequence, a critical region located at bases 2–8 of the duplex RNA. We had previously observed that introduction of a single mismatch at position 6 within the seed sequence abolishes post-transcriptional gene silencing (PTGS) when duplex RNAs target an mRNA in the cytoplasm (22). Just one mismatch within the seed sequence (709mm6) made the duplex inactive (Figure 1g, Supplementary
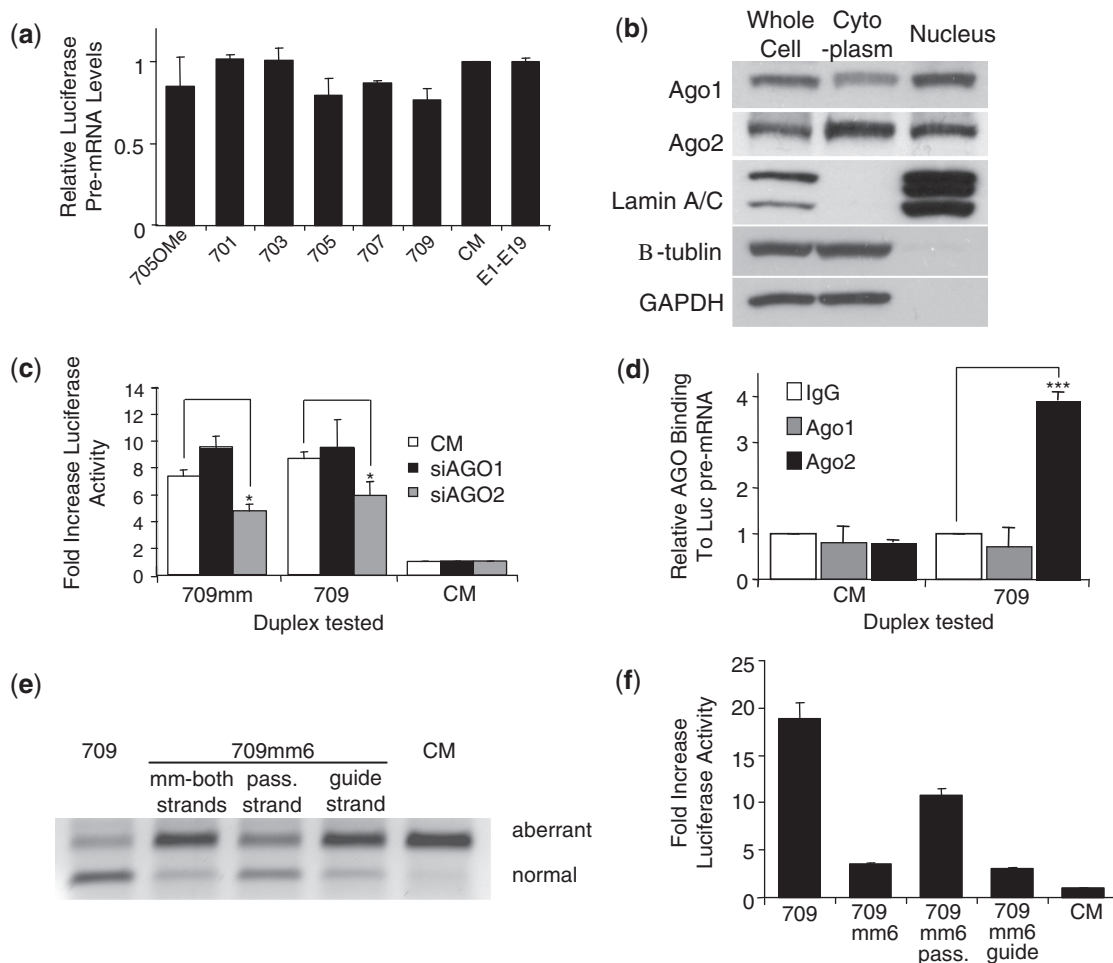
Figure 2. Mechanism of redirecting splicing by duplex RNAs. (**a**) Effect of duplex RNAs on levels of luciferase pre-mRNA. (**b**) Western blot showing localization of AGO1 and AGO2 in purified nuclei from HeLa pLuc/705 cells. (**c**) Effect of siRNA-mediated reduction of AGO1 or AGO2 levels on enhancement of luciferase expression by RNAs 709 and 709 mm. (**d**) RIP showing recruitment of AGO2 to Luc/705 pre-mRNA. RNA levels were measured by qPCR. (**e and f**) Effect of mismatches on either the guide or passenger strands of duplex RNAs (relative to the luciferase mRNA target). Unless otherwise noted, duplex RNAs were transfected into HeLa-derived pLuc/705 cells at 50 nM. CM: non-complementary control duplex. Student's *t*-test was performed for evaluating statistical significance between two study groups. *$P < 0.05$, ***$P < 0.001$. Error bars represent standard deviation (SD). Experiments were performed in triplicate or quadruplicate.

Figure S1g and h). The critical importance of position 6 for both RNA-mediated alteration of splicing and PTGS is consistent with both processes sharing similar protein machinery. The finding that a single mismatch (amid 18 fully complementary bases) abolishes activity also suggests a powerful selectivity for recognition of the target pre-mRNA.

Active RNAs do not cause cleavage of pre-mRNA

Recognition of mRNA by fully complementary duplex RNAs in the cytoplasm is associated with cleavage of the target RNA (14). To investigate the mechanism of RNA-mediated splice correction and the potential impact of RNA cleavage, we tested whether the duplex RNAs used in our experiment affect RNA levels. We observed several lines of evidence suggesting that RNAs can target introns without reducing RNA levels: (i) semi-quantitative PCR showed that levels of luciferase RNA remain relatively constant (Figure 1b, d, f and g);

(ii) measurement of luciferase activity indicate that the production of luciferase protein was being increased rather than reduced (Figure 1c and e; Supplementary Figure S1) and (iii) quantitative PCR reveals no significant change in pre-mRNA levels (Figure 2a). It is possible that some cleavage of target RNA sequences is occurring, but such cleavage does not affect the levels of RNA or increased production of active protein. Our data indicate that, unlike the well-known mechanism for standard post-transcriptional gene silencing (PTGS) in the cytoplasm, RNA-mediated alteration of splicing in the nucleus can avoid cleavage of the target transcript.

AGO2 plays primary role in pre-mRNA recognition

AGO proteins are critical components of the cellular machinery for recognizing small RNAs and we hypothesized that they might also be involved in RNA-mediated control of splicing. There are four AGO proteins in human cells (AGO1-4). AGO2 is responsible for post-transcriptional
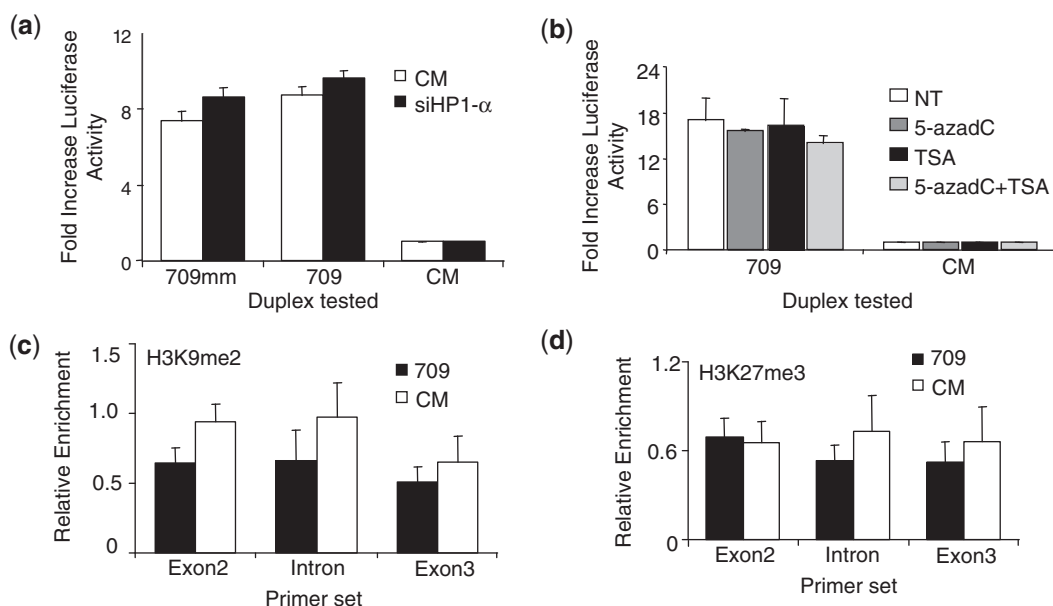
Figure 3. Altered splicing is not due to chromatin changes. (**a**) Effect of siRNA-mediated reduction of HP1α levels on the ability of RNAs 709 and 709 mm to enhance luciferase expression. (**b**) Effect of adding 100 nM TSA and/or 10 μM 5-AZA-dC on the ability of duplex RNA 709 to enhance luciferase activity. (**c** and **d**) ChIP showing the effect of RNA 709 on H3K9me2 or H3K27me3 modification, evaluated by primer sets designed to amplify three different regions of the gene. Unless otherwise noted, duplex RNAs were transfected into HeLa-derived pLuc/705 cells at 50 nM. CM: non-complementary control duplex. Error bars represent standard deviation (SD). Experiments were performed in triplicate.

gene silencing (14) but AGO1 has been reported to be involved in altering splicing through recognition of non-coding RNA (23). AGO2 is found in cytoplasmic p-bodies (24,25), but has also been reported to be in the nucleus (16,17,26,27). We identified both AGO1 and AGO2 within purified nuclei from HeLa pLuc/705 cells (Figure 2b).

To test which AGO variant was responsible for small RNA-mediated alteration of splicing, we used anti-AGO1 or anti-AGO2 siRNAs to deplete cellular AGO1 or AGO2 (Supplementary Figure S2a). Inhibition of AGO1 and AGO2 expression was not absolute, possibly because inhibition of AGO expression reduces the efficiency of gene silencing, and a total reversal of effects on RNA splicing would not be expected. Lowering levels of AGO2 protein reduced the efficiency of RNA-mediated splice correction and production of active luciferase (Figure 2c). Reduced expression of AGO1 had no effect on RNA-mediated alternative splicing or luciferase expression. We then used RNA immunoprecipitation (RIP) to examine recruitment of AGO1 and AGO2 to the luciferase transcript. AGO2, but not AGO1, was recruited to luciferase pre-mRNA (Supplementary Figures S1i and 2d). These data from two independent experimental tests point toward AGO2 being the best candidate for involvement in the observed RNA-mediated splice correction.

No evidence for chromatin modification

Kornblihtt has reported that duplex RNAs can induce inclusion of exons in an AGO1-dependent manner through complementarity to endogenous antisense transcripts, inducing changes in chromatin structure and affecting elongation of pre-mRNA by RNA polymerase

(23). The mechanism we observe is fundamentally different. Our data suggest a critical role for AGO2 rather than AGO1 (Figure 2c and d). Our data also suggest that the pre-mRNA is that molecular target rather than an antisense transcript. RNA 709mm6G with a mismatched base at position 6 of the strand complementary to the pre-mRNA lost the ability to redirect splicing (Figure 2e and f; Supplementary Figure S2b), suggesting that pre-mRNA is the molecular target. In contrast, RNA 709mm6P containing a seed-sequence mismatch relative to a putative antisense non-coding transcript retained the ability to alter splicing.

Inhibition of HP1α expression (Figure 3a and Supplementary Figure 2c) or synthetic epigenetic regulatory agents 5-aza deoxycytidine (5-AZA-dC) or trichostatin A (TSA) (Figure 3b) did not affect splice correction. We observed no alteration of histone marks H3K9me2 (Figure 3c) or H3K27me3 (Figure 3d) after treating with duplex RNA 709. Taken together, our data suggest that RNA-mediated alternative splicing does not necessarily require chromatin modifications or effects on transcription and that the mechanism that we observed differs from that previously reported.

RNA-mediated exon exclusion for SMN1 and dystrophin

To further characterize RNA-mediated alternative splicing, we examined a gene containing both exonic and intronic target sequences. Spinal muscular atrophy (SMA) is an inherited neurodegenerative disorder caused by loss or mutation of the survival motor neuron 1 (SMN1) gene (28). A second gene, SMN2, is closely related to SMN1 but its active spliceform is not efficiently expressed. Oligonucleotides that alter SMN2 splicing can increase
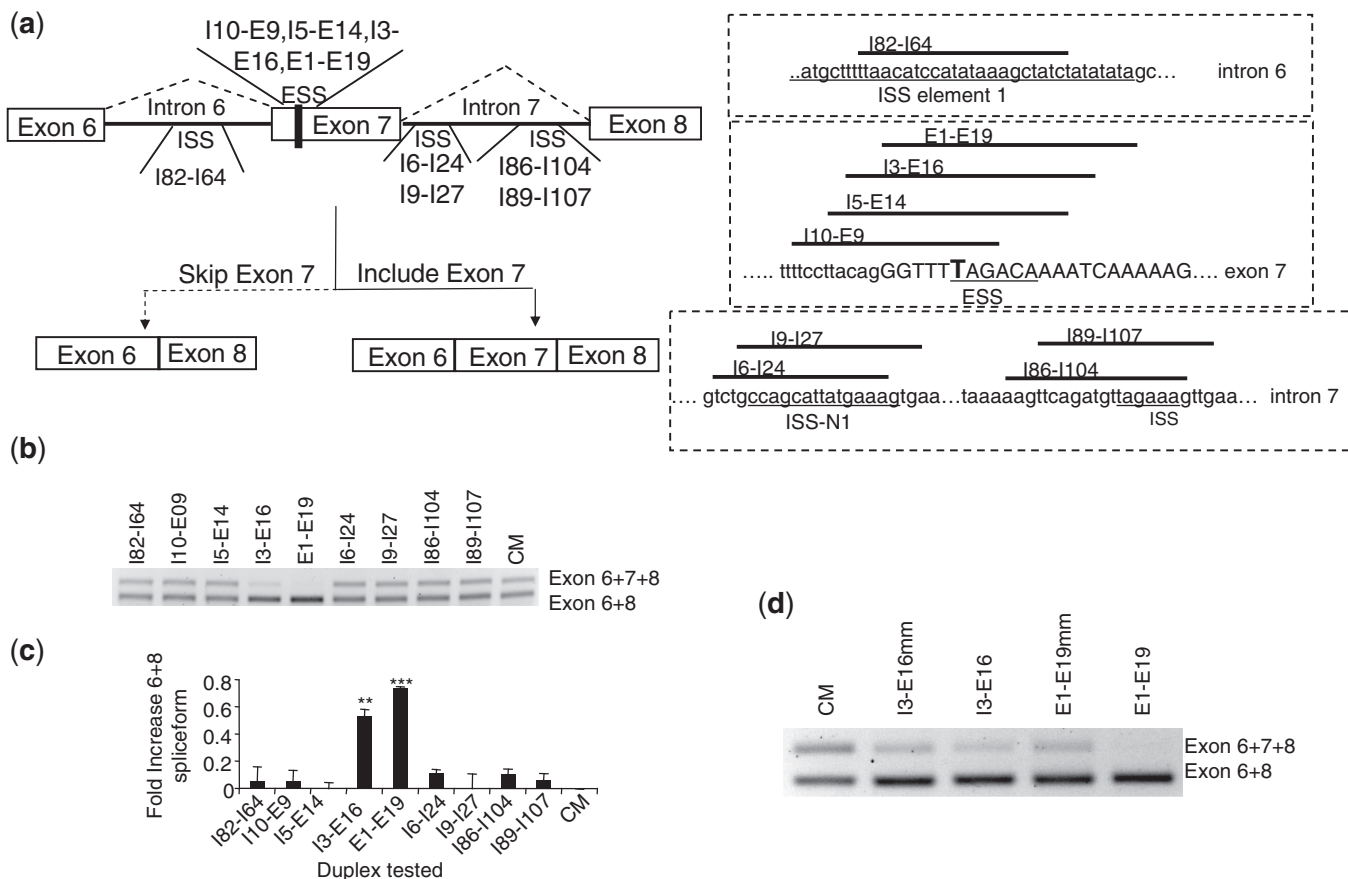
Figure 4. RNAs alter splicing of SMN2 in SMA type 1 fibroblast cells. (**a**) Left: schematic of SMN2 splicing. The dark bar within exon 7 shows the position of the C to T transition in SMN2 relative to SMN1. Right: schematic showing the binding sites for duplex RNAs. Sequences underlined indicate exonic splicing silencer (ESS) or intronic splicing silencer (ISS). Intron sequences are lower case, and exon sequences are upper case. The C to T transition is bold. (**b** and **c**) Semiquantitative RT–PCR showing the effect of fully complementary duplex RNAs (100 nM) on splicing of SMN2. (**d**) Semiquantitative RT–PCR comparing effects of fully complementary and mismatch-containing complementary duplex RNAs (100 nM) on splicing of SMN2. ISS: intronic splicing silencer; ESS: exonic splicing silencer. CM is a non-complementary RNA duplex. Student's *t*-test was performed for evaluating statistical significance between two study groups. **$P < 0.01$, ***$P < 0.001$. Error bars represent standard deviation (SD). Experiments were performed in duplicate or quadruplicate.

SMN2 protein to therapeutically useful levels that compensate for loss of SMN1 (29,30).

SMN2 can be spliced to directly join exons 6 and 8 or to produce an isoform that contains exons 6–8 (Figure 4a). We designed duplex RNAs to target sequences near intronic splice silencers (ISSs) or an exonic splice silencer (ESS). Duplexes I3-E16 and E1–E19 (numbering is relative to the intron exon junction) targeted near the intron 6/exon 7 junction, increased exclusion of exon 7 (Supplementary Table S2; Figure 4b and c). Both duplexes were fully complementary to the target sequence and these data demonstrated that duplex RNAs complementary to exons could redirect alternative splicing.

While our data suggest that AGO2 lacks the ability to induce cleavage of pre-mRNA in the nucleus, there is no doubt that duplex RNAs complementary to exons can induce AGO2-mediated cleavage of mature mRNA in the cytoplasm. The potential for cytoplasmic cleavage is important for the outcome of RNA-mediated exon exclusion because the target sequence is within exon 7 and

cleavage of mature mRNA will affect the ratio of the two spliceforms. To test whether we could manipulate the spliceform ratio of SMN2, we introduced mismatches at positions known to disrupt cleavage of target RNAs by AGO2.

Mismatch-containing duplexes I3–E16 mm and E1–E19 mm increased splicing of the $6+8$ spliceform (Supplementary Figure S3a–S3c). Exclusion of exon 7 was dose dependent (Supplementary Figure S3d–S3i). When we directly compared the fully complementary and mismatch-containing duplexes, we observed that $6+7+8$ spliceform was better preserved upon the addition of mismatched RNAs, consistent with our hypothesis that introduction of mismatches would reduce cleavage of exon-included mRNA in the cytoplasm (Figure 4d). It is important to note that the change in spliceform ratio shifts isoform production away from the potentially therapeutic variant. It is possible that more extensive testing of duplexes complementary to other sites might identify compounds that enhance production of the $6+7+8$ isoform.

As observed for duplex RNAs targeting engineered luciferase, the action of duplex RNAs targeting SMN2 was reversed by use of siRNAs to reduce AGO2 expression while reduction of AGO1 expression did not affect splicing (Supplementary Figure S4a and Figure 5a). The duplex RNAs that induced exon exclusion achieved this activity without reducing levels of SMN2 pre-mRNA (Figure 5b), providing additional evidence that AGO2 can function in the nucleus independent from RNA cleavage. Inhibition of HP1α, treatment with TSA or 5-AZA-dC had no effect on RNA-mediated alteration of splicing (Supplementary Figure S4b; Figure 5c and d). As with engineered luciferase, RNA-mediated splice modulation of SMN2 appears to produce by a mechanism that differs from that previously reported (23).

We also examined RNAs complementary to dystrophin pre-mRNA to test their effects on splicing of dystrophin (Figure 6a and Supplementary Table S3). We used a cell line (GM03429) with an exon 45–50 deletion. We targeted sequences within exon 51 because deletion of exon 51 removes a premature stop codon and can produce partially active dystrophin that may have value for treating patients with muscular dystrophy (6). Since the target sequence was within an exon, all duplex RNAs contained one centrally located mismatch at either position 10 (in one case) or 11. Two RNAs, 66 and 68/10, successfully induced exon exclusion and production of the truncated dystrophin characteristic of the more mild Becker's muscular dystrophy (Figure 6b and c). Altered splicing of dystrophin, engineering luciferase and SMN2 provides three examples of small-RNA mediated
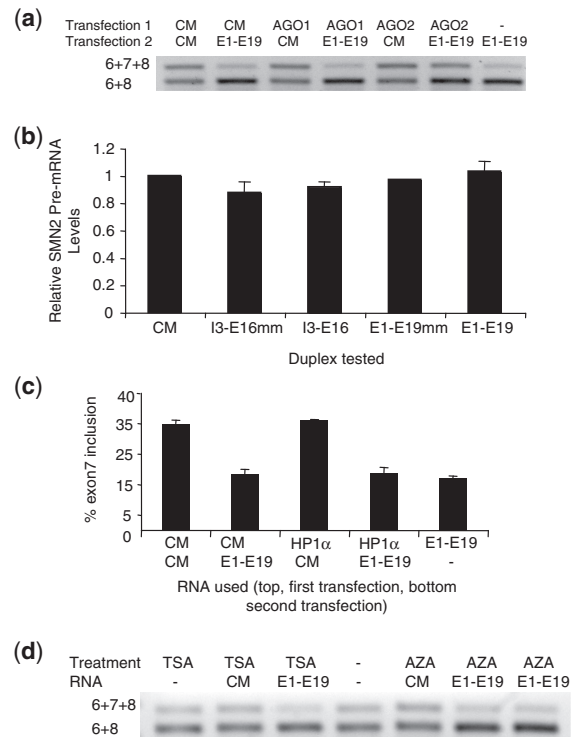
Figure 5. Mechanism of SMN2 splice alteration. (**a**) Effect of AGO1 or AGO2 knockdown on RNA-mediated alteration of splicing. (**b**) Effect of duplex RNAs on SMN2 pre-mRNA levels. (**c**) Effect of silencing of HP1α on RNA-mediated alteration of splicing. (**d**) Effect of adding TSA and/or 5-AZA-dC on RNA-mediated alteration of splicing. Error bars represent standard deviation (SD). Experiments were performed in duplicate or triplicate.
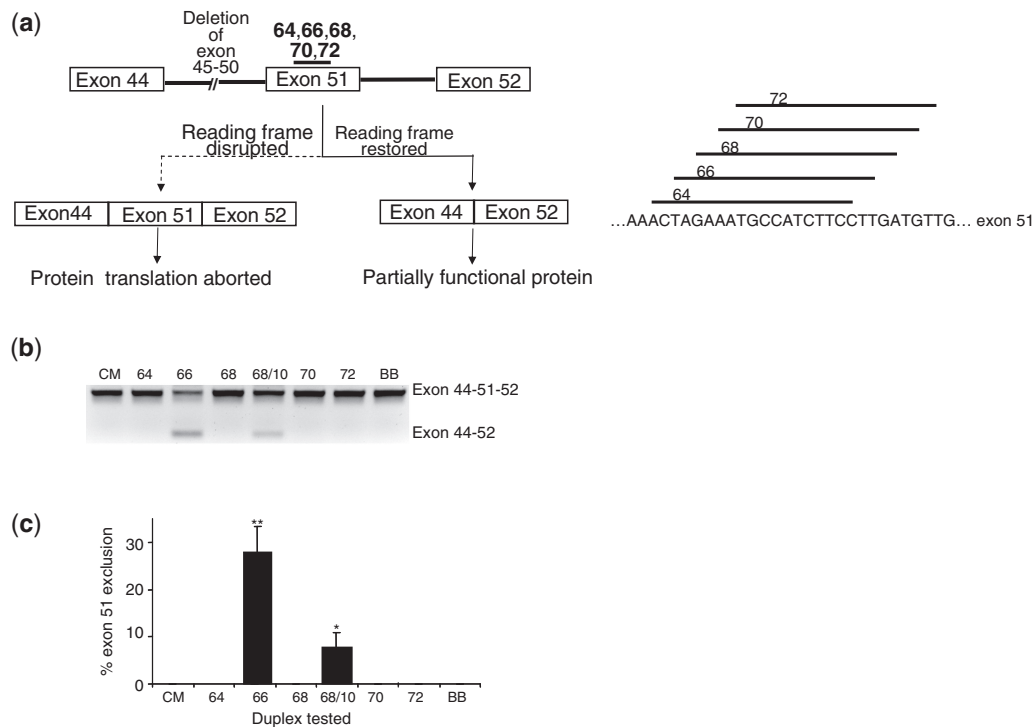
Figure 6. RNAs alter splicing of dystrophin. (**a**) Left: schematic of reading frame restoration by RNAs targeting exon 51. Out-of-frame splicing (dashed line) and in-frame splicing (solid line) are indicated. Right: binding sites for duplex RNAs used in DMD fibroblast cells. (**b**) Representative RT–PCR image showing effect of duplex RNAs on splicing of dystrophin. (**c**) Quantitation of data in Figure 6b and replicates. Averaged effects of duplex RNAs on exclusion of exon 51. CM and BB are non-complementary duplexes. Student's *t*-test was performed for evaluating statistical significance between two study groups. **P < 0.01, *P < 0.5. Error bars represent standard deviation (SD). Experiments were performed triplicate.

exon/intron exclusion. In all cases, we needed to screen only a handful of RNAs before identifying active agents, suggesting that the phenomenon is general and easily achievable.

DISCUSSION

Control of gene expression by small RNAs within mammalian cells is usually associated with gene silencing in the cytoplasm. miRNAs (31,32) and AGO protein (16,17,26,27), however, both exist in the nucleus, suggesting that recognition of nuclear RNA targets might also help regulate cellular function. We (15,16) and others (33,34) have observed that small RNAs can either silence or activate gene transcription through recognition of non-coding transcripts that overlap genes. These data and our demonstration that duplex RNAs can affect splicing through recognition of pre-mRNA establish the potential for potent RNA-mediated regulation in mammalian cell nuclei.

Our findings are the first example of RNA-mediated exclusion of introns or exons from mRNA. There has been one previous report describing RNAs that affect exon inclusion (23), but the mechanism appears different from the one suggested by our data. Given the rich variety of RNA targets in the nucleus, it should not be surprising that RNA can employ multiple mechanisms to regulate splicing. The precedent that gene silencing by small RNAs can be achieved by blocking transcription through binding to non-coding transcripts or post-transcriptionally through recognition of mRNA also supports the hypothesis that multiple routes may also exist for RNA-mediated control of splicing.

AGO2 is involved in RNA-mediated exon exclusion. This may be seen as surprising because the ability of AGO2 to induce cleavage of mRNA targets in the cytoplasm is well known. In nuclear extracts, however, Tuschl reported that cleavage was much weaker relative to cytoplasmic activity and cleavage site selectivity was greatly reduced (35). Fluorescence correlation spectroscopy suggests that nuclear AGO complexes are much smaller than AGO complexes in the cytoplasm (36), providing one explanation why activities might differ. Other reports have described post-translational modifications that affect AGO2 activity (37–39), and such

modifications may play a role in targeting and activity in the nucleus. Our results emphasize the need for experimenters to have an open mind regarding how AGO might function in the nucleus and design experiments accordingly.

We have not observed evidence of target transcript cleavage during our studies of AGO2-mediated transcriptional silencing and activation (15,16). Cleavage was not detected by 5′ RACE and expression of the targeted non-coding transcript was unchanged during gene activation. When RIP is performed with anti-AGO2 antibody, the target transcript is detected even though detection involves primers that flank the RNA binding site where cleavage would be predicted to occur.

Our observation that AGO2 mediates RNA-mediated exon exclusion without causing destruction of transcripts in the nucleus, regardless of whether the target sequence is within an intron or an exon, further establishes that nuclear AGO2 function can differ significantly from the function of AGO2 in the cytoplasm. Understanding the molecular basis for the different properties of AGO2 in the nucleus and cytoplasm will be an important goal for future studies. Nuclear AGO2 may have different post-translational modifications or may bind to different proteins or other cofactors. The duplex RNAs used in our studies target sequences near splice sites and it is likely that binding of the AGO2/RNA complex blocks the sites and alters splicing preferences.

Synthetic RNA duplexes are widely used to modulate gene expression and our results demonstrate that they can also control splicing. Typically, for gene silencing, duplexes are designed to be fully complementary and mismatch-containing duplexes are used as controls. We have previously shown that the introduction of mismatched bases relative to their mRNA targets can permit RNA duplexes to achieve high levels of allele selectivity (39). We now show that mismatched RNA duplexes can alter splicing and help tailor the ratio of spliced products. The use of mismatched RNA duplexes to achieve desired selectivities emphasizes the potential to achieve improved important properties of RNAs through mechanism-guided duplex design.

Our data demonstrating RNA-mediated alteration of splicing have several implications (Figure 7): (i) Small RNAs can function together with nuclear AGO2
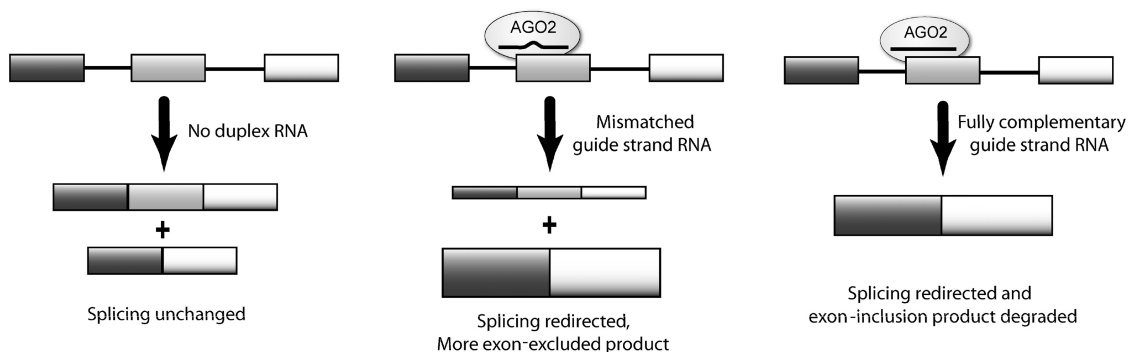

Figure 7. Scheme showing redirection of splicing by fully complementary and mismatch-containing duplex RNAs.

to recognize pre-mRNA transcripts and alter splicing. This finding expands the range of RNA-mediated control of gene expression to alternative splicing; (ii) regulation of splicing is robust and reproducible, consistent with the robustness found in endogenous regulatory pathways. (iii) Small miRNA-like mismatch-containing duplexes also alter splicing. miRNAs exist in the nucleus (31,32) and our data suggest that miRNAs have the potential to modulate splicing; (iv) redirecting splicing using duplex RNAs provides an alternative to using antisense oligonucleotides that may prove advantageous for development of nucleic acid therapeutics and (v) mismatched duplexes can be used if it is necessary to preserve spliceforms that retain targeted exons, while fully complementary duplexes can be used if maximal biasing toward exon-excluded spliceforms are required. The potential of small RNAs to modulate alternative splicing by small RNAs offers another layer to the subtle pattern of RNA-mediated regulation that exists inside cells and a new option for therapeutic development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online: Supplementary Tables S1–S3, Supplementary Figures S1–S4 and Supplementary reference [40].

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