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XRN2 is required for the degradation of target RNAs by RNase H1-dependent antisense oligonucleotides

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ABSTRACT

Antisense oligonucleotides (ASOs) can suppress the expression of a target gene by cleaving pre-mRNA and/or mature mRNA via RNase H1. Following the initial endonucleolytic cleavage by RNase H1, the target RNAs are degraded by a mechanism that is poorly understood. To better understand this degradation pathway, we depleted the expression of two major 5′ to 3′ exoribonucleases (XRNs), named XRN1 and XRN2, and analyzed the levels of 3′ fragments of the target RNAs *in vitro*. We found that the 3′ fragments of target pre-mRNA generated by ASO were almost completely degraded from their 5′ ends by nuclear XRN2 after RNase H1-mediated cleavage, whereas the 3′ fragments of mature mRNA were partially degraded by XRN2. In contrast to ASO, small interference RNA (siRNA) could reduce the expression level of only mature mRNA, and the 3′ fragment was degraded by cytoplasmic XRN1. Our findings indicate that the RNAs targeted by RNase H1-dependent ASO are rapidly degraded in the nucleus, contrary to the cytoplasmic degradation pathway mediated by siRNA.

1. Introduction

Antisense oligonucleotides (ASOs) have been widely used as laboratory tools and therapeutic agents because of their potent and specific ability to inhibit the expression of a target gene *in vitro* and *in vivo* [1–3]. It was first reported over 35 years ago that a short synthetic oligodeoxynucleotide can inhibit the replication and protein expression of Rous sarcoma virus [4,5]. In the early years of this technology, the inhibition activity of ASO was thought to be caused by a steric blocking effect upon hybridization to target RNA. Subsequently, it was reported that target RNAs are cleaved by RNase H at the site of the RNA/DNA heteroduplex [6]. Once ASOs bind via Watson-Crick hybridization to target RNAs, RNase H1 is recruited and hydrolyzes RNA in RNA/DNA hybrids through its endonucleolytic activity. Following endonucleolytic cleavage by RNase H1, the target RNA fragments immediately disappear, suggesting that they are rapidly degraded by intracellular nucleases [7], although the degradation mechanism is not understood in detail. In addition, since RNase H1 mainly resides in the cellular nucleus and mitochondria [8], both pre-mRNA and mature mRNA are likely targets for ASOs, but it is unknown if they are degraded by the same degradation pathway.

There are two major eukaryotic 5′ to 3′ exoribonucleases (XRNs), XRN1 and XRN2. XRN1 is primarily localized in the cytoplasm and has important roles in mRNA turnover and degradation, and it degrades decapped mRNA. In addition, various noncoding RNA transcripts, such as long non-coding RNA (lncRNA), small nucleolar RNA (snoRNA), and aberrant tRNA, are degraded by XRN1 [9]. In *Drosophila* cells, the 3′ fragments of target mRNA generated by siRNAs are decomposed by XRN1 after endonucleolytic cleavage by AGO2 [10]. In contrast, XRN2 is an evolutionarily conserved XRN related to XRN1, but is predominantly present in the nucleus. XRN2 has an important function in mRNA transcription termination. After RNA polymerase II transcribes a poly (A) signal, the transcript is cleaved and the 5′ fragment is polyadenylated. Although RNA polymerase II continues transcription beyond the poly (A) signal, XRN2 enters at the cleavage site and degrades the 3′ transcript until it dissociates RNA polymerase II from the DNA template. XRN2 also plays crucial roles in the maturation and quality control of several classes of RNA such as rRNA, snoRNA, and pre-mRNA [9,11].

The aim of this study was to better understand the degradation mechanism of RNAs targeted by ASOs, and thus we modified the expression of XRN1 and XRN2 to analyze the fate of 3′ fragment of the target pre-mRNA or mature mRNA generated by RNase H1-dependent ASO. We also investigated the XRN responsible for the degradation of target RNA mediated by siRNA in mammalian cells. We found that the 3′ fragments of target RNA generated by ASOs are rapidly degraded from their 5′ ends by nuclear XRN2, unlike in the case with siRNA. Moreover, our data suggest that there may be

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another degradation pathway related to the activity of ASOs but not mediated by RNase H1 and XRN2.

2. Materials and methods

2.1. siRNAs and antisense oligonucleotides

ApoB-ASO reported previously by Straarup et al. [2] and Her3-ASO reported previously by Zhang et al. [12] were prepared and used in this study. These ASOs were phosphorothioated and modified with locked nucleic acid (LNA) (also known as 2′-*O*,4′-*C*-methylene-bridged nucleic acid (2′,4′-BNA)) [13,14]. The sequences of the ASOs are shown in Table S1. These ASOs were synthesized and purified by Gene Design, Inc. (Osaka, Japan). XRN1 siRNA, XRN2 siRNA, RNase H1 siRNA, ApoB siRNA, and negative control (NC) siRNA with a medium GC content, were obtained from Invitrogen (Carlsbad, CA, USA) as Stealth RNAi siRNAs. The sequences of the siRNAs used in this study are shown in Table S2.

2.2. Cell culture

Huh-7 (a human hepatoma cell line) and HepG2 (a human hepatoblastoma cell line) cells were obtained from the Japanese Collection of Research Bioresources (JCRB; Osaka, Japan). These cell lines were maintained at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics/antimycotics (Sigma–Aldrich, St. Louis, MO, USA).

2.3. Transfections with siRNA and ASO

Huh-7 or HepG2 cells were seeded and transfected in 96-well plates (Corning; Corning, NY, USA). After seeding Huh-7 cells at 7.5×10^3 cells/well or HepG2 cells at 1.5×10^4 cells/well, the cells were reverse transfected with 20 nM siRNA for Huh-7 cells or 40 nM for HepG2 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The medium was changed the following day to fresh 10% FBS/DMEM. After 24 h, cells were transfected with 100 nM ApoB-ASO or Her3-ASO, or 20 nM ApoB siRNA, using Lipofectamine 3000 (Invitrogen).

2.4. Quantitative reverse transcription-polymerase chain reaction

Twenty four hours after the second transfection, total RNA was extracted using a CellAmp Direct RNA Prep Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio Inc.) and analyzed with a StepOnePlus system (Applied Biosystems; Foster City, CA, USA). The sequences of the primer set are shown in Table S3. The expression levels of the target RNA were quantitated in at least three independent experiments and normalized to those of *GAPDH*.

2.5. Western blot

Total cellular protein was prepared by cell lysis in radioimmunoprecipitation assay (RIPA) buffer (Sigma–Aldrich) with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Total protein concentrations were determined with a BCA protein

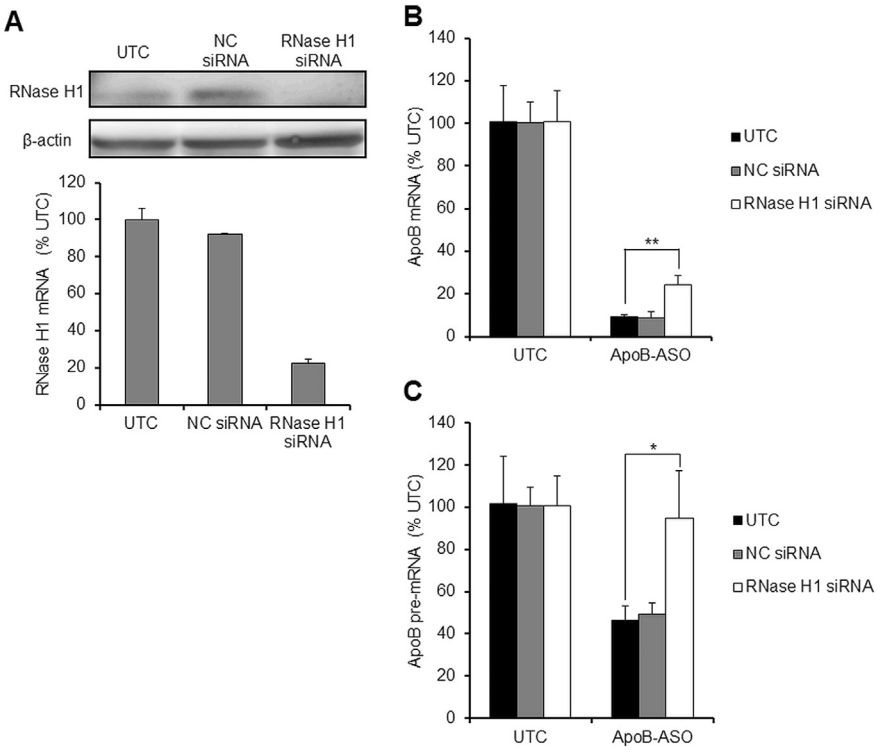

Fig. 1. Effects of RNase H1 siRNA on the potency of ApoB-ASO. (A) Huh-7 cells were transfected with RNase H1 or NC siRNA and the expression level of cellular RNase H1 was assessed by western blot and qRT-PCR. β-actin was used as a loading control for western blot. The level of RNase H1 mRNA was quantitated and normalized to that of GAPDH mRNA. These values were set to 100% in the untreated control (UTC). (B, C) Following siRNA treatment, cells were transfected with ApoB-ASO for 24 h. The expression level of ApoB mature mRNA (B) and pre-mRNA (C) was analyzed by qRT-PCR. The bar graph shows mean ± SD (n = 3). $^*p < 0.05$, $^{**}p < 0.005$ determined by Student's t-test compared to the siRNA untreated control.

assay reagent kit (Pierce/Thermo Scientific, Rockford, IL, USA) according to manufacturer's protocol. Aliquots corresponding to 6 μg total protein were separated by SDS-polyacrylamide gel electrophoresis (5–20% gradient gel) and blotted onto PVDF membranes. The membranes were blocked for 1 h in Blocking One (Nacalai Tesque) and incubated overnight at 4 °C in Tris-buffered saline and 0.1% Tween 20 (TBS-T) containing 5% bovine serum albumin and monoclonal mouse anti-XRN1 antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal rabbit anti-XRN2 antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA), monoclonal rabbit anti-RNase H1 antibody (1:1000) (ProteinTech, Chicago, IL, USA), or for 1 h at room temperature in Blocking One containing monoclonal mouse anti-β-actin (1:1000) (Sigma–Aldrich). Subsequently, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated antibodies (1:3000) (Amersham Biosciences, Buckinghamshire, UK). The blots were visualized using an ECL Prime kit (Amersham Biosciences) and ImageQuant LAS 4000 (Fuji Film, Tokyo, Japan).

2.6. 5′ Rapid amplification of cDNA ends

To identify the 3′ RNA fragments generated by RNase H1-mediated endonucleolytic cleavage, 5′ rapid amplification of the cDNA ends (5′ RACE) was performed using a GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Briefly, the GeneRacer RNA oligo was ligated to the 5′-end of the 3′ RNA fragments using T4 ligase. The ligated RNA was reverse transcribed by SuperScript III reverse transcriptase and the random primer. For the first amplification of 5′ RACE cDNA, touchdown PCR was performed using the GeneRacer 5′ primer and the ApoB pre-mRNA specific primer 5′-ACTGGGAGAATTCTATCCTAACCAGAT-3′ with initial denaturation (5 min at 98 °C), three cycles of annealing at 72 °C, three cycles at 69 °C, three cycles at 66 °C, three cycles at 63 °C, and 20 cycles at 60 °C. Each annealing was followed by a 2 min primer extension at 72 °C and a 10 s denaturation at 98 °C. The final extension time at 72 °C was 7 min. The second amplification was performed for an additional 25 cycles with the GeneRacer 5′ nested primer and the second ApoB pre-mRNA specific primer 5′-TCTAGGAGAGGAGGCAGGATATTTCT-3′. 5′ RACE products were visualized on 1% agarose gels stained with ethidium bromide. To determine the cleavage sites, the PCR product was cloned into p-GEM T easy vector (Promega, Madison, WI, USA) and sequenced.

2.7. Statistical analyses

Statistical comparisons of results were performed by Student's t-tests.

3. Results

3.1. Analysis of the expression level of target RNA after RNase H1 knockdown

To examine whether the reduction of target mature mRNA and pre-mRNA levels caused by the ASO was definitely through RNase H1 cleavage, we first investigated whether depletion of RNase H1 expression prevents ASO-mediated degradation of these transcripts. Huh-7 cells were treated with RNase H1 siRNAs, and after

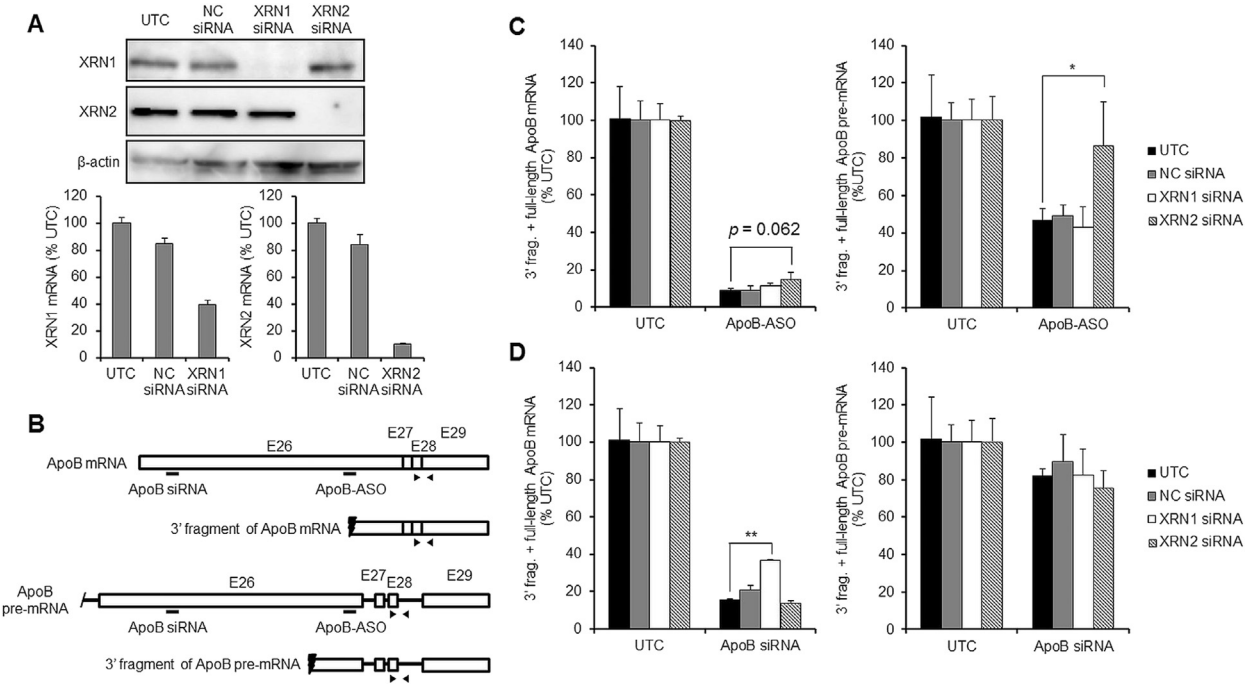

Fig. 2. Effects of XRN depletion on the degradation of the 3′ fragments of ApoB mature mRNA or pre-mRNA. (A) Huh-7 cells were transfected with XRN1 or XRN2 siRNA and the expression levels of cellular XRN1 and XRN2 were assessed by western blot and qRT-PCR. The level of XRN1 and XRN2 mRNA was quantitated and normalized to that of GAPDH mRNA. These values were set to 100% in the untreated control (UTC). (B) Schematic representation of ApoB transcripts, oligonucleotides (lines) and primer sets (arrowheads) for qRT-PCR analysis. Exon 26 is targeted by ApoB-ASO and ApoB siRNA. The forward primer was designed on exon 28 and the reverse primer was designed on exon 29 to detect intact ApoB mature mRNA and 3′ cleavage product. The forward primer was designed on exon 28 and the reverse primer was designed on intron 28 to detect the intact ApoB pre-mRNA and 3′ cleavage product. Each primer set amplified a single product of the expected size. (C) Following XRN1 or XRN2 siRNA treatment, cells were transfected with ApoB-ASO for 24 h. The levels of 3′ fragment (3′ frag.) and full-length ApoB mature mRNA (left panel) and pre-mRNA (right panel) generated by ApoB-ASO were assessed by qRT-PCR. (D) Following XRN1 or XRN2 siRNA treatment, cells were transfected with ApoB siRNA for 24 h. The levels of 3′ fragment (3′ frag.) and full-length ApoB mature mRNA (left panel) and pre-mRNA (right panel) generated by ApoB siRNA were assessed by qRT-PCR. The bar graph shows mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.005$ determined by Student's t-test compared to the siRNA untreated control.

48 h ApoB-ASO was transfected into the siRNA treated cells. The expression levels of ApoB transcripts were analyzed 24 h after transfection with ASO. RNase H1 siRNA promoted an approximately 80% reduction in RNase H1 mRNA and protein levels (Fig. 1A). Treatment with ApoB-ASO reduced the expression levels of ApoB mature mRNA by approximately 90% without pretreatment of RNase H1 siRNA, while RNase H1 depletion attenuated the knockdown effect of ApoB-ASO by approximately 75% (Fig. 1B). Considering that RNase H1 is mainly located in the nucleus and mitochondria [8], and also that ASOs targeting sites in introns can promote target reduction [15], it is likely that pre-mRNA is targeted by ASO as well. Therefore, we investigated the expression level of ApoB pre-mRNA in the same cell lysate. ApoB-ASO resulted in approximately 60% reduction in ApoB pre-mRNA without pretreatment of RNase H1 siRNA, while the knockdown activity of this ASO was almost entirely ablated in RNase H1 depleted cells (Fig. 1C). These results suggest that the reduction of ApoB pre-mRNA is almost completely due to direct cleavage by RNase H1, while the reduction of ApoB mature mRNA is due not only to pre-mRNA reduction, but also to direct cleavage of mature mRNA by RNase H1. Further, these data also suggest that there may be another degradation pathway leading to decreased mature mRNA because ApoB-ASO remained active at the level of mature mRNA, even though cellular RNase H1 was reduced by more than 80%.

3.2. Quantification of the 3′ fragments of target RNA generated by RNase H1-dependent ASO

Since the knockdown effect of ApoB-ASO was mediated by RNase H1, 5′ and 3′ degradation fragments of target RNAs are thought to be generated after endonucleolytic cleavage by RNase H1. However, following initial endonucleolytic cleavage by RNase H1, the transcripts targeted by ASOs are degraded by a poorly understood mechanism. XRN1 and XRN2 are two major 5′ to 3′ exoribonucleases for RNA processing in mammalian cells [9]. To investigate whether XRN1 or XRN2 is responsible for the degradation of the 3′ fragment generated by RNase H1 cleavage, we depleted these exonucleases in Huh-7 cells. XRN1 or XRN2 siRNA promoted approximately 60–90% reduction in target mRNA and protein levels (Fig. 2A). After siRNA treatment, ApoB-ASO was transfected into the cells and the expression levels of ApoB mature mRNA or pre-mRNA were analyzed 24 h later. In addition, we used ApoB siRNA instead of ApoB-ASO to compare the degradation pathway involving ASO or siRNA. Specific primer sets were designed in the 3′ downstream of the ApoB-ASO or ApoB siRNA target region on ApoB mature mRNA or pre-mRNA for qRT-PCR (Fig. 2B). These primer sets were used for amplification of both intact transcripts and 3′ cleavage fragments. When cells were treated with ApoB-ASO, depletion of XRN1 did not result in accumulation of the 3′ fragment of ApoB mature mRNA and pre-mRNA.

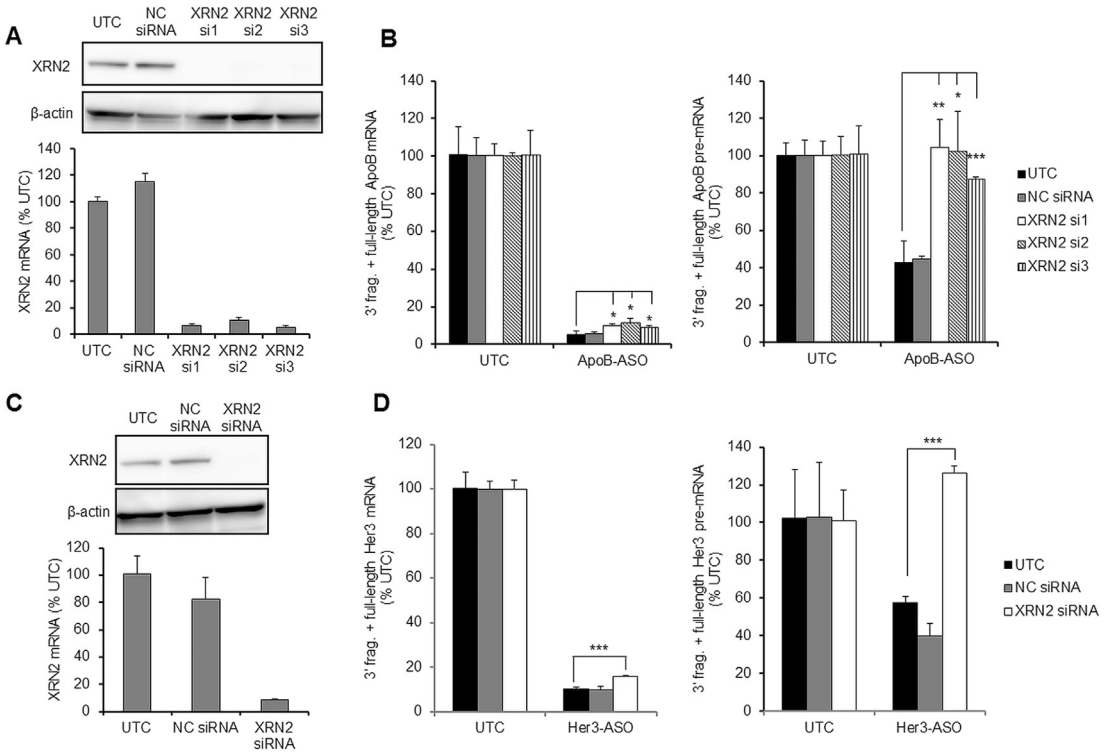

Fig. 3. Further evaluation of the knockdown effect of XRN2. (A) Huh-7 cells were transfected with each XRN2 siRNA or NC siRNA. The expression level of cellular XRN2 was assessed by western blot and qRT-PCR. The level of XRN2 mRNA was quantitated and normalized to that of GAPDH mRNA. These values were set to 100% in the untreated control (UTC). (B) Following siRNA treatment, cells were transfected with ApoB-ASO for 24 h. The levels of 3′ fragment (3′ frag.) and full-length ApoB mature mRNA (left panel) and pre-mRNA (right panel) generated by ApoB-ASO were assessed by qRT-PCR. (C) HepG2 cells were transfected with XRN2 siRNA or NC siRNA and the expression level of cellular XRN2 was assessed by western blot and qRT-PCR. The level of XRN2 mRNA was quantitated and normalized to that of GAPDH mRNA. These values were set to 100% in the untreated control (UTC). (D) Following siRNA treatment, cells were transfected with Her3-ASO for 24 h. The levels of 3′ fragment (3′ frag.) and full-length Her3 mature mRNA (left panel) and pre-mRNA (right panel) generated by Her3-ASO were assessed by qRT-PCR with primer sets downstream of the ASO binding site. The bar graph shows mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ determined by Student's t-test compared to the siRNA untreated control.

On the other hand, treatment with XRN2 siRNA did not significantly change the levels of the 3′ fragment of ApoB mature mRNA, but did show a slight tendency to increase ($p = 0.062$). Interestingly, depletion of XRN2 led to significant accumulation of the 3′ fragment of ApoB pre-mRNA (Fig. 2C). In contrast, when cells were treated with ApoB siRNA, only ApoB mature mRNA was reduced, and depletion of XRN1 led to accumulation of the 3′ fragment of ApoB mature mRNA (Fig. 2D).

3.3. Further evaluation of the knockdown effect of XRN2

We performed two additional experiments to support our finding that the RNase H1-mediated fragment of ApoB transcripts appears to accumulate by depletion of XRN2. Different sequences of XRN2 siRNA were used and the 3′ fragment of ApoB transcripts in Huh-7 cells was analyzed. We confirmed that all of the XRN2 siRNAs, including the preceding siRNA (XRN2 si1), resulted in more than 90% reduction of both mRNA and protein (Fig. 3A). Depletion of XRN2 resulted in significant accumulation of the 3′ fragment of ApoB pre-mRNA when cells were treated with any of the XRN2 siRNAs. Additionally, the levels of the 3′ fragment of mature mRNA slightly but significantly changed upon treatment with any XRN2 siRNA (Fig. 3B). These results proved that both pre-mRNA and mature mRNA are degraded by XRN2, and this change is not caused by off-target effects of a specific sequence of XRN2 siRNA. To investigate whether XRN2-mediated degradation is a general pathway for target RNAs by RNase H1-dependent ASO, we conducted a similar experiment using a different target gene (*Her3*) and cell line (HepG2). Specific primer sets were designed in the 3′ downstream of the Her3-ASO target region on Her3 mature mRNA or pre-mRNA for qRT-PCR (Fig. S1). HepG2 cells were treated with XRN2 or NC siRNA. After 48 h, Her3-ASO was transfected into the cells and the expression levels of Her3 mature mRNA and pre-mRNA were analyzed by qRT-PCR. XRN2 siRNA promoted greater than 90% reduction in mRNA and protein levels (Fig. 3C) and depletion of XRN2 led to the significant accumulation of the 3′ fragment of both Her3 mature mRNA and pre-mRNA (Fig. 3D). These findings indicate that XRN2 completely degrades the 3′ fragment of pre-mRNA and at least partly degrades that of mature mRNA targeted by ASO in any target and cell line.

3.4. 5′ RACE analysis of the 3′ RNA fragments generated by RNase H1-mediated cleavage

To identify the 3′ RNA fragments generated by RNase H1-mediated endonucleolytic cleavage, 5′ rapid amplification of cDNA ends (5′ RACE) was performed on RNA from cells transfected with ApoB-ASO (Fig. 4A). 5′RACE products were detected in cells treated with ApoB-ASO, and pretreatment with XRN2 siRNA increased the 5′RACE band intensity compared to pretreatment with NC siRNA (Fig. 4B). The 5′ end of RNase H1 cleaved products in XRN2-depleted cells were confirmed by sequencing 18 individual clones from 5′RACE; the results showed that all the 5′ ends of 3′ fragments are positioned within the binding site of ApoB-ASO (Fig. 4C). Human RNase H1 preferentially cleaves the heteroduplex substrate between 7 and 12 nucleotides from the 5′-RNA–3′-DNA terminus of the duplex [16]. Our results suggest that XRN2 acts as a primary 5′ exonuclease following endonucleolytic cleavage by RNase H1 because the cutting sites of RNase H1 are retained in XRN2-depleted cells.

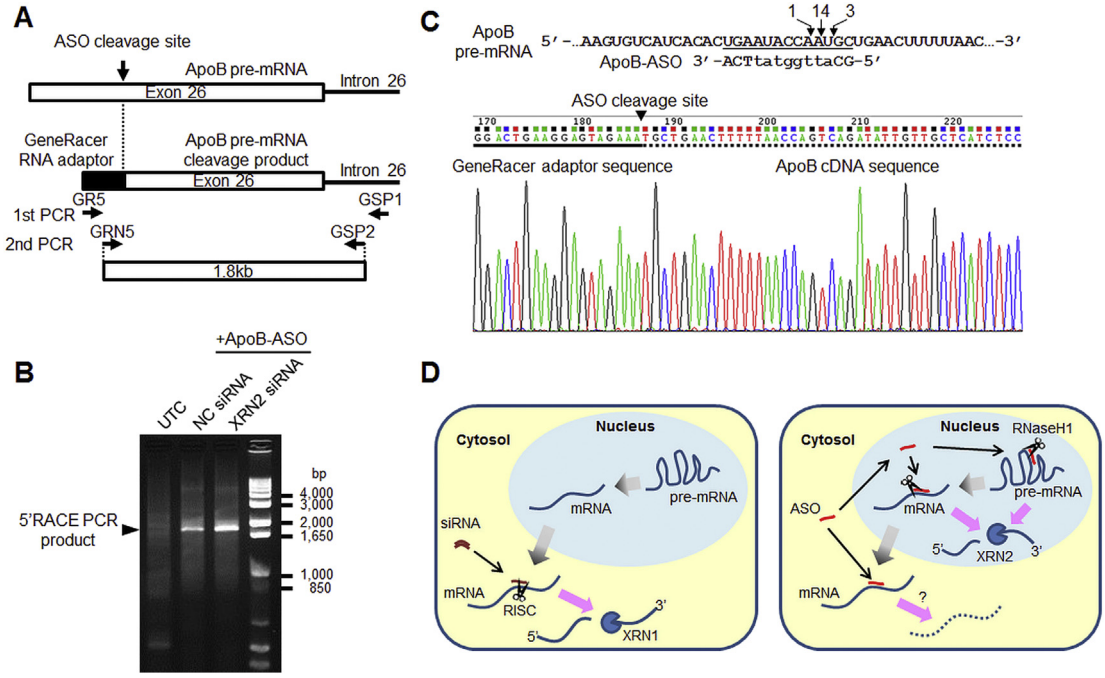

Fig. 4. 5′ RACE analysis of the 3′ fragments generated by RNase H1-mediated cleavage. (A) Schematic representation of ApoB pre-mRNA, illustrating the location of the ASO cleavage site and the primers used for PCR amplification of the cleavage fragment. GR5; GeneRacer 5′ primer, GRN5; GeneRacer 5′ nested primer; GSP1; ApoB pre-mRNA specific reverse primer; GSP2; ApoB pre-mRNA specific nested primer. (B) Agarose gel visualizing 5′ RACE PCR amplification products after siRNA and ASO treatment using the primer set GRN5 and GSP2. (C) The sequence of ApoB pre-mRNA containing the ApoB-ASO binding site (underlined) is illustrated. The arrows indicate the 5′ ends as determined by sequencing of individual clones from 5′ RACE derived from XRN2-depleted cells. The numbers above the arrows indicate the number of clones sequenced with that cleavage site. The sequence chromatograph containing the main cleavage site is illustrated in the lower panel. (D) A schematic diagram for the degradation pathway of 3′ fragments of target RNA generated by siRNA (left panel) and ASO (right panel).

4. Discussion

siRNA degrades the target RNA via intracellular endonuclease AGO2. Orban et al. reported that, following endonucleolytic cleavage by AGO2, the 3′ fragment of target RNA generated by siRNA is degraded by cytoplasmic 5′ to 3′ exoribonuclease XRN1 in *Drosophila* cells [10]. We likewise showed that depletion of XRN1 specifically results in accumulation of the 3′ fragment of target mRNA generated by siRNA in mammalian cells, consistent with the fact that siRNA can operate in the cytoplasm via RISC complexes containing AGO2. ASO likely operates through two degradation pathways. In one pathway, the fragment of target RNA is exported to the cytosol after cleavage by RNase H1 in the nucleus and is then degraded by the same pathway as siRNA. In the other, the fragment of target RNA is degraded in the nucleus. Both possibilities were examined by focusing on two 5′ to 3′ exoribonucleases, cytoplasmic XRN1, and nuclear XRN2. We found that depletion of XRN2 specifically results in accumulation of the 3′ fragment of target RNA generated by RNase H1-dependent ASO, indicating that the fragment cleaved by RNase H1-dependent ASO is degraded by XRN2 in the nucleus and identifies a new function of XRN2.

Our findings show that the cutting or degradation of pre-mRNA is almost completely suppressed by depletion of RNase H1 or XRN2, suggesting that pre-mRNAs are targeted by RNase H1 and that their fragments are degraded by XRN2 in the nucleus. This conclusion is compatible with the fact that ASO can induce exon skipping by targeting pre-mRNA, and ASO targeting the intron can cleave pre-mRNA via RNase H1 in the nucleus [17,18]. On the other hand, since the cutting and degradation of mature mRNA was slightly inhibited by depletion of RNase H1 or XRN2, ASO and RNase H1 may also interact with mature mRNA and this mature mRNA is at least partly degraded by XRN2. Further, our data suggest that there may be another degradation mechanism that contributes to the activity of ASO targeting mature mRNA because ApoB-ASO was still active at the level of mature mRNA, even though cellular RNase H1 was reduced by more than 80%. Previously, Wu et al. reported another RNase H which may play a role in the activity of ASOs [7]. In addition, since mature mRNA is believed to be present in both the nucleus and cytoplasm, it would be interesting to know whether there are different degradation pathways depending on the subcellular localization of the mRNA. We are currently investigating this possibility. A proposed mechanism for the degradation pathway of target RNA generated by siRNA and ASO is illustrated in Fig. 4D.

In this study, we focused on the 3′ fragment of target RNAs generated by RNase H1-dependent ASO. It has also been reported that the 5′ fragment of target RNA generated by siRNA can be degraded by the exosome complex present in the cytoplasm of *Drosophila* cells [10]. Exosome complex has been reported to also be in the nucleus [19]. Further investigation is needed to reveal how the 5′ fragments of RNA cleaved by RNase H1-dependent ASO are degraded in mammalian cells. In conclusion, we revealed that the 3′ fragments of target RNAs generated by RNase H1-dependent ASO are rapidly degraded from their 5′ ends by XRN2 in the nucleus, in contrast to the degradation pathway of siRNA. This finding will help shed light on the poorly understood degradation mechanisms of RNAs targeted by ASO.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.171.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.171.

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