Gene suppression via U1 small nuclear RNA interference (U1i) machinery using oligonucleotides containing 2′-modified-4′-thionucleosides

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ABSTRACT

Gene suppression via U1 small nuclear RNA interference (U1i) is considered to be one of the most attractive approaches, and takes the place of general antisense, RNA interference (RNAi), and anti-micro RNA machineries. Since the U1i can be induced by short oligonucleotides (ONs), namely U1 adaptors consisting of a 'target domain' and a 'U1 domain', we prepared adaptor ONs using 2′-modified-4′-thionucleosides developed by our group, and evaluated their U1i activity. As a result, the desired gene suppression via U1i was observed in ONs prepared as a combination of 2′-fluoro-4′-thionucleoside and 2′-fluoronucleoside units as well as only 2′-fluoronucleoside units, while those prepared as combination of 2′-OMe nucleoside/2′-OMe-4′-thionucleoside and 2′-fluoronucleoside units did not show significant activity. Measurement of T_m values indicated that a higher hybridization ability of adaptor ONs with complementary RNA is one of the important factors to show potent U1i activity.

1. Introduction

Nucleic acid-based therapeutics using antisense, short-interfering RNA (siRNA), and anti-micro RNA molecules are intensely interested in new generation of drug development.[1] Thus far, a number of chemically-modified oligonucleotides (ONs) have been developed toward not only in vitro, but also in vivo utility, and their activity of gene suppression has been evaluated.[2–4] To expand the possibilities of nucleic acids-based therapeutics, however, development of a new approach to gene suppression along with above-mentioned machineries is required. Gene silencing machinery using U1 small nuclear RNA (U1 snRNA), a component of U1 small nuclear ribonucleoprotein (snRNP), has been considered to be one of the most promising prospective approaches. The primary function of U1 snRNA is recognition of the 5′ splice site sequence during pre-mRNA splicing by base pairing in its 5′ end. In addition, U1 snRNA can suppress the gene expression via sequence-specific binding upstream of the poly(A) signal of pre-mRNA, which is termed U1 snRNA interference (U1i).[5,6] Beckley et al. have con-

structed expression plasmids that code 5′-end-mutated U1 snRNAs, including complementary sequences against exogenous and endogenous genes, and succeeded to suppress the target genes upon transfection of the constructed plasmids into cultured cells.[7,8] Although their approach seems to be attractive and effective for gene suppression, no further advance toward nucleic acid-based therapeutics was reported, because of the inconvenience of pre-pareing custom U1i targeting plasmids. However, Goraczniak et al. have recently succeeded to induce U1i using short ONs, U1 adaptors.[9] The U1 adaptors consists of two domains: a 'target domain' designed to base pair to the target gene's pre-mRNA in the 3′-terminal exon, and a 'U1 domain' that recruits U1 snRNP to the target pre-mRNA. In addition, the adaptors consist of 2′-OMeRNA and locked nucleic acid (LNA), which have a higher hybridization ability with the target RNA sequences. Accordingly, U1 snRNP was recruited on the pre-mRNA near the poly(A) signal with assistance of the adaptors to suppress gene expression. Since this phenomenon can be induced by readily available short ONs, U1i caused by the U1 adaptors may be an alternative approach to effective and sequence-specific gene suppression.

Meanwhile, we have been working on a project to develop chemically-modified ONs, which can be applicable to aptamer, antisense and RNAi machineries. Thus far, we have reported the synthesis of 4′-thioRNA[10] and 4′-thioDNA,[11] which possess a sulfur atom instead of sugar ring oxygen, and their utility toward the applications of nucleic acid-based therapeutics.[12–16] Furthermore,

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we recently developed 2′-modified-4′-thioRNAs,[17] that is, 2′-fluoro-4′-thioRNA (2′-F-4′-thioRNA) and 2′-OMe-4′-thioRNA, which have a hybrid chemical modification based on 2′-modified ONs and 4′-thioRNA (Fig. 1). In their hybridization with a complementary RNA, 2′-F-4′-thioRNA showed the highest T_m value among the ONs evaluated. On the other hand, 2′-OMe-4′-thioRNA showed the highest stability against nuclease digestion in a comprehensive comparison. Concerning 2′-OMe-4′-thioRNA, we showed that this modification induced potent and long-term RNAi activity compared with siRNAs modified with 4′-thioRNA and 2′-OMeRNA.[18] These preferable properties of 2′-modified-4′-thioRNAs prompted us to evaluate whether the ONs containing 2′-OMeRNA and 2′-F-4′-thioRNA act as U1 adaptors instead of those containing 2′-OMeRNA and LNA.

In this paper, we describe the synthesis of adaptor ONs using 2′-modified-4′-thionucleosides,[19] components of 2′-modified-4′-thioRNAs, and evaluated their gene suppression activity via U1i machinery.

2. Results and discussion

We first constructed reporter assay system to evaluate U1i activity of the U1 adaptors. As shown in Figure 2A, a plasmid, originating from pGL3-C firefly luciferase expression plasmid, was prepared by insertion of a consensus binding sequence for wild type U1 snRNA in the 3′ untranslated region (3′UTR) of luciferase coding sequence. This luciferase expression plasmid was represented as WTx1. In addition to the wild type sequence, plasmid containing a mutated U1 binding sequence was also prepared, which was represented as MTx1. Moreover, the U1snRNA binding sequence (or mutated U1snRNA binding sequence) was inserted in tandem to give WTx2 and WTx3 (and also MTx2 and MTx3) to show the required U1i activity more vividly.[7,9,20] Since the resulting pre-mRNAs from a series of WT plasmids have wild-type U1snRNP binding sequence, the luciferase activity is expected to decrease compared with control plasmid (pGL3-C without the U1snRNA binding sequence) arising from the desired U1i activity. On the other hand, the luciferase activity should be active upon transfection of a series of MT plasmids, because endogenous U1snRNP cannot bind to the resulting pre-mRNAs. After the desired plasmids were prepared, the function of the resulting plasmids was firstly tested. The luciferase activity after 24 h upon transfection of pGL3-C into HeLa cells was set as 100%, and relative activity of each plasmid was evaluated. As can be seen in Figure 3, the luciferase activity was reduced to approximately 40% when WTx1 plasmid was transfected into HeLa cells. The activity was completely lost when WTx2 and WTx3 plasmids were transfected. On the other hand, little gene suppression was observed upon transfection of MTx1, MTx2, or MTx3, since the endogenous U1snRNP cannot bind to the resulting pre-mRNAs from a series of MT plasmids. These results confirmed that the reporter assay system to evaluate U1i activity was successfully constructed.

As described in Section 1, Goraczniak et al. reported induction of U1i using U1 adaptors, which recruit U1snRNP on the target
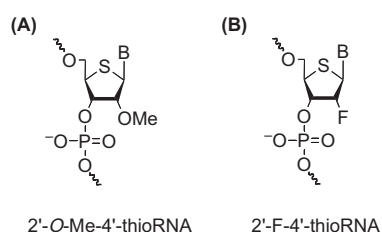

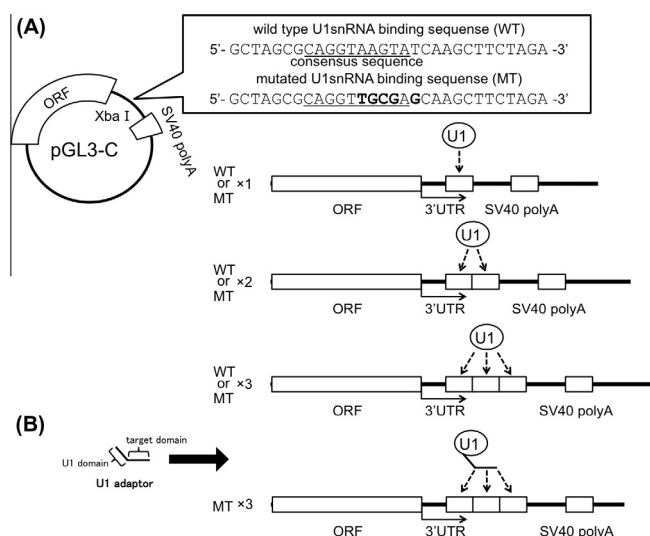Figure 1. Structures of 2′-modified-4′-thioRNAs.

Figure 2. Preparation of reporter assay system. (A) Structures and sequences of luciferase reporter plasmids. Details were described in the Section 3. (B) Recruitment of U1snRNP by U1 adaptor.
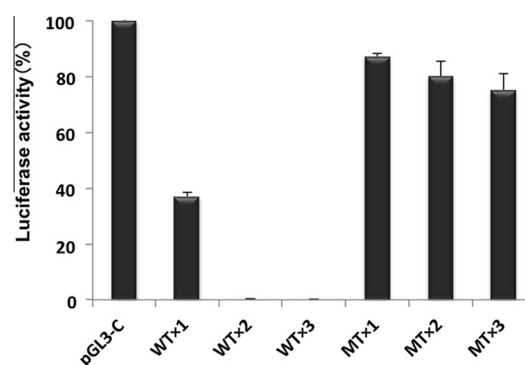

Figure 3. Luciferase activity upon transfection of a series of WT and MT plasmids. Each firefly luciferase activity is normalized to the cotransfected Renilla luciferase activity, and relative luciferase activity is shown based on the activity of pGL3-C.

pre-mRNA (Fig. 2B).[9] In their report, they carefully investigated the relationship between modification pattern of U1 adaptor and U1i activity. As a result, the adaptor ONs modified with uniform 2′-OMeRNA showed no U1i activity, while those containing 2′-OMeRNA and LNA, which would exhibit higher hybridization ability with complementary RNA, induced effective U1i. In addition, the best activity was obtained in U1 adaptor consisting of 15 mer of the target domain and 13 mer of the U1 domain. With these previous studies as references, we designed U1 adaptors containing various chemically-modified nucleoside units including 2′-modified-4′-thionucleosides. The sequence and modification pattern of the U1 adaptors are shown in Figure 4A. The adaptors are all 28 mer and consist of the target domain (5′-AAGCUUG-CUCGCAAC-3′) and the U1 domain (5′-GCCAGGUAAGUAU-3′). To append higher hybridization ability, purine units of U1 adaptors were all modified with 2′-fluoronucleoside (2′-F) units. On the other hand, pyrimidine units were modified with 2′-F, 2′-OMe, 2′-OMe-4′-thio, or 2′-F-4′-thio nucleoside units to give adaptors 1–4. From the data shown in Figure 3, the plasmid possessing multiple binding sites, that is, MTx3 seemed better to evaluate U1i activity by the adaptor ONs. Thus, each of adaptors 1–4 was cotransfected with MTx3 plasmid into HeLa cells, and the activity of gene suppression relative to that of 40 nM of T7 terminator sequence as a negative control was evaluated after 24 h. As shown in

Table 1
Hybridization ability of adaptors with RNA1 or RNA2

(A) duplex	T_m (°C)[a]	ΔT_m (°C)[b]	(B) duplex	T_m (°C)[a]	ΔT_m (°C)[b]
RNA:RNA1	73.8 ± 0.2	–	RNA:RNA2	64.2 ± 0.1	–
adaptor 1:RNA1	81.5 ± 0.1	7.7	adaptor 1:RNA2	71.1 ± 0.4	6.9
adaptor 2:RNA1	86.7 ± 0.1	12.9	adaptor 2:RNA2	74.5 ± 0.3	10.3
adaptor 3:RNA1	84.7 ± 0	10.9	adaptor 3:RNA2	75.3 ± 0.6	11.1
adaptor 4:RNA1	88.4 ± 0.2	14.6	adaptor 4:RNA2	76.9 ± 0.2	12.7

(A) T_m values of duplex between the target domain of each adaptor and a complementary RNA1 (15 mer). (B) T_m values of duplex between the U1 domain of each adaptor and a complementary RNA2 (13 mer).
[a] T_m values were given as an average of three independent experiments.
[b] T_m values were calculated based on the T_m values of RNA:RNA1 or RNA:RNA2.

Figure 4B, adaptors **1** and **3**, consisting 2′-OMe/2′-F[21] and 2′-OMe-4′-thio/2′-F[21] units, did not shown any significant gene suppression, although slight activity was observed at higher concentration (40 nM). Contrary to adaptors **1** and **3**, the desired gene suppression dependent on the concentration of adaptor transfected was obviously observed in adaptors **2** and **4**, which consisted of 2′-F/2′-F[21] and 2′-F-4′-thio/2′-F[21] units. The IC_{50} value of adaptor **4** was estimated as 9.6 nM, which was almost equal to that of adaptor **2** (IC_{50} = 6.7 nM). In addition, these activities were almost comparable to the results reported by Goraczniak et al.[9]

To consider the difference of gene suppression activity by adaptors **1**–**4**, we measured T_m value of the duplexes between the adaptor and a complementary RNA sequence. Since the adaptors consist of the target domain and the U1 domain, complementary RNA sequences, that is RNA1 (5′-GUUGCGAGCAAGCUU-3′; complementary to the target domain) and RNA2 (5′-AUACUUAC-

CUGGC-3′; complementary to the U1 domain), were prepared and T_m values of the resulting duplexes were measured (Table 1). The duplex of RNA, a natural 28 mer possessing the same sequence context as the adaptor ONs, and RNA1 showed T_m value of 73.8 °C. The T_m values of adaptors:RNA1 were all higher than that of RNA:RNA1 (ΔT_ms were from 7.7 to 14.6 °C). The same tendency was observed in the duplexes of adaptors:RNA2 (ΔT_ms were from 6.9 to 12.7 °C). In general, adaptor ONs consisting of 2′-F/2′-F and 2′-F-4′-thio/2′-F units formed thermally more stable duplexes than ONs consisting of 2′-OMe/2′-F and 2′-OMe-4′-thio/2′-F units.

Concerning stability against nuclease digestion, 2′-OMeRNA and 2′-OMe-4′-thioRNA were much more stable than 2′-FRNA and 2′-F-4′-thioRNA.[17] Therefore, it is expected that ONs consisting of 2′-OMe/2′-F and 2′-OMe-4′-thio/2′-F units would be more stable against nuclease digestion than those consisting of 2′-F/2′-F and 2′-F-4′-thio/2′-F units. As one explanation of the results in Figure 4B, therefore, it can be concluded that higher thermal stability of adaptors, rather than nuclease resistance, is required for effective gene suppression, at least in vitro.

By the way, the adaptors have complementary sequences against pre-mRNA. Therefore, there is a possibility that the adaptor ONs act as an antisense molecule to suppress gene expression, but not as the U1 adaptor. To exclude this possibility, we prepared two sequences, that is, ON1 corresponding to the target domain and
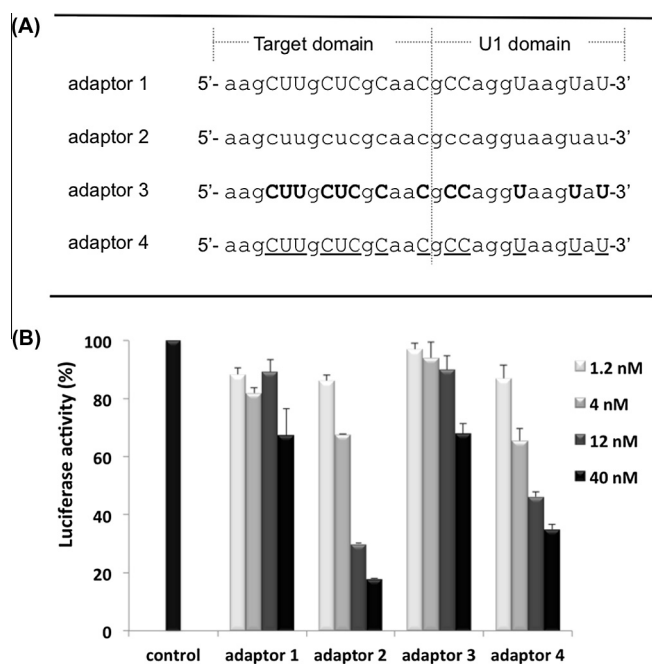

Figure 4. U1i activity by adaptor ONs modified with 2′-modified-4′-thionucleosides. (A) The sequence and modification pattern of the U1 adaptors. 2′-OMe units are uppercase, 2′-F units are lowercase, 2′-OMe-4′-thio units are bold uppercase, and 2′-F-4′-thio units are underlined uppercase. (B) Measurement of the U1i activity of adaptor ONs. Details were described in the Section 3.
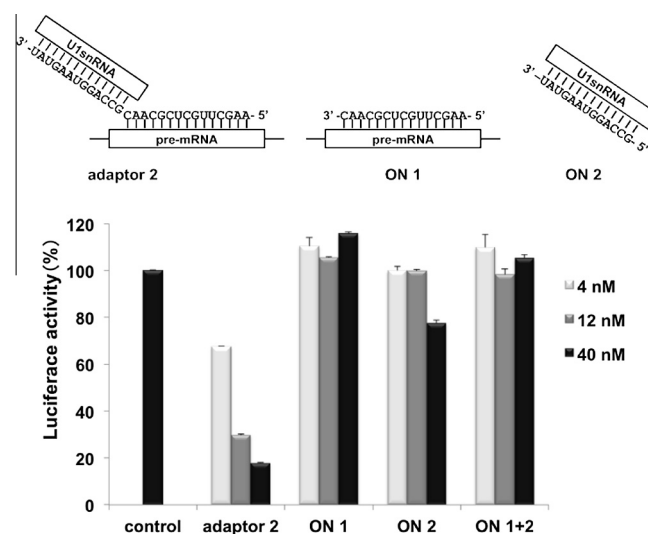

Figure 5. Comparison of gene suppression by adaptor **2**, ON1, and ON2. Details were described in the Section 3.

ON2 corresponding to the U1 domain. Both ONs were modified with 2′-F/2′-F units, and their activity was evaluated at 4, 12, and 40 nM, respectively. As can be seen in Figure 5, neither ON1 nor ON2 showed any significant gene suppression under the same conditions different from adaptor **2**. In addition, combined transfection of ON1 and ON2 into HeLa cells also showed no activity. These results strongly suggested that the adaptor ONs require both the target domain and the U1 domain in the molecule to suppress gene expression, and their activity comes from U1i, but not antisense mechanism.

In conclusion, we examined the gene suppression via U1i machinery using ONs modified with 2′-modified-4′-thionucleosides. The effective gene suppression was observed in ONs consisting of 2′-F/2′-F and 2′-F-4′-thio/2′-F units, while those consisting of 2′-OMe/2′-F and 2′-OMe-4′-thio/2′-F units did not show significant activity. Therefore, it was suggested that 2′-F and 2′-F-4′-thio modification is available for the gene suppression via U1i machinery. Contrary to the favorable results reported by us in this manuscript, Vickers et al. have reported that gene silencing mediated by U1 adaptor resulted in significant off-target silencing arising from sequestering of U1 snRNP.[22] In their report, the authors concluded that careful screening of U1 adaptor sequences is needed to minimize U1 snRNP sequestration and establish maximal specificity. Thus, further examination would be required to show the utility of U1i mediated by U1 adaptor. Since targeting the same gene either U1 adaptor or siRNA strongly enhances gene silencing,[9] development of not only siRNA but also U1 adaptor by 2′-modified-4′-thioRNAs should be attractive, and these are currently underway.

3. Experimental section

3.1. Synthesis of ONs

Support bound chemically modified ONs used in this study were synthesized on an H-6 DNA/RNA synthesizer (Nihon Techno Service) using the corresponding phosphoramidite units at a 0.2 µmol scale following the standard procedure described for oligoribonucleotides. Each of phosphoramidite units was used at concentration of 0.07 M in dry acetonitrile, and the coupling time was extended to 12 min for each step. After completion of the synthesis, the CPG support was treated with methanolic ammonia (saturated at 0 °C) at room temperature for 24 h. Then, the support was filtered off. The filtrate was concentrated and the ON protected by a DMTr group at the 5′-end was purified on reversed-phase HPLC, using a J'sphere ODS-M80 column (10 × 150 mm, YMC) with a linear gradient of acetonitrile (from 5% to 50%) in 0.1 N TEAA buffer (pH 7.0). The residue was concentrated and treated with aqueous acetic acid (70%) for 20 min at room temperature. The solution was concentrated and the residue was purified on reversed-phase HPLC, using a J'sphere ODS-M80 column (10 × 150 mm, YMC) with a linear gradient of acetonitrile (from 5% to 25%) in 0.1 N TEAA buffer (pH 7.0). The structure of each adaptor ON was confirmed by measurement of MALDI-TOF/MASS spectrometry on an ultraflex TOF/TOF (Buruker Daltonics). Adaptor 1; calculated mass, $C_{280}H_{341}F_{15}N_{108}O_{177}P_{27}$, 9174.4 (M−H); observed mass, 9174.0. Adaptor 2; calculated mass, $C_{267}H_{302}F_{28}N_{108}O_{164}P_{27}$, 9016.1 (M−H); observed mass, 9016.7. Adaptor 3; calculated mass, $C_{280}H_{341}F_{15}N_{108}O_{164}P_{27}S_{13}$, 9379.1 (M−H); observed mass, 9379.5. Adaptor 4; calculated mass, $C_{267}H_{302}F_{28}N_{108}O_{151}P_{27}S_{13}$, 9223.8 (M−H); observed mass, 9223.7. The structure of ON1 and ON2 was confirmed by measurement of LRMS (ESI-TOF) on Waters LCT Premier 2695. ON1; m/z calculated for $[M−3H]^{3−}$ 1586.2, found 1585.9. ON2; m/z calculated for $[M−4H]^{4−}$ 1045.9, found 1046.0.

3.2. Construction of plasmids for reporter assay

As shown in Figure 2A, double strand DNA of consensus binding sequence for wild type U1 snRNA was synthesized, digested with *Nhe*I and *Xba*I, and inserted into *Xba*I site located on the 3′ UTR of luciferase coding sequence of pGL3-C (Promega). The resulting plasmid was termed WTx1. To prepare WTx2, WTx1 was digested with *Xba*I and inserted above wild type U1 snRNA binding sequence. Using WTx2, WTx3 was also constructed. Similarly, by using mutated U1 snRNA binding sequence, a series of MT plasmids (MTx1, MTx2, and MTx3) were also prepared.

3.3. Evaluation of U1i activity

HeLa cells were cultured in Minimum Essential Medium containing 10% fetal bovine serum with 1% non-essential amino acids. About 3.5×10^5 cells were plated onto one well of 6-well culture plate. For plasmid transfection (Figs. 3–5), each WT or MT reporter plasmid (0.15 µg) was mixed with 0.6 µg of pRL-TK *Renilla* luciferase expression plasmid (Promega) to compensate transfection efficiency. Then, the combined plasmids were then transfected into cells using X-treme GENE HP (Roche) 12 h after plating. For adapter ONs transfection (Figs. 4 and 5), each adaptor was mixed with ineffective ON (T7 terminator sequence, 5′-GCCTCTAAACGGGTCTT-GAGG-3′) to maintain the ON concentration at 40 nM (100 pmols/2.5 mL culture medium). The combined ONs were then transfected into cells using Lipofectamine 2000 (Invitrogen) 6 h before plasmid transfection. Twenty-four hour after plasmid transfection, cell lysates were prepared and firefly and *Renilla* luciferase activities were obtained by Infinite 200 PRO (TECAN) using Dual-Luciferase Reporter Assay System (Promega) according to supplier's recommendation. All the transfection experiments were carried out three independent culture wells, and each firefly luciferase activity was normalized to *Renilla* luciferase activity.

3.4. Measurement of T_m values

Thermally induced transitions were monitored at 260 nm on a Shimazu UV-1800 Spectrophotometer. Samples were prepared as follows. Duplex formation: a solution containing an appropriate ON and a complementary sequence (1.5 µM each) in a buffer of 10 mM Na cacodylate (pH 7.0) containing 100 mM NaCl were heated at 90 °C for 3 min, then cooled gradually to room temperature and used for the thermal denaturation study. The sample temperature was increased 1.0 °C/min.

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21. Adaptor **1** consists of 2′-OMe nucleoside units for pyrimidine and 2′-F nucleoside units for purine, and represented as 2′-OMe/2′-F. Similarly, adaptor **3** consists of 2′-OMe-4′-thio nucleoside units for pyrimidine and 2′-F nucleoside units for purine, and represented as 2′-OMe-4′-thio/2′-F. This type of indication was also used for adaptors **2** and **4**.
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