RNA Probes

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An Activatable siRNA Probe: Trigger-RNA-Dependent Activation of RNAi Function**

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As the roles of RNA in complex cellular systems continue to be revealed, interest has focused on expansion of the repertoire of RNA tools for the artificial control of cellular functions.[1] Among the most promising tools are small-interfering-RNA-based modulators that use the powerful catalytic activity of cellular RNA interference (RNAi). Several research groups have reported approaches to the trigger-dependent control of RNAi[2,3] or Dicer-processing activity.[4,5] However, only small molecules have been identified as successful triggers, and all of the reported procedures operate under turn-OFF control, whereby RNAi activities are suppressed by the binding of triggers to RNA probes. One challenge to the more active use of such RNAi-based modulators is the establishment of a strategy to produce a new RNA tool whose RNAi activity can be enhanced in a trigger-dependent turn-ON manner. Herein, we report a newly designed synthetic hairpin-shaped (Hp) sense strand/antisense strand pair as an activatable small-interfering-RNA (siRNA) probe and demonstrate that this system enables the first turn-ON control of RNAi activity with an RNA trigger.

The cellular RNAi process in the cytoplasm is initiated by the presence of double-stranded RNA (dsRNA) through two activation steps: 1) Dicer processing of dsRNA to a 19–21 base pair (bp) sense strand (SS)/antisense strand (AS) with a two-nucleotide (nt) overhang, and 2) loading into an RNA-induced silencing complex (RISC) to cleave messenger RNA (mRNA) that has a complementary sequence to the AS (Figure 1a). We focused on the Dicer-processing step and designed an activatable siRNA system comprising Hp-SS and AS probes for the trigger-RNA-dependent control of siRNA function in a turn-ON manner (Figure 1b). The Hp-SS probe is a synthetic fusion of an SS domain of siRNA (red), a regulatory stem domain (pink), and a recognition loop domain (green) complementary to the trigger RNA (black; Figure 1b). In the absence of a trigger RNA, the regulatory stem is designed to inhibit SS/AS hybridization (OFF state), whereas the binding of the trigger RNA with concomitant

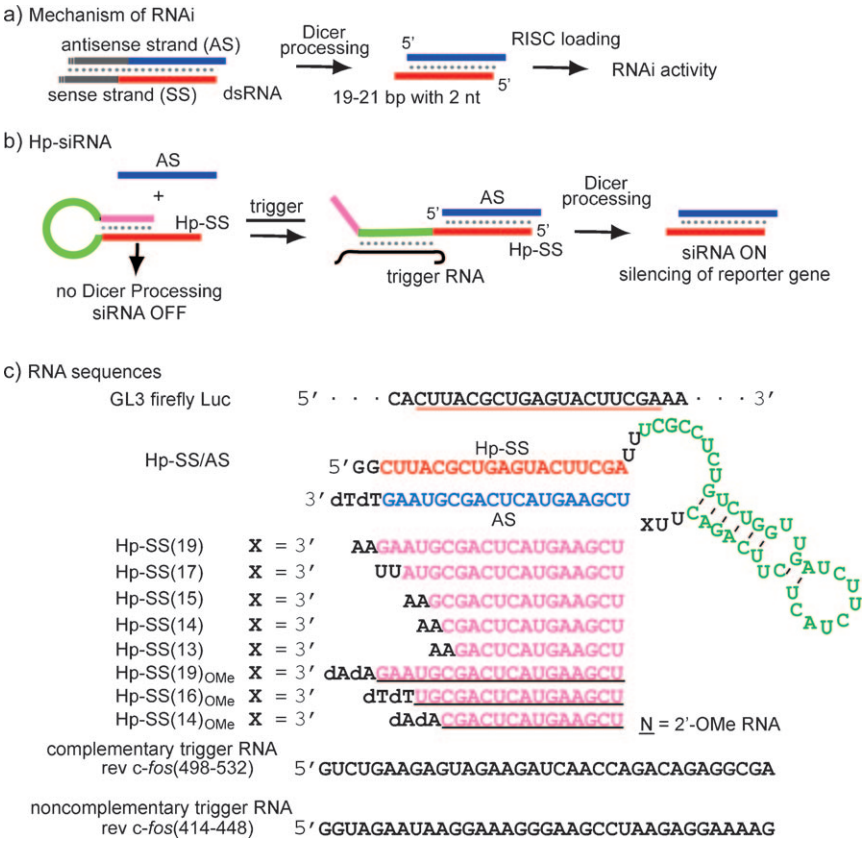

Figure 1. a,b) Schematic illustrations of the RNAi mechanism and the designed activatable siRNA system. c) RNA sequences used in this study. The sense strand (SS), antisense strand (AS), trigger-binding recognition loop (in a predicted secondary structure), and regulatory stem domain are shown in red, blue, green, and pink, respectively. The secondary structure of RNA was predicted by the RNAstructure program (version 4.5).

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liberation of the SS can induce SS/AS hybridization followed by Dicer processing and the ignition of siRNA activity (ON state). If the concept proves viable, the presence of the trigger RNA should induce RNAi function.

To demonstrate this concept, we performed gene-silencing experiments in HeLa cells. Firefly luciferase (pGL3-Control vector) was used as the target reporter gene, wherein the coding region 155–173 (underlined in red in Figure 1c) relative to the first nucleotide of the start codon serves as the complementary domain of AS (shown in blue) in the activated siRNA.[6] The trigger RNA was the inverse sequence of the coding region 498–532 of human c-*fos* mRNA (rev c-*fos* RNA(498–532)).[7] The recognition loop domain (shown in green) of Hp-SS is complementary to the trigger. Before examining the turn-ON control of RNAi activity, we checked whether the ternary complex Hp-SS/AS/trigger RNA (center in Figure 1b) was a possible substrate of Dicer and could silence reporter firefly luciferase activity in cells. As a positive control, Hp-SS(control) with no regulatory stem domain was prepared, subjected to ternary complexation with AS and loop-complementary trigger RNA, and then transfected into HeLa cells (see Figure S1 in the Supporting Information). The activity of firefly luciferase was reduced to 26% in the presence of the ternary complex, which suggests that the long sequence at the 3′ end of the SS is permissive, and that the complex could serve as a substrate of Dicer to give an active siRNA duplex. Furthermore, AS alone showed almost no silencing activity, which indicates that the observed silencing activity of the ternary complex was indeed due to RNAi by the activated siRNA.

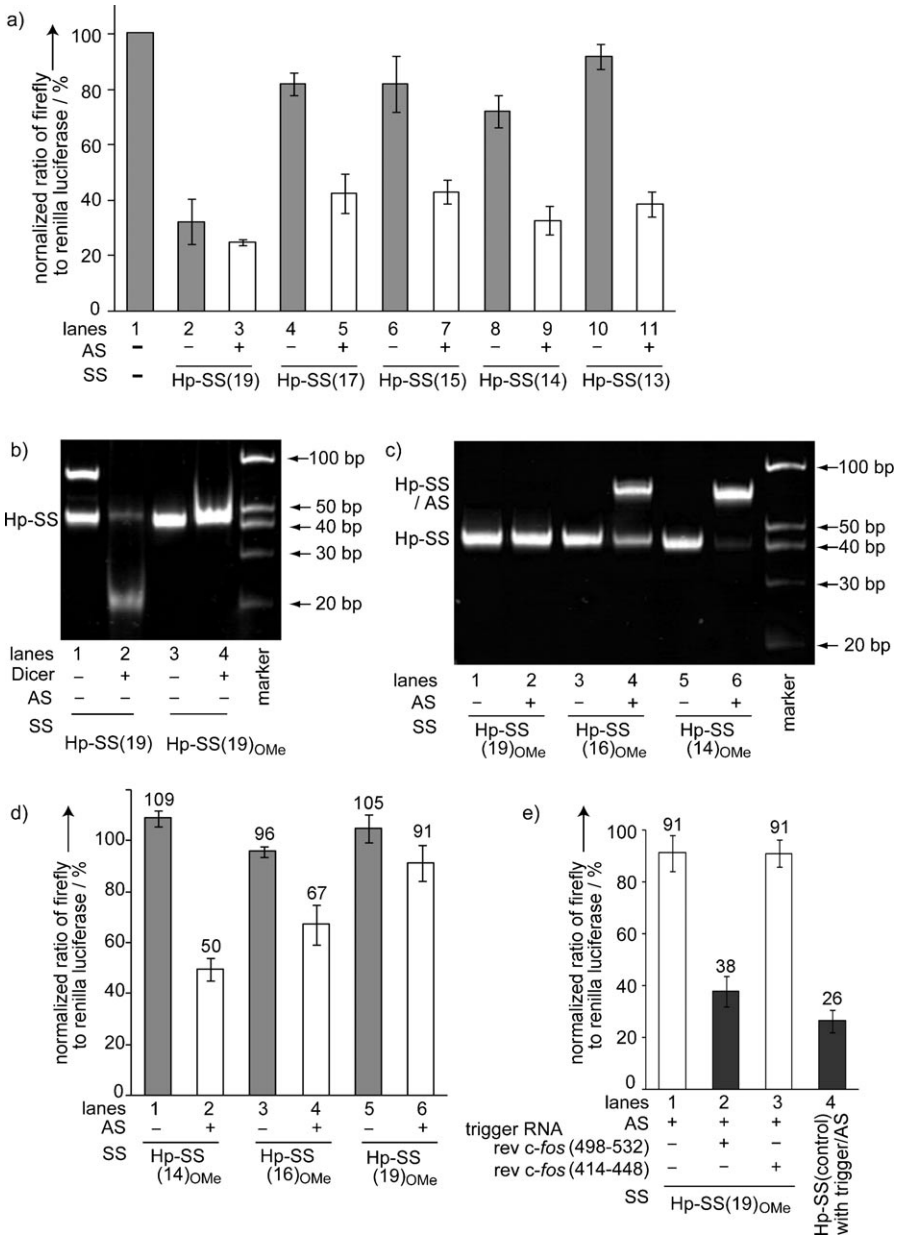

Figure 2. a,d,e) Relative firefly luciferase activity in HeLa cells transfected with pGL3-Control (firefly)/pRL-TK (renilla) vectors in the presence of different combinations of Hp-SS, AS, and trigger RNA molecules. The data for the transfection experiments are averages of the results of at least three independent experiments; error bars show the standard deviation. b) Native PAGE analysis of the Dicer processing of Hp-SS(19) (lanes 1 and 2) and Hp-SS(19)$_{OMe}$ (lanes 3 and 4) in the absence of AS. c) Native PAGE analysis of the hybridization of AS/Hp-SS$_{OMe}$ with different stem lengths.

We next examined the optimal length of the regulatory stem (shown in pink) of the Hp-SS for the suppression of hybridization with the AS. We prepared a series of Hp-SS probes, Hp-SS(13)–Hp-SS(19), with a 13–19 nt regulatory-stem length (Figure 1c). In the absence of the AS, Hp-SS(13)–Hp-SS(17), which have a $\leq$17 nt stem length, showed low RNAi activity (Figure 2a, lanes 4, 6, 8, and 10). However, stems containing 17 or fewer nucleotides were too short to inhibit hybridization of the SS/AS duplex; thus, nonnegligible silencing activity was observed upon the addition of the AS (lanes 5, 7, 9, and 11) without trigger RNA. These results indicated that a longer stem length is indispensable for the prevention of 19-mer SS/AS duplex hybridization in cells. However, Hp-SS(19) itself showed clear RNAi activity even in the absence of the AS (Figure 2a, lane 2), which suggests that Hp-SS(19) with a 19 nt stem could function as a substrate for Dicer (or Drosha) processing.

Facing difficulties in probe design, we explored a new Hp-SS design with an optimized regulatory stem domain which is long enough to prevent SS/AS hybridization without being a substrate for Dicer. Although it might be possible to design

suitable activatable siRNA by using only a natural RNA motif, careful estimations of the structure are essential. To simplify the design strategy, we planned to introduce modified nucleotides into the regulatory stem region. However, modification has so far been mainly to enable and not to inhibit Dicer processing.[8] After exploratory studies, we chose to use 2′-*O*-methyl (2′-OMe) modifications of the regulatory domain.

To clarify the effect of the 2′-OMe modification, the newly designed Hp-SS/AS siRNA probes were subjected to Dicer-processing experiments in vitro. Hp-SS(19) and Hp-SS(19)$_{OMe}$, which contain a natural and fully 2′-OMe modified 19 nt regulatory stem domain, respectively, and a fixed 35 nt recognition loop, were incubated with recombinant human Dicer, and the processing was analyzed by native polyacrylamide gel electrophoresis (PAGE; Figure 2b). Even in the absence of the AS, Hp-SS(19), which exists as both a monomer and a dimer, was processed by Dicer to give approximately 21-mer SS/AS siRNA (lane 2). This result is consistent with the in-cell silencing activity described above (Figure 2a, lane 2). Interestingly, Hp-SS(19)$_{OMe}$, which exists only as the monomer (Figure 2b, lane 3), was completely resistant to processing by Dicer under the same conditions (lane 4), which suggests that full 2′-OMe modification of only one strand of dsRNA can effectively inhibit Dicer processing of both strands. We then optimized the stem length for appropriate inhibition of Hp-SS/AS hybridization. Hp-SS(19)$_{OMe}$, Hp-SS(16)$_{OMe}$, and Hp-SS(14)$_{OMe}$ were incubated with an equal amount of the AS, and the Hp-SS/AS hybridization efficiency was analyzed by native PAGE (Figure 2c). Hp-SS(16)$_{OMe}$ and Hp-SS(14)$_{OMe}$ hybridized with the AS to give a new band (lanes 4 and 6). In marked contrast, Hp-SS(19)$_{OMe}$ remained unhybridized (Figure 2c, lane 2). These results indicate that a stem as long as 19 nt is required to prevent SS/AS hybridization. Indeed, Hp-SS(16)$_{OMe}$ and Hp-SS(14)$_{OMe}$ showed nonnegligible silencing activity in the presence of the AS in HeLa cells (Figure 2d, lanes 1–4).

With a potent 2′-OMe-modified regulatory stem domain in hand, we finally applied the activatable siRNA system to the trigger-RNA-dependent activation of RNAi in cells. As estimated from the results of Dicer processing, Hp-SS(19)$_{OMe}$, either alone or in the presence of the AS, showed almost no silencing activity under our experimental conditions (Figure 2d, lanes 5 and 6). The presence of trigger rev c-*fos* RNA(498–532) increased the silencing efficiency (lane 1 versus lane 2 in Figure 2e).[9] These results suggest that the otherwise inactive Hp-SS(19)$_{OMe}$/AS probe pair was activated upon hybridization with the trigger RNA to turn ON the RNAi. The activated siRNA suppressed the targeted firefly luciferase activity to 38%, a level similar to that observed with the ternary complex Hp-SS(control)/AS/trigger RNA, which was used as a positive control (26%; Figure 2e, lane 4; see also Figure S1, lane 2 in the Supporting Information). When the noncomplementary trigger RNA rev c-*fos*(414–448) (the inverse sequence of the coding region 414–448 of human c-*fos* mRNA) was used instead of the original trigger, no silencing enhancement was observed (Figure 2e, lane 1 versus lane 3). This result showed clearly that the observed activation was highly dependent on the trigger RNA sequence.

In summary, a proof of concept was demonstrated for an approach based on activatable siRNA for turn-ON control of RNAi activity. To our knowledge, turn-ON control of RNAi with an RNA trigger has not been reported previously. This general approach is applicable to any type of trigger RNA and can be adapted to other trigger RNA molecules simply by adjusting the recognition-loop sequence. An endogenous RNA molecule could potentially be used as the trigger, depending on its intracellular concentration. Thus, the present system may have various in-cell (e.g., in-cell gene sensing)[10,11] and pharmaceutical applications. Further studies along these lines are planned.

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