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Intracellular Build-up RNAi with Single-Strand Circular RNAs as siRNA Precursors

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We herein report a new approach for RNA interference, so-called "build-up RNAi" approach, where single-strand circular RNAs with a photocleavable unit or disulfide moiety were used as siRNA precursors. The advantages of using these circular RNA formats for RNAi were presented in aspects of immunogenicity and cellular uptake.

RNA interference (RNAi) suppresses the expression of target genes in a sequence-specific manner[1] and has potential use in therapeutic applications.[2, 3] The 20–23 base pairs of double-stranded natural RNA is generally used for RNAi and is called small interference RNA (siRNA). However, standard siRNAs cause undesired immune responses *in vivo* and exhibit instability and off-target effects. Various RNA molecular designs have been reported to solve these problems and fall under two major strategies: one is chemical modification of nucleic acid monomers[4-6] and the other is the nanostructure design of RNA oligomers. The canonical nanostructure design of RNA is double-stranded RNA (dsRNA) [7] or short hairpin RNA (shRNA) .[8] Conversely, short shRNA (sshRNA),[9] branched RNA,[10-12] small internally segmented interfering RNA (sisiRNA),[13, 14] asymmetric interfering RNA (aiRNA),[15, 16] dumbbell RNA,[17, 18] double stranded circular RNA[19, 20] and caged siRNA[21-25] are non-canonical nanostructure designs of RNA. When introduced into cells, these designed RNA molecules are cleaved by Dicer with a reduction in molecular size and converted into active siRNA species or act directly as active siRNAs. In contrast, we reported the first build-up reaction for RNAi, in which fragmented small RNAs were used to build a larger active siRNA species in cells

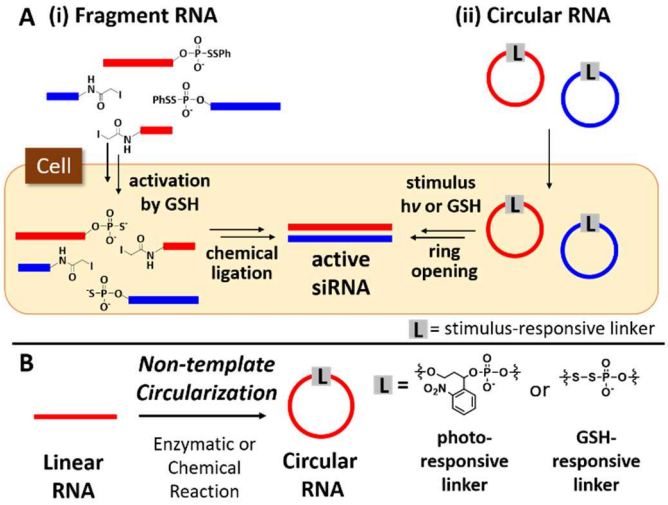

Figure 1. (A) Conceptual scheme of build-up RNA interference using circular RNA precursors. (B) Preparative methods of circular RNAs used in this study.

(Fig. 1A (i)).[26] In this example, linear fragmented RNAs were converted into longer active siRNA species by a chemical ligation reaction triggered by intracellular GSH (glutathione). This build-up process, i.e. the construction of active siRNAs from smaller precursors, is a key concept for improving the properties of siRNAs for medicinal applications. Moreover, a significant reduction of undesired immune responses to siRNA was achieved in this study. Encouraged by this successful embodiment of the build-up concept, we envision that other advantages can be endowed on siRNAs through various molecular designs of siRNA precursors based on the build-up concept. Here, we report a new type of build-up reaction for RNAi assembly, in which small circular precursor RNAs form a larger species of dsRNA in cells (Fig. 1A (ii)). Here, two circular single-stranded RNAs that do not interact with each other are converted into linear RNAs in the cell, and this process is triggered by light irradiation or by a reductive reaction by intracellular GSH. Subsequently, they form double-stranded RNA, namely active siRNA, thereby exhibiting an RNAi effect. Recently, the importance and advantages of circular RNA in

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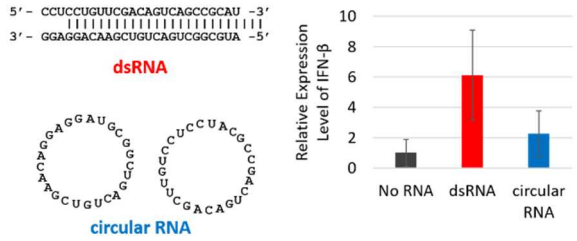

Figure 2. Immunostimulatory effect of dsRNA and circular RNA. The expression level of IFN-β mRNA was measured by quantitative RT-PCR at 24 h posttransfection of RNA (100 nM) in T98G cells. The expression level of IFN-β mRNA was normalized to that of β-actin. The relative expression of IFN-β mRNA in mock-transfected cells was set as 1. Error bars represent the standard deviation of three experiments.

biology and chemical biology have been widely recognized.[27-30] The circular siRNA precursors in this study were easily prepared by a non-template enzymatic or chemical reaction (Fig. 1B).

In starting this study, we examined whether the cyclic structure of RNA can reduce the immune response when compared with that of the corresponding linear structure. Double-stranded RNA of 25 bases and two cyclic RNAs with the same sequence were introduced into T98G cells, and the expression level of IFN-β mRNA, which is an indicator of an immune response, was analyzed using quantitative RT-PCR (Fig. 2). The cyclic structure RNA was found to express less than 50% IFN-β mRNA than the linear structure. Thus, the cyclic structure was expected to avoid stimulation of immune response receptors. Therefore, a cyclic structure as a substrate for a new build-up reaction was chosen.

In build-up reactions using cyclic RNA as a substrate, it is important to design a molecule such that two cyclic RNAs do not form a double strand before the build-up reaction and linear RNAs form dsRNA after the reaction has proceeded. Double strand formation was expected to occur as the molecular size of the circular RNA increased. Thus, cyclic RNAs of 21, 23 and 27 bases with relatively small molecular sizes were designed. Caged circular antisense nucleic acids[31-34] and caged circular siRNAs[19, 35-39] that exert their effect by a light trigger have been reported. Based on these precedents, the *o*-nitrobenzyl alcohol unit was used as a photocleavable linker (Fig. 3A). The RNA sequence was designed to target the firefly luciferase 849–869 regions for the following dual luciferase assays. We prepared three sets of siRNA precursors of different lengths, 21-mer (**21LS**, **21LA**), 23-mer (**23LS**, **23LA**) and 27-mer (**27LS**, **27LA**), using the corresponding commercially available phosphoramidite reagents. Those linear RNAs having a phosphate group at the 5′ terminus were treated with T4 RNA ligase to prepare target circular RNAs (**21CS**, **21CA**, **23CS**, **23CA**, **27CS**, and **27CA**). In order to restrain concatemerization in the circularization reaction, RNA concentration was set to be as low as 1 μM. The target circular RNAs were isolated by preparative denaturing (d)PAGE, achieving isolated yields of 9.1%–15% (Fig. S1, Table S1) Before evaluating RNAi activity, several molecular profiles were evaluated with the circular RNAs. Initially, the duplex-forming ability of the circular RNAs was evaluated by non-denaturing PAGE. The linear or circular RNA was mixed with the corresponding complementary RNA strand and the mixtures were analyzed (Fig. 3B). While the linear pair showed a single band with a significant band shift from that of each RNA fragment, the circular pair showed two discrete bands corresponding to the two RNA fragments. This result suggests that the 21-mer circular RNAs cannot form duplexes with the circular RNAs with complementary sequences. The same tendency was also observed for 23-mer and 27-mer circular RNAs (Fig. S8A). Next, the responsiveness to photoirradiation of the circular RNA was evaluated *in vitro*. After irradiating the RNA solution with 365 nm UV light for a certain period of time, the samples were analyzed by dPAGE (Fig. 3C). As the irradiation time increased, the band representing the circular RNA weakened and the band representing the linear RNA became stronger. The two newly formed bands after UV irradiation should be linear RNA with the nitrosobenzene unit at the 5′-

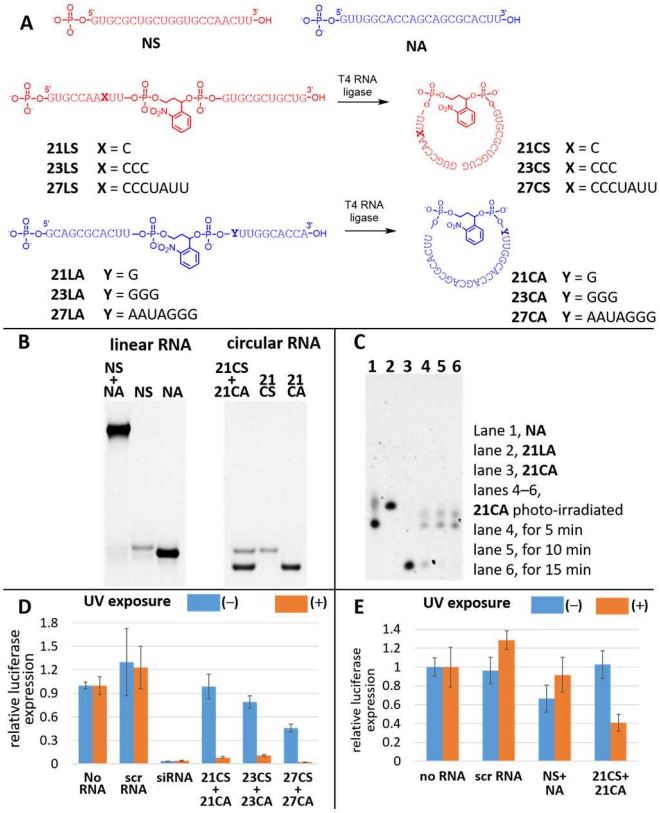

Figure 3. Synthesis and evaluation of circular RNAs with the photocleavable linker. (A) Structures and sequences of normal siRNA strands, circular RNAs with the photocleavable linker and their linear precursors. The circularization reactions were performed by treatment of the linear RNAs with T4 RNA ligase. (B) PAGE analysis of linear and circular RNA pairs of sense and anti-sense strands. Equimolar of sense and anti-sense strands were mixed and analyzed by 20% non-denaturing PAGE. The gels were stained with SYBR Green II. (C) Ring opening reaction by photoirradiation *in vitro*. Lane 1, **NA**; lane 2, **21LA**; lane 3, **21CA**; lanes 4–6, **21CA** photo-irradiated for a certain period of time, lane 4, 5 min; lane 5, 10 min; lane 6, 15 min. The samples were analyzed by 10% denaturing PAGE and the gel was stained with SYBR Green II. (D) Gene silencing effect of siRNA or circular RNAs in dual luciferase assays with or without photoirradiation. The luminescence signals for each sample were normalized to that of the no RNA conditions. Error bars represent the standard deviation of three experiments. (E) Gene silencing effect of siRNA or circular RNAs in dual luciferase assays without using lipofection. The luminescence signals for each sample were normalized to that of the no RNA conditions. Significant difference between **NA+NA** and **21CS+21CA** under UV-irradiation was confirmed by T-test ($P < 0.05$). Error bars represent the standard deviation of three experiments. scr RNA: scramble dsRNA.

phosphate terminus and linear RNA without the nitrosobenzene unit, which can be removed through the β-elimination reaction. The results suggest that circular RNAs were converted efficiently to linear RNAs by UV irradiation, which should be suitable for application in cells. The weaker band intensity after the UV-irradiation suggested that the RNAs might be partially degraded under this *in vitro* condition. After confirming these basic molecular profiles of the siRNA precursors, RNAi activity was evaluated. The dual luciferase assay was performed using HeLa cells administrated with both firefly and renilla luciferase vectors. RNA samples were added by lipofection and the relative luciferase activity (firefly/renilla) was evaluated (Fig. 3D). Four hours after RNA administration UV irradiation was performed and the luciferase activity was evaluated 20 h after the irradiation. For normal siRNAs (**NS** + **NA**), the silencing effect was more than 90%, independent of the UV irradiation. In the case of scramble RNA, no silencing effect was observed either with or without UV irradiation. For the 21-mer siRNA precursors (**21CS** + **21CA**), the silencing effect was highly dependent on UV irradiation; almost no silencing effect was observed when no UV irradiation was used, whereas a silencing effect comparable to that of normal siRNA was observed upon UV irradiation. The gene silencing activity of linear 23-mer and 27-mer siRNAs was confirmed (Fig. S8B). The switching of activity by photoirradiation did not work as effectively as the ring size increased. In the case of the 27-mer circular siRNA precursors (**27CS** + **27CA**), 54% silencing was observed without UV-irradiation. This may arise from the larger ring size of these circular RNAs being more susceptible to endonucleases, and thus were more prone to conversion to the linear RNA form, which caused irradiation-independent RNAi activity. There was also clear concentration-dependency for gene-silencing effects (Fig. S3). No gene silencing effect was observed when only the circular antisense strand was used (Fig. S3), which deny the possibility that the observed silencing effect was due to antisense mechanism. Additionally, this method was applicable to endogenous gene ApoB, [40] and the irradiation-dependent gene silencing effect was observed with reasonable concentration dependency (Figs. S4, S5, Table S3). Next, the dual luciferase assay was performed without using lipofection to evaluate the cellular uptake of the siRNA precursors. Here, HeLa cells were treated with RNA samples in a hypertonic solution and then water was added to the medium (Fig. 3E). [41, 42] Normal siRNA showed a 9%–34% silencing effect regardless of UV-irradiation, whereas the siRNA precursors showed a stronger silencing effect (60%) only with UV-irradiation. This higher RNAi activity could be attributed to the higher cellular uptake of circular siRNA precursors when compared with that of normal siRNA.

If the build-up reaction proceeds under stimulation with intracellular factors, it is more practical for therapeutic techniques. Therefore, GSH abundantly present in cells was selected as the intracellular stimulus and the phosphoryldithio linkage was selected as the GSH-responsive unit (Fig. 4A).

The RNA sequence was the same as that of the previous UV-mediated build-up siRNA. Linear RNA with an alkylthiol group at the 3'-terminus and a phosphorothioate group at the 5'-terminus was synthesized by an automatic synthesizer (**PS-LS**, **PS-LA**). After deprotection and cleavage from the CPG, RNAs were obtained with their 3′ termini protected as the disulfide form. The obtained RNAs were first treated with dithiothreitol (DTT) to cleave the disulfide bond, then with 2,2′-dithiodipyridine to achieve circularization. Target circular RNA (**PS-CS**, **PS-CA**) was isolated by dPAGE and subsequent gel extraction, achieving isolated yields around 7% (Fig. S2, Table S2). The disulfide-mediated circular RNA was treated with 10 mM DTT or GSH for 30 min and the reaction mixture was analyzed by dPAGE (Fig. 4B). By treatment with these thiol-based reductants, the circular RNA was converted to the corresponding linear RNA. Since the intracellular GSH concentration is typically 1–10 mM, this result suggests that the disulfide-based circular RNA can undergo the ring opening reaction efficiently in cells. After confirming the key properties of the siRNA precursors, siRNA activity was evaluated by the dual luciferase assays. When the RNA samples were administrated by lipofection, almost comparable silencing effects to that of normal siRNA were observed (Fig. 4C). The dual luciferase assays without lipofection were also performed (Fig. 4D). The superior silencing effect was also observed for circular siRNA precursors (50%) when compared with that of normal siRNA (43%). In both cases, the RNAi activity was independent of the external stimulus, demonstrating that this methodology should be suitable for therapeutic applications. In addition to luciferase assay system, endogenous ApoB-targeting system was evaluated (Figs. S6, S7), where comparable gene silencing effect was observed between

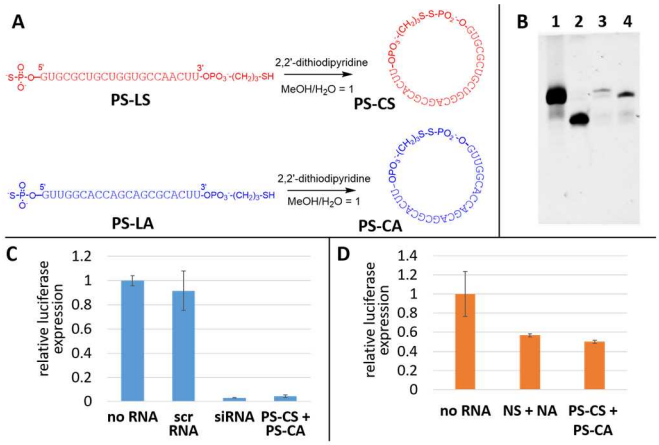

Figure 4. Synthesis and evaluation of disulfide circular RNAs. (A) Structures and sequences of disulfide circular RNAs and their linear precursors. The circularization reactions were performed by treatment with linear RNAs and 2,2'-dithiodipyridine. (B) Ring opening reaction by treatment with 10 mM GSH or DTT *in vitro*. Lane 1, **PS-LA**; lane 2, **PS-CA**; lanes 3–4; **PS-CA** treated with reductant: lane 3, treatment with 10 mM GSH; lane 4, treatment with 10 mM DTT. The samples were analyzed by 10% denaturing PAGE and the gel was stained with SYBR Green II. (C) Gene silencing effect of siRNA or circular RNAs in the dual luciferase assays. The luminescence signals for each sample were normalized to that of the no RNA conditions. Error bars represent the standard deviation of three experiments. (D) Gene silencing effect of siRNA or circular RNAs in the dual luciferase assays without using lipofection. The luminescence signals for each sample were normalized to that of the no RNA conditions. Significant difference between **NA+NA** and **PS-CS+PS-CA** was confirmed by T-test ($P < 0.05$). Error bars represent the standard deviation of three experiments. scr RNA: scramble dsRNA.

normal siRNAs and the disulfide circular RNA pairs. Importantly, the ApoB mRNA cleavage through RNA interference mechanism was confirmed by 5'-RACE experiments (Figs. S7C, S7D). It was also confirmed that the circular RNAs with photocleavable and disulfide linker had lower immunogenicity compared with normal siRNAs (Fig. S9).

In summary, we have developed a new build-up RNAi approach using circular siRNA precursors with a photocleavable unit or a disulfide moiety. These precursors can be converted efficiently to the corresponding linear RNAs by UV-irradiation or a high concentration of GSH or DTT *in vitro*. In dual-luciferase assays, the circular siRNAs showed comparable RNAi activity to that of normal siRNA, indicating that these precursor RNAs can be converted into active siRNA or its equivalent in cells in response to an external stimulus (photoirradiation) or the intracellular environment (GSH). When administrated to the cells without lipofection, the circular RNAs showed higher RNAi activity than normal siRNAs, suggesting that the circular RNAs had higher cellular uptake than normal siRNAs. The new RNAi method was also successfully applied to endogenous ApoB gene, demonstrating the versatility of the methodology. Based on these advantageous points of circular RNA precursors for build-up RNAi, a study for further improving the molecular design to yield better molecular properties and function for *in vivo* applications is underway.

Conflicts of interest

There are no conflicts to declare.

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