

DESCRIPTION

SELF-CIRCULARIZED RNA STRUCTURE

Technical Field

The following description relates to self-circularization RNA constructs.

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Background Art

Circular RNAs (circRNAs) are single-stranded transcripts covalently linked. Through RNA-seq data and a newly developed bioinformatics approach, more than tens of thousands of circRNA types have been identified in various living organisms. In eukaryotes, it is known that a circRNA may be generated through back-splicing from mRNA and may regulate gene expression by performing a microRNA sponge function *in vivo*. It is not known whether a circRNA induces immunogenicity, and it exists very stably *in vivo* due to its structural characteristics.

Recently, the development of therapeutic agents using messenger RNA (mRNA) is active. However, mRNA has a limitation in that it is easily degraded *in vivo* and has a relatively short half-life. In order to overcome this limitation, studies such as attaching poly(A)tail to mRNA to improve stability are in progress. In the same vein, US 10,953,033 discloses a circRNA for the purpose of gene expression *in vivo* based on the structural characteristics of the circRNA.

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Disclosure of the Invention

Technical Goals

A technical task of the present disclosure is to provide an RNA construct that is circularized by performing a self-targeting and splicing reaction.

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However, the technical task of the present disclosure is not limited to that mentioned above, and other tasks not mentioned herein will be clearly understood by those skilled in the pertinent technical field from the following description.

30 Technical Solutions

According to an aspect of the present disclosure, there is provided a self-circularization RNA construct having the following structure:

5' - IGS (internal guide sequence) - ribozyme - gene of interest - target site - 3'.

In one example embodiment of the present disclosure, the IGS region and the target site form a guanine (G) : uracil (U) wobble base pair, in which the guanine forming the wobble base pair may be located at the 5' end of the IGS region, and the
 5 uracil forming the wobble base pair may be located at the 3' end of a target site region.

In another example embodiment of the present disclosure, the IGS region may include or consist of a nucleotide sequence represented by 5'-GNNNNN-3', and the target site region may include or consist of a nucleotide sequence represented by 5'-
 10 N'N'N'N'N'U-3'. N of the IGS region and N' of the target site region may each independently be A, G, C, or U, but preferably, at least one nucleotide may be a nucleotide capable of complementary binding between the IGS region and the target site region.

In yet another example embodiment of the present disclosure, a nucleotide sequence of the IGS region may be reverse complementary to a nucleotide sequence of
 15 the target site region except for the guanine.

In another example embodiment of the present disclosure, the ribozyme may be a Group I intron ribozyme, and the ribozyme may include or consist of a nucleotide sequence represented by SEQ ID NO: 6.

In yet another example embodiment of the present disclosure, the construct may
 20 include a nucleotide extending in a 5' direction of the IGS region to form a P1 helix and a P10 helix. In this connection, the P1 helix may be formed in a region in which the complementary binding between the IGS region and the target site is formed together with a nucleotide extending in a 3' direction of the target site, and the P10 helix may be formed in a region in which the nucleotide extending in the 5' direction of the IGS
 25 region forms complementary binding to a sequence reverse complementary to the extended nucleotide located between ribozyme and a GOI region. The length of the extended nucleotide forming the P1 helix may be 3-nt, and the length of the extended nucleotide forming the P10 helix may be 6-nt.

In yet another example embodiment of the present disclosure, the construct may
 30 form a P1 helix but not a P10 helix.

In yet another example embodiment of the present disclosure, the construct may include an antisense sequence (AS) region and an antisense binding sequence (ABS)

region capable of complementary binding to each other at the 5' end and the 3' end.

In yet another example embodiment of the present disclosure, the ABS region may consist of a sequence reverse complementary to the AS region.

In yet another example embodiment of the present disclosure, the length of the AS region may vary depending on GOI, but may be 10-nt to 500-nt, preferably more than 50-nt and less than 400-nt, and more preferably 150-nt to 350-nt.

In yet another example embodiment of the present disclosure, the GOI region may include an internal ribosome entry site (IRES) region at the 5' end, and may include an initiation codon and a termination codon.

In yet another example embodiment of the present disclosure, the construct may further include a spacer region including a random nucleotide sequence, in which the spacer region may be located between the IGS region and the gene of interest region and/or between the gene of interest region and the target site region.

In yet another example embodiment of the present disclosure, the spacer region may include or consist of poly(A), and the poly(A) is a polynucleotide in which adenine (A) is repeatedly linked, in which the A may be linked repeatedly 10 to 50 times, and preferably linked repeatedly 30 times.

Advantageous Effects

The self-circularization RNA construct of an example embodiment of the present disclosure can be expressed in a DNA vector and simultaneously circularized through a self-targeting & splicing reaction without a separate GTP treatment to form a circRNA, and the circRNA can consist only of a gene of interest. The gene of interest has the advantage of being able to rapidly express a peptide or protein, including an IRES region, an initiation codon, and a termination codon. In addition, a circRNA has a circular structure and has a stable and high half-life because 5' and 3' ends are not exposed. Accordingly, functional RNA such as miRNA, anti-miRNA, shRNA, aptamer, mRNA vaccine, mRNA therapeutic agent, antibody, vaccine adjuvant, and CAR-T mRNA can be produced as a circRNA to have high stability in cells.

Brief Description of Drawings

FIGS. 1A and 1B are schematic diagrams of a process in which a self-

circularization RNA construct of an example embodiment of the present disclosure including only IGS, ribozyme, gene of interest, and target site region is formed into a circRNA by a self-targeting and splicing (STS) reaction.

FIG. 2A is a schematic diagram of a process in which a self-circularization RNA construct of an example embodiment of the present disclosure including AS, IGS, ribozyme, gene of interest, target site, and ABS region is formed into a circRNA by an STS reaction.

FIG. 2B is a schematic diagram of a self-circularization RNA construct of an example embodiment of the present disclosure including IRES and a termination codon in the gene of interest region to enable transgene translation, and further including nucleotides extending in a 5' direction to form P1 helix and P10 helix, and a process in which the construct is formed into a circRNA by an STS reaction.

FIG. 2C shows one form of a self-circularization RNA construct of an example embodiment of the present disclosure.

FIG. 2D shows a DNA template nucleotide sequence for producing a self-circularization RNA construct expression vector of an example embodiment of the present disclosure.

FIG. 3 shows the results of electrophoresis on a polyacrylamide gel to identify that a circRNA is generated immediately after *in vitro* transcription of the self-circularization RNA construct expression vector of an example embodiment of the present disclosure, without additional GTP treatment. Samples obtained immediately after transcription (direct STS) and samples after the first and second STS reaction stages in which additional GTP is treated to induce primary and secondary circularization reactions were used, and a portion of each sample was treated with RNase R to remove linear RNA, and a circRNA in the samples was concentrated and electrophoresed.

FIG. 4 is a verification that Candidate 1, which is assumed as a circRNA, is a circRNA from the results of FIG. 3. FIG. 4A shows a result of extracting RNA from the candidate 1 band and performing RT-PCR. FIG. 4B shows a nucleotide sequence analysis result of RNA extracted from the candidate 1 band.

FIG. 5 is a verification that the RNA of the candidate 1 band from the results of FIG. 4 is a monomer, and shows the results of electrophoresis after extracting RNA

from candidate 1 and 2 bands assumed as circRNAs from the results of FIG. 3 and inducing nick by treatment with Mg^{2+} .

FIG. 6 shows a result of identifying that the self-circularization RNA construct expression vector of an example embodiment of the present disclosure generates a circRNA in cells and the transgene included in the circRNA is expressed. Specifically, FIG. 6A shows a structure of the self-circularization RNA construct expression vector, FIG. 6B shows a result of identifying the expression of transgene through luciferase activity assay, and FIGS. 6C and 6D show RT-PCR and nucleotide sequence analysis results verifying that the transgene is expressed in a circRNA.

FIGS. 7 and 8 show circRNA purification conditions and purification results through HPLC.

Specifically, FIG. 7A shows HPLC analysis conditions using an Ultra HPLC system, FIG. 7B shows a result of column purification of a sample obtained immediately after *in vitro* transcription, and FIG. 7C shows a result of column purification after treating the sample with RNase R. In addition, FIG. 8A shows a result of identifying a peak in the fraction obtained through column purification, and FIG. 8B shows an electrophoresis result of fractions 7, 10, 11, 12, and 13 in which peaks are prominent in FIG. 8A.

FIGS. 9 and 10 identify the effect of an AS region on STS reaction and circularization in a self-circularization RNA construct of an example embodiment of the present disclosure. FIG. 9 shows a structure of a self-circularization RNA including AS regions of each different length. FIG. 10 shows an electrophoresis result of a sample obtained after *in vitro* transcription of a vector expressing the RNA.

FIGS. 11 and 12 identify the effect of a spacer region on STS reaction and circularization in a self-circularization RNA construct of an example embodiment of the present disclosure. FIG. 11 shows a structure of self-circularization RNA including a spacer of a control group (control spacer) and poly(A) spacers of various lengths. FIG. 12 shows an electrophoresis result of a sample obtained after *in vitro* transcription of a vector expressing the RNA.

FIG. 13 indicates a nucleotide sequence near a region cleaved by a ribozyme and each region of: a self-circularization RNA including only a P1 region; self-circularization RNA including only P1 and P10 regions; and a self-circularization RNA

including a P1 region, a P10 region, and an AS region.

FIG. 14 shows a nucleotide sequence of a DNA template for preparing a self-circularization RNA expression vector including only a P1 region without P10 and AS regions.

5 FIG. 15 shows an electrophoresis result of a sample obtained after *in vitro* transcription of each self-circularization RNA expression vector of FIG. 13.

FIGS. 16A and 16B show the results identified through electrophoresis that a self-circularization RNA construct is formed into a circRNA after *in vitro* transcription in a self-circularization RNA expression vector including only a P1 region without P10
10 and AS regions.

FIGS. 17 to 19 identify the effect of a nucleotide sequence of a P1 region on STS reaction and circularization in a self-circularization RNA construct of an example embodiment of the present disclosure. Specifically, FIG. 17 shows a design of the P1 region of each different nucleotide sequence, FIG. 18 shows an electrophoresis result of
15 a sample obtained after *in vitro* transcription of a self-circularization RNA expression vector having the P1 region of FIG. 17, and FIG. 19 shows an electrophoresis result of a sample obtained after *in vitro* transcription of a self-circularization RNA expression vector having an AU-rich P1 region and a self-circularization RNA expression vector having 2 sites for P1 formation, and shows an RT-PCR electrophoresis result identifying
20 circular RNA.

FIG. 20 is a succinct diagram of only the essential configuration of the self-circularization RNA construct. FIG. 20A shows that circRNA preparation is possible only with an RNA construct including only IGS, ribozyme and GOI region, and uracil base at the 3' end. FIG. 20B is a schematic diagram showing that circRNA preparation
25 is possible only with IGS, ribozyme, and RNA constructs of the GOI region when the uracil base is included at the 3' end of a GOI, in which the generated circRNA consists only of the GOI. In other words, FIG. 20B is a schematic diagram showing that when uracil is included after 5 unique nucleotide sequences at any site in the GOI, with that part as the 3' end, the rest of the 3' end side of the GOI is sent right behind the ribozyme,
30 so that circular RNA is configured only of the GOI without additional uracil.

Best Mode for Carrying Out the Invention

The present inventors used a trans-splicing ribozyme (T/S ribozyme) to prepare a circRNA, and devised a system in which the RNA containing a gene of interest performs a self-targeting and splicing reaction to be circularized (hereinafter referred to as “circularization system by self-targeting & splicing reaction”) (FIGS. 1A and 1B).

5 Group I intron ribozyme may induce trans-splicing by cleaving the target RNA through two successive trans-esterification reactions and then linking separate transcripts to each other at the cleaved 3' end.

 Accordingly, in the system of an example embodiment of the present disclosure, an internal guide sequence (IGS) is configured in the 5' direction of a gene of interest
10 (GOI) and a target site is configured in the 3' direction so that a circRNA may be produced by inducing cleavage and splicing by the ribozyme located between GOI and IGS by forming a guanine (G) : uracil (U) wobble base pair so that the IGS binds complementary to the target site (FIGS. 2A and 2B).

 The present inventors focused on the previous study (Mol Ther. 2005 Nov;
15 12(5): 824-34.) for improving the trans-splicing efficiency of Group I intron ribozyme, and as diagrammed in FIG. 2C, designed a self-circularization RNA construct such that an AS (antisense sequence) region and ABS (antisense binding sequence) capable of complementary binding to each other exist at the 5' and 3' ends of the self-circularization RNA construct, and P1 and P10 helixes are formed before/after the
20 secondary structure of the ribozyme, and prepared a DNA template capable of expressing the RNA construct under a T7 promoter (Example 1).

 Then, a vector into which the DNA template was inserted was produced and transcribed *in vitro* (IVT), and then the formation of a circRNA was identified. As a result, it was found that the vector expresses a self-circularization RNA construct, and
25 the RNA construct forms a monomeric circRNA through a self-targeting and splicing (STS) reaction immediately after transcription even without the addition of GTP (Example 2).

 Furthermore, the present inventors tried to identify whether the self-circularization RNA construct expression vector may express the RNA construct of an
30 example embodiment of the present disclosure even in cells, and whether the expressed RNA may express a gene of interest contained in the form of a circRNA. Specifically, a plasmid vector was produced by including IRES and a termination codon in a gene of

interest to enable translation of the gene (transgene) in the cells and using gaussia luciferase as a transgene in the gene of interest, and the plasmid vector was transformed into cells to identify the production of a circRNA and luciferase activity. As a result, it was identified at the molecular level that the produced plasmid vector expressed the self-circularization RNA construct in the cells, the construct was circularized into a circRNA by ribozyme, and the gene of interest was expressed in the circRNA (Example 3).

Subsequently, the present inventors attempted to optimize an RNA construct to efficiently form a circRNA through an STS (direct STS) reaction immediately on IVT.

First, the direct STS reaction rate according to a length of an AS region on IVT was identified through a specific experiment. Specifically, a self-circularization RNA expression vector was produced so that the lengths of the AS region and the ABS region were 50, 100, 150, 200, 250, or 300-nt, and the direct STS efficiency was identified after the vector was subjected to IVT. As a result, it was identified that the self-circularization efficiency was remarkably reduced at a length of 200-nt or more. Although the self-circularization efficiency was similar at 50, 100, and 150-nt lengths, it was identified that the *in vitro* transcription reaction itself decreased when the AS region and the ABS region of a 50-nt or 100-nt length are included. In terms of circRNA production efficiency, it was identified that the lengths of the AS region and the ABS region were preferably 150-nt (Example 5).

Similar to the identification of the circRNA production efficiency according to the lengths of the AS region and the ABS region, the circRNA production efficiency according to the length and nucleotide sequence of a spacer region on IVT was identified. As a result, it was found that although its length and nucleotide sequence did not significantly affect the circRNA production efficiency, the linking of 30 adenines (A) was sufficient not to create a structural conflict that may occur due to a narrow gap between ribozyme and IRES in the case of the ribozyme and the EMCV IRES (Example 6).

In an example embodiment of the present disclosure, the ribozyme was used as a Group I intron ribozyme capable of a continuous trans-esterification reaction. Group I intron ribozymes induce trans-splicing by linking separately existing transcripts to each other at the cleaved 3' end after cleavage of the target site, and link the 5' site of a

GOI to the cleaved 3' end, not a separate transcript in a self-circularization RNA construct. It was intended to identify whether the P1 and P10 helix regions, known to increase trans-splicing efficiency, and the AS region and ABS region at both ends of the self-circularization RNA construct also had a positive effect on circularization efficiency.

5 Specifically, the present inventors produced a self-circularization RNA expression vector including only a P1 helix region, only P1 and P10 helix regions, or both the P1 and P10 helix and AS regions, and identified direct STS efficiency after IVT of the vector. As a result, surprisingly, it was found that the P10 helix region in the self-circularization RNA construct reduced the circRNA production efficiency in the
10 presence of the P1 helix and P10 helix regions. Excellent circRNA production efficiency was exhibited even when only the P1 helix region was included without the AS region (Example 7).

Furthermore, the present inventors designed a self-circularization RNA construct so that only IGS forms a P1 helix so that only a gene of interest may be left in
15 the final product, circRNA, and identified whether an STS reaction occurs in various IGS sequences. As a result, surprisingly, even when only the IGS region formed the P1 helix, it was identified that the STS reaction of the self-circularization RNA construct expressed in DNA was induced, and furthermore, the circRNA was formed even though the IGS region and the target site region were not complementary to each
20 other. However, when the IGS region and the target site region are not complementary to each other, a non-specific reaction may occur at an undesired site, thereby generating an unwanted product. The IGS region of 5'-GNNNNN-3' and the target site region of 5'-N'N'N'N'N'U-3' showed that the circRNA production efficiency increased as the ratio of nucleotide sequences capable of complementary binding to each other increased, and
25 that the specific sequence exhibited higher circRNA production efficiency (Example 8).

From the above, the present inventors identified that it was possible to obtain a circRNA through an STS reaction only with the RNA construct diagrammed in FIG. 1A, and the self-circularization RNA construct diagrammed in FIG. 20A may form a circRNA configured only of a gene of interest including a U base at the GOI 3' end in
30 the circRNA precursor as shown in the schematic diagram of FIG. 20B.

In the P1 helix, when the secondary structure of the Group I intron ribozyme is formed, the nucleotide sequence linked to the front end (5' direction) of the ribozyme

and the nucleotide sequence in the 3' direction of the transcript in which conjugation is induced by the ribozyme refer to a helix structure formed through a complementary bond, and the P10 helix refers to a helix structure formed through a complementary bond between a front end region of the ribozyme and a nucleotide sequence in the 5' direction of the transcript cleaved by the ribozyme.

In an example embodiment of the present disclosure, the P1 helix may be formed through a complementary bond between the IGS at the 5' end and the target site at the 3' end in the self-circularization RNA construct of an example embodiment of the present disclosure, and the P10 helix refers to a helix structure formed through a complementary bond between a nucleotide sequence extended at the 5' end and a nucleotide sequence linked to the rear end (3' direction) of the ribozyme.

As used herein, the nucleotide sequence forming the P1 helix is referred to as a P1 region or P1 helix region, and the nucleotide sequence forming the P10 helix is also referred to as a P10 region or P10 helix region.

In an example embodiment of the present disclosure, the nucleotide sequence of the IGS region is 5'-GNNNNN-3', the nucleotide sequence of the target site region is 5'-N'N'N'N'U-3', and the P1 helix may be formed by complementarily binding the IGS region and the target site region to each other. In this connection, in order to exclude overlapping expression, the description of the P1 helix region may be used in combination with the nucleotide sequence of the IGS region.

In an example embodiment of the present disclosure, the P1 helix may be formed including the nucleotide sequence extended in the 5' direction of the IGS region, and the nucleotide sequence of the naturally extended nucleotide sequence and the reverse complementary sequence is extended in the 3' direction of the target site to configure the P1 helix. In this connection, when the extended nucleotide sequence exists, as used herein, the extended nucleotide sequence region is denoted as P1 to distinguish the extended nucleotide sequence from the IGS region. The same goes for the P10 helix.

In an example embodiment of the present disclosure, the target site region is intended to represent a nucleotide sequence that binds complementary to the IGS region, and may overlap with the gene of interest (GOI) region according to a nucleotide sequence design of the IGS region. In this connection, in order to specify the region

that binds complementary to the IGS region, the region that binds complementary to the IGS region in the gene of interest is classified and named as a target site. In other words, in an example embodiment of the present disclosure, the target site may overlap part or all of the gene of interest region, or may exist separately from the gene of interest.

In an example embodiment of the present disclosure, the AS (antisense sequence) region is located at the 5' end of the self-circularization RNA construct and aims at hydrogen bonding with the nucleotide sequence of the ABS (antisense binding sequence) region located at the 3' end of the RNA construct. In an example embodiment of the present disclosure, the AS region essentially coexists with the ABS region, and the absence of the AS region is understood to include the absence of the ABS region, and likewise, the RNA construct including the AS region is understood to also include the ABS region.

As a result of identifying the effect of the presence or absence of three types of configurations of the P1 region, P10 region, and AS region on the circularization efficiency by the STS reaction of the self-circularization RNA construct, the present inventors identified that an amount of circRNA production was large in the order of when both the P1 and P10 regions and the AS region were included, when only the P1 region was included, and when only the P1 and P10 regions were included. It was identified that the self-circularization RNA construct in the case of including only the P1 region was also circularized with sufficient efficiency to generate a circRNA. Accordingly, an example embodiment of the present disclosure provides a self-circularization RNA construct in the case of including only the P1 region.

Accordingly, as used herein, the self-circularization RNA construct including only the P1 region means that the P10 region and the AS region do not exist, and is not used in the meaning of excluding other configurations. Similarly, the self-circularization RNA construct including only the P1 region and the AS region means that the P10 region does not exist and is not used in the sense of excluding other configurations other than the P10 region.

Mode for Carrying Out the Invention

Hereinafter, example embodiments will be described in detail with reference to

the accompanying drawings. However, since various changes may be made to the example embodiments, the scope of right of the patent application is not limited or restricted by these example embodiments. It should be understood that all modifications, equivalents and substitutes for the example embodiments are included within the scope of right.

The terms used in the example embodiments are used for description purposes only, and should not be construed as being limited by these example embodiments. The terms in singular form may include plural forms unless otherwise specified. It will be understood that the terms “comprising” or “having,” when used herein, specify the presence of stated features, integers, steps, operations, elements, components, or combinations thereof, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, or combinations thereof.

Unless otherwise defined, all terms used herein, including technical or scientific terms, have the same meanings as commonly understood by a skilled person in the technical field to which the example embodiments belong. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meanings of the context of the relevant art, and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

In addition, in the description with reference to the accompanying drawings, the same reference numerals are assigned to the same components regardless of the reference numerals, and redundant descriptions thereof will be omitted. In describing the example embodiments, when it is determined that a detailed description of related known technologies may unnecessarily obscure the gist of the example embodiments, the detailed description thereof will be omitted.

[Examples]

Example 1. Design of Self-circularization RNA and Production of RNA Expression Vector

The self-circularization RNA was designed as shown in FIG. 2C, and a T7 promoter sequence was additionally included for *in vitro* transcription reaction in the DNA template for expressing the same (FIG. 2D). The DNA template was amplified

by PCR using T7 Circular Forward primer: 5'-GGGATTCTGAACATCGATTAATACGACTCACTATAGGGGCATCGATTGAATTGT CGA-3' ($T_m = 77.5^\circ\text{C}$) and T7 Circular Reverse primer: 5'-AGATCTCTCGAGCAGCGCTGCTCGAGGCAAGCTT-3' ($T_m = 79.4^\circ\text{C}$). The DNA
 5 template amplification product was inserted into the pTOP TA V2 cloning vector (Enzymonomics) using PstI restriction enzyme to produce a self-circularization RNA expression vector.

Example 2. Identification of *In Vitro* Transcription and Self-Circularization

10 2-1. *In Vitro* Transcription (IVT)

The self-circularization RNA expression vector produced in Example 1 above was transcribed *in vitro* using NEB's HiScribe T7 high yield RNA synthesis kit according to the manufacturer's protocol. Specifically, after 3 hours of reaction at 37°C with 20 uL scale (1 ug T7 DNA template, 1 X Reaction buffer, 10 mM each ATP,
 15 UTP, CTP, GTP, T7 RNA polymerase mix 2 ul), 29 uL of nuclease-free water was added, and then 1 uL of RNase-free DNase I (10 U/uL) was added and reacted at 37°C for 30 minutes to induce an immediate circularization (direct STS) reaction after transcription.

Subsequently, an additional circularization reaction was induced to identify the stage in which the self-circularization reaction was completed. Specifically, in order to
 20 induce the first self-targeting and splicing (1st STS) reaction, 28 uL of nuclease-free water and 20 uL of 5X STS buffer (50 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM MgCl₂), 2 uL of 100 mM GTP (final 2 mM) were further added to make a volume of 100 uL, followed by self-circularization reaction at 37°C for 1 hour. Then, after heating at 55°C for 15 minutes, column purification was performed using a Monarch
 25 RNA cleanup kit (NEB). A second STS (2nd STS) reaction was induced by adding 20 uL of 5X STS buffer, 100 mM GTP (final 2 mM), and 28 uL of nuclease-free water to 50 uL of the column-purified sample again to make a final 100 uL and performing a reaction at 37°C for 3 hours. After the reaction, heating was performed at 55°C for 8 minutes, then column purification was performed using a Monarch RNA cleanup kit
 30 (NEB), and the concentration was measured using Nanodrop (Thermo Fisher Scientific product) equipment.

In order to remove linear RNA from the reaction product, some of the column-

purified samples after the first and second STS reactions were treated with RNase R. Specifically, 10 uL of 10X RNase R reaction buffer (10X: 0.2 M Tris-HCl pH 8.0, 1 M KCl, 1 mM MgCl₂) and RNase R 20 Unit were added to column-purified IVT RNA of up to 100 ug (adjusting the volume to 100 uL with water) at 37°C for 30 minutes, and then RNase R 10 unit was further added for performing a reaction for another 30 minutes. Then, column purification was performed using a Monarch RNA cleanup kit (NEB) and a concentration was measured with Nanodrop.

250 ng each of the samples treated with RNase R on RNA obtained from each STS stage and RNA obtained from each stage was mixed 1 : 1 with 10 M Urea-BPB (1X TBE) dye, heated at 75°C for 5 minutes, and then 4% polyacrylamide-7 M urea denature PAGE (2 hours of electrophoresis at 50 W conditions while maintaining a temperature at 50°C) was performed, and the gel was stained with an SYBR Gold Nucleic Acid Stain product (Thermo Fisher Scientific) and analyzed using ImageQuant 800 (Cytiva product).

As a result, as can be seen in FIG. 3, when RNase R was treated, RNA bands that were not well cleaved by RNase R and thus were enriched were revealed (Candidate 1). In addition, bands that were clearly observable despite RNase R treatment were identified (bands presumed to be Candidate 2 and Nicked circular RNA). In addition, it was already identified that even without additional 1st and 2nd STS reactions, substances presumed to be circRNAs were sufficiently prepared only by a direct STS reaction, that is, an *in vitro* transcription reaction.

2-2. Identification of Self-Circularization

Subsequently, RT-PCR sequencing was performed to verify that Candidate 1 was a circRNA among the RNA bands not cleaved by RNase R from the PAGE results. Specifically, RT-PCR was performed using primers capable of PCR amplification only when circRNAs were made using a circular RNA sample purified by ethanol precipitation by cutting and crushing the band at Candidate 1 position in the PAGE and eluting the band in water at 37°C for 3 to 16 hours and a linear RNA that does not allow circularization due to the absence of a ribozyme site and an antisense site as controls.

For reverse transcription (RT), OneScript Plus RTase (Abm) was used, and 125 ng of each RNA (a sample with or without RNase R treatment on RNAs presumed to be

control RNA and circular RNA) was heated at 70°C for 5 minutes. Thereafter, the sample was put on ice, and was added with 5X RT buffer 4 uL, 10 mM dNTP mix 1 uL, 2 uM reverse primer (Circular STS R) 1 uL, and OneScript Plus RTase 200 U in an orderly manner. Then, the sample was reacted at 50°C for 15 minutes in a final
 5 volume of 20 uL, heated at 95°C for 5 minutes to inactivate the enzyme, and then stored on ice.

Subsequently, PCR was performed using AccuPower Taq PCR premix (Bioneer), 2 uL of an RT sample and 1 uL each of 20 uM of Circular STS F (5'-CCCTGAGTGGCTGAGCTCAGG-3') and Circular STS R (5'-
 10 CAGCAAGCATACTAAATTGCCAG-3') were added and adjusted to a volume of 20 uL with water, and PCR amplification was performed under the conditions of 95°C for 1 minute, [95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds], 35 cycles, and 72°C for 5 minutes. 5 uL of a PCR product was put into 1 uL of 6X DNA loading dye, electrophoresed at 150 V for 35 minutes, and images were analyzed using a gel
 15 imaging system (Davinch-Gel product of Youngin Scientific). The expected length of the STS PCR product was 479 bp, and when analyzed by GeneRuler 50 bp DNA ladder (Thermo Fisher Scientific) on 1.5% agarose gel (Intron Bio's product, RedSafe Nucleic Acid Staining Solution, is contained in a concentration of 1X), only in the case of an RNA sample presumed to be circular RNA, a specific PCR product of a predicted size
 20 was observed regardless of RNase R treatment (FIG. 4A).

In addition, the PCR product was isolated and purified according to the manufacturer's protocol using a gel extraction kit (Cosmogenetech product) from the band of the expected size obtained, and cloned using a TOPcloner TA-Blunt kit (Enzynomics product). DH5alpha *E. coli* (Chemically competent *E. coli*, Enzynomics
 25 product) was transformed to obtain *E. coli* colonies from LB-Agar (including Kanamycin) plates, and plasmid DNA was extracted and purified according to the manufacturer's protocol using a DNA purification kit (Cosmogenetech product). As a result of requesting sequencing (using M13R (-40) or M13F (-20) universal primer provided by Sanger sequencing service company of Cosmogenetech), it was identified
 30 that 3' of Gaussia Luciferase and 5' of IRES were correctly linked at an STS junction site (FIG. 4B).

From the above results, it can be seen that Candidate 1 is a circular RNA.

2-3. Revalidation of Self-Circularization

It was identified that Candidate 1 is a circRNA through RT-PCR, but theoretically, the possibility that Candidate 1 is in the form of a dimer may not be excluded. Accordingly, a nicking test was performed to re-verify that Candidate 1 is a circRNA.

MgCl₂ was mixed with purified circular RNA candidates 1 and 2 (100 ng) to the final 0, 2.5, and 5 mM, respectively, and the sample in the final 10 uL of water was heated at 65°C for 30 minutes and then stored on ice for a while, and then was mixed with 10 uL of 10 M Urea-BPB (1X TEB) loading dye.

After heating at 75°C for 5 minutes, 4% polyacrylamide-7 M urea denature PAGE (2 hours of electrophoresis at 50 W conditions while maintaining a temperature at 50°C) was performed, and gels were stained with an SYBR Gold Nucleic Acid Stain product (Thermo Fisher Scientific) and analyzed using ImageQuant 800 (Cytiva product). The results are shown in FIG. 5.

It was identified that in the case of Candidate 1, in the absence of Mg²⁺, a band with a size corresponding to a slight nicked circular RNA was included (1092-nt), and in the case of a 2.5 mM Mg²⁺ condition, the band at the position of Candidate 1, considered the position of a circular RNA, decreased. In addition, it was identified that a band of nicked circular RNA size was still present. In the case of a 5 mM Mg²⁺ condition, it was identified that even the nicked circular RNA band disappeared by hydrolysis. Thus, it was observed that Candidate 1, that is, circular RNA, proceeded from the size of 1092-nt expected as a monomer when nicking occurred (2.5 mM Mg²⁺) and eventually completely degraded (5 mM Mg²⁺). Accordingly, it was identified again that Candidate 1 is a circular RNA and a monomer.

Candidate 2 has a size similar to the intact RNA size of 1874-nt (around 2000-nt of the marker). Under mild nicking conditions of 2.5 mM Mg²⁺, unlike Candidate 1, it was identified that the 1092-nt band, the size of the nicked circular RNA, was not generated and disappeared, and thus that it was not a circular RNA.

Example 3. Identification of Intracellular Transcription and Self-Circularization

In Example 2, it was identified that the self-circularization RNA expression vector produced in Example 1 was transcribed *in vitro* to form a circRNA. Accordingly, it was intended to identify whether the vector also operated the same in cells, and furthermore, whether the gene of interest included in the circRNA was expressed smoothly in the cells.

For the expression of a gene of interest in cells, the gene of interest is designed with the structure of 5'-EMCV IRES-transgene-stop codon-3', and for easy identification of the expressed gene of interest, gaussia luciferase (G.luci) was used as a transgene.

The self-circularization RNA construct including the aforementioned gene of interest was designed, and a DNA template, which may express the same, was inserted into a plasmid to be expressed under a pCMV promoter (FIG. 6A). The plasmid vector was transfected into 293A cells, the generation of a circRNA was identified by RT-PCR and sequencing, and the expression of transgene was identified by performing luciferase activity assay.

Specifically, 293A cells were seeded at 2×10^5 /well in 6 well plates and were transformed into the plasmid vector using a lipofectamine 2000 transfection reagent after 24 hours. After 6 hours of transformation, a culture medium was replaced. After the transformation, 100 UL of culture medium was collected at 12, 24, and 48 hours to measure G.luci activity. It was identified that G.luci activity was detected in the culture medium and the activity increased over time, and thus it was identified that the transgene G.luci gene was expressed in the vector introduced into the cells (FIG. 6B).

It was identified at the molecular level through RT-PCR and sequencing whether transgene expression was caused by the formation of a circRNA. 48 hours after transformation, total RNA was extracted from the 293A cells introduced with the plasmid vector using trizol reagent, and then it was identified that circular RNA was generated by performing RT-PCR.

For reverse transcription (RT), OneScript Plus RTase (Abm product) was used, and 1 ug of total RNA was heated at 70°C for 5 minutes and then placed on ice. Then, 5X RT buffer 4 uL, 10 mM dNTP mix 1 uL, 2 uM reverse primer (Circular STS R) 1 uL, OneScript Plus RTase 200 U were added in an orderly manner, and the final volume of

20 uL was reacted at 50°C for 15 minutes, heated at 95°C for 5 minutes to inactivate the enzyme, and then stored on ice.

Subsequently, PCR was performed using AccuPower Taq PCR premix (Bioneer product), 2 uL of an RT sample and 1 uL each of 20 uM of Circular STS primer F (5' - caaggacttggagcccatggagcag – 3') and primer R (5' - tgtgccgcctttgcaggtgtatc – 3') were added, adjusted to a volume of 20 uL with water, and PCR amplification was performed under the conditions of 95°C for 1 minute, [95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds] 35 cycles, and 72°C for 5 minutes. A specific PCR product of the predicted size was identified only when the expected length of the STS PCR product was 479 bp and circular RNA is present when analyzed by GeneRuler 50 bp DNA ladder (Thermo Fisher Scientific product) in 1.5% agarose gel (Intron Bio's product, RedSafe Nucleic Acid Staining Solution, is contained in a 1X concentration). In this connection, 5 uL of PCR product was put into 1 uL of 6X DNA loading dye, and electrophoresed at 150 V for 35 minutes, and then the image was analyzed with a gel imaging system (Davinch-Gel product of Youngin Scientific) (FIG. 6C).

In addition, as to the obtained band of the expected size, the PCR product was isolated and purified according to the manufacturer's protocol by using a gel extraction kit (Cosmogenetech product), cloned using TOPcloner TA-Blunt kit (Enzynomics product), and transformed into DH5alpha *E. coli* (Chemically competent *E. coli*, Enzynomics product) to obtain *E. coli* colonies on LB-Agar (including Kanamycin) plates, and extract and purify plasmid DNA using a DNA purification kit (Cosmogenetech product) and manual. As a result of the sequencing request (using M13R (-40) or M13F (-20) universal primer provided by Sanger sequencing service company of Cosmogenetech Inc.), it was identified that the 3' of Gaussia Luciferase and the 5' of the IRES were precisely linked nucleotide sequences at the STS junction site (FIG. 6D).

From the above, it was identified at the molecular level that the produced plasmid vector expressed a self-circularization RNA construct in cells, the construct was circularized into a circRNA by ribozyme, and a gene of interest was expressed in the circRNA.

Example 4. circRNA Purification

4-1. Identification of circRNA Purification Potential by Performing HPLC

In order to minimize the occurrence of immunogenicity in the production of a circRNA for human injection, analysis and purification using HPLC were performed. Specifically, an Agilent 1290 Infinity II Bio UHPLC system was used and the analysis conditions are as shown in FIG. 7A. For analysis, the gradient conditions of [Analytical] were used, and the conditions of [Fraction collection] were used for sample fraction.

When RNA purified only with a column (a Monarch RNA cleanup kit (NEB)) after IVT (FIG. 7B) and RNA treated with RNase R (FIG. 7C) were analyzed, it was found that there was an increasing peak in the RNase R-treated sample, and that a circRNA could be isolated by HPLC without RNase R treatment.

4-2 Purification of circRNA by Performing HPLC

Through HPLC, fraction was performed using the gradient shown in the fraction collection conditions, and each fraction was obtained as shown in FIG. 8A. Electrophoresis was performed in the same manner as in the previous method by taking 200 ng each of fractions 7 and 10 to 13 with clear peaks on 4% denature PAGE.

As a result, as can be seen in FIG. 8B, clean circular RNA was isolated and purified from fraction 12.

Example 5. AS (antisense sequence) Region Optimization

It was intended to identify the effect of the lengths of an antisense sequence (AS) region and a reverse-complementary antisense binding sequence (ABS) region on the immediate circularization reaction during an *in vitro* transcription process. Accordingly, a DNA template having different lengths of the AS region and ABS region of 50, 100, 150, 200, 250, or 300-nt was prepared (FIG. 9). A vector capable of expressing each RNA construct was produced in the same manner as in Example 1, and *in vitro* transcription was performed at 37°C for 3 hours in the same manner as in Example 2. The degree of immediate STS reaction was compared and identified by relative band intensity by performing PAGE on 4% polyacrylamide-7 M urea gel (20 × 20 cm, 1 mm).

The nucleotide sequences of each AS region are shown in Table 1 below.

[Table 1]

Sections	Nucleotide sequences (5' --> 3')
AS50	AAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGTGGCTGACCC
AS100	ACTCGAAGTGGCTGCGTACCACACCCGTCGCATTGGAGAAGGGCACGT AGAAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGTGGCTGA CCC
AS150	GGCGGCCAGCATGGAGAACTGCCATGGCTCAGCCAGGTAGTACTGTGG GTACTCGAAGTGGCTGCGTACCACACCCGTCGCATTGGAGAAGGGCAC GTAGAAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGTGGCT GACCC
AS200	AGCGTGAGGAAGTTGATGGGGAAGCCCAGCACGATCAGCAGAAACATG TAGGCGGCCAGCATGGAGAACTGCCATGGCTCAGCCAGGTAGTACTGT GGGTACTCGAAGTGGCTGCGTACCACACCCGTCGCATTGGAGAAGGGC ACGTAGAAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGTGG CTGACCC
AS250	GGATGTAGTTGAGAGGCGTGCGCAGCTTCTTGTGCTGGACGGTGACGTA GAGCGTGAGGAAGTTGATGGGGAAGCCCAGCACGATCAGCAGAAACAT GTAGGCGGCCAGCATGGAGAACTGCCATGGCTCAGCCAGGTAGTACTG TGGGTACTCGAAGTGGCTGCGTACCACACCCGTCGCATTGGAGAAGGGC ACGTAGAAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGTGG CTGACCC
AS300	GGTGAAGCCACCTAGGACCATGAAGAGGTCAGCCACGGCTAGGTTGAG CAGGATGTAGTTGAGAGGCGTGCGCAGCTTCTTGTGCTGGACGGTGACG TAGAGCGTGAGGAAGTTGATGGGGAAGCCCAGCACGATCAGCAGAAAC ATGTAGGCGGCCAGCATGGAGAACTGCCATGGCTCAGCCAGGTAGTAC TGTGGGTACTCGAAGTGGCTGCGTACCACACCCGTCGCATTGGAGAAGG GCACGTAGAAGTTAGGGCCTTCTGTGCCATTCATGGCTGTGGCCCTTGT GGCTGACCC

As a result, as can be seen in FIG. 10 and Table 2 below, in the case of AS50, AS100, and AS150, the self-circularization efficiency was relatively excellent compared to the length of AS200 or longer. On the other hand, for AS50 and AS100, total RNA produced after the *in vitro* transcription reaction was significantly less. Accordingly, it was found that AS150 was the most optimal length for self-circularization RNA construct expression and circular RNA production.

[Table 2]

AS series	AS50	AS100	AS150	AS200	AS250	AS300
A: Amount of Total RNA produced (μg)	69.5 ± 1.77	9.15 ± 0.46	176.5 ± 3.89	140 ± 3.54	133 ± 2.83	141 ± 5.66
B: Relative band intensity of circular RNA (%)	6.35 ± 0.25	6.75 ± 0.04	5.95 ± 0.18	4.30 ± 0.07	4.20 ± 0.07	3.60 ± 0.14
Relative factor (A * B)	441	62	1050	602	559	508

Example 6. Spacer Region Optimization

Subsequently, in order to identify the effect of the length or type of the spacer region on the immediate circularization reaction during an *in vitro* transcription process, a DNA template including spacer regions of different lengths and nucleotide sequences was produced (FIG. 11). A vector capable of expressing each RNA construct was produced in the same manner as in Example 1, and *in vitro* transcription was performed at 37°C for 3 hours in the same manner as in Example 2. The degree of immediate STS reaction was compared and identified by relative band intensity by performing PAGE on 4% polyacrylamide-7 M urea gel (20 × 20 cm, 1 mm). The nucleotide sequences of each spacer region are shown in Table 3 below. In this experiment, the spacers of A10, A30, and A50 were used by adding a restriction recognition site at the 3' end for IRES insertion immediately after the spacer region. In this experiment, the AatII site (GACGTC) was added to the 3' end of the spacer and used.

15

[Table 3]

Sections	Nucleotide sequences (5' --> 3')
A10	AAAAAAAAAAAA
A30	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
A50	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
Control spacer 1	GGTAGTGGTGCTACTAACTTCAGCCTGCTGAAGCA
Control spacer 2	GGTAGTAACTACTAACTACAACCTGCTGAAGCA

As a result, as can be seen in FIG. 12 and Table 4 below, despite the difference in the length and sequence of the spacer, there was no significant difference in the immediate circularization efficiency in the *in vitro* transcription process, but it was found that A30 operated as the most optimal spacer for self-circularization RNA construct expression and circular RNA production.

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[Table 4]

AS150 Spacer version	A10	Control spacer 1	Control spacer 1	A30	A50
A: Amount of Total RNA produced (μg)	199.5 ± 9.19	199.5 ± 6.36	187 ± 5.66	190 ± 8.49	192.5 ± 0.71

B: Relative band intensity of circular RNA (%)	5.55 ± 0.35	6.7 ± 0.14	6.15 ± 0.07	6.5 ± 1.27	5.7 ± 0.85
Relative factor (A * B)	1106 ± 19	1311 ± 70	1150 ± 21	1240 ± 297	1097 ± 160

Example 7. Optimization of Self-circularization RNA Construct

7-1. AS Region, and P1 Helix and P10 Helix Regions

The self-circularization RNA construct designed in Example 1 includes an AS region, and P1 helix and P10 helix regions. Hereinafter, in order to identify the effect of each configuration on the immediate circularization reaction during the *in vitro* transcription process, as diagrammed in FIG. 13, a DNA template including only the P1 helix region, only the P1 and P10 helix regions, or both the P1 and P10 helices and the AS region was prepared. A vector capable of expressing each RNA construct was produced in the same manner as in Example 1, and *in vitro* transcription was performed at 37°C for 3 hours in the same manner as in Example 2. The degree of immediate STS reaction was compared and identified by relative band intensity by performing PAGE on 4% polyacrylamide-7 M urea gel (20 × 20 cm, 1 mm).

The nucleotide sequences of the T7 DNA template including only the P1 helix region are shown in FIG. 14.

As a result, as can be seen in FIG. 15 and Table 5 below, there was no significant difference in the total RNA transcribed and generated in each vector *in vitro*, but it was found that the circular RNA production amount was high in the order of including both the P1 and P10 helix and the AS regions, including only the P1 helix region, and including only the P1 and P10 regions.

[Table 5]

AS150	P1 (No AS)	P1&P10 (No AS)	P1&P10 (AS150)
A: Amount of Total RNA produced (μg)	200	196	182
B: Relative band intensity of circular RNA(%)	3.2	0.5	5.1
Relative factor (A * B)	640	98	928

7-2. Circularization Verification of Self-circularization RNA Construct that Does Not Include P10 and AS Regions

From the results of Example 7-1, it can be understood that sufficient circRNAs

could be produced without an additional circularization stage in the *in vitro* transcription process from a DNA template (P1 construct) that does not include P10 and AS regions. In order to verify this, the product after the STS reaction of Example 7-1 was treated with RNase R to remove linear RNA, PAGE was performed in the same manner as in the previous experiment, and RT-PCR and sequencing were performed in the same manner as in Example 2-2.

As a result, as can be seen in FIGS. 16A and 16B, even when self-circularization was performed using the P1 construct, when RNase R was treated, an RNA band that was not easily cut by RNase R and enriched was observed. As a result of performing RT-PCR and sequencing of the STS reaction product in the band, it was identified that it was a circRNA.

Example 8. Optimization of P1 Helix Region

From the results of Example 7, it was identified that the self-circularization construct without P10, AS, and ABS could also sufficiently make a circRNA. Accordingly, it was intended to verify assuming that a circular RNA would be generated only when the Internal Guide Sequence (IGS) present at the 5' end and the nucleotide sequence of the target site present at the 3' end configuring the P1 helix are complementarily bound to each other (U and G are wobble base pairs).

Since the IGS region is GNNNNN and the nucleotide sequence of the target site is N'N'N'N'N'U, when only one U nucleotide is added to the GOI, the finally generated circRNA consists only of one additional U base and the GOI region (FIGS. 1A and 1B). In addition, when the 3' end of the GOI ends with a U base, by designing the nucleotide sequence of the IGS region to be reverse-complementary to the GOI, the circRNA finally generated may be prepared to consist only of the GOI region (FIGS. 20A and 20B).

As shown in FIG. 17, various sequences of IGS and target site were designed, a vector was produced by the method of Example 1, and then the *in vitro* transcription of Example 2-1 was performed.

As a result, as can be seen in FIGS. 18-19 and Table 6 below, when both ends have complementary sequences, it was possible to identify an increase in the circRNA generated through the immediate STS reaction after *in vitro* transcription, and it was

found that the vector including the no-complement IGS region generated a very low level of a circRNA. From the above, it was found that both the IGS and the target site could efficiently induce a self-circularization reaction when they were complementary to each other.

5

[Table 6]

P1 variants (No AS)	AS150 (P1&P10)	RZ004	RZ001	RZ003	GC- rich	AU- rich	2 sites	No complement
Relative band intensity of circular RNA(%)	5.4	3.7	7.9	6.6	5.4	11.6	6.1	1

As described above, although the example embodiments have been described with reference to the limited drawings, those skilled in the pertinent technical field may apply various technical modifications and variations based on the above. For example, proper results may be achieved although the described techniques are performed in an order different from the described method, and/or the described components, such as a system, structure, device, circuit, etc., are coupled or combined in a form different from the described method or replaced or substituted by other components or equivalents.

Accordingly, other implementations, other example embodiments, and the equivalents of the claims also belong to the scope of the claims below.

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[Industrial Applicability]

The present disclosure may be used for the production of circRNAs for protein expression *in vitro*, in cells, and *in vivo*, and may be used to produce miRNA, anti-miRNA, shRNA, aptamer, mRNA vaccine, mRNA therapeutic agent, antibody, vaccine adjuvant, and functional RNA of CAR-T mRNA into a circRNA.

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CLAIMS

1. A self-circularization RNA construct, wherein:
the construct has a structure of 5' - IGS (internal guide sequence) - ribozyme -
5 gene of interest - target site - 3';
the IGS region and the target site forma guanine (G) : uracil (U) wobble base
pair;
the guanine forming the wobble base pair is located at the 5' end of the IGS
region; and
10 the uracil forming the wobble base pair is located at the 3' end of a target site
region.
2. The self-circularization RNA construct of claim 1, wherein the target site
region overlaps with a gene of interest region.
15
3. The self-circularization RNA construct of claim 1, wherein a nucleotide
sequence of the IGS region is reverse complementary to a nucleotide sequence of the
target site region except for the guanine.
- 20 4. The self-circularization RNA construct of claim 1, wherein the ribozyme is a
Group I intron ribozyme.
5. The self-circularization RNA construct of claim 1, wherein the ribozyme
includes a nucleotide sequence represented by SEQ ID NO: 6.
25
6. The self-circularization RNA construct of claim 1, wherein the construct
includes a nucleotide extending in a 5' direction of the IGS region to form a P10 helix.
7. The self-circularization RNA construct of claim 1, wherein the construct
30 includes a nucleotide extending in a 5' direction of the IGS region and a 3' direction of
the target site to form a P1 helix.

8. The self-circularization RNA construct of claim 7, wherein the construct does not form a P10 helix.

5 9. The self-circularization RNA construct of claim 1 or 6, wherein the construct further includes an antisense sequence (AS) region in a 5' direction of the IGS region and an antisense binding sequence (ABS) region capable of complementary binding to the AS region in a 3' direction of the target site.

10 10. The self-circularization RNA construct of claim 9, wherein a length of the AS region is 50-nt to 400-nt.

11. The self-circularization RNA construct of claim 1, wherein the gene of interest region includes an internal ribosome entry site (IRES) region at the 5' end.

15 12. The self-circularization RNA construct of claim 1, wherein the construct has a ribozyme region and a gene of interest region linked by a spacer region including a random nucleotide sequence.

20 13. The self-circularization RNA construct of claim 1, wherein the construct has a gene of interest region and a target site region linked by a spacer region including a random nucleotide sequence.

14. A vector expressing the self-circularization RNA construct of claim 1.

25 15. The vector of claim 14, wherein the vector includes a promoter operably linked to a gene encoding a self-circularization RNA.