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Methods for controlling the expression of target genes

[0001]

The present invention relates to a method for controlling the transcription of a target gene using oligonucleotides, and a method for activating the expression of a specific transcription factor in a living organism using this method, thereby effectively inducing life phenomena such as cell differentiation, reprogramming, cell proliferation, and hormone secretion in which the transcription factor is involved. In particular, this invention relates to a method for inducing the differentiation of mesenchymal stem cells into muscle cells, adipocytes, or osteocytes by expressing transcription factors involved in their differentiation, and to a method for producing iPS cells by expressing transcription factors for reprogramming somatic cells.

[0002]

The genome, which is the blueprint of life, is translated into proteins, the final functional molecules, through a process called gene expression. Transcription is the primary regulatory

step, and various methods for regulating transcription have been established to date. One method for suppressing transcription involves using nucleic acids, and one such method is the antisense method. When mRNA is used as the sense strand, and an antisense molecule complementary to its target sequence is double-stranded via a Watson-Crick type hydrogen bond, the translation of that mRNA into the corresponding protein is inhibited. When the antisense strand is DNA, RNase H cleaves mRNA and causes translation inhibition (Non-patent Literature 1). Furthermore, there is an antigene method that involves attaching a third strand to target double-stranded DNA via a Hoogsteen-type hydrogen bond, thereby inducing transcriptional inhibition (Non-Patent Literature 2). In ribozyme methods, which use ribozymes, which are RNA or DNA with enzymatic activity, the ribozyme hydrolyzes the target mRNA, causing translation inhibition (Non-Patent Literature 3). Furthermore, transcriptional regulation methods utilizing transcription factor binding sequences have also been developed. Gene transcription begins when a transcription factor recognizes and binds to a transcription factor binding sequence within its promoter region. Therefore, there is a technique that involves coexisting the target gene with a high concentration of short decoy DNA double strands (decoy DNA) having the same sequence as the transcription factor binding sequence of the target gene, thereby trapping the transcription factor with the decoy DNA and inhibiting the transcription of the target gene (Non-Patent Literature 4). Furthermore, in recent years, research and development of RNA interference (RNAi), which suppresses the expression of target genes using short double-stranded RNA (siRNA) (Non-Patent Literature 5), has advanced dramatically, and the practical application of siRNA has

been almost established (Non-Patent Literature 6). It has also been found that RNA interference can induce inhibition of target gene translation. Thus, in conventional methods for regulating the expression of target genes, all short-chain nucleotides used have a repressive effect. While there are indirect techniques that indirectly activate the expression of target genes by inhibiting the function of repressors such as miRNAs that intrinsically suppress gene expression in vivo, there were no techniques that directly act on target gene promoters to promote the transcriptional activity of target genes using short-chain nucleotides.

[0003]

On the other hand, methods for expressing target genes within cells using vectors have been established, and these methods include the use of viral vectors and non-viral vectors (Non-Patent Documents 7, 8).

In the case of viral vectors, the introduction efficiency and ability to induce target gene expression are very high. However, since genes introduced into the body can integrate into the genome, there is a problem that their expression can continue permanently and cause

cancer, and the development of leukemia in gene therapy using retroviral vectors has been reported (Non-Patent Literature 9). Furthermore, deaths have been reported even with the administration of adenovirus vectors that do not integrate into the host genome, so there are many safety issues that need to be addressed when using viral vectors. In contrast, non-viral vectors, such as plasmid DNA, are superior in terms of safety, but have the disadvantage of low delivery efficiency (Non-Patent Document 10). Therefore, there has been a need for a method of activating target gene transcription that can efficiently and reliably activate the transcription of the target gene, while also having an extremely low risk of causing cancer and being safe to activate.

[0004]

Stem cells play an extremely important role as a source of cells in realizing regenerative medicine.

There is a great deal of research underway to differentiate stem cells into specific tissue cells in order to regenerate desired tissues and organs, or to treat diseases and defects. In particular, the use of stem cells harvested from one's own body eliminates concerns about

immunological rejection, and the induction of differentiation from various stem cells into specific cells through transcriptional regulation is expected to have applications in regenerative medicine. There are high expectations for regenerative medicine using these differentiated cells, and numerous cell transplantation therapies, primarily focusing on blood vessels, nerves, and cardiac muscle, have already been attempted. Progress is being made in elucidating the transcriptional regulation of various transcription factors during cell differentiation and dedifferentiation. In particular, for mesenchymal stem cell differentiation, transcription factors known as master regulators, such as MyoD, Runx2, and PPAR γ 2, have been identified, and the transcriptional mechanism is being elucidated. To date, it has been known that MyoD, along with Myf5 and myogenin, are involved in the differentiation of mesenchymal stem cells into muscle cells (Non-Patent Literature 11), and that the expression of Osterix, along with Runx2, is essential for differentiation into osteoblasts (Non-Patent Literature 12). Furthermore, it is believed that C/EBP α , along with PPAR γ 2, is involved in the differentiation of adipocytes (Non-Patent Document 13). Of these factors, MyoD, Runx2, and PPAR γ 2, which are master regulators, have been confirmed to induce differentiation of mesenchymal stem cells into myocytes, osteoblasts, and adipocytes in three directions by inducing their expression using viral vectors, etc. (Non-Patent Literature 14, etc.)

[0005]

Furthermore, embryonic stem cells (ES cells) created from fertilized eggs are one type of pluripotent stem cell that is expected to have applications in regenerative medicine, but they raise bioethical issues because they involve human embryos.

In contrast, induced pluripotent stem cells (iPS cells) can be produced from somatic cells such as human skin cells, thus overcoming the ethical issues that plagued ES cells and making them highly promising for practical use (Non-Patent Documents 15, 16). Furthermore, iPS cells can be prepared from the patient's own cells, giving them an advantage over ES cells in terms of immunocompatibility during transplantation. However, because viral vectors are used as an efficient method for establishing iPS cells, adverse events such as cancer caused by the insertion of introduced genes into the host genomic DNA have become a problem (Non-patent documents 17, 18). Furthermore, it has been reported that in iPS cell establishment methods using viral vectors, the constitutive expression (ectopic expression) of the introduced gene continues, inhibiting differentiation induction from iPS cells and inducing cancer (Non-Patent Literature 19). As described above, the biggest obstacle to the differentiation induction of stem cells and the establishment of iPS cells, which are essential for the practical application of regenerative medicine, is the necessity of using vectors such as viral vectors, which can cause cancer due to their persistent expression. Therefore, in order to realize the clinical application of regenerative medicine, there was a strong need for a method

of transcriptional activation of target genes that could achieve the same expression efficiency as viral vectors, yet was safe, rapidly disappearing in a short time, and had an extremely low possibility of causing cancer.

[0006]

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[0007]

This invention provides a transcriptional activation method that uses oligonucleotides, which rapidly lose their activity in vivo, as an effective and safe means for temporarily activating gene expression, and that can achieve high expression efficiency of the target gene.

Furthermore, this method aims to provide an efficient and safe method for inducing stem cell differentiation and establishing iPS cells, which are essential for regenerative medicine using the transcriptional activation method described herein.

[0008]

While studying the transcriptional regulatory mechanism of promoters by transcription factors, the inventors focused on the fact that the transcription factor binding region, which is the region on the promoter that binds to transcription factors, attracts the transcription factor by van der Waals forces and binds by hydrogen bonding. Based on this, they conceived the idea of creating an oligonucleotide containing a transcription factor binding region that has transcription-inducing activity.

However, it was unclear whether the transcription factor binding region, when detached from the promoter region, would actually attract the transcription factor, and the methodology for selectively and reliably reaching the transcription initiation region of the promoter to be activated remained unknown.

As a result of diligent research, the inventors have constructed an oligonucleotide in which a single-stranded nucleotide homologous to a portion of the upstream region of a target gene is linked to a transcription factor binding region with a linker. By using this oligonucleotide, they have succeeded in selectively activating the transcription of any target gene.

In other words, specifically, a single-stranded nucleotide (anchor sequence) homologous to a portion of the upstream sequence of a target gene whose transcription is to be activated is created, and this single-stranded nucleotide is linked to a double-stranded nucleotide (transcription factor binding site) containing an arbitrary transcription factor binding region sequence using a spacer sequence (linker region), thereby creating an oligonucleotide ("gene jack oligonucleotide") containing both a single-stranded anchor site and a double-stranded transcription factor binding site.

) will be created.

Next, when the "gene jack nucleotide" is introduced into cells by lipofection or other methods, transcription of the target gene is induced or activated.

This phenomenon is thought to occur because the upstream sequence of a target gene on the genome recruits gene jack nucleotides containing the corresponding anchor site to the target gene's promoter. The transcription factor binding site, linked via the anchor and linker, then attracts intracellular transcription factors to the vicinity of the promoter, and the transcription of the target gene is induced or activated by the action of these transcription factors.

This transcriptional activation method is a groundbreaking novel technique that can selectively and efficiently activate only target genes within cells.

Conversely, by replacing the transcription factor binding region sequence with a double-stranded nucleotide containing a transcription repressor binding region, this versatile transcriptional control technique can also be used to specifically repress the transcription of a target gene. For this reason, the inventors have named this method of transcriptional control of a target gene the "gene jack oligonucleotide method."

In the "GeneJack Oligonucleotide Method" of the present invention, "GeneJack Oligonucleotides" (hereinafter sometimes simply referred to as "oligonucleotides") are used.

The substance is introduced directly into cells using methods such as lipofection without being inserted into a vector.

Therefore, it is not stably incorporated into the genome, and its effect on transcriptional regulation is transient.

Therefore, it is a highly safe technology that does not carry the risk of carcinogenesis due to constitutive transcriptional activation, as is the case with viral vector methods.

In this invention, in order to verify the effectiveness of the "Gene Jack Oligonucleotide Method," an experimental system utilizing the multidirectional differentiation ability of mesenchymal stem cells was first used.

When gene jack oligonucleotides targeting MyoD, a master regulator (transcription factor) that determines the differentiation direction of mesenchymal stem cells, were transfected into the mesenchymal stem cell line C3H10T1/2 by lipofection, it was confirmed that MyoD was selectively activated and differentiation into muscle was induced.

Similarly, it was confirmed that targeting other master regulators, such as Runx2, also induced differentiation into osteoblasts.

Next, we attempted to apply the gene jack oligonucleotide method to the development of a novel method for establishing iPS cells, which is a further objective of this invention.

By introducing four transcription factors known as Yamanaka factors (Oct3/4, Sox2, KLF4, c-Myc), which are used to generate iPS cells, into mouse embryonic fibroblasts (MEFs), and activating each transcription factor, we were able to confirm the expression of undifferentiated markers.

Among these transcription factors, the c-Myc factor is thought to be one of the causes of cancerous transformation in established iPS cells.

Therefore, the establishment of iPS cells without the c-Myc factor is being considered, but the low efficiency of this process is a problem.

On the other hand, with the gene jack oligonucleotide method, transcription induction is transient and does not involve integration into the genome, allowing the use of four factors, including the c-Myc factor.

Thus, the results obtained suggest that this method makes it possible to establish stable iPS cells with an extremely low probability of becoming cancerous without compromising establishment efficiency.

These findings led to the completion of the present invention.

[0009]

In other words, the present invention encompasses the following inventions.

[1] A reagent for controlling the transcription of a target gene, comprising as an active ingredient an oligonucleotide in which an anchor site consisting of a single-stranded nucleotide homologous to the base sequence of the upstream region of the target gene is bound via a linker to a transcription factor binding site consisting of a double-stranded nucleotide containing any transcription factor binding sequence or transcription repressor binding sequence.

[2] The transcriptional control reagent according to [1], characterized in that the anchor site consists of a base sequence that is 10 to 50 bases in common with the base sequence of the upstream region of the promoter of the target gene.

[3] The transcriptional control reagent according to [1] or [2], characterized in that the transcription factor binding site includes a base sequence in which a transcription factor binding sequence or a transcription repressor binding sequence are linked in tandem.

[4] A transcriptional regulatory reagent according to any one of [1] to [3] above, characterized in that the target gene is a transcription factor gene that determines the direction of

differentiation, and the transcription factor binding site includes a transcription factor binding sequence, for use as a differentiation inducer in stem cells.

[5] A transcriptional regulatory reagent according to any one of [1] to [3] above, characterized in that the target gene consists of three oligonucleotides, each of the three genes Oct3/4, Sox2, and KLF4, or four genes including the c-Myc gene, and the transcription factor binding site contains a transcription factor binding sequence, for use as a dedifferentiation agent for establishing iPS cells.

[6] A transcriptional regulatory reagent according to any one of [1] to [3] above, characterized in that the oligonucleotide targets the upstream region of a causative gene that causes disease due to deficiency or deletion in vivo, and the transcription factor binding site includes a transcription factor binding sequence, thereby improving or treating a disease by activating or inducing the transcription of the causative gene.

[7] A method for inducing or activating the transcription of a target gene within a cell, comprising: (1) constructing an oligonucleotide by linking a single-stranded nucleotide having a base sequence homologous to the upstream region sequence of the target gene to a double-stranded nucleotide containing a transcription factor binding region sequence via a linker; and (2) introducing the oligonucleotide into a cell containing the target gene.

[8] The method according to [7], characterized in that the cells are stem cells and the target gene is a transcription factor gene that determines the direction of differentiation, for differentiating stem cells into specific organ cells.

[9] The method according to [7], characterized in that the cells are somatic cells and the target gene is one or more transcription factor genes selected from the Oct3/4, Sox2, KLF4, and c-Myc genes, for reprogramming cells.

[10] A method for differentiating stem cells into specific organ cells, comprising the following steps (1) to (4): (1) selecting a transcription factor as a target that determines the direction of differentiation into the organ; (2) constructing an oligonucleotide by linking a single-stranded nucleotide having a base sequence homologous to the upstream region sequence of the transcription factor gene with a double-stranded nucleotide containing a transcription factor binding region sequence via a linker; (3) introducing the oligonucleotide constructed in step (2) into stem cells; and (4) culturing the stem cells obtained in step (3) in a medium suitable for differentiation into the specific organ.

[11] A stem cell differentiation inducer containing as an active ingredient an oligonucleotide in which a single-stranded nucleotide having a base sequence homologous to the sequence of

the upstream region of a transcription factor gene is linked to a double-stranded nucleotide containing a transcription factor binding region sequence via a linker.

[12] A method for establishing iPS cells, comprising the following steps (1) to (3): (1) Constructing oligonucleotides by linking single-stranded nucleotides, each consisting of a base sequence homologous to the upstream region sequence of three genes, Oct3/4, Sox2, and KLF4, or four genes including the c-Myc gene, to double-stranded nucleotides containing a transcription factor binding region sequence via a linker; (2) Introducing the three or four types of oligonucleotides constructed in step (1) into somatic cells; (3) Culturing the somatic cells obtained in step (2) in a medium suitable for cell reprogramming.

[13] A somatic cell dedifferentiation agent containing, as an active ingredient, an oligonucleotide in which a single-stranded nucleotide having a base sequence homologous to the upstream region sequence of each of three genes, Oct3/4, Sox2, and KLF4, or four genes including the c-Myc gene, is linked to a double-stranded nucleotide containing a transcription factor binding region sequence via a linker.

[14] A method for activating or inducing the transcription of a causative gene that causes disease due to deficiency or absence in the body, comprising the following steps (1) to (3): (1) a step of identifying a causative gene that causes disease and selecting it as a target gene; (2) a step of constructing an oligonucleotide by linking a single-stranded nucleotide having a

base sequence homologous to the upstream region sequence of the target gene with a double-stranded nucleotide containing a transcription factor binding region sequence via a linker; (3) a step of introducing the oligonucleotide constructed in step (2) into cultured cells or cells or tissues in the body.

[15] A gene therapy pharmaceutical composition containing, as an active ingredient, an oligonucleotide in which a single-stranded nucleotide having a base sequence homologous to the sequence of the upstream region of a causative gene that causes disease when deficient or missing in the body is linked to a double-stranded nucleotide containing a transcription factor binding region sequence via a linker.

[16] Oligonucleotides having transcriptional activation function of a target gene, characterized in that the nucleotides (a) to (c) below are chemically bonded in this order: (a) a single-stranded nucleotide consisting of a nucleotide sequence homologous to the sequence of the upstream region of the target gene; (b) a single-stranded nucleotide consisting of a nucleotide sequence that does not show complementarity with the nucleotide sequence of the adjacent region of (a); (c) a double-stranded nucleotide consisting of a nucleotide sequence including a transcription factor binding region sequence.

[0010]

The "GeneJack Oligonucleotide" used in this invention has high transcriptional activity efficiency for target genes, and its transcriptional activation is transient. Therefore, it does not pose the risk of side effects such as cancer caused by the integration of the introduced gene into the genome, which is a problem with gene transfer methods using viral vectors.

Furthermore, this invention is a highly versatile technology applicable to any gene expression in mammalian cells, and since it is possible to highly express a desired gene, it can be used in various gene therapies.

Furthermore, by applying this method to induce the expression of transcription factors that determine the differentiation direction of various stem cells, such as mesenchymal stem cells, it becomes possible to differentiate various stem cells into desired organs. Moreover, by applying it to induce the expression of a transcription factor called Yamanaka factor, which is used for somatic cell reprogramming, it becomes possible to establish iPS cells without the risk of cancer, thus representing a great boon for the field of regenerative medicine.

[0011]

The figure above shows a schematic diagram of the action of gene jack oligonucleotides.

The figure below shows examples of gene jack oligonucleotides used in the experiment.

[Anchor sequence]C (Control oligonucleotide): A portion of the Luciferase gene sequence (confirmed by Blast search to be a sequence not present in the mouse genome) is used.

T (Targeted oligonucleotide): This uses the positive chain sequence of the promoter of the target gene MyoD (the 20-nucleotide sequence from position -77 to -96 upstream of the transcription start site).

[TF (Transcription Factor) binding elements]WT CMV (CMV minimal promoter): Uses sequences derived from the minimal promoter of CMV (Cytomegalovirus).

SC CMV (Scramble mutated CMV): This type of CMV uses a sequence in which the base sequence of the CMV minimal promoter has been randomly rearranged and mutated.

Linker: A 5-base T is inserted between the anchor sequence and the TF binding elements.

C&WT CMV, T&SC CMV, and T&WT CMV oligonucleotides shown in Figure 1 (below) were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted MyoD mRNA was measured by quantitative PCR.

The expression levels were corrected using GAPDH as an internal control, and the relative expression levels were shown with the corrected value for C&WT CMV expression levels set to 1.

The target gene was changed to Runx2, and the anchor sequences of T&SC CMV and T&WT CMV were changed to the positive strand sequence of the Runx2 promoter sequence (-1469 to -1488).

These C&WT CMV, T&SC CMV, and T&WT CMV oligonucleotides were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted Runx2 mRNA was measured by quantitative PCR.

The values were corrected using GAPDH as an internal control, and, as in Figure 2, are shown as relative values with C&WT CMV set to 1.

The target gene was changed to Preproinsulin gene II (Insulin-II) or ribosomal RNA (rRNA), and the anchor sequence of Targeted oligonucleotide (Targeted) was changed to the forward-chain sequence of the upstream region of Insulin-II or rRNA.

These oligonucleotides were transfected into mouse liver cancer cell line Hepa1-6 cells, and the cells were harvested after 24 hours.

mRNA of the Insulin-II (left figure) or rRNA (right figure) gene extracted from these cells was measured by quantitative PCR.

Control (Cont.

The same Anchor sequence as in Figure 1 is used, but derived from the Luciferase gene sequence. All TF binding elements use the CMV promoter sequence from Figure 1.

GAPDH was used as an internal control for correction, and rRNA was placed in lane 1 (Cont.

The relative values were shown with the value of) set to 1.

Oligonucleotides were used in which the length of the anchor sequence bases was changed from the 20 bases used in previous experiments to 10, 14, 16, 18, 20, 25, and 30 bases.

Furthermore, a control sample was prepared for each base length, with the same base length as the Targeted sample.

These oligonucleotides were transfected into C3H10T1/2 cells, and the cells were harvested after 24 hours. The extracted MyoD mRNA was measured by quantitative PCR.

The expression levels were corrected using GAPDH as an internal control, and the corrected value for the expression level in lane 5 was set to 1, resulting in the relative expression levels shown.

Targeted and control oligonucleotides, in which the target gene Runx2 was changed and the base length of the anchor sequence was altered from 20 bases used in previous experiments to 16 and 18 bases, were transfected into C3H10T1/2 cells.

After 24 hours, the cells were collected, and the extracted Runx2 mRNA was measured by quantitative PCR.

The expression levels were corrected using GAPDH as an internal control, and the corrected value for the expression level in lane 1 was set to 1, resulting in the relative expression levels shown.

Oligonucleotides with a 4-base mutation introduced into the anchor sequence were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted MyoD mRNA was measured by quantitative PCR.

For each of the four-nucleotide-mutated oligonucleotides, three different mutation sites were created and used in the experiment.

In the diagram, WT is "T&WT CMV" as shown in the lower diagram of Figure 1, and Cont.

This is the "C&WT CMV".

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

When MyoD was the target gene, Targeted and Control oligonucleotides with linker base counts of 0, 3, 5, or 10 were transfected into C3H10T1/2 cells.

After 24 hours, the cells were collected, and the mRNA of the extracted MyoD was measured by quantitative PCR.

GAPDH is used as an internal control for correction, and lane 3 (T5 Cont.)

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

For the case where Runx2 was the target gene, Targeted and Control oligonucleotides, in which the number of linker bases was varied to 0, 3, 5, or 10, were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted Runx2 mRNA was measured by quantitative PCR.

GAPDH is used as an internal control for correction, and lane 3 (T5 Cont.)

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

Oligonucleotides with anchor sequences consisting of 20 base pairs from the transcription start site in the upstream region of the MyoD gene to positions -5, -25, -96, -322, -790, -953, -23321, or -23414 were transfected into C3H10T1/2 cells.

Note that in the diagram, Cont.

This is the same as "C&WT CMV" in Figure 1.

After 24 hours, the cells were collected, and the mRNA of the extracted MyoD was measured by quantitative PCR.

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

C3H10T1/2 cells were transfected with an oligonucleotide in which the 3' end of a transcription factor binding sequence derived from the CMV minimal promoter was modified with biotin. After 24 hours, the cells were harvested and chromatin immunoprecipitation was performed using streptavidin beads.

The anchor sequences are the same as in Figure 1, with the control using a sequence derived from the Luciferase gene and the target using a sequence derived from the promoter region of MyoD.

The precipitated product was measured quantitatively by PCR using a PCR primer set capable of detecting the precipitation of chromatin fragments including the vicinity of -77 to -96 upstream of the target MyoD (amplifying -375 to -545), and a PCR primer set designed distal to the target sequence as a negative control (amplifying -8539 to -8779).

Oligonucleotides, in which mutations were introduced into the transcription factor binding sequences present in the CMV promoter sequence used as TF binding elements, were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted MyoD mRNA was measured by quantitative PCR.

The anchor sequences are the same as in Figure 1, with the control using a sequence derived from the Luciferase gene and the target using a sequence derived from the promoter region of MyoD.

WT CMV refers to oligonucleotides using a TF sequence derived from the wild-type minimal

CMV promoter (same as in Figure 1), while MT CMV refers to oligonucleotides using a minimal CMV promoter-derived sequence with a mutation in the TF binding sequence.

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

Oligonucleotides, in which mutations were introduced into the transcription factor binding sequences present in the CMV promoter sequence used as TF binding elements, were transfected into C3H10T1/2 cells. After 24 hours, the cells were harvested, and the extracted Runx2 mRNA was measured by quantitative PCR.

The control group uses the same Luciferase gene-derived sequence as in Figure 1 for the anchor sequence, while the targeted group uses the same Runx2 promoter region-derived sequence as in Figure 3.

WT CMV and MT CMV are, as in Figure 12, TF sequences derived from the wild-type minimal CMV promoter region and sequences derived from the mutant CMV promoter, respectively.

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative expression levels are shown with the corrected value for the expression level of) set to 1.

For targeting the MyoD gene, instead of the CMV promoter-derived sequence shown in Figure 1, we used oligonucleotides consisting of eight tandem AP1 binding sequences or three each of the Sp/KLF family binding sequences (GTbox and GCbox/GC/GT box).

C3H10T1/2 cells were transfected, and after 24 hours, the cells were harvested. The extracted MyoD mRNA was measured by quantitative PCR.

The anchor sequences are the same as in Figure 1, with the control using a sequence derived from the Luciferase gene and the target using a sequence derived from the promoter region of MyoD.

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative values were shown with the value of) set to 1.

For the Runx2 gene target, the same experiment as in Figure 14 was performed using

oligonucleotides in which the AP1 binding sequence was tandemly attached to a sequence derived from the CMV promoter as TF binding elements.

C3H10T1/2 cells were transfected, harvested after 24 hours, and extracted Runx2 mRNA was measured by quantitative PCR.

For the anchor sequence, the Control group used the same Luciferase-derived gene sequence as in Figure 1, while the Target group used the Runx2 promoter-derived sequence as in Figure 3.

GAPDH is used as an internal control for correction, and lane 1 (Cont.

The relative values were shown with the value of) set to 1.

Oligonucleotides targeting the MyoD gene were transfected into C3H10T1/2 cells. The cells were harvested after 24, 72, or 120 hours, and the extracted MyoD mRNA was measured by quantitative PCR.

The anchor sequences are the same as in Figure 1, with the control sequence derived from the Luciferase gene and the target sequence derived from the promoter region of MyoD.

GAPDH is used as an internal control for correction, and lane 1, lane 3, or lane 5 (Cont.

The relative values were shown with the value of) set to 1.

Oligonucleotides targeting the Runx2 gene were transfected into C3H10T1/2 cells. Cells were harvested after 24, 72, or 120 hours, and the extracted Runx2 mRNA was measured by quantitative PCR.

For the anchor sequences, the control uses the same Luciferase-derived gene sequence as in Figure 1, while the targeted uses the same Runx2 promoter-derived sequence as in Figure 3.

GAPDH is used as an internal control for correction, and lane 1, lane 3, or lane 5 (Cont.

The relative values were shown with the value of) set to 1.

In this experiment, we used oligonucleotides in which some of the oligoDNA used in previous experiments was replaced with LNA (Locked Nucleic Acid), an artificial nucleic acid.

Note that, as with the Targeted case, the control (Cont.) has been replaced with an LNA.

I prepared (this).

These oligonucleotides were transfected into C3H10T1/2 cells, and the cells were harvested after 24 hours. The extracted MyoD mRNA was measured by quantitative PCR.

The expression levels were corrected using GAPDH as an internal control, and the corrected value for the expression level in lane 1 was set to 1, resulting in the relative expression levels shown.

This figure shows the selective differentiation induction of mouse mesenchymal stem cell lines (C3H10T1/2) by key transcription factors.

Mesenchymal stem cells are differentiated into muscle cells or osteocytes, respectively, by the expression of the key transcription factors MyoD or Runx2.

Oligonucleotides were transfected into C3H10T1/2 cells, and after differentiation induction in the direction of muscle cells and osteocytes, the cells were harvested, and the mRNA of MyoD and Runx2 genes, as well as each differentiation marker gene, was measured by quantitative PCR.

The Control model uses the same Anchor sequence as in Figure 1, but derived from the Luciferase gene. MyoD and Runx2 models use the promoter-derived sequences of their respective genes as Anchor sequences (same as in Figure 1 or Figure 3). All TF binding elements use the CMV promoter-derived sequences shown in Figure 1.

The values were corrected using GAPDH as an internal control, and the relative values were shown with the value of each lane 1 (Control) set to 1.

Transcription levels of the Myogenin and MHC genes were observed as muscle differentiation marker genes, and the ALP and OC genes were observed as osteocyte differentiation marker genes.

Oligonucleotides targeting the MyoD gene or the Runx gene were transfected into C3H10T1/2 cells, and after 72 hours, the culture medium was changed to muscle differentiation medium.

After 3 days of culture, immunostaining with myosin heavy chain (MHC) was performed to evaluate muscle differentiation (green).

At the same time, nuclear staining was performed using DAPI (blue).

The oligonucleotide used is the same as in Figure 20.

Similar to Figure 21, C3H10T1/2 cells were transfected using the same oligonucleotide as in Figure 20, and then the culture medium was changed to osteogenic differentiation medium after 24 hours.

Alizarin red staining was performed after 29 days of culture to evaluate ossification.

Mouse embryonic fibroblasts (MEFs) were transfected with oligonucleotides targeting transcription factors involved in iPS cell induction, one at a time. After approximately 24 hours, the cells were harvested, and the mRNA levels of each gene were measured using quantitative PCR.

The control uses the same luciferase-derived sequence as in Figure 1 as the anchor sequence, while Oct3/4, Sox2, KLF4, and c-Myc use the promoter-derived sequences of their respective genes as anchor sequences, and all TF binding elements use the CMV promoter-derived sequences shown in Figure 1.

The figures show the results when targeting the Oct3/4 gene, Sox2 gene, KLF4 gene, or c-Myc gene, respectively.

This shows the relative expression level with value 1.

Furthermore, GAPDH was used as an internal control to correct for this.

The same four oligonucleotides as in Figure 23 (Targeted Oct3/4, Sox2, KLF4, c-Myc; Targeted OSKM) were simultaneously transfected into MEF cells. After approximately 24 hours, the cells were harvested, and the mRNA levels of each gene were measured by quantitative PCR.

The control is the same as in Figure 23.

The figures show, from left to right, the relative expression levels when targeting the Oct3/4 gene, Sox2 gene, KLF4 gene, or c-Myc gene.

Furthermore, GAPDH was used as an internal control to correct for this.

The same four oligonucleotides as in Figure 23 (Targeted Oct3/4, Sox2, KLF4, c-Myc; Targeted OSKM) were simultaneously transfected into MEF cells, and alkaline phosphatase (AP) staining was performed approximately three days later (right figure).

The control is the same as in Figure 23.

The left figure shows the quantification of AP staining levels, Cont.

This shows the relative expression level with value 1.

[0012]

1 .

Gene Jack Oligonucleotide Method (1) What is the Gene Jack Oligonucleotide Method? For a target gene whose transcription is to be activated, a single-stranded nucleotide (anchor site) homologous to the upstream region sequence is created. This single-stranded nucleotide is then linked to a double-stranded nucleotide (transcription factor binding site) containing an arbitrary transcription factor binding region sequence using a spacer sequence (linker site) to create an oligonucleotide containing both a single-stranded anchor site and a double-stranded transcription factor binding site.

In this invention, this oligonucleotide is referred to as "GeneJack Oligonucleotide."

By introducing the "GeneJack Oligonucleotide" into cells using methods such as lipofection, transcription of the target gene is induced or activated.

This phenomenon is thought to occur because the anchor site in the gene jack oligonucleotide selectively recognizes and recruits a portion of the upstream region of the target gene, and the transcription factor binding site attached to the linker attracts the intracellular transcription factor to the vicinity of the target gene transcription start site, thereby inducing or activating the transcription of the target gene through the action of the transcription factor.

Methods that specifically induce or activate the transcription of such target genes are called "genejack oligonucleotide methods."

This transcriptional activation method allows for the selective and efficient activation of only target genes within cells.

Conversely, in the "gene jack oligonucleotide method" of the present invention, by using a double-stranded nucleotide containing a transcription repressor binding region instead of a double-stranded nucleotide corresponding to the transcription factor binding site, the transcription of the target gene can be selectively repressed.

Therefore, the "gene jack oligonucleotide method" of the present invention can be defined as a method for controlling the transcription of a target gene using an oligonucleotide in which a single-stranded anchor site homologous to the upstream region sequence of the target gene and a double-stranded transcription factor binding site or transcription repressor binding site are linked by a linker.

[0013]

(2) Design of Gene Jack Oligonucleotides (2-1) Target gene The target gene to be selected can be any gene that is present in the cell's genome.

In designing gene jack oligonucleotides, sequence information of 10 to 60 bases, preferably 17 to 50 bases, and more preferably 18 to 40 bases, from the upstream region of the target gene is utilized.

Sequence information for the upstream region can be obtained from publicly known sources such as databases like GenBank, academic literature, and patent documents.

If no publicly known information exists, the sequence information of the upstream region of the target gene can be appropriately identified based on the sequence information of the target gene and the conventional techniques of those skilled in the art.

Here, the term "cell" can refer to either a cell in a living organism or a cultured cell outside the organism. Typically, it refers to mammalian cells such as human cells, but given that the regulatory mechanism by promoter transcription factors is common from prokaryotes to eukaryotes, it is not limited to mammalian cells and also includes bacteria, yeast, insect cells, etc.

Target genes can include genes that are normally expressed in the body, genes with reduced expression levels, or dormant genes.

In other words, it can be used as a gene therapy tool administered to the affected area to treat diseases caused by excessively low expression levels within the body, or to increase expression levels within the body for health promotion or disease prevention.

For example, it can be used to enhance VEGF gene expression to promote vascular regeneration in infarcted areas of myocardial infarction patients, which has been considered as a form of gene therapy in the past; to treat adenosine deaminase (ADA) deficiency, a

genetic disease; to activate tumor suppressor genes in patients with cancers such as prostate cancer, breast cancer, and esophageal cancer; and to enhance gene expression in patients with diseases caused by weak expression of physiologically active proteins such as enzymes essential in the body.

Furthermore, by selecting a transcription factor gene that determines the direction of differentiation in stem cells as a target gene, it is possible to induce differentiation of stem cells in a specific direction by activating the transcription of that target gene.

For example, by targeting MyoD and Runx2, which are master regulators (transcription factors) of mesenchymal stem cells, differentiation into muscle cells and osteoblasts can be induced.

Furthermore, by selecting transcription factors necessary for somatic cell reprogramming (known as Yamanaka factors: Oct3/4, Sox2, KLF4, c-Myc) as target genes, iPS cells can be created by activating the transcription of these target genes.

[0014]

(2-2) Design Method for Gene Jack Oligonucleotides The gene jack oligonucleotide of the present invention has a structure in which a single-stranded "anchor site" and a double-stranded "transcription factor binding site" are linked by a "linker site (single-stranded)".

In addition to deoxyribonucleotides (DNA), ribonucleotides (RNA) and various other artificial nucleotides can be used as types of nucleotides in the gene jack oligonucleotide of the present invention.

Typically, DNA is used due to its availability and ease of synthesis, but for reagents administered into the body, artificial nucleotides such as cross-linked nucleotides (BNAs) are preferable because their half-life can be extended.

An "anchor site" is a single-stranded nucleotide selected from the vicinity of the transcription start region, such as the promoter region, in the upstream region of the target gene.

Specifically, these are single-stranded nucleotides of 17 to 60 bases, preferably 18 to 40 bases, that complement the base sequence approximately 30 to 950 bases, preferably 90 to 900 bases, upstream from the transcription start region.

The "transcription factor binding site" is a double-stranded nucleotide selected from any

"transcription factor binding region" that exhibits binding activity to transcription factors known to be present in target cells containing the target gene to be expressed.

The preferred base length of the "transcription factor binding region" is 20 to 100 bases, preferably about 40 to 60 bases.

If the "transcription factor binding region" is short, for example, in the case of the AP1 binding sequence, it can be tandem-linked to adjust the length to 20 to 100 base pairs.

Generally, a "transcription factor binding region" is a base sequence within the promoter region to which a transcription factor binds. Traditionally, it was considered typical for transcription factors to have a common binding region ("TATAAAA" or a similar sequence) called TATA-binding protein (TBP). However, various sequences, including CG-rich sequences, have been reported and compiled into databases.

Furthermore, each of these "transcription factor binding regions" has a specific base sequence for its respective transcription factor.

In other words, while there is specificity between the transcription factor binding region that constitutes the gene jack oligonucleotide of the present invention and the transcription factor attracted by said transcription factor binding region, there is universality in the

transcriptional induction activity of the gene and its promoter sequence activated by said transcription factor.

In this invention, this point is confirmed by using the transcriptional activity of the MyoD gene with a transcription factor binding region derived from the CMV promoter, which is originally unrelated to the MyoD promoter, an AP1 binding sequence arranged in tandem with it, and a GC/GTbox sequence.

The "linker region" is a spacer sequence commonly used as a linker, and is a single-stranded nucleotide consisting of an arbitrary base sequence that does not show complementarity with the nucleotide sequence of the adjacent region of the anchor site, and is composed of 3 to 15 bases, preferably 4 to 12 bases, and more preferably 5 to 10 bases.

[0015]

(2-3) Method for producing gene jack oligonucleotides Since the gene jack oligonucleotides of the present invention have short sequences, it is possible to produce them by separately synthesizing the single-stranded "anchor site" and "linker site (single-stranded)" and the

double-stranded "transcription factor binding site" and then joining them together. However, a preferred method is to prepare them by mixing equal amounts of a "forward oligonucleotide" corresponding to the entire length of the gene jack oligonucleotide and a complementary strand, a "reverse oligonucleotide," corresponding only to the double-stranded nucleotide region, and then annealing them.

[0016]

(3) Method for introducing gene jack oligonucleotides into cells In this example, lipofection is used, but the method is not limited to this, and various nucleic acid introduction methods such as electroporation and microinjection can also be used.

When introducing the gene jack oligonucleotide of the present invention into cells using the reverse transfection method, the target cells to be introduced are to be acted upon in a suspension state by the oligonucleotide mixed with the transfection reagent. Therefore, the target cells must be grown by suspension culture or grown in a fixed medium and then detached from the medium beforehand to be in a suspension state.

In the electroporation method, cells that have been previously cultured are detached and suspended, then mixed with a buffer such as MEF Nucleofector solution 1 (Lonza). The sample solution is then transferred to a cuvette and electroporation is performed.

[0017]

(4) Method for confirming transcriptional activation of target gene The activation of target gene transcription can be confirmed by measuring the amount of target gene mRNA in the total mRNA transcribed within the cell. Typically, quantitative PCR is used, but competitive PCR, HiCEP, microarrays, or Northern blotting may also be used.

Furthermore, if the target is a transcribed gene product, an assay system using an antibody that recognizes the final target gene product, for example, a physiologically active protein, can be used.

Furthermore, if the target gene is a transcription factor or group of transcription factors used to induce differentiation, and the cell morphology changes clearly, this can be confirmed not

only by observing the morphological change itself, but also by detecting cell surface markers after differentiation.

Quantitative PCR was performed using standard methods.

Specifically, total RNA was prepared from cells using the RNA extraction reagent Sepasol-RNA I SuperG (nacalai tesque), treated with DNase, single-stranded cDNA was synthesized using RTase (Takara Bio), and PCR was performed using SYBR Premix Ex taq (Takara Bio), a PCR premix reagent containing a fluorescent dye.

[0018]

2 .

Method for inducing differentiation of stem cells (1) Target stem cells In addition to pluripotent stem cells such as ES cells, iPS cells, and mesenchymal stem cells, any stem cell at any differentiation level may be used, including somatic stem cells such as myeloid stem cells, neural stem cells, hematopoietic stem cells, adipose stem cells, spermatogonial cells, and

erythroblasts. As long as the genes or gene groups that determine the direction of differentiation of the stem cells are known, the present invention can be applied by designating those genes or gene groups as target genes or target gene groups.

In particular, stem cells in which the direction of differentiation is determined by a single transcription factor called a master regulator are more preferable.

[0019]

(2) Differentiation induction of mesenchymal stem cells (2-1) Design of gene jack oligonucleotides for differentiation induction of mesenchymal stem cells As one embodiment of the present invention, mesenchymal stem cells in which the master regulator (transcription factor) that determines the direction of differentiation is clearly defined were selected as the model system.

The transcription factors that act as master regulators for mesenchymal stem cells are MyoD, Runx2, and PPAR γ 2, and it is known that the expression of each transcription factor induces differentiation in three directions: into muscle cells, osteoblasts, and adipocytes.

Specifically, to activate MyoD expression, for example, a gene jack oligonucleotide is introduced into mesenchymal stem cells. This gene jack oligonucleotide consists of a single-stranded nucleotide homologous to the upstream region-specific base sequence of the MyoD promoter region's transcription start sequence, and a linker region consisting of a single-stranded nucleotide that does not show complementarity with the nucleotide sequence of the adjacent region of the anchor site, which is linked to an appropriate double-stranded transcription factor binding region.

In in vitro culture, after introducing gene jack oligonucleotides into mesenchymal stem cells, MyoD expression may be sufficiently activated in a standard stem cell culture medium, and then the medium may be changed to a well-known muscle cell differentiation induction medium. However, by introducing gene jack oligonucleotides into mesenchymal stem cells and culturing them in a muscle cell differentiation induction medium simultaneously, mesenchymal stem cells can be rapidly, efficiently, and reliably differentiated into muscle cells.

These differentiated muscle cells are isolated from stem cells using methods such as flow cytometry or magnetic beads, and then used for cell transplantation to supplement muscle cells using well-known methods.

Furthermore, the gene jack oligonucleotides of the present invention can be introduced into the affected area of a living organism requiring transplantation, either directly or indirectly, such as by intravenous injection or topical application, along with mesenchymal stem cells, as a differentiation inducer containing the gene jack oligonucleotides targeting MyoD of the present invention. In such cases, it is preferable to use nucleotide analogs such as BNA as the type of nucleotide to enhance stability in vivo.

The same applies when other Runx2 genes or PPAR γ 2 genes are targeted.

[0020]

(2-2) Method for confirming differentiation induction Differentiation induction can be confirmed by observing the morphological changes that occur in differentiated cells, but more accurately, it can be confirmed by detecting surface markers specific to differentiated cells.

For example, in the case of muscle cells differentiated by MyoD, well-known muscle cell

differentiation markers such as myogenin or MHC can be identified using fluorescently labeled antibodies, and only differentiated muscle cells can be isolated using flow cytometry or magnetic beads based on these markers.

[0021]

3. iPS cell induction method (1) Differentiation induction factors for iPS cells The gene jack oligonucleotide method of the present invention is applied to each of the four genes of Yamanaka factor (Oct3/4, Sox2, KLF4, c-Myc), or to each of the three genes Oct3/4, KLF4, Sox2.

The four (or three) gene jack oligonucleotides created are introduced simultaneously or sequentially into somatic cells to be reprogrammed (dedifferentiated) (for example, patient-derived fibroblasts) using methods such as lipofection.

While it is possible to sufficiently activate the transcription of each gene in a standard cell culture medium and then switch to a stem cell medium containing a dedifferentiation-inducing agent, introducing gene jack oligonucleotides and culturing them in the

dedifferentiation-inducing medium simultaneously allows for rapid and efficient reprogramming into iPS cells.

[0022]

(2) Confirmation of somatic cell reprogramming To confirm that somatic cells have been reprogrammed, it is possible to observe the morphological changes themselves, as pluripotent stem cells undergo clear morphological changes. More precisely, it can be confirmed by detecting surface markers specific to reprogrammed stem cells.

Furthermore, this can be confirmed by testing the differentiation potential of stem cells after reprogramming.

Furthermore, iPS cells can be isolated using magnetic beads or flow cytometry, which have antibodies or lectins on their surface that recognize the undifferentiated cell marker.

4 .

Gene Therapy Pharmaceutical Compositions: As target genes for the gene jack oligonucleotides of the present invention, by selecting causative genes that cause disease due to deficiency or deletion in the body, or by selecting genes whose expression is to be enhanced in the body to treat disease, such as tumor suppressor genes, the transcription of these genes can be activated or induced. Therefore, the gene jack oligonucleotides for the present invention can be made into gene therapy pharmaceutical compositions by appropriately incorporating well-known pharmaceutically acceptable carriers and the like.

Examples of the former target genes include enzyme genes for treating diseases caused by weak expression of physiologically active proteins such as enzymes essential in the body, such as the ADA gene for treating adenosine deaminase (ADA) deficiency, a genetic disease.

Examples of the latter target genes include the VEGF gene, which promotes vascular regeneration in the infarcted area of myocardial infarction patients, and various tumor

suppressor genes for cancer patients such as prostate cancer, breast cancer, and esophageal cancer.

As a general rule, the preferred route of administration for the pharmaceutical composition containing the gene jack oligonucleotides of the present invention, which are produced using these genes as target genes, is one that can quickly reach the cells, tissues, or organs where the target gene to be activated is present.

Therefore, the most preferred method of administration is one in which the drug is directly introduced into the relevant tissue, while other suitable methods include intravenous administration, such as drip infusion, and parenteral administration, such as transdermal administration via patches.

The pharmaceutical composition of the present invention may optionally contain pharmaceutically acceptable carriers, excipients, diluents, additives, solubilizers, solvents, gelling agents, etc.

Here, the target of administration is typically humans, but it also includes non-human animals such as pets, laboratory animals, and livestock.

The amount, frequency, and duration of the pharmaceutical composition of the present

invention vary depending on the characteristics of the target gene, and also on the type and severity of the disease being treated, so it is not possible to give a general answer.

Furthermore, although it varies depending on the patient's age, weight, symptoms, etc., a preferred dosage example is 0.001 to 100 mg/day per adult, preferably 0.1 to 10 mg/day, and more preferably 1 to 10 mg/day, in terms of oligonucleotide equivalent.

However, the above-mentioned preferred dosage range is illustrative and not limiting.

Examples of administration frequency include once or multiple times a day, or continuous administration via intravenous infusion.

The duration of administration can also be determined by pharmacologists and clinicians in the relevant field using known methods based on diagnosable indicators.

The pharmaceutical compositions of the present invention can also be used in combination with other existing therapeutic and/or prophylactic agents.

Since the mechanisms of action of existing drugs and the pharmaceutical composition of the present invention are considered to be fundamentally different, additive, and in some cases synergistic, effects can be expected.

[0024]

The present invention will be described in more detail below with reference to examples, but the present invention is not limited in any way by these examples.

Other terms and concepts in this invention are based on the meanings of terms conventionally used in the art, and the various techniques used to carry out this invention, except for those techniques whose sources are specifically indicated, can be easily and reliably implemented by those skilled in the art based on known literature, etc.

Furthermore, various analyses were performed by following the methods described in the instruction manuals and catalogs of the analytical instruments, reagents, and kits used.

Furthermore, the contents of the technical documents, patent publications, and patent application specifications cited herein shall be referred to as the contents of the present invention.

[0025]

(Measurement methods, conditions, etc. used in the examples)

quantitative PCR

In this invention, the transcriptional activity of genes was measured using quantitative PCR (Real Time RT-PCR method: Murayama et al., (2008) Cell 133, 627-639), with modifications to the RNA extraction method.

First, the cells were homogenized in 1 ml of Sepazol (nacalai tesque), an RNA extraction reagent, and total RNA was extracted according to the attached instructions for use.

cDNA was synthesized from total RNA using the reverse transcriptase Revata Ace (Toyobo) and oligo-dT primers.

Quantitative PCR was performed using a Thermal Cycler Dice™ TP800 (Takara Bio) thermal

cycler and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), a premix reagent for quantitative PCR.

[0026]

Methods for introducing oligonucleotides into cells

After detaching proliferating C3H10T1/2 cells or MEF cells from the culture dish by trypsin treatment, a cell suspension was prepared in DMEM (high-glucose medium) to a concentration of 2×10^5 NER1 cells/ml. Oligonucleotides were then transfected using the lipofection reagent "Lipofectamine LTX (Invitrogen)" by reverse transfection according to the manufacturer's protocol.

[0027]

Differentiation into muscle cells

Semi-confluent cells were transferred from growth medium (containing DMEM, 10% bovine serum, and an antibiotic-antifungal mixture (nacalai tesque)) to differentiation medium (containing DMEM and 2% horse serum), and the medium was changed every 24 hours.

Immunostaining was performed five days after differentiation induction to observe the expression of nuclei (DNA) and myosin heavy chains (MHC) by immunohistochemistry.

[0028]

Differentiation into osteocytes

The day after oligonucleotide introduction, the cell culture medium was replaced with DMEM containing 10% bovine serum and an antibiotic-antifungal mixture (nacalai tesque) supplemented with 0.5 μ M dexamethasone, 25 μ g/ml ascorbic acid, and 2.5 mM glycerol phosphate.

Approximately 30 days later, C3H10T1/2 cells that had been differentiated into osteoblasts were fixed with 10% formalin.

Next, a 2% Alizarin Red solution (Sigma) was added to stain the calcified cells reddish-brown, confirming calcium deposition, an indicator of ossification.

[0029]

AP staining

After culturing, the cells were stained with alkaline phosphatase (AP), an undifferentiated marker, using the RED-Color AP Staining Kit (SBI) according to the manufacturer's protocol.

[0030]

(Example 1) Transcriptional activation of target genes by gene jack oligonucleotides (1-1)

Transcriptional activation of MyoD mRNA In this example, in order to investigate the action of gene jack oligonucleotides, we targeted "MyoD," a transcription factor called a master regulator that determines the differentiation direction into skeletal muscle cells in mesenchymal stem cells, and verified whether or not it selectively enhances the transcriptional activity of the MyoD gene.

Therefore, a gene jack oligonucleotide was constructed by linking a single-stranded nucleotide (anchor sequence) homologous to a portion of the MyoD promoter sequence with a double-stranded nucleotide sequence containing transcription factor binding regions (TF (Transcriptional Factor) binding elements) derived from the CMV minimal promoter, using a linker (a 5-base T) (Figure 1, bottom).

Here, regarding the anchor sequences, the original sequence for each is denoted as Targeted Oligo (T), and the random sequence used as a control is denoted as Control Oligo (C).

Anchor sequence (C) is a part of the Luciferase gene sequence, and Blast search has confirmed that it is a sequence not present in the mouse genome.

TF binding elements (WT CMV) are sequences containing transcription factor binding regions

derived from the CMV minimal promoter, while TF binding elements (SC CMV) are sequences in which the WT CMV sequence has been randomly rearranged and mutated.

The upper part of Figure 1 schematically illustrates the action of gene jack oligonucleotides.

The gene jack oligonucleotides designed as shown in the lower part of Figure 1 were prepared by dissolving and mixing equal amounts of the forward and reverse of the following synthetic oligonucleotides in TE buffer (Tris-EDTA buffer) to 100 μ M and then annealing them.

[0031]

Forward Oligo Nucleotide C&WT CMV 5'-

TGAACTTCCCGCCGCCGTTGTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGG/

[0032]

Reverse Oligonucleotide Targeted CMV minimal _R (WT CMV) 5'-

GACCTCCACCGTACACGCCTACCGCCCATTTGCGTCAATGGGGCGGAG-3' (SEQ ID NO: 4) Mutated

CMV minimal _R (SC CMV) 5'-

TACCTTTGCAACGCATCGCCTACCGGCGAGCACCCAGCGTGAGGCGTC-3' (SEQ ID NO: 5)

[0033]

Proliferating C3H10T1/2 cells (mesenchymal stem cells) were detached from the culture dish by trypsin treatment. A cell suspension was then prepared in DMEM (high-glucose medium) to a concentration of 2×10^5 cells/ml. Oligonucleotides were then transfected using "Lipofectamine LTX (Invitrogen)" by reverse transfection according to the manufacturer's protocol.

Cells were collected after 24 hours, and the amount of MyoD mRNA was measured by quantitative PCR (corrected using GAPDH as an internal control).

The same applies below.) We investigated the transcriptional activation effect on the target MyoD gene.

The C3H10T1/2 cells used in this example were obtained from the Human Science Promotion Foundation Research Resource Bank. The C3H10T1/2 cells were maintained at 37°C under 5% CONER conditions in DMEM (Dulbecc's modified Eagle medium, Sigma) containing 10% bovine serum and an antibiotic-antifungal mixed solution (nacalai tesque).

The culture conditions for these cells are the same in the following examples.

Next, the amount of MyoD mRNA was measured by quantitative PCR using the primers listed below.

MyoD fw: 5'-TGAGCAAAGTGAATGAGGCCTTCG-3' (SEQ ID NO: 6) MyoD rv: 5'-TGCAGACCTTCGATGTAGCGGAT-3' (SEQ ID NO: 7) The following primers were used for GAPDH amplification in the control.

GAPDH fw: 5'-CAGAACTACATCCCTGCATCC-3' (Sequence ID 8) GAPDH rv: 5'-CCACCTTCCTGATGTCATCA-3' (Sequence ID 9)

[0034]

The results of transcriptional activation of the MyoD gene showed that only oligonucleotides (T&WT CMV) in which both the anchor sequence and TF binding elements were target sequences and transcription factor binding region sequences derived from the CMV minimal promoter increased the amount of MyoD mRNA.

This demonstrates that the gene jack oligonucleotide of the present invention can increase the amount of mRNA in the target gene (Figure 2).

[0035]

(1-2) Transcriptional activation of Runx2 mRNA The same experiment as in (1-1) above was

performed using oligonucleotides designed in which the target gene was replaced with the Runx2 gene.

Specifically, the target gene was changed to Runx2, and the anchor sequence used in T&SC CMV and T&WT CMV was a sequence corresponding to the upstream region of Runx2 (-1469 to -1488). Equal amounts of the following synthetic oligonucleotides were dissolved and mixed in 100 μ M TE buffer (Tris-EDTA buffer) and prepared by annealing.

Forward Oligo nucleotides (Note: For C&WT CMV, the sequence shown in SEQ ID NO: 1 was used; for Targeted CMV minimal _R (WT CMV), the sequence shown in SEQ ID NO: 4 was used; and for Mutated CMV minimal _R (SC CMV), the sequence shown in SEQ ID NO: 5 was used.)

) T&SC CMV 5'-

CCATCGCTCCCAACTAATGATTTTTGACGCCTCACGCTGGGTGCTCGCCGGTGAGGCGATGCGTTGCAA

[0036]

These C&WT CMV, T&SC CMV, and T&WT CMV oligonucleotides were transfected into C3H10T1

/2 cells. After 24 hours, the cells were harvested, and Runx2 mRNA was measured by quantitative PCR using the following primers.

Runx2 fw: 5'-ACAACCACAGAACCACAAG-3' (Sequence ID 12) Runx2 rv: 5'-
TCTCGGTGGCTGGTAGTGA-3' (Sequence ID 13)

[0037]

The results showed that the amount of Runx2 mRNA could be increased by oligonucleotides designed to target Runx2 (Figure 3).

This indicates that the mechanism of target gene activation by oligonucleotides is not limited to MyoD.

[0038]

(1-3) Transcriptional activation of Insulin II or rRNA The same experiment as in (1-1) above was performed using oligonucleotides designed by replacing the target gene with Preproinsulin gene II (Insulin-II) or an rRNA gene.

Specifically, Targeted is an oligonucleotide formed by linking the WT CMV and linker sequence from Example 1 to an Anchor sequence in which the target gene has been changed to Insulin-II or an rRNA gene, and Control (Cont.

) corresponds to the C&WT CMV of Example 1.

Equal amounts of the following synthetic oligonucleotides, in their forward and reverse directions, were dissolved and mixed in TE buffer (Tris-EDTA buffer) to a concentration of 100 μ M and prepared by annealing.

Targeted (Insulin-II) 5'-

AACTGGTTCATCAGGCCATCTTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGA

[0039]

These insulin-II and rRNA oligonucleotides were transfected into Hepa1-6 cells, and the cells were harvested after 24 hours. Insulin-II RNA and rRNA were then measured quantitatively by PCR using the primers listed below.

Insulin-II fw: 5'-GGAGACCTTAATGGGCCAAACAG-3' (SEQ ID NO: 16) Insulin-II rv: 5'-GCCCACCAGCTTTATAGTCCAAA-3' (SEQ ID NO: 17) rRNA fw: 5'-TTTTTGGGGAGGTGGAGAGTC-3' (SEQ ID NO: 18) rRNA rv: 5'-CTGATACGGGCAGACACAGAAC-3' (SEQ ID NO: 19)

[0040]

The results showed that the amount of mRNA in either insulin-II, a type of hormone, or rRNA, which constitutes the ribosome responsible for translation, could be increased by oligonucleotides designed as target genes (Figure 4).

This indicates that the mechanism of target gene activation by oligonucleotides is not limited to transcription factors.

[0041]

(Example 2) Examination of the length of the "anchor site" (2-1) In the case of MyoD In this example, we examined the length of the upstream region-specific nucleotide sequence (Anchor sequence) of the target gene necessary for the activation of the target gene.

Similar to Example 1, the sequence of the linker region and the double-stranded transcription factor binding region was not changed, but the length of the anchor sequence was altered for the Targeted and Cont.

Gene jack oligonucleotides were synthesized, each prepared to contain 30 to 10 bases (30, 25, 20, 18, 16, 14, and 10 bases).

Of these, Targeted and Cont. correspond to "20 base pairs".

This corresponds to the T&WT CMV and C&WT CMV of Example 1.

The forward oligonucleotides used for the 30 and 25 base cases are shown below.

Note that "20 bases" refers to Targeted and Cont.

This corresponds to Sequence ID No. 3 and Sequence ID No. 1, and in the case of "18, 16, 14 and 10 bases", it is a base sequence obtained by sequentially deleting 2 or 4 bases from the 5' end of Sequence ID No. 3 and Sequence ID No. 1.

In the following, unless otherwise specified, when referring to the sequence of a gene jack oligonucleotide, it refers to the base sequence of the forward oligonucleotide.

(Note: Cont. consists of 30 bases and 25 bases.)

For this, instead of using a sequence derived from Luciferase, we used a randomly rearranged sequence of the Targeted cells.

The transcriptional activity of MyoD mRNA was measured under the same conditions as in Example 1.

[0042]

30 bases Cont.

5'-

CTGTGGCCCGGTCTACGCCAGCTATACTCCTTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTG

5'-

CTAGCCGGTCTCTCGCTGCGATCGCTTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGC

[0043]

As a result, it was revealed that in the case of MyoD, an anchor sequence length of 18 bases or more increases the amount of MyoD mRNA.

This suggests that anchor sequences of 18 bases or more are preferable for transcriptional activation of target genes (Figure 5).

[0044]

(2-2) In the case of Runx2, the results of Example (2-1) were used to verify Runx2 as the target gene, and the Targeted and Cont. of the 20-base Anchor sequences corresponding to T&WT CMV and C&WT CMV used in Example (1-2) were analyzed.

Gene jack oligonucleotides were synthesized by sequentially deleting two bases from the 5' end to obtain lengths of 18 and 16 bases.

In Figure 6, Targeted and Cont. correspond to "20 bases".

This uses sequence numbers 11 and 1.

Using these gene jack oligonucleotides, transcriptional activity was measured in the same manner as in Example (2-1) above.

[0045]

As a result, it was revealed that in the case of Runx2 as well, an anchor sequence length of 18 bases or more increases the amount of Runx2 mRNA.

These results suggest that anchor sequences of 18 bases or more are preferable for transcriptional activation of target genes (Figure 6).

[0046]

(2-3) Verification of the "anchor site" sequence in the case of MyoD As shown in (2-1) above, in

the case of MyoD, at least an 18-base sequence in the anchor sequence is required as the "anchor site". Therefore, we decided to verify whether the length of "18 bases" is important, or whether it is important that it complements at least 18 consecutive bases in the anchor sequence.

Using the anchor sequence corresponding to the 20-base T&WT CMV of Example 1, which showed the highest transcriptional activity, a gene jack oligonucleotide was prepared by introducing a 4-base mutation (the forward oligonucleotide is shown below), and the MyoD gene transcription level was measured in the same manner as in (2-1) above.

Note that in the case of 20 bases (Targeted), see Sequence ID 3, Cont.

This uses sequence number 1.

[0047]

4MT (The underlined part is where the mutation was introduced into sequence number 3.)

) #1 5'-

ATTGGCTTGAGGCGCCGGGCTTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGG.

[0048]

As a result, none of the mutated oligonucleotides were able to increase the amount of MyoD mRNA.

From this, it can be understood that, in order to activate the transcription of a target gene, it is important for the "anchor site" sequence to be completely complementary to a sequence of a certain length or longer within the anchor sequence.

Furthermore, in the case of MyoD, it was shown that it is important that the sequence is at least 18 consecutive base pairs that are completely complementary (Figure 7).

[0049]

(Example 3) Investigation of Linker Length Effective for Transcriptional Activation (3-1) In the case of MyoD In the gene jack oligonucleotide (T&WT CMV: SEQ ID NO: 3) targeting MyoD constructed in (1-1) of Example 1, a 5-base thymine (T) sequence was used as the spacer sequence of the linker portion.

We investigated whether a linker is necessary for transcriptional activation, and if so, what its optimal length is, by varying its length from 0 to 10 bases (in Figure 8, T5's Targeted corresponds to the case using SEQ ID NO: 3, and T5's Cont.

This corresponds to sequence number 1).

The results showed that the longest linker length was 5 bases, and that linkers of 5 bases or longer increased the amount of MyoD mRNA. Conversely, it was revealed that short linkers of 3 bases or less did not have a transcriptional activation effect.

This suggests that linkers are essential and require at least four base pairs (Figure 8).

[0050]

(3-2) In the case of Runx2, the effectiveness of Linker was also examined in Runx2.

The gene jack oligonucleotide (T&WT CMV: SEQ ID NO: 11) targeting Runx2, constructed in the above examples (1-2), was prepared by varying the linker length from 0 to 10 bases and used (in Figure 9, T5 Targeted corresponds to the case where SEQ ID NO: 11 is used, and T5 Cont.

This corresponds to sequence number 1).

As a result, similar to the results in (3-1) above, it became clear that only linker lengths of 5 or 10 bases increased the amount of Runx2 mRNA, indicating that even when targeting Runx2, linkers are essential and at least 4 bases are required (Figure 9).

[0051]

(Example 4) Investigation of the optimal upstream location to select as the "anchor site"

Using the case where MyoD is targeted as a model, the optimal upstream location for determining the anchor sequence for the target gene was investigated.

In Example 1 (1-1), the anchor sequence used to construct the gene jack oligonucleotide (T&WT CMV: SEQ ID NO: 3) was the sequence of 20 bases (up to position 77) from position 96 upstream of the transcription start site of MyoD. However, in this example, we used a single-stranded "anchor sequence" of 20 bases from positions 5 and 25 further downstream, and positions 322, 790, 953, 23321, and 23414 upstream.

The MyoD promoter sequence used was obtained from the database (DBTSS) (NM_010866), and the positions were counted based on that sequence information.

The respective base sequences are shown below as -5, -25, -322, -790, -953, -23321, and -23414.

Note that -96 is sequence number 3, and Cont.

This is sequence number 1.

[0052]

-5 5'-

AAGCTAGGGGCCAGGACGCCTTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGG,

[0053]

As a result, it was revealed that if the "Anchor sequence" of the GeneJack oligonucleotide is located between position -96 and -790 upstream of the target (MyoD) gene, the mRNA of MyoD can be increased by the GeneJack oligonucleotide.

This suggests that there is an optimal location for the anchor sequence in the upstream region of the target gene (Figure 10).

[0054]

(Example 5) Verification using chromatin immunoprecipitation To investigate whether the oligonucleotide was indeed recruited to the target gene promoter, an investigation was conducted using chromatin immunoprecipitation.

Specifically, in the gene jack oligonucleotide (T&WT CMV: SEQ ID NO: 3) constructed in Example 1 (1-1) above, the CMV minimal promoter sequence end of the "transcription factor binding region" was biotin-modified.

Cont.

This is sequence number 1, and each reverse oligonucleotide (sequence number 4) was also used after biotin modification of the 5' end.

The biotin-modified oligonucleotides described above were transfected into C3H10T1/2 cells,

and the cells were harvested after 24 hours. Chromatin immunoprecipitation experiments were then performed using streptavidin beads.

Chromatin immunoprecipitation was performed following the method of Murayama et al. (Murayama et al., (2008) Cell 133, 627-639), with some modifications.

TetraLink (trademark) Tetrameric is an avidin-containing reagent.

Biotinylated oligonucleotide complexes were immunoprecipitated using Avidin Resin (Promega).

Next, the precipitated product was detected by quantitative PCR.

We used primers targeting the upstream region of the MyoD gene, from -375 to -545, which is detectable for gene jack oligonucleotide recruitment, and primers targeting the region from -8539 to -8779, which is more than the length of the DNA fragment used for immunoprecipitation (approximately 0.5 kb) away from the target sequence, as a negative control group.

[0055]

The primer sequences used in the quantitative PCR of this example are as follows: -375 to -545 fw: 5'-CGCCCCCAGCCTCCCTTTCCAG-3' (SEQ ID NO: 34) -375 to -545 rv: 5'-TGTCAGAGGTGTGGTGAAGAAA-3' (SEQ ID NO: 35) -8539 to -8779 fw: 5'-GTCAGCGCTCTACATAGGGT-3' (SEQ ID NO: 36) -8539 to -8779 rv: 5'-TAGCTTCCCCTCATGCTCTG-3' (SEQ ID NO: 37)

[0056]

As a result, only in the case of gene jack oligonucleotides (T&WT CMVs) that are labeled as "Targeted" with the upstream region of the MyoD gene as the "Anchor sequence," was it possible to detect amplification products derived from significant precipitates using primers targeting the -375 to -545 region near the transcription start site of MyoD.

On the other hand, when the precipitated product was detected using a primer targeting a

region more than 8000 base pairs upstream from the -96 to -77 position of the MyoD gene, which was designated as the "Anchor sequence" of the Targeted gene, Cont.

No significant difference was observed.

This suggests that gene jack oligonucleotides are specifically recruited to regions corresponding to the "Anchor sequence" designed in the upstream region of the target gene (Figure 11).

[0057]

(Example 6) Examination of the "transcription factor binding site" sequence - 1 (6-1) In the case of MyoD The "transcription factor binding site" of the double strand of gene jack oligonucleotide used in each of the above examples contains a transcription factor binding sequence derived from the CMV minimal promoter.

To verify that MyoD transcription increases when transcription factors actually bind to the "transcription factor binding site," we investigated the effect of oligonucleotides containing a

transcription factor binding sequence derived from the CMV minimal promoter (WT CMV) and oligonucleotides containing a sequence in which a mutation has been introduced into the said sequence (MT CMV) (Targeted) on increasing MyoD mRNA transcription.

The Targeted WT CMV on the left side of Figure 12 is indicated by sequence number 3, and Cont.

This is indicated by sequence number 1.

The MT CMV on the right side of Figure 12 is the result of introducing a mutation, and the sequence used is as follows.

[0058]

MT CMV Cont.

5'-

TGAACTTCCCGCCGCCGTTGTTTTCTCCACACCATTGACGCAAATGAACGGTAGGCGTGTACGGTGGGA

[0059]

As a result, in the case of "MT CMV," in which a mutation was introduced into the transcription factor binding sequence, there was almost no increase in MyoD mRNA.

From the above, it was shown that transcription factors are attracted to the CMV minimal promoter in a manner dependent on the transcription factor binding sequence present, and that the transcriptional activation function by oligonucleotides is exerted (Figure 12).

[0060]

(6-2) In the case of Runx2, the same experiment as in (6-1) above was performed, but with Runx2 as the target gene.

At that time, the Targeted and Cont. of WT CMV.

This is shown in sequence numbers 11 and 1, and the sequence used in the case of MT CMV Targeted is as follows: Cont.

This refers to the MT CMV Cont. in the case of the above MyoD.

It is identical to [the other one].

MT CMV Targeted 5'-

CCATCGCTCCCAACTAATGATTTTTCTCCACACCATTGACGCAAATGAACGGTAGGCGTGTACGGTGGGA

[0061]

As a result, similar to the above findings, no increase in Runx2 mRNA was observed with oligonucleotides containing a CMV minimal promoter in which a mutation was introduced into the transcription factor binding sequence.

This indicates that even when Runx2 is targeted, the transcriptional activation function by oligonucleotides is exerted in a manner dependent on the transcription factor binding sequence present in the CMV minimal promoter (Figure 13).

[0062]

(Example 7) Examination of the "transcription factor binding site" sequence - 2 (7-1) In the case of MyoD In all of the gene jack oligonucleotides in the above examples, the "transcription factor binding site" is a transcription factor binding sequence derived from the "CMV minimal promoter".

Therefore, in this example, in order to verify that transcriptional activation effects can be observed even when other transcription factor binding sequences are used as the "transcription factor binding site," the effects of oligonucleotides in which typical transcription factor binding sequences, such as the "transcription factor AP-1 binding sequence (GACTCAT)" or the "Sp/KLF family binding sequence (GC/GT box)," were tandem-bound were investigated instead of the CMV minimal promoter.

The sequence used is shown below.

AP1 Control 5'-

TGAACTTCCCGCCGCCGTTGTTTTTTGACTCATGACTCATGACTCATGACTCATGACTCATGACTCATGAC

[0063]

As a result, it was revealed that oligonucleotides increase the mRNA level of target genes even when AP-1 binding sequences or Sp/KLF family binding sequences are used as transcriptional activation sequences (Figure 14).

From the above, it was shown that TF binding elements in oligonucleotides are not limited to those derived from the CMV minimal promoter, but can induce an increase in target gene mRNA if they are transcription factor binding sequences.

[0064]

(7-2) In the case of Runx2, when the same experiment as in (7-1) above was performed with Runx2 as the target gene, Runx2 transcription was activated by oligonucleotides linked to the AP-1 sequence, similar to the results for MyoD in Figure 14.

These results demonstrate that, not limited to MyoD, TF binding elements in oligonucleotides can induce an increase in target gene mRNA if they are transcription factor binding sequences, even if they are not derived from the CMV minimal promoter (Figure 15).

[0065]

(Example 8) Duration of transcriptional activity of gene jack oligonucleotides (8-1) In the case of MyoD In this example, we investigated how long the transcriptional activity of the gene jack oligonucleotide of the present invention lasts.

Gene jack oligonucleotides targeting the MyoD promoter constructed in Example 1 (1-1)

(Targeted in Figure 16 is SEQ ID NO: 3, Cont.

C3H10T1/2 cells were transfected with Sequence ID No. 1, and the cells were collected after 24 hours, 72 hours, and 120 hours. MyoD mRNA was measured by quantitative PCR.

As a result, the transcriptional activation ability of the oligonucleotide of the present invention was highest after 24 hours, then decreased, and completely disappeared after 120 hours.

These results indicate that the effects of oligonucleotides are transient (Figure 16).

[0066]

(8-2) In the case of Runx2, the same experiment as in (8-1) above was performed, but with Runx2 as the target gene.

The gene jack oligonucleotide used was Sequence ID No. 11 (Cont.

This is sequence number 1).

As a result, the same results as in the case of MyoD in Figure 16 were obtained.

This indicates that the transcriptional activation ability of gene-jack oligonucleotides of the present invention is transient, not limited to oligonucleotides targeting MyoD (Figure 17).

[0067]

(8-3) Transcriptional activation of MyoD by BNA (LNA) The same experiment as in (1-1) of Example 1 above was performed by replacing DNA with cross-linked nucleic acid (BNA) 2',4'-BNA (LNA (Gene Design Inc.)) and using oligonucleotides designed to target MyoD.

Specifically, Targeted is the three locations of T&WT CMV in Example 1, and Control (Cont.

This example uses an oligonucleotide in which three positions of the C&WT CMV from Example 1 are replaced with 2',4'-BNA (LNA).

These oligonucleotides were transfected into C3H10T1/2 cells, and the cells were harvested after 24 hours. The amount of extracted MyoD mRNA was measured by quantitative PCR.

Control 5'-TGA ACTTCCCGCCGCGTTGt(L)Tt(L)Tt(L)

GACGCCTCACGCTGGGTGCTCGCCGGTGAGGCGATGCGTTGCAAAGGTA-3' (SEQ ID NO: 47)

Targeted 5'-ATTGGCTTGAGGCGCCCCCGt(L)Tt(L)Tt(L)

GACGCCTCACGCTGGGTGCTCGCCGGTGAGGCGATGCGTTGCAAAGGTA-3' (SEQ ID NO: 48) n(L) =

LNA

[0068]

The results showed that MyoD mRNA levels could be increased by 2',4'-BNA (LNA) oligonucleotides (Figure 18).

This suggests that activation of target genes by oligonucleotides can be induced using artificial nucleic acids.

[0069]

(Example 9) Selective differentiation of mesenchymal stem cells (9-1) Observation of differentiation marker expression From the results of each of the above examples, the gene jack oligonucleotides of the present invention were demonstrated to increase transcriptional activity in mesenchymal stem cells (C3H10T1/2 strain). MyoD and Runx2, which were selected as targets in this case, are transcription factors that act as master regulators determining the direction of differentiation of mesenchymal stem cells (Figure 19).

In this example, we demonstrate that mesenchymal stem cells (C3H10T1/2 strain) can be selectively differentiated into muscle cells or osteocytes, respectively, by expressing the target MyoD or Runx2 using gene jack oligonucleotides.

To this end, C3H10T1/2 cells were first transfected with oligonucleotides targeting each gene,

and after 24 hours, the mRNA levels of MyoD and Runx2 were measured by quantitative PCR (Figure 20, left column).

Note that in the diagram, Cont.

MyoD in Targeted uses sequence number 1, MyoD uses sequence number 3, and Runx2 uses sequence number 11.

The results showed that oligonucleotides targeting each gene selectively activate only the targeted gene.

Next, C3H10T1/2 cells were transfected with oligonucleotides, and after 24 hours, the culture medium of the cells was changed to the respective differentiation medium, and the expression of differentiation marker genes was observed.

Specifically, in cells with increased MyoD expression, selective induction of the myocyte differentiation markers "Myogenin" and "MHC" genes was observed, and in cells with increased Runx2 expression, selective induction of the osteoblast differentiation markers "ALP" and "OC" genes was observed (Figure 20, middle and right columns).

Here, the "MHC" gene is a myosin heavy chain gene specific to muscle cells, while the "Myogenin" gene is an indicator gene for myotubes.

As a result, it was revealed that oligonucleotides targeting each gene specifically induce the differentiation of stem cells related to that targeted gene.

The induction of expression of these differentiation marker genes was measured by quantitative PCR using the following primer set.

Myogenin fw: 5'-CAACCAGGAGGAGCGCGATCTCCG-3' (SEQ ID NO: 49) Myogenin rv: 5'-AGGCGGCTGTGGGAGTTGCATTCACT-3' (SEQ ID NO: 50) MHC fw: 5'-CATCGTCCAGGAAGCCC-3' (SEQ ID NO: 51) MHC rv: 5'-TTCGCTTATGATTTTGGTGTGAACC-3' (SEQ ID NO: 52) ALP fw: 5'-ATCTTTGGTCTGGCTCCCATG-3' (SEQ ID NO: 53) ALP rv: 5'-TTTCCCGTTCACCGTCCAC-3' (SEQ ID NO: 54) OC fw: 5'-GACAAGTCCCACACAGCAAC-3' (SEQ ID NO: 55) OC rv: 5'-GGACATGAAGGCTTTGTCAG-3' (SEQ ID NO: 56)

[0070]

(9-2) Differentiation into muscle cells In this example, we investigated whether oligonucleotides can induce changes such as cell differentiation and morphological changes by activating target genes.

After transfection of C3H10T1/2 cells with an oligonucleotide targeting MyoD (T&WT CMV: SEQ ID NO: 3), the cells, which were in a semi-confluent state, were replaced from growth medium (containing DMEM, 10% bovine serum, and an antibiotic-antifungal mixture (nacalai tesque)) to differentiation medium (containing DMEM, 2% horse serum) after 24 hours, and the medium was changed every 24 hours.

Immunostaining was performed 5 days after differentiation induction to observe the expression of nuclei (DNA) and myosin heavy chains (MHC) by immunohistochemistry (Figure 21).

In addition, similar experiments were conducted using the Control (SEQ ID NO: 1) and Runx2 (SEQ ID NO: 11) oligonucleotides for comparison. Only when MyoD-targeted oligonucleotides were used did myosin heavy chain (MHC) expression get induced, and multinucleated cells characteristic of muscle cells were observed, confirming that differentiation into muscle cells occurred.

[0071]

(9-3) Differentiation into osteocytes In this example, we investigated whether osteogenic differentiation is induced by oligonucleotides, using the same oligonucleotides (SEQ ID NOs: 1, 3, 11) as in Example (9-2).

After transfection of each oligonucleotide into C3H10T1/2 cells, the cell culture medium was replaced 24 hours later with DMEM containing 10% bovine serum and an antibiotic-antifungal mixed solution (nacalai tesque) supplemented with 0.5 μ M dexamethasone, 25 μ g/ml ascorbic acid, and 2.5 mM glycerol phosphate.

Approximately 30 days later, calcium deposition, an indicator of ossification, was confirmed by Alizarin Red S (Sigma) staining. Alizarin red staining was observed only when oligonucleotides targeting Runx2 were introduced into the cells (Figure 22).

This revealed that oligonucleotides targeting Runx2 selectively induce osteogenic differentiation.

[0072]

(Example 10) iPS cell induction (10-1) MEF cells were transfected with oligonucleotides targeting iPS cell induction transcription factors (Targeted Oct3/4, Sox2, KLF4 and c-Myc).

The base sequence of the oligonucleotide used in that process is shown below.

Note: Cont.

This example uses Sequence ID No. 1, similar to Example 1.

Furthermore, mouse embryonic fibroblasts (MEFs) were used after being maintained at 37°C under 5% CO₂ conditions in DMEM (Dulbecc's modified Eagle medium, Sigma) containing 10% bovine serum and an antibiotic-antifungal mixed solution (nacalai tesque), similar to the C3H10T1/2 cells used in Example 1, etc. (The same procedure was followed in the following experiments.)

Cells were harvested 24 hours after transfection, and the mRNA levels of Oct3/4, Sox2, KLF4, and c-Myc were measured by quantitative PCR.

The results showed that each oligonucleotide activated the transcription of its target gene (Figure 23).

Targeted Oct3/4 5'-

GGAAGCAGGGTATCTCCATCTTTTCTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGG/

[0073]

The following primers were used to quantitatively measure the mRNA levels of Oct3/4, Sox2, KLF4, and c-Myc using PCR.

Oct3/4 fw: 5'-TTCATGTCCTGGGACTCCTC-3' (Sequence ID 61) Oct3/4 rv: 5'-

CACGAGTGGAAAGCAACTCA-3' (Sequence ID 62) Sox2 fw: 5'-GGCAGCTACAGCTAGATGCAGGAGC-3' (Sequence ID 63) Sox2 rv: 5'-CTGGTCATGGAGTTGTACTGCAGG-3' (Sequence ID 64) KLF4 fw:

5'-TCACACAGGCGAGAAACCTT-3' (Sequence ID 65) KLF4 rv: 5'-GAGCGGGCGAATTTCCA-3'
(Sequence ID 66) c-Myc fw: 5'-TGACCTAACTCGAGGAGGAGCTG-3' (Sequence ID 67) c-Myc rv: 5'-
AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC-3' (Sequence ID 68)

[0074]

(10-2) The four oligonucleotides used in (10-1) (Targeted Oct3/4, Sox2, KLF4, c-Myc; Targeted OSKM) were transfected into the same MEF cells, and the cells were harvested 24 hours later. The mRNA levels of Oct3/4, Sox2, KLF4, and c-Myc were measured by quantitative PCR.

The results showed that each oligonucleotide could simultaneously activate the transcription of four different target genes (Figure 24).

[0075]

(10-3) The four oligonucleotides used in (10-1) (Targeted Oct3/4, Sox2, KLF4, c-Myc; Targeted OSKM) were transfected into MEF cells, and after 3 days, the amount of alkaline phosphatase (AP), an indicator of undifferentiated cells, was quantified (Figure 25 left), and the amount of protein accumulation was examined by AP staining (Figure 25 right).

As a result, cells transfected with oligonucleotides targeting Oct3/4, Sox2, KLF4, and c-Myc showed high expression of AP protein (Figure 25).

Based on these results, we successfully dedifferentiated MEF cells by simultaneously activating four transcription factors (Oct3/4, Sox2, KLF4, c-Myc) in MEF cells using oligonucleotides, suggesting that the oligonucleotides of the present invention are also effective in establishing iPS cells.

[0076]

The "Gene Jack Oligonucleotide Method" of the present invention is a technology that can induce the expression of desired target genes without the risk of cancer or side effects such as

cytotoxicity. Therefore, it can be used as a gene therapy tool for diseases caused by various gene expression reductions, replacing conventional systems that control gene expression, such as viral vectors and non-viral vectors.

Furthermore, by inducing the expression of transcription factors that have the ability to differentiate mesenchymal stem cells and other types of stem cells in a specific direction, it is possible to induce the differentiation of various stem cells into specific organ cells.

Conversely, by applying this to the induction of the expression of transcription factors involved in the reprogramming of somatic cells in patients, it is possible to establish iPS cells, thus enabling organ transplantation without the risk of cancer.

Thus, significant contributions to the fields of gene therapy and regenerative medicine are expected in the future.

[0077]

SEQ ID NO: 1: C&WT CMV SEQ ID NO: 2: T&SC CMV SEQ ID NO: 3: T&WT CMV SEQ ID NO: 4:

Targeted CMV minimal_R (WT CMV) SEQ ID NO: 5: Mutated CMV minimal_R (SC CMV) SEQ ID

NO: 6: MyoD fw SEQ ID NO: 7: MyoD rv SEQ ID NO: 8: GAPDH fw SEQ ID NO: 9: GAPDH rv SEQ ID
NO: 10: T&SC CMV SEQ ID NO: 11: T&WT CMV SEQ ID NO: 12: Runx2 fw SEQ ID NO: 13: Runx2 rv
SEQ ID NO: 14: Targeted (Insulin-II) SEQ ID NO: 15: Targeted (rRNA) SEQ ID NO: 16: Insulin-II fw
SEQ ID NO: 17: Insulin-II rv SEQ ID NO: 18: rRNA fw SEQ ID NO: 19: rRNA rv SEQ ID NO: 20: MyoD
30b-Cont.

Sequence ID 21: MyoD 30b-Targeted; Sequence ID 22: MyoD 25b-Cont.

SEQ ID NO: 23: MyoD 25b-Targeted SEQ ID NO: 24: 4MT#1 SEQ ID NO: 25: 4MT#2 SEQ ID NO: 26:
4MT#3 SEQ ID NO: 27: MyoD (-5) SEQ ID NO: 28: MyoD (-25) SEQ ID NO: 29: MyoD (-322) SEQ ID
NO: 30: MyoD (-790) SEQ ID NO: 31: MyoD (-953) SEQ ID NO: 32: MyoD (-23321) SEQ ID NO: 33:
MyoD (-23414) SEQ ID NO: 34: -375 to -545 fw SEQ ID NO: 35: -375 to -545 rv SEQ ID NO: 36:
-8539 to -8779 fw SEQ ID NO: 37: -8539 to -8779 rv SEQ ID NO: 38: MT CMV Cont.

(MyoD) Sequence ID 39: MT CMV Targeted (MyoD) Sequence ID 40: MT CMV Targeted (Runx2)
Sequence ID 41: AP1 Control (MyoD) Sequence ID 42: AP1 Targeted (MyoD) Sequence ID 43: GC
/GT box Control (MyoD) Sequence ID 44: GC/GT box Targeted (MyoD) Sequence ID 45:
Targeted AP1 R Sequence ID 46: Targeted GC/GT box R Sequence ID 47: LNA Control Sequence
ID 48: LNA Targeted Sequence ID 49: Myogenin fw Sequence ID 50: Myogenin rv Sequence ID
51: MHC fw Sequence ID 52: MHC rv Sequence ID 53: ALP fw Sequence ID 54: ALP rv Sequence
ID 55: OC fw Sequence ID 56: OC rv Sequence ID 57: Targeted Oct3/4 Sequence ID 58: Targeted

Sox2 Sequence ID 59: Targeted KLF4 SEQ ID NO: 60: Targeted c-Myc SEQ ID NO: 61: Oct3/4 fw
SEQ ID NO: 62: Oct3/4 rv SEQ ID NO: 63: Sox2 fw SEQ ID NO: 64: Sox2 rv SEQ ID NO: 65: KLF4 fw
SEQ ID NO: 66: KLF4 rv SEQ ID NO: 67: c-Myc fw SEQ ID NO: 68: c-Myc rv