medium developed in recent years, the frequency of emergence of deviated cells is suppressed to some extent. Deviated cells, however, still spontaneously emerge, and in the case where the cells emerge, it is still essential to remove colonies that contain deviated cells.

[0005]

In order to maintain the undifferentiated state, colonies containing deviated cells are carefully removed by pipetting operations under a microscope apparatus upon subculture. A device performing such an operation of removing colonies, for example, an observing device combined with pipetting performed by robot handling, has been developed as well.

[0006]

Furthermore, Patent Document 1 discloses culture of pluripotent stem cells in the presence of activin for proliferating pluripotent stem cells such as iPS cells while maintaining undifferentiated states thereof.

[Prior Art Documents]

[Patent Document]

[0007]

[Patent Document 1] JP2012-143229A

[SUMMARY]

[Problem to be Solved by the Invention]

[0008]

As described above, a deviation phenomenon tends to occur in subculture of pluripotent stem cells such as human iPS cells, and maintaining the undifferentiated state is difficult. After several times of subculture, it is likely that the deviation phenomenon occurs in many iPS cell colonies and the colonies can become colonies that contain cells deviated from the undifferentiated state. Complicated operations such as careful culturing and careful colony sorting are therefore indispensable. From the viewpoint of promoting the stem cell industry as well, a method for maintaining undifferentiated states of pluripotent stem cells, which involves less complicated operations and can be performed by a non-expert, has been desired.

[0009]

Furthermore, with respect to pluripotent stem cells such as human iPS cells, in terms of practical applications thereof to regenerative medicine and drug discovery research, a reliable supply of a large amount of high-quality cells has been desired. Recently, therefore, suspension culture has been attempted but there are problems that, for example, cells are damaged when a cell cluster is divided. Therefore, there has been a demand for a method that enables a large amount of pluripotent stem cells such as human iPS cells to be cultured easily and efficiently.

[0010]

Hemagglutinin (HA) derived from *Clostridium botulinum* is a component of a botulinum neurotoxin complex. Recently, it has been discovered that the HA has a molecular mechanism in which the HA specifically binds to E-cadherin that is a cell adhesion molecule, and thereby breaks down the barrier between intestinal epithelial cells. The present inventors have been attempting to remove "cells deviated from the undifferentiated state," which emerge in colonies during the culture of stem cells having pluripotency, using the molecular mechanism of the HA.

[0011]

The present disclosure, in one aspect, provides a novel hemagglutinin that can remove "a cell deviated from the undifferentiated state", which emerges in a colony during culture of stem cells having pluripotency.

[0012]

The present disclosure, in one aspect, provides a novel method for culturing stem cells having pluripotency using hemagglutinin.

[Means for Solving Problem]

[0013]

In one aspect, the present invention refers to a mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B, the complex protein comprising: subcomponents HA1, HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B; wherein the mutant hemagglutinin complex protein has an amino acid corresponding to asparagine at position 286 in an amino acid sequence of a wild type of the subcomponent HA1 having been mutated. In another aspect, the present invention refers to a mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B, the complex protein consisting of subcomponents HA1, HA2, and HA3, and the complex protein comprising an amino acid corresponding to asparagine at position 286 in an amino acid sequence of a wild type of the

subcomponent HA1 and an amino acid corresponding to arginine at position 528 in an amino acid sequence of a wild type of the subcomponent HA3, with both of the amino acids having been mutated. In yet another aspect, the present invention refers to a method for culturing a stem cell having pluripotency, the method comprising: culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein according to the present invention. In still another aspect, the present invention refers to a method for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of a stem cell having pluripotency, the method comprising: culturing the stem cell having pluriopotency in the presence of a mutant hemagglutinin complex protein according to the present invention. In another aspect, the present invention refers to a a method for maintaining an undifferentiated state of a stem cell having pluripotency, the method comprising: culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein according to the present invention. In another aspect, the present invention refers to a method for culturing iPS cells, the method comprising: culturing the iPS cells in suspension culture in the presence of a mutant hemagglutinin complex protein according to the present invention. In another aspect, the present invention refers to a method for dividing a cell cluster of iPS cells, the method comprising: culturing the iPS cells in suspension culture in the presence of a mutant hemagglutinin complex protein according to the present invention. In another aspect, the present invention refers to a kit, comprising: a medium component for a stem cell having pluripotency; and a mutant hemagglutinin complex protein according to the present invention. In another aspect, the present invention refers to a composition comprising a mutant hemagglutinin complex protein according to the present invention, wherein the composition being used for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of a stem cell having pluripotency, or culturing in suspension culture an iPS cell. The present disclosure, in one example, relates to a mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B, the complex protein containing at least subcomponents HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B, and amino acids constituting a glycosylation site, with at least one of the amino acids having been mutated.

[0014]

The present disclosure, in one example, relates to a mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B, the complex protein consisting of

subcomponents HA1, HA2, and HA3, and one or two amino acids selected from an amino acid corresponding to aspartic acid at position 264 in a wild-type amino acid sequence of the subcomponent HA1, an amino acid corresponding to asparagine at position 286 in the wild-type amino acid sequence of the subcomponent HA1, and an amino acid corresponding to arginine at position 528 in a wild-type amino acid sequence of the subcomponent HA3 having been mutated.

[0015]

The present disclosure, in one example, relates to a method for culturing stem cells having pluripotency, the method including culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein of the present disclosure.

[0016]

The present disclosure, in one example, relates to a method for removing a cell deviated from the undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of stem cells having pluripotency, the method including culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein of the present disclosure.

[0017]

The present disclosure, in one example, relates to a method for culturing iPS cells of human origin, the method including culturing the iPS cells in suspension culture in the presence of hemagglutinin derived from *Clostridium botulinum*.

[0018]

The present disclosure, in one example, relates to a method for dividing a cluster of iPS cells of human origin, the method including culturing the iPS cells in suspension culture in the presence of hemagglutinin derived from *Clostridium botulinum*.

[Effects of the Invention]

[0019]

The present disclosure, in one example, can provide an effect that "a cell deviated from the undifferentiated state", which emerges in a colony during culture of stem cells having pluripotency, can be removed.

[0020]

The present disclosure, in one example, can provide an effect that stem cells having pluripotency can be cultured easily and efficiently in large quantities.

[0030]

The mutant HA complex protein of the present disclosure contains at least subcomponents HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B and, in one or more embodiments, may further contain a subcomponent HA1. In one or more embodiments, the mutant HA complex protein of the present disclosure is a complex consisting of two components, HA2 and HA3, and from the viewpoint that cell-cell adhesion can be inhibited further efficiently and deviated cells can be removed further efficiently, it is a complex consisting of three components, HA1, HA2, and HA3.

[0031]

In the mutant HA complex protein of the present disclosure, at least one of the amino acids constituting a glycosylation site has been mutated. In one or more embodiments, the mutant HA complex protein of the present disclosure can also be referred to as a mutant hemagglutinin complex protein in which at least part or all of the glycosylation activity of wild-type hemagglutinin derived from *Clostridium botulinum* type B has been deleted. As amino acids constituting the glycosylation site in wild-type hemagglutinin derived from *Clostridium botulinum* type B, the present inventors have found, for example, asparagine at position 286 in the amino acid sequence (SEQ ID NO: 1) of HA1 and arginine at position 528 in the amino acid sequence (SEQ ID NO: 3) of HA3. Furthermore, examples of other known amino acids include aspartic acid at position 264 in the amino acid sequence (SEQ ID NO: 1) of HA1 (for example, KwangKook Lee et al, Biochem Biophys Res Commun 2014 Apr 4, Vol 446, Issue 2, pp.568-573). In the mutant HA complex protein of the present disclosure, in one or more embodiments, these amino acids or amino acids corresponding to these amino acids have been mutated, and from the viewpoint that deviated cells can be efficiently removed from colonies, preferably arginine at position 528 in the amino acid sequence (SEQ ID NO: 3) of HA3 or an amino acid corresponding thereto has been mutated, and more preferably, arginine at position 528 in the amino acid sequence (SEQ ID NO: 3) of HA3 or an amino acid corresponding thereto and asparagine at position 286 in the amino acid sequence (SEQ ID NO: 1) of HA1 or an amino acid corresponding thereto have been mutated. In the present disclosure, "amino acids corresponding to these amino acids" denote amino acids located at conformationally equivalent positions to those of wild-type amino acids constituting

the above-mentioned glycosylation site. In the present disclosure, in one or more embodiments, mutation of amino acids includes substitution with amino acids that do not recognize a carbohydrate chain, preferably substitution with alanine.

[0032]

The mutant HA complex protein of the present disclosure has an amino acid sequence constituting an E-cadherin binding site in wild-type hemagglutinin derived from *Clostridium botulinum* type B. That is, in one or more embodiments, the mutant HA complex protein of the present disclosure has E-cadherin binding activity. Therefore, the present disclosure, in another aspect, relates to a mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B that contains at least subcomponents HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B, has E-cadherin binding activity, and contains amino acids constituting a glycosylation site, with at least one of the amino acids having been mutated. As the E-cadherin binding site, an E-cadherin binding site in wild-type hemagglutinin derived from *Clostridium botulinum* type A having E-cadherin binding activity in the wild-type hemagglutinin derived from *Clostridium botulinum* type B has been analyzed (for example, KwangKook Lee et al, Science 2014 Jun 20, Vol. 344, no. 6190 pp. 1405-1410).

[0033]

In one or more embodiments, the mutant HA complex protein of the present disclosure contains, as the subcomponent HA3, part or all of the amino acid sequence in which arginine at position 528 or an amino acid corresponding thereto has been mutated in the amino acid sequence (SEQ ID NO: 3) of a wild type of HA3 (wild-type HA3).

[0034]

In one or more embodiments, the mutant HA complex protein of the present disclosure contains, as the subcomponent HA2, part or all of the amino acid sequence (SEQ ID NO: 2) of a wild type of HA2 (wild-type HA2).

[0035] In one or more embodiments, the mutant HA complex protein of the present disclosure contains, as the subcomponent HA1, part or all of the amino acid sequence in which aspartic acid at position 264 or an amino acid corresponding thereto and/or asparagine at position 286 or an amino acid corresponding thereto have/has been mutated in the

amino acid sequence (SEQ ID NO: 1) of a wild type of HA1 (wild-type HA1).

[0036]

In one or more embodiments, the mutant HA complex protein of the present disclosure is a mutant hemagglutinin complex protein that consists of subcomponents HA1, HA2, and HA3, with one or both of an amino acid corresponding to asparagine at position 286 in an amino acid sequence of a wild type of the subcomponent HA1 and an amino acid corresponding to arginine at position 528 in an amino acid sequence of a wild type of the subcomponent HA3 having been mutated.

[0037]

Examples of a first embodiment of the mutant HA complex protein of the present disclosure include a mutant HA complex (a mutant HA complex 1) containing HA1 in which aspartic acid at position 264 and/or asparagine at position 286 have/has been mutated in the amino acid sequence (SEQ ID NO: 1) of wild-type HA1 (mutant HA1), wild-type HA2, and wild-type HA3. In one or more embodiments of the mutant HA complex 1, it is acceptable as long as at least one of the subcomponents HA1 contained in the mutant HA complex 1 is mutant HA1, and it is preferable that all HA1 be mutant HA1.

[0038]

Examples of a second embodiment of the mutant HA complex protein of the present disclosure include a mutant HA complex (a mutant HA complex 2) containing wild-type HA1, wild-type HA2, and HA3 in which arginine at position 528 or an amino acid corresponding thereto has been mutated in the amino acid sequence (SEQ ID NO: 3) of wild-type HA3 (mutant HA3). In one or more embodiments of the mutant HA complex 2, it is acceptable as long as at least one of the subcomponents HA3 contained in the mutant HA complex 2 is mutant HA3, and it is preferable that all HA3 be mutant HA3.

[0039]

Examples of a third embodiment of the mutant HA complex protein of the present disclosure include a mutant HA complex (a mutant HA complex 3) containing mutant HA1, wild-type HA2, and mutant HA3, and preferably containing HA1 (SEQ ID NO: 4) in which asparagine at position 286 in the amino acid sequence (SEQ ID NO: 1) of wild-type HA1 has been mutated to alanine, wild-type HA2 (SEQ ID NO: 2), and HA3

derived from *Clostridium botulinum* type A (hereinafter also referred to as a "type A mutant HA complex").

[0064]

In one or more embodiments, examples of the type A mutant HA complex include a known type A mutant HA complex, in which at least one of the amino acids constituting a glycosylation site has been mutated. Examples of the amino acids constituting a glycosylation site in wild-type hemagglutinin derived from *Clostridium botulinum* type A include asparagine at position 285 in the amino acid sequence (SEQ ID NO: 16) of HA1, as well as arginine at position 528 in the amino acid sequence (SEQ ID NO: 18) of HA3 and aspartic acid at position 263 in the amino acid sequence (SEQ ID NO: 16) of HA1.

[0065]

The type A mutant HA complex contains at least subcomponents HA2 and HA3 of the hemagglutinin derived from *Clostridium botulinum* type A and may further contain a subcomponent HA1 in one or more embodiments. In one or more embodiments, the type A mutant HA complex is a complex consisting of two components, HA2 and HA3, and from the viewpoint that cell-cell adhesion can be inhibited more efficiently and deviated cells can be removed more efficiently, it is a complex consisting of three components, HA1, HA2, and HA3.

[0066]

In the type A mutant HA complex, at least one of the amino acids constituting a glycosylation site has been mutated. In one or more embodiments, the type A mutant HA complex can also be referred to as a mutant hemagglutinin complex protein in which at least part or all of the glycosylation activity of wild-type hemagglutinin derived from *Clostridium botulinum* type A has been deleted. In one or more embodiments, in the type A mutant HA complex, amino acids constituting a glycosylation site or amino acids corresponding thereto have been mutated, and from the viewpoint that deviated cells can be efficiently removed from a colony, preferably arginine at position 528 in the amino acid sequence (SEQ ID NO: 18) of HA3 or an amino acid corresponding thereto has been mutated, and more preferably arginine at position 528 in the amino acid sequence (SEQ ID NO: 18) of HA3 or an amino acid corresponding thereto and asparagine at position 285 in the amino acid sequence (SEQ

ID NO: 16) of HA1 or an amino acid corresponding thereto have been mutated.

[0067]

The type A mutant HA complex has an amino acid sequence constituting the E-cadherin binding site in wild-type hemagglutinin derived from *Clostridium botulinum* type A. That is, in one or more embodiments, the type A mutant HA complex has E-cadherin binding activity.

[0068]

In one or more embodiments, the type A mutant HA complex contains, as the subcomponent HA3, part or all of the amino acid sequence in which arginine at position 528 in the amino acid sequence (SEQ ID NO: 18) of a wild type of type A HA3 (wild-type HA3) or an amino acid corresponding thereto has been mutated.

[0069]

In one or more embodiments, the type A mutant HA complex contains, as the subcomponent HA2, part or all of the amino acid sequence (SEQ ID NO: 17) of a wild type of type A HA2 (wild-type HA2).

[0070]

In one or more embodiments, the type A mutant HA complex contains, as the subcomponent HA1, part or all of the amino acid sequence in which aspartic acid at position 264 or an amino acid corresponding thereto and/or asparagine at position 286 or an amino acid corresponding thereto have/has been mutated in the amino acid sequence (SEQ ID NO: 16) of a wild type of type A HA1 (wild-type HA1).

[0071] In one or more embodiments, the type A mutant HA complex may has a tag binding to the C-terminal of the subcomponent HA1. In one or more embodiments, examples of the tag binding to the C-terminal include a FLAG-tag and a D4-tag (DDDD, SEQ ID NO: 15). In one or more embodiments, the mutant complex protein of the present disclosure has preferably no tag binding to the N-terminal of the subcomponent HA1 and has preferably no His-tag or FLAG-tag binding to the N-terminal of the subcomponent HA1.

[0072] In one or more embodiments, the present disclosure relates to a method for culturing stem cells having pluripotency using a culture composition of the present disclosure, a

method for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of stem cells having pluripotency, a method for maintaining the undifferentiated state of stem cells having pluripotency. In one or more embodiments, these methods include performing cell culture of stem cells having pluripotency in the presence of the culture composition of the present disclosure.

[0073] The present disclosure further relates to one or more non-limiting embodiments described below.

<A1> A mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B,

the complex protein containing:

at least subcomponents HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B; and

at least one of mutation s of amino acids constituting a glycosylation site of hemagglutinin derived from *Clostridium botulinum* type B.

<A2> A mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B,

the complex protein containing:

at least subcomponents HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B;

an amino acid sequence constituting an E-cadherin binding site; and

at least one of mutations of amino acids constituting a glycosylation site of hemagglutinin derived from *Clostridium botulinum* type B.

<A3> The mutant hemagglutinin complex protein according to <A1>, wherein the complex protein further contains a subcomponent HA1 of hemagglutinin derived from *Clostridium botulinum* type B.

<A4> The mutant hemagglutinin complex protein according to <A3>, wherein the amino acids constituting a glycosylation site are selected from the group consisting of an amino acid corresponding to aspartic acid at position 264 in an amino acid sequence of a wild type of the HA1, an amino acid corresponding to asparagine at position 286 in the amino acid sequence of a wild type of the HA1, and an amino acid corresponding to

at least one of mutations of amino acids constituting a glycosylation site of hemagglutinin derived from *Clostridium botulinum* type B, and

the composition being used for any one of methods selected from the group consisting of:

a method for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of a stem cell having pluripotency;

a method for maintaining an undifferentiated state of a stem cell having pluripotency;

a method for culturing iPS cells of human origin in suspension culture; and

a method for dividing a cell cluster of iPS cells of human origin.

<G2> The composition according to <G1>, wherein the *Clostridium botulinum* is *Clostridium botulinum* type A or *Clostridium botulinum* type B.

<G3> The composition according to <G1> or <G2>, wherein the complex protein further contains a subcomponent HA1 of hemagglutinin derived from *Clostridium botulinum*.

<G4> The composition according to <G3>, wherein the amino acids constituting a glycosylation site is selected from:

the group consisting of an amino acid corresponding to aspartic acid at position 263 in an amino acid sequence of a wild type of the HA1, an amino acid corresponding to asparagine at position 285 in the amino acid sequence of a wild type of the HA1, and an amino acid corresponding to arginine at position 528 in an amino acid sequence of a wild type of the HA3 in the case of *Clostridium botulinum* type A; and the group consisting of an amino acid corresponding to aspartic acid at position 264 in the amino acid sequence of a wild type of the HA1, an amino acid corresponding to asparagine at position 286 in the amino acid sequence of a wild type of the HA1, and an amino acid corresponding to arginine at position 528 in the amino acid sequence of a wild type of the HA3 in the case of *Clostridium botulinum* type B.

<H1> A method for culturing a stem cell having pluripotency, the method including culturing the stem cell having pluripotency in the presence of a composition according to any one of <G1> to <G4>.

<H2> The method according to <H1>, wherein the cell culture is adhesion culture or suspension culture.

CLAIMS

1. A mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B, the complex protein comprising:

subcomponents HA1, HA2 and HA3 of hemagglutinin derived from *Clostridium botulinum* type B;

wherein the mutant hemagglutinin complex protein has an amino acid corresponding to asparagine at position 286 in an amino acid sequence of a wild type of the subcomponent HA1 having been mutated.

2. The mutant hemagglutinin complex protein according to claim 1, wherein

the mutant hemagglutinin complex protein further has at least one ofan amino acid corresponding to aspartic acid at position 264 in an amino acid sequence of a wild type of the HA1 having been mutated and an amino acid corresponding to arginine at position 528 in an amino acid sequence of a wild type of the HA3 having been mutated.

3. A mutant hemagglutinin complex protein derived from *Clostridium botulinum* type B,

the complex protein consisting of subcomponents HA1, HA2, and HA3, and

the complex protein comprising an amino acid corresponding to asparagine at position 286 in an amino acid sequence of a wild type of the subcomponent HA1 and an amino acid corresponding to arginine at position 528 in an amino acid sequence of a wild type of the subcomponent HA3, with both of the amino acids having been mutated.

4. The mutant hemagglutinin complex protein according to any one of claims 1 to 3, wherein

the subcomponent HA1 is tagged at a C-terminal thereof.

5. A method for culturing a stem cell having pluripotency, the method comprising:

culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

6. The method according to claim 5, wherein
the culturing is performed by adhesion culture or suspension culture.

7. A method for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of a stem cell having pluripotency, the method comprising:
culturing the stem cell having pluriopotency in the presence of a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

8. A method for maintaining an undifferentiated state of a stem cell having pluripotency, the method comprising:
culturing the stem cell having pluripotency in the presence of a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

9. A method for culturing iPS cells, the method comprising:
culturing the iPS cells in suspension culture in the presence of a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

10. The method according to claim 9, wherein
the method comprises:
culturing a cell cluster of the iPS cells in suspension culture in the presence of the mutant hemagglutinin complex protein to divide the cell cluster into blobs, and
culturing the blobs to form a new cell cluster in suspension culture,
in the same medium as that in which the cell cluster is divided.

11. A method for dividing a cell cluster of iPS cells, the method comprising:
culturing the iPS cells in suspension culture in the presence of a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

12. The method according to any one of claims 9 to 11, wherein the mutant hemagglutinin complex protein is taken up by endocytosis.

13. A kit, comprising:
a medium component for a stem cell having pluripotency; and
a mutant hemagglutinin complex protein according to any one of claims 1 to 4.

14. A composition comprising a mutant hemagglutinin complex protein according to any one of claims 1 to 4
wherein,the composition being used for removing a cell deviated from an undifferentiated state, the cell being a cell that has emerged or may possibly emerge during culture of a stem cell having pluripotency, or culturing in suspension culture an iPS cell.