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(71) Demandeur/Applicant:
LSIP, LLC, JP

(72) Inventeurs/Inventors:
TAKAMATSU, TETSURO, JP;
DAI, PING, JP

(74) Agent: NORTON ROSE FULBRIGHT CANADA LLP/S.E.N.C.R.L., S.R.L.

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(54) Title: IPS CELLS AND METHOD FOR GENERATING SAME

(57) Abrégé/Abstract:
The present invention provides: a method for generating iPS cells, which is characterized by having at least one kind of connexin inhibitor factor and at least one kind of TGFβ signaling inhibitor factor act on cells; iPS cells which contain at least one kind of connexin inhibitor factor; an iPS cell inducing agent which contains at least one kind of inhibitor factor that is selected from the group consisting of connexin inhibitor factors and TGFβ signaling inhibitor factors; a culture medium for iPS cell induction, which contains at least one kind of inhibitor factor that is selected from the group consisting of connexin inhibitor factors and TGFβ signaling inhibitor factors; and a kit for iPS cell induction, which contains at least one kind of inhibitor factor that is selected from the group consisting of connexin inhibitor factors and TGFβ signaling inhibitor factors.

ABSTRACT

The present invention provides a method for producing iPS cells, comprising reacting cells with at least one connexin inhibitor and at least one TGFβ signaling inhibitor; iPS cells comprising at least one connexin inhibitor; an iPS cell inducer comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGFβ signaling inhibitors; a medium for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGFβ signaling inhibitors; and a kit for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGFβ signaling inhibitors.

DESCRIPTION

iPS CELLS AND METHOD FOR GENERATING SAME

TECHNICAL FIELD

[0001] The present invention relates to iPS cells (induced pluripotent stem cells) and a method for producing the same.

The present invention also relates to an iPS cell inducer, a medium for inducing iPS cells, and a kit for inducing iPS cells.

BACKGROUND ART

[0002] iPS cells are also referred to as artificial pluripotent stem cells or induced pluripotent stem cells, which are cells achieving pluripotent differentiation ability equivalent to that of embryonic stem (ES) cells.

[0003] Cells having pluripotent differentiation ability, such as ES cells and iPS cells, have the ability to differentiate into all organs and tissues constituting an organism. Accordingly, these cells are expected to be a highly effective means for reproducing organs, blood, bone marrow, tissue, etc., that are damaged by some diseases.

[0004] However, blood, bone marrow, tissue, organs, and other biological materials obtained from ES cells may cause transplant rejection. It is essential to solve such immunological problems, and there are also ethical problems. Conversely, iPS cells can be produced using a patient's own somatic cells; hence, there are neither immunological rejection problems nor ethical problems.

[0005] Professor Shinya Yamanaka and his group have succeeded in producing iPS cells by introducing genes of four types of reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc (so-called Yamanaka factors) into mouse fibroblasts (NPL 1 and NPL 2); however, clinical application of the iPS cells has the following problems:

1. There is a possibility that foreign genes, such as bacteria DNA, may be integrated into the genome of the host cell.

2. iPS cells produced by conventional methods have safety problems because they

become cancerous at a constant frequency.

3. iPS cells produced by overexpression of foreign genes have unexpected influences, other than pluripotency.

CITATION LIST

NON-PATENT LITERATURE

[0006] NPL 1: Takahashi et al., Cell 2006, 126: 663-676.

NPL 2: Okita et al., Nature 2007, 448: 313-317.

SUMMARY OF INVENTION

TECHNICAL PROBLEM

[0007] An object of the present invention is to provide safe iPS cells that have no risk of becoming cancerous.

SOLUTION TO PROBLEM

[0008] The present inventors conducted research in view of the above problems, and surprisingly found that iPS cells could be established, without introducing genes, by reacting cells with a connexin inhibitor and a TGF-β signaling inhibitor.

[0009] The present invention provides iPS cells, a method for producing the same, an iPS cell inducer, a medium for inducing iPS cells, and a kit for inducing iPS cells, as described below.

Item 1. A method for producing iPS cells, comprising reacting cells with at least one connexin inhibitor and at least one TGFβ signaling inhibitor.

Item 2. The method according to Item 1, wherein the connexin inhibitor is a connexin 43 inhibitor.

Item 3. The method according to Item 1 or 2, wherein the connexin inhibitor is siRNA, shRNA, miRNA, antisense RNA, peptide, antibody, or ribozyme against connexin, or an expression vector that can release RNA molecules thereof in the cells.

Item 4. The method according to any one of Items 1 to 3, wherein the TGF-β signaling inhibitor is a TGF-β receptor I inhibitor.

Item 5. The method according to any one of Items 1 to 4, wherein the TGF-β receptor

inhibitor is SB-431542, A-83-01, or ALK5 inhibitor.

Item 6. iPS cells comprising at least one connexin inhibitor.

Item 7. The iPS cells according to Item 6, wherein the connexin inhibitor is a connexin 43 inhibitor.

Item 8. The iPS cells according to Item 6 or 7, wherein the connexin inhibitor is siRNA, shRNA, miRNA, antisense RNA, peptide, antibody, or ribozyme against connexin, or an expression vector that can release RNA molecules thereof in the cells.

Item 9. An iPS cell inducer comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors.

Item 10. A medium for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors.

Item 11. A kit for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors.

ADVANTAGEOUS EFFECTS OF INVENTION

[0010] The method of the present invention can produce iPS cells that have a great advantage in their practical use for human, because introduction of foreign genes (particularly, oncogenes) is not required, any characteristics other than the iPS cells are not changed because no virus is used, and the iPS cells are so safe that they have very low risk of becoming cancerous or being infected.

[0011] The following four points regarding the iPS cells of the present invention have been confirmed:

1. Pluripotent cell marker factor expression is maintained at the genetic and protein levels.

2. Subcutaneous implantation of the iPS cells in mice leads to teratoma formation and induces differentiation into all three germ layers, including gland, cartilage, and neural tissues.

3. Embryoid bodies are formed, and are able to differentiate into endothelial cells, neural cells, and cardiomyocytes depending on differentiation-inducing conditions. The

differentiated cardiomyocytes function physiologically.

4. Chimeric mice can be produced.

[0012] In addition, the iPS cells of the present invention allow the production of germ lines.

[0013] Accordingly, the iPS cells provided by the present invention are very excellent in producing biological materials for use in, for example, regenerative medicine.

BRIEF DESCRIPTION OF DRAWINGS

[0014] Figure 1 shows the results regarding cells obtained in the examples of the present invention.

a. The morphology of siPSCs is similar to that of mouse ES cells.

b. ALP activity of siPSCs was observed.

c. Expression was confirmed at the protein level by immunostaining using various anti-pluripotency marker antibodies (Nanog, Oct4, Sox2, and SSEA1).

d. The expression of various anti-pluripotency marker genes was confirmed at the gene level by RT-PCR.

e. When the cells were implanted subcutaneously in SCID mice, teratomas were formed; and gland, cartilage/muscle, and nerve/epithelium were confirmed.

Figure 2 shows the morphology of siPSCs derived from EGFP mouse myocardium.

Figure 3 shows the production of chimeric mice from siPSCs cells.

a. Chimeric mice generated from siPSCs-#1.

b. Chimeric mice generated from siPSCs-#2.

Figure 4 schematically illustrates TGF-β signaling.

DESCRIPTION OF EMBODIMENTS

[0015] In the present specification, the term "iPS cells" refers to cells that have achieved pluripotent differentiation ability, which are also called artificial pluripotent stem cells or induced pluripotent stem cells. The iPS cells obtained in the present invention are able to differentiate into all of endodermal cells, ectodermal cells, and mesodermal cells (see Fig. 1), and allow the production of chimeric mice (Fig. 3) and germ lines.

[0016] The term "connexin (Cx)" is a generic name of proteins involved in gap junction,

and they are named, for example, Cx43 (43kDa), depending on their molecular weight. Specific examples of connexins include Cx26, Cx30, Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx50, etc. More than 20 types of connexins are currently known. The target connexins for inhibition in the present invention also include connexins to be discovered in the future. The type and expression of connexins are described in detail in a review paper (Oyamada et al., BBA, 2005, 1719: 6-23), and the description thereof is incorporated herein by reference. The type and expression site of connexins are summarized in the following table.

[0017] [Table 1]

Tissue or cell type	Expressed connexin	
	Human	Mouse
-	hCx23	mCx23
-	hCx25	-
Breast, cochlea, placenta, hepatocyte, skin, pancreas, kidney, intestine	hCx26	mCx26
Brain, cochlea, skin	hCx30	mCx30
Brain, spinal cord, Schwann cells	hCx30.2	mCx29
Skin, kidney	hCx30.3	mCx30.3
Cochlea, placenta, skin	hCx31	mCx31
Skin	hCx31.1	mCx31.1
-	hCx31.9	mCx30.2
Hepatocyte, secretory acinar cells, Schwann cells	hCx32	mCx32
Sertoli cells	-	mCx33
Neurons, pancreatic β-cells	hCx36	mCx36
Endothelium, granulosa cells, lung, skin	hCx37	mCx37
Cardiac conduction system, endothelium, lung	hCx40	mCx40
-	hCx40.1	mCx39
Many cell types	hCx43	mCx43
Cardiac conduction system, smooth muscle cells, neurons	hCx45	mCx45
Lens	hCx46	mCx46
Brain, spinal cord	hCx47	mCx47
Lens	hCx50	mCx50
-	hCx59	-
Retinal horizontal cells	hCx62	mCx57

The table above shows connexins mainly expressed in tissues. In the present invention, it is preferable to inhibit connexins mainly expressed in target cells. For example, in the ventricle muscle in which Cx43 is highly expressed, it is preferable to inhibit Cx43.

In addition, Cx45 is also expressed in the ventricle muscle; Cx45 may be inhibited, or Cx43 and Cx45 may be inhibited at the same time. Preferred connexins to be inhibited are connexins 26, 32, 43, 45, etc., that are expressed in many cell types. Particularly preferred is connexin 43. The amino acid sequences of connexin 43 are known, and some examples are shown below.

[0018] [Table 2]

Origin of Cx43	Database accession number of gene/amino acid sequence
Human	NM_000165
Rat	NM_000567
Mouse	NM_010288
Monkey	AB169817

Depending on the animal species of iPS cells, corresponding connexin inhibitors can be used.

[0019] The functions of connexin inhibitors are, for example, inhibition of connexin gene expression in cells (inhibition of transcription or translation), functional inhibition due to complex formation with intracellular connexins (proteins), and the like. Specific examples of connexin inhibitors include siRNA, shRNA, miRNA, antisense RNA, peptide, antibody, or ribozyme against connexins, and expression vectors that can release RNA molecules thereof in cells. Preferred among these are siRNA, shRNA, miRNA, antisense RNA, and ribozyme; and more preferred are siRNA, shRNA, and miRNA. Since they have different sequences depending on the type of connexin, the source of the cells, etc., it is necessary to select suitable sequences. Those skilled in the art can easily design or select suitable sequences of siRNA, shRNA, miRNA, antisense RNA, peptide, ribozyme, or expression vectors that can release RNA molecules thereof in cells. For example, preferred examples of siRNA against connexin 43 in human (the same sequences are applicable to monkey) are as follows:

Sense: 5'-CAA UUC UUC UUG CCG CAA TT-3' (SEQ ID NO: 1)

Antisense: 5'-UUG CGG CAA GAA GAA UUG TT-3' (SEQ ID NO: 2)

[0020] As connexin inhibitors, glycosaminoglycan having a sulfuric acid group (Japanese Unexamined Patent Publication No. 2003-119146; the description thereof is incorporated

herein by reference) can also be used.

[0021] In one embodiment, "connexin inhibitors" are compounds that affect or modulate a connexin, a connexin hemichannel (connexon), or the cell proliferative activity of a connexin. Connexin inhibitors include, without limitation, antisense compounds (e.g., antisense polynucleotides), RNAi and siRNA compounds, antibodies and binding fragments thereof, and peptides and polypeptides (including "peptidomimetics" and peptide analogs). A preferred connexin inhibitor is a connexin 43 inhibitor. Exemplary connexin inhibitors are described in further detail herein.

[0022] In one embodiment, the connexin inhibitors of the present invention can modulate or affect the transport of molecules into and out of cells (e.g., blocking, inhibition, or downregulation).

[0023] Certain connexin inhibitors provide downregulation of connexin expression, for example, by downregulation of mRNA transcription or translation, or decrease or inhibit a connexin protein, a connexin hemichannel or cell proliferative activity (Asazuma-Nakamura et al., Exp Cell Res., 2009, 315: 1190-1199; Nakano et al., Invest Ophthalmol Vis Sci., 2007, 49: 93-104; Zhang et al., Oncogene, 2001, 20: 4138-4149).

[0024] Examples of connexin inhibitors include agents that decrease or inhibit expression or function of connexin mRNA and/or protein, or that decrease a connexin, a connexin hemichannel or cell proliferative activity. Connexin inhibitors include anti-connexin polynucleotides, such as antisense polynucleotides and other polynucleotides (e.g., polynucleotides having siRNA or ribozyme functionalities), as well as antibodies and binding fragments thereof, and peptides and polypeptides (including peptidomimetics and peptide analogs that modulate hemichannel or cell proliferative activity).

[0025] Anti-Connexin Polynucleotides

Anti-connexin polynucleotides include connexin antisense polynucleotides as well as polynucleotides having functionalities that enable them to downregulate connexin expression. Other suitable anti-connexin polynucleotides include RNAi polynucleotides, siRNA polynucleotides, and shRNA polynucleotides.

[0026] Synthesis of antisense polynucleotides and other anti-connexin polynucleotides (RNAi, siRNA, and ribozyme polynucleotides as well as polynucleotides having modified and mixed backbones) is known to those of skill in the art. See, e.g., Stein C.A. and Krieg A.M. (Eds.), Applied Antisense Oligonucleotide Technology, 1998 (Wiley-Liss). Methods of synthesizing antibodies and binding fragments as well as peptides and polypeptides (including peptidomimetics and peptide analogs) are known to those of skill in the art. See, e.g., Lihu Yang et al., Proc. Natl. Acad. Sci. U.S.A., 1; 95(18): 10836-10841 (Sept 1 1998); Harlow and Lane (1988) "Antibodies: A Laboratory Manual" Cold Spring Harbor Publications, New York; Harlow and Lane (1999) "Using Antibodies" A Laboratory Manual, Cold Spring Harbor Publications, New York.

[0027] According to one aspect, the downregulation of connexin expression is based generally upon the use of antisense polynucleotides (such as DNA or RNA polynucleotides), and more particularly upon the use of antisense oligodeoxynucleotides (ODN). These polynucleotides (e.g., ODN) target the connexin protein(s) to be downregulated. Typically, the polynucleotides are single-stranded, but may be double-stranded.

[0028] The antisense polynucleotide may inhibit transcription and/or translation of a connexin. Preferably, the polynucleotide is a specific inhibitor of transcription and/or translation from the connexin gene or mRNA, and does not inhibit transcription and/or translation from other genes or mRNAs. The product may bind to the connexin gene or mRNA either (i) 5' to the coding sequence, and/or (ii) to the coding sequence, and/or (iii) 3' to the coding sequence.

[0029] The antisense polynucleotide is generally antisense to a connexin mRNA. Such a polynucleotide may be capable of hybridizing to the connexin mRNA, and may thus inhibit the expression of connexin by interfering with one or more aspects of connexin mRNA metabolism (including transcription, mRNA processing, mRNA transport from the nucleus, translation or mRNA degradation). The antisense polynucleotide typically hybridizes to the connexin mRNA to form a duplex that can cause direct inhibition of translation and/or destabilization of the mRNA. Such a duplex may be susceptible to degradation by

nucleases.

[0030] The antisense polynucleotide may hybridize to all or part of the connexin mRNA. Typically, the antisense polynucleotide hybridizes to the ribosome binding region or the coding region of the connexin mRNA. The polynucleotide may be complementary to all of or a region of the connexin mRNA. For example, the polynucleotide may be the exact complement of all or a part of connexin mRNA. However, absolute complementarity is not required, and polynucleotides that have sufficient complementarity to form a duplex having a melting temperature of greater than about 20°C, 30°C or 40°C under physiological conditions are particularly suitable for use in the present invention.

[0031] Thus, the polynucleotide is typically a homologue of a sequence complementary to the mRNA. The polynucleotide may be a polynucleotide that hybridizes to the connexin mRNA under conditions of medium to high stringency, such as 0.03 M sodium chloride and 0.03 M sodium citrate at from about 50°C to about 60°C.

[0032] For certain aspects, suitable polynucleotides are typically from about 6 to 40 nucleotides in length. Preferably, a polynucleotide may be from about 12 to about 35 nucleotides in length, or more preferably from about 18 to about 32 nucleotides in length. According to another aspect, the polynucleotide may be at least about 40, for example, at least about 60 or at least about 80 nucleotides in length; and up to about 100, about 200, about 300, about 400, about 500, about 1,000, about 2,000 or about 3,000 or more nucleotides in length.

[0033] The connexin protein or proteins targeted by the polynucleotide will be dependent upon the site at which downregulation is to be effected. The connexin is a connexin that naturally occurs in a human or animal in one aspect, or naturally occurs in the tissue in which connexin expression or activity is to be decreased. The connexin gene (including coding sequence) generally has homology with the coding sequence of one or more of the specific connexins mentioned herein, such as homology with the connexin 43 coding sequence. The connexin is typically an α or β connexin. Preferably, the connexin is an α connexin and is expressed in the cells used as the starting material for iPS.

[0034] Some connexin proteins are, however, more ubiquitous than others in terms of distribution in tissue. One of the most widespread is connexin 43. Polynucleotides targeted to connexin 43 are particularly suitable for use in the present invention. In other aspects, other connexins are targeted.

[0035] Anti-connexin polynucleotides include connexin antisense polynucleotides as well as polynucleotides having functionalities that enable them to downregulate connexin expression. Other suitable anti-connexin polynucleotides include RNAi polynucleotides, shRNA polynucleotides, and siRNA polynucleotides.

[0036] In one preferred aspect, the antisense polynucleotides are targeted to the mRNA of only one connexin protein. Most preferably, this connexin protein is connexin 43. In other aspects, the connexin protein is connexin 26, 30, 31.1, 32, 36, 37, 40, or 45. In still other aspects, the connexin protein is connexin 30.3, 31, 40.1, or 47. These are examples of human connexin proteins. In the case of other animal species, mRNA of the corresponding connexin is targeted.

[0037] It is also contemplated that polynucleotides targeted to separate connexin proteins may be used in combination (e.g., 1, 2, 3, 4 or more different connexins may be targeted). For example, polynucleotides targeted to connexin 43, and one or more other members of the connexin family (such as connexins 26, 30, 30.3, 31.1, 32, 36, 37, 40, 40.1, 45, and 47) can be used in combination.

[0038] Alternatively, the antisense polynucleotides may be part of compositions that may comprise polynucleotides to more than one connexin protein. Preferably, one of the connexin proteins to which polynucleotides are directed is connexin 43. Other connexin proteins to which oligodeoxynucleotides are directed may include, for example, connexins 26, 30, 30.3, 31.1, 32, 36, 37, 40, 40.1, 45, and 47. Suitable exemplary polynucleotides (and ODNs) directed to various connexins are set forth in SEQ ID NOs: 3 to 14.

[0039] Individual antisense polynucleotides may be specific to a particular connexin, or may target 1, 2, 3 or more different connexins. Specific polynucleotides will generally target sequences in the connexin gene or mRNA that are not conserved between connexins,

whereas non-specific polynucleotides will target conserved sequences for various connexins.

[0040] The polynucleotides for use in the invention may suitably be unmodified phosphodiester oligomers. Such oligodeoxynucleotides may vary in length. A 30 mer polynucleotide has been found to be particularly suitable.

[0041] Many aspects of the invention are described with reference to oligodeoxynucleotides. However, it is understood that other suitable polynucleotides (such as RNA polynucleotides) may be used in these aspects.

[0042] The antisense polynucleotides may be chemically modified. This may enhance their resistance to nucleases and may enhance their ability to enter cells. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'- phosphoramidates and oligoribonucleotide phosphorothioates, and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates. Alternatively, mixed backbone oligonucleotides ("MBOs") may be used. MBOs contain segments of phosphorothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy-or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides. Methods of preparing modified backbone and mixed backbone oligonucleotides are known in the art.

[0043] The precise sequence of the antisense polynucleotide used in the invention will depend upon the target connexin protein. In one embodiment, suitable connexin antisense polynucleotides can include polynucleotides (SEQ ID NOs: 3 to 14).

[0044] Suitable polynucleotides for the preparation of the combined polynucleotides described herein include, for example, polynucleotides to Connexin Cx43 and polynucleotides for connexins 26, 30, 31.1, 32 and 37.

[0045] Although the precise sequence of the antisense polynucleotide used in the invention will depend upon the target connexin protein, for connexin 43, antisense polynucleotides having the following sequences have been found to be particularly suitable:

GTA ATT GCG GCA AGA AGA ATT GTT TCT GTC (SEQ ID NO: 3);

GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC (SEQ ID NO: 4); and

GGC AAG AGA CAC CAA AGA CAC TAC CAG CAT (SEQ ID NO: 5).

[0046] For example, suitable antisense polynucleotides for connexins 26, 31.1 and 32 have the following sequences:

5' TCC TGA GCA ATA CCT AAC GAA CAA ATA (connexin 26) (SEQ ID NO: 6);

5' CGT CCG AGC CCA GAA AGA TGA GGT C (connexin 31.1) (SEQ ID NO: 11); and

5' TTT CTT TTC TAT GTG CTG TTG GTG A (connexin 32) (SEQ ID NO: 14).

[0047] Other connexin antisense polynucleotide sequences useful according to the methods of the present invention include:

5' CAT CTC CTT GGT GCT CAA CC 3' (connexin 37) (SEQ ID NO: 7);

5' CTG AAG TCG ACT TGG CTT GG 3' (connexin 37) (SEQ ID NO: 8);

5' CTC AGA TAG TGG CCA GAA TGC 3' (connexin 30) (SEQ ID NO: 9);

5' TTG TCC AGG TGA CTC CAA GG 3' (connexin 30) (SEQ ID NO: 10);

5' AGA GGC GCA CGT GAG ACA C 3' (connexin 31.1) (SEQ ID NO: 12); and

5' TGA AGA CAA TGA AGA TGT T 3' (connexin 31.1) (SEQ ID NO: 13).

[0048] Polynucleotides (including ODNs) directed to connexin proteins can be selected in terms of their nucleotide sequence by any convenient and conventional approach. For example, the computer programs MacVector and OligoTech (from Oligos Etc., Eugene, Oregon, USA) can be used. Once selected, the ODNs can be synthesized using a DNA synthesizer.

[0049] Polynucleotide Homologues

For example, the polynucleotide may be a homologue of a complement to a sequence in connexin mRNA. Such a polynucleotide typically has at least about 70% homology, preferably at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98% or at least about 99% homology with the relevant sequence, for example, over a region of more than at least about 15, at least about 20, at least about 40, at least about 100 contiguous nucleotides (of the homologous sequence).

[0050] Homology may be calculated based on any method in the art. For example, the UWGCG Package provides the BESTFIT program, which can be used to calculate homology (for example, used on its default settings) (Devereux et al. (1984) Nucleic Acids Research 12, p 387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (typically on their default settings), for example, as described in Altschul, S. F. (1993) J Mol Evol 36: 290-300; Altschul, S. F. et al. (1990) J MoI Biol 215: 403-10.

[0051] Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high-scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when:

the cumulative alignment score falls off by the quantity X from its maximum achieved value;

the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or

the end of either sequence is reached.

[0052] The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W), the BLOSUM62 scoring matrix (Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci USA 89: 10915-10919), alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

[0053] The BLAST algorithm performs a statistical analysis of the similarity between two sequences. See, e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5787.

One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to a second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[0054] The homologous sequence typically differs from the relevant sequence by at least about 2, 5, 10, 15, 20 or more mutations (which may be substitutions, deletions or insertions). These mutations may be measured across any of the regions mentioned above in relation to calculating homology.

[0055] The homologous sequence typically hybridizes selectively to the original sequence at a level significantly above background. Selective hybridization is typically achieved using conditions of medium to high stringency (for example, 0.03 M sodium chloride and 0.03 M sodium citrate at from about 50°C to about 60°C). However, such hybridization may be carried out under any suitable conditions known in the art (see Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual). For example, if high stringency is required, suitable conditions include 0.2 x SSC at 60°C. If lower stringency is required, suitable conditions include 2 x SSC at 60°C.

[0056] Peptide and Polypeptide Connexin Inhibitor

Binding proteins (including peptides, peptidomimetics, antibodies, antibody fragments, and the like) are also suitable inhibitors.

[0057] Binding proteins include, for example, monoclonal antibodies, polyclonal antibodies, antibody fragments (including, for example, Fab, $F(ab')_2$ and Fv fragments); single chain antibodies; single chain Fvs; and single chain binding molecules (those comprising, for example, a binding domain, hinge, CH2 and CH3 domains), recombinant antibodies and antibody fragments that are capable of binding an antigenic determinant (i.e., that portion of a molecule, generally referred to as an epitope) that makes contact with a particular antibody or other binding molecule. These binding proteins (including antibodies, antibody fragments,

and so on) may be chimeric or humanized, or otherwise made to be less immunogenic in the subject to whom they are to be administered, and may be synthesized, produced recombinantly, or produced in expression libraries. Any binding molecule known in the art or later discovered is envisioned, such as those referenced herein and/or described in greater detail in the art. For example, binding proteins include not only antibodies and the like, but also ligands, receptors, peptidomimetics, or other binding fragments or molecules (for example, produced by phage display) that bind to a target (e.g., connexin or associated molecules).

[0058] Binding molecules will generally have a desired specificity (including, but not limited to, binding specificity) and desired affinity. Affinity, for example, may be a Ka of greater than or equal to about 10^4M^{-1}, greater than or equal to about 10^6M^{-1}, greater than or equal to about 10^7M^{-1}, greater than or equal to about 10^8M^{-1}. Affinities of even greater than about 10^8M^{-1} (affinities equal to or greater than about 10^9M^{-1}, about $10^{10}M^{-1}$, about $10^{11}M^{-1}$, and about $10^{12}M^{-1}$) are suitable. Affinities of binding proteins according to the present invention can be readily determined using conventional techniques (for example, those described by Scatchard et al., 1949 Ann. NY. Acad. Sci. 51: 660).

[0059] By using data obtained from hydropathy plots, it has been proposed that a connexin contains four-transmembrane-spanning regions and two short extra-cellular loops. The positioning of the first and second extracellular regions of connexin was further characterized by the reported production of anti-peptide antibodies used for immunolocalization of the corresponding epitopes on split gap junctions. Goodenough D.A., J Cell Biol 107: 1817-1824 (1988); Meyer R.A., J Cell Biol 119: 179-189 (1992).

[0060] Connexin inhibitors include peptides comprising an amino acid sequence corresponding to a transmembrane region (e.g., 1st to 4th) of a connexin (e.g., connexins 45, 43, 26, 30, 31.1, and 37). Connexin inhibitors may comprise a peptide comprising an amino acid sequence corresponding to a portion of a transmembrane region of a connexin 45. Connexin inhibitors include a peptide having an amino acid sequence that comprises about 5 to 20 contiguous amino acids of accession number: AAA60458, a peptide having an amino

acid sequence that comprises about 8 to 15 contiguous amino acids of accession number: AAA60458, or a peptide having an amino acid sequence that comprises about 11 to 13 contiguous amino acids of accession number: AAA60458. Other embodiments are directed to a connexin inhibitor that is a peptide having an amino acid sequence that comprises at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 20, at least about 25, or at least about 30 contiguous amino acids of accession number: AAA60458. In certain connexin inhibitors provided herein, the extracellular domains of connexin 45 corresponding to the amino acids at positions 46-75 and 199-228 of accession number: AAA60458 may be used to develop the particular peptide sequences. Certain peptides described herein have an amino acid sequence corresponding to the regions at positions 46-75 and 199-228 of accession number: AAA60458. The peptides need not have an amino acid sequence identical to those portions of accession number: AAA60458, and conservative amino acid changes may be made such that the peptides retain binding activity or functional activity. Alternatively, the peptide may target regions of the connexin protein other than the extracellular domains (e.g., the portions of accession number: AAA60458 not corresponding to positions 46-75 and 199-228).

[0061] Also, suitable connexin inhibitors comprise a peptide comprising an amino acid sequence corresponding to a portion of a transmembrane region of a connexin 43. Connexin inhibitors include peptides having an amino acid sequence that comprises about 5 to 20 contiguous amino acids of accession number: NP_034418, peptides having an amino acid sequence that comprises about 8 to 15 contiguous amino acids of accession number: NP_034418, or peptides having an amino acid sequence that comprises about 11 to 13 contiguous amino acids of accession number: NP_034418. Other connexin inhibitors include a peptide having an amino acid sequence that comprises at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 20, at least about 25, or at least about 30 contiguous amino acids of accession number: NP_034418.

Other connexin inhibitors comprise the extracellular domains of connexin 43 corresponding to the amino acids at positions 37-76 and 178-208 of accession number: NP_034418. Connexin inhibitors include peptides described herein that have an amino acid sequence corresponding to the regions at positions 37-76 and 178-208 of accession number: NP_034418. The peptides need not have an amino acid sequence identical to those portions of accession number: NP_034418, and conservative amino acid changes may be made such that the peptides retain binding activity or functional activity. Alternatively, peptides may target regions of the connexin protein other than the extracellular domains (e.g., the portions of accession number: NP_034418 not corresponding to positions 37-76 and 178-208).

[0062] The anti-connexin peptides may comprise sequences corresponding to a portion of the connexin extracellular domains with conservative amino acid substitutions such that peptides are functionally active connexin inhibitors. Exemplary conservative amino acid substitutions include, for example, the substitution of a nonpolar amino acid with another nonpolar amino acid, the substitution of an aromatic amino acid with another aromatic amino acid, the substitution of an aliphatic amino acid with another aliphatic amino acid, the substitution of a polar amino acid with another polar amino acid, the substitution of an acidic amino acid with another acidic amino acid, the substitution of a basic amino acid with another basic amino acid, and the substitution of an ionizable amino acid with another ionizable amino acid.

[0063] Examples of peptides targeted to connexin 43 are shown below as SEQ ID NOs: 15 to 23:

FEVAFLLIQWI (SEQ ID NO: 15)

LLIQWYIGFSL (SEQ ID NO: 16)

SLSAVYTCKRDPCPHQ (SEQ ID NO: 17)

VDCFLSRPTEKT (SEQ ID NO: 18)

SRPTEKTIFII (SEQ ID NO: 19)

LGTAVESAWGDEQ (SEQ ID NO: 20)

QSAFRCNTQQPG (SEQ ID NO: 21)

QQPGCENVCYDK (SEQ ID NO: 22)

VCYDKSFPISHVR (SEQ ID NO: 23)

[0064] Examples of peptides targeted to Cx32, Cx40, or Cx43 are shown below as SEQ ID NOs: 24 to 37:

Peptides targeted to Cx32 (SEQ ID NO: 34 is targeted to Cx32/Cx43)

ESVWGDEKSSFI (SEQ ID NO: 24)

ICNTLQPGCNSV (SEQ ID NO: 25)

SVCYDHFFPISH (SEQ ID NO: 26)

RLVKCEAFPCPNTVDCFVSRPTEKT (SEQ ID NO: 27)

VKCEAFPCPNTV (SEQ ID NO: 28)

VDCFVSRPTEKT (SEQ ID NO: 29)

VCYDHFFPISHVR (SEQ ID NO: 30)

VWGDEKSSFICNTLQPGY (SEQ ID NO: 31)

DEKSSFICNTLQPGY (SEQ ID NO: 32)

SRPTEKTVFTV (SEQ ID NO: 33)

SRPTEKT (SEQ ID NO: 34)

ICNTLQPGCNSV (SEQ ID NO: 35)

Peptide targeted to Cx40

FLDTLHVCRRSPCPHP (SEQ ID NO: 36)

Peptide targeted to Cx43

KRDPCHQVDCFLSRPTEK (SEQ ID NO: 37)

[0065] SEQ ID NOs: 38 to 65 indicate inhibitors of human connexins (huCx) 26, 30, 30.3, 31.1, 32, 36, 37, 40, 40.1, 43, 45, 46, and 46.6 for use as described herein.

huCx26 KEVWGDEQADFVCNTLQPGCKNVCYDHYFPISHIR (SEQ ID NO: 38)

huCx30 QEVWGDEQEDFVCNTLQPGCKNVCYDHFFPVSHIR (SEQ ID NO: 39)

huCx30.3 EEVWDDEQKDFVCNTKQPGCPNVCYDEFFPVSHVR (SEQ ID NO: 40)

huCx31 ERVWGDEQKDFDCNTKQPGCTNVCYDNYFPISNIR (SEQ ID NO: 41)

huCx31.1 ERVWSDDHKDFDCNTRQPGCSNVCFDEFFPVSHVR (SEQ ID NO: 42)

huCx32 ESVWGDEKSSFICNTLQPGCNSVCYDQFFPISHVR (SEQ ID NO: 43)

huCx36 ESVWGDEQSDFECNTAQPGCTNVCYDQAFPISHIR (SEQ ID NO: 44)

huCx37 ESVWGDEQSDFECNTAQPGCTNVCYDQAFPISHIR (SEQ ID NO: 45)

huCx40.1 RPVYQDEQERFVCNTLQPGCANVCYDVFS PVSHLR (SEQ ID NO: 46)

huCx43 ESAWGDEQSAFRCNTQQPGCENVCYDKSFPISHVR (SEQ ID NO: 47)

huCx46 EDVWGDEQSDFTCNTQQPGCBNVCYBRAFPISHIR (SEQ ID NO: 48)

huCx46.6 EAIYSDEQAKFTCNTRQPGCDNVCYDAFAPLSHVR (SEQ ID NO: 49)

huCx40 ESSWGDEQADFRCDTIQPGCQNVCTDQAFPISHIR (SEQ ID NO: 50)

huCx45 GESIYYDEQSKFVCNTEQPGCENVCYDAFAPLSHVR (SEQ ID NO: 51)

huCx26 MYVFYVMYDGFSMQRLVKCNAWPCPNTVDCFVSRPTEKT (SEQ ID NO: 52)

huCx30 MYVFYFLYNGYHLPWVLKCGIDPCPNLVDCFISRPTEKT (SEQ ID NO: 53)

huCx30.3 LYIFHRLYKDYDMPRVVACSVEPCPHTVDCYISRPTEKK (SEQ ID NO: 54)

huCx31 LYLLHTLWHGFNMPRLVQCANVAPCPNIVDCYIARPTE (SEQ ID NO: 55)

huCx31.1 LYVFHSFYPKYILPPVVKCHADPCPNIVDCFISKPSEKN (SEQ ID NO: 56)

huCx32 MYVFYLLYPGYAMVRLVKCDVYPCPNTVDCFVSRPTEKT (SEQ ID NO: 57)

huCx36 LYGWTMEPVFVCQRAPCPYLVDCFVSRPTEKT (SEQ ID NO: 58)

huCx37 LYGWTMEPVFVCQRAPCPYLVDCFVSRPTEKT (SEQ ID NO: 59)

huCx40.1 GALHYFLFGFLAPKKFPCTRPPCTGVVDCYVSRPTSKS (SEQ ID NO: 60)

huCx43 LLIQWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKT (SEQ ID NO: 61)

huCx46 IAGQYFL YGFELKPLYRCDRWPCPNTVDCFISRPTEKT (SEQ ID NO: 62)

huCx46.6 LVGQYLLYGFEVRPFFPCSRQPCPHVVDCFVSRPTEKT (SEQ ID NO: 63)

huCx40 IVGQYFIYGIFLTTLHVCRRSPCPHPVNCYVSRPTEKN (SEQ ID NO: 64)

huCx45 LIGQYFLYGFQVHPFYVCSRLPCHPKIDCFISRPTEKT (SEQ ID NO: 65)

[0066] SEQ ID NOs: 18 to 19 and 66 to 79 provide the extracellular domains of connexin family members (human connexins (huCx) 26, 30, 30.3, 31.1, 32, 36, 37, 40, 40.1, 43, 45, 46, and 46.6) that constitute connexin inhibitors (peptides). Such connexin inhibitors may comprise from about 8 to about 15, or from about 11 to about 13 contiguous amino acids of the peptides in SEQ ID NOs: 18 to 19 and 66 to 79. Conservative amino acid changes may

be made to the peptides or fragments thereof.

huCx43 LLIQWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKTIFII (SEQ ID NO: 66)

huCx26 MYVFYVMYDGFSMQRLVKCNAWPCPNTVDCFVSRPTEKTVFTV (SEQ ID NO: 67)

huCx30 YVFYFLYNGYHLPWVLKCGIDPCPNLVDCFISRPTEKTVFTI (SEQ ID NO: 68)

huCx30.3 LYIFHRLYKDYDMPRVVACSVEPCPHTVDCYISRPTEKKVFTY (SEQ ID NO: 69)

huCx31 LYLLHTLWHGFNMPRLVQCANVAPCPNIVDCYIARPTEKKTY (SEQ ID NO: 70)

huCx31.1 LYVFHSFYPKYILPPVVKCHADPCPNrVDCFISKPSEKNIFTL (SEQ ID NO: 71)

huCx32 MYVFYLLYPGYAMVRLVKCDVYPCPNTVDCFVSRPTEKTVFTV (SEQ ID NO: 72)

huCx36 LYGWTMEPVFVCQRAPCPYLVDCFVSRPTEKTIFII (SEQ ID NO: 73)

huCx37 LYGWTMEPVFVCQRAPCPYLVDCFVSRPTEKTIFII (SEQ ID NO: 74)

huCx40.1 GALHYFLFGFLAPKKFPCTRPPCTGVVDCYVSRPTEKSLLML (SEQ ID NO: 75)

huCx46 IAGQYFLYGFELKPLYRCDRWPCPNTVDCFISRPTEKTIFII (SEQ ID NO: 76)

huCx46.6 LVGQYLLYGFEVRPFFPCSRQPCPHVVDCFVSRPTEKTVFLL (SEQ ID NO: 77)

huCx40 IVGQYFIYGIFLTTLHVCRRSPCPHPVNCYSRPTEKNVFIV (SEQ ID NO: 78)

huCx45 LIGQYFLYGFQVHPFYVCSRLPCHPKIDCFISRPTEKTIFLLC (SEQ ID NO: 79)

[0067] SEQ ID NOs: 80 to 92 and partial peptides thereof shown below provide peptides inhibitors of connexin 40 shown with reference to the extracellular loops (E1 and E2) of connexin 40.

[0068] E1

LGTAAESSWGDEQADFRCDTIQPGCQNVCTDQAFPISHIRFWVLQ (SEQ ID NO: 80)

LGTAAESSWGDEQA (SEQ ID NO: 81)

DEQADFRCDTIQP (SEQ ID NO: 82)

TIQPGCQNVCTDQ (SEQ ID NO: 83)

VCTDQAFPISHIR (SEQ ID NO: 84)

AFPISHIRFWVLQ (SEQ ID NO: 85)

[0069] E2

MEVGFIVGQYFIYGIFLTTLHVCRRSPCPHPVNCYVSRPTEKNVFIV (SEQ ID NO: 86)

MEVGFIVGQYF (SEQ ID NO: 87)

IVGQYFIYGIFL (SEQ ID NO: 88)

GIFLTTLHVCRRSP (SEQ ID NO: 89)

RRSPCPHPVNCY (SEQ ID NO: 90)

VNCYVSRPTEKN (SEQ ID NO: 91)

SRPTEKNVFIV (SEQ ID NO: 92)

[0070] SEQ ID NOs: 93 to 105 provide peptides inhibitors of connexin 45 shown with reference to the extracellular loops (E1 and E2) of connexin 45.

[0071] E1

LTAVGGESIYYDEQSKFVCNTEQPGCENVCYDAFAPLSHVRFWVFQ (SEQ ID NO: 93)

LTAVGGESIYYDEQS (SEQ ID NO: 94)

DEQSKFVCNTEQP (SEQ ID NO: 95)

TEQPGCENVCYDA (SEQ ID NO: 96)

VCYDAFAPLSHVR (SEQ ID NO: 97)

APLSHVRFWVFQ (SEQ ID NO: 98)

[0072] E2

FEVGFLIGQYFLYGFQVHPFYVCSRLPCHPKIDCFISRPTEKTIFLL (SEQ ID NO: 99)

FEVGFLIGQYF (SEQ ID NO: 100)

LIGQYFLYGFQV (SEQ ID NO: 101)

GFQVHPFYVCSRLP (SEQ ID NO: 102)

SRLPCHPKIDCF (SEQ ID NO: 103)

IDCFISRPTEKT (SEQ ID NO: 104)

SRPTEKTIFLL (SEQ ID NO: 105)

[0073] Examples of inhibitors (peptides) of connexin 45 are as follows:

YVCSRLPCHP (SEQ ID NO: 106)

QVHPFYVCSRL (SEQ ID NO: 107)

FEVGFLIGQYFLY (SEQ ID NO: 108)

GQYFLYGFQVHP (SEQ ID NO: 109)

GFQVHPFYVCSR (SEQ ID NO: 110)

AVGGESIYYDEQ (SEQ ID NO: 111)

YDEQSKFVCNTE (SEQ ID NO: 112)

NTEQPGCENVCY (SEQ ID NO: 113)

CYDAFAPLSHVR (SEQ ID NO: 114)

FAPLSHVRFWVF (SEQ ID NO: 115)

LIGQY (SEQ ID NO: 116)

QVHPF (SEQ ID NO: 117)

YVCSR (SEQ ID NO: 118)

SRLPC (SEQ ID NO: 119)

LPCHP (SEQ ID NO: 120)

GESIY (SEQ ID NO: 121)

YDEQSK (SEQ ID NO: 122)

SKFVCN (SEQ ID NO: 123)

TEQPGCEN (SEQ ID NO: 124)

VCYDAFAP (SEQ ID NO: 125)

LSHVRFWVFQ (SEQ ID NO: 126)

[0074] The antibodies and peptides used as connexin inhibitors may be commercial products. Examples of Cx43 antibodies include Cat. No: Sigma, C-6219; Zymed, 13-8300; Santa Cruz, sc-6560; etc. Examples of Cx43-inhibiting peptides include Cat. No: Santa Cruz, sc-6560 P; BD Transduction Laboratories, 610063; and Abcam, ab56616.

[0075] TGFβ signaling inhibitors are defined as agents that inhibit TGF-β signal transduction, including TGF-β antagonists known in the art. For example, agents that bind to TGF-β receptor I and prevent TGF-β from binding to the TGF-β receptor act as TGF-β signaling inhibitors.

[0076] Other non-limiting examples include blocking (neutralizing) antibodies specific for a human TGF-β (NAb), such as those described by Dasch et al. (J. Immunol. (1989) 142: 1536) and Lucas et al. (J. Immunol. (1990) 145: 1415), soluble TGF-β receptors, membrane-bound TGF-β receptors, protease inhibitors that inactivate a protease responsible for activating a precursor TGF-β into mature TGF-β, antibodies that are specific to TGF-β receptors (Type I, II, or III) and prevent TGF-β binding to the receptor, and combinations thereof.

[0077] The TGF-β signal transduction is described with reference to Fig. 4.

[0078] Ligand TGF-β binds to TGF-β receptor II (TβRII) to thereby activate TβRII. The activated TβRII is polymerized with TGF-β receptor I (TβRI) to form a tetramer composed of two TβRI and two TβRII, and phosphorylation occurs in the C terminus of TβRI. The phosphorylated TβRI becomes an active form. To the C terminus of the phosphorylated TβRI, Smad2 and Smad3 (mediators of TGF-β signaling) are supplied and phosphorylated. The phosphorylated Smad2 and Smad3 bind to Smad4 localized in the cytoplasm, and nuclear translocation occurs, followed by binding to the target gene, thereby affecting the expression of the target gene.

[0079] The TGF-β signaling inhibitors of the present invention inhibit any one of the above signal transduction pathways. Accordingly, substances having functions, such as inhibition of TGF-β expression, inhibition of TβRI or TβRII expression, inhibition of the binding of TGF-β to TβRII, inhibition of the formation of a tetramer composed two TβRI and two TβRII, inhibition of the phosphorylation of the C terminus of TβRI, dephosphorylation of the C terminus of TβRI, ubiquitination of TβRI, inhibition of the phosphorylation of Smad2 and Smad3, inhibition of the expression of Smad2 and Smad3, ubiquitination of Smad2 and Smad3, dephosphorylation of phosphorylated Smad2 and Smad3, and inhibition of the

binding of phosphorylated Smad2 and Smad3 to Smad4, can function as TGF-β signaling inhibitors in the present invention.

[0080] The TGF-β signaling inhibitors may be commercial products. Such commercial products are divided into (1) those inhibiting receptors, and (2) those inhibiting downstream signal transduction of the receptors.

(1) Receptor I (ALK 5) inhibitor:

SB425342, A-83-01, and ALK5 inhibitors

(2) Signal transduction inhibitor:

Inhibitor Smad: Smad7 binds to TβRI so as to inhibit the binding of Smad2 and Smad3 to TβRI.

[0081] Signaling mediator inhibition: siRNA or antisense oligo DNA inhibits the expression of Smad2, Smad3, and Smad4.

[0082] The TGF-β signaling inhibitors widely include commercially available Receptor I (ALK 5) inhibitors (e.g., SB425342, A-83-01, and ALK5 inhibitors) and substances that mimic the function of signal transduction inhibitors.

[0083] The TGF-β signaling inhibitors include antibodies against TGFβ, TβRI, and TβRII. The antibodies include, as described regarding connexin inhibitors, monoclonal antibodies, polyclonal antibodies, antibody fragments (including, for example, Fab, $F(ab')_2$, and Fv fragments); single-chain antibodies; single-chain Fvs; and single-chain binding molecules (e.g., those comprising, for example, a binding domain, hinge, CH2 and CH3 domains), recombinant antibodies and antibody fragments that are capable of binding an antigenic determinant (i.e., that portion of a molecule, generally referred to as an epitope) that makes contact with a particular antibody or other binding molecule.

[0084] The TGF-β signaling inhibitors include substances that prevent the conversion of TGF-β precursor into mature TGF-β, and also include soluble TGF-β receptors.

[0085] TGF-β is generally secreted as a latent precursor consisting of TGF-β non-covalently associated with a protein designated latency-associated protein (LAP; Harpel et al. (1992) Prog. Growth Factor Res. 4: 321). This latent complex requires enzymatic cleavage

of carbohydrate groups or transient acidification to release the active cytokine. Purified LAP by itself binds active TGF-β with high affinity to form a latent complex. A DNA encoding a 278 amino acid peptide corresponding to pre-pro-TGF-β, terminating just prior to the mature form of TGF-β and containing a Cys33 to Ser33 substitution has been expressed (Derynck et al. (1985) Nature 316: 701), and found to bind TGF-β and render it latent.

[0086] Soluble forms of TGF-β receptors will also bind TGF-β and prevent binding to membrane-associated TGF-β receptors. TGF-β receptors are described by Wang et al. (Cell (1991) 67: 797) and Lin et al. (Cell (1992) 68: 775). Soluble forms of TGF-β receptors may be prepared by methods that are known in the art. For example, deletion mutants lacking the transmembrane domain of a TGF-β receptor can be prepared, which will express a soluble TGF-β binding protein (Miyazono et al. (Adv. Immunol. (1994) 55: 181).

[0087] Other types of TGF-β antagonists that function as TGF-β signaling inhibitors are also known in the art. For example, Yamaguchi et al. (Nature (1990) 346: 281) discuss decorin, a small chondroitin-dermatan sulphate proteoglycan that binds TGF-β and modulates the activity of this growth factor. Ohtsuki and Massague (Mol. Cell. Biol. 12: 261-265, 1992) disclose protein kinase inhibitors that block certain biological activities of TGF-β. The design and use of protease inhibitors as drugs is well known in the art (Design of Enzyme Inhibitors as Drugs; Sandler and Smith, eds; 1989, Oxford University Press; Proteinase Inhibitors Medical and Biological Aspects; Katunuma, Umezawa and Holzer, eds., 1983, Springer-Verlag); thus, inhibitors of cruzain van be prepared and will be useful as TGF-β antagonists.

[0088] Still other TGF-β antagonists and methods for their production are well known in the art, with many more currently under development. The specific TGF-β antagonist employed is not a limiting feature, as any effective TGF-β antagonist may be useful in this invention. Examples of such antagonists include monoclonal and polyclonal antibodies directed against one or more isoforms of TGF-β (U.S. Pat. No. 5,571,714 and WO 97/13844), TGF-β receptors, fragments thereof, derivatives thereof and antibodies directed against TGF-β receptors (U.S. Pat. Nos. 5,693,607, 6,008,011, 6,001,969 and 6,010,872; and WO

92/00330, WO 93/09228, WO 95/10610 and WO 98/48024); latency-associated peptide (WO 91/08291), large latent TGF-β (WO 94/09812), fetuin (U.S. Pat. No. 5,821,227), decorin and other proteoglycans, such as biglycan, fibromodulin, lumican and endoglin (U.S. Pat. Nos. 5,583,103, 5,654,270, 5,705,609, 5,726,149, 5,824,655, 5,830,847, and 6,015,693; and WO 91/04748, WO 91/10727, WO 93/09800 and WO 94/10187).

[0089] Further examples of such antagonists include somatostatin (WO 98/08529), mannose-6-phosphate or mannose-1-phosphate (U.S. Pat. No. 5,520,926), prolactin (WO 97/40848), insulin-like growth factor II (WO 98/17304), IP-10 (WO 97/00691), arg-gly-asp-containing peptides (U.S. Pat. No. 5,958,411 and WO 93/10808), extracts of plants, fungi and bacteria (European Patent Application No. 813875, Japanese Patent Application No. 81-19984 and U.S. Pat. No. 5,693,610), antisense oligonucleotides (U.S. Pat. Nos. 5,683,988, 5,772,995, 5,821,234 and 5,869,462; and WO 94/25588), and other proteins involved in TGF-β signaling, including SMADs and MADs (European Patent Application EP 874046; WO 97/31020, WO 97/38729, WO 98/03663, WO 98/07735, WO 98/07849, WO 98/45467, WO 98/53068, WO 98/55512, WO 98/56913, WO 98/53830 and WO 99/50296; and U.S. Pat. Nos. 5,834,248, 5,807,708 and 5,948,639) and Ski and Sno (G. Vogel, Science, 286: 665 (1999) and Stroschein et al., Science, 286: 771-74 (1999)) and fragments and derivatives of any of the above molecules that retain the ability to inhibit the activity of TGF-β.

[0090] TGF-β receptors and TGF-β-binding fragments of TGF-β receptors, especially soluble fragments, are useful TGF-β antagonists in the methods of the present invention. TGF-β receptors and the nucleic acids encoding them are well known in the art. The nucleic acid sequence encoding TGF-β type 1 receptor is disclosed in GenBank accession number L15436 and in U.S. Pat. No. 5,538,892 of Donahoe et al. The nucleic acid sequence of TGF-β type 2 receptor is publicly available under GenBank accession numbers AW236001; A135790; AI279872; AI074706; and AA808255. The nucleic acid sequence of TGF-β type 3 receptor is also publicly available under GenBank accession numbers NM003243; AI887852; AI817295; and AI681599. In a preferred embodiment, the TGF-β antagonist is an antibody that blocks TGF-β binding to its receptor, or fragments thereof,

such as $F(ab)_2$ fragments, Fv fragments, single-chain antibodies and other forms of "antibodies" that retain the ability to bind to TGF-β. The antibody may be chimerized or humanized.

[0091] Other examples include an RNA sequence that acts as RNAi against TGFβ1 receptor II (Japanese Patent Publication No. 2005-253344), siRNA for silencing of TGFβII receptor expression (Japanese Patent Publication No. 2007-502284), pyrimidinylimidazole as a TGF-β inhibitor (Japanese Patent Publication No. 2008-511631), pyrimidinylpyrazole (Japanese Patent Publication No. 2008-511630), and the like.

[0092] Preferred TGF-β signaling inhibitors are SB-431542, A-83-01, and ALK5 inhibitors.

[0093] The TGF-β signaling inhibitors can be added to the medium of the cells. The concentration is, for example, an effective amount of the TGF-β signaling inhibitor used. In the case of SB-431542, for example, the concentration is about 1 to 100 μM, and preferably about 10 μM.

[0094] The connexin inhibitor can be incorporated into the cells by adding the connexin inhibitor in an amount not less than the effective amount to the medium. For example, in the case of siRNA, about 10 to 1,000 nM, and preferably about 100 nM of the connexin inhibitor can be added to the medium. The amounts of TGF-β signaling inhibitors and connexin inhibitors other than those mentioned above can be easily determined by those skilled in the art.

[0095] Examples of the cells to be used as the starting material of the iPS cells include all types of somatic cells, such as cardiomyocytes, fibrocytes, skin cells, muscle cells, and fat cells. Such somatic cells are preferably those that express connexins, and more preferably those that highly express connexins.

[0096] The iPS cells can be obtained from mammalian cells (e.g., human, mouse, rat, cattle, horse, pig, rabbit, sheep, goat, monkey, dog, and cat) or animal cells (e.g., chicken, duck, and xenopus).

[0097] The connexin inhibitor and TGFβ signaling inhibitor may be reacted with the cells simultaneously or sequentially. Preferably, these inhibitors are simultaneously reacted with

the cells.

[0098] From the iPS cells obtained by reacting the cells with the connexin inhibitor and TGF-β signaling inhibitor, a group of iPS cells can be separated and purified by removing non-iPS cells according to a general method.

[0099] At the time when the iPS cells of the present invention are induced by reacting the cells with the connexin inhibitor and TGF-β signaling inhibitor, either one or both of the connexin inhibitor and TGF-β signaling inhibitor is presumably present in the cells. In particular, when these inhibitors are molecules that inhibit gene expression in the cells, they are considered to be present in the cells at the moment when the cells turn into iPS cells. In particular, the connexin inhibitor is not conventionally used in the production and culture of iPS, and the iPS cells comprising the connexin inhibitor are novel.

[0100] The connexin inhibitor and TGF-β signaling inhibitor both can be used to induce iPS cells, and are thus useful as iPS cell inducers.

[0101] The medium comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGFβ signaling inhibitors can induce iPS cells, and is thus useful as a medium for inducing iPS cells.

[0102] Furthermore, at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors can induce iPS cells, and is thus useful as a kit for inducing iPS cells.

EXAMPLES

[0103] The present invention is described in detail below according to Examples.

Example 1: Reprogramming of cardiomyocytes derived from neonatal mouse ventricles

Target and Method

1) Isolation of Cardiomyocytes

Two-day-old neonatal C57BL/6 or C57BL/6-Tg (CAG-EGFP) mouse hearts including ventricles were minced into approximately 2-mm cubes with dissecting scissors by a conventional method. They were treated with 0.2% collagenase (type II; Worthington Biochemical Corp. U.S.; dissolved in PBS) to dissociate cardiomyocytes. The dissociated

cardiomyocytes were centrifuged, and incubated for 45 minutes in DMEM containing 10% FBS at 37°C with 5% CO_2. After the incubation, the supernatant was further incubated under the same conditions. This process was repeated four times. Thus, fibroblasts were removed, and the cardiomyocytes were isolated.

[0104] 2) Reprogramming from Cardiomyocytes

The cardiomyocytes were seeded at 2 x 10^5 cells in a 6-cm dish, and cultured in the above DMEM medium. On day 7 after culture, the cardiomyocytes were switched from 15% FBS medium to mouse ES media containing either a TGF-β receptor inhibitor SB-431542 (10 μM; Tocris, USA), siRNA Cx43 (100 nM), or both compounds.

The mouse Cx43 siRNA sequences used are as follows:

[0105] sense: 5'-CAA UUC CUC CUG CCG CAA TT-3'

antisense: 5'-UUG CGG CAG GAG GAA UUG TT-3'

[0106] 3) Evaluation of Pluripotency

(i) Cell Immunostaining of Early Marker of Es Cells

Two iPS cell colonies were obtained after about two weeks from cardiomyocytes that were selectively cultured in the ES medium containing both 10 μM SB-431542 and 100 nM siRNA Cx43. iPS-like cells grown by culturing on feeder cells under the same conditions as mouse ES cell culture conditions were used for immunostaining, etc., and for confirmation of teratoma formation.

[0107] Measurement of alkaline phosphatase (ALP) was performed using an Alkaline Phosphatase Detection Kit (Millipore, USA).

[0108] The antibodies used in cell immunostaining are as follows:

[0109] rabbit anti-Nanog antibody (diluted 200 times, Cosmo Bio.; REC-RAB004PF),

rabbit anti-Oct4 antibody (diluted 500 times; sc-9081),

goat anti-Sox2 antibody (diluted 200 times; sc-17320), and

mouse anti-SSEA1 antibody (diluted 200 times; sc-21702).

(ii) Teratoma Formation

The iPS-like cells (2 x 10^5) were implanted subcutaneously in SCID mice. After

five weeks, tumors were removed, and whether teratomas would be formed was histologically confirmed by HE staining.

(iii) Production of Chimeric Mice

The iPS-like cells were implanted in eight-celled fertilized eggs derived from ICR mice, and the eggs were returned to the ICR mice. Then, it was confirmed whether chimeric mice would be produced.

[0110] Results

1. Expression of ES Early Marker Gene

The two iPS cells derived from mouse cardiomyocytes produced in the present invention, siPSCs (silencing-induced pluripotent stem cells), were morphologically similar to a mouse ES cell colony (Fig. 1a); ALP activity was confirmed (Fig. 1b); and the expression of the pluripotency marker factors, Nanog, Oct4, Sox2, and SSEA1, was confirmed at the protein level (Fig. 1c). Moreover, the expression of various pluripotency marker genes was also confirmed at the gene level (Fig. 1d). Furthermore, the induction of the iPS-like cells from the EGFP mouse cardiomyocytes was also confirmed (Fig. 2).

[0111] 2. Verification of Differentiation Potency of siPSCs

iPSCs were implanted subcutaneously in SCID mice. After five weeks, teratomas consisting of all three embryonic germ layers, such as epidermal and neural tissues (ectoderm), muscles and cartilage (mesoderm), and gland (endoderm), were formed (Fig. 1e). Although no data is provided, it was confirmed that siPSCs formed blastoderms, which were able to differentiate into neurons, cardiomyocytes, or endothelial cells, depending on differentiation-inducing conditions. Beat and conduction were observed in the cardiomyocytes. Further, in the cells differentiation-induced by immunostaining and RT-PCR, the expression of three embryonic germ layer marker gene was confirmed at both protein and gene levels.

[0112] 3. Production of Chimeric Mice

The two iPS cells derived from mouse cardiomyocytes produced in the present invention were injected into blastocysts to thereby generate adult chimeric mice containing a

mixture of cells derived from the iPS cells. This result suggests that the iPS cells have differentiation pluripotency equivalent to that of ES cells (Fig. 3).

[0113] The patent documents and papers referred to herein are incorporated herein by reference in their entireties.

INDUSTRIAL APPLICABILITY

[0114] The method of the present invention can produce iPS cells safely and readily; therefore, the present invention provides a highly effective means for clinical application of iPS cells in the future.

CLAIMS

1. A method for producing iPS cells, comprising reacting cells with at least one connexin inhibitor and at least one TGFβ signaling inhibitor.

2. The method according to claim 1, wherein the connexin inhibitor is a connexin 43 inhibitor.

3. The method according to claim 1, wherein the connexin inhibitor is siRNA, shRNA, miRNA, antisense RNA, peptide, antibody, or ribozyme against connexin, or an expression vector that can release RNA molecules thereof in the cells.

4. The method according to claim 1, wherein the TGFβ signaling inhibitor is a TGF-β receptor I inhibitor.

5. The method according to claim 1, wherein the TGF-β receptor inhibitor is SB-431542, A-83-01, or ALK5 inhibitor.

6. iPS cells comprising at least one connexin inhibitor.

7. The iPS cells according to claim 6, wherein the connexin inhibitor is a connexin 43 inhibitor.

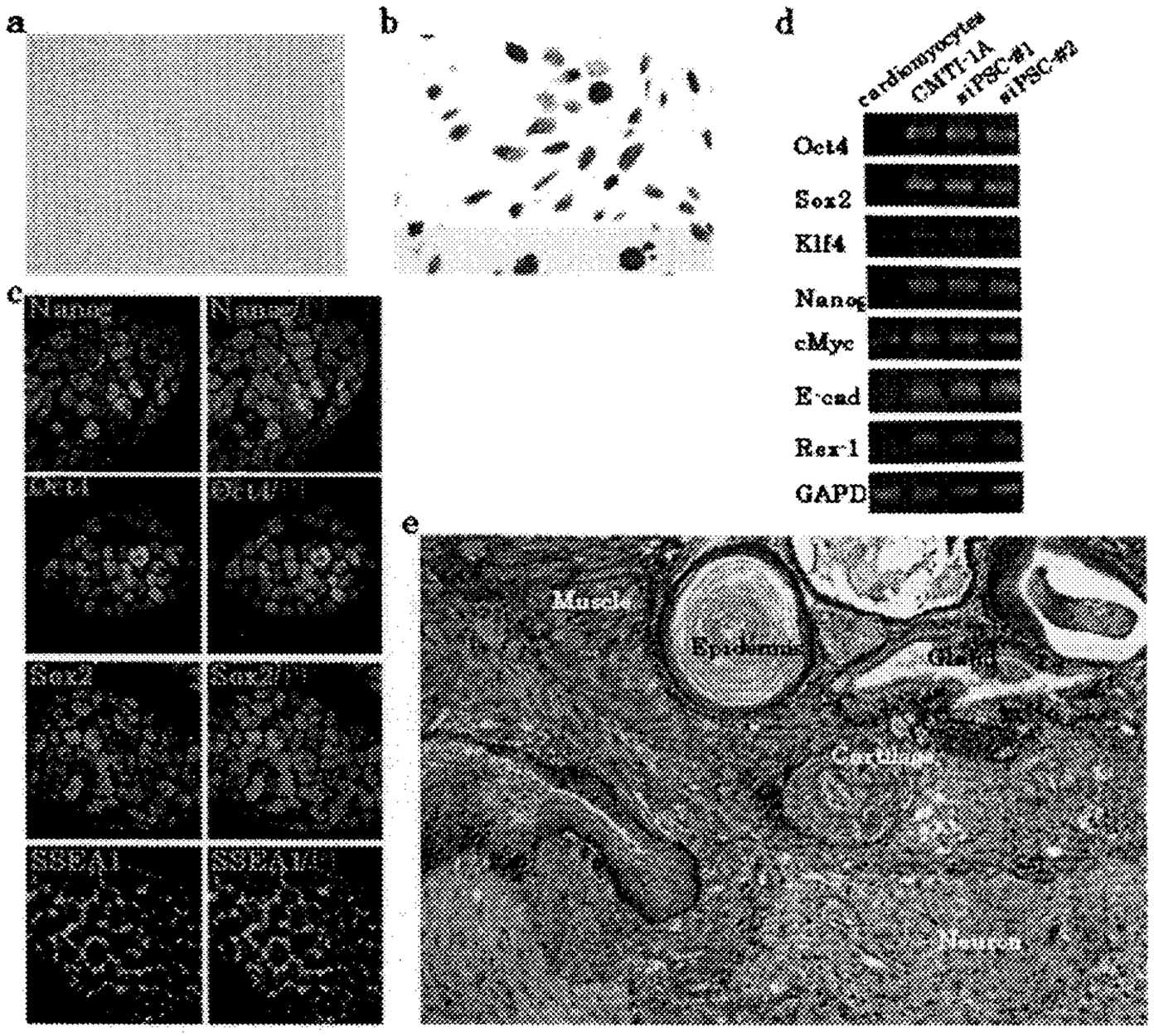
8. The iPS cells according to claim 6, wherein the connexin inhibitor is siRNA, shRNA, miRNA, antisense RNA, peptide, antibody, or ribozyme against connexin, or an expression vector that can release RNA molecules thereof in the cells.

9. An iPS cell inducer comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGFβ signaling inhibitors.

10. A medium for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors.

11. A kit for inducing iPS cells, comprising at least one inhibitor selected from the group consisting of connexin inhibitors and TGF-β signaling inhibitors.

[図1]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Neuron

[図2]


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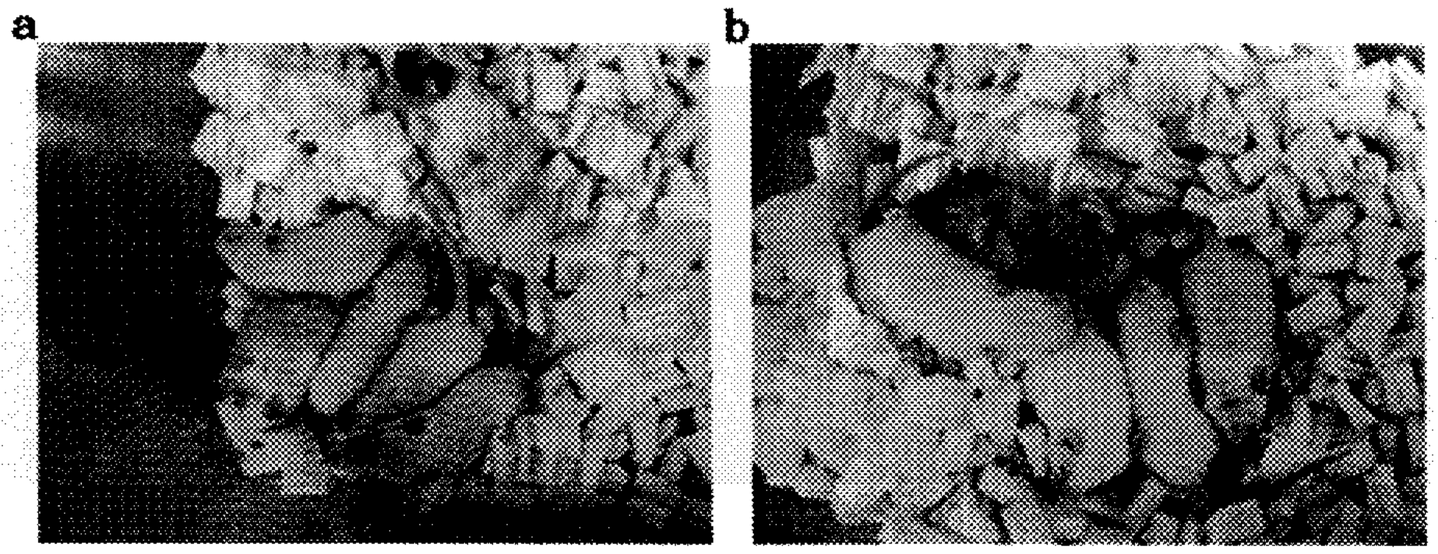
Merge

[図3]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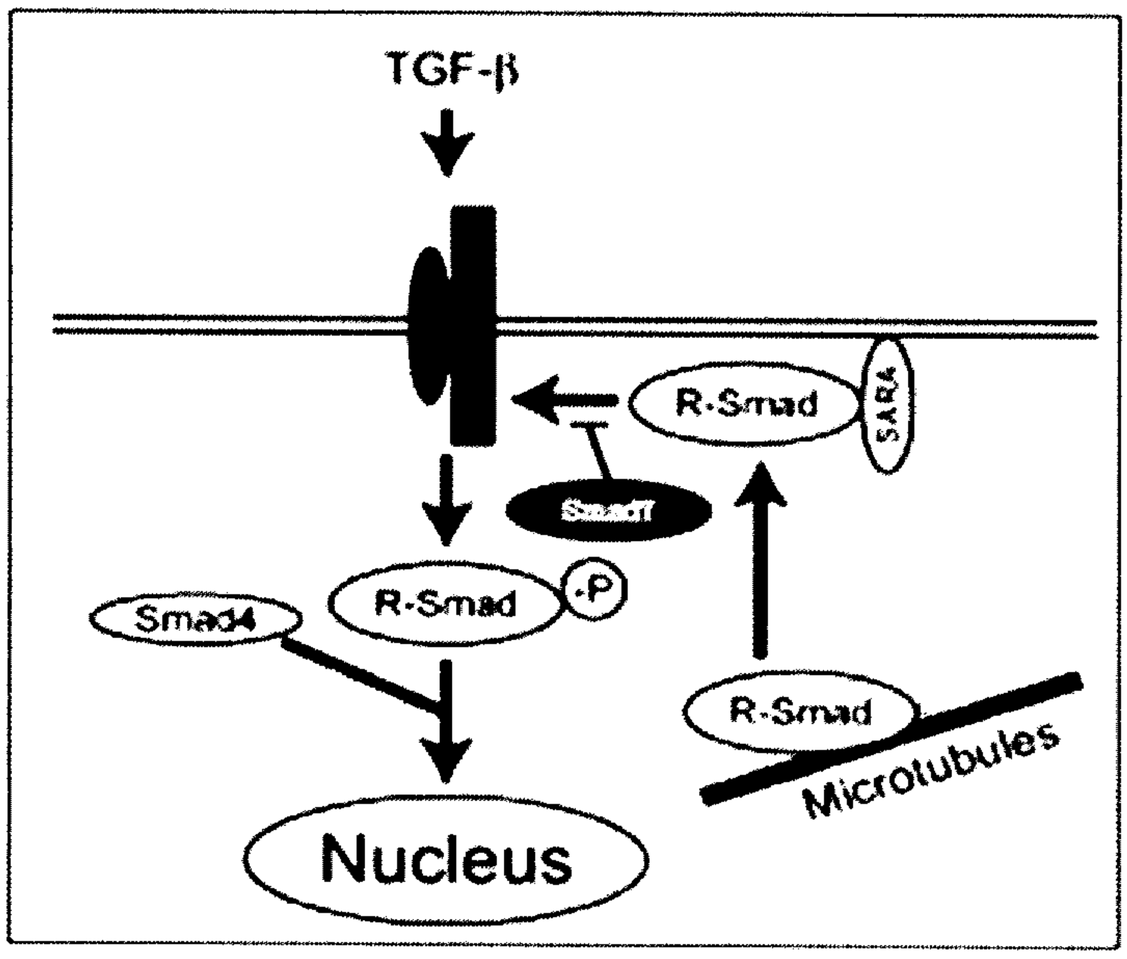
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SARA
Smad7
R-Smad
·P
Smad4
R-Smad
Microtubules
Nucleus