HUMAN BMP-7 PROMOTER AND METHOD FOR EXPLORING BONE-RELATED SUBSTANCE BY USING THE SAME

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to a 5' upstream region DNA containing a promoter of a human bone morphogenetic protein (hereafter referred to as BMP-7). Further, the present invention relates to a method for exploring a low molecular weight compound positively or negatively which regulates the expression of human BMP-7 by using a mass of animal or yeast cells that are introduced with a recombinant expression vector harboring a 5' upstream region DNA containing the human BMP-7 promoter integrated into a suitable reporter gene, and by using a reporter activity as an indicator.

(2) Description of the Related Art

At present, a bone morphogenetic activity has been reported for a bone morphogenetic factor, BMP, belonging to TGF (transforming growth factor) -β superfamily (Science 150, 893-897, 1965; Science 242, 1528-1534, 1988). Known species of BMP are BMP-1 to BMP-14. Among them, the members from BMP-2 to BMP-14 have been known as showing the bone morphogenetic activity. BMPs ranging from BMP-2 to BMP-14 are considered as effective to therapeutic and preventive treatment for various bone dysfunction and bone diseases, however, they exist in very small amount in nature. Therefore, an available large quantity from BMP-2 to BMP-14 used for these treatments requires production of recombinant protein. The production of the recombinant protein generally is very expensive compared with a low molecular weight compound. Furthermore, there are many restrictions as a medical drug in terms of physical properties and administration methods due to proteinic characteristics. Considering these points, a small molecular organic compound having the activity equal to that of the BMP protein, if any, should be a highly promising medical drug. The substance obtainable with the exploring method provided by the present

invention has the activity to induce the expression of human BMP-7, a bone osteogenesis factor, and also has the efficacy equal to that of human BMP-7, representing very high usefulness. On the contrary, if human BMP-7 is concerned with bone and cartilage hyperplasia, inhibiting the expression may prevent osteohyperplasia. The present invention is able to detect the inhibition of the human BMP-7 expression and provides a method for exploring a substance to prevent hyperplasia. In addition, it is known that human BMP-7 has the ability to enhance the differentiation of kidney cells (Proc. Natnl. Acad. Sci., U.S.A., Vol. 93, p. 9021-9026, 1996). Thus, the experimental system provided by the present invention can be applied to a method for exploring the agent for the treatment of the kidney disorder.

For such an exploring method, an example has been so far only reported using a murine BMP-2 promoter (W097/15308), and there is no example of using the human BMP-7 promoter. In addition, since the materials of the exploring method provided by the present invention are all derived from human sources, it can be expected that discovered substances should show the effects at clinic practically.

SUMMARY OF THE INVENTION

The present invention provides a 5' upstream region DNA containing a promoter of human BMP-7. By using 5' upstream region gene containing the human BMP-7 promoter and an animal cell introduced with a recombinant expression vector that has been connected to an appropriate reporter gene, the low molecular weight compounds which regulate positively or negatively the expression of human BMP-7 can be explored with reference to a reporter activity. The low molecular weight compounds and their derivatives have morphogenetic activity and inhibiting activity for bone and cartilage through the expression of human BMP-7 and are effective as preventive or therapeutic agents for cartilage and bone diseases, remedies for osteometastasis, or therapeutic and preventive agents for excess osteogenesis. Furthermore, these low molecular weight compounds and their derivatives are useful as preventive or therapeutic agents for kidney disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an exon-intron structure of 10.8 kb 5' upstream region of human BMP-7 gene and a restriction enzyme map. A net shape shows an exon region and an open square shows an intron region.

Fig. 2 is a recombinant expression vector (pMSS115) containing a 5' upstream region of human BMP-7 gene. A promoter region (4.4 kb) (base No. from 3813 to 8222 shown in SEQ ID NO. 1 of the Sequence Listing, referring from the 2nd *Xba*I to the 3rd *Xba*I from 5' terminal in Fig. 1) was inserted to *Nhe*I restriction enzyme site of pGL3-basic.

Fig. 3 is a result of measuring human BMP-7 promoter activity (transiently expression).

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a DNA whose nucleotide sequence is represented by the base sequence No. from 1 to 10877 shown in SEQ ID NO. 1 of the Sequence Listing that encodes a human bone morphogenetic protein-7 promoter region, or a fragment thereof. SEQ ID NO. 1 of the Sequence Listing shows the 5' upstream region sequence of the human BMP-7 gene.

The present invention relates to a method for preparing the DNA shown in SEQ ID NO. 1 of the Sequence Listing by conducting the steps of:

(1) digestion of a human placenta genomic DNA with a *Hin*dIII restriction enzyme,

(2) isolation by agarose gel electrophoresis,

(3) cloning of the isolated DNA fragment digested with *Hin*dIII into a lambda phage vector λDASH II treated with the same enzyme,

(4) packaging of said vector into the phage,

(5) establishment of genomic DNA library by infecting *Escherichia coli* with the phage,

(6) screening by PCR, and

(7) subcloning into a plasmid vector.

The plasmid vector used herewith is not restricted and can be used among ones commercialized. A pUC18 vector can be a preferable example.

The present invention relates to a recombinant expression vector characterized by integration of the full length or a part of DNA shown in SEQ ID NO. 1 of the Sequence Listing into a reporter gene. In detail, the recombinant expression vector is constructed to locate a suitable region of 5' upstream region of the human BMP-7 gene, that is represented by SEQ ID NO. 1 of the Sequence Listing, in front of a reporter gene. The reporter gene such as luciferase or β-galactosidase gene shows an expressing status on behalf an original product. The vector as the original for the recombination expression vector is not specially restricted to allow to use a plasmid vector commercialized. The present invention used pGL3-basic as a preferable example. The use of pGL3-basic yielded pMSS115 (9.2 kb) that is a recombination expression vector containing the human BMP-7 promoter and a luciferase gene. The present invention assigned it to the recombination expression vector. It is necessary to introduce the vector to mammalian cells, preferably a human osteoblast-like cells, such as SaOS-2 cells, with a liposome. The animal cells stably transfected with the recombinant expression vector are selected by using a resistance marker.

The present invention relates to a method for exploring a bone-related substance, characterized by using the recombinant expression vector characterized by integration of the full length or a part of DNA shown in SEQ ID NO. 1 of the Sequence Listing into a reporter gene. It relates to the method for exploring a bone-related substance wherein the bone-related substance is osteogenesis inducing substance or a bone-related substance wherein a bone-related substance is osteogenesis inhibiting substance. A low molecular weight compound which induces or inhibits the expression of human BMP-7 can be obtained by isolating the promoter which regulates the expression of the gene, by connecting it to a suitable reporter gene and by introducing the gene structure to a suitable mammal cell to make an exploring system. The substance which regulates the expression of human BMP-7 in the exploring system works on the promoter to increase or

decrease the expression level of the reporter gene. Therefore, a simple and easy measurement of the reporter activity makes an exploration of the aimed substance possible.

The animal cell transfected with said vector can be used for a method for screening a chemical compound library by high throughput screening (Nature, Vol. 384, Suppl., p. 14-16, (1996) and exploring an active substance from natural substances. The substance which increases or decreases an activity is searched by treating the cell with a substance for an appropriate time period and thereafter measuring the reporter activity. The substance obtained hereby can regulate the expression by working directly on a transcription factor or indirectly on the promoter of human BMP-7 through regulating a signal transduction system. Therefore, these compounds are effective as a therapeutic agent for osteocartilaginous diseases, cancer metastasis to bone, or osteohyperplasia.

Furthermore, these compounds are useful as a therapeutic agent for kidney disorders.

The substance obtained by the present invention has bone or cartilage morphogenetic activity and is effective as an agent for therapeutic and preventive treatment in the fields of orthopedic surgery (fracture, osteoarthritis such as joint osteoarthritis and hip joint osteoarthritis, arthrosteitis, damage of cartilage such as damage of meniscus, regeneration of bone and cartilage deficit caused by injury and tumor dissection, bone reconstruction such as spinal fusion and vertebral canal enlargement, and congenital cartilage and bone diseases such as dysoteogenesis and achondroplasia), or dental fields (bone reconstruction such as palatoschisis, mandible reconstruction, and residual ridge construction), and osteoporosis. Moreover, the substance of the present invention can be used for bone graft in aesthetic surgery. These therapeutic treatments are effective to therapies in the fields of veterinary surgery. On the other hand, the present invention can provide a substance to inhibit bone or cartilage morphogenesis. In this case, the substance is

applied as an agent for prevention and therapy of bone and cartilarge hyperplasia.

In addition, the present invention can provide a substance with ability to enhance the differentiation of kidney cells and it can be applied to an agent for the treatment and prevention of the kidney disorder.

EXAMPLES

This invention shall be more illustratively explained by way of the following Examples. The following Examples are to be considered in all respects as illustrative and not restrictive.

Example 1 Isolation of 5' upstream region of human BMP-7 gene

A human placenta genomic DNA (a product of CloneTech) was digested by using various kinds of restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Sph*I, and *Xba*I), separated by agarose gel electrophoresis, transferred to a nylon membrane, and subjected to the Southern hybridization using BMP-7 cDNA (EMBO J. 9: 2085-2093, 1990) as a probe. As the result, it was found that digestion by the restriction enzyme *Hin*dIII among restriction enzymes used yielded a DNA fragment of ca. 11 kb containing the longest human BMP-7 gene. Then, a human placenta genomic DNA was digested by the restriction enzyme *Hin*dIII and separated by agarose gel electrophoresis to extract a DNA fragment of ca. 11 kb from the agarose gel. The DNA fragment obtained was cloned to lambda phage vector λDASH II (Stratagene Ltd. made) digested by the restriction enzyme *Hin*dIII. The vector was in vitro packaged by Gigapack III XL Extract (Stratagene Ltd. made), infected to *Escherichia coli* XL1-Blue MRA (Stratagene Ltd. made) to make a genomic DNA library. The library was divided into pools. Each pool was amplified by a screening (Nucleic Acids Res. 21: 2627-2631, 1993) using PCR; namely, the PCR method by using PCR primers (SEQ ID NO. 2 and SEQ ID NO.3 of the Sequence Listing) corresponding to translation region to select the objective pool, to obtain finally 5' upstream region (10.8 kb) of human BMP-7 gene. In addition, the 5' upstream 10.8 kb fragment

was subcloned to a pUC18 vector (a product of Amersham Pharmacia Biotech). The vector was named *E. coli* pKOT 314. The *E. coli* pKOT 314 was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry 1-3, Higashi 1-chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan, in March 30, 1998 with depository number FERM P-16737 and transferred to the International Depository Authority under Budapest Treaty on February 17, 1999 (Deposit No. FERM BP-6651).

Example 2 Determination of DNA sequence of 5' upstream region of human BMP-7 gene

The sequence of 5' upstream region of human BMP-7 gene obtained was determined by Amersham Pharmacia Biotech's ALF DNA Sequencer according to the method of Sanger et al. (Proc. Natl Acad. Sci. USA 74: 5463-5467. 1977). The sequence thus analyzed is described in SEQ ID NO. 1 of the Sequence Listing. The base sequence No. from 5557 to 10780 of SEQ ID NO. 1 was already been reported (EMBO J. 9: 2085-2093, 1990). However, there are many differences from the sequence of this invention.

Example 3 Construction of a recombinant expression vector containing the human BMP-7 promoter and a luciferase reporter gene

As shown in Fig. 1, the promoter of human BMP-7 exists in the upstream of exon 1. Then, a region of 4.4 kb containing a promoter from *Xba*I of the second position to *Xba*I of the third position from the 5' terminal described in Fig. 1 were - to align in the upstream of the reporter gene - inserted in the restriction enzyme site, *Nhe*I, of a luciferase reporter vector pGL3-basic (a product of Pro Mega Ltd.) to construct a recombinant expression vector pMSS115 (9.2 kb). This is presented in Fig. 2.

Example 4 Measurement of the activity of the human BMP-7 promoter (introduction of a recombinant expression vector to a human cell and transient expression)

In order to express transiently the human BMP-7 recombinant expression vector, said recombinant expression

vector (pMSS115) was mixed with a vector, pRL-SV40 (a product by Pro Mega Co.) containing sea pansy luciferase gene as an internal control for measurement of efficiency of gene introduction in equal quantity. Then, cationic liposome lipofectamine (a product of Lifetech Oriental Co.) was added to the mixture solution to add to human osteosarcoma cells HOS, MG63, and SaOS-2 for transfection. Fire fly luciferase activity and sea pansy luciferase activity were measured by Pikka Gene Dual Kit (a product of Toyo Ink Co.). The result is presented in Fig. 3. The promoter activity was expressed as a ratio of fire fly luciferase activity to sea pansy luciferase activity. From the result, it has been known that the DNA of SEQ ID NO. 1 of the Sequence Listing has a promoter activity.

Example 5 Introduction of the recombinant expression vector to a human cell and stabilized expression

In order to express the human BMP-7 recombinant expression vector stably, said vector was mixed with a vector pPUR (a product of CloneTech Ltd.) containing puromycin resistant gene in the proportion of 10 : 1 and also mixed with cationic liposome lipofectamine (a product of Lifetech Oriental Co.) to add to a human osteosarcoma cell HOS for transfection. The cells to which the aimed gene had been introduced were selected from a culture medium containing puromycin (a product of Sigma Ltd.).

Example 6 Screening of active low molecular weight compound

The established cells selected were inoculated in a 96-well plate, treated with substances of various chemical compound libraries for 1 - 3 days, dissolved with a cytolytic agent (a product of Pro Mega Ltd.), and measured for enzyme activity by employing a luciferase assay kit (a product of Pro Mega Ltd.). By such processes, various substances inducing or inhibiting the expression of human BMP-7 are obtained.

Sequence Listing Free Text

<210> 1
<223> Human BMP-7 5' upstream gene sequence including the exon 1 regions.

<210> 2
<223> Sense PCR primer for cloning 5' upstream human BMP-7 gene sequence corresponding to the exon 1 region.

<210> 3
<223> Reverse PCR primer for cloning 5' upstream human BMP-7 gene sequence corresponding to the exon 1 region.

What is claimed is:

1. A DNA whose nucleotide sequence is represented by the base sequence No. from 1 to 10877 shown in SEQ ID NO. 1 of the Sequence Listing that encodes a human bone morphogenetic protein-7 promoter region, or a fragment thereof.

2. A method for preparing the DNA shown in SEQ ID NO. 1 of the Sequence Listing comprising the steps of:

(1) digestion of a human placenta genomic DNA with a *Hin*dIII restriction enzyme,

(2) isolation by agarose gel electrophoresis,

(3) cloning of the isolated DNA fragment digested with *Hin*dIII into a lambda phage vector λDASH II treated with the same enzyme,

(4) packaging of said vector into the phage,

(5) establishment of genomic DNA library by infecting *Escherichia coli* with the phage,

(6) screening by PCR, and

(7) subcloning into a plasmid vector.

3. A recombinant expression vector characterized by integration of the full length or a part of DNA shown in SEQ ID NO. 1 of the Sequence Listing into a reporter gene.

4. The method for exploring a bone-related substance characterized by using the recombinant expression vector according to claim 3.

5. The method for exploring a bone-related substance according to claim 4, wherein the bone-related substance is osteogenesis inducing substance.

6. The method for exploring a bone-related substance according to claim 4, wherein the bone-related substance is osteogenesis inhibiting substance.

7. The method for exploring a substance effective at the treatment of a kidney disorder by using a recombinant expression vector according to claim 3.