

15 October 2002

HUMAN BMP-4 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 (BMP, hereafter-4). 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-4 by using a mass of animal or yeast cells that are introduced with a 5' upstream region DNA containing the human BMP-4 promoter and a recombinant expression vector 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. On the other hand, there are many restrictions as a medical drug in terms of physical properties and administration due to its proteinic characteristics. Considering these points, a small molecular organic compound having the equal activity to that of said BMP protein, if any, will become a very promising medical drug. The substance obtained by the exploring method provided by the present invention has the activity to induce the expression



of human BMP-4, a bone morphogenetic protein, and also has the same activity as that of human BMP-4 to be a very useful application. On the contrary, there is a report (New Engl. J. Med., Vol. 335, p. 555-561, 1996) suggesting that human BMP-4 causes bone and cartilage hyperplasia. In this case, inhibiting the expression of human BMP-4 may prevent osteohyperplasia. The present invention is able to detect the inhibition of the expression of human BMP-4 and provides a method for exploring a substance which prevents such hyperplasia.

For such an exploring method, an example was so far only reported using a murine BMP-2 promoter (W097/15308); there is no example of using a human BMP-4 promoter. The region of the murine BMP-4 promoter has been already cloned (Biochem. Biophys. Acta, Vol. 1218, p. 221-224, 1994), but no detailed sequence data of the human BMP-4 promoter has been elucidated. The present invention first disclosed the sequence of the human BMP-4 promoter. The homology of the entire sequence of 5' upstream region DNA between human and murine BMP-4 (J. Biol. Chem., Vol. 270, p. 28364-28373, 1995) is 52.2%. The materials for the exploring method provided by the present invention are all derived from human sources so that the substance obtained by the exploration would be a clinical application practically. It is expected to obtain a substance capable of more accurate regulation of the human BMP-4 expression by transfecting two promoters (promoter 1 and promoter 2) separately or simultaneously in the recombinant expression vector into the host cells.

SUMMARY OF THE INVENTION

The present invention provides a 5' upstream region DNA containing a promoter of human BMP-4. By using 5' upstream region gene containing the human BMP-4 promoter and an animal cell introduced with a recombinant expression vector which has been integrated in an appropriate reporter gene, the low molecular weight compounds which regulate positively or negatively the expression of human BMP-4 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-4 and are useful as preventive or therapeutic agents for cartilage and bone diseases, remedies for osteometastasis, or therapeutic and preventive agents for osteohyperplasia.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an exon-intron structure of 6.7 kb 5' upstream region of human BMP-4 gene and a restriction enzyme map.

Fig. 2 is a recombinant expression vector (pMSS116) containing the promoter 1 of 5' upstream region of human BMP-4 gene. The promoter 1 region (base No. from 1 to 3361 shown in SEQ ID NO. 1 of the Sequence Listings, from 5' terminal to *Xho*I shown in Fig. 1) was inserted to the *Nhe*I and *Xho*I restriction enzyme site of pGL3-basic.

Fig. 3 is a recombinant expression vector (pMSS118) containing the promoter 2 of 5' upstream region of human BMP-4 gene. The promoter 2 region (2.3 kb) (base No. from 3361 to 5645 shown in SEQ ID NO. 1 of the Sequence Listings, from *Xho*I to *Bgl*II of exon 2 shown in Fig. 1) was inserted to the *Xho*I and *Bgl*II restriction enzyme site of pGL3-basic.

Fig. 4 is a recombinant expression vector (pMSS119) containing the promoter 1 and the promoter 2 of 5' upstream region of human BMP-4 gene. The promoter 1 and the promoter 2 region (base No. from 1 to 5645 shown in SEQ ID NO. 1 of the Sequence Listings, from 5' terminal to *Bgl*II of exon 2 shown in Fig. 1) were inserted to the *Nhe*I and *Bgl*II restriction enzyme site of pGL3-basic.

Fig. 5 is a result of measuring a human BMP-4 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 6774 in SEQ ID NO. 1 of the Sequence Listing that encodes a human bone morphogenetic protein-4 promoter region, or a fragment thereof. SEQ ID NO. 1 of the Sequence Listing shows the 5' upstream region sequence of the human BMP-4 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 *Eco*RI restriction enzyme,

(2) isolation by agarose gel electrophoresis,

(3) cloning of the isolated DNA fragment digested with *Eco*RI 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 by locating a suitable region of 5' upstream region of the human BMP-4 gene which 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 of an original product. The vector as the original for the recombination expression vector is not specially restricted but to allow to use a plasmid vector commercialized. The present invention used pGL3-basic as a preferable example. The use of pGL3-basic yielded pMSS116 (8.2 kb), pMSS118 (7.1 kb), and pMSS119 (10.5 kb) that are the recombination expression vectors containing the human BMP-4 promoter and luciferase reporter genes. The present invention assigned them to the recombination expression vectors. It is necessary to introduce the recombinant expression vector to mammalian cells, preferably human osteoblast-like cells, such as SaOS-2 cells, with a liposome. The animal cells stably transfected with the recombinant expression vectors 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-4 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-4 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 vectors 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 human BMP-4 promoter through regulating a signal transduction system. Therefore, these compounds are effective as a therapeutic agent for osteocartilaginous diseases, cancer metastasis to bone, or osteohyperplasia.

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 cartilage hyperplasia.

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-4 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 under a standard condition using BMP-4 cDNA (Science 242, 1528-1534, 1988) as a probe. As the result, it was found that digestion by the restriction enzyme *Eco*RI among restriction enzymes used yielded a DNA fragment of ca. 7 kb containing the longest human BMP-4 gene. Then, a human placenta genome DNA was digested by the restriction enzyme *Eco*RI and separated by agarose gel electrophoresis to extract a DNA fragment of ca. 7 kb from the agarose gel. The DNA fragment obtained was cloned to a lambda phage vector λDASH II (Stratagene Ltd.) digested by the restriction enzyme

*Eco*RI. The vector was in vitro packaged by Gigapack III XL Extract (Stratagene Ltd.), infected to *Escherichia coli* XL1-Blue MRA (Stratagene Ltd.) to make a genomic DNA library. The library was divided into pools. Each pool was amplified by a screening (Nucleic Acids Research 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 the exon 1 region to select the objective pool, to yield finally 5' upstream region (6.8 kb) of human BMP-4 gene. In addition, the 5' upstream 6.8 kb fragment was subcloned to a pUC18 vector (a product of Amersham Pharmacia Biotech). The vector was named *E. coli* pKOT 312. The *E. coli* pKOT 312 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-16736 and transferred to the International Depository Authority under Budapest Treaty on February 17, 1999 (Deposit No. FERM BP-6650).

Example 2 Determination of DNA sequence of 5' upstream region of human BMP-4 gene

The sequence of 5' upstream region of human BMP-4 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.

Example 3 Construction of a recombinant expression vector containing the human BMP-4 promoter and a luciferase reporter gene

As shown in Fig. 1, human BMP-4 has two promoters before exon 1 (promoter 1) and after exon 2 (promoter 2) similar to the promoter structure of mouse BMP-4. Then, a region containing the promoter 1 (from 5' terminal to *Xho*1 described in the Fig. 1) was inserted into the upstream restriction enzyme sites, *Nhe*I and *Xho*I, of the reporter gene of a luciferase reporter vector pGL3-basic (a product of Pro Mega

Ltd.) by using *Xba*I restriction enzyme site derived from the pUC18 vector existing in the 5' terminal to construct a recombinant expression vector pMSS116 (8.2 kb). This is shown in Fig. 2. Further, a recombinant expression vector pMSS118 (7.1 kb) was obtained by inserting a region (from *Xho*1 to *Bgl*II in exon 2 described in Fig. 1) containing the promoter 2 into the restriction enzyme sites, *Xho*I and *Bgl*II, of pGL3-basic. This is shown in Fig. 3. Furthermore, a region (from 5' terminal to *Bgl*II in exon 2 described in Fig. 1) containing both the promoter 1 and the promoter 2 was inserted into the restriction enzyme sites, *Nhe*I and *Bgl*II, of pGL3-basic by using *Xba*I site derived from the pUC18 vector, as in the case of pMSS116 to construct a recombinant expression vector pMSS119 (10.5 kb). This is shown in Fig. 4.

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

In order to express transiently the human BMP-4 recombinant expression vector, above-described recombinant expression vectors (pMSS116, pMSS118, pMSS119) were mixed with a vector, pRL-SV40 (a product of 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 mixed with said DNA 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 shown in Fig. 5. 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 human BMP-4 recombinant expression vector to a human cell and stabilized expression

In order to express the human BMP-4 recombinant expression vector stably, above-described vectors were 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 has been introduced were selected from a culture medium containing puromycin (a product of Sigma Ltd.).

Example 6 Screening of active low molecular weight compound

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-4 can be explored.

Sequence Listing Free Text

<210> 1
<223> Human BMP-4 5' upstream gene sequence including the exon 1 through exon 3 regions.

<210> 2
<223> Sense PCR primer for cloning 5' upstream human BMP-4 gene sequence corresponding to the exon 1 region

<210> 3
<223> Reverse PCR primer for cloning 5' upstream human BMP-4 gene sequence corresponding to the exon 1 region

CLAIMS

1. A DNA whose nucleotide sequence is represented by the base sequence No. from 1 to 6774 shown in SEQ ID NO. 1 of the Sequence Listing that encodes a human bone morphogenetic protein-4 promoter region.

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

(1) digesting a human placenta genomic DNA with a *Eco*RI restriction enzyme,

(2) subjecting the *Eco*RI-digesting mixture to agarose gel electrophoresis to isolate a DNA fragment of ca. 7 kb,

(3) cloning of the isolated DNA fragment digested with *Eco*RI into a lambda phage vector λDASH II treated with *Eco*RI,

(4) packaging of said vector into the phage,

(5) establishing a genomic DNA library by infecting *Escherichia coli* with the phage,

(6) screening by PCR method by using PCR primers whose nucleotide sequence are represented by the base sequence shown in SEQ ID Nos. 2 and 3 of the Sequence Listing corresponding to the exon 1 region, and

(7) subcloning the 6.8 kb of gene fragment located 5′ upstream of human bone morphogenetic protein-4 gene to a plasmid vector to isolate the DNA shown in SEQ ID No: 1.

3. A recombinant expression vector characterized in that the human bone morphogenetic protein-4 promoter region according to claim 1 is integrated in an upstream of a reporter gene.

4. A method for screening a bone-related substance that regulates the expression level of human bone morphogenetic protein-4 gene, comprising introducing the recombinant expression vector of claim 3 into a mammal cell and then

measuring the expression level of the reporter gene in the vector.

5. A method for screening a bone-related substance of claim 4, wherein the bone-related substance is an osteogenesis inducing substance that increases the expression level of the reporter gene in the vector having a promoter region of human bone morphogenetic protein-4 gene.

6. A method for screening a bone-related substance of claim 4, wherein the bone-related substance is an osteogenesis inhibiting substance that decreases the expression level of the reporter gene in the vector having a promoter region of human bone morphogenetic protein-4.