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(54) Benævnelse: **GDF-5 MUTANT TIL AT INDUCERE BRUSKDANNELSE**

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DESCRIPTION

[0001] The present invention is directed to GDF-5 related proteins having an improved capability of inducing cartilage formation and a reduced capability of inducing bone formation. The novel proteins are particularly useful in the treatment of cartilage defects, wherein the formation of bone tissue is undesirable.

[0002] Synovial joints are essential for the biomechanical function of the skeleton. An improper function as observed in arthritic diseases directly results in a severe loss of life quality. Therefore, joint biology has been in focus of extensive research for years leading to an understanding of joint anatomy and histology as well as the biomechanical properties and roles of articular cartilage and other components in joint function and maintenance.

[0003] GDF-5 (Hötten et al. 1994, Biochem. Biophys Res. Commun. 204, 646-652) is a morphogen which has been shown to promote cell proliferation, differentiation and/or tissue formation in several tissues. The protein is also known as morphogenic protein MP52, bone morphogenetic protein-14 (BMP-14) or cartilage-derived morphogenetic protein-1 (CDMP-1). GDF-5 shows chondrogenic activity and congenital GDF-5 mutations cause defects in digit, wrist and ankle joints in mice and humans (Storm et al., 1994; Thomas et al., 1997). The expression of GDF-5 is most strikingly limited to regions where joints will develop and is one of the earliest markers of joint formation (Storm and Kingsley, 1999). BMP receptor signaling is required for postnatal maintenance of articular cartilage (Rountree, 2004, PLoS Biol. 2004 November, 2(11))

[0004] GDF-5 is closely related to GDF-6 and GDF-7. These three proteins form a distinct subgroup of the TGF-ß superfamily, thus displaying comparable biological properties and an extraordinary high degree of amino acid sequence identity (see i.e. Wolfman et al. 1997, J. Clin. Invest. 100, 321-330). All family members are initially synthesized as larger precursor proteins which subsequently undergo proteolytic cleavage at a cluster of basic residues approximately 110-140 amino acids from the C-terminus, thus releasing the C-terminal mature protein parts from the N-terminal prodomain. The mature polypeptides are structurally related and contain a conserved bioactive domain comprising six or seven canonical cysteine residues which is responsible for the characteristical three-dimensional "cystine-knot" motif of these proteins. Native GDF-5 related proteins are homodimeric molecules and act mainly through interaction with specific receptor complexes which are composed of type I and type II serine/threonine receptor kinases. The receptor kinases subsequently activate Smad proteins, which then propagate the signals into the nucleus to regulate target gene expression.

[0005] It has repeatedly been demonstrated that members of the GDF-5/-6/-7 subgroup are primarily important inducers and regulators of bone and cartilage (Cheng et al. 2003, J. Bone & Joint Surg. 85A, 1544-1552; Settle et al. 2003, Developm. Biol. 254, 116-130). GDF-5 and related proteins bind to and oligomerize two types of membrane bound serine-threonine kinase receptors termed type I and II. Upon ligand binding, these complexes transduce signals by phosphorylating members of the SMAD family of transcription factors, which upon activation enter the nucleus and regulate transcription of responsive genes (Massague, 1996). Recent experiments have implicated two different type I receptors in skeletal patterning, BMPR-IA and BMPR-IB. Both receptors are expressed in dynamic patterns during normal development. In several limb structures, for example, in joint interzones and perichondrium, an overlapping expression of BMPR-IA and BMPR-IB is observed (Mishina et al., 1995; Zou et al, 1997; Baur et al, 2000). With regard to the BMPR-IA and BMPR-IB expression patterns, GDF-5 signal transduction should be accomplished by the interaction with both BMPR-IA and BMPR-IB (Chang et al., 1994; Zou et al., 1997). Null mutations in the bmpr-1 b gene produce viable mice with defects in bone and joint formation that closely resemble those seen in mice missing GDF-5 (Storm and Kingsley, 1996; Yi et al, 2000), whereas bmpr-Ia/ mice are known to die early in embryogenesis (Mishina et al, 1995). However, a conditional knockout of BMPR-IA under the control of a GDF-5-Cre driver bypasses embryonic lethality and produces viable mice with normally formed joints. But, after birth articular cartilage within the joints wears away in a process reminiscent to osteoarthritis, which points at the importance of this receptor in cartilage homoeostasis and repair (Rountree et al., 2004).

[0006] The activity of the wild-type proteins of GDF-5 related protein family generally results in the formation of cartilage and bone. However, different medical conditions exist, wherein a formation of cartilage is desirable, however, the formation of bone tissue is undesired. For example, it is evident that in case of joint defects, the formation of cartilage is desirable whereas ossification should be avoided.

[0007] Therefore, the object of the present invention is to specifically use the effect of inducing cartilage formation of GDF-5 related proteins and to turn off the inducing effect of bone formation. This object was solved by providing variant proteins of mature human GDF-5 as represented by amino acids 382 to 501 of SEQ ID NO: 2 comprising an amino acid substitution selected from R399E, W417F and W417R. Surprisingly, it was found out that it is possible to provide variants of GDF-5 related proteins having an improved capability of inducing cartilage formation and a reduced capability of inducing bone formation. This can be

achieved by modifying GDF-5 related proteins such that they have an increased affinity for the BMPR-IB and/or a reduced affinity for the BMPR-IA.

[0008] Wild-type GDF-5 binds BMPR-IB in vitro with about 40- to 120-fold higher affinity (K_D ~ 8-27 pM) as compared with BMPR-IA (K_D ~ 1-1,1 nM). It was found that by modifying the binding affinity of GDF-5 related proteins such that the affinity for BMPR-IB is increased while the affinity for BMPR-IA is reduced, cartilage formation is facilitated while the formation of bone is reduced. This can be achieved by specific substitutions of one or more amino acid residues relating to a BMPR-IB and/or BMPR-IA binding site in the amino acid sequence of a GDF-5 related protein.

[0009] The binding affinity of GDF-5 related proteins having specific substitutions is compared to the binding affinity of human wild-type GDF-5 related protein, in particular human wild-type GDF-5.

[0010] In order to avoid misunderstandings and ambiguities, some frequently used terms herein are defined and exemplified as follows:

The term "cystine-knot domain" as used herein means the well known and conserved cysteine-rich amino acid region which is present in the mature parts of TGF-beta superfamily proteins such as i.e. human GDF-5 and forms a three-dimensional protein structure known as cystine-knot. In this domain the respective location of the cysteine residues to each other is important and is only allowed to vary slightly in order not to lose the biological activity. It has been demonstrated that the cystine-knot domain alone is sufficient for the biological function of the protein (Schreuder et al. (2005), Biochem Biophys Res Commun. 329, 1076-86). Consensus sequences for cystine-knot domains are well known in the state of the art. According to the definition defined herein the cystine-knot-domain of a protein starts with the first cysteine residue participating in the cystine-knot of the respective protein and ends with the residue which follows the last cysteine participating in the cystine-knot of the respective protein. For example, the cystine-knot domain of the human GDF-5 precursor protein (SEQ ID NO: 2) consists of the amino acids 400-501 (see also FIG. 1).

[0011] The term "GDF-5-related protein" as used herein means any naturally occurring or artificially created protein which is very closely related to human growth/differentiation factor 5 (hGDF-5). Common feature of all GFD-5-related proteins is the occurrence of a cystine-knot-domain with an amino acid identity of at least 60% to the 102 aa cystine-knot domain of human GDF-5 (amino acids 400-501 of SEQ ID NO: 2), which is sufficient for the biological function of the protein. The term "GDF-5-related proteins" includes proteins belonging to the group of GDF-5, GDF-6 and GDF-7 proteins from vertebrate or mammalian species as well as recombinant variants thereof as long as these proteins show the above mentioned percentage of identity with the cystine-knot domain of human GDF-5. The limiting value of 60% is well suitable to separate members of the GDF-5/-6/-7 group of proteins as well as variants thereof from further proteins such as more distantly related GDFs and BMPs. A comparison of the 102 aa cystine-knot-domains of human GDF-5, human GDF-6 and human GDF-7 (see FIG. 2) reveals the high grade of amino acid identity between these proteins. Human GDF-6 shares 87 (85%) and human GDF-7 shares 83 (81 %) identical residues with the cystine-knot-domain of human GDF-5. The respective domains of GDF-5/-6/- 7 molecules from other vertebrate and mammalian species which have been identified so far also show very high identity percentages of at least 75% (between 79% and 99%), when compared with human GDF-5. In contrast, GDFs and BMPs not belonging to the GDF-5/-6/-7 subgroup display much lower identity values below 60%.

[0012] The determination of corresponding amino acid positions in related amino acid sequences as well as the calculation of percentages of identity can be easily performed with the help of well known alignment algorithms and optionally computer programs using these algorithms. For example, the amino acid identities in this patent application (i.e. FIG. 2) have been calculated by aligning sequences with the freeware program ClustalX (Version 1.81) with default parameters and subsequent counting of identical residues by hand. Default settings for pairwise alignment (slow-accurate) are: gap opening parameter: 10.00; gap extension parameter 0.10; Protein weight matrix: Gonnet 250. The ClustalX program is described in detail in Thompson, J. D., Gibson, T.J., Plewniak.F., Jeanmougin.F. and Higgins.D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24:4876-4882. ClustalX is a windows interface for the ClustalW multiple sequence alignment program and is i.e. available from various sources, i.e. by anonymous ftp from ftp-igbmc.u-strasbg.fr, ftp.embl-heidelberg.de, ftp.ebi.ac.uk or via download from the following webpage: http://www-igbmc.u-strasbg.fr/BioInfo/. The ClustalW program and algorithm is also described in detail in Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994): CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680. Especially preferred GDF-5-related proteins display amino acid identities of at least 70%, 80%, 90% or 95% to the 102 aa cystine-knot domain of human GDF-5.

[0013] Non-limiting examples for vertebrate and mammalian GDF-5-related proteins are precursors and mature proteins of human GDF-5 (disclosed as MP52 in WO95/04819 and as human GDF-5 in Hotten et al. 1994, Biochem. Biophys Res. Commun. 204, 646-652), recombinant human (rh) GDF-5/MP52 (WO96/33215), MP52 Arg (WO97/06254); HMW human MP52s (WO97/04095), CDMP-1 (WO96/14335), mouse (Mus musculus) GDF-5 (US 5,801 ,014), rabbit (Oryctolagus cuniculus) GDF-5 (Sanyal et al. 2000, Mol Biotechnol. 16, 203-210), chicken (Gallus gallus) GDF-5 (NCBI accession no. NP_989669), african clawed frog (Xenopus laevis) GDF-5 (NCBI accession no. AAT99303), monomeric GDF-5 (WO 01/1 1041 and WO 99/6161 1), human GDF-6/BMP-13 (US 5,658,882), mouse GDF-6 (NCBI accession no NP_038554), GDF-6/CDMP-2 (WO96/14335), human GDF-7/BMP-12 (US 5,658,882), mouse GDF-7 (NCBI accession no AAP97721), GDF-7/CDMP-3 (WO96/143335). Covered by the invention are also GDF-5-related proteins having additional mutations such as substitutions, additions and deletions, as long as these additional mutations do not completely abolish the biological protein activity.

[0014] The present invention is based on the finding of the inventors that it is possible by specific modifications in the region of the amino acid sequence of a GDF-5 related protein which is involved in the binding to BMPR-IB and/or BMPR-IA to change the protein in such a way that same has an improved ability of inducing cartilage formation and a reduced ability for inducing bone formation.

[0015] It was found out that proteins having an increased affinity for BMPR-IB and/or proteins having a reduced affinity for BMPR-IA are better capable for inducing cartilage formation while the formation of bone is reduced. These properties are especially pronounced in proteins showing both an increased affinity for BMPR-IB and a reduced affinity for BMPR-IA.

[0016] The GDF-5 related proteins of the present invention can be obtained by chemical modification or genetic engineering technology with recombinant proteins being preferred. The proteins can be obtained by replacing at least one amino acid residue relating to a BMPR-IB and/or BMPR-IA binding site in the amino acid sequence of a GDF-5 related protein.

[0017] An inventive protein is a variant of human GDF-5, whereby the tryptophan residue at position 414 is exchanged against arginine (W414R). Referring to the mature sequence of GDF-5 (for mature monomeric GDF-5 shown in SEQ ID NO:4), this corresponds to a substitution at position 33. Surprisingly, it was found that this protein variant has a considerably reduced affinity for the BMPR-IA. In contrast, the affinity for the BMPR-IB is almost unaffected.

[0018] A further inventive variant protein comprises the amino acid substitution R399E. Referring to the mature sequence of GDF-5 (for mature monomeric GDF-5 shown in SEQ ID NO: 4), this corresponds to a substitution at position 18. Said protein variant has a reduced affinity for the BMPR-IA.

[0019] Preferably, the GDF-5 related proteins of the present invention are present as "isolated" proteins. This means that the protein of the present invention is substantially separated from other proteins and peptide molecules which are present in the natural source of the isolated protein (e.g. other polypeptides of the protein of the natural source). For example, a recombinant expressed peptide is considered isolated. According to a preferred embodiment of the invention, the variant protein is a recombinant protein. Further, a peptide is also considered isolated, if it has been altered by human intervention or expressed by an organism that is not its natural source. Moreover, an "isolated" protein is free from some of the other cellular material with which it is naturally associated or cell culture medium, when produced by recombinant techniques or chemical precursors or other chemicals when chemically synthesized. Specifically excluded from the definition of "isolated" protein, are unpurified mixtures or compositions.

[0020] According to another embodiment, the present invention relates to a nucleic acid encoding a protein of the present invention. The nucleic acid has a sequence such that a substitution of certain residues relating to a BMPR-IB and/or BMPR-IA binding site of the respective wild-type GDF-5 related protein is achieved. The base triplets coding for these amino acids and the degeneracy of the genetic code are generally known. The nucleic acid can be a DNA-sequence and/or a RNA-sequence as long as the protein according to the invention can be obtained from this nucleic acid upon expression in a suitable system. The nucleic acid of the invention may be wholly or partially synthetic. The nucleic acids comprise single stranded and/or wholly or partially double stranded polynucleotide sequences. The nucleic acid may be produced by any means including genomic preparations, cDNA preparations, in vitro synthesis, PCR, RT-PCR and/or in vitro or in vivo transcription.

[0021] Particularly preferred are "isolated" nucleic acids, which are substantially separated from nucleic acid molecules which are present in the natural source of the nucleic acid (e.g. sequences encoding other polypeptides). Preferably, an "isolated" nucleic acid is free of at least some of the sequences which naturally flank the nucleic acid (i.e. sequences located at the 5' and 3' ends of the nucleic acid) in its naturally occurring replicon. For example, a cloned nucleic acid is considered isolated. A nucleic acid is also considered isolated, if it has been altered by human intervention or placed in a locus or location that is not its natural side or

if it is introduced into a cell. Moreover, an isolated nucleic acid can be free from some of the other cellular material with which it is naturally associated or culture medium when produced by recombinant techniques or chemical precursors or other chemicals when chemically synthesized.

[0022] In a preferred way, the nucleic acids of the invention can be prepared by a total gene synthesis or by side-directed mutagenesis of the nucleic acid encoding wild-type or modified GDF-5 related proteins. Methods include template directed ligation, recursive PGR, cassette mutagenesis, side directed mutagenesis or other techniques that are well-known in the art may be utilized.

[0023] The nucleic acids of the present invention may comprise further nucleic acid sequences which may add further functions to the isolated nucleic acid of the invention. For example, such additional nucleic acid sequences may comprise nucleic acid sequences that allow for proper expression of a protein of the invention and may encompass promoter sequences, regulatory sequences, stop signals, replication origins and like. The skilled person is well aware of such functional nucleic acid sequences and of how to arrange them in order to arrive at a nucleic acid molecule with the desired properties.

[0024] Expression vectors are also disclosed wherein the nucleic acid is inserted in a suitable vector system, the vector system being selected according to the desired expression of the protein. The vector system can be a eukaryotic vector system but preferably is a prokaryotic vector system with which the proteins can be produced in a particularly easy and pure manner. A suitable expression vector is for example shown in WO 96/33215. The expression vector can also be a viral vector which can be used for example in gene therapy approaches.

[0025] Host cells and transgenic organisms are also disclosed. The host cells and transgenic organisms are characterized in that they contain a nucleic acid or an expression vector according to the invention and that they are able to use the information present in the nucleic acids and in the expression vector, respectively for the expression of the proteins according to the invention. Thus, the present disclosure relates to transgenic organisms or cells transiently or stably transformed or transfected with at least one nucleic acid or at least one vector encoding a protein of the invention or to a progeny of such transgenic organisms or cells. Furthermore, the present disclosure relates to cells, cell cultures, tissues and/or parts of transgenic organisms. It is understood that for the purpose of the present disclosure the term "transgenic organism" not only encompasses the organism where the nucleic acid of the invention has been transiently or stably introduced but also refers to the progeny of such organisms irrespective of the generation distance, provided that these organisms still comprise the nucleic acid of the invention and express the protein of the invention.

[0026] Preferably, the transgenic organism or cell is of prokaryotic or eukaryotic origin. Preferably, the transgenic organism is a microorganism. Preferred microorganisms are bacteria, yeasts, algae or fungi. Suitable host cells are preferably prokaryotic cells, in particular E.coli strains. Particularly useful host cells are defendants of E.coli W31 10 as shown for example in WO 96/33215. In a preferred embodiment, host cells, preferably of human origin, may also be useful for a transplantation to patients in need thereof.

[0027] The preparation of a transformed organism or of a transformed cell requires introducing the appropriate DNA into the appropriate host organism or cell. A multiplicity of methods is available for this process which is referred to as transformation. Thus, by way of example, the DNA may be introduced directly by microinjection or by bombardment with DNA coated microparticles or nanoparticles. The cell may also be permeabilized chemically, for example using polyethylene glycol, so that the DNA can enter the cell via diffusion. The DNA may also be transformed via protoplast fusion with other DNA-containing units such as minicells, cells, lysosomes or liposomes. Another suitable method for introducing DNA is electroporation in which the cells are reversibly permeabilized by an electric impulse.

[0028] Also disclosed herein is a method for producing a protein having an improved capability of inducing cartilage formation and a reduced capability of inducing bone formation, comprising the steps of:

1. (i) randomizing at least one amino acid position in a region of a GDF-5 related protein relating to a BMPR-IB and/or BMPR-IA binding site in order to obtain protein variants,
2. (ii) analyzing the protein variants obtained in (i) with respect to their affinity to the BMPR-IB and/or BMPR-IA,
3. (iii) selecting those protein variants which provide an increased affinity for the BMPR-IB and/or a reduced affinity for the BMPR-IA.

[0029] The regions of a GDF-5 related protein involved in binding to BMPR-IA or BMPR-IB are known in the art. In step (i) at

least one amino acid position in one or both of these regions is randomized. It is preferred to randomize at least two, three or more amino acid positions. The amino acids present in the wild-type sequence of a GDF-5 related protein are replaced by other amino acids by chemical modifications or preferably by genetical engineering technology. Methods for producing the randomized protein variants of step (i) encompass the synthetic de novo synthesis of the proteins and/or the expression of the proteins from a nucleic acid encoding therefore. In a particular preferred way, the protein variants of step (i) are prepared by expression using the respective nucleic acids.

[0030] Preferably, protein variants are obtained for all other possible amino acids at the relevant position. However, it is also possible to carry out only a specific replacement of one or more amino acids against other amino acids. For example, hydrophilic amino acids can be replaced by hydrophobic amino acids. Alternatively, hydrophobic amino acids can be replaced by hydrophilic amino acids. A conservative substitution, wherein the hydrophilic or hydrophobic character is kept, is also possible. By the substitution, preferably an exchange against an amino acid having another steric demand is carried out.

[0031] The plurality of protein variants obtained in step (i) is then analyzed with respect to their affinity to BMPR-IB and/or to BMPR-IA. This can be effected in a way which is known and usual in the technical field. Methods for assessing protein-receptor interactions are common practice.

[0032] In step (iii), those protein variants which provide an increased affinity for BMPR-IB and/or a reduced affinity for BMPR-IA are selected. It was surprisingly found that these particular proteins have an improved capability of inducing cartilage formation and a reduced capability of inducing bone formation.

[0033] Further disclosed are antibodies against the described GDF-5 related proteins. These antibodies are specific for the claimed recombinant GDF-5 related proteins. Preferably, they are specific for the regions of GDF-5 related proteins containing one or more of the amino acid replacements described herein. Preferably, the antibodies are specific for a region of a recombinant protein derived from a GDF-5 related protein relating to a BMPR-IB and/or BMPR-IA binding site. These antibodies can be generated by using those fragments of the proteins as described above as immunogens to generate antibodies by known methods. The antibodies can be monoclonal or polyclonal and they can be any isotypes. Also disclosed are antibody fragments such as Fab fragments or Fab_2 fragments. The antibodies can also be humanized antibodies or generic antibodies etc.

[0034] The antibodies are, inter alia, suitable as an analytic tool. They can be used for investigating the absorption and distribution of a protein according to the invention in the body. Furthermore, the above antibodies are suitable for studying release kinetics.

[0035] Further subject matter of the present application is a pharmaceutical composition comprising the recombinant GDF-5 related protein or a nucleic acid or a vector or a host cell according to the invention. In principle, any pharmaceutical compositions which have already been published in context with GDF-5 related proteins are suitable. An expression vector or a host cell can be considered to be advantageous as active substances in a pharmaceutical composition. Also combinations of a protein according to the invention with other proteins can be used in preferred pharmaceutical compositions. Of course, the invention also comprises pharmaceutical compositions containing further substances like e.g. pharmacologically acceptable additives or carriers. The formulation may include antioxidants, preservatives, colouring, flavouring and emulsifying agents, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, excipients and/or pharmaceutical adjuvants. For example, a suitable carrier or vehicle may be water for injection, physiological saline solution or a saline solution mixed with a suitable carrier protein such as serum albumin. A preferred antioxidant for the preparation of the composition of the present invention is ascorbic acid.

[0036] The solvent or diluent of the pharmaceutical composition may be either aqueous or non-aqueous and may contain other pharmaceutically acceptable excipients which are capable of modifying and/or maintaining a pH, osmolarity, viscosity, clarity, scale, sterility, stability, rate of dissolution or odour of the formulation. Similarily other components may be included in the pharmaceutical composition according to the present invention in order to modify and/or maintain the rate of release of the pharmaceutically effective substance. Such modifying components are substances usually employed in the art in order to formulate dosages for parenteral administration in either unit or multi-dose form.

[0037] The finally formulated pharmaceutical composition prepared according to the present invention may be stored in sterile vials in form of a solution, suspension, gel, emulsion, solid or dehydrated or lyophilized powder. These formulations may be stored either in a ready-to-use form or in a form, e.g. in case of a lyophilized powder, which requires reconstitution prior to administration. The above and further suitable pharmaceutical formulations are known in the art and are described in, for example, Gus Remington's Pharmaceutical Sciences (18th Ed. , Mack Publishing Co., Eastern, Pa., 1990, 1435-1712). Such

formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the pharmaceutically effective component.

[0038] Other effective administration forms comprise parenteral slow-release, i.e. retarded, formulations, inhalent mists, or orally active formulations. For example, a slow-release formulation may comprise proteins bound to or incorporated into particulate preparations of polymeric compounds (such as polylactic acid, polyglycolic acid, etc.) or liposomes.

[0039] The pharmaceutical composition according to the present invention may also be formulated for parenteral administration, e.g., by infusion or injection, and may also include slow-release or sustained circulation formulations. Such parenterally administered therapeutic compositions are typically in the form of pyrogen-free, parenterally acceptable aqueous solutions comprising the pharmaceutically effective component(s) in a pharmaceutically acceptable carrier and/or diluent.

[0040] The pharmaceutical composition may comprise a matrix material, e.g. in cases where regeneration of cartilage is intended. It is advantageous to the protein, the nucleic acid, the expression vector or the host cell when they are applied in and/or on a biocompatible matrix material. Matrix material as used herein means a carrier or matrix acting as a scaffold for cell recruitment, attachment, proliferation and differentiation and/or as a potential delivery and storage device for the recombinant GDF-5 related proteins of the invention. In contrast to the solid matrices, carriers consist of amorphous materials having no defined surfaces and lacking a specific shape, i.e. alkyl cellulose, pluronics, gelatins, polyethylene glycols, dextrins, vegetable oils, sugars and other liquid and viscous substances.

[0041] Exemplary matrix materials are for example described in WO 98/21972. These matrix materials are equally suitable for the proteins according to the invention. The matrix material can be transplanted into the patient, e.g. surgically, wherein the protein or the DNA encoding the protein can be slowly released from the matrix material and then be effective over a long period of time. All types of matrix materials are useful in accordance with the present invention as long as they are biocompatible and selected for the intended area or indication of use. The matrix material can be a natural material, a modified natural material as well as a synthetic material. All already known matrices for morphogenetic proteins are encompassed. The extracellular matrix comprises for examples various collagens as for example types I, II, V, IX, X, XI and XIII, further proteoglycans and glycosamine glycans as for example chondroitin sulfates, biglycans, decorines and/or hyaluronic acid or non-collageneous proteins as e.g. osteopontin, laminin, fibronectin, vitronectin and cartilage matrix protein. All mentioned natural materials may also be used in artificially modified forms. For a non-limiting list of useful carriers and matrices (see further Kirker-Head, 2000, Advanced Drug Delivery 43, 65-92).

[0042] A further possibility concerns liposomal formulations comprising the recombinant GDF-5 related protein according to the invention. Liposomes used in said formulations are commonly known to the person skilled in the art. In particular, preferred liposomal formulations are disclosed in WO 2008/049588. More preferred liposomal formulations are described on pages 9 to 13 of WO 2008/049588.

[0043] Furthermore, the GDF-5 variant proteins of the invention can be administered in combination with other pharmaceutically active substances. Said pharmaceutically active substances can be, for example, painkillers such as locally effective painkillers or other substances that have a positive effect on diseases, wherein the formation of cartilage is desired, like protease inhibitors. These are only examples of possible additives, and a worker skilled in the art can easily add other excipients which are in use in pharmaceutical preparations or which are generally regarded as safe.

[0044] Due to their improved capability of inducing cartilage formation, the recombinant GDF-5 variant proteins of the present invention are particularly suitable for use in the treatment of diseases, wherein the formation of cartilage is desired but the formation of bone is undesirable. Thus another aspect of the present invention is the use of the present proteins, nucleic acids, vectors or host cells in the treatment of these diseases. In particular, the present proteins, nucleic acids, vectors or host cells are for use in the treatment of cartilage defects or for the treatment of traumatic rupture or detachment of cartilage,
in particular age-related cartilage defects for example due to wear, osteoarthritis, rheumatoid arthritis, sports related injuries,
diseases which can affect the cartilage like chondrodystrophies, diseases characterized by disturbance of growth and subsequent ossification of cartilage, achondroplasia, costochondritis, spinal disc herniation and spinal disc repair, relapsing polychondritis,
repair of cartilage defects associated with tumors, either benign or malignant, like chondroma or chondrosarcoma.

[0045] Another aspect is a method for the treatment of diseases, wherein the formation of cartilage is desired but the formation of bone is undesirable comprising the step of administering a protein, nucleic acid, vector or host cell according to the invention to a patient in need of such treatment.

[0046] As used herein, the term "treating" refers to reversing, alleviating or inhibiting the progress of a disease, disorder or

condition or one or more symptoms of such disease, disorder or condition to which such term applies. As used herein, treating may also refer to decreasing the probability or incidence of the occurrence of a disease, disorder or condition in a mammal as compared to an untreated control population or as compared to the same mammal prior to treatment. For example, as used herein, treating may refer to preventing a disease, disorder or condition and may include delaying or preventing the onset of a disease, disorder or condition or delaying or preventing the symptoms associated with a disease, disorder or condition. As used herein, treating may also refer to reducing the severity of a disease, disorder or condition or symptoms associated with such disease, disorder or condition prior to a mammal's affliction with the disease, disorder or condition. Such prevention or reduction of the severity of a disease, disorder or condition prior to affliction relates to the administration of the composition of the present invention as described herein to a subject that is not at the time of administration afflicted with the disease, disorder or condition. As used herein, treating may also refer to preventing the recurrence of a disease, disorder or condition or of one or more symptoms associated with such disease, disorder or condition.

[0047] The following Examples together with the Figures and Sequence Protocols are intended to further illustrate the invention.

[0048] SEQ ID NO: 1 shows the DNA sequence, and SEQ ID NO: 2 shows the protein sequence of the human GDF-5 precursor.

[0049] SEQ ID NO: 3 shows the DNA sequence and SEQ ID NO: 4 shows the protein sequence of the human mature monomeric GDF-5.

Figures

[0050]

FIG. 1 shows additional features of the human GDF-5 precursor protein according to SEQ ID NO:2:

aa 001-381 pre-prodomain (bold letters)

aa 001-027 signal peptide (bold and underlined)

aa 382-501 mature protein part

aa 400-501 cystine-knot-domain (underlined)

FIG. 2 shows a comparison of the 102 aa cystine-knot domains of human GDF-5 (SEQ ID NO:2), human GDF-6 (sequence 26 from U.S. Pat. No. 5,658,882) and human GDF-7 (sequence 2 from U.S. Pat. No. 5,658,882).

Amino acid residues which are identical in all three molecules are highlighted by borders.

FIG. 3 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant W414R (as described in example 2).

FIG. 4 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant I449V (as described in example 3).

FIG. 5 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant R399E (as described in example 3).

FIG. 6 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant S439E (as described in example 3).

FIG. 7 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant R399M (as described in example 3).

FIG. 8 shows the results of an alkaline phosphatase assay (ALP) with recombinant human GDF-5 mutant W414R (as described in example 3).

Example 1: Creation, expression and purification of GDF-related proteins

[0051] DNAs coding for the mature parts of human GDF-5, human GDF-6 and human GDF-7 proteins have been isolated from human ROB-C26 osteoprogenitor cells (Yamaguchi et al. 1991, Calcif. Tissue Int. 49, 221-225) via RT-PCR technique and subsequently ligated into prokaryotic plasmid vectors. In order to identify functionally important amino acid residues in the mature parts of GDF-5, -6 and -7, various single mutations have been introduced into these sequences via site directed mutagenesis.

[0052] All individual mutations were created by using the QuickChangeTM site-directed mutagenesis kit with the PfuTurboTm DNA polymerase and the DPN I endonuclease from Stratagene according to the instruction manual of the manufacturer.

[0053] Using the bacterial strain W3110BP transformed with the plasmids and induced with IPTG, the proteins were expressed in inclusion bodies. These inclusion bodies were isolated using a homogenization buffer (25 mM Tris HCl pH 7.3, 10 mM EDTA NaOH pH 8, 8 M Urea) and wash buffer (1 M Urea, 20 mM Tris HCl, pH 8.3, 10 mM EDTA NaOH pH 8.0) according to standard procedures. Further purification was carried out on a reversed phase column Aquapore Octyl (Applied Biosys, (CV = 7,8 ml) 100x10, 20µ, No 186470) with a gradient from 100% of Eluent A (0.1 % TFA, HPLC H_2O) to 100% Eluent B (0.1 % TFA, 90 % CH_3N, HPLC H_2O) in 104 minutes (flow rate: 3 ml/min). After a western blot control, the fractions containing the mutant protein were pooled and lyophilized.

[0054] The mutant proteins were dissolved in dissolving buffer (6 M Guanidin HCl, 50 m M Tris, 150 mM NaCl, 3 mM DTT, pH = 8.0), the protein concentration was exactly adjusted to 2.6 mg/ml and the pH was adjusted between 8 and 9. After 2 h incubation at room temperature, refolding buffer (1 M NaCl, 50 mM Tris, 5 mM EDTA, 1 mM GSSG, 2 mM GSH, 33 mM Chaps, pH = 9.5) was added under gentle agitation to reach a final concentration of 0.16 mg/ml.

[0055] The solution was then incubated for 48 h at 22°C and the refolding was stopped by changing the pH to 3-4 by adding 18% HCl. After centrifugation, the non-refolded monomer was separated from the dimer form by carrying out a second RP-HPLC under the same conditions. The fractions containing the dimerized protein were pooled, lyophilized and stored at -70°C.

Example 2: Measurement of the biological activity of different variants of GDF-related proteins *in vitro* by ALP assay

[0056] $2.0x10^5$ cells of C2C12-Ib (a cell line stably overexpressing the BMPR-IB receptor) and cells of C2C12 were incubated for 3-4 days in 20 ml cell culture medium (alpha-MEM, Penicilline/Streptomycine, 2 mM L-glutamine, 10% FCS) at 37°C, 5% CO_2, H_2O-saturated. The cells were subsequently washed with PBS (phosphate buffered saline), trypsinated and resuspended in culture medium to a density of $3x10^4$ cells/ml. 150 µl were transferred to each well of a 96 well culture plate and incubated for 24 h at 37° C, 5% CO_2, H_2O-saturated. After washing with medium the wells were filled with 120µl of new culture medium. 40 µl of different dilutions of mutant or wild type protein (dissolved in 10 mM HCl and diluted at least 250fold in medium) were added, followed by another incubation step for 72 h at 37°C, 5% CO_2, H_2O-saturated. After washing with PBS, 150 µl of lysis solution (0,2% Nonidet P40, 0,2g $MgCl_2$ x $6H_2O$, adjusted to 1000 ml with water) was added, followed by 15-18h incubation at 37°C, 5% CO2, H_2O-saturated. 50 µl of each well were subsequently transferred to a new 96 well plate. 50 µl of substrate solution (2,5x concentrated diethanolamine substrate buffer + 148g/l PNPP (sodium p-nitrophenyl-phosphate)) was then added to each well and the plates were incubated for 4 min at 37°C, 5% CO_2, H_2O-saturated. The ALP-reaction was stopped afterwards with 100 µl of 30g/l NaOH and finally the optical density was measured with an automatic microplate reader at 405 nm under consideration of blank value subtraction.

[0057] As an example, results (average values of 2 independent experiments) regarding hGDF-5 mutant W414R for C2C12-Ib cells are shown in FIG. 3. Five different protein concentrations (14 ng/mL, 44.5 ng/mL, 133.2 ng/mL, 400 ng/mL and 1200 ng/mL) have been used in this assay. The mutant protein W414R exhibits biological activity in cells where the BMPR-IB receptor (C2C12-Ib cells) is overexpressed, indicating that the BMPR-IB binding site of W414R is functional active. Wildtype protein (rhGDF-5) served as a control in the assay system.

[0058] Further results of the biological activity of further hGDF-5 mutants for the cell lines C2C12 and C2C12-Ib are shown in table 1.

Example 3: Measurement of the biological activity of different variants of GDF-related proteins in vitro by ALP assay

[0059] 5x10^5 cells of ATDC-5 cells and 5x10^5 cells for MCHT1/26 were incubated for 3-4 days in 20 ml cell culture medium (alpha-MEM, 2 mM L-glutamine, 10% FCS, for MCHT1/26; DMEM/F12 (1:1), 5% FCS) at 37°C, 5% CO_2, H_2O-saturated. The cells were subsequently washed with PBS (phosphate buffered saline), trypsinated and resuspended in culture medium to a density of 3x104 cells/ml. 150 µl were transferred to each well of a 96 well culture plate and incubated for 24 h at 37° C, 5% CO_2, H_2O-saturated. After washing with medium the wells were filled with 120 µl of new culture medium for MCHT1/26 and 120 µl assay medium for ATDC-5 (DMEM/F12 (1:1), 0.5% FCS) plus 40 µl of different dilutions of mutant or wild type protein (dissolved in 10 mM HCl and diluted at least 250fold in medium) were added, followed by another incubation step for 72 h at 37°C, 5% CO_2, H_2O-saturated. After washing with PBS, 150 µl of lysis solution (MCHT1/26 lysis solution: 0,2% Nonidet P40, 1 mM $MgCl_2$; ATDC-5 lysis solution: 100 mM Na-Glycine, 1% Nonidet P40, 1 mM $MgCl_2$) was added, followed by 1 h incubation for ATDC-5 and 15 - 18h for MCHT1/26 at 37°C, 5% CO_2, H_2O-saturated. 50 µl of each well were subsequently transferred to a new 96 well plate. 50 µl of substrate solution (2,5x concentrated diethanolamine substrate buffer + 148g/l PNPP (sodium p-nitrophenyl-phosphate)) was then added to each well and the plates were incubated for another 60 min at 37°C, 5% CO_2, H_2O-saturated. The ALP-reaction was stopped afterwards with 100 µl of 30g/l NaOH and finally the optical density was measured with an automatic microplate reader at 405 nm under consideration of blank value subtraction.

[0060] Exemplary results (average values of 2 independent experiments) regarding the hGDF-5 mutants I449V, R399E, S439E, R399M, W414R are shown in FIG. 4-8, respectively. Five different protein concentrations (14.8 ng/ml, 44.5 ng/ml, 133.2 ng/ml, 400 ng/ml, 1200 ng/ml) have been used in this assay. Compared to wild-type GDF-5 the mutant proteins exhibit a higher biological activity on ATDC-5 cells compared to MCHT1/26 cells in this assay system.

Example 4: Biacore Affinity measurement of GDF-5-related proteins

[0061] A BiacoreT100 system (Biacore, GE Healthcare, Chalfont St. Giles, GB) was used for all biosensor experiments. Approximately 200 resonance units (RU) of the Fc-fusion protein receptor ectodomains of BMPR-IB, BMPR-IA, or BMPR-II were immobilized to protein G CM5 biosensor chips. Interaction sensorgrams were recorded at a flow rate of 60 µl/min at 30°C in 10 mM HEPES (pH 7.4), 300 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20. The experiments were carried out in duplicate using ligand concentrations of 0.05 to 100 nM. All apparent binding affinities were obtained using BIAevaluation v. 2.2.4 (Biacore, GE Healthcare, Chalfont St. Giles, GB). The affinities for ligand type I receptor interaction were derived by fitting the kinetic data to a 1:1 Langmuir binding model (*KD* (kin)). Due to too fast binding kinetics (exceeding 106 M-1 s-1 (for *k*on) and 10-2 s-1 (for *k*off)) the apparent binding affinities for the ligand:BMPR-II interaction were determined from the dose dependency of equilibrium binding (*K*D (eq)).

[0062] The results of the Biacore affinity measurements for different variants of human GDF-5 are shown in table 1.

	ALP				Biacore (KD)				
	MCHT1/26	ATDC-5	C2C12	C2C12-Ib	BMPR-IA	BMPR-IB	BMPR-II	KD(A)/KD(B) (M)	ratio/wild type
GDF-5 WT	+++	+++	0	+++	1* - 1,1** nM	8* - 27** pM	32 nM	46** - 122*	1
R399M	+++	++++	+	++	0.54 nM*	2.5 pM*	32 nM	216*	1.8
R399E	0	+++	0	++	22.5 nM*	172 pM*	32 nM	130*	1.1
M412V	0	++	0	++	13 nM**	39 pM**	n.d.	333**	8.3
W414R	0	+	0	+++	20.3 nM*	30 pM*	no binding	668*	5.5
V417R	0/+	+	0	+++	27 nM**	46 pM**	n.d.	587**	14.7
V417R	0	+	0	+++	98 nM**	37 pM**	n.d.	2649**	66.2
F436K	+	++	0	++(+)	32.5 nM*	45 pM*	32 nM	717*	5.9
S439E	0	+	0	++	43.4 nM*	10 pM*	10 nM	2400*	19.7
S439E	0	++	0	++(+)	25 nM**	43 pM**	n.d.	581**	14.5
I449V	0/+	+	0	+(+)	5.7 nM**	26 pM**	n.d.	219**	5.5

* = Results of affinity measurement 1 concerning GDF-5 wild-type, affinity to BMPR-IA: 1 nM, affinity to BMPR-IB: 8 pM
** = Results of affinity measurement 2 concerning GDF-5 wild-type, affinity to BMPR-IA: 1,1 nM, affinity to BMPR-IB: 27 pM
0 = No ALP activity
+ to ++++ = ALP activity, number + represents the intensity of the ALP activity
n.d = not determined

SEQUENCE LISTING

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470                 475                 480                 485

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Phe Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met
            100                 105                 110

gtc gtg gag tcg tgt ggc tgt agg                                      360
Val Val Glu Ser Cys Gly Cys Arg
        115                 120
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<210> 4

<211> 120

<212> PRT

<213> Homo sapiens

<400> 4

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Ala Pro Leu Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys
1               5                   10                  15

Ala Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly
            20                  25                  30

Trp Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys
        35                  40                  45

Glu Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn
    50                  55                  60

His Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr
65                  70                  75                  80

Pro Pro Thr Ala Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu
                85                  90                  95

Phe Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met
            100                 105                 110

Val Val Glu Ser Cys Gly Cys Arg
        115                 120
```

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Proteinvariant af modent, humant GDF-5, som er repræsenteret ved aminosyrerne 382 - 501 af SEQ ID NO: 2 og som omfatter en aminosyresubstitution, der er valgt fra gruppen bestående af R399E, W417F og W417R.

2. Nukleinsyre, der koder for proteinvarianten ifølge krav 1.

3. Proteinvarianten ifølge krav 1 eller nukleinsyren ifølge krav 2 til anvendelse i behandlingen af sygdomme, hvor dannelsen af brusk er ønsket, men dannelsen af knogle er uønsket.

4. Proteinvarianten eller nukleinsyren til anvendelse ifølge krav 3 til anvendelse ved behandling af bruskdefekter eller til behandling af traumatisk ruptur eller løsrivelse af brusk, navnlig aldersbetingede bruskdefekter for eksempel på grund af slitage, osteoarthritis, rheumatoid arthritis, sport-relaterede skader, sygdomme, der kan påvirke brusken såsom chondrodystrofier, sygdomme karakteriseret af forstyrrelse af vækst og efterfølgende forbening af brusk, achondroplasi, costochondritis, diskusprolaps og diskusreparation, recidiverende polychondritis, reparation af bruskdefekter associeret med tumorer, enten godartede eller ondartede, ligesom chondrom eller chondrosarkom.

5. Farmaceutisk sammensætning, der omfatter en proteinvariant ifølge krav 1, en nukleinsyre ifølge krav 2, en vektor omfattende en nukleinsyre ifølge krav 2 eller en værtscelle omfattende nukleinsyren ifølge krav 2 som det aktive middel, eventuelt i kombination med farmaceutisk acceptable tilsætningsstoffer eller bærere.

DRAWINGS

FIGURES:

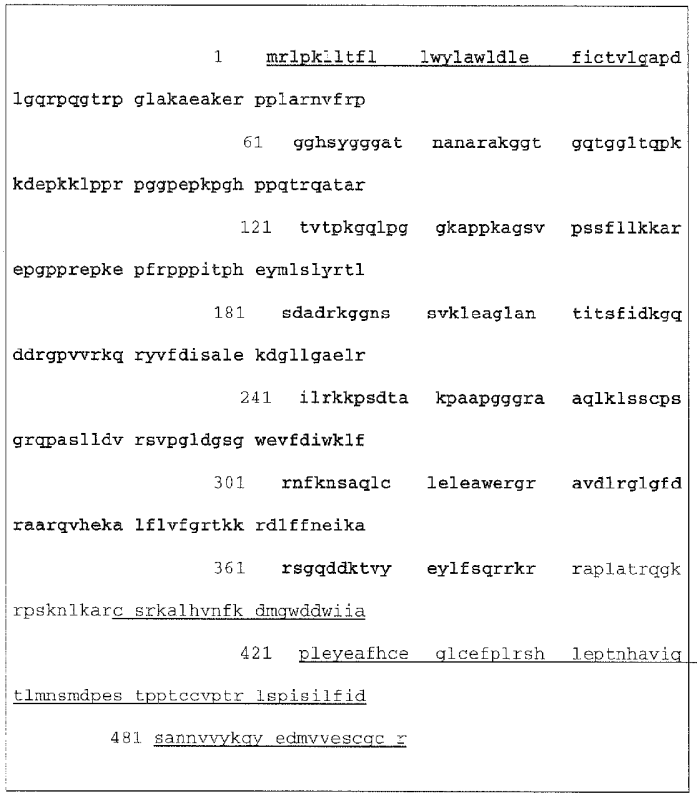


FIG. 1

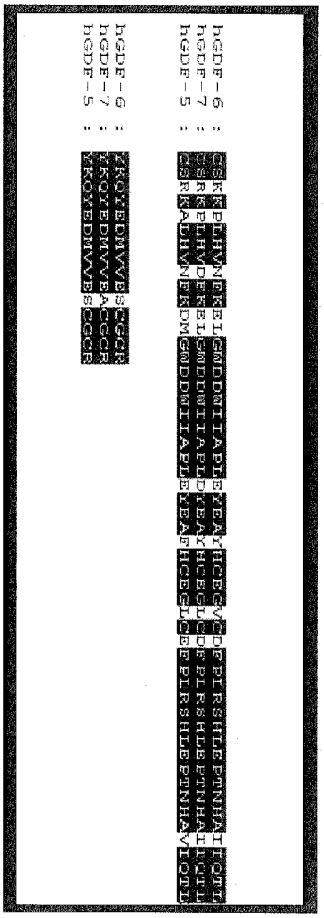
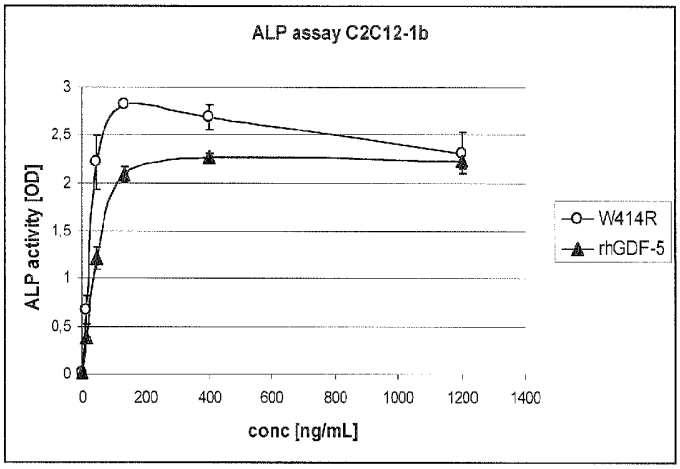


Fig. 2

FIG. 3


ALP assay C2C12-1b
ALP activity [OD]
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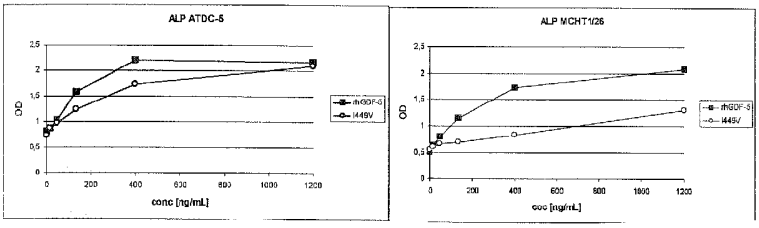
2,5
2
1,5
1
0,5
0
0
200
400
600
800
1000
1200
1400
conc [ng/mL]
W414R
rhGDF-5

FIG. 4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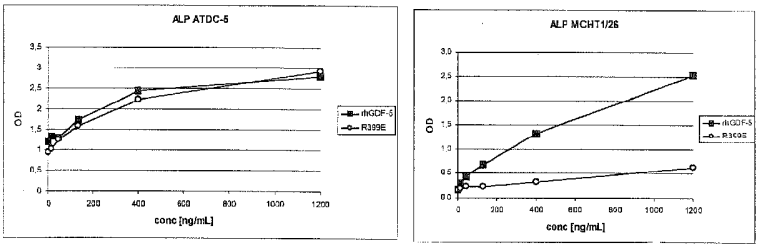
conc [ng/mL]
rhGDF-5
I445V

Fig. 5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conc [ng/mL]
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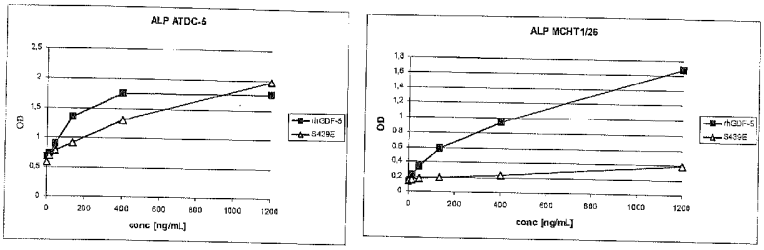
R399E

Fig. 6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conc [ng/mL]
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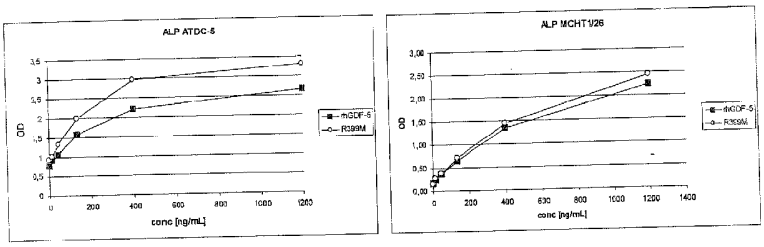
B439E

Fig. 7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ALP MCHT1/26
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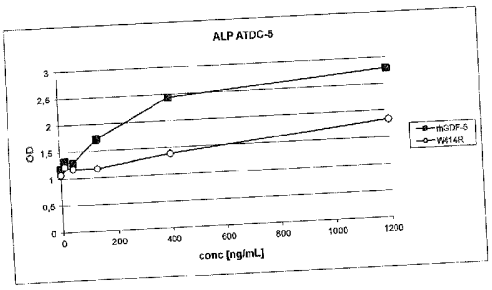
R99M

Fig. 8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OD
conc [ng/mL]
rhGDF-5
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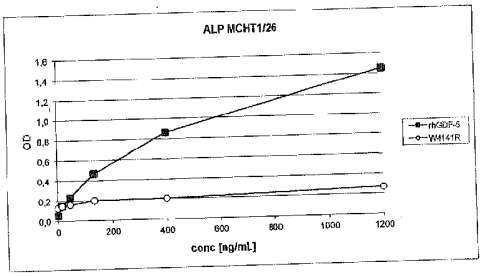
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