Title: USE OF A TIMP-2 SECRETED PROTEIN PRODUCT FOR PREVENTING AND TREATING PANCREATIC DISEASES AND/OR OBESITY AND/OR METABOLIC SYNDROME

Abstract: The present invention discloses TIMP-2 proteins secreted by the developing pancreas, and polynucleotides, which identify and encodes these proteins. The invention also relates to the use of these sequences in the diagnosis, study, prevention, and treatment of pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome.
Use of a TIMP-2 secreted protein product for preventing and treating pancreatic diseases and/or obesity and/or metabolic syndrome

Description

This invention relates to the use of low molecular weight TIMP-2 protein and to the use of effectors/modulators thereof in the diagnosis, study, prevention, and treatment of pancreatic diseases (e.g. diabetes mellitus), obesity and/or metabolic syndrome and to the use in regeneration of tissues such as pancreatic tissues and others.

Many human proteins serve as pharmaceutically active compounds. Several classes of human proteins that serve as such active compounds include hormones, cytokines, cell growth factors, and cell differentiation factors. Most proteins that can be used as a pharmaceutically active compound fall within the family of secreted proteins. Secreted proteins are generally produced within cells at rough endoplasmic reticulum, are then exported to the golgi complex, and then move to secretory vesicles or granules, where they are secreted to the exterior of the cell via exocytosis. Examples for commercially used secreted proteins are human insulin, thrombolytic agents, interferons, interleukins, colony stimulating factors, human growth hormone, transforming growth factor beta, tissue plasminogen activator, erythropoetin, and various other proteins. Receptors of secreted proteins, which are membrane-bound proteins, also have potential as therapeutic or diagnostic agents. It is, therefore, important for developing new pharmaceutical compounds to identify secreted proteins that can be tested for activity in a variety of animal models. Thus, in light of the pervasive role of secreted proteins in human physiology, a need exists for identifying and characterizing novel functions for human secreted proteins and the genes that encode them. This knowledge will allow one to detect, to treat, and to prevent medical diseases, disorders, and/or conditions by using secreted proteins or the genes that encode them.
The pancreas is an essential organ possessing both an exocrine function involved in the delivery of enzymes into the digestive tract and an endocrine function by which various hormones are secreted into the blood stream. The exocrine function is assured by acinar and centroacinar cells that produce various digestive enzymes and intercalated ducts that transport these enzymes in alkaline solution to the duodenum. The functional unit of the endocrine pancreas is the islet of Langerhans. Islets are scattered throughout the exocrine portion of the pancreas and are composed of four cell types: alpha-, beta-, delta- and PP-cells, reviewed for example in Kim S.K. and Hebrok M., (2001) Genes Dev. 15: 111-127. Beta-cells produce insulin, represent the majority of the endocrine cells and form the core of the islets, while alpha-cells secrete glucagon and are located in the periphery. Delta-cells and PP-cells are less numerous and secrete somatostatin and pancreatic polypeptide, respectively.

Early pancreatic development has been well studied in different species, including chicken, zebrafish, and mice (for an detailed review, see Kim & Hebrock, 2001, supra). The pancreas develops from distinct dorsal and ventral anlagen. Pancreas development requires specification of the pancreas structure along both anterior-posterior and dorsal-ventral axes. A number of transcription factors, which are critical for proper pancreatic development have been identified (see Kim & Hebrock, 2001, supra; Wilson M.E. et al., (2003) Mech Dev. 120: 65-80).

In humans, the acinar and ductal cells retain a significant proliferative capacity that can ensure cell renewal and growth, whereas the islet cells become mostly mitotically inactive. This is in contrast to rodents where beta-cell replication is an important mechanism in the generation of new beta cells. It has been suggested, that during embryonic development, pancreatic islets of Langerhans originate from differentiating duct cells or other cells with epithelial morphology (Bonner-Weir S. and Sharma A., (2002) J Pathol. 197: 519-526; Gu G. et al., (2003) Mech Dev. 120: 35-43). In adult humans, new beta cells arise in the vicinity of ducts (Butler A.E. et al., (2003) Diabetes 52: 102-110;

Pancreatic beta-cells secrete insulin in response to rising glucose levels and other secretagogues such as arginine. Insulin amongst other hormones plays a key role in the regulation of the fuel metabolism. Insulin stimulates the storage of glycogen and triglycerides and also the synthesis of proteins. The entry of glucose into muscles and adipose cells is stimulated by insulin. In patients who suffer from diabetes mellitus the amount of insulin produced by the pancreatic islet cells is too low, resulting in elevated blood glucose levels (hyperglycemia). In diabetes type 1 beta cells are lost due to autoimmune destruction. In type 2 diabetic patients, liver and muscle cells loose their ability to respond to normal blood insulin levels (insulin resistance). High blood glucose levels (and also high blood lipid levels) lead to an impairment of beta-cell function and to an increase in beta-cell apoptosis. It is interesting to note that the rate of beta-cell neogenesis does not appear to change in type 2 diabetics (Butler et al., 2003, supra), thus causing a reduction in total beta-cell mass over time. Eventually the application of exogenous insulin becomes necessary in type 2 diabetics.

Improving metabolic parameters such as blood sugar and blood lipid levels (e.g. through dietary changes, exercise, medication or combinations thereof) before beta cell mass has fallen below a critical threshold leads to a relatively rapid restoration of beta cell function. However, after such a treatment the pancreatic endocrine function would remain impaired due to the only slightly increased regeneration rate.
In type 1 diabetics, the lifespan of pancreatic islets is dramatically shortened due to autoimmune destruction. Treatments have been devised which modulate the immune system and may be able to stop or strongly reduce islet destruction (Raz I. et al., (2001) Lancet 358: 1749-1753; Chatenoud L. et al., (2003) Nat Rev Immunol. 3: 123-132). However, due to the relatively slow regeneration of human beta cells such treatments could only be successful if they are combined with an agent that can stimulate beta cell regeneration.

A variety of model organisms has been used to study the formation of beta cells and to analyze the effect of treatments aimed at the improvement of diabetic conditions. Zebrafish has become a popular model vertebrate for the study of developmental processes as well as for pharmacological and toxicological studies over the last decade (Rubinstein, 2003, Curr Opin Drug Discov Devel. 6: 218-223; Grunwald & Eisen, 2002, Nat Rev Genet. 3: 717-724). In this organism, large numbers of transparent embryos which rapidly develop outside of their mother are readily available. Transgenic lines expressing marker proteins under the control of tissue-specific promoters allow to rapidly assess the effects of pharmacological treatments or gene loss- and gain-of-function treatments. Zebrafish islets contain the same cell types in a similar spatial organization as mammalian islets. A large number of genes which control pancreatic development in mammals also control pancreatic development in zebrafish (Biemar et al., 2001, Dev Biol. 230: 189-203; Ober et al., 2003, Mech Dev. 120: 5-18). Suppressing gene function in zebrafish embryos using antisense oligonucleotides, modified Peptide Nucleic Acids (mPNAs) or other antisense compounds with good efficiency and specificity yields phenotypes which are usually indistinguishable from genetic mutants in the same gene (Nasevicius et al., Nat Genet. 2000 26: 216-220; Effimov et al., NAR 26: 566-575; Urtishak et al., 2003, Dev Dyn. 228: 405-13). Thus, zebrafish embryos represent a relevant model to identify genes or compounds which control beta cell formation in humans.

Diabetes is a very disabling disease, because insulin therapy or today's
common anti-diabetic drugs do not control blood sugar levels well enough to completely prevent the occurrence of high and low blood sugar levels. Chronically elevated blood sugar levels are toxic and cause long-term complications such as renopathy, retinopathy, neuropathy and peripheral vascular disease. There is also a host of related conditions, such as obesity, hypertension, heart disease and hyperlipidemia, for which persons with diabetes are substantially at risk.

Apart from the impaired quality of life for the patients, the treatment of diabetes and its long term complications presents an enormous financial burden to our healthcare systems with rising tendency. Thus, for the prevention or treatment of diabetes mellitus type 1, LADA, and diabetes mellitus type 2 there is a strong need in the art to identify factors that induce regeneration of pancreatic insulin producing beta-cells.

Obesity is one of the most prevalent metabolic disorders in the world. It is still a poorly understood human disease that becomes as a major health problem more and more relevant for western society. Obesity is defined as a body weight more than 20% in excess of the ideal body weight, frequently resulting in a significant impairment of health. Obesity may be measured by body mass index, an indicator of adiposity or fatness. Further parameters for defining obesity are waist circumferences, skinfold thickness and bioimpedance. It is associated with an increased risk for cardiovascular disease, hypertension, diabetes mellitus type 2, hyperlipidaemia and an increased mortality rate. Obesity is influenced by genetic, metabolic, biochemical, psychological, and behavioral factors and can be caused by different reasons such as non-insulin dependent diabetes, increase in triglycerides, increase in carbohydrate bound energy and low energy expenditure (Kopelman P.G., (2000) Nature 404: 635-643).

The concept of 'metabolic syndrome' (syndrome x, insulin-resistance syndrome, deadly quartet) was first described 1966 by Camus and

The molecular factors regulating food intake and body weight balance are incompletely understood. Even if several candidate genes have been described which are supposed to influence the homeostatic system(s) that regulate body mass/weight, like leptin or the peroxisome proliferator-activated receptor-gamma co-activator, the distinct molecular mechanisms and/or molecules influencing obesity or body weight/body mass regulations are not known.

There is a need in the prior art for candidate genes that are specifically expressed in early development in certain pancreatic tissues. These genes and the thereby encoded proteins can provide tools to the diagnosis and treatment of severe pancreatic disorders and related diseases. Therefore, this invention describes a secreted protein that is specifically expressed in pancreatic tissues early in the development. The invention further discloses the role of this protein in the regulation of glucose metabolism. The invention relates to the use of this protein in the diagnosis, prevention and/or treatment of pancreatic dysfunctions, such as diabetes, and other related diseases such as obesity and/or metabolic syndrome. These proteins and genes are especially useful in regeneration processes, such as regeneration of the pancreatic islet cells, in particular beta cells.
The protein of the invention is a member of the TIMP gene family and corresponds to human tissue inhibitor of metalloproteinase 2 (TIMP-2). The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases (MMP), which have been associated directly and indirectly with many developmental processes such as branching morphogenesis, regulation of cell migration, apoptosis, angiogenesis, and regulation of innate immunity. In addition to their link with developmental events, MMPs have been implicated in several disease processes such as tumor metastasis, arthritis, and emphysema. Family members of TIMPs have pluripotential effects on cell growth, apoptosis and differentiation. For example, cytokine-mediated DNA damage is decreased by TIMP-1 and TIMP-2 in isolated rat islet and INS-1 cells in vitro (Han X, Diabetes 2001). The TIMP-2 gene displays several features of a housekeeping gene and is likely to play a role significantly different from that of other family members (Hammani K. et al., (1996) J Biol Chem. 271: 25498-25505). TIMP2 was described to abrogate angiogenic factor-induced endothelial cell proliferation in vitro and angiogenesis in vivo independent of MMP inhibition (see Seo D.W. et al., (2003) Cell 114: 171-180).

The TIMP-2 protein and possible uses in different diseases are disclosed in several patent applications. EP-A-0398753 and EP-A-0464147 disclose the cDNA sequence and the amino acid sequence of human TIMP-2. According to EP-A-0464147, TIMP-2 and analogs can be used therapeutically in diseases characterized by the uncontrolled activity of matrix metalloproteinases including arthritis, diabetes, cancer, ulcers of mucosa and epithelial tissues, autoimmune mediated inflammation, lung injury, granulomatous diseases, myocardial infarctions and others. There is, however, no experimental evidence that a lack of TIMP-2 is indeed associated with diabetes. TIMP-2 is also described as metalloproteinase inhibitor polypeptide useful for inhibiting tumor cell dissemination, for treating rheumatoid arthritis, Paget's disease, osteoporosis, anemia and immunological disorders (see US 2002090654-A1). Patent US 5,714,465 describes TIMP-2 as useful for the inhibition of tumour cell dissemination.
Patent application WO 02/00677 describes TIMP-2 protein as useful in the prevention, treatment and diagnosis of cancer, immune disorders, cardiovascular disorders and neurological diseases. A composition useful for treating pulmonary inflammation e.g. chronic obstructive pulmonary disease comprising a lung surfactant polypeptide and a protease inhibitor (TIMP-2) is described in WO 03/090682. TIMP-2 is further described in patent US 5,914,392 and patent applications EP 1041083 and WO 95/05478 for treating cancer and inhibiting metastasis of cancer. A 194 amino acid fragment of TIMP-2 is described in WO 03/103705 as being useful for the treatment of wounds and in WO 03/057884 for treating diseases related to angiogenesis, e.g. arthritis, psoriasis or retinopathy) and/or metastasis of cancer cells.

It was shown by immunocytochemically staining that TIMP-2 is expressed in human pancreatic islets (Tomita T et al, 1997, Mod Pathol 10: 47-54). TIMP-2 is involved in the efficient activation of pro matrixmetalloproteinase (MMP)-2, which plays a role in the morphogenesis of tissues (Wang Z et al, 2000, JBC 275, 26411; Miralles F et al, 2004, JCB 143, 827). Furthermore, TIMP-2 was strongly increased during regeneration after acute cerulein-induced pancreatitis in rats (Muller-Pillasch et al, 1997, Pancreas 15, 168). Han et al. show that low doses of TIMP-1 and very high doses of TIMP-2 decrease cytokine-induced DNA fragmentation (Han et al., 2001, Diabetes 50:1047-1055). They conclude that TIMP-1 but not TIMP-2 prevent cytokine-induced apoptosis in rat islets and beta-cells.

To the best of our knowledge, no disclosure has been made in the prior art that provides evidence for a function of TIMP-2 in the regeneration of tissues such as pancreatic islet tissues and others and thus in the treatment of diabetes. In addition, no function of TIMP-2 has been described in the regulation of body weight. Thus, since secreted proteins are a major target for drug action and development, it is of high value to identify and characterize novel functions for secreted proteins. The present invention advances the state of the art by providing previously unknown functions for the low molecular weight human
secreted protein TIMP-2. This satisfies a need in the art by providing new therapeutic strategies for treating pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, for example, treating diabetes by regeneration of pancreatic islet cells. The protein of the invention can be used to promote a regeneration of, for example, pancreatic islet cells which then start producing insulin on their own. Therefore, further discovery and development of such novel functions for secreted proteins like TIMP-2 would have a strong beneficial effect on medical services and healthcare.

Accordingly, the present invention relates to TIMP-2 protein with novel functions in the human metabolism, regeneration and pancreatic developmental processes. The present invention discloses TIMP-2 secreted protein and effectors/modulators thereof involved in the regulation of pancreatic function and metabolism, especially in pancreas diseases such as diabetes mellitus, e.g. insulin dependent diabetes mellitus and/or non-insulin dependent diabetes mellitus, and/or metabolic syndrome, obesity, and/or related disorders such as coronary heart disease, eating disorder, cachexia, hypertension, hypercholesterolemia (dyslipidemia), liver fibrosis, and/or gallstones. Further, the present invention discloses TIMP-2 polypeptides, TIMP-2 nucleic acids and effectors/modulators thereof involved in the modulation, e.g. stimulation, of pancreatic development and/or regeneration of pancreatic cells or tissues, e.g. cells having endocrine functions, particularly cells in islets such as beta-cells. In addition, we describe a protein that is clearly involved in glucose-metabolism and body-weight regulation.

The invention also relates to functional fragments of TIMP-2, i.e. Fragments capable of modulating, particularly stimulating pancreatic development. Such functional fragments may be identified by the methods as described in the EXAMPLES sections.

TIMP-2 homologous proteins and nucleic acid molecules coding therefor are obtainable from vertebrate species. Particularly preferred are nucleic acids encoding the human TIMP-2 protein and variants thereof. The invention
particularly relates to nucleic acid molecules encoding polypeptides contributing to regulating the energy homeostasis and the mammalian metabolism, wherein said nucleic acid molecules comprises the nucleotide sequence of human TIMP-2 (SEQ ID NO: 1; Genbank Accession Number NM_003255) and/or a sequence complementary thereto.

Before the present invention is described in more detail, it is understood that all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

In this invention, we used a screen for secreted factors expressed in developing mammalian (mouse) pancreas, as described in more detail in the Examples section (see Example 1). This screen identified TIMP-2 as secreted factor expressed in developing mouse pancreas. The present invention describes mammalian TIMP-2 protein and the polynucleotides encoding it, in particular human TIMP-2, as being involved in the conditions and processes mentioned above.

We further show that TIMP-2 induces the differentiation of insulin-producing cells. In connection with the present invention, the term "progenitor cells" relates to undifferentiated cells capable of being differentiated into insulin producing cells. The term particularly includes stem cells, i.e. undifferentiated or immature embryonic, adult, or somatic cells that can give rise to various specialized cell types. The term "stem cells" can include embryonic stem cells (ES) and primordial germ cells (EG) cells of mammalian, e.g. human or animal origin. Isolation and culture of such cells is well known to those skilled in the art (see, for example, Thomson et al., (1998) Science 282: 1145-1147; Shamblott et al., (1998) Proc. Natl. Acad. Sci. USA 95: 13726-13731; US 6,090,622; US 5,914,268; WO 00/27995; Notarianni et al., (1990) J. Reprod. Fert. 41: 51-56; Vassilieva et al., (2000) Exp. Cell. Res. 258: 361-373). Adult or somatic stem cells have been identified in numerous different tissues such as intestine, muscle, bone

Embryonic stem cells can be isolated from the inner cell mass of pre-implantation embryos (ES cells) or from the primordial germ cells found in the genital ridges of post-implanted embryos (EG cells). When grown in special culture conditions such as spinner culture or hanging drops, both ES and EG cells aggregate to form embryoid bodies (EB). EBs are composed of various cell types similar to those present during embryogenesis. When cultured in appropriate media, EB can be used to generate in vitro differentiated phenotypes, such as extraembryonic endoderm, hematopoietic cells, neurons, cardiomyocytes, skeletal muscle cells, and vascular cells. We have previously described a method that allows EB to efficiently differentiate into insulin-producing cells (as described in patent application PCT/EP02/04362, published as WO 02/086107 and by Blyszczuk et al., (2003) Proc Natl Acad Sci USA 100: 998-1003, which are incorporated herein by reference).

The results shown in Fig. 3 clearly demonstrate an induction of the differentiation of insulin-producing cells by TIMP-2. Thus, TIMP-2 can stimulate the differentiation of pancreatic insulin-producing beta-cells and is therefore a strong candidate for therapeutic uses in the treatment of diabetes, for example, when regeneration of cells, in particular beta-cells, is required.

In the present invention the term "beta-cell regeneration" refers to an at least partial restoration of normal beta-cell function by increasing the number of functional insulin secreting beta-cells and/or by restoring normal function in functionally impaired beta-cells.
Furthermore, evidence of the effect of TIMP-2 on pancreatic function is provided. Fertilized one-cell stage embryos of zebrafish carrying the transgene with insulin regulatory sequences linked to a marker protein cDNA were injected with TIMP-2 or control antisense oligonucleotides (as described in more detail in EXAMPLES. Figure 4 shows the effect of complete inhibitor of TIMP-2 on the development of pancreatic islets. The figure clearly shows, that the formation of two or more smaller islets (referred to as dispersed islet phenotype) was induced after injection of TIMP-2 antisense oligonucleotides. If TIMP-2 is inactive, the formation of the dispersed islet phenotype is trigged. Therefore, TIMP-2 is involved in development of the pancreas and thus, it plays a important role in regeneration of pancreatic islet cells.

Furthermore, we could show that TIMP-2 reduces significantly the severity of streptozotocin (STZ)-induced diabetes in vivo. First, diabetic mice treated with TIMP-2 constantly have lower blood glucose levels than carrier treated mice. This effect is strong especially in early stages of diabetes development (FIGURE 5). Second, animals with TIMP-2 containing pumps were able to gain bodyweight, while all other diabetic animals were not (FIGURE 6). In addition, TIMP-2 was not toxic to the animals, whereas in other treatment groups, some animals died throughout the experiment. Third, glucose challenge in these mice was markedly improved and was not different from control animals in the early phase of diabetes development (d5). In later stages (d12), TIMP-2 treated animals still displayed similar glucose tolerance curves to control animals (FIGURE 7). Fourth, diabetes incidence after TIMP-2 treatment was reduced compared to carrier treatment reflected by the observation, that pancreatic insulin content were increased (FIGURE 8).

Combined treatment with gastrin and epidermal growth factor (EGF) was shown recently to ameliorate severity of diabetes by inducing sufficient regeneration of a functional islet mass and thereby restoring glucose homeostasis (Rooman & Bouwens: Diabetologia 2003 46(7):926-33). We
can show in this invention, that TIMP-2 was significantly more effective than the combined gastrin and EGF treatment on all parameters tested. TIMP-2 could significantly improve glucose-stimulated insulin release and pancreatic insulin content. Furthermore, TIMP-2 treatment in mice did not induce significant changes in pancreas morphology. This invention discloses that TIMP-2 treatment in STZ-diabetic mice is very efficient. TIMP-2 treatment in diabetic mammals (mice) reduces significantly the severity of diabetes. This can be explained by an effect on the regeneration of functional pancreatic beta cells (islet cells) as shown in this invention in the stem cell and zebrafish experiments. The regenerated beta cells can restore glucose homeostasis in mammals.

The function of the mammalian TIMP-2 in metabolism was validated by analyzing the role in adipocyte differentiation. As described in more detail in Example 3, TIMP-2 shows differential expression in adipocytes. The expression of TIMP-2 is down-regulated during mammalian adipocyte differentiation, as shown for murine 3T3L-1 (Fig. 2A) and human adipocytes (see Fig. 2B and 2C). The TIMP-2 protein in preadipocytes is involved in adipose differentiation at a very early stage, and is suggested to play an essential role in adipogenesis. The results are confirming a role of TIMP-2 in the regulation of human metabolism, for example, as effector/modulator (for example, inhibitor) of adipogenesis. Thus, TIMP-2 is considered as a strong candidate for the manufacture of a pharmaceutical composition and a medicament for the treatment of conditions related to human metabolism, such as diabetes, obesity, and/or metabolic syndrome.

The invention also encompasses polynucleotides that encode TIMP-2. Accordingly, any nucleic acid sequence, which encodes the amino acid sequences of TIMP-2, can be used to generate recombinant molecules that express TIMP-2. In a particular embodiment, the invention encompasses nucleic acids encoding TIMP-2. It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding the proteins, some bearing minimal homology to the
nucleotide sequences of any known and naturally occurring gene, may be produced. The invention contemplates each and every possible variation of nucleotide sequence that can be made by selecting combinations based on possible codon choices.

Also included within the scope of the present invention are alleles of the genes encoding TIMP-2. As used herein, an 'allele' or 'allelic sequence' is an alternative form of the gene, which may result from at least one mutation in the nucleic acid sequence. Alleles may result in altered mRNAs or polypeptides whose structures or function may or may not be altered. Any given gene may have none, one or many allelic forms. Common mutational changes, which give rise to alleles, are generally ascribed to natural deletions, additions or substitutions of nucleotides. Each of these types of changes may occur alone or in combination with the others, one or more times in a given sequence.

The nucleic acid sequences encoding TIMP-2 and homologous proteins may be extended utilizing a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements.

In order to express a biologically active protein, the nucleotide sequences encoding the proteins or functional equivalents, may be inserted into appropriate expression vectors, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods, which are well known to those skilled in the art, may be used to construct expression vectors containing sequences encoding the proteins and the appropriate transcriptional and translational control elements. Regulatory elements include for example a promoter, an initiation codon, a stop codon, a mRNA stability regulatory element, and a polyadenylation signal. Expression of a polynucleotide can be assured by (i) constitutive promoters such as the Cytomegalovirus (CMV) promoter/enhancer region, (ii) tissue specific promoters such as the insulin promoter (see, Soria et al., 2000, Diabetes 49: 157), SOX2 gene promoter (see Li et al., (1998) Curr. Biol. 8: 971-974), Msi-1
promotor (see Sakakibara et al., (1997) J. Neuroscience 17: 8300-8312),
alpha-cardia myosin heavy chain promotor or human atrial natriuretic factor
Biol. Chem. 264: 6472-6479) or (iii) inducible promoters such as the
tetracycline inducible system. Expression vectors can also contain a selection
agent or marker gene that confers antibiotic resistance such as the neomycin,
hygromycin or puromycin resistance genes. These methods include in vitro
recombinant DNA techniques, synthetic techniques, and in vivo genetic
recombination. Such techniques are described in Sambrook, J. et al. (1989)
Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview,
John Wiley & Sons, New York, N.Y.

In a further embodiment of the invention, natural, modified or recombinant
nucleic acid sequences encoding TIMP-2 may be ligated to a heterologous
sequence to encode a fusion protein.

A variety of expression vector/host systems, as known in the art, may be
utilized to contain and express sequences encoding the proteins or fusion
proteins. These include, but are not limited to, micro-organisms such as
bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA
expression vectors; yeast transformed with yeast expression vectors; insect
cell systems infected with virus expression vectors (e.g., baculovirus,
adenovirus, adeno-associated virus, lentivirus, retrovirus); plant cell systems
transformed with virus expression vectors (e.g., cauliflower mosaic virus,
CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g.,
Ti or PBR322 plasmids); or animal cell systems.

The presence of polynucleotide sequences of the invention in a sample can be
detected by DNA-DNA or DNA-RNA hybridization and/or amplification using
probes or portions or fragments of said polynucleotides. Nucleic acid
amplification based assays involve the use of oligonucleotides or oligomers
based on the sequences specific for the gene to detect transformants
containing DNA or RNA encoding the corresponding protein. As used herein 'oligonucleotides' or 'oligomers' refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe or amplifier.

The presence of TIMP-2 in a sample can be determined by immunological methods or activity measurement. A variety of protocols for detecting and measuring the expression of proteins, using either polyclonal or monoclonal antibodies specific for the protein or reagents for determining protein activity are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the protein is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul, Minn.) and Maddox, D.E. et al. (1983; J. Exp. Med. 158: 1211-1226).

Suitable reporter molecules or labels, which may be used, include radionuclides, enzymes, fluorescent, chemiluminescent or chromogenic agents as well as substrates, co-factors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding a protein of the invention may be cultured under conditions suitable for the expression and recovery of said protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence or/and the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides, which encode the protein may be designed to contain signal sequences, which direct secretion of the protein through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding the protein to
nucleotide sequence encoding a polypeptide domain, which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAG extension/affinity purification system (Immunex Corp., Seattle, Wash.) The inclusion of cleavable linker sequences such as those specific for Factor XA or Enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and the desired protein may be used to facilitate purification.

The data disclosed in this invention show that the TIMP-2 secreted protein and effector/modulator molecules thereof are useful in diagnostic and therapeutic applications implicated, for example, but not limited to, pancreatic diseases (e.g. diabetes mellitus such as insulin dependent diabetes mellitus and/or non insulin dependent diabetes mellitus an/or LADA), obesity, metabolic syndrome, eating disorder, cachexia, hypertension, coronary heart disease, hypercholesterolemia (dyslipidemia), and/or gallstones. Further, the data show that TIMP-2 and effector/modulator molecules thereof are useful for the modulation, e.g. stimulation of pancreatic development and/or for the regeneration of pancreatic cells or tissues, e.g. cells having endocrine functions, particularly cells in Langerhans islets such as beta -cells. Hence, diagnostic and therapeutic uses for the proteins of the invention nucleic acids and proteins of the invention are, for example but not limited to, the following: (i) tissue regeneration in vitro and in vivo (regeneration for all these tissues and cell types composing these tissues and cell types derived from these tissues), (ii) small molecule drug target, (iii) antibody target (therapeutic, diagnostic, drug targeting/cytotoxic antibody), (iv) diagnostic and/or prognostic marker, (v) protein therapy, (vi) gene therapy (gene delivery/gene ablation), and/or (vii) research tools.

According to this invention TIMP-2 may be administered

i) as a pharmaceutical composition e.g. enterally, parenterally or topically,
preferably directly to the pancreas,

ii) via implantation of TIMP-2 protein product expressing cells, and/or

iii) via gene therapy

as described in more detail below.

Further, the TIMP-2 expression level in a patient might be influenced by a TIMP-2 modulator/effecter, preferably a TIMP-2 activator administered

i) as a pharmaceutical composition e.g. enterally, parenterally or topically, preferably directly to the pancreas,

ii) via cell based therapy, and/or

iii) via gene therapy

as described in more detail below.

The TIMP-2 product or the TIMP-2 modulator/effecter, i.e. a pharmaceutically active substance influencing, particularly increasing the TIMP-2 expression level or function may be administered in the above described manner alone or in combination with another pharmaceutical composition useful to treat beta-cell degeneration, for example hormones, growth factors or immune modulating agents.

A TIMP-2 product or a modulator/effecter thereof may be administered in patients suffering from a disease going along with impaired beta-cell function, for example but not limited to diabetes type 1, LADA, or progressed diabetes type 2. It is further contemplated that a TIMP-2 product or the modulator/effecter thereof may be administered preventively to patients at risk to develop beta-cell degeneration, like for example but not limited to patients suffering from diabetes type 2 or LADA in early stages. A variety of pharmaceutical formulations and different delivery techniques are described in further detail below.

The present invention also relates to methods for differentiating progenitor cells into insulin-producing cells in vitro comprising

(a) activating one or more pancreatic genes in a progenitor, e.g. stem cell
(optional step, particularly if embryonic stem cells are used)

(b) aggregating said cells to form embryoid bodies (optional step, particularly if embryonic stem cells are used)

(c) cultivating embryoid bodies or cultivating adult stem cells (e.g., duct cells) in specific differentiation media containing a TIMP-2 protein product and/or a modulator/effecter thereof under conditions wherein beta-cell differentiation is significantly enhanced, and

(d) identifying and selecting insulin-producing cells.

Activation of pancreatic genes may comprise transfection of a cell with pancreatic gene operatively linked to an expression control sequence, e.g. on a suitable transfection vector, as described in WO 03/023018, which is herein incorporated by reference. Examples of preferred pancreatic genes are Pdx1, Pax4, Pax6, neurogenin 3 (ngn3), Nkx 6.1, Nkx 6.2, Nkx 2.2, HB 9, BETA2/Neuro D, Isl 1, HNF1-alpha, HNF1-beta and HNF3 of human or animal origin. Each gene can be used individually or in combination with at least one other gene. Pax4 is especially preferred.

TIMP-2 products, e.g. TIMP-2 protein or nucleic acid products, are preferably produced via recombinant techniques because such methods are capable of achieving high amounts of protein at a great purity, but are not limited to products expressed in bacterial, plant, mammalian, or insect cell systems.

Further, the data show that the TIMP-2 nucleic acids and proteins and effector/modulator molecules thereof are useful for the modulation, e.g. stimulation of pancreatic development and/or for the regeneration of pancreatic cells with endocrinous functions, particularly cells in Langerhans islets such as insulin-producing beta-cells.

For example, but not limited to, cDNAs encoding the proteins of the invention and particularly their human homologues may be useful in stimulating, enhancing or regulating the regeneration of tissues, and the proteins of the
invention and particularly their human homologues may be useful when administered to a subject in need thereof. By way of non-limiting example, the compositions of the present invention will have efficacy for treatment of patients suffering from, for example, pancreatic diseases (e.g. diabetes mellitus type I, late stages of diabetes mellitus type II, LADA), obesity, and/or metabolic syndrome as described above.

Administration of a TIMP-2 protein product and/or modulators/effectors thereof, particularly activators, in a pharmaceutical composition to a subject in need thereof, particularly a human patient, leads to an at least partial regeneration of pancreatic cells. Preferably, these cells are insulin producing beta-cells that will contribute to the improvement of a diabetic state. With the administration of this composition e.g. on a short term or regular basis, an increase in beta-cell mass can be achieved. This effect upon the body reverses the condition of diabetes partially or completely. As the subject's blood glucose homeostasis improves, the dosage administered may be reduced in strength. In at least some cases further administration can be discontinued entirely and the subject continues to produce a normal amount of insulin without further treatment. The subject is thereby not only treated but could be cured entirely of a diabetic condition. However, even moderate improvements in beta-cell mass can lead to a reduced requirement for exogenous insulin, improved glycemic control and a subsequent reduction in diabetic complications. In another example, the compositions of the present invention will also have efficacy for treatment of patients with other pancreatic diseases such as pancreatic cancer, dysplasia, or pancreatitis, if beta-cells are to be regenerated.

Beside diabetes, the compositions of the present invention will also have efficacy for treatment of patients with other pancreatic diseases such as pancreatic cancer, dysplasia or pancreatitis.

The TIMP-2 nucleic acids and proteins and effectors/modulators thereof are useful in diagnostic and therapeutic applications implicated in various
embodiments as described below. For example, but not limited to, cDNAs encoding the proteins of the invention and particularly their human homologues may be useful in gene therapy, and the proteins of the invention and particularly their human homologues may be useful when administered to a subject in need thereof. By way of non-limiting example, the compositions of the present invention will have efficacy for treatment of patients suffering from, for example, pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome as described above.

TIMP-2 product cell therapy, i.e. pancreatic implantation of cells producing TIMP-2 protein product, is also contemplated. This embodiment would involve implanting cells capable of synthesizing and secreting a biologically active form of TIMP-2 protein product into patients. Such TIMP-2 protein product-producing cells may be cells that are natural producers of TIMP-2 protein product or may be cells that are modified to express the protein. Such modified cells include recombinant cells whose ability to produce a TIMP-2 protein product has been augmented by transformation with a gene encoding the desired TIMP-2 protein product in a vector suitable for promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered TIMP-2 protein product of a foreign species, it is preferred that the cells producing TIMP-2 protein product be of human origin and produce human TIMP-2 protein product. Likewise, it is preferred that the recombinant cells producing TIMP-2 protein product be transformed with an expression vector containing a gene encoding a human TIMP-2 protein product. Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or nonhuman animal cells may be implanted in patients in biocompatible, semipermeable polymeric enclosures or membranes that allow release of TIMP-2 protein product, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue.

Alternatively, TIMP-2 protein product secreting cells may be introduced into a patient in need intraportally via a percutaneous transhepatic approach

Further, the invention relates to a cell preparation comprising differentiated progenitor cells, e.g. stem cells exhibiting insulin secretion, particularly an insulin-producing cell line obtainable by the method described above. The insulin-producing cells may exhibit a stable or a transient expression of at least one pancreatic gene involved in beta-cell differentiation. The cells are preferably human cells that are derived from human stem cells. For therapeutic applications the production of autologous human cells from adult stem cells of a patient is especially preferred. However, the insulin producing cells may also be derived from non-autologous cells. If necessary, undesired immune reactions may be avoided by encapsulation, immunosuppression and/or modulation or due to non-immunogenic properties of the cells.

The insulin producing cells of the invention preferably exhibit characteristics that closely resemble naturally occurring beta-cells. Further, the cells of the invention preferably are capable of a quick response to glucose. After addition of 27.7 mM glucose, the insulin production is enhanced by a factor of at least 2, preferably by a factor of at least 3. Further, the cells of the invention are capable of normalizing blood glucose levels after transplantation into mice.

The invention further encompasses functional pancreatic cells obtainable or obtained by the method according to the invention. The cells are preferably of mammalian, e.g. human origin. Preferably, said cells are pancreatic beta-cells, e.g. mature pancreatic beta-cells or stem cells differentiated into pancreatic beta-cells. Such pancreatic beta cells preferably secrete insulin in
response to glucose. Moreover, the present invention provides functional pancreatic cell that express glucagon in response to glucose. A preparation comprising the cells of the invention may additionally contain cells with properties of other endocrine cell types such as alpha-cells, delta-cells and/or PP-cells. These cells are preferably human cells.

The cell preparation of the invention is preferably a pharmaceutical composition comprising the cells together with pharmacologically acceptable carriers, diluents and/or adjuvants. The pharmaceutical composition is preferably used for the treatment or prevention of pancreatic diseases, e.g. diabetes.

According to the present invention, the functional insulin producing cells treated with TIMP-2 may be transplanted preferably intrahepatic, directly into the pancreas of an individual in need, or by other methods. Alternatively, such cells may be enclosed into implantable capsules that can be introduced into the body of an individual, at any location, more preferably in the vicinity of the pancreas, or the bladder, or the liver, or under the skin. Methods of introducing cells into individuals are well known to those of skill in the art and include, but are not limited to, injection, intravenous or parenteral administration. Single, multiple, continuous or intermittent administration can be effected. The cells can be introduced into any of several different sites, including but not limited to the pancreas, the abdominal cavity, the kidney, the liver, the celiac artery, the portal vein or the spleen. The cells may also be deposited in the pancreas of the individual.

The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Patent Numbers 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference.
See also, PCT Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 1 13: 322-329, 1991, Aebischer et al., Exper. Neurol., 11 1:269-275, 1991; Tresco et al., ASAIO, 38:17-23, 1992, each of which is specifically incorporated herein by reference. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible particles or beads and depot injections, are also known to those skilled in the art.

In another embodiment gene therapy ex vivo is envisioned, i.e. the patient's own cells may be transformed ex vivo to produce a TIMP-2 protein product or a protein stimulating TIMP-2 expression and would be directly reimplanted. For example, cells retrieved from the patient may be cultured and transformed with an appropriate vector. After an optional propagation/expansion phase, the cells can be transplanted back into the same patient's body, particularly the pancreas, where they would produce and release the desired TIMP-2 protein product. Delivery by transfection and by liposome injections may be achieved using methods, which are well known in the art. Any of the therapeutic methods described above may be applied to any suitable subject including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

TIMP-2 product gene therapy in vivo is also envisioned, by introducing the gene coding for a TIMP-2 protein product into targeted pancreas cells via local injection of a nucleic acid construct or other appropriate delivery methods (Hefti, J. Neurobiol., 25:1418-1435, 1994). For example, a nucleic acid sequence encoding a TIMP-2 protein product may be contained in an adeno-associated virus vector or adenovirus vector for delivery to the pancreas cells. Alternative viral vectors include, but are not limited to, retrovirus, herpes simplex virus and papilloma virus vectors. Physical transfer, either in vivo or ex vivo as appropriate, may also be achieved by liposome-mediated transfer, direct injection (naked DNA), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation or microparticle bombardment (gene gun).
The active agents of the invention may be administered alone or in combination with another medicament useful to prevent or treat pancreatic disorders or metabolic syndrome, particularly beta-cell degeneration, for example hormones, growth factors or antioxidants such as GLP-1 and stabilized forms of GLP-1, GLP-1 analogues, DPP-IV inhibitors, nicotinamide, vitamin C, INGAP peptide, TGF-alpha, gastrin, prolactin, members of the EGF-family, or immune modulating agents such as anti-CD3 antibodies, DiaPep277 or anti-inflammatory agents such as Cox2 inhibitors, acetyl-salicylic acid, or acetaminophen. The agents may be administered in combination with the beta cell regenerating proteins, nucleic acids and effectors/modulators thereof described in PCT/EP2004/007917, e.g. pleiotrophin and agonists thereof, or in PCT/EP2004/013175, PCT/EP2004/013535, PCT/EP 2005/000545, PCT/EP 2005/0017111 and EP 04018751.0, which are herein incorporated by reference.

More particularly, the agents of the invention may be administered together with beta cell mitogens and/or beta cell protective agents such as GLP-1 or derivatives thereof such as GLP-1 or derivatives thereof, e.g. GLP-1 (7-36 amide), exendin-4, prolactin or neurotrophins such as NGF.

The agents are preferably administered together with pharmaceutical agents which have an immunosuppressive activity, e.g. antibodies, polypeptides and/or peptidic or non-peptidic low molecular weight substances.

Preferred examples of immunosuppressive agents are listed in the following Table 1.
Table 1: Exemplary agents for immune suppression

<table>
<thead>
<tr>
<th>Names</th>
<th>Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-amino-1,3-propanediol derivatives</td>
<td>Used for preventing or treating chronic rejection in a patient receiving an organ or tissue allo- or xenotransplant</td>
</tr>
<tr>
<td>2-amino-2(2-(4-octylphenyl)ethyl)propane-1,3-diol hydrochloride</td>
<td>Immunosuppression, from accelerated lymphocyte homing</td>
</tr>
<tr>
<td>4-thiophenoxy-n-(3,4,5-trialkoxyphenyl) pyrimidine-2-amines</td>
<td>Lck inhibitors</td>
</tr>
<tr>
<td>40-O-(2-hydroxyethyl)-rapamycin, SDZ-RAD, Everolimus</td>
<td>Sirolimus (rapamycin) derivative, used for acute kidney rejection; reduces rejection and graft vasculopathy following heart transplantation by inhibiting cell proliferation</td>
</tr>
<tr>
<td>6-(3-dimethyl-aminoproplonyl) forskolin</td>
<td>Immunosuppressing action useful also for treating autoimmune disease</td>
</tr>
<tr>
<td>6-mercaptopurine (6-MP)</td>
<td>Used to treat Crohn's disease, inflammatory bowel disease and for organ transplant therapy</td>
</tr>
<tr>
<td>A-420983</td>
<td>Lck-inhibitor</td>
</tr>
<tr>
<td>ABX-CBL (CBL-1)</td>
<td>Mouse monoclonal AB targeted against human T-cell, B-cells, NK-cells and monocytes, for treatment of steroid-resistant graft-vs-host diseases, potential use in treatment of inflammatory and autoimmune disorders</td>
</tr>
<tr>
<td>Alfaccept (human LFA-3 IgG1 fusion protein)</td>
<td>Knocks out causative memory T-lymphocytes; used to treat psoriasis, a T-cell mediated inflammatory disorder</td>
</tr>
<tr>
<td>Antisense ICAM-1 inhibitor (ISIS 2302), Enlimomab, BIRR1, Alfacorsen</td>
<td>Mouse monoclonal AB blocks white blood cell adhesion to T-cell surface molecule (ICAM-1r); treatment of kidney transplant rejection</td>
</tr>
<tr>
<td>Antithymocyte immunoglobulin (ATGAM)</td>
<td>Anti-human thymocyte, immunoglobulin; used in reversal of acute kidney transplant rejection and will likely be used off-label for transplant induction therapy</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Treatment of rheumatoid arthritis and prevention of kidney transplant rejection, and other autoimmune or inflammatory disorders such as inflammatory bowel disease</td>
</tr>
<tr>
<td>Baohuodsie-1</td>
<td>Flavonoid; inhibits lymphocyte activation; Ma et al., Transplantation 78:831-838, (2004)</td>
</tr>
<tr>
<td>basiliximab</td>
<td>Monoclonal AB that binds to receptor sites on T-cells, preventing activation by transplanted tissue (renal transplant)</td>
</tr>
<tr>
<td>BMS-279700</td>
<td>Lck-inhibitor</td>
</tr>
<tr>
<td><strong>BTI-322</strong></td>
<td>Mouse derived monoclonal AB targeted to CD2 receptor; used for prevention of first-time kidney rejection, and treatment of resistant rejection</td>
</tr>
<tr>
<td><strong>Cladribine</strong></td>
<td>Antimetabolite and immunosuppressive agent that is relatively selective for lymphocytes; used to treat lymphoid malignancies, e.g. hairycell leukemia</td>
</tr>
<tr>
<td><strong>CP-690550</strong></td>
<td>JAK-3 inhibitor</td>
</tr>
<tr>
<td><strong>Cyclophosphamide (CTX)</strong></td>
<td>Immunosuppressant for treatment of arthritis and other auto-immune disorders and cancers</td>
</tr>
<tr>
<td><strong>Cyclosporine (cyclosporin A, cyclosporin)</strong></td>
<td>11 amino acid cyclic peptide; blocks helper T-cell, immunosuppressant used in organ transplant therapy and other immune diseases</td>
</tr>
<tr>
<td><strong>Daclizumab, HAT (Humanized Anti-Tac), SMART anti-Tac, anti-CD25, and humanized anti-IL2-receptor</strong></td>
<td>Monoclonal AB inhibits binding of IL-2 to IL-2 receptor by binding to IL-2 receptor; suppresses T-cell activity against allografts (renal transplant)</td>
</tr>
<tr>
<td><strong>Dexamethasone (Decadron, Dexone, Dexasone)</strong></td>
<td>An adrenocorticoid, effective immunosuppressant in various disorders</td>
</tr>
<tr>
<td><strong>DIAPEP-277</strong></td>
<td>Immunomodulatory properties</td>
</tr>
<tr>
<td><strong>DiaMyd peptide</strong></td>
<td>GAD-derived immunomodulatory peptide</td>
</tr>
<tr>
<td><strong>Dipeptide Boronic Acid (DPBA)</strong></td>
<td>Proteasome inhibitor; Wu et al., Transplantation 78: 360-366, (2004)</td>
</tr>
<tr>
<td><strong>Docosahexaenoic acid (DHA)</strong></td>
<td>Immunosuppressant that lowers the proportion of T-cells expressing CD4 or CD8, blocks antigen recognition process; Taku et al., Journal of Agricultural and Food Chemistry 48: 1047, (2000)</td>
</tr>
<tr>
<td><strong>efalizumab</strong></td>
<td>T-cell modulator that target T-cells through interactions with adhesion molecules on endothelial cell surface, target migration of T-cells into the skin and target activation of T-cells; used to treat Psoriasis</td>
</tr>
<tr>
<td><strong>Efomycine M</strong></td>
<td>Leukocyte adhesion inhibitor, Anti-inflammatory</td>
</tr>
<tr>
<td><strong>FTY720 (oral myriocin derivative)</strong></td>
<td>Alters lymphocyte infiltration into grafted tissues; used for prevention of organ rejection in kidney transplants</td>
</tr>
<tr>
<td><strong>GAD-based vaccine/immunemodulator, e.g. from Diamyd company</strong></td>
<td>Prevention and treatment of insulin-dependent diabetes</td>
</tr>
<tr>
<td><strong>Glatiramer acetate (co-polymer-1)</strong></td>
<td>Synthetic peptide copolymer; decoy that mimics structure of myelin so immune cells bind Copaxone instead of myelin; for multiple sclerosis</td>
</tr>
<tr>
<td>Substance</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Gusperimus (15-deoxypergualin)</td>
<td>Intravenous immunosuppressant; suppresses production of cytotoxic T-cells, neutrophils and macrophages</td>
</tr>
<tr>
<td>HLA-B2702 peptide</td>
<td>Human peptide, blocks action of NK cells and T-cell mediated toxicities, used for prevention of first kidney allograft rejection</td>
</tr>
<tr>
<td>hu1124(anti-CD11a)</td>
<td>Humanized monoclonal antibody; targets CD11a receptor on surface of T-cells to selectively inhibit immune system rejection of transplanted organs</td>
</tr>
<tr>
<td>hOKT31gamma (Ala-Ala)</td>
<td>Non Fc-binding humanized anti CD3 antibody</td>
</tr>
<tr>
<td>IBC-VSO1</td>
<td>A synthetic, metabolically inactive form of insulin designed to prevent pancreatic beta cell destruction (vaccine)</td>
</tr>
<tr>
<td>IGRP-derived peptides</td>
<td>T-cell modulator</td>
</tr>
<tr>
<td>Imatinib (STI571, Glivec or Gleevac)</td>
<td>Lck inhibitor</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Monoclonal AB, binds and inactivates human TNFalpha; used to treat Crohn's disease and rheumatoid arthritis</td>
</tr>
<tr>
<td>Interferon</td>
<td>Immunomodulatory properties</td>
</tr>
<tr>
<td>ISAbx247</td>
<td>Used to treat autoimmune diseases such as rheumatoid arthritis and psoriasis</td>
</tr>
<tr>
<td>L-683,742: also described as 31-desmethyl-31-hydroxy-L-683,590</td>
<td>Treatment of autoimmune diseases, infectious diseases and/or prevention of organ transplant rejections</td>
</tr>
<tr>
<td>Leflunomide (ARAVA)</td>
<td>Antiinflammatory agent</td>
</tr>
<tr>
<td>Medi-500 (T10B9)</td>
<td>Intravenous monoclonal AB that targets human T-cells; treats acute kidney rejection and graft-vs-host disease</td>
</tr>
<tr>
<td>Medi-507</td>
<td>Intravenous humanized AB directed against CD2 T-cell; used to treat corticosteroidresistant graft-vs-host disease and prevention of kidney rejection</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Antimetabolite used to treat Crohn's disease, severe psoriasis, and adult rheumatoid arthritis (and as an anti-cancer drug)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Antiproliferative effect on cellular immune system including T-cells, B-cells and macrophages; used to treat hormone-refractory prostate cancer, acute myelogenous leukemia and multiple sclerosis</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>Proliferation of T and B lymphocytes by blocking the synthesis of purine nucleotides; used in organ transplant therapy and inflammatory bowel disease</td>
</tr>
<tr>
<td>Medicine</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>OKT4A</td>
<td>Mouse monoclonal AB targeted against human CD4 T-cell; used for prevention of kidney transplant rejection when used in combination with other immunosuppressant drugs</td>
</tr>
<tr>
<td>Oral interferon-alpha (IFN-alpha)</td>
<td>Early onset type 1 diabetes</td>
</tr>
<tr>
<td>Muromonab-CD3</td>
<td>Monoclonal AB that binds to receptor sites on T-cells, preventing activation by transplanted tissue</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Corticosteroid, suppresses inflammation associated with transplant rejection</td>
</tr>
<tr>
<td>Psora-4</td>
<td>Kv1.3-blocker</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Antibiotic; has immunomodulatory properties</td>
</tr>
<tr>
<td>Rituximab</td>
<td>CD20 antibody</td>
</tr>
<tr>
<td>S100beta</td>
<td>Possesses immunosuppressive activities in diabetic animal models</td>
</tr>
<tr>
<td>Sirolimus, Rapamycin</td>
<td>Immunosuppressant and potent inhibitor of cytokine (e.g. IL-2)-dependent T-cell proliferation (kidney transplant)</td>
</tr>
<tr>
<td>Tacrolimus (Prograf, FK-506)</td>
<td>Interferes with IL-2 TCR communication</td>
</tr>
</tbody>
</table>

Preferred immunosuppressive agents are DiaPep277, anti-CD3-antibodies such as hOKT31 gamma (Ala-Ala) and GAD peptides such as DiaMyd GAD peptides.

The combination therapy may comprise coadministration of the medicaments during the treatment period and/or separate administration of single medicaments during different time intervals in the treatment period.

The administration of immunosuppressive drugs, such as cyclosporin, can be used to reduce the host reaction versus graft. Allografts using the cells obtained by the methods of the present invention are also useful because a single healthy donor could supply enough cells to regenerate at least partial pancreas function in multiple recipients.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such
pharmaceutical compositions may consist of TIMP-2 nucleic acids and the proteins and homologous nucleic acids or proteins, antibodies to TIMP-2, mimetics, agonists, antagonists or inhibitors of TIMP-2 or nucleic acids. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone or in combination with other agents, drugs or hormones. The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations, which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art. For any compounds, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of preadipocyte cell lines or in animal models, usually mice, rabbits, dogs or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. A therapeutically effective dose refers to that amount of active ingredient, for example the TIMP-2 nucleic acids or proteins or fragments
thereof or antibodies, which is sufficient for treating a specific condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions, which exhibit large therapeutic indices, are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage from employed, sensitivity of the patient, and the route of administration. The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors, which may be taken into account, include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week or once every two weeks depending on half-life and clearance rate of the particular formulation. Normal dosage amounts may vary from 0.1 to 100,000 microg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

The agents of the invention may be administered in patients suffering from a disease going along with reduced beta cell number and/or impaired beta-cell function, for example but not limited to one of the diseases for which a pro-
proliferative effect on pancreatic beta cells and/or an anti-apoptotic/pro-
survival effect on pancreatic beta cells and/or a beta cell neogenesis-
promoting effect would be beneficial:

- Type 1 diabetes: new onset, established, prevention in high-risk
  patients (identified e.g. via screening for multiple autoantibodies)
- LADA: new onset and established
- Type 2 diabetes: when loss of beta cell mass occurs
- MODY (Maturity Onset Diabetes of the Young, all forms)
- Gestational diabetes
- Islet + duct cell transplantation – treatment of recipients before or
  after transplantation
- Treatment of islets before transplantation/during pre-
  transplantation culture
- Pancreatitis-associated beta cell loss

The compositions are also useful for in vitro and ex vivo applications for
which a pro-differentiation effect on pancreatic beta cells and precursors
thereof would be beneficial:

- In vitro differentiation of stem cells into beta cells
- In vitro transdifferentiation of duct or exocrine cells into beta cells
- MODY (all forms)
- Persistent Hyperinsulinemic Hypoglycemia of Infancy

More particularly, the compositions may be administered in diabetes type 1,
LADA or prognosed diabetes type 2, but also preventively to patients at risk to
develop complete beta-cell degeneration, like for example but not limited to
patients suffering from diabetes type 2 or LADA and type 1 diabetes in early
stages, or other types of diseases as indicated above. The compositions may
also be used to prevent or ameliorate diabetes in patients at risk for type 1
diabetes or LADA (identified e.g. by screening for autoantibodies, genetic
predisposition, impaired glucose tolerance or combinations thereof).
Finally, the invention also relates to a kit comprising at least one of
(a) a nucleic acid molecule coding for a protein of the invention or a
functional fragment thereof;
(b) a protein of the invention or a fragment or an isoform thereof;
(c) a vector comprising the nucleic acid of (a);
(d) a host cell comprising the nucleic acid of (a) or the vector of (c);
(e) a polypeptide encoded by the nucleic acid of (a);
(f) a fusion polypeptide encoded by the nucleic acid of (a); and
(g) an antibody, an aptamer or another effector/modulator, particularly an
activator of the nucleic acid of (a) or the polypeptide of (b), (e) or (f) and
(h) an anti-sense oligonucleotide of the nucleic acid of (a).

The kit may be used for therapeutic purposes or for screening applications as
described above. The kit may further contain user instructions.

The Figures show:

**Fig. 1: Human TIMP-2 nucleic acid and proteins**
Fig. 1A shows the nucleic acid sequence of human TIMP-2 protein (SEQ ID
NO: 1; GenBank Accession Number NM_003255).
Fig. 1B shows the amino acid sequence (one-letter code) of human TIMP-2
protein (SEQ ID NO: 2; GenBank Accession Number NP_003246).

**Fig. 2: Analysis of TIMP-2 protein expression in mammalian adipocytes**
The relative RNA-expression is shown on the Y-axis, In Fig. 2A-C, the X-axis
represents the time axis. 'd0' refers to day 0 (start of the experiment), "d12"
refers to day 12 of adipocyte differentiation.

Fig. 2A shows the microarray analysis of TIMP-2 expression in mouse
fibroblast (3T3-L1) cells during the differentiation from preadipocytes to mature
adipocytes.
Fig. 2B shows the microarray analysis of TIMP-2 expression in human SGBS
cells, during the differentiation from preadipocytes to mature adipocytes.
Fig. 2C shows the microarray analysis of TIMP-2 expression in human abdominal adipocyte cells, during the differentiation from preadipocytes to mature adipocytes.

**Fig. 3: TIMP-2 dependent induction of the differentiation of insulin producing cells**

Mouse embryonic stem (ES) cells were differentiated as described previously (patent application PCT/EP02/04362, published as WO 02/086107, which is incorporated herein by reference). At the end of the differentiation procedure, cells were harvested and total RNA was isolated. The abundance of insulin mRNA (Fig. 3) was determined using quantitative RT-PCR in an Applied Biosystems 7000 sequence detection device. Levels were normalized using 18S RNA as control and a cycle number of 36 as reference. The numbers on the vertical line refer to the abundance of the indicated transcripts relative to an abundance for which 36 cycles are necessary for detection. 'R1' refers to unmodified mouse R1 embryonic stem (ES) cells; 'Pax4' refers to R1 mouse embryonic stem (ES) cells stably transfected with a CMV-Pax4 expression construct; 'insulin expression rel. to ∆Ct36' refers to expression of insulin in Fig. 3; 'control, supernatant 293 cells' refers to the differentiation protocol as described in Example 6, with the addition of supernatant of 293 cells without TIMP-2; 'TIMP-2 enriched supernatant' refers to the differentiation protocol as described in Example 7, with the addition of TIMP-2 enriched supernatant of 293 cells to differentiated cells.

**Fig. 4: Effect of TIMP-2 antisense oligonucleotides on the pancreatic development**

A 24 hr old zebrafish embryo, injected with control antisense oligonucleotides (upper panel, referred to as control) or TIMP-2 antisense oligonucleotides (lower panel, referred to as TIMP-2). Pancreatic islets are visualised by a marker protein (green). Note one bigger islet in the control antisense oligonucleotide injected and several smaller islets in TIMP-2 antisense oligonucleotide injected embryos (dispersed islet phenotype).
Fig. 5: TIMP-2 significantly lowers blood glucose levels in STZ-diabetic mice

Time course of non-fasted blood glucose levels in C57/Bl6 mice (mean ± SE). STZ-diabetes was induced at d0. Alzet osmotic minipumps containing carrier, EGF/Gastrin or TIMP-2 were implanted i.p. at d1 post STZ treatment, and were actively pumping for 1 week. Animals with carrier containing pumps developed hyperglycemia within 3 days after STZ-treatment (square; n = 12). A combination of EGF/Gastrin slightly decreased blood glucose levels to mild hyperglycemia (triangle; n = 12), while application of TIMP-2 resulted in significant reduction of blood glucose levels during early stages of treatment (circle; n = 12) compared to carrier treated animals (* p < 0.05). On d2 post STZ, these animals were not significantly different from untreated non-diabetic control animals (diamond; n = 12).

Figure 6: TIMP-2 significantly increases body weight in STZ-diabetic mice

Time course of body weight in C57/Bl6 mice (mean ± SE). Animals with carrier containing pumps gradually lost body weight and during the first week after STZ treatment, then started to recover slowly (square; n = 12), while EGF/Gastrin treated animals continuously lost body weight throughout the experiment (triangle; n = 12). In contrast to carrier treated animals, the application of TIMP-2 resulted in a significant increase in body weight during the treatment period (circle; n = 12; * p < 0.05). They did not reach body weight values of untreated non-diabetic control animals (diamond; n = 12).

Figure 7: TIMP-2 improves glucose tolerance in STZ-diabetic mice

Figure 7A. Intraperitoneal glucose tolerance test in C57/Bl6 mice on d5 post STZ. Blood glucose levels at t=0 were defined as 100% of each animal, respectively. Shown are relative values in percentage of basal (mean ± SE). Impaired glucose tolerance developed in carrier treated (square; n = 11) as well as in EGF/Gastrin treated animals (triangle; n = 10). Application of TIMP-2 resulted in improved glucose tolerance (circle; n = 12), which was identical to untreated non-diabetic control animals (diamond; n = 12).
**Figure 7B.** Intraperitoneal glucose tolerance test in C57/B16 mice on d12 post STZ. Blood glucose levels at t=0 were defined as 100% of each animal, respectively. Shown are relative values in percentage of basal (mean ± SE). Carrier treated animals still displayed impaired glucose tolerance (square; n = 8). Similar curves were obtained from EGF/Gastrin treated animals (triangle; n = 6). Application of TIMP-2 still improved glucose tolerance on d12 (circle; n = 12), which was not different from untreated non-diabetic control animals (diamond; n = 12; t > 15 min).

**Figure 8: TIMP-2 reduces diabetes incidence**

Pancreatic insulin content (mean ± SE) in C57/B16 mice on d13 post STZ. Shown is amount of insulin per amount of protein in total pancreas extracts. All diabetic animals had significantly reduced pancreatic insulin content compared to untreated non-diabetic control animals (first bar; n = 9). TIMP-2 treated animals (fourth bar; n = 9) had increased pancreatic insulin content compared to carrier (second bar; n = 8) or EGF/Gastrin treated animals (third bar; n = 6).

The examples illustrate the invention:

**Example 1: Identification of secreted factors expressed in pancreas**

A screen for secreted factors expressed in developing mouse pancreas was carried out according to methods known by those skilled in the art (see, for example Pera E.M. and De Robertis E.M., (2000) Mech Dev 96: 183-195) with several modifications.

Expression cDNA library:
embryogenesis, the innervation and vascularization of the pancreas can be observed. Therefore, the tissue used in the screen might have contained besides pancreatic cells some adipocyte precursors, blood vessels, as well as neuronal cells.

A mouse embryonic stage 9.5-15 pancreatic bud library was prepared in pCMVSPORT-6 vector using SUPERSCRIPT Plasmid System from Invitrogen according to the manufacturer's instructions. The non-amplified library was electroporated into MaxEff DH10B cells (Invitrogen).

Secretion cloning
Bacterial clones were picked with sterile toothpicks from agar plates and cultured in 96-deep-well microtiter plates in LB-ampicillin (see Sambrook et al., supra). Aliquots of 8 cultures were pooled, and plasmid DNA was isolated using the BioRobot_9600 apparatus according to the manufacturer's instructions (Qiagen; QIAprep(r) Turbo BioRobot Kit. Human 293 cell culture cells were cultured in 75 ml tissue culture flasks in DMEM and 10% fetal calf serum. At 90-99% confluence, the cells were splitted at 1:3 ratio and plated onto poly-D-lysine (Sigma) coated 96-well plates. Cells were transfected with 100-500 ng plasmid using lipofectamine 2000 (Invitrogen). After 6 hours, the medium was exchanged for fresh complete growth medium. 24 hours after transfection, the cells were washed twice with DMEM without cysteine and methionine (Invitrogen), supplemented with 1% dialysed Bovine serum (Sigma) with 50 microgram per ml Heparin (Sigma) and glutamine. The cells were labeled radioactively ('S35 Met-label', from Hartmann Analytic GmbH). After 12 hours, aliquots of the supernatants were harvested in 96-well PCR plates and subjected to SDS gel electrophoresis in precast, 20% gradient polyacrylamide Criterion gels (Biorad) under reducing conditions, using Criterion Dodeca Cell gel running chamber (Biorad). The gels were fixed in 10% acetic acid, 25% isopropanol for 30 min, soaked 15-30 min in AMPLIFY reagent (Amersham), dried and exposed to X-OMAT (AR) film (Kodak). Positive clones were identified and regrown in 96-well-plates. DNA of individual clones was prepared and used for transfection as described above.
If one of the clones yielded proteins of the same size as that of the original pool, a positive clone was identified. Positive clones were partially sequenced from the 5' end (SEQLAB, Goettingen).

**Example 2: Identification of the human TIMP-2 homologous nucleic acids and proteins**

The term "polynucleotide comprising the nucleotide sequence as shown in GenBank Accession number" relates to the expressible gene of the nucleotide sequences deposited under the corresponding GenBank Accession number. The term "GenBank Accession number" relates to NCBI GenBank database entries (Ref.: Benson et al., Nucleic Acids Res. 28 (2000) 15-18).

TIMP-2 homologous proteins and nucleic acid molecules coding therefore are obtainable from insect or vertebrate species, e.g. mammals or fish. Particularly preferred are nucleic acids and proteins encoded thereby comprising human TIMP-2 homologs. The following mouse sequence was identified in the secreted factor screen: *Mus musculus* tissue inhibitor of metalloproteinase 2 (Timp2), GenBank Accession Number NM_011594 (3624 base pairs mRNA) and GenBank Accession Number NP_035724 (220 amino acid protein).

Sequences homologous to mouse TIMP-2 were identified using the publicly available program BLASTP 2.2.3 of the non-redundant protein database of the National Center for Biotechnology Information (NCBI) (see, Altschul et al., 1997, Nucleic Acids Res. 25: 3389-3402). The best human homolog of mouse TIMP-2 is *Homo sapiens* tissue inhibitor of metalloproteinase 2 (TIMP2) gene, GenBank Accession Number NM_003255 (1075 base pairs mRNA; SEQ ID NO: 1; see Fig. 1A) and GenBank Accession Number NP_003246 (220 amino acid protein; SEQ ID NO: 2; see Fig. 1B).

To identify possible zebrafish orthologues of human and mouse TIMP-2, sequence databases (NCBI non redundant protein database [ftp://ftp.ncbi.nih.gov/blast/db/], EST section of NCBI GenBank (see Boguski
et al., 1993, Nat Genet. 4: 332-333), dbEST--database for "expressed sequence tags", and zebrafish genome draft assembly 2 [http://www.ensembl.org/Danio_rerio/] were searched using the blastall program (version 2.2.6, Altschul et al. 1997, supra). Starting from the blast hits candidate genes were assembled and translated as necessary using the programs genewise (version 2.2.0, see http://www.ebi.ac.uk/Wise2/), getorf, est2genome and showseq (from the EMBOSS package version 2.7.1, see http://www.hgmp.mrc.ac.uk/Software/EMBOSS/). The resulting candidate protein sequences were compared to similar mouse and human proteins in multiple alignments made with the ClustalW program (version 1.83, see Thompson et al., 1994, Nucleic Acids Research, 22: 4673-4680) to verify the homology to mouse TIMP-2. If available from the assembly data, translation start sites were selected for antisense oligonucleotides targeting. Otherwise splice donor sites identified by alignment of zebrafish EST data or mouse protein data to zebrafish genomic sequence were used for antisense oligonucleotides targeting.

Example 3: Expression of TIMP-2 in mammalian adipocytes

For analyzing the role of the protein disclosed in this invention in the in vitro differentiation of different mammalian cell culture cells for the conversion of pre-adipocytes to adipocytes, mammalian fibroblast (3T3-L1) cells (e.g., Green & Kehinde, Cell 1: 113-116, 1974) were obtained from the American Tissue Culture Collection (ATCC, Hanassas, VA, USA; ATCC-CL 173). 3T3-L1 cells were maintained as fibroblasts and differentiated into adipocytes as described in the prior art (e.g., Qiu et al., J. Biol. Chem. 276: 11988-11995, 2001; Slieker et al., BBRC 251: 225-229, 1998). In brief, cells were plated in DMEM/10% FCS (Invitrogen, Karlsruhe, Germany) at 50,000 cells/well in duplicates in 6-well plastic dishes and cultured in a humidified atmosphere of 5% CO₂ at 37°C. At confluence (defined as day 0: d0) cells were transferred to serum-free (SF) medium, containing DMEM/HamF12 (3:1; Invitrogen), fetuin (300 μg/ml; Sigma, Munich, Germany), transferrin (2 μg/ml; Sigma), pantothenate (17 μM; Sigma), biotin (1 μM; Sigma), and EGF (0.8nM; Hoffmann-La Roche, Basel,
Switzerland). Differentiation was induced by adding dexamethasone (DEX; 1 μM; Sigma), 3-methyl-isobutyl-1-methylxanthine (MIX; 0.5mM; Sigma), and bovine insulin (5 μg/ml; Invitrogen). Four days after confluence (d4), cells were kept in SF medium, containing bovine insulin (5 μg/ml) until differentiation was completed. At two time points of the differentiation procedure, at day 0 (day of confluence) and day 12 of differentiation, suitable aliquots of cells were taken.

The adipose tissue was isolated according to standard procedures known to those skilled in the art, snap frozen in liquid nitrogen and stored at −80°C until needed.

RNA was isolated from human primary adipose tissue and cell culture cells using Trizol Reagent (for example, from Invitrogen, Karlsruhe, Germany) and further purified with the RNeasy Kit (for example, from Qiagen, Germany) in combination with an DNase-treatment according to the instructions of the manufacturers and as known to those skilled in the art. Total RNA was reverse transcribed (preferably using Superscript II RNaseH- Reverse Transcriptase, from Invitrogen, Karlsruhe, Germany) and subjected to microarray analysis.

The target preparation, hybridization and scanning was performed as described in the manufactures manual (see Affymetrix Technical Manual, 2002, obtained from Affimetrix, Santa Clara, USA).

The expression analysis of the TIMP-2 gene using 3T3L-1, SGBS, and primary human abdominal adipocyte differentiation clearly shows differential expression of murine and human TIMP-2 in adipocytes. Several independent experiments were done, respectively. The experiments show that the TIMP-2 transcripts are most abundant at day 0 compared to day 12 during differentiation (Fig. 2A-C).

Thus, the TIMP-2 protein has to be decreased in order for the preadipocytes to differentiate into mature adipocyte. The TIMP-2 protein in preadipocytes has the potential to inhibit adipose differentiation. Therefore, the TIMP-2...
protein might play an essential role in the regulation of human metabolism, in particular in the regulation of adipogenesis and thus it might be an essential role in pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome.

Example 4. Antisense-inhibition of TIMP-2 in zebrafish

To study the effect of TIMP-2 on pancreatic function, several experiments were performed in zebrafish. Zebrafish were raised, maintained, and crossed as described (see, Westerfield, 1995, The Zebrafish Book. Eugene, Oregon: Univ.of Oregon Press). Staging was performed according to Kimmel et al., 1995, Dev Dyn 203:253-310. Development of zebrafish embryos was carried out at 28°C. The age of embryos is indicated as hours post fertilization (hpf), the age of larvae as days post fertilization (dpf). Zebrafish carrying the transgene with insulin regulatory sequences linked to a marker protein cDNA were used for the experiments. As control, progeny of crosses between AB and TL strain fish (Westerfield et al., supra; http://www.zfin.org) were used for injections.

Zebrafish has two closely related TIMP-2 homologues. The following TIMP-2 specific antisense oligonucleotide sequences were used:
TIMP-2-1 5’-GAC ATA CTC ATC TTC AGC GGA CAC T-3’ (SEQ ID NO: 3) and TIMP-2-2 5’-CAA TAC AGC TCC TGA CGC TCT TCA T-3’(SEQ ID NO: 4).

TIMP-2 or control antisense oligonucleotides were injected into fertilized one-cell stage embryos as described (see, for example, Nasevicius & Ekker, 2000, Nat Genet 26: 216–220; Utrishak et al., 2003, Dev Dyn. 228: 405-413). Images of zebrafish embryos were taken using a MZFLIII stereomicroscope (Leica) equipped with epifluorescence.

Figure 4 shows the effect of loss-of-function of TIMP-2 on the development of pancreatic islets. The formation of 2 or more smaller islets (dispersed islet
phenotype) was observed after injection of TIMP-2 antisense oligonucleotides.

Example 5: Generation of ES cells expressing the Pax4 gene.

Mouse R1 ES cells (Nagy et al., (1993) Proc. Natl. Acad. Sci. USA 90: 8424-8428) were electroporated with the Pax4 gene under the control of the CMV promoter and the neomycin resistance gene under the control of the phosphoglycerate kinase I promoter (pGK-1).

ES cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10⁻⁴ M beta-mercaptoethanol, 2 nM glutamine, 1% non-essential amino acids, 1 nM Na-pyruvate, 20% FCS and 500 U/ml leukaemia inhibitory factor (LIF). Briefly, approximately 10⁷ ES cells resuspended in 0.8 ml phosphate buffered saline (PBS) were subjected to electroporation with 25 µg/ml of linearized expression vector (Joyner, Gene Targeting: A Practical Approach, Oxford University Press, New York, 1993). Five minutes after electroporation, ES cells were plated on petri dishes containing fibroblastic feeder cells previously inactivated by treatment with 100 µg/ml mitomycin C. One day after electroporation, culture medium was changed to medium containing 450 µg/ml G418. Resistant clones were separately isolated and cultured 14 days after applying the selection medium. Cells were always cultured at 37°C, 5% CO₂. These untreated and undifferentiated ES cells were used as control the experiment.

Example 6: Differentiation of ES cells into insulin-producing cells (referred to as 'control, supernatant 293 cells' in Fig. 3)

The ES cell line R1 (wild type, 'R1' in Fig. 3) and ES cells constitutively expressing Pax4 (‘Pax4’ in Fig. 3) were cultivated as embryoid bodies (EB) by the hanging drop method, as described in patent application PCT/EP02/04362, published as WO 02/086107, which is incorporated herein by reference, with media as described below. The embryoid bodies were
allowed to form in hanging drop cultures for 2 days and then transferred for three days to suspension cultures in petri dishes. At day 5, EBs were plated separately onto gelatin-coated 6 cm cell culture dishes containing a differentiation medium prepared with a base of Iscove modified Dulbecco's medium. After dissociation and replating at day 14 cells were cultured up to 40 days in the differentiation medium prepared with a base of Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) with the addition of supernatant of 293 cells without TIMP-2.

Example 7: Expression of pancreas specific genes after differentiation of ES cells into insulin-producing cells

Expression levels of pancreas specific genes was measured by Taqman analysis. Total RNA was isolated from undifferentiated R1 and Pax4+ ES cells (control ES cells) at day 0 and differentiated R1 ES and Pax4+ ES cells at day 40 and further purified with the RNeasy Kit (for example, from Qiagen, Germany) in combination with an DNase-treatment according to the instructions of the manufacturers and as known to those skilled in the art. Total RNA was reverse transcribed (preferably using Superscript II RNaseH-Reverse Transcriptase, from Invitrogen, Karlsruhe, Germany) and subjected to Taqman analysis preferably using the Taqman 2xPCR Master Mix (from Applied Biosystems, Weiterstadt, Germany; the Mix contains according to the Manufacturer for example AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference Rox and optimized buffer components) on a GeneAmp 5700 Sequence Detection System (from Applied Biosystems, Weiterstadt, Germany).

Results show that markers for beta-cell differentiation function were expressed at higher levels in Pax4+ differentiated ES cells than in differentiated wild type ES cells demonstrating that activation of a pancreatic developmental control gene renders differentiation more efficient than for wild type ES cells (Fig. 3). Expression of substantial amounts of insulin in differentiated stem cells indicates that differentiated cells show a phenotype
similar to beta-cells.

**Example 8: Induction of differentiation of insulin-producing cells by TIMP-2 (referred to as 'TIMP-2-enriched supernatant' in Fig. 3)**

In order to study the effect of TIMP-2 to induce beta-cell differentiation in vitro, stable mouse embryonic stem (ES) cells expressing the Pax4 under the control of the cytomegalovirus (CMV) early promoter/enhancer region were generated as described in Example 5. Pax4 and wild type ES cells were then cultured in hanging drops or spinner cultures to allow the formation of embryoid bodies. Embryoid bodies were subsequently plated, enzymatically dissociated, and replated. After dissociation, cells were cultured in a differentiation medium containing various growth factors. Additionally TIMP-2 enriched supernatant of 293 cells was added every second day until day 40. Under such conditions, the expression of insulin was induced by TIMP-2 (Fig. 3). By comparison, untreated ES cells did contain only very small numbers of insulin-producing cells at the same stage. These data demonstrate that TIMP-2 can significantly promote and enhance ES cells differentiation into insulin-producing cells compared to untreated ES cells.

The results shown in Figure 3 clearly demonstrate a significant induction of the differentiation of insulin-producing cells, if TIMP-2 is added at later stages of differentiation. Thus, TIMP-2 has a strong stimulative effect on the differentiation of insulin-producing beta cells.
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Example 9: Functional characterisation of the differentiated insulin-producing cells

One important property of beta-cells is glucose responsive insulin secretion. To test whether the Pax4 derived insulin-producing cells possessed this glucose responsive property, an *in vitro* glucose responsive assay can be performed on the differentiated cells. On the day of the assay, the differentiation medium of 12 or 6 well plate is removed and the cells are washed 3 times with Krebs Ringer Bicarbonate Hepes Buffer (KRHB; 125 mM NaCl, 4.74 mM KCl, 1 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 25 mM Hepes, pH 7.4 and 0.1% BSA) supplemented with 2.8 mM Glucose. For Preincubation cells were incubated in KRHB + 2.8 mM Glucose for 2 hours at 37°C. Afterwards cells were incubated in 500 ml KRHB + 2.8 mM Glucose for 1 hour and the supernatant is then kept for measurement of basal insulin secretion. For the stimulated insulin release 500 ml KRHB containing 27.7 mM glucose is added to the cells. After 1 hour incubation at 37°C, the KRHB is recovered for measurement of glucose-induced insulin secretion and the cells were extracted with acid-ethanol. (see also Irminger, J.-C. et al., 2003, Endocrinology 144: 1368-1379). Insulin levels can be determined by an Enzyme-Linked Immunosorbent Assay (ELISA) for mouse insulin (Mercodia) and performed according to the manufacturer's recommendations.

Example 10: Transplantation of Pax4 ES derived insulin-producing cells in STZ diabetic mice

The therapeutic potential of TIMP-2 induced insulin-producing cells to improve and cure diabetes can be investigated by transplanting the cells into streptozotocin induced diabetic mice. Streptozotocin is an antibiotic which is cytotoxic to beta-cells when administered at certain dosage (see Rodrigues et al.: Streptozotocin-induced diabetes, in McNeill (ed) Experimental Models of Diabetes, CRC Press LLC, 1999). Its effect is rapid, rendering an animal severely diabetic within 48 hours.
Non-fasted Male BalbC mice can be treated with STZ to develop hyperglycaemia after STZ treatment. Mice are considered diabetic if they have a blood glucose level above 10 mmol/l for more than 3 consecutive days. Cells are transplanted under the kidney capsule and into the spleen of animals. The presence of the insulin-producing cells can be confirmed by immunohistological analysis of the transplanted tissue. Results are expected to demonstrate that the transplanted cells can normalise blood glucose in diabetic animals.

Example 11. Effects of TIMP-2 in adult C57/Bl6 mice with moderate Type 1 Diabetes

At 9 weeks of age, male C57Bl6 mice received i.p. 120 mg/kg body weight (BW) of streptozotocin (STZ) after overnight fasting (= day 0). Alzet osmotic minipumps (model 1007D) releasing human EGF (38.7 nmol/kg BW/d; Sigma) and rat gastrin I 60 nmol/kg/d; Sigma), human TIMP-2 (0.218 nmol/kg/d; R&D Systems) or carrier (saline, 0.01% BSA) for one week were implanted i.p. at day 1 (d1) post STZ treatment in groups of 12 animals each. The study has been carried out according to the ‘Principles of laboratory animal care’.

Non-fasted blood glucose levels (BG) from the tail vein and BW were monitored daily. Animals with non-fasted blood glucose levels above 300 mg/dL were defined as ‘diabetic’. Fasted blood glucose levels were determined on d0, d5 and d12. Intraperitoneal glucose tolerance tests (IPGTT) were performed after a 7h fasting period on d5 and d12. At t = 0, 1.5 g glucose/kg bodyweight was injected i.p. Blood glucose levels were determined after 15, 30, 60, 90, 120 and 180 min.

After completion of factor treatment on d8, pancreata of selected mice were fixed in parafomaldehyde and embedded in paraffin for Haematoxilin-Eosin staining, using standard protocols. Immunofluorescence was performed on paraffin sections, using C-peptide and Ki67 as primary antibodies for islet
staining and proliferation marker, respectively. Ducts and nucleus were stained with markers as known to those skilled in the art.

On d13, blood was collected by retroorbital bleeding. Pancreatic insulin was extracted in acid alcohol using standard protocols, followed by insulin ELISA. Protein concentrations were obtained using the principles of Bradford assay.

**TIMP-2 significantly lowers blood glucose levels in STZ-diabetic mice**

Non-fasted blood glucose levels of mice treated with TIMP-2 were significantly lower on d3 and d4 post STZ than those of mice treated with carrier only (d3: 219.5 mg/dl vs. 344.3 mg/dl; d4: 229.6 mg/dl vs. 355.2 mg/dl, p < 0.05, respectively). On d2, blood glucose levels of TIMP-2 treated mice were not significantly different from non-diabetic control animals. EGF/Gastrin treated mice displayed similar blood glucose levels like TIMP-2 treated animals, but were not significantly different from carrier treated mice (Fig. 5). TIMP-2 treatment resulted in lower fasted blood glucose levels on d5 and d12 compared to carrier treatment.

**TIMP-2 significantly increases body weight in STZ-diabetic mice**

All animals lost body weight on d0, since STZ was applied in the fasted stage. Animals gained body weight during TIMP-2 treatment, while carrier and EGF/Gastrin treated mice did not (TIMP-2 vs carrier: d3, d5, d6: p < 0.01; d4, d7, d8: p < 0.05; TIMP-2 vs EGF/Gastrin: d6, d8, d9, d11: p < 0.05; d10, d13: p < 0.01; Fig. 6). All TIMP-2 treated mice survived, while 3/12 EGF/Gastrin and 1/12 carrier treated mice died (EGF/Gastrin: d2, d3, d11; carrier: d4).

**TIMP-2 improves glucose tolerance in STZ-diabetic mice**

In early stages of STZ-diabetes (d5 post STZ), when the implanted pump was still active, TIMP-2 treated mice displayed improved absolute glucose levels during IPGTT compared to carrier treated mice. In order to compare the different treatment groups, blood glucose levels at t=0 were defined as 100% for each group, respectively. Mice with TIMP-2 containing pumps had
identical relative blood glucose levels as control animals on d5 (Fig. 7A). On d12, when factor treatment was completed, TIMP-2 treated mice still had slightly improved relative blood glucose levels compared to carrier treated mice, while there was no significant difference to control animals (Fig. 7B).

TIMP-2 reduces diabetes incidence
Diabetes incidence was reduced in TIMP-2 treated mice compared to carrier treated mice (6/12 vs 9/12). Therefore, pancreatic insulin content on d13 showed increased levels in TIMP-2 treated mice compared to carrier treated mice (Fig. 8, respectively).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.
Claims

1. A pharmaceutical composition comprising a TIMP-2 secreted protein and/or a functional fragment thereof, a nucleic acid molecule encoding a TIMP-2 protein and/or a functional fragment thereof and/or an effector/modulator of said protein or protein fragment or said nucleic acid molecule.

2. The composition of claim 1, wherein the composition contains pharmaceutically acceptable carriers, diluents, and/or additives.

3. The composition of claim 1 or 2, wherein the nucleic acid molecule is a mammalian TIMP-2 nucleic acid as shown in SEQ ID NO: 1, particularly encoding the human TIMP-2 polypeptide as shown in SEQ ID NO: 2 and/or a nucleic molecule which is complementary thereto or a fragment thereof or a variant thereof.

4. The composition of any one of claims 1-3, wherein the nucleic acid molecule is a DNA molecule, particularly a cDNA or a genomic DNA.

5. The composition of any one of claims 1-4, wherein said nucleic acid encodes a polypeptide contributing to regulating the metabolism, in particular human metabolism.

6. The composition of any one of claims 1-5, wherein said nucleic acid molecule is a recombinant nucleic acid molecule.

7. The composition of any one of claims 1-6, wherein the nucleic acid molecule is a vector, particularly an expression vector.

8. The composition of any one of claims 1-7, wherein the polypeptide is a recombinant polypeptide.
9. The composition of claim 8, wherein said recombinant polypeptide is a fusion polypeptide.

10. The composition of any one of claims 1-9 which is a therapeutic composition.

11. The composition of any one of claims 1-10 for administration
   i) directly to the pancreas,
   ii) via implantation of TIMP-2 protein product expressing cell and/or
   iii) via gene therapy

12. The composition of any one of claims 1-11 for the manufacture of an
    agent for detecting and/or verifying, for the treatment, alleviation and/or
    prevention of pancreatic diseases (e.g. diabetes such as diabetes
    mellitus type I and/or late stages of diabetes mellitus type 2 or LADA),
    obesity, metabolic syndrome and/or other metabolic diseases or
    dysfunctions.

13. The composition of any one of claims 1-12 for the manufacture of an
    agent for the modulation of pancreatic development.

14. The composition of any one of claims 1-13 for the manufacture of an
    agent for the regeneration of pancreatic tissues or cells, particularly
    pancreatic islet cells, preferably beta cells.

15. The composition of any one of claims 1-14 for application in vivo.

16. The composition of claim 15 for administration to a patient who is to
    receive or has received transplantation of pancreatic tissue.

17. The composition of any one of claims 1-15 for application in vitro or ex
    vivo.
18. The composition of claim 17 for administration to beta cells or progenitor cells.

19. The composition of claim 18 for generating replacement material for dysfunctional and/or destroyed beta cells.

20. The composition of claim 18 or 19 for the manufacture of a transplantable beta cell preparation.

21. The composition of any one of claims 1-20 in combination with an immunosuppressive agent, which is preferably selected from the compounds as shown in Table 1 or combinations thereof, particularly from DiaPep277, anti-CD-3-antibodies and/or GAD peptides.

22. The composition of claim 21 in combination with at least one further beta cell mitogen and/or beta cell protective agent, particularly GLP-1 or derivatives thereof, exendin, prolactin, neurotrophins or combinations thereof.

23. Use of a TIMP-2 nucleic acid molecule or a polypeptide encoded thereby or a fragment or a variant of said nucleic acid molecule or said polypeptide and/or an effector/modulator of said nucleic or polypeptide for the manufacture of a medicament for the treatment of pancreatic diseases (e.g. diabetes such as diabetes mellitus type 1 and/or late stages of diabetes mellitus type 2 or LADA), obesity, metabolic syndrome and and/or other metabolic diseases or dysfunction for controlling the function of a gene and/or a gene product which is influenced and/or modified by a TIMP-2 polypeptide.

24. Use of a TIMP-2 nucleic acid molecule or use of a polypeptide encoded thereby, or use of a fragment or a variant of said nucleic acid molecule or said polypeptide, or use of an effector/modulator of said nucleic acid molecule or said polypeptide for identifying substances capable of
interacting with a TIMP-2 polypeptide in vitro and/or in vivo.


26. The animal of claim 25, wherein the expression of the TIMP-2 polypeptide is increased and/or reduced.

27. A recombinant host cell exhibiting a modified expression of a TIMP-2 polypeptide, or a recombinant host cell which comprises a nucleic acid molecule as defined in any one of claims 1 to 7.

28. The cell of claim 27 which is a human cell.


30. A method of identifying a (poly)peptide involved in the regulation of energy homeostasis and/or metabolism in a mammal comprising the steps of
   (a) contacting a collection of (poly)peptides with a TIMP-2 homologous polypeptide or a fragment thereof under conditions that allow binding of said (poly)peptides;
   (b) removing (poly)peptides which do not bind and
   (c) identifying (poly)peptides that bind to said TIMP-2 homologous polypeptide.

31. A method of screening for an agent which effects/modulates the regulation of energy homeostasis and/or metabolism in a mammal comprising the steps of
   (a) incubating a mixture comprising
       (aa) a TIMP-2 polypeptide or a fragment thereof;
       (ab) a binding target/agent of said TIMP-2 polypeptide or fragment thereof; and
(a) a candidate agent under conditions whereby said polypeptide or fragment thereof specifically binds to said binding target at a reference affinity;
(b) detecting the binding affinity of said TIMP-2 polypeptide or fragment thereof to said binding target to determine an affinity for the agent; and
(c) determining a difference between affinity for the agent and reference affinity.

32. A method for screening for an agent, which effects/modulates the regulation of energy homeostasis and/or metabolism in a mammal, comprising the steps of
(a) incubating a mixture comprising
   (aa) a TIMP-2 polypeptide or a fragment thereof; and
   (ab) a candidate agent
   under conditions whereby said TIMP-2 polypeptide or fragment thereof exhibits a reference activity,
(b) detecting the activity of said TIMP-2 polypeptide or fragment thereof to determine an activity for the agent; and
(c) determining a difference between activity for the agent and reference activity.

33. A method of producing a composition comprising the (poly)peptide identified by the method of claim 30 or the agent identified by the method of claim 31 or 32 with a pharmaceutically acceptable carrier and/or diluent.

34. The method of claim 33 wherein said composition is a pharmaceutical composition for preventing, alleviating or treating of diseases and disorders, including pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome.
35. Use of a (poly)peptide as identified by the method of claim 30 or of an agent as identified by the method of claim 31 or 32 for the preparation of a pharmaceutical composition (i) for the treatment, alleviation and/or prevention of pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, (ii) for the modulation of pancreatic development and/or (iii) for the regeneration of pancreatic cells or tissues.

36. Use of a nucleic acid molecule as defined in any one of claims 1 to 7 for the preparation of a medicament (i) for the treatment, alleviation and/or prevention of diseases or dysfunctions, including pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, (ii) for the modulation of pancreatic development and/or (iii) for the regeneration of pancreatic cells or tissues.

37. Use of a polypeptide as defined in any one of claims 1 to 6, 8 or 9 for the preparation of a medicament (i) for the treatment, alleviation and/or prevention of pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, (ii) for the modulation of pancreatic development and/or (iii) for the regeneration of pancreatic cells or tissues.

38. Use of a vector as defined in claim 7 for the preparation of a medicament (i) for the treatment, alleviation and/or prevention of pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, (ii) for the modulation of pancreatic development and/or (iii) for the regeneration of pancreatic cells or tissues.

39. Use of a host cell as defined in claim 27 or 28 for the preparation of a medicament (i) for the treatment, alleviation and/or prevention of pancreatic diseases (e.g. diabetes), obesity, and/or metabolic syndrome, (ii) for the modulation of pancreatic development and/or (iii) for the regeneration of pancreatic cells or tissues.
40. Use of a TIMP-2 nucleic acid molecule or of a fragment thereof for the production of a non-human transgenic animal which over- or under-expresses the TIMP-2 gene product.

41. Kit comprising at least one of

(a) a TIMP-2 nucleic acid molecule or a functional fragment or an isoform thereof;
(b) a TIMP-2 amino acid molecule or a functional fragment or an isoform thereof;
(c) a vector comprising the nucleic acid of (a);
(d) a host cell comprising the nucleic acid of (a) or the vector of (c);
(e) a polypeptide encoded by the nucleic acid of (a), expressed by the vector of (c) or the host cell of (a);
(f) a fusion polypeptide encoded by the nucleic acid of (a);
(g) an antibody, an aptamer or another effector/modulator against the nucleic acid of (a) or the polypeptide of (b), (e), or (f) and/or
(h) an anti-sense oligonucleotide of the nucleic acid of (a).
Fig. 1A

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Fig. 1B
Fig. 2A

Fig. 2B
Fig. 3
Mean non-fasted blood glucose

Blood glucose (mg/dL)

- no STZ, no treatment (n = 12)
- STZ, carrier (n = 12)
- STZ, EGF/Gastrin (n = 12)
- STZ, TIMP-2 (n = 12)

Fig. 5
Fig. 6
IPGTT on d5: mean relative blood glucose

- no STZ, no treatment (n = 12)
- STZ, carrier (n = 11)
- STZ, EGF/Gastrin (n = 10)
- STZ, TIMP-2 (n = 12)

Fig. 7A

IPGTT on d12: Mean relative blood glucose

- no STZ, no treatment (n = 9)
- STZ, carrier (n = 8)
- STZ, EGF/Gastrin (n = 6)
- STZ, TIMP-2 (n = 9)

Fig. 7B
Mean pancreatic insulin/protein on d13

- no STZ no treatment, n = 9
- STZ carrier, n = 8
- STZ EGF/G., n = 6
- STZ TIMP-2, n = 9

Fig. 8
DeveloGen Aktiengesellschaft für entwicklungsbioiogische Forschung

Use of a TIMP-2 secreted protein product for preventing and treating pancreatic diseases and/or obesity and/or metabolic syndrome

33730P WO_GE

EP 04 018 751.0
2004-08-06
4
PatentIn version 3.3
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DNA
Homo sapiens

 gene
(1). (1075)
nucleic acid sequence of human TIMP-2 protein

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Ala Val Ser Glu Lys Glu Val Asp Ser Gly Asn Asp Ile Tyr Gly Asn
50 55 60
Pro Ile Lys Arg Ile Gln Tyr Glu Ile Lys Gln Ile Lys Met Phe Lys
65 70 75 80
Gly Pro Glu Lys Asp Ile Glu Phe Ile Tyr Thr Ala Pro Ser Ser Ala
85 90 95
Val Cys Gly Val Ser Leu Asp Val Gly Gly Lys Gly Glu Tyr Leu Ile
100 105 110
Ala Gly Lys Ala Ala Gly Asp Gly Lys Met His Ile Thr Leu Cys Asp
115 120 125
Phe Ile Val Pro Trp Asp Thr Leu Ser Thr Thr Gln Lys Lys Ser Leu
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Asn His Arg Tyr Gln Met Gly Cys Glu Cys Lys Ile Thr Arg Cys Pro
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Trp Val Thr Glu Asn Ile Asp Gly His Gln Ala Lys Phe Phe Ala
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Cys Ile Lys Arg Ser Asp Gly Ser Cys Ala Trp Tyr Arg Gly Ala Ala
195 200 205
Pro Pro Lys Gln Glu Phe Leu Asp Ile Glu Asp Pro
210 215 220
antisense oligonucleotide sequence

TIMP-2 specific antisense oligonucleotide sequence

catactca tttcagcgg acact

catacagct cctgacgctc ttcat


### INTERNATIONAL SEARCH REPORT

#### A. CLASSIFICATION OF SUBJECT MATTER

**IPC 7**  
A61K38/57  
A01K67/027  
G01N33/68

According to international Patent Classification (IPC) or to both national classification and IPC

#### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC 7**  
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

#### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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| X         | US 6 673 623 B1 (HUBERMAN ELIEZER)  
6 January 2004 (2004-01-06)  
column 3, line 15 - column 10, line 48 | 1-41                  |
| X         | WO 90/11287 A (US HEALTH)  
4 October 1990 (1990-10-04)  
cited in the application  
page 24, lines 7-11; claims | 1-41                  |

Further documents are listed in the continuation of box C.  
Patent family members are listed in annex.

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier document but published on or after the international filing date
  * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  * "O" document referring to an oral disclosure, use, exhibition or other means
  * "P" document published prior to the international filing date but later than the priority date claimed

**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**"R"** document member of the same patent family

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**Date of the actual completion of the international search**  
19 October 2005

**Date of mailing of the international search report**  
25/10/2005

**Name and mailing address of the ISA**  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70-340-2040), TX 31 651 epo nl,  
Fax: (+31-70-340-3016)

**Authorized officer**  
Durrenberger, A
INTERNATIONAL SEARCH REPORT

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 33–35 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

   see FURTHER INFORMATION sheet PCT/ISA/210

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

□ The additional search fees were accompanied by the applicant's protest.

□ No protest accompanied the payment of additional search fees.
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<td>RIGG ANNE S ET AL: &quot;Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect in vivo&quot; CANCER GENE THERAPY, vol. 8, no. 11, November 2001 (2001-11), pages 869-878, XP002313228 ISSN: 0929-1903 page 876, right-hand column, last paragraph - page 877</td>
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Continuation of Box II.2

Claims Nos.: 33-35

Present claims 33-35 relate to the use of compounds defined by reference to a desirable characteristic or property, namely their ability to modulate TIMP-2 activity. The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for these claims.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.
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<td>AU 634533 B2</td>
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