

(19) **DANMARK**

(10) **DK/EP 1885747 T3**



(12)

Oversættelse af
europæisk patentskrift

Patent- og
Varemærkestyrelsen

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- (51) Int.Cl.: **C 07 K 14/505 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2014-02-24**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2013-11-20**
- (86) Europæisk ansøgning nr.: **06753630.0**
- (86) Europæisk indleveringsdag: **2006-05-15**
- (87) Den europæiske ansøgnings publiceringsdag: **2008-02-13**
- (86) International ansøgning nr.: **EP2006004564**
- (87) Internationalt publikationsnr.: **WO2006120030**
- (30) Prioritet: **2005-05-13 EP 05010473**
- (84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR**
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- (54) Benævnelse: **ERYTHROPOIETINVARIANTER**
- (56) Fremdragne publikationer:
WO-A-2004/043382
WO-A-2005/103076
DATABASE GENESEQ [Online] EBI; 11 February 2000 (2000-02-11), XP002394132 Database accession no. AAY43803
ERBYRAKTAR S, ET AL.: "ASIALOERYTHROPOIETIN IS A NONERYTHROPOIETIC CYTOKINE WITH BROAD NEUROPROTECTIVE ACTIVITY IN VIVO" PNAS, vol. 100, no. 11, 27 May 2005 (2005-05-27), pages 6741-6746, XP009070746

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Description

Field of the Invention

[0001] The present invention relates to novel endogenous variants of erythropoietin (EPO) and their use for treatment or prevention of a condition associated with tissue damage due to cell death (apoptosis, necrosis) and inflammation, in particular for neuroprotection, e.g. treatment of acute (for example stroke) and chronic disease (for example ALS) of the nervous system.

Background of the Invention

[0002] Stroke is a debilitating disease which affects more than 400,000 persons per year in the United States and is the third most common cause of death in the United States. In addition one-half of neurology inpatients have stroke related problems. At current trends, this number is projected to jump to one million per year by the year 2050. When the direct costs (care and treatment) and the indirect costs (lost productivity) of strokes are considered together, strokes put a burden of \$43.3 billion per year on the society of the United States alone. About 1/3 of patients die in the first three months, 1/3 remain with severe disabilities, and only 1/3 recover with acceptable outcome. In 1990 cerebrovascular diseases were the second leading cause of death worldwide, killing over 4.3 million people world wide. Thus, from a public health perspective, stroke is one of the most relevant diseases.

[0003] Stroke is characterized by the sudden loss of circulation to an area of the brain, resulting in a corresponding loss of neurologic function. Also called cerebrovascular accident or stroke syndrome, stroke is a nonspecific term encompassing a heterogeneous group of pathophysiologic causes, including thrombosis, embolism, and hemorrhage. Strokes currently are classified as either hemorrhagic or ischemic. Acute ischemic stroke refers to strokes caused by thrombosis or embolism and account for 80% of all strokes.

[0004] Ischemic strokes result from blockage of the arteries that supply the brain, most commonly in the branches of the internal carotid arteries. The blockage usually results when a piece of a blood clot (thrombus) or of a fatty deposit (atheroma) due to atherosclerosis breaks off (becoming an embolus), travels through the bloodstream, and lodges in an artery that supplies the brain. Blood clots may form when a fatty deposit in the wall of an artery ruptures. The rupture of such a fatty deposit may also form when a large fatty deposit slows blood flow, reducing it to a trickle. Blood that flows slowly is more likely to clot. Thus, the risk of a clot forming in and blocking a narrowed artery is high. Blood clots may also form in other areas, such as in the heart or on a heart valve. Strokes due to such blood clots are most common among people who have recently had heart surgery and people who have a heart valve disorder or an abnormal heart rhythm (arrhythmia), especially atrial fibrillation. Also, in certain disorders such as an excess of red blood cells (polycythemia), the risk of blood clots is increased because the blood is thickened.

[0005] An ischemic stroke can also result, if the blood flow to the brain is reduced, as may occur when a person loses a lot of blood or has very low blood pressure. Occasionally, an ischemic stroke occurs when blood flow to the brain is normal but the blood does not contain enough oxygen. Disorders that reduce the oxygen content of blood include severe anemia (a deficiency of red blood cells), suffocation, and carbon monoxide poisoning. Usually, brain damage in such cases is widespread (diffuse), and coma results. An ischemic stroke can occur, if inflammation or infection narrows blood vessels that supply the brain. Similarly, drugs such as cocaine and amphetamines can cause spasm of the arteries, which can lead to a narrowing of the arteries supplying the brain to such an extent that a stroke is caused.

[0006] The brain requires glucose and oxygen to maintain neuronal metabolism and function. The inadequate delivery of oxygen to the brain leads to a hypoxia and ischemia results from insufficient cerebral blood flow. The consequences of cerebral ischemia depend on the degree and the duration of reduced cerebral blood flow. Neurons can tolerate ischemia for 30-60 minutes. If flow is not re-established to the ischemic area, a series of metabolic processes ensue. The neurons become depleted of ATP and switch over to anaerobic glycolysis, a much less efficient pathway. Lactate accumulates and the intracellular pH decreases. Without an adequate supply of ATP, ion pumps in the plasma membrane fail. The resulting influx of sodium, water, and calcium into the cell causes rapid swelling of neurons and glial cells. Membrane depolarization also stimulates the massive release of the amino acids glutamate and aspartate, both of which act as excitatory neurotransmitters in the brain. Glutamate further activates sodium and calcium ion channels in the neuronal cell membrane namely the well characterized N-methyl-D-aspartate (NMDA) calcium channel. Excessive calcium influx causes the disordered activation of a wide range of enzyme systems (proteases, lipases, and nucleases). These enzymes and their metabolic products, such as oxygen free radicals, damage cell membranes, genetic material, and structural proteins in the neurons, ultimately leading to the cell death of neurons (Dirnagl, U. et al. (1999) Trends Neurosci. 22: 391-397).

[0007] Strokes begin suddenly, develop rapidly, and cause death of brain tissue within minutes to days. In the ischemic brain, we commonly distinguish two tissue volumes - the core of the infarction and the surrounding zone, known as ischemic penumbra - the underperfused and metabolically compromised margin surrounding the irrevocably damaged

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core. Core and penumbra are characterized by two different kinds of cell death: necrosis and apoptosis (which is also called programmed cell death or delayed neuronal cell death). The severe perfusion deficit in the core causes a breakdown of metabolic processes, cellular energy supply and ion homeostasis, which causes the cells to lose their integrity within minutes. Thus, acute necrosis of cell and tissue prevails in the core. In the penumbra, some residual perfusion is maintained by collateral vessels, which may be unable to maintain the full functional metabolism, but prevents immediate structural disintegration. However, over time (hours to several days), the alteration of cellular homeostasis causes more and more cells to die, and the volume of the infarction increases. The penumbra has thus to be considered as tissue at risk during the maturation of the infarct. In this region, apoptosis and inflammatory signaling cascades play an important role. It may initially constitute 50% of the volume that will end up as infarction. The mechanisms that lead to delayed cell death provide targets for a specific neuroprotective therapy in brain regions challenged by ischemia, but which are still viable.

[0008] Therapeutic options so far are highly disappointing: Thrombolysis with rtPA, the only therapy with proven efficacy in a major clinical trial (NINDS), is only effective within a three hour time window, limiting its application to only a few percent of patients with ischemic stroke. In other words, besides basic supportive therapy, at present more than 95 % of strokes cannot be treated specifically. This is in sharp contrast to our knowledge concerning the basic pathophysiology of this disease, which has emerged over the last decade. In particular, extensive knowledge has accumulated on mechanisms of parenchymal brain damage and endogenous neuroprotection, as well as functional and structural reorganization.

[0009] Recently, attention has focused on potential therapeutic roles for endogenous brain proteins possessing neuroprotective properties. EPO, a glycoprotein hormone produced primarily by cells of the peritubular capillary endothelium of the kidney, which is a member of the growth hormone/prolactin cytokine family (Zhu Y. and D'Andrea A.D. (1994) *Curr. Opin. Hematol.* 1: 113-118) is a promising candidate. Although EPO was first characterized and is now widely known for its role as a haematopoietic hormone the detection of EPO and its receptor (EPOR) in rodent and human brain tissue as well as in cultured neurons and astrocytes expanded the search for other biological roles of EPO.

[0010] In the brain, a paracrine EPO/(Epo-R)₂ system exists independent of the endocrine system of adult erythropoiesis; neurones express (Epo-R)₂ and astrocytes produce EPO (Ruscher et al. (2002) *J. Neurosci.* 22, 10291-301; Prass et al. (2003) *Stroke* 34,1981-1986). It was demonstrated *in vitro* and *in vivo* that EPO is a potent inhibitor of neuronal apoptosis induced by ischemia and hypoxia (Ruscher et al. (2002) *J. Neurosci.* 22, 10291-301; Bernaudin, M., et al. (1999) *J Cereb Blood Flow Metab.* 19: 643-51; Morishita, E., et al. (1997) *Neuroscience.* 76: 105-16). It was reported by several groups that addition of EPO to neuronal cultures protects against hypoxic and glutamic acid toxicity (Henn F.A. and Braus D.F. (1999) *Eur. Arch. Psychiatry Clin. Neurosci.* 249: 48-56, Vogeley K. et al. (2000) *Am. J. Psychiatry* 157: 34-39) and reduces neurologic dysfunction in rodent models of stroke (Brines M.L. et al. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97: 10526-10531 and Bernaudin et al. (1999) *J. Cereb. Blood Flow Metab.* 10: 643-651). The promising results of these experiments have been corroborated in human studies wherein it was shown that EPO therapy for acute stroke is safe and might be beneficial (Ehrenreich H. et al. (2002) *Mol. Medicine* 8: 495-505) and WO 00/35475 A2. These cell and more particular neuroprotective properties of EPO have led to further research in this area to substantiate these findings in larger trial and the use of EPO is now proposed in other indication as well including, for example, schizophrenia (Ehrenreich H et al. (2004) *Molecular Psychiatry* 9: 42-54 and WO 02/20031 A2).

[0011] For the application of EPO to prevent tissue damage the hematopoietic activity is often not required and might be detrimental if large amounts of EPO are administered to treat or ameliorate the effects of, hypoxia or ischemia induced tissue damage. Therefore, attempts have been made to create EPO variants, which only exhibit the cell protective property but not the hematopoietic properties. US 2003/0130197 describes peptide mimetics of EPO for the treatment of neurodegenerative disorders, which bear no sequence homology to naturally occurring EPO or fragments thereof. US 6,531,121 discloses a asialoerythropoietin which is generated by complete desialylation of recombinant EPO showed an increased ability to cross the endothelial cell barrier and had a decreased hematopoietic activity. Carbamylated erythropoietin (CEPO) was also shown to exhibit a tissue protective effect but no erythropoietic effect (Leist et al. (2004) *Science* 305: 239-242 and WO 2005/025606 A1).

[0012] WO 2004/043382 discloses human erythropoietin polypeptide variants having altered erythropoietic activity. The disclosed EPO variants contain amino acid differences in two or more different EPO-modification regions compared to wild-type human EPO sequences. Such regions include the carbohydrate content region (A30, H32, P87, W88, P90), the aggregation region (N24, N38, N83), and the EPOR binding region (T44, T48, N147, and L155). In some of the disclosed variants, the erythropoietic activity was shown to be decreased, or the time required to reach the maximal level of such activity was extended. However, there is no teaching or suggestion of any EPO variants whose hematopoietic activity is essentially removed while the cell or neuroprotective activity is retained. Finally, it was shown that a 17-mer peptide of EPO inhibited cell death of two neuronal cell lines, SK-N-MC and NS20Y (Campana W.M. et al. (1998) *Int. J. Mol. Medicine* 1: 235-241), while at the same time having no hematopoietic activity. However, 1 ng/ml of the EPO peptide was needed to elicit the same antiapoptotic effect as 100 pg/ml recombinant EPO (rhEPO) in NS20Y cells and as 400 pg/ml rhEPO in SK-N-MC cells. Given the apparent molecular weight of rhEPO of about 66.000 g/mol (the

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calculated molecular weight is about 33.000 g/mol but does not include the weight of oligosaccharide residues comprised in rhEPO) and of about 1.900 g/mol of the EPO peptide a concentration of 1,52 pmol/l and 6,06 pmol/l, respectively, of rhEPO and 1 nmol/l of the EPO peptide elicited the same level of a cell protective effect. Consequently, the EPO peptide is between 650-fold to 165-fold less active than rhEPO in prevention of cell death. It is evident from this figures that the EPO region comprised in the 17-mer does not play a major role in the cell protective function of EPO. Therefore, all EPO variants, which have a decreased hematopoietic activity known in the prior art suffer from the disadvantage that they are not natural occurring since they have either lost their natural glycosylation or they are artificial truncations and/or they have vastly diminished cell protective activity, if compared to rhEPO. Therefore, there is a need in the prior art to provide an EPO derivative, which is close to the naturally occurring EPO and which has the same or better tissue protecting activity as rhEPO but less or no hematopoietic, in particular no erythropoietic activity.

[0013] This problem is solved by the provision of new EPO variants, which were found to occur naturally in human and mouse tissue (brain, kidney) and which exhibit a cell protective activity similar or better to rhEPO but which do not exhibit any significant hematopoietic activity.

Summary of the Invention

[0014] In one aspect the present invention is concerned with an EPO variant encoding polynucleotide selected from the group consisting of:

- (a) polynucleotides encoding the mature form of the polypeptides termed hs3, h1-4, h1-5, hs4, h1-1, h2-1, mS, mG3, mG5, m301, mK3, ha, hAma, hAmE, and hA-10 having the deduced amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 50, 51, 52, and 53, respectively;
- (b) a polynucleotide encoding the mature form of the polypeptide termed ha-sequence without leader consisting of the deduced amino acid sequence as shown in SEQ ID NO: 61;
- (c) polynucleotides having the coding sequence, as shown in SEQ ID NOs: 1,3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 55, 56, 57, and 58 encoding at least the mature form of the polypeptide;
- (d) a polynucleotide consisting of the coding sequence, as shown in SEQ ID NO: 60 encoding the mature form of the polypeptide termed ha-sequence without leader;
- (e) polynucleotide encoding a humanized version of the polypeptides mS, mG3, mG5, m301 and mK3 consisting of the deduced amino acid sequence as shown in SEQ ID NOs: 14, 16, 18, 20, and 22;
- (f) polynucleotides encoding a polypeptide comprising a fusion of an amino acid sequence selected from the group of amino acid sequences as shown in SEQ ID NO: 24, 26, 28, and 30, at the N-terminus of an amino acid sequence selected from the group of amino acid sequences as shown in SEQ ID NO: 32, 34, 36, and 38, wherein said fusion has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (g) polynucleotides comprising a fusion of polynucleotide sequences selected from the group of polynucleotide sequences as shown in SEQ ID NO 23, 25, 27, and 29, 5' of a polynucleotide sequence selected from the group of polynucleotide sequences as shown in SEQ ID NO: 31, 33, 35, and 37, wherein said fusion has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (h) polynucleotides encoding a derivative of a polypeptide encoded by a polynucleotide of any one of (a) to (g), wherein in said derivative between 1 and 10 amino acid residues are conservatively substituted compared to said polypeptide, and said derivative has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (i) polynucleotides encoding a fragment of n polypeptide encoded by a polynucleotide of any one of (a) to (h), wherein in said fragment between 1 and 10 amino acid residues are N-and/or C-terminally deleted and/or between 1 and 10 amino acids are deleted N- and/or C-terminally of the junction compared to said polypeptide, and said fragment has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (j) polynucleotides which are at least 95% identical to a polynucleotide as defined in any of (a) to (d) and which at the same time have cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (k) polynucleotides the complementary strand of which hybridizes under stringent conditions to a polynucleotide as defined in any one of (a) to (j) and which code for a polypeptide having cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;
- (l) polynucleotides encoding an EPO variant polypeptide, which comprises the N-terminal part of full length EPO including helix A and which lacks at least one of the following:

- (i) a fragment of at least 10 amino acids between helix A and helix B;
- (ii) a fragment of at least 10 amino acids of helix B;
- (iii) a fragment of at least 6 amino acids between helix B and helix C;
- (iv) a fragment of at least 10 amino acids of helix C;

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(v) a fragment of at least 20 amino acids between helix C and D; and/or

(vi) a fragment of at least 10 amino acids of helix D;

wherein said variant has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity.

(m) polynucleotides encoding a derivative of a polypeptide encoded by a polynucleotide of any one of (l), wherein in said derivative between 1 and 10 amino acid residues are conservatively substituted compared to said polypeptide, and said derivative has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

(n) polynucleotides the complementary strand of which hybridizes under stringent conditions to a polynucleotide as defined in any one of (l) to (m) and which code for a polypeptide having cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

or the complementary strand of such a polynucleotide.

[0015] A further aspect of the present invention is a homolog of an erythropoietin (EPO) variant encoding polynucleotides from another higher eukaryotic species.

[0016] In a preferred aspect the polynucleotide of the present invention which is DNA, genomic DNA or RNA.

[0017] In another aspect the present invention is concerned with a vector containing the polynucleotide of the present invention. It is preferred that the polynucleotide contained in the vector is operatively linked to expression control sequences allowing expression in prokaryotic and/or eukaryotic host cells.

[0018] Another aspect of the invention is a host cell genetically engineered with the polynucleotide of the present invention or the vector of the present invention.

[0019] Another aspect of the invention is a transgenic non-human mammal selected from the group of non-human primate, horse, bovine, sheep, goat, pig, dog, cat, rabbit, mouse, rat, guinea pig, hamster and gerbil containing a polynucleotide of the present invention, a vector of the present invention and/or a host cell of the present invention.

[0020] Another aspect of the invention is a process for producing an EPO variant polypeptide encoded by the polynucleotide of the present invention comprising: culturing the host cell of the present invention and recovering the polypeptide encoded by said polynucleotide.

[0021] In a preferred embodiment the process of the present invention, further comprises the step of modifying said EPO variant, wherein the modification is selected from the group consisting of oxidation, sulfation, phosphorylation, addition of oligosaccharides or combinations thereof.

[0022] Another aspect of the invention is a process for producing cells capable of expressing at least one of the EPO variants comprising genetically engineering cells in vitro with the vector of the invention, wherein said EPO variant polypeptide(s) is(are) encoded by a polynucleotide of the invention.

[0023] Another aspect of the invention is a polypeptide having the amino acid sequence encoded by a polynucleotide of the present invention or obtainable by the process of the present invention.

[0024] Disclosed in the context of the invention is an antibody specifically binding to the polypeptide of the present invention.

[0025] Another aspect of the invention is a pharmaceutical composition comprising a polynucleotide of the present invention, a vector of the present invention, a host cell of the present invention, a polypeptide of the present invention and/or an antibody of the present invention and a one or more pharmaceutically acceptable carrier.

[0026] Another aspect of the invention is the use of a polynucleotide of the present invention, a vector of the present invention, a host cell of the present invention, a polypeptide of the present invention for the manufacture of a medicament for the treatment or prevention of a condition associated with tissue damage due to cell death, e.g. apoptosis and necrosis as well as by inflammation.

[0027] In a preferred use of the present invention cell death is induced by ischemia, hypoxia, bacterial infection, viral infection, autoimmunologically, traumatically, chemically (e.g. metabolically, toxically) induced, or radiation induced.

[0028] In a preferred use of the present invention the condition is an acute neurodegenerative/neuroinflammatory disorder or a chronic neurodegenerative/neuroinflammatory disorder, is an acute or chronic disorder of the heart (e.g. myocardial infarction), lung (e.g. asthma, chronic obstructive lung disease), kidney (e.g. glomerulonephritis), liver (e.g. chronic liver failure) or pancreas (e.g. pancreatitis) or said condition is associated with an organ (e.g. kidney or liver) or cell transplantation (e.g. stem cell)..

[0029] Preferably the acute neurodegenerative and/or neuroinflammatory disorder is selected from the group consisting of cerebral ischemia or infarction including embolic occlusion and thrombotic occlusion, reperfusion following acute ischemia, perinatal hypoxic-ischemic injury, cardiac arrest, intracranial hemorrhage, subarachnoid hemorrhage and intracranial lesions (e.g. CNS trauma), spinal cord lesions, intravertebral lesions, whiplash shaken infant syndrome, infectious encephalitis (e.g. herpes encephalitis), meningitis (e.g. bacterial), headache (e.g. migraine).

[0030] Preferably the chronic neurodegenerative/neuroinflammatory disorder is selected from the group consisting of

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dementias (e.g. Alzheimer's disease, vascular dementias), Pick's disease, diffuse Lewy body disease, progressive supranuclear palsy (Steel-Richardson syndrome), multiple sclerosis, multiple system atrophy (including Shy-Drager syndrome), chronic epileptic conditions associated with neurodegeneration, motor neuron diseases, degenerative ataxias, cortical basal degeneration, ALS-Parkinson's Dementia complex of Guam, subacute sclerosing panencephalitis, Huntington's disease, Parkinson's disease, synucleinopathies, primary progressive aphasia, striatonigral degeneration, Machado-Joseph disease/spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, Gilles De La Tourette's disease, bulbar and pseudobulbar palsy, spinal and spinobulbar muscular atrophy (Kennedy's disease), primary lateral sclerosis, familial spastic paraplegia, Werdnig-Hoffmann disease, KugelbergWielander disease, Tay-Sach's disease, Sandhoff disease, familial spastic disease, spastic paraparesis, progressive multifocal leukoencephalopathy, familial dysautonomia (Riley-Day syndrome), polyneuropathies (e.g. diabetic, alcohol-toxic, Guillain-Barre-Syndrome, chronic inflammatory demyelinating polyneuropathy), prion diseases, addiction, affective disorders (e.g. depression), schizophrenic disorders, chronic fatigue syndrome, chronic pain (e.g. lower back pain).

[0031] In a preferred use of the present invention the condition is aging.

[0032] In a preferred use of the present invention the medicament is administered prior to or after the onset of said condition.

Detailed Description of the Invention

[0033] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0034] The present invention is based on the surprising observation that EPO variants are expressed in neuronal tissue and the determination that the variants protected neurons from damage induced by oxygen and glucose deprivation but did not show hematopoietic activity. This behavior makes them suitable for use as therapeutics in situations where the hematopoietic function of EPO is not required or deleterious. Accordingly a first aspect of the present invention is an EPO variant encoding polynucleotide selected from the group consisting of:

(a) polynucleotides encoding the mature form of the polypeptides termed hs3, h1-4, h1-5, hs4, h1-1, h2-1 mS, mG3, mG5, m301 and mK3 having the deduced amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22, respectively;

(b) polynucleotides having the coding sequence, as shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 encoding at least the mature form of the polypeptide;

(c) polynucleotide encoding a humanized version of the polypeptides mS, mG3, mG5, m301 and mK3 consisting of the deduced amino acid sequence as shown in SEQ ID NOs: 14, 16, 18, 20, and 22;

(d) polynucleotides encoding a polypeptide comprising a fusion of an amino acid sequence selected from the group of amino acid sequences as shown in SEQ ID NO 24, 26, 28, and 30, at the N-terminus, preferably directly, i.e. without any intervening amino acids, of an amino acid sequence selected from the group of amino acid sequences as shown in SEQ ID NO 32, 34, 36, and 38, wherein said fusion has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

(e) polynucleotides comprising a fusion of polynucleotide sequences selected from the group of polynucleotide sequences as shown in SEQ ID NO 23, 25, 27, and 29, 5', preferably directly 5', i.e. without any intervening polynucleotides, of a polynucleotide sequence selected from the group of polynucleotide sequences as shown in SEQ ID NO 31, 33, 35, and 37;

(f) polynucleotides encoding a derivative of a polypeptide encoded by a polynucleotide of any one of (a) to (e), wherein in said derivative between 1 and 10 amino acid residues are conservatively substituted compared to said polypeptide, and said derivative has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

(g) polynucleotides encoding a fragment of a polypeptide encoded by a polynucleotide of any one of (a) to (f), wherein in said fragment between 1 and 10 amino acid residues are N- and/or C-terminally deleted and/or between 1 and 10 amino acids are deleted N- and or C-terminally of the junction compared to said polypeptide, and said fragment has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

(h) polynucleotides which are at least 95% identical to a polynucleotide as defined in any one of (a) to (b) and which at the same time have cell protective and in particular neuroprotective activity but essentially no hematopoietic activity; and

(i) polynucleotides the complementary strand of which hybridizes under stringent conditions to a polynucleotide as

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defined in any one of (a) to (h) and which code for a polypeptide having cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

or the complementary strand of such a polynucleotide

5 **[0035]** The invention further relates to an EPO variant encoding polynucleotide selected from the group consisting of:

(a) polynucleotides encoding the polypeptides termed ha, hAma, hAmE, hA-10 and ha-sequence without leader, having the deduced amino acid sequence as shown in SEQ ID NOs: 50, 51, 52, 53 and 61 respectively;

10 (b) polynucleotides having the coding sequence, as shown in SEQ ID NOs: 55, 56, 57, 58 and 61 encoding at least the mature form of the polypeptide;

(c) polynucleotides encoding a derivative of a polypeptide encoded by a polynucleotide of any one of (a) to (b), wherein in said derivative between 1 and 10 amino acid residues are conservatively substituted compared to said polypeptide, and said derivative has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

15 (d) polynucleotides encoding a fragment of a polypeptide encoded by a polynucleotide of any one of (a) to (b), wherein in said fragment between 1 and 10 amino acid residues are N- and/or C-terminally deleted and/or between 1 and 10 amino acids are deleted N- and/or C-terminally of the junction compared to said polypeptide, and said fragment has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

20 (e) polynucleotides which are at least 95% identical to a polynucleotide as defined in any one of (a) to (b) and which at the same time have cell protective and in particular neuroprotective activity but essentially no hematopoietic activity; and

(f) polynucleotides the complementary strand of which hybridizes under stringent conditions to a polynucleotide as defined in any one of (a) to (e) and which code for a polypeptide having cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

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or the complementary strand of such a polynucleotide.

[0036] In a further aspect the polynucleotides of the present invention comprise homologs of the EPO variants of the present invention derived from another higher eukaryotic species, in particular from mammals, more preferably from non-human primates; from rodents, e.g. rat, or guinea pig; ruminant, e.g. cow; or sheep; horse; pig; rabbit; dog; or cat, which have cell protective and in particular neuroprotective activity but essentially no hematopoietic activity. In this context the term homolog refers to a polynucleotide encoding a EPO variant derived from another species, which comprises essentially the same deletion as the polynucleotides according to SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 55, 56, 57, 58 or 60. A deletion of a polynucleotide is considered to be essentially the same, if it involves the deletion of polynucleotides encoding a polypeptide, which is homologous to the respectively deleted polypeptides in the EPO variant polypeptides according to SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 50, 51, 52, 53 or 61. The criteria for determining homology between two peptide sequences are well established. For this purpose programs as BLASTP can be used. A deletion is still considered to be essentially the same if it involves 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 55, 56, 57, 58 or 61 more or less nucleotides as the respective deletion in SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or 21, which are also depicted in Fig. 2 and 3.

40 **[0037]** A further aspect of the present invention is an EPO variant encoding polynucleotide selected from the group consisting of:

(a) polynucleotides encoding an EPO variant polypeptide, which comprises the N-terminal part of full length EPO including helix A and which lacks at least one of the following:

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(i) a fragment of at least 10 amino acids, preferably 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids between helix A and helix B;

(ii) a fragment of at least 10 amino acids, preferably 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28 amino acids of helix B;

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(iii) a fragment of at least 6 amino acids between helix B and helix C;

(iv) a fragment of at least 10 amino acids, preferably 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 amino acids of helix C;

(v) a fragment of at least 20 amino acids, preferably 21, 22, 23, 24, 25, 26, or 27 amino acids between helix C and D; and/or

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(vi) a fragment of at least 10 amino acids, preferably 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 of helix D;

wherein said variant has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity.

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(b) polynucleotides encoding a derivative of a polypeptide encoded by a polynucleotide of any one of (a), wherein in said derivative between 1 and 10 amino acid residues are conservatively substituted compared to said polypeptide, and said derivative has cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

5 (c) polynucleotides the complementary strand of which hybridizes under stringent conditions to a polynucleotide as defined in any one of (a) to (b) and which code for a polypeptide having cell protective and in particular neuroprotective activity but essentially no hematopoietic activity;

or the complementary strand of such a polynucleotide.

10 **[0038]** In this context helix A, B, C, and D of the EPO polypeptide are regions homologous to the respective helix A, B, C, and D regions of full length EPO from mouse and human as outlined in Fig. 4. It is well known in the art how to determine homologies between two polypeptide sequences and someone of skill in the art will be capable to align a given EPO polypeptide sequence derived, e.g. from another species, and to determine the respective position of helix A, B, C, and D in this EPO polypeptide. It is preferred that the EPO variant polynucleotide is derived from a higher
15 eukaryote, in particular a mammal or bird. Preferred mammals are humans, non-human primates; rodents, e.g. rat, or guinea pig; ruminant, e.g. cow; or sheep; horse; pig; rabbit; dog; or cat. A larger number of such full length EPO encoding polynucleotides from various species are known, including without limitation cat (Gene Bank Acc. L10606), pig (Gene Bank Acc. 10607), sheep (Gene Bank Acc. 10610), dog (Gene Bank Acc. L13027), macaque (Gene Bank Acc. M18189), rhesus monkey (Gene Bank Acc. L10609), mouse (Gene Bank Acc. 12930), rat (Gene Bank Acc. L10608), human (Gene Bank Acc. M11319) Bos taurus (Gene Bank Acc. U44762) and Bos indicus (Gene Bank Acc. L41354).

20 **[0039]** Preferably the polynucleotides encoding an EPO variant polypeptide lacks the following: (i); (ii); (iii); (iv); (v); (vi); (i) and (ii); (i) and (iii); (i) and (iv); (i) and (v); (i) and (vi); (ii) and (iii); (ii) and (iv); (ii) and (v); (ii) and (vi); (iii) and (iv); (iii) and (v); (iii) and (vi); (iv) and (v); (iv) and (vi); (v) and (vi); (i), (ii) and (iii); (i), (ii) and (iv); (i), (ii) and (v); (i), (ii), (vi), (i), (iii) and (iv); (i), (iii) and (v); (i), (iii) and (vi); (i), (iv) and (v); (i), (iv) and (vi); (i), (v) and (vi); (ii), (iii) and (iv); (ii), (iii) and (v); (ii), (iii) and (vi); (ii), (iv) and (v); (ii), (iv) and (vi); (ii), (v) and (vi); (iii), (iv) and (v); (iii), (iv) and (vi); (iii), (v) and (vi); or (iv), (v) and (vi).

25 **[0040]** A polypeptide that exhibits cell protective activity is a polypeptide that has at least 50% (e.g., at least: 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 98%; 99%; 99.5%; or 100% or even more) of the ability of the respective EPO variant to protect neurons from damage by apoptosis, wherein the apoptosis is induced by oxygen or glucose deprivation, by chemical or radiation exposure or by viral or bacterial infection. Assays to determine damage to cells, in particular to neuronal cells are known in the art. A suitable assays is the oxygen glucose deprivation assay described herein below. In the described assay the readout is the amount of lactate dehydrogenase activity (LDH). However, a variety of other methods exist, which allow assessing the damage induced in a cell and in particular the amount of cell death (e.g. apoptosis, necrosis). These assays include without limitation Tunnel assays, MTT-assay, life/death assay by staining (e.g. Ethidium bromide and acridine orange staining), caspase assay, electron microscopy, DNA-laddering,
30 which are all well known in the art.

35 **[0041]** An EPO variant polypeptide that exhibits essentially no hematopoietic activity is a polypeptide, which elicits in art known colony formation assays, an example of which is described below, at the same molar concentration as the rhEPO and wt mEPO, respectively, less than 10% of the CFU-E (Colony forming unit-erythroblast), preferably less than
40 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%. The respective CFU-E numbers are calculated for a given rhEPO, wt mEPO or EPO variant by subtracting from each value the number of CFU-E observed in a control reaction (without wt or EPO variant).

45 **[0042]** In the context of the polypeptides of the present invention the term "junction" refers to the site wherein two amino acids follow each other which are not consecutive in the rhEPO or mouse wt EPO and which are potentially the result of splice events or other rearrangements in the EPO mRNA. The respective junction of the EPO variants of the present invention can be derived from Fig. 4, e.g. is ENIT | VGQQ for hS3, VGQQ | ALLV for h1-4, VNFY | ALL for h1-5, KRME|PWEF for hS4, ITVP|GPVG for h1-1, LLEN|NHC for h2-1, KRME|KELM for mS, LLAN|FLRG for mG3, DT-FC|RRGD for mG5, KVN|LRGK for m301 or LSEA|VHGR for mK3.

50 **[0043]** The polynucleotide molecules of the invention can be synthesized *in vitro* (for example, by phosphoramidite-based synthesis) or can be obtained from a cell, such as the cell of a mammal.

55 **[0044]** The EPO variants termed mS, mG3, mG5, m301 and mK3 having the deduced amino acid sequence as shown in SEQ ID NOs 14, 16, 18, 20, and 22, respectively were isolated from mouse. The mouse sequence is highly homologous to the human sequence. An alignment of the amino acid sequences of EPO derived from humans and mouse is provided in Fig. 4. As is apparent the mouse sequence is distinguished from the human sequence by the lack of an alanine residue at position 8 and by the following 39 substitutions (the numbering is according to the respective amino acid position in the human EPO, the first amino acid indicated is the human amino acid at that position and the second is the corresponding mouse amino acid): 4H → 4P, 6C → 6R, 9W → 9T, 11W → 11L, 18S → 18L, 19L → 19I, 27G → 27C, 43L → 43I, 52I → 52V, 54T → 54M, 60H → 60G, 61C → 61P, 62S → 62R, 64N → 64S, 84G → 84E, 85Q → 85E, 95A → 95S, 101V → 101I, 103R

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→ 103Q; 104G → 104A; 109V → 109A; 115W → 115P; 117P → 117T; 122V → 122I; 126V → 126I; 134T → 134S; 138A → 138V; 145A → 145L; 146I → 146M; 151A → 151T; 152A → 152T; 153S → 153P; 154A → 154P; 160I → 160L; 162A → 162V; 166R → 166C; 173S → 173A; 187A → 187V and 190T → 190R. A humanized mS, mG3, mG5, m301 or mK3 carries the additional alanine residue at position 8 and/or at one or more preferably 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 or 37 positions the human rather than the mouse amino acid sequence. It is particularly preferred that mS, mG3, mG5, m301 and mK3 are fully humanized, i.e. that every amino acid at the above outlined positions, in as far as they are present in the respective variant, is of the human sequence rather than the mouse sequence. It is expected that the humanization of the mouse variants will diminish any immunological problems, which might be encountered when using in the treatment of humans.

[0045] The EPO variant nucleic acid molecules of the invention can be DNA, cDNA, genomic DNA, synthetic DNA, or, RNA, and can be double-stranded or single-stranded, the sense and/or an antisense strand. These molecules can be produced by, for example, polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription.

[0046] The polynucleotide molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide, i.e. the polypeptides with SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22. In addition, these nucleic acid molecules are not limited to coding sequences, e.g., they can include some or all of the non-coding sequences that lie upstream or downstream from a coding sequence.

[0047] In addition, the isolated nucleic acid molecules of the invention can encompass segments that are not found as such in the natural state. Thus, the invention encompasses recombinant nucleic acid molecules incorporated into a vector (for example, a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location). Recombinant nucleic acid molecules and uses therefore are discussed further below.

[0048] In preferred embodiments the polynucleotides of the present invention also comprise nucleic acid molecules which are at least 95%, preferably 96%, 97%, 98%, or 99% identical to: (a) a nucleic acid molecule that encodes the polypeptide of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 50, 51, 52, 53 or 61 and (b) the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 55, 56, 57, 58 or 60 respectively and which at the same time cell protective and in particular neuroprotective activity but essentially no hematopoietic activity

The determination of percent identity between two sequences is accomplished using the mathematical algorithm of Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTP programs of Altschul et al. (1990) J. Mol. Biol. 215: 403-410. BLAST nucleotide searches are performed with the BLASTN program, score = 100, word length = 12, to obtain nucleotide sequences homologous to the EPO variant polypeptide encoding nucleic acids. BLAST protein searches are performed with the BLASTP program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to the EPO variant polypeptide, respectively. To obtain gapped alignments for comparative purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs are used.

[0049] Hybridization can also be used as a measure of homology between two nucleic acid sequences. A nucleic acid sequence encoding any of the EPO variants disclosed herein, or a derivative or fragment thereof, can be used as a hybridization probe according to standard hybridization techniques. The hybridization of an EPO variant probe to DNA or RNA from a test source (e.g., a mammalian cell) is an indication of the presence of the relevant EPO DNA or RNA in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by a wash in 0.2 X SSC, 0.1 % SDS at 65°C. When selecting a probe specific for a variant carrying an internal deletion it is preferred that the probe used to detect homologous nucleic acids overlaps the boundaries of the deletion, e.g. hs3, hi -4, hi -5, hS4, mS, mG3, mG5 or m301. In cases where the splicing leads to an alternate C-terminus of the protein, e.g. hi -1, h2-l or mK3 it is preferred that the probe used to detect homologous DNA sequences overlaps the boundaries between the known EPO sequence and the alternate C-terminus. For example, a probe could be designed, which comprises 10 complementary bases 5' of the splice site and 10 complementary bases 3' of the splice site.

[0050] An "isolated DNA" is either (1) a DNA that contains sequence not identical to that of any naturally occurring sequence, or (2), in the context of a DNA with a naturally-occurring sequence (e.g., a cDNA or genomic DNA), a DNA free of at least one of the genes that flank the gene containing the DNA of interest in the genome of the organism in which the gene containing the DNA of interest naturally occurs. The term therefore includes a recombinant DNA incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote. The term also includes a separate molecule such as a cDNA where the corresponding genomic DNA has introns and therefore a different sequence; a genomic fragment that lacks at least one of the flanking genes; a fragment of cDNA or genomic DNA produced by polymerase chain reaction (PCR) and that lacks at least one of the flanking

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genes; a restriction fragment that lacks at least one of the flanking genes; a DNA encoding a non-naturally occurring protein such as a fusion protein, mutein, or fragment of a given protein; and a nucleic acid which is a degenerate variant of a cDNA or a naturally occurring nucleic acid. In addition, it includes a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a non-naturally occurring fusion protein. It will be apparent from the foregoing that isolated DNA does not mean a DNA present among hundreds to millions of other DNA molecules within, for example, cDNA or genomic DNA libraries or genomic DNA restriction digests in, for example, a restriction digest reaction mixture or an electrophoretic gel slice.

[0051] A further aspect of the present invention is a vector containing the polynucleotide(s) of the present invention or a protein encoded by a polynucleotide of the present invention. The term "vector" refers to a protein or a polynucleotide or a mixture thereof which is capable of being introduced or of introducing the proteins and/or nucleic acid comprised into a cell. It is preferred that the proteins encoded by the introduced polynucleotide are expressed within the cell upon introduction of the vector.

[0052] In a preferred embodiment the vector of the present invention comprises plasmids, phagemids, phages, cosmids, artificial mammalian chromosomes, knock-out or knock-in constructs, viruses, in particular adenoviruses, vaccinia viruses, attenuated vaccinia viruses, canary pox viruses, lentivirus (Chang, L.J. and Gay, E.E. (2000) *Curr. Gene Therap.* 1:237-251), herpes viruses, in particular Herpes simplex virus (HSV-1, Carlezon, W.A. et al. (2000) *Crit. Rev. Neurobiol.*), baculovirus, retrovirus, adeno-associated-virus (AAV, Carter, P.J. and Samulski, R.J. (2000) *J. Mol. Med.* 6:17-27), rhinovirus, human immune deficiency virus (HIV), filovirus and engineered versions thereof (see, for example, Cobinger G. P. et al (2001) *Nat. Biotechnol.* 19:225-30), virosomes, "naked" DNA liposomes, and nucleic acid coated particles, in particular gold spheres. Particularly preferred are viral vectors like adenoviral vectors or retroviral vectors (Lindemann et al. (1997) *Mol. Med.* 3:466-76 and Springer et al. (1998) *Mol. Cell.* 2:549-58). Liposomes are usually small unilamellar or multilamellar vesicles made of cationic, neutral and/or anionic lipids, for example, by ultrasound treatment of liposomal suspensions. The DNA can, for example, be ionically bound to the surface of the liposomes or internally enclosed in the liposome. Suitable lipid mixtures are known in the art and comprise, for example, DOTMA (1, 2-Dioleoyloxypropyl-3-trimethylammoniumbromid) and DPOE (Dioleoylphosphatidyl-ethanolamin) which both have been used on a variety of cell lines.

[0053] Nucleic acid coated particles are another means for the introduction of nucleic acids into cells using so called "gene guns", which allow the mechanical introduction of particles into the cells. Preferably the particles itself are inert, and therefore, are in a preferred embodiment made out of gold spheres.

[0054] In a further aspect the polynucleotide of the present invention is operatively linked to expression control sequences allowing expression in prokaryotic and/or eukaryotic host cells. The transcriptional/translational regulatory elements referred to above include but are not limited to inducible and non-inducible, constitutive, cell cycle regulated, metabolically regulated promoters, enhancers, operators, silencers, repressors and other elements that are known to those skilled in the art and that drive or otherwise regulate gene expression. Such regulatory elements include but are not limited to regulatory elements directing constitutive expression like, for example, promoters transcribed by RNA polymerase III like, e.g., promoters for the snRNA U6 or scRNA 7SK gene, the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, viral promoter and activator sequences derived from, e.g., NBV, HCV, HSV, HPV, EBV, HTLV, MMTV or HIV; which allow inducible expression like, for example, CUP-1 promoter, the tet-repressor as employed, for example, in the tet-on or tet-off systems, the lac system, the trp system; regulatory elements directing tissue specific expression, preferably nerve cell specific expression, e.g. promoter (e.g. Thy-1.2, NSE, , myosin light chain II, tyrosine hydroxylase, CaMKIIalpha promoter, platelet-derived growth factor beta-chain (PDGF), dopamine beta-hydroxylase, Tau, regulatory elements (e.g. NRSE/RE-1; neuron-restrictive silencing element/ repressor element 1) directing cell cycle specific expression like, for example, cdc2, cdc25C or cyclin A; or the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α - or α -mating factors.

[0055] As used herein, "operatively linked" means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest.

[0056] Similarly, the polynucleotides of the present invention can form part of a hybrid gene encoding additional polypeptide sequences, for example, a sequence which encodes a protein that functions as a marker or reporter. The hybrid gene can lead to a fusion protein or the two or more parts can be separated by internal ribosomal entry sites (IRES) sequence, which lead to the expression of two or more separate proteins. Examples of marker and reporter genes include β -lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, G418^r), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β -galactosidase), green fluorescent protein (GFP) and variants thereof and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, additional sequences that can serve the function of a marker or reporter. If the expression of the hybrid gene leads to one polypeptide the hybrid

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polypeptide will usually include a first portion and a second portion; the first portion being a EPO variant polypeptide and the second portion being, for example, the reporter described above or an Ig constant region or part of an Ig constant region, e.g., the CH2 and CH3 domains of IgG2a heavy chain. Other hybrids could include a heterologous peptide sequence to facilitate purification and/or detection, e.g. an antigenic tag like, for example, a myc tag, or a tag with preferential binding to a region, e.g. chitin tag or His tag. Recombinant nucleic acid molecules can also contain a polynucleotide sequence encoding a EPO variant polypeptide operatively linked to a heterologous signal sequence. Such signal sequences can direct the protein to different compartments within the cell and are well known to someone of skill in the art. A preferred signal sequence is a sequence that facilitates secretion of the resulting protein. Preferably these signal and/or tag sequences are designed in such that they can be cleaved of the EPO variant after purification to provide an essentially pure protein without too many amino acids, preferably not more than 10 additional amino acids to the final EPO. Such cleavage sites are well known in the art and comprise, e.g. endopeptidase cleavage sites and intein cleavage sites.

[0057] Another aspect of the present invention is a host cell genetically engineered with the polynucleotide or the vector as outlined above. The host cells that may be used for purposes of the invention include but are not limited to prokaryotic cells such as bacteria (for example, *E. coli* and *B. subtilis*), which can be transformed with, for example, recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing the polynucleotide molecules of the invention; simple eukaryotic cells like yeast (for example, *Saccharomyces* and *Pichia*), which can be transformed with, for example, recombinant yeast expression vectors containing the polynucleotide molecule of the invention; insect cell systems like, for example, Sf9 of Hi5 cells, which can be infected with, for example, recombinant virus expression vectors (for example, baculovirus) containing the polynucleotide molecules of the invention; *Xenopus* oocytes, which can be injected with, for example, plasmids; plant cell systems, which can be infected with, for example, recombinant virus expression vectors (for example, cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (for example, Ti plasmid) containing a EPO variant nucleotide sequence; or mammalian cell systems (for example, COS, CHO, BHK, HEK293, VERO, HeLa, MDCK, Wi38, Swiss 3T3 and NIH 3T3 cells), which can be transformed with recombinant expression constructs containing, for example, promoters derived, for example, from the genome of mammalian cells (for example, the metallothionein promoter) from mammalian viruses (for example, the adenovirus late promoter, CMV IE and the vaccinia virus 7.5K promoter) or from bacterial cells (for example, the tet-repressor binding is employed in the tet-on and tet-off systems). Also useful as host cells are primary or secondary cells obtained directly from a mammal and transfected with a plasmid vector or infected with a viral vector. Depending on the host cell and the respective vector used to introduce the polynucleotide of the invention the polynucleotide can integrate, for example, into the chromosome or the mitochondrial DNA or can be maintained extrachromosomally like, for example, episomally or can be only transiently comprised in the cells.

[0058] Since EPO is heavily glycosylated in vivo it is desirable to choose an expression system, which provides faithful glycosylation of the protein. Consequently, it is preferred to introduce the polynucleotides encoding the EPO slice variants of the present invention into higher eukaryotic cells, in particular into mammalian cells, e.g. COS, CHO, BHK, HEK293, VERO, HeLa, MDCK, Wi38, Swiss 3T3 or NIH 3T3 cells.

[0059] A further aspect of the present invention is a transgenic non-human mammal containing a polynucleotide, a vector and/or a host cell as described above. The animal can be a mosaic animal, which means that only part of the cells making up the body comprise polynucleotides, vectors, and/or cells of the present invention or the mammal can be a transgenic animal which means that all cells of the animal comprise the polynucleotides and/or vectors of the present invention or are derived from a cell of the present invention. Mosaic or transgenic animals can be either homo- or heterozygous with respect to the polynucleotides of the present invention contained in the cell. In a preferred embodiment the transgenic animals are either homo- or heterozygous knock-out or knock-in animals with respect to the genes which code for the proteins of the present invention. The mammal is selected from the group of non-human primate horse, bovine, sheep, goat, pig, dog, cat, goat, rabbit, mouse, rat, guinea pig, hamster, and gerbil.

[0060] Another aspect of the present invention is a process for producing an EPO variant polypeptide encoded by a polynucleotide of the present invention comprising: culturing the host cell described above and recovering the polypeptide encoded by said polynucleotide. Preferred combinations of host cells and vectors are outlined above and further combination will be readily apparent to someone of skill in the art. Depending on the intended later use of the recovered peptide a suitable cell type can be chosen. As outlined above eukaryotic cells are preferably chosen, if it is desired that the proteins produced by the cells exhibit an essentially natural pattern of glycosylation and prokaryotic cells are chosen, if, for example, glycosylation or other modifications, which are normally introduced into proteins only in eukaryotic cells, are not desired or not needed.

[0061] It is known in the prior art that the pharmacokinetic of protein drugs can be significantly altered by modification of the protein. For full length EPO it has been described that glycosylation, in particular the presence of sialic acid residues at the end of the oligosaccharide side chains attributes to the circulation time (WO 95/05465) and that removal of sialic acid groups exposes galactose residues, which increases clearance by the liver. Therefore, one approach taken to enhance EPO circulation time was the increase in sialic acid residues. Several approaches, thus, involve the provision

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of additional glycosylation sites (see e.g. WO 91/05867, WO 94/09257 and WO 01/81405. Such modified EPO analogs may have at least one additional N-linked and/or O-linked carbohydrate chain. Other attempts to improve the half life of EPO involved the addition of polyethylene glycol residues (PEG) of varying length the amino acid backbone (see e.g. WO 00/32772, WO 01/02017, WO 03/029291. Another attempt used the modification of EPO molecules with at least one N-linked and/or O-linked oligosaccharide which were further modified with oxidation, sulfation, phosphorylation PEGylation or a combination thereof (see WO 2005/025606). All these approaches can equally be employed to extend the half life of the EPO variants of the present invention and accordingly in a preferred embodiment above process further comprising the step of modifying the EPO variant, wherein the modification is selected from the group consisting of oxidation, sulfation, phosphorylation, addition of oligosaccharides or combinations thereof. If the addition of further N-linked or O-linked oligonucleotides is desired it is possible to introduce them by introducing additional glycosylation sites as has been described in the prior art, e.g. at positions 30, 51, 57, 69, 88, 89, 136 and/or 138, if the respective position is present in the variant of the present invention (see WO 01/81405).

[0062] A further aspect of the invention is a process for producing cells capable of expressing at least one of the EPO variants comprising genetically engineering cells *in vitro* with the vector of claim 3 or 4, wherein said EPO variant polypeptide(s) is(are) encoded by a polynucleotide of the present invention.

[0063] Another aspect of the invention is a polypeptide having the amino acid sequence encoded by a polynucleotide of the invention or obtainable by the process mentioned above. The polypeptides of the invention include all those disclosed herein and fragments of these polypeptides, which carry between 1 and 10 N- and/or C-terminal deletions. Preferably, the deletions are less than 10, less than 9, less than 8, less than 7, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3, less than 2, less than 1 amino acids. The polypeptides embraced by the invention also include fusion proteins that contain either the EPO slice variant as indicated in SEQ ID Nos 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 or humanized version of 14, 16, 18, 20 and 22 or a fragment thereof as defined above fused to an unrelated amino acid sequence. The unrelated sequences can comprise additional functional domains or signal peptides. Signal peptides are described in greater detail and exemplified below.

[0064] The polypeptides can be any of those described above but with not more than 10 (e.g., not more than: 10, nine, eight, seven, six, five, four, three, two, or one) conservative substitutions. Conservative substitutions are known in the art and typically include substitution of, e.g. one polar amino acid with another polar amino acid and one acidic amino acid with another acidic amino acid. Accordingly, conservative substitutions preferably include substitutions within the following groups of amino acids: glycine, alanine, valine, proline, isoleucine, and leucine (non polar, aliphatic side chain); aspartic acid and glutamic acid (negatively charged side chain); asparagine, glutamine, methionine, cysteine, serine and threonine (polar uncharged side chain); lysine, histidine and arginine; and phenylalanine, tryptophane and tyrosine (aromatic side chain); and lysine, arginine and histidine (positively charged side chain). It is well known in the art how to determine the effect of a given substitution, e.g. on pK_i, etc. All that is required of a polypeptide having one or more conservative substitutions is that it has at least 50% (e.g., at least: 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 98%; 99%; 99.5%; or 100% or more) of the ability of the unaltered EPO variant to protect neurons from damage/cell death (e.g. by apoptosis or necrosis), wherein the cell death is induced by oxygen and/or glucose deprivation, by toxic, chemical, physical, mechanical, inflammatory or radiation exposure or by viral or bacterial infection.

[0065] Both polypeptides and peptides can be produced by standard *in vitro* recombinant DNA techniques and *in vivo* transgenesis, using nucleotide sequences encoding the appropriate polypeptides or peptides. Methods well-known to those skilled in the art can be used to construct expression vectors containing relevant coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.) [Cold Spring Harbor Laboratory, N.Y., 1989], and Ausubel et al., *Current Protocols in Molecular Biology* [Green Publishing Associates and Wiley Interscience, N.Y., 1989].

[0066] Polypeptides and fragments of the invention also include those described above, but modified for *in vivo* use by the addition, at the amino- and/or carboxyl-terminal ends, of blocking agents to facilitate survival of the relevant polypeptide *in vivo*. This can be useful in those situations in which the peptide termini tend to be degraded by proteases prior to cellular uptake. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxyl terminal residues of the peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology by methods familiar to artisans of average skill.

[0067] Alternatively, blocking agents such as pyroglutamic acid or other molecules known in the art can be attached to the amino and/or carboxyl terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxyl terminus can be replaced with a different moiety. Likewise, the peptides can be covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

[0068] The term "isolated" polypeptide or peptide fragment as used herein refers to a polypeptide or a peptide fragment which either has no naturally-occurring counterpart or has been separated or purified from components which naturally accompany it, e.g., in tissues such as tongue, pancreas, liver, spleen, ovary, testis, muscle, joint tissue, neural tissue, gastrointestinal tissue or tumor tissue, or body fluids such as blood, serum, or urine. Typically, the polypeptide or peptide

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fragment is considered "isolated" when it is at least 70%, by dry weight, free from the proteins and other naturally-occurring organic molecules with which it is naturally associated. Preferably, a preparation of a polypeptide (or peptide fragment thereof) of the invention is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, the polypeptide (or the peptide fragment thereof), respectively, of the invention. Thus, for example, a preparation of polypeptide x is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, polypeptide x. Since a polypeptide that is chemically synthesized is, by its nature, separated from the components that naturally accompany it, the synthetic polypeptide is "isolated."

[0069] An isolated polypeptide (or peptide fragment) of the invention can be obtained, for example, by extraction from a natural source (e.g., from tissues or bodily fluids); by expression of a recombinant nucleic acid encoding the polypeptide; or by chemical synthesis. A polypeptide that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will necessarily be free of components which naturally accompany it. The degree of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

[0070] Disclosed in the context of the invention is an antibody, which specifically binds to the EPO variant polypeptide encoded by polynucleotides of the invention or obtainable by the process mentioned above. The term "antibody" comprises monoclonal and polyclonal antibodies and binding fragments thereof, in particular Fc-fragments as well as so called "single-chain-antibodies" (Bird R. E. et al (1988) Science 242:423-6), chimeric, humanized, in particular CDR-grafted antibodies, and dia or tetrabodies (Holliger P. et al (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6444-8). Also comprised are immunoglobulin like proteins that are selected through techniques including, for example, phage display to specifically bind to the polypeptides of the present invention. In this context the term "specific binding" refers to antibodies raised against peptides derived from splice junctions or junctions created by other processes, e.g. ENIT | VGQQ of hS3, VGQQ | ALLV of h1-4, VNFY | ALLV of h1-5, KRME | PWEP of hS4, |TVP | GPVG of h1-1, LNEN | NHC of h2-1, KRME | KELM of mS, LLAN | FLRG of mG3, DTFC | RRGD of mG5, KVNF | LRGK of m301 or LSEA | VHGR of mK3. Such peptides can comprise additional or less N- or C-terminal amino acids. An antibody is considered to be specific to the EPO variant, if its affinity towards the variant is at least 50-fold higher, preferably 100-fold higher, more preferably at least 1000-fold higher than towards the full length human or murine EPO. Preferably specific antibodies of the present invention do not or essentially do not bind to full length human or murine EPO. It is well known in the art how to make antibodies and to select antibodies with a given specificity.

[0071] A further aspect of the present invention concerns the use of a polynucleotide, a vector, a host cell or a polypeptide of the present invention for the manufacture of a medicament for the treatment or prevention of a condition associated with tissue damage due to cell death (e.g. apoptosis and necrosis) . The apoptosis or necrosis leads to the cell destruction, which can be prevented or ameliorated when using the polynucleotide, vector, host cell or polypeptide of the present invention. Cell death can be induced by many different internal and external stimuli and include preferably ischemia, hypoxia, bacterial or viral infection, radiation, or induced by metabolic, toxic, chemical, autoimmune, or traumatic stimuli. It is well known in the art how to detect cell death like, for example, using morphological criteria, a TUNNEL assay, MTT-assay, life/death assay by staining (e.g. Ethidium bromide and acridine orange staining), caspase assay, electron microscopy, DNA-laddering or the LDH release assay described below. For example, apoptosis is characterized by chromatin fragmentation, extravasation of cellular contents and eventually death of the cell. It has been recognized to play a role in many acute or chronic pathologic processes. Accordingly, a preferred use of the present invention comprises the administration of polynucleotides, vectors, host cells or polypeptides of the present invention to prevent, treat or ameliorate acute and chronic neurodegenerative or neuroinflammatory disorders, acute or chronic disorder of the heart (e.g. myocardial infarction), lung (e.g. asthma, chronic obstructive lung disease), kidney (e.g. glomerulonephritis), liver (e.g. chronic liver failure) or pancreas (e.g. pancreatitis), as well as conditions associated with cell (e.g. stem cell) or organ transplantation (e.g. kidney or liver). In this respect is also envisioned that the EPO variants of the present invention can be included in storage solutions used for storing organs or limbs for transport and/or after traumatic injury.

[0072] Acute neurodegenerative disorders include, but are not limited to, various types of acute neurodegenerative disorders associated with neuronal cell death including cerebrovascular insufficiency, focal or diffuse brain trauma, diffuse brain damage, and spinal cord injury. Examples of acute neurodegenerative disorders are: cerebral ischemia or infarction including embolic occlusion and thrombotic occlusion, reperfusion following acute ischemia, perinatal hypoxic-ischemic injury, cardiac arrest, as well as intracranial hemorrhage of any type (such as epidural, subdural, subarachnoid and intracerebral), and intracranial and intravertebral lesions (such as contusion, penetration, shear, compression and laceration), whiplash shaken infant syndrome infectious encephalitis (e.g. herpes encephalitis), meningitis (e.g. bacterial), headache (e.g. migraine).

[0073] Chronic neurodegenerative disorders that can be treated with the EPO variants of the present invention include, but are not limited to, dementias (e.g. Alzheimer's disease, vascular dementias), Pick's disease, diffuse Lewy body disease, progressive supranuclear palsy (Steel-Richardson syndrome), multiple sclerosis, multiple system atrophy (including Shy-Drager syndrome), chronic epileptic conditions associated with neurodegeneration, motor neuron diseases

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including amyotrophic lateral sclerosis, degenerative ataxias, cortical basal degeneration, ALS-Parkinson's-Dementia complex of Guam, subacute sclerosing panencephalitis, Huntington's disease, Parkinson's disease, synucleinopathies (including multiple system atrophy), primary progressive aphasia, striatonigral degeneration, Machado-Joseph disease/spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, Gilles De La Tourette's disease, bulbar and pseudobulbar palsy, spinal and spinobulbar muscular atrophy (Kennedy's disease), primary lateral sclerosis, familial spastic paraplegia, Werdnig-Hoffmann disease, Kugelberg-Welander disease, Tay-Sach's disease, Sandhoff disease, familial spastic disease, spastic paraparesis, progressive multifocal leukoencephalopathy, familial dysautonomia (Riley-Day syndrome), and prion diseases (including, but not limited to Creutzfeldt-Jakob, Gerstmann-Strussler-Scheinker disease, Kuru and fatal familial insomnia), polyneuropathies (e.g. diabetic, alcohol-toxic, Guillain-Barré-Syndrome, chronic inflammatory demyelinating polyneuropathy), prion diseases, addiction, affective disorders (e.g. depression), schizophrenic disorders, chronic fatigue syndrome, chronic pain (e.g. lower back pain).

[0074] A further aspect of the present invention concerns the use of a polynucleotide, a vector, a host cell or a polypeptide of the present invention for the manufacture of an anti-aging medication. The basis for this application of the EPO variants of the present invention is the fact that the progressing deterioration of most bodily functions, which accompanies aging has been associated with cell death and it is, therefore, envisioned that the EPO variants of the present invention, which only provide the beneficial cell protective effect can be taken continuously without suffering the side effects usually associated with the continuous administration of EPO, which, however, can be attributed to the erythropoietic effect of EPO.

[0075] The inventors have astonishingly found that the nucleic acids and proteins according to the invention possess astonishing anti-inflammatory properties (see figures and experiments). Thus, these EPO variants are useful in treatment of inflammatory and degenerative diseases. Inflammatory diseases are diseases such as but not limited to multiple sclerosis, viral and bacterial infections or sepsis. Degenerative diseases are diseases such as but not limited to stroke, myocardial infarctions.

[0076] The invention also relates to all kinds of forms of in vivo expression of the nucleic acids according to the invention. It further relates to transformed cells, in particular stem cells which are used as therapeutic agents. Such cells may be stably transformed with a nucleic acid according to the invention. The nucleic acid may in a cassette where it is operably linked to a promoter. The promoter may be capable of driving the expression only in particular tissues, such as but not limited to neuronal tissue or the brain or tissue which exhibits inflammation or degeneration. Respective teaching may be taken from WO 97/14307.

[0077] The activity (in units) of EPO polypeptide is traditionally defined based on its effectiveness in stimulating red cell production in rodent models (and as derived by international standards of EPO). One unit (U) of regular EPO (MW of about 34,000) is about 10 ng of protein (1 mg protein is approximately 100,000 U). However, as mentioned the invention involves the use of non-hematopoietic forms of erythropoietin, and as such, this definition based on hematopoietic activity is inappropriate. Thus, as used herein, the activity unit of EPO variant is defined as the amount of protein required to elicit the same activity in neural or other erythropoietin-responsive cellular systems as is elicited by native EPO in the same system. The skilled artisan will readily determine the units of a non-hematopoietic EPO following the guidance herein.

[0078] In a further aspect the present invention provides a pharmaceutical composition comprising a polynucleotide, a vector, a host cell, a polypeptide of the present invention and a one or more pharmaceutically acceptable carrier.

[0079] In the practice of one aspect of the present invention, a pharmaceutical composition as described above may be administered to a mammal by any route which provides a sufficient level of an erythropoietin variant. It can be administered systemically or locally. Such administration may be parenterally, transmucosally, e.g., orally, nasally, rectally, intravaginally, sub-lingually, submucosally or transdermally. Preferably, administration is parenteral, e.g., via intravenous or intraperitoneal injection, and also including, but is not limited to, intra-arterial, intramuscular, intradermal and subcutaneous administration. If the pharmaceutical composition of the present invention is administered locally it can be injected directly into the organ or tissue to be treated. In cases of treating the nervous system this administration route includes, but is not limited to, the intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration, which can employ intracranial and intravertebral needles, and catheters with or without pump devices.

[0080] In a preferred embodiment of pharmaceutical composition comprises an EPO variant polypeptide in a dosage unit form adapted for protection or enhancement of EPO-responsive cells, tissues or organs which comprises, per dosage unit, an effective non-toxic amount within the range from about 0.5 mg to 5 mg of EPO variants; 0.6 mg to 5 mg of EPO variants; 0.7 mg to 5 mg of EPO variants; 0.8 mg to 5 mg of EPO variants; 0.9 mg to 5 mg of EPO variants; 1 to 5 mg of EPO variants; 1.5 to 5 mg of EPO variants; 2 to 5 mg of EPO variants; 2.5 to 5 mg of EPO variants; 3.5 to 5 mg of EPO variants; 4 mg to 5 mg of EPO variants; or 4.5 to 5 mg of EPO variants and a pharmaceutically acceptable carrier.

[0081] In a preferred embodiment, an EPO variant polypeptide may be administered systemically at a dosage between 100 nanograms to about 50 micrograms per kg body weight, preferably about 20 micrograms to about 50 micrograms per kg-body weight. Such serum levels may be achieved at about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 hours post-administration.

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Such dosages may be repeated as necessary. For example, administration may be repeated daily, or every other, third, fourth, fifth, sixth, or seventh day, as long as clinically necessary, or after an appropriate interval, e.g., every 1 to 12 weeks, preferably, every 3 to 8 weeks. In one embodiment, the effective amount of EPO variant and a pharmaceutically acceptable carrier may be packaged in a single dose vial or other container. Depending on the respectively treated disease or condition the EPO variant can be administered in a single dose, for a predetermined period of time or continuously. When an acute condition or disease is treated it might be sufficient to provide the patient with a single dose of EPO variant or for a period of, e.g. for 2 days to 12 months, preferably 1 week to 6 months, more preferably 2 weeks to 3 months. If a chronic disease or condition is treated or if the EPO variant is used to prevent or reduce the deterioration associated with aging the EPO variant can be administered continuously. If the EPO variant of the present invention is administered for a given time period or continuously it is preferably administered in the intervals and preferred intervals indicated above. The intervals necessary will depend in part on the serum level of the EPO variant necessary to treat or ameliorate the respective disease and on the pharmacokinetic of the respective EPO variant, which will in part depend on modifications of EPO by, for example, PEG. It will be in the discretion of the practitioner to determine the exact duration, dose and type of EPO variant taking into consideration, e.g. the condition of the patient to be treated, the severity of the condition etc.

[0082] For other routes of administration, such as by use of a perfusate, injection into an organ, or other local administration, a pharmaceutical composition will be provided which results in similar levels of an EPO variant as described above. A level of about 10 pg/ml to about 1000 ng/ml is desired.

[0083] The pharmaceutical compositions of the invention may comprise a therapeutically effective amount of a compound, e.g. polynucleotide, polypeptide, cell or vector, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0084] Pharmaceutical compositions adapted for oral administration may be provided as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or nonaqueous liquids); as edible foams or whips; or as emulsions. Tablets or hard gelatine capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc. Solutions and syrups may comprise water, polyols and sugars.

[0085] An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract (e.g., glyceryl monostearate or glyceryl distearate may be used). Thus, the sustained release of an active agent may be achieved over many hours and, if necessary, the active agent can be protected from being degraded within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

[0086] Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Pharmaceutical compositions adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For topical administration to the skin, mouth, eye or other external tissues a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration

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to the eye include eye drops. In these compositions, the active ingredient can be dissolved or suspended in a suitable carrier, e.g., in an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouthwashes.

[0087] Pharmaceutical compositions adapted for nasal administration may comprise solid carriers such as powders (preferably having a particle size in the range of 20 to 500 microns). Powders can be administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nose from a container of powder held close to the nose. Alternatively, compositions adapted for nasal administration may comprise liquid carriers, e.g., nasal sprays or nasal drops. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for administration by inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient. In a preferred embodiment, pharmaceutical compositions of the invention are administered via the nasal cavity to the lungs.

[0088] Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations. Pharmaceutical compositions adapted for parenteral administration include aqueous and nonaqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially isotonic with the blood of an intended recipient. Other components that may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, e.g., sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. In one embodiment, an autoinjector comprising an injectable solution of an EPO variant may be provided for emergency use by ambulances, emergency rooms, and battlefield situations, and even for self-administration in a domestic setting, particularly where the possibility of traumatic amputation may occur, such as by imprudent use of a lawn mower. The likelihood that cells and tissues in a severed foot or toe will survive after reattachment may be increased by administering an EPO variant to multiple sites in the severed part as soon as practicable, even before the arrival of medical personnel on site, or arrival of the afflicted individual with severed toe in tow at the emergency room.

[0089] In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile saline can be provided so that the ingredients may be mixed prior to administration.

[0090] Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

[0091] A perfusate composition may be provided for use in transplanted organ baths, for in situ perfusion, or for administration to the vasculature of an organ donor prior to organ harvesting

[0092] Such pharmaceutical compositions may comprise levels of an EPO variant or a form of an EPO variant not suitable for acute or chronic, local or system administration to an individual, but will serve the functions intended herein in a cadaver, organ bath, organ perfusate, or in situ perfusate prior to removing or reducing the levels of the EPO variant contained therein before exposing or returning the treated organ or tissue to regular circulation.

[0093] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container (s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0094] In another embodiment, for example, EPO variant can be delivered in a controlled-release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Sefton (1987) CRC Crit. Ref. Biomed. Eng. 14: 201; Buchwald et al. (1980) Surgery 88:507; Saudek et al. (1989) N. Eng. J. Med. 321: 574). In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533; Treat et al. (1989) in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, N.Y., 353-365; WO 91/04014; U.S. 4,704,355). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release (1974) Langer and Wise (eds.), CRC Press: Boca Raton, Fla.; Controlled Drug Bioavailability, Drug Product Design and Performance, (1984) Smolen and Ball (eds.), Wiley: N.Y.;

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Ranger and Peppas (1953) J. Macromol. Sci. Rev. Macromol. Chem. 23: 61; see also Levy et al. (1985) Science 228: 190; During et al. (1989) Ann. Neurol. 25: 351; Howard et al. (1989) J. Neurosurg. 71: 105).

[0095] In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the target cells, tissue or organ, thus requiring only a fraction of the systemic dose (see, e.g., Goodson (1984) 115-138 in Medical Applications of Controlled Release, vol. 2). Other controlled release systems are discussed in the review by Langer (1990, Science 249: 1527-1533).

[0096] In another embodiment, EPO variant, as properly formulated, can be administered by nasal, oral, rectal, vaginal, or sublingual administration.

[0097] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers.

[0098] Selection of the preferred effective dose will be determined by a skilled artisan based upon considering several factors which will be known to one of ordinary skill in the art. Such factors include the particular form of the pharmaceutical composition, e.g. polypeptide or vector, and its pharmacokinetic parameters such as bioavailability, metabolism, half-life, etc., which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and each patient's circumstances, e.g., depending upon the condition and the immune status of the individual patient, according to standard clinical techniques.

[0099] In another aspect of the invention, a perfusate or perfusion solution is provided for perfusion and storage of organs for transplant, the perfusion solution including an amount of an pharmaceutical compositions effective to protect EPO variant-responsive cells and associated cells, tissues or organs.

[0100] Transplant includes but is not limited to xenotransplantation, where a organ (including cells, tissue or other bodily part) is harvested from one donor and transplanted into a different recipient; and autotransplant, where the organ is taken from one part of a body and replaced at another, including bench surgical procedures, in which an organ may be removed, and while ex vivo, resected, repaired, or otherwise manipulated, such as for tumor removal, and then returned to the original location. In one embodiment, the perfusion solution is the University of Wisconsin (UW) solution (U.S. 4,798,824) which contains from about 1 to about 25 U/ml erythropoietin, 5% hydroxyethyl starch (having a molecular weight of from about 200,000 to about 300,000 and substantially free of ethylene glycol, ethylene chlorohydrin, sodium chloride and acetone); 25 mM KH_2PO_4 ; 3 mM glutathione; 5 mM adenosine; 10 mM glucose; 10 mM HEPES buffer; 5 mM magnesium gluconate; 1.5 mM CaCl_2 . 105 mM sodium gluconate; 200,000 units penicillin; 40 units insulin; 16 mg Dexamethasone; 12 mg Phenol Red; and has a pH of 7.4-7.5 and an osmolality of about 320 mOsm/l. The solution is used to maintain cadaveric kidneys and pancreases prior to transplant. Using the solution, preservation may be extended beyond the 30-hour limit recommended for cadaveric kidney preservation. This particular perfusate is merely illustrative of a number of such solutions that may be adapted for the present use by inclusion of an effective amount of the pharmaceutical composition. In a further embodiment, the perfusate solution contains the equivalent from about 5 to about 35 U/ml erythropoietin, or from about 10 to about 30 U/ml erythropoietin.

[0101] While the preferred recipient of an EPO variant for the purposes herein throughout is a human, the methods herein apply equally to other mammals, particularly domesticated animals, livestock, companion and zoo animals. However, the invention is not so limiting and the benefits may be applied to any mammal.

[0102] If a person is known to be at risk of developing a stroke a prophylactic administration of the pharmaceutical composition of the present invention is possible. In these cases the pharmaceutical compositions, in particular EPO variant polypeptide is preferably administered in above outlined preferred and particular preferred doses on a daily basis. Preferably, between 100 nanograms to about 50 micrograms per kg body weight, preferably about 20 micrograms to about 50 micrograms per kg-body weight. This administration can be continued until the risk of developing a stroke has lessened. In most instances, however, the pharmaceutical composition will be administered once a stroke has been diagnosed. In these cases it is preferred that a first dose of the pharmaceutical composition is administered for the first time within 24 hours after the first symptoms of a stroke are evident, preferably within 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 hour or less. Preferably the administration is then continued for preferably at least 7, more preferably at least 14 and more preferably for at least 21 days. The doses are administered preferably once a day and preferably in above indicated doses.

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Brief Description of the Figures and Drawings

[0103]

- 5 **Fig. 1:** Comparison of EPO PCR products: Panel A depicts the DNA products of various PCR reactions performed with either pure plasmid comprising the different murine EPO variants or cDNA from mouse brain of kidney, which are separated on a 1.2% agarose gel. From the left to the right the lanes comprise: 1 kb molecular weight marker, the product of pure mK3, pure mG3, pure mG5, pure m301, pure mS, pure mWT, brain cDNA, kidney cDNA. Panel B depicts the DNA product of a PCR performed with cDNA from human brain. From the left to the right the lanes comprise 1 kb molecular weight standard and the PCR product of human brain cDNA.
- 10 **Fig. 2:** Alignment of nucleotide sequences of the EPO variants identified in murine brain cDNA and "wild type" murine EPO, i.e. the sequence of the previously described EPO.
- 15 **Fig. 3:** Alignment of nucleotide sequences of the EPO variants identified in human brain cDNA and "wild type" human EPO.
- Fig. 4:** Alignment of the amino acid sequences of the EPO variants identified in mouse and human with the respective "wild type" EPO.
- 20 **Fig. 5:** Hematopoietic activity of murine and human EPO and the EPO variants of the present invention. Panel A depicts the results of a colony forming assay for murine EPO and EPO variants and Panel B depicts the of a colony forming assay for human EPO and EPO variants.
- 25 **Fig. 6:** Experimental setup for neuroprotection assays with rhEPO and EPO-isomers.
- Fig. 7:** Panel A shows an experiment with 1 h 40 min and 1 h 50 min oxygen glucose deprivation (OGD) length. At both time-points a protection rate of 40-50% for the neuroguardians, but no protection with mEPO and rhEPO was observed. Panel B shows an experiment with two different time-points (OGD length between two experiments varies according to density of neurons). At 2 h 45min only weak protection is achieved with wtEPO (20-30%) compared to neuroguardians (60-70%). Full protection capacity of rh EPO is only observed at higher damage levels (3 h 15min).
- 30 **Fig 8:** Panel A shows an experiment with 2 h 00 min, 2 h 15 min and 2 h 20 min OGD length with a protein concentration equalling 100 U/l hEPO. At all three time-points a protection rate of 40-50% for the human, but no protection with mEPO and rhEPO. Panel B shows an experiment with two different time-points (OGD length between two experiments varies according to density of neurons). At 2 h 45 min only weak protection is achieved with wtEPO (20-30%) compared to neuroguardians (60-70%). Full protection capacity of EPO is only observed at higher damage levels (3 h 15 min).
- 35 **Fig 9:** Panel A shows a Western Blot from medium of HEK293-cells transfected with pcDNA3.1-V5/His-hEPO, pcDNA3.1-V5/His-hS3 or pcDNA3.1-V5/His-hS4, respectively. These media were used for experiments shown in Fig. 8. Concentration of hEPO, quantified by the mouse-EPO-ELISA (R&D) was 2U/ml. rhEPO (=2.5 ng were loaded on the gel), hEPO (=0.4 ng were loaded on the gel), hS3 and hS4: each 20 µl medium (collected 2 days after transfection). Marker = 5 µl BenchMark™ His-tagged Protein Standard (Invitrogen). Panel B shows a Western Blot of His-Tag-purified mouse wild type EPO (mEPO), human hS3 and hS4 EPO variants. mEPO was quantified with the EPO-mouse-ELISA. 130 pg mEPO were loaded onto the gel. (primary antibody: rabbit anti-rhEPO-Antikörper; Santa-Cruz).
- 40 **Fig. 10:** Alignment of the amino acid sequences of the EPO variants created recombinantly (alpha-helix mutants) and identified in vivo. Herein SEQ ID NO 50 is the human alpha helix wild type sequence; SEQ ID NO 51 is hAmA (point mutation Alanin); SEQ ID NO 52 is hAmE (point mutation glutamic acid); SEQ ID NO 53 is hA-10 (deletion mutant) and SEQ ID NO 54 is hA-20 (deletion mutant).
- 45 **Fig. 11:** hEPO and hS3 mediated cytoprotection in a model of ischemia consisting of serum deprivation and hypoxia in H9c2 cardiac myoblasts. H9c2 cells were incubated in serum-deprived DMEM-medium either in normoxic or hypoxic conditions for 24h. Apoptosis was assessed 24h later by LDH assay. Data were normalized by setting the delta LDH release of untreated cells in normoxic and hypoxic conditions to 100%. **A:** Column

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diagram representing the average values of normalized LDH release. **B:** Data were presented as box plot diagram showing the median (line across the box), the 25th percentile (lower hinge), the 75th percentile (upper hinge), the maximum and the minimum value. Number of experiments n=7. $P^* < 0,001$ (ANOVA1).

- Fig. 12:** Immunoprecipitation of EPO variants using an anti-mEPO antibody from R&D (goat, biotin-labelled); **A:** Detection of a second EPO isoform (30kDa) in a kidney protein extract of CoCl₂-treated mice (12956). **B:** Blocking of the antibody-antigen interaction by DarbpoietinA.
- Fig. 13:** Neuroprotection mediated by Erythropoietin Alpha-helix (hA; n=4). hA 100U/l: 30pM; hA 50 U/l: 15pM; hEPO: 30pM=100U/l; $P^* < 0,05$; ANOVA1 versus control
- Fig. 14:** Neuroprotection mediated by several human EPO-isoforms (n=6) $P^* < 0,05$; ANOVA1 versus control
- Fig. 15:** Neuroprotection mediated by Erythropoietin Alpha-helix deletion variants (n=6) $P^* < 0,05$; ANOVA1 versus control **A:** column diagram showing the average values of normalized LDH release. **B:** Box plot showing the medians and percentiles (25%, 75%) values of normalized LDH release
- Fig.16.** Effects of human EPO variants and full length human EPO on LPS-induced cytokine production by human macrophages. Purified human monocytes were differentiated into macrophages in the presence of rhu M-CSF (50 ng/ml) for 6 days. Macrophages (1x10⁶/ml) were pre-incubated with hS4, hS3 or hEPO (300 mU/ml each) for 3h and then stimulated with 10 ng/ml endotoxin (LPS from E.coli 0127:B8) for 4h. Cytokine concentration in supernatants was determined by ELISA (Cytometric Beads Array, Becton Dickinson, Heidelberg, Germany). Data are shown as mean \pm SD. ** Results differed from Control (PBS) group ($p < 0.01$; Mann-Whitney U test; n=3-9 per group).
- Fig. 17:** Alignment of nucleic acid sequences of EPO deletion variants.
- Fig. 18:** DNA sequences of mutants and deletion variants created recombinantly as well as wild type Helix A (hWT-EPO Helix A). Herein SEQ ID NO 55 is hA (Wild type Helix A), SEQ ID NO 56 is hAmA (deletion mutant with Alanin), SEQ ID NO 57 is hAmE (deletion mutant with glutamic acid), SEQ ID NO 58 is hA-10 (deletion mutant Helix A minus 10 aa) and SEQ ID NO 59 is hA-20 (deletion mutant Helix A minus 20 aa).
- Fig. 19:** A preferred embodiment wherein the leader transport sequences are deleted is depicted. A shows the hA DNA sequence without leader as SEQ ID NO 60. This is the mature exported protein. It also shows the leader-sequence (SEQ ID NO 63). B shows hA amino acid without leader as SEQ ID NO 61 and the amino acid sequence of the leader-sequence as SEQ ID NO 62.

Examples

Synthesis of murine EPO cDNA

[0104] RNA was isolated from kidneys of wild type C57BL/6 or SV129S6 mice or from two different mouse brains (1 hour after stroke) by trizol extraction. The RNA was precipitated with chloroform and isopropanol and finally dissolved in DEPC-H₂O. DNA was digested to the RQ1 RNase-free DNase protocol from Promega. The reaction was stopped by addition of 200 μ l phenol/chloroform/isopropyl alcohol (25/24/1) to the reaction mix and centrifugation for 10 min at 10000 rpm and 10°C. The supernatant was mixed with 200 μ l chloroform/isopropyl alcohol (24/1) and centrifuged for 10 min at 10000 rpm and 10°C. 20 μ l 8 M lithium chloride and 550 μ l absolute ethanol were added to the supernatant. This mix was then incubated for 1 h at -70°C and subsequently precipitated for 30 min by centrifugation at 11000 rpm and 0°C. The resulting pellet was washed with 600 μ l 75% ethanol, centrifuged at 8000 rpm (4°C, 10 min) and dried at room temperature. The RNA was dissolved in 20 μ l DEPC-H₂O.

[0105] Moloney murine leukemia virus reverse transcriptase (MuLV, RNase H minus, purchased from Promega) was employed in first strand cDNA synthesis in a 15 μ l reaction volume with DEPC-H₂O comprising 3 μ g RNA and 3 μ l random hexamer primer (10 μ M). Reverse transcription was carried out with 6 μ l M-MuLV reaction buffer (5x), 2 μ l dNTP (2,5 mM each), 1 μ l RNase inhibitor (1U/ μ l), 1 μ l M-MuLV reverse transcriptase and 5 μ l DEPC-H₂O in a PCR machine running the following program: 5 min at 21 °C; 1 h at 37°C; 5 min at 95°C.

[0106] The resulting cDNA pool was used to amplify the complete EPO cDNA by a Nested PCR approach. The first step employed primers lying outside of the coding region of the EPO gene (genepo_sense (SEQ ID NO 39) gaa ctt cca agg atg aag act tgc age and genepo_antisense; (SEQ ID NO 40): gtg gca gca gca tgt cac ctg tc). The second step used

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primers designed to amplify the gene from start to stop codon, with attached *Bam*HI cleaving sites for the subsequent cloning (epo_sense (SEQ ID NO 41: tat gga tcc atg ggg gtg ccc gaa cgt ccc ac and epo_antisense (SEQ ID NO 42: tat gga tcc tca cct gtc ccc tct cct gca gac). All primers were from MWG-Biotech AG. A nested PCR was performed in a Hybaid PCR machine in two steps, first PCR (3 min at 95°C; 35 cycles: 30 sec at 65°C, 1 min at 72°C, 30 sec at 95°C; 10 min at 72°C; 4°C hold) and second PCR (3 min at 95°C; 5 cycles: 30 sec at 67°C, 1 min at 72°C, 30 sec at 95°C; 15 cycles: 30 sec at 70°C, 1 min at 72°C, 30 sec at 95°C; 10 min at 72°C; 4°C).

[0107] In both PCRs, *Pfu* Turbo Hotstart DNA Polymerase (Stratagene) was used according to the manufacturer's protocol. The PCR product of the first step was diluted 1:50 for the second PCR. A second cDNA synthesis protocol was performed using the Access RT-PCR System (Invitrogen) with the following parameters: 48°C 5 min; 94°C 2 min; 40 cycles: 94°C 30 sec, 65°C 1 min, 70°C 2 min; 70°C 7 min; 4°C. The second PCR was performed as described above.

[0108] The amplified full-length EPO cDNA and the EPO isomers were separated on a 1.2% TAE-agarose gel. A picture of the various PCR products is shown in Fig. 1a. The fragments were then purified using the Wizard SV-Gel Cleanup System (Promega) or the Gel Extraction Kit (Qiagen, Hilden, Germany). As *Pfu* Polymerase generates blunt end products, the cDNA was subcloned in the pCR-Blunt II-TOPO Vector using chemically competent Top10 One Shot Cells from (both Invitrogen).

[0109] Plasmid-DNA was isolated out of single colonies by usage of the Qiagen QIA prep Kit. Inserts were sequenced on an ALFexpress™ DNA Sequencer (Pharmacia Biotech) using the Thermo Sequenase™ Primer Cycle Sequencing Kit (Amersham Biosciences). The primers M13FWDCY (SEQ ID NO 43: gtc gtg act ggg aaa acc ctg gcg) and M13REVCY (SEQ ID NO 44: agc gga taa caa ttg cac aca gga) were labelled with Cy5. The parameters for sequencing were: t=900 min; T=55°C; 800V; 55 mA and 30 W. The sequence analysis revealed the existence of a novel variant of EPO lacking exon 4 and three internally deleted variants. The nucleotide sequences are depicted in Fig. 2a and Fig. 2b and the encoded peptide sequences are depicted in Fig. 4. The nucleotide and peptide sequence of the EPO variant mS corresponds to SEQ ID NO 13 and SEQ ID NO 14, respectively. The nucleotide and peptide sequence of the EPO variant mG3 corresponds to SEQ ID NO 15 and SEQ ID NO 16, respectively. The nucleotide and peptide sequence of the EPO variant mG5 corresponds to SEQ ID NO 17 and SEQ ID NO 18, respectively. The nucleotide and peptide sequence of the EPO variant m301 corresponds to SEQ ID NO 19 and SEQ ID NO 20, respectively. The nucleotide and peptide sequence of the EPO variant mK3 corresponds to SEQ ID NO 21 and SEQ ID NO 22, respectively.

Synthesis of human EPO cDNA

[0110] Human adult kidney (male) and fetal brain (male) poly A+ RNA was purchased from Stratagene. cDNA was generated from 250 ng kidney RNA or 200 ng brain RNA according to the Moloney murine leukaemia virus reverse transcriptase (MuLV, RNase H minus) as described above. The resulting cDNA pool was used to amplify the complete EPO cDNA using *Pfu* Polymerase (Stratagene) with the following primers: Hepo_sense (SEQ ID NO 45): gat ggg ggt gca cga atg tcc tgc and Hepo_antisense (SEQ ID NO 46): cac acc tgg tca tct gtc ccc tgt c.

[0111] The PCR was performed in a PCR machine from Invitrogen (3 min at 95°C; 35 cycles: 30 sec at 67°C, 1 min at 72°C, 30 sec at 95°C; 10 min at 72°C). In the case of the fetal brain cDNA a Nested PCR approach was used, performing a second amplifying step on the PCR product of 20 cycles. The amplified PCR products were separated on a 1.2% TAE-agarose gel (Fig. 1b) and purified using the Gel Extraction Kit (Qiagen, Hilden, Germany). The purified cDNA was subcloned in the pCR-Blunt II-TOPO Vector using chemically competent Top10 One Shot Cells (both from Invitrogen). Plasmid-DNA was isolated out of single colonies by usage of the QIA prep Kit (Qiagen, Hilden, Germany). Inserts were sequenced on an ALFexpress™ DNA sequencer (Pharmacia Biotech) using the Thermo Sequenase™ Primer Cycle Sequencing Kit (Amersham Biosciences). The primers M13FWDCY (SEQ ID NO 43) and M13REVCY (SEQ ID NO 44) were labelled with Cy5. The parameters for sequencing were: t=900min; T=55°C; 800 V; 55 mA and 30 W. The sequence analysis revealed the existence of two novel variants of human EPO missing exon 3 and the first half of exon 4, respectively, and a number of variants that follows the rule of repeated trimers or hexamers as detected in the mouse. The nucleotide sequences are depicted in Fig. 3a and Fig. 3b and the encoded peptide sequences are depicted in Fig. 4. The nucleotide and peptide sequence of the EPO variant hS3 corresponds to SEQ ID NO 1 and SEQ ID NO 2, respectively. The nucleotide and peptide sequence of the EPO variant h1-4 corresponds to SEQ ID NO 3 and SEQ ID NO 4, respectively. The nucleotide and peptide sequence of the EPO variant h1-5 corresponds to SEQ ID NO 5 and SEQ ID NO 6, respectively. The nucleotide and peptide sequence of the EPO variant hS4 corresponds to SEQ ID NO 7 and SEQ ID NO 8, respectively. The nucleotide and peptide sequence of the EPO variant h1-1 corresponds to SEQ ID NO 9 and SEQ ID NO 10, respectively. The nucleotide and peptide sequence of the EPO variant h2-1 corresponds to SEQ ID NO 11 and SEQ ID NO 12, respectively.

Expression of His-tagged proteins in HEK cells

[0112] *Bam*HI and *Eco*RI restriction sites for cloning were added to both the mouse and the human EPO variants by

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using overhang sense primers and overhang antisense primers without stop codon (for mouse variants: epo_sense (SEQ ID NO 41) and epoeco_antisense (SEQ ID NO 47): aaa gaa ttc cct gtc ccc tct cct gca gac etc; for human variants: hepobam_se (SEQ ID NO 48): tat gga tcc atg ggg gtc cac gaa tgt cc, hepoeeco_as [SEQ ID NO 49]: aga gaa ttc tct gtc ccc tgt cct gca g). The PCR products were cloned into pcDNA-3.1-HIS/V5 A (Invitrogen) using BamHI and EcoRI restriction sites. Plasmids were amplified in XL-1 Blue Competent Cells (recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lac [F' proAB lacI^qZΔM15 Tn10 (Tet^R)] (Stratagene). The XL-1 Blue Competent Cells transformation protocol was performed without β-mercaptoethanol and with a prolonged heat pulse of 60 seconds. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). For transfection into mammalian cells DNA was extracted using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). HEK 293 cells (BD biosciences) were grown for 18 days in Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin, 1 g/l glucose, 3.7 g/l NaHCO₃; supplemented with 10% fetal calf serum GOLD, 1% penicillin/streptavidine and 1% L-glutamine) in tissue culture flasks (25 cm²) at 37°C and 5% CO₂. Cells were split every 2-3 days after reaching 80-90% confluence. At DIV18 120,000 cells were plated per well in a 12 well plate containing Dulbecco's modified Eagle's medium without antibiotics. Cells were grown for approximately 48 h till 50% confluence. Transfection was performed with Lipofectamine 2000 (Invitrogen) adapting the provided protocol for HEK cells.

[0113] Plating medium of HEK-cells was replaced 10 min before transfection by serum-free DMEM without antibiotics. Cells were incubated 5 h at 37°C with DNA-Lipofectamine complexes. Medium was then changed to fresh serum-containing DMEM without antibiotics. At DIV2 cells were split and plated in Dulbecco's modified Eagle's with antibiotics.

Expression and Purification of His-tagged EPO variants

[0114] His-tagged proteins were transiently expressed in HEK-cells. Medium from HEK293 cells was harvested 2-6 days after transfection with pcDNA-3.1-HIS/V5 A - constructs. Cell debris was pelleted at 3500 rpm, 4°C for 15 min. BD TALON™ Metal Affinity Resin (BD Biosciences) was used for purification of his-tag proteins. All steps (equilibration, washing and elution) were performed at pH 7.1. The provided protocol was modified to a prolonged over-night binding step at 4°C. Eluate was collected in 500 μl-fractions. Fractions were analysed by Western Blots using an anti-rhEPO antibody from Santa-Cruz or a murine EPO ELISA-Kit (R&D). Imidazole was removed from protein-containing fractions using HiTrap™ Desalting columns (5 ml) from Amersham Biosciences according to the manufactures protocol. This included a change of buffer to PBS.

Western Blot

[0115] A 16% SDS-Gel was prepared using standard-protocols and run at 110 V. Blotting was done on nitrocellulose-membranes for 45 min at 200 mA. The blot was blocked for at least one hour in blocking buffer containing 5% non-fat dry milk powder in 0,1% Tween-20. Incubation with the first antibody (EPO (H-162) sc-7956 rabbit polyclonal IgG, Santa Cruz, 1:500) was performed over-night at 4°C. The secondary antibody (goat anti-rabbit HRP; 1:1000) was added for 2 hours at room-temperature. The blot was revealed by use of Luminol; photos were exposed for 2 minutes. Membranes were stained with Ponceau Red. The EPO specific antibody was capable of detection all EPO variants.

Erythroid Colony formation assay

[0116] Bone marrow cells were harvested from tibia and femur of male C57BL/6 mice (8-11 weeks) and resuspended in α-medium (supplemented with 10% fetal calf serum GOLD, 1% penicillium/streptavidine and 1% L-glutamine). Cells were seeded in 35 mm² Petri dishes (225.000 cells/dish) containing 8 parts Metho Cult SF 3236 methyl cellulose (StemCell Technologie Inc), 1 part cells and 2 parts α-medium mixed with HEK-cell preconditioned medium containing the EPO derivatives (150 U/l in the case of murine EPO). 150 U/l of rhEPO (Roche) was used as positive control. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 48 hours. For evaluation only reddish colonies containing at least 6 hemoglobinised cells were taken into account.

Hematopoietic potential of the EPO variants

[0117] Metho Cult SF 3236 triggers the formation of colonies (CFU-M, CFU-G or CFU-E) only after addition of the appropriate cytokines. Formation of CFU-E (Colony forming unit-erythroblast) can be observed, after addition of erythropoietin, after 2 days. The small irregular reddish colonies disappear by day 3.

[0118] In this assay, the hematopoietic potential of the variants was tested and compared to wild type form of EPO as well as rhEPO. The following conditions were prepared for comparison: medium from HEK cells transfected with pZ/EG as negative control, medium from HEK cells transfected with pZ/EG cells plus 150U/l rhEPO (Roche) as a positive control, and medium from HEK cells transfected with either pZ/EG-EPO-IRES (150U/l murine EPO), pZ/EG-Splice-IRES

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(variant S; mS) or pZ/EG-G3-IRES (variant G3; mG3). At DIV2 only reddish colonies were counted containing at least 6 hemoglobinised cells. The results of three independent experiments are depicted in Fig. 5.

[0119] In comparison to murine EPO and rhEPO the murine EPO variants (mS and mG3-variant) lacked haematopoietic potential.

Primary neuronal cultures

[0120] Rat primary neuronal cultures were obtained from E16 to early E19 embryos of Wistar rats (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany). Cre-expressing mouse neurons were obtained from E16 embryos of heterozygous transgenic mice expressing Cre-recombinase under the control of the tubulin α -1 promoter (provided by Dr. U. Schweitzer; Experimental Endocrinology, Charité). Murine and rat cultures were prepared according to a modified protocol from Brewer (1995) J Neurosci Res. 42: 674-83. Cerebral cortex was isolated after removal of meninges and rinsed twice in PBS (Biochrom, Berlin, Germany). After 15 min incubation in trypsin/EDTA (0.05/0.02% w/v in PBS) at 37°C, tissues were rinsed twice in N-Med (modified Eagle's medium from Gibco with 10% fetal calf serum, 100 U penicillin plus streptomycin from Biochrom, 2 mM L-glutamine, 100 IE insulin/I, 10 mM HEPES and 44 mM glucose) and dissociated carefully in a small volume of N-Med using a Pasteur pipette. Cells were pelleted at room temperature by 2 min centrifugation at 210 g and resuspended in NBM starter medium (Neurobasal medium from Gibco with 2% B27 supplement from Gibco, 1% Pen/Strep, 0,5 mM L-glutamine and 25 μ M glutamate).

Preparation of culture plates

[0121] 24-well plates and 6-well plates were pretreated by over-night incubation at 4°C with poly-L-lysine from Biochrom (2.5 μ g/ml in PBS). Rinsing of the wells with PBS was followed by 1h incubation at 37°C with coating medium (modified Eagle's medium with 5% FCS Gold from PAA, 1% Pen/Strep, 10mM HEPES and 0,03 w/v collagen G from Biochrom), then the wells were carefully rinsed twice with PBS. Volume and type of plating medium was chosen depending on experimental procedure.

Oxygen glucose deprivation in rat primary cortical neurons - a cell culture model of cerebral ischemia

[0122] For OGD the culture medium was washed out by rinsing once with PBS. OGD was induced with 500 μ l of a deoxygenated aglycemic solution (BSS₀-O₂; 143.8 mM Na⁺, 5.5 mM K⁺, 1.8 mM Ca²⁺, 0.8 mM Mg²⁺, 125.3 mM Cl⁻, 26.2 mM HCO₃⁻ and 0.8 mM SO₄²⁻, pH 7.4) in a hypoxic atmosphere generated by a dedicated, humidified gas-tight incubator (Concept 400, Ruskinn Technologies, Bridgend, UK) flushed with a gas mix containing 5% CO₂, 85% N₂ and 10% H₂. OGD-time depended on the density and the age of the culture and varied between 2 h 30min and 2 h 40min. In control experiments the wells were treated with 500 μ l of the oxygenated glycolic BSS₀ solution (BSS₀+O₂; 143.8 mM Na⁺, 5.5 mM K⁺, 1.8 mM Ca²⁺, 0.8 mM Mg²⁺, 125.3 mM Cl⁻, 26.2 mM HCO₃⁻, 0.8 mM SO₄²⁻, and 20 mM glucose, pH 7.4) and incubated at 37°C in a normoxic atmosphere containing 5% CO₂. Immediately after OGD, treated cells and controls were changed from BSS solution to 500 μ l of medium containing 40% conditioned NBM plus 60% fresh NBM. After 24h, lactate dehydrogenase (LDH) activity was measured in the supernatants as an indicator of cell death.

[0123] For LDH measurement 25 μ l of medium was mixed with 100 μ l fresh β -NADH solution (0.15 mg/ml in 1xLDH-buffer; Sigma, reduced form) in a 96 wells plate (Greiner). 25 μ l of 22.7 mM pyruvate (Sigma) was added immediately before placing the plate into the Reader (Thermo Labsystems; MRXTC Revelation). Parameters were chosen as follows: filter: 340 nm, shake time: 5 sec, interval: 30 sec, counts: 10. LDH-concentration was calculated proportionally to the LDH-standard (Greiner, system calibrator).

Induction of neuroprotection by conditioned medium from transfected HEK293 cells expressing EPO variants

[0124] In the following experiments rhEPO (recombinant human EPO from Sigma Aldrich, Deisenhofen, Germany) was used as a positive control. Neuroprotection assays are schematically depicted in Fig. 6. Neurons were plated in 24-well plates at a density of 300,000 cells in a final volume of 600 μ l NBM starter medium. After 4 days, 200 μ l of the medium was replaced by 250 μ l fresh NBM (same as NBM starter without glutamate).

[0125] For pretreatment with rhEPO, wild type mEPO, wild type hEPO or EPO variants the medium was removed to an end volume of 200 μ l and filled up with 200 μ l fresh NBM+B27 containing equimolar amounts (corresponding to 200 U/l rhEpo) of EPO or EPO variants, respectively. Equivalent concentrations of the various EPO variants (as well as mEPO and hEPO) in the conditioned medium from HEK293 cells were estimated by Western blot and EPO-Elisa. Thereafter neurons were grown for 48 h under normoxic, humidified conditions at 37°C before oxygen glucose deprivation (OGD) was performed (OGD interval as indicated). Cell death was assessed 24 h after OGD by measurement of LDH release. Reduction in LDH release, compared to mock-treated neurons (medium from HEK293 cells transfected with

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the backbone plasmid; = ko; 100%), is a quantitative measure of neuroprotection. In all experiments we observed a more robust neuroprotective effect provided by the EPO variants, if compared to wt EPO (see Fig. 7 Panel A and B for murine EPO and variants thereof and Fig. 8 Panel A and B for human EPO and variants thereof).

[0126] The neuroprotection induced by the murine EPO variants is more robust than that induced by EPO (rhEPO as well as wild type mouse EPO). For example, neuroprotection mediated by EPO can only be observed in a clearly defined window of OGD length (corresponding to a clearly defined damage level). At low concentration the neuroprotection by hS3 and hS4 was equal or better than the neuroprotection of wt hEPO. Overall, neuroprotection induced by the variants is stronger than that induced by rhEPO. In addition, variants have an higher neuroprotective potential than both wild type forms mEPO and hEPO, which were produced by the same procedure as the EPO variants.

H9c2 - model of ischemia

[0127] The rat BDIX heart myoblast cell line (obtained from European Collection of Cell Cultures) was cultured in DMEM (Biocrom) containing 4.5g/l glucose supplemented with 2mM L-glutamine, 10% inactivated fetal calf serum and 1% penicillin-streptavidin. Subconfluent cultures (70%) were subcultured 1:4. Cells were plated in 400µl medium containing 120pM hEPO or hS3 respectively in a density of 15,000 cells per well in 24-well plates and cultured for 48 hours. Hypoxia was achieved by culturing the cells in 400µl serum-deficient DMEM containing 4.5g/l glucose supplemented with 2mM L-glutamine and 1% penicillin-streptavidin and leaving them for 24h in an anaerobic workstation (Concept 400, Ruskinn Technologies, Bridgend, UK) saturated with a gas mix containing 5% CO₂, 85% N₂ and 10% H₂ at 37°C. Control cells were left in serum-deficient DMEM in a normoxic incubator. At the end of the experiment medium was replaced to 400µl fresh serum-deficient DMEM and LDH was measured according to standard protocols 24h later.

Immunoprecipitation

[0128] Male 129S6 mice or male C57Bl6 mice (8-10 weeks, Bundesinstitut für Risikobewertung, Berlin) having free access to food and water were used for the experiments. CoCl₂ was injected subcutaneously in a dose of 60mg/kg and animals were killed 18 hours later. Protein expression was measured in serum, kidney and brain protein extracts by a commercial available ELISA (R&D, mEPO).

[0129] Antibodies for immunoprecipitation were purchased from R&D (anti-mEPO antibody, goat, biotin-labelled) and Santa-Cruz (anti-rhEPO, rabbit). Immunoprecipitation was performed according to standard protocols and evaluated by western blot.

[0130] Blocking of the western blot detection antibody was achieved by two hours incubation with 10µg DarbpoietinA at room temperature prior to blot incubation.

Generation of alpha-helix-mutants (Fig. 10)

[0131] Human alpha-helix-mutants were all generated by PCR based approaches using standard protocols.

[0132] Mutant A (hAmA) and mutant E (hAmE) are variants of the alpha-helix with amino acid exchange at position 41 (arginine). cDNA sequence was changed from AGG to GCG for mutant A (alanine) or to GAG for mutant E (glutamate). -20aa and -10aa are deletion variants of the alpha-helix missing 20 amino acids or 10 amino acids respectively at the c-terminus. All mutants were generated without V5 and His-tag and expressed in HEK 293 cells. Neuroprotection experiments were performed as described previously using medium of transfected HEK cells expressing the different variants.

hEPO and hS3 mediated cytoprotection in a model of ischemia in H9c2 cells (Fig. 11)

[0133] The cytoprotective potential of the EPO variants was shown exemplarily for purified hEPO and hS3 in a model of ischemia consisting of serum deprivation and hypoxia in H9c2 cardiac myoblasts (Figure 1). LDH release was assessed as a marker of apoptotic cell death. We found significant cytoprotective capacities for both variants (approximately 20% and 25% for hEPO and hS3).

Immunoprecipitation reveals EPO splicing isoform in kidney protein extracts of CoCl₂-treated mice (Fig. 12)

[0134] To strengthen our finding of EPO splicing isoforms in human and murine tissues by a PCR-based approach we performed immunoprecipitations on murine serum, brain and kidney protein extracts of CoCl₂ treated mice using antibodies tested to recognize both isoforms. Subcutane injection of CoCl₂ is known to increase erythropoietin levels in several mouse tissues, namely blood, brain, liver and kidney.

[0135] We were able to precipitate erythropoietin (approximately 40kDa) from serum, brain and kidney protein extracts

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of CoCl₂ treated mice (Figure2); precipitation of erythropoietin from a kidney protein extract of an untreated mouse failed due to the low expression level. Furthermore we were able to prove the existence of a second smaller protein (approximately 30kDa) in the kidney protein extract of CoCl₂ treated mice. This protein is specifically recognized by the anti-rhEPO antibody as shown by complete blocking of the antibody-antigene interaction with Darbpoietin A. These findings strongly support the existence of a murine erythropoietin splicing isoform. These results were reproduced in a second mouse strain, namely C57Bl6.

Neuroprotection mediated by different isoforms of the erythropoietin alpha-helix (Fig. 13)

[0136] Analysing the neuroprotective potentials of the so far identified erythropoietin variants we suggested the alpha helix to be the functionally important domain for the neuroprotective character of erythropoietin. In order to test this hypothesis we expressed a shortened form of human erythropoietin, namely the alpha-helix domain, in HEK 293 cells and tested this peptide in our OGD-model. We found an equivalent protective potential with 30pM and 15pM of this peptide to 30pM of hEPO as shown in Figure 13.

[0137] In order to identify the functional important residues in the alpha helix domains of human erythropoietin we generated different erythropoietin mutants containing either amino acid exchanges (hAmA and hAmE) or complete domain deletions (hA-10 and hA-20).

[0138] Neither the neutral nor the acidic amino acid exchange at position 41 was able to destroy the neuroprotective potential of the alpha-helix in our OGD model (Figure 14).

Neuroprotection mediated by several human EPO-isoforms (n=6) $P^* < 0,05$; ANOVA1 versus control (Fig. 14)

[0139] Deletion variants missing 10 or 20 amino acids at the c-terminus of the alpha-helix were expressed in HEK293 cells and also tested in the OGD-model. The deletion variant hA-10 had still neuroprotective properties comparable to the hS3 splice isoform. Deletion of 20 amino acids (hA-20) led to a peptide that was not protective anymore (Fig. 15).

Immunomodulation by human erythropoietin variants (Fig. 16)

[0140] The human EPO variants hS3 and hS4 exhibit strong immunomodulatory effects. In human macrophages stimulated with the endotoxin lipopolysaccharide (LPS) hS3 and hS4 induce the anti-inflammatory cytokine IL-10 and reduce the expression of the pro-inflammatory cytokines IL-6 and IL-8. Compared to EPO (hWT) anti-inflammatory effects of hS3 and hS4 are much more pronounced. These anti-inflammatory properties of EPO variants are useful in treatment of inflammatory (e.g. Multiple Sclerosis, viral and bacterial infections, sepsis) and degenerative diseases (e.g. stroke, myocardial infarctions).

SEQUENCE LISTING

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 40 Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu
 65 70 75 80

45

50

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Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys
85 90 95

5 Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly
100 105 110

10 Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro
115 120 125

15 Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr
130 135 140

Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys
145 150 155

20 Arg Thr Gly Asp Arg
160 164

<210> 3
25 <211> 525
<212> DNA
<213> Homo sapiens

<400> 3

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35 aggtacctct tggaggccaa ggaggccgag aatatcacga cgggctgtgc tgaacactgc 180
agcttgaatg agaatatcac tgtcccagac accaaagtta atttctatgc ctggaagagg 240
atggaggctcg ggcagcaggc cctgttggtc aactcttccc agcctgaggga gcccctgcag 300
40 ctgcatgtgg ataaagccgt cagtggcctt cgcagcctca ccactctgct tcgggctctg 360
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ctgaagctgt acacagggga ggcctgcagg acaggggaca gatga 525

<210> 4
50 <211> 174
<212> PRT
<213> Homo sapiens

<400> 4

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EP 1 885 747 B1

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
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 Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30
 10
 Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45
 Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60
 15
 Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
 65 70 75 80
 20
 Met Glu Val Gly Gln Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp
 85 90 95
 25
 Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser
 100 105 110
 30
 Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser
 115 120 125
 35
 Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp
 130 135 140
 40
 Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys
 145 150 155 160
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<210> 5

<211> 495

<212> DNA

45 <213> Homo sapiens

<400> 5

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EP 1 885 747 B1

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10 cgcagcctca ccactctgct tcgggctctg ggagccaga aggaagccat ctccctcca 360
gatgcggcct cagctgctcc actccgaaca atcactgctg acactttccg caaactcttc 420
cgagtctact ccaatttcct ccggggaaag ctgaagctgt acacagggga ggcctgcagg 480
15 acaggggaca gatga 495

<210> 6
20 <211> 164
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<213> Homo sapiens
25 <400> 6

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EP 1 885 747 B1

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Ser Leu
 1 5 10 15
 5 Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30
 10 Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45
 Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60
 15 Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Leu Leu Val
 65 70 75 80
 20 Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala
 85 90 95
 25 Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala
 100 105 110
 Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu
 115 120 125
 30 Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser
 130 135 140
 35 Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg
 145 150 155 160
 Thr Gly Asp Arg
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 <210> 7
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 <212> DNA
 <213> Homo sapiens
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 aggtacctct tggaggccaa ggaggccgag aatatcacga cgggctgtgc tgaacactgc 180
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EP 1 885 747 B1

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 5 ctcaccactc tgcttcgggc tctggggagcc cagaaggaag ccattctccc tccagatgag 360
 gcctcagctg ctccactccg aacaatcact gctgacactt tccgcaaact cttccgagtc 420
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 10 gacagatga 489

<210> 8
 <211> 162
 15 <212> PRT
 <213> Homo sapiens

<400> 8

20 Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
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 25 Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30
 30 Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45
 Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60
 35 Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
 65 70 75 80
 40 Met Glu Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser
 85 90 95
 Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys
 100 105 110
 45 Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr
 115 120 125
 50 Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe
 130 135 140
 55 Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly
 145 150 155 160
 Asp Arg

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<210> 9
 <211> 475
 <212> DNA
 <213> Homo sapiens

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<400> 9

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 agcttgaatg agaatatcac tgtcccaggc cctgttggtc aactcttccc agcctgggga 240
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 tcgggctctg ggagcccaga aggaagccat cccccctcca gatgcggcct cagctgctcc 360
 actccgaaca atcactgctg acactttccg caaactcttc cgagtctact ccaatttcct 420
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<210> 10
 <211> 87
 <212> PRT
 <213> Homo sapiens

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<400> 10

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Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
 1 5 10 15

35

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30

40

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45

45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60

50

Asn Ile Thr Val Pro Gly Pro Val Gly Gln Leu Phe Pro Ala Val Gly
 65 70 75 80

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Ala Pro Ala Ala Ala Cys Gly
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<210> 11
 <211> 301
 <212> DNA
 <213> Homo sapiens

<400> 11

EP 1 885 747 B1

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 5 ctgggcctcc cagtcctggg cgcctcatct gtgacagccg agtcctggag 120
 aggtacctct tggaggccaa ggaggccgag aatatcacga cgggctgtgc tgaacctgc 180
 agcttgaatg agaacaatca ctgctgacac tttccgcaa ctcttccgag tctactccaa 240
 10 tttcctccgg gaaagctga agctgtacac aggggaggcc tgcaggacag gggacagatg 300
 a 301

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 15 <211> 68
 <212> PRT
 <213> Homo sapiens
 <400> 12

20 Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
 1 5 10 15
 25 Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30
 30 Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45
 Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60
 35 Asn Asn His Cys
 65

<210> 13
 40 <211> 399
 <212> DNA
 <213> Mus musculus
 <400> 13

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 50 tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180
 ctgagtgaat atattacagt ccagatacc aaagtcaact tctatgcttg gaaaagaatg 240
 gagaaggaat tgatgtgcc tccagatacc accccacctg ctccactccg aacactcaca 300
 55 gtggatactt tctgcaagct cttccgggtc tacgccaaact tctccgggg gaaactgaag 360
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EP 1 885 747 B1

<210> 14
 <211> 132
 <212> PRT
 <213> Mus musculus

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<400> 14

Met Gly Val Pro Glu Arg Pro Thr Leu Leu Leu Leu Leu Ser Leu Leu
 1 5 10 15

10

Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30

15

Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45

20

Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60

25

Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met
 65 70 75 80

30

Glu Lys Glu Leu Met Ser Pro Pro Asp Thr Thr Pro Pro Ala Pro Leu
 85 90 95

35

Arg Thr Leu Thr Val Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr Ala
 100 105 110

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Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Val Cys Arg
 115 120 125

Arg Gly Asp Arg
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<210> 15
 <211> 387
 <212> DNA
 <213> Mus musculus

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<400> 15

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EP 1 885 747 B1

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 5 tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180
 ctgagtgaaa atattacagt cccagatacc aaagtcaact tctatgcttg gaaaagaatg 240
 gaggtggaag aacaggccat agaagtttgg caaggcctgt ccctgctctc agaagccatc 300
 10 ctgcaggccc aggcctgct agccaacttc ctccggggga aactgaagct gtacacggga 360
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15 <210> 16
 <211> 128
 <212> PRT
 <213> Mus musculus

20 <400> 16

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 1 5 10 15

25 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30

30 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45

35 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60

40 Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met
 65 70 75 80

Glu Val Glu Glu Gln Ala Ile Glu Val Trp Gln Gly Leu Ser Leu Leu
 85 90 95

45 Ser Glu Ala Ile Leu Gln Ala Gln Ala Leu Leu Ala Asn Phe Leu Arg
 100 105 110

50 Gly Lys Leu Lys Leu Tyr Thr Gly Glu Val Cys Arg Arg Gly Asp Arg
 115 120 125

55 <210> 17
 <211> 513
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<400> 17

EP 1 885 747 B1

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5 tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180
ctgagtgaaa atattacagt cccagatacc aaagtcaact tctatgcttg gaaaagaatg 240
gaggtggaag aacaggccat agaagtttgg caaggcctgt ccctgctctc agaagccatc 300
10 ctgcaggccc aggccctgct agccaattcc tcccagccac cagagaccct tcagcttcat 360
atagacaaag ccatcagtgg tctacgtagc ctcaactcac tgcttcgggt actgggagct 420
cagaagggaat tgatgtcgcc tccagatacc accccacctg ctccactccg aacactcaca 480
15 gtggatactt tctgcaggag aggggacagg tga 513

<210> 18
20 <211> 170
<212> PRT
<213> Mus musculus

<400> 18
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EP 1 885 747 B1

Met Gly Val Pro Glu Arg Pro Thr Leu Leu Leu Leu Ser Leu Leu
 1 5 10 15

5 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30

10 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45

15 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60

Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met
 65 70 75 80

20 Glu Val Glu Glu Gln Ala Ile Glu Val Trp Gln Gly Leu Ser Leu Leu
 85 90 95

25 Ser Glu Ala Ile Leu Gln Ala Gln Ala Leu Leu Ala Asn Ser Ser Gln
 100 105 110

30 Pro Pro Glu Thr Leu Gln Leu His Ile Asp Lys Ala Ile Ser Gly Leu
 115 120 125

Arg Ser Leu Thr Ser Leu Leu Arg Val Leu Gly Ala Gln Lys Glu Leu
 130 135 140

35 Met Ser Pro Pro Asp Thr Thr Pro Pro Ala Pro Leu Arg Thr Leu Thr
 145 150 155 160

40 Val Asp Thr Phe Cys Arg Arg Gly Asp Arg
 165 170

<210> 19

<211> 279

<212> DNA

45 <213> Mus musculus

<400> 19

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tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180

55 ctgagtgaat atattacagt ccagataacc aaagtcaact tcctccgggg gaaactgaag 240

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EP 1 885 747 B1

<210> 20
 <211> 92
 <212> PRT
 <213> Mus musculus

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<400> 20

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 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30
 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45
 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60
 Ile Thr Val Pro Asp Thr Lys Val Asn Phe Leu Arg Gly Lys Leu Lys
 65 70 75 80
 Leu Tyr Thr Gly Glu Val Cys Arg Arg Gly Asp Arg
 85 90

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<210> 21
 <211> 591
 <212> DNA
 <213> Mus musculus

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<400> 21

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 tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180
 ctgagtgaat atattacagt ccagataacc aaagtcaact tctatgcttg gaaaagaatg 240
 gaggtggaag aacaggccat agaagtttgg caaggcctgt ccctgctctc agaagctgta 300
 cacgggagag gtctgcagga gaggggacag gtgacatgct gctgccaccg tgggtggaccg 360
 acgaacttgc tccccgtcac tgtgtcatgc caaccctcca ccactcccaa ccctcatcaa 420
 acgggtcatt accttcttac cagtctgtcc catggacact ccagcaccag cagtgcacac 480
 ctctggggcca gaagaacttc ccagagctcc attctgaaat ctaaagatgt cgctggacaa 540
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<210> 22
 <211> 196
 <212> PRT

EP 1 885 747 B1

<213> Mus musculus

<400> 22

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 1 5 10 15
 10 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30
 15 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45
 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60
 20 Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met
 65 70 75 80
 25 Glu Val Glu Glu Gln Ala Ile Glu Val Trp Gln Gly Leu Ser Leu Leu
 85 90 95
 30 Ser Glu Ala Val His Gly Arg Gly Leu Gln Glu Arg Gly Gln Val Thr
 100 105 110
 Cys Cys Cys His Arg Gly Gly Pro Thr Asn Leu Leu Pro Val Thr Val
 115 120 125
 35 Ser Cys Gln Pro Ser Thr Thr Pro Asn Pro His Gln Thr Gly His Tyr
 130 135 140
 40 Leu Leu Thr Ser Leu Ser His Gly His Ser Ser Thr Ser Ser Asp Ile
 145 150 155 160
 45 Leu Gly Ala Arg Arg Thr Ser Gln Ser Ser Ile Leu Lys Ser Lys Asp
 165 170 175
 Val Ala Gly Gln Ala Arg Gly Pro Arg Glu Glu Glu Pro Gln Asn Gln
 180 185 190
 50 Leu Gly Phe Val
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<210> 23

<211> 159

55 <212> DNA

<213> Homo sapiens

<400> 23

EP 1 885 747 B1

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 5 aggtacctct tggaggccaa ggaggccgag aatatcacg 159

<210> 24

<211> 53

<212> PRT

10 <213> Homo sapiens

<400> 24

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 1 5 10 15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
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20

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 35 40 45

Ala Glu Asn Ile Thr
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<210> 25

<211> 246

30 <212> DNA

<213> Homo sapiens

<400> 25

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 35 aggtacctct tggaggccaa ggaggccgag aatatcacga cgggctgtgc tgaacactgc 180
 40 agcttgaatg agaatatcac tgtcccagac accaaagtta atttctatgc ctggaagagg....240
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45 <210> 26

<211> 82

<212> PRT

<213> Homo sapiens

50 <400> 26

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EP 1 885 747 B1

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Ser Leu
 1 5 10 15
 5 Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
 20 25 30
 10 Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
 Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
 50 55 60
 15 Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
 65 70 75 80
 20 Met Glu
 20 <210> 27
 <211> 243
 <212> DNA
 <213> Mus musculus
 25 <400> 27
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 35 gag 243
 40 <210> 28
 <211> 81
 <212> PRT
 <213> Mus musculus
 <400> 28
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EP 1 885 747 B1

Met Gly Val Pro Glu Arg Pro Thr Leu Leu Leu Leu Ser Leu Leu
 1 5 10 15

5 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
 20 25 30

10 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
 35 40 45

15 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
 50 55 60

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 65 70 75 80

20 Glu

<210> 29
 <211> 326
 <212> DNA
 25 <213> Mus musculus

<400> 29

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 tacatcttag aggccaagga ggcagaaaat gtcacgatgg gttgtgcaga aggtcccaga 180
 35 ctgagtgaag atattacagt cccagatacc aaagtcaact tctatgcttg gaaaagaatg 240
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 ctgcaggccc aggccctgct agccaa 326

40 <210> 30
 <211> 109
 <212> PRT
 <213> Mus musculus

45 <400> 30

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EP 1 885 747 B1

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1 5 10 15

5 Leu Ile Pro Leu Gly Leu Pro Val Leu Cys Ala Pro Pro Arg Leu Ile
20 25 30

10 Cys Asp Ser Arg Val Leu Glu Arg Tyr Ile Leu Glu Ala Lys Glu Ala
35 40 45

15 Glu Asn Val Thr Met Gly Cys Ala Glu Gly Pro Arg Leu Ser Glu Asn
50 55 60

Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met
65 70 75 80

20 Glu Val Glu Glu Gln Ala Ile Glu Val Trp Gln Gly Leu Ser Leu Leu
85 90 95

25 Ser Glu Ala Ile Leu Gln Ala Gln Ala Leu Leu Ala Asn
100 105

<210> 31
<211> 336
<212> DNA
30 <213> Homo sapiens

<400> 31

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cggggccagg cctgttggt caactcttcc cagccgtggg agcccctgca gctgcatgtg 120
gataaagccg tcagtggcct tcgcagcctc accactctgc ttcgggctct gggagcccag 180

40 gataaagccg tcagtggcct tcgcagcctc accactctgc ttcgggctct gggagcccag 180
aaggaagcca tctcccctcc agatggggcc tcagctgctc cactccgaac aatcactgct 240
gacactttcc gcaaactctt ccgagtctac tccaatttcc tccggggaaa gctgaagctg 300

45 tacacagggg aggcctgcag gacaggggac agatga 336

<210> 32
<211> 111
50 <212> PRT
<213> Homo sapiens

<400> 32

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EP 1 885 747 B1

Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu
 1 5 10 15

5 Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser Gln
 20 25 30

10 Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly Leu
 35 40 45

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10 Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr
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35

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 0035475 A2 [0010]
- WO 0220031 A2 [0010]
- US 20030130197 A [0011]
- US 6531121 B [0011]
- WO 2005025606 A1 [0011]
- WO 2004043382 A [0012]
- WO 9505465 A [0061]
- WO 9105867 A [0061]
- WO 9409257 A [0061]
- WO 0181405 A [0061]
- WO 0032772 A [0061]
- WO 0102017 A [0061]
- WO 03029291 A [0061]
- WO 2005025606 A [0061]
- WO 9714307 A [0076]
- WO 9104014 A [0094]
- US 4704355 A [0094]
- US 4798824 A [0100]

Non-patent literature cited in the description

- DIRNAGL, U. et al. *Trends Neurosci.*, 1999, vol. 22, 391-397 [0006]
- ZHU Y. ; D'ANDREA A.D. *Curr. Opin. Hematol.*, 1994, vol. 1, 113-118 [0009]
- RUSCHER et al. *J. Neurosci.*, 2002, vol. 22, 10291-301 [0010]
- PRASS et al. *Stroke*, 2003, vol. 34, 1981-1986 [0010]
- BERNAUDIN, M. et al. *J Cereb Blood Flow Metab.*, 1999, vol. 19, 643-51 [0010]
- MORISHITA, E. et al. *Neuroscience*, 1997, vol. 76, 105-16 [0010]
- HENN F.A ; BRAUS D.F. *Eur. Arch. Psychiatry Clin. Neurosci.*, 1999, vol. 249, 48-56 [0010]
- VOGLEY K. et al. *Am. J. Psychiatry*, 2000, vol. 157, 34-39 [0010]
- BRINES M.L. et al. *Proc. Natl. Acad. Sci. U.S.A.*, 2000, vol. 97, 10526-10531 [0010]
- BERNAUDIN et al. *J. Cereb. Blood Flow Metab.*, 1999, vol. 10, 643-651 [0010]
- EHRENREICH H. et al. *Mol. Medicine*, 2002, vol. 8, 495-505 [0010]
- EHRENREICH H et al. *Molecular Psychiatry*, 2004, vol. 9, 42-54 [0010]
- LEIST et al. *Science*, 2004, vol. 305, 239-242 [0011]
- CAMPANA W.M. et al. *Int. J. Mol. Medicine*, 1998, vol. 1, 235-241 [0012]
- KARLIN ; ALTSCHUL. *Proc. Natl. Acad. Sci. USA*, 1993, vol. 90, 5873-5877 [0048]
- ALTSCHUL et al. *J. Mol. Biol.*, 1990, vol. 215, 403-410 [0048]
- ALTSCHUL et al. *Nucleic Acids Res.*, 1997, vol. 25, 3389-3402 [0048]
- Current Protocols in Molecular Biology. John Wiley & Sons, 1991, 6.3.1-6.3.6 [0049]
- CHANG, L.J. ; GAY, E.E. *Curr. Gene Therap.*, 2001, vol. 1, 237-251 [0052]
- CARLEZON, W.A. et al. *Crit. Rev. Neurobiol.*, 2000 [0052]
- AAV, CARTER, P.J. ; SAMULSKI, R.J. *J. Mol. Med.*, 2000, vol. 6, 17-27 [0052]
- COBINGER G. P. et al. *Nat. Biotechnol.*, 2001, vol. 19, 225-30 [0052]
- LINDEMANN et al. *Mol. Med.*, 1997, vol. 3, 466-76 [0052]
- SPRINGER et al. *Mol. Cell*, 1998, vol. 2, 549-58 [0052]
- SAMBROOK et al. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, 1989 [0065]
- AUSUBEL et al. *Current Protocols in Molecular Biology*. Green Publishing Associates and Wiley Interscience, 1989 [0065]
- BIRD R. E. et al. *Science*, 1988, vol. 242, 423-6 [0070]
- HOLLIGER P. et al. *Proc. Natl. Acad. Sci. U.S.A.*, 1993, vol. 90, 6444-8 [0070]
- SEFTON. *CRC Crit. Ref. Biomed. Eng.*, 1987, vol. 14, 201 [0094]
- BUCHWALD et al. *Surgery*, 1980, vol. 88, 507 [0094]
- SAUDEK et al. *N. Eng. J. Med.*, 1989, vol. 321, 574 [0094]
- LANGER. *Science*, 1990, vol. 249, 1527-1533 [0094]
- TREAT et al. *Liposomes in the Therapy of Infectious Disease and Cancer*. Liss, 1989, 353-365 [0094]
- Medical Applications of Controlled Release. CRC Press, 1974 [0094]
- Controlled Drug Bioavailability, Drug Product Design and Performance. Wiley, 1984 [0094]
- RANGER ; PEPPAS. *J. Macromol. Sci. Rev. Macromol. Chem.*, 1953, vol. 23, 61 [0094]
- LEVY et al. *Science*, 1985, vol. 228, 190 [0094]
- DURING et al. *Ann. Neurol.*, 1989, vol. 25, 351 [0094]

EP 1 885 747 B1

- **HOWARD et al.** *J. Neurosurg.*, 1989, vol. 71, 105 [0094]
- **GOODSON.** *Medical Applications of Controlled Release*, 1984, vol. 2, 115-138 [0095]
- **BREWER.** *J Neurosci Res.*, 1995, vol. 42, 674-83 [0120]

ERYTHROPOIETINVARIANTER

PATENTKRAV

- 5 1. Polynukleotid, der koder for erythropoietin (EPO)-variant, hvilket polynukleotid er udvalgt fra gruppen bestående af:
- (a) polynukleotider, der koder for den modne form af polypeptiderne betegnet hs3, h1-4, h1-5, hs4, h1-1, h2-1, mS, mG3, mG5, m301, mK3, ha, hAma, hAmE og hA-10 med den afledte aminosyresekvens ifølge henholdsvis
10 SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 50, 51, 52 og 53;
 - (b) et polynukleotid, der koder for den modne form af polypeptidet betegnet ha-sekvens uden leder bestående af den afledte aminosyresekvens ifølge SEQ ID NO: 61;
 - (c) polynukleotider med den kodende sekvens ifølge SEQ ID NO: 1, 3, 5, 7, 9,
15 11, 13, 15, 17, 19, 21, 55, 56, 57 og 58, der koder for mindst den modne form af polypeptidet;
 - (d) et polynukleotid, der består af den kodende sekvens ifølge SEQ ID NO: 60, hvilket polynukleotid koder for den modne form af polypeptidet betegnet ha-sekvens uden leder;
 - 20 (e) polynukleotid, der koder for en humaniseret version af polypeptiderne mS, mG3, mG5, m301 og mK3, som består af den afledte aminosyresekvens ifølge SEQ ID NO: 14, 16, 18, 20 og 22,
 - (f) polynukleotider, der koder for et polypeptid omfattende en fusion af en aminosyresekvens udvalgt fra gruppen af aminosyresekvenser ifølge SEQ
25 ID NO: 24, 26, 28 og 30 ved N-terminus af en aminosyresekvens udvalgt fra gruppen af aminosyresekvenser ifølge SEQ ID NO: 32, 34, 36 og 38, hvor fusionen har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
 - (g) polynukleotider, som omfatter en fusion af polynukleotidsekvenser udvalgt
30 fra gruppen af polynukleotidsekvenser ifølge SEQ ID NO: 23, 25, 27 og 29 i 5'-enden af en polynukleotidsekvens udvalgt fra gruppen af polynukleotidsekvenser ifølge SEQ ID NO: 31, 33, 35 og 37, hvor fusionen har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;

- (h) polynukleotider, der koder for et derivat af et polypeptid kodet for af et polynukleotid ifølge et hvilket som helst af (a) til (g), hvor, i derivatet, mellem 1 og 10 aminosyrerester er traditionelt substitueret sammenlignet med polypeptidet, og derivatet har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- (i) polynukleotider, der koder for et fragment af n polypeptid kodet for af et polynukleotid ifølge et hvilket som helst af (a) til (h), hvor, i fragmentet, mellem 1 og 10 aminosyrerester er deleteret N- og/eller C-terminalt og/eller mellem 1 og 10 aminosyrer er deleteret N- og/eller C-terminalt i forhold til fusionsstedet sammenlignet med proteinet, og fragmentet har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- (j) polynukleotider, som er mindst 95% identiske med et polynukleotid som defineret ifølge et hvilket som helst af (a) til (d), og som samtidig har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- (k) polynukleotider, hvis komplementære streng under stringente betingelser hybridiserer til et polynukleotid som defineret ifølge et hvilket som helst af (a) til (j), og som koder for et polypeptid med cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- (l) polynukleotider, der koder for et EPO-variant-polypeptid, som omfatter den N-terminale del af fuldlængde-EPO indbefattende helix A, og som mangler mindst én af følgende:
- (i) et fragment på mindst 10 aminosyrer mellem helix A og helix B;
 - (ii) et fragment på mindst 10 aminosyrer fra helix B;
 - (iii) et fragment på mindst 6 aminosyrer mellem helix B og helix C;
 - (iv) et fragment på mindst 10 aminosyrer fra helix C;
 - (v) et fragment på mindst 20 aminosyrer mellem helix C og helix D;
- og/eller
- (vi) et fragment på mindst 10 aminosyrer fra helix D;
- hvor varianten har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;

- (m) polynukleotider, der koder for et derivat af et polypeptid kodet for af et polynukleotid ifølge et hvilket som helst af (l), hvor, i derivatet, mellem 1 og 10 aminosyrerester er traditionelt substitueret sammenlignet med polypeptidet, og derivatet har cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- (n) polynukleotider, hvis komplementære streng under stringente betingelser hybridiserer til et polynukleotid som defineret ifølge et hvilket som helst af (l) til (m), og som koder for et polypeptid med cellebeskyttende og navnlig neurobeskyttende aktivitet, men i det væsentlige ingen hæmatopoietisk aktivitet;
- eller et sådant polynukleotids komplementære streng.
2. Polynukleotid ifølge krav 1, som er DNA, genomisk DNA eller RNA.
3. Vektor, som indeholder polynukleotidet ifølge krav 1 eller 2.
4. Vektor ifølge krav 3, hvori polynukleotidet er operativt bundet til ekspressionskontrolsekvenser, der muliggør ekspression i prokaryote og/eller eukaryote værtsceller.
5. Værtscelle, der er genetisk modificeret med polynukleotidet ifølge krav 1 eller 2 eller vektoren ifølge krav 3 eller 4.
6. Transgent ikke-humant pattedyr udvalgt fra gruppen af ikke-human primat, hest, kvæg, får, ged, gris, hund, kat, kanin, mus, rotte, marsvin, hamster og ørkenrotte, hvilket pattedyr omfatter polynukleotidet ifølge krav 1 eller 2, en vektor ifølge krav 3 eller 4 og/eller en værtscelle ifølge krav 5.
7. Fremgangsmåde til frembringelse af et EPO-variant-polypeptid kodet for af polynukleotidet ifølge krav 1 eller 2, hvilken fremgangsmåde omfatter: dyrkning af værtscellen ifølge krav 5 og indvinding af polypeptidet kodet for af polynukleotidet.
8. Fremgangsmåde ifølge krav 7, hvilken fremgangsmåde endvidere omfatter trinnet med modificering af EPO-varianten, hvor modifikationen er udvalgt fra

gruppen bestående af oxidation, sulfatering, phosphorylering, addition af oligo-saccharider og kombinationer deraf.

9. Fremgangsmåde til frembringelse af celler, der kan udtrykke mindst én af
5 EPO-varianterne, hvilken fremgangsmåde omfatter genetisk modificering af celler in vitro med vektoren ifølge krav 3 eller 4, hvor EPO-variant-polypeptidet eller EPO-variant-polypeptiderne kodes for af polynukleotidet ifølge krav 1 eller 2.
10. Polyprotein med aminosyresekvensen kodet for af polynukleotidet ifølge
10 krav 1 eller 2 eller opnåeligt ved fremgangsmåden ifølge krav 7 eller 8.
11. Farmaceutisk sammensætning, som omfatter polynukleotidet ifølge krav 1 eller 2, en vektor ifølge krav 3 eller 4, en værtselle ifølge krav 5 og/eller et poly-peptid ifølge krav 10 og en eller flere farmaceutisk acceptable bærere.
15
12. Anvendelse af polynukleotidet ifølge krav 1 eller 2, en vektor ifølge krav 3 eller 4, en værtselle ifølge krav 5 eller et polyprotein ifølge krav 10 til fremstilling af et lægemiddel til behandling eller forebyggelse af en tilstand associeret med vævsbeskadigelse som følge af celledød.
20
13. Anvendelse ifølge krav 12, hvor celledøden er induceret af iskæmi, hypoksi, bakterieinfektion, virusinfektion eller er induceret autoimmunologisk, traumatisk, kemisk eller ved stråling.
- 25 14. Anvendelse ifølge krav 12 eller 13, hvor tilstanden er en akut eller kronisk neurodegenerativ og/eller neuroinflammatorisk lidelse, en akut eller kronisk hjerte-, lunge-, nyre-, lever- eller pancreaslidelse, eller tilstanden er associeret med en organ- eller celled transplantation.
- 30 15. Anvendelse ifølge krav 14, hvor den akutte neurodegenerative og/eller neuroinflammatoriske lidelse er udvalgt fra gruppen bestående af cerebral iskæmi eller cerebralt infarkt indbefattende embolisk okklusion og trombotisk okklusion, reperfusion efter akut iskæmi, perinatal hypoksisk-iskæmisk skade, hjertestop, intrakraniell blødning, subarahnoidalblødning og intrakranielle læsioner, ryg-

marvslæsioner, intravertebrale læsioner, piskesmæld, rystet baby-syndrom, infektiøs encephalitis, meningitis og hovedpine.

16. Anvendelse ifølge krav 14, hvor den kroniske neurodegenerative og/eller
5 neuroinflammatoriske lidelse er udvalgt fra gruppen bestående af demens, Picks sygdom, diffus Lewy-legeme-sygdom, progressiv supranukleær lammelse (Steel-Richardsons syndrom), multipel sklerose, multipel systematrofi, kroniske epileptiske tilstande associeret med neurodegeneration, motorneuronsygdomme, degenerative ataksier, corticobasal degeneration, ALS-Parkinson-demenskompleks fra
10 Guam, subakut skleroserende panencephalitis, Huntingtons sygdom, Parkinsons sygdom, synucleinopatier, primær progressiv afasi, striatonigral degeneration, Machado-Josephs sygdom/spinocerebellar ataksi type 3 og olivopontocerebellare degenerationer, Gilles De La Tourettes sygdom, bulbær og pseudobulbær lammelse, spinal og spinobulbær muskelatrofi (Kennedys sygdom), primær lateralsk-
15 leroase, familiær spastisk paraplegi, Werdnig-Hoffmanns sygdom, Kugelberg-Welanders sygdom, Tay-Sachs sygdom, Sandhoffs sygdom, familiær spastisk sygdom, spastisk paraparese, progressiv multifokal leukoencephalopati, familiær dysautonomi (Riley-Days syndrom), polyneuropatier, prionsygdomme, afhængighed, affektive lidelser, skizofrenilidelser, kronisk træthedssyndrom og kroniske
20 smerter.

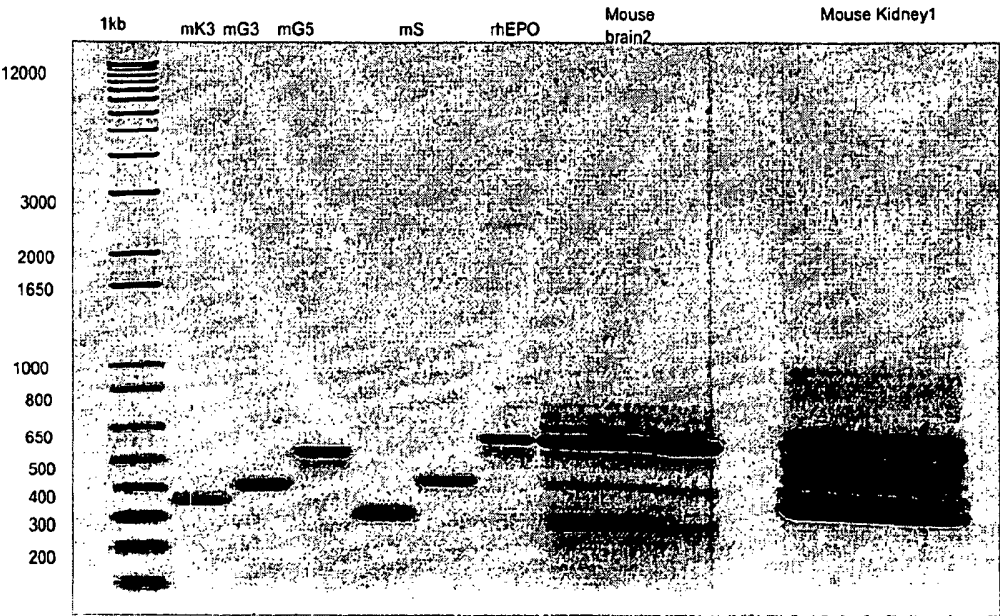
17. Anvendelse ifølge krav 13 eller 14, hvor tilstanden er aldrig.

18. Anvendelse ifølge krav 13 eller 14, hvor lægemidlet administreres før eller
25 efter tilstandens opståen.
-

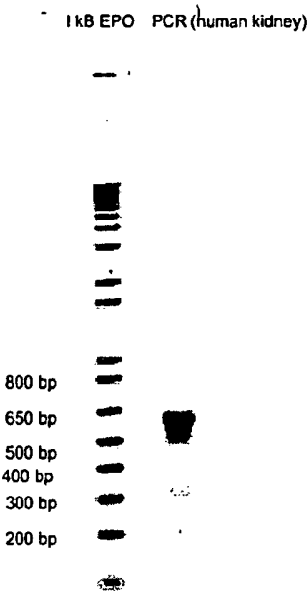
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Fig. 1

A



B



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Fig. 2a

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Fig. 2b

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mK3	-----	-
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mK3	-----	-
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Fig. 3a

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 hS4 tatgcctggaagaggatggag-----
 h1-1 -----
 h2-1 -----

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418

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Fig 3b

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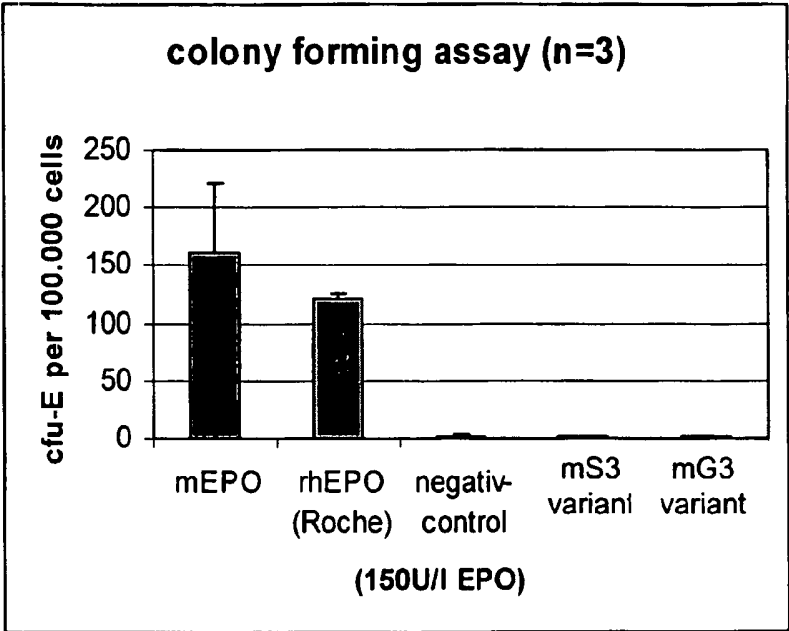

Fig. 4

Helix Affinity	Helix A	B'	Helix B
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hS3	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
h1-4	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
h1-5	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
hS4	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
h1-1	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
h2-1	MGVHECPAWLWLLLSLLSLPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
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mS	MGVPERP-TLLLLLSILLIPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
mG3	MGVPERP-TLLLLLSILLIPLGLPVLCA	PPRLICDSRV	LERYLLEAKAE
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h1-4	SSQPWEPIQLHVDKAVSGLRSITTLRA	LGAKQKEAISP	PDAAASAAPLRTITADTFKLF
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Fig. 5

A



B

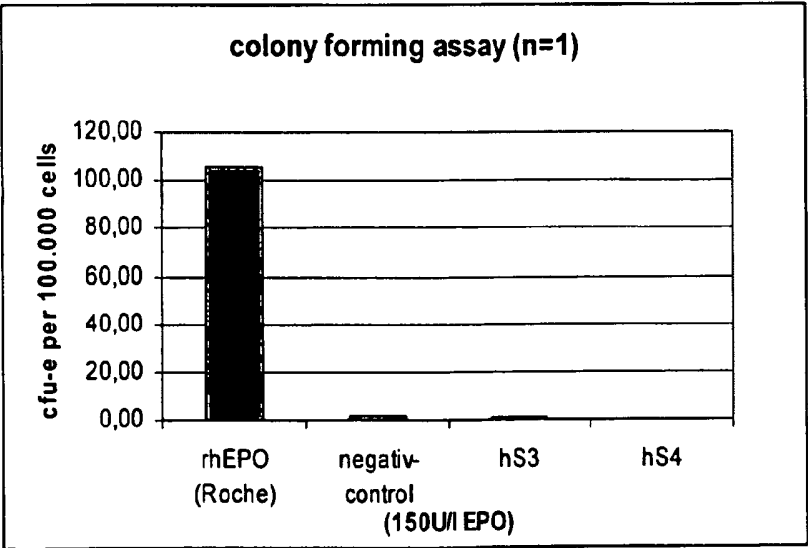
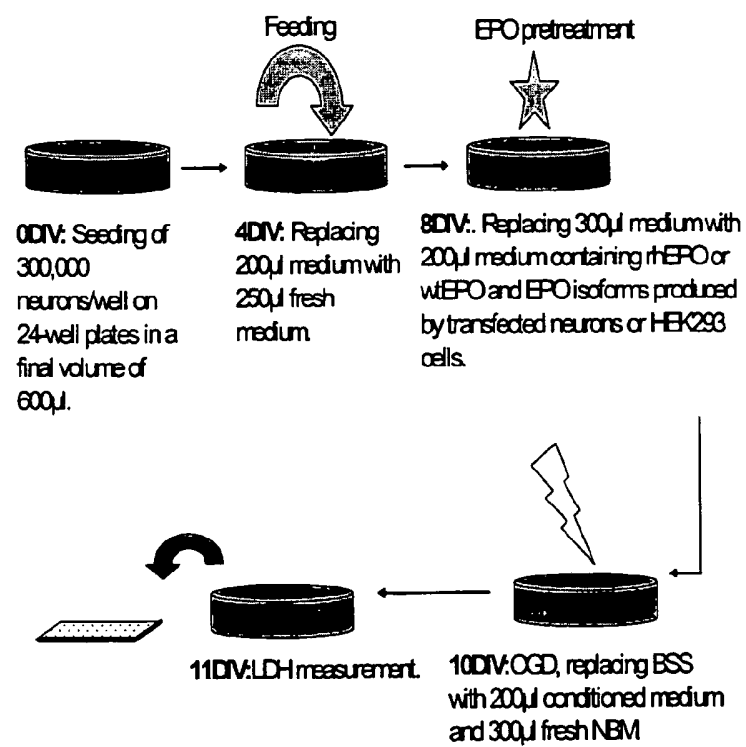


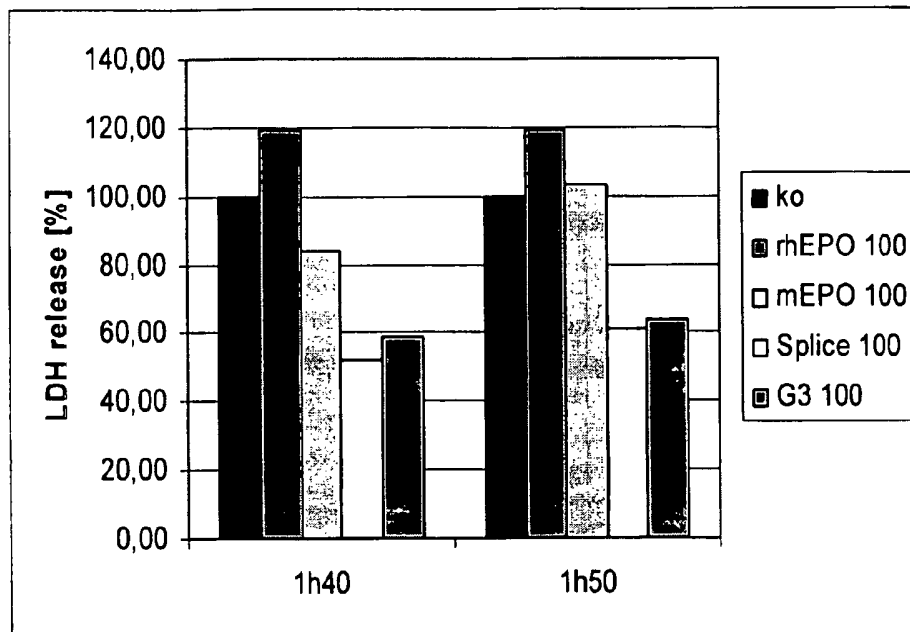
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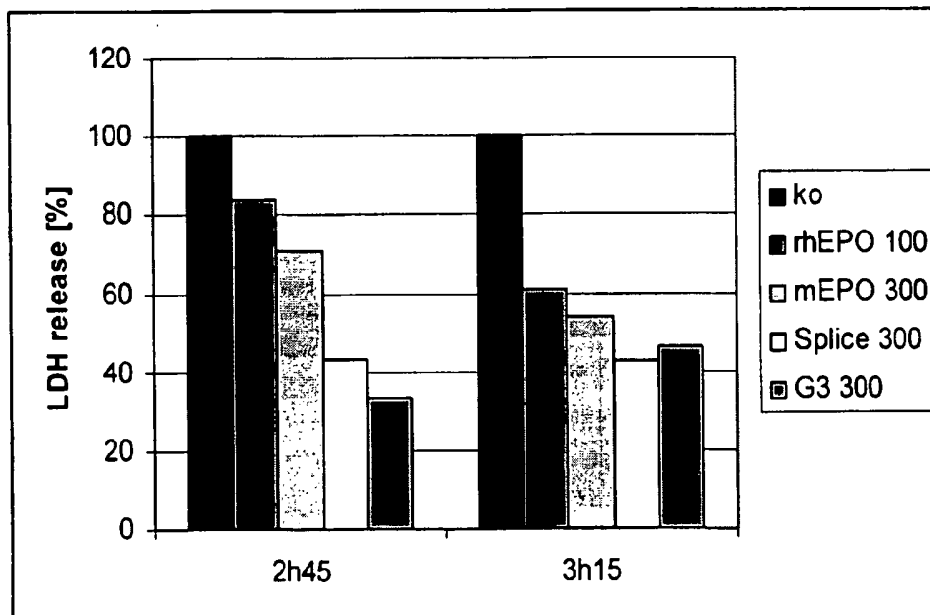
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Fig. 7

A



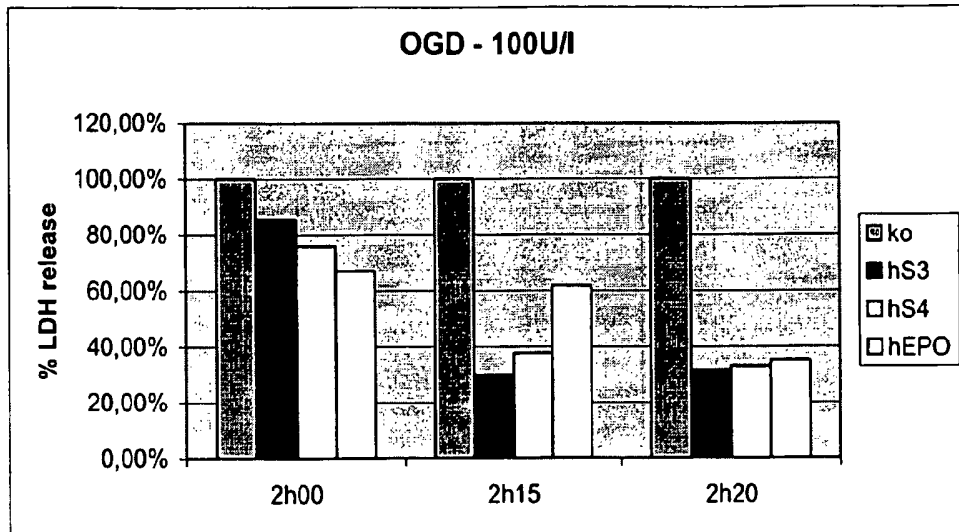
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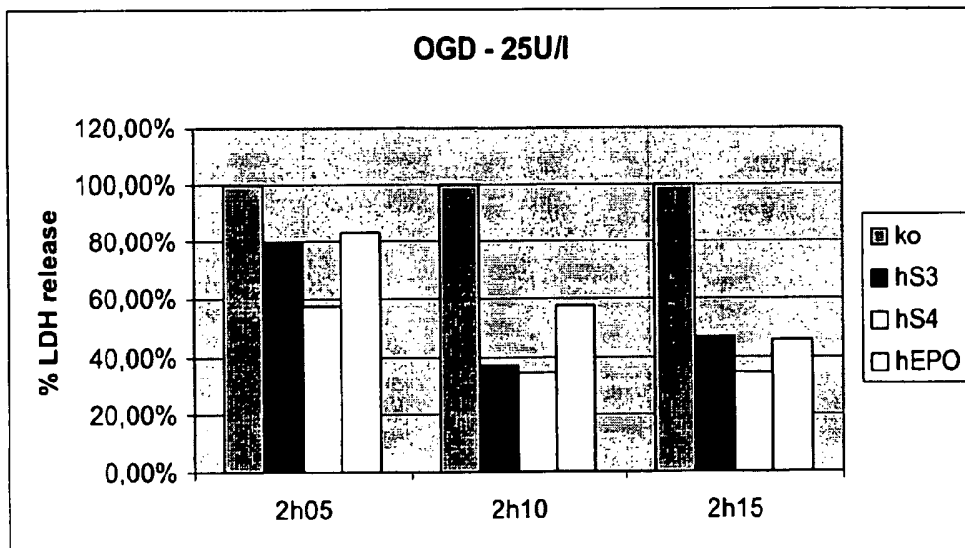
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Fig. 8

A



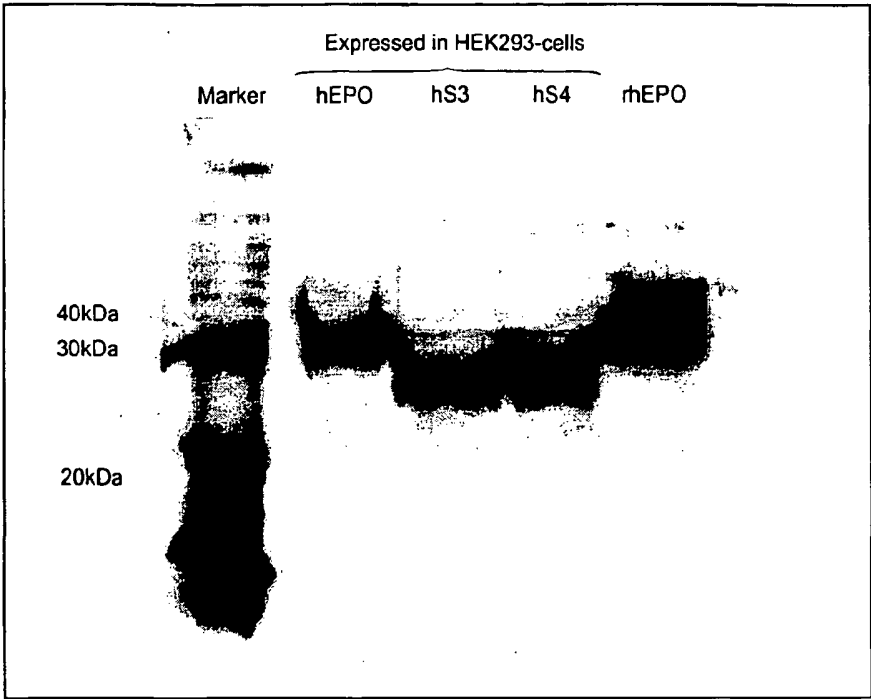
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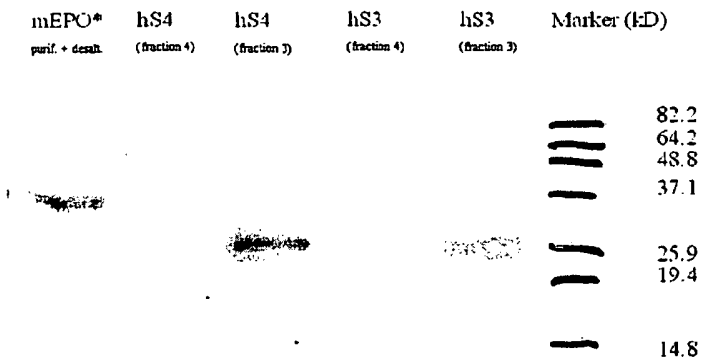
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Fig. 9

A



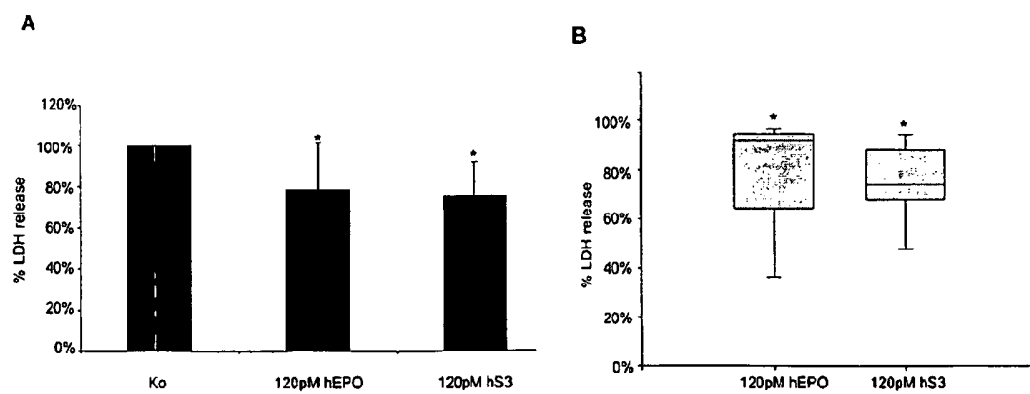
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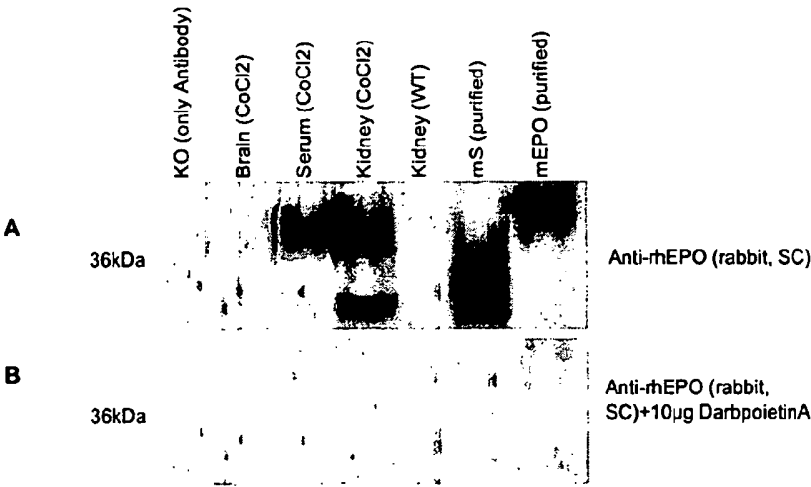
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Fig. 11



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Fig. 12



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Fig. 13

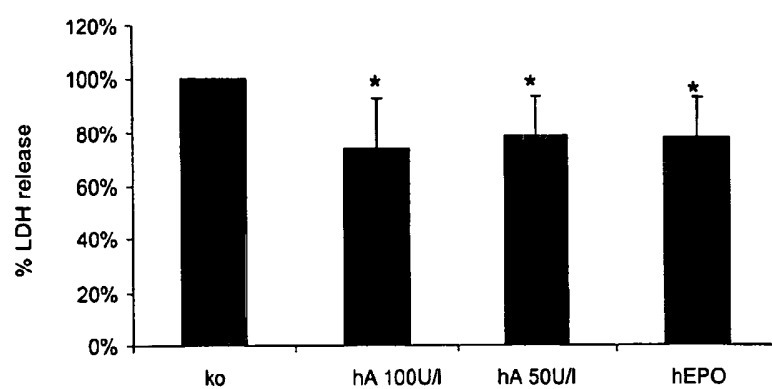
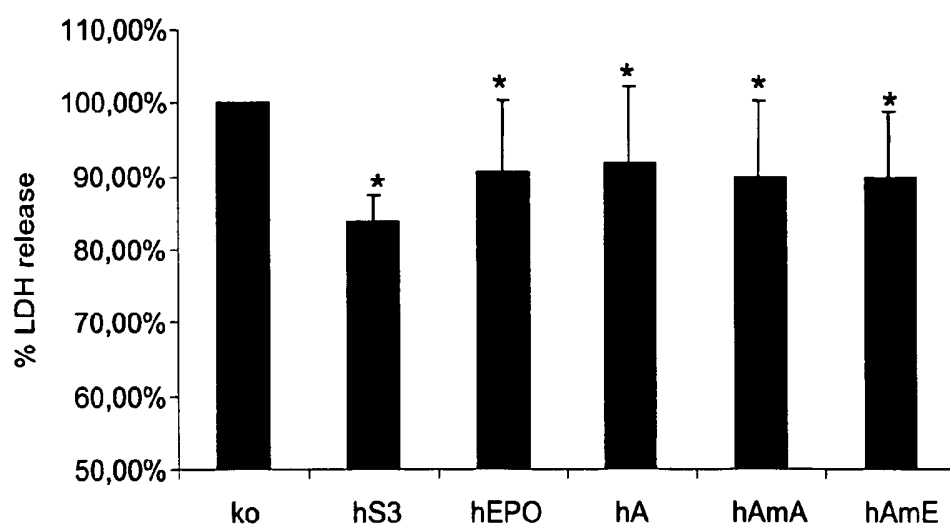


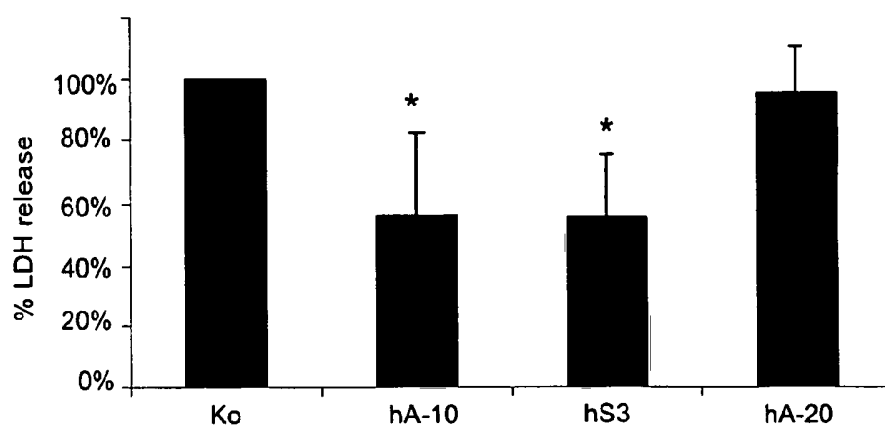
Fig. 14



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Fig. 15

A



B

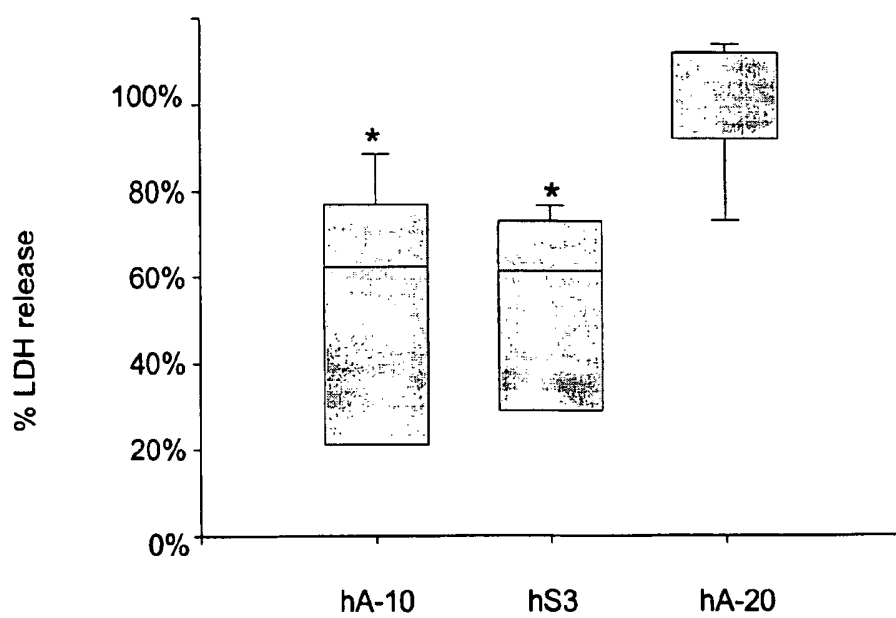
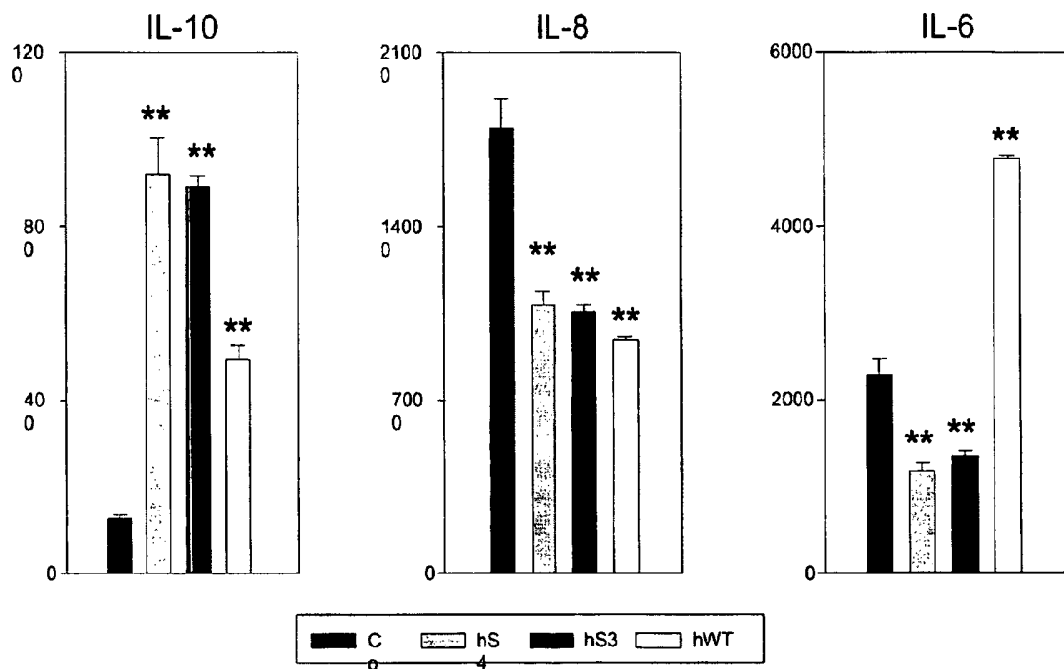


Fig. 16



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Fig. 17

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gtcctgcggggccaggccctgttgggtcaactcttccagccgtgggagccctgcagctgcatgtggataaagcc

gtcagtgcccttcgcagcctcaccactctgcttcgggctctgcgagcccagaaggaagccatctccctccagat

gcggcctcagctgtccactccgaacaatcactgctgacactttccgcaaactcttcgagctctactccaatttc

ctccggggaaagctgaagctgtacacaggggaggcctgcaggacaggggacagatga

hWT
 hA
 hAmA
 hAmE
 hA-10
 hA-20

Fig. 18

hA (hWT-EPO Helix A) - SEQ ID NO 55

atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctct
gggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggagaggt
acctcttgaggccaaggaggccgagaatatcacg

hAmA (Mutant Alanin hWT-EPO Helix A) SEQ ID NO 56

atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctct
gggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggagggcgt
acctcttgaggccaaggaggccgagaatatcacg

hAmE (Mutant Glutamic-Acid hWT-EPO Helix A) SEQ ID NO 57

atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctct
gggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggaggagt
acctcttgaggccaaggaggccgagaatatcacg

hA-10 (hWT-EPO Helix A minus 10aa)SEQ ID NO 58

atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctct
gggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggagaggt
acctc

hA-20 (hWT-EPO Helix A minus 20aa)SEQ ID NO 59

atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgtcgctccctct
gggcctcccagtcctgggcgccccaccacgcctcatc

Fig. 19

A - hA DNA without leader:

(hWT-EPO Helix A without leader transport sequence) – SEQ ID NO 60 – (mature exported protein)

5'-
gccccaccacgcctcatctgtgacagccgagtcctggagaggtacctctggaggccaaggaggccgagaatatcacg....-3'

Leader-Sequence (SEQ ID NO 63):

5'-atgggggtgcacgaatgtcctgcctggctgtggcttctcctgtccctgctgctcctctgggcctccagtcctgggc-3'

B - hA amino acid without leader:

(hWT-EPO Helix A without leader transport sequence) – SEQ ID NO 61 (mature exported proteins):

APRLICDSRVLERYLLEAKEAENIT

Leader-Sequence (SEQ ID NO 62): MGVHECPAWLWLLLSLLSLPLGLPVLG