The present invention relates to thymosin β4 (TB4) fusion polypeptides and in particular to TB4 fusion polypeptides having a longer serum half-life than wild-type TB4. The present invention also relates to TB4 fusion polypeptides for use as a medication, and to methods of treatment using said TB4 fusion polypeptides. In particular, the present invention provides TB4 fusion polypeptides for use in methods of prevention or treatment of myocardial infarction, muscle wasting conditions and diseases and diabetic nephropathy.

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THYMOSIN-BETA-FOUR FUSION PROTEINS

FIELD
The present invention relates to thymosin β4 (TB4) fusion polypeptides. More specifically, the present invention relates to TB4 fusion polypeptides having a longer serum half-life than wild-type TB4. The present invention also relates to TB4 fusion polypeptides for use as a medicament, and to methods of treatment using said TB4 fusion polypeptides. In particular, the present invention provides TB4 fusion polypeptides for use in methods of prevention or treatment of myocardial infarction, muscle wasting conditions and diseases and www.diabetic nephropathy.

BACKGROUND
TB4 is a naturally occurring peptide of 43 amino acids in length that is highly conserved between species. TB4 affects a number of biological activities such as wound healing, angiogenesis, migration and tissue integrity. TB4 is also thought to be involved in stem cell recruitment, migration and differentiation. In addition, TB4 has been shown to have anti-inflammatory, anti-fibrotic and anti-apoptotic effects (Goldstein et al. Expert Opin. Biol. Ther. 2012 12:37-51; all references cited in this application are expressly incorporated herein by reference).

The tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (ac-SDKP) released by cleavage of the N-terminal sequence of TB4 by endoproteinase has also been shown to stimulate endothelial cell migration and differentiation. Previous studies have suggested that thymosin β4 (TB4) could be a potential target for stimulating a regenerative approach in the heart after myocardial infarction. For instance, preclinical myocardial infarction studies in mice have shown that TB4 activates epicardial progenitor cells (EPDCs) in the heart, leading to cardiac repair with improved cardiac function and new vessel formation. (Smart et al. Nature. 2007;445:177-82; Bock-Marquette et al. JACC 2009;46:728-738; Smart et al. Ann. N.Y. Acad.Sci 2010; 1194:97-104; Smart et al. Nature. 2011;474:640-644; Mummery & Passier. Circ Res 2011;109:828-829; Zhou et al. J Clin Invest. 2011;121:1894-1904). TB4 has been demonstrated to protect cardiomyocytes from apoptosis, inhibit inflammation and prevent fibrotic gene expression in cardiac fibroblasts, thus reducing fibrosis. (Bock-Marquette et al; Nature 2004;432:466-472;

The mechanism of action for the regenerative properties of TB4 in the heart is still unknown. Without wishing to be bound by any theory, it can be speculated that TB4 present in the extracellular space constitutes a repair signal.

In addition, in a recent study in non-ischemic rat hearts, gene transfer of TB4 stimulated the proliferation and differentiation of resident cardiac progenitor cells into cardiomyocyte, vascular smooth muscle and endothelial cells, indicating that TB4 may be of therapeutic value also in chronic heart failure. (Chen et al, Gene Ther. 2013;20:225-233).

The therapeutic use of TB4 also includes treatment of eye dryness and epidemiolyta bullosa. In a recent phase 2 clinical trial in severe dry eye, TB4 peptide has been shown to significantly improve signs and symptoms of Severe Dry Eye (http://www.regenerx.com/wt/page/pr_1340196791).

However, therapeutic uses of TB4 are hindered by the very short half-life of TB4 peptide in human blood circulation (~2 hours). This short half-life limits the usefulness of TB4 in indications requiring systemic exposure and means that repeated administrations and/or extremely high doses of TB4 peptide are required in order to obtain any therapeutic benefit. Treatment with TB4 thus requires frequent, specialist care. These issues represent severe constraints on the clinical efficacy of TB4 peptide.

There is therefore a need for a TB4 polypeptide that has a longer half-life but which retains a similar level of biological activity to existing TB4 peptides. There is a need for a TB4 polypeptide which is clinically effective at lower concentrations and/or less frequent dosing regimens than existing TB4 peptides. There is a need for a TB4 polypeptide with a longer half-life for use as a medicament. There is also a need for a TB4 polypeptide with a longer half-life for use in the prevention and treatment of myocardial infarction, a muscle wasting disease or diabetic nephropathy.

**SUMMARY OF INVENTION**

As described below, the present invention features thymosin β4 (TB4) fusion polypeptides as well as methods of prevention or treatment of myocardial infarction, muscle
wasting conditions and diseases and diabetic nephropathy and methods of treatment using the
TB4 fusion polypeptides.

In one embodiment, the invention is a thymosin β4 (TB4) fusion polypeptide comprising:
(i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ
ID NO: 1, optionally having a Glycine residue at the N terminus; and (ii) an Fc domain at the C
terminus, the Fc domain optionally comprising one or more mutations which reduce and/or
ablate effector functions.

In another embodiment, the fusion polypeptide has an Fc domain with a FQQ triple
mutation. In still another embodiment, the fusion polypeptide has an Fc domain with a YTE
triple mutation.

In other embodiments, the fusion polypeptide can have an Fc domain that is IgG1, IgG2,
IgG3 or IgG4.

In some embodiments, the fusion polypeptide comprises all of the residues of the full-
length mature TB4 polypeptide as defined in SEQ ID NO: 1. In other embodiments, the fusion
polypeptide comprising residues 1-18 (SDKPDMAEIEKFSDKLK) of the full-length mature
TB4 polypeptide as defined in SEQ ID NO: 1.

In still other embodiments, the fusion polypeptide further comprises a linker peptide
located between the TB4 polypeptide residues and the Fc domain.

In some embodiments, the fusion polypeptide has a linker peptide comprising at least one
repeat of SEQ ID NO: 2 (GGGGX) where X is an amino acid. In other embodiments, the fusion
polypeptide has at least one repeat of the linker peptide of GGGGG, GGGGS or GGGGA.

In other embodiments, the fusion polypeptide also comprises a hinge peptide located
between the TB4 polypeptide residues and the Fc domain; the hinge peptide can be THTCPPC
(SEQ ID NO: 8), ATHTCPPC (SEQ ID NO: 9); or ACPPC (SEQ ID NO: 10).

In some embodiments, the fusion polypeptide further comprising a hinge peptide located
between the linker peptide and the Fc domain; the hinge can be THTCPPC (SEQ ID NO: 8),
ATHTCPPC (SEQ ID NO: 9); or ACPPC (SEQ ID NO: 10).

In some embodiments, the fusion polypeptide has an amino acid substitution at residue 30
numbered according to SEQ ID NO: 1. In some embodiments that substation is either S30A or
S30T.
In some embodiments, the fusion polypeptide has a hinge peptide that is either THTCPPC (SEQ ID NO: 8) or ACPPC (SEQ ID NO: 10).

In some embodiments, the fusion polypeptide has a lysine residue at the C terminus of the Fc domain.

In some embodiments, the fusion polypeptide has a linker peptide and the linker peptide is 1 to 10 repeats of SEQ ID NO: 2 (GGGGX) where X is an amino acid. In other embodiments, X is a serine residue.

In some embodiments, the fusion polypeptide has a linker peptide and the linker peptide is at least two repeats of SEQ ID NO: 2 (GGGGX), where X is an amino acid. In other embodiments, X is a serine residue.

In some embodiments, the fusion polypeptide has a linker peptide and the linker peptide is five or six repeats of SEQ ID NO: 2 (GGGGX), where X is an amino acid. In other embodiments, X is a serine residue.

In one embodiment, the fusion polypeptide has the sequence according to SEQ ID NO: 5.

In one embodiment, the fusion polypeptide has the sequence according to SEQ ID NO: 7.

In one embodiment, the fusion polypeptide has the sequence according to SEQ ID NO: 8.

In one embodiment, the fusion polypeptide has the sequence according to SEQ ID NO: 9.

In some embodiments, the invention is a dimer comprising: (i) a first subunit comprising a fusion polypeptide as described above; and (ii) a second subunit comprising a fusion polypeptide as described above. In other embodiments the two subunits are identical.

In some embodiments, the invention is a nucleic acid encoding: a fusion polypeptide as described above, or a dimer as described above.

In some embodiments, the invention is a host cell containing a fusion polypeptide as described above, or a dimer as described above.

In some embodiments, the fusion polypeptide and the dimer can be used as a medicament.

In some embodiments, the fusion polypeptide or dimer can be used in the prevention or treatment of myocardial infarction.

In some embodiments, the fusion polypeptide or dimer can be used in the prevention or treatment of a muscle wasting disease.
In some embodiments, the fusion polypeptide or dimer can be used in the prevention or treatment of diabetic nephropathy.

In some embodiments, the fusion polypeptide, or dimer are administered in a single dose to a subject in need thereof.

In some embodiments, the invention is a pharmaceutical composition comprising the fusion polypeptide and/or the dimer, together with one or more pharmaceutically acceptable excipients.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1**: Depicts the semilogarithmic plot of concentration-time data after intraperitoneal doses of i) TB4-Fc fusion protein at 3 mg/kg; ii) TB4-HSAwt fusion protein at 3.9 mg/kg; iii) TB4-HSA-RG fusion protein at 3.6 mg/kg or iv) TB4-HSA-RGY fusion protein at 3.0 mg/kg. Solid lines correspond to mean values (n=3).

**Figure 2**: Depicts effects of TB4 peptide and fusion protein in the acute myocardial infarction (MI) setting. Mice were subjected to myocardial infarction by a permanent ligation of the coronary artery, and treated with daily injections of TB4 peptide or a single intravenous injection of TB4-Fc or TB4-HSA-wt. After 7 days, scar area in the epicardium was evaluated in the hearts.

**Figure 3**: (A) and (B) Depicts the effect of TB4-Fc fusion protein on an established scar. Mice were subjected to myocardial infarction by a transient ligation of the coronary artery, and treated with a single intravenous injection of TB4-Fc at day 21. One week later mice were sacrificed and A) scar area and B) Wt-1 positive EPDCs were evaluated in the hearts.

**Figure 4**: (A) and (B) Depicts the effect of different TB4-Fc variants on scar area and vessel formation following myocardial infarction. Mice were subjected to a 60-minute ischemia followed by reperfusion by transient ligation of the coronary artery, and treated with i) placebo (PBS); ii) TB4-Fc fusion protein (T15) at a dose of 0.5 mg/kg; iii) TB4-Fc fusion protein (T15) at a dose of 3 mg/kg; iv) GSDKP-Fc fusion protein (T39) at a dose of 3 mg/kg or v) truncated TB4 (aa 1-18)-Fc fusion protein (T46) at a dose of 3 mg/kg 15 minutes after reperfusion. Four weeks later mice were sacrificed and A) scar area and B) blood vessel density were evaluated in the hearts.

**Figure 5**: (A), (B), and (C) Depicts the effect of different TB4-Fc variants on skeletal muscle weight in MI mice. Mice were subjected to a 60-minute ischemia followed by
reperfusion by transient ligation of the coronary artery, and treated with i) placebo (PBS); ii) TB4-Fc fusion protein (T15) at a dose of 0.5 mg/kg; iii) TB4-Fc fusion protein (T15) at a dose of 3 mg/kg; iv) GSDKP-Fc fusion protein (T39) at a dose of 3 mg/kg or v) truncated TB4 (aa 1-18)-Fc fusion protein (T46) at a dose of 3 mg/kg 15 minutes after reperfusion. Four weeks later mice were sacrificed and skeletal muscle weight was evaluated.

**Figure 6**: Depicts the expression of WT-1 in the epicardium after myocardial infarction in combination with various TB4-FC variants.

**Figure 7**: Depicts the left ventricle ejection factor (LVEF) adjusted for day-1 LVEF of the mice treated for 28 days with different TB4-Fc variants.

**Figure 8**: Depicts the increased blood vessel formation, as measured by percent volume density, at the cardiac infarction border zone in mice treated with various TB4-Fc variants.

**BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS**

SEQ ID NO: 1 - Sequence of the full-length mature TB4 polypeptide. A TB4 fusion polypeptide of the present invention comprises at least residues 1-4 (SDKP) of the full length mature TB4 polypeptide.

SEQ ID NO: 2 - Sequence of the first, linking, peptide. A TB4 fusion polypeptide of the invention may comprise at least one repeat of this first, linking, peptide.

SEQ ID NO: 3 - Sequence of the Fc domain. This sequence is a triple mutant Fc domain, having residues 5, 6 and 102 which are mutated relative to the wild-type Fc domain.

SEQ ID NO: 4 - Sequence of a particular TB4 fusion polypeptide of the invention (designated T15), comprising a glycine residue at the N terminus, a full-length TB4 sequence, 5 repeats of the GGGGS linker peptide, the sequence ATHTCPPC in the hinge region, a triple mutant Fc domain and a lysine residue at the C terminus.

SEQ ID NO: 5 - Sequence of a particular TB4 fusion polypeptide of the invention (designated T39), comprising a glycine residue at the N terminus, residues 1-4 (SDKP) of the full length mature TB4 sequence, 2 repeats of the GGGGS linker peptide, the sequence THTCPPC in the hinge region, a triple mutant Fc domain and a lysine residue at the C terminus.

SEQ ID NO: 6 - Sequence of a particular TB4 fusion polypeptide of the invention (designated T46), comprising a glycine residue at the N terminus, residues 1-18 of the full-length mature TB4 polypeptide, 2 repeats of the GGGGS linker peptide, the sequence THTCPPC in the hinge region, a triple mutant Fc domain and a lysine residue at the C terminus.
SEQ ID NO: 7 - Sequence of a particular TB4 fusion polypeptide of the invention (designated T47), comprising a glycine residue at the N terminus, residues 1-4 of the full-length mature TB4 polypeptide, 5 repeats of the GGGGS linker peptide, a further GGGS peptide linker, the sequence ACPPC in the hinge region and a triple mutant Fc domain.

SEQ ID NO: 8 - Sequence of a second, hinge, peptide.
SEQ ID NO: 9 - Sequence of an alternative second, hinge, peptide.
SEQ ID NO: 10 - Sequence of an alternative second, hinge, peptide.
SEQ ID NOS: 11-15 - Sequences of signal proteins of the present invention.

SEQUENCE LISTINGS

SEQ ID NO: 1
SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES

SEQ ID NO: 2
GGGGS

SEQ ID NO: 3
PAPEFEGGPSVFLFPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVSVLTVLHQLDNLNGKEYKCKVSNKALPASIEKTISKAKGQPREPQVYTLPSSREEMTKNQVSLTCLVKGFYPSIDIAVEWESNGQPENNYKTTPPVLDSDGFFLYSKLTVDKSRWQQGNSCVSCVMHEALHNHYTQKSLSLPG

SEQ ID NO: 4
GSDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGESGGGSGGGGSATHTCPPCPAPEFEGGPSVFLFPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVSVLTVLHQLDNLNGKEYKCKVSNKALPASIEKTISKAKGQPREPQVYTLPSSREEMTKNQVSLTCLVKGFYPSIDIAVEWESNGQPENNYKTTPPVLDSDGFFLYSKLTVDKSRWQQGNSCVSCVMHEALHNHYTQKSLSLPGK
SEQ ID NO: 5
GSDKPGGGGSGGGSGGGSTHTCPPCPAPEFEGGSPVFLFPPKPKDRTLMI
SRTPETCVVVVDSHEDPVEFKNWYVDGVEVHNAKTPREEQYNSTYRVVSLTVLH
QDWLNGKEYKCKVSNKALPASIEKTISAKAGQPREPQVYTLPPSREEMTKNQVSLTCLV
KGFPSDIAVEWESNGQPPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLPGK

SEQ ID NO: 6
GSDKPDMAEIEKFDKSLLKGGGGSGGGSTHTCPPCPAPEFEGGSPVFLFPPKPDKTLMIS
SRTPETCVVVVDSHEDPVEFKNWYVDGVEVHNAKTPREEQYNSTYRVVSLTVLH
QDWLNGKEYKCKVSNKALPASIEKTISAKAGQPREPQVYTLPPSREEMTKNQVSLTCLV
KGFPSDIAVEWESNGQPPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLPGK

SEQ ID NO: 7
GSDKPGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGG
ACPPCPAPEFEGGSPVFLFPPKDTRLMI
SRTPETCVVVVDSHEDPVEFKNWYVDGVEVHNAKTPREEQYNSTYRVVSLTVLH
QDWLNGKEYKCKVSNKALPASIEKTISAKAGQPREPQVYTLPPSREEMTKNQVSLTCLV
KGFPSDIAVEWESNGQPPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLPG

SEQ ID NO: 8
THTCPPC

SEQ ID NO: 9
ATHTCPPC

SEQ ID NO: 10
ACPPPC

SEQ ID NO: 11
The present invention meets one or more of the above needs by providing a thymosin β4 (TB4) fusion polypeptide comprising:

(i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ ID NO: 1, optionally having a Glycine residue at the N terminus; and

(ii) an Fc domain at the C terminus, the Fc domain optionally comprising one or more mutations that reduce and/or ablate effector functions.

(iii) or HSA domain at the C terminus, the HSA domain optionally comprising one or more mutations (HSA-Y, HSA-RG, HSA-RGY)

A TB4 fusion polypeptide of the present invention has surprisingly been found to have a longer half-life compared to a full-length wild-type mature TB4 peptide lacking an Fc domain, while maintaining activity.

Advantageously, the TB4 fusion proteins of the present invention retain a similar level of biological activity to the wild-type TB4 peptide. In particular, the present inventors have found that the TB4 fusion polypeptides of the invention, comprising a TB4 fragment at the N terminus and an Fc domain at the C terminus, increase the half-life of the protein when compared to a TB4 polypeptide lacking the Fc domain.
Surprisingly, the present inventors have also found that the fusion polypeptides of the invention (conjugated to an Fc domain) provide a much longer half-life compared to conjugation with an HSA polypeptide.

A TB4 fusion polypeptide of the invention may comprise the full-length mature TB4 polypeptide, with the N-terminal methionine residue removed, as defined in SEQ ID NO: 1. Alternatively, a TB4 fusion polypeptide of the invention may comprise residues 1-18 (SDKPDM AEIEKFDKS KLK) of the full-length mature TB4 polypeptide.


A TB4 fusion polypeptide of the invention may comprise a TB4 peptide sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence of a full-length mature TB4 polypeptide as defined in SEQ ID NO: 1; or to a fragment thereof. The fragment thereof may comprise at least residues 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29, 1-30, 1-31, 1-32, 1-33, 1-34, 1-35, 1-36, 1-37, 1-38, 1-39, 1-40, 1-41 or 1-42 of a full length mature TB4 polypeptide as defined in SEQ ID NO: 1.

A TB4 fusion polypeptide of the invention may have an amino acid substitution at residue 30, numbered according to SEQ ID NO: 1. The amino acid substitution at residue 30 may be S30A or S30T. The S30A substitution removes the O-glycosylation site at position 30 while the S30T substitution increases O-glycosylation at position 30. Thus, the TB4 fusion polypeptide of the present invention may have an altered glycosylation pattern relative to the wild-type full length mature TB4 polypeptide as defined by SEQ ID NO: 1.

A TB4 fusion polypeptide of the invention comprises an Fc domain. The Fc domain may be a modified Fc which lacks the effector functions of a wild-type Fc domain. Effector functions include recruitment of immune cells and complement proteins, stimulation of phagocytosis and/or degranulation of mast cells, basophils and eosinophils, antibody-dependent cell-mediated
cytotoxicity ("ADCC"), complement dependent cytotoxicity ("CDC"), and antibody-dependent cell-mediated phagocytosis ("ADCP").

As used herein the Fc region includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain, and fragments thereof. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and optionally the hinge region N-terminal to these domains. For IgA and IgM the Fc region can include the J chain. For IgG, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cy2 and Cy3) and optionally the hinge region between Cgamma2 (Cy1) and Cgamma2 (Cy2). Although the boundaries of the Fc region can vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as set forth in Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Fc can refer to this region in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion protein.

Polymorphisms have been observed at a number of different Fc positions, including but not limited to positions 270, 272, 312, 315, 356, and 358 as numbered by the EU index, and thus slight differences between the presented sequences and sequences in the prior art may exist. Polymorphic forms of human immunoglobulins have been well-characterized. At present, 18 Gm allotypes are known: Glm (1, 2, 3, 17) or Glm (a, x, f, z), G2m (23) or G2m (n), G3m (5, 6, 10, 11, 13, 14, 15, 16, 21, 24, 26, 27, 28) or G3m (bl, c3, b3, bo, b3, b4, s, t, gl, c5, u, v, g5) (Lefranc, et al., The human IgG subclasses: molecular analysis of structure, function and regulation. Pergamon, Oxford, pp. 43-78 (1990); Lefranc, G. et al., 1979, Hum. Genet.: 50, 199-211). It is specifically contemplated that the TB4 fusion polypeptides of the present invention may incorporate the Fc region, or fragment thereof, of any allotype, isoallotype, or haplotype of any immunoglobulin gene, and are not limited to the allotype, isoallotype or haplotype of the sequences provided herein.

As used herein, the term "Fc fusion protein" encompasses proteins (e.g., conjugate compounds of the present disclosure) comprising a full length Fc domain as well as proteins comprising Fc domain fragments (e.g., a full CH2 domain, a full CH3 domain, a CH2 fragment,
a CH3 fragment, or combinations thereof). An Fc fusion protein may optionally comprise all or a portion of the hinge region.

In some aspects, the TB4 fusion polypeptides of the present invention comprise an Fc domain. In some aspects, the Fc domain comprises a CH2 region, and/or a CH3 region, and/or fragments thereof. In some specific aspects, the Fc domain comprises a CH2 region and a CH3 region. In some aspects, the Fc domain consists of a CH2 region and a CH region. In some aspects, the Fc domain is an IgG Fc domain, from example, an Fc domain from an IgGl, IgG2, IgG3, or IgG4. In some aspects, the IgG Fc domain is a human or humanized IgG Fc domain. In some aspects, the Fc domain is an IgGl Fc domain.

In some aspects, the IgG Fc domain, for example an IgGl Fc domain, is a native (wild type) domain. In some aspects, the native IgGl Fc domain comprises the amino acids of SEQ ID NO: #. In other aspects, the native IgGl Fc domain consists of the amino acids of SEQ ID NO: #. In other aspects, the Fc domain is a mutant IgG domain, for example, a mutant IgGl, IgG2, IgG3, or IgG4 domain. In some specific aspects, the mutant Fc domain is a mutant IgGl Fc domain.

A person skilled in the art would understand that in some aspects, an Fc Fusion protein can form dimers due to the homodimeric nature of molecules comprising an Fc region. In some aspects the Fc regions of a TB4 fusion polypeptide may be differentially engineered with mutations to: promote and/or maintain heterodimerization (e.g., chimeric mutations, complementary mutations, lock and dock mutations, knobs into holes mutations, strand-exchange engineered domain (SEED) mutations, etc., see for example, US Patent No. 7,183,076; Merchant et al. (1998) Nat. Biotech 16:677-681; Ridgway et al. (1996) Protein Engineering 9:617-621; Davis et al. (2010) Prot. Eng. Design & Selection 23:195-202; WO 2007/110205; WO 2007/147901; Gunasekaran et al. (2010) JBC 285:19637-46). Accordingly, a TB4 fusion polypeptide can be engineered to form a heterodimer comprising for example a first TB4 peptide fused to a first Fc region or fragment thereof, and a second (i.e., different) TB4 peptide fused to a second Fc region or fragment, wherein the first and second Fc regions, or fragments thereof have been engineered to heterodimerize.

In some aspects, the Fc domain of a TB4 fusion protein of the invention has reduced binding to an Fc receptor to reduce cytotoxicity, e.g., via ADCC. In some aspects, the Fc domain of a TB4 fusion protein of the invention has increased binding to an Fc receptor to
enhance half-life, e.g., via FcRn recycling. Certain modifications can provide desired effector functions or serum half-life. Where it is desirable to eliminate or reduce effector function, so as to minimize side effects or therapeutic complications, certain other Fc regions can be used. The Fc region can be also be modified to increase the binding affinity for FcRn and thus increase serum half-life. Accordingly, in some aspects, the Fc domain of the cysteine-engineered antibody or Fc fusion protein has increased binding to the Fc receptor FcRn.

In some aspects, the Fc domain of a TB4 fusion protein of the invention comprises a non-naturally occurring ADCC reducing amino acid residue at one or more positions selected from the group consisting of 234, 235, 236, 237, 238, 239, 240, 241, 243, 244, 245, 247, 251, 252, 254, 255, 256, 262, 263, 264, 265, 266, 267, 269, 279, 280, 284, 292, 296, 297, 298, 299, 305, 313, 316, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 339, 341, 343, 370, 373, 378, 392, 416, 419, 421, 440 and 443 as numbered by the EU index as set forth in Kabat. Numerous specific Fc region mutations capable of reducing ADCC activity are known in the art and include, for example 234F, 235E, 235F, 235Q, 235Y, 239A, 332Q, 333IS, 332Q and combinations thereof. For example, see the mutations described in WO8807089, W09958572, W09951642, WO2012175751, WO201 1149999, WO201 1066501, WO2000042072, WO201 1120134, which are herein incorporated by reference in their entireties. Fc comprising molecules with reduced ADCC effector function also include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Pat. No. 6,737,056). Such Fc mutants also include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581). Optionally, mutations which reduce both ADCC and CDC may be incorporated.

In one embodiment, the present invention provides a TB4 fusion polypeptide, wherein the Fc region is an IgGl, IgG2 or IgG3 Fc region and comprises at least one modification at one or more positions selected from the group consisting of 234, 235, and 331 as numbered by the EU index as set forth in Kabat. In still another specific embodiment, the Fc region is an IgGl, IgG2 or IgG3 Fc region and the non-naturally occurring amino acids are selected from the group consisting of 234F, 235E, 235F, 235Q (or 235Y), 239A, 332Q, 333IS, 332Q as numbered by the EU index as set forth in Kabat.
In another embodiment, the present invention provides a TB4 fusion polypeptide, wherein the Fc region is an IgG4 Fc region and comprises at least one modification at one or more positions selected from the group consisting of 228 and 235 as numbered by the EU index as set forth in Kabat. In still another specific embodiment, the Fc region is an IgG4 Fc region and the non-naturally occurring amino acids are selected from the group consisting of 228P, 235E and 235Y as numbered by the EU index as set forth in Kabat.

In still other embodiments the modifications at one or more positions are selected from the group consisting of 234F/235E/331S, 234F/235F/331S, and 234F/235Q/322Q for IgGl, IgG2, and IgG3 as numbered by the EU index as set forth in Kabat. In another embodiment the modification is at positions 228P/235E for IgG4 as numbered by the EU index as set forth in Kabat.

In some aspects, the Fc domain of a TB4 fusion protein of the invention comprises a non-naturally occurring half-life extending amino acid residue at one or more positions selected from the group consisting of 238, 250, 252, 254, 256, 257, 256, 265, 272, 286, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428, 433, 434, as numbered by the EU index as set forth in Kabat. Numerous specific mutations capable of increasing the half-life of an Fc containing molecule are known in the art and include, for example 252Y, 254T, 256E, and combinations thereof. For example, see the mutations described in U.S. Patent Nos. 6,277,375, 7,083,784; 7,217,797, 8,088,376; U.S. Publication Nos. 2002/0147311; 2007/0148164; and International Publication Nos. WO 98/23289 and WO 09/058492, which are herein incorporated by reference in their entireties. Fc comprising molecules with enhanced half-life also include those with substitution two or more of Fc region residues 250, 252, 254, 256, 257, 309, 311, 428, 433, 434, (U.S. Pat. Nos. 7,083,784 and 8,088,376).

Specific combinations of the present invention include, but are not limited to, 252Y/254T/256E and 250Q/428L for all IgG types. In addition, where the IgG is IgG 3 and embodiment of the invention includes 435H.

The serum half-life of proteins comprising Fc regions may be increased by increasing the binding affinity of the Fc region for FcRn. The term "half-life" as used herein means a pharmacokinetic property of a molecule that is a measure of the mean survival time of the molecules following their administration. Half-life can be expressed as the time required to
eliminate 50 percent of a known quantity of the molecule from the subject's body (e.g., human patient or other mammal) or a specific compartment thereof, for example, as measured in serum, i.e., circulating half-life, or in other tissues. In general, an increase in half-life results in an increase in mean residence time (MRT) in circulation for the molecule administered.

The modified Fc domain may comprise or consist of SEQ ID NO: 3, which has three amino acid substitutions relative to the wild-type Fc sequence at positions 5, 6 and 102 of SEQ ID NO: 3. In the unmodified Fc domain, positions 5 and 6 are both leucine and position 102 is proline.

The Fc domain of a TB4 fusion polypeptide of the invention may comprise a sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 3. Such variants may also lack the effector function of a wild-type Fc domain, in that they may also have mutations at residues 5, 6 and 102, numbered relative to SEQ ID NO: 3. Such variants may comprise phenylalanine at residue 5, glutamic acid at residue 6 and serine at residue 102, numbered relative to SEQ ID NO: 3.

A TB4 fusion polypeptide of the invention may further comprise a first, linking, peptide, located between the TB4 polypeptide residues and the Fc domain. The first, linking, peptide may comprise at least one repeat of SEQ ID NO: 2 (GGGGX). X is an amino acid selected from the group consisting of G, A and S.

The linker peptide may comprise from 1 to 10 repeats of SEQ ID NO: 2 (GGGGX). The linker peptide may comprise one, two, three, four, five, six, seven, eight, nine or ten repeats of SEQ ID NO: 2. The linker peptide may comprise at least two repeats of SEQ ID NO: 2 (GGGGX). The linker peptide may comprise five or six repeats of SEQ ID NO: 2 (GGGGX). X is an amino acid selected from the group consisting of G, A and S.

Other sequences such as repeats of a tetra-peptide sequence SGTA (residues Serine, Glycine, Threonine, Alanine) may also be used as a suitable first, linking, peptide.

The linker peptide may comprise at least one, at least two, at least three, at least four, at least five, or at least six repeats of a short peptide sequence. Further linker peptides may also be known to those skilled in the art of protein engineering.

A TB4 fusion polypeptide of the invention may comprise a hinge peptide selected from THTCPPC (SEQ ID NO: 12), ATHTCPPC (SEQ ID NO: 13) and ACPPC (SEQ ID NO: 14). The hinge peptide is located between the TB4 polypeptide residues and the Fc domain; where the
linker peptide is also present, the hinge peptide is located between the first peptide and the Fc domain. The hinge peptide may be THTCPPC (SEQ ID NO: 12). Alternatively, the hinge peptide may be ACPPC (SEQ ID NO: 14).

A TB4 fusion polypeptide of the present invention may further comprise a lysine residue at the C terminus of the Fc domain. The lysine residue at the C terminus of the Fc domain is often removed on production by the cellular machinery.

A TB4 fusion polypeptide of the present invention may have a sequence according to SEQ ID NO: 4. A TB4 fusion polypeptide of the invention may have a sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence as defined in SEQ ID NO: 4.

A TB4 fusion polypeptide of the present invention may have a sequence according to SEQ ID NO: 5. A TB4 fusion polypeptide of the invention may have a sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence as defined in SEQ ID NO: 5.

A TB4 fusion polypeptide of the present invention may have a sequence according to SEQ ID NO: 6. A TB4 fusion polypeptide of the invention may have a sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence as defined in SEQ ID NO: 6.

A TB4 polypeptide of the present invention may have a sequence according to SEQ ID NO: 7. A TB4 polypeptide of the invention may have a sequence that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence as defined in SEQ ID NO: 7.

A person skilled in the art would understand that in some aspects, an Fc Fusion protein can form dimers due to the homodimeric nature of molecules comprising an Fc region. Therefore, in some aspects, a TB4 fusion polypeptide of the present invention may be dimerised with another TB4 fusion polypeptide of the present invention. Accordingly, the present invention provides a dimer comprising (i) a first subunit comprising or consisting of a TB4 fusion polypeptide as defined above; and (ii) a second subunit comprising or consisting of a TB4 fusion polypeptide as defined above. A TB4 fusion polypeptide of the present invention may exist as a homodimer.
In some aspects the Fc regions of a TB4 fusion polypeptide may be differentially engineered with mutations to: promote and/or maintain heterodimerization (e.g., chimeric mutations, complementary mutations, lock and dock mutations, knobs into holes mutations, strand-exchange engineered domain (SEED) mutations, etc., see for example, US Patent No. 7,183,076; Merchant et al. (1998) Nat. Biotech 16:677-681; Ridgway et al. (1996) Protein Engineering 9:617-621; Davis et al. (2010) Prot. Eng. Design & Selection 23:195-202; WO 2007/110205; WO 2007/147901; Gunasekaran et al. (2010) JBC 285:19637-46). Accordingly, a TB4 fusion polypeptide can be engineered to form a heterodimer comprising for example a first TB4 peptide fused to a first Fc region or fragment thereof, and a second (i.e., different) TB4 peptide fused to a second Fc region or fragment, wherein the first and second Fc regions, or fragments thereof have been engineered to heterodimerize.

The present invention further provides a pharmaceutical composition comprising a TB4 fusion polypeptide of the invention together with one or more pharmaceutically acceptable excipients. Suitable pharmaceutically acceptable excipients may facilitate processing of the active compounds into preparations suitable for pharmaceutical administration. The pharmaceutical composition may take the form of an aqueous solution and may include physiologically compatible buffers such as Hank’s solution, Ringer’s solution, or physiologically buffered saline. The pharmaceutical composition may additionally or alternatively contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. The pharmaceutical composition may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the pharmaceutical composition may contain suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

The present invention further provides nucleic acids encoding a TB4 fusion polypeptide comprising:

(i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ ID NO: 1, optionally having a Glycine residue at the N terminus; and an Fc domain at the C terminus, the Fc domain optionally comprising one or more mutations which reduce or ablate effector function.

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Thus, a nucleic acid of the present invention may encode a TB4 fusion polypeptide having the sequence according to SEQ ID NO: 4. A nucleic acid of the present invention may encode a TB4 fusion polypeptide having the sequence according to SEQ ID NO: 5. A nucleic acid of the present invention may encode the TB4 fusion polypeptide having the sequence according to SEQ ID NO: 6. A nucleic acid of the present invention may encode a TB4 fusion polypeptide having the sequence according to SEQ ID NO: 7. A nucleic acid of the present invention may encode a TB4 fusion polypeptide that is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the sequence according to any of SEQ ID NOs 4 to 7.

Table 1 below provides the sequences of representative constructs of the present invention.

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</table>
The present invention further provides a host cell comprising a nucleic acid of the invention, i.e. a nucleic acid encoding a TB4 fusion polypeptide comprising (i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ ID NO: 1, optionally having a Glycine residue at the N terminus; and (ii) an Fc domain at the C terminus, the Fc domain optionally comprising one or more mutations which reduce or ablate effector function.

The present invention further provides a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide, for use as a medicament. The present invention further provides methods of treatment using a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide. Thus, the present invention provides a TB4 fusion polypeptide comprising:

(i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ ID NO: A, optionally having a Glycine residue at the N terminus; and

(ii) an Fc domain at the C terminus, the Fc domain optionally comprising one or more mutations which reduce or ablate effector function;

or a composition comprising said TB4 fusion polypeptide, for use as a medicament.

The present invention also includes a TB4-PEG (polyethylene glycol) conjugate. The PEG is of any molecular weight. In one embodiment the PEG is 40kDa. In some embodiments the PEG is attached to TB4 at a variety of locations. In one embodiment the PEG is attached at the C-terminus of TB4. In one embodiment the PEG is attached to the mid-chain of TB4.
The present invention further provides a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide, for use in the prevention or treatment of myocardial infarction; and a method of treating or preventing myocardial infarction using a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide.

The present invention further provides a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide, for use in the prevention or treatment of a muscle wasting disease; and a method of treating or preventing a muscle wasting disease using a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide. Specific muscle wasting diseases include, but are not limited to, Duchenne muscular dystrophy and cachexia that is often associated with chronic diseases (e.g. COPD and chronic heart failure).

The present invention further provides a TB4 fusion polypeptide as defined above, or a composition comprising said TB4 fusion polypeptide, for use in the prevention or treatment of diabetic nephropathy; and a method of treating or preventing diabetic nephropathy using the TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide.

Additionally and/or alternatively, the present invention provides a TB4 fusion polypeptide as defined above, or a composition comprising said TB4 fusion polypeptide, for use in a therapeutic method of tissue regeneration; and/or a therapeutic method of tissue regeneration using a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide.

The tissue to be regenerated may be selected from the group consisting of cardiac muscle, skeletal muscle and renal epithelium.

When used as a medicament or in a particular method of prevention or treatment as defined above, a TB4 fusion polypeptide of the present invention, or a composition comprising said TB4 fusion polypeptide, may be administered together with one or more further therapeutic agents. Thus the present invention provides a TB4 fusion polypeptide, or a composition comprising said TB4 fusion polypeptide, together with one or more further therapeutic agents for simultaneous, sequential, or separate use. The present invention further provides a therapeutic method using a TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide, and one or more further therapeutic agents. The further therapeutic agents
may be administered simultaneously, sequentially, or separately from the TB4 fusion polypeptide of the invention, or a composition comprising said TB4 fusion polypeptide.

The present inventors have surprisingly found that a single dose of a TB4 fusion polypeptide of the present invention induces a greater clinical efficacy relative to a single dose of a wild-type control TB4 polypeptide, lacking an Fc domain.

Without being bound by any theory, the present inventors suggest that the greater clinical efficacy of the TB4 fusion polypeptides of the present invention is a direct result of their markedly longer half-life and/or stability compared to a wild-type TB4 peptide lacking an Fc domain. Thus, as seen in the examples described below, a single dose of a TB4 fusion polypeptide of the present invention may be sufficient to reduce the scar area following myocardial infarction.

Therapeutic efficacy, such as reduction in tissue scar area, may be determined in a patient using a variety of standard, known techniques.

The present invention further provides a method of treatment or prevention of a disease comprising administering a TB4 fusion polypeptide as defined above to a subject. The subject may be a human subject. In particular, the present invention provides a method of treatment or prevention of a disease selected from myocardial infarction, muscle wasting diseases and diabetic nephropathy.

**EXAMPLES**

Aspects of the present disclosure can be further defined by reference to the following non-limiting examples, which describe in detail preparation of certain antibodies of the present disclosure and methods for using antibodies of the present disclosure. It will be apparent to those skilled in the art that many modifications, both to materials and methods, can be practiced without departing from the scope of the present disclosure.

**Characterization data of various TB4 fusion proteins of the present invention:**

TB4-Fc - 286 aa; MW 31713.57 (+~1500glycosylation); Isoelectric Point 6.44; Charge at pH 7 - 1.6; aa sequence:

GSDKPDMAEIEKDSDKKLKKTETQEKEKNPLPSKETIQEKEKQAGGSESGGGSSEPKSC
DHTHTCPPCPAPELGGPSVFLFPPKDTLMSRTPEVTVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSEEMTNQVSLTCLVKGFYPSDIAVEWESNQPPENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQGGNVFSCSVMHEALHNHYTQKSLSLPGK

HSA Fusions

TB4-WT-HAS - 649aa; MW 73430.05; Isoelectric Point 5.9; Charge at pH7 -15.17; aa sequence:
GSDKPDMAIEIKFDKSKLKKTETQEKNPLSKETIEQEKOAGESGSGGGSGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLYQQFPFEDHVKLNVETEFAKTVADESAENCDSKLHTLFGDKLCTVATLRETYGEMADGCAKQEPERNCLQLHDDNPLPRLVRVEVDVMCTAFHDEENFLKLYEIARRHPYFYAPELLLFFAKRYKAFTECCQAADKAACLLLPLKDELDRDEGKASSAKQRLCASKLQQKFGERAFKAWAVARLSQRPFAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENODISKLKCCCEKPLEKSHCIAEVENDMPADLPSLAADFVESDVCNYEAEKDVFLGMFLYEYARRPDYSVVLRLRLAKYSETLEKCCAAAADPHEYAKVFDFEKPLLVEEPQNLIKQNCLEQLFEGYKCFQNALLVRYTSDKPQVSTPLVEVSRNLKGVSKCCKHPEAKRMPCADYLTVVLNLQCVLHKTPVSDRVTCKCTESLVNRPFCASELVEDETVPKENAEFTFHADICTLSEKERQIKKQKTLVHELKHKPKATKEQLKAVMDDFAAFVEKKCADDKETCFACEEGK KlVAASQALGLHHH

TB4-WTY-HAS
GSDKPDMAIEIKFDSKKLKKTETQEKNPLSKETIEQEKOAGESGSGGGSGGGSDAHKSEVAHRFKDLGEENFKALVLIAFAQYLYQQFPFEDHVKLNVETEFAKTVADESAENCDSKLHTLFGLKCTVATLRETYGEMADGCAKQEPERNCLQLHDDNPLPRLVRVEVDVMCTAFHDEENFLKLYEIARRHPYFYAPELLLFFAKRYKAFTECCQAADKAACLLLPLKDELDRDEGKASSAKQRLCASKLQQKFGERAFKAWAVARLSQRPFAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENODISKLKCCCEKPLEKSHCIAEVENDMPADLPSLAADFVESDVCNYEAEKDVFLGMFLYEYARRPDYSVVLRLRLAKYSETLEKCCAAAADPHEYAKVFDFEKPLLVEEPQNLIKQNCLEQLFEGYKCFQNALLVRYTSDKPQVSTPLVEVSRNLKGVSKCCKHPEAKRMPCADYLTVVLNLQCVLHKTPVSDRVTCKCTESLVNRPFCASELVEDETVPKENAEFTFHADICTLSEKERQIKKQKTLVHELKHKPKATKEQLKAVMDDFAAFVEKKCADDKETCFACEEGK KlVAASQALGLHHH
VTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVEL
VKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGYKLVAASQAALGL
HHHHHHHHHHH

TB4-RG-HSA
GSDKPDMAIEIKFDKSKLKKTETQEKNPLPSKETIEQEKEKQAGESGGGSGGGGSDAHKS
EVAHRFKDLGGEENFKALVLIAFAQYLQQCPFEDHVKLNVETEFAKTCVADESAENC
KSLHTLFGDKLCTVATLRETYGEMADCCAQEPERNECFLQHKDDNPNLPRLVREVD
VMCTAFHDNEETFLKKLYEIARRHPYFYAPELLEFFAKRYKAATTECCQAADKACLLLP
KLDELREDEGKASSAKQRLKCASLQKFGERAFAKAWVARLSQRFPKAEFAEVSKLVTDL
TKVHTECCHGDLECCADDRAOLAKYICENGQDISSSKLKECEKKPLEKSHCIAEVENDE
MPADLPSLAADFVESKDVCNKAYEAKDVFLGMFLYEYARRHPDYSVLNNLQCVLHEKTPVSDR
VTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFRFHADICTLSEKERQGKQKQTALVEL
VKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGYKLVAASQAALGLHHHHHHHHHHHHHHHHHHHHHHHHHHH

TB4-RGY-HSA
GSDKPDMAIEIKFDKSKLKKTETQEKNPLPSKETIEQEKEKQAGESGGGSGGGGSDAHKS
EVAHRFKDLGGEENFKALVLIAFAQYLQQCPFEDHVKLNVETEFAKTCVADESAENC
KSLHTLFGDKLCTVATLRETYGEMADCCAQEPERNECFLQHKDDNPNLPRLVREVD
VMCTAFHDNEETFLKKLYEIARRHPYFYAPELLEFFAKRYKAATTECCQAADKACLLLP
KLDELREDEGKASSAKQRLKCASLQKFGERAFAKAWVARLSQRFPKAEFAEVSKLVTDL
TKVHTECCHGDLECCADDRAOLAKYICENGQDISSSKLKECEKKPLEKSHCIAEVENDE
MPADLPSLAADFVESKDVCNKAYEAKDVFLGMFLYEYARRHPDYSVLNNLQCVLHEKTPVSDR
VTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFRFHADICTLSEKERQGKQKQTALVEL
VKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGYKLVAASQAALGLHHHHHHHHHHHHHHHHHHHHHHHHHHH
TB4L2-delTHTFc TM - 284aa; MW 31168.81 (+~1500glycosylation) - as monomer; Isoelectric Point 6.14, 1 A280 corresponds to 0.89 mg/ml; Charge at pH 7 -2.73; aa sequence:
GSDKPDMAIEIKFDKS KLKKTETQEKNPLPS KETIEQEKQAGES GGGGS GGGGSATGGG GAACPPCPAPEFGGPSVFLPPPKDKTLMISRTPEVTVCVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTWLHQDWLNGKEYKCKVSNKALPASIEKTISKA KGQPREPQVYTLPPSREEMTKNQVSLTCLKGFYPSDIAVEWESNGQPENN YKTT PVL DSDGSFLYSLKTVDKSRQQQNVFCSVMHEALNHYTQKSLSLSPGK

TB4 G4SxFc TM - 295aa; MW 31982.52 (+~1500glycosylation) - as monomer; Isoelectric Point 6.22; 1 A280 corresponds to 0.91 mg/ml; Charge at pH 7 -2.64, aa sequence:
GSDKPDMAIEIKFDKS KLKKTETQEKNPLPS KETIEQEKQAGES GGGGS GGGGS GGGGS GGGGS GGGGSATHTCPPCP APEFEGGPSVFLPPPKDKTLMIS RTPEVTVCVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTWLHQDWLNGKEYKCKVSNKALPASIEKTISKA KGQPREPQVYTLPPSREEMTKNQVSLTCLKGFYPSDIAVEWESNGQP ENNYKTT PVLDS DSGSFLYSLKTVDKSRQQQNVFCSVMHEALNHYTQKSLSLSPGK

Example 1; Pharmacokinetic studies in healthy mice

MATERIALS AND METHODS

Production of TB4 fusion proteins

DNA sequences encoding the TB4 peptide sequence N-terminally fused to either the constant part of human immunoglobulin (TB4-Fc) or human serum albumin (TB4-HSA) were cloned into a mammalian expression vector (pOE) to provide a TB4-Fc fusion polypeptide; and a TB4-HSA fusion polypeptide. The pOE vectors contain the following elements; AmpR, BGH polyA, oriP, pUC-ori and/or EBNA-1. DNA fragments corresponding to TB4-Fc and TB4-HSA were cloned and assembled using High Fidelity PCR cloning techniques.

The TB4-Fc and TB4-HSA fusion polypeptides were transiently expressed in HEK293F cells in suspension using 293fectin™ (Invitrogen) as the transfection reagent and grown in
serum-free Freestyle™ medium (Invitrogen). The supernatant was harvested 10 days post transfection.

Alternatively TB4-Fc and TB4-HAS fusion polypeptides were transiently expressed in CHO-based cells (CEP6 or G22) for 6-14 days.

The TB4-Fc fusion polypeptide was purified from the medium by standard Protein A affinity chromatography using MAbSelect SuRe (GE Healthcare) followed by SEC when required. The supernatant was harvested 10 days post transfection. The TB4-Fc fusion polypeptide was purified by standard Protein A affinity chromatography (GE Healthcare). The TB4-HSA fusion polypeptide was purified from the medium by a two-step process consisting of first passing the supernatant through a HiTrap Cibacron Blue column (GE Healthcare) that selectively binds HSA followed by a polish step using an anion exchange HiTrap Q column (GE Healthcare). The purified fusion polypeptides were buffer-exchanged into PBS using dialysis or PD-10 columns and the purity of the constructs was verified using SDS-PAGE and by analytical size-exclusion chromatography, mass spectrometry, and N-terminal sequencing. The resulting proteins were pure and highly monomeric.

**Pharmacokinetic analysis**

Healthy C57/B16 mice (8-12 weeks old; males, Charles River), non-fasted, with body weight of 20-22 g were used in single dose pharmacokinetic (PK) studies of a wild-type TB4 peptide, TB4-Fc fusion polypeptide, and TB4-HSAwt fusion polypeptide. All animal experiments were approved by the animal ethical committee in Gothenburg. The wild-type TB4 peptide was given with a single intraperitoneal injection at 12 mg/kg. The TB4-Fc and TB4-HSAwt fusion polypeptides were administered with a single intravenous injection at a dose level of 3.0 mg/kg for TB4-Fc; and 3.9 mg/kg for TB4-HSAwt.

Mice (n=3) were sacrificed at each of the following time points: predose, 0.5, 1, 2, 4 or 6.5 hrs post-injection. Mice were anaesthetized with isoflurane (Forene, Abbot, Scandinavia, Sweden) and the blood was collected via heart puncture.

Plasma samples were taken at seven time points (0.083, 6, 24, 72, 168 or 336 hr) via vena saphena puncture (according to Etics 168-2009). 20μL of blood was withdrawn at each time-
point, and collected in microvette CB K2E Di-calcium-EDTA tubes (Microvette CB300, serum brown, Sarstedt). Finally the mice were terminated (504 hr) with isoflurane and the blood was collected via heart puncture. The plasma samples were centrifuged at 10000 x g for 5 min at 4 °C and stored in plastic Eppendorf tubes placed on ice during handling and stored at -80 °C pending quantification of the biologic compound.

The concentration of TB4 peptide was measured in mouse plasma samples using a LC-MS method. Rabbit plasma was used as surrogate matrix and the stable isotope labelled analyte [13C15N3-Pro]-Thymosine Beta 4 was used as a surrogate analyte. Plasma and standard samples (50 µL) were mixed with 100 µL of internal standard (stable isotope labelled-TB4 =134nM), into a low binding plate. The plasma proteins were then precipitated with an equal volume (150µL) of acetonitrile. The samples were mixed for 5 minutes and centrifuged at 4000*g for 20 minutes. The supernatant (50µL) was mixed with 50µL of water/0.1%FA in a new plate, and injected onto a UPLC (I-class)-MS Waters system for analysis. The injection volume was 5µL.

Two sandwich-ELISA assays were set up and qualified to measure levels of either TB4-Fc fusion polypeptide or TB4-HSAwt fusion polypeptide in mouse plasma samples using the Gyrolab platform (Gyros AB, Uppsala, Sweden). Briefly both TB4-Fc fusion polypeptide and TB4-HSAwt fusion polypeptide were captured by the biotinylated sheep anti-human thymosine beta-4 affinity purified polyclonal antibody (AF6796 R&D systems). TB4-Fc fusion polypeptide was then detected by Alexa labelled goat anti-human IgG, Fc gamma fragment specific (Jackson; 109-605-098), whereas the TB4-HSAwt fusion polypeptide was detected by Alexa - labelled mouse anti-human IgGl (USBiological; MAb A1274-87K).

RESULTS

The exposure profiles for all compounds, except TB4, are shown in Figure 1. TB4 basal levels before dosing were approximately around 70nM. The terminal half-life of the TB4 peptide was estimated at 1-2 h. Terminal half-life was markedly prolonged for the TB4-HSA fusion polypeptide to around 30-40 h. However, it was surprisingly found that the TB4-Fc fusion polypeptide has a significantly longer half-life of around 300 hours (see Table 1).

Table 1: Terminal half-life of TB4 and TB4 fusion proteins
<table>
<thead>
<tr>
<th>Compound</th>
<th>Terminal half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB4 peptide</td>
<td>2 hours</td>
</tr>
<tr>
<td>TB4-HSA fusion polypeptide</td>
<td>1.2-1.7 days</td>
</tr>
<tr>
<td>TB4-Fc fusion polypeptide</td>
<td>12 days</td>
</tr>
</tbody>
</table>

It can be seen from Table 1 that although TB4-HSA fusion polypeptide has a longer half-life than the TB4 peptide alone, the TB4-Fc fusion polypeptide provides a much greater increase in the half-life of the protein. Furthermore, the TB4-Fc fusion polypeptide shows a much longer half-life even when compared to the TB4-HSA fusion polypeptide.

Thus, the present inventors have surprisingly found that Fc conjugation provides a much greater effect on the half-life of TB4 compared to HSA conjugation.

**Example 2: Acute myocardial infarction studies in mice**

**MATERIALS AND METHODS**  
Acute MI model in mice - permanent ligation of the coronary artery

Male C57BL/6 mice were used as in Example 1 above. For the permanent ligation model, mice were anaesthetised with isofluran and were intubated using an intravenous plastic catheter (ref 381 134, BD Angiocath) and connected to a ventilator (Mini Vent, type 845, Hugo Sachs Electronic) supplied with 2.5-3% isofluran mixed with air and oxygen (80/20%) (ca 150 strokes/minute, 200µl tidal volume).

Rectal temperature was maintained at 37.5 °C by a heating operation table and a heating lamp. Mice were shaved on the chest and ECG needles were inserted in the paws for detection of heart rate. An incision was made in the skin, and the chest muscles were carefully separated and the 4th intercostal space was opened; and a chest retractor inserted. The pericardium was gently dissected and a 7-0 silk ligature was placed around the left coronary artery just under the atrium. The artery was permanently occluded by leaving the ligature around the vessel throughout the study period. Confirmation of ischemia was seen through paleness of the heart distal from suture and ST-elevation of the ECG signal. The ribs and skin was closed with 6-0 absorbable sutures. Analgesic (0.05mg/kg Temgesic, 10mL/kg) was given subcutaneously and mice were allowed to recover in its cage on an electric heating pad.
Treatment with TB4 peptide and fusion proteins

To investigate the effects of TB4 treatments in an acute MI setting, mice were subjected to myocardial infarction by ligating the coronary artery permanently (as described above). Mice were treated with daily intraperitoneal (i.p.) injections of TB4 peptide (12 mg/kg/day or 36 mg/kg/day); or with a single intravenous (i.v.) injection of TB4-Fc fusion polypeptide (3 mg/kg) or TB4-HSA fusion polypeptide (3.9 mg/kg); or with a PBS control. The first i.p. injection of native TB4 and i.v. injection of TB4-Fc and TB4-HSA fusion polypeptides were given just after surgery before the mice had woken up. Blood was collected at day 4 from vena saphena and at day 7 by heart puncture, when the mice were sacrificed. Hearts were removed, rinsed in saline and fixed in formalin for 48 hours before paraffin embedding.

Histology

Formalin-fixed hearts were sliced transversely into 1 mm slices from apex to the top. The heart slices were dehydrated in ethanol and xylene and finally embedded into paraffin. 4 µm thick sections were stained with Masson’s trichrome (MTC) for detection of fibrosis to evaluate the scar area. Stained sections were scanned with Mirax scanner and imported into Biopix. Scar area was determined in the left ventricle as damaged area divided by the total left ventricle area (scar area (% of LV) = scar area/ total left ventricle area).

RESULTS

Plasma concentration of TB4-Fc fusion polypeptide was measured on day 3 and day 7 and was found to range between 25 and 28 µg/mL, as expected according to the pharmacokinetic data in healthy mice (see Example 1). The corresponding values for TB4-HSAwt fusion polypeptide were 2.0-5.3 µg/mL on day 3 and 0.4-0.8 µg/mL on day 7, also in line with the pharmacokinetic data obtained in healthy mice.

Treatment with daily doses of native TB4 peptide or with a single intravenous injection of TB4 fusion polypeptide (either TB4-Fc or TB4-HSA) reduced the scar area compared to no treatment (Figure 2).

The TB4-Fc fusion polypeptide and the TB4-HSA-wt fusion polypeptide were superior to native TB4 peptide in reducing the scar area after a myocardial infarction (Figure 2). After
treatment with a single intravenous injection of TB4-Fc fusion polypeptide or the TB4-HSA-wt fusion polypeptide, reduction of the scar area was more pronounced when compared to mice treated with daily intraperitoneal injections of the native non-conjugated TB4 peptide (Figure 2). Furthermore, the reduction in the scar area was more pronounced in the TB4-Fc-treated mice than in the TB4-HSA-treated mice (Figure 2).

It can therefore be seen from the data that the TB4-Fc fusion polypeptide had a greater effect in vivo than the TB4-HSA fusion polypeptide. These results are in line with the pharmacokinetic data showing that TB4-Fc fusion polypeptides have a significantly longer half-life than the TB4-HSA fusion polypeptide.

**Example 3: Chronic myocardial infarction studies in mice**

To investigate if administration of TB4-Fc fusion polypeptides could also have positive effects on an established scar in the reperfused MI model, mice were dosed 21 days post-MI and scar area was evaluated at day 28.

**MATERIALS AND METHODS**

**Chronic MI model in mice - transient ligation of the coronary artery**

For the transient ligation model, mice were treated as described in Example 2, with the exception that pentobarbital was used to anaesthetise the mice and the artery was occluded transiently by removing the ligature after 45 minutes of ischemia.

To investigate the effects of TB4-Fc fusion polypeptide on an established scar, mice subjected to permanent or transient ligation were administered TB4-Fc fusion polypeptide or a PBS control 21 days after the infarction. One week later, at day 28, mice were sacrificed and hearts removed and fixated in formalin for 48 hours.

**Histology and immunohistochemistry**

Histology was performed as described above. Immunohistochemistry for Wt-1 (a marker for epicardially derived cells (EPDCs)) was performed in a Ventana Discovery XT autostainer, by using a rabbit polyclonal antibody against anti-Wt-1 (dilution 1:200, CA1026, Calbiochem, Merck Millipore, San Diego, CA, USA). All reagents were Ventana products (Roche). Wt-1 positive cells were evaluated blindly with a manual scoring system were 0=no positive Wt-1
cells; 1 = rare number of Wt-1 positive cells; 2 = few Wt-1 positive cells in a single layer, located to a specific level in the heart; 3 = moderate number of Wt-1 positive cells, located to several levels; 4 = extensive number of Wt-1 positive cells in a thick layer, located to several levels.

RESULTS

The TB4-Fc fusion polypeptide was found to have a positive effect on an established scar following MI. Firstly, after treatment with a single dose of the TB4-Fc fusion polypeptide, a statistically significant reduction of the scar area was observed (Figure 3A).

Secondly, treatment with the TB4-Fc fusion polypeptide was found to induce the expression of Wt-1 in the epicardium. Wt-1 is a marker of epicardially derived cells (EPDCs). After treatment with a single intravenous injection of TB4-Fc fusion polypeptide, there was a statistically significant increase in the number of Wt-1 positive cells (Figure 3B), suggesting ongoing cardiac repair.

Thus the present inventors have shown that even an established post-MI scar benefits from the treatment with the TB4-Fc fusion polypeptide of the invention.

Example 4: TB4-Fc fusion polypeptides efficacy study in MI mice

MATERIALS AND METHODS

Myocardial infarction was induced in mice by transient ligation of the coronary artery as described above. The ligature was removed after 60 minutes of ischaemia. Fifteen minutes after reperfusion, mice were administered i) placebo (PBS); ii) TB4(full-length)-Fc fusion polypeptide at a dose of 0.5 mg/kg; iii) TB4(full-length)-Fc fusion polypeptide at a dose of 3 mg/kg; iv) GSDKP-Fc fusion polypeptide (designated T46) at a dose of 3 mg/kg; or v) TB4 (aa 1-18)-Fc at a dose of 3 mg/kg. The animals were sacrificed after 28 days and their hearts were analysed. The scar area was determined as described in Example 2.

Blood vessel formation in the infarct border zone of the heart was assessed by measuring the percent volume density. These results are shown in Figure 6.

Furthermore, 28 days after MI, different types of skeletal muscle were harvested and their weights were measured. The muscle weights of mice treated with TB4-Fc fusion polypeptides were compared to the muscle weights of the PBS-treated mice.
RESULTS

A statistically significant reduction of the scar area was observed after treatment with a single 3 mg/kg dose of the TB4(full-length)-Fc fusion polypeptide shortly after reperfusion (Figure 4A). These data are in agreement with the results from the acute MI mouse study presented in Example 2.

Furthermore, there was a marked increase in the blood vessel density in the infarct border zone in mice treated with a single 3 mg/kg dose of the TB4(full-length)-Fc fusion polypeptide compared to placebo-treated animals (Figure 4B). These data indicate that the TB4(full-length)-Fc fusion polypeptide induces blood vessel formation in the infarct border zone after myocardial infarction.

Analysis of skeletal muscle weight showed a statistically significant (i.e. P=0.05 or less) increase in the weight of certain types of muscle in mice treated with the TB4(full-length)-Fc fusion polypeptide (administered at a dose of 3 mg/kg) and mice treated with the TB4 (aa 1-18)-Fc fusion polypeptide, when compared to the placebo-treated mice (Figure 5). For instance, the soleus muscle had a statistically significant gain in weight in both the mice treated with TB4(full-length)-Fc (3 mg/kg) and the mice treated with TB4 (aa 1-18)-Fc. Furthermore, the mice treated with TB4 (aa 1-18)-Fc also demonstrated a statistically significant increase in the weight of the extensor digitalis longus muscle and the gastrocnemius muscle. There was a statistically significant overall increase in the skeletal muscle weight in the mice treated with TB4 (aa 1-18)-Fc. A marked overall increase in the skeletal muscle weight was also observed in the mice treated with TB4(full-length)-Fc fusion polypeptide.

Thus the present inventors have surprisingly found that conjugation of an Fc domain to a TB4 peptide substantially extends the half-life of the TB4 peptide when compared to unconjugated TB4 peptide and even when compared to HSA-conjugated TB4 peptide. The inventors have also demonstrated herein that the TB4-Fc fusion polypeptides of the present invention have a positive effect in an acute MI setting as well as in a chronic MI setting with an established scar. The present inventors have also shown that treatment with the TB4-Fc fusion polypeptides of the present invention results in an increase in skeletal muscle weight.

Other Embodiments
From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents, publications, CAS, and accession numbers mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent, publication, and accession number was specifically and individually indicated to be incorporated by reference.
Claims

1. A thymosin β4 (TB4) fusion polypeptide comprising:
   (i) at least residues 1-4 (SDKP) of a full-length mature TB4 polypeptide as defined in SEQ ID NO: 1, optionally having a Glycine residue at the N terminus; and
   (ii) an Fc domain at the C terminus, the Fc domain optionally comprising one or more mutations which reduce and/or ablate effector functions.

2. The fusion polypeptide of claim 1, wherein the Fc domain comprises an FQQ triple mutation.

3. The fusion polypeptide of claim 1, wherein the Fc domain comprises a YTE triple mutation.

4. The fusion polypeptide of claim 1, wherein the Fc domain is IgGl, IgG2, IgG3 or IgG4.

5. The fusion polypeptide of claim 1, comprising all residues of the full-length mature TB4 polypeptide as defined in SEQ ID NO: 1.

6. The fusion polypeptide of claim 1, comprising residues 1-18 (SDKPDM AEIEKFDKS KLK) of the full-length mature TB4 polypeptide as defined in SEQ ID NO: 1.

7. The fusion polypeptide as claimed in any one of claims 1 to 4, further comprising a linker peptide located between the TB4 polypeptide residues and the Fc domain.

8. The fusion polypeptide as claimed in claim 5, wherein the linker peptide comprises at least one repeat of SEQ ID NO: 2 (GGGGX).
9. The fusion polypeptide of claim 6, wherein the linker peptide comprises at least one repeat of GGGGG, GGGGS or GGGGA.

10. The fusion polypeptide as claimed in any one of claims 1 to 4, further comprising a hinge peptide located between the TB4 polypeptide residues and the Fc domain; the hinge peptide being selected from THTCPPC (SEQ ID NO: 8), ATHTCPPC (SEQ ID NO: 9); and ACPPC (SEQ ID NO: 10).

11. The fusion polypeptide as claimed in claim 5 or claim 7, further comprising a hinge peptide located between the linker peptide and the Fc domain; the hinge peptide being selected from THTCPPC (SEQ ID NO: 8), ATHTCPPC (SEQ ID NO: 9); and ACPPC (SEQ ID NO: 10).

12. The fusion polypeptide as claimed in claim 3, having an amino acid substitution at residue 30 numbered according to SEQ ID NO: 1.

13. The fusion polypeptide as claimed in claim 10, wherein the amino acid substitution is selected from S30A and S30T.

14. The fusion polypeptide as claimed in claim 8 or claim 9 wherein the hinge peptide is THTCPPC (SEQ ID NO: 8).

15. The fusion polypeptide as claimed in claim 8 or claim 9 wherein the hinge peptide is ACPPC (SEQ ID NO: 10).

16. The fusion polypeptide as claimed in any one of claims 1 to 13, further comprising a lysine residue at the C terminus of the Fc domain.

17. The fusion polypeptide as claimed in any one of claims 5 to 14, wherein the linker peptide comprises 1 to 10 repeats of SEQ ID NO: 2 (GGGGX), optionally wherein X is a serine residue.
18. The fusion polypeptide as claimed in any one of claims 5 to 14, wherein the linker peptide comprises at least two repeats of SEQ ID NO: 2 (GGGGX), optionally wherein X is a serine residue.

19. The fusion polypeptide as claimed in any one of claims 5 to 14, wherein the linker peptide comprises five or six repeats of SEQ ID NO: 2 (GGGGX), optionally wherein X is a serine residue.

20. The fusion polypeptide as claimed in claim 1, having the sequence according to SEQ ID NO: 5.

21. The fusion polypeptide as claimed in claim 1, having the sequence according to SEQ ID NO: 7.

22. The fusion polypeptide as claimed in claim 1, having the sequence according to SEQ ID NO: 8.

23. The fusion polypeptide as claimed in claim 1, having the sequence according to SEQ ID NO: 9.

24. A dimer comprising:
   (i) a first subunit comprising a fusion polypeptide as claimed in any one of the preceding claims; and
   (ii) a second subunit comprising a fusion polypeptide as claimed in any one of the preceding claims.

25. A dimer as claimed in claim 20, where the two subunits are identical.

26. A nucleic acid encoding:
   (i) a fusion polypeptide as claimed in any one of claims 1 to 19; or
   (ii) a dimer as claimed in claim 20 or claim 21.
27. A host cell comprising the nucleic acid of claim 22.

28. The fusion polypeptide as claimed in any one of claims 1 to 19, or the dimer as claimed in claim 20 or claim 21, for use as a medicament.

29. The fusion polypeptide as claimed in any one of claims 1 to 19, or the dimer as claimed in claim 20 or claim 21, for use in the prevention or treatment of myocardial infarction.

30. The fusion polypeptide as claimed in any one of claims 1 to 19, or the dimer as claimed in claim 20 or claim 21, for use in the prevention or treatment of a muscle wasting disease.

31. The fusion polypeptide as claimed in any one of claims 1 to 19, or the dimer as claimed in claim 20 or claim 21, for use in the prevention or treatment of diabetic nephropathy.

32. The fusion polypeptide, or dimer, for use according to any one of claims 24 to 30 wherein said use comprises administration of a single dose of the fusion polypeptide, or dimer, to a subject in need thereof.

33. A pharmaceutical composition comprising the fusion polypeptide as claimed in any one of claims 1 to 19, or the dimer as claimed in claim 20 or claim 21, together with one or more pharmaceutically acceptable excipients.
FIG. 1

- TB4-Fc 3.0 mg/kg (n=3)
- TB4-HSAwt- 3.9 mg/kg (n=2)
- TB4-HSA-RG- 3.6 mg/kg (n=3)
- TB4-HSA-RGY- 3.0 mg/kg (n=2)

Conc (nmol/L) vs Time (h)
Figure 2

![Graph showing scar area (% of LV) with various treatments: PBS, TB4 (12 mg/kg/d), TB4 (36 mg/kg/d), TB4-Fe (3.0 mg/kg), TB4-HSA (3.9 mg/kg/d). The graph indicates a significant difference (P<0.05) between treatments.]
Figure 3

A

\[ \text{scar area (\% LV)} \]

\[ \begin{array}{c}
\text{control} \\
\text{TB4-Fc}
\end{array} \]

\[ p < 0.05 \]

B

\[ \text{WT-1 scoring} \]

\[ \begin{array}{c}
\text{control} \\
\text{TB4-Fc}
\end{array} \]

\[ p < 0.05 \]
Figure 4

A  Scar area adjusted for day-1 LVEF

B  Blood vessels in infarct border zone adjusted for day-1 LVEF
Figure 5

A

TB4-Fc 3 mg

% increase compared to vehicle

GN  Sol  QS  EDL  TA

P=0.1  P<0.01  P=0.07  P=0.06

B

TB4 (aa 1-19)-Fc 3 mg/kg

% increase compared to vehicle

GN  Sol  QS  EDL  TA

P=0.04  P<0.01  P=0.07  P=0.05  P=0.1

GN = Gastrocnemius
Sol = Soleus
QS = Quadriceps
EDL = Extensor Digitalis Longus
TA = Tibialis Anterior

C

Muscle Weight Difference

% increase compared to vehicle

Vehicle  TB4 (aa 1-19) - Fc 3 mg/kg  TB4-Fc 3 mg/kg  TB4-Fc 3 mg/kg

P=0.04

P=0.06
Figure 6

The graph shows the Wt-1 scoring for different treatments. The x-axis represents various treatments including sham+untreated, MI+untreated, MI+TB4 12 mg/kg/d, MI+TB4 36 mg/kg/d, MI+TB4-Fc 3 mg/kg, and MI+TB4-HSA 3.9 mg/kg. The y-axis represents the Wt-1 scoring ranging from 0 to 5.
Figure 7

![Graph showing adjusted EF (%) for different treatments. The x-axis represents various treatments including PBS, GSDKP-Fc (3 mg/kg), TB4-Fc (0.5 mg/kg), TB4-Fc (3 mg/kg), and TB4(aa 1-19)-Fc (3 mg/kg). The y-axis represents adjusted EF (%) ranging from 0 to 50%.]
A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/22, C07K 14/575 (2016.01)
CPC - A61K 38/2292, C07K 14/57581, C07K 2319/30

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

B. FIELD(S) SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 38/22, C07K 14/575 (2016.01 )
CPC - A61K 38/2292, C07K 14/57581 , C07K 2319/30

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C07K 2319/00
(keyword limited; terms below)

Electronic data base consulted during the international search (name of database and, where practicable, search terms used)
PatBase, PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents, Google Scholar
Search terms: thymosin, thymosin beta 4, thymosin beta 4, thymosin B-4, thymosin B4, TB4, fusion, fused, linked, Fc, hinge, linker, peptide, polypeptide, FQX, Phe-Gln-Gln, Phe Gin Gin, YTE, Tyr-Thr-Glu, Tyr Thr Glu, IgG1, IgG2, IgG3, IgG4

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>US 2006/0264360 A1 (GIRARDI et al.) 23 November 2006 (23.1.2006) para [0010], [0041], [0042], [0059], [0237], [0517]; SEQ ID NO: 1; SEQ ID NO: 10</td>
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<td>A</td>
<td>US 2013/0039884 A1 (BOGIN et al.) 14 February 2013 (14.02.2013) para [0094], [0157], [0279], [0319], [0366], [0399], [0468], [0517]</td>
<td>1-10, 12-15, 22-23, 20-21</td>
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<td>Y</td>
<td>US 2015/0017169 A1 (UCB PHARMA S.A.) 15 January 2015 (15.01.2015) para [0156], [0206]; SEQ ID NO: 173</td>
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<td>Y</td>
<td>US 201 1/0305697 A1 (WALCZAK) 15 December 2011 (15.12.2011) abstract; para [0060]; claim 7; SEQ ID NO: 58</td>
<td>23</td>
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</table>

Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search: 27 April 2016
Date of mailing of the international search report: 17 MAY 2016

Name and mailing address of the ISA/US: Lee W. Young
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PCT Helpdesk: 571-273-4300
PCT OSP: 571-273-7774

Form PCT/ISA/2 10 (second sheet) (January 2015)
### DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 2013/0216513 A1 (SALAS et al.) 22 August 2013 (22.08.2013) SEQ ID NO: 121</td>
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</tbody>
</table>
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. \(\square\) Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. \(\square\) Claims Nos.: 11, 16-19, 24-26, 27-33
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

1. \(\square\) As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. \(\square\) As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. \(\square\) As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. \(\square\) No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
\(\square\) The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
\(\square\) The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
\(\square\) No protest accompanied the payment of additional search fees.