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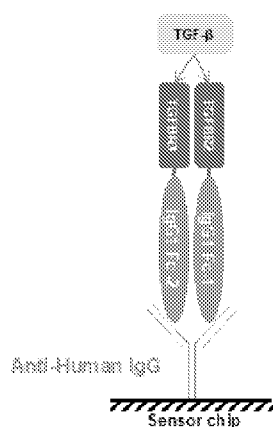
(54) Title: TGF- β INHIBITOR COMPOSITION AND USE THEREOF

Fig. 7

(57) Abstract: A therapeutic composition is described that can be used for treating or prevention of diseases association with modulation of activity of human TGF- β . In certain aspects, the disclosed invention is based, on engineering of a heterodimeric protein assembly that is capable of binding to human TGF- β and attenuating its function. The heterodimeric protein assembly comprises extracellular portions of human TGFR1, TGFR2 and TGFR3, or their functional fragments. Each extracellular TGFR portion is fused to a distinct mutant of Fc portion of the human Ig Gamma-1. The two distinct Fc mutants in the heterodimeric protein assembly are engineered as to favor the heteromeric dimer formation between the two Fc mutants over any homomeric assembly. DNA expression vectors and expression systems for the polypeptides in mammalian cells are also provided for.

TGF- β INHIBITOR COMPOSITION AND USE THEREOF

FIELD OF THE INVENTION

Generally, the invention relates to the field of biological pharmaceuticals as well as their use in
5 in oncology, e.g. prostate cancer, lung cancer, etc. More specifically, the invention relates to a heterodimeric TGFBR2/TGFBR3 -derived composition that is capable of inhibiting TGF- β cytokine.

BACKGROUND

10 Transforming growth factor beta (TGF- β) isoforms, TGF- β 1, β 2, and β 3, are small (25 kDa) homodimeric signaling proteins. They are secreted in a latent form and are activated by multiple mechanisms, including integrin binding and proteolysis. They form a complex with the TGF- β type I and type II receptors (T β RI and T β RII) for signal transduction, in which T β RII phosphorylates and activates T β RI. The phospho-T β RI then phosphorylates intracellular Smad2
15 and Smad3, which form a complex with the common-mediator Smad, Smad4, to regulate gene expression.

TGF- β s are potent growth inhibitors in normal epithelial cells, including normal prostate epithelial cells, by stimulating apoptosis and inhibiting G1 to S cell cycle progression. The deletion of Smad4 has been furthermore shown to drive the invasion and metastasis of indolent prostate
20 tumors with Pten deletion in a mouse model, demonstrating the tumor suppressive activity of the TGF- β /Smad pathway in the prostate gland. Thus, it is not surprising that carcinoma cells in general and prostate cancer (PCa) cells in particular are resistant to TGF- β -induced growth inhibition and that loss of or reduced expression of the signaling receptors, T β RI, T β RII, or the non-signaling TGF- β type III receptor, also known as betaglycan, is often observed during the
25 progression of human PCa. (see Qin T, et al., Oncotarget, 2016, Vol. 7, (No. 52), pp: 86087-86102, the entire teachings of which are incorporated by reference herein).

Prostate carcinoma cells, while responding poorly to TGF- β -mediated growth inhibition, often produce much higher levels of TGF- β isoforms than their normal counterparts. Furthermore, latent TGF- β is activated by the protease prostate specific antigen (PSA), which is an androgen receptor
30 (AR) target gene abundantly secreted by advanced androgen-independent PCa cells. Serum TGF- β 1 levels have been shown to correlate with tumor burden, metastasis, and serum PSA in PCa

patients and an increased level of TGF- β 1 is strongly associated with PCa progression and poor clinical outcome. Excessive levels of TGF- β may act on tumor stromal cells in a paracrine fashion to promote disease progression. TGF- β 's tumor promoting activity may be related to its ability to generate and maintain cancer stem cells, including PCa stem cells, which are AR negative and presumably sensitive to TGF- β . TGF- β s are also known to stimulate the conversion of CD4+CD25- T cells to CD4+CD25+Foxp3+ regulatory T-cells, which inhibit anti-tumor immunity. Treatments with TGF- β inhibitors, such as soluble betaglycan or a pan-isoform neutralizing antibody, have been shown to have beneficial effects in animal models of PCa, including inhibition of the growth and angiogenesis of tumors formed by AR negative human PCa cells or inhibition of regulatory T-cell production and tumor progression. Thus, there are multiple mechanisms by which TGF- β s promote the progression of advanced disease and treatment with TGF- β inhibitors appears to be a viable strategy for attenuating disease progression. The TGF- β pathway is known however to be tumor suppressive in normal and some experimental models of early stage adenocarcinomas as mentioned above, and even advanced tumors may contain early and late stages of lesions due to tumor heterogeneity. Thus, the greatest perceived risk of TGF- β antagonists in treating late stage PCa is the potential progression of early-stage tumor cells in which TGF- β pathway is still tumor suppressive.

TGFBR1, TGFBR2 and TGFBR3 proteins are single spanning membrane receptors. They consist of signal peptide, extracellular domain, transmembrane and cytoplasmic domains.

TGFBR1 exists in two isoforms, Isoform-1 and Isoform-3, where Isoform-1 is commonly accepted as the canonical form and is four amino acid residues shorter than the non-canonical Isoform 3. Extracellular portion of TGFBR1 consists of 125 and 129 amino acid residues for Isoform-1 and Isoform-2, respectively, while extracellular domain of TGFBR2 contains 144 amino acids. Extracellular domain of TGFBR3 consists of 767 amino acid residues and includes N-terminal BGo and C-terminal BGzp domains (Fig. 1). BGo domain binds TGF- β whereas BGzp binds both TGF- β and activins.

SUMMARY OF THE INVENTION

This summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This summary is not intended to identify key

features or essential features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

In certain aspects, the present invention provides for a heterodimeric recombinant protein composition capable of binding human TGF- β . The recombinant protein composition included a
5 first polypeptide which includes amino acid sequence of SEQ ID NO. 1 or of SEQ ID NO. 2; and a second polypeptide which includes amino acid sequence of SEQ ID NO. 3, or of SEQ ID NO. 4, or of SEQ ID NO. 5.

In certain aspects, the present invention provides for a therapeutic composition which contains a recombinant protein composition capable of binding human TGF- β . The recombinant
10 protein composition included a first polypeptide which includes amino acid sequence of SEQ ID NO. 1 or of SEQ ID NO. 2, a second polypeptide which includes amino acid sequence of SEQ ID NO. 3, or of SEQ ID NO. 4, or of SEQ ID NO. 5, and a pharmaceutically acceptable carrier or excipient. The therapeutic composition may further comprising about 0.02% PS-20, about 3% D-mannitol, about 20 mM NaCl, about 70 mM L-proline, and about 25 mM histidine at pH 6.0 .

15 The first polypeptide of the recombinant heterodimeric protein may contain amino acid sequence of SEQ ID NO. 2, while the second polypeptide includes amino acid sequence of SEQ ID NO. 5.

In certain aspects, the present invention provides for an isolated nucleic acid encoding a polypeptide containing amino acid sequence of: SEQ ID NO. 1, or of SEQ ID NO. 2, or of SEQ ID
20 NO. 3, or of SEQ ID NO. 4, or of SEQ ID NO. 5. The nucleic acid may have its codon usage optimized for high expression of the polypeptide in a mammalian cell.

The isolated nucleic acid may contain a DNA sequence of SEQ ID NO. 6, or of SEQ ID NO. 7, or of SEQ ID NO. 8, or of SEQ ID NO. 9.

In certain aspects, the present invention provides for an isolated nucleic acid containing a
25 DNA sequence of SEQ ID NO. 10. The nucleic acid may be further incorporated into a mammalian cell. The mammalian may be a CHO-K1 cell.

In certain aspects, the present invention provides for a composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5, for use in the treatment of idiopathic
30 pulmonary fibrosis, systemic scleroderma, pulmonary arterial hypertension, or a fibrotic disease of heart, liver or kidney.

In certain aspects, the present invention provides for a method of treating or preventing idiopathic pulmonary fibrosis, systemic scleroderma, pulmonary arterial hypertension, or a fibrotic disease of heart, liver or kidney. The method includes administering to a patient in need for treating or preventing idiopathic pulmonary fibrosis, systemic scleroderma, pulmonary arterial
 5 hypertension, or a fibrotic disease of heart, liver or kidney, a therapeutically effective amount of a pharmaceutical composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings and descriptions are provided to aid in the understanding of the invention:

Fig. 1 is a schematic diagram of TGFBR3 (betaglycan) domain structure, with the N-terminal orphan domain (BGO) and the N- and C-terminal zona pellucida domains, BGZP-N and BGZP-C, respectively, glycosaminoglycan chains attached to two residues in the ZP-N subdomain are
 15 shown schematically as beads on a string, disulfide bonds are represented by S-S, while free cysteines are represented by -SH;

Fig. 2 shown SDS-PAGE analysis of TGFBR2/TGFBR3/Fc heterodimer next to molecular marker, 10 µg per lane was analyzed under reducing and non-reducing conditions to identify
 20 potential homo- and hetero-dimers, as a loading control, a mouse IgG2a (m-IgG2a) and a human IgG4 (h-IgG4) were also loaded, markers, kDa are indicated on the right;

Fig. 3 shows a titration curve for TGF-β, MDA-MD-231 cells in serum-free medium were treated with various concentrations of TGF-β and 24 hours post-treatment cell supernatant was quantified for IL-11, EC50 value was calculated from variable slope four parameter algorithm;

Fig. 4 shows inhibition of TGF-β-induced IL-11 secretion by TGFBR2/TGFBR1/Fc chimeras, MDA-MB-231 cells were treated with constant concentration of TGF-β, 2 ng/ml, in the presence of varying concentration of TGFBR2/TGFBR1-Isoform-1/Fc and TGFBR2/TGFBR1-Isoform-3/Fc, IC50 values were calculated from variable slope four parameter algorithm;

Fig. 5 shows Inhibition of TGF-β-induced IL-11 secretion by TGFBR2/TGFBR3/Fc chimera. MDA-MB-231 cells were treated with constant concentration of TGF-β, 2 ng/ml, in the presence of varying concentration of TGFBR2/TGFBR3/Fc, the experiment was repeated twice,

left and right panes, and the corresponding IC₅₀ values were calculated from variable slope four parameter algorithm;

Fig. 6 shows inhibition of TGF- β -induced IL-11 secretion by TGFBR2/TGFBR3/Fc chimera, MDA-MB-231 cells were treated with two concentrations of TGF- β , 1 ng/ml and 2 ng/ml, in the presence of varying concentration of TGFBR2/TGFBR3/Fc, data for two TGF- β concentrations are shown on left and right panes, respectively and the corresponding IC₅₀ values were calculated from variable slope four parameter algorithm;

Fig. 7 shows schematic diagram of TGFBR2/TGFBR3/Fc capturing approach, anti-human IgG were chemically crosslinked to the chip surface, TGFBR2/TGFBR3/Fc was captured by the immobilized antibodies and various concentrations of TGF- β were applied to the chip, at regeneration step, TGFBR2/TGFBR3/Fc along with TGF- β bound to it was removed from the capturing antibodies using 3 M magnesium chloride;

Fig. 8 shows sensograms of binding of TGF- β to TGFBR2/TGFBR3/Fc captured by immobilized anti-human antibodies in experiment 1, the curves were analyzed using 1:1 binding model;

Fig. 9 shows sensograms of binding of TGF- β to TGFBR2/TGFBR3/Fc captured by immobilized anti-human antibodies in experiment 2, the curves were analyzed using 1:1 binding model;

Fig. 10 shows sensograms of binding of TGF- β to TGFBR2/TGFBR3/Fc captured by immobilized anti-human antibodies in experiment 3, the curves were analyzed using 1:1 binding model;

Fig. 11 shows the plasmid map of TGFBR2-Fc1 fusion protein expression construct for transient transfection in mammalian cells;

Fig. 12 shows the plasmid map of TGFBR1-Fc2, Isoform 1, fusion protein expression construct for transient transfection in mammalian cells;

Fig. 13 shows the plasmid map of TGFBR1-Fc2, Isoform 3, fusion protein expression construct for transient transfection in mammalian cells; and

Fig. 14 shows the plasmid map of TGFBR3-Fc2 fusion protein expression construct for transient transfection in mammalian cells;

Fig. 15 shows the plasmid map of pAG12B-TGFBR2-Fc1 for facilitating plasmid generation for expression of TGFBR2/TGFBR3/Fc in mammalian cells;

Fig. 16 shows the plasmid map of pAG12B-TGFBR3-Fc2 for facilitating plasmid generation for expression of TGFBR2/TGFBR3/Fc in mammalian cells;

Fig. 17 shows the plasmid map of pAG12B- TGFBR2/TGFBR3/Fc for co-expressing both pAG12B-TGFBR2-Fc1 and TGFBR2/TGFBR3/Fc in mammalian cells, each mediated by its own promoter regulatory region and BGH poly A region;

Fig. 18 shows Western blot analysis of selected 12 clones for the first screen, 7.5ul of cell medium from each clone was mixed with 7.5ul of reduced (A. left panel) of non-reduced (B. right panel), analyzed on a 4-12% SDS gel and detected using the HRP-conjugated anti-human IgG, double bands (corresponding to TGFBR2-Fc1 and TGFBR3-Fc2) were detected under the reduced conditions and a single band (~125kDa) was detected under non-reduced conditions;

Fig. 19 shows 2nd screen of single cell clones. Clone #31 and clone #36 from the 1st screen were diluted to average 0.5-1 cell/well for 4x 96-well plates, 10ul of cell medium from 96 single cell clones was analyzed by dot blot;

Fig. 20 shows validation of 12 single cell clones, 7.5ul of cell medium from each of 12 selected single cell clones was analyzed by Western blot using anti-human IgG-HRP;

Fig. 21 shows documentation of monoclonality, imaging of colony formation from single cell of clone #A6 and #G9. During the 2nd screen, clones A6 and G9 with obvious single healthy cell were traced everyday by micro-photographing to monitor the growth of two clones (A for clone A6 and B for clone G9);

Fig. 22 shows stability test results: five clones (A6, D10, D11, F7 and G9) that were selected from the second screen of single cell clones were used for the stability test for a total ten passages, each clone was seeded in a 96-well plate in triplets with the same amount of cells and passaged twice a week (3-4 days), conditioned media were collected from each passage and analyzed by ELISA assay and detected by using HRP-conjugated anti-human IgG;

Fig. 23 shows SDS-PAGE analysis of purified from conditioned medium TGFBR2/TGFBR3/Fc heterodimeric protein: the conditioned media of clones A6, D10 and G9 were collected at day 10 of post-passaging, the heterodimeric protein was purified by passing the conditioned medium through a Protein A sepharose column and analyzed by SDS-PAGE under non-reducing conditions.

DETAILED DESCRIPTION OF THE INVENTION

The teachings disclosed herein are based, in part, upon engineering of a heterodimeric protein assemblies that are capable of binding to human TGF- β and attenuating its function. The
5 heterodimeric protein assemblies of the present teachings are two-subunit chimeric proteins, each subunit comprised of a cytoplasmic fragment of human TGFBR1, TGFBR2, or TGFBR3, fused with a Fc fragment of human IgG.

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are
10 discussed below or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and how to make and use them. The scope or meaning of any use of a term will be apparent from the specific context in which the term is used. "About" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically,
15 exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Alternatively, and particularly in biological systems, the terms "about" and "approximately" may mean values that are within an order of magnitude, preferably within 5- fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term
20 "about" or "approximately" can be inferred when not expressly stated.

The methods of the invention may include steps of comparing sequences to each other, including wild-type sequence to one or more mutants (sequence variants). Such comparisons typically comprise alignments of polymer sequences, e.g., using sequence alignment programs and/or algorithms that are well known in the art (for example, BLAST, FASTA and MEGALIGN,
25 to name a few). The skilled artisan can readily appreciate that, in such alignments, where a mutation contains a residue insertion or deletion, the sequence alignment will introduce a "gap" (typically represented by a dash, or "A") in the polymer sequence not containing the inserted or deleted residue.

The methods of the invention may include statistical calculations, e.g. determination of IC₅₀
30 or EC₅₀ values, etc.. The skilled artisan can readily appreciate that such can be performed using a

variety of commercially available software, e.g. PRISM (GraphPad Software Inc, La Jolla, CA, USA) or similar.

"Homologous," in all its grammatical forms and spelling variations, refers to the relationship between two proteins that possess a "common evolutionary origin," including proteins from
5 superfamilies in the same species of organism, as well as homologous proteins from different species of organism. Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions. However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to
10 sequence similarity and may or may not relate to a common evolutionary origin.

The term "sequence similarity," in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin.

The terms "protein" and "polypeptide" are used interchangeably. The polypeptides described
15 herein may be comprised of more than one contiguous amino acid chain, thus forming dimers or other oligomeric formations. In general, the polypeptides of the present teachings for use in mammals are expressed in mammalian cells that allow for proper post-translational modifications, such as CHO or HEK293 cell lines, although other mammalian expression cell lines are expected to be useful as well. It is therefore anticipated that the polypeptides of the present teachings may be
20 post-translationally modified without substantially effecting its biological function.

In certain aspects, functional variants of the heterodimeric protein assemblies of the present teachings include fusion proteins having at least a biologically active portion of the human TGFBR1, TGFBR2, or TGFBR3 or a functional fragment thereof, and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to,
25 polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin heavy chain constant region (e.g., an Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, the TGFBR1, TGFBR2, or TGFBR3 polypeptide portions may be fused with a domain that stabilizes the TGFBR1, TGFBR2, or TGFBR3 polypeptides *in vivo* (a "stabilizer" domain),
30 optionally via a suitable peptide linker. The term "stabilizing" means anything that increases the half life of a polypeptide in systemic circulation, regardless of whether this is because of decreased

destruction, decreased clearance, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on certain proteins. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (e.g., dimerizing, tetramerizing) domains and functional domains that confer an additional biological function, e.g. promoting accumulation at the targeted site of action *in vivo*.

In an exemplary embodiment, the first subunit is a fusion of a cytoplasmic fragment of human TGFBR2 with a Fc-1 portion of human IgG1 (GenBank: J00228.1). The IgG-Fc-1 fragment has five amino acid substitutions to improve heterodimer formation and other functions. The second subunit is a fusion protein of an extracellular domain of either TGFBR1 L34-E125, for Isoform-1, or TGFBR1 L34-E129, for Isoform-2, or an extracellular fragment G21-D379 of human TGFBR3, fused with Fc-2 fragment of IgG1. The Fc-2 fragment fused to TGFBR1 or TGFBR3 extracellular domains has three mutations for function modifications.

The extracellular fragment of TGFBR2 extends from T23 to Q166. However, last 7 amino acid residues of this domain are LLLVIFQ, wherein 6 out of 7 residues are hydrophobic. These residues were therefore removed, and thus the construct ends with residue D159, resulting in a 137 amino acid fragment. Therefore, in one exemplary embodiment TGFBR2-Fc-1 fusion protein may comprise the amino acid sequence of SEQ. ID NO. 1

TGFBR2-Fc-1 fusion polypeptide1 (SEQ ID NO. 1)

```

MKWVTFISLL FLFSSAYSTI PPHVQKSVNN DMIVTDNNGA VKFPQLCKFC DVRFSTCDNQ 60
KSCMSNCSIT SICEKPQEVV VAVWRKNDEN ITLETVCHDP KLPYHDFILE DAASPKCIMK 120
EKKKPGETFF MCSCSSDECN DNIIFSEEYN TSNPDGSGGG DKTHTCPPCP APELLGGPSV 180
FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY 240
RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVCT LPPSRDELTK 300
NQVSLSCAVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFKLVSKL TVDKSRWQQG 360
NVFSCSVMHE ALHNHYTQKS LSLSPGK 387

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In another exemplary embodiment TGFBR2-Fc-1 fusion protein may comprise the amino acid sequence of SEQ. ID NO. 2

TGFBR2-Fc-1 fusion polypeptide2 (SEQ ID NO. 2)

5
 MYRMQLLS CI ALSLALVTNS TIPPHVQKSV NNDMIVTDNN GAVKFPQLCK FCDVRFSTCD 60
 NQKSCMSNCS ITSICEKPQE VCVAVWRKND ENITLETVCH DPKLPYHDFI LEDAASPKCI 120
 MKEKKKPGET FFMCS CSSDE CNDNIIFSEE YNTSNPDGSG GGDKTHTCPP CPAPELLGGP 180
 SVFLFPPKPK DTL MISRTPE VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS 240
 10 TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV CTLPSPSRDEL 300
 TKNQVSLSCA VKGFYPSDIA VEWESNGQPE NNYKTTPPV L DSDGSFKLVS KLTVDKSRWQ 360
 QGNVFSCSVM HEALHNHYTQ KSLSLSPGK 389

15 To generate TGFBR1-Fc-1 fusion protein, both isoforms were used where coding sequences of extracellular domains of TGFBR1 were fused with a modified human IgG1-Fc fusion protein. Native coding sequence of TGFBR1, L34-E125 or L34-E129 fragments (Isoform-1 and Isoform-2, respectively) were used for fusion with modified IgG-Fc-2 that includes three mutations: S237C, T249W and K292A.

20 In one exemplary embodiment TGFBR1-Fc-2 fusion protein (Isoform-1) may comprise the amino acid sequence of SEQ. ID NO. 3.

TGFBR1-Fc-2 fusion polypeptide (SEQ ID NO. 3)

25 MYRMQLLS CI ALSLALVTNS LQCFCHLCTK DNFTCVTDGL CFVSVTETTD KVIHNSMCIA 60
 EIDLIPRDRP FVCAPSSKTG SVTTTYCCNQ DHCNKIELPT TVKSSPGLGP VEGSGGGDKT 120
 HTCPCCPAPE LLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE 180
 VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAKGQP 240
 REPQVYTLPP CRDELTKNQV SLWCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDS DGS 300
 30 FFLYSALTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK 344

In another exemplary embodiment TGFBR1-Fc-2 fusion protein (Isoform-3) may comprise the amino acid sequence of SEQ. ID NO. 4.

TGFBR13-Fc-2 fusion polypeptide (SEQ ID NO. 4)

5 MYRMQLLS CI ALSLALVTNS LQCFCHLCTK DNFTCVTDGL CFVSVTETTD KVIHNSMCIA 60
 EIDLIPDRP FVCAPSSKTG SVTTTYCCNQ DHCNKIELPT TGPFSVKSSP GLGPVEGSGG 120
 GDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV 180
 DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA 240
 10 KGQPREPQVY TLPPCRDELT KNQVSLWCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD 300
 SDGSFFLYSA LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK 348

To generate TGFBR3-Fc-2 fusion molecule, G21-D379 was fused to a modified human IgG1-
 15 Fc-2 fusion protein which includes three mutations S237C, T249W and K292A.

In an exemplary embodiment TGFBR3-Fc-2 fusion protein may comprise the amino acid sequence of SEQ. ID NO. 5.

20 TGFBR3-Fc-2 fusion polypeptide (SEQ ID NO. 5)

MYRMQLLS CI ALSLALVTNS GPEPGALCEL SPVSASHPVQ ALMESFTVLS GCASRGTTGL 60
 PQEVHVLNLR TAGQGPGQLQ REVTLHLNPI SSVHIHHKSV VLLNSPHPL VWHLKTERLA 120
 TGVSRFLFLVS EGSVVQFSSA NFSLTAETEE RNFPHGNEHL LNWARKEYGA VTSFTELKIA 180
 25 RNIYIKVGED QVFPPKCNIG KNFLSLNYLA EYLQPKAAEG CVMSSQPQNE EVHIIELITP 240
 NSNPYSAFQV DITIDIRPSQ EDLEVVKNL I LILKCKKSVN WVIKSFDVKG SLKIIAPNSI 300
 GFGKESERSM TMTKSIRDDI PSTQGNLVKW ALDNGYSPIT SYTMAPVANR FHLRLNNAE 360
 EMGDDEEVHTI PPELRILLDG SGGGDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT 420
 PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG 480
 30 KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPCRD ELTKNQVSLW CLVKGFYPSD 540
 IAVEWESNGQ PENNYKTTTP VLDSDGSFFL YSALTVDKSR WQQGNVFSCS VMHEALHNHY 600
 TQKSLSLSPG K 611

Using well established molecular biology techniques, synthetic gene corresponding to the protein designs of the fusion proteins of the present teachings have been codon-optimized for expression in mammalian cells and synthesized. Invitrogen pTT vector was digested with EcoRI and BamHI restriction enzymes and corresponding constructs digested with EcoRI and BamHI
 5 were cloned into the vector. All constructs were verified by sequencing of the entire coding region to ensure correct cloning and coding sequence integrity.

In an exemplary embodiment the DNA fragment with an open reading frame corresponding to TGFBR2-Fc-1 fusion protein may comprise the DNA sequence of SEQ. ID NO. 6.

10 TGFBR2-Fc-1 DNA1 (SEQ ID NO. 6)

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AGCTGAATTC ACCGCCATGT ACCGGATGCA GCTGCTGAGC TGTATCGCCC TGTCTCTGGC 60
CCTGGTCACC AATAGCACAA TCCCTCCACA CGTGCAGAAA AGCGTGAACA ACGACATGAT 120
15 CGTGACCGAC AACAAATGGCG CCGTGAAGTT CCCTCAGCTG TGCAAGTTCT GCGACGTGCG 180
GTTTCAGCACC TGTGACAACC AGAAAAGCTG CATGAGCAAC TGCAGCATCA CCAGCATCTG 240
CGAGAAGCCC CAAGAAGTGT GCGTCGCCGT CTGGCGGAAG AACGACGAGA ACATCACCCCT 300
GGAAACCGTG TGTCACGACC CCAAGCTGCC CTACCACGAC TTCATCCTGG AAGATGCCGC 360
CTCTCCTAAG TGCATCATGA AGGAAAAGAA GAAGCCCGGC GAGACATTCT TCATGTGCAG 420
20 CTGCTCCAGC GACGAGTGCA ACGACAACAT CATCTTCAGC GAAGAGTACA ACACCAGCAA 480
TCCCGACGGA AGCGGCGGAG GCGATAAGAC CCATACCTGT CCTCCATGTC CTGCTCCAGA 540
GCTGCTCGGA GGCCCTTCCG TGTTTCTGTT CCCTCCAAAG CCTAAGGACA CCCTGATGAT 600
CAGCAGAACC CCTGAAGTGA CCTGCGTGGT GGTGGATGTG TCTCACGAGG ACCCCGAAGT 660
GAAGTTCAAT TGGTACGTGG ACGGCGTGGA AGTGCACAAC GCCAAGACCA AGCCTAGAGA 720
25 GGAACAGTAC AACAGCACCT ACAGAGTGGT GTCCGTGCTG ACCGTGCTGC ACCAGGATTG 780
GCTGAACGGC AAAGAGTATA AGTGCAAGGT GTCCAACAAG GCCCTGCCTG CTCCTATCGA 840
GAAAACCATC AGCAAGGCCA AGGGCCAGCC TCGCGAACCT CAAGTCTGTA CACTGCCTCC 900
TAGCCGGGAC GAGCTGACCA AGAATCAGGT GTCCCTGTCC TGTGCCGTGA AGGGCTTCTA 960
CCCTTCCGAT ATCGCCGTGG AATGGGAGAG CAATGGCCAG CCTGAGAACA ACTACAAGAC 1020
30 CACACCTCCT GTGCTGGACA GCGACGGCTC CTTTAAGCTG GTGTCCAAGC TGACAGTGGA 1080
CAAGAGCAGA TGGCAGCAGG GCAACGTGTT CAGCTGCAGC GTGATGCACG AGGCCCTGCA 1140
CAACCACTAC ACCCAGAAGT CCCTGAGCCT GTCTCCTGGC AAGTGATAGG ATCCTCGA 1198
  
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In another exemplary embodiment the DNA fragment with an open reading frame corresponding to TGFBR2-Fc-1 fusion protein may comprise the DNA sequence of SEQ. ID NO. 7.

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TGFBR2-Fc-1 DNA2 (SEQ ID NO. 7)

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10  AATTAAGCCA CCATGTACCG TATGCAACTC CTGTCTTGCA TTGCACTAAG TCTTGCCCTT 60
    GTCACGAATT CGACGATCCC ACCTCACGTT CAGAAGTCGG TTAATAACGA CATGATAGTC 120
    ACTGACAACA ACGGTGCAGT CAAGTTTCCA CAACTGTGTA AGTTTTGTGA TGTGAGATTT 180
    TCCACCTGTG ACAACCAGAA GTCCTGCATG TCTAACTGCT CCATCACCTC CATCTGTGAA 240
    AAGCCACAGG AAGTCTGTGT CGCTGTCTGG AGAAAGAATG ACGAGAACAT AACACTAGAG 300
15  ACAGTTTGCC ATGACCCCAA GCTCCCCTAC CATGACTTTA TTCTGGAAGA TGCTGCTTCT 360
    CCAAAGTGCA TTATGAAGGA AAAAAAAAAAG CCTGGTGAGA CTTTCTTCAT GTGTTCCCTGT 420
    TCTTCTGATG AGTGCAATGA CAACATCATC TTCTCCGAAG AATATAACAC CTCTAATCCT 480
    GACGGTTCTG GTGGAGGGGA CAAACTCAC ACATGCCCAC CGTGCCAGC ACCTGAACTC 540
    CTGGGGGGAC CGTCAGTCTT CCTCTTCCCC CAAAACCCA AGGACACCCT CATGATCTCC 600
20  CGGACCCCTG AGGTCACATG CGTGGTGGTG GACGTGAGCC ACGAAGACCC TGAGGTCAAG 660
    TTCAACTGGT ACGTGGACGG CGTGGAGGTG CATAATGCCA AGACAAAGCC GCGGGAGGAG 720
    CAGTACAACA GCACGTACCG TGTGGTCAGC GTCCTCACCG TCCTGCACCA GGAAGTGGCTG 780
    AATGGCAAGG AGTACAAGTG CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA 840
    ACCATCTCCA AAGCCAAAGG GCAGCCCCGA GAACCACAGG TGTGCACCCT GCGGGCATCC 900
25  CGGGATGAGC TGACCAAGAA CCAGGTCAGC CTGTCTTGCG CTGTCAAAGG CTTCTATCCC 960
    AGCGACATCG CCGTGGAGTG GGAGAGCAAT GGGCAGCCGG AGAACAACTA CAAGACCACG 1020
    CCTCCCGTGC TGGACTCCGA CGGCTCCTTC AAGCTCGTCA GCAAGCTCAC CGTGGACAAG 1080
    AGCAGGTGGC AGCAGGGGAA CGTCTTCTCA TGCTCCGTGA TGCATGAGGC TCTGCACAAC 1140
    CACTACACGC AGAAGAGCCT CTCCCTGTCT CCGGGTAAAT GAGAGCTCGC C 1191

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In an exemplary embodiment the DNA fragment with an open reading frame corresponding to TGFBR3-Fc-2 fusion protein may comprise the DNA sequence of SEQ. ID NO. 8.

TGFBR3-Fc-2 DNA1 (SEQ ID NO. 8)

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5
AGCTGAATTC ACCGCCATGT ACCGGATGCA GCTGCTGAGC TGTATCGCCC TGTCTCTGGC 60
CCTGGTCACA AATAGCGGAC CTGAACCTGG CGCTCTGTGC GAGCTGTCTC CTGTGTCTGC 120
TTCTCATCCT GTGCAGGCCC TGATGGAAAG CTTACAGTG CTGAGCGGCT GTGCCAGCAG 180
AGGCACAACA GGA CTGCCTC AAGAGGTGCA CGTGCTGAAC CTGAGAACCG CCGGACAAGG 240
10 ACCTGGACAG CTGCAGAGAG AAGTGACCCT GCACCTGAAT CCTATCAGCT CCGTGCACAT 300
CCACCACAAG AGCGTGGTGT TTCTGCTGAA CAGCCCTCAT CCTCTCGTGT GGCACCTGAA 360
AACCGAGAGA CTGGCTACCG GCGTGTCCAG ACTGTTTCTG GTGTCTGAGG GCAGCGTGGT 420
GCAGTTCAGC AGCGCCAATT TCAGCCTGAC CGCCGAGACA GAGGAACGGA ATTTCCCTCA 480
CGGCAACGAG CATCTGCTGA ATTGGGCCAG AAAAGAATAT GGCGCCGTGA CCAGCTTCAC 540
15 CGAGCTGAAG ATCGCCCGGA ACATCTACAT CAAAGTGGGC GAAGATCAGG TGTTCCTCC 600
AAAGTGCAAT ATCGGCAAGA ACTTCCTGAG CCTGAACTAC CTGGCCGAGT ACCTGCAGCC 660
TAAAGCCGCC GAAGGCTGCG TGATGAGTAG CCAGCCTCAG AACGAGGAAG TGCACATCAT 720
CGAGCTGATC ACCCCTAACA GCAACCCCTA CAGCGCCTTC CAGGTGGACA TCACCATCGA 780
CATCAGACCC AGCCAAGAGG ATCTGGAAGT GGTCAAGAAC CTGATCCTGA TCCTCAAGTG 840
20 CAAGAAAAGC GTGAACTGGG TCATCAAGAG CTTGACGTG AAGGGCAGCC TGAAGATCAT 900
TGCCCCAAAC AGCATCGGCT TCGGCAAAGA AAGCGAGCGG AGCATGACCA TGACCAAGAG 960
CATCAGGGAC GACATCCCCA GCACACAGGG CAACCTGGTT AAGTGGGCCC TCGACAATGG 1020
CTACAGCCCC ATCACCAGCT ACACAATGGC CCCTGTGGCC AACCGGTTCC ACCTGAGACT 1080
GGAAAACAAC GCCGAGGAAA TGGGCGACGA AGAGGTCCAC ACAATCCCTC CAGAGCTGAG 1140
25 AATCCTGCTG GATGGATCTG GCGGCGGAGA CAAGACCCAC ACCTGTCCCTC CATGTCCTGC 1200
ACCAGAACTG CTCGGCGGAC CTTCCGTGTT CCTGTTTCCA CCTAAGCCTA AGGACACCCT 1260
GATGATCAGC AGAACCCCTG AAGTGACCTG CGTGGTGGTG GACGTGTCCC ACGAAGATCC 1320
CGAAGTGAAG TTCAATTGGT ACGTGGACGG CGTCGAGGTG CACAACGCCA AGACAAAGCC 1380
CAGAGAGGAA CAGTACAACA GCACCTACAG AGTGGTGTCC GTGCTGACCG TGCTGCACCA 1440
30 GGATTGGCTG AACGGCAAAG AGTACAAGTG CAAAGTGTCC AACAAAGGCC TGCCTGCTCC 1500
TATCGAGAAA ACCATCAGCA AGGCCAAGGG CCAGCCTAGA GAACCCAGG TGTACACACT 1560
GCCTCCATGC AGAGATGAGC TGACCAAGAA CCAGGTGTCA CTGTGGTGCC TGGTCAAGGG 1620
CTTCTACCCC TCTGATATCG CCGTGGAATG GGAGAGCAAT GGACAGCCCG AGAACAACTA 1680
CAAGACAACC CCTCCTGTGC TGGACAGCGA CGGCTCATTC TTTCTGTACA GCGCCCTGAC 1740

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CGTGGACAAG AGCAGATGGC AGCAGGGCAA CGTGTTCAGC TGCAGCGTGA TGCACGAGGC 1800
 CCTGCACAAC CACTACACCC AGAAGTCTCT GAGTCTGAGC CCCGGCAAGT GATAGGATCC 1860
 TCGA 1864

- 5 In another exemplary embodiment the DNA fragment with an open reading frame corresponding to TGFBR3-Fc-2 fusion protein may comprise the DNA sequence of SEQ. ID NO. 9.

TGFBR3-Fc-2 DNA2 (SEQ ID NO. 9)

10 TTGCGGCCGC CACCATGTAC CGTATGCAAC TCCTGTCTTG CATTGCACTA AGTCTTGCCC 60
 TTGTCACGAA TTCGGGTCCA GAGCCTGGTG CACTGTGTGA ACTGTCACCT GTCAGTGCCT 120
 CCCATCCTGT CCAGGCCCTTG ATGGAGAGCT TCACTGTTTT GTCAGGCTGT GCCAGCAGAG 180
 GCACAACTGG GCTGCCACAG GAGGTGCATG TCCTGAATCT CCGCACTGCA GGCCAGGGGC 240
 15 CTGGCCAGCT ACAGAGAGAG GTCACACTTC ACCTGAATCC CATCTCCTCA GTCCACATCC 300
 ACCACAAGTC TGTTGTGTTC CTGCTCAACT CCCACACCC CCTGGTGTGG CATCTGAAGA 360
 CAGAGAGACT TGCCACTGGG GTCTCCAGAC TGTTTTTTGGT GTCTGAGGGT TCTGTGGTCC 420
 AGTTTTTCATC AGCAAACTTC TCCTTGACAG CAGAAACAGA AGAAAGGAAC TTCCCCCATG 480
 GAAATGAACA TCTGTTAAAT TGGGCCCGAA AAGAGTATGG AGCAGTTACT TCATTCACCG 540
 20 AACTCAAGAT AGCAAGAAAC ATTTATATTA AAGTGGGGGA AGATCAAGTG TTCCCTCCAA 600
 AGTGCAACAT AGGGAAGAAT TTTCTCTCAC TCAATTACCT TGCTGAGTAC CTTCAACCCA 660
 AAGCAGCAGA AGGGTGTGTG ATGTCCAGCC AGCCCCAGAA TGAGGAAGTA CACATCATCG 720
 AGCTAATCAC CCCCAACTCT AACCCTACA GTGCTTTCCA GGTGGATATA ACAATTGATA 780
 TAAGACCTTC TCAAGAGGAT CTTGAAGTGG TCAAAAATCT CATCCTGATC TTGAAGTGCA 840
 25 AAAAGTCTGT CAACTGGGTG ATCAAATCTT TTGATGTAA GGGAAGCCTG AAAATTATTG 900
 CTCCTAACAG TATTGGCTTT GGAAAAGAGA GTGAAAGATC TATGACAATG ACCAAATCAA 960
 TAAGAGATGA CATTCCCTTCA ACCCAAGGGA ATCTGGTGAA GTGGGCTTTG GACAATGGCT 1020
 ATAGTCCAAT AACTTCATAC ACAATGGCTC CTGTGGCTAA TAGATTTTCAT CTTCCGGCTTG 1080
 AAAATAATGC AGAGGAGATG GGAGATGAGG AAGTCCACAC TATTCCTCCT GAGCTACGGA 1140
 30 TCCTGCTGGA CGGTTCTGGT GGAGGGGACA AAACTCACAC ATGCCACCG TGCCCAGCAC 1200
 CTGAACTCCT GGGGGGACCG TCAGTCTTCC TCTTCCCCC AAAACCCAAG GACACCCTCA 1260
 TGATCTCCCG GACCCCTGAG GTCACATGCG TGGTGGTGGA CGTGAGCCAC GAAGACCCTG 1320
 AGGTCAAGTT CAACTGGTAC GTGGACGGCG TGGAGGTGCA TAATGCCAAG ACAAAGCCGC 1380
 GGGAGGAGCA GTACAACAGC ACGTACCGTG TGGTCAGCGT CCTCACCGTC CTGCACCAGG 1440

ACTGGCTGAA TGGCAAGGAG TACAAGTGCA AGGTCTCCAA CAAAGCCCTC CCAGCCCCCA 1500
 TCGAGAAAAC CATCTCCAAA GCCAAAGGGC AGCCCCGAGA ACCACAGGTG TACACCCTGC 1560
 CCCCATGCCG GGATGAGCTG ACCAAGAACC AGGTCAGCCT GTGGTGCCTG GTCAAAGGCT 1620
 TCTATCCCAG CGACATCGCC GTGGAGTGGG AGAGCAATGG GCAGCCGGAG AACAACTACA 1680
 5 AGACCACGCC TCCCGTGCTG GACTCCGACG GCTCCTTCTT CCTCTACAGC GCTCTCACCG 1740
 TGGACAAGAG CAGGTGGCAG CAGGGGAACG TCTTCTCATG CTCCGTGATG CATGAGGCTC 1800
 TGCACAACCA CTACACGCAG AAGAGCCTCT CCCTGTCTCC GGGTAAATGA CTCG 1854

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EXAMPLES

The following Examples illustrate the forgoing aspects and other aspects of the present teachings. These non-limiting Examples are put forth so as to provide those of ordinary skill in the art with illustrative embodiments as to how the compounds, compositions, articles, devices, and/or methods claimed herein are made and evaluated. The Examples are intended to be purely

15 exemplary of the inventions disclosed herein and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for.

20 **Example 1:** Construction of plasmids for transient expression of polypeptides of the present teachings.

Plasmid maps for expression of TGFBR2-Fc-1, TGFBR11-Fc-1, TGFBR13-Fc-1 and TGFBR3-Fc-2 constructs for transient transfection in mammalian cells are shown illustratively in Fig. 11, 12, 13, and 14.

25 **Example 2:** Transient protein expression of polypeptides of the present teachings.

Transient transfection in Expi293F cells was carried out for protein production. Cells were transfected according to manufacturer's protocol. Plasmid DNA was isolated using CsCl density gradient centrifugation and ethidium bromide was removed by ion exchange column with Dowex

30 AG50W-X8 resin.

Expi293F cells were propagated in Freestyle Expi293F Expression Medium to a density of $2.00\text{E}+06$ - $2.50\text{E}+06$ cell/ml and then diluted to $1.20\text{E}+06$ cells/ml by Freestyle Expi293F Expression Medium 18-20 hours before transfection.

At the day of transfection, cell density should be at $2.20\text{E}+06$ - $2.50\text{E}+06$ cell/ml. A 125-
5 ml batch of Expi293F cells was used as a standard transfection reaction

The cells were transfected with equivalent amounts of constructs containing TGFBR2-Fc-1 DNA1 (SEQ ID NO. 6) and TGFBR3-Fc-2 DNA1 (SEQ ID NO. 8) (Fig. 11 and Fig. 14) in pTT5 vector ($62.5\text{ }\mu\text{g}$ + $62.5\text{ }\mu\text{g}$). DNA for both constructs was diluted in 3 ml of PBS

Linear Polyethyleneimine (LPEI), 3 mg/ml stock solution, was diluted in 3 ml of PBS to a
10 final concentration of 0.21 mg/ml. Total amount of LPEI added to transfection was $625\text{ }\mu\text{g}$ i.e. 5-fold the mass of combined DNA

To 3 ml of DNA solution, 3 ml of diluted LPEI solution was added, vortexed and incubated at room temperature for 10 min

Mixture of DNA:LPEI, 6 ml was added dropwise to 125 ml of cell suspension
15 24 hours post-transfection, 3.3 ml of 40% Tryptone N1 in PBS was added to the transfected cell suspension to a final concentration of 1%

On Day 5 after transfection, cell density of transfected cells was usually $4.50\text{E}+06$ - $5.50\text{E}+06$ cell/ml and viability - 70% - 88%

20 **Example 3:** Purification of polypeptides of the present teachings.

Transiently expressed, as described of foregoing Example 2, TGFBR2-Fc-1/ TGFBR3-Fc-2 (also referred to as TGFBR2/TGFBR3/Fc) heterodimer protein was purified using Protein A Sepharose. Cells were harvested on Day 5 by centrifugation at 300 g for 10 min and the resulting
25 supernatant was centrifuged at 35,000 g for 30 min. Cleared supernatant was filtered through $0.2\text{ }\mu$ filter to remove cell debris and protein aggregates. Protein A Sepharose, 1 ml, was washed with PBS to remove preservatives and was added to cleared and filtered culture medium. The resin was incubated with the culture medium at 4°C on a shaker for overnight. After incubation, Protein A resin was packed into a column, washed with 10 column volumes of PBS. Protein A-bound
30 proteins were eluted with 100 mM Gly-HCl pH 3.0 and the eluate was immediately neutralized by adding 1/10th of the eluate volume of 1 M Tris-HCl pH 8.8. Eluted protein was extensively

dialyzed against PBS, filter sterilized through 0.2 μ filter and stored at 4°C. Purity of the resulted TGFBR2/TGFBR3/Fc preparation was analyzed by SDS-PAGE analysis (Fig. 2)

5 **Example 4:** Functional characterization of TGFBR2/TGFBR3/Fc heterodimer protein.

Functional properties of purified TGFBR2/TGFBR3/Fc protein were tested a cell-based assay. MDA-MB-231 cells respond to TGF- β treatment by secretion of IL-11.

Cell maintenance:

- 10 1. Propagate MDA-MB-231 cells in the DMEM medium containing 10% FBS on T75;
2. Trypsinize cells, re-suspend in DMEM containing 10% FBS;
3. Test cell viability and count cell numbers using CytoSmart cell counter or equivalent.
Standard viability should be >90%, if less, do not use this batch of cells;
4. Prepare dilutions of the cells for plating at cell density of 2.00E+05 cells/ml. Plate 1 ml of
15 the cell suspension per well of 24-well plate;
5. Allow cells to attach for 16-24 hours at 37°C, 5% CO₂, then remove medium;
6. Wash wells with fresh serum-free DMEM, 1.0 ml per well twice, add 1ml of serum-free
DMEM and incubate 20-24 hours at 37°C, 5% CO₂;
7. Wash wells with fresh serum-free DMEM, 1.0 ml per well twice
20 8. Prepare test substances according to experimental design using serum-free DMEM. If
both TGF- β and TGFBR2/TGFBR3/Fc are used, incubate the mixture for 10-15 min at room temp
prior to adding to the cells.
9. Aspirate medium from the wells and add pre-incubated samples and appropriate controls,
0.5 ml per well
25 10. Incubate the cells after treatment for 24 hours then harvest the supernatants;
11. Centrifuge the supernatants at 300 x g for 10 min, collect cleared supernatants.

Optimization of assay conditions:

- 30 Quantitation of IL-11 secretion by MDA-MB-231 cells was carried out using anti-human IL-
11 ELISA kit. All steps of the procedure were performed according to manufacturer's
recommendations.

To identify suitable conditions for IL-11 production assay, dose-response curve for TGF- β was generated. Starting from 5 ng/ml and using 2-fold serial dilutions, TGF- β was titrated down to 5 pg/ml. Variable slope (four parameter) curve fitting algorithm was used for calculation of EC50 value that was 0.219 ng/ml (Fig. 3). Due to the nature of the ELISA kit used for these series of experiments, absorbance at EC50 resulted only in 0.25-0.30 AU. To study TGFBR2/TGFBR3/Fc inhibition of TGF- β activity, higher concentration of TGF- β were used that are specified for each experiment.

To investigate inhibitory properties of other TGFBR2/TGFBR1/Fc chimeras, MDA-MB-231 cells were treated with TGF- β in the presence of varying concentrations of TGFBR2/TGFBR1-Isoform-1/Fc and TGFBR2/TGFBR1-Isoform-3/Fc. For this experiment, TGF- β was added at a fixed concentration of 2 ng/ml and TGFBR2/TGFBR1/Fc chimeras were titrated from 1,000 ng/ml to 1 ng/ml. Both chimeras demonstrated concentration-dependent inhibition of TGF- β -induced IL-11 secretion. Calculated IC50 values for TGFBR2/TGFBR1-Isoform-1/Fc and TGFBR2/TGFBR1-Isoform-3/Fc chimeras were 23.1 ng/ml and 12.3 ng/ml, respectively (Fig. 4).

To investigate inhibitory properties of TGFBR2/TGFBR3/Fc fusion protein, MDA-MB-231 cells were treated with TGF- β in the presence of varying concentrations of TGFBR2/TGFBR3/Fc. For this titration experiment, TGF- β was added at a constant concentration of 2 ng/ml and TGFBR2/TGFBR3/Fc fusion protein was titrated from 1,000 ng/ml to 1 ng/ml.

TGFBR2/TGFBR3/Fc demonstrated monotonous concentration-dependent inhibition of TGF- β -induced IL-11 secretion. The experiment was repeated twice and calculated IC50 values were 4.78 ng/ml and 2.35 ng/ml, respectively (Fig. 5).

Inhibition of TGFBR2/TGFBR3/Fc was studied at two different concentrations of TGF- β , 1 ng/ml and 2 ng/ml. Maximal concentration of TGFBR2/TGFBR3/Fc was reduced from 1,000 ng/ml used in previous experiments to 100 ng/ml. TGFBR2/TGFBR3/Fc displayed monotonous inhibition of IL-11 secretion for both TGF- β concentrations. TGFBR2/TGFBR3/Fc IC50 values for 1 ng/ml and 2 ng/ml were 2.9 ng/ml and 5.3 ng/ml, respectively (Fig. 6).

Molecular mass of TGF- β is 24 kDa and estimate molecular mass of TGFBR2/TGFBR3/Fc is about 120 – 130 kDa. In terms of molar ratios between TGF- β and TGFBR2/TGFBR3/Fc, for TGF- β concentration of 1 ng/ml or 42 pM, IC50 for TGFBR2/TGFBR3/Fc is 2.9 ng/ml or 24 pM.

For 2 ng/ml or 84 pM TGF- β , IC₅₀ value is 5.2 ng/ml or 43 pM. For both concentrations, TGF- β is present in apparent 2-fold molar excess over TGFBR2/TGFBR3/Fc. If TGFBR2/TGFBR3/Fc is capable of acting like a dimer and to bind two dimeric TGF- β molecules, then at both TGF- β concentrations TGFBR2/TGFBR3/Fc:TGF- β molar ratios are roughly 2:1. Given that the TGF- β :TGFBR2/TGFBR3/Fc ratio remains the same, it could be suggested that lowering concentrations of TGF- β and TGFBR2/TGFBR3/Fc further would result in apparently lower IC₅₀ values. Due to the nature of this particular assay, it is not practical to use TGF- β concentration below 1 ng/ml because IL-11 secretion response is diminished, and this affects data reliability. Therefore, for this particular assay under tested concentrations of TGF- β , IC₅₀ value for TGFBR2/TGFBR3/Fc could be as low as 2.9 ng/ml (24 pM).

Example 5: Characterization of TGFBR2/TGFBR3/Fc – TGF- β interaction in surface plasmon resonance.

To study interaction between TGF- β to TGFBR2/TGFBR3/Fc using surface plasmon resonance, the nature of TGFBR2/TGFBR3/Fc of being human IgG1-Fc fusion protein has been utilized. One of the standard approaches to capture IgG-containing molecules is immobilization of species-specific capturing antibodies on a chip with subsequent loading of the test fusion proteins followed by ligand application. This approach was used for measuring binding parameters for TGFBR2/TGFBR3/Fc and TGF- β as schematically shown in Fig. 7.

Protein conjugation to the chip

Anti-human antibodies were covalently attached to the chip the following protocol.

1. SR7000 Gold Sensor Slide was placed into the instrument and primed with Running Buffer, (1x PBS supplemented with 0.005% Tween 20), for 5 min at 250 μ l/min, then allowed to stabilize at 25 μ l/min. All steps were carried out at 25 °C;
2. Protein preparations were diluted using Immobilization Buffer (10 mM Na-acetate pH 5.0) to a final concentration of 25 μ g/ml;

3. Reagents for immobilization procedure were prepared as follows. EDC/NHE activation agent consisting of EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) at 40 mg/ml and NHS (N-hydroxysuccinimide) at 10 mg/ml in water. 1 M Ethanolamine-HCl pH 8.5 in water;
4. Activation: EDC/NHS activation agent was injected into the chip at 10 μ l/min for 8 min
5 followed by 5 min wash with Running Buffer;
5. Immobilization: Anti-Human IgG or Fc receptors at a final concentration of 25 μ g/ml were injected into the chip at 10 μ l/min for 9 min;
6. Deactivation: Unreacted active groups on the chip surface were blocked by injection of 1 M Ethanolamine-HCl at 10 μ l/min for 8 min;
- 10 7. After antibody or Fc receptors conjugation, the chip was washed with Running Buffer for 15 min at 25 μ l/min.

Determination of binding constants for TGF- β and TGFBR2/TGFBR3/Fc interaction

To study interaction of TGF- β and TGFBR2/TGFBR3/Fc, antibody-capturing approach
15 was used. Ant-human IgG were covalently attached to the chip and used for capturing TGFBR2/TGFBR3/Fc (Fig. 7).

1. Chip with immobilized anti-human IgG was equilibrated with Running Buffer at a flow rate of 25 μ l/min for 15 min;
2. TGFBR2/TGFBR3 at a final concentration of 25 μ g/ml, was loaded at 25 μ l/min for 2
20 min, then the chip was washed for 3 min with Running Buffer to remove unbound material;
3. A series of 2-fold dilutions of TGF- β were prepared using Running Buffer starting from 100 nM concentration. Seven concentrations were used: 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 nM. The ligand was loaded at 25 μ l/min for 2 min;
- 25 4. After ligand loading, dissociation phase of the experiment was carried out using Running Buffer at 25 μ l/min flow rate for 4 min;
5. Regeneration step, dissociation of protein complexes bound by immobilized anti-human IgG was carried out by 3 M MgCl₂ running through the chip at 25 μ l/min for 30 sec;
- 30 6. Series of sensograms for series of TGFBR2/TGFBR3/Fc at different concentrations of TGF- β were generated and used for analysis;

7. A kinetic evaluation of 1:1 binding model was used for the analysis of TGF- β interaction with TGFBR2/TGFBR3/Fc.
8. Dissociation constant, K_D , (M), was calculated from association, k_a , (1/(M*s)) and dissociation, k_d , (1/s) constants generated from analysis of series of the concentration curves. K_D (M) is a quotient of division of k_d , (1/s) by k_a , (1/(M*s)).

After capturing TGFBR2/TGFBR3/Fc by anti-human antibodies immobilized on chip (Fig. 7), varying concentrations of TGF- β were loaded and binding sensograms were generated (Fig. 7 and Fig. 8). The series of the sensograms were analyzed by kinetic evaluation using 1:1 binding model. SPR binding experiment was repeated three times to demonstrate reproducibility. Dissociation constants were calculated from association constant K_a (1/M*s) and dissociation constant K_d (1/s) (Tables 1, 2 and 3). K_D , (M) is equal K_a divided by K_d .

According to SDS-PAGE analysis of TGFBR2/TGFBR3/Fc under non-reduced conditions (Figure 6), the preparation used for this study contained more than single band indicating a presence of various homo- and/or hetero-dimers. As the result, association phase of sensograms was not monotonous and thus more difficult to analyze. However, dissociation phase of all sensograms demonstrates remarkable lack of dissociation thus indicating high affinity interaction.

The results are summarized in Tables 1, 2 and 3 and the corresponding series of sensograms are shown on Fig. 8, 9 and 10. Summary table (Table 4) show mean K_D , (M) values, and standard deviation (SD) for each experiment. Median value for each experiment and corresponding average is shown as well.

Table 1: Experiment 1: Binding parameters for binding of TGF- β to captured TGFBR2/TGFBR3/Fc. Binding constant, K_D (M), was averaged for all 7 curves and also median was calculated.

Concentration, nM	TGF- β	Bmax ([Signal (uRIU)])	k_a (1/(M*s))	k_d (1/s)	KD (M)	BI ([Signal (uRIU)])	Chi2 ([Signal (uRIU)]^2)
1.56	Cycle 2 ChLeft-ChRight - Reference curve fitted	5.89	1.87E+05	1.06E-05	5.65E-11	0	109.47
3.13	Cycle 3 ChLeft-ChRight - Reference curve fitted	7.18	1.38E+05	6.37E-04	4.62E-09	0	109.47
6.25	Cycle 4 ChLeft-ChRight - Reference curve fitted	12.77	2.26E+06	1.89E-06	8.38E-13	0	109.47
12.50	Cycle 5 ChLeft-ChRight - Reference curve fitted	31.41	1.24E+06	1.93E-06	1.56E-12	0	109.47
25.00	Cycle 6 ChLeft-ChRight - Reference curve fitted	133.22	5.05E+05	3.41E-07	6.75E-13	0	109.47
50.00	Cycle 7 ChLeft-ChRight - Reference curve fitted	184.16	3.80E+05	1.90E-07	4.98E-13	0	109.47
100.00	Cycle 8 ChLeft-ChRight - Reference curve fitted	173.16	3.10E+05	2.64E-04	8.52E-10	0	109.47
	Average				9.13E-10		
	StdDev				1.69E-09		
	Median				1.56E-12		

5 **Table 2:** Experiment 2: Binding parameters for binding of TGF- β to captured TGFBR2/TGFBR3/Fc. Binding constant, KD (M), was averaged for all 7 curves and also median was calculated.

Concentration, nM	TGF- β , Experiment 2	Bmax ([Signal (uRIU)])	k_a (1/(M*s))	k_d (1/s)	KD (M)	BI ([Signal (uRIU)])	Chi2 ([Signal (uRIU)]^2)
1.56	Cycle 2 ChLeft-ChRight - Reference curve fitted	2.97	3.11E+05	1.52E-03	4.90E-09	0	135.58
3.13	Cycle 3 ChLeft-ChRight - Reference curve fitted	10.59	1.55E+05	8.26E-04	5.33E-09	0	135.58
6.25	Cycle 4 ChLeft-ChRight - Reference curve fitted	18.77	2.34E+06	5.06E-06	2.17E-12	0	135.58
12.50	Cycle 5 ChLeft-ChRight - Reference curve fitted	16.01	1.72E+06	2.38E-06	1.38E-12	0	135.58
25.00	Cycle 6 ChLeft-ChRight - Reference curve fitted	165.38	6.70E+05	8.81E-07	1.32E-12	0	135.58
50.00	Cycle 7 ChLeft-ChRight - Reference curve fitted	177.29	4.30E+05	1.80E-06	4.18E-12	0	135.58
100.00	Cycle 8 ChLeft-ChRight - Reference curve fitted	204.29	3.43E+05	5.51E-04	1.61E-09	0	135.58
	Average				1.16E-09		
	StdDev				1.96E-09		
	Median				4.18E-12		

10

Table 3: Experiment 3: Binding parameters for binding of TGF- β to captured TGFBR2/TGFBR3/Fc. Binding constant, KD (M), was averaged for all 7 curves and also median was calculated.

Concentration, nM	TGF- β , Experiment 2	Bmax ([Signal (uRIU)])	k_a (1/(M*s))	k_d (1/s)	KD (M)	BI ([Signal (uRIU)])	Chi2 ([Signal (uRIU)]^2)
1.56	Cycle 2 ChLeft-ChRight - Reference curve fitted	7.87	5.27E+06	1.69E-03	3.20E-10	0	159.27
3.13	Cycle 3 ChLeft-ChRight - Reference curve fitted	11.75	8.50E+06	4.56E-03	5.37E-10	0	159.27
6.25	Cycle 4 ChLeft-ChRight - Reference curve fitted	17.81	2.53E+06	2.06E-05	8.16E-12	0	159.27
12.50	Cycle 5 ChLeft-ChRight - Reference curve fitted	47.83	1.25E+06	1.31E-05	1.04E-11	0	159.27
25.00	Cycle 6 ChLeft-ChRight - Reference curve fitted	156.93	5.56E+05	9.90E-07	1.78E-12	0	159.27
50.00	Cycle 7 ChLeft-ChRight - Reference curve fitted	180.85	3.99E+05	1.53E-08	3.85E-14	0	159.27
100.00	Cycle 8 ChLeft-ChRight - Reference curve fitted	203.67	3.14E+05	5.60E-04	1.78E-09	0	159.27
	Average				3.90E-10		
	StDev				6.51E-10		
	Median				1.04E-11		

Table 4: Summary of binding constant, KD (M), for binding of TGF- β to

5 TGFBR2/TGFBR3/Fc calculated from 3 independent experiments.

	Average	StDev	CV, %	Median
Experiment 1	7.90E-10	1.59E-09	201%	1.56E-12
Experiment 2	1.16E-09	1.96E-09	169%	4.18E-12
Experiment 3	3.90E-10	6.51E-10	167%	1.04E-11
Average	7.79E-10	1.57E-09	201%	5.38E-12

10 **Example 6:** Generation of stable cell line for overexpression of TGFBR2/TGFBR3/Fc heterodimer protein.

In this Example, the amino acid sequences of TGFRB2-Fc1 fusion polypeptide2 (SEQ ID NO. 2) and TGFRB3-Fc2 fusion polypeptide (SEQ ID NO. 5) for the composition of

15 TGFBR2/TGFBR3/Fc fusion protein were codon optimized for mammalian cell expression. DNAs encoding for codon optimized TGFRB2-Fc1 DNA2 (SEQ ID NO. 7) with an additional PacI site at the 5' end and a BGH poly A region plus an AscI site at the 3' end and TGFRB3-Fc2 DNA2 (SEQ ID NO. 9) were then chemically synthesized. The DNA fragment that contains the coding region of TGFRB2-Fc1 was cloned into an intermediate vector pAG12B between NheI and XhoI sites to

20 generate pAG12B-TGFRB2-Fc1 vector (Fig 15). The DNA fragment that contains the coding region of TGFRB3-Fc2 was cloned into pAG12B vector between the NheI and XhoI sites to generate pAG12B-TGFRB3-Fc2 vector (Fig 16). To generate vector pAG12B-

TGFR2/TGFR3/Fc (Fig 17), the MluI-XhoI fragment from the vector pAG12B-TGFR3-Fc2 that contains the entire promoter region and TGFR3-Fc2 was cloned into vector pAG12BTGFR2-Fc1 between AscI and XhoI sites. The isolated positive clones from each stage were validated by DNA sequencing analysis using a variety of primers. The full sequence of the pAG12B- TGFR2/TGFR3/Fc comprises SEQ. ID NO. 10.

pAG12B- TGFR2/TGFR3/Fc DNA (SEQ ID NO. 10)

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CHO-K1 cells (ATCC CCL-61) were maintained in DMEM/F12 media supplemented with 10% FBS at 37°C with 5% CO₂. Transfection of CHO-K1 cells with pAG12B-

TGFBR2/TGFBR3/Fc vector in the presence of transfection reagent “SuperFect” was performed according to the manufacturer’s protocol (Qiagen #301305). In brief, 2ug of DNA, 8ul of Superfect and 200ul of DMEM medium (no FBS) were mixed and incubated at room temperature for 15min. CHO-K1 cells were seeded in a 6-well plate at least 2h prior transfection and the transfection mix was added drop-wise to the surface of each well of freshly seeded cells. Transfected cells were then passaged 48h post-transfection. Stably transfected cells were 1:10 diluted and selected by adding hygromycin B to final 300ug/ml in complete medium. The hygromycin B-containing medium was changed twice a week until individual colonies became visible (3-4 weeks). For the first screen, 96 individual clones derived from single cells (most likely) were picked and amplified in a 96-well plate. After incubated at 37oC/5% CO2 for 10-14 days, conditioned media from each well were collected and analyzed by dot blot (or Western blot) using HRP-conjugated goat antihuman IgG (Pierce) (Fig 18). Two highly expressed clones were picked for the second single cell clone screen. To make sure the final developed TGFBR2/TGFBR3/Fc/CHO cell line compliance to the clonality requirement, limiting dilution cloning (LDC) method was employed. Two clones (#31 and #36) from the first screen were passaged twice with >98% cell viability. The procedure described below using one 50ml conical tube is sufficient to seed 2 x 96-well plate for each of the two clones isolated from the first screen.

a) Treat passaged cells with trypsin-EDTA and dilute the cells with the complete medium to a final concentration of 1000 viable cells/ml;

b) After carefully counting, further dilute cells with 50ml of complete medium to 0.5-1 viable cell/0.2ml;

c) Using the multiple channel pipettor to transfer 0.2ml of the diluted cells into each well of the 96-well plate (2 plates for #31 and 2 plates for #36);

d) After 12h of plating, examine the plates and label those wells that contain a single viable cell;

e) Monitor two wells with single healthy cell by daily microimaging (Fig 19 and 20);

f) Incubate the plates at 37oC and 5% CO2 for 12-14 days

g) Transfer 5ul of conditioned medium from each of the wells that were labeled from single cells to a new 96-well dot blot apparatus;

h) Examine the expression of TGFBR2/TGFBR3/Fc from single cell clones (Fig. 21)

Cells from clones #A6 and #G9 were amplified in the complete medium and the productivity of the two clones were assessed by ELISA assay. Comparing to the purified

5 TGFBR2/TGFBR3/Fc, an estimate production for clone A6 and G9 is 50-100mg/L.

For the stability test, five clones from the second screen (A6, D10, D11, F7 and G9) were selected and tested for their stability. 10,000 cells/well was seeded in a 96-well plate with triplicates for each clone. Cultured media were collected from each passage (twice a week) and the expression of TGFBR2/TGFBR3/Fc was monitored for 10 passages by ELISA assay using the
10 primary antibody against human IgG (Fig. 22).

TGFBR2/TGFBR3/Fc protein heterodimer of this example was purified from conditioned media essentially as described in the forgoing Example 3. Purity of purified TGFBR2/TGFBR3/Fc heterodimer protein was assessed by SDS-PAGE under non-reducing conditions (Fig. 23). Purified protein was equilibrated against a buffer containing 0.02% PS-20, 3% D-mannitol, 20 mM NaCl,
15 70 mM L-proline, 25 mM histidine, pH 6.0, and aliquoted for storage at 4°C.

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

20 While specific embodiments of the subject matter have been discussed, the above specification is illustrative and not restrictive. Many variations will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

25

What is claimed is:

1. A recombinant heterodimeric protein capable of binding human TGF- β comprising:
a first polypeptide comprising amino acid sequence of SEQ ID NO. 1 or of SEQ ID NO. 2; and
5 a second polypeptide comprising amino acid sequence of SEQ ID NO. 3, or of SEQ ID NO. 4,
or of SEQ ID NO. 5.
2. A therapeutic composition comprising a heterodimeric protein of claim 1 and a
pharmaceutically acceptable carrier or excipient.
10
3. A recombinant heterodimeric protein of claim 1, wherein said first polypeptide comprises
amino acid sequence of SEQ ID NO. 2, and said second polypeptide comprises amino acid
sequence of SEQ ID NO. 5.
- 15 4. The therapeutic composition of claim 2, further comprising about 0.02% PS-20, about 3%
D-mannitol, about 20 mM NaCl, about 70 mM L-proline, and about 25 mM histidine at pH 6.0
.
5. An isolated nucleic acid encoding a polypeptide comprising amino acid sequence selected
20 from the group consisting of: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4,
and SEQ ID NO. 5.
6. The nucleic acid of claims 4, wherein the codon usage is optimized for high expression of
said polypeptide in a mammalian cell.
25
7. The nucleic acid of claim 4, wherein said isolated nucleic acid comprises a DNA sequence
selected from the group consisting of: SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, and SEQ
ID NO. 9.
- 30 8. An isolated nucleic acid comprising a DNA sequence of SEQ ID NO. 10.
9. A mammalian cell harboring the nucleic acid of claim 8.

10. The mammalian cell of claim 9, wherein said mammalian cell is a CHO-K1 cell.
11. A composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5, for use in the treatment of idiopathic pulmonary fibrosis.
12. A composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5, for use in the treatment of systemic scleroderma.
13. A composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5, for use in the treatment of pulmonary arterial hypertension.
14. A composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5, for use in the treatment of fibrotic disease of heart, liver or kidney.
15. A method of treating or preventing idiopathic pulmonary fibrosis, the method comprising administering to a patient in need for treating or preventing idiopathic pulmonary fibrosis a therapeutically effective amount of a pharmaceutical composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5.
16. A method of treating or preventing systemic scleroderma, the method comprising administering to a patient in need for treating or preventing systemic scleroderma a therapeutically effective amount of a pharmaceutical composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5.
17. A method of treating or preventing pulmonary arterial hypertension, the method comprising administering to a patient in need for treating or preventing pulmonary arterial

hypertension a therapeutically effective amount of a pharmaceutical composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5.

- 5 18. A method of treating or preventing fibrotic disease, the method comprising administering to a patient in need for treating or preventing fibrotic disease of heart, liver or kidneys a therapeutically effective amount of a pharmaceutical composition comprising a first polypeptide comprising amino acid sequence of SEQ ID NO. 2, and a second polypeptide comprising amino acid sequence of SEQ ID NO. 5.

10

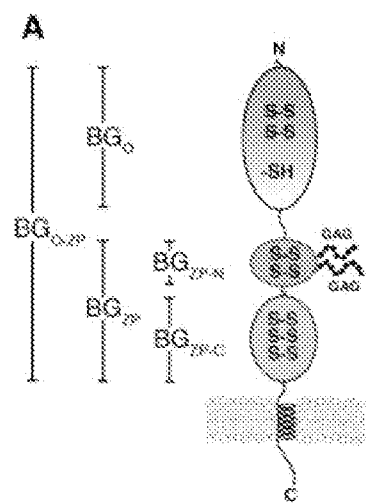


Fig. 1

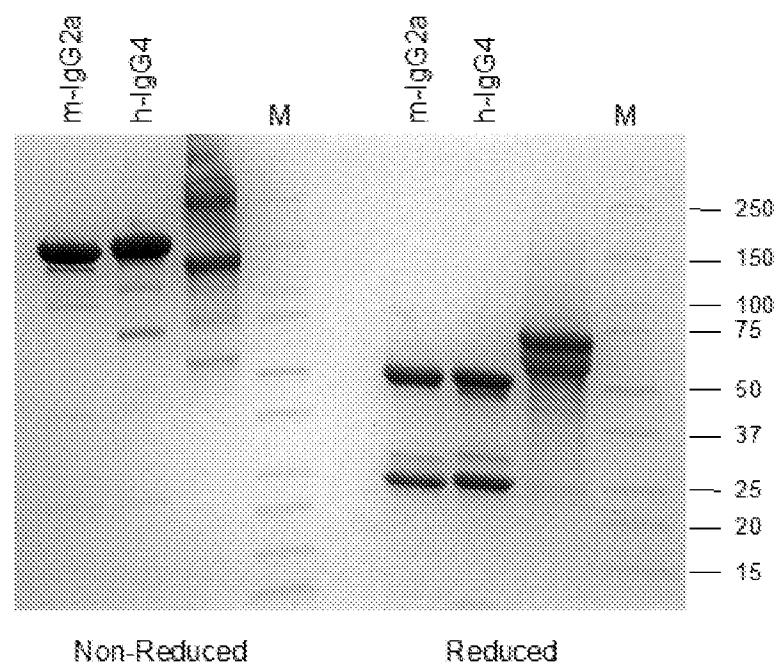


Fig. 2

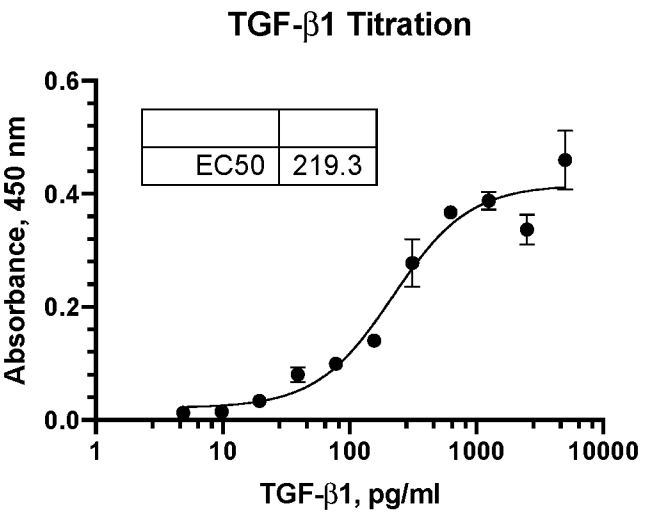


Fig. 3

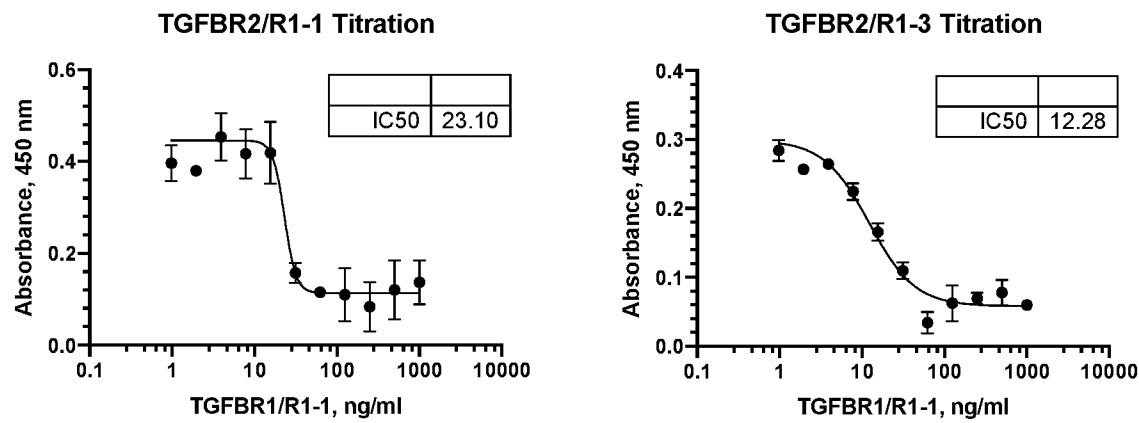


Fig. 4

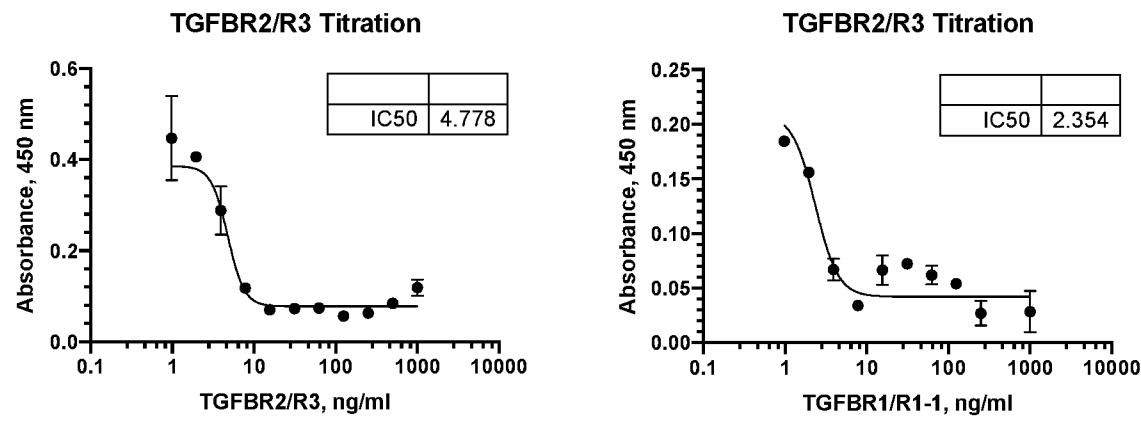


Fig. 5

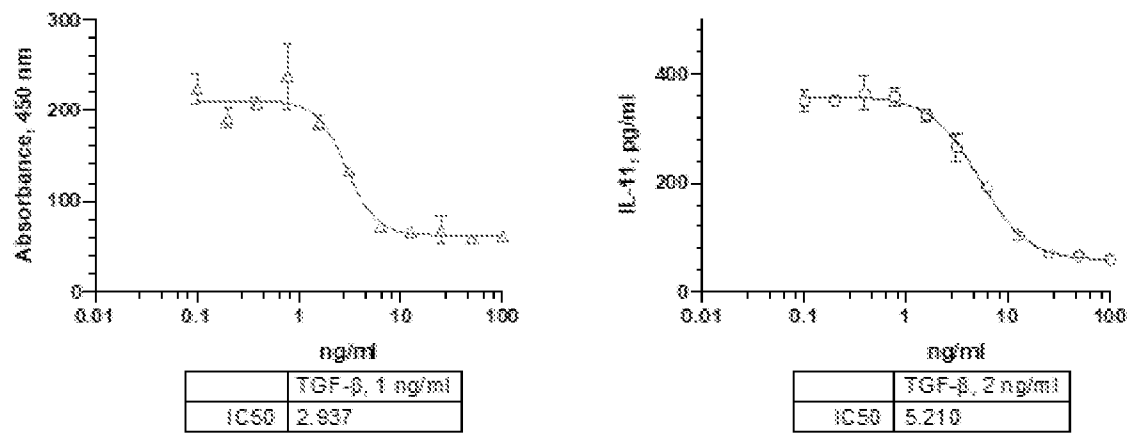


Fig. 6

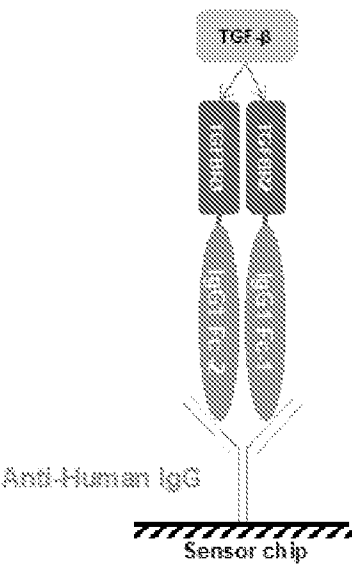


Fig. 7

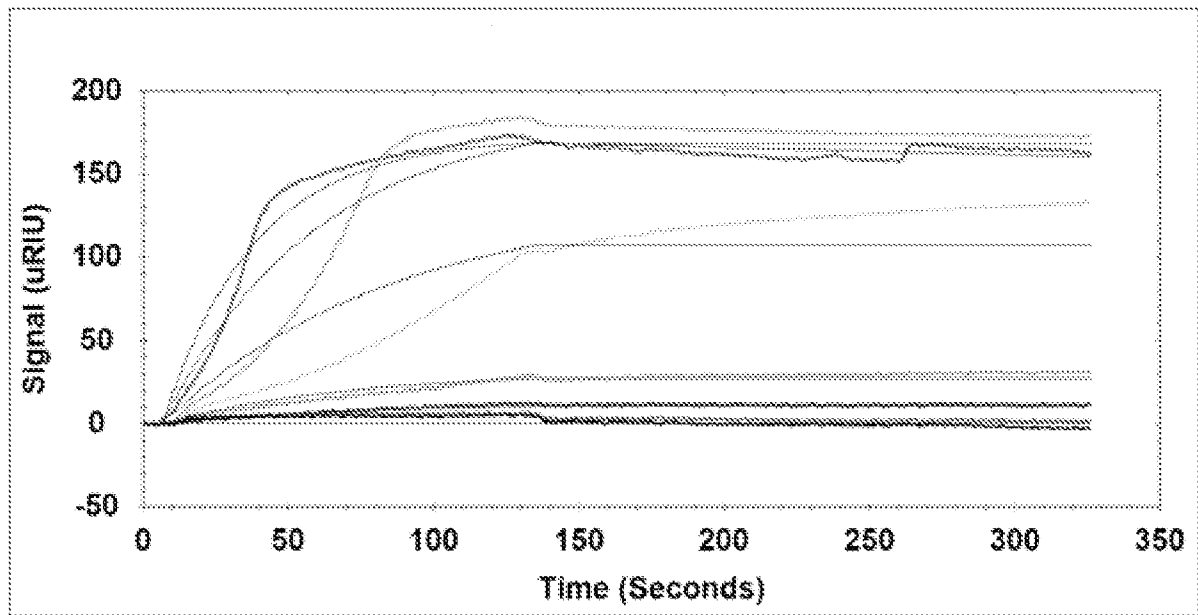


Fig. 8

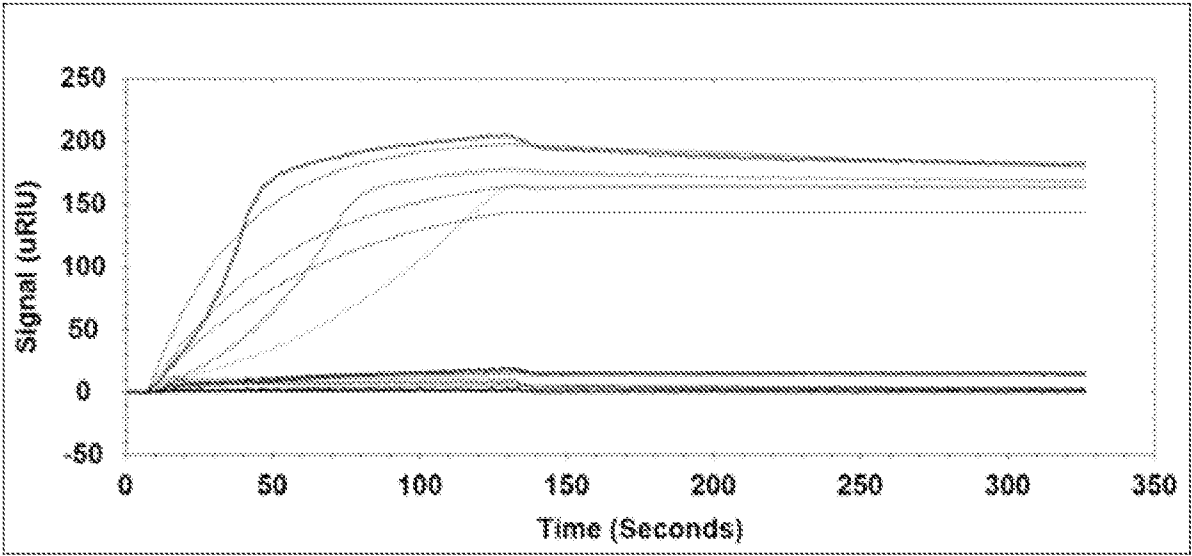


Fig. 9

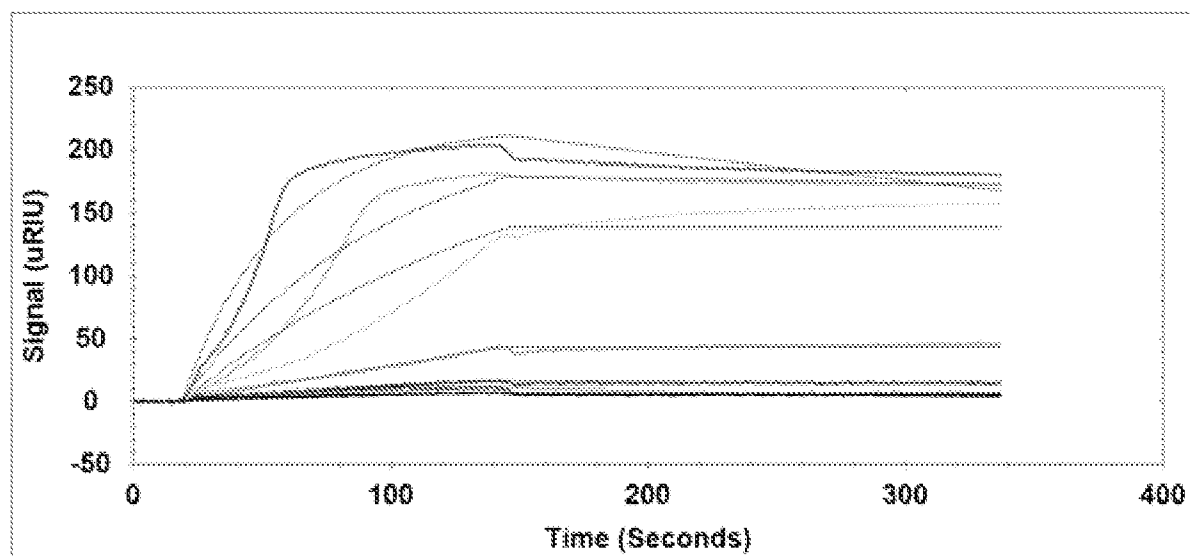


Fig. 10

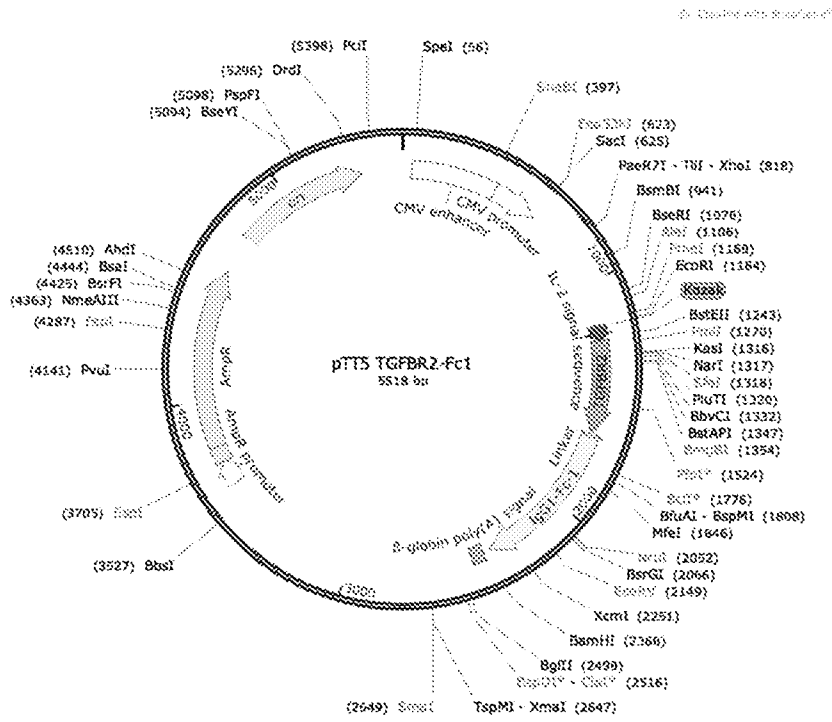
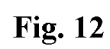


Fig. 11



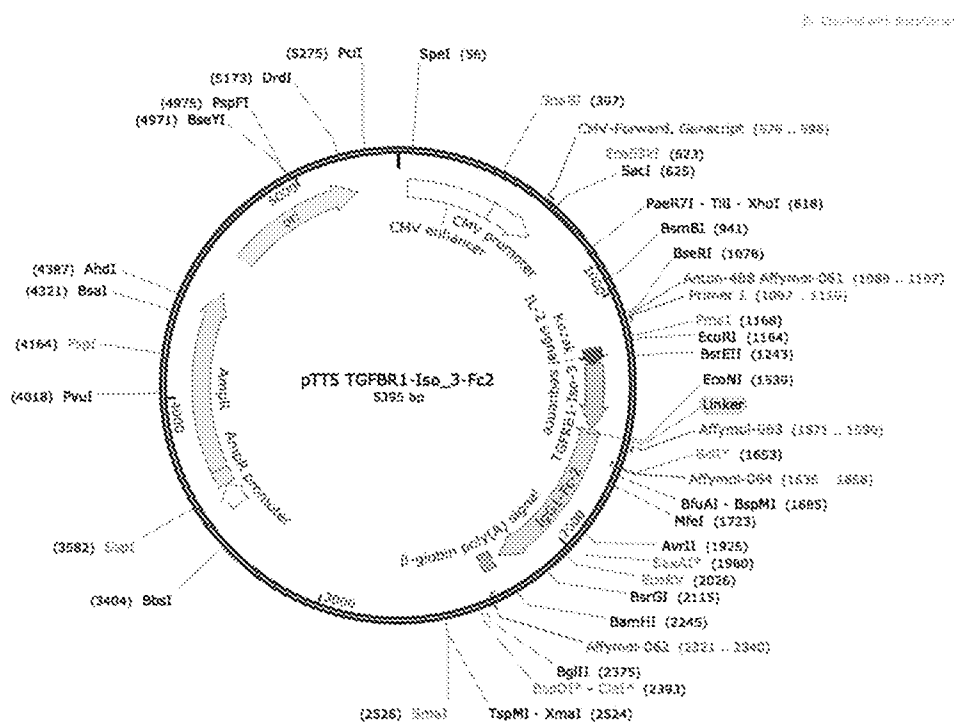


Fig. 13

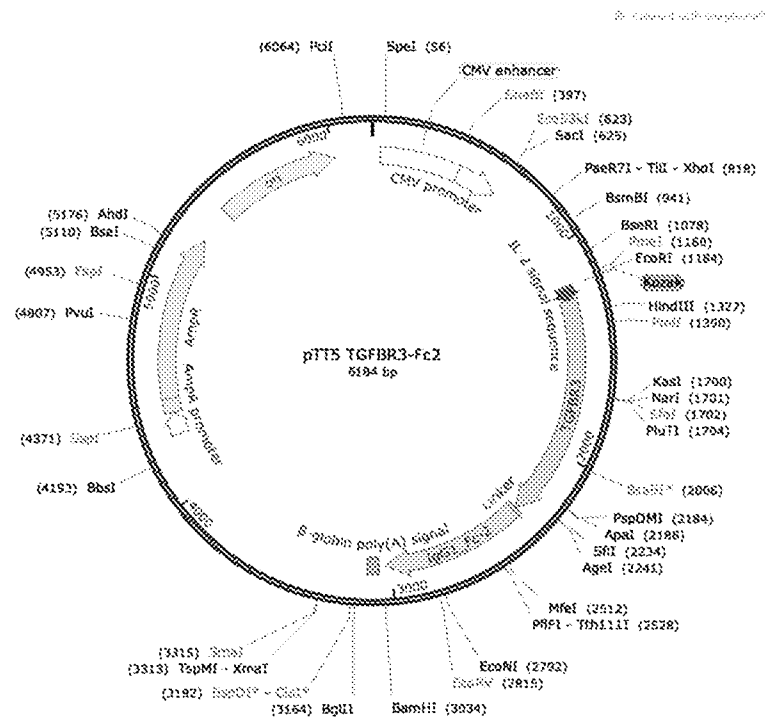


Fig. 14

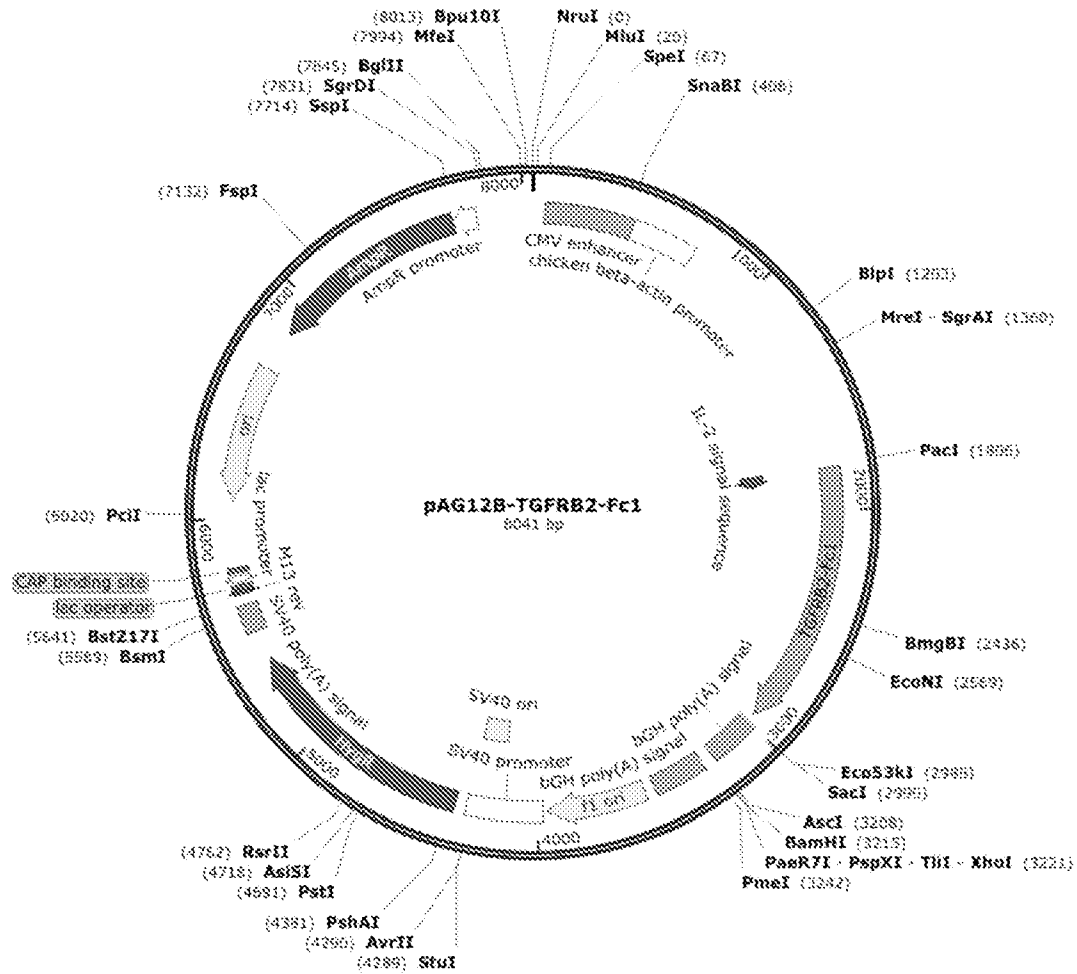


Fig. 15

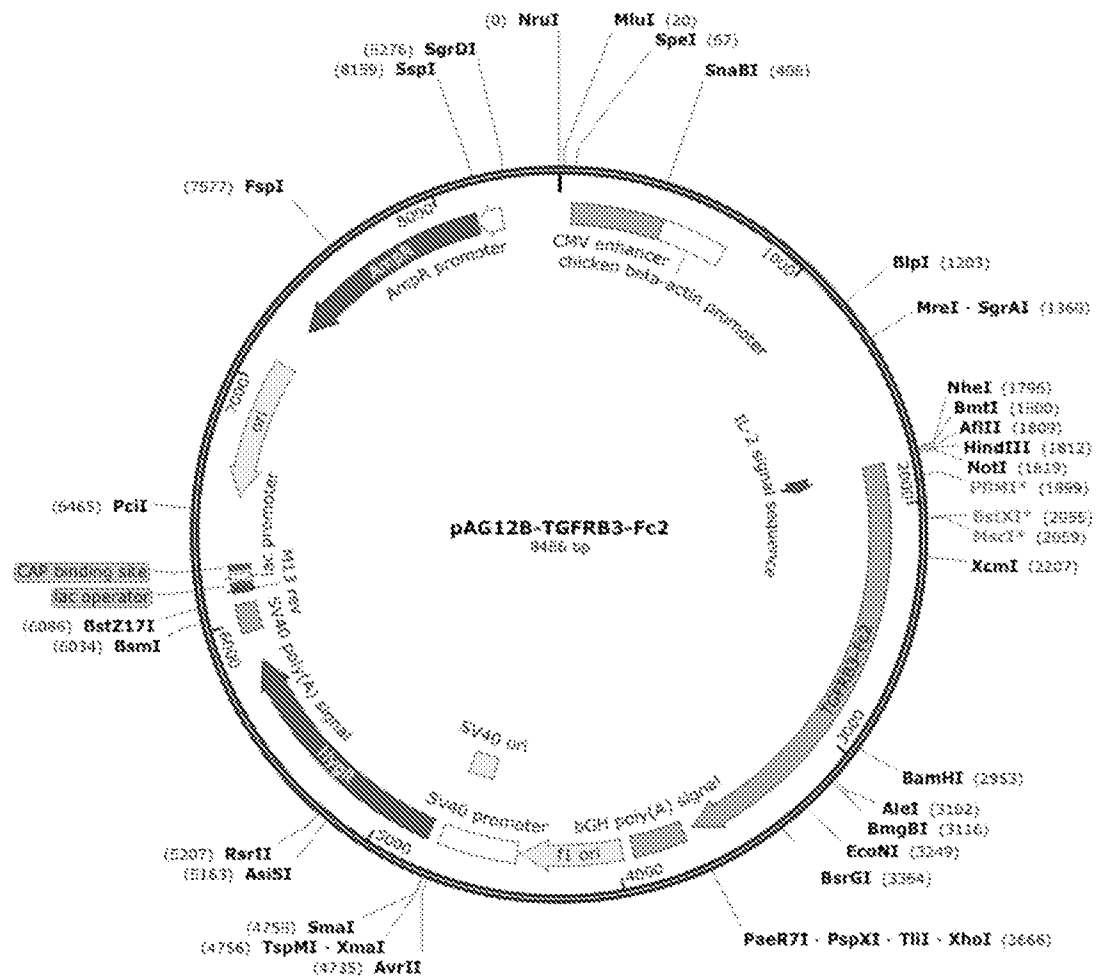


Fig. 16

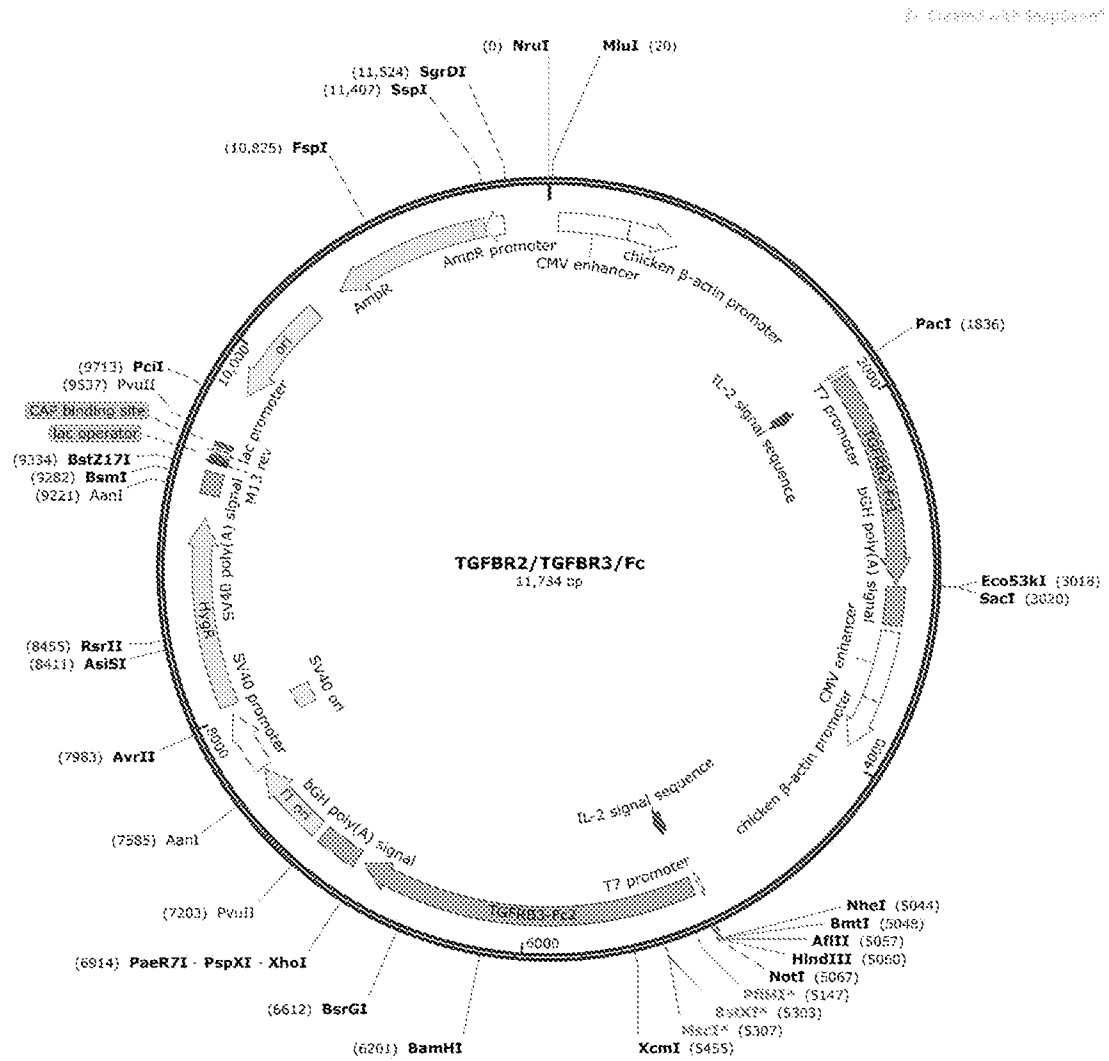


Fig. 17

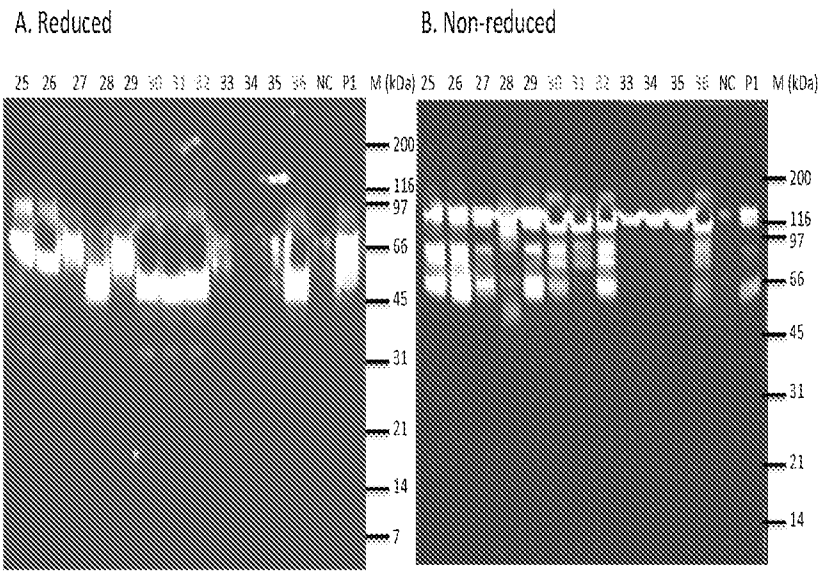


Fig. 18

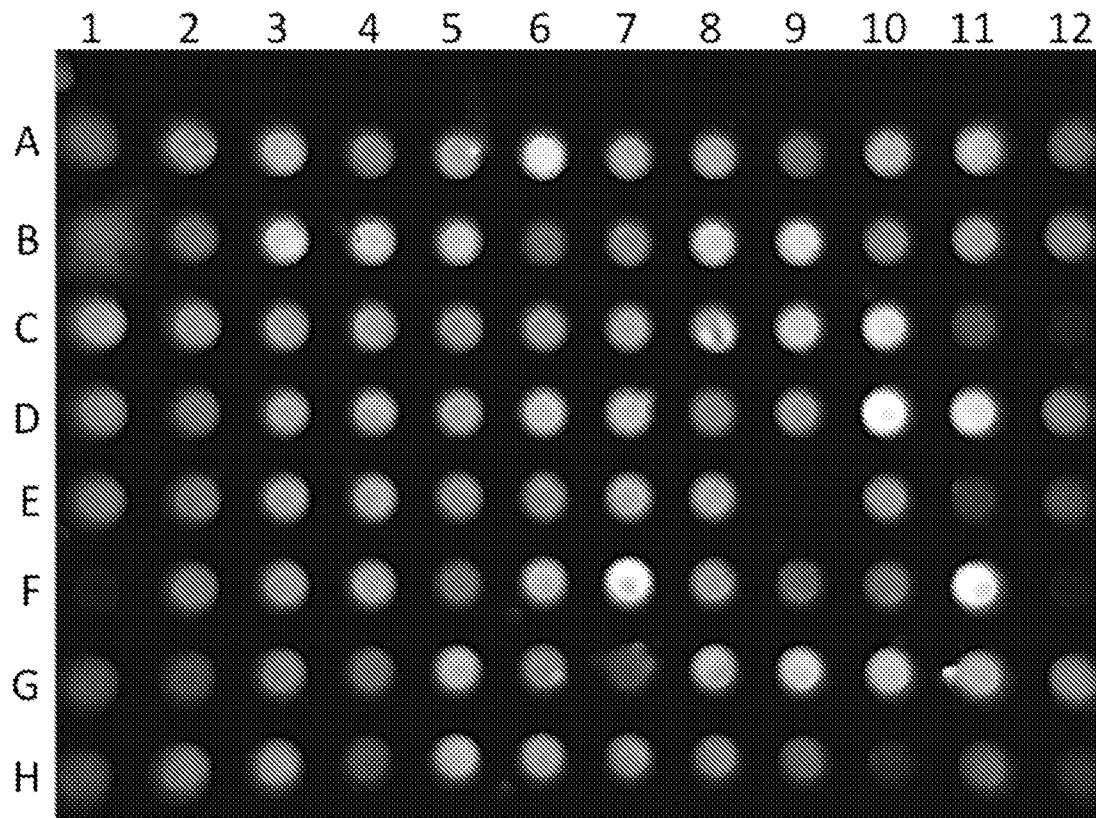


Fig. 19

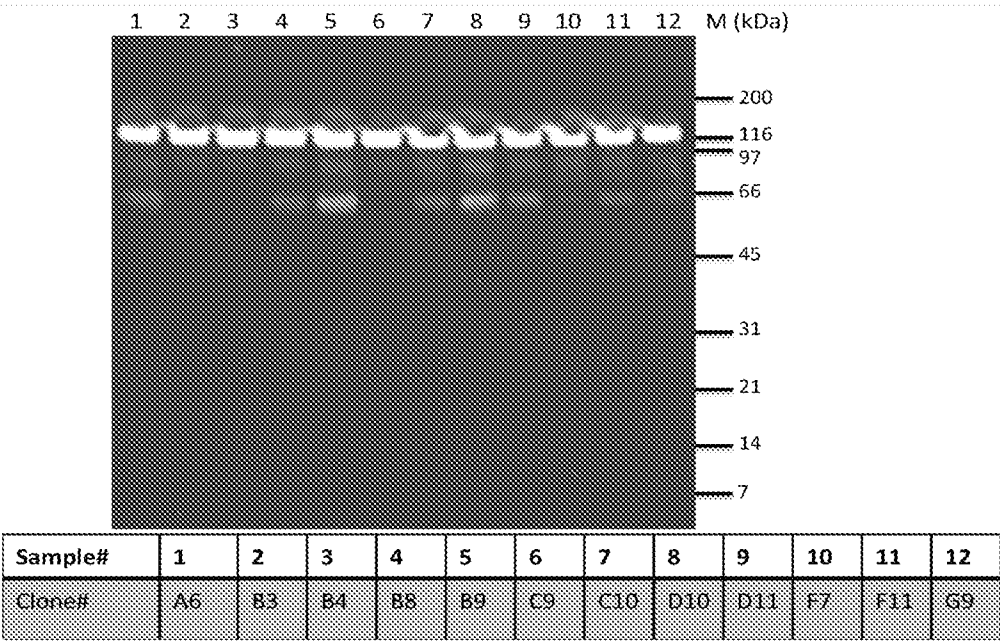
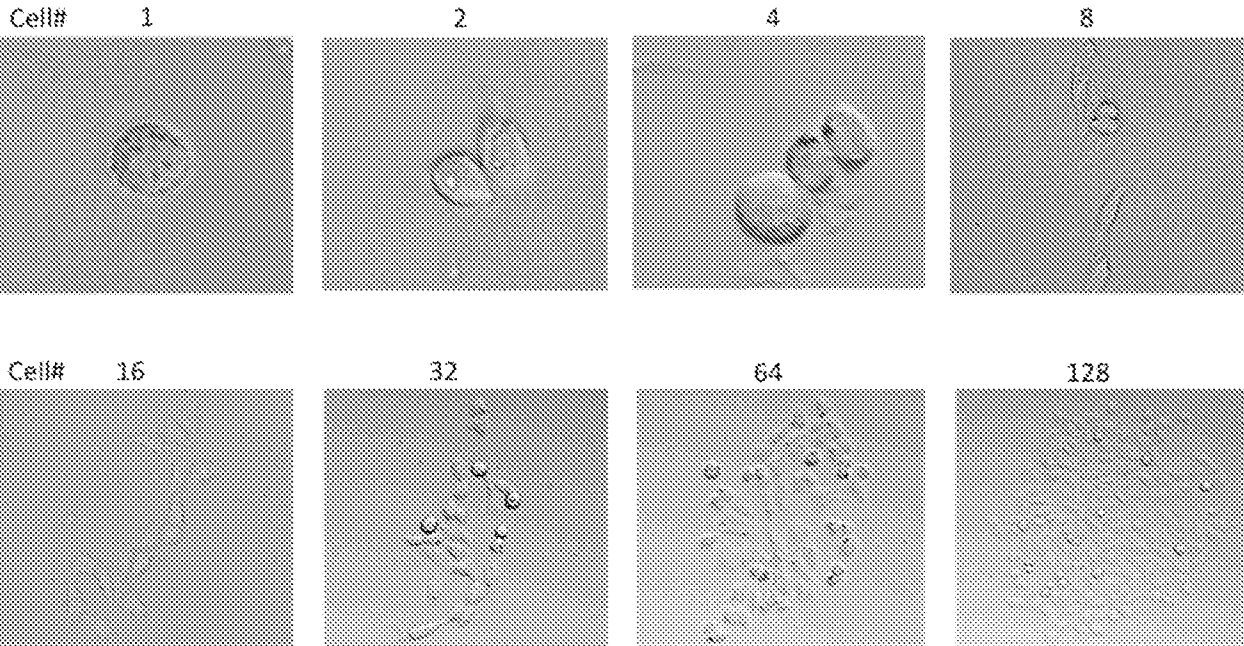


Fig. 20

(A)



(B)

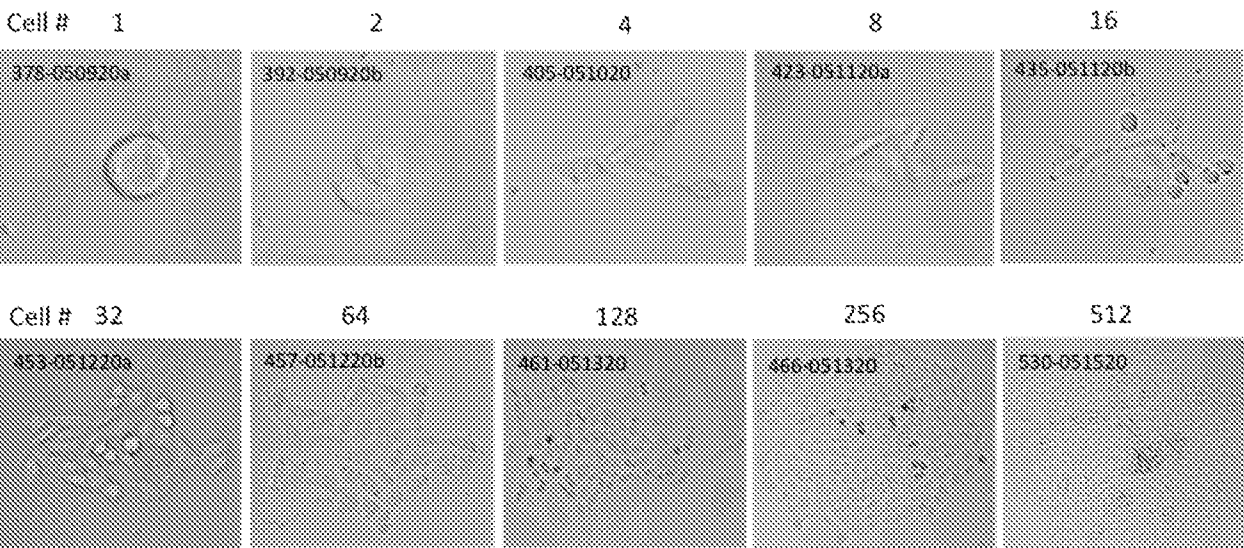


Fig. 21

		A6	D10	D11	F7	G9
051820 (d0)	1st	0.710	1.386	1.292	0.730	1.327
052220 (d4)	2nd	0.650	1.286	1.334	0.569	1.298
052620 (d4)	3rd	0.592	1.269	1.236	0.598	1.359
052920 (d4)	4th	0.397	0.891	1.122	0.437	0.947
060120 (d3)	5th	0.412	0.800	0.884	0.353	0.909
060420 (d3)	6th	0.315	0.780	0.897	0.331	0.753
060820 (d3)	7th	0.463	1.199	1.076	0.493	1.345
061220 (d4)	8th	0.505	1.159	1.234	0.514	1.370
061520 (d3)	9th	0.434	0.997	0.684	0.290	1.018
061820 (d4)	10th	0.539	0.858	0.651	0.231	0.778

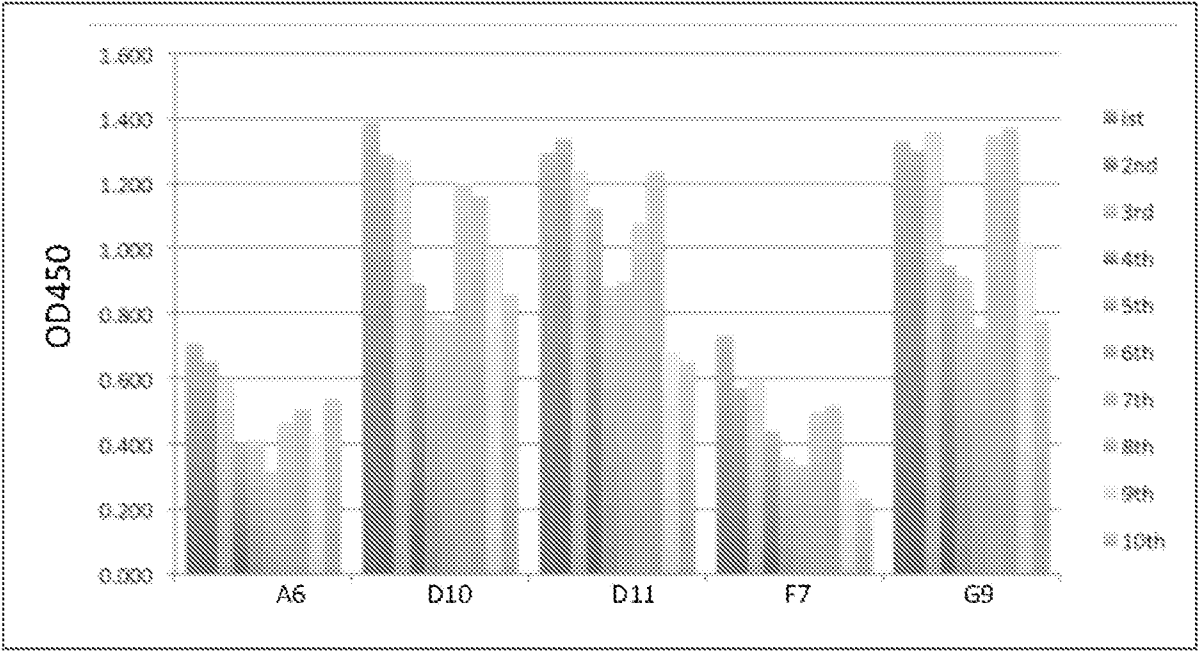


Fig. 22

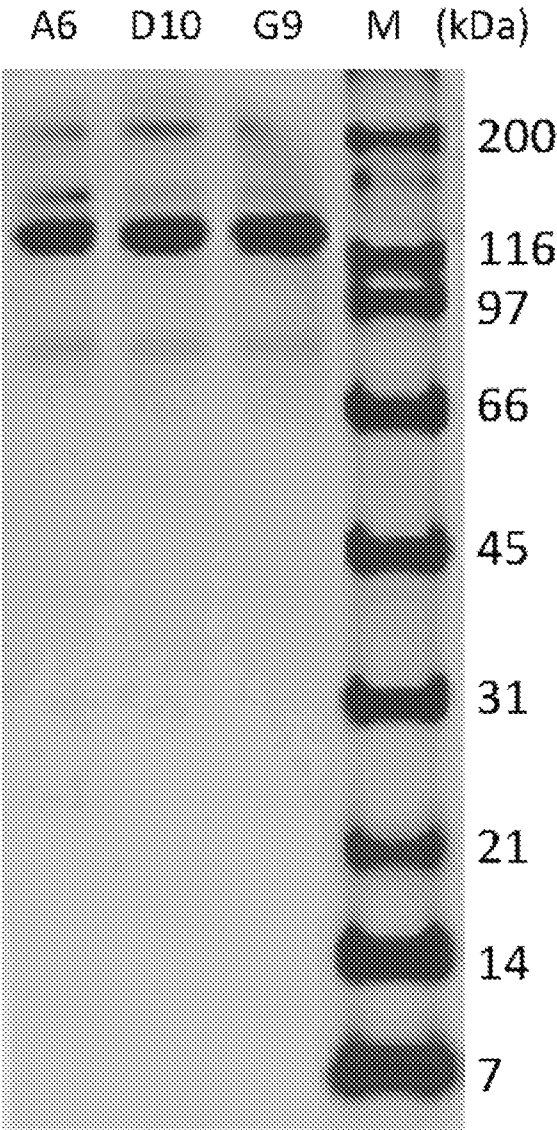


Fig. 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2021/065834

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2021/065834

A. CLASSIFICATION OF SUBJECT MATTER (see extra sheet) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K, A61K, C12N, C12P, A61P Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatSearch, EMBL, NCBI, Espacenet, DWPI, USPTO DP, CIPO (Canada PO), SIPO DB		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2019/113464 A1 (ELSTAR THERAPEUTICS, INC.), 13.06.2019, sections SUMMARY OF THE INVENTION, TGF-beta inhibitor, Nucleic Acids, example 3, table.4, claims 1-13,52-62, figs.1-7, SEQ ID NO: 143, 144, 157, 158, 189	1-18
A	CN 110354260 A (QICHENSHENG BIOTECHNOLOGY ZHUHAI CO LTD), 22.10.2019, claims 5-6, 10, section MODE FOR INVENTION, SEQ ID NO: 3	1-18
A	US 2021/0114982 A1 (IAIN WALTERS et al.), 22.04.2021, section DETAILED DESCRIPTION OF THE INVENTION, claims 60-77	11-18
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 22 February 2022 (22.02.2022)		Date of mailing of the international search report 09 June 2022 (09.06.2022)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer I. Goretova Telephone No. (8-499) 240-25-91

INTERNATIONAL SEARCH REPORT

Classification of subject matter

International application No.

PCT/US 2021/065834

C07K 14/71 (2006.01)***C07K 19/00*** (2006.01)***A61K 38/18*** (2006.01)***A61K 47/68*** (2017.01)***C12N 15/62*** (2006.01)***C12N 5/10*** (2006.01)***C12P 21/02*** (2006.01)***A61P 9/00*** (2006.01)***A61P 11/00*** (2006.01)***A61P 13/00*** (2006.01)***A61P 19/04*** (2006.01)