

[8a] In one aspect of the present disclosure, there is provided an isolated fusion protein comprising, in an N- to C- terminal orientation:

(i) a portion consisting of in an N- to C-terminal orientation:

(a) a soluble portion of an extracellular domain of a human TGF β type II receptor;

(b) an amino acid linker; and

(c) an immunoglobulin (Ig) hinge region; and

(ii) an immunoglobulin (Ig) Fc domain;

wherein the amino acid linker consists of from 10 to 40 amino acid residues and wherein the amino acid linker comprises a C-terminal portion of an immunoglobulin (Ig) C_H1 domain.

[8b] In another aspect of the present disclosure, there is provided a human TGF β type II binding protein comprising two fusion proteins disclosed herein, wherein each fusion protein comprises an extracellular domain of a human TGF β type II receptor, wherein the two fusion proteins are covalently linked together, and wherein the two extracellular domains together define a binding site for binding a human TGF β type II cytokine.

[8c] In a further aspect of the present disclosure, there is provided an isolated nucleic acid comprising a nucleotide sequence encoding the fusion protein disclosed herein.

[8d] In another aspect of the present disclosure, there is provided an expression vector comprising the nucleic acid disclosed herein.

[8e] In a further aspect of the present disclosure, there is provided a host cell comprising the expression vector disclosed herein.

[8f] In another aspect of the present disclosure, there is provided a pharmaceutical composition comprising: (i) the expression vector disclosed herein; and (ii) at least one pharmaceutically acceptable carrier or diluent.

[8g] In a further aspect of the present disclosure, there is provided a use of a fusion protein disclosed herein in the manufacture of a medicament for treating cancer in a subject in need thereof.

[36] **FIGURE 2** depicts a sequence alignment of the amino acid sequences of the human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM CH1 domains (top) and CH2 domains (bottom). SEQ ID NOs for the domain sequences from top to bottom are: 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, and 81.

5 [37] **FIGURE 3** depicts a Western blot for phosphorylated Smad2 following treatment of reporter cells with TGF β and/or the TGF β type II receptor fusion proteins hTGF β R-IgG-1 and hTGF β R-Fc as indicated. Total Smad2 and Smad3 were used as a loading control. TGF β activity was markedly reduced by hTGF β R-IgG-1 compared to hTGF β R-Fc.

10 [38] **FIGURE 4** depicts a Western blot for phosphorylated Smad2 following treatment of reporter cells with TGF β and/or the TGF β type II receptor fusion proteins hTGF β R-IgG1-1 (1), hTGF β R-IgG1-2 (2), hTGF β R-IgG1-3 (3), and hTGF β R-IgG1-4 (4) as indicated. B-actin was used as a loading control.

[39] **FIGURES 5A-5C** depict tumor volumes in mice following treatment with the indicated virus. Each line represents the tumor volume of one mouse.

15 [40] **FIGURES 6A-6B** depict Western blots for phosphorylated Smad2 following treatment of the indicated cell lines with TGF β and/or the indicated virus. Total Smad2 and Smad3 were used as a loading control.

DETAILED DESCRIPTION

[41] The invention provides an isolated fusion protein for use in the treatment of a variety of medical conditions, for example, in inhibiting proliferation of a tumor cell, inhibiting tumor growth, treating cancer, treating an inflammatory condition, or treating an infection, in a subject. Exemplary fusion proteins comprise: a first portion of an extracellular domain, transmembrane domain, or intracellular domain of a cytokine, cytokine receptor, or immunomodulatory protein; an amino acid linker; and at least one of, a second portion of an extracellular domain, transmembrane domain, or intracellular domain of a cytokine, cytokine receptor, or immunomodulatory protein; an immunoglobulin (Ig) hinge region; and an immunoglobulin (Ig) Fc domain. In certain embodiments, the linker comprises from about 5 to about 40 amino acid residues. Exemplary fusion proteins of the invention include cytokine traps.

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[42] A cytokine trap, *e.g.* a TGF β trap, is a molecule containing a soluble portion of the

Example 1: TGFβR Fusion Protein Plasmid and Adenovirus Construction

[125] This Example describes the production of plasmids and viral expression vectors that encode TGFβR fusion proteins.

[126] To construct a nucleotide sequence encoding a mouse TGFβR-IgG1 fusion protein (mTGFβR-IgG1), plasmids pORF9-mIL10RA, pUNO1-mTGFBR2, and pFUSEss-CH1g-mG1 were purchased from Invivogen. The pUNO1-mTGFBR2 plasmid was cleaved with KasI and NheI to release a 1.7 kb fragment with the coding region of the mouse TGFβ type 2 receptor. The pORF9-mIL10RA plasmid was cleaved with KasI and NheI to release a 3 kb fragment containing the vector backbone. Those two fragments were ligated to generate the plasmid pORF9-TGFBR2.

[127] The plasmid pORF9-TGFBR2 was amplified with primers flanking the KasI site 5' of the coding region and either a primer corresponding to the 3' end of the extracellular domain followed by an NheI site to produce only the extracellular domain, or a primer corresponding to the 3' end of the extracellular domain followed by a portion of the mouse IgG1 (mIgG1) CH1 domain to produce the 5' half of a fusion gene. The plasmid pFUSEss-CH1g-mG1 was amplified with primers corresponding to the 3' end of the mIgG1 gene followed by a NheI site, and the 3' end of the extracellular domain of the mTGFβR followed by a portion of the mIgG1 CH1 domain. Fusion genes were generated by combining these PCR products in a second round PCR reaction. PCR products were then cleaved with KasI and NheI and ligated into a pORF9 backbone cleaved with the same enzymes to generate pORF9 plasmids carrying either the extracellular domain or the mIgG1 fusion genes. The resulting nucleotide sequence encoded a fusion protein (mTGFβR-IgG, SEQ ID NO: 33) including residues 1-159 of the mTGFβR sequence (ending in TSSPD (SEQ ID NO: 82)) immediately followed by residues 90-324 of the mIgG1 sequence, starting at the beginning of the final β strand of the second immunoglobulin fold (beginning with STKVD (SEQ ID NO: 83)).

[128] To construct nucleotide sequences encoding human TGFβR-IgG1 fusion proteins, plasmids carrying cDNA of human IgG1 (hIgG1, Accession BC072419 in pCMV-SPORT6) and human TGFβ receptor type 2 (Accession BC040499 in pBluescriptR) were purchased from Thermo Scientific. PCR amplification using a 5' primer carrying a SalI site, a 3' primer carrying an XhoI site, and linking primers carrying a sequence from the 3' end of hTGFβR and the 5' end of hIgG1 was performed as described for the mouse genes.

[129] Nucleotide sequences encoding a series of fusion proteins were generated. A first fusion protein, hTGFβR-IgG1-1 (SEQ ID NO: 22), included residues 1-159 of hTGFβR (ending in TSNPD (SEQ ID NO: 84)), immediately followed by residues 88-330 of hIgG1, starting at the beginning of the final β strand of the second immunoglobulin fold (beginning at KPSNT (SEQ ID NO: 85)). A second fusion protein, hTGFβR-IgG1-2 (SEQ ID NO: 62), included residues 1-159 of hTGFβR (ending in TSNPD (SEQ ID NO: 84)), immediately followed by residues 90-330 of hIgG1 (beginning at SNTKV (SEQ ID NO: 86)). A third fusion protein, hTGFβR-IgG1-3 (SEQ ID NO: 63), included residues 1-159 of hTGFβR (ending in TSNPD (SEQ ID NO: 84)), immediately followed by residues 92-330 of hIgG1 (beginning at TKVDK (SEQ ID NO: 87)). A fourth fusion protein, hTGFβR-IgG1-4, included residues 1-159 of hTGFβR (ending in TSNPD (SEQ ID NO: 84)), immediately followed by residues 94-330 of hIgG1 (beginning at VDKRV (SEQ ID NO: 88)). A fifth fusion protein, hTGFβR-Fc (SEQ ID NO: 48), included residues 1-159 of TGFβR (ending in TSNPD (SEQ ID NO: 84)), immediately followed by residues 100-330 of hIgG1 (beginning at PKSCD (SEQ ID NO: 89)). The fifth fusion protein was referred to as hTGFβR-Fc because it included only the Fc domain and hinge region of the immunoglobulin, in contrast to hTGFβR-IgG-1, hTGFβR-IgG-2, hTGFβR-IgG-3, and hTGFβR-IgG-4, which included from six to twelve additional amino acids from hIgG1. Details of the fusion proteins are shown in **TABLE 3**.

TABLE 3

Fusion Protein	hTGFβR Residues	hIgG1 Residues	hTGFβR- hIgG1 Junction
hTGFβR-IgG1-1	1-159	88-330	TSNPD-KPSNTKVDKRVEPKSCD (SEQ ID NO: 90)
hTGFβR-IgG1-2	1-159	90-330	TSNPD-SNTKVDKRVEPKSCD (SEQ ID NO: 91)
hTGFβR-IgG1-3	1-159	92-330	TSNPD-TKVDKRVEPKSCD (SEQ ID NO: 92)
hTGFβR-IgG1-4	1-159	94-330	TSNPD-VDKRVEPKSCD (SEQ ID NO: 93)
hTGFβR-Fc	1-159	100-330	TSNPD-PKSCD (SEQ ID NO: 94)

[130] Nucleotide sequences encoding the fusion proteins were cloned into plasmids for downstream applications as appropriate. For adenovirus construction, nucleotide sequences were cloned into a derivative of pXC1 (which carries the 5' portion of the adenovirus genome), modified to carry a SalI site at the start site of the E1B-19k region and an XhoI site 200 base pairs 3' of the SalI site. When indicated, pXC1 was further modified at the E1A promoter region to produce the plasmid pXC1-TAV-255, which renders E1A expression cancer-selective (as previously described in U.S. Patent No. 9,073,980). PCR products were cloned into the pXC1 (or pXC1-TAV) backbone using InFusion (Clontech) according to the manufacturer's instructions.

[131] Where indicated, the pXC1 plasmids were cotransfected with the plasmid pJM17 in HEK-293A cells to allow homologous recombination to rescue recombinant virus. Virus was collected and underwent two rounds of plaque purification and sequencing to confirm presence of the fusion gene and test for presence of the TAV-255 deletion as necessary. The virus carrying the mouse isoform was grown in 293 cells, and the virus carrying the human isoform was plaque purified and grown exclusively in A549 cells after the initial viral rescue in 293 cells. Virus to be used in animal experiments was purified using Fast-Trap adenovirus purification kits (Millipore), dialyzed into viral storage buffer (25 mM NaCl, 10 mM Tris pH 8, 5% glycerol), and stored at -80° until use. Details of the viruses tested are shown in **TABLE 4**.

TABLE 4

Virus	E1A Promoter	E1B-19k Modification
Wild-type	Wild-type	Wild-type
Ad-Control	Wild-type	Deleted
Ad-mTGFβR-IgG1	Wild-type	Deleted and replaced with mTGFβR-IgG1
Ad-hTGFβR-IgG1-1	TAV-255	Deleted and replaced with hTGFβR-IgG1-1

Example 2: Inhibition of TGFβ signaling

[132] This Example describes a comparison between disclosed hTGFβR-IgG1 fusion proteins and conventional hTGFβR-IgG1 fusion proteins.

[133] As described in Example 1, plasmids were generating encoding a series of human TGFβ trap fusion proteins: hTGFβR-IgG1-1, hTGFβR-IgG1-2, hTGFβR-IgG1-3, hTGFβR-IgG1-4, and hTGFβR-Fc.

[134] hTGFβR-Fc (SEQ ID NO: 48) contains amino acids Thr23 to Asp159 of the human TGFβ type II receptor and amino acids Pro100 to Lys330 of human IgG1. This sequence is identical that used in a commercially available TGFβ trap fusion protein (R&D Systems).

[135] In contrast to the conventional TGFβ trap fusion protein, hTGFβR-IgG1-1 (SEQ ID NO: 22), hTGFβR-IgG-2 (SEQ ID NO: 62), hTGFβR-IgG-3 (SEQ ID NO: 63), and hTGFβR-IgG-4, contain twelve, ten, eight, or six amino acids, respectively, from the CH1 domain of IgG1 that serve as a flexible, non-immunogenic linker between the TGFβ type II receptor and the hinge and Fc region of the IgG1.

WHAT IS CLAIMED IS:

1. An isolated fusion protein comprising, in an N- to C- terminal orientation:
 - (i) a portion consisting of in an N- to C-terminal orientation:
 - (a) a soluble portion of an extracellular domain of a human TGF β type II
5 receptor;
 - (b) an amino acid linker; and
 - (c) an immunoglobulin (Ig) hinge region; and
 - (ii) an immunoglobulin (Ig) Fc domain;wherein the amino acid linker consists of from 10 to 40 amino acid residues and
10 wherein the amino acid linker comprises a C-terminal portion of an immunoglobulin (Ig) C_H1 domain.
2. The isolated fusion protein of claim 1, wherein the amino acid linker consists of from 10 to 30 amino acid residues.
3. The isolated fusion protein of claim 1, wherein the amino acid linker consists of from
15 10 to 20 amino acid residues.
4. The isolated fusion protein of claim 1, wherein the amino acid linker consists of from 10 to 15 amino acid residues.
5. The isolated fusion protein of claims 1-4, wherein the Ig C_H1 domain is selected from an IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM C_H1 domain.
- 20 6. The isolated fusion protein of claim 5, wherein the Ig C_H1 domain is an IgG1 C_H1 domain.
7. The isolated fusion protein of claim 6, wherein the amino acid linker comprises an amino acid sequence selected from SEQ ID NO: 1 and SEQ ID NO: 60.
8. The isolated fusion protein of claim 1, wherein the soluble portion of an extracellular
25 domain of a human TGF β type II receptor comprises amino acid residues 23-159 of SEQ ID NO: 12.

9. The isolated fusion protein of any one of claims 1-8, wherein the Ig Fc domain and hinge region are selected from a human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM Fc domain and hinge region.
10. The isolated fusion protein of claim 9, wherein the Ig Fc domain and hinge region are a human IgG1 Fc domain and hinge region.
11. The isolated fusion protein of claim 10, wherein the Ig Fc domain and hinge region comprise the amino acid sequence of SEQ ID NO: 13.
12. The isolated fusion protein of any one of claims 1-11, wherein the Ig Fc, Ig hinge region, and Ig C_H1 domain are obtained from a single immunoglobulin.
13. The isolated fusion protein of any one of claims 1-12, wherein the fusion protein comprises an amino acid sequence selected from SEQ ID NO: 22 and SEQ ID NO: 62.
14. A human TGF β type II binding protein comprising two fusion proteins of any one of claims 1-13, wherein each fusion protein comprises an extracellular domain of a human TGF β type II receptor, wherein the two fusion proteins are covalently linked together, and wherein the two extracellular domains together define a binding site for binding a human TGF β type II cytokine.
15. An isolated nucleic acid comprising a nucleotide sequence encoding the fusion protein of any one of claims 1-13.
16. An expression vector comprising the nucleic acid of claim 15.
17. The expression vector of claim 16, wherein the expression vector is an oncolytic virus.
18. The expression vector of claim 17, wherein the oncolytic virus is an adenovirus.
19. The expression vector of claim 18, wherein the adenovirus is a type 5 adenovirus .
20. The expression vector of claim 19, wherein the nucleotide sequence encoding the fusion protein is inserted into an E1b-19K insertion site located between the start site of E1b-19K and the start site of E1b-55K.
21. The expression vector of claims 19 or 20, wherein the adenovirus comprises a deletion of a Pea3 binding site, or a functional fragment thereof.
22. A host cell comprising the expression vector of any one of claims 16-21.

23. A pharmaceutical composition comprising: (i) the expression vector of any one of claims 16-21; and (ii) at least one pharmaceutically acceptable carrier or diluent.

24. Use of a fusion protein of any one of claims 1-13 in the manufacture of a medicament for treating cancer in a subject in need thereof.

5 25. Use of an expression vector comprising an isolated nucleic acid comprising a nucleotide sequence encoding the fusion protein of any one of claims 1-13 in the manufacture of a medicament for treating cancer in a subject in need thereof.

26. The use of claim 24 or 25, wherein the cancer is selected from melanoma, squamous cell carcinoma of the skin, basal cell carcinoma, head and neck cancer, breast cancer, anal
10 cancer, cervical cancer, non-small cell lung cancer, mesothelioma, small cell lung cancer, renal cell carcinoma, prostate cancer, gastroesophageal cancer, colorectal cancer, testicular cancer, bladder cancer, ovarian cancer, liver cancer, hepatocellular carcinoma, cholangiocarcinoma, brain and central nervous system cancer, thyroid cancer, parathyroid cancer (*e.g.*, parathyroid carcinoma), endometrial cancer, neuroendocrine cancer, lymphoma (*e.g.*, Hodgkin and non-
15 Hodgkin), leukemia, merkel cell carcinoma, gastrointestinal stromal tumors, multiple myeloma, uterine cancer, a sarcoma, kidney cancer, ocular cancer, pancreatic cancer, and a germ cell cancer (*e.g.*, ovarian germ cell cancer).

27. The fusion protein of any one of claims 1-13 or an expression vector comprising an isolated nucleic acid comprising a nucleotide sequence encoding the fusion protein of any one
20 of claims 1-13, for use in treating cancer.