

CLAIMS

1. A method of producing a protein comprising a target-specific extravesicular domain (TED) of an extracellular vesicle (EV) surface protein comprising modifying a polynucleotide comprising a nucleotide sequence encoding the extravesicular domain (ED) of an EV surface protein by a mutagenesis method to engineer a target binding site comprising at least two modified regions within the ED amino acid sequence, each with a length of 3-20 contiguous amino acids flanked by regions of the wild-type ED sequence at its N-terminus and C-terminus, the modified regions obtained through substitution mutations, thereby producing a repertoire of polynucleotides encoding a variety of TEDs, each comprising a different target binding site, and selecting a TED specifically recognizing a predetermined target, and producing the protein comprising the selected TED, wherein said EV surface protein is a tetraspanin.

2. The method of claim 1, wherein the repertoire of polynucleotides is comprised in a variety of TEDs displayed on the outer surface, using a display system which is selected from the group consisting of a yeast, phage, bacterium, ribosome, mRNA or mammalian cell display.

3. The method of any one of claims 1 or 2, wherein the TED comprises at least 70% sequence identity to the wild-type ED.

4. The method of any one of claims 1 to 3, wherein the wild-type ED originates from a tetraspanin, selected from the group consisting of:

- a) CD81 comprising the amino acid sequence identified as SEQ ID NO:87;
- b) CD9 comprising the amino acid sequence identified as SEQ ID NO:89;
- c) CD53 comprising the amino acid sequence identified as SEQ ID NO:90;
- d) TSPAN32 comprising the amino acid sequence identified as SEQ ID NO:91;
- e) CD82 comprising the amino acid sequence identified as SEQ ID NO:92;
- f) CD63 comprising the amino acid sequence identified as SEQ ID NO:93;
- g) CD151 comprising the amino acid sequence identified as SEQ ID NO:94; and
- h) CD37 comprising the amino acid sequence identified as SEQ ID NO:95

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5. The method of any one of claims 1 to 4, wherein the wild-type ED is of any one of:

- a) CD81, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:130 or SEQ ID NO:131;
- b) CD9, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:132, SEQ ID NO:182 or SEQ ID NO:133;
- c) CD53, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:134 or SEQ ID NO:135;
- d) TSPAN32, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:136 or SEQ ID NO:137;
- e) CD82, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:138 or SEQ ID NO:139;
- f) CD63, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:140 or SEQ ID NO:141;
- g) CD151, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:142 or SEQ ID NO:143; or
- h) CD37, wherein the ED comprises any one of the amino acid sequences identified as SEQ ID NO:144 or SEQ ID NO:145;

6. The method of any one of claims 1 to 5, wherein the protein comprises a loop structure in the ED amino acid sequence which is stabilized by one or more cysteine(s) at position(s) to allow the formation of one or more disulfide bonds, preferably wherein the modified regions are positioned within a loop region of the ED.

7. The method of any one of claims 1 to 6, wherein

- a) the ED is of CD81 and characterized by one or both of the following features:
 - i) the amino acid sequence is modified to introduce cysteines to allow formation of one or more disulfide bonds not naturally-occurring in the wild-type ED sequence, preferably between positions 134 and 144 and/or between 130 and 146 and/or 135 and 168, wherein numbering is of human CD81 identified as SEQ ID NO:87;
 - ii) one of the modified regions is positioned within positions 160 and 172, preferably wherein the TED comprises at least one further binding region positioned

between positions 132 and 141, or between positions 180 and 189, wherein numbering is of human CD81 identified as SEQ ID NO:87;

or

b) the ED is of CD9 and characterized by one or both of the following features:

i) the amino acid sequence is modified to introduce cysteines to allow formation of one or more disulfide bonds not naturally-occurring in the wild-type ED sequence, preferably between positions 20 and 28, wherein numbering of the positions is of the CD9 large extracellular loop (LEL, SEQ ID NO:118)

ii) the modified regions are positioned within positions 155-166, positions 128-142, positions 130-140, or positions 169-180.

8. The method of any one of claims 1 to 7, wherein the protein comprising the TED is a target-specific EV surface protein (TSP) comprising said TED and at least one transmembrane domain

wherein the transmembrane domain is of any one of:

a) CD81, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:147, SEQ ID NO:148, SEQ ID NO:149 or SEQ ID NO:150;

b) CD9, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153 or SEQ ID NO:154;

c) CD53, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157 or SEQ ID NO:158;

d) TSPAN32, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:159, SEQ ID NO:160, SEQ ID NO:161 or SEQ ID NO:162;

e) CD82, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165 or SEQ ID NO:166;

f) CD63, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:167, SEQ ID NO:168, SEQ ID NO:169 or SEQ ID NO:170;

g) CD151, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173 or SEQ ID NO:174; or

h) CD37, wherein the transmembrane domain comprises any one of the amino acid sequences identified as SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177 or SEQ ID NO:178.

9. The method of any one of claim 8, wherein both, the ED and the transmembrane domain, originate from the same EV surface protein.

10. A method of producing a target-specific extracellular vesicle (TEV) preparation by

a) providing a polynucleotide encoding a protein comprising the TED obtainable by a method of any one of claims 1 to 9;

b) introducing said polynucleotide into a source cell or source cell mixture;

b) culturing said cell(s) under conditions producing extracellular vesicles;

c) isolating a fraction comprising a TEV comprising the target binding site of the TED; and

d) producing a preparation of the TEV comprised in said fraction

wherein in method step a) the polynucleotide encodes said protein comprising said TED and at least one transmembrane domain, and in method step c) a fraction is isolated comprising such TEV displaying the target binding site on the outer surface of the vesicular membrane.

11. The method of claim 10, which further comprises loading the TEV with an intravesicular load, wherein the load comprises any one or more of peptides, polypeptides, protein domains, proteins, lipids, genes, nucleic acids such as mRNAs, miRNAs, RNAi mediating molecules in particular locked nucleic acids or phosphorothioates, DNA, DNA fragments, plasmids such as minicircle DNA, drugs such as small molecules, in particular chemotherapeutics or senolytics.

12. The method of any one of claims 10 to 11, wherein the source cell or source cell mixture is originating from a eukaryotic or prokaryotic source, preferably of body

tissue, body fluid, or a cell culture, wherein the source cell or source cell mixture is obtained from a subject and the TEV preparation is formulated for autologous use.

13. A protein comprising a target-specific extravesicular domain (TED) of an extracellular vesicle (EV) surface protein obtainable by a method of any one of claims 1 to 9.

14. A target-specific extravesicular domain (TED) of an extracellular vesicle (EV) surface protein comprising at least 70% sequence identity to a wild-type extravesicular domain (ED) of a mammalian EV surface protein sequence and comprising an artificial target binding site comprising at least two modified regions, each with a length of 3-20 contiguous amino acids flanked by regions of the wild-type ED sequence at its N-terminus and C-terminus, the modified regions obtained through substitution mutations,

wherein the ED originates from a tetraspanin selected from the group consisting of:

- a) CD81 comprising the amino acid sequence identified as SEQ ID NO:87;
- b) CD9 comprising the amino acid sequence identified as SEQ ID NO:89;
- c) CD53 comprising the amino acid sequence identified as SEQ ID NO:90;
- d) TSPAN32 comprising the amino acid sequence identified as SEQ ID NO:91;
- e) CD82 comprising the amino acid sequence identified as SEQ ID NO:92;
- f) CD63 comprising the amino acid sequence identified as SEQ ID NO:93;
- g) CD151 comprising the amino acid sequence identified as SEQ ID NO:94; and
- h) CD37 comprising the amino acid sequence identified as SEQ ID NO:95.

15. A target-specific EV surface protein (TSP) comprising at least one transmembrane domain and an extravesicular domain (ED) comprising at least 70% sequence identity to a wild-type ED of a mammalian EV surface protein sequence and comprising an artificial target binding site comprising at least two modified region with a length of 3-20 contiguous amino acids flanked by regions of the wild-type ED sequence at its N-terminus and C-terminus, the modified regions obtained through substitution mutations,

wherein the ED originates from a tetraspanin selected from the group consisting of:

- a) CD81 comprising the amino acid sequence identified as SEQ ID NO:87;
- b) CD9 comprising the amino acid sequence identified as SEQ ID NO:89;
- c) CD53 comprising the amino acid sequence identified as SEQ ID NO:90;
- d) TSPAN32 comprising the amino acid sequence identified as SEQ ID NO:91;
- e) CD82 comprising the amino acid sequence identified as SEQ ID NO:92;
- f) CD63 comprising the amino acid sequence identified as SEQ ID NO:93;
- g) CD151 comprising the amino acid sequence identified as SEQ ID NO:94; and
- h) CD37 comprising the amino acid sequence identified as SEQ ID NO:95.

16. A polynucleotide encoding any one of the protein of claim 13, the TED of claim 14, or the TSP of claim 15.

17. A target-specific extracellular vesicle (TEV) comprising a membrane and a displaying a target-specific molecule on the outer surface of the membrane, wherein the target-specific molecule is any one of the protein of claim 13, the TED of claim 14, or the TSP of claim 15.

18. An extracellular vesicle library comprising a variety of at least 10^2 TEVs of claim 17 with different target specificity.