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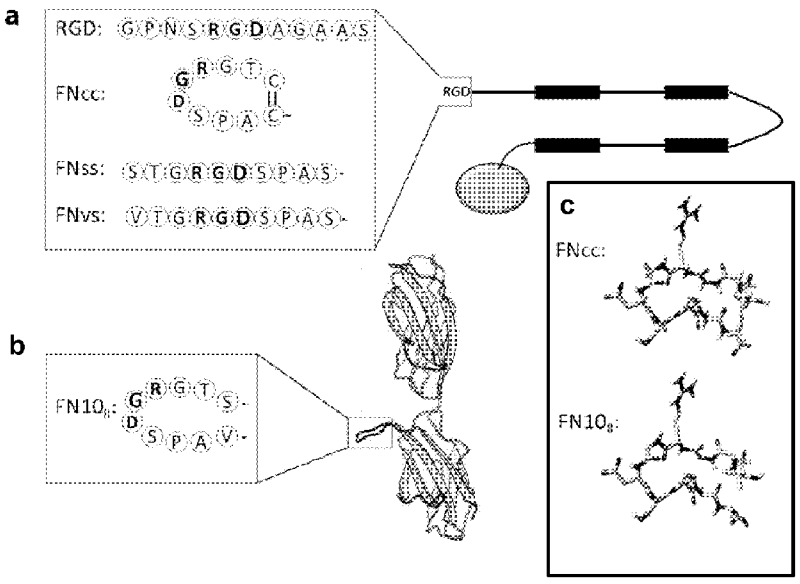
(54) **Title**: CYCLIC RGD CELL-BINDING MOTIF AND USES THEREOF

(57) **Abstract**: A recombinant fusion protein is comprising a spider silk fragment and a cyclic RGD cell-binding motif with selectivity for integrins, such as for α5β1 integrins. The fusion protein is useful as a cell scaffold material and for the cultivation of cells displaying integrins on their cell surface.

Fig 1



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CYCLIC RGD CELL-BINDING MOTIF AND USES THEREOF

Technical field of the invention

The present invention relates to the fields of eukaryotic cell culture and tissue engineering. The invention provides new proteins, a cell scaffold material comprising the proteins, and a method for cultivation of cells wherein polymers of the new proteins are used as a cell scaffold material.

Background to the invention

The phenotype of a cell is largely influenced by its display of integrins. By expressing several types of integrins on its surface, the cell is able to bind multiple kinds of ligands and thereby interpret parallel signals from the surrounding extracellular matrix (ECM). Cells cultured *in vitro* often express a different kind of integrin pattern than corresponding cells *in vivo*. In order to maintain the original phenotype of cells, or to accomplish a specific cellular response (e.g. differentiation, proliferation), it is important to enable integrin binding also during *in vitro* culture. This is most commonly done by coating cell culture plastics with ECM proteins like laminin, fibronectin, collagen or vitronectin, or mimics thereof. The ECM coatings will provide ligands for various integrins, with activation of different cellular pathways as a result. However, within several cell culture disciplines it is desirable to find ways to accomplish this on a defined matrix without the use of animal derived substrates.

WO 2011/129756 discloses methods and a cell scaffold material based on a miniature spider silk protein for eukaryotic cell culture. The protein may contain various short (3-5 amino acid residues) cell-binding peptides.

WO 2012/055854 discloses polymers consisting of a fusion protein containing a miniature spider silk protein and a large non-spidroin protein fragment of more than 30 amino acid residues which provides affinity to another molecule. The fusion protein may additionally contain various cell-binding peptides.

WO 2015/036619 discloses polymers consisting of a fusion protein containing a miniature spider silk protein and a cell-binding peptide comprising the amino acid residues RGD. The fusion protein is useful for cultivation of human pluripotent stem cells (hPSCs).

Several strategies have been attempted in order to accomplish ligands with high affinity and selectivity for specific integrins. For instance, phage libraries expressing RGD-containing peptides have been used in panning experiments. The outcome of such experiments is however dependent on limitations of the sequence coverage in the phage library. Moreover, epitopes that promote cell adherence might be missed when using a selection method that is based on inhibition of binding to coated integrins by peptides in solution. The interaction between a cell and the surrounding ECM is a cross-talk where initial binding causes intracellular signaling resulting in integrin activation and conformational changes that affects the affinity to the ligand. Thus, a cell-free system with coated integrins might miss the peptides with highest affinity to the activated form of the integrin. Ivanov, B. *et al.*, Bioconjugate Chem. 6: 269-277 (1995) and Koivunen E. *et al.*, Biotechnology 13(3): 265-270 (1995) disclose various RGD-containing peptides.

Several peptidomimetics and non-peptidic small molecules have been designed and synthesized with the purpose to find potent and selective integrin ligands. Rational design of ligands for certain integrins has been hampered by the lack of determined structures.

In most previous studies the goal has been to obtain a potent inhibitor of a specific integrin binding, for example with the purpose to hinder tumor cell invasion or unwanted angiogenesis. In those cases, a functional integrin binding is not required; rather the goal is a soluble molecule that is a potent integrin antagonist. WO 2013/185027 discloses soluble variants of human fibronectin with integrin antagonist activity, i.e. blocking or reducing activities of integrin, such as cell adhesion.

Despite these advances in the field, there is still a need for new cell scaffolds in the field, in particular since various cell types may have preference for different scaffolds and since there is a need for efficient cell scaffolds for wound healing.

Summary of the invention

It is an object of the present invention to provide proteins and a cell scaffold that promotes proliferation, differentiation and migration of cells, in particular primary cells.

It is in particular an object of the present invention to provide proteins and a cell scaffold which support proliferation, differentiation and migration of keratinocytes.

It is a further object of the present invention to achieve increased cell adhesion efficacy to a cell scaffold.

It is in particular an object of the present invention to provide proteins and a cell scaffold which provides early attachment of adherent cells.

It is also an object of the present invention to provide proteins and a cell scaffold that are useful for efficient expansion of adherent cells *in vitro*.

It is also an object of the present invention to provide proteins and a cell scaffold that are useful for transferring cells as a cell sheet, e.g. to a wound area *in vivo*.

Is it an object of the present invention to provide proteins and a cell scaffold that attract inherent cells for migration into a wound area, e.g. from the wound edges from where dermal keratinocytes are usually recruited during wound healing.

For these and other objects that will be evident from the following disclosure, the present invention provides a cyclic RGD cell-binding motif comprising the amino acid sequence

$$C^1X^1X^2RGDX^3X^4X^5C^2$$

wherein

each of X^1, X^2, X^3, X^4 and X^5 are independently selected from natural amino acid residues other than cysteine; and

C^1 and C^2 are connected via a disulphide bond. The cell-binding motif has selectivity for integrins, such as for $\alpha 5\beta 1$ integrins.

It has surprisingly been found that recombinant proteins containing this cyclic RGD cell-binding motif are useful for the cultivation of cells displaying integrins on their cell surface.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

Without wishing to be bound to any specific theory, it is contemplated that the cell-binding motif presented herein imitates the α5β1-specific RGD loop motif of fibronectin by positioning cysteines adjacent to the RGD sequence to allow formation of a disulphide-bridge to constrain the chain into a similar type of turn loop. This cyclic RGD cell-binding motif increases the cell adhesion efficacy to a matrix made of a protein containing the cell-binding motif, such as a recombinantly produced spider silk protein.

The present invention provides according to an aspect a recombinant protein comprising said cell-binding motif with selectivity for integrins, such as for $\alpha 5\beta 1$ integrins. This recombinant protein is surprisingly useful for the cultivation of cells displaying integrins on their cell surface.

The present invention provides according to a one aspect a recombinant fusion protein comprising a spidroin fragment and said cell-binding motif with selectivity for integrins, such as for $\alpha 5\beta 1$ integrins. This recombinant fusion protein is surprisingly useful for the cultivation of cells displaying integrins on their cell surface.

. In preferred embodiments of the invention, each of X^1, X^2, X^3, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, D, E, M, P, N and Q.

In other preferred embodiments of the invention, each of X^1 and X^3 are independently selected from the group of amino acid residues consisting of: G, S, T, M, N and Q; and each of X^2, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, P, N and Q.

In certain preferred embodiments of the invention, X^1 is selected from the group of amino acid residues consisting of: G, S, T, N and Q; X^3 is selected from the group of amino acid residues consisting of: S, T and Q; and each of X^2, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, P and N.

In some preferred embodiments of the invention, X^1 is S or T; X^2 is G, A or V; preferably G or A; more preferably G; X^3 is S or T; preferably S; X^4 is G, A, V or P; preferably G or P; more preferably P; and X^5 is G, A or V; preferably G or A; more preferably A.

In certain preferred embodiments of the invention, the cell-binding motif is comprising the amino acid sequence CTGRGDSPAC (SEQ ID NO: 10).

Further preferred cyclic RGD cell-binding motifs according to the invention display at least 60%, such as at least 70%, such as at least 80%, such as at least 90% identity to CTGRGDSPAC (SEQ ID NO: 10), with the proviso that position 1 and 10 are always C; position 4 is always R; position 5 is always G; position 6 is always D; and positions 2-3 and 7-9 are never cysteine. It is understood that the non-identical positions among positions 2-3 and 7-9 can be freely selected as set out above.

In some preferred fusion proteins according to the invention, the cell-binding motif is arranged N-terminally of the spidroin fragment.

In certain preferred fusion proteins according to the invention, the spidroin fragment is comprising the protein moieties **REP** and **CT**, wherein

REP is a repetitive fragment of from 70 to 300 amino acid residues, selected from the group consisting of $\mathbf{L(AG)_nL}$, $\mathbf{L(AG)_nAL}$, $\mathbf{L(GA)_nL}$, and $\mathbf{L(GA)_nGL}$, wherein

n is an integer from 2 to 10;

each individual **A** segment is an amino acid sequence of from 8 to 18 amino acid residues, wherein from 0 to 3 of the amino acid residues are not Ala, and the remaining amino acid residues are Ala;

each individual **G** segment is an amino acid sequence of from 12 to 30 amino acid residues, wherein at least 40% of the amino acid residues are Gly; and

each individual **L** segment is a linker amino acid sequence of from 0 to 30 amino acid residues; and

CT is a fragment of from 70 to 120 amino acid residues, having at least 70% identity to SEQ ID NO: 3.

In some preferred fusion proteins according to the invention, the spidroin fragment has at least 70% identity to SQ ID NO: 2 or to amino acid residues 18-277 of SEQ ID NO: 13.

According to a further aspect, the present invention provides a cell scaffold material comprising a protein polymer which as a repeating unit is containing the recombinant fusion protein according to the invention.

In a preferred embodiment of the cell scaffold material according to the invention, the protein polymer is in a physical form selected from the group consisting of film, coating, foam, fiber and fiber-mesh.

In one preferred embodiment of the cell scaffold material according to the invention, the protein polymer is in a physical form of a free-standing matrix.

According to a related aspect, the present invention provides a method for the cultivation of cells, comprising the steps of

- providing a sample of cells;
- applying the sample to a cell scaffold material; and
- maintaining the cell scaffold material having the cells applied thereto under conditions suitable for cell culture;

wherein

the cell scaffold material comprises a protein polymer, which is containing the recombinant protein, such as the recombinant fusion protein, according to the invention as a repeating structural unit.

It has surprisingly been found that recombinant proteins containing this cyclic RGD cell-binding motif are useful for the cultivation of cells displaying integrins on their cell surface. Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

According to a further aspect, the present invention provides use of the recombinant fusion protein according to the invention, the cell scaffold material according to the invention, or the recombinant protein according to the invention for the cultivation of cells displaying integrins on their cell surface.

It has surprisingly been found that recombinant proteins, such as recombinant fusion proteins, containing this cyclic RGD cell-binding motif are

useful for the cultivation of cells displaying integrins on their cell surface. The immobilized (i.e. not in solution) cell-binding motif promotes integrin activation and cell binding.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

In preferred embodiments of the method or the use according to the invention, the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface; and the cell-binding motif of the recombinant fusion protein has selectivity for $\alpha 5\beta 1$ integrins.

Brief description of the drawings

Fig. 1 illustrates silk constructs with cell binding motifs derived from fibronectin.

Fig. 2 shows micrographs and FTIR spectra of FN_{CC} silk (SEQ ID NO: 13) matrices.

Fig. 3 shows micrographs and coverage density of endothelial cells (EC), mesenchymal stem cells (MSC) and keratinocytes (KC) after 1 h adhesion to film of WT silk (SEQ ID NO: 2) or silk functionalized with RGD (SEQ ID NO: 16) or FN_{CC} (SEQ ID NO: 13).

Fig. 4 shows micrographs and cell coverage area of keratinocytes (KC) after 1 h adhesion to either silk functionalized with FN_{CC} (SEQ ID NO: 13), a bovine fibronectin coated surface (BFN) or tissue culture treated cell plastic (TCT).

Fig. 5 shows micrographs and cell coverage area of keratinocytes (KC) after 1 h adhesion to WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16).

Fig. 6 shows cell coverage area and stress fiber ranking of keratinocytes (KC) after 3 h adhesion to WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16).

Fig. 7 shows graphs of formation of focal adhesions in keratinocytes after adherence for 3 h onto films of WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16).

Fig. 8 shows a graph of an Alamar blue viability assay of keratinocytes seeded on films of WT-silk (SEQ ID NO: 2) or FN_{CC} silk (SEQ ID NO: 13).

Fig. 9 shows a sequence alignment of spidroin C-terminal domains.

List of appended sequences

SEQ ID NO:	
1	RepCT (4RepCT, WT) (DNA)
2	RepCT (4RepCT, WT)
3	CT
4	consensus CT sequence
5	repetitive sequence from Euprosthenops australis MaSp1
6	consensus G segment sequence 1
7	consensus G segment sequence 2
8	consensus G segment sequence 3
9	FN_{VS}, native fibronectin RGD cell-binding motif
10	FN_{CC}
11	FN_{SS}
12	linear RGD cell-binding motif, Widhe *et al*. (2013)*
13	FN_{CC}-4RepCT
14	FN_{SS}-4RepCT
15	FN_{VS}-4RepCT
16	RGD-4RepCT, Widhe *et al*. (2013)*
17	FN_{CC}-4RepCT (DNA)
18	FN_{SS}-4RepCT (DNA)
19	FN_{VS}-4RepCT(DNA)
20	RGD-4RepCT, Widhe *et al*. (2013) (DNA)*
21-24	RGD peptides with glycine spacer
25-28	Linker peptides
29	CT *Euprosthenops sp* MaSp1
30	CT *Euprosthenops australis* MaSp1

SEQ ID NO:	
31	CT *Argiope trifasciata* MaSp1
32	CT *Cyrtophora moluccensis* Sp1
33	CT *Latrodectus geometricus* MaSp1
34	CT *Latrodectus hesperus* MaSp1
35	CT *Macrothele holsti* Sp1
36	CT *Nephila clavipes* MaSp1
37	CT *Nephila pilipes* MaSp1
38	CT *Nephila madagascariensis* MaSp1
39	CT *Nephila senegalensis* MaSp1
40	CT *Octonoba varians* Sp1
41	CT *Psechrus sinensis* Sp1
42	CT *Tetragnatha kauaiensis* MaSp1
43	CT *Tetragnatha versicolor* MaSp1
44	CT *Araneus bicentenarius* Sp2
45	CT *Argiope amoena* MaSp2
46	CT *Argiope aurantia* MaSp2
47	CT *Argiope trifasciata* MaSp2
48	CT *Gasteracantha mammosa* MaSp2
49	CT *Latrodectus geometricus* MaSp2
50	CT *Latrodectus hesperus* MaSp2
51	CT *Nephila clavipes* MaSp2
52	CT *Nephila madagascariensis* MaSp2
53	CT *Nephila senegalensis* MaSp2
54	CT *Dolomedes tenebrosus* Fb1
55	CT *Dolomedes tenebrosus* Fb2
56	CT *Araneus diadematus* ADF-1
57	CT *Araneus diadematus* ADF-2
58	CT *Araneus diadematus* ADF-3
59	CT *Araneus diadematus* ADF-4
60	STGRGDSPAV ($FN10_{II}$)

* Widhe M *et al.*, Biomaterials 34(33): 8223-8234 (2013)

Detailed description of the invention

Recombinantly produced spider silk and numerous other materials are useful as matrices for culture of mammalian cells. The inclusion of cell adhesion motifs derived from the extracellular matrix (ECM) into such materials increases cell attachment and proliferation by interaction with integrins on the cell surface. The integrins do not just confer the physical connection between cells and the surrounding, but also mediate signals controlling for example cell growth, polarity, proliferation and survival. Moreover, the integrins are essential for cell migration by acting as the cells' “feet”.

The most widely characterized cell adhesion motif is the RGD peptide, first discovered in fibronectin. The RGD motif is found also in many other molecules of the natural ECM, for example in vitronectin, fibrinogen and in cryptic sites of both collagen I and several of the laminin α chains. Almost half of the known integrins, including α3β1, α5β1, α8β1, αvβ1, αIIbβ3, αvβ3, αvβ5, αvβ6a and αvβ8, have been shown to bind ECM in a RGD-dependent manner. However, after initial proofs of RGD as general cell adhesion motif, it soon became clear that integrins in general bind with magnitudes higher affinity to larger RGD containing proteins than to short RGD peptides. The preferred conditions for binding also seem to vary between different integrins.

The present invention is based on a designed cell-binding motif. Without wishing to be bound to any specific theory, it is contemplated that the cell-binding motif presented herein imitates the α5β1-specific RGD loop motif of fibronectin by positioning cysteines in precise positions adjacent to the RGD sequence to allow formation of a disulphide-bridge to constrain the chain into a similar type of turn loop. This cyclic RGD cell-binding motif increases the cell adhesion efficacy to a matrix made of a protein containing the cell-binding motif, such as a recombinantly produced spider silk protein or a synthetic peptide.

The term "cyclic" as used herein refers to a peptide wherein two amino acid residues are covalently bonded via their side chains, more specifically through a disulfide bond between two cysteine residues.

It is shown herein that the cell adhesive properties of a material is significantly enhanced by introducing the cyclic RGD cell-binding motif on a cysteine linked loop compared to when a linear RGD peptide is added. In addition, the cyclic RGD cell-binding motif presented herein promotes both proliferation of and migration by primary cells. Human primary cells cultured on a cell scaffold material containing the cyclic RGD cell-binding motif showed increased attachment, spreading, stress fiber formation and focal adhesions compared to the same material containing a linear RGD peptide.

The cyclic RGD cell-binding motif presented herein is also suitable for preparing free-standing matrices, in particular matrices containing spider silk, on which cells could readily form a monolayer culture. Such free-standing matrices are useful for cell sheet transfer. Thus, a material containing the cyclic RGD cell-binding motif presented herein, such as a spider silk material, is useful for both an *in vitro* setting, where adherent cells need to be expanded efficiently, and in an *in vivo* situation where cells need to be transferred as a cell sheet to e.g. a wound area. The results also support that a material containing the cyclic RGD cell-binding motif presented herein, such as a spider silk material, can efficiently attract inherent cells for migration into a wound area, e.g. from the wound edges from where dermal keratinocytes are usually recruited during wound healing. Cell binding to a cell scaffold containing the cyclic RGD cell-binding motif presented herein is demonstrated to involve the α5β1 integrin, and to support proliferation and migration of keratinocytes.

The present inventor used DNA technology to modify the cell-binding motif of fibronectin, where the RGD motif is presented on a turn loop. This was accomplished with the amino acid sequence flanking RGD in the tenth type III domain of fibronectin as base (Fig. 1b). Firstly, the same decapeptide (VTGRGDSPAS; SEQ ID NO: 9) as in the turn loop of fibronectin was introduced N-terminally to a protein to yield a construct denoted FN_{VS} (Fig. 1a). Without wishing to be bound to any specific theory, it was hypothesized that the cell-binding motif could be made more efficient by positioning the valine and serine residue situated 3 positions before and 4 positions after the RGD motif respectively, spatially very close to each other. The present

inventor therefore mutated these two residues to cysteines (Fig. 1a, c), so that the RGD containing motif is flanked by one cysteine on each side. The cysteines are spatially less than 2 Å apart, and thus connect the peptide chain into a disulphide bridged loop (denoted FN_{CC}; SEQ ID NO: 10). As control, a variant with the two cysteines exchanged to serines was also constructed (denoted FN_{SS}; SEQ ID NO: 11). The present inventor investigated the effect of these FN motifs, when introduced into protein matrices, on various mechanisms of early attachment (including spreading, stress fiber formation, focal adhesions and integrin binding) in primary adherent cells of human origin. It was found that the FN_{CC} variant containing a cyclic RGD cell-binding motif increases the cell adhesion efficacy to a matrix made of a protein containing the cell-binding motif as compared to the controls FN_{VS} and FN_{SS}.

It can be seen from the crystal structure of the ninth and tenth domain of fibronectin determined by Leahy DJ *et al.*, Cell 84(1): 155-164 (1996), that the valine and serine residue situated 3 positions before and 4 positions after the RGD motif respectively, are located spatially very close to each other (Fig. 1c). Again without wishing to be bound to any specific theory, it is therefore considered that the cell-binding motif presented herein imitates the α5β1-specific RGD loop motif of fibronectin by positioning cysteines adjacent to the RGD sequence to allow formation of a disulphide-bridge to constrain the chain into a similar type of turn loop. As a consequence, it is concluded that the cell-binding motif presented herein is in particular selective for $\alpha 5\beta 1$ integrins.

Thus, the relevant silk constructs with cell binding motifs derived from fibronectin are illustrated in Fig. 1. Fig. 1a schematically shows the silk protein 4RepCT with different RGD motifs genetically introduced to its N-terminus. "RGD" in Fig 1a denotes the RGD containing peptide (SEQ ID NO 12) used in Widhe M *et al.*, Biomaterials 34(33): 8223-8234 (2013). "FN_{VS}" denotes the RGD-containing decapeptide from fibronectin (SEQ ID NO: 9). "FN_{CC}" denotes the same peptide with V and S exchanged to C (SEQ ID NO: 10). "FN_{SS}" denotes the same peptide with V and S exchanged to S (SEQ ID NO: 11). Fig. 1b shows the structure of the 9th and 10th domain of fibronectin, displaying the turn loop containing the RGD motif (SEQ ID NO: 60). Fig. 1c

shows a structure model of the RGD loop taken from fibronectin, with the residues V and S mutated to C (adapted from 1FNF.pdb).

The cell-binding motif presented herein is selective for binding to integrins presented on the cell surface, such as and preferably to $\alpha 5\beta 1$ integrins. In the context of the present invention, "specific" or "selective" interaction of the cell-binding motif with its target integrin means that the interaction is such that a distinction between specific and non-specific, or between selective and non-selective, interaction becomes meaningful. The interaction between two proteins is sometimes measured by the dissociation constant. The dissociation constant describes the strength of binding (or affinity) between two molecules. Typically the dissociation constant between an antibody and its antigen is from 10^{-7} to 10^{-11} M. However, high specificity does not necessarily require high affinity. Molecules with low affinity (in the molar range) for its counterpart have been shown to be as specific as molecules with much higher affinity. In the case of the present invention, a specific or selective interaction refers to the extent to which a particular method can be used to preferentially bind to a specific protein or cell type, displaying the target integrin or a fragment thereof, under given conditions in the presence of other proteins or cells in a sample of a naturally occurring or processed biological or biochemical fluid. In other words, specificity or selectivity is the capacity to distinguish between related proteins and cell types displaying the related proteins. Specific and selective are sometimes used interchangeably in the present description.

The cyclic RGD cell-binding motif is comprising, or consisting of, the amino acid sequence

$$C^1X^1X^2RGDX^3X^4X^5C^2$$

wherein

each of X^1, X^2, X^3, X^4 and X^5 are independently selected from natural amino acid residues other than cysteine; and

C^1 and C^2 are connected via a disulphide bond.

It is preferred that each of X^1, X^2, X^3, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, D, E, M, P, N and Q.

It is more preferred that each of X^1 and X^3 are independently selected from the group of amino acid residues consisting of: G, S, T, M, N and Q; and that each of X^2, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, P, N and Q. The resulting cell-binding motif does not contain any charged or bulky residues which could be disadvantageous for the cell-binding efficacy.

It is in particular preferred that:

- X^1 is selected from the group of amino acid residues consisting of: G, S, T, N and Q;
- X^3 is selected from the group of amino acid residues consisting of: S, T and Q; and
- each of X^2, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, P and N.

It is more preferred that

- X^1 is S or T;
- X^2 is G, A or V; preferably G or A; more preferably G;
- X^3 is S or T; preferably S;
- X^4 is G, A, V or P; preferably G or P; more preferably P; and
- X^5 is G, A or V; preferably G or A; more preferably A.

A particularly preferred cyclic RGD cell-binding motif is comprising, or consisting of, the amino acid sequence CTGRGDSPAC (FN_{CC}; SEQ ID NO: 10).

Further preferred cyclic RGD cell-binding motifs according to the invention display at least 60%, such as at least 70%, such as at least 80%, such as at least 90% identity to CTGRGDSPAC (FN_{CC}; SEQ ID NO: 10), with the proviso that position 1 and 10 are always C; position 4 is always R; position 5 is always G; position 6 is always D; and positions 2-3 and 7-9 are never cysteine. It is understood that the non-identical positions among positions 2-3 and 7-9 can be freely selected as set out above.

The thus identified cyclic RGD cell-binding motif is useful in any recombinant or synthetic protein or peptide so as to provide selective binding to integrins, in particular $\alpha 5\beta 1$ integrins. Thus, there is provided a recombinant protein comprising the cell-binding motif with selectivity for

integrins, such as for $\alpha 5\beta 1$ integrins. The recombinant protein is useful for the cultivation of cells, e.g. mammalian cells, displaying integrins, in particular $\alpha 5\beta 1$ integrins, on their cell surface.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

Fibronectin is recognized by at least ten of the cell surface receptors of the integrin family, among which five (α3β1, α4β1, α5β1, α8β1, αvβ1) include the β1 subunit. The α5 subunit is found only in combination with β1 and the α5β1 integrin is unique since it is specialized for binding of fibronectin only, and therefore originally denoted the fibronectin receptor. The specific interaction between α5β1 and fibronectin seem to be fundamental for vertebrate development since lack of either α5β1 or fibronectin results in early embryonic lethality. Fibronectin and α5β1 has also been shown important in the wound repair process of airway epithelium, where both have been observed to be exclusively expressed by the migratory cells in the wounded area, and to play a critical role in endothelial cell migration *in vitro* and angiogenesis *in vivo*.

There is provided a recombinant or synthetic protein or peptide comprising a cell-binding motif with selectivity for integrins, such as for $\alpha 5\beta 1$ integrins, wherein the cell-binding motif is as set out above.

A preferred recombinant protein is comprising a cell-binding motif with selectivity for integrins, such as for $\alpha 5\beta 1$ integrins, wherein the cell-binding motif has the amino acid sequence

$$C^1X^1X^2RGDX^3X^4X^5C^2$$

wherein

X^1 is selected from the group of amino acid residues consisting of: G, S, T, N and Q;

X^3 is selected from the group of amino acid residues consisting of: S, T and Q; and

each of X^2, X^4 and X^5 are independently selected from the group of amino acid residues consisting of: G, A, V, S, T, P and N; and

C^1 and C^2 are connected via a disulphide bond.

Preferred embodiments of the cell-binding motif is presented herein. In particular, it is preferred that:

X^1 is S or T; preferably T;

X^2 is G, A or V; preferably G or A; more preferably G;

X^3 is S or T; preferably S

X^4 is G, A, V or P; preferably G or P; more preferably P;

X^5 is G, A or V; preferably G or A; more preferably A.

A specific preferred cell-binding motif is comprising the amino acid sequence CTGRGDSPAC (FN_{CC}; SEQ ID NO: 10).

The recombinant protein is useful in cell scaffold materials. It is also useful for the cultivation of cells displaying integrins on their cell surface, in particular, wherein the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

The recombinant or synthetic protein may also be constituted by a shorter peptide comprising or even consisting of the cell-binding motif, e.g. containing 10-50, or 10-30 amino acid residues. These peptides may be chemically coupled or immobilized to a surface as is well-known in the art. Advantageously, the peptide contains or is coupled to a spacer which allows greater accessibility to the cell-binding motif. The thus immobilized (i.e. not in solution) recombinant protein is surprisingly useful for the cultivation of cells displaying integrins on their cell surface, in particular, wherein the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface.

The cell-binding motif is advantageously presented as part of a fusion protein together with a spider silk protein, in particular a miniature spider silk protein. The terms "spidroins" and "spider silk proteins" are used interchangeably throughout the description and encompass all known spider silk proteins, including major ampullate spider silk proteins which typically are abbreviated "MaSp", or "ADF" in the case *of Araneus diadematus*. These major ampullate spider silk proteins are generally of two types, 1 and 2. These terms furthermore include non-natural proteins with a high degree of identity and/or similarity to the known spider silk proteins.

There is provided a recombinant fusion protein comprising a spidroin fragment and the cell-binding motif with selectivity for integrins, such as for $\alpha5\beta1$ integrins, set out above. The spidroin fragment is preferably comprising, or consisting of, the protein moieties **REP** and **CT**, wherein

REP is a repetitive fragment of from 70 to 300 amino acid residues, selected from the group consisting of $\mathbf{L(AG)_nL}$, $\mathbf{L(AG)_nAL}$, $\mathbf{L(GA)_nL}$, and $\mathbf{L(GA)_nGL}$, wherein

n is an integer from 2 to 10;

each individual **A** segment is an amino acid sequence of from 8 to 18 amino acid residues, wherein from 0 to 3 of the amino acid residues are not Ala, and the remaining amino acid residues are Ala;

each individual **G** segment is an amino acid sequence of from 12 to 30 amino acid residues, wherein at least 40% of the amino acid residues are Gly; and

each individual **L** segment is a linker amino acid sequence of from 0 to 30 amino acid residues; and

CT is a fragment of from 70 to 120 amino acid residues, having at least 70% identity to SEQ ID NO: 3.

The fusion protein according to the invention harbors both a desired selective cell-binding activity in the cell-binding motif and an internal solid support activity in the spidroin fragment. The binding activity of the fusion protein is maintained when it is structurally rearranged to form polymeric, solid structures. These protein structures, or protein polymers, also provides a high and predictable density of the cell-binding motif with selective interaction activity towards integrins, e.g. $\alpha5\beta1$ integrins. The thus immobilized cell-binding motif promotes integrin activation and cell binding. The way biomaterials functionalized with RGD stimulate different cell responses is not only affected by the type of RGD motif used, but also the resulting surface concentrations of ligands. Since the rather small silk proteins used in the present study self-assemble into multilayers where each molecule carries an RGD motif, a dense surface presentation is expected. However, if a more sparse surface concentration is desired, any possible surface density can be achieved simply by mixing silk proteins with and without the cyclic RGD cell-

binding motif disclosed herein at different ratios, thereby directing the cellular response of interest.

In most of the proteins that have been engineered to contain RGD, the motif has been added as a linear extension either to the N- or C-terminus, thus with a high possibility of exposure and flexibility due to minimal constrain of the chain from the rest of the protein. Several constructs with the RGD motif placed within a protein fold have been made to reduce the flexibility of the RGD motif, but at the same time also reducing its exposure. The cyclic RGD cell-binding motif disclosed herein can advantageously be presented as a linear extension either to the N- or C-terminus, thus with a high possibility of exposure. At the same time, its cyclic properties limit the flexibility and is believed to contribute to highly useful cell binding properties. Furthermore, the covalent incorporation of the peptide into a folded protein chain might have contributed to the apparently efficient integrin-mediated cell binding, involving α5β1.

The term "fusion protein" implies here a protein that is made by expression from a recombinant nucleic acid, i.e. DNA or RNA that is created artificially by combining two or more nucleic acid sequences that would not normally occur together (genetic engineering). The fusion proteins according to the invention are recombinant proteins, and they are therefore not identical to naturally occurring proteins. In particular, wildtype spidroins are not fusion proteins according to the invention, because they are not expressed from a recombinant nucleic acid as set out above. The combined nucleic acid sequences encode different proteins, partial proteins or polypeptides with certain functional properties. The resulting fusion protein, or recombinant fusion protein, is a single protein with functional properties derived from each of the original proteins, partial proteins or polypeptides. Furthermore, the fusion protein according to the invention and the corresponding genes are chimeric, i.e. the protein/gene moieties are derived from at least two different species.

The fusion protein typically consists of from 170 to 2000 amino acid residues, such as from 170 to 1000 amino acid residues, such as from 170 to 600 amino acid residues, preferably from 170 to 500 amino acid residues,

such as from 170 to 400 amino acid residues. The small size is advantageous because longer proteins containing spider silk protein fragments may form amorphous aggregates, which require use of harsh solvents for solubilisation and polymerisation.

The fusion protein may contain one or more linker peptides, or **L** segments. The linker peptide(s) may be arranged between any moieties of the fusion protein, e.g. between the **REP** and **CT** moieties, at either terminal end of the fusion protein or between the spidroin fragment and the cell-binding motif. The linker(s) may provide a spacer between the functional units of the fusion protein, but may also constitute a handle for identification and purification of the fusion protein, e.g. a His and/or a Trx tag. If the fusion protein contains two or more linker peptides for identification and purification of the fusion protein, it is preferred that they are separated by a spacer sequence, e.g. His_6-spacer-His_6-. The linker may also constitute a signal peptide, such as a signal recognition particle, which directs the fusion protein to the membrane and/or causes secretion of the fusion protein from the host cell into the surrounding medium. The fusion protein may also include a cleavage site in its amino acid sequence, which allows for cleavage and removal of the linker(s) and/or other relevant moieties. Various cleavage sites are known to the person skilled in the art, e.g. cleavage sites for chemical agents, such as CNBr after Met residues and hydroxylamine between Asn-Gly residues, cleavage sites for proteases, such as thrombin or protease 3C, and self-splicing sequences, such as intein self-splicing sequences.

The spidroin fragment and the cell-binding motif are linked directly or indirectly to one another. A direct linkage implies a direct covalent binding between the moieties without intervening sequences, such as linkers. An indirect linkage also implies that the moieties are linked by covalent bonds, but that there are intervening sequences, such as linkers and/or one or more further moieties, e.g. 1-2 NT moieties.

The cell-binding motif may be arranged internally or at either end of the fusion protein, i.e. C-terminally arranged or N-terminally arranged. It is preferred that the cell-binding motif is arranged at the N-terminal end of the fusion protein. If the fusion protein contains one or more linker peptide(s) for

identification and purification of the fusion protein, e.g. a His or Trx tag(s), it is preferred that it is arranged at the N-terminal end of the fusion protein.

A preferred fusion protein has the form of an N-terminally arranged cell-bonding motif, coupled by a linker peptide of 0-30 amino acid residues, such as 0-10 amino acid residues, to a **REP** moiety. Optionally, the fusion protein has an N-terminal or C-terminal linker peptide, which may contain a purification tag, such as a His tag, and a cleavage site.

The recombinant protein is useful in cell scaffold materials. It is also useful for the cultivation of cells displaying integrins on their cell surface, in particular wherein the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

Without wishing to be bound to any specific theory, it is contemplated that the cell-binding motif is functionally displayed on the surface of the resulting cell scaffold material, which is herein surprisingly shown to be advantageous for the binding capacity with respect to mammalian cells, c.f. Examples 6-9.

The prominent positive effect of the cell scaffold material containing the cyclic RGD cell-binding motif presented herein is evident already at initial attachment (within 0.5-3 h) of primary cells. Strong and rapid attachment of cells onto a material has been suggested to be of considerable importance when it comes to various clinical applications, where the present environment for cells is far from optimal, and fast establishment is necessary for cell survival. One example is the stressful milieu of a chronic wound, often with high bacterial load and necrosis. Here, migrating keratinocytes might benefit from the support of a suitably designed biomaterial constituting containing the cyclic RGD cell-binding motif, such as as a spider silk fusion protein. Also in clinical settings where the close surroundings imply physical stress, like velocity of passing fluids, e.g. blood passing the stent in a heart or a vessel implant, a material that facilitates for the endothelial cells to rapidly and firmly

attach to an implant could be critical, and thus even decisive for a successful outcome.

A scaffold intended for tissue engineering will obviously be subjected to harsher handling and environments than in a cell culture setting, why the observed improved stability of the spider silk material containing the cyclic RGD cell-binding motif is valuable. This increase in stability compared to the wild type silk allows preparation of transferable scaffolds, e.g. free-standing films as demonstrated herein.

The protein moiety **REP** is fragment with a repetitive character, alternating between alanine-rich stretches and glycine-rich stretches. The **REP** fragment generally contains more than 70, such as more than 140, and less than 300, preferably less than 240, such as less than 200, amino acid residues, and can itself be divided into several **L** (linker) segments, **A** (alanine-rich) segments and **G** (glycine-rich) segments, as will be explained in more detail below. Typically, said linker segments, which are optional, are located at the **REP** fragment terminals, while the remaining segments are in turn alanine-rich and glycine-rich. Thus, the **REP** fragment can generally have either of the following structures, wherein n is an integer:

L(AG)$_n$L, such as **LA$_1$G$_1$A$_2$G$_2$A$_3$G$_3$A$_4$G$_4$A$_5$G$_5$L**;

L(AG)$_n$AL, such as **LA$_1$G$_1$A$_2$G$_2$A$_3$G$_3$A$_4$G$_4$A$_5$G$_5$A$_6$L**;

L(GA)$_n$L, such as **LG$_1$A$_1$G$_2$A$_2$G$_3$A$_3$G$_4$A$_4$G$_5$A$_5$L**; or

L(GA)$_n$GL, such as **LG$_1$A$_1$G$_2$A$_2$G$_3$A$_3$G$_4$A$_4$G$_5$A$_5$G$_6$L**.

It follows that it is not critical whether an alanine-rich or a glycine-rich segment is adjacent to the N-terminal or C-terminal linker segments. It is preferred that n is an integer from 2 to 10, preferably from 2 to 8, also preferably from 4 to 8, more preferred from 4 to 6, i.e. n=4, n=5 or n=6.

In some embodiments, the alanine content of the **REP** fragment is above 20%, preferably above 25%, more preferably above 30%, and below 50%, preferably below 40%, more preferably below 35%. It is contemplated that a higher alanine content provides a stiffer and/or stronger and/or less extendible fiber.

In certain embodiments, the **REP** fragment is void of proline residues, i.e. there are no Pro residues in the **REP** fragment.

Turning now to the segments that constitute the **REP** fragment, it is emphasized that each segment is individual, i.e. any two **A** segments, any two **G** segments or any two **L** segments of a specific **REP** fragment may be identical or may not be identical. Thus, it is not a general feature of the spidroin that each type of segment is identical within a specific **REP** fragment. Rather, the following disclosure provides the skilled person with guidelines how to design individual segments and gather them into a **REP** fragment, which is a part of a functional spider silk protein useful in a cell scaffold material.

Each individual **A** segment is an amino acid sequence having from 8 to 18 amino acid residues. It is preferred that each individual **A** segment contains from 13 to 15 amino acid residues. It is also possible that a majority, or more than two, of the **A** segments contain from 13 to 15 amino acid residues, and that a minority, such as one or two, of the **A** segments contain from 8 to 18 amino acid residues, such as 8-12 or 16-18 amino acid residues. A vast majority of these amino acid residues are alanine residues. More specifically, from 0 to 3 of the amino acid residues are not alanine residues, and the remaining amino acid residues are alanine residues. Thus, all amino acid residues in each individual **A** segment are alanine residues, with no exception or with the exception of one, two or three amino acid residues, which can be any amino acid. It is preferred that the alanine-replacing amino acid(s) is (are) natural amino acids, preferably individually selected from the group of serine, glutamic acid, cysteine and glycine, more preferably serine. Of course, it is possible that one or more of the **A** segments are all-alanine segments, while the remaining **A** segments contain 1-3 non-alanine residues, such as serine, glutamic acid, cysteine or glycine.

In an embodiment, each **A** segment contains 13-15 amino acid residues, including 10-15 alanine residues and 0-3 non-alanine residues as described above. In a more preferred embodiment, each **A** segment contains 13-15 amino acid residues, including 12-15 alanine residues and 0-1 non-alanine residues as described above.

It is preferred that each individual **A** segment has at least 80%, preferably at least 90%, more preferably 95%, most preferably 100% identity to an amino acid sequence selected from the group of amino acid residues 7-19, 43-56, 71-83, 107-120, 135-147, 171-183, 198-211, 235-248, 266-279, 294-306, 330-342, 357-370, 394-406, 421-434, 458-470, 489-502, 517-529, 553-566, 581-594, 618-630, 648-661, 676-688, 712-725, 740-752, 776-789, 804-816, 840-853, 868-880, 904-917, 932-945, 969-981, 999-1013, 1028-1042 and 1060-1073 of SEQ ID NO: 5. Each sequence of this group corresponds to a segment of the naturally occurring sequence of *Euprosthenops australis* MaSp1 protein, which is deduced from cloning of the corresponding cDNA, see WO2007/078239. Alternatively, each individual **A** segment has at least 80%, preferably at least 90%, more preferably 95%, most preferably 100% identity to an amino acid sequence selected from the group of amino acid residues 25-36, 55-69, 84-98, 116-129 and 149-158 of SEQ ID NO: 2. Each sequence of this group corresponds to a segment of expressed, non-natural spider silk proteins, which proteins have the capacity to form silk fibers under appropriate conditions. Thus, in certain embodiments of the spidroin, each individual **A** segment is identical to an amino acid sequence selected from the above-mentioned amino acid segments. Without wishing to be bound by any particular theory, it is envisaged that **A** segments according to the invention form helical structures or beta sheets.

Furthermore, it has been concluded from experimental data that each individual **G** segment is an amino acid sequence of from 12 to 30 amino acid residues. It is preferred that each individual **G** segment consists of from 14 to 23 amino acid residues. At least 40% of the amino acid residues of each **G** segment are glycine residues. Typically the glycine content of each individual **G** segment is in the range of 40-60%.

It is preferred that each individual **G** segment has at least 80%, preferably at least 90%, more preferably 95%, most preferably 100% identity to an amino acid sequence selected from the group of amino acid residues 20-42, 57-70, 84-106, 121-134, 148-170, 184-197, 212-234, 249-265, 280-293, 307-329, 343-356, 371-393, 407-420, 435-457, 471-488, 503-516, 530-552, 567-580, 595-617, 631-647, 662-675, 689-711, 726-739, 753-775, 790-

803, 817-839, 854-867, 881-903, 918-931, 946-968, 982-998, 1014-1027, 1043-1059 and 1074-1092 of SEQ ID NO: 5. Each sequence of this group corresponds to a segment of the naturally occurring sequence of *Euprosthenops australis* MaSp1 protein, which is deduced from cloning of the corresponding cDNA, see WO2007/078239. Alternatively, each individual **G** segment has at least 80%, preferably at least 90%, more preferably 95%, most preferably 100% identity to an amino acid sequence selected from the group of amino acid residues 1-24, 37-54, 70-83, 99-115 and 130-148 of SEQ ID NO: 2. Each sequence of this group corresponds to a segment of expressed, non-natural spider silk proteins, which proteins have the capacity to form silk fibers under appropriate conditions. Thus, in certain embodiments of the spidroin in the cell scaffold material, each individual **G** segment is identical to an amino acid sequence selected from the above-mentioned amino acid segments.

In certain embodiments, the first two amino acid residues of each **G** segment are not -Gln-Gln-.

There are three subtypes of the **G** segment. This classification is based upon careful analysis of the *Euprosthenops australis* MaSp1 protein sequence (see WO2007/078239), and the information has been employed and verified in the construction of novel, non-natural spider silk proteins.

The first subtype of the **G** segment is represented by the amino acid one letter consensus sequence GQG(G/S)QGG(Q/Y)GG (L/Q)GQGGYGQGA GSS (SEQ ID NO: 6). This first, and generally the longest, **G** segment subtype typically contains 23 amino acid residues, but may contain as little as 17 amino acid residues, and lacks charged residues or contain one charged residue. Thus, it is preferred that this first **G** segment subtype contains 17-23 amino acid residues, but it is contemplated that it may contain as few as 12 or as many as 30 amino acid residues. Without wishing to be bound by any particular theory, it is envisaged that this subtype forms coil structures or 3_1-helix structures. Representative **G** segments of this first subtype are amino acid residues 20-42, 84-106, 148-170, 212-234, 307-329, 371-393, 435-457, 530-552, 595-617, 689-711, 753-775, 817-839, 881-903, 946-968, 1043-1059 and 1074-1092 of SEQ ID NO: 5. In certain embodiments, the first two amino

acid residues of each **G** segment of this first subtype according to the invention are not -Gln-Gln-.

The second subtype of the **G** segment is represented by the amino acid one letter consensus sequence GQGGQGQG(G/R)Y GQG(A/S)G(S/G)S (SEQ ID NO: 7). This second, generally mid-sized, **G** segment subtype typically contains 17 amino acid residues and lacks charged residues or contain one charged residue. It is preferred that this second **G** segment subtype contains 14-20 amino acid residues, but it is contemplated that it may contain as few as 12 or as many as 30 amino acid residues. Without wishing to be bound by any particular theory, it is envisaged that this subtype forms coil structures. Representative **G** segments of this second subtype are amino acid residues 249-265, 471-488, 631-647 and 982-998 of SEQ ID NO: 5.

The third subtype of the **G** segment is represented by the amino acid one letter consensus sequence G(R/Q)GQG(G/R)YGQG (A/S/V)GGN (SEQ ID NO: 8). This third **G** segment subtype typically contains 14 amino acid residues, and is generally the shortest of the **G** segment subtypes. It is preferred that this third **G** segment subtype contains 12-17 amino acid residues, but it is contemplated that it may contain as many as 23 amino acid residues. Without wishing to be bound by any particular theory, it is envisaged that this subtype forms turn structures. Representative **G** segments of this third subtype are amino acid residues 57-70, 121-134, 184-197, 280-293, 343-356, 407-420, 503-516, 567-580, 662-675, 726-739, 790-803, 854-867, 918-931, 1014-1027 of SEQ ID NO: 5.

Thus, in preferred embodiments of the spidroin in the cell scaffold material, each individual **G** segment has at least 80%, preferably 90%, more preferably 95%, identity to an amino acid sequence selected from SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

In an embodiment of the alternating sequence of **A** and **G** segments of the **REP** fragment, every second **G** segment is of the first subtype, while the remaining **G** segments are of the third subtype, e.g. ...$\mathbf{A}_1\mathbf{G}_{short}\mathbf{A}_2\mathbf{G}_{long}\mathbf{A}_3\mathbf{G}_{short}\mathbf{A}_4\mathbf{G}_{long}\mathbf{A}_5\mathbf{G}_{short}$... In another embodiment of the **REP** fragment, one **G** segment of the second subtype interrupts the **G** segment regularity *via* an insertion, e.g. ...$\mathbf{A}_1\mathbf{G}_{short}\mathbf{A}_2\mathbf{G}_{long}\mathbf{A}_3\mathbf{G}_{mid}\mathbf{A}_4\mathbf{G}_{short}\mathbf{A}_5\mathbf{G}_{long}$...

Each individual **L** segment represents an optional linker amino acid sequence, which may contain from 0 to 30 amino acid residues, such as from 0 to 20 amino acid residues. While this segment is optional and not critical for the function of the spider silk protein, its presence still allows for fully functional spider silk proteins and polymers thereof which form fibers, films, foams and other structures. There are also linker amino acid sequences present in the repetitive part (SEQ ID NO: 5) of the deduced amino acid sequence of the MaSp1 protein from *Euprosthenops australis*. In particular, the amino acid sequence of a linker segment may resemble any of the described **A** or **G** segments, but usually not sufficiently to meet their criteria as defined herein.

As shown in WO 2007/078239, a linker segment arranged at the C-terminal part of the **REP** fragment can be represented by the amino acid one letter consensus sequences ASASAAASAA STVANSVS (SEQ ID NO: 22) and ASAASAAA (SEQ ID NO: 23), which are rich in alanine. In fact, the second sequence can be considered to be an **A** segment according to the definition herein, whereas the first sequence has a high degree of similarity to **A** segments according to this definition. Another example of a linker segment has the one letter amino acid sequence GSAMGQGS (SEQ ID NO: 24), which is rich in glycine and has a high degree of similarity to **G** segments according to the definition herein. Another example of a linker segment is SASAG (SEQ ID NO: 25).

Representative **L** segments are amino acid residues 1-6 and 1093-1110 of SEQ ID NO: 5; and amino acid residues 159-165 of SEQ ID NO: 2, but the skilled person will readily recognize that there are many suitable alternative amino acid sequences for these segments. In one embodiment of the **REP** fragment, one of the **L** segments contains 0 amino acids, i.e. one of the **L** segments is void. In another embodiment of the **REP** fragment, both **L** segments contain 0 amino acids, i.e. both **L** segments are void. Thus, these embodiments of the **REP** fragments according to the invention may be schematically represented as follows: $(\mathbf{AG})_n\mathbf{L}$, $(\mathbf{AG})_n\mathbf{AL}$, $(\mathbf{GA})_n\mathbf{L}$, $(\mathbf{GA})_n\mathbf{GL}$; $\mathbf{L}(\mathbf{AG})_n$, $\mathbf{L}(\mathbf{AG})_n\mathbf{A}$, $\mathbf{L}(\mathbf{GA})_n$, $\mathbf{L}(\mathbf{GA})_n\mathbf{G}$; and $(\mathbf{AG})_n$, $(\mathbf{AG})_n\mathbf{A}$, $(\mathbf{GA})_n$, $(\mathbf{GA})_n\mathbf{G}$. Any

of these **REP** fragments are suitable for use with any **CT** fragment as defined below.

The **CT** fragment of the spidroin in the cell scaffold material has a high degree of similarity to the C-terminal amino acid sequence of spider silk proteins. As shown in WO2007/078239, this amino acid sequence is well conserved among various species and spider silk proteins, including MaSp1 and MaSp2. A consensus sequence of the C-terminal regions of MaSp1 and MaSp2 is provided as SEQ ID NO: 4. In Fig. 9, the following MaSp proteins are aligned, denoted with GenBank accession entries where applicable:

TABLE 1 - Spidroin CT fragments

Species and spidroin	Entry
Euprosthenops sp MaSp1 (Pouchkina-Stantcheva*)	Cthyb_Esp
Euprosthenops australis MaSp1 (SEQ ID NO: 3)	CTnat_Eau
Argiope trifasciata MaSp1	AF350266_At1
Cyrtophora moluccensis Sp1	AY666062_Cm1
Latrodectus geometricus MaSp1	AF350273_Lg1
Latrodectus hesperus MaSp1	AY953074_Lh1
Macrothele holsti Sp1	AY666068_Mh1
Nephila clavipes MaSp1	U20329_Nc1
Nephila pilipes MaSp1	AY666076_Np1
Nephila madagascariensis MaSp1	AF350277_Nm1
Nephila senegalensis MaSp1	AF350279_Ns1
Octonoba varians Sp1	AY666057_Ov1
Psechrus sinensis Sp1	AY666064_Ps1
Tetragnatha kauaiensis MaSp1	AF350285_Tk1
Tetragnatha versicolor MaSp1	AF350286_Tv1
Araneus bicentenarius Sp2	ABU20328_Ab2
Argiope amoena MaSp2	AY365016_Aam2
Argiope aurantia MaSp2	AF350263_Aau2
Argiope trifasciata MaSp2	AF350267_At2
Gasteracantha mammosa MaSp2	AF350272_Gm2

Species and spidroin	Entry
Latrodectus geometricus MaSp2	AF350275_Lg2
Latrodectus hesperus MaSp2	AY953075_Lh2
Nephila clavipes MaSp2	AY654293_Nc2
Nephila madagascariensis MaSp2	AF350278_Nm2
Nephila senegalensis MaSp2	AF350280_Ns2
Dolomedes tenebrosus Fb1	AF350269_DtFb1
Dolomedes tenebrosus Fb2	AF350270_DtFb2
Araneus diadematus ADF-1	U47853_ADF1
Araneus diadematus ADF-2	U47854_ADF2
Araneus diadematus ADF-3	U47855_ADF3
Araneus diadematus ADF-4	U47856_ADF4

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It is not critical which specific **CT** fragment is present in the spider silk protein in the cell scaffold material. Thus, the **CT** fragment can be selected from any of the amino acid sequences shown in Fig. 9 and Table 1 or sequences with a high degree of similarity. A wide variety of C-terminal sequences can be used in the spider silk protein.

The sequence of the **CT** fragment has at least 50% identity, preferably at least 60%, more preferably at least 65% identity, or even at least 70% identity, to the consensus amino acid sequence SEQ ID NO: 4, which is based on the amino acid sequences of Fig. 9.

A representative **CT** fragment is the *Euprosthenops australis* sequence SEQ ID NO: 3 or amino acid residues 180-277 of SEQ ID NO: 13. Thus, in one embodiment, the **CT** fragment has at least 70%, such as at least 80%, such as at least 85%, preferably at least 90%, such as at least 95%, identity to SEQ ID NO: 3, amino acid residues 180-277 of SEQ ID NO: 13, or any individual amino acid sequence of Fig. 9 and Table 1. For example, the **CT** fragment may be identical to SEQ ID NO: 3, amino acid residues 180-277 of SEQ ID NO: 13, or any individual amino acid sequence of Fig. 9 and Table 1.

The **CT** fragment typically consists of from 70 to 120 amino acid residues. It is preferred that the **CT** fragment contains at least 70, or more than 80, preferably more than 90, amino acid residues. It is also preferred that the **CT** fragment contains at most 120, or less than 110 amino acid residues. A typical **CT** fragment contains approximately 100 amino acid residues.

The term "% identity", as used herein, is calculated as follows. The query sequence is aligned to the target sequence using the CLUSTAL W algorithm (Thompson *et al*, Nucleic Acids Research, 22:4673-4680 (1994)). A comparison is made over the window corresponding to the shortest of the aligned sequences. The amino acid residues at each position are compared, and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

The term "% similarity", as used herein, is calculated as described above for "% identity", with the exception that the hydrophobic residues Ala, Val, Phe, Pro, Leu, Ile, Trp, Met and Cys are similar; the basic residues Lys, Arg and His are similar; the acidic residues Glu and Asp are similar; and the hydrophilic, uncharged residues Gln, Asn, Ser, Thr and Tyr are similar. The remaining natural amino acid Gly is not similar to any other amino acid in this context.

Throughout this description, alternative embodiments according to the invention fulfill, instead of the specified percentage of identity, the corresponding percentage of similarity. Other alternative embodiments fulfill the specified percentage of identity as well as another, higher percentage of similarity, selected from the group of preferred percentages of identity for each sequence. For example, a sequence may be 70% similar to another sequence; or it may be 70% identical to another sequence; or it may be 70% identical and 90% similar to another sequence.

In a preferred fusion protein according to the invention, the **REP-CT** fragment has at least 70%, such as at least 80%, such as at least 85%, preferably at least 90%, such as at least 95%, identity to SEQ ID NO: 2 or to amino acid residues 18-277 of SEQ ID NO: 13.

In one preferred fusion protein according to the invention, the protein has at least 70%, such as at least 80%, such as at least 85%, preferably at

least 90%, such as at least 95%, identity to SEQ ID NO: 13. In a particularly preferred embodiment, the fusion protein according to the invention is SEQ ID NO: 13.

The cell scaffold material according to the invention comprises a protein or peptide according to the invention displaying the cyclic RGD cell-binding motif. The cyclic RGD cell-binding motif may be exposed from short synthetic peptides or longer synthetic or recombinant proteins, which may in turn be attached to or associated with a matrix or support.

The cell scaffold material preferably comprises a protein polymer, which protein polymer in turn is containing the recombinant fusion protein according to the invention as a repeating structural unit, i.e. the protein polymer contains or consists of a polymer of the recombinant fusion protein according to the invention. This implies that the protein polymer contains or consists of an ordered plurality of fusion proteins according to the invention, typically well above 100 fusion protein units, e.g. 1000 fusion protein units or more. In a preferred embodiment, the cell scaffold material according to the invention consists of the protein polymer.

The magnitude of fusion protein units in the polymer implies that the protein polymer obtains a significant size. In a preferred embodiment, the protein polymer has a size of at least 0.01 µm in at least two dimensions. Thus, the term "protein polymer" as used herein relates to fusion protein polymers having a thickness of at least 0.01 µm, preferably macroscopic polymers that are visible to the human eye, i.e. having a thickness of at least 1 µm. The term "protein polymer" does not encompass unstructured aggregates or precipitates. While monomers/dimers of the fusion protein are water soluble, it is understood that the protein polymers according to the invention are solid structures, i.e. not soluble in water. The protein polymers are comprising monomers of the recombinant fusion proteins according to the invention as a repeating structural unit.

The protein polymer according to the invention is typically provided in a physical form selected from the group consisting of fiber, film, coating, foam, net, fiber-mesh, sphere and capsule. According to one embodiment, it is

preferable that the protein polymer according to the invention is a fiber, film or fiber-mesh. According to certain embodiments, it is preferable that the protein polymer has a three-dimensional form, such as a foam or a fiber-mesh. One preferred embodiment involves thin (typically 0.01-0.1 µm thickness) coatings made of the protein polymer, which are useful for coating of stents and other medical devices. The term "foam" is comprising a porous foam with channels connecting the bubbles of the foam, sometimes to the extent that it can even be regarded as a three-dimensional net or mesh of fibers.

In a preferred embodiment, the protein polymer is in a physical form of a free-standing matrix, such as a free-standing film. This is highly useful as it allows for transfer of a cell sheet where needed, e.g. in an *in vivo* situation where cells need to be transferred as a cell sheet to e.g. a wound area.

The fiber, film or fiber-mesh typically has a thickness of at least 0.1 µm, preferably at least 1 µm. It is preferred that the fiber, film or fiber-mesh has a thickness in the range of 1-400 µm, preferably 60-120 µm. It is preferred that fibers have a length in the range of 0.5-300 cm, preferably 1-100 cm. Other preferred ranges are 0.5-30 cm and 1-20 cm. The fiber has the capacity to remain intact during physical manipulation, i.e. can be used for spinning, weaving, twisting, crocheting and similar procedures. The film is advantageous in that it is coherent and adheres to solid structures, e.g. the plastics in microtiter plates. This property of the film facilitates washing and regeneration procedures and is very useful for separation purposes.

The fusion protein according to the invention harbors both the desired cell-binding activity in the cyclic RGD cell-binding motif and an internal solid support activity in the **REP-CT** moieties, and these activities are employed in the cell scaffold material. The cell scaffold material provides a high and predictable density of the selective interaction activity towards an organic target. Losses of valuable protein moieties with selective interaction activity are minimized, since all expressed protein moieties are associated with the cell scaffold material.

The polymers which are formed from the fusion proteins according to the invention are solid structures and are useful for their physical properties, especially the useful combination of high strength, elasticity and light weight.

A particularly useful feature is that the **REP-CT** moieties of the fusion protein are biochemically robust and suitable for regeneration, e.g. with acid, base or chaotropic agents, and suitable for heat sterilization, e.g. autoclaving at 120°C for 20 min. The polymers are also useful for their ability to support cell adherence and growth.

The properties derived from the **REP-CT** moities are attractive in development of new materials for medical or technical purposes. In particular, the cell scaffold materials according to the invention are useful as scaffolds for cell immobilization, cell culture, cell differentiation, tissue engineering and guided cell regeneration. They are also useful in preparative and analytical separation procedures, such as chromatography, cell capture, selection and culture, active filters, and diagnostics. The cell scaffold materials according to the invention are also useful as in medical devices, such as implants and stents, e.g. as coatings.

In a preferred embodiment, the cell scaffold material comprises a protein polymer, which is consisting of a recombinant fusion protein according to the invention as a repeating structural unit. And in a further preferred embodiment, the cell scaffold material is a protein polymer, which is consisting of a recombinant fusion protein according to the invention as a repeating structural unit.

According to a further aspect, the present invention provides a method for the cultivation of cells, comprising the steps of

- providing a sample of cells;
- applying the sample to a cell scaffold material; and
- maintaining the cell scaffold material having the cells applied thereto under conditions suitable for cell culture;

wherein

the cell scaffold material comprises a protein polymer, which is containing a recombinant protein, such as recombinant fusion protein, according to the invention as a repeating structural unit.

In a preferred embodiment, the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface; and the cell-binding motif of the recombinant fusion protein has selectivity for $\alpha 5\beta 1$ integrins.

In preferred embodiments, the recombinant protein containing this cyclic RGD cell-binding motif is immobilized, such as to a solid support (i.e. not in solution), e.g. to the surface of a cell cultivation device or any type of surface where cell binding and growth is desirable. The resulting exposure of the thus immobilized cyclic RGD cell-binding motif surprisingly promotes integrin activation and cell binding to the immobilized recombinant protein containing this cyclic RGD cell-binding motif.

Recombinant fusion proteins containing this cyclic RGD cell-binding motif are particularly useful for the cultivation of cells displaying integrins on their cell surface, since the internal spidroin fragment allows the fusion protein to be brought into ordered polymers and thereby provides an internal solid support to the immobilized (i.e. not in solution) cell-binding motif. The resulting exposure of the immobilized cyclic RGD cell-binding motif surprisingly promotes integrin activation and cell binding to polymers of the recombinant fusion proteins.

Without limitation thereto, preferred cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines, in particular of human origin.

Without being limited thereto, the method is useful for cultivation of endothelial cells, human mesenchymal stem cells and keratinocytes, in particular of human origin. It is particularly useful for cultivation of keratinocytes.

The cell cultivation method may advantageously be performed both *in vitro* and *in vivo*.

The present invention will in the following be further illustrated by the following non-limiting examples.

Examples

Statistics

One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 6.05 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Example 1 - Genetic incorporation of fibronectin-derived cell-binding motifs into recombinant spider silk

The recombinant spider silk protein 4RepCT (SEQ ID NO: 2, herein denoted WT) was genetically functionalized with the RGD containing cell binding motif from the fibronectin type III module 10, in four slightly different versions (Fig. 1). In the first (FN_{CC}-4RepCT; SEQ ID NO: 13), two amino acids flanking the RGD sequence were substituted for cysteines to enable loop formation of the motif (CTGRGDSPAC; SEQ ID NO: 10). In the second (FN_{SS}-4RepCT; SEQ ID NO: 14), the introduced cysteines were substituted for serines to create a linear control (STGRGDSPAS; SEQ ID NO: 11). Here the amino acid serine was selected due to its resemblance to cysteine, while lacking the ability to form disulfide bonds. In the third (FN_{VS}-4RepCT; SEQ ID NO: 15), the original sequence of the fibronectin motif (VTGRGDSPAS; SEQ ID NO: 9) was used as a linear, native control. In the fourth (RGD-4RepCT; SEQ ID NO: 16), the RGD containing peptide (SEQ ID NO 12) used in Widhe M *et al.*, Biomaterials 34(33): 8223-8234 (2013) was used as a further linear control.

The genes encoding the functionalized variants (FN_{CC}-4RepCT DNA - SEQ ID NO: 17; FN_{SS}-4RepCT DNA - SEQ ID NO: 18; FN_{VS}-4RepCT DNA - SEQ ID NO: 19; and RGD-4RepCT DNA - SEQ ID NO: 20) were made by cloning of oligos encoding the different motifs into the vector encoding 4RepCT (4RepCT DNA -SEQ ID NO: 1) and using restriction enzymes. The new sequences were introduced N-terminally to 4RepCT and confirmed by sequencing.

Example 2 - Expression of fusion proteins containing fibronectin-derived cell-binding motifs

Protein production in *E. coli* of the genetic constructs obtained in Example 1 and the following purification were done essentially as described in Hedhammar M *et al.*, Biochemistry 47(11):3407-3417 (2008) and Hedhammar M *et al.*, Biomacromolecules 11: 953-959 (2010).

Briefly, *Escherichia coli* BL21(DE3) cells (Merck Biosciences) with the expression vector for the target protein were grown at 30°C in Luria-Bertani medium containing kanamycin to an OD_{600} of 0.8-1 and then induced with isopropyl β-D-thiogalactopyranoside and further incubated for at least 2 h. Thereafter, cells were harvested and resuspended in 20 mM Tris-HCl (pH 8.0) supplemented with lysozyme and DNase I. After complete lysis, the supernatants from centrifugation at 15,000 *g* were loaded onto a column packed with Ni Sepharose (GE Healthcare, Uppsala, Sweden). The column was washed extensively before elution of bound proteins with 300 mM imidazole. Fractions containing the target proteins were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0). The target protein was released from the tags by proteolytic cleavage. To remove the released HisTrxHis tag, the cleavage mixture was loaded onto a second Ni Sepharose column and the flowthrough was collected. The protein content was determined from the absorbance at 280 nm.

The protein solutions obtained were purified from lipopolysaccharides (lps) as described in Hedhammar *et al.*, Biomacromolecules 11:953–959 (2010). The protein solutions were sterile filtered (0.22 µm) before being used to prepare scaffolds (film, foam, coatings or fibers).

The recombinant spider silk proteins were successfully expressed in *E coli* and purified with similar yield and purity as the original 4RepCT (WT; SEQ ID NO: 2).

Example 3 - Fabrication of cell culture matrices

After purification, the protein solutions obtained in Example 2 were filter sterilized (0.22 µm) and concentrated by centrifugal filtration (Amicon Ultra, Millipore) before preparation of films, as described in Widhe M *et al.*,

Biomaterials 31(36): 9575-9585 (2010) and Widhe M *et al.*, Biomaterials 34(33): 8223-8234 (2013).

Briefly, petri dishes were coated at room temperature with recombinant spider silk solution at a concentration of 0.3 mg/ml to generate films. Foams were made by rapid pipetting of the silk solution, and fibers were formed by gentle wagging in 15 ml tube followed by cutting into smaller pieces.

For studies of early attachment and repopulation, solutions of a protein concentration of 0.3 mg/ml were casted into films in 96- and 24 well cell culture plates respectively (Sarstedt, suspension cells) precoated with 1% pluronic to limit cell adhesion to the plastic surface. In control experiments, a reducing agent (either 5 mM Dithiothreitol, 20 mM β-mercaptoethanol or 10 mM Tris(2-carboxyethyl)phosphine HCl) were added to the protein solutions directly before films were prepared.

For microscopic studies, the proteins were casted as films in chamber glass slides (LabTekII). For Alamar blue experiments, where whole well coverage is desired, the cell culture wells were coated with a covering protein solution of 0.3 mg/ml for 2 h before the liquid was removed. Films and coated surfaces were allowed to dry over night at 25°C and 30% rh under sterile conditions, then washed twice with sterile 20 mM phosphate buffer, pH 7.4, and pre-incubated with complete cell culture medium for 1 h at 37°C with 5% CO_2 before cell seeding.

Free-standing films were prepared by applying of a droplet of protein solution (3 mg/ml) onto a ~3 mm wide frame of metal wire hanging hooked up in a well of a 96-well plate and allowed to dry over night at 25°C and 30% rh under sterile conditions.

The control Bovine Fibronectin (Sigma-Aldrich F1137) was coated at recommended concentration (5 µg/cm^2) overnight at 37°C.

It was observed that a spider silk protein functionalized with a disulfide-looped RGD motif self-assembles into stable matrices. As shown by the micrographs in Fig. 2a, the FN_{CC}-4RepCT (SEQ ID NO: 13) protein could be presented as matrices in the format of fiber (upper), film (middle) and free standing film (lower). Scale bars in Fig. 2a indicate 500 µm (upper & middle) and 1000 µm (lower). Surprisingly, the FN_{CC}-4RepCT protein could form

fibers, film and foam with appeared higher stability and integrity than noted for linear RGD silk proteins (RGD-4RepCT, SEQ ID NO: 16) and WT silk proteins (4RepCT, SEQ ID NO: 2). With the FN_{CC}-4RepCT protein, it was even possible to form free-standing films. The smooth film formats (casted and free-standing) were used in the subsequent cell adhesion experiments to rule out the effects of matrix morphology.

Example 4 - Structural analysis of matrices

Fourier Transform Infrared Spectroscopy (FTIR) spectra of the fibers, casted films and free-standing films obtained in Example 3 were recorded on a FTIR spectrometer (Bruker). The films were placed on a crystal for measuring IR spectra by attenuated total reflection. For each spectrum 100 scans were averaged. The amide I region was further analyzed to compare the peak height of α-helical (1654 cm^{-1}) and β-sheet (1629 cm^{-1}) structures, respectively.

Fig. 2b shows FTIR spectra of FN_{CC}-4RepCT (SEQ ID NO: 13) silk matrices in the format of fiber (upper), film (middle) and free standing film (lower). Peaks for typical signal of α-helix and β-sheet respectively are indicated by lines. Interestingly, the FTIR data in Fig. 2b show that the free-standing films have, oppositely to the casted films, completely converted to β-sheet structure.

Example 5 - Cell culture

Human dermal microvascular endothelial cells (EC), (HDMEC, PromoCell GmbH, Germany) isolated from dermis from adult donor were grown in culture flasks coated with gelatin (Sigma Aldrich) in complete endothelial cell media MV, containing 5% fetal bovine serum (PromoCell GmbH, Germany).

Human mesenchymal stem cells (hMSC, Gibco) from bone marrow were grown in culture flasks coated with CELLstart (Gibco) in complete StemPro MSC serum free medium CTS (Gibco) containing 25 ng/µl fibroblast growth factor β (Gibco) and 2 mM Glutamax (Gibco).

Normal human epidermal keratinocytes from adult skin (NHEK-ad) were purchased from Lonza. Subculture, proliferation and migration experiments were done in KGM-Gold (Lonza), containing bovine pituitary extract, whereas adhesion experiments were performed in KGM-CD (chemically defined), supplemented with $CaCl_2$ to give 1.2 mM Ca^{2+}.

Keratinocyte and mesenchymal stem cell cultures, as well as experiments, were performed under serum-free conditions to avoid possible interactions between the matrices and serum proteins that potentially could give rise to increased cell adherence.

Medium was changed every 2-3 days. Cells were harvested with TrypLE (Life Technologies) when reaching a confluency of 80% for subculture or experiments. All experiments were performed at 37 °C with 5% CO2 and 95% humidity.

Example 6 - Effect of matrices on early attachment of adherent cells

A. Early attachment Assay

Cells were harvested at passage 3-8, seeded at 20 000/cm^2 and allowed to adhere to the films or controls for 1 h in a cell incubator before gentle washing twice with pre-warmed phosphate buffered saline (PBS) followed by 10 min fixation with 96% ethanol. After three washings in water, cells were stained for 30 min with 0.1% Crystal Violet in H_2O. Plates were dried after extensive washing in water.

Attachment and morphology of cells bound to the films obtained in Example 3 were documented by taking micrographs at 2x and 10x magnification in an inverted bright field microscope. The color was then dissolved in 40 µL 20% acetic acid for 10 min, and 35 µL of the solution was transferred to a 384-well plate for optical density measurement at 595 nm (TECAN Infinite M200). Wells with cells fixed without pre-washing was used as positive control. Wells with no cells were used as blank. Experiments were run in hexaplicates and repeated three times.

Determination of cell coverage area within a defined region (9.12 mm^2) of the micrographs (at 2x magnification) was done using the software NIS elements BR (Nikon).

B. Cellular stainings

Cells were harvested at passage 3-8, seeded at 3500/cm^2 and allowed to adhere onto films for 20 min, 1 or 3 hours in chamber slides. After gentle washing, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 1% bovine serum albumin (BSA, AppliChem) in PBS.

Primary antibody were used at the following concentrations in 1% BSA: mouse anti human vinculin (Sigma V9131) at 9.5 µg/ml, mouse-anti human beta1-integrin (activated conformation, clone HUTS-4) at 3.3 µg/mL, or mouse-anti human alpha5-integrin (ligand bound conformation, clone SNAKA-51) at 2.5 µg/mL, both Millipore.

Secondary antibody was AlexaFlour488 goat anti mouse IgG (H+L), cross adsorbed (Invitrogen), used at 1:500. Phalloidin-AlexaFluor594 (Life Technologies) were used at 1:40 to detect filamentous actin. DAPI was used for nuclear staining. Slides were mounted in Fluorescence mounting medium (Dako, Copenhagen).

The stained cells were analyzed using an inverted microscope (Nikon Eclipse Ti) at 4x and 10x magnification. Excitation at 563/45 nm and detection at 625/50 nm was used for red fluorescence, whereas excitation with 387/11 nm and detection at 447/60 nm was used to monitor blue fluorescence.

For microscopic analysis of cell adhesion (formation of focal adhesions and stress fibers), a confocal microscope was used (Carl Zeiss LSM 710) at 10x and 63x magnification.

Presence of stress fibers were defined as strongly stained prominent and thick f-actin filaments, and graded from 0-4, where 0=none, 1=few-some, 2=many, 3=most, and 4=all cells exhibit stress fibers.

Presence of focal adhesions were estimated as percent of cells exhibiting focal adhesions. Quality of focal adhesions were graded from 1-4 regarding presence of small and dim focal adhesion (=1p), small and distinct

focal adhesions (=2p), abundant focal adhesions (=3p) and large and bright focal adhesions (=4p), and multiplied with the portion of positive cells expressing this specific type of focal adhesions (0-4, where 0≈none of, 1≈one fourth of, 2≈half of, 3≈three fourths of, and 4≈all of the focal adhesion-positive cells.)

C. FN_{CC}-silk promotes early attachment of adherent cells

First, we wanted to investigate how well adherent cells attach and spread on the FN_{CC}-silk (FN_{CC}-4RepCT, SEQ ID NO: 13) compared to linear RGD proteins (RGD-4RepCT, SEQ ID NO: 16) and WT silk proteins (4RepCT, SEQ ID NO: 2) obtained in Example 3. Silk films of the three different variants were prepared in cell culture plates, and human primary endothelial cells (EC), mesenchymal stem cells (MSC) or keratinocytes (KC) were allowed to adhere for 1 h before fixation and staining.

Fig. 3a shows micrographs of EC, MSC and KC after 1 h adhesion to a film of WT silk (SEQ ID NO: 2) or silk functionalized with RGD (SEQ ID NO: 16) or FN_{CC} (SEQ ID NO: 13), followed by staining with crystal violet (10x magnification). Scale bar 50 µm.

Fig. 3b shows the OD of crystal violet dissolved from cells adhered to different silk variants for EC (upper panel), and MSC (middle panel), and cell coverage area by KC (lower panel) within a defined region (9.12 mm^2). EC and MSC: triplicates or duplicates, KC: quadruplicates. All cell types n=3. Seeding density 20 000/cm2. Boxplot: line= median, box: 25%-75%, whiskers= mean and max. Statistics: * P<0.05, ** P<0.01, **** P<0.0001.

From the micrographs shown in Fig. 3a, a clear improvement of attachment is seen on the FN_{CC} films compared to both RGD and WT for all three cell types. After imaging, the color trapped by the EC and MSC respectively was dissolved and OD was captured and used as a measure of the number of bound cells (Fig. 3b, upper and middle panel). For both cell types significantly more cells had bound to the FN_{CC}-silk after 1 h compared to WT silk (P<0.01 for EC and P<0.05 for MSC). Significantly more EC had attached to FN_{CC} also compared to RGD silk (p<0.01). This colorimetric method was less suitable for KC since with this cell type, some cells also

attached outside the film surface, thus contributing to the OD value although not bound to the silk film. Instead, the area of cells bound to the film was measured by image analysis at 2x magnification, as shown in figure 3b (lower panel). The area of KC bound to FN_{CC} was significantly larger than on both WT - and RGD -silk ($P<0.0001$).

D. Primary KC adhere equally well to FN_{CC}-silk and bovine fibronectin

After seeing this positive effect of the introduced FN_{CC} motif, we wanted to find out how well the FNCC-silk would compare to native, full length fibronectin, where the RGD is presented on a turn loop constrained by the structure. We therefore used fibronectin from bovine plasma (BFN) to coat cell culture wells, as well as naked cell culture treated plastic (TCT), on which KC can be cultured, as a control. Serum-free experimental conditions was chosen to avoid possible interactions between the matrices and serum proteins that potentially could give rise to increased cell adherence.

The results for KC after 1 h adhesion to either silk functionalized with FN_{CC}, a bovine fibronectin coated surface (BFN) or tissue culture treated cell plastic (TCT) are presented in Fig. 4. Fig. 4a shows micrographs at 10x magnification after staining with crystal violet. Seeding density 40 000/cm^2. Scale bar 50 µm. Fig. 4b shows cell coverage area within a defined region (9.12 mm^2), (Quadruplicates, n=3). Seeding density 20 000/cm2. Boxplot: line= median, box: 25%-75%, whiskers= mean and max. Statistics (vs TCT): **** $P<0.0001$.

When comparing cell coverage area, it was evident that KC bound equally well to the BFN and the FN_{CC}-silk after 1 h adherence, and importantly both significantly better than TCT ($P<0.0001$) (Fig. 4).

Example 7 - Impact of a cysteine-looped conformation for RGD presentation in FN_{CC}-silk

Encouraged by these results, we wanted to go further and look into the mechanism by which FN_{CC}-silk (SEQ ID NO: 13) creates an attractive surface for the cells. For this purpose, we used two FN-silk variants where a linear RGD presentation is expected (Fig. 1a). The first variant (FN_{VS}; SEQ ID NO:

15), contains the original sequence of the RGD-containing motif in fibronectin, to show the effect of the native flanking amino acids without influence of the loop conformation. In the second variant (FN_{SS}; SEQ ID NO: 14), the two flanking cysteines in FN_{CC} were substituted for serine, which resembles cysteine but lacks the –SH-group and is therefore unable to form disulfide bridges. The different FN-silk variants, as well as RGD-silk (SEQ ID NO: 16) and WT-silk (SEQ ID NO: 2), were evaluated with primary KC. Cells were analyzed both for early attachment (Fig. 5), spreading and formation of stress fibers (Fig. 6), and focal adhesions (Fig. 7). Early attachment assay and cellular stainings were performed as detailed in Example 6.

A. Early attachment

The results for KC after 1 h adhesion to films of WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16) are presented in Fig. 5. Fig. 5a shows micrographs at 10x magnification after staining with crystal violet. Seeding density 20 000/cm^2. Fig. 5b shows cell coverage area within a defined region (9.12 mm^2), (Quadruplicates, n=3). Boxplot: line= median, box: 25%-75%, whiskers= mean and max. Statistics: **** P<0.0001.

In initial experiments, KC were allowed to adhere for 1 h onto films of WT, RGD- and FN-silk variants, and stained with crystal violet for detection and morphology (Fig. 5a). When pooling data from image analysis of 3 experiments (hexaplicates), FN_{CC}-silk showed increased attachment (i.e. area covered by cells) compared to both FN_{SS} and FN_{VS} (P<0.0001, Fig. 5b). FN_{CC}-silk also gave significantly higher adhesion of KC compared to RGD-silk (P<0.0001). All FN-silk variants showed significantly increased adhesion compared to WT-silk (P<0.0001).

Moreover, pooled data from 8 experiments, where the crystal violet was dissolved from the cells and the OD thereof measured in a plate reader, showed very similar results (FNCC versus FN-controls, P<0.0001), despite that cells in these experiments to some degree also adhered to the cell plastic outside the silk-films (data not shown).

B. Cell spreading and formation of stress-fibers

The results for KC after 3 h adhesion to films of WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16) are presented in Fig. 6. Fig. 6a shows cell coverage area, duplicates, n=4. Boxplot: line= median, box: 25%-75%, whiskers= mean and max. Fig. 6b shows stress fiber ranking (mean and standard deviation, single wells, n=3). Seeding density 3 500/cm^2. Statistics: ***P<0.001, *P<0.05.

By staining for F-actin, cell spreading and formation of stress fibers in KC after 3 h adhesion were investigated (Fig. 6). The results show that FN_{CC} film, but not FN_{SS} or FN_{VS} films, gave a significantly increased spreading of KC compared to RGD-film ($p<0.05$) and WT-film ($p<0.001$), when measuring total cell area in 4x micrographs (n=4, duplicates), (Fig. 6a). The spreading of KC on FN_{CC}-silk was also significantly increased compared to FN_{SS}-silk ($p<0.05$). The KC on RGD, FN_{SS}- and FN_{VS}-silk showed a higher proportion of cells with a rounded appearance, whereas on FN_{CC}-silk most cells had a nice spread-out morphology with distinct actin filaments.

KC stained for F-actin were also analyzed for the presence of stress fibers, as an indicator of established attachment (Fig. 6b). Presence of stress fibers was defined as thick and brightly stained actin filaments (bundles), and the analysis was done by inspection at 63x magnification (n=3). This analysis showed similar results as the area measurement, but no statistically significant differences were found.

C. Formation of focal adhesions

Formation of focal adhesions within the cells was analyzed after 3 h by staining for F-actin in combination with vinculin, which is one of the major components of the focal adhesion complex. Co-staining of F-actin and vinculin is thus a sign of integrin-involved, well established binding of cells to the underlying substrate. Focal adhesions appear as yellow-greenish elongations of the F-actin filaments, often situated close to the cell membrane.

The results from the analysis of formation and characterization of focal adhesions in KC after adherence for 3 h onto films of WT-silk (SEQ ID NO: 2) or silk functionalized with FN_{CC} (SEQ ID NO: 13), FN_{VS} (SEQ ID NO: 15), FN_{SS} (SEQ ID NO: 14) or RGD (SEQ ID NO: 16) is presented in Fig. 7. Slides were scanned with a confocal microscope at 10x for overview (Fig. 7a) and at 63x for details (Fig. 7b). Two types of grading of the focal adhesions in the cells were performed.

Firstly, the percentage of cells exhibiting focal adhesions was assessed by visual examination of the entire film in each well at 10x magnification. Pooled data from three experiments showed a significant increase in percentage of cells expressing focal adhesions on FN_{CC}-silk, compared to RGD and WT ($p<0.05$), (Fig. 7a). Fig. 7a is a graph showing percentage of cells exhibiting focal adhesions (mean and standard deviation). Experiments were run in duplicates, n=3.Statistics: * $P<0.05$.

Secondly, since not only the abundance of cells exhibiting focal adhesions, but also the characteristics of the focal adhesions seen in the cells appeared to differ between the different silk variants, we decided to examine this further. The appearance of the focal adhesions within each positive cell was therefore evaluated according to a grading system. Briefly, grading spanned from small and dim, appearing sparsely within the cell (“subtle”), to large and bright, appearing abundantly within the cell (“prominent”). In this way we could judge the quality of the focal adhesions independently of how many of the cells on the film that exhibited these structures. The outcome of this analysis showed a tendency of more prominent focal adhesions in cells attached to FN_{CC}-silk compared to the other silk types. Fig 7b is a graph showing grading of the focal adhesions, independently of total number of positive cells found, (mean and standard deviation). Grading was done in single wells, n=3.

The results show that the variation of focal adhesion quality is larger in cells on RGD, FN_{SS} and FN_{VS} than on FN_{CC}-silk, reflecting the presence of both prominent and subtle focal adhesions in cells on FN_{SS} and FN_{VS}, but almost only prominent focal adhesions on FN_{CC}-silk. Interestingly, such

prominent focal adhesions appeared as early as 20 minutes after seeding onto FN_{CC}.

In each individual experiment, FN_{CC}-silk, without exception, gave the most efficient adhesion of the tested films. In contrast, the attachment onto FN_{SS}- and FN_{VS}-silk varied from being similar to RGD-silk to being only somewhat lower than on FN_{CC}-silk.

With the aim to further elucidate the role of the cysteine linked loop for presentation of the RGD motif, we performed experiments where reducing agents were added to the FN_{CC}-silk solution directly before films were casted. The idea was to prevent disulphide formation in these films, generating a linear, non-looped motif. However, no differences compared to non-reduced FN_{CC} film were detected. When considering that the films are completely dried during the production process, one can assume that the reducing agent, in the lack of buffer, can no longer prevent disulfide formation to occur. We therefore consider FN_{SS} the most proper non-looped control accomplishable.

Example 8 - Engagement of integrin α5β1 in KC adhering to FN_{CC}-silk

Since the integrin α5β1 is known to selectively bind to fibronectin, we decided to investigate if this integrin is involved in the binding of KC to FN_{CC}-silk (SEQ ID NO: 13). To do this, we selected two monoclonal antibodies, developed to specifically recognize the ligand bound conformation of α5 integrin (SNAKA-51) and the activated conformation of β1 integrin (HUTS-4), respectively, and used them in combination for staining of KC adhering to FN_{CC}-silk for 3 h, in combination with staining with phalloidin for F-actin, as set out in Examples 6-7. Analysis of the cells revealed a week but distinct staining pattern resembling the pattern seen when staining for vinculin.

Example 9 - Applications of FN_{CC}-silk

Intrigued by the findings of such excellent binding properties of FN_{CC}-silk regarding early attachment of adherent cells, we performed a few pilot studies to get a picture of its ability to support various cell culture applications. Firstly, we wanted to evaluate the effect of the FN_{CC} motif on cell proliferation.

A. Cell viability analysis with Alamar blue

Cell growth of primary keratinocytes (NHEK) after initial low seeding density 3 500 cells/cm^2 on wells coated with films of WT-silk (SEQ ID NO: 2) or FN_{CC}-silk (SEQ ID NO: 13) in 96-well plates was monitored with Alamar Blue cell viability assay (Molecular Probes) every third day during the culture period. After 4 h incubation with Alamar blue (diluted 1:10 in cell culture medium), fluorescence intensity of 90 µL supernatants from the cultures was measured with a fluorescence plate reader (CLARIOstar, BMG Labtech) using excitation at 544 nm and emission at 595 nm. Two independent experiments were performed where films were analysed in hexaplicates. Fluorescence intensities, correlating the number of living cells in each well, were plotted over time to yield growth profiles of cells seeded on silk with different cell binding motifs. The results presented in Fig. 8 show an increased level of viable cells on FN_{CC}-silk compared to WT-silk ($P<0.001$ day 3 and $P<0.0001$ day 6; ****$P<0.0001$, *** $P<0.001$), indicating an improved ability to support cell proliferation conveyed by the FN_{CC} motif.

B. Repopulation Assay

To evaluate the ability of the different silk variants to support repopulation of an open would field, dermal keratinocytes (NHEK) were stained with Oregon green cell trace (Life Technologies) before seeded onto films of FN_{CC}-silk (SEQ ID NO: 13) and WT-silk (SEQ ID NO: 2) at 20 000 cells/cm^2 in 24 well plates. Wound field inserts (CytoSelectTM Wound healing assay, Cell Biolabs) were added into the wells before cell seeding to generate a 0.9 mm wide open would field in the cell monolayer, while keeping the film intact. After 16 h the inserts were removed, and the repopulation process were followed each day and documented by inverted fluorescence microscopy at day 0 (insert removal), day 2 and 4. At day 6 cells were fixed and stained according to the assay protocol, and imaged by inverted bright field microscopy.

Thus, green-traced cells were seeded at high density into wells with inserts preventing cells to reach a defined part of the silk-film, the “wound field”. After monolayer formation outside the wound field, the insert was

removed, and repopulation of the gap was documented during 6 days of culture, thus allowing both migration and proliferation of cells. Keratinocytes efficiently repopulated the wound field on FN_{CC}-silk, which was almost completely covered with cells at the end of the experiment.

C. Transferable cell monolayers

NHEK were harvested and traced with AMCA orange cell tracker (Life Technologies) before seeded onto free-standing films of FN_{CC}-silk silk (SEQ ID NO: 13) mounted on metal frames at 20 000 cells/cm2. The formed monolayer was documented by inverted fluorescence microscopy.

Primary keratinocytes seeded onto such free-standing films formed a monolayer that could easily be transferred between culture wells.

Example 10 - Cell adherence to surfaces with immobilized peptides

A silicon (SiO) surface is activated using an organosilane (e.g. 3-aminopropyltriethoxysilane APTES) to thereafter immobilize aminoreactive peptides (via their N-terminus) using e.g. EDC/NHS chemistry.

The peptides used for immobilization are designed with a glycine spacer, as follows:

1. GGGGGCTGRGDSPAC (SEQ ID NO: 21)
2. GGGGGVTGRGDSPAS (SEQ ID NO: 22)
3. GGGGGSTGRGDSPAS (SEQ ID NO: 23)
4. GGGGGCDWRGDNQFC (SEQ ID NO: 24)

Early attachment to the surfaces with immobilized peptides is analyzed using human keratinocytes (HaCAT) seeded at 20 000/cm^2. The cells are then allowed to adhere for 1 h in a cell incubator before gentle washing twice with pre-warmed phosphate buffered saline (PBS) followed by 10 min fixation with 96% ethanol. After three washings in water, cells are stained for 30 min with 0.1% Crystal Violet in H_2O.

Attachment and morphology of cells are documented by taking micrographs at 2x and 10x magnification in an inverted bright field microscope. The Crystal violet color is then dissolved in 40 µL 20% acetic

acid for 10 min, and 35 µL of the solution is transferred to a 384-well plate for optical density measurement at 595 nm (TECAN Infinite M200). Cells fixed without pre-washing are used as positive control (reference).

Example 11 - Cell culture on FNcc silk matrices

After purification, solutions of FNcc-silk protein (SEQ ID NO: 13) were used to coat cell culture plates (Sarstedt, hydrophobic plates for suspension cells). Briefly, the protein solutions were diluted to 0.1 mg/ml in Tris buffer, and allowed to incubate at room temperature for 30 minutes before removal and wash.

Cells were harvested using trypsination (TrpLE) and seeded onto the FNcc-silk coatings at suitable cell density (3-10 000 cells/cm2). Cell growth was monitored with Alamar Blue cell viability assay (Molecular Probes) regularly (every 2-3 day). At the end point, after 7-14 days, Live/dead staining was performed. The following cell types showed positive growth profile and a majority (>80%) of viable cells at the end point:

Human Skeletal Muscle Satellite Cells
Human Dermal Microvascular Endothelial Cells
Human Mesenchymal stem cells
Mouse Mesenchymal stem cells
Human Dermal fibroblasts
HaCaT Keratinocytes
MIN6-m9 pancreatic cell line

CLAIMS

1. A recombinant fusion protein comprising a spidroin fragment and a cell-binding motif with selectivity for integrins, wherein the cell-binding motif is comprising the amino acid sequence

$$C^1X^1X^2RGDX^3X^4X^5C^2$$

wherein

X^1 is S or T;

X^2 is G, A or V;

X^3 is S or T;

X^4 is G, A, V or P; and

X^5 is G, A or V; and

C^1 and C^2 are connected via a disulphide bond;

and wherein the spidroin fragment is comprising the protein moieties **REP** and **CT**, wherein

REP is a repetitive fragment of from 70 to 300 amino acid residues, selected from the group consisting of **L(AG)$_n$L**, **L(AG)$_n$AL**, **L(GA)$_n$L**, and **L(GA)$_n$GL**, wherein

n is an integer from 2 to 10;

each individual **A** segment is an amino acid sequence of from 8 to 18 amino acid residues, wherein from 0 to 3 of the amino acid residues are not Ala, and the remaining amino acid residues are Ala;

each individual **G** segment is an amino acid sequence of from 12 to 30 amino acid residues, wherein at least 40% of the amino acid residues are Gly; and

each individual **L** segment is a linker amino acid sequence of from 0 to 30 amino acid residues; and

CT is a fragment of from 70 to 120 amino acid residues, having at least 70% identity to SEQ ID NO: 3.

2. A recombinant fusion protein according to claim 1, wherein X^2 is G or A.

3. A recombinant fusion protein according to claim 2, wherein X^2 is G.

4. A recombinant fusion protein according to any preceding claim, wherein X^3 is S.

5. A recombinant fusion protein according to any preceding claim, wherein X^4 is G or P.

6. A recombinant fusion protein according to claim 5, wherein X^4 is P.

7. A recombinant fusion protein according to any preceding claim, wherein X^5 is G or A.

8. A recombinant fusion protein according to claim 7, wherein X^5 is A.

9. A recombinant fusion protein according to claim 1 wherein the cell-binding motif is comprising the amino acid sequence CTGRGDSPAC (SEQ ID NO: 10).

10. A recombinant fusion protein according to any preceding claim, wherein the cell-binding motif has selectivity for for $\alpha 5\beta 1$ integrins.

11. A cell scaffold material comprising a protein polymer which as a repeating unit is containing a recombinant fusion protein according to any one of claims 1-10.

12. A cell scaffold material according to claim 11, wherein the protein polymer is in a physical form selected from the group consisting of film, coating, foam, fiber and fiber-mesh.

13. A cell scaffold material according to any one of claims 11-12, wherein the protein polymer is in a physical form of a free-standing matrix.

14. A method for the cultivation of cells, comprising the steps of
- providing a sample of cells;
- applying the sample to a cell scaffold material; and
- maintaining the cell scaffold material having the cells applied thereto under conditions suitable for cell culture;
wherein
the cell scaffold material comprises a protein polymer, which is containing a recombinant fusion protein according to any one of claims 1-10 as a repeating structural unit.

15. A method according to claim 14, wherein the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface; and wherein the cell-binding motif of the recombinant fusion protein has selectivity for $\alpha 5\beta 1$ integrins.

16. A method according to any one of claims 14-15, wherein the cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

17. Use of an immobilized recombinant fusion protein comprising the amino acid sequence

$$C^1X^1X^2RGDX^3X^4X^5C^2$$

wherein
X^1 is S or T;
X^2 is G, A or V;
X^3 is S or T;
X^4 is G, A, V or P; and
X^5 is G, A or V; and
C^1 and C^2 are connected via a disulphide bond;
for the cultivation of cells displaying integrins on their cell surface.

18. Use of the recombinant fusion protein according to any one of claims 1-10, or the cell scaffold material according to any one of claims 11-13 for the cultivation of cells displaying integrins on their cell surface.

19. Use according to any one of claims 17-18, wherein the cell-binding motif of the cell scaffold material or the recombinant protein has selectivity for $\alpha 5\beta 1$ integrins; and wherein the cells are displaying $\alpha 5\beta 1$ integrins on their cell surface.

20. Use according to any one of claims 17-19, wherein the cells are selected from skeletal muscle cells, endothelial cells, stem cells, fibroblasts, keratinocytes and cell lines.

Fig 1

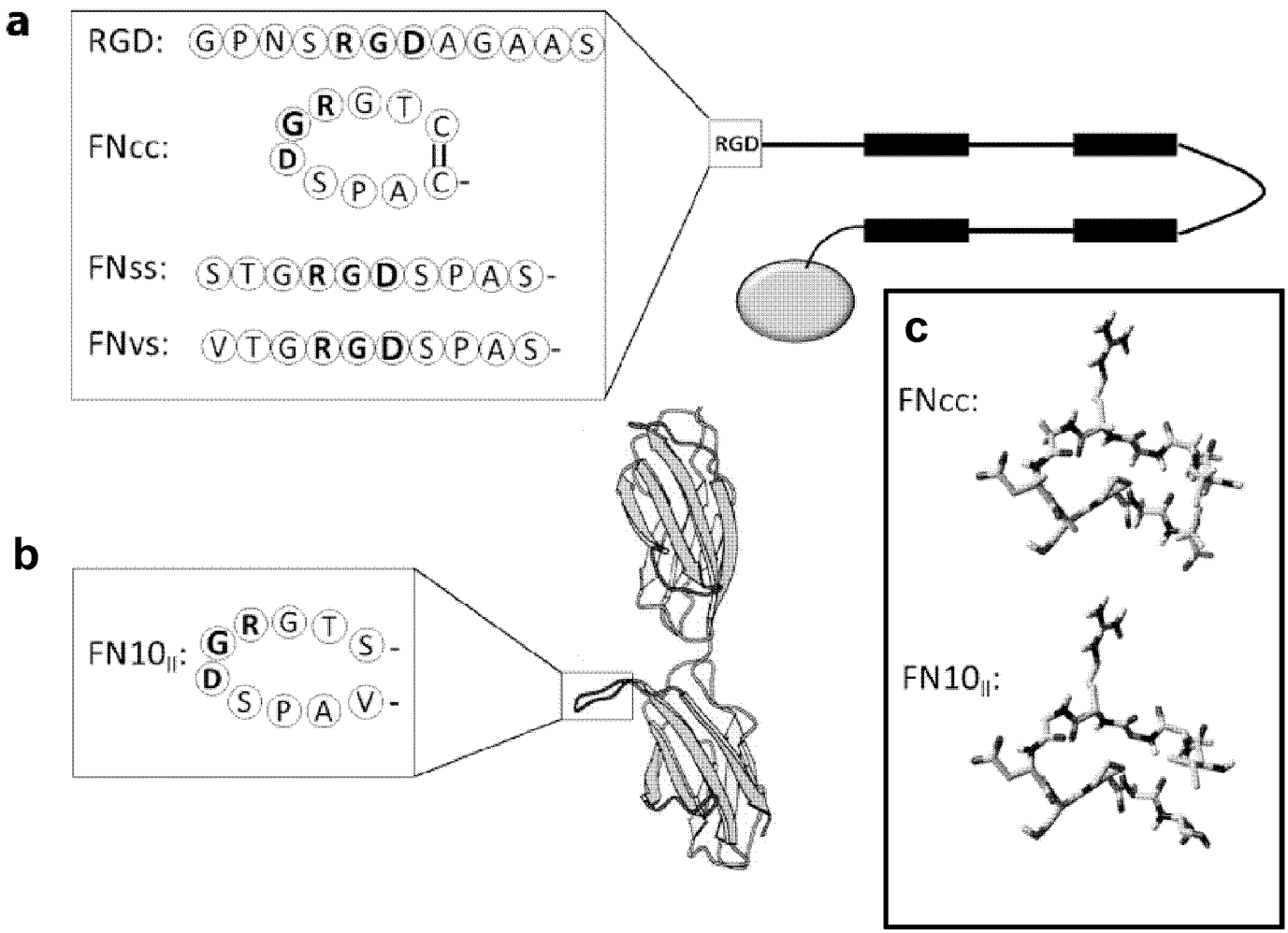


Fig 2

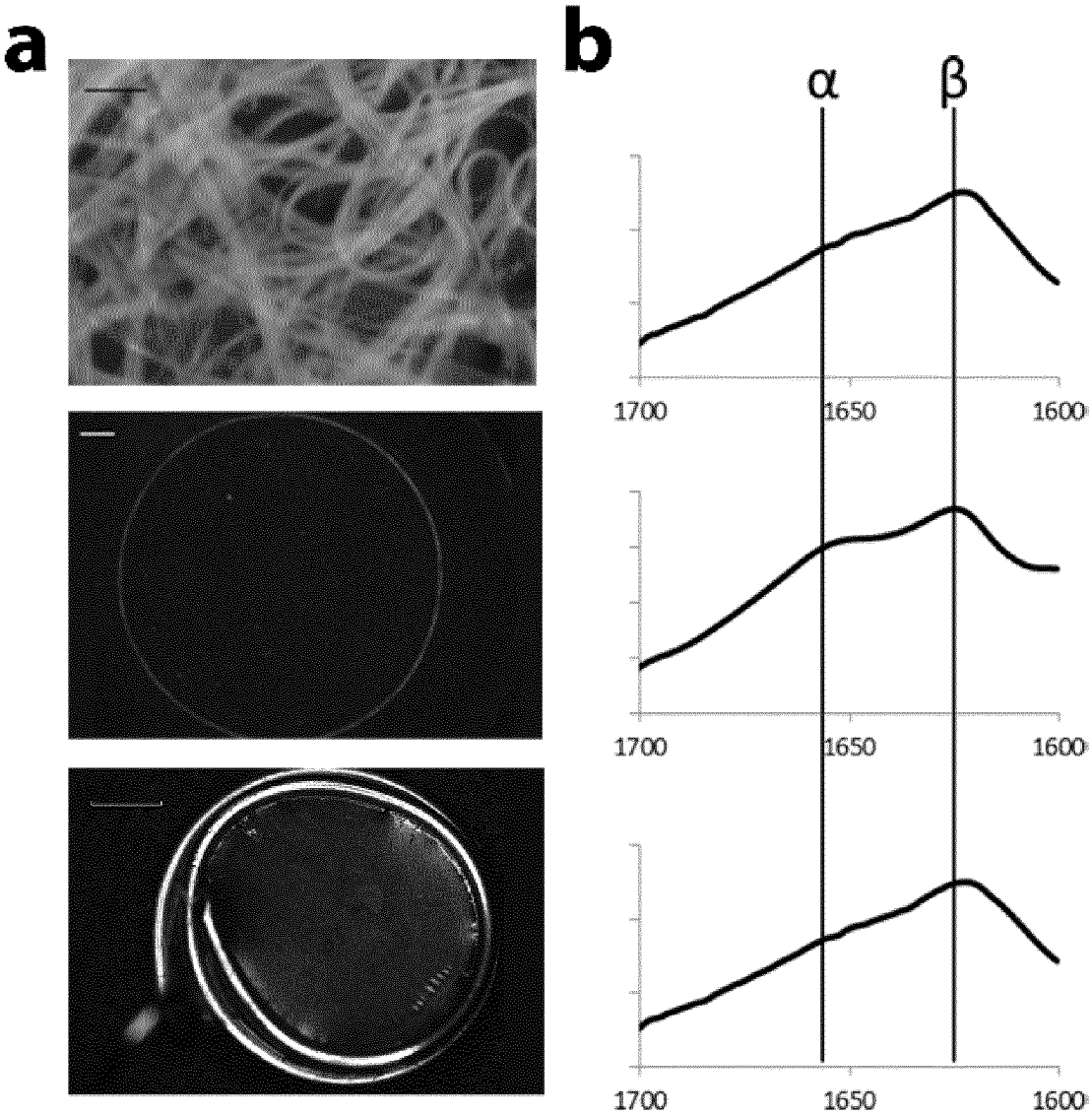


Fig 3a

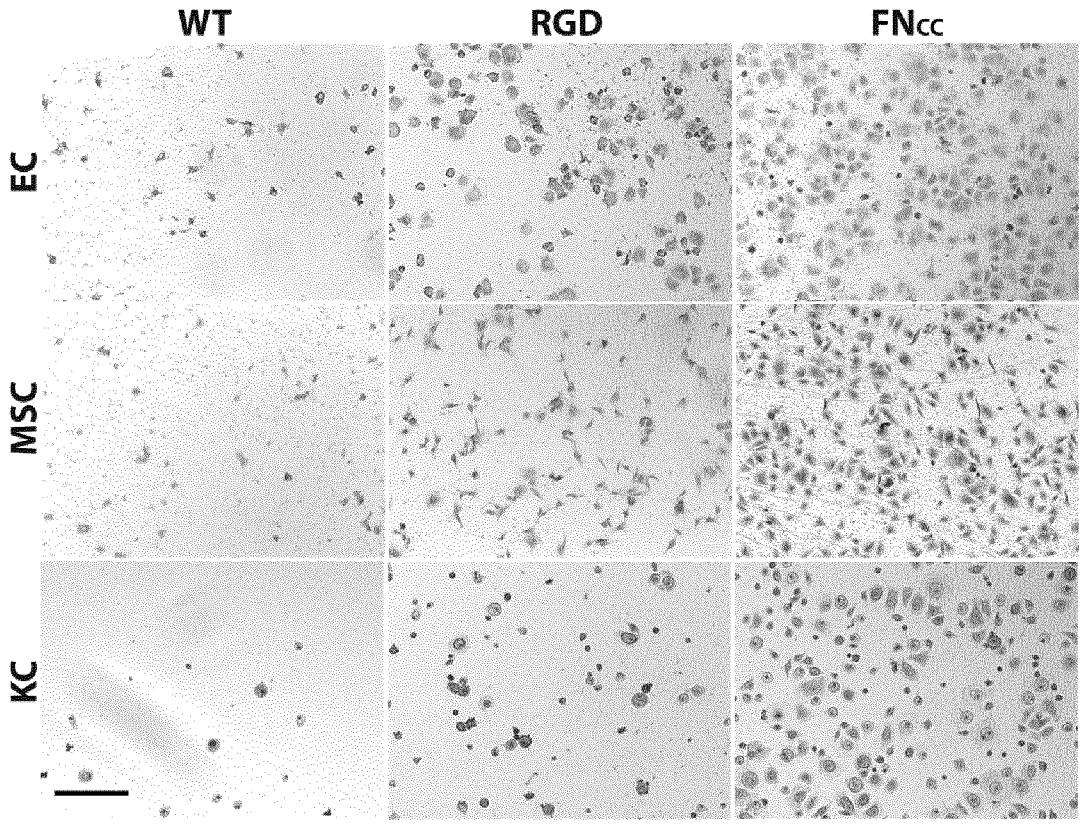
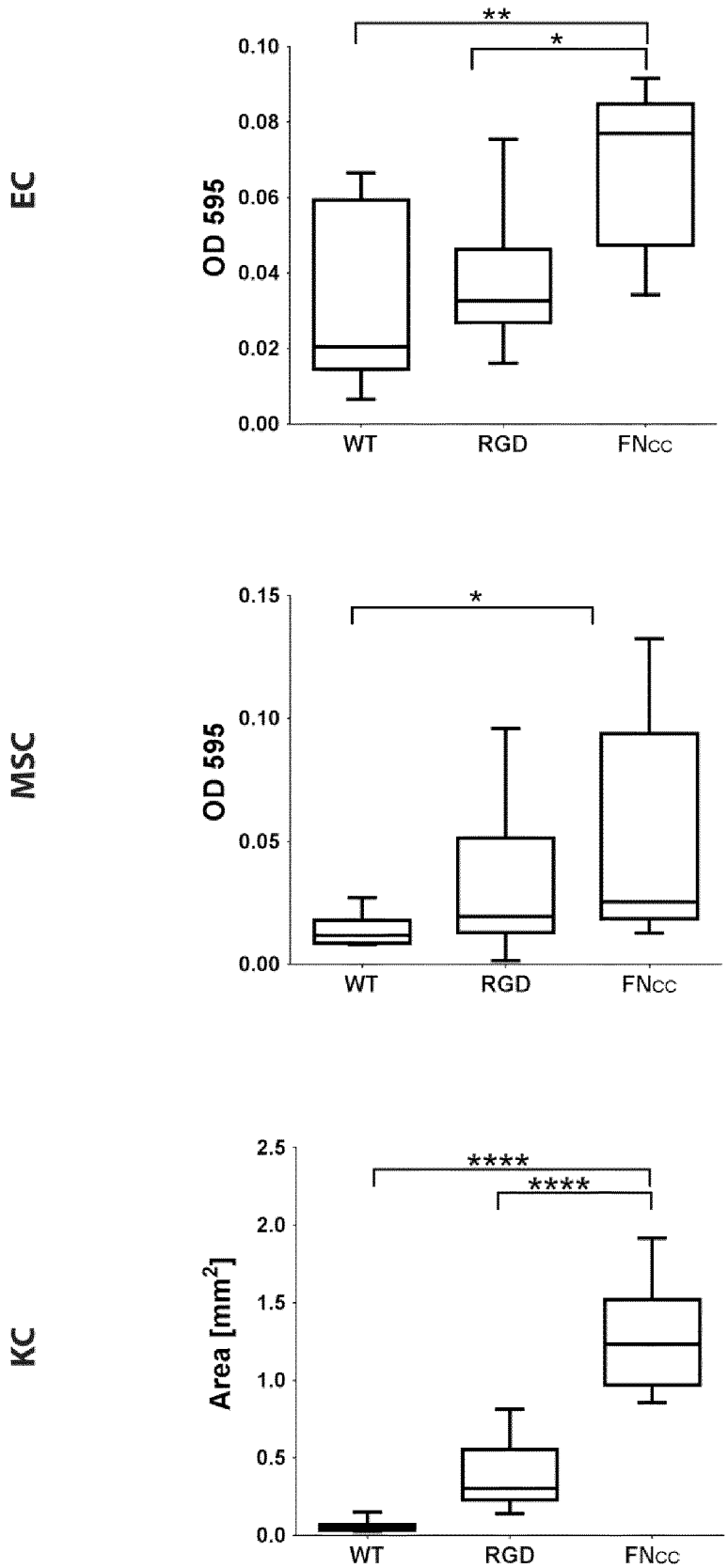


Fig 3b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OD 595
0.10
0.08
0.06
0.04
0.02
0.00
**
*
WT
RGD
FNcc
MSC
OD 595
0.15
0.10
0.05
0.00
*
WT
RGD
FNcc
KC
Area [mm²]
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2.0
1.5
1.0
0.5
0.0
****
****
WT
RGD
FNcc

Fig 4a

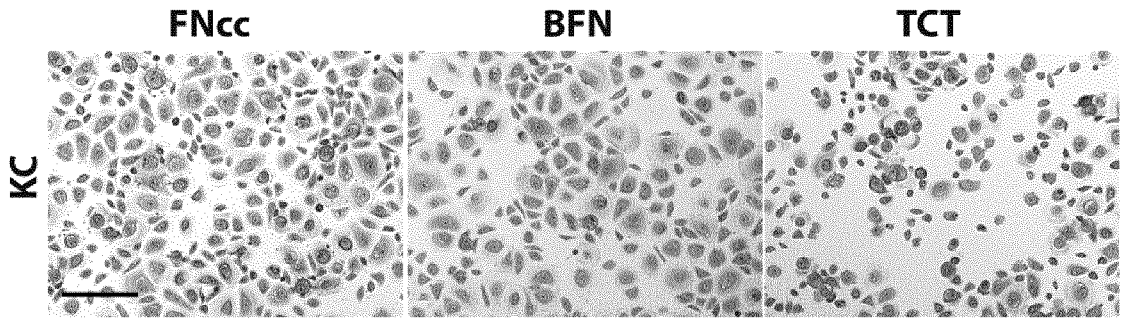


Fig 4b

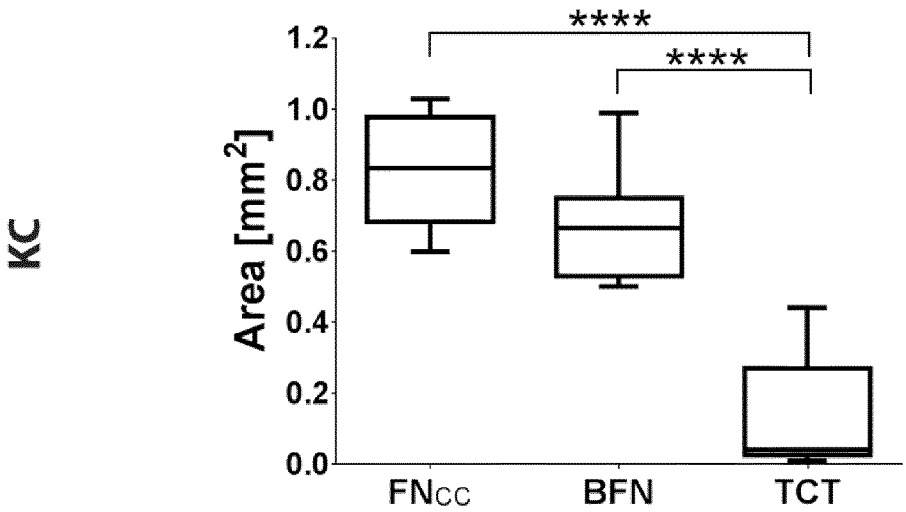


Fig 5a

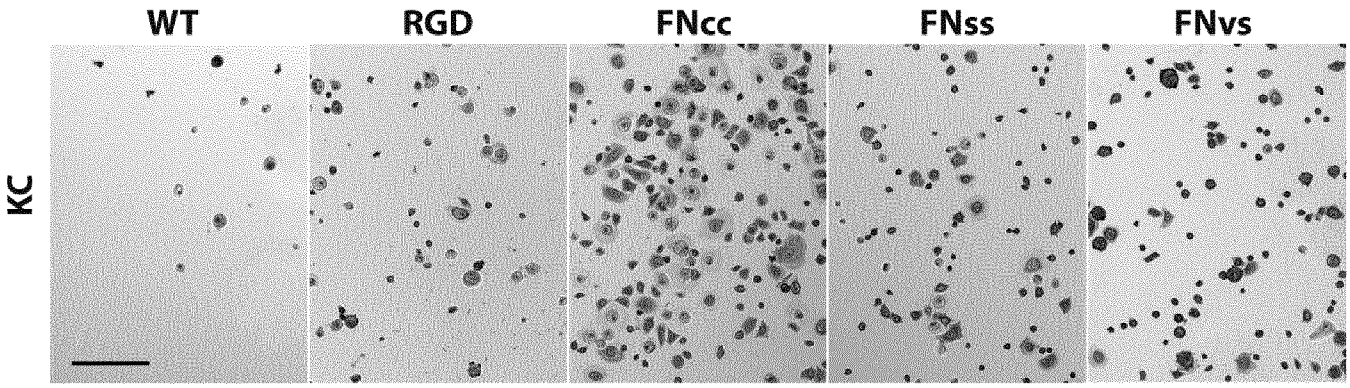
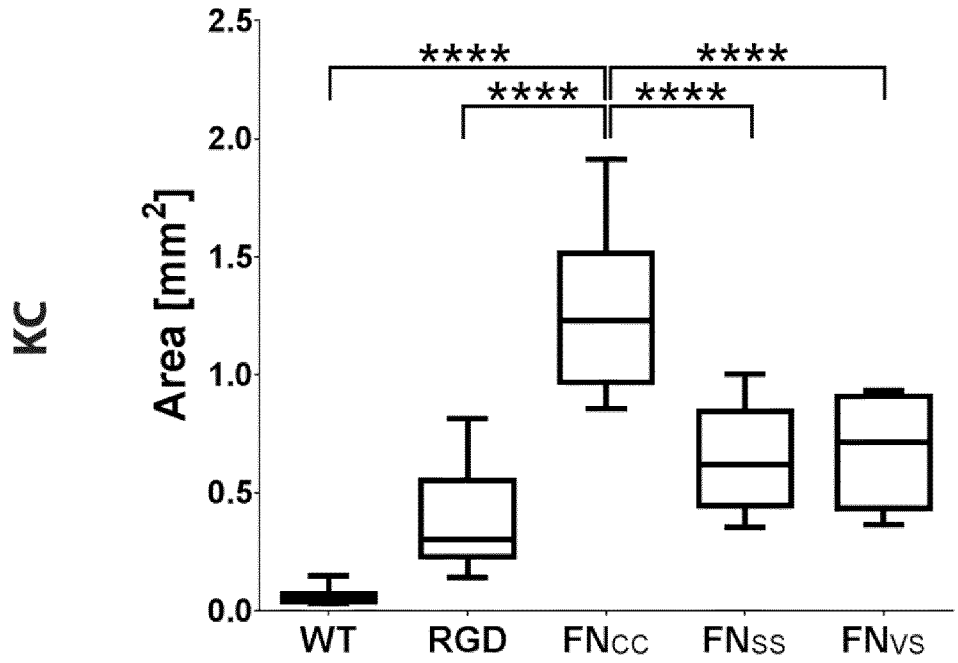

WT
RGD
FNcc
FNss
FNvs
KC

Fig 5b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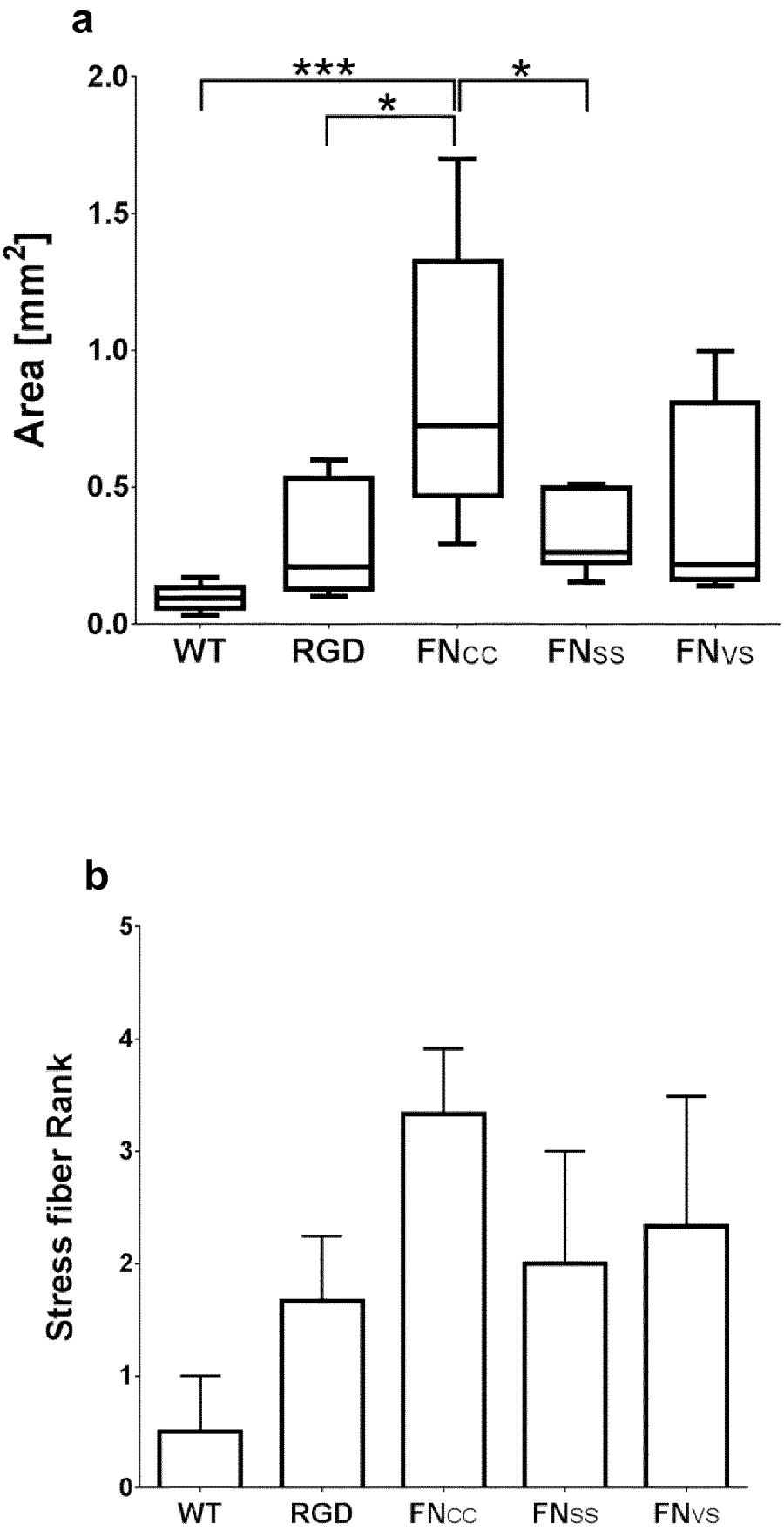
1.0
0.5
0.0
****
****
****
****
WT
RGD
FNcc
FNss
FNvs

Fig 6


a
***
*
*
2.0
1.5
1.0
0.5
0.0
Area [mm2]
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RGD
FNCC
FNSS
FNVS
b
5
4
3
2
1
0
Stress fiber Rank
WT
RGD
FNCC
FNSS
FNVS

Fig 7

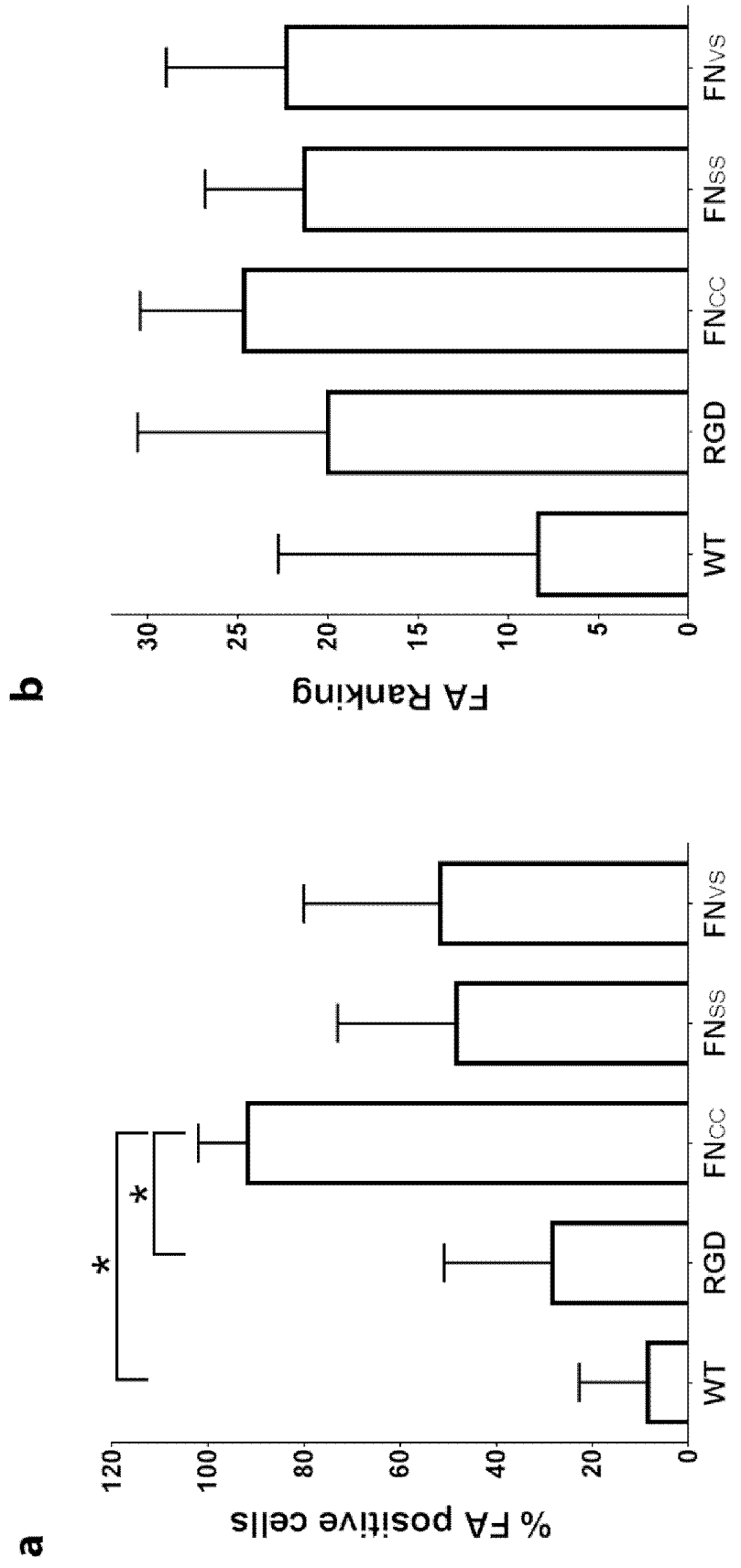
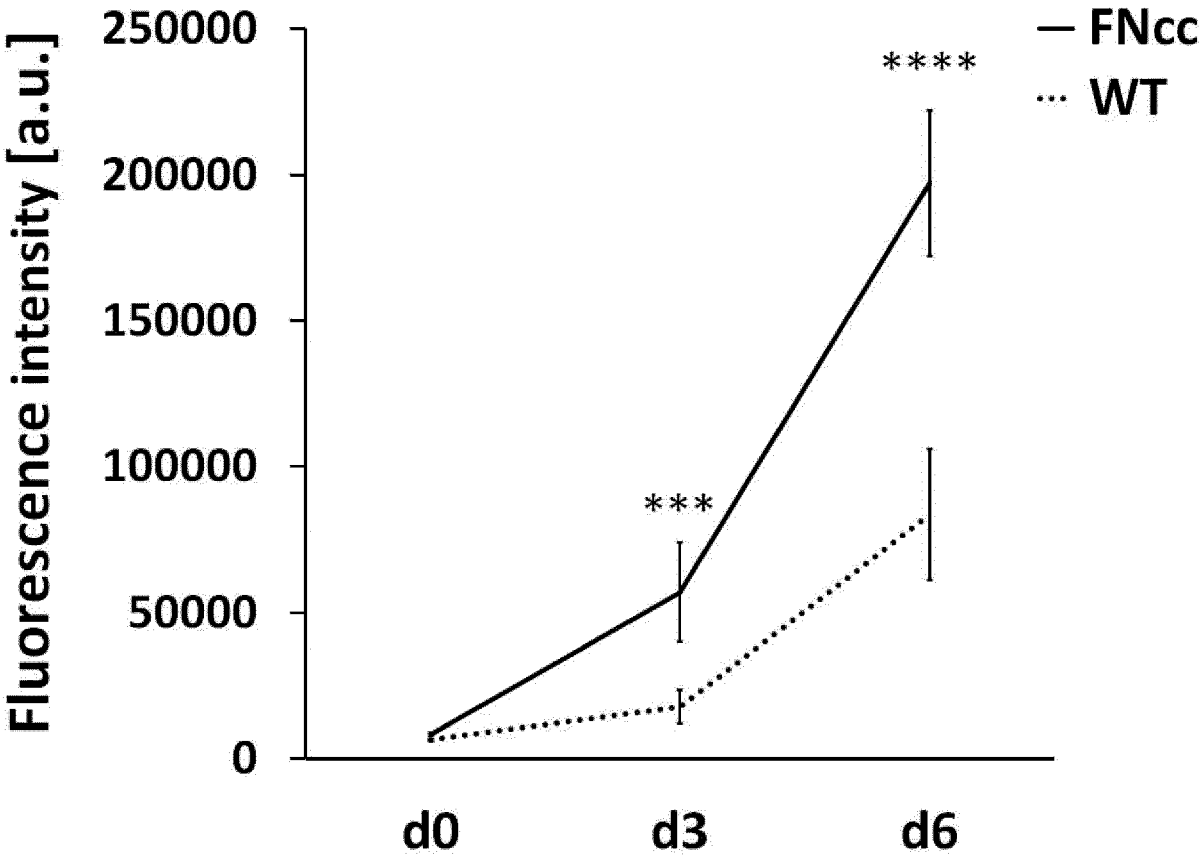

a
% FA positive cells
0
20
40
60
80
100
120
*
*
WT
RGD
FNcc
FNss
FNvs
b
FA Ranking
0
5
10
15
20
25
30
WT
RGD
FNcc
FNss
FNvs

Fig 8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150000
100000
50000
0
d0
d3
d6
***
****
FNcc
WT

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AF350266_At1    SRLSSPGAAS RVSSAVTSLV SSGGPTNSAA LSNTISNVVS QISSSNPGLS
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AF350267_At2    SRLSSPQASS RVSSAVSTLV SSG-PTNPAS LSNAISSVVS QVSSSNPGLS
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AF350275_Lg2    SALSSPTTHA RISSHASTLL SSG-PTNSAA ISNVISNAVS QVSASNPGSS
AY953075_Lh2    SALSSPTTHA RISSHASTLL SSG-PTNAAA LSNVISNAVS QVSASNPGSS
AY654293_Nc2    SRLASPDSGA RVASAVSNLV SSG-PTSSAA LSSVISNAVS QIGASNPGLS
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AF350270_DtFb2  SRLSSPQAAS RVSSAVSSLV SNG-QVNVAA LPSIISSLSS SISASSTAAS
U47853_ADF1     NRLSSAGAAS RVSSNVAAIA SAG----AAA LPNVISNIYS GVLSS--GVS
U47854_ADF2     SRLSSPSAAA RVSSAVS-LV SNGGPTSPAA LSSSISNVVS QISASNPGLS
U47855_ADF3     SRLSSPAASS RVSSAVSSLV SSG-PTKHAA LSNTISSVVS QVSASNPGLS
U47856_ADF4     SVYLRLQPRL EVSSAVSSLV SSG-PTNGAA VSGALNSLVS QISASNPGLS

Consensus       SRLSSPQASS RVSSAVSNLV SSG-PTNSAA LSNTISNVVS QISASNPGLS
```

Fig 9

```
CThyb_Esp       GCDVLVQALL EVVSALIHIL GSSSIGQVNY GSAGQATQLV GQSVYQALGE F
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AF350266_At1    GCDVLVQALL EIVSALVHIL GSANIGQVNS SGVGRSASIV GQSINQAFS- -
AY666062_Cm1    GCDVLVQALL EVVSALIHIL GSSSIGQVNY GSAGQATQIV ---------- -
AF350273_Lg1    SCDVLVQALL ELVTALLTII GSSNVGNVNY DSSGQYAQVV SQSVQNAFV- -
AY953074_Lh1    ACDVLVQALL ELVTALLTII GSSNIGSVNY DSSGQYAQVV TQSVQNVFG- -
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AY666076_Np1    GCDVLVQALL EVVSALIHIL GSSSIGQVNY GSAGQATQIV ---------- -
AF350277_Nm1    GCDVLIQALL EVVSALIHIL GSSSIGQVNY GSAGQATQ-- ---------- -
AF350279_Ns1    GCDVLIQALL EVVSALVHIL GSSSIGQVNY GSAGQATQ-- ---------- -
AY666057_Ov1    GCDVLVQALL EVVSAPIHIL GSSSIGQVNY GSAGQATQIV ---------- -
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AF350286_Tv1    GCDTVVQALL EVAAALVHVL ASSNIGQVNL NTAGYTSQL- ---------- -
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AY365016_Aam2   SCDVLVQALL EIVSALVHIL GSSSIGQINY SASSQYARLV GQSIAQALG- -
AF350263_Aau2   GCDVLVQALL ELVSALVHIL GSSSIGQINY AAS------- ---------- -
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AF350280_Ns2    GCDVLIXALL EIVSACVTIL SSSSIGQVNY GAA------- ---------- -
AF350269_DtFb1  DCEVLVQVLL EVVSALVQIV CS-------- ---------- ---------- -
AF350270_DtFb2  DCEVLVQVLL EIVSALVQIV SSANVGYINP EASGSLN-AV GSALAAAMG- -
U47853_ADF1     SSEALIQALL EVISALIHVL GSASIGNVSS VGVNSALNAV QNAVGAYAG- -
U47854_ADF2     GCDILVQALL EIISALVHIL GSANIGPVNS SSAGQSASIV GQSVYRALS- -
U47855_ADF3     GCDVLVQALL EVVSALVSIL GSSSIGQINY GASAQYTQMV GQSVAQALA- -
U47856_ADF4     GCDALVQALL ELVSALVAIL SSASIGQVNV SSVSQSTQMI SQALS----- -

Consensus       GCDVLVQALL EVVSALVHIL GSSSIGQVNY GSAGQATQIV GQSVAQALGE F
```

Fig 9 (continued)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/064543

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435 C12N15/62
ADD.

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 C12N

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)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/185027 A2 (DCB USA LLC [US]; UNIV NAT CHENG KUNG) 12 December 2013 (2013-12-12)	17
A	seq id no.18	1-16, 18-20

A	WO 2015/036619 A1 (SPIBER TECHNOLOGIES AB [SE]) 19 March 2015 (2015-03-19) cited in the application seq ID No:19; claims 1-19	1-20

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] 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

"E" earlier application or patent 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	Date of mailing of the international search report
8 September 2016	21/10/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hoff, Céline

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International application No
PCT/EP2016/064543

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