Title: INVASIN INTEGRIN BINDING DOMAINS IN CELL CULTURE

FIELD OF THE INVENTION

[001] This invention pertains to improved culture methods for culturing and expanding cells, including epithelial and endothelial cells, and obtaining organoids. The invention also pertains to particular integrin-binding proteins used in such culture methods, carriers coated with such integrin-binding proteins, and tissue culture systems comprising such carriers.

BACKGROUND OF THE INVENTION

[002] The background description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[003] Cell culture is a widely used *in vitro* tool for improving understanding of cell biology, gene function, tissue morphology, and mechanisms of diseases, drug action, protein production and the development of tissue engineering. Cell culture is often used in the development of drugs.

[004] The most commonly used types of cell culture are 2D and 3D models (reviewed by Kapałczyńska et al. Arch Med Sci. 2018 Jun; 14(4): 910–919. doi: 10.5114/aoms.2016.63743). Historically, cell culture is carried out under adherent 2D conditions wherein cells are attached to a glass or plastic dish. However, nowadays cells, such as epithelial cells, are often cultured in the presence of an exogenous (artificial) extracellular matrix (ECM). Growth in 2D can be performed on ECM-coated surfaces, including, for example permeable supports such as Transwell-inserts based on e.g., PTFE membranes. A frequently used other option is to present the ECM proteins in a 3D hydrogel (i.e., highly hydrated polymer network). These procedures attempt to mimic interactions that cells normally have with the extracellular matrix found *in vivo*. Both ECM-based 2D, and 3D approaches have been demonstrated to provide a more relevant representation of natural environment compared to growing cells on, for example, plastic surfaces.

[005] *In vivo*, the extracellular matrix (ECM) is a dynamic 3-dimensional network of macromolecules that provides structural support for cells and tissues. Amongst the

many distinct roles, the ECM orchestrates cell signaling leading to apical-basal polarity, inhibition of anoikis, and growth. Extracellular soluble proteins/glycoproteins, such as collagen type IV, RGD-class proteins like fibronectin, and various classes of laminins, are the main players. These proteins are ligands of αß heterodimeric Integrins, that mediate the signals driving the vitality of all epithelial tissues. The so-called matrisome, its components and functions, as well as interactions between the various components have been reviewed by Karamanos et al. (FEBS J (2021);288(24):6850-6912. doi: 10.1111/febs.15776 and FEBS J (2023) 290(22):5238-5247. Doi: 10.1111/febs.16778).

[006] *In vivo*, each tissue type has its own distinct ECM, which is still incompletely understood. However, mimicking or approximating the *in vivo* ECM has been demonstrated to improve *in vitro* cell culture, as well as to provide a more relevant representation of the natural*, in vivo*, characteristics of such cells. However, the *in vivo* ECM is not easily replicated in the laboratory settings and often researchers have to resort to the use of ECM directly extracted from animal sources.

[007] One of the most *in vitro* used exogenous (artificial) extracellular matrix material is Matrigel®. Another well-known extracellular matrix material is basement membrane extracts (BME). Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cell line, a tumor rich in ECM proteins including laminin-1 (a major component), collagen IV, heparan sulfate proteoglycans, entactin/nidogen, and a number of growth factors. For large-scale production of Matrigel, this tumor is inoculated in mice. The resulting ECM-based hydrogel is among the most widely used models for 2D and 3D cell cultures *in vitro* and enables improvement of attachment and differentiation of both normal and transformed cells, including polarized cells such as epithelial and endothelial cells.

[008] Matrigel, as well as other hydrogels, are also used as exogenous (artificial) extracellular matrix in stem cell-based organoid cultures. Organoids are (self-organized) 3D tissue-engineered cell-based *in vitro* models that recapitulate many aspects of the complex structure and function of the corresponding *in vivo* tissue. They can be dissected and interrogated for fundamental mechanistic studies on development, regeneration, and repair in human tissues, and can also be used in diagnostics, disease modelling, drug discovery and personalized medicine. Typically, organoids are derived from either pluripotent or tissue-resident stem (embryonic or

adult) or progenitor or differentiated cells from healthy or diseased tissues, including tumors. As a consequence, organoids are a promising cell source for tissue regeneration, tissue repair, and could be applied as a therapeutic tool for various disease models. To date, numerous organoid engineering strategies that support organoid culture and growth, proliferation, differentiation, and maturation have been reported, and the rationale underlying these strategies have been reviewed (e.g., Zhao et al. (2022) Nature Reviews Methods Primers 2:94 (2022) doi: 10.1038/s43586-022-00174-y).

[009] Although Matrigel can support organoid culture, the composition is inherently heterogeneous and poorly defined and offers little control over the biochemical and biophysical cues that are necessary for improving organoid culture. Indeed, ECMs such as Matrigel have well-known limitations, including lot-to-lot variability, excessive cost, and discrepancies in composition and structure.

[010] Therefore, other matrices with defined compositions have been explored as alternative matrices to Matrigel, such as human collagen, fibrin, or synthetic hydrogels decorated with peptides mimicking ECM proteins. Organoid growth in the presence of various synthetic matrices is still less efficient than Matrigel-cultured organoids. There is therefore an unmet demand to develop an alternative matrix approach to culturing cells, for example epithelial cells, for example organoids, using ECMs such a Matrigel.

[011] Another essential constraint that is unmet relates to the ability to expand or culture cell systems, including organoids, under conditions that are GMP (good manufacturing practice)-compliant. Most cell systems, including organoids, are generated by, for example, expansion of (stem) cells in 2D or 3D structures in the presence of artificial ECMs such as Matrigel. In addition, due to its mouse sarcoma origin, and its in-mouse production, the danger of pathogenic contaminations make it not suitable for human *in vivo* translation. Until to date, known synthetic matrices appear to not have solved the issues with Matrigel in a sufficient way.

[012] It can thus be seen that there remains a need for methods that would allow for culturing cell systems, including organoids, and that would reproduce features of the native ECM, without being dependent on ECMs such as Matrigel, while also being potentially GMP-compliant and capable of being translated into a clinical environment.

[013] In light of this, new products, compositions, and methods for use in culturing (including 2D and 3D cell culturing) cells (such as epithelial cells, for example in

organoids), would be highly desirable but are not yet readily available. In particular, there is a clear need in the art for reliable, efficient, and reproducible products, compositions, methods and uses that allow to be used in cell culturing. Accordingly, a technical problem underlying the present invention can been seen in the provision of such products, compositions, methods and uses for complying with any of the aforementioned needs, or at least providing the public with a useful choice. This technical problem and others apparent from the current disclosure, may be solved by the embodiments characterized in the claims and herein below.

SUMMARY OF THE INVENTION

[014] As embodied and broadly described herein, the present invention is directed to the surprising finding that an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus (also referred to herein as “the isolated protein according to the invention”) allows cells or cell systems, including organoids, stem cells, epithelial cells, primary tissues and induced pluripotent stem cells (iPSCs) to be grown without requirement for an basal membrane extract, such as Matrigel or Basal Membrane Extract (BME)./pct

[015] In addition, it was surprisingly found that the isolated protein according to the invention can be used to replace basal membrane extracts, such as Matrigel, in cell culture. In addition, it was surprisingly found that the isolated protein according to the invention can be used to replace natural or synthetic ECMs commonly used in cell culture. Furthermore, it was found that the isolated protein according to the invention can act like common ECM ligands such as laminin, collagen and/or fibronectin, in particular laminin and/or fibronectin. As shown in the Examples herein, and/or demonstrated in experiments by the inventors, the isolated protein may be used to replace or supplement commonly used ECM ligands and/or substrates such as Matrigel, for example, during culturing of cells. It was surprisingly found that with the isolated protein according to the invention it has become possible to culture cells with a performance efficiency that is close to, equal to or better than that of commonly used basal membrane extracts including Matrigel. It was surprisingly found that in the presence of the isolated protein during culturing of cells, it has become possible to culture cells with a performance efficiency that is close to, equal to or better than that of commonly used basal membrane extracts including Matrigel. In particular, it is

believed that the recognition of ECM ligand-specific (such as laminin specific and/or fibronectin specific) integrin receptors in a manner that support efficient culturing of the cells (e.g., in the form of organoids) makes the isolated protein according to the invention an alternative ECM source.

[016] Therefore, in accordance with the invention, there is provided for an *in vitro* method of culturing cells (such as expanding, growing, proliferating, differentiating or maintaining cells), the method comprising a) exposing the cells to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus; and b) culturing the exposed cells in the presence of a culture medium, preferably wherein the culturing of the exposed cells is in the presence of an isolated protein as defined in step a) or the isolated protein of step a) to which the cells have been exposed.

[017] In accordance with the invention, there is also provided for the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, preferably wherein the protein is coated on a carrier, as well as isolated nucleic acids encoding such proteins, and host cells comprising such isolated proteins and/or nucleic acids, and carriers coated with such isolated protein.

[018] In accordance with the invention, there is also provided for the use of the isolated protein in cell culture, and use of a carrier coated with such isolated protein in cell culture (such as a tissue culture or an organoid culture).

[019] In accordance with the invention, there is also provided for a method of preparing a carrier coated with the isolated protein according to the invention, as well as to a cell culture system comprising such carriers coated with the isolated protein, preferably also comprising cells.

[020] This and other aspects and embodiments of the invention will be described in detail herein below.

BRIEF DESCRIPTION OF THE DRAWINGS

[021] Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

[022] Figure 1: Integrins are a family of cell adhesion receptors that play a crucial role in the attachment of cells to the extracellular matrix and in cell-cell interaction, *in vivo* and *in vitro* (e.g., culturing organoids).

[023] Figure 2: Integrin receptor types and their ligand specificity: Invasin is recognized by multiple integrin receptors.

[024] Figure 3: Invasin induces an open-active conformation of integrins on K562 cells.

[025] Figure 4: Epithelial cells derived from the lung, colon and liver organoids adhere to BME and bacterial-produced Invasin.

[026] Figure 5: Intestinal epithelial cells cultured on Invasin in 2D, demonstrate normal apical-basal polarity.

[027] Figure 6: Vinculin-TdTomato reporter organoids, grown as 2D on the isolated protein of the invention (e.g., Invasin) or BME, show active integrin signaling.

[028] Figure 7: Human fetal hepatocytes can be expanded as 2D cells on the isolated protein according to the invention (e.g., Invasin) for multiple passages.

[029] Figure 8: Human cells, from different origins, can be expanded long term as 2D cells on Invasin.

[030] Figure 9: Intestinal epithelial cells, grown in 2D on Invasin in 2D, can be differentiated towards well-known functional intestinal cells.

[031] Figure 10: Integrin α/β1 pairs and their ligands. The universal integrin β1 associates with different integrin α subunits with unique ligand specificities: Collagens, Laminins, proteins containing the amino acid sequence RGD like fibronectin, or leucocyte-specific cell adhesion molecules (CAM; not expressed in intestinal epithelial cells). The integrin-binding domain of Invasin interacts with the heterodimers in the boxes. Asterisks denote the integrins expressed in colon epithelial organoids.

[032] Figure 11: Invasin supports the growth of primary human liver tissue. Primary human liver tissue was processed, and single cells were plated on Inv497-coated wells. After 10 days, the resulting 2D organoid sheet was analyzed using immunofluorescence. Staining revealed liver-specific markers, including albumin and hepatocyte nuclear factor 4 (HNF4), and a nuclear marker DAPI was used.

[033] Figure 12: 7500 single cells of wild type (WT), ITGA6 and ITGAV knockout (KO) colon organoids were cultured in BME® hydrogels in standard medium, with Rho-kinase inhibitor (Y-27) or the β1-integrin allosterically inhibiting antibody AIIB2. Cultures were cultured for 7 days. Quantification of viable cells using ATP-sensitive luminescent assay (CellTiter-GLO). Mean ± SD, n=4.

[034] Figure 13: Quantification by CellTiter-GLO of colon and ileum organoid-derived single cells adhering to coats of Inv497 or Inv192, either as an isolated fragment or fused to Maltose binding protein (MBP). Identical protein concentrations (5 ug/ml)) where used for coating. RLU are given for four technical replicates for each condition. Means and standard deviation are indicated.

[035] Figure 14: A) Quantification of adherence of organoid-derived colon/ileum single cells to coated BME, recombinant laminin521 (LN521) or Inv192 fragments from Y. pseudotuberculosis and enterocolitica, using a cellular ATP-driven luminescent assay (CellTiter-GLO). Relative light unit (RLU). Mean ± SD, n=3 B) Quantification of 2D growth of BME-established colon and ileum organoids on different coated wells, all started from 50.000 cells/well. The coats are BME, Invasin from Y. pseudotuberculosis (Inv192 Y. pseu), Y. enterocolitica (Inv192 Y. ent) or no coat. Mean ± SD, n=8. C) Transepithelial electrical resistance (TEER) measurement of colon and ileum cells grown on Inv497, BME, or BSA coats, measured on days 0 and 7. Mean ± SD, n=3.

[036] Figure 15: A) Human ileum organoid cells cultured for the indicated days; quantification of live cell numbers was done using trypan blue counting with a hemocytometer (n=3), values are in thousands. Mean and standard deviation are indicated. Of note, cells did not adhere and rapidly died on non-coated plates B) Human ileum 2D organoid sheets cultured on Invasin (Inv497) for 14 passages maintain polarity. Basal marker integrin β1 (bottom), apical marker F-actin (upper) and nuclear marker DAPI (middle).

[037] Figure 16: Legends: A) Airway epithelial organoid cells (line LU30) were cultured for 9 passages on Inv497 coat and stained for integrin β1, proliferation marker Ki67, apical marker F-actin and DAPI. Images represents side view of immunofluorescent staining presented. Scalebar is 50 µm. B) Human airway epithelial cells (LU30) were cultured on Inv497, BME or no coat. CellTiter-Glo was performed over multiple days. Standard deviation and mean are indicated of five technical replicates. C) Freshly isolated human airway cells were cultured for 3 passages on Invasin and BME. After 3 passages, cells were replated, and TEER was measured over three days. Standard deviation and mean of three technical replicates are indicated.

[038] Figure 17: A) 50.000 cells mouse lacrimal gland organoid-derived single cells adhering to Inv497, BME or no coat. Assayed by CellTiterGLO. The mean of the

relative light units (RLU) ± SD, are given for four technical replicates. B) Quantification of viable cells of mouse lacrimal gland after 7 days using CellTiterGLO. Mean ± SD, n=3. Started from 25.000 cells/well. C) TEER of mouse lacrimal gland cultures over time. Cultures were started with 25.000 cells/well. Means ± SD, n=3. D) Timeline of long-term culture of mouse lacrimal gland organoid-derived cells on Inv497, with every dot indicating a passage. The line could be passaged for at least 18 times and growth remained exponential. At the time of writing, cells were still in culture with a weekly 1:3 passage ratio.

[039] Figure 18: A) Snake epithelial cells recognize Invasin as extracellular matrix protein using an adhesion assay. Adhesion assay was performed with 30.000 cells/well. Figure represents quantification of Aspidelaps lubricus cowles (A. l. cowlesi) and Naja naja venom gland organoid-derived single cells adhering to a coat of Invasin (Inv497), of BME or no coat, using CellTiterGlo. Mean ± SD, n=4. B) Quantification of growth of Aspidelaps lubricus cowlesi (A. l. cowlesi) and Naja naja venom gland cells after seven days of culture on Inv497, BME or no coat. C) Timeline of venom gland cells of the indicated snake species in culture on Inv497 coat, with each dot indicating a passage. At the time of writing, exponentially growing cells were still in culture with a weekly 1:3 passage ratio.

[040] Figure 19: A) Multiple passages in 2D on Inv497 of airway (LU30 and LU31), ileum (N39), colon (P26N) 3D-established organoids. Dots represent passages. Passage ratio 1:2 for intestine and 1:3 for airway epithelial cells. B) Biopsies of healthy colon tissue cultured on BME, Inv497 or no coat. After 10 days, numbers of viable cells were quantified using CellTiter-GLO. Mean ± SD, n=3. C) Freshly isolated, primary airway epithelial cells grown on Inv497 or BME, stained for basal cells (KRT5); club cells (CC10); and nuclear marker DAPI. Scale bar: 25 µm. D) Multiple passages in 2D on Inv497 of primary epithelial cells from healthy colon biopsies from three different donors (lines C#1, C#2, C#3) and airway biopsies from three different donors (lines A#1, A#2, A#3). Dots represent passages. Weekly passage ratio for airway epithelial sheets was 1:3 and for colon 1:2.

[041] Figure 20: A-C Freshly isolated epithelial cells from mouse duodenum, jejunum and ileum adhere to Invasin (left, first panel). Mean ± SD n=4. Viable cells were measured using CellTiterGLO after 4 days of culture on Invasin (Inv497), BME or no coat (second panel). Mean ± SD, n=3, N=3.

[042] Figure 21: Transmission electron microscopy (TEM) images of goblet cells, enteroendocrine cells (EEC) and Paneth cells upon differentiation after 12 passages on Invasin or BME. Scalebar is indicated.

[043] Figure 22: Transmission electron microscopy images (TEM) of differentiated airway cells cultured on INV or BME showing a polarized pseudostratified epithelium with prominent ciliated cells.

[044] Figure 23: A) Brightfield images of affigel10 beads covered with proteins BSA or Invasin and as negative control PBS solution. The cultures were imaged on Day0 and Day8. Cells and resins are indicated. With the higher magnification at day 8 in Affigel Invasin condition a clear epithelial layer is observed around the resin. B) After 8 days the cultures were analyzed using confocal microscopy highlighting nuclear marker DAPI, apical marker F-actin and ZO-I, and basal marker Integrin a6, proliferative marker Ki67. Beads were covered with Invasin as confirmed with a specific antibody against the integrin-binding domain of Invasin.

[045] Figure 24: A) Brightfield images of colon ITGA2-/- organoids cultured for 8 days in collagen-I supplemented with Affigel beads covered with different proteins (BSA or Invasin/protein according to the invention) or BME. Arrows indicate epithelial sheets around Affigel-Invasin beads which was not observed in the Affigel-PBS or Affigel-BSA condition. B) Immunofluorescent confocal images of epithelial colon cells grown on Affigel-Invasin beads.

DESCRIPTION

Definitions

[046] A portion of this disclosure contains material that is subject to copyright protection (such as, but not limited to, diagrams, device photographs, or any other aspects of this submission for which copyright protection is or may be available in any jurisdiction.). The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or patent disclosure, as it appears in the Patent Office patent file or records, but otherwise reserves all copyright rights whatsoever.

[047] Various terms relating to the methods, compositions, uses and other aspects of the present invention are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art to which the invention pertains, unless otherwise indicated. Other specifically defined terms are to be construed in a manner

consistent with the definition provided herein. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein.

[048] For purposes of the present invention, the following terms are defined below.

[049] As used herein, the singular form terms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “an isolated protein” includes a combination of two or of such isolated proteins, and the like. For example, a method for coating a carrier with an isolated protein according to the invention, includes coating the carrier with plurality of such isolated proteins or combination of more than one type of isolated protein.

[050] As used herein, “about” and “approximately", when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed invention. Unless otherwise clear from context, all numerical values provided herein include numerical values modified by the term “about”.

[051] As used herein, “and/or” refers to a situation wherein one or more of the stated cases may occur, alone or in combination with at least one of the stated cases, up to with all of the stated cases.

[052] As used herein, "at least" a particular value means that particular value or more. For example, "at least 2" is understood to be the same as "2 or more" i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, …, etc. As used herein, the term "at most" a particular value means that particular value or less. For example, "at most 5" is understood to be the same as "5 or less" i.e., 5, 4, 3, ….-10, -11, etc.

[053] As used herein, “comprising” or “to comprise” is construed as being inclusive and open ended, and not exclusive. Specifically, the term and variations thereof mean the specified features, steps or components are included. These terms are not to be interpreted to exclude the presence of other features, steps, or components. It also encompasses the more limiting “to consist of”.

[054] As used herein, “conventional techniques” or “methods known to the skilled person” refer to a situation wherein the methods of carrying out the conventional techniques used in methods of the invention will be evident to the skilled worker. The practice of conventional techniques in molecular biology, biochemistry, cell culture,

genomics, sequencing, medical treatment, pharmacology, immunology, and related fields are well-known to those of skill in the art and are discussed, in various handbooks and literature references, including those cited herein.

[055] As used herein, “culturing,” “cultivating”, “growing” or variations thereof refer, when directed to a cell or cells, to a method step to propagate, expand, maintain, or differentiate a population of cells in culture media. Conventional methods and techniques are well-known to the skilled person in the field of molecular biology, biology, biochemistry, genomics, cell culturing and the like. Although the term “culturing” is generally understood to include the proliferation or division of cells, it also includes methods of differentiating cells in culture medium.

[056] For example, and as the skilled person knows, culturing cells may mean maintaining, propagating, differentiating cells under conditions that support their survival, growth, proliferation, differentiation. At the same time, for example, the skilled person knows that culturing cells excludes, for example, incubation of cells for purposes unrelated to the culturing of the cells, such as transient exposure for analytical, testing, or treatment purposes.

[057] As used herein, the term “culture media” includes media that are suitable for the *in vitro* cell culture of human or animal cells for a prolonged period of time. Such culture media comprises sufficient components to allow the cells to maintain, grow, proliferate and/or differentiate over longer period of, for example, for example, at least a day. A “defined culture medium” refers to a (growth) medium suitable for the *in vitro* cell culture of human or animal cells and in which all of the chemical components are known. Such defined media does not or essentially not comprise any ill-defined source of nutrients and/or other ill-defined factors. A culture medium may be serum-free.

[058] As used herein the term “fusion protein” refer to any polypeptide which is not normally found in nature is a species, in particular a polypeptide in which one or more part of the amino acids sequence are not associated with each other in nature. For example, a fusion protein may comprise a N-terminal part consisting of a first sequence of amino acids and a C-terminal part consisting of a second sequence of amino acids that are not associated with each other in nature and/or are not associated with each other in nature in this order. A fusion protein may for example be obtained from transcription and translation of a fusion gene of nucleic acid. Such fusion gene may be created by joining parts of two different genes/nucleic acid sequences.

[059] As used herein, the term “isolated” when referring to a polynucleotide (nuclei acid) or polypeptide (protein), refers to nucleic acids or present being present in a non-naturally occurring environment, e.g., are separated from their naturally occurring environment. For example, an isolated protein or polypeptide according to the invention relates to a protein which is no longer in its natural environment, for example, is no longer present in the species of the *Yersinia* genus from which it was derived, or, for example, (outer) membrane factions thereof. In other words, in the context of the invention, in those embodiments wherein the protein according to the invention is a naturally occurring protein, the protein according to the invention is no longer present in the species of the *Yersinia* genus from which it was derived and is, for example, no longer part of a *Yersinia* genus cell. Is some embodiments the protein according to the invention may be artificially present in another cell of another species, such as eukaryote cell (i.e., animal cell), in which this protein does not naturally occur. However, preferably the protein according to the invention (or isolated protein) is not part of a cell (or cell membrane) and is, for example, present outside a cell.

[060] The terms, next to being isolated from naturally occurring source, may also refer to such protein or nucleic acid being artificially or synthetically produced. Within the context of the current invention, it will be clear for the skilled person if a reference to a protein, polypeptide, nucleic acid, or polynucleotide includes reference to an “isolated” protein, polypeptide, nucleic acid, or polynucleotide. Within the context of the current invention, it will be clear for the skilled person, that non-natural occurring nucleic acids or proteins, such a fusion protein or the like, or shorter versions of naturally occurring proteins or the like do not have a naturally occurring environment. These are still referred to as isolated proteins according to the invention.

[061] As used herein “identity” or “sequence identity” refers to the degree of relatedness between two or more amino acid sequences, or two or more nucleic acid sequences (polynucleotide sequences), as determined by comparing the sequences. The comparison of sequences and determination of sequence identity may be accomplished using a mathematical algorithm; those skilled in the art will be aware of computer programs available to align two sequences and determine the percent identity between them. The skilled person will appreciate that different algorithms may yield slightly different results.

[062] Thus, the “percent identity” between a query nucleic acid sequence and a subject nucleic acid sequence is the “identities” value, expressed as a percentage, which is calculated by, for example, the BLASTN algorithm when a subject nucleic acid sequence has 100% query coverage with a query nucleic acid sequence after a pairwise BLASTN alignment is performed. Such pairwise BLASTN alignments between a query nucleic acid sequence and a subject nucleic acid sequence are performed by using the default settings of the BLASTN algorithm available on the National Center for Biotechnology Institute's website with the filter for low complexity regions turned off.

[063] Similarly, the “percent identity” between a query amino acid sequence and a subject amino acid sequence is the “identities” value, expressed as a percentage, which is calculated by the BLASTP algorithm when a subject amino acid sequence has 100% query coverage with a query amino acid sequence after a pairwise BLASTP alignment is performed. Such pairwise BLASTP alignments between a query amino acid sequence and a subject amino acid sequence are performed by using the default settings of the BLASTP algorithm available on the National Center for Biotechnology Institute's website with the filter for low complexity regions turned off.

[064] The query sequence may be 100% identical to the subject sequence, or it may include up to a certain integer number of amino acid or nucleotide alterations as compared to the subject sequence such that the % identity is less than 100%. For example, the query sequence is at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to the subject sequence. Such alterations include at least one amino acid deletion, substitution (including conservative and non-conservative substitution), or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the query sequence or anywhere between those terminal positions, interspersed either individually among the amino acids or nucleotides in the query sequence or in one or more contiguous groups within the query sequence.

Detailed description

[065] The invention is defined herein, and in particular in the accompanying claims. Subject-matter which is not encompassed by the scope of the claims does not form part of the present claimed invention.

[066] It is contemplated that any method, use, or composition described herein can be implemented with respect to any other method, use or composition described

herein. Embodiments discussed in the context of methods, use and/or compositions of the invention may be employed with respect to any other method, use or composition described herein. Thus, an embodiment pertaining to one method, use or composition may be applied to other methods, uses and compositions of the invention as well.

[067] As embodied and broadly described herein, the present invention is directed to the surprising finding that an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus (also referred to herein as "the isolated protein according to the invention") allows cells or cell systems, including organoids, stem cells, primary tissues and induced pluripotent stem cells (iPSCs) to be grown without requirement for an basal membrane extract, such as Matrigel.

[068] In addition, it was found that the isolated protein according to the invention allows cells or cell systems of various sources or origins and etiologies to be grown without requirement for a basal membrane extract. The cell or cell systems include primary tissues (for example of biopsies) of, for example, liver and lung (including airway epithelial cells) cells, intestine cells including ileum and colon, lacrimal gland cells, cells from distinct animals, such as snakes, mouse, and human. The cultured cells can form pseudostratified epitheliums like *in vivo* tissue, for example in 2D culture and in 3D culture. The cells cultured according to the invention form organoids. The cells retain proliferative capacity and can form tight epitheliums in Transwell systems, which make these systems useful for, for example, providing tissue for regenerative medicine and/or for the study of such tissues (e.g., *in vitro* models). Moreover, the distinct types of tissues can be maintained long-term on substrates coated with the protein according to the invention (e.g., invasin), since the cells support multiple passages. The cells exposed to the protein according to the invention (e.g., invasin) during culturing of the cells can differentiate toward specialized cells. For example, intestinal epithelial cells may be differentiated towards well-known functional intestinal cells, including goblet cells, enteroendocrine cells and Paneth cells with the methods disclosed herein. Advantageously also provided is for co-cultures of cells, for example of human cells, with microbes (e.g., bacteria and viruses), or with or cell types, for example with (human) immune cells with the invention disclosed herein. In some embodiments, the cells are epithelial cells.

[069] In addition, it was surprisingly found that the isolated protein according to the invention can be used to replace basal membrane extracts, such as Matrigel, in cell culture. In addition, it was surprisingly found that the isolated protein according to the invention can be used to replace natural or synthetic ECMs commonly used in cell culture. Furthermore, it was found that the isolated protein according to the invention can act like common ECM ligands such as laminin, collagen and/or fibronectin, in particular laminin and/or fibronectin. As shown in the Examples herein, and/or demonstrated in experiments by the inventors, the isolated protein may be used to replace or supplement commonly used ECM ligands and/or substrates such as Matrigel.

[070] It was surprisingly found that with the isolated protein according to the invention it has become possible to culture cells, including organoids, with a performance efficiency that is close to, equal to or better than that of commonly used basal membrane extracts including Matrigel. In particular, the recognition of ECM ligand-specific (such as laminin specific and/or fibronectin specific) integrin receptors in a manner that support efficient culturing of the cells (e.g., in the form of organoids) makes the isolated protein according to the invention a strong candidate as an alternative ECM source. The isolated protein according to the invention can be used in culturing cells, for example in the form of organoids, for example in 2D cultures (for example by coating cell culture devices, such as plates or Transwell membranes, with the isolated protein according to the invention. The isolated protein according to the invention can also be used in culturing cells, for example in the form of organoids, for example in 3D cultures (for example by coating hydrogels, with the isolated protein according to the invention; or for example by coating beads with the isolated protein according to the invention (see Example 24).

[071] Typically, 2D cell culture in the context of the current invention is to be understood to relate to culturing the cells on a two-dimensional substrate. The two-dimensional substrate may, for example, be coated with the isolated protein according to the invention, and the cells to be cultured in accordance with the method of the invention are seeded on the coated two-dimensional substrate.

[072] The two-dimensional substrate may be any kind of substrate, for rigid substrates like plastic or glass (for example coated with the isolated protein according to the invention) or (semi)-permeable membranes, such as, for example used in

Transwell culture systems, but may also include thin layer or film of an hydrogel, such as, for example a basal membrane extract, Matrigel, a collagen hydrogel, a fibrin hydrogel, an alginate hydrogel, polyacrylamide (PA) hydrogel, polyethylene glycol (PEG) hydrogels, hyaluronic acid based hydrogels (HA), polypeptide based hydrogels, protein hydrogels, e.g. elastin like protein (ELP) hydrogels, silk hydrogels, agarose hydrogels, gelatine based hydrogels, synthetic polymer hydrogels such as polyisocyanopeptide based hydrogels (PIC), ureidopyrimidinone based (Upy) hydrogels, cellulose based hydrogels, starch based hydrogels, chitosan based hydrogels, dextran based hydrogels, polylactic acid (PLA), polyglycolic acid (PGA), polylactic acid-co-glycolic acid (PLGA), PLGA-PEG (poly(ethylene glycol))-PGLA copolymers and/or decellularized ECM hydrogels. Culturing cells on 2D hydrogel films has some of the same advantages as disadvantages of as conventional 2D methods not using hydrogels but permits user-defined control of the substrate stiffness and adhesive ligand presentation. In accordance with the invention, the 2D substrate is considered a carrier that may be coated with the isolated protein according to the invention, independent of whether the 2D substrate is a rigid material or a hydrogel. Thus, in such embodiments, the two-dimensional structure is to be considered to act or function as a carrier of the isolated protein according to the invention.

[073] Typically, 3D cell culture in the context of the current invention is to be understood to relate to culturing the cells on and/or in a three-dimensional substrate. The three-dimensional substrate may, for example, be coated with the isolated protein according to the invention, and the cells to be cultured in accordance with the method of the invention are seeded on and/or in the three-dimensional substrate. Within the context of the current invention, coating of a three-dimensional substrate with the isolated protein according to the invention is meant to indicate that the isolated protein according to the invention has become associated with the three-dimensional substrate, for example hydrogel.

[074] Association may, for example be by binding or linking of the isolated peptide according to the invention with components, for example polymers, that form or will form the three-dimensional substrate, for example hydrogel. Consequently, the isolated protein according to the invention is not only present on the surface of the three-dimensional structure, but also in the interior parts of the three-dimensional structure, for example hydrogel. This thus allows cell that are present in the three-

dimensional substrate to be exposed to the isolated protein according to the invention, and wherein, in a preferred embodiment, the isolated protein according to the invention is bound to or associated with the three-dimensional substrate or components therein. In the context of the current invention, such three-dimensional structure is to be considered to act or function as a carrier of the isolated protein according to the invention. In embodiments according to the invention, association of the protein according to the invention may, for example be with beads, therewith allowing 3D culturing of the cells. In embodiments according to the invention, such beads (or other 3D substrates) may be comprised in or on a three-dimensional substrate, for example a gel (see below), therewith allowing 3D culturing of the cells (as shown in the Examples).

[075] In embodiments of the invention, the three-dimensional structure is a gel, preferably a hydrogel, suitable for culturing cell. Typical examples that may be used in accordance with the current invention include hydrogels such as basal membrane extracts, Matrigel, a collagen hydrogel, a fibrin hydrogel, an alginate hydrogel, polyacrylamide (PA) hydrogel, polyethylene glycol (PEG) hydrogels, hyaluronic acid-based hydrogels (HA), polypeptide-based hydrogels, protein hydrogels, e.g. elastin like protein (ELP) hydrogels, silk hydrogels, agarose hydrogels, gelatine based hydrogels, synthetic polymer hydrogels such as polyisocyanopeptide based hydrogels (PIC), ureidopyrimidinone based (Upy) hydrogels, cellulose based hydrogels, starch based hydrogels, chitosan based hydrogels, dextran based hydrogels, polylactic acid (PLA), polyglycolic acid (PGA), polylactic acid-co-glycolic acid (PLGA), PLGA-PEG (poly(ethylene glycol))-PGLA copolymers and/or decellularized ECM hydrogels, for example Affigel.

[076] In embodiments of the invention, the three-dimensional structure is a bead. The bead may, for example, be a resin bead, a gel bead, a glass bead, an iron bead, or any other suitable bead for using the culturing method according to the invention.

[077] The isolated protein according to the invention can be used for maintaining, proliferating, or differentiating cells, for example cells in an organoid. This and other aspects, embodiments, technical effects, and advantages according to the invention become apparent based on the disclosure provided herein.

[078] Therefore, and in accordance with the invention, there is provided for the use of an isolated protein comprising at least an integrin binding domain with at least 90%

identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus in cell culture. Cells to be cultured are exposed to the isolated protein according to the invention and subsequently cultured, preferably while maintaining exposure of the cells to the isolated protein according to the invention. Therefore, in accordance with the invention, there is provided for the use of an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus in cell culture. Cells to be cultured are exposed to the isolated protein during at least part of the (subsequent) culturing of the cells.

[079] Therefor, and in accordance with the invention, there is provided for an *in vitro* method of culturing cells, and wherein the method comprises a) exposing the cells to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus; and b) culturing the exposed cells in the presence of a culture medium.

[080] Therefor, and in accordance with the invention, there is provided for an *in vitro* method of culturing cells, and wherein the method comprises a) exposing the cells for at least 12 hours, at least 24 hours, at least 2, 3, 4, 5, 6, or 7 days, for example at least one week, for example at least 2, 3, 4 or more weeks to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus; and b) culturing the exposed cells in the presence of a culture medium.

[081] Within the context of the current invention the *in vitro* method of culturing the cells may be any typical method of culturing known to the skilled person.

[082] In some embodiment the method according to the invention is for expanding cells, i.e., a method wherein at least part of the cells are multiplied or increased in number. In some embodiments, and wherein a combination of different cells are used in the method of the invention, the method may be for expanding at least part of one type of cell in the cell culture but may also be for expanding at least part of all types of cells that are used in the method according to the invention.

[083] In some embodiment the method according to the invention is for maintain cells, i.e., a method wherein at least part of the cells are preserved or kept alive. In some embodiments, and wherein a combination of different cells are used in the

method of the invention, the method may be for maintaining at least part of one type of cell in the cell culture but may also be for maintaining at least part of all types of cells that are used in the method according to the invention.

[084] In some embodiment the method according to the invention is for growing cells, i.e., a method wherein at least part of the cells are grown. In some embodiments, and wherein a combination of different cells are used in the method of the invention, the method may be for growing at least part of one type of cell in the cell culture but may also be for growing at least part of all types of cells that are used in the method according to the invention. Growing cells may include proliferation of at least part of the cells and/or differentiation of at least part of the cells into specific cells or into more mature cells.

[085] Therefore, in some embodiment the method according to the invention is for proliferating cells, i.e., a method wherein at least part of the cells are proliferated. In some embodiments, and wherein a combination of different cells are used in the method of the invention, the method may be for proliferating at least part of one type of cell in the cell culture but may also be for proliferating at least part of all types of cells that are used in the method according to the invention.

[086] Therefore, in some embodiment the method according to the invention is for differentiating cells, i.e., a method wherein at least part of the cells are differentiated. In some embodiments, and wherein a combination of different cells are used in the method of the invention, the method may be for differentiating at least part of one type of cell in the cell culture but may also be for differentiating at least part of all types of cells that are used in the method according to the invention.

[087] The terms “culturing,” “expanding”, “maintaining”, “growing”, “proliferating” and “differentiating” are well-known and understood by the skilled person in the context of the current invention, and wherein these all are examples or embodiments of culturing according to the invention.

[088] For example, and as the skilled person understands, culturing cells in the presence of the isolated protein according to the invention can mean maintaining, propagating, differentiating cells under conditions that support their survival, growth, proliferation, differentiation, wherein the isolated protein according to the invention is included in the culturing with the purpose of influencing or sustain these processes. Thus, the skilled person understands this excludes, for example, mere incubation of

the cells with a protein according to the invention for purposes unrelated to the culturing of the cells, such as transient exposure for analytical, testing, or treatment purposes.

[089] The method of the invention provides that the cells to be cultured are exposed to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus. Within the context of the current invention, “exposing” is meant to indicate that the cells according to the invention are allowed to contact and interact with the isolated protein of the invention. The skilled person thus understands, for example, that ”exposing” in the context of the invention means that the protein according to the invention is included as part of the culture medium to influence or sustain the processes of maintaining, propagating, or differentiating the cells under conditions that support their survival, growth, proliferation, differentiation of these cells. Thus, the person skilled in the art understands that culturing includes exposure of the cells to the protein according to the invention during the culturing.

[090] The skilled person understands that exposure of the cell to an isolated protein according to the invention is preferably under conditions that are suitable for culturing the cells, in particular because cells are exposed to the isolated protein according to the invention during culturing.

[091] Thus, in some embodiments, the *in vitro* method of culturing cells comprises culturing cells in the presence of a culture medium, and wherein the cells are exposed to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, for example, for at least 12 hours.

[092] Exposure to a particular cell may be temporarily (for example for period of 1 hour or more, for example 5, 6, 7 or 8 hours, for example 12 hours), for example during the beginning of the culturing of the cells, or for a prolonged period of time (for example for at least 12 hours, for example, at least 24 hours, at least 2, 3, 4, 5, 6, or 7 days, for example at least one week, for example at least 2, 3, 4 or more weeks) during the culturing of the cells. Preferably, the cells are exposed to the isolated protein according to the invention for the full duration of the culturing.

[093] Exposure to a particular cell may be temporarily, for example for a period of time to allow cultured cells to propagate, expand, maintain or differentiate.

[094] For example, in preferred embodiments, the cells are exposed to the isolated protein according to the invention, for a period of at least 12 hours, at least 24 hours, at least 2, 3, 4, 5, 6, or 7 days, for example at least one week, for example at least 2, 3, 4 or more weeks. In other words, in such embodiments, the cells are exposed to the isolated protein according to the inventions during culturing for a period of at least 12 hours, at least 24 hours, at least 2, 3, 4, 5, 6, or 7 days, for example at least one week, for example at least 2, 3, 4 or more weeks.

[095] Preferably the cells are exposed to the isolated protein according to the invention, and wherein the cells are present in a culture medium, preferably the culture medium used for culturing (in step b). Exposing the cells to the isolated protein according to the invention may be by, for example, first providing a cell culture system (e.g., a culture disk or flask) with the isolated protein according to the invention but may also be by adding the isolated protein to a cell culture already comprising the cells to be cultured in accordance with the method according to the invention.

[096] Therefore, step a) of the method according to the invention is in embodiments of the invention a step of exposing the cells in the presence of a culture medium to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus.

[097] The isolated protein according to the invention comprises at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus. The *Yersinia* genus is a genus of facultative anaerobe Gram-negative, coccobacilli bacteria. Some members of *Yersinia* are pathogenic in humans, such as *Yersinia pestis*, which causes plague, *Yersinia pseudotuberculosis*, and *Yersinia* enterocolitica. The remaining species are generally considered non-pathogenic to humans; and genomic resource and analysis platforms for comparative analysis of *Yersinia* (see for example, Le-Bury et al (2023) Microbiol Spectr11(2):e0382622. doi: 10.1128/spectrum.03826-22). Currently, the genus *Yersinia* comprises 26 Gram-negative bacterial species, which include *Y. aldovae, Y. aleksiciae, Y. alsatica, Y. artesiana, Y. bercovieri, Y. canariae, Y. enterocolitica, Y. entomophaga, Y. frederiksenii, Y. hibernica, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretii, Y. nurmii, Y. pekkanenii, Y. pestis, Y.*

proxima, Y. pseudotuberculosis, Y. rochesterensis, Y. rohdei, Y. ruckeri, Y. similis, Y. thracica, Y. vastinensis, and Y. wautersii.

[098] Each of the species may be the species for which the isolated protein according to the invention displays at least 90% identity to the integrin binding domain of an invasin protein of that species. For example, the species may be *Y. enterocolitica.* In such embodiment, the isolated protein according to the invention comprises at least an integrin binding domain that has at least 90% identify compared to the integrin binding domain of an invasin protein that is encoded and/or expressed in *Y. enterocolitica.* In another example, the species may be *Y. pestis.* In such embodiment, the isolated protein according to the invention comprises at least an integrin binding domain that has at least 90% identify compared to the integrin binding domain of an invasin protein (also referred to in the art as intimin) that is encoded and/or expressed in *Y. pestis.* In the same way, each of the species disclosed herein may be the species for which the isolated protein according to the invention displays at least 90% identity to the integrin binding domain of an invasin protein of that species, as the skilled person understands.

[099] The invasin proteins of the *Yersinia* genus are bacterial membrane bound proteins that enable infiltration of, for example, human cells by bacteria expressing such protein. The interplay between invasin and the, for example, human cells, via β1 integrins, culminates in the internalization of bacteria through endosomes by the cell. In *Yersinia* species*,* for example *Yersinia pseudotuberculosis,* the extracellular region of invasin is composed of the COOH-terminal 497 residues of the protein and can be expressed as a soluble protein (referred to as Inv497, Inv-497 or Invasin 497; see also the Examples). This protein binds to integrins and facilitates uptake when attached to bacteria or beads. The shortest invasin fragment capable of integrin binding (e.g., the integrin binding domain) consists of the COOH-terminal 192 amino acids of an invasin protein (referred to as Inv192, Inv-192 or Invasin 192). In the case of *Yersinia* enterocolitica it consists of the COOH-terminal 193 amino acids of the invasin protein. When reference is made herein to the COOH-terminal 192 amino acids of an invasin protein, also to be understood to be included is the COOH-terminal 193 amino acids of the invasin protein of *Yersinia* enterocolitica (SEQ ID NO 10).

[100] In preferred embodiments, the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus is exposed to the cells in a manner that does not allow the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus to be internalized by the cells. This may for example, be achieved by coating or attaching the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus to a surface, object, molecule or network of molecules (e.g. polymers and the like forming a hydrogel) or any other carrier that cannot be internalized by the cell (for example, due to size or charge thereof). The skilled person knows how to achieve this. Thus, in a preferred embodiment, the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus is coated on a surface.

[101] In some embodiment of the invention, the integrin binding domain of an invasin protein of a species of the *Yersinia* genus consist or comprises of the COOH-terminal 192 amino acids of an invasin protein (In the case of *Yersinia* enterocolitica it consists of the COOH-terminal 193 amino acids of the invasin protein. When reference is made herein to the COOH-terminal 192 amino acids of an invasin protein, also to be understood to be included in the COOH-terminal 193 amino acids of the invasin protein of *Yersinia* enterocolitica (SEQ ID NO 10)). In some embodiment of the invention, the isolated protein according to the invention consists or comprises of the COOH-terminal 192 amino acids of an invasin protein (in which case there is 100% sequence identity with the integrin binding domain of an invasin protein of a species of the *Yersinia* genus; for example, Inv192). Notably, the 192 amino acid fragment lacks homology with the integrin-binding domains of fibronectin.

[102] In some embodiment of the invention, the integrin binding domain of an invasin protein of a species of the *Yersinia* genus consist or comprises of the COOH-terminal 497 amino acids of an invasin protein. In some embodiment of the invention, the isolated protein according to the invention consists or comprises of the COOH-terminal 497 amino acids of an invasin protein (in which case there is 100% sequence identity with the integrin binding domain of an invasin protein of a species of the *Yersinia*

genus; for example, Inv497). Notably, the 497 amino acid fragment lacks homology with the integrin-binding domains of fibronectin.

[103] The isolated protein according to the invention comprises at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus. In some embodiments, the isolated protein according to the invention comprises more than one integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus. The more than one integrin binding domains may be the same integrin binding domains but may also differ.

[104] The integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus is able to bind to an integrin, more particular to an αβ1 Integrin, such as α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combinations thereof, and as disclosed herein.

[105] In preferred embodiments, the integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus is able to bind to an integrin expressed by a cell, for example of a vertebrate cell, an animal cell, a mammalian cell, or snake cell, a rodent cell, or a human cell, the integrin preferably being an αβ1 Integrin, such as α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combinations thereof, or homologs/orthologs thereof. The skilled person knowns how to determine such binding, using conventional techniques readily available in the prior art.

[106] The integrin binding domain displays at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus. In addition to the integrin binding domain, the isolated protein according to the invention may comprise additional stretches of amino acids, either providing further functionality to the isolated protein according to the invention. In such embodiments, the isolated protein according to the invention may be referred to as an isolated fusion protein according to the invention. Examples of functionalities that may be contemplated in the isolated protein according to the invention are detectability, stability, facilitation purification, facilitation linking, associating, or binding to other compounds, including proteins, or facilitating coating of materials such as plastics, beads, glasses, and the like). For example, the isolated protein according to the invention may be a fusion

protein further comprising an antibody and/or further comprising an ECM ligand and so on.

[107] However, the 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus relates to the integrin binding domain of the isolated protein according to the invention. The skilled person will understand that it suffices if the integrin binding domain of the isolated protein according to the invention has at least 90% identity to the integrin binding domain of at least on invasin protein of at least one species of the *Yersinia* genus. It is not required for the integrin binding domain of the isolated protein to have at least 90% identity to the integrin binding domain of an invasin protein of more than one, or all species of the *Yersinia* genus.

[108] With increasing preference, the integrin binding domain of the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus.

[109] In embodiments wherein the integrin binding domain of the isolated protein according to the invention is shorter in length than the integrin binding domain of an invasin protein of a species of the *Yersinia* genus, sequence identity is determined for the overlapping amino acids only and using best fit.

[110] In embodiments wherein the integrin binding domain of the isolated protein according to the invention is longer in length than the integrin binding domain of an invasin protein of a species of the *Yersinia* genus, sequence identity is determined for the overlapping amino acids only and using best fit.

[111] In some embodiments, the isolated protein comprises at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, and wherein the integrin binding domain of the isolated protein comprises the cysteine (C) at position 907, the cysteine at position 982, the aspartic acid (D) at position 911 and the aspartic acid as position 811 of the invasin protein of *Yersinia pseudotuberculosis*, or at corresponding position in the invasin protein of any other species of the *Yersinia* genus (as the skilled person is able to determine). In some embodiments, although less preferred, one or more of these amino acids may be substituted with a functional similar amino acid such as glutamic acid (E), Asparagine (N) or Glutamine (Q) in case of the

aspartic acid (at position 911 and/or 811 indicated above). The above-indicated amino acids are conserved amino acids located in the integrin binding domain of invasin. Thus, in some embodiments, the integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus comprised in the isolated protein according to the invention comprises these four conserved amino acids cysteine, cysteine, aspartic acid, aspartic acid, at a position corresponding to position 907, position 982, position 911 and position 811 of the invasin protein of *Yersinia pseudotuberculosis* (or corresponding position in the invasin protein of any other species of the *Yersinia* genus).

[112] The integrin binding domain of the isolated protein according to the invention may, in the case of a fusion protein, be present at the N-terminal of the isolated peptide according to the invention, at the C-terminal of the isolated peptide according to the invention, or within the amino acid sequence of the isolated peptide according to the invention. In a preferred embodiment, the integrin binding domain of the isolated protein according to the invention is at the C-terminal of the isolated protein according to the invention.

[113] As defined herein, the term “isolated” when referring to a polynucleotide (nuclei acid) or polypeptide (protein), refers to proteins or nucleic acids being present in a non-naturally occurring environment, e. g. are separated from their naturally occurring environment. The isolated protein according to the invention is, in embodiments wherein the isolated protein is identical to an invasin protein of the *Yersinia* genus, not present in said species, or, for example, membrane fragments thereof.

[114] The cells that are exposed to the isolated protein according to the invention are subsequently cultured in the presence of a culture medium suitable for culturing the cells. In an embodiment, the culture medium is an aqueous culture medium.

[115] In the practice of the invention, any culture medium known to be suitable for the cells that are to be cultured in the culturing method according to the invention may be used. The culture media include any of the conventional culture media used in the art, including, for example DMEM (Dulbecco’s Modified Eagle Medium), RPMI 1640 Medium, DMEM/F12 Medium, MEM (Minimum Essential Medium), Ham’s F-10 and F12 Medium, Medium 199 (M199) and the like.

[116] As detailed herein, in a preferred embodiment, during the culturing of the cells in step b), the cells are continued to be exposed to the isolated protein according to the invention. In other words, during at least part, preferably all, of the culturing of the cells, the cells are cultured in the presence of the isolated protein according to the invention.

[117] In accordance with the invention, and in a preferred embodiment, there is provided that the isolated protein according to the invention is coated on a carrier.

[118] The skilled person will understand the carrier should be a carrier that is suitable for use in cell culture. Such suitable carriers for use in accordance with the invention are well-known to the skilled person.

[119] Within the context of the current invention, coating of the carrier with the isolated protein according to the invention is meant to indicate that the isolated protein according to the invention has become associated with the carrier, for example by direct binding or by indirect binding, for example via a linker. The binding to the carrier may be by covalent binding or may be by non-covalent binding.

[120] Association with the carrier may, for example, be by binding or linking of the isolated peptide directly to a surface of a substrate, for example to glass or plastics suitable for culturing. Coating may also be binding of the isolated polypeptide according to the invention to a component comprised in the carrier. For example, when the carrier is a gel or hydrogel, the isolated protein according to the invention may be linked to components, for example polymers, in the gel or hydrogel and that form or will form the two-dimensional or three-dimension gel. Consequently, the isolated protein according to the invention is not only present on the surface of the gel but is also included in the interior parts of the gel, hydrogel, or any other three-dimensional structure. This thus allows cells that are present in the three-dimensional substrate (for example, in 3D culture, for example when culturing organoids) to be exposed to the isolated protein according to the invention. Therefore, in a preferred embodiment the isolated protein according to the invention is linked to or bound to the gels or components present in the gel. In the context of the current invention, such gel is to be considered to act or function as a carrier of the isolated protein according to the invention. Thus, in such embodiments the isolated protein is bound or linked to the gel, including hydrogel, or to a component that is present is such gel or hydrogel. In these embodiments, the isolated protein

according to the invention is available throughout the gel, including its surface and/or its interior.

[121] The carrier may be a non-porous surface. In some embodiments, the carrier is glass, the glass being suitable for use in cell culture. In some embodiments, the carrier is a plastic, the plastic being suitable for cell culture. Examples of such plastic or glass carrier include culturing dishes or culturing flasks. Other examples include glass beads and plastic beads. These and other non-porous surfaces suitable for use in the culturing of cells are well-known to the skilled person.

[122] In some embodiments, the carrier may be a porous surface, the porous surface being suitable for cell culture. In some embodiments, the carrier is a membrane, the membrane being suitable for cell culture. An example of such membrane includes membranes as used in Transwell inserts. The membranes may, for example, have a pore size of about 0.1 - 0.8 μm. An example of a membrane suitable for cell culture are Polytetrafluoroethylene membranes and the like.

[123] In some embodiments the carrier is a gel being suitable for cell culture. In some embodiments the carrier is a hydrogel gel being suitable for cell culture.

[124] The skilled person is well-aware of gel or hydrogels that may suitable be used in the context of the current invention. Examples of such gels and hydrogels include gel based on basal membrane extracts, Matrigel, a collagen hydrogel, a fibrin hydrogel, an alginate hydrogel, polyacrylamide (PA) hydrogel, polyethylene glycol (PEG) hydrogels, hyaluronic acid-based hydrogels (HA), polypeptide-based hydrogels, protein hydrogels, e.g. elastin like protein (ELP) hydrogels, silk hydrogels, agarose hydrogels, gelatine based hydrogels, synthetic polymer hydrogels such as polyisocyanopeptide based hydrogels (PIC), ureidopyrimidinone based (Upy) hydrogels, cellulose based hydrogels, starch based hydrogels, chitosan based hydrogels, dextran based hydrogels, polylactic acid (PLA), polyglycolic acid (PGA), polylactic acid-co-glycolic acid (PLGA), PLGA-PEG (poly(ethylene glycol))-PGLA copolymers, and/or decellularized ECM hydrogels. An example is Affigel. As will be understood by the skilled person, the gels or hydrogel may also contain other components, such as other proteins like fibronectin. The skilled person is well aware of how to prepare and use such gels in culturing cells, and in accordance with the current invention. In some embodiments, the carrier is an extracellular matrix. In some embodiments, the carrier is Matrigel. In some embodiments, the carrier is a

basal membrane extract. In some embodiments, the carrier prevents internalization of the isolated protein according to the invention by the cells.

[125] In accordance with the invention, and in a preferred embodiment, there is provided that the isolated protein according to the invention is coated on a carrier by covalent binding or wherein the isolated protein is coated on a carrier by non-covalent binding and/or wherein the isolated protein is coated on a carrier by direct binding or by indirect binding through an intermediary.

[126] The isolated protein according to the invention may bind to the carrier directly or indirectly through an intermediary. The intermediary may be a chemical linker or another protein or a combination thereof. The linker may be photo or chemically sensitive. Furthermore, the isolated protein according to the invention may be non-specifically adsorbed to the carrier or may be covalently coupled or attached to the carrier, for example through affinity tag-binding partner interaction. The isolated protein according to the invention may also be linked to a polymer present or included in the carrier. The skilled person is well-aware of conventional methods readily available in the art that allow to achieve the above.

[127] As indicated herein, the carrier may, in preferred embodiments, be gel or hydrogel. In such embodiments, the isolated protein according to the invention may be included throughout the gel or hydrogel, and wherein the isolated protein according to the invention is linked to the gel or hydrogel by covalent binding or by non-covalent biding, and/or by direct binding or by indirect binding through an intermediary, and as already detailed above.

[128] As will be understood by the skilled person, the concentration, amount, or density of the isolated protein according to the invention to be used should be sufficient to allow providing a cell sufficient signal as to allow it to be cultivated in accordance with the method according to the invention. The inventors have demonstrated in experiments that if, for example, density of the isolated protein, when coated on the surface of a plastic culture disk, is low, interaction with the cells (exposure) may not be sufficient to allow the beneficial culturing in accordance with the invention. The skilled person thus understand that in such case, the density of the isolated protein on the plastic surface should be increased and can do so by routine experimentation. For example, for coating 2D surfaces, e.g., flasks or Transwell inserts, provided with a hydrogel film or not, sufficient coating can be

achieved by incubating the 2D surface at a concentration of about 1 – 50 micrograms of the isolated protein according to the invention per ml, for example between 5 and 35 microgram per ml.

[129] Likewise, in case the isolated protein according to the invention is included in a hydrogel, for example in 3D culturing, the concentration or density should be adapted in case the concentration or density appears to be low.

[130] The inventors believe that a minimal density and/or concentration is required in order to have sufficient exposure of the isolated protein according to the invention to the cells, to allow the cells to be cultivated.

[131] In some embodiments, it is thereof contemplated that during culturing in accordance with the inventions, additional isolated protein according to the invention is provided to the culture system (e.g., flask, bottle, sac, or bioreactor), for example coated to a carrier.

[132] It is believed that it is important that isolated proteins according to the invention of ECM can simultaneously bind a multitude of integrin receptors present on the cells. This multivalence stabilizes the binding between ligand and integrin. In vivo the multivalence is achieved because proteins such as collagen or laminins form a mutual network. In the present invention, the ligand molecules (i.e., the isolated proteins according to the invention) are indirectly presented multivalently by linking (coating) them to a (synthetic network) or coating them on a carrier. All this leads, among other things, to the strengthening of intracellular signals that ensure the polarity of the cells.

[133] In 3D organoids growing in Matrigel or any other gel, the basal side is therefore directed outwards. The apical side is directed towards the lumen of the organoid (spherical shape). The basal side contains all receptors for growth factors (Wnt signal, epidermal growth factor, and so on). In the absence of Matrigel, or other ECMs such as collagen, the polarity will immediately reverse, resulting in loss of growth. During 3D growth in Matrigel, ECM proteins are present around the growing organoid. That enforces the correct polarity. With 2D growth in Transwell systems, this polarity is enforced by the coating. Cells align themselves with the basal side towards the coat. Growth factors are now offered through the medium in the bottom compartment. Laminins are very capable of forming a network among themselves. When part of a laminin protein (E8 laminins) is removed, network formation fails.

They are then useless for organoid growth. However, if you coat the same E8 molecules on a plastic surface, they function fine.

[134] It has now been found that the isolated protein according to the invention has the same properties as ligand for integrin receptors and may be used as an alternative of, for example, Matrigel or BME.

[135] In accordance with the invention, and in a preferred embodiment, there is provided that the cell culture is a 2D cell culture, a 3D cell culture, an organoid, a tissue, or a primary tissue.

[136] Therefore, there is provided for a method according to the invention and wherein the method of culturing the cells is a method for culturing a 2D cell culture, a 3D cell culture, an organoid, a tissue, or a primary tissue.

[137] It was surprisingly found that the isolated protein according to the invention may be used in culturing a wide variety of cells and in a variety of models, including 2D and 3D cell culture models.

[138] It was in particular found that the isolated protein according to the invention may suitably be used in the culture of organoids, including tumoroids. Preferably, the organoid or tumoroid comprises at least epithelial cells. Preferably, the organoid or tumoroid comprises at least endothelial cells.

[139] It was in particular found that the isolated protein according to the invention may suitably be used in the culture of a tissue. The tissue may, for example, be obtained from a healthy or diseased subject, and may be obtained from a diseased tissue. The tissue may also have been cultivated *in vitro*. Preferably, the tissue comprises at least epithelial cells. Preferably, the tissue comprises at least endothelial cells.

[140] It was in particular found that the isolated protein according to the invention may suitably be used in the culture of a primary tissue.

[141] In accordance with the invention, and in a preferred embodiment, there is provided that the isolated protein according to the invention is a protein that binds or activates an αβ1 Integrin expressed on the cell surface of the exposed cells, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

[142] Therefore, there is provided for the method according to the invention, and wherein the isolated protein binds or activates an αβ1 Integrin expressed on the cell

surface of the exposed cells, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof. Moreover, there is provided for the method according to the invention, and wherein the cells are cells expressing an αβ1 Integrin, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

[143] Integrins are heterodimeric cell surface receptors which are known to facilitate attachment of cells to their surrounding tissues, including *in vivo* extracellular matrix (ECM) structures such as laminins, collagen and which play a significant role in cell signaling and signal transduction from the *in vivo* ECM to cells, involving cell growth, division, differentiation, survival, or death. Integrins are vitally important to a wide range of multicellular organisms, since cell attachment to the ECM is a basic requirement to create a multicellular organism.

[144] The integrins are a family of heterodimeric cell surface receptors involved in cell-cell and cell-substrate adhesion. They act as bridging molecules that link intracellular signaling molecules to the extracellular matrix through bi-directional signaling and control cell behavior and tissue architecture.

[145] As shown in the Examples herein, and/or demonstrated by the inventors in experiments, it was surprisingly found that the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia genus* according to the invention allows cells to be cultured in the absence of any ECM ligand, such as laminin, fibronectin, or collagen, or Matrigel.

[146] In a preferred embodiment, the cells express at least one αβ1 Integrin, i.e., at least one integrin comprising a β1-subunit and an α-subunit. As is known to the skilled person, various isoforms of the α-subunit and of the β-subunit exist. For example, in mammals, integrins are assembled from at least eighteen α-subunits and at least eight β-subunits. At least four integrins, α1β1, α2β1, α10β1 and α11β1, have been shown to bind collagens. At least three integrins, α3β1, α6β1, and α7β1, have been shown to bind laminins. α5β1 is an example of an integrin that binds with fibronectin.

[147] In another preferred embodiment, the cells express at least one α3β1 Integrin. In another preferred embodiment, the cells express at least one α6β1 Integrin. In another embodiment, the cells express at least one α5β1 Integrin. In another preferred

embodiment, the cells express at least one α3β1 Integrin and a α6β1 Integrin. In another preferred embodiment, the cells express at least one α3β1 Integrin and a α5β1 Integrin. In another preferred embodiment, the cells express at least one α6β1 Integrin and a α5β1 Integrin. In another preferred embodiment, the cells express at least one α3β1 Integrin, a α6β1 Integrin, and a α5β1 Integrin. It was surprisingly found that the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus according to the invention is able to bind and activate an αβ1 Integrin expressed on the cell surface of the exposed cells. Preferably, the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus according to the invention is able to bind an α3β1 Integrin. Preferably, the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus according to the invention is able to bind an α6β1 Integrin. Preferably, the isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus according to the invention is able to bind an α5β1 Integrin.

[148] In a preferred embodiment, the isolated protein bind at least a α3β1 Integrin and a α6β1 Integrin. In a preferred embodiment, the isolated protein bind at least a α3β1 Integrin and a α5β1 Integrin. In a preferred embodiment, the isolated protein bind at least a α6β1 Integrin and a α5β1 Integrin. In a preferred embodiment, the isolated protein bind at least a α3β1 Integrin, a α6β1 Integrin and a α5β1 Integrin.

[149] The term “binding or activating” an αβ1 integrin, and the like, is to indicate that the isolated protein according to the invention is able to interact with the αβ1 integrin, thereby prompting the αβ1 integrin to activate signal transduction pathways in the cell, including pathways that mediate cellular signals such as regulation of the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane.

[150] In some embodiments, the cells, for example epithelial cells, express the αβ1 Integrin according to the invention before the cells are exposed to the isolated protein according to the invention. However, it is also contemplated that the cells first express the αβ1 Integrin according to the invention shortly after the cells have been exposed

to the isolated protein according to the invention, or during exposure to the isolated protein according to the invention.

[151] In accordance with the invention, and in a preferred embodiment, there is provided that the cells, i.e., the cells exposed to the isolated protein according to the invention, are cells selected from the group consisting of vertebrate cells, mammalian cells or human cells.

[152] Therefore, there is provided for the method according to the invention, and wherein the cells, i.e., the cells exposed to the isolated protein according to the invention, are cells selected from the group consisting of vertebrate cells, mammalian cells or human cells. The cells may, for example, be snake cells, pig cells, rodent cells, primate cells, and human cells. As shown in the Examples herein, and/or demonstrated by the inventors in experiments, it was surprisingly found that the isolated protein according to the invention was able to support culturing of cells from different vertebrates, including snake and humans, supporting the broad applicability of the invention disclosed herein.

[153] In addition, in accordance with the invention, and in a preferred embodiment, there is provided that the cells, i.e., the cells exposed to the isolated protein according to the invention, are cells selected from the group consisting of stem cells, induced-pluripotent stem cells, adult stem cells, primary cells, epithelial cells, and endothelial cells. Therefore, there is provided for the method according to the invention, and wherein the cells, i.e., the cells exposed to the isolated protein according to the invention, are cells selected from the group consisting of stem cells, induced-pluripotent stem (iPS) cells, adult stem cells, primary cells, a cell line, epithelial cells, and endothelial cells. The cells may be undifferentiated or differentiated cells. Preferably the cells are polarized cells. Preferably, the cells are epithelial cells. Preferably, the cells are endothelial cells. Preferably, the cells are stem cells. Preferably, the cells are induced iPS cells. Preferably, the cells, including the stem cells, are epithelial in nature in that they already express αβ1 integrin. Therefore, in a preferred embodiment, the cells are cells that express αβ1 integrin.

[154] The cells may be obtained from a tissue from a subject, or may, for example, be obtained from prior cell culturing, for example, from prior organoids. As will be understood by the skilled person, such prior culturing may be any type of culturing,

including culturing that, for example, includes the use of basal membrane extracts such as Matrigel.

[155] The cells may be one type of cells but may also be a combination of distinct types of cells. For example, is some embodiments, the cells may be obtained from a tissue sample of a subject, including for example, a tumor sample, and which may comprise more than one type of cell. For example, it is contemplated that the cells used in the invention is a combination of at least epithelial cells and epithelial stem cells, or of epithelial cells and unipotent or multipotent progenitor cells of such epithelial cells. In another example, it is contemplated that the cells used in the invention is a combination of at least epithelial cells and mesenchymal cells, or epithelial cells, epithelial stem cells and mesenchymal cells. In other examples, part of the cells express αβ1 integrin (receptors). The skilled person can envisage similar combinations of cells while using endothelial cells instead of epithelial cells.

[156] The cells according to the invention may be obtained or derived for different part of a body. For example, and preferably, the cells may be any cell from liver, pancreas, intestinal tract, lung, kidney, adrenal gland, breast, prostate, skin, eye, oral cavity, central nervous system, or any carcinoma. Preferably the cell is an epithelial cell. Preferably the cells is an epithelial liver cell, epithelial pancreas cell, epithelial intestinal tract cell, epithelial lung cell, epithelial kidney cell, epithelial adrenal gland cell, epithelial breast cell, epithelial prostate cell, epithelial skin cell, epithelial eye cell, epithelial oral cavity cell, or any epithelial carcinoma cell.

[157] In some embodiments the cells are diseased cells. In some embodiments, the cells are healthy cells. In some embodiments the cells have been obtained from a healthy subject, or the cells are derived from cells obtained from a healthy subject. In some embodiments, the cells have been obtained from a diseased subject, or the cells are derived from cells obtained from a diseased subject. For example, the cells may be obtained from a diseased tissue or may be derived from cells obtained from a diseased subject. The term “derived” is here meant to indicate that the cells are cells that have been cultured from cells initially obtained from a subject, by one passage or more.

[158] In accordance with the invention, and in a preferred embodiment, there is provided that the species of the *Yersinia* genus is selected from the group consisting of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*,

and *Yersinia enterocolitica* cells, i.e. the isolated protein according to the invention is a protein comprising an integrin binding domain with at least 90% identity to an integrin binding domain of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*, or *Yersinia enterocolitica* cells.

[159] Therefore, there is provided for the method according to the invention, and wherein the species of the *Yersinia* genus is selected from the group consisting of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*, and *Yersinia enterocolitica*.

[160] In a preferred embodiment, the species of the *Yersinia* genus is *Yersinia pseudotuberculosis.* In a preferred embodiment, the species of the *Yersinia* genus is *Yersinia pestis.* In a preferred embodiment, the species of the *Yersinia* genus is *Yersinia similis.* In a preferred embodiment, the species of the *Yersinia* genus is *Yersinia wautersii*. In a preferred embodiment, the species of the *Yersinia* genus is *Yersinia enterocolitica.* Most preferred, the species is *Yersinia pseudotuberculosis.*

[161] As shown in the Examples herein, and/or demonstrated in experiments, or contemplated by the inventors, it is viewed that an isolated protein according to the invention that comprising at least an integrin binding domain and wherein that integrin binding domain has at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus is useful in the invention as disclosed herein.

[162] In particular, based on the amino acid sequence of the integrin binding domains of an invasin protein of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*, and *Yersinia enterocolitica* is it easily contemplated by the skilled person, and based on the disclosure herein, that an isolated protein according to the invention that comprising at least an integrin binding domain and wherein that integrin binding domain has at least 90% identity to an integrin binding domain of an invasin protein of these species may be used in the current invention.

[163] In accordance with the invention, and in a preferred embodiment, there is provided that the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 – 5.

[164] Therefore, there is provided for the method according to the invention, and wherein the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

[165] In an embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 1. SEQ ID NO: 1 corresponds with the amino acid sequence of the invasin protein of *Yersinia pseudotuberculosis* (see, for example EMBL GenBank CCH50678.1, CCH50686.1, CCH50688.1, CCH50690.1, CCH50693.1).

[166] In an embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 2. SEQ ID NO: 2 corresponds with the amino acid sequence of the invasin protein of *Yersinia pestis* (see, for example, NCBI sequence WP_244662257.1).

[167] In an embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 3. SEQ ID NO: 3 corresponds with the amino acid sequence of the invasin protein of *Yersinia similis* (see, for example, NCBI sequence WP_025383552.1).

[168] In an embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 4. SEQ ID NO: 4 corresponds with the amino acid sequence of the invasin protein of *Yersinia wautersii* (see, for example, NCBI REF. SEQ. WP_155979599.1).

In an embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 5. SEQ ID NO: 5 corresponds with the amino acid sequence of the invasin protein of *Yersinia enterocolitica* (see, for example, EMBL protein sequence CAA37448.1).

[169] In a preferred embodiment, the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence according to SEQ ID NO: 1.

[170] In some embodiments, any of the SEQ ID NO:1 – 5 may comprise one, two, three of four amino acid modifications, for example a deletion, a substitution, or an insertion.

[171] In accordance with the invention, and in a preferred embodiment, there is provided that the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to at least 50, 100, 150, 180 or

more adjacent amino acids of the last 250 amino acids of the C-terminus of an amino acid sequence selected from the group consisting of SEQ ID NO : 1 - 5.

[172] Therefore, there is provided for the method according to the invention and wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to at least 50, 100, 150, 180 or more adjacent amino acids of the last 250 amino acids of the C-terminus of an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

[173] In accordance with the invention, and in a preferred embodiment, there is provided that the integrin binding domain comprised in the isolated protein according to the invention comprises or consists of an amino acid sequence with at least 90% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

[174] In an embodiment, the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 6. SEQ ID NO: 6 corresponds with the amino acid sequence of the integrin binding domain of *Yersinia pseudotuberculosis* (192 amino acids at the C-terminal of the invasin protein).

[175] In an embodiment, the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 7. SEQ ID NO: 7 corresponds with the amino acid sequence of the integrin binding domain of *Yersinia pestis* (192 amino acids at the C-terminal of the invasin protein).

[176] In an embodiment, the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 8. SEQ ID NO: 8 corresponds with the amino acid sequence of the integrin binding domain of *Yersinia similis* (192 amino acids at the C-terminal of the invasin protein).

[177] In an embodiment, the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 9. SEQ ID NO: 9 corresponds with the amino acid sequence of the integrin binding domain of *Yersinia wautersii* (192 amino acids at the C-terminal of the invasin protein).

[178] In an embodiment, the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 10. SEQ ID NO: 10 corresponds with the amino acid sequence of the integrin binding domain of *Yersinia* enterocolitica (193 amino acids at the C-terminal of the invasin protein).

[179] With increasing preference, the integrin binding domain comprised in the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:6.

[180] With increasing preference, the integrin binding domain comprised in the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:7.

[181] With increasing preference, the integrin binding domain comprised in the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:8.

[182] With increasing preference, the integrin binding domain comprised in the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:9.

[183] With increasing preference, the integrin binding domain comprised in the isolated protein according to the invention has at least 90%, 91%, 92%, 93%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:10.

[184] In some embodiments, any of the SEQ ID NO: 6 – 10 may comprise one, two, three of four amino acid modifications, for example a deletion, a substitution, or an insertion. Preferably the one, two, three or four amino acid modifications do not substantially modify the integrin binding functionality of the integrin binding domain comprised in the isolated protein according to the invention. The skilled person is well aware how to establish this using conventional techniques readily available.

[185] In a preferred embodiment, there is provided for the method according to the invention wherein the integrin binding domain comprised in the isolated protein comprises or consist of an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

[186] In a preferred embodiment, the isolated protein according to the invention comprises or consist of a protein with the amino acid sequence of SEQ ID NO: 6.

[187] In a preferred embodiment, the isolated protein according to the invention comprises or consist of a protein with the amino acid sequence of SEQ ID NO: 7.

[188] In a preferred embodiment, the isolated protein according to the invention comprises or consist of a protein with the amino acid sequence of SEQ ID NO: 8.

[189] In a preferred embodiment, the isolated protein according to the invention comprises or consist of a protein with the amino acid sequence of SEQ ID NO: 9.

[190] In a preferred embodiment, the isolated protein according to the invention comprises or consist of a protein with the amino acid sequence of SEQ ID NO: 10.

[191] Preferably, the isolated protein according to the invention consist of a protein with the amino acid sequence of SEQ ID NO: 6.

[192] In accordance with the invention, and in a preferred embodiment, there is provided that the isolated protein according to the invention is produced in a host cell or chemically. The skilled person is well aware of conventional techniques to produce the proteins, such as the isolated protein according to the invention in a host cell or by using chemical means not involving any cell. Host cells may be any type of host cell, including for example bacterial cells such as E.coli, or yeast cells. Another suitable host cell could be a baculovirus or viral like baculovirus system. However, in a preferred embodiment, the host cell is of the same species as the species of the cells that will be exposed in the method according to the invention to the isolated protein. For example, in case in the method of the invention culturing of human cells is contemplated, in a preferred embodiment the host cell used to produce the isolated protein according to the invention for use in the method, is a human host cell as well. However, any other type of suitable host cells may be used in producing the isolated protein according to the invention.

[193] Once produced by the host cell, the isolated protein according to the invention may be isolated and purified, using conventional techniques known to the skilled person, and directly used or stored before using in the invention.

[194] In accordance with the invention, and in a preferred embodiment, there is provided that culturing of the exposed cells is for a period selected from the group consisting of at least one day, at least 2 days, at least 3 days, at least one week, or at least two weeks or more.

[195] In accordance with the invention, and in another preferred embodiment, there is provided that culturing of the exposed cells is for a period to allow cultured cells to propagate, expand, maintain or differentiate.

[196] In a preferred embodiment of the invention, the culturing of the exposed cells in step b) of the invention is in the presence of the isolated protein according to the invention. In other words, in a preferred embodiment of the invention, the cells are exposed to the isolated protein according to the invention also during step b), or at least during at least part of step b). In such embodiments, step b) would comprise culturing the exposed cells in the presence of a culture medium in the (continued) presence of the isolated protein of step a).

[197] As will be understood by the skilled person, during the culturing of the exposed cells, the culture media may be refreshed or may be replaced with another culture media (e.g., with different composition) as may be desired, and depending on the particular cell culture. The culture media may be any suitable media for culturing the cells and may include growth factors such a Wnt signaling agonists or antagonists, BMP signaling agonists or antagonists, EGF signaling agonists or antagonists, FGF signaling agonists or antagonists, cAMP modulating compounds, and the like. The culture media may comprise serum. The culture media may be free of serum.

[198] As was demonstrated by experiments performed by the inventors, including experiments shown herein, the method according to the invention is in particular suitable for culturing epithelial cells and/or endothelial cells. In particular it was found that by using the isolated protein according to the invention, correct apical-basal polarity of the epithelial cells and/or the endothelial cells was found.

[199] Therefore, and in accordance with the invention, in a preferred embodiment, the method according to the invention is for promoting polarization of epithelial cells and/or endothelial cells.

[200] Cell polarity (or polarization) refers to spatial differences in shape, structure, and function within a cell. Almost all cell types exhibit some form of polarity, which enables them to carry out specialized functions. Classical examples of polarized cells include epithelial cells with apical-basal polarity. Cells can polarize in response to chemical, electrical, mechanical, or other physical stimuli. There are conventional techniques known by the skilled person to achieve polarity of for example epithelial

and/or endothelial cells. The method of the invention, in which a cell is exposed (during culturing) to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, provides a new tool to the field.

[201] In accordance with the invention, and in a preferred embodiment, there is provided that method further comprises drug screening, drug toxicity assessment, or drug dosing assessment.

[202] The invention provides the use of the method or use of the cells thus obtained in drug screening, drug toxicity assessment, drug dosing assessment, drug target validation, drug target discovery, toxicology and toxicology screens, personalized medicine, regenerative medicine and/or as ex vivo cell/organ models, such as disease models. Another use provided for is for the use of the cells obtained with the method according to the invention, for example an organoid, in cell therapy, for example for replacement of tissue via transplant or regrowth.

[203] Therefore, the invention provides a method of drug screening, and/or of drug toxicity assessment, and/or of drug dosing assessment, in which the method of the invention, comprising (a) exposing the cells to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, and (b) culturing the exposed cells (i.e. the cells being exposed to the protein according to the invention) in the presence of a culture medium, is carried out; and further a drug is contacted with the cells previously obtained.

[204] The cells cultured according to the invention are thought to faithfully represent the *in vivo* situation. Therefore, as well as providing normal ex vivo cell/organ models, the cells of the invention can be used as ex vivo disease models.

[205] Diseases that can be studied with the cells of the invention may include genetic diseases, metabolic diseases, pathogenic diseases, inflammatory diseases etc., for example including, but not limited to cystic fibrosis, inflammatory bowel disease (such as Crohn's disease), carcinoma, adenoma, adenocarcinoma, cancer, diabetes (such as type I or type II), hypercholesterolemia, etc.

[206] In accordance with the invention, there is provided for the isolated protein as defined herein, preferably wherein the isolated protein is coated on a carrier.

[207] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is coated to a plastic surface (plastic substrate or carrier).

[208] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is coated to a glass surface (glass substrate or carrier).

[209] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is coated to a (semi) porous membrane, for example a PTFE membrane, for example as used in Transwell inserts.

[210] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is linked to or bound to a hydrogel.

[211] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is present throughout a hydrogel, including these disclosed herein.

[212] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is not an invasin protein.

[213] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is a fusion protein.

[214] In some embodiments, there is provided for the isolated protein according to the invention, and wherein the isolated protein according to the invention is a non-natural protein.

[215] Also provided is for a nucleic acid encoding an isolated protein according to the invention. Preferably, the nucleic acid encodes for a fusion protein according to the invention.

[216] Also provided is for a host cell comprising the isolated protein as disclosed herein, or a nucleic acid as disclosed herein. Preferably, the isolated protein is a fusion protein.

[217] Also provided is for a carrier coated with the isolated protein according to the invention, preferably wherein the carrier is sterile. The skilled person will understand

the carrier should be a carrier that is suitable for use in cell culture. Such suitable carriers for use in accordance with the invention are well-known to the skilled person. Within the context of the current invention, coating of the carrier with the isolated protein according to the invention is meant to indicate that the isolated protein according to the invention has become associated with the carrier, for example by direct binding or by indirect binding, for example via a linker. The binding to the carrier may be by covalent binding or may be by non-covalent binding .

[218] Association with the carrier may, for example, be by binding or linking of the isolated peptide directly to a surface of a substrate, for example to glass or plastics suitable for culturing. Coating may also be binding of the isolated polypeptide according to the invention to a component comprised in the carrier. For example, when the carrier is a gel or hydrogel, the isolated protein according to the invention may be linked to components, for example polymers, in the gel or hydrogel and that form or will form the two-dimensional or three-dimension gel. Consequently, the isolated protein according to the invention is not only present on the surface of the gel but is also included in the interior parts of the gel, hydrogel, or any other three-dimensional structure. This thus allows cells that are present in the three-dimensional substrate (for example, in 3D culture, for example when culturing organoids) to be exposed to the isolated protein according to the invention. Therefore, in a preferred embodiment the isolated protein according to the invention is linked to or bound to the gels or components present in the gel. In the context of the current invention, such gel is to be considered to act or function as a carrier of the isolated protein according to the invention. Thus, in such embodiments the isolated protein is bound or linked to the gel, including hydrogel, or to a component that is present is such gel or hydrogel. In these embodiments, the isolated protein according to the invention is available throughout the gel, including its surface and/or its interior.

[219] The carrier may be a non-porous surface. In some embodiments, the carrier is glass, the glass being suitable for use in cell culture. In some embodiments, the carrier is a plastic, the plastic being suitable for cell culture. Examples of such plastic or glass carrier include culturing dishes or culturing flasks. Other examples include glass beads and plastic beads. These and other non-porous surfaces suitable for use in the culturing of cells are well-known to the skilled person.

[220] In some embodiments, the carrier may be a porous surface, the porous surface being suitable for cell culture. In some embodiments, the carrier is a membrane, the membrane being suitable for cell culture. An example of such membrane includes membranes as used in Transwell inserts. The membranes may, for example, have a pore size of about 0.1 - 0.8 µm. An example of a membrane suitable for cell culture are Polytetrafluoroethylene membranes and the like.

[221] In some embodiments the carrier is a gel being suitable for cell culture. In some embodiments the carrier is a hydrogel gel being suitable for cell culture.

[222] The skilled person is well-aware of gel or hydrogels that may suitable be used in the context of the current invention. Examples of such gels and hydrogels include gel based on basal membrane extracts, Matrigel, a collagen hydrogel, a fibrin hydrogel, an alginate hydrogel, polyacrylamide (PA) hydrogel, polyethylene glycol (PEG) hydrogels, hyaluronic acid-based hydrogels (HA), and polypeptide-based hydrogels. Other examples include Affigel and PIC gels as discussed herein elsewhere. As will be understood by the skilled person, the gels or hydrogel may also contain other components, such as other proteins like fibronectin. The skilled person is well aware of how to prepare and use such gels in culturing cells, and in accordance with the current invention. In some embodiments, the carrier is an extracellular matrix. In some embodiments, the carrier is Matrigel. In some embodiments, the carrier is a basal membrane extract.

[223] In accordance with the invention, there is provided for the use of the isolated protein as defined and disclosed herein for use in cell culture. The use may be according to the embodiments disclosed herein.

[224] There is also provided for a method of preparing a carrier coated with a protein according to the invention, and wherein the method comprises contacting the isolated protein according to the invention with the carrier thereby allowing the carrier to be coated with the isolated protein (in accordance with what has been disclosed herein with respect to the coating for the various carries, for example hydrogels).

[225] Finally, there is provided for a cell culture system that comprises a carrier, and wherein the carrier has been coated with the isolated protein ((in accordance with what has been disclosed herein with respect to the coating of the various carrier, for example hydrogels). Preferably the cell culture system is sterile.

Preferably the cell culture system further comprises cells, for example epithelial cells, for example an organoid.

[226] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein.

[227] All references cited herein, including journal articles or abstracts, published or corresponding patent applications, patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

[228] It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

[229] It will be understood that all details, embodiments, and preferences discussed with respect to one aspect of embodiment of the invention is likewise applicable to any other aspect or embodiment of the invention and that there is therefore not need to detail all such details, embodiments, and preferences for all aspect separately.

[230] As regards the embodiments characterized in this specification, it is intended that each embodiment be read independently as well as in combination with another embodiment. For example, in case of an embodiment 1 reciting 3 alternatives A, B and C, an embodiment 2 reciting 3 alternatives D, E and F and an embodiment 3 reciting 3 alternatives G, H and I, it is to be understood that the specification unambiguously discloses embodiments corresponding to combinations A, D, G; A, D, H; A, D, I; A, E, G; A, E, H; A, E, I; A, F, G; A, F, H; A, F, I; B, D, G; B, D, H; B, D, I; B, E, G; B, E, H; B, E, I; B, F, G; B, F, H; B, F, I; C, D, G; C, D, H; C, D, I; C, E, G; C, E, H; C, E, I; C, F, G; C, F, H; C, F, I, unless specifically mentioned otherwise.

[231] Having now generally described the invention, the same will be more readily understood through reference to the following examples which is provided by way of illustration and is not intended to be limiting of the present invention. Further aspects and embodiments will be apparent to those skilled in the art.

CLAUSES

[232] The current invention can be summarized as follows:

1. An *in vitro* method of culturing cells, the method comprising exposing the cells, preferably for at least 12 hours, to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus (a); and culturing the exposed cells in the presence of a culture medium (b).

2. The method of clause 1 wherein the isolated protein is coated on a carrier, preferably wherein the carrier is selected from the group consisting of a non-porous surface, a porous surface, a membrane, a gel, a hydrogel, or a extracellular matrix, more preferably wherein the isolated protein is present throughout a gel or wherein the isolated protein is present throughout a hydrogel.

3. The method of any of the previous clauses wherein the isolated protein is coated on a carrier by covalent binding or wherein the isolated protein is coated on a carrier by non-covalent binding and/or wherein the isolated protein is coated on a carrier by direct binding or by indirect binding through an intermediary.

4. The method of any of the previous clauses wherein the method of culturing the cells is a method for culturing a 2D cell culture, a 3D cell culture, an organoid, a tissue, or a primary tissue.

5. The method of any of the previous clauses, wherein the isolated protein binds or activates an αβ1 Integrin expressed on the cell surface of the exposed cells, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

6. The method of any of the previous clauses wherein the cells are cells expressing an αβ1 Integrin, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

7. The method of any of the previous clauses wherein the cells are cells selected from the group consisting of vertebrate cells, mammalian cells or human cells and/or wherein the cells are cells selected from the group consisting of stem cells, induced-pluripotent stem cells, adult stem cells, primary cells, a cell line, epithelial cells, endothelial cells and cells that express αβ1 integrin.

8. The method of any of the previous clauses wherein the species of the *Yersinia* genus is selected from the group consisting of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*, and *Yersinia* enterocolitica.

9. The method of any of the previous clauses wherein the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

10. The method of any of the previous clauses wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to at least 50, 100, 150, 180 or more adjacent amino acids of the last 250 amino acids of the C-terminus of an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

11. The method of any of the previous clauses wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

12. The method of any of the previous clauses wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

13. The method of any of the previous clauses wherein the isolated protein is produced in a host cell or chemically.

14. The method of any of the previous clauses wherein culturing the exposed cell is for a period selected from the group consisting of at least one day, at least 2 days, at least 3 days, at least one week, or at least two weeks.

15. The method of any of the previous clauses wherein the method is for promoting polarization of epithelial cells and/or endothelial cells.

16. The method of any of the previous clauses wherein the method further comprises drug screening, drug toxicity assessment, or drug dosing assessment.

17. An isolated protein as defined in any of the previous clauses 1 - 16, preferably wherein the isolated protein is coated on a carrier.

18. An isolated nucleic acid encoding the isolated protein of clause 17.

19. A host cell comprising the isolated protein of clause 17 or the isolated nucleic acid of clause 18.

20. A carrier coated with the isolated protein of clause 17, preferably wherein the carrier is sterile.

21. Use of the isolated protein of clause 17 or the isolated nucleic acid of clause 18 or the carrier of clause 20 in cell culture.

22. A method of preparing a carrier of clause 20, the method comprising contacting the isolated protein of clause 17 with the carrier thereby allowing the carrier to be coated with the isolated protein.

23. A cell culture system comprising the carrier of clause 20 or clause 22.

24. A cell culture system according to clause 23 further comprising cells.

EXAMPLES

Example

Introduction

[233] For organoid technology, integrin αβ1 receptors on, for example epithelial cells and endothelial cells, need to interact with protein ligands (extracellular matrix proteins (ECM)) normally found in the *in vivo* basal membrane, to ensure proper basal-apical polarity, survival, and growth. The specificity of the integrin heterodimer receptor is mediated by various α-components, generating signaling through the shared integrin β1 chain. For example, the α6β1dimer forms the major receptor for the ECM ligand laminin, being one of the main components of the basal lamina.

[234] Matrigel is a commercial hydrogel of ECM proteins produced by the murine Engelbreth-Holm-Swarm (EHS) tumor providing the laminin ligand as the most prominent constituent. Its production in mice is incompatible with clinical applications, requires a lot of laboratory animals, is expensive, and poorly defined.

[235] For that reason, the inventors set out to identify new ways to allow organoid technology, and which do not depend on ECM proteins or ECM hydrogels such as Matrigel.

[236] The inventors have surprisingly found that integrin binding domains of species of the *Yersinia* genus, and derivates thereof with high amino acid identity, may be used as alternative for ECM proteins or ECM hydrogels such as Matrigel, or in addition to such ECM proteins or ECM hydrogels. The experiments performed by the inventors, including those included herein, demonstrate that truncated versions of the invasin of species of the genus *Yersinia* and that only contains the integrin-binding domain thereof promote adhesion and proliferation of gastrointestinal and

airway organoid models. The integrin binding domain, moreover, supports the direct outgrowth of organoids from primary tissues.

[237] Subsequent organoid cultures can be passaged over extended periods without losing their potential to grow or to differentiate. Importantly, the growth kinetics observed do not differ from those seen with Matrigel/BME.

[238] The invention thus enables the culture of adult stem cell-derived organoids starting from a broad spectrum of tissues under molecularly defined, inexpensive, and xenogeneic-free conditions.

[239] **Material & Methods & Results**

[240] *Culture medium compositions used in the Examples.*

[241] Table 1: Medium composition used for culturing human epithelial cells of different origins.

Reagent	Source	Conc.	Colon Ileum	Airway	Liver hepatocyte
F12 advanced	Gibco	NA	+	+	+
Hepes	Gibco	10 nM	+	+	+
Glutamax	Gibco	1:100	+	+	+
N-acetylcysteine (Nac)	Sigma-Aldrich	1,25 mM	+	+	+
Nicotinamide (Nic)	Sigma-Aldrich	10 µM	+	+	+
B27	Sigma-Aldrich	1:50	+	+	+
p38 inhibitor SB202190	Sigma-Aldrich	10 µM	+	+ (1 µM)	
A83-01 inhibitor TGF-β type I receptor	Tocris	0,5 µM	+	+	+ (2 µM)
PGE2	Tocris	1 µM	+		
Gastrin	Tocris	10 nM			+
TGFa	Peprotech	20ng/ml			+
EGF	Peprotech	50 ng/ml	+		+
hFGF10	Peprotech	0,1 µg/ml		+	+
hHGF	Peprotech	25 ng/ml			+

FGF7	Peprotech	100 ng/ml		+ (25 ng/ml)	+
CHIR	Sigma	3 uM			+
NGS Wnt Surrogate-Fc	#: N001 IpA (Immunoprecise)	0,5 nM	+		
Rspondin3-Fc	#: R001 IpA (Immunoprecise)	3%	+	+ (1% vol/vol)	
Rspondin-1 conditioned medium	Made in house	15%			+
Noggin-Fc	#: N002 IpA (Immunoprecise)	2%	+	+	
Penecillin Streptomycin	Gibco	100U/ml	+	+	+
Primocin	Invivogen	50 µg/ml	+	+	+

[242] Cell culture: 2D epithelial cells and organoid models of different tissue origins were grown in specific, optimized media, as published: Colon organoids as previously described in Drost J, van Jaarsveld RH, Ponsioen B, et al. Sequential cancer mutations in cultured human intestinal stem cells. Nature. 2015;521(7550):43-47. doi:10.1038/nature14415. Human ileum organoids as previously described in Pleguezuelos-Manzano C, Puschhof J, van den Brink S, Geurts V, Beumer J, Clevers H. Establishment and Culture of Human Intestinal Organoids Derived from Adult Stem Cells. Curr Protoc Immunol. 2020;130(1):e106. doi:10.1002/cpim.106. Hepatocyte liver organoids as previously described in Hu H, Gehart H, Artegiani B, et al. Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. Cell. 2018;175(6):1591-1606.e19. doi:10.1016/j.cell.2018.11.013 and Airway organoids as previously described in Sachs N, Papaspyropoulos A, Zomer-van Ommen DD, et al. Long-term expanding human airway organoids for disease modeling. EMBO J. 2019;38(4). doi:10.15252/embj.2018100300.

[243] Table 2: Medium composition used for culturing human epithelial cells (colon/ileum) and epithelial cells of different origins.

Reagent	**Source**	**Conc.**	**Colon Ileum**	**Airway**	**Mouse lacrimal gland**	**Snake Venom gland**	**Mouse Intestine**
F12 advanced	Gibco	NA	+	+	+	+	+

Hepes	Gibco	10 nM	+	+	+	+	+
Glutamax	Gibco	1:100	+	+	+	+	+
N-acetylcysteine (Nac)	Sigma-Aldrich	1,25 mM	+	+	+	+	+
Nicotinamide (Nic)	Sigma-Aldrich	10 mM	+	+ (5 mM)	+	+	
B27	Sigma-Aldrich	1:50	+	+ (B27 + vitA,)	+(B27+ vitA)	+	+
p38 inhibitor SB202190	Sigma-Aldrich	10 µM	+	+ (1 µM)			
A83-01 inhibitor TGF-β type I receptor	Tocris	0,5 µM	+	+	+	+	
PGE2	Tocris	1 µM	+		+	+	
Gastrin	Tocris	10 nM				+ (100 nM)	
TGFa	Peprotech	20ng/ml					
EGF	Peprotech	50 ng/ml	+			+	+
FGF10	Peprotech	0,1 µg/ml		+	+	+	
hHGF	Peprotech	25 ng/ml					
FSK	Peprotech	1 nM			+		
FGF7/KGF	Peprotech	100 ng/ml		+ (25 ng/ml)			
CHIR	Sigma	3 µM					
NGS Wnt Surrogate-Fc	#: N001 IpA	0,5 nM	+				+
Rspondin3- Fc	#: R001 IpA	3%	+	+ (1%)	+(1%)	+ (2%)	+
Noggin-Fc	#: N002 IpA	2%	+	+ (1%)	+ (1%)	+	+
Penecillin Streptomycin	Gibco	100U/ml	+	+		+	+
Primocin	Invivogen	50 µg/ml	+	+		+	

[244] Cell culture: 2D epithelial cells and organoid models of different tissue origins were grown in specific, optimized media, as published: Colon organoids as previously described in Drost J, van Jaarsveld RH, Ponsioen B, et al. Sequential cancer mutations in cultured human intestinal stem cells. Nature. 2015;521(7550):43-47. doi:10.1038/nature14415. Human ileum organoids as previously described in Pleguezuelos-Manzano C, Puschhof J, van den Brink S, Geurts V, Beumer J, Clevers H. Establishment and Culture of Human Intestinal

Organoids Derived from Adult Stem Cells. Curr Protoc Immunol. 2020;130(1):e106. - Hepatocyte liver organoids as previously described in Hu H, Gehart H, Artegiani B, et al. Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. Cell. 2018;175(6):1591-1606.e19. Airway organoids as previously described in Sachs N, Papaspyropoulos A, Zomer-van Ommen DD, et al. Long-term expanding human airway organoids for disease modeling. EMBO J. 2019;38(4). Snake venom gland organoids as previously described in Post, Y. et al. Snake Venom Gland Organoids. Cell 180, 233-247.e21 (2020). Mouse lacrimal gland organoids as previously described in Bannier-Hélaouët, M. et al. Exploring the human lacrimal gland using organoids and single-cell sequencing. Cell Stem Cell 28, 1221-1232.e7 (2021). Mouse intestinal organoids as previously described in Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. Nature 459, 262–265 (2009). New lines from human primary biopsies were processed as described in Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat. Protoc. 11, 1724–1743 (2016).

Example 1:

[245] Figure 3 shows that the isolated protein of the invention (e.g., invasin) induces an open-active conformation of integrins on K562 cells.

[246] *Background and results explained.*

[247] K562 cells almost exclusively express the integrin α5ß1 on their surface. Integrins are in a bent-closed conformation, i.e., they are in the OFF state. An interaction of these integrins with classical ligands like Fibronectin induces a conformational change to an ON-state. This active conformation can be detected specifically by the integrin ß1-specific HUTS4 antibody. As a positive control, we monitored HUTS4 binding to K562 cells using FACS analysis with a recombinant fibronectin version (FNIII9-10). We then tested whether incubation with a recombinantly-produced soluble short version of Invasin would similarly lead to increased HUTS4 binding.

[248] *Results*

[249] An equal number of K562 wild-type cells were incubated with a human IgG antibody directed against HUTS4 together with 150nM fibronectinIII9-10, as a positive control, or 150 nM Invasin or medium. After washing, cells were incubated

with Alexa fluor 488 goat anti-human antibody (aH488). The open-active ligand binding of Invasin and fibronectinIII9-10 F was analyzed, based on mean fluorescent intensity of Alexa488, via FACS analysis. The results showed that fibronectinIII9-10 and Invasin, based on HUTS4 binding to K562 cells, induce an open-active integrin conformation on K562 cells.

[250] *Conclusion*

[251] Invasin is recognized as a genuine ligand for the integrin α5ß1 on K562 cells, as shown via FACS analysis, as this interaction resulted in an open-active conformation of this integrin.

[252] *Material and Methods*

[253] *Bacterial expression and purification of Invasin and Fibronectin.*

[254] An E.coli codon-optimized coding sequence of the C-terminal 192 amino acids of the Inv gene of *Yersinia pseudotuberculosis* was synthesized by GeneArt (ThermoFisher) and cloned, using standard cloning protocols, into a pMalC2x vector (Addgene). An 8x-His tag, H3V-3C protease cleavage site, transglutaminase FXIII sequence and a FLAG-tag were introduced at the C-terminus of the MPB-tag. Sequence-verified pMalC2x-Invasin192 vector was transformed into SHuffle T7 K12 Competent E.coli cells (New England Biolabs). When the medium (Terrific Broth) with the bacteria reached an OD600 optical density of 0.8, protein expression was induced by adding 1mM IPTG (Sigma) for 4 hours at 26°C. Bacterial cells were collected using centrifugation (10.000 rpm, 10 min.). The obtained bacterial pellets were resuspended in lysis buffer (50 mM HEPES, 200 mM NaCl (pH 7.4), 1mg/ml lysozyme (ThermoFisher), 25 U/ml benzonase (Sigma), protease inhibitor cocktail (Roche) and subsequently sonicated. Upon centrifugation for 20 minutes at 11.000 rpm, the soluble protein fraction (upper phase) was collected and used for Ni-Nta (Qiagen) affinity purification based on the 6xHIS tag present in the protein. Further purification was performed by size exclusion chromatography using a Superdex S200 increase 10/300 GL size-exclusion column (Cytiva) equilibrated with buffer (50 mM HEPES, pH 7.4, 200 mM NaCl). The purified proteins were analyzed under reducing and non-reducing conditions on a 4-15% TGX precast SDS-PAGE (BioRad) gel and visualized using InstantBlue Coomassie Protein stain (Abcam). Protein concentration was based on protein MW (28kDa) and extinction coefficient (calculated by entering the sequence into online software ProtParam) combined with

NanoDrop measuring absorbance at 280. Purified proteins were incubated with HRV-3C (1 µl cleavage enzyme / 200 µg Invasin protein) (Pierce) O/N at 4°C. MBP and HRV-3C_His were removed using Ni-Nta beads (Qiagen) to obtain purified Inv192 protein. Cleaved products were finally analyzed on 4-15% TGX precast SDS-PAGE (BioRad) gel as described above.

[255] A similar procedure was performed for expressing and purifying the integrin-binding domain of fibronectin (FibronectinIII-9-10). In short, E.coli-codon optimized coding sequence of residues 1326-1509 of fibronectin was synthesized by GeneArt and cloned in a pET21 vector harboring a C-terminal 6x-HIS-tag (Novagen). Protein expression was carried out in Escherichia coli BL21 (DE3) grown in standard LB medium. Protein induction was started with 1 mM IPTG when cells were grown until the OD600 reached 0.6 at 37°C. After induction, cells were pelleted and resuspended in lysis buffer (50 mM Tris (pH 7.4), 200 mM NaCl, 1mg/ml lysozyme, 25 U/ml Benzonase and protease inhibitor cocktail). Cells were further lysed by sonication. The FibronectinIII-9-10 was purified using Ni-NTA (Qiagen). The fractions were eluted with 250 mM imidazole (Sigma) buffer and subsequently subjected to SEC in 50 mM tris (pH 7.5) and 200 mM NaCl using a Superdex 75 column on an ÄKTA pure machine (GE Healthcare). The protein purification quality was analyzed upon running samples on a 4-15% TGX precast SDS-PAGE (Biorad) gel. Proteins were visualized using InstantBlue Coomassie Protein stain (Abcam).

[256] *Binding of Invasin and activation of α5β1 on K562 cells measured by flow cytometry.*

[257] K562 wild-type cells, grown under standard conditions (DMEM Glutamax (Gibco) with 100 U/ml Penicillin-Streptomycin and 5% FBS (Sigma-Aldrich) in a 5% CO2 incubator at 37°C), were washed with DMEM/F12 (Gibco) to remove FBS. An equal number of cells were incubated with 150 nM of the LIBS (Ligand-induced binding site) human IgG antibody HUTS4 together with 150 nM recombinant fibronectinIII-9-10, 150 nM Invasin, or medium for 90 minutes at room temperature while rotating. After incubation, cells were washed 3x with DMEM/F12 and incubated with 5 ug/ml Alexa fluor 488 goat anti-human IgG (aH488) (Life technologies, A11013) for 30 minutes at room temperature while rotating. Subsequently, cells were washed 3x with DMEM/F12, and ligand binding was analyzed using FACS (BD Fortessa), based on mean fluorescent intensity of Alexa488.

Example 2:

[258] Figure 4 shows that epithelial cells derived from the lung, colon and liver organoids adhere to BME and to the isolated protein according to the invention (e.g., bacterial-produced Invasin).

[259] *Background and results explained.*

[260] An adhesion assay was conducted to see if organoid-derived epithelial cells derived from the liver, intestine, and lung could recognize Invasin as an extracellular matrix protein.

[261] *Results*

[262] The adherence of isolated single cells derived from hepatocyte, airway or colon organoids added to wells coated with BME as a positive control or Invasin or uncoated wells as a negative control was quantified. Moreover, the specificity of the adherence of these epithelial cells to Invasin or BME was tested by adding an integrin β1 blocking AIIB2 antibody.

[263] Upon washing, an ATP-driven luminescent assay (CellTiterGLO) was used to quantify the remaining adherent cells. The lung, colon and liver epithelial cells could specifically adhere to the BME and Invasin coat. Adhesion to BME or Invasin could be partially inhibited by the allosteric integrin β1 blocking antibody AIIB2. The minus AIIB2 condition is the spontaneous adhesion towards the indicated coat.

[264] *Conclusion*

[265] Epithelial cells derived from the lung, colon and liver organoids adhere to BME and bacterial produced invasin protein. The adherence to Invasin or BME can be partially blocked by the integrin β1 blocking AIIB2 antibody.

[266] *Material and Methods*

[267] *Quantifying the adherence of isolated single cells from hepatocyte, airway, or colon organoids towards coated BME or Invasin.*

[268] Epithelial cells, derived from established liver, intestine, and lung organoids, were incubated for 1.5 hrs. on uncoated wells or wells coated with BME (2% in PBS) or Invasin (10 ug/ml diluted in PBS) with or without the integrin β1 blocking AIIB2 antibody (1 ug/ml (Kind gift from Arnoud Sonnenberg)) and subsequently washed extensively with DMEM/F12. An ATP-driven luminescent assay (CellTiterGLO) (Promega) was used to quantify the remaining adherent cells.

Example 3:

[269] Figure 5 shows that intestinal epithelial cells cultured on the isolated protein of the invention (e.g., Invasin) in 2D, demonstrate normal apical-basal polarity.

[270] *Background and result explained.*

[271] Many tissues are lined by epithelia with apical cell membranes facing the lumen. Polarization allows the epithelial cells to transport molecules across the surface in a directional manner. It also determines the localization of the adhesion molecules that hold the cells together laterally acting as a barrier to paracellular diffusion and various pathogens (e.g., bacteria and viruses). Polarization, moreover, is essential to ensure that growth factor receptors are at the basal site of the cells where they are in contact with the bloodstream. To determine if intestinal organoid-derived epithelial cells remain a correct apical-basal polarity when grown in 2D on Invasin or BME, an immunofluorescent staining was performed to analyze the localization of apical-expressed proteins villin and F-actin.

[272] *Results*

[273] Epithelial cells, derived from human ileum organoids, were grown in 2D in a Transwell system coated with 2% BME or 10 ug/ml recombinant Invasin protein and analyzed by confocal imaging for fluorescent Villin and F-actin expression. The results showed the apical expression of Villin and F-actin when grown as 2D on Invasin and BME.

[274] Conclusion

[275] Intestinal epithelial cells cultured as 2D on Invasin remain correctly polarized, as shown by the apical expression of villin and F-actin.

[276] *Material & Methods*

[277] *Confocal imaging of intestinal epithelial cells*

[278] Confocal imaging of ileum epithelial cells grown in 2D in a Transwell system (Greiner 12-well translucent, 0.3 µm pore size) coated O/N at 4°C with 2% BME (R&D systems) or 10 ug/ml recombinant Invasin protein. 2D-grown organoids were fixed with 4% formaldehyde (pH 7.4) at room temperature (RT) for 30 minutes. Subsequently, the fixed cells were washed with PBS 0.1% Triton (PBST) (ThermoFisher), permeabilized, using PBS 1% Triton, for 30 minutes at RT, followed by blocking of the cells with 2% BSA (Sigma) for 60 min at RT. The fixed and permeabilized cells were incubated O/N at 4°C with a primary antibody directed against Villin (Santa Cruz, sc-58897) (1:1000). Upon extensive washing with PBST,

the binding of the primary antibodies was visualized after 2 hours of incubation at RT with the secondary antibody: Donkey a-Mouse568 (Life technologies A10037, 1:1000).

[279] Phalloidin fluor conjugates Phalloidin647 (Sigma, 65906) (1:1000) were used to visualize F-actin directly. DNA was stained with DAPI (ThermoFisher) (1:1000). 2D-grown epithelial cells were imaged on a confocal SP8 (Leica) microscope and analyzed using Imaris software.

Example 4:

[280] Figure 6 shows that Vinculin-TdTomato reporter organoids, grown as 2D on the isolated protein of the invention (e.g., Invasin) or BME, show active integrin signaling.

[281] *Background and result explained.*

[282] A mouse Vinculin-TdTomato reporter organoid model derived from the lacrimal gland was used to analyze downstream integrin signaling upon growth on Invasin. The integrated Vinculin-TdTomato reporter visualizes the expression and location of the vinculin protein. Vinculin links integrin receptors on the cell surface to the actin cytoskeleton inside the cell.

[283] *Results*

[284] Confocal imaging of 2D cultured lacrimal gland-derived mouse epithelial cells containing the integrated Vinculin-TdTomato reporter on Invasin and, as a positive control, BME coated well both showed strong downstream signaling of integrin as shown by the localization of the Vinculin-TdT reporter protein, in fire, at the basal side. The nucleus in the cross-section is white.

[285] *Conclusion*

[286] 2D grown epithelial cells, derived from lacrimal gland-derived mouse epithelial cells containing the integrated Vinculin-TdTomato reporter, show active downstream integrin signaling when grown on Invasin.

[287] *Material & Methods*

[288] Glass 96-well plates (Greiner) were O/N coated at 4°C with 10 ug/ml Invasin protein or 2% BME® (R&D system) in PBS. The next day, the solution was removed, and epithelial mouse lacrimal gland organoids, grown as 3D, containing the downstream integrin signaling reporter (vinculinTdTomato) were completely released from the BME matrix using 1:100 Dispase (ThermoFisher) for 30 minutes at 37°C.

Subsequently, organoids were washed twice with cold DMEM/F12 and treated with TryplE (Gibco) for 15 minutes at 37°C, with repeated mechanical shearing using P1000 to dissociate organoids into single cells. The single cells were washed twice with cold DMEM/F12, and the number of life cells was determined upon trypan blue (VWR) staining. An equal number of cells were added to the BME or Invasin-coated wells. After five days of culturing, the 2D-grown epithelial Vinculin-TdTomato reporter cells were fixed with 4% formaldehyde (pH 7.4) at room temperature (RT) for 30 minutes. Subsequently, the fixed cells were washed with PBS and incubated with DAPI (ThermoFisher) (1:1000), imaged using a confocal SP8 (Leica) microscope and analyzed using ImageJ software.

Example 5:

[289] Figure 7 shows that human fetal hepatocytes can be expanded as 2D cells on the isolated protein according to the invention (e.g., Invasin) for multiple passages.

[290] *Results*

[291] Human fetal hepatocytes were cultured, as 2D, on Invasin or, as a positive control, on BME for multiple passages. After every passage, three individual wells of a 96-well plate were used to quantify the amount of genomic DNA to indicate the number of cells growing on Invasin or, as a positive control, BME upon passaging. The results showed that human fetal hepatocytes can be cultured and passaged in 2D on Invasin and BME.

[292] *Conclusion*

[293] Human fetal hepatocytes can be expanded as 2D cells on Invasin for multiple passage.

[294] *Material and Methods*

[295] *Growing epithelial 2D cultures on Invasin and BME*

[296] 3D-established human fetal hepatocyte organoids grown in BME were treated with 1:100 Dispase (ThermoFisher) to remove all traces of BME components. After washing the Dispase-treated organoids with cold DMEM/F12, organoids were dissociated with TryplE to single cells; this reaction was stopped by washing cells 2x with DMEM/F12. Equal amounts of epithelial cells were cultured on O/N-coated plates with 10 ug Invasin or 2% BME diluted in PBS. When plating hepatocyte epithelial cells on the coats, 10 uM Y-27 (stem cell technologies) was supplemented to the media, for the first 2 days. Wells with growing epithelial cells were refreshed

with 200 ul hepatocyte expansion medium every two days. When epithelial cells reached 80 to 100% confluency, cells were passaged by adding pre-warmed TrypLE (Gibco) to the wells. Cells covered with TrypLE were collected by scraping the epithelial layer with a pipet tip. The culture plates were checked using a brightfield microscope to confirm that all cells were successfully collected. After removing cells from the wells, cells were washed twice with cold DMEM/F12. Epithelial cell pellets were resuspended in corresponding media and plated on freshly BME or Invasin-coated wells. Three wells that were not used for passaging were used for genomic DNA quantification. DNA was obtained using ZYMO Research DNA isolation kit and DNA/well was quantified using Nanodrop apparatus (Thermo Scientific).

Example 6:

[297] Figure 8 shows that human cells, from different origins, can be expanded long term as 2D cells on the isolated protein according to the invention (e.g., Invasin)

[298] *Results*

[299] Epithelial cells of different origins (hepatocytes, colon, ileum, and airway), derived from established organoids, could be passaged on Invasin in 2D for at least five passages. The ratio of splitting is indicated.

[300] *Conclusion*

[301] Human epithelial cells of different origins can be expanded long-term on Invasin in 2D.

[302] *Material and Methods*

[303] Culturing of human epithelial cells, from different origins, as 2D cultures on the isolated protein according to the invention (e.g., Invasin).

[304] Organoids (colon, ileum, hepatocyte, airway) grown in BME were treated with 1:100 Dispase (ThermoFisher) to remove all traces of BME. After washing the Dispase-treated organoids with cold DMEM/F12, organoids were dissociated with TrypLE to single cells. This reaction was stopped by washing cells 2x with DMEM/F12. Equal amounts of epithelial cells were cultured on plates coated (O/N) with 10 ug Invasin or 2% BME in PBS. 10 uM Y-27 (Stem Cell Technologies) was added to the media (Table 1) during the first two days of and upon passaging. Wells with growing epithelial cells were refreshed every 2 days with 200 ul of the corresponding mediums. When epithelial cells reached 80 to 100% confluency, cells were passaged by adding pre-warmed TrypLE (Gibco) to the wells upon removal of

the medium. Cells covered with TrypLE were collected by scraping the epithelial layer using a pipet tip. Wells were checked using a brightfield microscope to confirm that all cells were collected. After removing cells from the wells, cells were washed twice with cold DMEM/F12. Epithelial cell pellets were resuspended in the corresponding media and plated on freshly invasin-coated wells.

Example 7:

[305] Figure 9 shows that intestinal epithelial cells, grown in 2D on the isolated protein according to the invention (e.g., Invasin) in 2D, can be differentiated towards well-known functional intestinal cells.

[306] *Results*

[307] Epithelial ileum cells grown in 2D on Invasin or, as a positive control, BME were differentiated towards specialized intestinal cells by changing the culture medium composition. The ileum cells have, therefore, integrated fluorescent reporters to analyze the presence of the induced differentiated cells (Goblet cells; MUC2-GFP, enteroendocrine (EEC) cells; CHGA-iRFP and Paneth cells: DEFA5-dsRED) via FACS analysis. The difference between cells cultured in expansion and differentiation medium on Invasin and BME was analyzed by FACS. The FACS plots and percentages of the differentiated cells are mentioned in the Figure. Ileum cells cultured in 2D on Invasin or BME could be differentiated towards functional intestinal cells such as goblet and enteroendocrine cells.

[308] *Conclusion*

[309] Intestinal epithelial cells grown in 2D on Invasin can be differentiated towards well-known functional intestinal cells: goblet cells and enteroendocrine cells.

[310] *Materials and Methods*

[311] *Differentiation of the triple reporter epithelial cells grown on Invasin.*

[312] The N39 triple reporter organoid model (Goblet cells; MUC2-GFP, enteroendocrine (EEC) cells; CHGA-iRFP and Paneth cells: DEFA5-dsRED) (see He G-W, Lin L, DeMartino J, et al. Optimised human intestinal organoid model reveals interleukin-22-dependency of Paneth cell formation. *Cell Stem Cell*. 2022;29(9):1333-1345.e6. doi:10.1016/j.stem.2022.08.002) were grown on coated Invasin to test if the epithelial cells could retain the full functionality of an intestinal epithelium. The epithelial cells were, therefore differentiated towards functional and well-known specialized intestinal cell types like goblet, enteroendocrine and Paneth

cells. This was carried out as described (see He G-W, Lin L, DeMartino J, et al. Optimised human intestinal organoid model reveals interleukin-22-dependency of Paneth cell formation. *Cell Stem Cell*. 2022;29(9):1333-1345.e6. doi:10.1016/j.stem.2022.08.002) with minor differences. The differences were as follows: the epithelial cells were cultured on invasin-coated Transwell until 90-100% confluency. Differentiation was induced using a 2-step differentiation protocol. The first step is to add 500 ul of differentiation medium, including 250 nM forkhead box O (FOXO) inhibitor (AS1842856, Sigma) (see Eng SJ, Nonnecke EB, de Lorimier AJ, et al. FOXO inhibition rescues α-defensin expression in human intestinal organoids. *Proc Natl Acad Sci*. 2023;120(47):e2312453120. doi:10.1073/pnas.2312453120) to the lower and upper compartment of the Transwell system for 7 days. Every 2 days new differentiation media was added. The second step to induce Paneth cells, 2ng/ml of IL-22 (Peprotech) was used in a maturation medium (as described in He G-W, Lin L, DeMartino J, et al. Optimized human intestinal organoid model reveals interleukin-22-dependency of Paneth cell formation. Cell Stem Cell. 2022;29(9):1333-1345.e6. doi:10.1016/j.stem.2022.08.002), and 500 ul was added to the lower compartment of the Transwell system, whereas 250 ul of DMEM/F12 was added to the upper compartment of the Transwell system. This culture condition was maintained for 7 days with refreshing differentiation medium and DMEM/F12 every 2 days. Quantification of the differentiated epithelial cells was analyzed using FACS (BD fortessa).

[313] It was demonstrated by the inventors, the same or identical results are obtained when another isolated protein according to the invention is used, and that only comprised of the integrin binding domain of the invasin protein of a species of the *Yersinia* genus, for example the integrin binding domain of the invasin protein of *Yersinia pseudotuberculosis,* herein also referred to as invasin-192.

[314] Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

[315] Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect,

description, or embodiment of the present invention is disclosed, taught, or suggested in the relevant art.

Example 8:

[316] Figure 11 shows the establishment of adult human liver tissue cultures on the isolated protein according to the invention (e.g., Invasin).

[317] *Result*:

[318] Primary human liver tissue was processed, and single cells were plated on Invasin coated wells. After 10 days, the resulting 2D organoid sheet was analyzed using immunofluorescence. Staining revealed liver-specific markers, including albumin and hepatocyte nuclear factor 4 (HNF4), and a nuclear marker DAPI was used.

[319] *Conclusion*:

[320] Invasin supports the growth of primary human liver tissue.

Example 9:

[321] Airway cultures started from tissue biopsies were passaged multiple times on the isolated protein according to the invention (e.g., Invasin) and could be genetically modified using CRISPR-Cas9.

[322] *Background*: One of the key applications of organoid technology in regenerative medicine is the ability to introduce or repair disease-related genes. The principle involves modifying genes outside the human body and reintroducing repaired tissue to cure diseases. This process requires a well-defined, contamination-free human culture system. Invasin provides such a system, as it is a simple protein compared to the undefined, xenogeneic nature of Matrigel, which consists of over 8,000 components. We first tested whether it was possible to introduce or modify genes in human epithelial cells cultured on Invasin.

[323] *Results*: Airway epithelial cells obtained from lung tissue were successfully cultured on Invasin for five passages. After these passages, the epithelial cells were electroporated with DNA constructs for the life-actin gene and a CRISPR-Cas9 base-editing system to introduce a TP53 mutation. Life-actin is a gene construct that fluorescently labels actin filaments within the cell, introduced via the transposase technique. TP53 is an oncogene frequently mutated in cancers; using CRISPR-Cas9 base editing, we altered a single nucleotide, converting an amino acid into a stop codon.

[324] Selection for the life-actin construct was performed using puromycin, as a resistance cassette was introduced alongside the gene. TP53 mutation selection was conducted using Nutlin-3, which eliminates healthy cells while allowing TP53-mutant cells to survive. Surviving cells were DNA-sequenced to confirm the introduction of the stop mutation. The progression of puromycin selection for the life-act cassette was monitored over time via red fluorescence emitted by the actin-reporter. The selection methods resulted in homogenous 2D airway cultures that harbored the genetic modifications (data not shown).

[325] *Conclusion*: Primary airway cultures initiated on Invasin can be successfully genetically modified. This demonstrates a potential application for regenerative medicine using organoid technology in a well-defined culture system.

[326] *Material & Methods*

[327] Human airway cells after passage 5 were used for experiments. These cells were cultured in 24-well plates (Greiner) on 24-well Transwell inserts with 3.0 µm pore size with a coating of 5 µg/ml Invasin or 1% for seven days before transfection. Twenty-four hours prior to infection 5 nM HRG1-beta 1 EGF (R&D systems) was added. Single cells were prepared as described in previous examples in Invasin-based two-dimensional organoid culturing. These single cells were then resuspended in Lipofectamine LTX Plus (Invitrogen). A mixture of 5 µg transposase and 5 µg Lifeact was prepared, and the volume was adjusted to 100 µl with the single cell suspension. This mixture was then transferred to a MicroPulser electroporation cuvette (BIO-RAD). Cells were subsequently electroporated with parameters for poring pulse set at 175 volt (V), 5 ms for pulse length, 50 ms for pulse interval, 2 pulses with a 10% decay rate and a positive polarity. Parameters for transfer pulse were set at 20 V for voltage, 50 ms for pulse length, 50 ms for pulse interval, 5 pulses with a 40% decay rate and a positive/negative polarity. 500 µl of DMEM was added and the mixture was rested at RT for 30 minutes. Following incubation, cells were washed and plated with growth medium. After 24 hours, Y-27632 was removed from the expansion medium, and 20 µM puromycin (Invivogen) or Nutlin-3 (for P53 selection) added. Medium was refreshed every two days.

Example 10:

[328] *Background:* Conjunctiva and cornea tissue transplantation hold great potential for curing eye diseases upon transplantation of organoids. We investigated the

possibility of culturing limbal stem cells and conjunctival cells from the human eye on isolated protein according to the invention (e.g., Invasin).

[329] *Results:* We successfully cultured human epithelial eye cells on the defined Invasin protein in transwell systems. The cultured limbal stem cells and conjunctival cells could be maintained on Invasin and differentiated into specific cell types. This defined culture system opens new possibilities for transplantation applications of these cells in human eyes.

[330] *Conclusion*: Invasin can be used to culture human epithelial eye cells in a defined system, facilitating their use in transplantation procedures.

Example 11:

[331] *Background*: Functional studies with human epithelium are predominantly conducted in 2D culture setups due to easy access to both the apical and basal sides of cell layers. This is particularly relevant for infection studies involving bacteria or viruses. To assess whether Invasin could maintain the functional barrier properties of epithelial cells, we co-cultured human stomach epithelial cells with bacteria.

[332] *Results:* Human stomach organoids were successfully passaged multiple times on isolated protein according to the invention (e.g., Invasin). After several passages, the stomach cells were co-cultured with bacteria. As a positive control, BME cultures were also analyzed. Fluorescently labeled bacteria were analyzed, indicated similar invasion rates between BME and Invasin stomach cultures. These results demonstrate that co-cultures with bacteria can be performed using Invasin-cultured cells.

[333] *Conclusion:* Invasin-based 2D cultures can be utilized to study pathogen interactions with human epithelial cells.

Example 12:

[334] *Background:* In the context of CAR-T therapy and immune-epithelial interactions, organoids are often co-cultured with immune cells. To evaluate the interaction between immune cells and tumor organoids, we cultured pancreatic tumor organoids on isolated protein according to the invention (e.g., Invasin) to test these abilities.

[335] *Results:* Pancreatic tumor epithelial cells could be cultured on Invasin for long-term maintenance. Immune cells were introduced to the apical side upon reaching confluency as 2D epithelial organoid sheets. Immune cells successfully induced apoptosis in cancer cells, while healthy epithelial cells remained intact.

[336] *Conclusion:* Invasin (and the method according to the invention) provides a viable system for co-culturing tumor organoids with immune cells, facilitating immune-mediated tumor cell apoptosis studies.

Example 13:

[337] *Background:* Cancer research often requires the controlled culture of tumor cells to study their proliferation, behavior, and treatment response. Moreover, 2D cultures could hold many advantages for drug screens as the cells are simply accessible. We explored whether on isolated protein according to the invention (e.g., Invasin) could support the growth of cancer cells and serve as a defined platform for studying tumor cell behavior.

[338] *Results:* Cancerous epithelial tissues, including colon cancer tissue, were successfully cultured on Invasin. These tumor cells proliferated and formed structured epithelial layers in 2D culture conditions. This indicates that Invasin can be used to study tumor tissue in 2D set up with the potential for drug response studies.

[339] *Conclusion:* Invasin provides a robust platform for culturing and studying cancer cells, enabling the use of this system for high-throughput drug screening.

Example 14 (Comparative):

[340] Figure 12 shows that Integrin combination α6β1 is essential for colon organoid growth in Matrigel/BME.

[341] *Background and results explained.*

[342] A single cell RNA sequencing data set was used to determine which integrin subunits are expressed in colon organoids (data not shown).

[343] To determine which integrin is crucial for the formation of colon organoids in Matrigel/BME, we used CRISPR-Cas9 base editing technique to introduce a stop-codon in integrin receptors, which were highly expressed in colon organoids.

[344] In addition, we used the antibody AIIB2, which mechanistically blocks the function of integrin β1 and thereby stops the function of integrin β1.

[345] *Results*

[346] The expression levels of β1 integrin subunit and α-integrin subunits were analyzed, selected for their interaction with collagens, laminins, or proteins containing the amino acid sequence RGD. All the presented α-integrins dimerize with integrin β1.

[347] Results indicated that integrin β1 together with α6 and αV integrins show the highest expression level in colon epithelial organoids.

[348] To analyze the individual function of these integrins, we knocked-out integrin α6 and αV using CRISPR-Cas9 base editing and blocked integrin β1 using the antibody AIIB2. As the integrins α6 and αV expectedly generate essential growth signals, we added Rho-kinase inhibitor (Y-27) to these mutant organoid cultures to circumvent the loss of these signals.

[349] Conclusion: α6β1 integrin heterodimer, and to a lesser extend αVβ1, is crucial for the generation and/or maintenance of intestinal colon organoids when cultured under Matrigel/BME culture condition.

Example 15:

[350] Figure 13 shows that gut epithelial cells adhere equally to Invasin192 (short version, SEQ ID NO: 6) and Invasin497 from *Yersinia pseudotuberculosis*, and that there is no effect of the MBP-fusion protein.

[351] *Background and results explained:*

[352] Invasin's shortest known integrin-binding domain is 192 amino acids long, starting from its C-terminus. This truncated version is the minimum required to stimulate integrins. The research has also focused on the 497-amino acid version (also from the C-terminus). It was aimed to assess if there are differences between the two versions of Invasin in stimulating adhesion of intestinal epithelial cells. Additionally, Invasin was produced with an N-terminal maltose-binding protein (MBP) tag, stabilizing and facilitating bacteria production. The MBP tag can be enzymatically removed. To evaluate whether the MBP tag affects the functionality of Invasin, both the MBP-Invasin version and the Invasin protein without the MBP tag were tested in an adhesion assay using isolated intestinal epithelial cells.

[353] The adherence of single intestinal epithelial cells derived from ileum and colon organoids was quantified after being added to wells coated with MBP-Invasin497, Invasin497, MBP-Invasin192, Invasin192, or uncoated wells (negative control). Following washing, an ATP-driven luminescent assay (CellTiter-Glo) was used to measure the remaining adherent cells. Intestinal epithelial cells adhered equally well to all coated Invasin variants.

[354] *Conclusion:*

[355] Epithelial cells derived from colon and ileum organoids adhered equally to Invasin variants 192 and 497, and the presence of the MBP tag did not affect this adhesion.

Example 16:

[356] Figure 14 shows the integrin-binding domain of *Yersinia* Invasin supports epithelial cell adhesion, growth, junction formation and polarity.

[357] *Background and results explained.*

[358] 1. The enteropathogenic *Yersinia* bacterium employs its surface-expressed Invasin protein to enter its host through a rare intestinal epithelial cell type, the microfold (M) cell which overlies lymph node-like structures called Peyer's patches. M-cells uniquely express integrins on their luminal (rather than basal) surface. *Yersinia* can reportedly bind and activate integrin-α3β1, -α4β1, -α5β1, -α6β1, and -αVβ1, through the small 25 kD C-terminal domain of its outer membrane protein Invasin (Inv192). This interaction is well studied from the perspective of allowing *Yersinia* to invade through the luminal surface of M-cells. The specific interaction of Invasin with integrin complexes has previously been studied almost exclusively in immune cells. At least 17 *Yersinia* species have been described. Among those, three are pathogenic in man: Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica. Most *Yersinia* species harbor homologs of the Invasin (Inv) gene.

[359] The two enteropathogenic Y. pseudotuberculosis and Y. enterocolitica, which are also distantly related to each other, both invade M-cells using their Invasin protein. Of note, Y. pestis is unable to invade epithelial cells due to an insertion of an unrelated nucleotide sequence into the central region of its -otherwise intact- Inv gene. Therefore, we would like to see if the Invasin protein from a different species could equally function in the adhesion and growth of epithelial intestinal cells. So, we recombinantly produce the Invasin192 version of *Yersinia pseudotuberculosis* and *Yersinia* enterocolitica and test in an adherence assay and growth assay the functionality of Invasin proteins from the different *Yersinia* species.

[360] 2. The growth of epithelial cells was also quantified using a Transepithelial Electrical Resistance (TEER) measurement on day 0 and day 7. A TEER measurement measures the confluency of 2D epithelial sheets in Transwell systems. High TEER values indicate a confluent epithelial layer with functional cell-cell junctions.

[361] 3. We tested whether it is possible to grow organoids from a single cell on Invasin in the appropriate growth factor cocktail. Therefore, we sorted one cell per well in a 96-well plate coated with Invasin. We followed the cell growth over time using intestinal epithelial cells endogenously tagged with a fluorescent-ubiquitination-based

cell cycle indicator (FUCCI)-reporter. This study showed that organoids can be generated from single cells on an Invasin.

[362] 4. One hallmark of epithelial cells is an apical-basal polarity to form a defensive barrier for pathogens (e.g., bacteria and viruses). To determine whether intestinal epithelial cells remain a correct apical-basal polarity, when grown in 2D on Invasin or BME, an immunofluorescent staining was performed to analyze the localization of apical-expressed proteins Villin and F-actin (data not shown).

[363] *Result*:

[364] 1. Epithelial intestinal cells adhere and grow equally on Invasin from *Yersinia pseudotuberculosis* and enterocolitica. This was compared to Matrigel/BME (contains only embryonic extracellular matrix proteins) and a mature extracellular matrix protein laminin521. The adherent cells and growth of epithelial sheets was quantified using an ATP-driven luminescent assay (CellTiterGLO).

[365] 2. Growth was also observed using TEER measurements on day 0 and day 7 on Invasin from *Yersinia pseudotuberculosis*. TEER measurements also indicated confluent epithelial 2D organoid sheets in the Transwell system, with tight cell-cell interactions. Creating a barrier (similar to *in vivo*) for transepithelial, infectious and drug screen studies.

[366] 3. It was possible to grow 2D epithelial organoid sheets from 1 single cell. Growth of the epithelial cell was analyzed over 10 days. The FUCCI-reporter indicate the phase of the cell-cycle based on the different color in the nucleus of the cells.

[367] 4. Epithelial cells, derived from human ileum organoids, were grown in 2D in a Transwell system coated with 2% BME or 10 ug/ml recombinant Invasin protein and analyzed by confocal imaging for fluorescent Villin and F-actin expression. The results showed the apical expression of Villin and F-actin when grown as 2D on Invasin and BME, in the same way as showed in Figure 5 for Example 3.

[368] *Conclusion:*

[369] 1. Epithelial cells derived from colon and ileum organoids adhere and grow on Invasin from *Yersinia pseudotuberculosis* and *Yersinia* enterocolitica.

[370] 2. The formed epithelial sheets on Invasin were confluent and formed a tight cell-cell junction. This is a crucial characteristic of epithelial tissue. This barrier function could be helpful for several functional studies.

[371] 3. Epithelial sheets could be formed from 1 single cell. We observed an average plating efficiency of 38.5% on an Inv497 coat, compared to our positive control Matrigel/BME coat at 45.8%, while no clones grew out under 'no coat' or bovine serum albumin (BSA)-coat conditions (n = 3, a total of 288 wells per condition was quantified)

[372] 4. The confluent epithelial sheets formed on Invasin were also correctly polarized, as detected with the apical markers Villin and F-actin facing towards the lumen.

[373] *Material & Methods*

[374] 1. Growth assay of epithelial cells in 2D

[375] Plastic 96-well plates (Greiner) were O/N coated at 4°C with 5 µg/ml Invasin protein in PBS or 2% BME® (R&D system) diluted in PBS. Next day solution was removed, and epithelial organoids (3D-established colon, ileum, airway, mouse intestine, lacrimal gland, snake venom gland organoids) were released from the BME® matrix with 10U/ml Dispase (Thermofisher), 30 minutes at 37°C (32°C for snake venom organoids). Subsequently, organoids were washed twice with cold DMEM/F12 and treated with TrypLE (Gibco), 15 minutes at 37°C, with repeated mechanical shearing using P1000 to dissociate organoids to single cells (The dissociation of single cells was carefully monitored using a brightfield microscope). Single cells were washed twice with cold DMEM/F12 and the number of dissociated cells were counted with trypan blue (VWR) cell counting using a hemocytometer. The same number of cells (50.000 cells / well) per epithelial organoid line were added in the coated wells. Growth was quantified using CellTiterGLO on different days and luminescence was analyzed on a Tristar multimode plate reader (Berthold). The same procedure was described above to measure TEER over multiple days in Transwell systems or quantify the number of cells growing on the Invasin coat or BME®. Quantification of the number of cells on different days was carried out using Trypan blue cell (VWR) counting and hemocytometer.

[376] 2. Trans-epithelial electrical resistance (TEER) measurement

[377] 3D-established organoid lines (colon, ileum, snake venom gland, mouse lacrimal gland) were removed from BME® using 10U/ml dispase for 30 min. at 37°C (32°C for snake venom gland organoids). Dispase-treated organoids were washed twice with cold DMEM/F12 using centrifugation (500 rpm, 5 min, 4°C), to remove the last traces of BME®. Subsequently, organoids were dissociated with TrypLE (Gibco)

at 37°C for 15 min. Single cells were washed twice with cold DMEM/F12. Single cells were suspended in expansion medium, supplemented with 10 uM Y-27, and plated on a pre-coated (similar as described before, 5 µg/ml Invasin and 2% BME®, O/N, 4°C) transwell system (Greiner 12-well transparent, 3 µm pore size). TEER measurements were done using Millicell ERS-2 volt ohmmeter, protein coated Transwells without cells were used as control samples for TEER quantification.

[378] 3. Colony forming assay on Invasin protein

[379] A 96-well plate (Greiner) was coated O/N at 4°C with 5 µg/ml of Invasin protein or 2% BME® diluted in PBS. Ileum N39 organoids that have been growing in BME® droplets for 7-days, were treated with 1:10 (5U/ml in culture medium) Dispase (Thermofisher) at 37°C for 30 minutes, washed twice with cold DMEM/F12 medium, and dissociated to single cells using TrypLE (Gibco). DAPI-negative cells were FACS (BD, influx) sorted (1 cell/well) into the protein coated 96-well plates containing 100 µl intestinal organoid growth media supplemented with 10 µM Y-27 (Stem cell technologies). Growth of colonies were analyzed using brightfield and fluorescent images with EVOS microscope. After 25 days, the total number of colonies was counted blinded and later quantified based on an ATP-sensitive luminescent assay (CellTiterGLO, Promega).

[380] 4. Confocal imaging of intestinal epithelial cells

[381] Confocal imaging of ileum epithelial cells grown in 2D for 10 days in a Transwell system (Greiner 12-well translucent, 0.3 µm pore size) coated O/N at 4°C with 2% BME (R&D systems) or 10 ug/ml recombinant Invasin protein. 2D-grown organoids were fixed with 4% formaldehyde (pH 7.4) at room temperature (RT) for 30 minutes. Subsequently, the fixed cells were washed with PBS 0.1% Triton (PBST) (ThermoFisher), permeabilized, using PBS 1% Triton, for 30 minutes at RT, followed by blocking of the cells with 2% BSA (Sigma) for 60 min at RT. The fixed and permeabilized cells were incubated O/N at 4°C with a primary antibody directed against Villin (Santa Cruz, sc-58897) (1:1000). Upon extensive washing with PBST, the binding of the primary antibodies was visualized after 2 hours of incubation at RT with the secondary antibody: Donkey a-Mouse568 (Life technologies A10037, 1:1000). Phalloidin fluor conjugates Phalloidin647 (Sigma, 65906) (1:1000) were used to visualize F-actin directly. DNA was stained with DAPI (ThermoFisher) (1:1000). 2D-

grown epithelial cells were imaged on a confocal SP8 (Leica) microscope and analyzed using Imaris software.

Example 17:

[382] In Figure 15 the characterization and growth conformation of intestinal epithelial cells on Invasin is depicted.

[383] *Results*:

[384] Intestinal epithelial cells from the colon and ileum were cultured on isolated protein according to the invention (e.g., Invasin), with growth confirmed using Ki67 immunofluorescent staining (data not shown). Cell counts of ileum epithelial cells were performed over multiple days to confirm growth. Ileum epithelial cells also maintained apical-basal polarity, as shown by immunofluorescent staining with apical marker F-actin and basal marker Integrin β1.

[385] *Conclusion*:

[386] Intestinal epithelial cell growth was confirmed by Ki67 staining and cell counting. Invasin-cultured epithelial cells retain their apical-basal polarity.

Example 18

[387] Figure 16 shows the characterization and growth conformation of airway epithelial cells on isolated protein according to the invention (e.g., Invasin).

[388] *Result*:

[389] Airway epithelial cells (LU30 line) cultured on Invasin exhibited growth, confirmed by immunofluorescent staining for the proliferative marker Ki67. Growth was further validated using an ATP-sensitive luminescent assay and TEER measurements over several days, indicating the rapid formation of a tight epithelial barrier. The cells maintained their polarity, as shown by F-actin staining of the apical marker. The cultured airway epithelial cells formed a pseudostratified epithelium, characteristic of airway tissue.

[390] *Conclusion*:

[391] Airway epithelial cells cultured on Invasin form a pseudostratified epithelium like *in vivo* airway tissue. These cells retain proliferative capacity and form a tight epithelium in Transwell systems.

Example 19

[392] Figure 17 shows that mouse lacrimal gland cells adhere, grow and can be passaged on isolated protein according to the invention (e.g., Invasin) for multiple times.

[393] *Result*:

[394] Mouse lacrimal gland cells adhered and grew on Invasin-coated surfaces, confirmed by an adhesion assay and CellTiter-Glo viability analysis after 7 days. TEER measurements indicated cell growth and tight epithelial formation. The cells were passaged multiple times.

[395] *Conclusion:*

[396] Invasin supports mouse lacrimal gland cells' growth and long-term maintenance as 2D organoid sheets.

Example 20

[397] Figure 18 shows that snake venom gland cells from two distinct species cells adhere, grow and could be passaged on isolated protein according to the invention (e.g., Invasin) for multiple times.

[398] *Result:*

[399] Snake venom gland cells from A. l. cowlesi and Naja naja adhered and grew on Invasin-coated surfaces. Adhesion was confirmed by an assay, and cell viability was assessed using CellTiter-Glo after 7 days. TEER measurements indicated cell growth and tight epithelial formation (data not shown). The cells could be passaged multiple times.

[400] *Conclusion:*

[401] Invasin supports the growth and long-term maintenance of snake venom gland cells as 2D organoid sheets.

Example 21

[402] Figure 19 shows long-term growth of epithelial colon, ileum, and airway cells on isolated protein according to the invention (e.g., Invasin). Establishment of cultures from primary colon and airway tissue biopsies took also place on Invasin.

[403] *Background:*

[404] Culturing epithelial cells from primary tissue biopsies is essential for organoid technology and various clinical and regenerative applications. This enables drug testing outside the body, biobank creation, ex vivo gene editing, and transplantation of repaired cells. We first assessed the ability to maintain epithelial cells on Invasin for

multiple passages from established 3D organoids. Next, we investigated whether primary biopsies could initiate 2D cultures on Invasin.

[405] *Results:*

[406] Epithelial cells from different origins including, colon, ileum, and airway [LU30, LU31]), derived from 3D-estalished organoids were successfully passaged on Invasin in 2D. The splitting ratio was 1:2 for intestinal and 1:3 for airway cultures. Colon and airway tissue biopsies also established 2D cultures on Invasin, confirmed for colon using CellTiter-Glo after 8 days. RNA sequencing of colon cultures from two donors showed similarity, corroborated by immunofluorescence (colon and airway). Primary colon and airway cultures remained viable for multiple passages.

[407] *Conclusion:*

[408] Human primary epithelial cells from various tissues can be maintained long-term in 2D on isolated protein according to the invention (e.g., Invasin).

Example 22

[409] Figure 20 shows mouse intestinal primary tissue cultures on Invasin.

[410] *Results:* Mouse intestinal tissue (duodenum, jejunum, ileum) was processed into single cells and cultured in Invasin-coated wells. Invasin recognition as an extracellular matrix protein was confirmed via an adhesion assay. Cell growth on Inv497 was verified after four days using CellTiter-Glo. Additionally, TEER measurements confirmed the formation of tight epithelial 2D organoid sheets over time (data not shown).

[411] *Conclusion:* Mouse intestinal primary tissue can be successfully cultured on Invasin.

Example 22

[412] Figure 21 shows that intestinal epithelial cells, grown on Invasin in 2D, can be differentiated towards well-known functional intestinal cells using air-liquid interphase differentiation procedure.

[413] *Results*: Ileum epithelial cells were cultured in 2D on Invasin or, as a control, BME for 12 passages before differentiation into specialized intestinal cells by modifying the culture medium. Fluorescent reporters were integrated to track the differentiation of goblet cells (MUC2-GFP), enteroendocrine (EEC) cells (CHGA-iRFP), and Paneth cells (DEFA5-dsRED). Differentiation capacity after 12 passages was analyzed using confocal microscopy (data not shown), single-cell RNA

sequencing (data not shown), and transmission electron microscopy (TEM). Ileum cells cultured on Invasin or BME successfully differentiated into functional intestinal cell types.

[414] *Conclusion:* Long-term culture on Invasin preserves the ability of intestinal epithelial cells to differentiate into mature and functional intestinal cell types.

[415] Material & Methods

[416] Differentiation of triple reporter ileum organoid line.

[417] Ileum N39 triple reporter line (Goblet cells; MUC2-GFP, enteroendocrine (EEC) cells; CHGA-iRFP and Paneth cells: DEFA5-dsRED) obtained from10 were passaged on the integrin-binding domain of Invasin (5 µg/ml) in Transwell system with 3 µm pore size (Greiner Bio-one, 662630) using expansion medium. To analyze if small intestinal epithelial cells could retain the full functionality of an intestinal epithelium when growing on Invasin, epithelial cells were differentiated towards functional and well-known intestinal cell types like goblet, enteroendocrine and Paneth cells. This was done as described by He G-W, Lin L, DeMartino J, et al. Optimized human intestinal organoid model reveals interleukin-22-dependency of paneth cell formation. Cell Stem Cell. 2022;29(9):1333-1345.e6., with minor differences. The differences were as follows: Invasin-grown epithelial cells were cultured on Transwell systems until 100% confluency was achieved. Differentiation was induced using a 2-step differentiation protocol. The first step is to add 500 µl of patterning medium to the lower compartment of the Transwell system for 7 days (refresh medium every 2 days with patterning medium); the upper compartment is just air. The second step is to induce Paneth cells, 500 µl maturation medium (as described in He G-W et al., 2022, *supra*) was added to the lower compartment of the Transwell system, with just air in the upper compartment. This culture condition was maintained for 7 days with refreshing maturation medium in the lower compartment daily. Differentiated cells were imaged with Leica stellaris8 STED microscope and analyzed using Imaris software.

[418] Single-cell RNA sequencing of ileum triple reporter line

[419] For single-cell RNA sequencing, the triple reporter ileum N39 line, late passage (passage 12) and early passage (passage 0) were used for differentiation. The ileum epithelial cells were differentiated in Transwell systems (Greiner 24-well inserts 3 um pore size). The ileum intestinal sheet was treated with TrypLE (Gibco) until cells round up and detach from the well. Single cells were collected and washed with cold

DMEM/F12 and resuspended in cold PBS 0.04% BSA (FACS buffer). DAPI-negative cells were sorted with the BDinflux. Cells were again counted using a hemocytometer and around 1000 cells/µl, total 40.000 cells were loaded to droplet-based scRNA-seq using the 10x genomics platform. Libraries were prepared using the 10x genomics chromium 3' gene expression solution v3.1 and sequenced on a NovaSeq6000 (Illumina). Read counts were analyzed using Seurat (v5). The full script is available at https://github.com/GJFvanSon/Hubrecht_clevers/Invasin_sc. In short, read matrix was filtered, allowing a minimum of 1500 features per cell. Cells were then clustered using standard settings and annotated based on known marker gene expression like described in10. Public single-cell expression data was loaded into seurat and integrated with our dataset using the seurat intergratelayers function with standard settings.

[420] Transmission Electron Microscopy (TEM)

[421] For Transmission EM, airway and ileum epithelial cell cultures were cultured on Invasin coated Transwell system (Greiner 12-well translucent, 3 µm pore size). Ileum organoids were differentiated as described above. Airway organoids were differentiated using PneumaCult airway medium and protocols (from Stem cell technologies). Differentiated cells were fixed with fixative (1.5% glutaraldehyde (sigma) / 0.067 M cacodylate (sigma) buffered to pH 7.4 / 1% sucrose (sigma), washed in washing buffer (0.1 M cacodylate (pH 7.4) / 1% sucrose) and postfixed with 1% osmium tetroxide in the same buffer containing 1.5% potassium ferrocyanide for 1 hour (dark) at 4°C. Samples were dehydrated in ethanol, infiltrated with Epon resin, embedded in the same resin, and polymerized at 60°C for 48 hours. Ultrathin sections were obtained using a Leica Ultracut UCT ultramicrotome (Leica Microsystems) and mounted on Formvar-coated copper grids. Sections were stained with 2% uranyl acetate in water and lead citrate. Then, sections were analyzed in a Tecnai Spirit T12 Electron microscope equipped with an Eagle 4kx4k CCD camera (Thermo Fisher Scientific).

Example 23

[422] Figure 22 shows how airway cultures started from tissue biopsies were passaged multiple times on Invasin and retained differentiation capacity to mature cell types using air-liquid interphase differentiation set-up.

[423] *Results:* Airway epithelial cells obtained from lung tissue were successfully cultured on Invasin for five passages. After these passages, the differentiation potential of the airway epithelium into mature cell types was assessed using an air-liquid interface (ALI) set-up. In this system, differentiation medium was placed in the basal compartment of a Transwell system, while the apical compartment was exposed to air. Under these conditions, airway epithelial cells successfully differentiated into ciliated cells and club cells. The presence of these differentiated cell types was confirmed using immunofluorescent staining (data not shown) and transmission electron microscopy.

[424] *Conclusion*: Long-term airway cultures initiated from primary tissue maintain their ability to differentiate into functional airway cell types when subjected to an air-liquid interface differentiation set-up. This demonstrates the feasibility of using Invasin to support airway epithelial cell differentiation for potential therapeutic applications.

[425] *Material & methods*

[426] Differentiation airway 2D sheets

[427] Freshly isolated airway tissue cells (two donors) were cultured for at least five passages on the integrin-binding domain of Invasin (Inv497, 5 ug/ml) in Transwell systems with 3 µm pore size (Greiner Bio-one, 662630) using expansion medium added to the upper and lower compartment of the Transwell system. When the epithelial sheets reached 100% confluency, differentiation was induced by adding complete PneumaCult Air-liquid interface medium (stemcell technology, #05001), including Heparin solution (stemcell technology, #07980) and hydrocortisone solution (stemcell technology, #07925) to the lower compartment of the Transwell system. The upper compartment remained air. Protocol and concentrations used for media were described by stemcell technologies. After 2-3 weeks of culturing epithelial cells with complete PneumCult air-liquid interface medium, with every 2-3 days changing medium, differentiated cells were imaged using confocal SP8 (Leica) microscope and analyzed using Imaris software.

<u>Example 24 - 3D-suspension cultures of colon organoids was obtained on affigel10 beads decorated with Invasin.</u>

[428] Background: Human intestinal organoids often require a functional substrate to support their growth. This substrate can take various forms as long as it provides the necessary stimuli to promote polarity and proliferation of organoid cells. Here we

show that the protein according to the invention, comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus, is such protein capable of stimulating growth. By covalently coupling of the invasin-derived protein to a resin matrix—using its free amine groups—it is possible to create a 3D scaffold that facilitates organoid development around the resin beads, mimicking a supportive microenvironment for tissue-like organization *in vitro*.

[429] Results: Single colon (line P26N) cells were mixed with Affigel10 resins covered by the Invasin derived proteins. After 8 days of culture in the presence of the beads covered with the Invasin derived protein, we observed an epithelial layer around the Affigel10 beads covered with the protein, but not in the control condition (PBS) or beads covered with control protein BSA. That the organoids maintained epithelial polarity on the Affigel10 resins covered by the Invasin derived proteins was observed with fluorescent staining marking apical marker F-actin and basal marker integrin A6. Conformation of the protein according to the invention coupled to the beads was also done using immunofluorescent staining based on an antibody specifically targeting the integrin-binding domain of invasin. Representative results are shown in Figure 23.

[430] Material and Methods

[431] Preparation of Invasin coated Affigel10 beads.

[432] The preparation of MBP_Inv497-coupled Affi-Gel 10 beads was performed according to the manufacturer's protocol (Bio-Rad). Specifically, 50 µl of Affi-Gel 10 resin was incubated with 350 µg MBP_Inv497 in 50 mM HEPES buffer containing 100 mM NaCl and 80 mM $CaCl_2$ for over 16 hours at 4°C with continuous rotation. The ligand concentration exceeded 100 µg/ml resin to ensure efficient coupling. Prior to coupling, Affi-Gel beads were washed with three bed volumes of cold deionized water to remove the isopropanol storage solution. No Tris or glycine was added, as these could interfere with the coupling chemistry. After Invasin incubation with AffiGel 10, beads were washed with HEPES buffer and, for a subset, with DMEM/F12 (Gibco) medium before use.

[433] Suspension culture of colon organoids with Affigel10-Invasin beads

[434] Colon (line P26N) organoids were dissociated into single cells using TrypLE incubation (37°C, 10 min, with repeatedly mechanically dissociation using pipet).

Following enzymatic digestion, cells were washed with DMEM/F12 (Gibco) to stop the TrypLE reaction. The washed single cells were then resuspended in complete expansion medium supplemented with 10 µM Y-27. The single cells in expansion medium were incubated with pre-prepared Affi-Gel10 beads coupled with Invasin, BSA or empty. The beads with the cells were incubated for 1 hour at 37°C in a 15 mL tube, with every 15 minutes the tube containing cells and beads were tilted to maximize cell-bead interaction. After 1 hour the beads and cells were plated and monitored every day using brightfield microscopy.

Example 25 - Invasin-coated beads support growth of collagen-adhesion-deficient colon organoids collagen-I culture.

[435] Background and results: Colon organoids lacking the ITGA2 integrin receptor, essential and only integrin receptor for collagen-I recognition, were used to assess whether Invasin-coated AffiGel beads could rescue growth in a collagen-I hydrogel. While ITGA2 knockout (KO) organoids failed to grow in collagen-I alone or with control beads (PBS or BSA-coated), Invasin-coated beads successfully supported growth after 8 days. As a positive control BME was taken along. Confocal microscopy confirmed proper apical-basal polarity, with ZO-1 (apical) and ITGA6 (basal) markers of these ITGA2 knockout organoids grown on Affigel-Invasin beads (Figure 24).

[436] Material and Methods

[437] Preparation of Invasin coated Affigel10 beads: The preparation of MBP_Inv497-coupled Affigel 10 beads was performed according to the manufacturer's protocol (Bio-Rad). Specifically, 50 µl of Affi-Gel 10 resin was incubated with 350 µg MBP_Inv497 in 50 mM HEPES buffer containing 100 mM NaCl and 80 mM $CaCl_2$ for over 16 hours at 4°C with continuous rotation. The ligand concentration exceeded 100 µg/ml resin to ensure efficient coupling. Prior to coupling, Affi-Gel beads were washed with three bed volumes of cold deionized water to remove the isopropanol storage solution. No Tris or glycine was added, as these could interfere with the coupling chemistry. After Invasin incubation with AffiGel 10, beads were washed with HEPES buffer and, for a subset, with DMEM/F12 (Gibco) medium before use.

[438] 3D collagen culture of ITGA2 knockout colon organoids in presence of Affigel10-Invasin beads: Colon (line P26N) ITGA2 knockout organoids, which are

unable to grow in Collagen-I due to the absence of the only collagen integrin receptor Integrin α2, were used as a model system to evaluate organoid survival in a collagen-based culture supplemented with Invasin-coated Affi-gel 10 beads. Invasin could be recognized by the other available integrin receptors in the ITGA2 knockout organoids. The ITGA2-/- organoids maintained in BME were released from the extracellular matrix by incubating the cultures with Dispase (10 U/mL) at 37°C for 20 minutes. Following enzymatic digestion (monitored using brightfield microscopy), organoids were washed twice with cold DMEM/F12 by centrifugation (500 rpm, 4°C, 5 min) to remove residual matrix components and dispase enzyme. The washed organoids in DMEM/F12 were mechanically fragmented to obtain smaller organoid fragments. The fragmented organoids were then pelleted, resuspended, and incubated in collagen-I (EzCol, Advanced biomatrix) mixed with Invasin-coated AffiGel10 beads at a 1:5 ratio (beads: collagen-I). The collagen-organoid mixture was plated and polymerized under standard culture conditions for 1 hour. Organoid cultures were carefully monitored over seven days, and at day 7 images were acquired using EVOS brightfield microscopy to assess organoid growth.

CLAIMS

1. An *in vitro* method of culturing cells, the method comprising
   - exposing the cells for at least 12 hours to an isolated protein comprising at least an integrin binding domain with at least 90% identity to an integrin binding domain of an invasin protein of a species of the *Yersinia* genus; and
   - culturing the exposed cells in the presence of a culture medium.

2. The method of claim 1 wherein the isolated protein is coated on a carrier, preferably wherein the carrier is selected from the group consisting of a non-porous surface, a porous surface, a membrane, a gel, a hydrogel, or a extracellular matrix, more preferably wherein the isolated protein is present throughout a gel or wherein the isolated protein is present throughout a hydrogel.

3. The method of any of the previous claims wherein the isolated protein is coated on a carrier by covalent binding or wherein the isolated protein is coated on a carrier by non-covalent binding and/or wherein the isolated protein is coated on a carrier by direct binding or by indirect binding through an intermediary.

4. The method of any of the previous claims wherein the method of culturing the cells is a method for culturing a 2D cell culture, a 3D cell culture, an organoid, a tissue, or a primary tissue.

5. The method of any of the previous claims, wherein the isolated protein binds or activates an αβ1 Integrin expressed on the cell surface of the exposed cells, preferably wherein the αβ1 Integrin is selected from the group consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

6. The method of any of the previous claims wherein the cells are cells expressing an αβ1 Integrin, preferably wherein the αβ1 Integrin is selected from the group

consisting of an α3β1 Integrin, α6β1 Integrin, α5β1 Integrin, or any combination thereof.

7. The method of any of the previous claims wherein the cells are cells selected from the group consisting of vertebrate cells, mammalian cells or human cells and/or wherein the cells are cells selected from the group consisting of stem cells, induced-pluripotent stem cells, adult stem cells, primary cells, a cell line, epithelial cells, endothelial cells and cells that express αβ1 integrin.

8. The method of any of the previous claims wherein the species of the *Yersinia* genus is selected from the group consisting of *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia similis*, *Yersinia wautersii*, and *Yersinia enterocolitica.*

9. The method of any of the previous claims wherein the invasin protein of a species of the *Yersinia* genus comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

10. The method of any of the previous claims wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to at least 50, 100, 150, 180 or more adjacent amino acids of the last 250 amino acids of the C-terminus of an amino acid sequence selected from the group consisting of SEQ ID NO: 1 - 5.

11. The method of any of the previous claims wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence with at least 90% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

12. The method of any of the previous claims wherein the integrin binding domain comprised in the isolated protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 6 - 10.

13. The method of any of the previous claims wherein the isolated protein is produced in a host cell or chemically.

14. The method of any of the previous claims wherein culturing the exposed cell is for a period selected from the group consisting of at least one day, at least 2 days, at least 3 days, at least one week, or at least two weeks.

15. The method of any of the previous claims wherein the method is for promoting polarization of epithelial cells and/or endothelial cells.

16. The method of any of the previous claims wherein the method further comprises drug screening, drug toxicity assessment, or drug dosing assessment.

17. An isolated protein as defined in any of the previous claims 1 - 16, preferably wherein the isolated protein is coated on a carrier.

18. An isolated nucleic acid encoding the isolated protein of claim 17.

19. A host cell comprising the isolated protein of claim 17 or the isolated nucleic acid of claim 18.

20. A carrier coated with the isolated protein of claim 17, preferably wherein the carrier is sterile.

21. Use of the isolated protein of claim 17 or the isolated nucleic acid of claim 18 or the carrier of claim 20 in cell culture.

22. A method of preparing a carrier of claim 20, the method comprising contacting the isolated protein of claim 17 with the carrier thereby allowing the carrier to be coated with the isolated protein.

23. A cell culture system comprising the carrier of claim 20 or claim 22.

24. A cell culture system according to claim 23 further comprising cells.

5