MODULATING THE INTERACTION BETWEEN ZO-2/TJP2 AND A SNAIL ZINC FINGER TRANSCRIPTION FACTOR FAMILY MEMBER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of Singapore patent application no. 201201458-5, filed February 29, 2012, the contents of it being hereby incorporated by reference in its entirety for all purposes.

Technical Field

The present invention generally relates to the field of biotechnology. Specifically, it refers to the interaction between ZO-2/TJP2 and members of the Snail zinc finger transcription factor family.

Background

Epithelia serve as the first line of defense of an organism that is constantly exposed to microbes, viruses and toxins. As a first line of defense, any open wound will need to be quickly repaired. Hence it will be advantageous for epithelial cells to be exposed to growth factors so that a quick encounter with these growth factors is possible when the integrity of the epithelial sheet is compromised. This will require such epithelial cells to position their growth factor receptors strategically on their surfaces.

The tight junction (TJ) is the topmost intercellular junction in epithelial cells that is linked to the regulation of paracellular permeability and signal transduction. The TJ is composed of membrane-associated guanylate kinase proteins (MAGUKs) that include Zonula Occluden proteins (ZO-1, ZO-2 and ZO-3). These proteins have been shown to contain nuclear sorting signals and

are capable of shuttling between the membrane and nucleus depending on cell density.

In particular, ZO-2 has been shown to be capable of shuttling between the nucleus and cytoplasm. In sparse Madin-Darby Canine Kidney (MDCK) cell cultures, ZO-2 tends to accumulate in the nucleus. In addition, when pig kidney epithelial cells were subjected to environmental stress or growth at 42°C, an increased nuclear staining of ZO-2 was observed. Furthermore, in some tumor cells, TJ proteins were also found inside the nucleus. It has been found that ZO-2 may function as a tumor suppressor by blocking cell cycle progression at the transcription and protein level. Support for the role of ZO-2 as a tumor suppressor also comes from observations that its expression is either lost or decreased in a majority of breast cancer cell lines and adenocarcinomas.

Accordingly, it appears that ZO proteins have been found to translocate to the nucleus when epithelial cells are subjected to external stress such as mechanical injury, heat shock and chemical insults. However, the biological relevance of this accumulation of ZO-2 in the nucleus under such circumstances has however remained somewhat elusive.

Slug is a member of the Snail superfamily of zinc finger transcription factors. Snail and Slug were shown to elicit epithelial-to-mesenchymal transition (EMT) through the direct repression of E-cadherin expression. This process is critical in developmental processes such as gastrulation, neural crest cell migration, organogenesis, as well as in the metastasis of tumors derived from epithelial tissues. EMT is also linked to wound healing, fibroblastic remodeling in mature tissues after injury and tubulogenesis. Hence, studying the

mechanisms that regulate the Snail superfamily of transcription factors is important.

There is therefore a need to establish a relationship between ZO-2 and the Snail superfamily of transcription factors and uses for such a relationship.

Summary

In a first aspect, there is provided a method of identifying candidate agents capable of modulating interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof, the method comprising: a. contacting the first polypeptide with the second polypeptide and a candidate agent; and b. determining whether: i. the binding of the first polypeptide with the second polypeptide is decreased or increased in the presence of said candidate agent when compared with a control; or ii. whether the affinity, extent or amount of the binding between the first polypeptide and the second polypeptide is decreased or increased in the presence of said candidate agent when compared with a control.

In a second aspect, there is provided a method of predicting the likelihood of tumor metastasis development or the effectiveness of a cancer treatment, the method comprising determining a change in: a. the binding of a first polypeptide with a second polypeptide when compared with a control; or b. the affinity, extent or amount of the binding between a first polypeptide and a second polypeptide when compared with a control; wherein the first polypeptide is ZO-2/TJP2 or a functional variant

thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

In a third aspect, there is provided a kit for identifying candidate agents capable of modulating the interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof, the kit comprising the first polypeptide, the second polypeptide and at least one candidate agent.

In a fourth aspect, there is provided a method of treating, inhibiting or suppressing tumor growth comprising: administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

In a fifth aspect, there is provided a method of inhibiting tumor metastasis comprising: administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

In a sixth aspect, there is provided a method of enhancing wound healing comprising: administering an agent which increases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the

second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

In a seventh aspect, there is provided an isolated complex between ZO-2/TJP2 and a Snail zinc finger transcription factor family member.

In an eighth aspect, there is provided the use of the isolated complex as defined herein as a biomarker for detecting cancer.

In a ninth aspect, there is provided an antibody directed against the binding portion of ZO-2/TJP2 and SNAI2 (Slug) at the guanylate kinase (GUK) domain of ZO-2/TJP2.

Detailed Description

It has now been discovered that nuclear ZO-2 directly interacts with the Snail superfamily of zinc finger transcription factors. In particular, the interaction of ZO-2 with a Snail zinc finger transcription factor enhances the nuclear retention of the ZO-2/Snail complex and hence protection from proteasomal degradation. It has been found that during early stages of epithelial-to-mesenchymal transition (EMT), nuclear translocation of ZO-2 may sequester Slug into the nucleus, which would then trigger a full or partial EMT to enhance migratory properties of cells by down-regulating epithelial markers. As it has been found that Slug and SNAI1 (Snail) were capable of down-regulating many epithelial markers, the entry of ZO-2 into the nucleus may serve to feed-forward a positive feedback loop that favored the initiation of a full or partial EMT.

This finding may allow for the synthesis of small drug targets that can be used to modulate this interaction between ZO-2 and Slug or any members of the Snail superfamily. With the discovery that the ZO-2/Snail complex possesses enhanced nuclear retention, it is now possible to tailor a method to modulate the interaction between ZO-2 and a Snail zinc finger transcription factor to exploit the effects of these proteins when bound.

Accordingly, there is provided a method of identifying candidate agents capable of modulating interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof. The method can comprise the step of a. contacting the first polypeptide with the second polypeptide and a candidate agent. The method can also comprise the step of b. determining whether: i. the binding of the first polypeptide with the second polypeptide is decreased or increased in the presence of said candidate agent when compared with a control; or ii. whether the affinity, extent or amount of the binding between the first polypeptide and the second polypeptide is decreased or increased in the presence of said candidate agent when compared with a control.

As used in the context of the specification, the term "ZO-2" refers to the protein Zonula Occludens-2, also known as tight junction protein 2 (TJP2). Hence, the terms "ZO-2" and "ZO-2/TJP2" as used in the specification are interchangeable, unless indicated otherwise.

In some instances, the first polypeptide comprises ZO-2/TJP2 including, but not limited to, SEQ ID NO: 1,

SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. The different sequences for ZO-2/TJP2 arise due to the different isoforms of the protein derived from different organisms.

The ZO-2/TJP2 may be derived from any organism as long as the ZO-2/TJP2 derived therefrom possesses the guanylate kinase (GUK) domain. The different organisms include, but are not limited to, humans, mouse and canines. In one example, the different organisms come from the genus including, but not limited to, Canis, Mus and Homo. In another example, the different organisms include, but are not limited to, *Canis lupus familiaris*, *Mus musculus* and *Homo sapiens*.

In some instances, the first polypeptide comprises ZO-2/TJP2 derived from *Homo Sapiens* including, but not limited to, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. In other instances, the first polypeptide comprises ZO-2/TJP2 derived from *Mus musculus* including, but not limited to, SEQ ID NO: 2 and SEQ ID NO: 3. In yet another instance, the first polypeptide comprises ZO-2/TJP2 derived from *Canis lupus familiaris* including, but not limited to, SEQ ID NO: 1.

In some instances, the ZO-2/TJP2 may be artificially created as study models. In other instances, the ZO-2/TJP2 may be derived from *Homo sapiens* for clinical studies and therapeutic applications.

The Snail superfamily is divided into two families, namely Snail and Scratch. The Snail family is further branched into the Snail and Slug subfamilies. Smuc is a more recent isolate in the family tree of the Snail superfamily. As used in the context of the specification,

the terms "Snail zinc finger transcription factors" and "Snail superfamily of zinc finger transcription factors", or variants thereof, are interchangeable, unless indicated otherwise. The term "Snail" as used in the context of the specification refers to any one of the members of the Snail superfamily of zinc finger transcription factors, unless otherwise indicated.

In instances, the second polypeptide comprises a Snail zinc finger transcription factor family member including, but not limited to, SNAI1 (Snail), SNAI2 (Slug), SNAI3 (Smuc), Scratch 1 and Scratch 2. The Snail zinc finger transcription factor family member may be derived from different organisms. The different organisms include, but are not limited to, humans, mouse and canines. In one example, the different organisms come from the genus including, but not limited to, Canis, Mus and Homo. In another example, the different organisms include, but are not limited to, *Mus musculus* and *Homo sapiens*. The Snail zinc finger transcription factor family member may be derived from any organism as long as the Snail zinc finger transcription factor family member derived therefrom possesses the binding domain exemplified by the tryptophan (W) amino acid in position 199 of the zinc finger 3 of *Mus musculus* Slug or the equivalent zinc finger domain of other Snail family members.

In instances, the second polypeptide comprises a Snail zinc finger transcription factor family member including, but not limited to, SNAI1 (Snail) and SNAI2 (Slug). In instances, the second polypeptide comprises a Snail zinc finger transcription factor family member including, but not limited to, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID

NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

In an example, SNAI1 (Snail) includes, but is not limited to, SEQ ID NO: 12 and SEQ ID NO: 13. In another example, SNAI2 (Slug) includes, but is not limited to, SEQ ID NO: 10 and SEQ ID NO: 11. In yet another example, SNAI3 (Smuc) includes, but is not limited to, SEQ ID NO: 18 and SEQ ID NO: 19. In another example, Scratch 1 includes, but is not limited to, SEQ ID NO: 14 and SEQ ID NO: 16. In yet another example, Scratch 2 includes, but is not limited to, SEQ ID NO: 15 and SEQ ID NO: 17. In another example, the second polypeptide comprises a Snail zinc finger transcription factor family member derived from *Homo sapiens* including, but not limited to, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 18. In yet another example, the second polypeptide comprises a Snail zinc finger transcription factor family member derived from *Mus musculus* including, but not limited to, SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 18.

In some instances, the Snail zinc finger transcription factor family member may be artificially created as study models. In other instances, the Snail zinc finger transcription factor family member may be derived from *Homo sapiens* for clinical studies and therapeutic applications.

A "functional variant" of a polypeptide describes a polypeptide which has at one or more positions an amino acid insertion, deletion, or substitution, either conservative or non-conservative, and wherein each of these types of changes may occur alone, or in combination with one or more of the others, one or more times in a given sequence. Preferably, a "functional variant" has at

least 80%, 82%, 85%, 87%, 90%, 92%, 95%, 97%, 98%, 99% or 99.5% sequence identity (optionally determined using Clustal W algorithm with the alignment default parameters, and default parameters) with the reference polypeptide.

The term "conservative" when used to describe variants or substitutions denotes the replacement of an amino acid residue by another, biologically similar residue with respect to hydrophobicity, hydrophilicity, cationic charge, anionic charge, shape, polarity and the like. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids, which can be substituted for one another, include asparagine, glutamine, serine and threonine. The term "conservative substitution" also includes the use of a substituted or modified amino acid in place of an unsubstituted parent amino acid provided that substituted peptide reacts with hK2. By "substituted" or "modified", the present invention includes those amino acids that have been altered or modified from naturally occurring amino acids. The term "non-conservative" will be construed accordingly.

In some instances, the first polypeptide is a functional variant of ZO-2/TJP2. The functional variant of the first polypeptide may have at least 80%, or at least 82%, or at least 85%, or at least 87%, or at least 90%, or at least 92%, or at least 95%, or at least 97%,

or at least 98%, or at least 99%, or at least 99.5% sequence identity with ZO-2/TJP2. In one example, the functional variant of ZO-2/TJP2 has at least 80% sequence identity with said ZO-2/TJP2.

In some instances, the second polypeptide is a functional variant of the Snail zinc finger transcription factor family member. The functional variant of the second polypeptide may have at least 80%, or at least 82%, or at least 85%, or at least 87%, or at least 90%, or at least 92%, or at least 95%, or at least 97%, or at least 98%, or at least 99%, or at least 99.5% sequence identity with the Snail zinc finger transcription factor family member. In one example, the functional variant of the Snail zinc finger transcription factor family member has at least 80% sequence identity with said Snail zinc finger transcription factor family member.

The term "interaction" as used herein is to be interpreted broadly to mean coupling of two molecules via non-covalent bonding, covalent bonding, electrostatic interactions due to hydrogen bonding or van der Waals forces, and non-electrostatic interactions due to hydrophobic effects. The term "interaction" also includes hybridization which is a complementary bonding between nucleic acids (nucleotide chains), for example.

It has been found that the interaction between the first polypeptide and the second polypeptide occurs in the guanylate kinase (GUK) domain of ZO-2/TJP2. In some instances, the binding occurs in the hydrophobic pocket formed by the region between helix 8 (N848 D849 F852) and helix 2 (I726 A727) of the GUK domain of *Canis lupus familiaris* ZO-2/TJP2. In other instances, the binding occurs in the equivalent hydrophobic pocket of the GUK

domain of ZO-2/TJP2 from a species other than *Canis lupus familiaris*, such as in members of the genus Homo and Mus.

In one example, the interaction between the first polypeptide and the second polypeptide occurs in the guanylate kinase (GUK) domain of ZO-2/TJP2 comprising SEQ ID NO: 26.

In some instances, the GUK domain of postsynaptic density protein 95 (PSD-95) is A534 to V563 (SEQ ID NO: 27), and V613 to L724 (SEQ ID NO: 28). The classification of other domains of postsynaptic density protein 95 (PSD-95) include, but is not limited to, SH3 Domain (G430 to K503), HOOK Domain (W507 to Y533), GUK domain (A534 to V563, V613 to L724) and NMP Domain (part of GUK domain) (P564 to S612). In some instances, the GUK domain may comprise of the region between helix 8 (N848 D849 F852) and helix 2 (I726 A727).

In some instances, a portion of Snail interacts with ZO-2. In one example, the ring side chain of the tryptophan (W) amino acid in position 199 interacts with the GUK domain. In another example, the ring side chain of W199 of Snail interacts with the residues N848, D849, F852, I726 and A727 of ZO-2.

In some instances, the interaction occurs at the tryptophan (W) amino acid in position 199 of the zinc finger 3 domain (ZNF3) (SEQ ID NO: 29) of *Mus musculus* Slug. In other instances, the interaction occurs at W198 in *Homo sapiens* Slug. In other instances, the binding occurs in the corresponding zinc finger domain of another Snail family member.

It has also been found that there is no association of members of the Snail zinc finger transcription factor family member, e.g. Slug, with either the protein Zonula Occludens-1 (ZO-1) or Zonula Occludens-3 (ZO-3).

The term "contacting" refers to the act of touching, making contact, or of bringing to immediate or close proximity, including at the cellular or molecular level, for example, to bring about a physiological reaction, a chemical reaction, or a physical change, e.g., in a solution or in any reaction mixture, including a 'neat' mixture of reactants. In instances where the first polypeptide, second polypeptide and candidate agent are in the form of a solution, the contacting step is performed by mixing the solutions comprising one or more of the first polypeptide, second polypeptide and candidate agent.

In instances, the first and second polypeptides and the candidate agent are contacted with each other under conditions wherein, in the absence of the candidate agent, the first and second polypeptide are able to bind with each other. In some instances, *in vitro* binding conditions include, but are not restricted to, about 4°C to about 37°C. In an example, *in vitro* binding conditions include, but are not restricted to: 4°C, 3h, PBS with 1%TX-100, 1mM EDTA, complete EDTA free protease inhibitor cocktail, 1mM DTT, 300μg/ml Benzamidine. Binding is also known to occur *in vivo* in, for example, yeast and mammalian cells.

The first polypeptide, second polypeptide and the candidate agent may be contacted in any order, or simultaneously. In some instances, the first polypeptide, the second polypeptide and the candidate agent are contacted separately or simultaneously. In one example, the first and second polypeptides are contacted simultaneously, followed by the candidate agent. In another example, the first polypeptide and the candidate agent are contacted simultaneously, followed by the

second polypeptide. In another example, the second polypeptide and the candidate agent are contacted simultaneously, followed by the first polypeptide.

The term "candidate agent" as used in the context of the specification refers to any molecule that is capable of modulating interaction between the first polypeptide and the second polypeptide. The terms "candidate agent", "test compound", "agent" and "drug target" are used interchangeably in the context of the specification. In some instances, the candidate agent includes, but is not limited to: small organic or inorganic molecules (e.g. having a molecular weight less than 1 kDa, or more preferably less than 500 Da), aptamers, polypeptides, nucleic acids, antibodies (including fragments thereof), phospholipids and other lipid derivatives.

As used in the context of the specification, the term "modulation", or grammatical variants thereof, refers to up-regulation (i.e., activation or stimulation), down-regulation (i.e., inhibition or suppression) of a biological activity, or the two in combination or apart. Modulation includes, for example, an effect due to competition or steric hindrance by binding of a compound to the interacting regions or vicinity, or by binding of the compound to other regions and affecting conformation or structure and thereby binding.

Accordingly, in some instances, the candidate agent may either up-regulate or down-regulate interaction between the first polypeptide and the second polypeptide when compared to a control. An up-regulation of the interaction promotes the interaction, while a down-regulation of the interaction inhibits the interaction. In some instances, the candidate agent is an agonist of

the interaction between the first polypeptide and the second polypeptide, thereby up-regulating the interaction. In other instances, the candidate agent is an antagonist of the interaction between the first polypeptide and the second polypeptide, thereby down-regulating the interaction.

In instances where the binding of the first polypeptide and the second polypeptide is decreased in the presence of the candidate agent, the candidate agent down-regulates the interaction. In such instances, the candidate agent is considered an antagonist of the interaction. In instances where the binding of the first polypeptide and the second polypeptide is increased in the presence of the candidate agent, the candidate agent up-regulates the interaction. In such instances, the candidate agent is considered an agonist of the interaction. A decrease or increase in the binding between the first and second polypeptide may be determined in relation to a control in which the candidate agent is absent. Accordingly, a control assay may also be performed to provide a baseline for comparison.

In instances where the binding of the first polypeptide and the second polypeptide is decreased in the presence of the candidate agent, the affinity of the binding of the first and second polypeptides is decreased. In instances where the binding of the first polypeptide and the second polypeptide is increased in the presence of the candidate agent, the affinity of the binding of the first and second polypeptides is increased.

Similarly, in instances where the binding of the first polypeptide and the second polypeptide is decreased

in the presence of the candidate agent, the amount of the binding of the first and second polypeptides is decreased. In instances where the binding of the first polypeptide and the second polypeptide is increased in the presence of the candidate agent, the amount of the binding of the first and second polypeptides is increased.

Further, in instances where the binding of the first polypeptide and the second polypeptide is decreased in the presence of the candidate agent, the extent of the binding of the first and second polypeptides is decreased. In instances where the binding of the first polypeptide and the second polypeptide is increased in the presence of the candidate agent, the extent of the binding of the first and second polypeptides is increased.

The affinity, extent or amount of the binding may be determined by the dissociation constant, K_D. The dissociation constant varies depending on the assay method used. An example of a method to measure the dissociation constant is Surface Plasmon Resonance (SPR, for example BiaCore). In the SPR technique, one binding partner is coupled to a sensor chip and then binding and dissociation of the second partner (either alone or in presence of an antagonist or agonist) is measured in real time. The complex can also be coupled and the dissociation determined in real time after addition of the antagonist or agonist. Other examples of assay methods to determine the dissociation constant include, but are not limited to, quantification by fluorimetry, flow cytometry, Fluorescence Resonance Energy Transfer (FRET), titration calorimetry or fluorescence polarization.

The dissociation constant of ZO-2/TJP2 and Snail, in the absence of the candidate agent, may be anywhere from about 10^{-9}M to about 10^{-3}M when compared to the dissociation constant of a control. The control may be, for example, a Slug mutant lacking a ZNF3 domain or a Slug mutant having a W199A mutation. The dissociation constant of ZO-2/TJP2 and Snail may increase in the presence of a candidate agent that is an agonist. Alternatively, the dissociation constant of ZO-2/TJP2 and Snail may decrease in the presence of a candidate agent that is an antagonist.

The change of affinity, extent or amount of the binding may be determined by a method including, but not limited to, a yeast two-hybrid system, a glutathione S-transferase (GST) binding assay, immunoprecipitation, immunofluorescence and combinations thereof. In other instances, a change in the binding is determined by a method including, but not limited to, a yeast two-hybrid system, a glutathione S-transferase (GST) binding assay, immunoprecipitation, immunofluorescence and combinations thereof.

In some instances, the candidate agent may have no effect on the interaction between the first polypeptide and the second polypeptide. That is, the binding between the first polypeptide and the second polypeptide are neither increased nor decreased in the presence of the candidate agent as compared to when the candidate agent is absent.

Since the interacting domains of Slug and ZO-2 have been identified, it is envisioned that test compounds modeled from these domains can be used as specific agonists or antagonists. In some instances, a test compound that inhibits the interaction between ZO-2 and

Snail decreases their binding. In other instances, a test compound that promotes the interaction between ZO-2 and Snail increases their binding. In some instances, a test compound that decreases the binding of ZO-2 and Snail possesses an aromatic ring similar to that in tryptophan. A fragment or peptide of Snail containing tryptophan at amino acid position 199 and that binds to ZO-2 may be an example of a candidate agent. An example of a fragment of Snail is the ZNF 3 domain as it is the smallest domain capable of binding to ZO-2, thereby interfering the interaction between Snail and ZO-2.

As mentioned above, epithelial-to-mesenchymal transition (EMT) is linked to wound healing and an increase in the binding of ZO-2 with Snail triggers EMT. In particular, it has been found that wounding the cell monolayer concurrently raises the levels of nuclear ZO-2 and Slug, whilst closure of the wound leads to the diminishing of ZO-2 in the nucleus and the rapid degradation of Slug. Thus, the shuttling of ZO-2 into the nucleus at the wound edge could serve to stabilize Slug by nuclear sequestration, thereby making Slug less prone to proteasomal degradation.

In an example, an increase in the binding of the first polypeptide and the second polypeptide indicates that the candidate agent is useful for enhancing wound healing. The term "wound" as used herein is to be interpreted broadly to include, but is not limited to, a cut or tear in tissue (lacerations) such as a surgical incision, a catheter insertion sit or a medical implants site, scrapes (abrasions), punctures caused by bites or other injuries, trauma or burn. Hence, in some instances, the candidate agent is useful for promoting the healing of wounds.

Further, as mentioned above, the finding that the ZO-2/Snail complex possesses enhanced nuclear retention may allow for the synthesis of small drug targets that can be used to modulate this interaction between ZO-2 and Slug or any members of the Snail superfamily, thus leading to a more favorable prognosis for cancer patients.

Based on this discovery, in an example, a decrease in the binding of the first polypeptide and the second polypeptide indicates that the candidate agent is useful for treating cancer.

A cancer is a group of cells (usually derived from a single cell) that has lost its normal control mechanisms and thus has unregulated growth. Cancerous (malignant) cells can develop from any tissue within any organ. As cancerous cells grow and multiply, they form a mass of cancerous tissue, called a tumor, that invades and destroys normal adjacent tissues. The term "tumor" refers to an abnormal growth or mass; tumors can be cancerous or noncancerous. Cancerous cells from the primary (initial) site can spread (metastasize) throughout the body. In some instances, the cancer includes, but is not limited to, lung cancer, breast cancer, metastatic breast cancer, cervical cancer, colorectal carcinoma, liver cancer, head and neck cancer, pancreatic cancer, gastric cancer, prostate cancer, renal cancer, sarcoma, multiple myeloma, leukemia, lymphoma, esophageal cancer, brain tumor, glioma, bladder cancer, endometrial cancer, thyroid cancer, bile duct cancer, bone cancer, eye cancer (retinoblastoma), gallbladder cancer, pituitary cancer, rectal cancer, salivary gland cancer, and nasal pharyngeal cancer. In other instances, the cancer

includes, but is not limited to, colon carcinoma, chondrosarcoma, breast cancer and adenocarcinoma.

In another example, a decrease in the binding of the first polypeptide and the second polypeptide indicates that the candidate agent is useful for inhibiting tumor metastasis.

In an embodiment, there is provided a method of predicting the likelihood of tumor metastasis development or the effectiveness of a cancer treatment. The method may comprise the step of determining a change in: a. the binding of a first polypeptide with a second polypeptide when compared with a control; or b. the affinity, extent or amount of the binding between a first polypeptide and a second polypeptide when compared with a control; wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

As mentioned above, the binding of the first polypeptide and the second polypeptide enhances the nuclear retention of the ZO-2/Snail complex. Accordingly, a change in the binding of the first polypeptide and the second polypeptide when compared with a control indicates a change in the localization of the ZO-2/Snail complex.

The method may comprise the step of obtaining a biological sample. The biological sample may be tumor cells, such as from a tumor biopsy or circulating tumor cells from the patient. An assay may be performed to determine localization of ZO-2 or Snail or ZO-2/Snail complex in the sample. The localization may be, for example, nuclear, cytosolic or membrane localization.

In an example, the method includes, but is not limited to, a yeast two-hybrid system, a glutathione S-

transferase (GST) binding assay, immunoprecipitation, immunofluorescence and combinations thereof.

A change in affinity, extent or amount of binding can be determined, for example, *in vitro* or *in vivo*. For example, a change in affinity, extent or amount may be determined by: (a) labeling one of the polypeptides, e.g. with fluorescence or antibodies etc; (b) contacting the labeled polypeptide with the other polypeptide which is immobilized, e.g. to a solid surface such as well plates. A change in affinity, extent or amount induced by a candidate agent will result in a decrease in fluorescence intensity if the agent disrupts binding.

The method may further comprise the step of comparing the localization of the ZO-2/Snail complex in the sample with an appropriate control, e.g. one or more samples from one or more individuals who do not have tumor cells or who do not have metastatic tumor cells.

Alternatively, most biopsies of patients will include tumor tissue as well as surrounding normal tissue, the latter providing an appropriate control.

In some instances, an increase in the binding of the first polypeptide and the second polypeptide indicates an increase in tumor metastasis development. That is, if the nuclear localization or cytosolic localization is determined to be greater than the level in the control sample due to the increase in binding, then the patient has an increased likelihood of developing a metastatic condition. Alternatively, if the membrane localization is determined to be less than the level in a control sample, then the patient has an increased likelihood of developing a metastatic condition. In other instances, an ~~increase in the binding of the first polypeptide and the~~

second polypeptide indicates a decrease in effectiveness in cancer treatment.

The cytosolic localization, or nuclear localization, or membrane localization, or combinations thereof, may be used as a biomarker or diagnostic tool to detect cancer or tumor metastasis.

Further, since metastasis is a late event in carcinogenesis, the cytosolic localization, or nuclear localization, or membrane localization, or combinations thereof may also be used as a biomarker to grade a tumor. Alternatively, the cytosolic localization, or nuclear localization, or membrane localization, or combinations thereof may also be used to predict the outcome of a treatment (e.g. tumor treatment). The use of biomarkers as disclosed herein may optionally comprise their use as biomarkers in combination with one or more other markers.

In some instances, a decrease in the binding of the first polypeptide and the second polypeptide indicates a decrease in tumor metastasis development. In other instances, a decrease in the binding of the first polypeptide and the second polypeptide indicates an increase in effectiveness in cancer treatment.

Determining the likelihood of tumor metastasis development or the effectiveness of a cancer treatment is of great value to the medical practitioner. For example, low effectiveness of a cancer treatment means that a longer or higher dose treatment should be given, and the patient should be more closely monitored for signs of recurrence of the cancer. An accurate prognosis is also of benefit to the patient. It allows the patient, along with their partners, family, and friends to also make decisions about treatment, as well as decisions about their future and lifestyle changes.

In one embodiment, there is provided a kit for identifying candidate agents capable of modulating the interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof. The kit may comprise the first polypeptide, the second polypeptide and at least one candidate agent.

In an example, the at least one candidate agent may be a library of candidate agents.

In one embodiment, there is provided a method of treating, inhibiting or suppressing tumor growth. The method may comprise the step of administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

The term "inhibiting" refers to barring the organism from acquiring the abnormal condition, while the term "treating" refers to a method of alleviating or abrogating the abnormal condition in the organism. The term "suppressing" refers to reducing the possibility of the organism from acquiring the abnormal condition.

In one embodiment, there is provided a method of inhibiting tumor metastasis. The method may comprise the step of administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

In one embodiment, there is provided a method of enhancing wound healing. The method may comprise the step of administering an agent which increases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

The agent may be administered in combination with cancer therapies, either separately or simultaneously. In some instances, the agent can be used in combination with conventional cancer therapies or pharmaceutical formulations useful for treating cancer. These treatments can include surgical procedures, radiation therapy and/or ablation therapy (e.g., laser therapy, infrared therapy and the like). Cancer therapies including dendritic cell therapy, chemokines, cytokines, tumor necrosis factors (e.g., TNF-α), chemotherapeutic agents (e.g., adenosine analogs (e.g., cladribine, pentostatin), alkyl sulfanates (e.g., busulfan)), anti-tumoral antibiotics (e.g., bleomycin, dactinomycin, daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, mitomycin), aziridines (e.g., thiotepa), camptothecin analogs (e.g., irinotecan, topotecan), cryptophycins (e.g., cryptophycin 52, cryptophicin 1), dolastatins (e.g., dolastatin 10, dolastatin 15), enedyine anticancer drugs (e.g., esperamicin, calicheamicin, dynemicin, neocarzinostatin, neocarzinostatin chromophore, kedarcidin, kedarcidin chromophore, C- 1027 chromophore, and the like), epipodophyllotoxins (e.g., etoposide, teniposide), folate analogs (e.g., methotrexate), maytansinoids (e.g., maytansinol and maytansinol analogues), microtubule agents (e.g., docetaxel, paclitaxel, vinblastine, vincristine, vinorelbine), nitrogen mustards (e.g.,

chlorambucil, cyclophosphamide, estramustine, if osf amide, mechlorethamine, melphalan), nitrosoureas (e.g., carmustine, lamustine, streptoxacin), nonclassic alkylators (e.g., altretamine, dacarbazine, procarbazine, temozolamide), platinum complexes (e.g., carboplatin, cisplatin), purine analogs (e.g., fludarabine, mercaptopurine, thioguanine), pyrimidine analogs (e.g., capecitabine, cytarabine, depocyt, floxuridine, fluorouracil, gemcitabine), substituted ureas (e.g., hydroxyurea)]; anti-angiogenic agents (e.g., canstatin, troponin I,), biologic agents (e.g., ZD 1839, virulizin and interferon), antibodies and fragments thereof (e.g., anti EGFR, anti-HER-2/neu, anti-KDR, 1MC-C225), anti-emetics (e.g., lorazepam, metroclopramide, and domperidone), epithelial growth factor inhibitors (e.g., transforming growth factor beta 1), anti-mucositic agents (e.g., dyclonine, lignocaine, azelastine, glutamine, corticoid steroids and allopurinol), anti-osteoclastic agents (e.g., bisphosphonates {e.g., etidronate, pamidronate, ibandronate, and osteoprotegerin}), hormone regulating agents (e.g., anti-androgens, LHRH agonists, anastrozole, tamoxifen), hematopoietic growth factors, anti-toxicity agents (e.g., amifostine), kinase inhibitors (gefitinib, imatinib), and mixtures of two or more thereof.

In one embodiment, there is provided an isolated complex between ZO-2/TJP2 and a Snail zinc finger transcription factor family member. The Snail zinc finger transcription factor family member may be any member of the Snail zinc finger transcription factor family. In one example, the Snail zinc finger transcription factor family member is SNAI2 (Slug).

The binding portion is as disclosed herein. In some instances, the binding portion of ZO-2/TJP2 and SNAI2 (Slug) is at the guanylate kinase (GUK) domain of ZO-2/TJP2. In an example, the guanylate kinase (GUK) domain of ZO-2/TJP2 comprises SEQ ID NO: 26.

In an embodiment, there is provided the use of the isolated complex as defined herein as a biomarker. In an example, the biomarker may be used for detecting cancer. In another example, the biomarker may be used for grading tumors. In yet another example, the biomarker may be used for tumor metastasis. In another example, the biomarker may be used for predicting the outcome of a treatment (e.g. tumor treatment).

In an embodiment, there is provided an antibody directed against the binding portion of ZO-2/TJP2 and SNAI2 (Slug) at the guanylate kinase (GUK) domain of ZO-2/TJP2. In an example, the guanylate kinase (GUK) domain of ZO-2/TJP2 comprises SEQ ID NO: 26.

Unless specified otherwise, the terms "comprising" and "comprise", and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, unrecited elements.

As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood

that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Brief Description Of Drawings

The accompanying drawings illustrate a disclosed embodiment and serves to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Fig. 1 shows the SH3-GUK-Acidic (SGA) domains of the bait used in Example 2, as well as of ZO-1, ZO-2 and ZO-3.

Fig. 2 shows the alignments of the zinc finger domains and the DNA base contact residues -1, 2, 3 and 6 for Slug and Zif268 in Example 8.

Fig. 3 shows the hydrophobic pocket within the GUK domain formed by the region between helix 8 (N848 D849 F852) and helix 2 (I726 A727) of ZO-2, where the ring side chain of W199 is shown buried inside the hydrophobic pocket of ZO-2 GUK formed mainly by residues N848, D849, F852, I726 and A727.

Fig. 4 shows the number of dots in the cell, nucleus and cytoplasm, respectively, in GFP-Slug MDCK cells seeded at a low cell density in Example 9. It can be seen that the count fell greatly when the cell number increased to 40-69 cells per image field and decreased down to a low background level once the cell number exceeded 70 cells per image field.

Fig. 5 shows the statistical analysis of the nuclear ZO-2 fragment and nuclear GFP-Slug staining in Example 11, evidencing that there is a positive relationship between the enhanced staining of GFP-Slug in the nucleus and the entry of Nu Flag ZO-2 3PSGAP into the nucleus. Null hypothesis is GFP-Slug enhanced staining in the nucleus is independent of Nu Flag ZO-2 3PSGAP entry into the nucleus.

Fig. 6 shows the immunoblot referred to in Example 11, evidencing that expression of Nu Flag-ZO2 3PSGAP caused a dramatic increase in the nuclear and cytosolic protein levels for GFP-Slug, while no effect was observed on the levels of GFP-Slug W199A or the GFP control. Equal loading was monitored by probing for α-tubulin or lamin B1 in the cytosolic or nuclear fractions, respectively. Anti-Flag antibodies were used to detect the presence of the transfected Nu Flag-ZO2 3PSGAP.

Fig. 7 shows a RT-PCR analysis referred to in Example 11, evidencing that Nu-ZO-2 3PSGAP expression did not affect GFP-Slug mRNA levels, thereby ruling out an effect on transcription.

Fig. 8 shows the immunoblot referred to in Example 12, evidencing that knocking down ZO-2 caused an effective reduction of endogenous ZO-2 level both in MDCK cells and MDCK GFP-Slug. There was also a corresponding decrease in endogenous Slug and GFP-Slug level in the two

respective experiments showing that a decrease in ZO-2 level did destabilize Slug and GFP-Slug in the two systems.

Figs. 9a and 9b show that there was a more rapid turnover of both endogenous Slug and GFP-Slug when siRNA against ZO-2 was introduced in Example 12. The prior treatment with proteasome inhibitor MG132 delayed the turnover of both endogenous Slug and GFP-Slug by increasing their initial amount at time zero, thus evidencing the absence of off-targeting effect by showing the continued translation of Slug and GFP-Slug in the presence of ZO-2 siRNA. The stabilizing effect ZO-2 had on Slug and GFP-Slug was reaffirmed.

Fig. 10 shows the immunoblot referred to in Example 12, evidencing that constructs of si-ZO-2 MDCK transfected with either pcDNA3 Nu Flag ZO-2 3PSGAP or Nu Flag ZO-2 3PSGA retained the GUK domain to which Slug interacts.

Fig. 11 shows that knocking down of ZO-2 led to a further increase in the proliferation rate for the GFP-Slug expressing cells demonstrated in Example 12. In contrast, proliferation of the GFP and GFP-W199A expressing cells was not significantly affected and remained below that of the GFP-Slug expressing cells treated with the control siRNA.

Fig. 12 shows the immunoblot referred to in Example 13, evidencing that the construct of mCherry tagged ZO-2 from mouse is resistant to knock down by si-ZO-2 #3 which was designed specifically against canine ZO-2.

Fig. 13 shows the results of the co-immunoprecipitation (IP) assay of Example 15, evidencing that Flag ZO-2 and HA-Slug co-immunoprecipitate.

Fig. 14 shows that a combination of GFP-Slug and mcherry ZO2 enhances the decrease in E-cadherin promoter activity in Example 17.

Examples

Non-limiting examples of the invention and a comparative example will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

Example 1

ZO-1, 2 & 3 Constructs

The templates used to generate the various ZO-1, 2 and 3 domain constructions used in the following examples were from Reichert et al. These various domains of ZO-1, 2 and 3 namely: Human ZO-1 SGA (amino acids 491-883), Canine ZO-2SGA (amino acids 575-947), ZO-2SH3 (amino acids 575-713), ZO-2GA (amino acids 714-947) and Canine ZO-3 SGA (amino acids 451-898), were cloned into pGBKT7 (Clontech) with primers designed to introduce a 5′ EcoRI and a 3′ SalI restriction site.

For the cloning of the various domains of ZO-2 namely: Canine ZO-2SGA (amino acids 575-947), ZO-2GA (amino acids 714-947), ZO-2AP (amino acids 883-1174) and ZO-2P (amino acids 948-1174) into pGEX-6P-1 (Amersham Biosciences), primers were designed to introduce a 5′ EcoRI and a 3′ SalI restriction site.

For the cloning of the various point mutations of ZO-2 namely: Canine ZO-2SGA I726V, F852Y, N848S F852Yand N848S F852Y I726V in pGEX-6P-1 (Amersham Biosciences), suitable primers were designed based on manufacturer's

instructions (Phusion® Site-Directed Mutagenesis Kit). Sequences of the primers used are available upon request.

For the cloning of the MYC tagged GA domain of ZO-1, ZO-2 and ZO-3 namely: Canine ZO-1GA (amino acids 631-883), ZO-2GA (amino acids 713-947), ZO-3GA (amino acids 606-899) into pGBKT7 for *in vitro* translation, primers were designed to introduce a 5′ EcoRI and a 3′ NotI restriction site. pcDNA3-Flag-ZO2 (amino acids 1-1175) was provided by Reichert et al and pcDNA3-Flag-ZO2ΔGA was obtained by deleting amino acids 713-947 using suitable overlapping primer pairs.

For the cloning of the nucleus targeting, membrane targeting and non-targeting truncated ZO-2 constructs namely: Canine ZO-2 3PSGA (amino acids 495-947) and ZO-2 3PSGAP (amino acids 495-1444) into pcDNA3 (Invitrogen), primers were designed to introduce a 5′ EcoRI and a 3′ XbaI restriction site. Nuclear and membrane targeting signals were introduced by PCR. The nucleus targeting signal was a three tandem repeat of the nuclear localization signal (NLS) of the simian virus 40 T-antigen. The sequence was obtained from the pDsRed2-Nuc vector (Clontech) (nucleotide position: 1298-1387), while the membrane targeting sequence was from the N-terminal 20 amino acids of neuromodulin, also called GAP-43, it contained a signal for post-translational palmitoylation of cysteines 3 and 4 that targeted the fusion protein to the membranes. This sequence was obtained from the pEYFP-Mem vector (Clontech) (nucleotide position: 679-738). All constructs were verified by sequencing.

Slug Constructs

The full-length mouse Slug cDNA was obtained from the I.M.A.G.E Consortium cDNA clone 2225875 by using suitable primers covering the 5′ and 3′ coding region of

the cDNA. The full length Slug cDNA, as well as the C'-terminus and N'-terminus truncation of Slug, to be named as EX (short for excluding the zinc finger domains) and ZNF (the 5 zinc finger domains of Slug) respectively, was cloned into pGEX4T-1 (Amersham Biosciences; 5'-BamHI/3'-SalI or EcoRI), pMAL (New England BioLabs; 5'-BamHI/3'-SalI), pEGFP, and pDHA (a modified pCI-neo vector (Promega) with two HA-Tags added to the 5' end of its MCS, 5'-EcoRI/3'-NotI) expression vectors. The GST-W199A construct was made by mutating trytophan at position 199 to an alanine using suitable primer pairs. Suitable overlapping primer pairs were used to generate substitutions in amino acids to disrupt the cysteine residues (C188A, C191A) involved in maintaining the zinc finger structures of zinc finger 3. Sequences of the primers used are available upon request.

Cell lines and transfection

The cell lines commonly used during the study were COS-1, 293T and MDCK. They were cultured in Dulbecco's Modified Essential Medium (DMEM-low glucose) with 10% FCS and supplemented with Penicillin/Streptomycin and L-Glutamine, at 37°C, 5% CO_2 and 95% humidity. MDCK cells were either seeded sparsely ($1x10^5$ cells/per well) or densely ($4x10^5$ cells/per well) on a 6 well plate with a coverslip in each well and grown overnight.

The plasmid DNA from midiprep was used for transfection of cells. A combination of lipofectamine plus and reagent or lipofectamine 2000 along with optimem I (Invitrogen) were used, following the manufacturer's protocol. Transfected cells were analyzed 24 or 48 hrs after transfection. For the generation of stable cell lines, cDNAs in mammalian expression vectors were transfected into MDCK cells using Lipofectamine 2000

(Invitrogen) and maintained for 24 hr prior to treatment with G418 (Calbiochem) and a selection period of 3-4 weeks until clones appear. Multiple clones were picked with cloning rings, combined and expanded until they reach a number suitable for further concentration by cell sorting. These combined positive clones were further screened with Western blot and immunofluorescence.

Antibodies

All antibodies used for Western blotting were diluted in 2% BSA in PBS 0.1% tween buffer with the indicated dilution factor. The following commercially available primary antibodies were used: rabbit anti-ZO-1 (Zymed Laboratories, South San Francisco, CA, USA, Cat. #61-7300) 1:1000, rabbit anti-ZO-2 (Zymed Laboratories, South San Francisco, CA, USA, Cat. #71-1400) 1:1000, rabbit anti-ZO-3 (Zymed Laboratories, South San Francisco, CA, USA, Cat. #36-4000) 1:1000, rabbit anti-Slug Ctr antibody (Abgent, Cat. #AP2053a) 1:200, goat polyclonal anti-Slug antibody (Santa Cruz, Cat. #SC-10436) 1:200, rabbit anti-Slug C19G7 (Cell signaling, 9585S) 1:1000, mouse anti-laminB1 (Abcam, Cat. #3046-100) 1:3000, rabbit anti-alpha tubulin (Abcam, Cat. #ab15246-500) 1:3000, mouse anti-GAPDH (Chemicon, Cat. #MAB374) 1:20000, rat monoclonal anti-Hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN, USA, Cat. #1867423) 1:1000, mouse anti-Flag (Sigma, Cat. #F3165) 2µg/10ml, mouse anti-Myc antibodies (Roche Diagnostics, Indianapolis, IN, USA, Cat. #11667149001) 1:2000, rabbit anti-GST (Santa Cruz, Cat. #SC-459) 1:1000, mouse anti-MBP (abcam, Cat. #R29.6) 1:1000, rabbit anti-GFP (Biovision, Cat. #3999-100) 1:1000.

As for the secondary antibodies, the following commercially available secondary antibodies were used at

1:5000: bovine anti-goat horseradish peroxidase (HRP)-labeled secondary antibodies (Santa Cruz, Cat. #SC-2350), donkey anti-goat HRP (Santa Cruz, Cat. #SC-2020), goat anti-rat HRP (Santa Cruz, Cat. #SC-2006), goat anti-rabbit HRP (Bio-Rad, Cat. #170-6515) and goat anti-mouse HRP (Bio-Rad, Cat. #170-6516).

All antibodies used for immunofluorescence were diluted in 1% BSA PBS. The following commercially available primary antibodies were used: mouse anti-ZO-2 (Zymed Laboratories, South San Francisco, CA, USA, Cat. #37-4700) 1:100, mouse anti-SC-35 (Sigma-Aldrich, Cat. #S4045) 1:300, rabbit anti-Slug Ctr antibody (Abgent, Cat. #AP2053a) 1:100, rabbit anti-Slug C19G7 (Cell signaling, 9585S) 1:100, rat monoclonal anti-Hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN, USA, Cat. #1867423) 1:100, and mouse anti-Flag (Sigma-Aldrich, Cat. #F3165; 2µg/10ml).

Fluorescently labeled (Alexa 488 and 594) secondary antibodies were from Molecular Probes (Eugene, OR, USA) 1:1000, and 4-6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Cat. #D1306) with a concentration of 300nM in PBS was used to stain the nuclei of cultured cells.

Example 2

Yeast two-hybrid system

A yeast two-hybrid screen was performed using the SH3-GUK-Acidic (SGA) domain of canine ZO-2 (amino acids 575-945) fused in-frame to the GAL4 DNA binding domain of the yeast expression vector pGBKT7 (Clontech) as a bait and a pre-transformed mouse 17 day embryo cDNA library fused to the GAL4 transactivator domain in the yeast expression vector pGADT7 (MATCHMAKER Two Hybrid System;

Clontech). The SGA domains of the bait, as well as the SGA domains of ZO-1, ZO-2 and ZO-3 are shown in Fig. 1.

All experimental details were according to the manufacturer's protocols. Clones were screened at high stringency with a quadruple (-Trp, -Leu, -His, -Ade) dropout media and then tested for α- and β-galactosidase activity. Library plasmids with the cDNA inserts from positive clones were isolated and sequenced, resulting in the identification of the entire Slug cDNA. The interaction was retested by transforming this Slug cDNA plasmid with bait vectors carrying the human ZO-1 SGA domain (amino acids 490-883), the canine ZO-2 SGA (amino acids 575-945), SH3 (amino acids 575-713), as well as the GA domain (amino acids 714-945) or the canine ZO-3 SGC domain (amino acids 450-899). Empty pGBKT7 and a bait plasmid with laminin cDNA (Clontech) were used as negative controls.

The yeast two-hybrid screen resulted in the isolation of full-length Slug, which was further tested by co-transformation with either pGBKT7 or pGBKT7-ZO-2SGA using QDO which lacked leucine, tryptophan, histidine and adenine as well as, α- and β-galactosidase assays. The presence of 'bait' and 'prey' plasmids in co-transformed cells was shown by growth on double drop-out (DDO) plates lacking leucine and tryptophan. pGADT7-Laminin C + pGBKT7-p53, pGBKT7 + pGADT7, pGBKT7-ZO-2SGA + pGADT7, and pGBKT7 + pGADT7-Slug, were all used as negative controls, while pGADT7-T-Ag + pGBKT7-p53 served as the positive control.

One particular clone named 30A was found to grow on quadruple dropout media (QDO) media and turned blue in both α-Gal Assay and β-Gal assays. This indicated the binding of the ZO-2 SGA bait to an unknown prey from the library leading to the activation of various promoters

for survival in QDO media and expression of α-galactosidases and β-galactosidases that elicited the color change in X-gal media. The library plasmid of this positive clone 30A was isolated, sequenced and was found to harbor the entire Slug cDNA.

Accordingly, it was shown that Slug is a potential interacting partner of ZO-2SGA verified by QDO selection, α-gal assay and β-gal assay.

The interaction was retested by transforming this Slug cDNA plasmid with bait vectors carrying the human ZO-1SGA domain (amino acids 490-883), the canine ZO-2SGA (amino acids 575-945), SH3 (amino acids 575-713), GA domain (amino acids 714-945), as well as the canine ZO-3SGA domain (amino acids 450-899). Interactions were determined by monitoring the growth of the co-transformed yeasts in on selective media & β-galactosidase activity. It was observed that only yeast co-transformed with pGBKT7-ZO-2SGA and pGADT7-Slug or pGBKT7-ZO-2GA and pGADT7-Slug grew on QDO selective media and produced blue color colonies, an indication of β-galactosidase activity. Hence, Slug did discriminately interact with the SGA domain of ZO-2 and not with those of ZO-1 or ZO-3, thus showing the existence of non-redundant features within the ZO family members.

Empty pGBKT7 or a bait plasmid with laminin cDNA (Clontech) was used as negative controls. No interaction was detected between the Slug and laminin, nor was there any interaction of Slug with an empty library vector comprising of the Gal4 DNA binding domain alone. Hence auto-activation and non-specific activation were checked and found to be negative.

Example 3

***In Vitro* Binding Assay**

Since the binding of ZO-2 was mapped to the GA domain, only this region would be used for this binding. An *in vitro* translation system was used to make the ZO-1, ZO-2 and ZO-3 GA domain separately so that their binding to Slug can be reconfirmed and verified individually in a system with minimum complexity.

GST, GST-Slug, GST-EX, GST-ZNF and various GST-tagged ZNF truncated Slug protein were produced, purified and bound to glutathione Sepharose-4B (Amersham Biosciences, Piscataway, NJ, USA) following standard protocols. Bound proteins were quantified by SDS-PAGE by comparing with known amounts of BSA as standards. A 15cm dish of MDCK cells were grown to 80-90% confluence and lysed in cold buffer B (PBS with 1% TX-100 PBS, 1mM EDTA, Complete EDTA free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), 1mM dithiothreitol (DTT), 300µg/ml Benzamidine, and put through a single freeze-thaw cycle. The lysate was centrifuged at 13000rpm for 10min at 4°C to obtain a clear supernatant. 30µl bed volume of glutathione Sepharose-4B carrying 20µg of GST or GST fused Slug derived, full length or truncated proteins were incubated with 1.5mg of the MDCK cell lysate, for 3hrs at 4°C. The beads were washed with 500µl of cold buffer B for 3 washes. This was followed by resuspension in 30µl of SDS sample buffer and heating of the sample at 95°C for 5 min. The analysis of the bound proteins were carried out by running 25µl of the sample through a 10% SDS-PAGE gel followed by autoradiography using suitable antibodies.

It was found that MYC-ZO-2GA can be captured by GST-Slug and C-terminal portion GST-ZNF. Neither MYC-ZO-1GA

nor MYC-ZO-3GA can be captured down by GST-Slug or GST-ZNF. Hence the findings confirmed the earlier observations that Slug binds mainly to ZO-2 but not ZO-1 or ZO-3.

Example 4

Immunoprecipitation

The interaction between ZO-2 and Slug *in vivo* was demonstrated by immunoprecipitating endogenous ZO-2 from the MDCK lysate.

pDHA-Slug and pcDNA-Flag-ZO2 or pcDNA-Flag-ZO2ΔGA were co-transfected into COS1 cells and then lysed after 24 hrs using lysis buffer (10mM Tris HCl pH7.9, 150mM NaCl, 0.02% sodium azide, 1% Trition X-100, 1% sodium deoxycholate, 1%BSA, protease inhibitor cocktail tablet). 2mg of the lysates were then incubated with the 30µl bed volume of the immobilized anti-HA resins for 3 hrs followed by 3 washes with lysis buffer. This was followed by resuspension in 30µl of SDS sample buffer without any reducing agents and heating of the sample at 95°C for 5 min. The analysis of the bound proteins were carried out by running all 25µl of the sample through a 10% SDS-PAGE gel followed by detection with the appropriate antibodies.

MDCK cells ($3x10^6$/150mm petri dish) were lysed at 0°C in 1ml of lysis buffer (20mM Tris HCl pH8.0, 137mM NaCl, 10% glycerol, 1% Trition X-100, 0.5% Sodium deoxycholate, 15U/ml DNase I, protease inhibitor cocktail tablet) and briefly sonicated. 2mg of total protein were incubated with 10µg of mouse anti-ZO2 or mouse IgG control at 4°C for 1hr followed by another 2hrs incubation with 80µl of suspended (25% v/v) IP matrix (Santa Cruz, sc-45060). They were then washed with 1ml PBS each time for 2 times, followed by a final wash with 1ml PBS with 0.1% Tween.

The precipitates were boiled in sample buffer for 5 min and subjected to electrophoresis and immunoblotting with the relevant antibodies.

As mentioned above, mouse preimmune serum was used as a negative control for immunoprecipitation. The samples were then electrophoresed, blotted and probed with anti-Slug and anti-ZO-2 antibodies. The result showed that Slug specifically interacted with ZO-2 and an absence of Slug in the negative control where pre-immune serum was used for immunoprecipitation.

Example 5

TNT *in vitro* translation binding assay

In an attempt to map the exact binding domain of ZO-2SGA for Slug, GST-ZO-2SGA, GST-ZO-2SH3, GST-ZO-2GA, GST-ZO-2AP (acidic and proline rich domain) and GST-ZO-2P (proline rich domain) were used for binding to HA-Slug, HA-EX (the N-terminus region of Slug excluding the zinc finger domain) and HA-ZNF (the C-terminus zinc finger domain).

In vitro translation was carried out using Quick Coupled T7 TNT System (Promega, Madson, WI) according to the manufacturers protocol. Slug, EX and ZNF cloned into the pDHA vector were *in vitro* translated and labeled with 35S Methionine. GST, GST-ZO-2 SGA, GST-ZO-2 SH3, GST-ZO-2 GA, GST-ZO-2 AP and GST-ZO-2 P were produced, purified as described above and were bound to glutathione Sepharose-4B (Amersham Biosciences, Piscataway, NJ, USA) following standard protocols.

Purified GST-fusion proteins were quantified by SDS-PAGE by comparing with known amounts of BSA as standards. 20 µl bed volume of beads carrying 20 µg of GST or the above GST fused ZO-2 truncated proteins were incubated with 10 µl of *in vitro* translated 35S Methionine labeled,

HA tagged Slug, EX or ZNF and topped up with 200 µl of buffer B (PBS with 1% TX-100 PBS, 1 mM EDTA, Complete EDTA free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 1 mM dithiothreitol (DTT), 300 µg/ml Benzamiidine) for 3 h at 4°C. The beads were washed three times with 500 µl of buffer B each time. This was followed by suspension in 30µl of SDS sample buffer and the analysis of the bound proteins by running through a 15% SDS-PAGE gel and autoradiography, with a 2 to 3 day exposure period. To reciprocate the binding, GST, GST-Slug and GST-ZNF were used as baits. They were incubated with *in vitro* translated MYC-ZO-1GA, MYC-ZO-1GA or MYC-ZO-3GA and probed for the presence of the different MYC-ZOs GA domains using mouse anti-myc antibodies (Roche Diagnostics, Indianapolis, IN, USA).

The results showed that the SH3 and the P do not bind to Slug or its ZNF domain. Instead it is the GA domain that exhibits the strongest binding effect comparable with signals obtained from binding of ZO-2SGA to GST-Slug and GST-ZNF. Binding of Slug and ZNF to GST-ZO-2AP can be detected but obviously weaker. GST-ZO-2P does not bind to Slug or ZNF.

Taken together, it goes to show that GUK is the more potent interacting domain, whilst the acidic domain only binds weakly to the ZNF domain of Slug.

Example 6

To verify the binding of full length ZO-2 to full length Slug *in vivo*, HA-Slug and Flag-ZO-2 or Flag-ZO-2ΔGA constructs were co-transfected into COS-1 cells.

This is followed by immunoprecipitating using the method in Example 4 with either anti-Flag and probing by anti-HA, or with anti-HA antibodies following which anti-Flag will be used for probing instead. Equal amounts of

total protein were used to immunoprecipitate HA-Slug or Flag-ZO-2. Following SDS-PAGE, the co-precipitation of ZO-2 or Slug, respectively, was monitored by Western blot analysis using antibodies to the Flag or HA tags, respectively. When Flag-ZO-2 was immunoprecipitated, HA-Slug could be detected with anti-HA but not so when the GA truncated form of Flag-ZO-2 was used.

Taken together, it showed that HA-Slug could bind to Flag-ZO-2 but not to Flag-ZO-2ΔGA in an *in vivo* setting. When HA-Slug was immunoprecipitated instead, Flag-ZO-2 could be detected with anti-Flag but not for Flag-ZO-2ΔGA. Hence, it reconfirmed the binding of HA-Slug to Flag-ZO-2 but not to Flag-ZO-2ΔGA.

Example 7

The ZNF region of Slug is required for binding to ZO-2. However, there are 5 zinc finger domains (ZNF1-5) within the ZNF region of Slug. Hence, in order to narrow down the exact zinc finger domain(s) responsible for initiating the interaction with ZO-2, a GST binding assay was carried out in accordance with the procedure in Example 3 using various zinc finger truncated mutants of Slug.

The series of GST-Slug derived truncated protein ranging from 0 to 28 were incubated with MDCK cell lysate and probed for the presence of endogenous ZO-2 using specific antibodies against ZO-2, where L is 15% loading input.

It was observed that ZNF3 was critical and sufficient for binding to ZO-2 since all constructs that harbored ZNF3 can capture ZO-2. Even that of construct 15 that comprised of merely the N-terminus EX domain and ZNF3 is sufficient to capture down ZO-2.

Example 8

Fine mapping of the interaction domain in ZO-2 and Slug

Mapping of the crucial amino acids involved in binding to ZO-2 GUK was aided by molecular dynamics simulations using the known crystal structures of closely related proteins such as the zinc finger protein Zif268 and the GUK containing PSD95 as templates for the zinc finger 3 of SNAI2 and the GUK of ZO-2.

To elaborate on this process, well-studied crystallized structure of PSD-95 (PDB file 1KJW) was used for modeling the docking site for Slug on ZO-2. Although there are several domains in PSD-95, only the well-conserved GUK domain (A534 to V563, and V613 to L724) was used in this case for modeling ZO-2 GUK. The classification of the various domains of PSD-95 is as follows: SH3 Domain (G430 to K503), HOOK Domain (W507 to Y533), GUK domain (A534 to V563, V613 to L724) and NMP Domain (part of GUK domain) (P564 to S612).

In the case of Slug, the C_2H_2 zinc finger Zif268 in complex with DNA from PDB 1A1H, was used as template for Slug ZNF3, 4 and 5 in modeling its binding to ZO-2 GUK. This was due to availability of its crystal structure and its exhibition of good homology with Slug. In the alignment, the Zif268 (1A1H) consists of 3 C_2H_2 zinc fingers. Full length Slug consists of 4 C_2H_2 zinc fingers while zinc finger 5 is a C_2HC zinc finger. The alignments of their zinc finger domains and the DNA base contact residues -1, 2, 3 and 6 (these numbers denote the positions from the start of each alpha helix) are shown in Fig. 2.

With the PSD-95 GUK serving as a template for ZO-2 GUK and the Zif268 ZNFs as the template for Slug ZNF3, 4, 5, the model reveals a hydrophobic pocket within the GUK domain formed by the region between helix 8 (N848 D849

F852) and helix 2 (I726 A727) of ZO-2 as shown in Fig. 3. In Fig. 3, the ring side chain of W199 is shown buried inside the hydrophobic pocket of ZO-2 GUK formed mainly by residues N848, D849, F852, I726 and A727.

There are reasons to suspect that the tryptophan (W) amino acid in position 199 of Slug ZNF3 can fit very well into this hydrophobic pocket of ZO-2, based on the assumption that when two proteins interact, there will be a pocket or complementary surface to make the interaction stronger. W199 is a good target as there are evolutionary evidences that support a ring structure amino acid like tryptophan wedging into such a pocket in GUK domain.

In order to verify if the tryptophan residue at position 199 plays a role here in the binding of Slug to ZO-2, a W199A point mutation was made prior to doing the GST-binding assay in accordance with the procedure in Example 3. In addition two cystenine to alanine point mutations were made on cystenine 188 and 191 (C188A and C191A) to check if the zinc finger structure plays a role in the binding of Slug to ZO-2.

As it turned out, W199 is critical for binding but not C188 and C191 that help maintain the zinc finger structure of Slug ZNF3.

Further support of the model comes from binding assays carried out using the following ZO-2 point mutants that affect the crucial amino acids surrounding the hydrophobic pocket within the GUK domain of ZO-2, namely GST-ZO2 SGA I726V, F852Y, N848S+F852Y and N848S+F852Y+I726V. The rationale behind the selection of these mutations is that since ZO-1 and ZO-3 do not bind Slug, unlike ZO-2, and that they differ in the above amino acids that projects into the hydrophobic pocket, mutating these amino acids in ZO-2 to those corresponding to either ZO-1 and/or ZO-3 should abolish the binding of

Slug to these ZO-2 mutants. The point mutated GST-ZO2 proteins were incubated with GFP-Slug MDCK lysate and probed for the presence of both GFP-Slug and endogenous Slug using specific antibodies against Slug.

As expected, the single point ZO-2 mutants showed weakened binding to GST-Slug, while a combination of 2 or all 3 mutations virtually abolished the interaction with GST-Slug. No interaction was detected with the GFP negative control while strong interaction was readily seen with GST-ZO2 SGA WT positive control.

Interestingly, mutating the corresponding amino acids in ZO-1 to those of ZO-2 did not render an effective binding of this ZO-1 mutant to Slug. Hence it hints of amino acids within ZO-1 that may yet interfere with such an interaction.

Example 9

Localization of ZO2 & Slug in MDCK culture

To determine if ZO-2 found in the nucleus of sparse MDCK cells is associated with Slug, MDCK cells were grown in both sparse and dense conditions as follows.

Further, a linear wound to the cell monolayer was inflicted in one of the dense cultures to visualize their localization in the region proximal to the wound in comparison to those at a distal area.

Proximity Ligation Assay (PLA)

This technology is based on two unique probes provided with the Duolink kit that consist of a secondary antibody attached to a unique synthetic oligonucleotide serving as a reporter. The proximity of the probes allows for DNA hybridization and ligation at the exact site where these probes are in close proximity. The distance at which this can happen is merely 40nm or less. Hence

only proteins that interact can hybridize, ligate, get amplified for detection by hybridizing to fluorescent probes. This method gives the specificity and sensitivity needed via DNA-DNA hybridization and DNA fold amplification of the initial ligation event respectively.

Since the limitation of this technique depends on the quality of the primary antibodies used for recognizing the interacting proteins, the quality and reliability of the antibodies were assessed by using the GFP-Slug MDCK as a positive control and GFP-W199A MDCK as a negative control.

MDCK cells ($4x10^4$ - $1x10^5$cells/per chamber) were grown on chamber slides (Lab-Tek 8 well glass slide system 177402) for at least 16 h, washed twice with PBS, and fixed in 3.7% formaldehyde in PBS for 30 min at room temperature. Subsequently, the slides were washed with PBS; incubated for 10 min in 50mM NH4Cl, PBS; washed with PBS; permeabilized for 5 min in 0.2% Triton X-100 in PBS; and washed three times with PBS with 0.05% Tween 20. The slides were then blocked for 30 min with 2% BSA in PBS at room temperature.

After blocking, the appropriate combinations of antibodies in blocking buffer were added in a humidified chamber at 37°C and incubated for 1hr. After washing with Duolink II wash buffer A, proximity ligation was performed using the Rabbit MINUS and Mouse PLUS Duolink in situ PLA kits (OLINK Bioscience) according to the manufacturer's protocol. Subsequently, slides were stained with DAPI before embedding in Crystal/Mount™ (Biomeda). Fluorescence was detected using a Zeiss Axioplan microscope equipped with the Zeiss AxioVision Imaging System or Zeiss LSM 700 laser scanning microscope.

Antibodies used for PLA were Zymed mouse anti-ZO2 and cell signaling rabbit anti-Slug. Single antibody incubations were performed as negative controls. These negative controls did not give significant PLA signals.

The images (not shown) were acquired with a 40x objective, a 1.6x magnifier and a 10x eye-piece to obtain images that gave a wide range of cell number per captured field. The spots per cell were counted by semiautomated image analysis using the single cell analysis function of BlobFinder software.

It was shown that the number of nuclear PLA spots pick up is much higher in the case of GFP-Slug MDCK than those seen in GFP-W199A MDCK.

Further, ZO-2 and Slug were seen clearly inside the nucleus where the cells were seeded sparsely. While the border staining of ZO-2 was also clearly visible in these cells, the border staining of Slug was only weakly visible. Accordingly, ZO2 and Slug co-localize to the nucleus in sparse MDCK cells. The nuclear stains of ZO2 and Slug decrease and disappear as the monolayer becomes confluence and subsequently adopt a more cytoplasmic localization.

In contrast, when the MDCK cells were seeded densely, the nuclear staining of Slug became perinuclear while border staining diminished further. Similarly, the staining of nuclear ZO-2 also turned perinuclear but a taint of it was still observable within the nucleus. The border staining of ZO-2 was still detectable.

To rule out the absence of signals due to substrate limitation at high cell number, the detection of ZO-1-ZO-2 complexes was included as a positive control. Strong PLA signals of ZO-1-ZO-2 complexes were shown to line the cell borders even at high cell densities, thereby ruling out substrate limitation as the reason for an absence of

PLA signals for ZO-2-Slug complexes at high cell densities.

Accordingly, it was demonstrated that the PLA signals tended to decrease as the cell density increased.

The ZO-2-Slug PLA signals in MDCK cells of various densities were quantitated by counting the spots on the images taken with the BlobFinder software. The number of cells subjected to analysis per cell number group, ranged from around 200 to 350. The parameters were set so that only the cells that fell within the 25th to the 75th percentile of the cytoplasmic and nuclear area were automatically counted and scored. This would filter out cells that were not fully captured at the edge of the images as well as those that were abnormally large or small.

The results shown in Fig. 4 indicate a high score of PLA signals in GFP-Slug MDCK cells seeded at a low cell density of 1-39 cells per image field. The PLA signals were counted with BlobFinder software and the number of spots per cell, per nucleus and per cytoplasm were presented in the graph of Fig. 4. The data points were grouped by the cell number per field captured using a Zeiss Axioplan microscope with a 40x objective, a 1.6x magnifier and a 10x eye-piece. The use of primary antibodies against ZO-2 alone, Slug alone or the omission of the primary antibodies was used as controls.

As can be seen in Fig. 4, the PLA signal count fell greatly when the cell number increased to 40-69 cells per image field and decreased down to a low background level once the cell number exceeded 70 cells per image field. This pattern was mirrored in the case of MDCK cells, although the initial count in the sparsely seeded cell group was much lower than the GFP-Slug MDCK.

Wound Assay

MDCK cells were seeded onto 10cm petri dish or coverslips in 24 wells-plate and grown until completely confluent. The cells were either left unwounded or wounded. Cells on the 10cm plates were wounded by scrapping the monolayer with cress-crossing lines while those on coverslips were inflicted with a single scratch wound. In both instances a 1ml pipette tip was used.

They were incubated overnight before being treated for immunofluorescence microscopy (as described in Example 10 below) or lysed with RIPA buffer (50mM Tris HCl pH7.9, 150Mm NaCl, 1mM EDTA, 1% Trition X-100, 0.1% SDS, protease inhibitor cocktail tablet) for Western blot analysis.

In the cells proximal to the wound edge, both ZO-2 and Slug were clearly seen within the nucleus. However in cells that were a couple of layers distal from the wound edge, a reduced level of both nuclear ZO-2 and Slug was seen, evidenced by weakened nuclear stains of ZO-2 and Slug in cells distal from the wound.

To further demonstrate this temporal relationship of Slug and ZO-2 in cells that were wounded, two confluent plates of MDCK cells were grown with one subjected to numerous scratches while the other unscratched.

Nuclear and cytosolic fractions were prepared in equal amounts of proteins (40µg) for each fraction analyzed by SDS-PAGE and Western blotting using an antibody to Slug. Lamin B1 and α-tubulin were used as markers and loading controls for the nuclear and cytosolic fraction, respectively.

It was shown that wounding of the monolayer caused an increase in the level of ZO-2 in the nuclear fraction while the amount in the cytosol remained comparable. A corresponding increase in nuclear Slug level was also

discernable with wounding but not so in the cytosolic fraction.

Example 10

Immunofluorescence Microscopy

MDCK cells grown on coverslips or in chamber slides were washed three times with phosphate-buffered saline (PBS) followed by fixing for 30 min in 4% paraformaldehyde (PFA) pH7.4 on ice. Fixation step was stopped by quenching with 50mM NH_4Cl-PBS for 10 min. This was followed by permeabilization with 0.2% Trition X-100 PBS for 5 min followed by 3 subsequent washes with PBS within 5 min. They were then blocked for 30 min in 1% BSA in PBS at room temperature. Following which, the fixed cells were incubated with the relevant antibodies at room temperature for 1hr. After 3 washes, the cells were incubated with the Alexa FluorR secondary antibodies (1:1000) in blocking buffer for 1hr at room temperature. After a final three washes with PBS, the coverslips were mounted with Crystal/Mount (Biomeda). The images were captured using a Zeiss Axioplan microscope equipped with the Zeiss AxioVision Imaging System.

Example 11

Presence of ZO-2 in the nucleus stabilizes Slug

As described, Slug and ZO-2 can interact. This association occurs between the ZNF domains of Slug and a C-terminal region comprising amino acids 495-1444 of ZO-2, and is abolished by substituting Trp199 in Slug to Ala (W199A). Since ZO-2 can localize either to the plasma membrane or the nucleus, the N-terminus of ZO-2 were coupled to known membrane or nuclear targeting signals to test if these constructs could influence the subcellular localization of Slug.

The ZO-2 constructs targeted to membranes were co-transfected with different Slug mutant into MDCK cells (results not shown). All Slug constructs harboring zinc finger domain 3, which carries W199 important for binding to ZO-2, accumulated together with the ZO-2 fragment in uncharacterized membranous structures in the cytoplasm. This co-localization with the ZO-2 fragment was abolished for constructs that either lack zinc finger 3 or carry the W199A mutation.

A ZO-2 truncation mutant containing the GUK domain important for the interaction with Slug was generated and fused to a nuclear localization signal (Nu-ZO2 3PSGAP). In contrast to wt ZO-2, which displays different subcellular localizations depending on experimental conditions, this nuclear targeting truncated ZO-2 is expected to simulate the enhanced nuclear accumulation of ZO-2 observed in sparse cultures and during wound healing. Cells stably expressing GFP, GFP-Slug or GFP-Slug W199A were transfected with this vector containing Nu-ZO2 SPSGAP.

It was shown that expression of Nu-ZO2 3PSGAP resulted in an increase in the nuclear staining of GFP-Slug when visualized by immunofluorescence imaging in accordance with the procedure in Example 10. This reflected the enhanced retention of Slug in the nucleus through its binding to the ZO-2 construct. Consistent with this interpretation, a stronger nuclear GFP-Slug staining was not observed in cells that did not express the nuclear ZO-2 fragment or in cells expressing GFP-Slug W199A.

Statistical significance of the correlation between expression of nuclear ZO-2 fragment and enhanced nuclear GFP-Slug staining was confirmed by examining the images of cells taken following their fixation. While the images

are not shown, the statistical analysis is shown in Fig. 5. Fig. 5 shows a positive relationship between the enhanced staining of GFP-Slug in the nucleus and the entry of Nu Flag ZO-2 3PSGAP into the nucleus. Null hypothesis is GFP-Slug enhanced staining in the nucleus is independent of Nu Flag ZO-2 3PSGAP entry into the nucleus.

$X^2=(ad-bc)^2(a+b+c+d)/(a+b)(c+d)(b+d)(a+c)$

Chi square statistical=125.4376

Degree of freedom = (no. of row-1) (no. of column-1) =1

Chi square critical = 3.84 (with alpha of 0.05)

It is thus shown that Chi square statistical is much higher than Chi square critical. The data presents a statistically significant relationship between the variables in the table, hence null hypothesis is rejected.

An alternative hypothesis is that GFP-Slug enhanced staining in the nucleus is dependent on Nu Flag ZO-2 3PSGAP which binds to and retains GFP-Slug in the nucleus hence protecting it from proteasomal degradation.

There was a correlation between the nuclear expression of Nu-ZO2 3PSGAP and an enhanced nuclear GFP signal intensity for cells expressing GFP-Slug as seen in Fig. 5. These data suggest that binding of GFP-Slug to Nu-ZO2 SPSGAP results in its nuclear retention and hence protection from proteasomal degradation.

Cells stably expressing GFP-Slug, GFP Slug W199A or GFP were transfected or not with a vector carrying Flag tagged Nu-ZO-2 3PSGAP. Cells were then lysed and cytosol and nuclear fractions analyzed by Western blot using antibodies to GFP.

As shown in Fig. 6, expression of Nu Flag-ZO2 3PSGAP caused a dramatic increase in the nuclear and cytosolic

protein levels for GFP-Slug. In contrast, no effect was observed on the levels of GFP-Slug W199A or, as a control, GFP. Equal loading was monitored by probing for α-tubulin or lamin B1 in the cytosolic or nuclear fractions, respectively. Anti-Flag antibodies were used to detect the presence of the transfected Nu Flag-ZO2 3PSGAP.

The RT-PCR analysis is shown in Fig. 7. Total RNA extracted from Nu Flag-ZO2 3PSGAP transfected and non-tranfected MDCK cells stably expressing GFP-Slug was amplified using specific primers for Slug and, as a control, GAPDH. Nu-ZO-2 3PSGAP expression did not affect GFP-Slug mRNA levels as assessed by RT-PCR shown in Fig. 7, ruling out an effect on transcription.

Taken together, these data are consistent with a stabilizing effect of Nu-ZO-2 3PSGAP on Slug.

Example 12

The stabilizing effect of Nu Flag ZO-2 3PSGAP on GFP-Slug has been demonstrated. It will therefore be interesting to examine whether the knock down of endogenous ZO-2 will have a destabilizing effect on GFP-Slug and/or endogenous Slug. Hence, the effectiveness of the siRNA against ZO-2 was tested here.

siRNA Knock down

$1x10^5$ GFP-Slug MDCK cells were seeded in each of the wells in a 6 well plate and siRNA duplexes (60 pmol) were forward transfected and incubated for 48 h. siRNAs were synthesized by Invitrogen: (1) si-control (SEQ ID NOs: 20 and 21); (2) si-ZO-2 #2 (si GUK) (SEQ ID NOs: 22 and 23); and (3) si-ZO-2 #3 (si PDZ2) (SEQ ID NOs: 24 and 25).

A portion of the GFP-Slug MDCK cells were treated with MG132 (1:20000) for 16 h after the 48 h incubation.

This was followed by a change to complete culture medium with cycloheximide (1:1000) and sampling at time zero and thereafter every 2 h. For ZO-2 rescue, $1.2x10^6$ MDCK cells were seeded on 10cm petri dishes and transfected with either si-ZO-2 #3 or si-control (370 pmol), along with either pcDNA3, pcDNA3-Nu Flag ZO-2 3PSGAP or pcDNA3-Nu Flag ZO-2 3PSGA constructs at 24μg per plate. The cells were harvested after 48 h with RIPA buffer (50mM Tris HCl pH7.9, 150Mm NaCl, 1mM EDTA, 1% Trition X-100, 0.1% SDS, protease inhibitor cocktail tablet) and analyzed by immunoblot.

The immunoblot results shown in Fig. 8 evidence that knocking down ZO-2 caused an effective reduction of endogenous ZO-2 level both in MDCK cells and MDCK GFP-Slug. There was also a corresponding decrease in endogenous Slug and GFP-Slug level in the two respective experiments showing that a decrease in ZO-2 level did destabilize Slug and GFP-Slug in the two systems.

Once verified for its effectiveness of the siRNA against ZO-2, GFP-Slug MDCK cells was subjected to the same ZO-2 siRNA or a non-targeting siRNA control and tracked the degradation of both endogenous Slug and GFP-Slug in a time course assay lasting for 6 h and 2 h sampling intervals.

Analysis of cell lysate by immunoblotting following transfection of ZO-2 siRNA or a non-targeting control siRNA into GFP-Slug MDCK cells grown on 6 well plates. A portion of these cells were treated with MG132 (1:20000) after 48 h for a further 16 h. Degradation of GFP-Slug and Slug were tracked every 2 h for a total of 6 h, following a change to complete culture medium with cycloheximide. Cycloheximide was added to block further protein synthesis.

To demonstrate the absence of any off-targeting effect of ZO-2 siRNA on Slug or GFP-Slug, a parallel set of the same experiment was carried out but was subjected to the proteasome inhibitor MG132 treatment prior to a change of medium that contained cycloheximide.

The results shown in Figs. 9a and 9b revealed a more rapid turnover of both endogenous Slug and GFP-Slug when siRNA against ZO-2 was introduced. As shown in Figs. 9a and 9b, this prior treatment with MG132 delayed the turned over of both endogenous Slug and GFP-Slug by increasing their initial amount at time zero.

Thus it demonstrated the absence of off-targeting effect by showing the continued translation of Slug and GFP-Slug in the presence of ZO-2 siRNA and reaffirmed the stabilizing effect ZO-2 had on Slug and GFP-Slug.

When ZO-2 siRNA transfected MDCK was simultaneously transfected with either pcDNA3 Nu Flag ZO-2 3PSGAP or Nu Flag ZO-2 3PSGA, it was shown that the rescue was effective in increasing the amount of Slug. Both of these constructs retained the GUK domain to which Slug interacts. Analysis of cell lysate by immunoblotting following the transfection of ZO-2 siRNA or a non-targeting control siRNA along with pcDNA3, pcDNA Nu Flag ZO-2 3PSGAP or pcDNA Nu Flag ZO-2 3PSGA into MDCK cells grown on 10cm plates is shown in Fig. 10. The levels of endogenous Slug in each case were analyzed by immunoblot after 48 h of incubation.

Knock down of ZO-2 stimulates the proliferation rate of MDCK GFP-Slug cells

The effect of knocking down ZO-2 on the proliferation rate of MDCK GFP-Slug cells was assessed here.

MDCK cells were transfected with siRNA specific against ZO-2 or a non-targeting control siRNA. The transfected were grown for 3 days before they were processed for Western analysis and immunofluorescence microscopy. GFP and GFP-W199A were used as controls for the knock down experiment.

Cell lines stably expressing GFP, GFP-Slug and GFP-W199A were subjected to siRNA against ZO-2 or a non-targeting siRNA as a control. Their rate of proliferation was then assessed via the WST-1 based assay. The results are shown in Fig. 11.

As shown in Fig. 11, knocking down of ZO-2 led to a further increase in the proliferation rate for the GFP-Slug expressing cells. In contrast, proliferation of the GFP and GFP-W199A expressing cells was not significantly affected and remained below that of the GFP-Slug expressing cells treated with the control siRNA.

Example 13

A rescue was also carried out using a full length mCherry tagged ZO-2 from mouse in this example. This construct is resistant to knock down by si-ZO-2 #3 which was designed specifically against canine ZO-2. Knock down of Slug was also carried out to confirm the target influenced by the knock down and replacement of ZO-2 was indeed Slug and GFP-Slug.

GFP-Slug MDCK cells were seeded at $1.5x10^5$ cells in each well of a 6 well plate. siRNAs including si-control and si-ZO-2 #3 (30pmol) were transfected following 3h of incubation. This was followed by transfecting mCherry Mm Tjp2 constructs (4μg) and a further 3h incubation before a final change to complete media.

Analysis of the cell lysate by immunoblotting followed the transfection of ZO-2 siRNA or a non-

targeting control siRNA into GFP-Slug MDCK cells grown on 6 well plates. The levels of GFP-Slug and endogenous Slug in each case were analysed by immunoblot after 24h of incubation and the results are shown in Fig. 12.

Example 14

In this example, presence of nuclear ZO2 and Slug was demonstrated in some clinical samples derived from the multiple cancer tissue array.

A multiple tissue array (Cybrdi™ Human Derived Biological Products CC00-10-001) slide was baked at 56°C for 10 min, followed by treatment with xylene for 15 min. This was followed by rehydration using 95%, 90% and 70% ethanol for 2, 2 and 3 min respectively and finally distilled water for 2 times 5 min. The slide was then subjected to antigen retrieve using a 2100 retriever (ProteoGenix) in sodium citrate pH 6.0 for 12 min. After cooling, the slide was rinsed with PBS and subjected to PFA fixation (see immunofluorescence at Example 10 for details). The primary antibodies used were rabbit anti-Slug (Cell Signalling, 1:50) and mouse anti-ZO2 (Invitrogen, 1:50). The secondary antibodies used were Alexa FluorR 594 donkey anti-mouse IgG (1:250) and Alexa FluorR 488 donkey anti-rabbit IgG (1:250).

The most prominent demonstration was from the ovarian section of a 35 year old female with invasive colon carcinoma infiltrating her ovary. The invasive colon carcinoma in ovary section was stained with anti-ZO2 and anti-Slug antibodies. Comparing this section with normal ovary section stained with the above antibodies showed the presence of large number of carcinoma cells with large nuclei and elevated levels of nuclear ZO2 and Slug not observable in the normal ovary section.

It is thus evidenced that metastatic cancers derived from colon carcinoma in ovary section showed an elevated level of ZO2 and Slug in the nucleus.

Similar observation was seen in a chondrosarcoma sample derived from the bone of a 59 year old female but not in another from a 48 year old female. The results (not shown) evidence that only certain chondrosarcoma sections showed an elevated level of nuclear ZO2 and Slug but not in the other.

Example 15

Co-immunoprecipitation (IP) assays of Slug and ZO-2 were carried out, in accordance with the procedure in Example 4, using COS1 cells transfected with HA-Slug and Flag-ZO-2 or Flag-ZO-2ΔGA constructs.

Equal amounts of total protein were used to immunoprecipitate HA-Slug or Flag-ZO-2. Following SDS-PAGE, the co-precipitation of ZO-2 or Slug, respectively, was monitored by Western blot analysis using antibodies to the Flag or HA tags, respectively.

The results shown in Fig. 13 evidence that Flag ZO-2 and HA-Slug co-immunoprecipitate.

Example 16

A GST binding assay in accordance with the procedure in Example 3 was performed using a series of point mutated GST-ZO2 proteins. They were incubated with GFP-Slug MDCK lysate and probed for the presence of both GFP-Slug and endogenous Slug using specific antibodies against Slug.

The results (not shown) evidence that ZO2 mutants abolish binding to GFP-SLUG.

Example 17

In this example, a combination of GFP-Slug and mcherry ZO2 was shown to enhance the decrease in E-cadherin promoter activity.

GFP and GFP-Slug stably expressing MDCK cells were seeded at $8x10^5$ cells per 10 cm tissue culture dish and knocked down with either 33 pmol/ml of si Ctrl or si ZO2 for 72h using Lipofectamine™ RNAiMax according to the manufacturer instruction. They were reseeded into 24 welled plates at $1x10^5$ cells per well and transfected with 1μg of either empty vector or either DsRed or mCherry tagged ZO-2 constructs using Lipofectamine™ 2000 along with 0.2 μg of pGaussia-Luciferase hu E-cadherin promoter reporter and 0.02 μg of pCMV-Vargula-Luciferase. Readings were taken in quadruplets after 24h using the DLAR4 kit (targetingsystems) according to the manufacturer instructions.

It was shown that both GFP-Slug and mcherry ZO2 can repress the E-cadherin promoter individually. The former can act directly while the latter may act through endogenous Slug, but when both are present they can repress the promoter activity even further. The results are shown in Fig. 14.

Claims

1. A method of identifying candidate agents capable of modulating interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof, the method comprising:
   a. contacting the first polypeptide with the second polypeptide and a candidate agent; and
   b. determining whether the binding of the first polypeptide with the second polypeptide is decreased or increased in the presence of said candidate agent when compared with a control.

2. The method of claim 1, wherein the ZO-2/TJP2 is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9.

3. The method of claim 1, wherein the Snail zinc finger transcription factor family member is selected from the group consisting of SNAI1 (Snail), SNAI2 (Slug), SNAI3 (Smuc), Scratch 1 and Scratch 2.

4. The method of claim 3, wherein the Snail zinc finger transcription factor family member is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13,

SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

5. The method of any one of claims 1-2, wherein the functional variant of ZO-2/TJP2 has at least 80% sequence identity with said ZO-2/TJP2.

6. The method of any one of claims 3-4, wherein the functional variant of the Snail zinc finger transcription factor family member has at least 80% sequence identity with said Snail zinc finger transcription factor family member.

7. The method of any one of the preceding claims, wherein the first polypeptide, the second polypeptide and the candidate agent are contacted separately or simultaneously.

8. The method of any one of the preceding claims, wherein a decrease in the binding of the first polypeptide and the second polypeptide indicates that the candidate agent is useful for treating cancer.

9. The method of any one of the preceding claims, wherein a decrease in the binding of the first polypeptide and the second polypeptide indicates that the candidate agent is useful for inhibiting tumor metastasis.

10. The method of any one of the preceding claims, wherein an increase in the binding of the first polypeptide and the second polypeptide indicates

that the candidate agent is useful for enhancing wound healing.

11. The method of any one of the preceding claims, wherein a change in the binding is determined by a method selected from the group consisting of a yeast two-hybrid system, a glutathione S-transférase (GST) binding assay, immunoprecipitation, immunofluorescence and combinations thereof.

12. A method of predicting the likelihood of tumor metastasis development or the effectiveness of a cancer treatment, the method comprising determining a change in the binding of a first polypeptide with a second polypeptide when compared with a control; wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

13. The method of claim 12, wherein a decrease in the binding of the first polypeptide and the second polypeptide indicates a decrease in tumor metastasis development or an increase in effectiveness in cancer treatment.

14. The method of any one of claims 12 or 13, wherein the method is selected from the group consisting of a yeast two-hybrid system, a glutathione S-transferase (GST) binding assay, ~~immunoprecipitation, immunofluorescence~~ and combinations thereof.

15. A kit for identifying candidate agents capable of modulating the interaction between a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof, the kit comprising the first polypeptide, the second polypeptide and at least one candidate agent.

16. A method of treating, inhibiting or suppressing tumor growth comprising: administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

17. A method of inhibiting tumor metastasis comprising: administering an agent which decreases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

18. A method of enhancing wound healing comprising: administering an agent which increases the binding of a first polypeptide and a second polypeptide, wherein the first polypeptide is ZO-2/TJP2 or a functional variant thereof and the second

polypeptide is a Snail zinc finger transcription factor family member or a functional variant thereof.

19. An isolated complex between ZO-2/TJP2 and a Snail zinc finger transcription factor family member.

20. The complex of claim 19, wherein the Snail zinc finger transcription factor family member is SNAI2 (Slug).

21. The complex of claim 20, wherein the binding portion of ZO-2/TJP2 and SNAI2 (Slug) is at the guanylate kinase (GUK) domain of ZO-2/TJP2.

22. The complex of claim 21, wherein the guanylate kinase (GUK) domain of ZO-2/TJP2 comprises SEQ ID NO: 26.

23. Use of the isolated complex of any one of claims 19-22 as a biomarker for detecting cancer.

24. An antibody directed against the binding portion of ZO-2/TJP2 and SNAI2 (Slug) at the guanylate kinase (GUK) domain of ZO-2/TJP2.

25. The antibody of claim 24, wherein the guanylate kinase (GUK) domain of ZO-2/TJP2 comprises SEQ ID NO: 26.