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(54) Title: RELEASE OF EXTRACELLULAR MEMBRANE PARTICLES CARRYING THE STEM CELL MARKER PROMININ-1 (CD133) FROM NEURAL PROGENITORS AND OTHER EPITHELIAL CELLS

(57) **Abstract:** The present invention relates to an extracellular membrane particle carrying integrated into the membrane prominin-1 molecules. In accordance with the present invention, theses particles also referred to as prominin-1 -containing membrane particles. The membrane particles according to the present invention are distinct from exosomes. The invention in a further aspect relates to a method of diagnosing an epithelial cancer or a hematopoietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of the invention wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer or a hematopoietic disease. Alternatively, an increased level of said particles is indicative of the presence of such a disease in a mammal. Additionally, the present invention relates to a method of prognosing a solid tumor in a mammal, comprising determining the change in the number of the particles of the invention in a body fluid of said mammal over time. Finally, the present invention relates to a method of diagnosing a degeneration of the retina in a mammal or a predisposition thereto comprising assessing for the presence of prominin-1 on the surface of the particles of the invention in a body fluid from said mammal wherein the absence of said particles or a reduced amount of prominin-1 on the surface of said particles in comparison to a control is indicative of degeneration of the retina or a predisposition thereto.

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Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells

The present invention relates to an extracellular membrane particle carrying integrated into the membrane prominin-1 molecules. In accordance with the present invention, these particles also referred to as prominin-1-containing membrane particles. The membrane particles according to the present invention are distinct from exosomes. The invention in a further aspect relates to a method of diagnosing an epithelial cancer or a hematopoietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of the invention wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer or a hematopoietic disease. Alternatively, an increased level of said particles is indicative of the presence of such a disease in a mammal. Additionally, the present invention relates to a method of prognosing a solid tumor in a mammal, comprising determining the change in the number of the particles of the invention in a body fluid of said mammal over time. Finally, the present invention relates to a method of diagnosing a degeneration of the retina in a mammal or a predisposition thereto comprising assessing for the presence of prominin-1 on the surface of the particles of the invention in a body fluid from said mammal wherein the absence of said particles or a reduced amount of prominin-1 on the surface of said particles in comparison to a control is indicative of degeneration of the retina or a predisposition thereto.

A variety of documents is cited throughout this specification. The disclosure content of these documents, including manufacturer's manuals and catalogues, is herewith incorporated by reference.

The proliferation *versus* differentiation of stem cells is a fundamental issue of biology and medicine. Yet, the underlying cell biological mechanisms are poorly understood. A key aspect in this context is cell polarity, which has moved into the focus especially in the case of mammalian neuroepithelial cells (Chenn et al., 1998; Huttner and Brand, 1997; Wodarz and Huttner, 2003), the somatic stem cells from which – directly or indirectly – all neurons of the central nervous system derive (Alvarez-Buylla et al., 2001; Fishell and Kriegstein, 2003; Kriegstein and Götz, 2003).

As is the case for other polarized epithelial cells, a characteristic feature of neuroepithelial stem cells is an apical plasma membrane (Aaku-Saraste et al., 1997; Huttner and Brand, 1997). Focusing on this membrane, it was recently reported that the switch of mouse neuroepithelial stem cells from symmetric, proliferative to asymmetric, neuron-generating cell divisions is associated with a halvening of the size of the apical plasma membrane (Kosodo et al., 2004). Moreover, as hypothesized previously (Huttner and Brand, 1997), apical plasma membrane is inherited by both daughter cells upon symmetric, proliferative division of neuroepithelial stem cells but by only one daughter cell upon asymmetric, neuron-generating division (Kosodo et al., 2004). These studies suggest a critical role of apical plasma membrane constituents in maintaining neuroepithelial cells in a stem cell state (Huttner and Brand, 1997; Kosodo et al., 2004; Wodarz and Huttner, 2003).

A notable constituent of the apical plasma membrane of neuroepithelial stem cells is prominin-1 (CD133), a pentaspan membrane glycoprotein expressed on the surface of many somatic stem cells (Bussolati et al., 2005; Alessandri et al., 2004; Corbeil et al., 2001b; Richardson et al., 2004; Weigmann et al., 1997; Yin et al., 1997), embryonic stem cell-derived progenitors (Kania et al. in press) and cancer stem cell (Singh et al., 2004). Remarkably, prominin-1 is specifically associated with plasma membrane protrusions, irrespective of the cell type (Corbeil et al., 2001b; Corbeil et al., 2000; Giebel et al., 2004; Maw et al., 2000; Röper et al., 2000a; Weigmann et al., 1997; Fargeas et al, 2004; reviewed in Fargeas et al., 2006).

Thus, in a number of issues associated with the differentiation and proliferation of cells and in particular stem cells have been clarified as is evidenced by the quoted literature. The proliferation of stem cells as well as cancer stem cells might be associated with the presence of the stem cell marker prominin-1 (Fargeas et al., 2006), and the lack of this specific molecule due to, for instance, its specific release as prominin-1-containing particles might correlate with the differentiation of these cells. Prominin-1-containing plasma membrane domain or microdomain might thus contain specific elements (e.g. prominin-1 and other protein and/or lipid) that allow the stem cell to keep its properties, i.e. proliferate and differentiate. Identification of membrane particles containing a stem cell marker released from stem cells will be suitable to follow the cellular differentiation process. In view of the above, it would be advantageous to have further means available that may guide the skilled person in his quest for being able to clearly distinguish phenomena associated with proliferation over those associated with differentiation.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims. Accordingly, the present invention relates to an extracellular membrane particle carrying integrated into the membrane prominin-1 molecules.

The term “extracellular membrane particle” describes a particle that occurs extracellularly, and in particular in body fluids of mammals such as mouse and human but may nevertheless - and is in fact hypothesized to be - derived from a cell such as a stem cell. Specifically, as has been demonstrated in accordance with the present invention, during the early stages of neurogenesis, neuroepithelial stem cells reduce the extent, and reorganize the nature, of their apical plasma membrane protrusions, giving rise to such prominin-1-containing membrane particles which appear in the lumen of the neural tube. Release of prominin-1-containing membrane particles by epithelial cells appears to be a widespread phenomenon.

The particle of the invention is further covered by a membrane that has integrated thereto prominin-1 molecules. By way of their integration, prominin-1 molecules display their extracellular portion(s) to the environment whereas the intracellular portion(s) are retained in the interior of the particles. The display of the extracellular portion of the prominin-1 molecules allows for the convenient detection of the particles and also of course of the prominin-1 molecules. The latter is important for diagnostic applications as will be shown herein below.

The term "prominin-1" refers to a protein first described by Weigmann et al., 1997. The amino acid sequence of this membrane protein in accordance with the present invention is available from Weigmann et al., 1997; Fargeas et al., 2003b. The term "prominin-1" refers to a protein with as well as without post-translational modifications. For example, in embryonic and adult tissues, prominin-1 has been shown to be glycosylated.

In accordance with the present invention, the existence of novel extracellular membrane particles that carry a surface marker of stem cells, prominin-1 (CD133) is reported. Although prominin-1 expression in embryonic and adult tissues has previously been studied by immunohistochemistry (Corbeil et al., 2000; Kosodo et al., 2004; Weigmann et al., 1997), these particles have never been detected before. In view of the extensive research carried out on the development and differentiation of embryonic cells, this result must be regarded as surprising.

The membrane particles of the present invention display the further features that upon differential centrifugation whereby a first centrifugation is performed at 300g for five minutes the pellet which does not contain extracellular membrane particle followed by a centrifugation at 1200g for twenty minutes, the pellet does contain extracellular membrane particle. Alternatively, the extracellular membrane particle is further characterized in that upon differential centrifugation whereby the extracellular membrane particle is not pelleted at centrifugal forces up to 10,000g for 30 minutes said particles are pelleted after centrifugation at 110,000g for 60 minutes.

In other terms, the membrane particle of the invention is found, e.g., in mammalian body fluids in two different forms, namely the larger form having an average diameter of 600 ± 100 nm (also referred to as P2 membrane particles or P2 particles throughout this specification) and the smaller form having an average diameter of 50 - 80 nm (also referred to as P4 membrane particles or P4 particles throughout this specification). It is well-known in the art of differential centrifugation that formation of a certain pellet depends both on the amount of g and the centrifugation time. Accordingly, the person skilled in the art is in the position to change both parameters in a way that a pellet of the same constitution is obtained by differential centrifugation. In other terms, the number of g may e.g. be lowered but the duration of centrifugation may be increased and the same pellet is obtained. The present invention does comprise all embodiments that are formed by changing the g-values and the time values whereby, nevertheless, a pellet of the same constitution is obtained, i.e. a pellet comprising as the decisive feature either embodiment of the particle of the invention. The particle having an average diameter of 50 - 80 nm has a vesicular structure as observed under the electron microscope.

The particles of the present invention may conveniently be employed in a variety of applications. Whereas the applicant does not wish to be bound by any theory, it is suggested that the membrane particles of the present invention may be employed to shed more light on the following biological issues of interest. First, prominin-1–containing membrane particles may function in intercellular communication. In this context, it is worth noting that the small prominin-1–containing membrane particles in the ventricular fluid increase during a period (E10.5-E12.5) when apical microvilli are most abundant in the floorplate. Given the role of the floorplate as a signaling center (Dodd et al., 1998; Marti and Bovolenta, 2002; Marti et al., 1995), this not only raises the possibility that the 50 - 80 nm particles, at least at these stages of central nervous system development, originate from the floorplate, but also that they may exert a signaling role. In accordance with the invention, it is considered that the P4 particles thus (i) have mitogenic and/or differentiating properties on receiving cells, and (ii) contain signaling molecules and therefore can be vehicles for extracellular transport and targeting receiving cells.

Second, the release of prominin-1–containing membrane particles by cells may be a means of disposal of membrane microdomains that endow these cells with certain properties. For example, given that prominin-1 is a stem cell marker (Alessandri et al., 2004; Corbeil et al., 2001b; Richardson et al., 2004; Weigmann et al., 1997; Yin et al., 1997), the release of prominin-1–containing membrane particles from cells may contribute to a down-regulation of their stem cell properties, or to their differentiation. The developmental regulation of the prominin-1–containing membrane particles in the ventricular fluid during neurogenesis, as well as their release concomitant with the differentiation of Caco-2 cells, are consistent with (but do not prove) such a role.

In addition, the prominin-1-containing membrane particles such as the vesicular structures of the present invention have some important implications in the diagnosis of diseases or predispositions of diseases. Corresponding embodiments will be discussed herein below.

In a preferred embodiment of the method of the present invention, the extracellular membrane particle and in particular the P2 particle is further characterized by the absence of cadherin immunoreactivity.

The term "cadherin immunoreactivity" is intended to mean that an antibody directed to cadherin (such as specifically raised against cadherin or cross-reacting with cadherin) binds to cadherin and that such binding can be detected by suitable means. For example, binding can be detected by using a detectably labeled antibody (e.g. an antibody labeled by a radioactive, luminescent, fluorescent label (or a tag such as HIS-tag, FLAG-tag or MYC-tag) or by using a secondary, labeled antibody directed to the first antibody specific for cadherin. The absence of cadherin immunoreactivity thus means that a binding/interaction between an antibody directed to cadherin and cadherin cannot be detected on the particles of the invention using conventional means of detection.

The prominin-1–containing membrane particles of the present invention lack other plasma membrane markers such as cadherin, and only few of them were weakly immunostained for actin. These observations suggest that the appearance of prominin-1–containing membrane particles in the extracellular medium does not simply reflect indiscriminate cell fragmentation but, rather, a specific release process. The latter conclusion is also supported by findings made in accordance with the invention that prominin-1 associated with the particles having a average diameter of 50 – 80 nm released by Caco-2 cells showed the same detergent solubility/insolubility (see Fig. 11) and cholesterol dependence (see Figs. 9 and 10) that is characteristic for prominin-1 associated with apical plasma membrane protrusions and distinguishes it from other membrane proteins which are associated with the planar regions of the apical plasma membrane (Röper et al., 2000a and b).

In another preferred embodiment of the present invention the extracellular membrane particle, and in particular the 50 – 80 nm particle is further characterized by the absence of CD63 immunoreactivity.

The term "CD63" is a protein that has (first) been described in Leucocyte Typing V (1995); Azorta et al., 1995. Its amino acid sequence has been published in Hotta et al., 1988; Miyamoto et al., 1994. CD63 is known in the art as a marker for exosomes (Théry et al., 2002).

The term "CD63 immunoreactivity" is intended to mean that an antibody directed to CD63 (such as specifically raised against CD63 or cross-reacting with CD63) binds to CD63 and that such binding can be detected by suitable means. Again, binding can be detected by using a detectably labeled antibody (e.g. an antibody labeled by a radioactive, luminescent, fluorescent label (or a tag) or by using a secondary, labeled antibody directed to the first antibody specific for CD63. The absence of CD63 immunoreactivity thus means that a binding/interaction between an antibody directed to CD63 and CD63 cannot be detected on the particles of the invention using conventional means of detection.

Importantly, various lines of evidence indicate that neither of the two size classes of prominin-1–containing membrane particles described here are exosomes, i.e. the internal membrane vesicles of multivesicular bodies that are released into the extracellular space by exocytosis (Février and Raposo, 2004; Stoorvogel et al., 2002; Théry et al., 2002), a process proposed to occur in the floorplate neuroepithelium (Tanaka et al., 1988). The particles with an average diameter of 600 nm, are much larger than exosomes, which typically have a diameter of 50-90 nm (Février and Raposo, 2004; Stoorvogel et al., 2002; Théry et al., 2002). The prominin-1–containing particles of 50 – 80 nm, though similar in size to exosomes, in fact represent a subpopulation of small membrane vesicles distinct from exosomes, lacking immunoreactivity for CD63, a tetraspan membrane protein highly enriched in exosomes from virtually any cell type (Théry et al., 2002). Moreover, prominin-1 has been characteristically found on protrusions of the plasma membrane (Weigmann et al., 1997; Corbeil et al., 2001b) (rather than in multivesicular bodies), which also does not support the prominin-1–containing smaller particles being multivesicular body-derived exosomes.

This finding provides further unambiguous support for the fact that the prominin-1-containing membrane particle of the present invention is in fact novel of the prior art.

In accordance with the present invention, two types of membrane particles have been identified that differ in size. Accordingly, in one further preferred embodiment of the present invention, the extracellular membrane particle has an average diameter of 600 ± 100 nm when analysed by electron microscopy. A suitable way to prepare for and perform EM is to resuspend particles (P2 and P4) in fixative, absorb them to 400-mesh formvar/carbon-coated grids, immunolabel them with anti-prominin-1 antibody and subject them to negative contrasting using a mixture of 1.9% methyl cellulose plus 0.1% uranyl acetate .These prominin-1–containing 600-nm P2 particles which have a ring-like appearance can be found in the ventricular fluid, and hence said membrane particles may arise from apical protuberances of neuroepithelial cells.

In an additional preferred embodiment of the present invention, the extracellular membrane particle of the invention has an average diameter of 50 to 80 nm when analysed by electron microscopy.

In accordance with the invention, it is proposed that the prominin-1-containing 50 - 80 nm P4 particles in the ventricular fluid originate from microvilli, as well as other small protrusions, of the apical plasma membrane of neuroepithelial cells (including the cells of the floorplate). In contrast to the larger membrane particles, these particles have a vesicular structure

Prominin-1 occurs throughout the animal kingdom (Fargeas et al., 2003a). In accordance with the present invention, it is suggested that the membrane particles of the present invention are found in all mammals.

In a particularly preferred embodiment of the present invention, the extracellular membrane particle is of human origin. In this regard, the human prominin-1 amino acid sequence is available from Miraglia et al.; 1997 and Fargeas et al., 2003b. Since the release of prominin-1-containing membrane particles is concomitant with the differentiation of human Caco-2 cells (ATCC HTB-37), the data obtained with mouse particles are believed to be immediately transferable to humans.

In an alternative preferred embodiment of the present invention, the extracellular membrane particle is of mouse origin.

In one further preferred embodiment of the invention, the extracellular membrane particle is further characterized in that it is comprised in a body fluid. When humans are concerned, it is understood that they donate a body fluid only after giving informed consent. In particular, when the presence of the particles is to be assessed in body fluids, this can conveniently be done e.g. by differential centrifugation or by immunological assays relying on antibodies to prominin-1.

The widespread occurrence of prominin-1–containing membrane particles in various human body fluids found in accordance with the present invention deserves comment. These body fluids constitute an easily accessible source of material for a protein-based diagnosis of human diseases involving prominin-1 (see, for example, (Maw et al., 2000)). As a broader perspective, quantitative and qualitative analyses of the prominin-1–containing membrane particles in such human body fluids may provide relevant medical information about the epithelial tissues concerned.

In particular, the prominin-1-containing membrane particles found in body fluids of humans will allow to conveniently diagnose hematopoietic diseases or cancer, in addition to certain genetic diseases.

In another preferred embodiment the extracellular membrane particle in said body fluid, is in saliva, urine, seminal fluid, lacrimal fluid, milk, blood, serum or cerebrospinal fluid.

The invention also relates to a method of diagnosing an epithelial cancer in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer.
Preferably, the method of the invention is an in vitro method. (This hold also true for other embodiments of the method of the invention discussed below).

The term "epithelial cancer" is a cancer derived from an epithelium.

The presence of the extracellular membrane particle in a body fluid can be assessed by various means. For example, the methods for the detection of these particles that have been employed in the dependent examples can conveniently be used. Thus, in the one embodiment, differential centrifugation in combination with immuno-blotting can be employed. Alternatively, other antibody-dependent methods may be employed. These include ELISA, EIA, RIA, immunofluorescence and the like. Where high through-put-screening methods are envisaged, microtiter plates having 96, 384 or 1536 wells may be employed. In general, the adaptation of the detection method in HTS-formats is within the skill of the person skilled in the art.

The amount of extracellular membrane particles in the body fluid can be assessed by conventional means, e.g. by the staining intensity employing the read-out system in assay formats such as ELISA, immuno-blot, RIA immunofluorescence etc. The amount in the body fluid tested is preferably over time so that at least two test points are available. As regards the time span between test points and the number of samples to be taken, it is referred to passages in the specification relating to the last described embodiment where these issues are explained in detail and which essentially also apply here. In a less preferred embodiment, the control value is obtained from a mammal or a plurality of mammals of the same species not suffering from an epithelial cancer. The various assay formats and further methods described for the present embodiment of the invention can also be applied to the other methods of the invention described further below. Naturally, if an antibody-dependent assay is employed, then not only antibodies derived from an animal or human may be employed but also derivatives of such antibody such as chimeric antibodies, humanized antibodies, scFvs, or antibody fragments retaining the binding sites such as Fab or F(ab$_2$)' fragments.

A decreased level is established if the amount/level of particles is at least 20%, preferably at least 40%, more preferred at least 60% and most preferred at least 80% below that of the control value. This holds also true for other embodiments measuring decreased levels as discussed in this specification.

Moreover, the invention relates to a method of diagnosing an epithelial cancer in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of the invention wherein an increased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer.

In this alternative of the present invention, an increased level of extracellular membrane particles is indicative of an epithelial cancer. An increased level in accordance with the present invention (also relating to other embodiments described herein) is established if at least 20%, preferably at least 40%, more preferred at least 75% and most preferred at least 100% such as 200% or even 500% of the extracellular membrane particles observed in the control are recorded. This embodiment of the invention as well as the further diagnostic embodiments of cancer, including solid tumors or hematopoietic diseases of the invention advantageously rely on the determination of prominin-1 which is associated with the membrane of the particles using, e.g. antibody specific for prominin-1.

Furthermore, the invention relates to a method of diagnosing an hematopoietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of the invention wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of a hematopoietic disease.

The term “hematopoietic disease” refers to diseases such as neoplastic diseases of the hematopoietic system. Preferably, the hematopoietic disease is a malignant disease such as leukemia or lymphoma.

All methods of detection explained in detail for the preceding embodiments, as well as establishment of control values mutates mutandis apply to the present embodiment.

Additionally, the invention relates to a further method of diagnosing an hematopietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of the invention wherein an increased level of said extracellular membrane particles as compared to a control value is indicative of the presence of a hematopoietic disease.

All methods of detection explained in detail for the preceding embodiments, as well as establishment of control values mutates mutandis apply to the present embodiment.

Apart from that, the invention also relates to a method of diagnosing an epithelial cancer in a mammal comprising

(a) determining in a body fluid from said mammal the marker profile of the extracellular membrane particle; and

(b) comparing said marker profile with the marker profile of a control marker profile wherein a deviation in the marker profile is indicative of the presence of an epithelial cancer or a predisposition thereto.

In variation of the above-recited methods, an epithelial cancer can also be diagnosed by determining the marker profile of the extracellular membrane particle. It is envisaged in accordance with the invention that the prominin-1-containing membrane particles/ vesicles contain markers in addition to prominin-1. The combination of markers found on the particle in toto provides the marker profile. A deviation of the marker profile of the test sample from the marker profile of the control is an indication of the presence of an epithelial cancer. It is noteworthy that some of the markers on the test sample as compared with the control may be found in abundance as others may found in reduced amounts. Also, the present invention envisages that some of the markers are absent whereas new markers may appear that a not found on the control or vice versa. Any deviation from the control, i.e. any combination of reduced or increased amount of marker, absence or presence of (new) markers is an indication of the presence of an epithelial cancer.

Moreover, the invention also relates to an additional method of diagnosing an hematopoietic disease in a mammal comprising

(a) determining in a body fluid from said mammal the marker profile of the extracellular membrane particle; and

(b) comparing said marker profile with the marker profile of a control marker profile wherein a deviation in the marker profile is indicative of the presence of a hematopoietic disease.

In variation to the previous embodiments, the method of the invention can also be employed for diagnosing hematopoietic diseases in a mammal. Further considerations and guidance how to perform the method of the invention, are referred to the preceding embodiment.

In a preferred embodiment of the additional method of the present invention, the marker profile includes or consists of the marker prominin-1.

The marker prominin-1 is assessed in accordance with this invention to be the pre-eminent marker for carrying out the present invention. In certain cases, the diagnostic method may be based on the sole assessment of the presence or amount of prominin-1 on the surface of the particles. This can be done, for example, by employing the methods described in the appended examples. Yet, it is preferred that the method provides for the analysis of at least one additional marker.

In another preferred embodiment of the additional method of the present invention, the deviation is a deviation in quantity.

This embodiment of the present invention is particularly advantageous for collecting information on the potential presence of a cancer or a hematopoietic disease in a mammal. Once a marker profile has been established, quantitation of the presence of a marker can be easily established, for example by assessing for the intensity in ELISA, immunoblot, or immunofluorescence methods.

In a further preferred embodiment of the additional method of the present invention, the deviation is a deviation in quality.

In this alternative of the method of the invention, the presence of absence of markers is investigated. The presence or absence of markers can of course be assessed in conjunction with the quantitative amount of different markers present preferably on the surface of the particles as has been outlined herein before.

In another preferred embodiment of the first and of the additional methods of the present invention, said epithelial cancer is prostate cancer, kidney cancer, breast cancer, brain cancer or colorectal cancer.

Moreover, the invention relates to a method of diagnosing a degeneration of the retina in a mammal or a predisposition thereto comprising assessing for the presence of prominin-1 on the surface of these particles in a body fluid from said mammal wherein the absence or a reduced amount of prominin-1 on the surface of said particles in comparison to a control is indicative of degeneration of the retina or a predisposition thereto.

In variation of the methods of the invention described herein above, this embodiment of the invention makes use of the fact that in certain genetic diseases such as degeneration of the retina or predispositions thereto, prominin-1 appears in an reduced amount on the surface of the membrane particles of the invention or is even absent therefrom. This is essentially due to the fact that a mutation in the prominin-1 gene may be associated with retinal degeneration. Accordingly, depending on where the mutation occurs, prominin-1 may appear in reduced amounts at the surface, e.g. due to an incorrect folding as a consequence of the mutation. Alternatively, if a mutation results in a truncation of a protein, the protein may no longer be anchored in the membrane and therefore will not be detectable on the surface at all. In other terms, the particle of the invention is employed as a vehicle that carries the marker for the disease, prominin-1. Due to the fact that the particle of the invention can be detected in body fluids, a convenient diagnostic means is herewith established. Further due to the fact that the genetic disposition is present even before the onset of the disease may occur, the convenient diagnostic method of the invention may allow the investigating physician to timely instigate counter-measures.

Whereas certain mutations will not interfere with the presence or amount of prominin-1 on the surface, in a preferred embodiment of the method of the present invention said prominin-1 is full-length or unmutated prominin-1.

In a further preferred embodiment of the method of the present invention, said degeneration is autosomal recessive retinal degeneration (see, e.g., Maw et al., 2000) or autosomal dominant Stargard-like degeneration (see, e.g., Michaelides et al., 2003).

This embodiment advantageously makes use of the fact that in autosomal recessive retinal degeneration, a deletion of nucleotide 1878 of the prominin-1-protein has been detected. This leads to a frameshift at codon 614 with the premature termination of translation. As result, the truncated protein does not reach the cell surface; see Maw et al., Human and Molecular Genetics 9 (2000), 27-34. The method of the present invention may also be applicable to the determination of autosomal dominant Stargard-like degeneration.
In a further preferred embodiment of the method of the present invention said mammal is a human.

In another preferred embodiment of the method of the present invention, said body fluid is saliva, urine, seminal fluid, lacrimal fluid, milk, blood, serum or cerebrospinal fluid.

In another preferred embodiment of the method of the present invention, the assessment or the determination of the presence of prominin-1 is effected by using an antibody specifically recognizing prominin-1.

The use of antibodies or fragments or derivatives thereof has been detailed to some extent in connection with the previously described embodiments. Antibodies to prominin-1 can readily make by the person skilled in the art using conventional technology (see Harlow & Lane, "Antibodies, a Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, 1988). In fact, antibodies are available in the art and specifically bind to prominin-1. These monoclonal antibodies recognize the AC133 and AC141 epitope (commercially available from Miltenyi Biotec), see also Yin et al., Blood 90 (1997), 5002-5012.Additional, monoclonal antibodies include rat antibody 13A4 directed against mouse prominin-1 and commercially available from eBiosecience..

Finally, the present invention relates to a method of prognosing a solid tumor in a mammal comprising determining the change in the number of the particles of the invention in a body fluid (i.e. preferably a sample of a body fluid) of said mammal over time. Preferably, said mammal is a human and said body fluid is as outlined herein above for other embodiments of the invention. As regards determination methods, those that have been described for other embodiments of the invention including those described in the appended examples may also applied here, in particular these depending on the assessment of prominin-1 such as employing antibodies specific for prominin-1. Comparison to a control is preferred. Alternatively, the determination of the marker profile (see above) on the particles of the invention may be determined over time and compared with a control.

"Over time" means that at least two determinations are effected. Preferably, the time span between determinations is at least one week, such as two or three weeks, and more preferred at least one month such as two, three or four months. In this way, for example, the efficacy of a medicament used for treating the solid tumor may be monitored. More preferred is that at least three, such as at least four, five, six, or more such as at least 10 determinations are effected. The time span between the at least three determinations may vary or may be constant. The decision in this regard will usually be taken by the attending physician.

An approximation of the number of particles over time to the control value is indicative of a good prognosis of the solid tumor. Note that in the occurrence of a solid tumor, the number of said particles may be higher or lower as compared to a healthy individual, i.e. an individual not suffering from a solid tumor. Most preferred is that said number is lower.

Solid tumors include, but are not limited to, brain cancer, kidney cancer, prostate cancer and breast cancer.

The figures show:

Fig. 1. Occurrence of prominin-1–containing particles in the ventricular fluid of the embryonic mouse brain.
Double immunofluorescence of a cryosection (20 μm) of an E11.5 mouse embryo using mAb 13A4 (b, d, f, red) and antiserum αE3 (a, c, f, green) directed against distinct extracellular epitopes of prominin-1. Single 5-μm optical sections of the midbrain were obtained by confocal microscopy in the middle of the cryosection (a, b) and at the level of the glass slide (c, d, f), as outlined in (e). (f) Merge of the area indicated by the white squares in (c and d); note the co-localisation of the 13A4 and αE3 immunoreactivities. Inset in (f), higher magnification of the particle indicated by the arrowhead in (f). Bars, 10 μm.

Fig. 2. Isolation of large and small prominin-1–containing membrane particles from the embryonic ventricular fluid.
(a) Ventricular fluid of E10.5-E11 mouse embryos was subjected to differential centrifugation for 5 min at 300 g (P1), 20 min at 1,200 g (P2), 30 min at 10,000 g (P3) and 1 h at 110,000 g (P4). The entire P1-P4 pellets were analyzed by immunoblotting for prominin-1. (b and c) Immunoflorescence for prominin-1 of P2 and P4 particles obtained by differential centrifugation of E13 ventricular fluid for 15 min at 10,000 g (b) and 1 h at 200,000 g (c), respectively. The inset in (b) shows a P2 particle at higher magnification, which is very similar to the one shown in the inset of Fig. 1f. Bar, 10 μm. (d-f) Negative staining EM of prominin-1 immunogold labeled P4 (d) and P2 (e, f) particles obtained by differential centrifugation of E11.5 ventricular fluid as in (a).

Fig. 3. Time course of appearance of P2 and P4 prominin-1–containing membrane particles in the ventricular fluid during embryonic mouse brain development.

(a and b) Ventricular fluid of E10.5-E13.5 mouse embryos was subjected to differential centrifugation as in Fig. 2a followed by immunoblotting of the pellets for prominin-1. Data are the mean of 3 independent experiments; bars indicate SD. For any given experiment, the same volume of ventricular fluid was analyzed at the various developmental stages. (a) Total prominin-1 immunoreactivity (sum of P1-P4 pellets, triangles) and prominin-1 immunoreactivity in the P2 pellet (squares) and the P4 pellet (circles) at the various developmental stages. (b) For each developmental stage, prominin-1 immunoreactivity in the P2 pellet (squares) and the P4 pellet (circles) is expressed as percentage of total (sum of P1-P4 pellets). (c) Cryosections of E7-E12.5 mouse embryos were subjected to immunofluorescence for prominin-1. The number of the ring-like prominin-1–containing particles (see inset in Fig. 1f) in the ventricular fluid was quantitated per unit area in single optical sections at the level of the glass slide. E7, amniotic fluid near the neural plate (19 x 10^4 µm^2); E9.5-E12.5, mean of forebrain and hindbrain ventricular fluids, bars indicate the variation of the forebrain/hindbrain values from the mean (E9.5, 66 x 10^4 µm^2; E10.5, 139 x 10^4 µm^2; E12.5, 134 x 10^4 µm^2).

Fig. 4. Prominin-1–containing protrusions on the apical plasma membrane of neuroepithelial cells at various stages of embryonic mouse brain development.

(a-d) Prominin-1 immunofluorescence at the apical surface of neuroepithelial cells at E8.5 (a, c; future hindbrain) and E 11.5 (b, d; dorsal telencephalon), viewed from the lateral (a, b) and lumenal (c, d) side of the neuroepithelium. Bar in c, 10 µm. (e-n) Prominin-1 immunogold EM of the apical plasma membrane of neuroepithelial cells at E8.5 (e-g) and E11.5 (h-n) (h, i, k, l, n: midbrain; j, m: forebrain). Bars, 300 nm. Some of the gold particles (12 nm) are indicated by arrowheads and junctional complexes by asterisks. (e, f) Microvilli. (g-k) Large pleiomorphic protuberances; note the thin stalks in (g, j), the vicinity of the junctional complexes in (j, k), the abundant prominin-1 immunoreactivity in (h), and the small prominin-1–labeled membrane buds (arrows) in (k); (i) shows the prominin-1–labeled cup-shape surface structure of (n) at higher magnification. (l) Short, thin protrusions; note the concentration of prominin-1 immunoreactivity at the tips (arrows). (m, n) Overviews of the apical plasma membrane of a single (m) and several (n) neuroepithelial cells; note the prominin-1–labeled protuberance in (m) and the cilium (arrow, note the basal body), but lack of microvilli, in (n). (o, p) Scanning EM of the apical plasma membrane of E11.5 neuroepithelial cells in the forebrain. The box in (o) indicates the area shown at higher magnification in (p). Open arrow, cilium; double-headed arrow, cauliflower-like protuberance; triple-headed arrow, cup-shape protuberance. Bar in o, 5 µm and in p, 1 µm. (q-t) Floorplate of the E11.5 spinal cord. (q) Prominin-1 immunofluorescence at relatively low exposure to show the more intense immunoreactivity of the floorplate cells (FP). Bar, 10 µm. (r) Overview prominin-1 immunogold EM; note the microvilli of the floorplate cells (FP) and the pleiomorphic protuberances but lack of microvilli on the neuroepithelial cells adjacent to the floor plate (double-asterisks); the bracket indicates the area shown at higher magnification in (s). Bar, 300 nm. (s) Prominin-1-labeled microvilli of the floorplate (12 nm gold). Bar, 300 nm. (t) Scanning EM of the apical plasma membrane. Note the bending of the lumenal surface, resulting in a lateral view of the microvilli-bearing floorplate cells and a lumenal view of the adjacent neuroepithelial cells. The black triangle and white bar at the left margin indicate the transition from floorplate to adjacent neuroepithelial cells and an apparent boundary between neuroepithelial cells with abundant vs. sparse protrusions, respectively. Bar, 10 µm.

Fig. 5. Occurrence of prominin-1–containing membrane particles in various human body fluids.

Various human body fluids were subjected to differential centrifugation for 5 min at 300 g (300 g), 30 min at 10,000 g (10,000 g), 1 h at 200,000 g (200,000 g) and 1 h at 400,000 g (400,000 g). The resulting pellets were analyzed by immunoblotting for prominin-1.

Fig. 6. The P4 prominin-1–containing membrane particles are distinct from exosomes carrying the tetraspanin CD63.

(a) 24-h-conditioned medium of Caco-2 cells grown for 10 days post-confluency was subjected to differential centrifugation as in Fig. 2a, and the resulting pellets were analyzed by immunoblotting for prominin-1. (b) 24-h-conditioned medium of Caco-2 cells grown for 14 days post-confluency was centrifuged for 30 min at 10,000 g, and the resulting supernatant, containing the P4 particles, was concentrated and subjected to equilibrium sucrose gradient centrifugation. Aliquots of the fractions (1.5%) and the pellet (50%) were analyzed by immunoblotting for prominin-1. (c, d) Negative staining and prominin-1 immunogold (12-nm) labeling EM of the P4 particles present in fraction 7 of the sucrose gradient. Note the presence of both prominin-1–positive (arrows, indicated only in c) and prominin-1–negative (open arrows) small vesicles. (e, f) Negative staining EM of prominin-1 (6-nm gold) and CD63 (12-nm gold) double-immunogold labeled particles present in fraction 7 of the sucrose gradient. Note that the small vesicles showing prominin-1 immunoreactivity are distinct from those showing CD63 immunoreactivity.

Fig. 7. Appearance of prominin-1–containing membrane particles in the culture medium upon differentiation of Caco-2 cells.

Caco-2 cells were grown for up to 21 days post-confluency. Cells received fresh medium 24 h before analysis. At the indicated time points, cells and the P4 pellet obtained from the conditioned medium were analyzed by immunoblotting. (a) Immunoblots. Top, prominin-1 in the P4 pellet of the medium; middle, prominin-1 in the cells; bottom, α-tubulin in the cells. (b, c) Quantitation of immunoblots for day 1 and day 8 post-confluency. (b) Prominin-1 immunoreactivity in the P4 pellet of the medium is expressed as percentage of total (sum of cells plus P4). Data are the mean of three independent experiments; bars indicate S.D. (c) Prominin-1 immunoreactivity in the cells was normalized to that of α-tubulin. Normalized prominin-1 immunoreactivity in the cells at day 8 post-confluency is expressed relative to that at day 1, which was arbitrarily set to 100%. Data are the mean of two independent experiments; bars indicate the variation of the individual values from the mean.

Fig. 8. Prominin-1–containing particles in the ventricular fluid of the embryonic mouse brain lack cadherin.

Double immunofluorescence of a cryosection (20 μm) of an E11.5 mouse embryo using mAb 13A4 against prominin-1 (b, c, e, f, pseudocolored in red) and either TRITC-phalloidin (0.2 μg/ml; Sigma) (a, c, pseudocolored in green) or mouse mAb anti-pan-cadherin (1:200; Sigma) (d, f, pseudocolored in green). Single 5-μm optical sections obtained by confocal microscopy at the level of the glass slide. (c) A few of the actin-positive particles (which were detected only upon amplification of the original signal) coincide with a small fraction of the prominin-1–positive particles (arrows). (f) Note the lack of cadherin staining. Bar, 10 μm.

Fig. 9. Cholesterol depletion of Caco-2 cells increases the release of prominin-1–containing P4 membrane particles into the medium.

Caco-2 cells 10 days post-confluency were incubated from 0-24 h and subsequently from 24-48 h in the indicated media; DL-FCS, medium supplemented with delipidated (rather than normal) fetal calf serum. Lovastatin was used at 10 μM and methyl-β-cyclodextrin (mβCD) at 2 mM. Prominin-1 immunoreactivity in the P4 pellet obtained from the 24-48 h medium (top panel) is expressed as percentage of total (sum of cells plus P4) (bottom panel).

Fig. 10. Microvilli of Caco-2 cells are affected by cholesterol depletion.

Electron micrographs of microvilli from 14 days post-confluency Caco-2 cells grown on filter and cultured 48 h in distinct media; (a) medium supplemented with normal fetal calf serum; (b) medium supplemented with delipidated fetal calf serum (DL-FCS) and 10 µM Lovastatin; (c and d) from 0-24 h medium supplemented with DL-FCS and 10 µM Lovastatin and subsequently from 24-48 h medium supplemented with DL-FCS, 10 µM Lovastatin and methyl-β-cyclodextrin (mβCD) at 2 mM. Note that cholesterol depletion caused a decrease in the length and density of microvilli and frequently induced microvillar microvesiculation.

Fig. 11. Prominin-1 in P4 membrane particles released by Caco-2 cells is associated with a Triton X-100–soluble, Lubrol WX–insoluble cholesterol-based microdomain.

The P4 pellet obtained from 24-h conditioned medium of Caco-2 cells 10 days post-confluency was incubated for 30 min at 4°C without (top) or with (bottom) 10 mM methyl-β-cyclodextrin (mβCD), incubated for 30 min at 4°C without detergent (PBS), with 1% Triton X-100 (TX-100) or with 1% Lubrol WX (L-WX), centrifuged for 1 h at 100,000 g, and supernatant (S) and pellet (P) were analysed by immunoblotting for prominin-1.

The examples illustrate the invention.

Example 1: Methods

Immunofluorescence microscopy

E7-E12.5 NMRI mouse embryos were fixed by immersion for 24 h at 4°C in 4% paraformaldehyde, 150 mM sodium phosphate buffer, pH 7.4. (In some experiments, 1% rather than 4% paraformaldehyde was used, without any obvious difference in the results obtained.) The fixed embryos were infiltrated overnight at 4°C with 30% sucrose in phosphate buffer, embedded in TissueTek and frozen on dry-ice. Cryosections (20 µm) were collected onto HistoBond microscope slides (Paul Marienfeld GmbH). The sections, dried overnight at 4°C, were hydrated with PBS and permeabilised for 15 min with 0.3% Triton X-100 in

PBS. Residual aldehyde was quenched for 15 min with 50 mM NH_4Cl in PBS. Sections were blocked for 1 h with buffer A (1% BSA, 5% fetal calf serum in PBS) and incubated overnight at 4°C in buffer A containing primary antibody against mouse prominin-1 (rat mAb 13A4 (Weigmann et al., 1997) at 8 µg/ml and rabbit antiserum αE3 (Maw et al., 2000) at 1:300 dilution). Sections were extensively rinsed in buffer A, incubated in buffer A containing secondary antibody (affinity-purified goat anti-rat or anti-rabbit IgG conjugated either to Cy2 or Cy3), rinsed several times with buffer A, with PBS and once with distilled water, and mounted in Moviol 4.88.

Stained sections were observed using either an Olympus epifluorescence microscope with a 100X oil immersion objective connected to a CCD camera, or a Zeiss or Leica confocal laser scanning microscope. The confocal microscope settings were such that 5 µm-thick optical sections were obtained in the middle of the cryosection or at the level of the glass slide, as shown schematically in Fig. 1e. The images shown were prepared from the digital data files using Adobe Photoshop and Illustrator. For the quantification of the ring-like particles in the neural tube lumen, the area analyzed in the optical sections (4-14 sections per developmental stage and brain region) was estimated using the Leica confocal software.

Isolation of prominin-1–containing membrane particles

VENTRICULAR FLUID. For each preparation, 10-30 E10.5-E13.5 NMRI mouse embryos were dissected free of embryonic membranes in ice-cold PBS. After removal of the ectodermal layer at the level of the hindbrain (E10.5-12.5) or midbrain (E13.5), the ventricular fluid (1-3 µl per embryo, depending on the age) was collected using a glass capillary connected to a micromanipulator. (see video in Supporting Information) The pooled ventricular fluid (20-50 µl) was diluted with 0.5-1 ml ice-cold PBS containing protease inhibitors (Sigma P8340 diluted 1:500). The diluted ventricular fluid was subjected to differential centrifugation as follows (all steps at 4°C): 5 min at 300 g, supernatant 20 min at

1,200 g, supernatant 30 min at 10,000 g, supernatant 1 hour at 110,000 g. The resulting pellets (P1-P4, respectively) were resuspended in SDS sample buffer and analyzed by immunoblotting for mouse prominin-1 (Corbeil et al., 2001a) using mAb 13A4 at 0.8 µg/ml.

For immunofluorescence analysis of prominin-1–containing particles in the ventricular fluid, diluted ventricular fluid from 20 E13 NMRI mouse embryos was centrifuged for 15 min at 10,000 g to obtain a pellet enriched in P2 particles, and the resulting supernatant for 1 hour at 200,000 g to obtain a pellet enriched in P4 particles. Pellets were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffer, resuspended in fixative, spotted onto polylysine-coated glass slides, allowed to dry, and subjected to immunofluorescence analysis as described above for cryosections.

For immunogold labeling and negative staining EM of prominin-1–containing particles in the ventricular fluid, diluted ventricular fluid from 10-20 E10.5-11.5 NMRI mouse embryos was subjected to differential centrifugation to obtain P1-P4 pellets. The P2 and P4 pellets were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffer and processed for EM as described below.

BODY FLUIDS. Seminal fluid, saliva, urine and lacrimal fluid were obtained from healthy volunteers with informed consent. Protease inhibitors were added to each sample. Seminal fluid (2-3 ml) was kept for 30 min at room temperature to allow liquefaction. Saliva (2 ml) was mixed with an equal volume of ice-cold PBS. Both seminal fluid and saliva were then filtered through gauze. Urine (4 ml) was used directly. Lacrimal fluid (50 µl) was collected with a Pasteur pipette after stimulation with onion juice. Samples were subjected to differential centrifugation as follows: 5 min at 300 g, supernatant 30 min at 10,000 g, supernatant 1 hour at 200,000 g, supernatant 1 hour at 400,000 g. The resulting pellets were resuspended in SDS sample buffer, and aliquots corresponding to 25-400 µl original fluid were analyzed by immunoblotting for human prominin-1 using rabbit antiserum hE2 (Florek et al., 2005) (1:3000-1:5000).

CACO-2 CELLS. Caco-2 cells were grown at 37°C in a 5% CO_2 atmosphere in MEM supplemented with 20% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was changed every 2-3 days.

For immunoblot analysis of prominin-1–containing particles in the medium, Caco-2 cells grown for 9 days post-confluency received fresh medium, which was collected after 24 h (24 h-conditioned medium). The conditioned medium was subjected to differential centrifugation as described above for ventricular fluid, followed by immunoblotting of the resulting P1-P4 pellets for human prominin-1 using rabbit antiserum hE2.

For the isolation of P4 particles from Caco-2 cell–conditioned medium, cells grown for 14 days post-confluency received fresh, serum-free medium (OptiMEM, Gibco) otherwise supplemented as above. After 24 h, the conditioned medium was centrifuged for 30 min at 10,000 g and the resulting supernatant (30 ml) concentrated to 0.5 ml using Centricon plus-20 (Millipore). The sample was subjected to equilibrium sucrose gradient (50-800mM) centrifugation as described (Huttner et al., 1983). Fraction 7, containing part of the peak of prominin-1 as revealed by immunoblotting (see Fig. 6b below), was diluted 1:2 with PBS and centrifuged for 30 min at 110,000 g onto a 20 µl 800 mM sucrose cushion, and the bottom 30 µl were analyzed by EM.

For the differentiation experiments, 40,000 cells/cm^2 were plated onto 5-cm dishes. Cells received fresh medium 16 h after plating, when they typically had reached confluency (t = 0 post-confluency). Cells were grown for up to 21 days post-confluency and received fresh medium 24 h before analysis. The 24 h-conditioned medium was centrifuged for 30 min at 10,000 g and the resulting supernatant for 1 h at 110,000 g to obtain the P4 pellet. In parallel, the cell monolayer was washed with ice-cold PBS and the cells were lysed for 30 min at 4°C in 200 µl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) containing protease inhibitors. The cell lysate was centrifuged for 15 min at 10,000 g and the resulting supernatant analyzed. In some experiments, cells were directly dissolved into SDS sample buffer. Cell lysates and P4 pellets were analyzed by immunoblotting for human prominin-1 using rabbit antiserum hE2 and for α-tubulin using a mouse mAb (Sigma T-9026, 1:5000). Chemiluminescence was detected using Hyperfilm (Amersham) and quantitated after scanning using MacBAS software.

Electron microscopy

NEUROEPITHELIUM. E8.5-11.5 NMRI mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffer and, in some experiments, additionally in 4% paraformaldehyde, 0.05% glutaraldehyde in 100 mM sodium phosphate buffer pH 7.4. Fixed embryos were cut into pieces containing defined parts of the central nervous system, which were infiltrated with gelatine at 37°C, embedded in gelatine and infiltrated with sucrose at 4°C, and cryosectioned as described previously (Wucherpfennig et al., 2003). After removal of the gelatine from the cryosections by incubation on a drop of PBS at 37°C, they were subjected to immunolabeling (Wucherpfennig et al., 2003), using mAb 13A4 (25-50 µg/ml) followed by 12 nm gold-coupled secondary antibody (Dianova) or, in some experiments, rabbit antiserum to rat IgG (Cappel#55704) followed by protein A/10-nm gold. Cryosections were contrasted for 10 min on ice with a mixture of 1.9% methyl cellulose / 0.3% uranyl acetate. Samples were viewed in a Morgagni electron microscope (FEI).

P2 AND P4 PARTICLES FROM VENTRICULAR FLUID. Fixed P2 and P4 pellets were resuspended in fixative, adsorbed to 400-mesh formvar/carbon-coated grids, processed through immunolabeling for prominin-1 as described above, and subjected to negative contrasting using a mixture of 1.9% methyl cellulose plus either 0.1% uranyl acetate (P2) or 0.3% uranyl acetate (P4).

P4 PARTICLES FROM CACO-2 CELLS. The particles in suspension were adsorbed to 400-mesh formvar/carbon-coated grids, fixed with 4% paraformaldehyde in phosphate buffer, subjected to either single-immunolabeling using the mouse mAb AC133 against human prominin-1 (10 µg/ml, Miltenyi) or double-immunolabeling using rabbit antiserum hE2 against human prominin-1 (Florek et al., 2005) (1:150) and mouse mAb CLB-gran/12, 425 against CD63 (10 µg/ml, Sanquin), followed by 6 nm gold-coupled anti-rabbit and 12 nm gold-coupled anti-mouse secondary antibodies (Dianova), and negatively contrasted using a mixture of 1.9% methyl cellulose and 0.3% uranyl acetate.

SCANNING EM. E11.5 NMRI mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde, 2% glutaraldehyde in 150 mM sodium phosphate buffer pH 7.4. Fixed embryos were dehydrated in ascending acetone (30%, 50%, 70%, 90% and 100%) and subjected to critical point drying using liquid carbon dioxide (CPD 030, BAL-TEC). The samples were mounted on aluminium stubs, coated with gold in a sputter coater (SCD 050, BAL-TEC) and viewed in a scanning electron microscope (XL 30 ESEM FEG, FEI).

Example 2: Occurrence of membrane particles carrying the stem cell marker prominin-1 in the ventricular fluid of the embryonic mouse brain

Immunofluorescence analysis of the developing mouse brain revealed that prominin-1 immunoreactivity was not only concentrated at the apical surface of the neuroepithelium, as observed previously (Kosodo et al., 2004; Weigmann et al., 1997), but was also associated with particles present in the ventricular fluid (Fig. 1). These prominin-1–containing particles escaped detection when optical sections were taken by confocal microscopy in the middle of the cryosection (Fig. 1a, b, e), but were readily observed in optical sections taken at the bottom of the cryosection (Fig. 1c-e). Presumably, only those membrane particles present in the lumen of the neural tube at the time of fixation were recovered with the cryosection at the end of the immunofluorescence procedure that sedimented onto, and adsorbed to, the glass slide. The prominin-1–containing particles in the ventricular fluid were observed with two different antibodies against distinct extracellular epitopes of prominin-1 (Fig. 1c, d, f) and often had a ring-like appearance (Fig. 1f inset). They lacked immunoreactivity for cadherin, a protein on the lateral plasma membrane of neuroepithelial cells (Aaku-Saraste et al., 1996; Kosodo et al., 2004; Nose and Takeichi, 1986), and only few of them were weakly stained for actin (Figure 8).

The physiological occurrence of prominin-1–containing particles in the lumen of the neural tube was corroborated by biochemistry. Ventricular fluid was collected from E10.5-11.0 mouse embryos by introducing, after removal of the ectodermal layer, a glass capillary into the neural tube lumen at the level of the hindbrain, followed by gentle aspiration of the liquid. Differential centrifugation of the ventricular fluid followed by immunoblotting for prominin-1 revealed the existence of two distinct populations of prominin-1–containing particles, which were largely recovered in the P2 pellet after centrifugation for 20 min at 1,200 g and in the P4 pellet after centrifugation for 1 h at 110,000 g, respectively (Fig. 2a).

The prominin-1–containing particles found in the P2 and P4 pellets were isolated from E13 ventricular fluid (centrifugation for 15 min at 10,000 g and 1 h at 200,000 g, respectively, to increase the yield) and analyzed by immunofluorescence for prominin-1. This showed that the ring-like appearing particles observed in the neural tube lumen (Fig. 1) were recovered in the P2 fraction (Fig. 2b) and were substantially larger than the particles recovered in the P4 fraction (Fig. 2c), consistent with the g force required for their sedimentation (Fig. 2a). Both populations of prominin-1–containing particles, from now on referred to in short as P2 and P4 particles, appeared to be relatively homogeneous.

Upon immunogold EM for prominin-1, the P2 particles isolated from E11.5 ventricular fluid appeared as electron-dense membrane structures with an average diameter of 600±100 nm, which showed abundant prominin-1 immunoreactivity on their surface (Fig. 2e, f). In contrast, the P4 particles appeared as ≈50-80 nm membrane vesicles with prominin-1 surface labeling (Fig. 2d).

Example 3: Differential appearance of prominin-1–containing P2 and P4 particles in the ventricular fluid during the development of the embryonic mouse brain

Immunoblotting for prominin-1 of ventricular fluid collected at various embryonic stages (E10.5-E13.5) showed a rise in total prominin-1–containing particles at early stages (E10.5-E12.5) followed by a decline thereafter (E12.5-E13.5) (Fig. 3a, triangles). The rise in total prominin-1 immunoreactivity was due to an increase in the prominin-1 recovered in the P4 pellet (reflecting an increase in either the number of P4 particles or the concentration of prominin-1 on them), while the prominin-1 recovered in the P2 pellet remained largely constant. The subsequent decline of total prominin-1 immunoreactivity was due to a decrease of prominin-1 in both, the P2 and P4 pellet (Fig. 3a, squares and circles, respectively). Comparison of the relative proportion of prominin-1 associated with either P2 or P4 particles at any one stage revealed a decrease in P2-associated prominin-1 and an increase in P4-associated prominin-1 during the period of brain development analyzed (Fig. 3b). Given that at the earliest developmental stage studied by immunoblotting, E10.5, the bulk of prominin-1 was associated with P2 particles, we chose immunofluorescence for prominin-1 on cryosections (see Fig. 1) to obtain information about their appearance in the ventricular fluid at stages earlier than E10.5, when the isolation and biochemical analysis of this fluid is not feasible. This showed the progressive appearance of the P2 particles in the ventricular fluid concomitant with the transition from the neural plate (E7) to the neural tube and the onset of neurogenesis (E9.5-E10.5) (Fig. 3c). Between E10.5 and E12.5, the concentration of P2 particles in the ventricular fluid as determined by prominin-1 immunofluorescence remained constant (Fig. 3c). Together with the results of immunoblotting (Fig. 3a, squares), this also indicates that there was no significant change in the concentration of prominin-1 per P2 particle between E10.5 and E12.5.

Example 4: Analysis of the prominin-1–containing protrusions of the apical plasma membrane of neuroepithelial cells during early development of the embryonic mouse brain

In light of the developmental regulation of the appearance of prominin-1–containing membrane particles in the ventricular fluid (Fig. 3), we investigated the morphology of apical plasma membrane protrusions of neuroepithelial cells before (E8.5) and after (E11.5) the onset of neurogenesis. While a lateral view of the neuroepithelium did not reveal a major difference in the prominin-1 immunostaining of the apical surface at the two developmental stages (Fig. 4a, b), a view onto the apical surface from the lumen did. At E8.5, prominin-1 immunoreactivity showed a fine punctate pattern (Fig. 4c), whereas at E11.5, the pattern was dominated by larger structures that often had a ring-like appearance (Fig. 4d), reminiscent of the P2 particles found in the ventricular fluid (Fig. 1f inset, Fig. 2b).

At the EM level, the predominant prominin-1–labeled structures of the apical plasma membrane of E8.5 neuroepithelial cells were microvilli (Fig. 4e, f), consistent with previous observations (Weigmann et al., 1997). In contrast, at E11.5, the apical surface of neuroepithelial cells largely lacked microvilli (Fig. 4m, n), and prominin-1 surface immunoreactivity was mostly associated with large electron-dense pleiomorphic protuberances that emerged from the apical plasma membrane via a thin stalk (Fig. 4h-k). Similar protuberances were already detected at E8.5 (Fig. 4g), albeit at much lower abundance. In addition to these large protuberances, prominin-1 immunoreactivity at E11.5 was associated with short, thin apical plasma membrane protrusions (Fig. 4l). Scanning EM of the apical surface of E11.5 neuroepithelial cells corroborated this morphology and in addition confirmed the presence of cilia (Nonaka et al., 1998) (Fig. 4n, p).

Example 5: Persistence of prominin-1–containing microvilli after the onset of neurogenesis in the floorplate

A notable exception with regard to the lack of apical microvilli after the onset of neurogenesis was the floorplate. At E11.5, consistent with the strong prominin-1 immunofluorescence of the floorplate (Fig. 4q), the corresponding cells showed abundant prominin-1–containing microvilli in immunogold EM (Fig. 4r, s). In contrast, in both transmission (Fig. 4r) and scanning (Fig. 4t) EM, the apical surface of the adjacent neuroepithelial cells was dominated by the presence of large pleiomorphic protuberances.

Example 6: Occurrence of prominin-1–containing particles in various body fluids

Given the widespread occurrence of prominin-1 in various embryonic and adult tissues (Corbeil et al., 2001b; Corbeil et al., 2000; Weigmann et al., 1997), we investigated whether prominin-1–containing particles are present in body fluids other than the ventricular fluid of the developing brain. Examination of various body fluids of adult humans by differential centrifugation followed by immunoblotting revealed the occurrence of prominin-1–containing particles in seminal fluid, saliva, urine and lacrimal fluid (Fig. 5). In all of these body fluids (except perhaps lacrimal fluid), the prominin-1–containing particles were of the P4 type as they were sedimented by centrifugation for 1 h at 200,000 g but not 30 min at 10,000 g. (It remains to be investigated whether the sedimentation of the prominin-1–containing particles in lacrimal fluid after 30 min at 10,000 g reflects a larger size or some kind of membrane aggregation.)

Example 7: Prominin-1–containing P4 particles lack the exosomal marker CD63

Given that prominin-1 is endogenously expressed by Caco-2 cells (Corbeil et al., 2000), a human colon carcinoma-derived cell line, we explored the use of these cells as a model to study the nature and release of prominin-1–containing particles from epithelial cells into the extracellular fluid. Indeed, analysis of 24-h-conditioned medium of Caco-2 cells, grown for 10 days post-confluency, by differential centrifugation followed by immunoblotting showed that these cells released prominin-1–containing particles into the medium (Fig. 6a). These particles were mostly of the P4 type, being sedimented by centrifugation for 1 h at 110,000 g but not 20 min at 1,200 g (Fig. 6a). Upon further fractionation by equilibrium sucrose gradient centrifugation, the prominin-1–containing P4 particles (as revealed by immunoblotting) yielded a discrete peak of relatively low buoyant density (Fig. 6b), similar to that of synaptic vesicles (Huttner et al., 1983).

Interestingly, immunogold labeling followed by negative staining EM showed that only a subpopulation of the small, ≈50-80 nm membrane vesicles present in the peak fraction of the sucrose gradient contained prominin-1, while other, similarly small vesicles lacked prominin-1 immunoreactivity on their surface (Fig. 6, c and d). This second, distinct subpopulation of small prominin-1–negative membrane vesicles, but not the small prominin-1–positive membrane vesicles (Fig. 6, e and f, small gold particles), were immunoreactive for CD63 (Fig. 6, e and f, large gold particles), a tetraspan membrane protein highly enriched in exosomes from virtually any cell type (Théry et al., 2002; van Niel et al., 2001). These data indicate that the small prominin-1–containing P4 particles are distinct from exosomes.

Example 8: Release of prominin-1–containing P4 particles by Caco-2 cells upon differentiation

Caco-2 cells are known to undergo enterocytic differentiation several days after reaching confluency (Louvard et al., 1992; Pinto et al., 1983). Given that the prominin-1–containing P4 particles in the ventricular fluid increased concomitant with the progression of neurogenesis (Fig. 3a, b), i.e. the differentiation of neuroepithelial cells (Alvarez-Buylla et al., 2001; Haubensak et al., 2004; Kriegstein and Götz, 2003), it was of interest to investigate whether the release of the prominin-1–containing P4-type particles from Caco-2 cells into the medium might be related to their differentiation. We therefore analyzed Caco-2 cells and the medium conditioned for 24 h by these cells at various time points during a 21-day period after the cells had reached confluency (Fig. 7). Analysis of the P4 pellets prepared from the media by immunoblotting for prominin-1 showed that the release of P4 particles by Caco-2 cells into the medium showed a steep increase between 6 and 8 days post-confluency (Fig. 7a, top), consistent with a possible relationship between P4 particle release and the differentiation of Caco-2 cells. The prominin-1 released by the cells as P4 particles at day 8 post-confluency amounted to ≈10% of total (Fig. 7b). In line with previous observations (Florek et al., 2005), the level of cell-associated prominin-1 between day 1 and day 8 post-confluency remained constant (Fig. 7c) and decreased by ≈25% at day 21 post-confluency.

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Claims

1. An extracellular membrane particle carrying integrated into the membrane prominin-1 molecules.

2. The extracellular membrane particle of claim 1 further characterized by the absence of cadherin immunoreactivity.

3. The extracellular membrane particle of claim 1 or 2 further characterized by the absence of CD63 immunoreactivity.

4. The extracellular membrane particle of any one of claims 1 to 3 having an average diameter of 600 ± 100 nm when analysed by electron microscopy.

5. The extracellular membrane particle of any one of claims 1 to 3 having an average diameter of 50 to 80 nm when analysed by electron microscopy.

6. The extracellular membrane particle of any one of claims 1 to 5 which is of human origin.

7. The extracellular membrane particle of any one of claims 1 to 5 which is of mouse origin.

8. The extracellular membrane particle of any one of claims 1 to 7 further characterized in that it is comprised in a body fluid.

9. The extracellular membrane particle of claim 8 wherein said body fluid is saliva, urine, seminal fluid, lacrimal fluid, milk, blood, serum or cerebrospinal fluid.

10. A method of diagnosing an epithelial cancer in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of any one of claims 1 to 9 wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer.

11. A method of diagnosing an epithelial cancer in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of any one of claims 1 to 9 wherein an increased level of said extracellular membrane particles as compared to a control value is indicative of the presence of an epithelial cancer.

12. A method of diagnosing a hematopoietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of any one of claims 1 to 9 wherein a decreased level of said extracellular membrane particles as compared to a control value is indicative of the presence of a hematopoietic disease.

13. A method of diagnosing a hematopietic disease in a mammal comprising assessing in a body fluid of said mammal for the presence of the extracellular membrane particle of any one of claims 1 to 9 wherein an increased level of said extracellular membrane particles as compared to a control value is indicative of the presence of a hematopoietic disease.

14. A method of diagnosing an epithelial cancer in a mammal comprising
    (a) determining in a body fluid from said mammal the marker profile of the extracellular membrane particle of any one of claims 1 to 9; and

(b) comparing said marker profile with the marker profile of a control marker profile wherein a deviation in the marker profile is indicative of the presence of an epithelial cancer.

15. A method of diagnosing a hematopoietic disease in a mammal comprising
    (a) determining in a body fluid from said mammal the marker profile of the extracellular membrane particle of any one of claims 1 to 9; and
    (b) comparing said marker profile with the marker profile of a control marker profile wherein a deviation in the marker profile is indicative of the presence of a hematopoietic disease.

16. The method of claim 14 or 15 wherein the marker profile includes or consists of the marker prominin-1.

17. The method of any one of claims 14 to 16 wherein the deviation is a deviation in quantity.

18. The method of claim 14 to 16 wherein the deviation is a deviation in quality.

19. The method of any one of claims 10, 11, 14 and 16 to 18 wherein said epithelial cancer is prostate cancer, kidney cancer, breast cancer, brain cancer or colorectal cancer.

20. A method of diagnosing a degeneration of the retina in a mammal or a predisposition thereto comprising assessing for the presence of prominin-1 on the surface of the particles of any one of claims 1 to 9 in a body fluid from said mammal wherein the absence or a reduced amount of prominin-1 on the surface of said particles in comparison to a control is indicative of degeneration of the retina or a predisposition thereto.

21. The method of claim 20 wherein said prominin-1 is full-length or unmutated prominin-1.

22. The method of claim 20 or 21 wherein said degeneration is autosomal recessive retinal degeneration or autosomal dominant Stargard-like degeneration.

23. A method for prognosing a solid tumor in a mammal comprising determining the change in the number of membrane particles of any one of claims 1 to 9 in a body fluid of said mammal over time.

24. The method of any one of claims 10 to 22 wherein said mammal is a human.

25. The method of any one of claims 10 to 23 wherein said body fluid is saliva, urine, seminal fluid, lacrimal fluid, milk, blood, serum or cerebrospinal fluid.

26. The method of any one of claims 16 to 25 wherein the assessment or the determination of the presence of prominin-1 is effected by using an antibody specifically recognizing prominin-1.

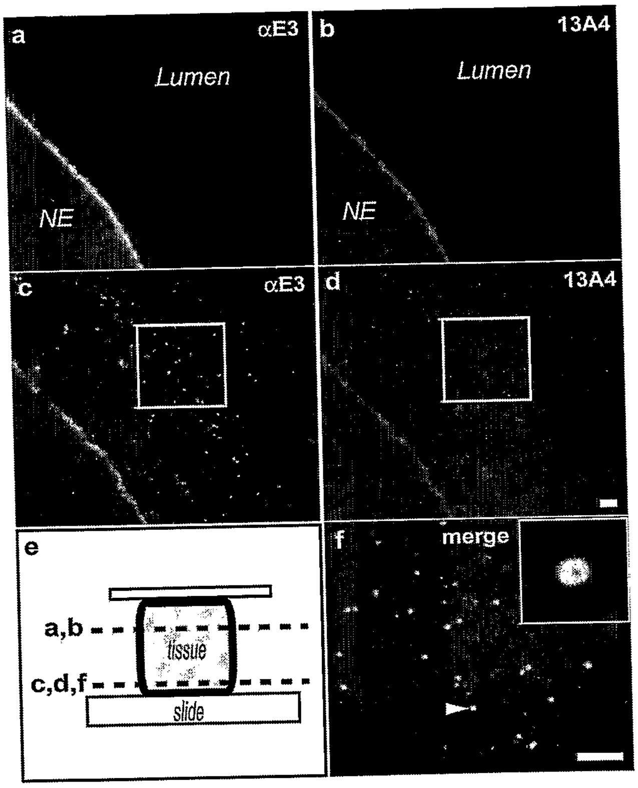
a
αE3
Lumen
NE
b
13A4
Lumen
NE
c
αE3
d
13A4
e
a,b
tissue
c,d,f
slide
f
merge

Fig. 1

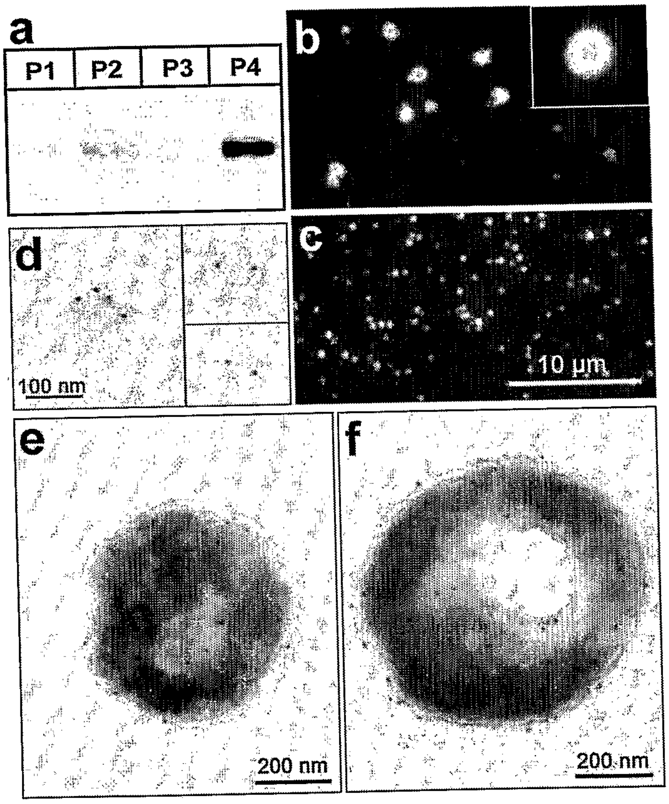

Fig. 2

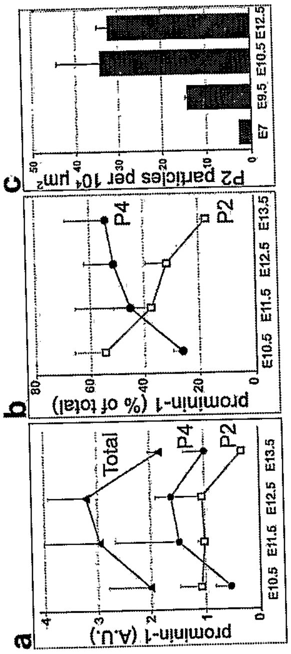
a
prominin-1 (A.U.)
0
1
2
3
4
E10.5
E11.5
E12.5
E13.5
Total
P4
P2
b
prominin-1 (% of total)
0
20
40
60
80
E10.5
E11.5
E12.5
E13.5
P4
P2
c
P2 particles per 10⁴ µm²
0
10
20
30
40
50
E7
E9.5
E10.5
E12.5

Fig. 3

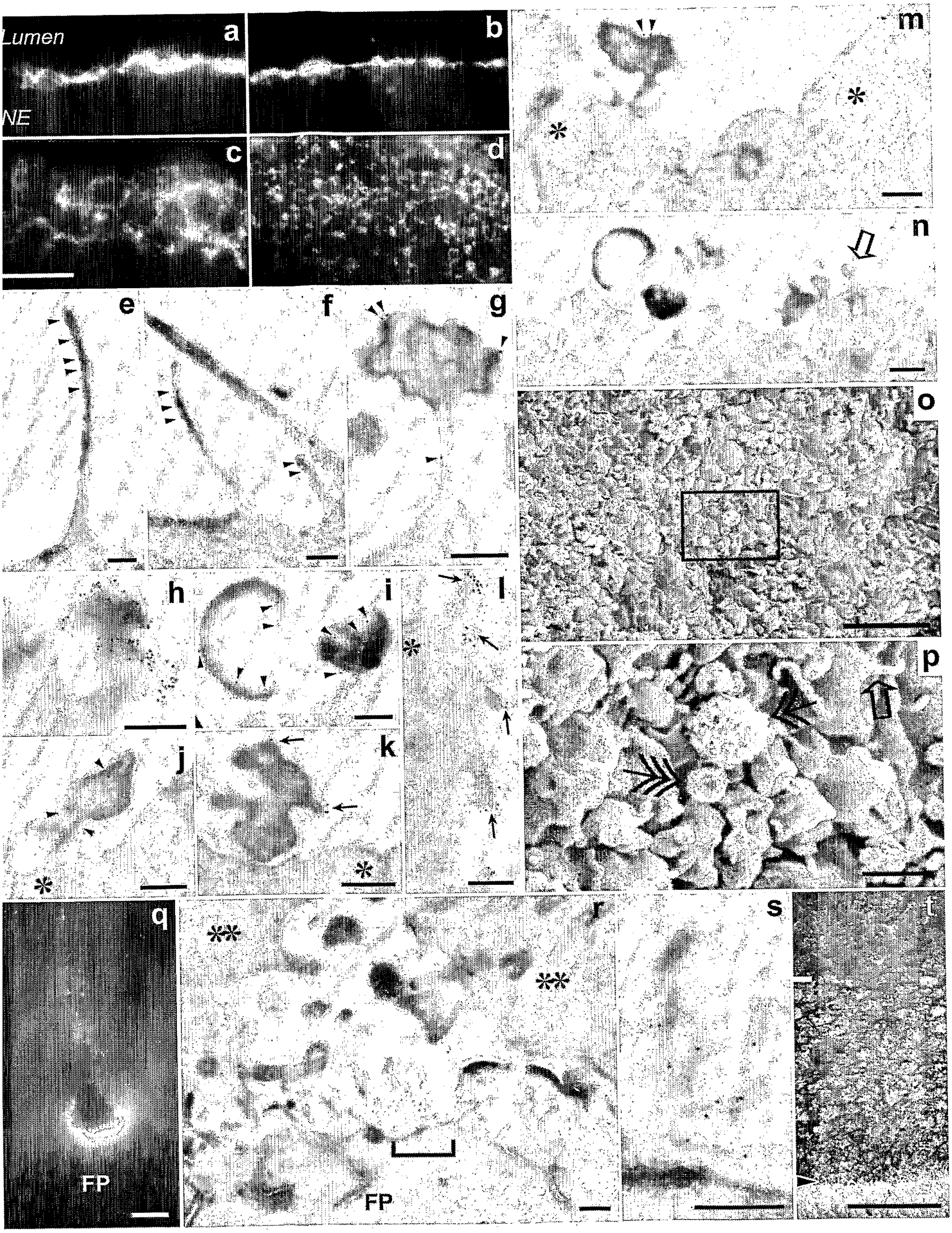

Fig. 4

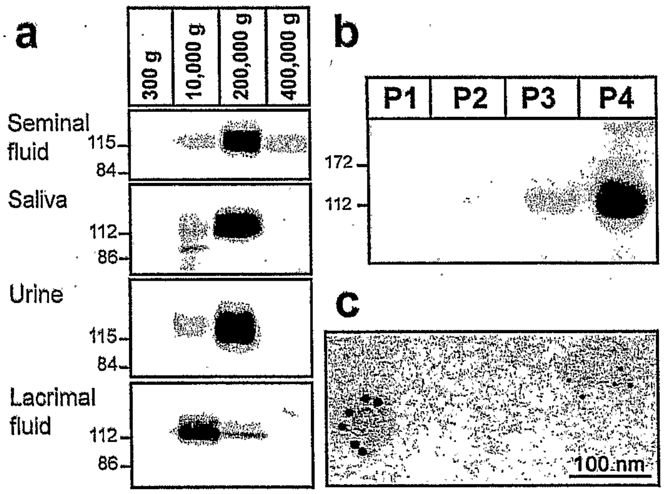
a
300 g
10,000 g
200,000 g
400,000 g
Seminal fluid
115
84
Saliva
112
86
Urine
115
84
Lacrimal fluid
112
86
b
P1
P2
P3
P4
172
112
c
100 nm

Fig. 5

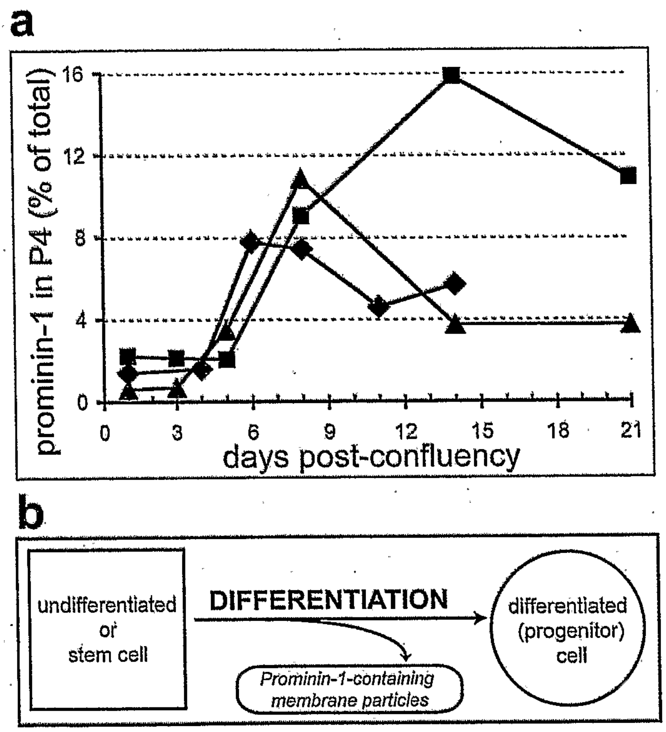
a
prominin-1 in P4 (% of total)
16
12
8
4
0
0
3
6
9
12
15
18
21
days post-confluency
b
undifferentiated or stem cell
DIFFERENTIATION
Prominin-1-containing membrane particles
differentiated (progenitor) cell

Fig. 6

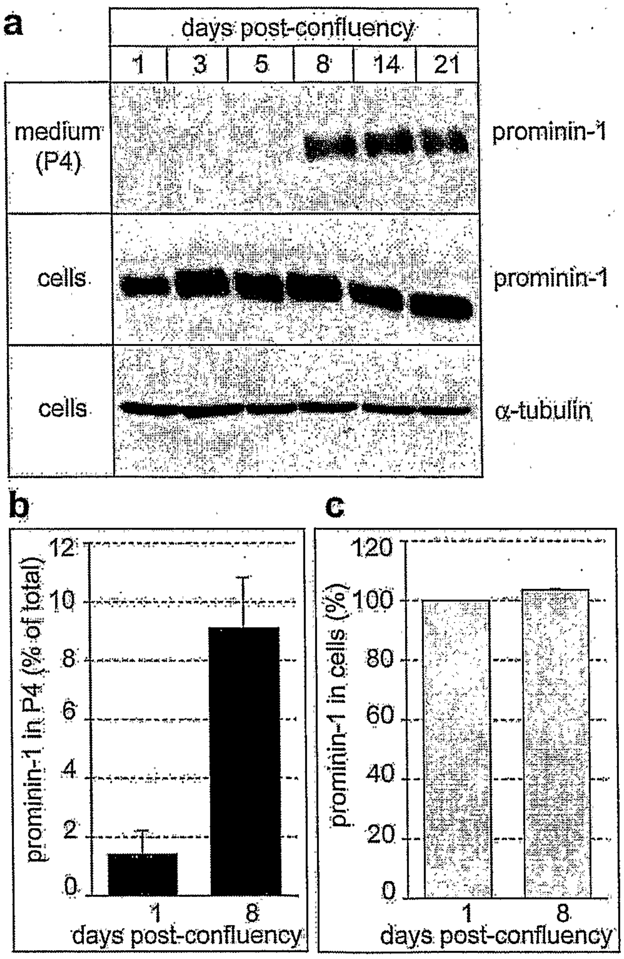
a
days post-confluency
1
3
5
8
14
21
medium (P4)
cells
cells
prominin-1
prominin-1
α-tubulin
b
prominin-1 in P4 (% of total)
0
2
4
6
8
10
12
1
8
days post-confluency
c
prominin-1 in cells (%)
0
20
40
60
80
100
120
1
8
days post-confluency

Fig. 7

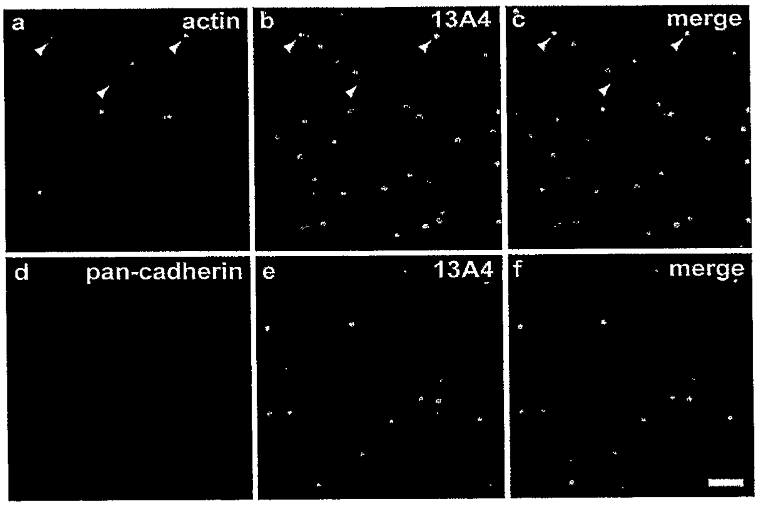

Fig. 8

Conditions	0-24h	24-48h
1	normal medium	normal medium
2	DL-FCS	DL-FCS
3	DL-FCS + Lovastatin	DL-FCS + Lovastatin
4	DL-FCS + Lovastatin	DL-FCS + Lovastatin + mβCD

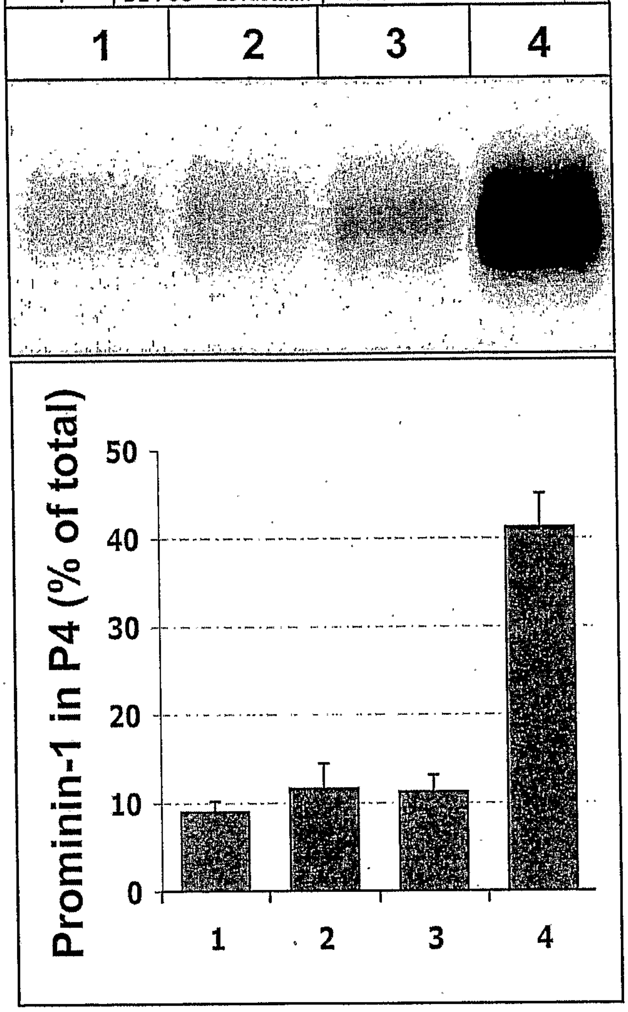

Fig. 9

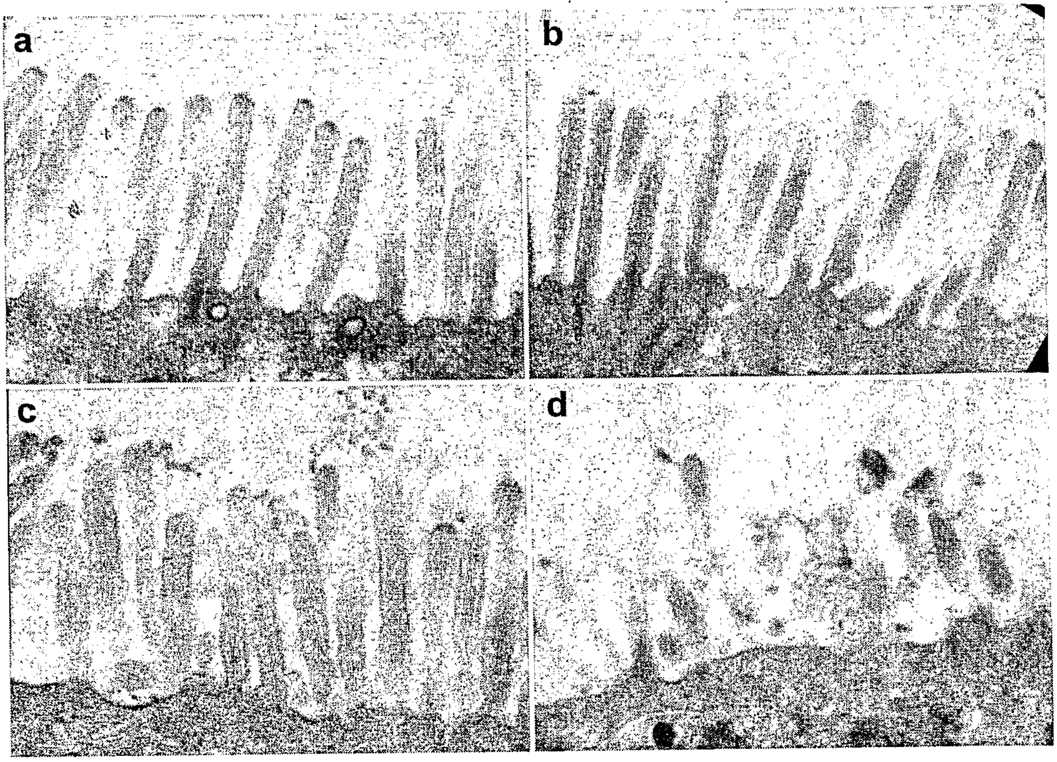

Fig. 10

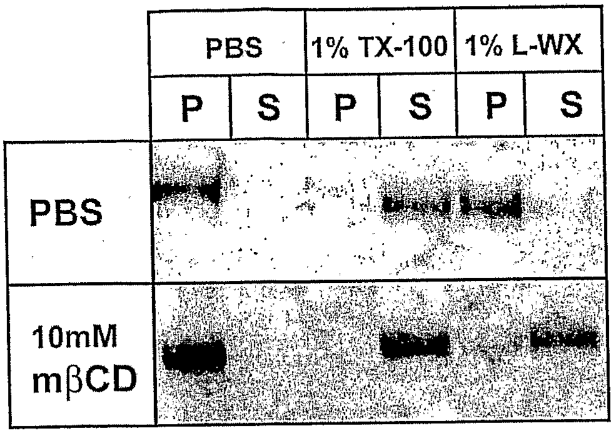

Fig. 11