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(54) Title: METHOD FOR PROGNOSING AND/OR DIAGNOSING THE QUALITY OF THE BARRIER FUNCTION OF THE SKIN

(57) Abstract: The present invention relates to a method for diagnosing an impaired or altered cutaneous barrier function comprising measuring the level of expression of prolyl oligopeptidase protease (PREP) and/or its enzymatic activity in a skin sample of a subject. It also relates to a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject.

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METHOD FOR PROGNOSING AND/OR DIAGNOSING THE QUALITY OF THE BARRIER FUNCTION OF THE SKIN

The present invention relates to a method for diagnosing an impaired or altered cutaneous barrier function comprising measuring the level of expression of prolyl oligopeptidase protease (PREP or POP) and/or its enzymatic activity in a skin sample of a subject. It also relates to a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject.

Human skin is constituted of two tissue compartments, namely a superficial compartment, the epidermis, and a deep compartment, the dermis. It also comprises sebaceous glands, sweat glands and hair follicles.

The epidermis is a tissue of which the cells are joined and connected to each other. The epidermis is a continually renewed epithelium. Renewal is a coordinated and finely regulated process that results in the insensitive and invisible removal of superficial cells by the desquamation mechanism.

The epidermis is conventionally divided into a basal layer of keratinocytes constituting the germinative layer of the epidermis, a so-called spinous layer constituted of several layers of polyhedral cells disposed on the germinative layer, one to three so-called granular layers constituted of flattened cells containing distinct cytoplasmic inclusions, keratohyalin grains, and finally, a set of superficial layers called the corneal layer (or stratum corneum) formed from keratinocytes at the terminal stage of their differentiation called corneocytes.

Corneocytes are non-nucleated cells mainly constituted of fibrous material containing keratins and surrounded by a corneal envelope. New keratinocytes are permanently produced to compensate for the continuous loss of epidermal cells at the level of the corneal layer during the desquamation process.

However, an imbalance between cell production at the level of the basal layer and the rate of desquamation may notably lead to the formation of flakes on the skin surface. Similarly, a deficiency in terminal differentiation of stratum corneum cells, for various reasons, can lead to the formation of cell clusters that are large, thick, visible with the naked eye, and called "scales" or, in other situations, to thinning of the stratum

corneum. This may lead to fragility of the barrier properties of the epidermis, chronic dehydration of the stratum corneum, loss of mechanical elasticity, stretching, as well as a lack of radiance and transparency of the skin.

5 As examples of factors favoring this impairment of skin surface quality, mention may be made of stress, the winter period, ageing, UV exposure, use of anionic detergents, a lack of sebum or a lack of hydration.

10 Thus, fragility of the cutaneous barrier may occur in the presence of external aggressions such as irritants (detergents, acids, bases, oxidants, reducers, concentrated solvents, harmful gases or fumes), thermal or climatic imbalances (cold, drought, radiation), xenobiotics (unwanted microorganisms, allergens) or internal aggressions such as psychological stress.

15 One of the critical steps in the terminal keratinocyte differentiation process is the cross-linking of protein precursors of the corneal envelope. This phenomenon plays an essential role in the development and maintenance of cutaneous cohesion, the physical properties of the skin as in the barrier function. The corneal envelope is an essential component of corneocytes.

20 Finally, dry skin, which lacks radiance, is often treated with moisturizing active ingredients that have an effect on the differentiation and maturation of the stratum corneum.

25 Filaggrin (FLG) is an essential protein for the regulation of epidermal homeostasis. It is a protein that can be incorporated into the corneal envelope or bind to keratin fibers to facilitate the formation of the fibrous matrix of corneocytes. The degradation of FLG allows a release into the upper corneal layer of free amino acids that contribute to the production of the Natural Moisturizing Factor (NMF) responsible
30 for hydration of the corneal layer. The FLG degradation process is thus a preferred target for cosmetics to favor skin hydration, in particular by stimulating enzymes favoring this degradation.

35 FLG is derived from a large precursor, profilaggrin, which is synthesized by granular keratinocytes and stored in the form of granules, keratohyalin grains, in the cytoplasm of these cells. Profilaggrin is formed from 10 to 12 FLG subunits bound to

each other by a short hydrophobic binding peptide (amino acid sequence FLYQVST, SEQ ID NO:1) and flanked by two truncated subunits, an NH₂-terminal domain homologous to S100A proteins and a single COOH-terminal tail (Rawlings et al., J. Invest. Dermatol., 2005; Sandilands et al., J. Cell Sci., 2009; Le Lamer et al., Biol. Chem., 2015). At the granular layer/corneal layer transition, profilaggrin is cut at the level of the binding peptides to form the basic monomers of FLG (324 amino acids) according to at least two proteolysis steps performed by endo- and exo-peptidases whose nature is not yet fully known (Thulin et al., Biochemistry, 1995). Calpain 1 and SASPase could be involved (Resing et al., J. Biol. Chem., 1993; Bernard et al., Identification and characterization of a novel retroviral-like aspartic protease specifically expressed in human epidermis. J Invest Dermatol 2005;125(2):278-87. Epub 2005/08/16). After facilitating the aggregation of keratin filaments to form the intracorneocyte matrix, FLG is degraded into amino acids that contribute to the formation of NMF (Rawlings et al., 2005; Sandilands et al., 2009; Le Lamer et al. 2015). While calpain 1, caspase 14 and bleomycin hydrolase, three cytosolic cysteine proteases, appear to be involved (Le Lamer et al., Biol. Chem. 2015; Denecker et al. 2007; Kamata et al. J. Biol. Chem., 2011; Hsu et al., J. Biol. Chem., 2011; Mechin et al., Cell. Mol. Life Sci., 2005), however, they alone are not sufficient to account for this total degradation called filaggrinolysis.

There is thus a need for methods for evaluating the cutaneous barrier function, which are efficient and reliable. A need also exists for cosmetic active ingredients capable of improving hydration and/or strengthening the barrier function of the epidermis.

The present invention meets this need.

In a surprising and unexpected manner, the Applicant has discovered that the expression of a protease, prolyl oligopeptidase protease (PREP), evidenced by measuring its activity and/or its immunodetection, is linked to the state of the barrier function of the skin of a subject.

As shown in the examples, PREP is a new player in filaggrinolysis. Indeed, PREP is co-localized with FLG. When the expression of PREP is silenced in three-dimensionally reconstructed human epidermis (RHEs), the epidermal barrier measured by transepidermal electrical resistance (TEER) decreases dramatically. Similarly, the abundance of key enzymes in the metabolism of filaggrin is reduced in

these models. In addition, when PREP is silenced, a change in the expression and abundance of numerous proteins expressed during the late stages of keratinocyte differentiation and important for the formation of the corneal envelope is observed.

Prolyl oligopeptidase protease (POP) or prolyl endopeptidase protease (PE or PREP) is expressed more strongly in granular keratinocytes. It cleaves the peptide bonds on the C-terminal side of proline residues and is coded by the PREP gene in humans. The human PREP gene is accessible under GenBank ID 5550 in NCBI (and its mRNA is available under the reference NM_002726 in the NCBI Reference Sequence). Its activity is limited to the action on oligopeptides of less than 10 kDa and it has an absolute requirement for the trans-configuration of the peptide bond preceding the proline. Its optimum pH of 5.8 and the content of FLG in proline also make PREP a very good candidate for its proteolysis.

All these results make PREP an excellent target both for the typology of the cutaneous barrier function, dry and/or dehydrated skin, and also for their cosmetic treatment.

The subject matter of the present invention is thus a method for prognosing and/or diagnosing an impaired or altered cutaneous barrier function, comprising a) measuring the level of expression of prolyl oligopeptidase protease (PREP) and/or its enzymatic activity in a skin sample of a subject.

The subject matter of the invention is also a method for evaluating the efficacy of a cosmetic treatment aimed at preventing the impairment of the cutaneous barrier function and/or aimed at strengthening the cutaneous barrier function in a subject, comprising the following steps:

- a) the cosmetic treatment of a subject, then measuring the level of expression and/or the enzymatic activity of PREP in a skin sample of said subject; then
- b) comparison of the measurement of step a with a control sample.

The subject matter of the invention is also a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the following steps:

- 5
- a) contacting at least one candidate compound with a skin sample of the subject, or with a skin or epidermis model in which the level of expression and/or the enzymatic activity of PREP is reduced, and then measuring the level of expression and/or the enzymatic activity of PREP in said sample or model;
- b) comparing the expression measured in step a) to the expression of PREP in a skin sample of the subject, or with a skin or epidermis model, not exposed to said compound; and
- 10 c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the expression of PREP in the skin sample of the subject or the skin or epidermis model that has been exposed to said candidate compound is detected, relative to the expression of PREP in the skin sample of the subject or the skin or
- 15 epidermis model that has not been exposed to the candidate compound.

The subject matter of the invention is also a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the

20 following steps:

- a) contacting at least one candidate compound with a recombinant PREP in the presence of filaggrin or one of its peptide fragments containing a proline, then measuring the level of the enzymatic activity of PREP;
- 25 b) comparing the enzymatic activity measured in step a) to the enzymatic activity of PREP in the absence of said compound; and
- c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the enzymatic activity of PREP is detected, relative to the enzymatic activity of PREP that has not
- 30 been contacted with the candidate compound.

The subject matter of the invention is also a skin equivalent or an epidermal equivalent, in which the level of expression and/or the enzymatic activity of PREP is reduced, for example following a knock-down of the PREP gene.

35 Finally, the subject matter of the invention is the cosmetic use of at least one active ingredient making it possible to stimulate the level of expression of PREP

and/or the enzymatic activity of PREP, to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function.

All the methods described in the present invention are *in vitro* methods.

5

Method for prognosing and/or diagnosing

The subject matter of the present invention is a method for prognosing and/or diagnosing an impaired or altered cutaneous barrier function in a subject, comprising measuring the level of expression of PREP and/or its enzymatic activity in a skin sample of said subject.

10

In particular, the impaired or altered cutaneous barrier function is related to dry and/or dehydrated skin.

15

“Cutaneous barrier function” is taken to mean the barrier function of the epidermis.

The subject is preferably a human subject, preferably a human subject aged between 20 and 80 years, preferably between 40 and 75 years.

20

The skin sample of the subject is an *ex vivo* skin sample. Typically, the skin sample of the subject may be taken by biopsy or sampling, the latter is preferably performed non-invasively, and in particular does not require local anesthesia. According to one preferred embodiment, the step of taking the sample is performed by rubbing the surface of the skin or using an adhesive surface such as a D-squame® disc.

25

“Expression level of PREP” is taken to mean the amount of mRNA produced, or the amount of protein produced, by the expression of this PREP gene. This amount may be expressed as such, or as a concentration or ratio.

30

“Enzymatic activity of PREP” is taken to mean any enzymatic activity of PREP in filaggrinolysis, notably cleavage of peptide bonds on the C-terminal side of proline residues. Thus, the enzymatic activity of PREP may be measured by the amount of cleaved filaggrin or the amount of cleaved filaggrin fragments. This amount may be expressed as such, or as a concentration or ratio.

The level of expression of PREP or its enzymatic activity may be measured by any suitable technique known from the prior art.

To measure the amount of mRNA, the RNAs may be extracted by any method of extraction of RNA from the skin, for example by the method described in the Materials and Methods section of the example below, and then may be processed for a specific quantification of mRNA by any mRNA quantification method, for example by quantitative PCR.

To measure the amount of PREP protein or the enzymatic activity of PREP, mention may be made of Western-blot, Slot-blot, Dot-blot, ELISA (Enzyme Linked Immuno-Sorbent Assay) methods of the singleplex or multiplex type, proteomic or glycomic methods, staining of polypeptides in a polyacrylamide gel by a silver-based dye, by Coomassie blue or by SYPRO, immunofluorescence, UV absorption, immunohistochemical methods in conventional, electronic or confocal microscopy, FRET (fluorescence resonance energy transfer), TR-FRET (time resolved FRET), FLIM (fluorescence lifetime imaging microscopy), FSPIM (fluorescence spectral imaging microscopy) methods, FRAP (fluorescence recovery after photobleaching) methods, reporter gene methods, atomic force microscopy (AFM) methods, surface plasma resonance methods, microcalorimetry methods, flow cytometry methods, biosensor methods, radioimmunoassay (RIA) methods, isoelectric focusing methods, and enzymatic tests notably using fluorescent peptides, methods using peptide chips, sugar chips, antibody chips, quantitative mass spectrometry methods such as iTRAQ, MS/MS, MRM or SWATH, MALDI-TOF type spectrometry methods.

To measure the enzymatic activity of PREP, the amount of peptide fragments of filaggrin containing proline may be evaluated. "Filaggrin peptide fragments" is taken to mean all peptides derived from the amino acid sequence of filaggrin containing a proline. Since filaggrin is very polymorphic, numerous sequences exist. The human profilaggrin sequence may be accessed in UniProtKB under the number P20930, or in GnomAD browser/FLG.

Preferably, the fragment is chosen from the following fragments:

SHTTPQGRS	(SEQ ID NO: 2),
GQSAPSTGG	(SEQ ID NO: 3),
GWTGPSTRG	(SEQ ID NO: 4),
GWTGPSTGV	(SEQ ID NO: 5),
IRGHPGSSR	(SEQ ID NO: 6),

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	GSRHPRSHH	(SEQ ID NO: 7),
	QSGTPHAET	(SEQ ID NO: 8),
	ARSSPGERH	(SEQ ID NO: 9),
	HSGIPRRQA	(SEQ ID NO: 10),
5	GQDGPHQQS	(SEQ ID NO: 11),
	IRAHPGSRR	(SEQ ID NO: 12),
	GSRHPGFHQ	(SEQ ID NO: 13),
	GQAGPHQQS	(SEQ ID NO: 14),
	GQAGPHQQS	(SEQ ID NO: 15),
10	GQTAPSTGG	(SEQ ID NO: 16),
	GQSGPRSAS	(SEQ ID NO: 17),
	RHHEPSTRA	(SEQ ID NO: 18),
	GSRNPRSHQ	(SEQ ID NO: 19),
	GRTGPSTGG	(SEQ ID NO: 20),
15	QSSGPRTSR	(SEQ ID NO: 21),
	GSRHPGSHH	(SEQ ID NO: 22),
	GKAGPHQQS	(SEQ ID NO: 23),
	IRGHGPGSRGG	(SEQ ID NO: 24),
	RTSRPRGSS	(SEQ ID NO: 25),
20	IHGHPGSSS	(SEQ ID NO: 26),
	QSEGPRTSR	(SEQ ID NO: 27),
	GSRHPRSHQ	(SEQ ID NO: 28),
	HSGIPRGQA	(SEQ ID NO: 29),
	QSAGPRTSR	(SEQ ID NO: 30), and
25	GSRHPTSHH	(SEQ ID NO: 31).

Preferably, the fragment is chosen from SHTTPQGRS fragments (SEQ ID NO: 2), GQSAPSTGG (SEQ ID NO: 3) and GWTGPSTRG (SEQ ID NO: 4).

30 In particular, the level of expression and/or the enzymatic activity of PREP is measured by quantitative PCR, Western blot, quantitative mass spectrometry methods of the iTRAQ, MS/MS type, enzymatic tests notably using fluorescent peptides or ELISA.

35 In one particular embodiment, the method for prognosing and/or diagnosing according to the invention further comprises the following steps:

- b) deducing from step a) whether the cutaneous barrier function of said subject is impaired or altered; and
- c) if the cutaneous barrier function is identified as impaired or altered in step b), treating the skin with a cosmetic composition against the impairment or alteration of the barrier function.

In one particular embodiment, the skin of said subject is diagnosed as having an impaired or altered cutaneous barrier function when the level of expression and/or the enzymatic activity of PREP measured in step a) in the skin sample of the subject is less, in particular significantly less, than a control.

In one particular embodiment, the control is a reference value.

In one particular embodiment, the reference value is determined by the average value of the level of expression and/or the enzymatic activity of PREP, measured in a determined population, for example a population aged 20 to 80 years, and/or having a satisfactory cutaneous barrier function.

Preferably, the skin of said subject is diagnosed as having an impaired or altered cutaneous barrier function when the level of expression and/or the enzymatic activity of PREP measured in step a) in the skin sample of the subject is at least 1.2 times less than a control.

In one particular embodiment, the reference value is determined by a ROC study ("*La courbe ROC (receiver operating characteristic) principes et principales applications en biologie clinique*", H. Delacour et al., Ann Biol Clin 2005 ; 63 (2): 145–54).

"Significantly lower", in the sense of the invention, is taken to mean a statistically significant variation in the level of expression and/or the enzymatic activity of PREP.

Cosmetic treatment method

The subject matter of the present invention is also a method for prognosing and/or diagnosing an impaired or altered cutaneous barrier function in a subject, said method comprising the following steps:

- a) measuring the level of expression and/or the enzymatic activity of prolyl oligopeptidase protease (PREP) in a skin sample of said subject;
- b) deducing from step a) whether the cutaneous barrier function of said subject is impaired or altered; and

- c) if the cutaneous barrier function is identified as impaired or altered in step b), treating the skin with a cosmetic composition against the impairment or alteration of the barrier function.

5 The measurement of step a) is preferably performed as described in the *"Method for prognosing and/or diagnosing"* section above.

The skin sample of said subject and the subject may be as described in the *"Method for prognosing and/or diagnosing"* section above.

10 In one particular embodiment, the skin of said subject is diagnosed as having an impaired or altered cutaneous barrier function when the level of expression and/or the enzymatic activity of PREP measured in step a) in the skin sample of the subject is less, in particular significantly less, than a control.

15 If the cutaneous barrier function is identified as impaired or altered in step b), then the skin is treated with a cosmetic composition against the impairment or alteration of the barrier function: this is step c). Preferably, this composition comprises at least one active ingredient for stimulating the activity of PREP.

20 "Cosmetic composition against the impairment or alteration of the barrier function" is here taken to mean any type of cosmetic composition (topical or oral for example) expected to improve the cutaneous barrier function.

The cosmetic composition may be applied by any suitable route of administration, in particular by topical or oral route. It may be for example in the form
25 of a cream, lotion, gel or suspension.

In one particular embodiment, the cosmetic composition of step c) comprises at least one active ingredient for stimulating the activity of PREP. Preferably, the active ingredient making it possible to stimulate the activity of PREP is D-aspartic acid (or D-aspartate) or a recombinant PREP protein.

30 Cosmetic compositions making it possible to strengthen the cutaneous barrier function thus preferably comprise at least one active ingredient making it possible to stimulate the activity of PREP such as D-aspartic acid (or D-aspartate) or a recombinant PREP protein. Such a recombinant PREP protein is for example marketed under the name Tolerase G by Therascience.

Method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function

The present invention also relates to a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the following steps:

- a) contacting at least one candidate compound with a skin sample of the subject, or with a skin or epidermis model in which the level of expression and/or the enzymatic activity of PREP is reduced, and then measuring the level of expression and/or the enzymatic activity of PREP in said sample or model;
- b) comparing the expression measured in step a) to the expression of PREP in a skin sample of the subject, or with a skin or epidermis model, not exposed to said compound; and
- c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the expression of PREP in the skin sample of the subject or the skin or epidermis model that has been exposed to said candidate compound is detected, relative to the expression of PREP in the skin sample of the subject or the skin or epidermis model that has not been exposed to the candidate compound.

The method of identification of the invention is preferably implemented *ex vivo*.

According to one embodiment, the skin sample used in the identification method according to the invention is as described above.

The skin or epidermis model in which the level of expression and/or the enzymatic activity of PREP is reduced is any *in vitro* model of reconstructed skin or reconstructed epidermis, in which the level of expression and/or the enzymatic activity of PREP is reduced.

The measurement of step a) is preferably performed as described in the "*Method for prognosing and/or diagnosing*" section above.

In one alternative, the invention relates to a method for evaluating the efficacy of a cosmetic treatment against the impairment of the cutaneous barrier function and/or for

the strengthening of the cutaneous barrier function in a subject, said method comprising, after a step of applying the cosmetic composition, a step (a) of measuring the level of expression and/or the enzymatic activity of PREP, in a skin sample of said subject.

Preferably, this evaluation method further comprises the steps consisting of:

- b) comparing said quantity measured in step a) with a control;
- c) determining whether said cosmetic treatment against the impairment of the cutaneous barrier function and/or for the strengthening of the cutaneous barrier function is effective in said subject.

The efficacy evaluation method is preferably implemented *in vitro* or *ex vivo*.

The subject is preferably as described above.

Preferably, the impaired or altered cutaneous barrier function is observed in a subject with dry and/or dehydrated skin.

In one particular embodiment, the control is the level of expression and/or the enzymatic activity of PREP in a skin sample of said subject prior to application of said cosmetic treatment.

Preferably, said cosmetic treatment is evaluated as effective in said subject if the level of expression and/or the enzymatic activity of PREP measured in step a) in the skin sample of the subject is higher, in particular significantly higher, than the control.

The subject matter of the invention is also a method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the following steps:

- a) contacting at least one candidate compound with a recombinant PREP in the presence of filaggrin or one of its peptide fragments containing a proline, then measuring the level of the enzymatic activity of PREP;
- b) comparing the enzymatic activity measured in step a) to the enzymatic activity of PREP in the absence of said compound; and
- c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the enzymatic activity of PREP is detected, relative to the enzymatic activity of PREP that has not been contacted with the candidate compound.

The method of identification of the invention is preferably implemented *ex vivo*.

The measurement of step a) is preferably performed as described in the
"Method for prognosing and/or diagnosing" section above. Preferably, the filaggrin
peptide fragments containing a proline are as described above.

Cosmetic use

The invention also relates to the cosmetic use of at least one active ingredient
making it possible to stimulate the level of expression of PREP and/or the enzymatic
activity of PREP, or of a recombinant PREP protein, to prevent the impairment of the
cutaneous barrier function and/or to strengthen the cutaneous barrier function.

Preferably, the active ingredient making it possible to stimulate the level of
expression of PREP is D-aspartic acid (or D-aspartate).

Indeed, D-aspartic acid or D-aspartate is described as an active ingredient
making it possible to stimulate the level of expression of PREP in Leydig and Sertoli
cells and spermatogonia in rats (Santillo A et al., Reproduction, 2019).

As an example of a recombinant PREP protein, mention may be made for
example of the protein marketed under the name of Tolerase G by Therascience, as
well as the Gluterase composition marketed by Energetica Natura.

Skin equivalent and epidermal equivalent

The subject matter of the invention is also a skin equivalent or an epidermal
equivalent, in which the level of expression and/or the enzymatic activity of PREP is
reduced, for example following a knock-down of the *PREP* gene. Said skin equivalent
or epidermal equivalent, in which the level of expression and/or the enzymatic activity
of PREP is reduced, may be obtained by a method comprising the knock-down of the
PREP gene.

In the present application, the skin equivalent and the epidermal equivalent are
also referred to as skin model and epidermal model, respectively. They may be used
in the *"Method for identifying a compound making it possible to prevent the impairment
of the cutaneous barrier function and/or to strengthen the cutaneous barrier function"*
described above.

The skin equivalent comprises a dermal equivalent and an epidermal equivalent. In particular, the skin equivalent according to the invention comprises, on the dermal equivalent, an epidermal equivalent comprising at least keratinocytes.

The epidermis equivalent comprises at least keratinocytes.

The keratinocytes may come from any origin but are preferably keratinocytes of human origin. They may be prepared by any method well known to those skilled in the art. Thus, the keratinocytes may be prepared by culture of dissociated epidermis from normal skin sampling or by culture of keratinocytes from the hair follicle sheath. Preferably, the keratinocytes are keratinocytes of normal human skin.

The skin equivalent and the epidermal equivalent according to the invention have a reduced level of expression and/or enzymatic activity of PREP.

“Reduced level of expression and/or enzymatic activity of PREP” is taken to mean a significantly reduced level of expression and/or enzymatic activity of PREP compared to a control. Preferably, the control is a sample of normal skin, preferably normal human skin (i.e. having a barrier function). For example, the level of expression and/or the enzymatic activity of PREP is reduced following a knock-down of the PREP gene.

“Knock-down” is taken to mean an artificial reduction in the expression and/or the enzymatic activity of PREP, preferably a significant reduction, compared to a control, preferably the control is the level of expression and/or the enzymatic activity of PREP in normal human skin. This reduction may be done either by genetic modification or by treatment with a short DNA or RNA oligonucleotide of which the sequence is complementary to the PREP gene or the PREP mRNA. The knock-down may notably be done by “transient knockdown”, by interfering RNA or by using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) or TALENs (transcription activator type effector nucleases) technologies.

The present invention also relates to a skin equivalent or epidermal equivalent in which the level of expression and/or the enzymatic activity of PREP is reduced relative to a control (preferably the control is a sample of normal skin; preferably a sample of normal human skin i.e. having a barrier function), which may be obtained by a method comprising a knock-down of the *PREP* gene.

The present invention will be described in more detail by the examples below.

FIGURES

[Fig 1] **Figure 1 shows the fact that PREP and filaggrin colocalize in the interfollicular epidermis.** Normal human skin sections were analyzed by dual marking by confocal microscopy with a monoclonal antibody specific for profilaggrin and filaggrin and with an anti-PREP antibody. The profile of the two fluorochromes was recorded along the white line on the magnification of the overlap of the two markings (merge). The Pearson and Manders coefficients were calculated from 30 different images produced from 3 donors.

[Fig 2] **Figure 2 shows the efficacy of the extinction of *PREP*.** 3D-reconstructed human epidermis (RHEs) were produced with keratinocytes transduced with shRNAs targeting PREP (sh-PREP) or a control shRNA (Ctrl). The expression of *PREP* was analyzed by RT-qPCR. n = 5 different RHEs.

[Fig 3] **Figure 3 shows the efficacy of the extinction of PREP at the protein level.** 3D reconstructed human epidermis (RHEs) were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP-1, shPREP-2 and shPREP-3) or a control shRNA (Ctrl). The expression of PREP was analyzed by Western blotting. The total proteins were separated by SDS-PAGE, transferred to membranes and stained with Ponceau Red or immunodetected with an anti-PREP antibody and an anti-actin antibody. An example is shown on the left, the quantification on the right. n = 8 control RHEs produced with keratinocytes from 2 different donors, and 10 RHEs produced with keratinocytes from 2 different donors and transduced with shPREPs.

[Fig 4] **Figure 4 shows the fact that the extinction of PREP profoundly affects the epidermal barrier function.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP-1, shPREP-2 and shPREP-3) or a control shRNA (Ctrl). The transepithelial electrical resistance (TEER) was measured.

[Fig 5] **Figure 5 shows the fact that the extinction of PREP affects the morphology of the epidermis.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP) or a control shRNA (Ctrl). They were analyzed by transmission electron microscopy. Representative images of the granular layer are shown, with the arrows indicating granular keratinocytes and the scale bar corresponding to 5 μ m. The number of granular keratinocyte bases was quantified.

[Fig 6] **Figure 6 shows the fact that the extinction of PREP affects filaggrin expression.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP) or a control shRNA (Ctrl). Filaggrin (FLG) gene expression was analyzed by RT-qPCR (left side); n=5. Profilaggrin (proFLG) and filaggrin (FLG) were immunodetected: an example is shown as well as the quantification of immunoreactive bands (relative to actin detection). n.s., not significant. n = 3.

[Fig 7] **Figure 7 shows the fact that the extinction of PREP affects the expression of the genes of the enzymes involved in the degradation of filaggrin.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP) or a control shRNA (Ctrl). Gene expression of bleomycin hydrolase (*BLMH*), caspase 14 (*CASP14*), calpain 1 (*CAPN 1*), kallikrein 7 (*KLK 7*) and peptidyl-arginine desiminases type 1 and 3 (*PAD1* and *PAD3*) was analyzed by RT-qPCR. n = 5.

[Fig 8] **Figure 8 shows the fact that the extinction of PREP affects gene expression of corneal envelope proteins.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP) or a control shRNA (Ctrl). Gene expression of transglutaminases (*TGM*) 1, 3 and 5, involucrin (*IVL*), loricrin (*LOR*), and corneodesmosin (*CDSN*) was analyzed by RT-qPCR. n = 5.

[Fig 9] **Figure 9 shows the fact that the extinction of PREP affects the expression of bleomycin hydrolase (BLMH) and loricrin (LOR) at the protein level.** 3D reconstructed human epidermis were produced with keratinocytes transduced with shRNAs targeting PREP (shPREP) or a control shRNA (Ctrl). Involucrin (IVL), BLMH and LOR were immunodetected: one example is shown and the intensity of the immunoreactive bands was quantified (relative to actin detection). n = 3.

[Fig 10] **Figure 10 shows the fact that PREP cleaves several peptides derived from the human filaggrin sequence and containing a proline.** The peptides of which the sequence (the sequence corresponding to the filaggrin or the binding peptide is underlined) is indicated above the graphs, were incubated in the absence or presence of PREP for 1h at 37°C and fluorescence was measured every 5 minutes. n = 3.

[Fig 11] **Figure 11 shows the fact that PREP cleaves peptides derived from the human filaggrin sequence and containing a proline.** The peptides of which the sequence is indicated (dabcyl and EDANS groups as well as C-ter glutamate are not shown; proline when present is underlined) were incubated in the presence of PREP

for 1h at 37°C and fluorescence was measured every 5 minutes. The average slope of each of the curves was calculated. n = 3.

EXAMPLES: EVALUATION ACCORDING TO THE INVENTION

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1. Colocalization of PREP with filaggrin

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Double-marking confocal microscopy analysis was performed to determine the degree of colocalization of PREP with filaggrin. Analysis by confocal microscopy showed that PREP has robust colocalization coefficients [Fig 1]: a Pearson mean coefficient equal to 0.40; and Manders mean coefficients between 0.25 and 0.52. Occasional or partial colocalization of this protease with (pro)filaggrin may thus be discussed. The occasional colocalization between PREP and (pro)filaggrin was distributed for 60% at the level of the granular layer, 30% at the level of the corneal layer and 10% at the interface of the two layers.

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2. Several synthetic peptides derived from the human filaggrin sequence are *in vitro* PREP substrates

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In order to study the *in vitro* proteolysis of filaggrin by PREP, five fluorogenic peptides of 7 to 8 amino acids long coupled to a Dabcyl group in N-ter and an EDANS group in C-ter via a glutamic acid (Table 1) were synthesized (JPT Peptide Technologies) according to the following rationale. Peptide 1 corresponds to a repeated sequence of filaggrin (Swiss-Prot #P20930; positions 2754–2761 and 3078–3085) with a central proline (Pro). Peptide 3 is the binding peptide between filaggrin subunits. Peptides 4 and 6 correspond to two other regions of the filaggrin monomer sequence (Swiss-Prot #P20930; positions 1522–1528 and 2106–2113) containing a proline, and thus potential PREP substrates. Peptide 7 is derived from peptide 1 in which the proline has been substituted with a serine (Ser). When the peptides are excited at 340 nm in the absence of enzymatic cut-off, Dabcyl (4-(dimethylaminoazo)benzene-4-carboxylic acid) plays the acceptor (quencher) role in the fluorescence resonance energy transfer reaction (FRET) and there is no light emitted. When the peptides are cleaved, the fluorescence emission by the EDANS at 490 nm is then quantifiable.

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[Table 1]

Sequence of fluorogenic peptides.

Peptide	sequence	note
1	Dabcyl-WTGPSTRG-Glu-EDANS	Drift of the filaggrin sequence
3	Dabcyl-FLYQVST-Glu-EDANS	Binding peptide between filaggrin monomers
4	Dabcyl-TTPQGRS-Glu-EDANS	Drift of another part of the filaggrin sequence
6	Dabcyl-QSAPSTGG-Glu-EDANS	Drift of another part of the filaggrin sequence
7	Dabcyl-WTG <u>S</u> STRG-Glu-EDANS	Peptide 1 with the proline substituted by a <u>serine</u>

The peptides are incubated in the absence or presence of PREP and the fluorescence is recorded over time. When peptide 1 was incubated in the absence of protease, no fluorescence emission was observed. However, the fluorescence increased as a function of time in the presence of PREP [Fig 10]. When the proline from peptide 1 was replaced with a serine (peptide 7), the fluorescence remained at the background noise level, confirming the specificity of protease for proline. PREP cuts the peptides 4 and 6, which indicates that PREP has a broad spectrum of action on several peptides from filaggrin. The inventors did not observe any cleavage of peptide 3. The calculation of the average slopes confirms all these results [Fig 11].

3. Extinction of the expression of PREP in RHEs

To definitively prove the involvement of PREP in filaggrinolysis and epidermal homeostasis, its expression was individually inhibited by the RNA interference technique, as previously described (Pendaries et al, J Invest Dermatol, 2014) using shRNAs (small hairpin RNA) transduced into primary keratinocytes using lentiviral particles (Merck-Sigma-Aldrich). RHEs were next produced at reduced relative humidity, a condition in which the metabolism of filaggrin is increased (Cau et al, J Dermatol Sci, 2017). Three different shRNAs targeting distinct exons of the *PREP* gene were used (Table 2), as well as a control shRNA (noted Ctrl) not targeting any human sequence. The extinction efficacy was analyzed by Western Blot and confirmed by RT-qPCR. The consequences of this inhibition were analyzed at the morphological, molecular and functional levels.

[Table 2]

Characteristics of the shRNAs used to silence the expression of PREP;
shRNA, small hairpin RNA.

Name	Transcript ID	#sh-RNA-1* target sequence (target exon)	#sh-RNA-2* target sequence (target exon)	sh-RNA-3* target sequence (target exon)
PREP	NM_002726	shPREP-1	shPREP-2	shPREP-3

		CCAGCTTTCTTAT ATGGCTAT (exon 11)	CCCAACATACTGT CTGACGAT (exon 4)	CCTGACCTCTTTG GTTGTT (exon 14)
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The three shRNAs induced a very sharp decrease in the expression of PREP protease [Fig 2 and Fig 3], both at the RNA level (decrease of 85%) and protein level (decrease of 78%). This led to a very sharp decrease in TEER [Fig 4]. The morphology of the RHEs, after staining with hematoxylin and eosin (H&E), was normal. *FLG* mRNA expression was slightly reduced by about 40% and detection of profilaggrin and filaggrin was reduced [Fig 6]. Analysis by transmission electron microscopy shows a decrease in the number of granular keratinocyte bases and possibly the size of the keratohyalin granules [Fig 5].

To find out whether the extinction of PREP impacts the enzymes of the metabolism of profilaggrin and filaggrin and more broadly the differentiation of keratinocytes, the expression of bleomycin hydrolase (BLMH), caspase-14, kallikrein 7 and calpain 1 proteases; peptidylarginine desiminases, transglutaminases and some other markers of differentiation was analyzed by RT-qPCR [Fig 7 and Fig 8] and/or Western blot [Fig 9]. The mRNA levels of BLMH and caspase-14 decrease by 60% and 50% respectively, those of calpain-1 and kallikrein-7 decrease slightly (30%). This decrease was confirmed by Western blot for BLMH. At the mRNA level, the expression of involucrin, hornerin and corneodesmosin, three components of corneal envelopes, is unchanged, while loricrin is less expressed (decrease of 60%). The same results are obtained at the protein level. A decrease in the expression of mRNA encoding transglutaminases 3 (less 60%) and 5 (less 40%) is noted while the expression of transglutaminase 1 is not modified.

Conclusions:

This study has made it possible to highlight a new protease important for the production of the natural moisturizing factor and, more generally, cornification: PREP.

PREP is a new player in filaggrinolysis. When PREP is silenced in RHEs, the abundance of key enzymes of the filaggrin metabolism, such as BLMH and caspase 14, is reduced.

In addition, when PREP is silenced, a change in expression and abundance of many proteins expressed during the late stages of keratinocyte differentiation is observed, suggesting that PREP could also be involved beyond, in controlling cornification.

CLAIMS

1. Method for prognosing and/or diagnosing an impaired or altered cutaneous barrier function, comprising a) measuring the level of expression and/or the enzymatic activity of prolyl oligopeptidase protease (PREP) in a skin sample of a subject.

2. Method according to claim 1, further comprising the following steps:

b) deducing from step a) whether the cutaneous barrier function of said subject is impaired or altered; and

c) if the cutaneous barrier function is identified as impaired or altered in step b), treating the skin sample of the subject with a cosmetic composition against the impairment or alteration of the barrier function.

3. Method according to claim 1 or 2, wherein the skin of said subject is diagnosed as having an impaired or altered cutaneous barrier function when the level of expression and/or the enzymatic activity of PREP measured in step a) in the skin sample of the subject is less, in particular significantly less, than a control.

4. Method according to claim 3, wherein the control is a reference value determined by the average value of the level of expression and/or the enzymatic activity of PREP, measured in a determined population, for example a population aged 20 to 80 years, and/or having a satisfactory cutaneous barrier function.

5. Method according to one of claims 2 to 4, wherein the cosmetic composition against the impairment or alteration of the barrier function comprises at least one active ingredient making it possible to stimulate the activity of PREP, preferably the active ingredient making it possible to stimulate the activity of PREP is D-aspartic acid (or D-aspartate) or a recombinant PREP protein.

6. Method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the following steps:

a) contacting at least one candidate compound with a skin sample of the subject, or with a skin or epidermis model in which the level of expression and/or the enzymatic activity of PREP is reduced, then

measuring the level of expression and/or the enzymatic activity of PREP in said sample or model;

- b) comparing the expression measured in step a) to the expression of PREP in a skin sample of the subject, or with a skin or epidermis model, not exposed to said compound; and
- c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the expression of PREP in the skin sample of the subject or the skin or epidermis model that has been exposed to said candidate compound is detected, relative to the expression of PREP in the skin sample of the subject or the skin or epidermis model that has not been exposed to the candidate compound.

7. Method for identifying a compound making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function in a subject, said method comprising the following steps:

- a) contacting at least one candidate compound with a recombinant PREP in the presence of filaggrin or one of its peptide fragments containing a proline, then measuring the level of the enzymatic activity of PREP;
- b) comparing the enzymatic activity measured in step a) to the enzymatic activity of PREP in the absence of said compound; and
- c) identifying said candidate compound as making it possible to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function, when an activation of the enzymatic activity of PREP is detected, relative to the enzymatic activity of PREP that has not been contacted with the candidate compound.

8. Method according to one of the preceding claims, wherein the impaired or altered cutaneous barrier function is observed in a subject having dry and/or dehydrated skin.

9. Method according to one of the preceding claims, wherein the skin sample of a subject is a sample previously obtained using a biopsy or by sampling.

10. Method according to one of the preceding claims, wherein the level of expression is either the amount of mRNA produced, or the amount of protein produced, by expression of the PREP gene; and/or the enzymatic activity of PREP is the amount of cleaved filaggrin, or the amount of cleaved filaggrin fragments, preferably the fragment is chosen from the fragments of sequence SEQ ID NO:2 to 4.

11. Method according to one of the preceding claims, wherein the level of expression and/or the enzymatic activity of PREP is measured by quantitative PCR, Western blot, by quantitative mass spectrometry methods of the iTRAQ, MS/MS type, by enzymatic tests notably using fluorescent peptides or ELISA.

12. Cosmetic use of at least one active ingredient making it possible to stimulate the level of expression and/or the enzymatic activity of PREP, to prevent the impairment of the cutaneous barrier function and/or to strengthen the cutaneous barrier function.

13. Use according to claim 12, wherein the active ingredient is selected from a recombinant PREP protein and D-aspartic acid.

14. Skin equivalent or epidermal equivalent, wherein the level of expression and/or the enzymatic activity of PREP is reduced following a knock-down of the *PREP* gene, preferably relative to a control.

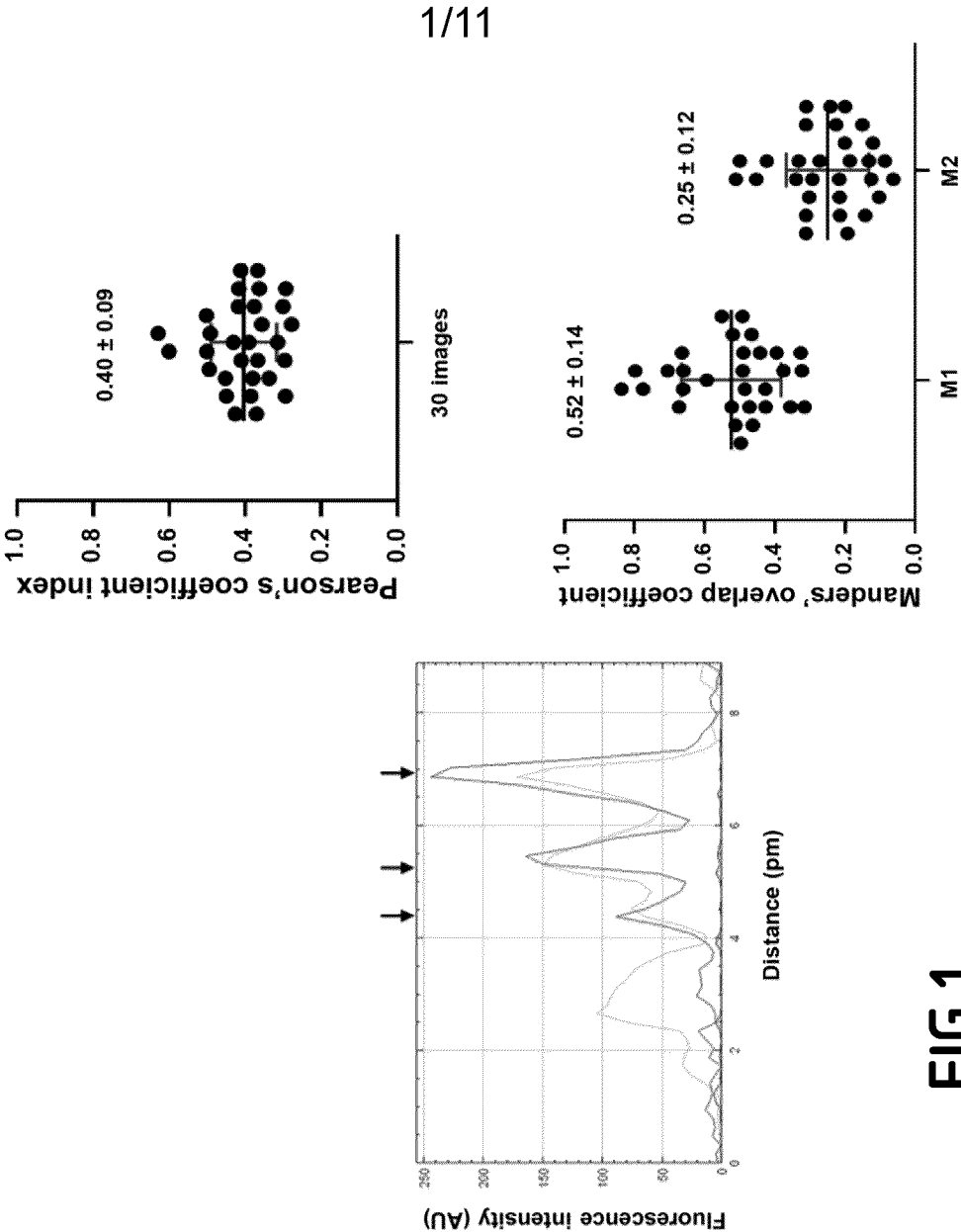


FIG.1

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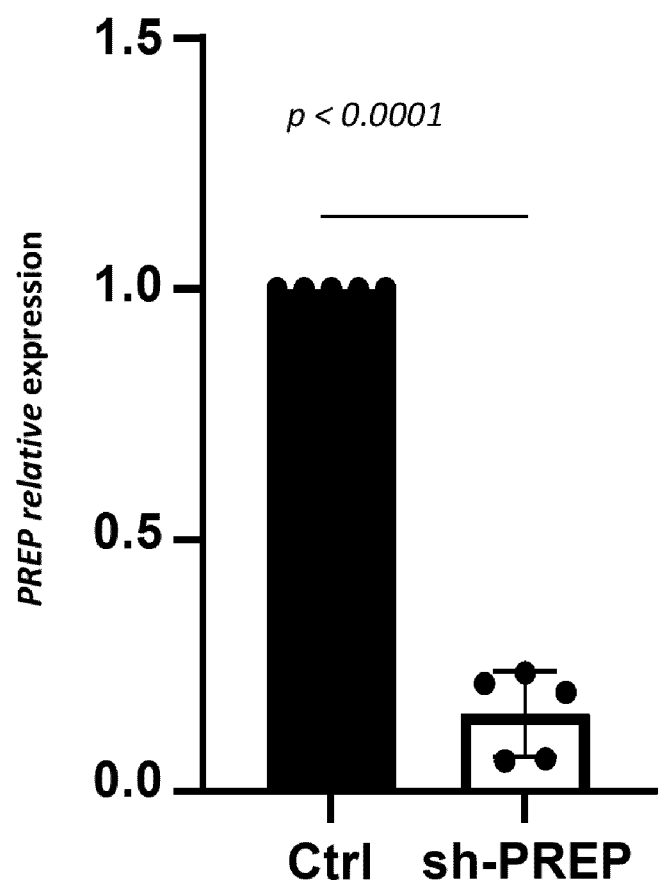


FIG.2

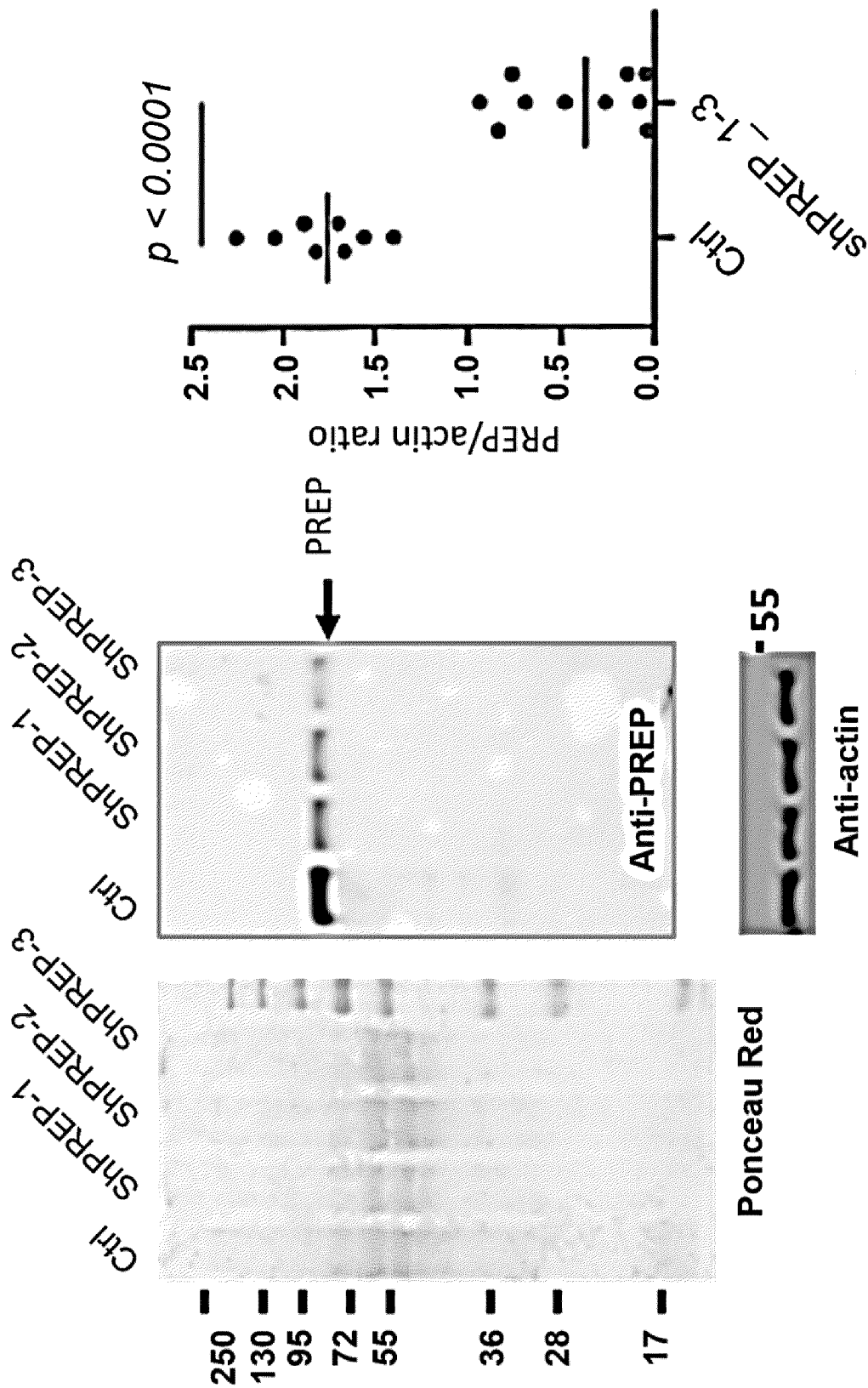


FIG.3

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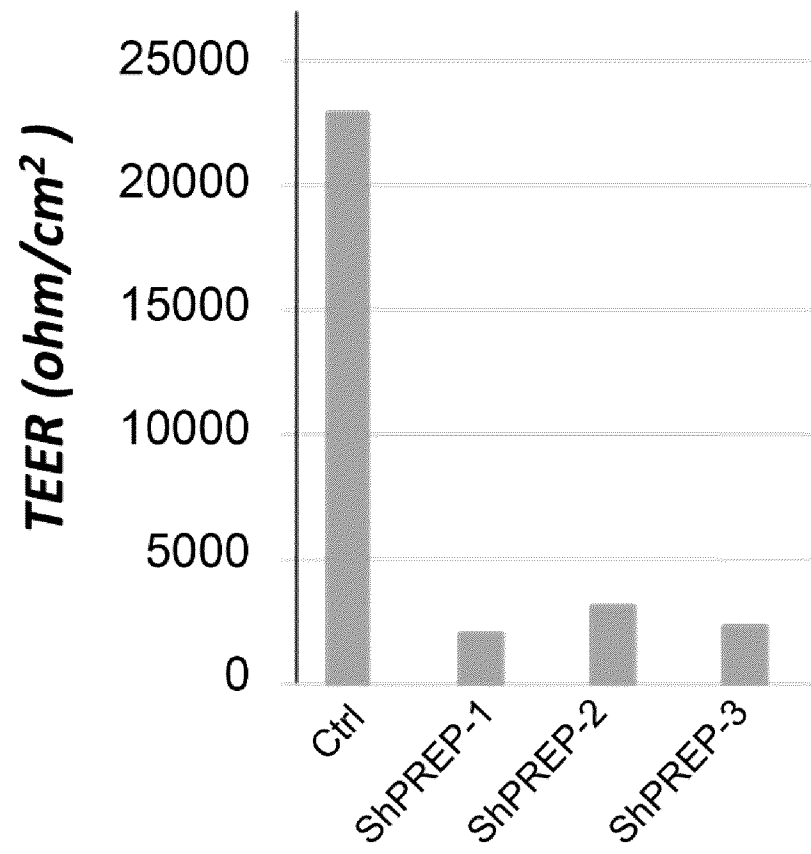


FIG.4

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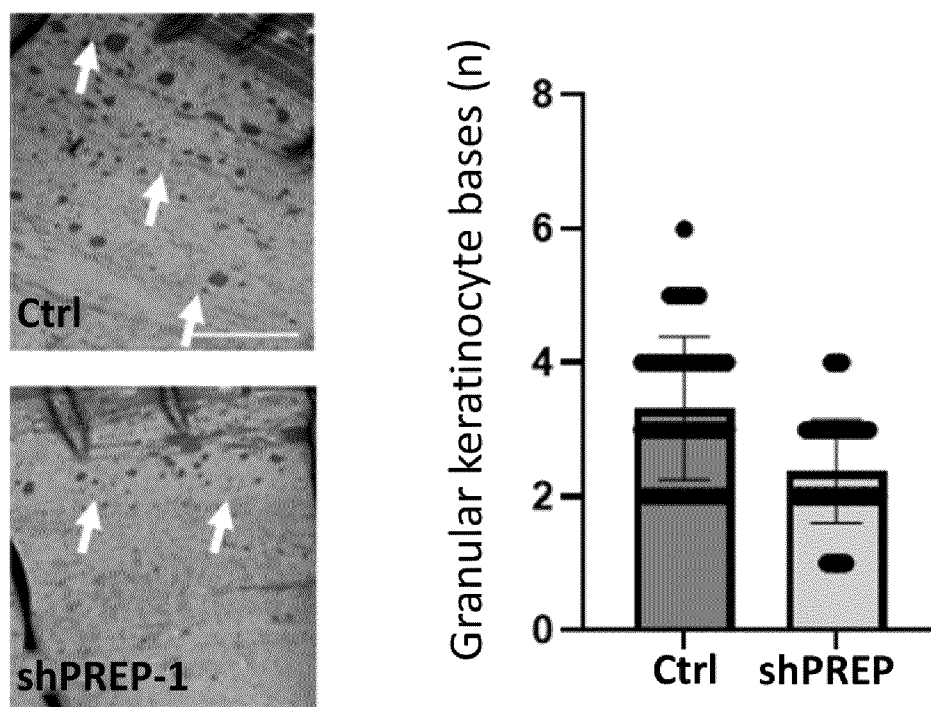


FIG.5

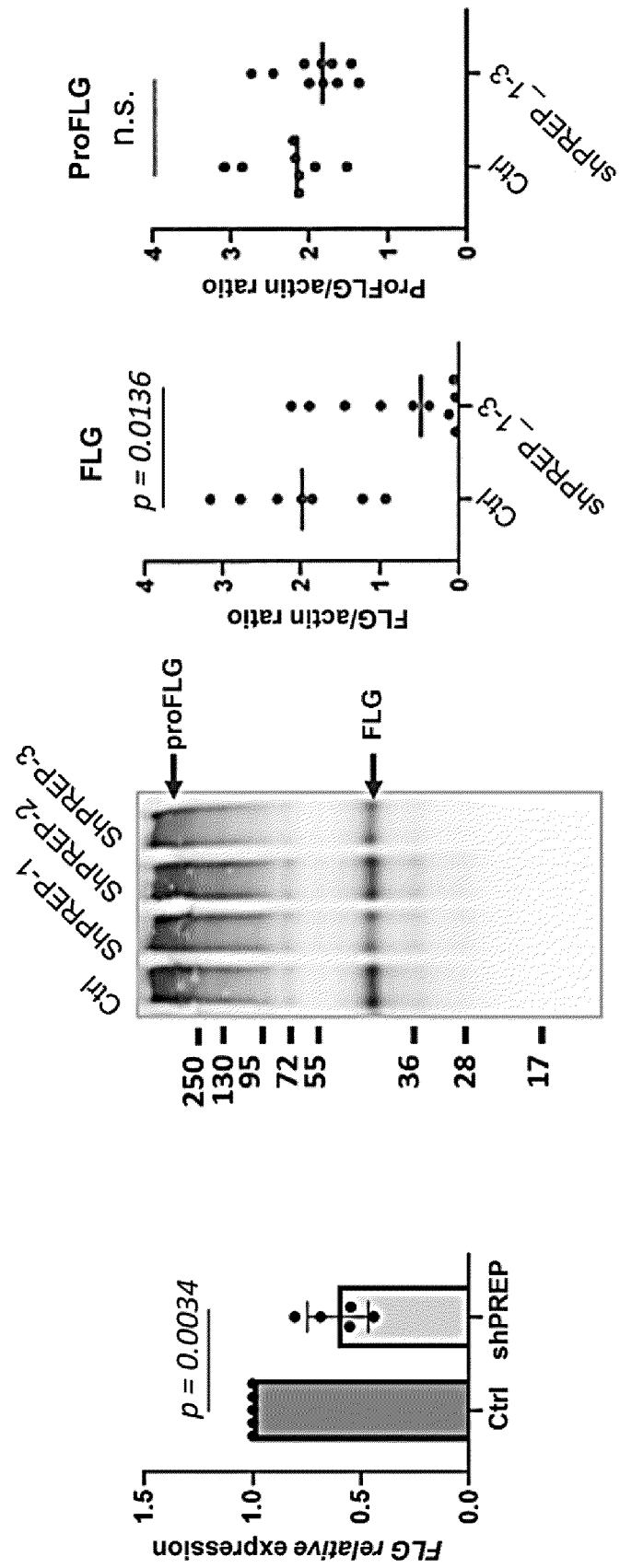


FIG.6

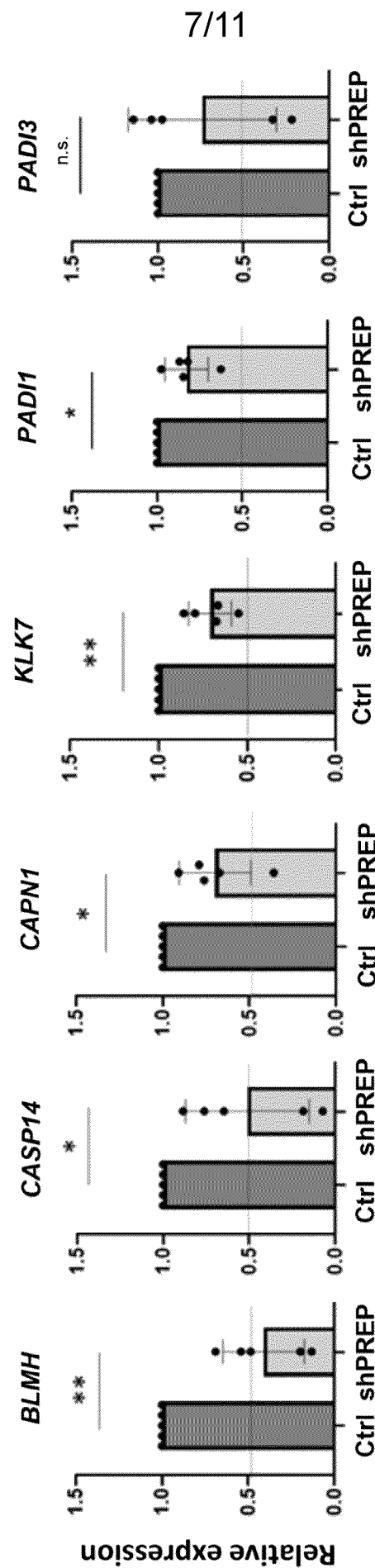


FIG.7

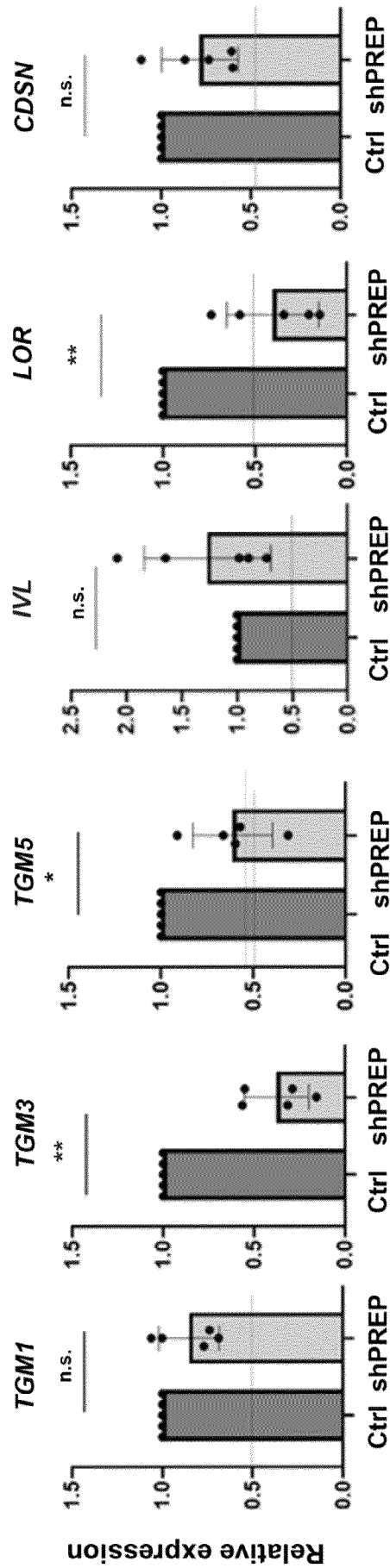


FIG.8

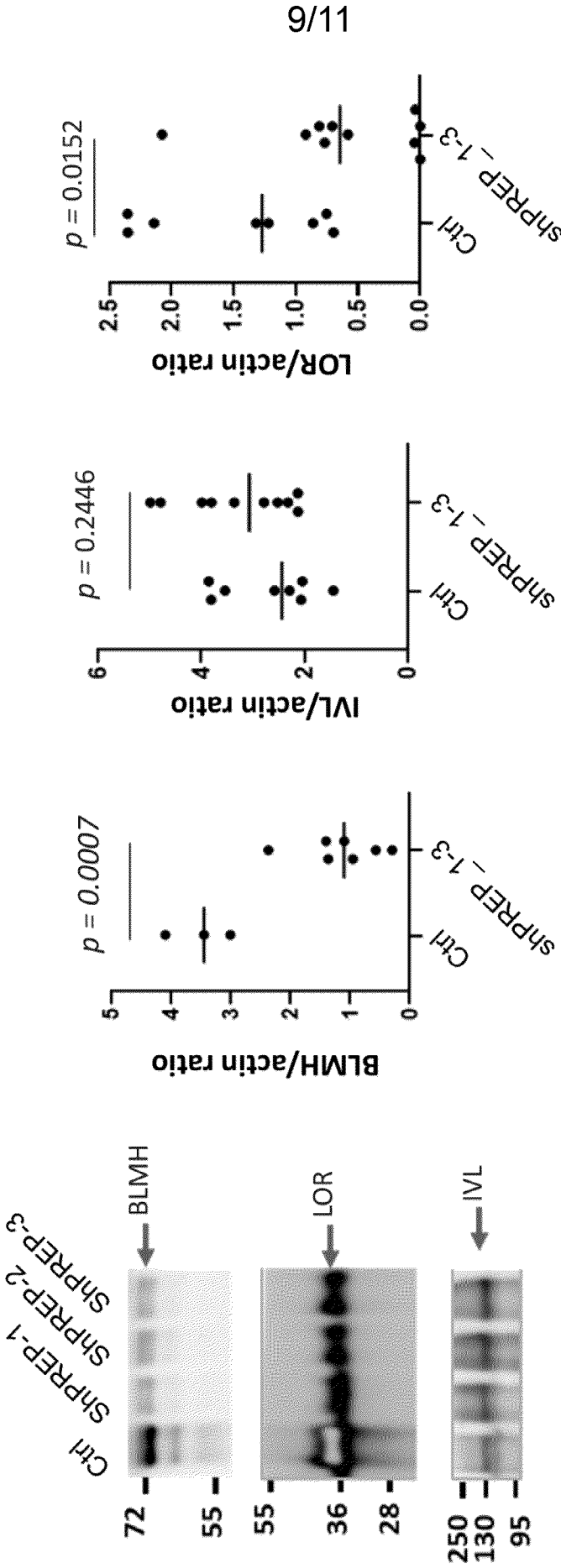


FIG.9

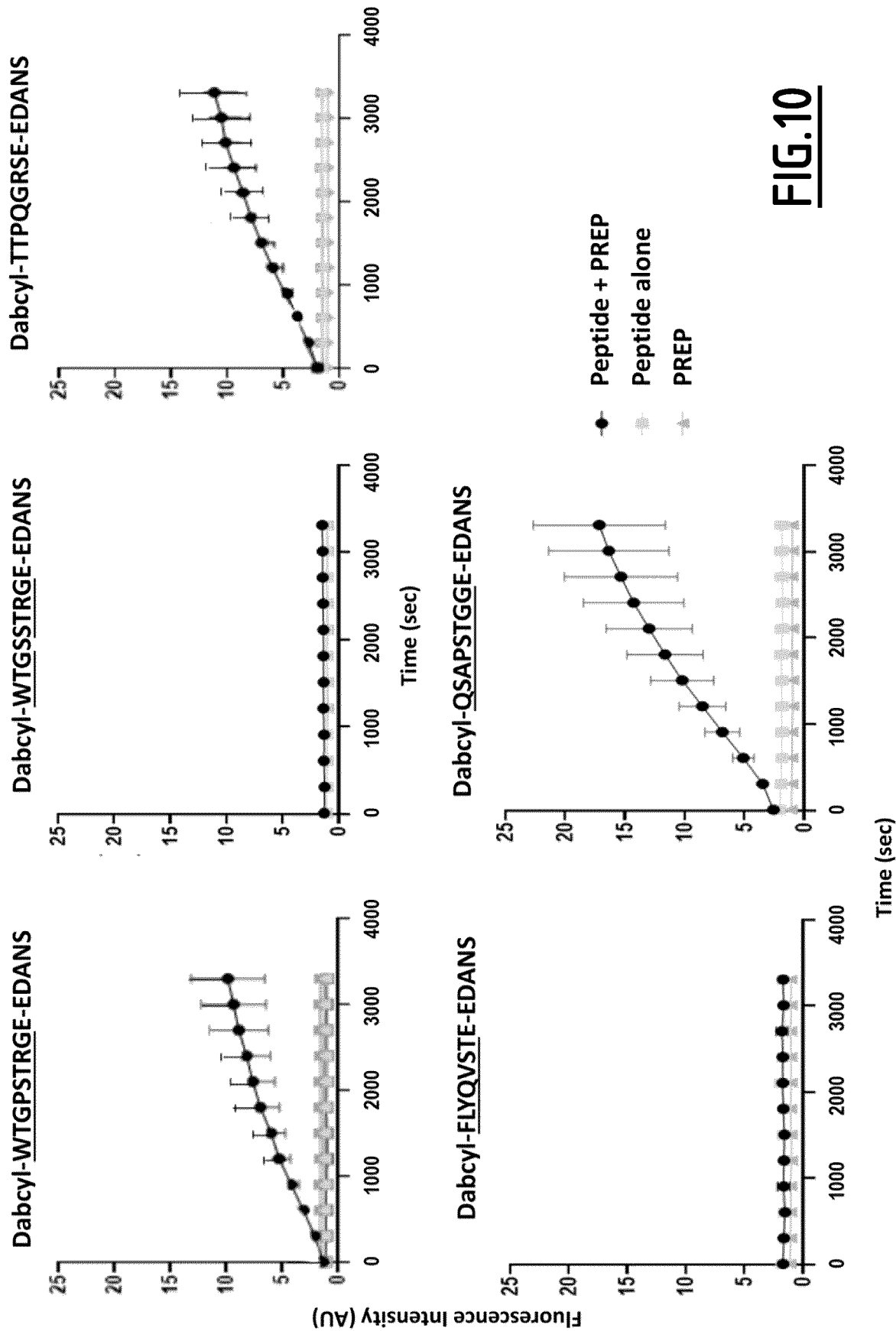


FIG.10

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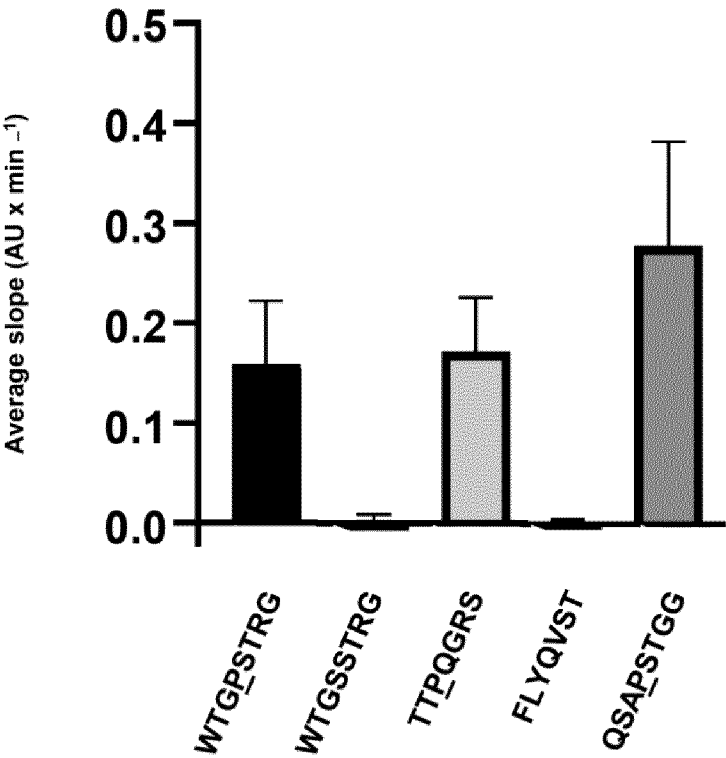


FIG.11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/058795

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/058795

A. CLASSIFICATION OF SUBJECT MATTER INV. C12Q1/37 G01N33/68 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12Q A61Q A61K G01N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 761 362 A1 (OREAL [FR]) 2 October 1998 (1998-10-02) revendication 18-19 -----	12
X	CN 108 403 527 A (FOSHAN YUNSHANG COSMETIC CO LTD) 17 August 2018 (2018-08-17) exemple 1-6 -----	12
X	US 2012/184620 A1 (TOJO YOSUKE [JP] ET AL) 19 July 2012 (2012-07-19) revendication 9-10, 13 ; para 1 ----- - / - -	12, 13
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 4 July 2024		Date of mailing of the international search report 16/07/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Vadot-Van Geldre, E

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/058795

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MYÖHÄNEN TIMO T. ET AL: "Distribution of Prolyl Oligopeptidase in Human Peripheral Tissues and in Ovarian and Colorectal Tumors", JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY , vol. 60, no. 9 1 September 2012 (2012-09-01), pages 706-715, XP093120347, US ISSN: 0022-1554, DOI: 10.1369/0022155412453051 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3524555/pdf/10.1369_0022155412453051.pdf Fig 1 ; pg 708, col 2, para 5 -----	14
X	ZHENG ZHANG ET AL: "Tissue Engineered Human Skin Equivalents", PHARMACEUTICS, vol. 4, no. 1, 6 January 2012 (2012-01-06) , pages 26-41, XP055488788, DOI: 10.3390/pharmaceutics4010026 abrégé ; Tableau 1 -----	14
A	SON E D ET AL: "Skin dryness in apparently healthy human skin is associated with decreased expression of bleomycin hydrolase in the stratum corneum", CLINICAL AND EXPERIMENTAL DERMATOLOGY, BLACKWELL SCIENTIFIC PUBLICATIONS, GB, vol. 40, no. 3, 11 December 2014 (2014-12-11), pages 247-253, XP071608679, ISSN: 0307-6938, DOI: 10.1111/CED.12520 abrégé ; Fig 1-4 ; pg 252, col 1, para 2-3 -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/058795

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