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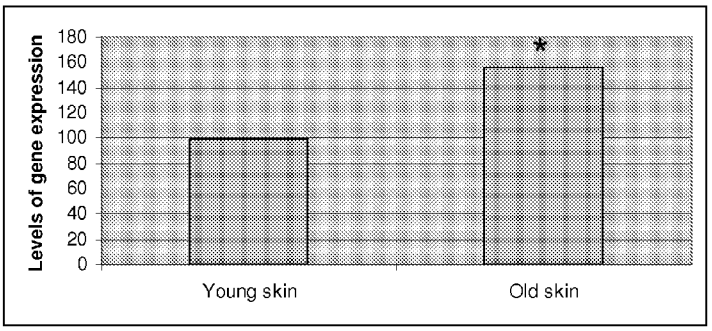
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(54) Title: ANTI-AGEING AGENTS



* p<0.05

Figure 2

(57) Abstract: This invention relates to a method for identifying an anti-ageing agent for use in skincare compositions, by determining the ability of a material or combination of materials to modulate the expression of molecules associated with microfibril or elastic fibres. More specifically, the invention relates to a method for identifying an anti-ageing agent by determining the ability of a material or combination of materials to modulate LOXL-1, LTBP-2 and/or LTBP-3 expression. The invention also relates to methods for assessing the anti-ageing effect of a material or combination of materials on the skin, anti-ageing agents identified by such methods, and compositions containing them.

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Title – Anti-ageing agents

Field of the invention

The invention relates to a method for identifying an anti-ageing agent for use in skincare compositions, by determining the ability of a material or combination of materials to modulate the expression of molecules associated with microfibril or elastic fibres. More specifically, the invention relates to a method for identifying an anti-ageing agent by determining the ability of a material or combination of materials to modulate LOXL-1, LTBP-2 and/or LTBP-3 expression. The invention also relates to methods for assessing the anti-ageing effect of a material or combination of materials on the skin, anti-ageing agents identified by such methods, and compositions containing them.

Background of the invention

Cutaneous ageing is the result of two distinct, biological processes which may occur concurrently: i) the passage of time, termed intrinsic ageing, and ii) environmental influences, termed extrinsic ageing.

Anti-ageing agents are commonly used in skincare compositions to reduce or prevent the signs of extrinsic ageing. In particular, anti-ageing agents are included to protect the skin against the adverse effects of free radicals, eg effects mediated by UV-radiation, or other sources of oxidative stress. Such agents include sunscreening agents and antioxidant agents.

It would clearly be advantageous to provide an anti-ageing agent that targets intrinsic ageing to improve the condition and aesthetic appearance of skin, whether or not it has been exposed to environmental stress. It would also be advantageous to use anti-ageing agents that target intrinsic ageing in combination with current agents that reduce the signs of extrinsic ageing, to improve the overall anti-ageing benefit afforded by current skincare compositions.

The clinical features of intrinsically aged skin are not usually evident until old age when, although smooth and unblemished, the skin surface appears pale and is characterised by fine wrinkles. Functionally, intrinsically aged skin is dry and less elastic than more youthful skin. The dermal elastic fibre network is responsible for providing the skin with its recoil capacity and loss of this property contributes to an aged appearance. Dermal elastic fibres have a complex biology due to their multiple components yet the effect of intrinsic ageing on all such molecules has not previously been explored.

The present inventors have used an Affymetrix® microarray-based approach to detect age-related changes in gene expression of molecules known to contribute to the elastic fibre network. Samples of photoprotected skin from young (24-26 years) and old (74-75 years) individuals were analysed. A comparison of the gene expression profiles from the young and old skin samples revealed that the majority of elastic fibre network components were unchanged with age. However, three genes were identified which showed significant differential expression. Given that intrinsic ageing of the skin is a slow and subtle process it is surprising that a significant alteration in expression level has been identified for these genes. This finding indicates that the identified genes and their expression products impact greatly on the organisation of elastic fibres in intrinsically aged skin. Thus, expression of these genes can be used to indicate the efficacy of a material or combination of materials as an anti-ageing agent.
The genes the inventors have identified are those which encode LOXL-1 (Lysyl oxidase-like 1), LTBP-2 (latent transforming growth factor beta-binding protein-2) and LTBP-3 (latent transforming growth factor beta-binding protein-3).

The term “LOXL-1” is used herein to denote, as the context requires, either the LOXL-1 enzyme or the gene that encodes it. Expression of LOXL-1 was found to be significantly reduced in old skin compared to young skin. Thus it appears that the level of LOXL-1 in our skin decreases as part of the intrinsic ageing process. Materials that are able to delay or prevent this natural decrease in LOXL-1 necessarily hinder the intrinsic ageing process and therefore act as anti-ageing agents.

The term "LTBP-2" is used herein to denote, as the context requires, either the LTBP-2 protein or the gene that encodes it. Expression of LTBP-2 was found to be significantly increased in old skin compared to young skin. Thus it appears that the level of LTBP-2 in our skin increases as part of the intrinsic ageing process. Materials that are able to delay or prevent this natural increase in LTBP-2 necessarily hinder the intrinsic ageing process and therefore act as anti-ageing agents.

The term "LTBP-3" is used herein to denote, as the context requires, either the LTBP-3 protein or the gene that encodes it. Expression of LTBP-3 was found to be significantly reduced in old skin compared to young skin. Thus it appears that the level of LTBP-3 in our skin decreases as part of the intrinsic ageing process. Materials that are able to delay or prevent this natural decrease in LTBP-3 necessarily hinder the intrinsic ageing process and therefore act as anti-ageing agents.

Quantitative polymerase chain reaction (qPCR) analysis may also be used to confirm changes in gene expression.

Brief summary of the invention

According to a first aspect of the invention, there is provided a method for identifying an anti-ageing agent for use in skincare compositions, which method comprises determination of the ability of a material or combination of materials to modulate the expression of LOXL-1, LTBP-2 and/or LTBP-3.

According to a second aspect of the invention, there is provided a method for evaluating the anti-ageing effect of a material or combination of materials by assessing the ability of the material or combination of materials to modulate the expression of LOXL-1, LTBP-2 and/or LTBP-3.

By a "material" is meant in the context of the present invention any single compound, or any mixture or extract. Preferred materials are commercially available cosmetic ingredients.

Due to the surprising identification in the differential expression of LOXL-1, LTBP-2 and LTBP-3 in young compared to old skin, the inventors have discovered that measurement of LOXL-1, LTBP-2 and/or LTBP-3 expression may be used to assess the potential usefulness of a material or combination of materials as an anti-ageing agent, and for screening large numbers of materials, both on their own and as combinations, for their anti-ageing effect.

It is relatively easy to determine the expression of a gene or gene product (ie protein) and methods for doing so are known in the art. Therefore, the present invention is advantageous because, due to the identification of LOXL-1, LTBP-2 and LTBP-3 as markers, the anti-ageing potential of a material may be determined quickly and easily. As a result, the invention provides useful methods for screening large numbers of materials, both on their own and as combinations, for their anti-ageing effect. Various methods are known for measuring gene or protein expression, including both *in vitro* and *in vivo* methods. The employment of both *in vitro* and *in vivo* testing enables the user to screen large numbers of materials for the desired effect *in vitro* before progressing to testing *in vivo*. This process means that unpromising agents can be eliminated from the search early, thereby saving time and resources.

The anti-ageing agents of the present invention are particularly advantageous because they target the intrinsic ageing process, and in particular the dermal elastic fibre network, which provides skin with its recoil capacity. In terms of visible signs, effects may be seen on the elasticity of the skin, its moisture balance and texture, and by the reduction or prevention of the appearance of fine lines and wrinkles.

Because they target the intrinsic ageing process, anti-ageing agents of the present invention may benefit those people who already protect their skin from

environmental factors, such as UV light, and who would consequently gain little benefit from the use of anti-ageing products which target extrinsic ageing. In addition, by targeting intrinsic ageing, the use of agents identified by the methods detailed here will benefit those areas of skin which are not normally exposed to environmental factors, for example areas of the body that are covered by clothes and therefore not exposed to sunlight.

The intrinsic anti-ageing agents identified by this method can also be used in combination with anti-ageing agents that target extrinsic ageing affects, for example with sunscreening agents and antioxidants. As complementary processes, this would allow the consumer to derive the benefit from both the intrinsic and extrinsic anti-ageing agents.

The inventors have found that there is a greater amount of LOXL-1 and LTBP-3 present in young skin compared to old skin, and hence that expression of LOXL-1 and LTBP-3 decreases with age. Materials or combinations of materials that upregulate the expression of LOXL-1 and/or LTBP-3 may therefore be useful as anti-ageing agents.

Thus, according to a further aspect of the invention, there is provided a material or combination of materials which increases the expression of LOXL-1 and/or LTBP-3 for use as an anti-ageing agent in a skincare composition.

The inventors have found that there is a greater amount of LTBP-2 in old skin compared to young skin, and hence that expression of LTBP-2 increases with age. Materials or combinations of materials that downregulate the expression of LTBP-2 may therefore be useful as anti-ageing agents.

Hence, according to a further aspect of the invention, there is provided a material or combination of materials which downregulate the amount of LTBP-2 for use as an anti-ageing agent in a skincare composition.

The expression of the LOXL-1, LTBP-2 and LTBP-3 genes may be determined by measuring the amount of corresponding mRNA. However, gene expression is more conveniently evaluated by analysis of the protein product. Thus, expression of the LOXL-1, LTBP-2 and LTBP-3 genes is most conveniently evaluated by measurement of the amount of the LOXL-1, LTBP-2 and LTBP-3 proteins respectively.

Evaluation of protein expression may be carried out by any suitable method known in the art. Typically, *in vitro* methods are carried out on cultured cells (keratinocytes). Protein expression can be determined by assaying the target proteins released from the cells into the growth media, for example using western blotting techniques.

Alternatively, the test material may be applied to the skin *in vivo*, in which case gene expression studies are most conveniently carried out on biopsied tissue samples. In preferred methods, biopsied tissue is frozen prior to immunohistochemical analysis. Target proteins may be identified using labeled-antibodies, in particular dye-labelled antibodies. Following incubation of the tissue sample with the appropriate unlabelled antibody, a dye-labelled secondary antibody is used to detect the presence of the first. Degree of antibody staining may be used to provide an indirect measure of protein expression.

The anti-ageing agents of the present invention are intended for use in skincare compositions. By “skincare composition” is meant in the context of the present invention a composition intended to benefit the skin. Skincare compositions may be oral compositions, eg food supplements. However, they are typically topical compositions, which are applied directly to the skin.

The anti-ageing agents of the present invention can be delivered to the skin via any of the conventional formulations known to those skilled in the art. The anti-ageing agents of the present invention are primarily intended for use in topical compositions, and in particular for use in topical compositions marketed as moisturisers, cleansers, toners, masks or scrubs etc, for the face or body.

The skincare compositions may be “cosmetic” compositions that provide skincare benefits in addition to their principal cosmetic effect. Cosmetic skincare formulations may be marketed, for example, as foundations, powders, lipsticks, eyeliners, eyeshadows, blushers, concealers etc.

Skincare compositions provided in accordance with the present invention comprise an anti-ageing ingredient identified in accordance with the present invention and a pharmaceutically acceptable carrier. Typically, said anti-ageing agent may be present in an amount of 0.0001-10% (w/w). Optionally, the composition may include additional active ingredients. The carrier may form the remainder of the composition up to 100% w/w. Compositions according to the present invention may be formulated in numerous forms for topical application known to those skilled in the art. The composition may often take the form of an aqueous or oily solution or dispersion or emulsion or a gel, including creams, serums, pastes, lotions, milks, ointments, salves, sticks, spray, roll-on, powders, aqueous gels, suspension dispersions and emulsions. Preferably, any solvent system comprises water. An emulsion may be an oil-in-water emulsion, a water-in-oil emulsion, an oil-in-water-in-oil emulsion or a water-in-oil-in-water emulsion. Preferred emulsions are oil-in-water emulsions.

Additional skincare actives known to have benefit on the skin may also be added. Examples include anti-acne actives, vitamins, anti-fungals, skin conditioners, organic or inorganic sunscreens, plant extracts and the active principle from these, skin identical lipids such as ceramides, moisturisers and other materials to promote the repair of the skin barrier such as hyaluronic acid and other suitable materials that are known to those skilled in the art. If desired, further anti-wrinkle or anti-ageing actives may be included, such as peptides and promoters of collagen and/or elastin production, hydroxy acids, anti-oxidants, anti-inflammatories and anti-microbials.

The composition may additionally comprise other conventional topical skincare ingredients known to those skilled in the art. These include, but are not limited to, one or more of the following: emollients, humectants or moisturizers, surfactants,

thickeners/viscosity modifying agents, emulsifiers and/or emulsion stabilizers, pH adjusters, perfumes and/or colourings. Such topical ingredients will be incorporated in conventional amounts known to those skilled in the art.

The compositions according to the invention may be applied to and left on the skin.

Detailed description

1 Expression of elastic fibre components in intrinsically aged skin

The inventors of the present invention used an Affymetrix® microarray-based approach to detect age-related changes in gene expression of molecules known to contribute to the elastic fibre network.

1.1 Skin biopsy procurement

Skin biopsies (6mm diameter) were obtained from the photoprotected buttock of three young (24-26 years) and three aged (74-75 years) healthy male volunteers. Each punch biopsy was obtained under 1% lignocaine local anaesthesia and bisected. Half of the biopsy was embedded in optimal cutting temperature compound (OCT, Miles Laboratories, Elkhart, IN, USA), snap frozen in liquid nitrogen and stored at –70°C for immunohistochemical analysis. The other half of the biopsy was immersed in RNA*later* solution (Ambion, Foster City, CA, USA), incubated at 4°C overnight to allow the solution to permeate the tissue and stored at –70°C for subsequent RNA extraction and Affymetrix® analysis.

An additional twelve volunteers were recruited to provide tissue for validation of the Affymetrix® results by quantitative real-time PCR (qPCR) and immunohistochemical analysis. Skin biopsies (6mm diameter) were obtained from photoprotected buttock of six young (two male, four female, age range 18-29 years) and six aged (two male, four female, age range 65-70 years) healthy volunteers. Each punch biopsy was embedded in OCT, snap frozen in liquid

nitrogen and stored at –70°C. Local ethical committee approval was obtained for all human studies and all subjects gave written, informed consent.

1.2 Affymetrix® study

1.2.1 RNA Extraction

Reagents and protocols from the TRIzol® Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) were used for total RNA extraction. Briefly, biopsies were removed from –70°C storage and RNA*later* solution was discarded. Each biopsy was placed in a separate 2ml tube with 800µl TRIzol®. Biopsies were homogenised for 1 minute using an Ultra-Turrax T10 Basic homogeniser (IKA® Werke GmbH & Co. KG, Staufen, Germany) with a dispersing element attached (S 10 N - 5 G; IKA® Werke GmbH & Co. KG). The lysate was incubated at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. To each lysate, 160µl chloroform was added and the tube shaken vigorously by hand for 15 seconds. Samples were incubated at room temperature for 3 minutes and centrifuged at full speed for 15 mins at room temperature. The colourless, upper phase (approximately 300µl) was transferred to a fresh RNase-free tube and an equal volume of 70% ethanol was added. The sample was mixed by vortexing and transferred to a spin cartridge (with a collection tube). Cartridges were centrifuged at full speed for 15 seconds and the flow through was discarded. The spin cartridge was re-inserted into the same collection tube and 350µl Wash buffer I was added. The spin cartridges (with a collection tube) were centrifuged at full speed for 15 seconds and the flow through and collection tube were discarded. The spin cartridge was inserted into a new collection tube and 80µl DNase mix (8µl DNase reaction buffer, 10µl DNase, 62µl RNase-free water; Invitrogen) was added directly onto the surface of the spin cartridge membrane. Samples were incubated at room temperature for 15 minutes. At the end of the incubation period, 350µl Wash Buffer I was added to the spin cartridge and samples were centrifuged at full speed for 15 seconds. The flow through and collection tube were discarded and the spin cartridge was inserted into a new tube. Wash buffer II (500µl) was added to the spin cartridge and centrifuged at full speed for 15

seconds. The flow through was discarded and the spin cartridge was re-inserted into the same collection tube. The spin cartridge membrane was washed again in 500µl Wash buffer II and centrifuged at full speed for 15 seconds. The flow through was discarded and the spin cartridge was centrifuged at full speed for 1 minute to dry the membrane. The spin cartridge was inserted into a recovery tube and 30µl RNase-free water was added to the centre of the spin cartridge. Samples were incubated at room temperature for 2 minutes and centrifuged at full speed for 1 minute to elute the RNA.

1.2.2 Microarray analysis

Microarray experiments were performed using the human genome U133A oligonucleotide array (Affymetrix Inc, High Wycombe, UK) according to the manufacturer's instructions. Total RNA (100 ng) from three young and three aged male volunteers was used with the Two-Cycle cDNA Synthesis Kit (P/N 900432 Affymetrix Inc; one sample hybridized per array). Technical quality control was performed with dChip. Background correction, quantile normalization, and gene expression analysis were performed using GCRMA. Differential expression between the young and old groups was tested statistically with CyberT on logarithmic scale data. False-discovery correction was performed with Q-value software. Significantly changed probe sets were selected on p-value (<0.05) and expression level (>50).

1.3 Immunohistochemical Analysis

1.3.1 Cryosectionning

Frozen tissue sections (5µm) were cut on a cryostat (OTF 5000; Bright Instrument Co Ltd, Huntingdon, Cambridgeshire, UK) and mounted on poly-L-lysine coated slides (VWR, Leicestershire, UK). Slides were stored at –70°C prior to use.

1.3.2 LOXL-1 immunostaining

Sections were defrosted at room temperature for 10 minutes and fixed in ice-cold acetone for 10 minutes. Sections were washed for 2 minutes in two changes of TBS. To prevent non-specific binding of the secondary antibody, 5% normal rabbit serum (Vector Laboratories Inc) in TBS was applied to each section for 30 minutes. Blocking serum was removed and mouse anti-human LOXL-1 primary antibody (Santa Cruz Biotechnology Inc., product # sc-166632, clone H-11, dilution 1:10 in 5% normal rabbit serum) was applied to each section for 1 hour at room temperature. Primary antibody was omitted from selected sections as a negative control. After washing twice in TBS for 2 minutes, sections were incubated for 30 minutes in rabbit anti-mouse Alexafluor 647 secondary antibody (diluted 1:200 in TBS). After washing twice in TBS for 2 minutes, sections were incubated for 2 minutes in DAPI (1µg/ml in TBS). After washing twice in TBS for 2 minutes, sections were mounted and coverslipped in Fluoromount-G.

1.3.3 LTBP-2 immunostaining

Sections were defrosted at room temperature for 10 minutes and fixed in ice-cold acetone for 10 minutes. Sections were washed for 2 minutes in two changes of TBS. To prevent non-specific binding of the secondary antibody, 5% normal goat serum (Vector Laboratories Inc) in TBS was applied to each section for 30 minutes. Blocking serum was removed and rabbit anti-human LTBP-2 primary antibody (Sigma Prestige Antibodies™, Poole, UK, product # HPA003415, diluted 1:100 in 5% normal goat serum) was applied to each section for 1 hour at room temperature. Primary antibody was omitted from selected sections as a negative control. After washing twice in TBS for 2 minutes, sections were incubated for 30 minutes in goat anti-rabbit Alexafluor 647 secondary antibody (diluted 1:200 in TBS). After washing twice in TBS for 2 minutes, sections were incubated for 2 minutes in DAPI (1µg/ml in TBS). After washing twice in TBS for 2 minutes, sections were mounted and coverslipped in Fluoromount-G.

1.3.4 LTBP-3 immunostaining

Sections were defrosted at room temperature for 10 minutes and fixed in ice-cold acetone for 10 minutes. Sections were washed for 2 minutes in two changes of TBS. To prevent non-specific binding of the secondary antibody, 5% normal goat serum in TBS was applied to each section for 30 minutes. Blocking serum was removed and rabbit anti-human LTBP-3 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, product # sc-98276, clone H-210, dilution 1:10 in 5% normal goat serum) was applied to each section for 1 hour at room temperature. Primary antibody was omitted from selected sections as a negative control. After washing twice in TBS for 2 minutes, sections were incubated for 30 minutes in goat anti-rabbit Alexafluor 647 secondary antibody (diluted 1:200 in TBS). After washing twice in TBS for 2 minutes, sections were incubated for 2 minutes in DAPI (1µg/ml in TBS). After washing twice in TBS for 2 minutes, sections were mounted and coverslipped in Fluoromount-G.

1.3.5 Image Capture and analysis

Fluorescent images were captured using All-in-one Type Fluorescence Microscope Biozero-8000 (Keyence, Osaka, Japan).

Plot profiles of fluorescence intensity were compiled using ImageJ software, and area under the curve analysis and t-tests were performed to detect changes in fluorescence intensity and therefore LOXL-1, LTBP-2 or LTBP-3 expression.

1.4 Results

1.4.1 Microarray data

Microarray data was examined for expression levels of reported microfibril and elastic fibre associated molecules (Kielty *et al.* Journal of Cell Science, 2002).

Molecules that were tested included Fibrillin-1, Fibrillin-2, Fibrillin-3, Latent TGFB Binding Protein-1, Latent TGFB Binding Protein-2, Latent TGFB Binding Protein-3, Latent TGFB Binding Protein-4, Decorin, Biglycan, Versican, MFAP-1, MFAP-3, MFAP-4 (MAGP-36), Elastin, Lysyl Oxidase, LOXL (LOXL-1), LOXL-2, LOXL-3, Fibulin-2, Fibulin-5, Emilin-1, Emilin-2, Vitronectin, Endostatin, Collagen VIII, Collagen XVI and Collagen VI.

Significantly changed probe sets were selected on p-value (<0.05) and expression level (>50). LOXL-1, LTBP-2 and LTBP-3 showed significantly changed expression.

Figure 1 shows the expression of LOXL-1 gene in young versus old skin.

Figure 2 shows the expression of LTBP-2 gene in young versus old skin.

Figure 3 shows the expression of LTBP-3 gene in young versus old skin.

2 Identifying materials that may reverse effects of intrinsic ageing

A two phase approach may be used to screen potential active ingredients for activity at the target binding sites. *In vitro* testing on cultured skin cells (keratinocytes) allows for the screening of large numbers of materials both on their own and as combinations. Ingredients with the desired effect *in vitro* can then be incorporated into simple o/w emulsion vehicles before being tested in a 12 day *in vivo* patch test. In both cases, the aim is to identify whether the selected ingredients have changed the expression of LOXL-1, LTBP-2 and/or LTBP-3 protein, either from the cultured keratinocytes or in the patch test biopsies.

2.1 *In vitro* screening

Keratinocyte cell lines (eg normal human epidermal keratinocytes (NHEK) (Promocell) and the immortalized human cell lines HaCaT) are grown at 37°C in a humidified incubator with 5% CO_2, undergoing passage at regular intervals. Cells

are cultured in growth medium with appropriate supplements (eg NHEK cells can be cultured in the Keratinocyte growth medium 2 kit with supplements (Promocell). Samples are then trypsinised using 1xTrypsin-EDTA (0.5g/L trypsin, 0.2g/L EDTA) (Lonza).

2.1.1 Western blotting

To identify LOXL-1, LTBP-2 and/or LTBP-3 release into the media, western blotting of conditioned media can be performed. Briefly, 10µl of media is mixed with 2xSDS loading buffer (100mM Tris-HCl pH6.8, 200mM DTT, 4% SDS, 0.2% Bromophenol blue, 25% glycerol), heated to 90°C for 3mins and run using 12% SDS polyacrylamide gels with a 4% stacking gel. Samples are run under 1xTris Glycine SDS (TGS) buffer at 125V for 1hour 30 minutes in an X-cell sure lock electrophoresis tank (Invitrogen). Following separation, samples are transferred to Immobilion-P nitrocellulose membranes (Millipore) and then transferred by a semi wet method using an X-cell II blot module (Invitrogen) transfer chamber and transferred at 25V for 1.5 hours as described by the manufacturer. Blots are then blocked in 5% milk. Once transferred, membranes are probed using a primary antibody to LOXL-1, LTBP-2 or LTBP-3. Following incubation with appropriate secondary antibody, membranes are then investigated using horse radishes peroxidise and the degree of staining assessed.

2.2 *In vivo* screening

10 healthy subjects are recruited in the age range 60-80. 20µl of test product is applied to the photoprotected buttock under standard 6mm Finn chambers (Scanpore, Tuulsula, Finland). An area is left untreated but occluded to provide a baseline control. Test products are applied on days 1, 4 and 8 of the assay. On day 12 of the assay, the Finn chambers are removed and 3mm punch biopsies taken under 1% lignocaine local anaesthesia from the test sites. Biopsied tissue is embedded in optimal cutting temperature compound (Tissue Tek, Miles Laboratory, Elkhart, IN, U.S.A.), snap frozen in liquid nitrogen and stored at –70°C prior to immunohistochemical analysis. 10µm frozen sections are then prepared

using a Cryostat and mounted onto gelatine coated slides prior to histological analysis. A primary antibody to LOXL-1, LTBP-2 or LTBP-3 is applied overnight at 4°C. A negative control is by incubation of the isotype sera at the appropriate concentration or by omission of the primary antibody. Sections are washed in TBS prior to incubation with the appropriate biotinylated secondary antibody for 30 minutes. Antibody staining is visualised using a well characterised immunoperoxidase reaction or more preferably using immunofluorescence. Sections are then lightly stained with nuclear fast red before being dehydrated and permanently mounted. A semi-quantitative scale can be used for grading the degree of antibody staining.

Claims

1. Method for identifying an anti-ageing agent for use in skincare compositions, which method comprises determination of the ability of a material or combination of materials to modulate the expression of LOXL-1, LTBP-2 and/or LTBP-3.

2. Method for evaluating the anti-ageing effect of a material or combination of materials by assessing the ability of the material or combination of materials to modulate the expression of LOXL-1, LTBP-2 and/or LTBP-3.

3. A method as claimed in claim 1, wherein the anti-ageing agent is identified by an increase in LOXL-1 expression.

4. A method as claimed in claim 1, wherein the anti-ageing agent is identified by a decrease in LTBP-2 expression.

5. A method as claimed in claim 1, wherein the anti-ageing agent is identified by an increase in LTBP-3 expression.

6. A method as claimed in claim 2, wherein the anti-ageing effect is evaluated by assessing the ability of the material or combination of materials to upregulate expression of LOXL-1.

7. A method as claimed in claim 2, wherein the anti-ageing effect is evaluated by assessing the ability of the material or combination of materials to downregulate expression of LTBP-2.

8. A method as claimed in claim 2, wherein the anti-ageing effect is evaluated by assessing the ability of the material or combination of materials to upregulate expression of LTBP-3.

9. A method as claimed in any preceding claim, which involves determining the amount of LOXL-1 protein, LTBP-2 protein and/or LTBP-3 protein present in a sample.

10. A method as claimed in any preceding claim, which is an *in vitro* method.

11. A method as claimed in claim 10, which uses cultured cell lines.

12. A method as claimed in claim 10 or claim 11, wherein western blot analysis is used to identify and quantify the release of LOXL-1, LTBP-2 and/or LTBP-3.

13. A method as claimed in any one of claims 1 to 9, in which the material under test is applied to the skin *in vivo.*

14. A method as claimed in claim 13, which method uses biopsied tissue.

15. A method as claimed in claim 13, wherein the biopsied tissue is frozen prior to immunohistochemical analysis.

16. A method as claimed in any one of claims 13 to 15, wherein the protein is identified using a labeled antibody.

17. An anti-ageing agent identified by the method of any preceding claim.

18. A skincare composition comprising an anti-ageing agent identified by the method of any one of claims 1 to 16.

19. A material or combination of materials which increases the expression of LOXL-1, for use as an anti-ageing agent in a skincare composition.

20. A material or combination of materials which decreases the expression of LTBP-2, for use as an anti-ageing agent in a skincare composition.

21. A material or combination of materials which increases the expression of LTBP-3, for use as an anti-ageing agent in a skincare composition.

22. Use of a material or combination of materials which increases the expression of LOXL-1 as an anti-ageing agent in the manufacture of a skincare composition.

23. Use of a material or combination of materials which decreases the expression of LTBP-2 as an anti-ageing agent in the manufacture of a skincare composition.

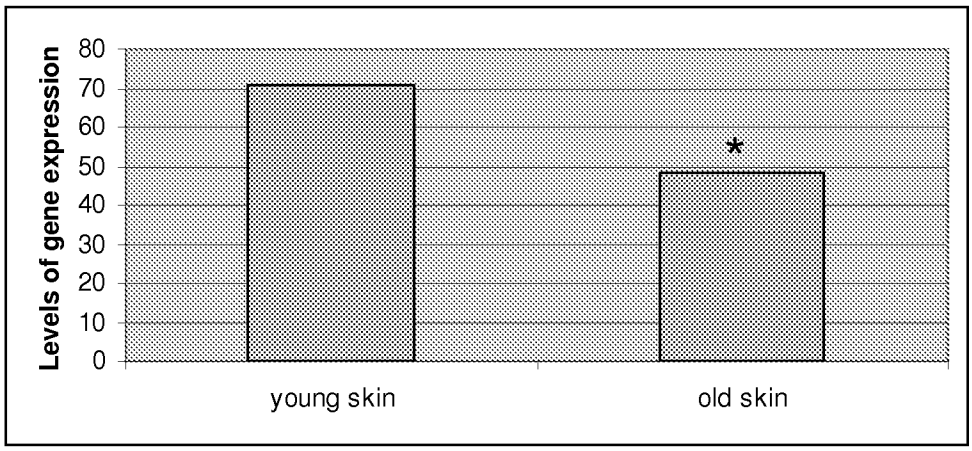
24. Use of a material or combination of materials which increases the expression of LTBP-3 as an anti-ageing agent in the manufacture of a skincare composition.

25. Use of LOXL-1, LTBP-2 and/or LTBP-3 as a marker for screening for anti-ageing agents.

26. Any novel method for identifying an anti-ageing agent generally as herein described.

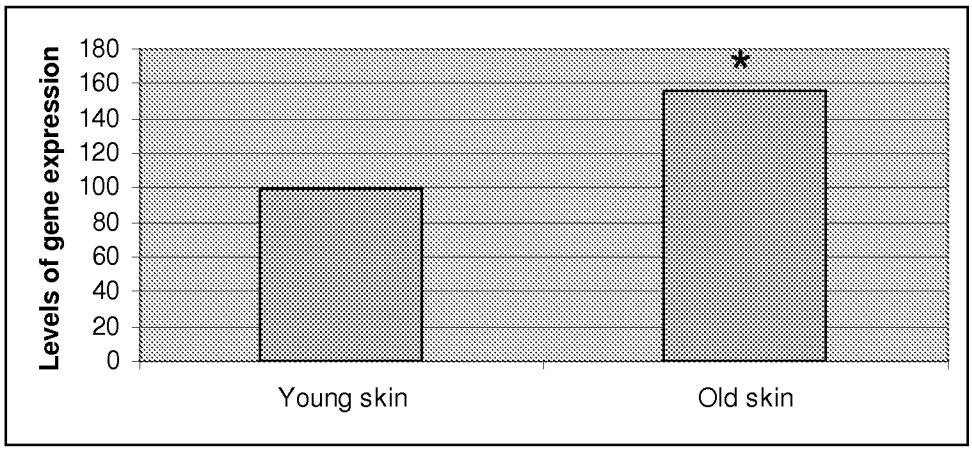
Drawings

Figure 1



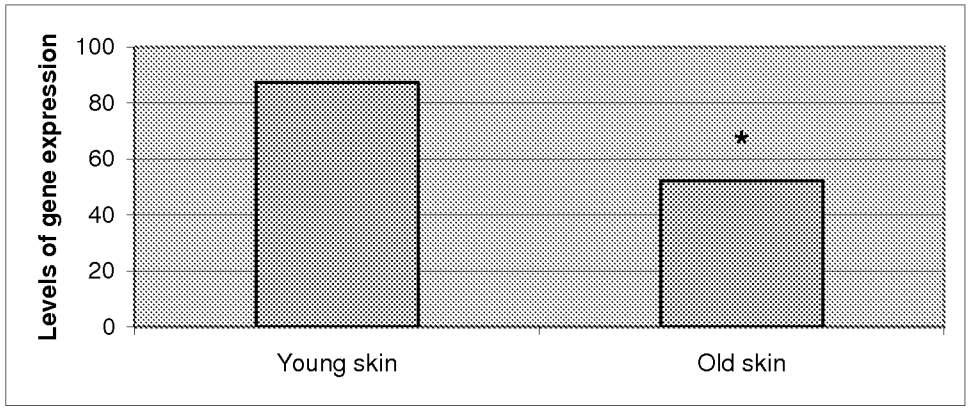
* $p<0.05$

Figure 2



* $p<0.05$

Figure 3



* $p<0.05$

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/051056

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53 G01N33/58 A61K8/00
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)
G01N A61K

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 practical, 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	WO 2005/069975 A2 (MASSACHUSETTS EYE & EAR INFIRM [US]; LI TIANSEN [US]; LIU XIAOQING [US) 4 August 2005 (2005-08-04)	1-3,6, 9-14,16, 25
Y	page 4, lines 11-23; claims 23-27, 35-37; figure 5C page 8, line 6 page 11, lines 24-25 page 12, lines 17-18 page 14, lines 20-22 page 15, lines 24-33 page 18, line 32 - page 20, line 33 page 25, line 13 - page 34, line 34	4,7,15

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

[X] See patent family annex.

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"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

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2011	23/08/2011

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/051056

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X Y	EP 1 762 610 A1 (UNIV KYOTO [JP]) 14 March 2007 (2007-03-14) paragraphs [0002], [00 3], [00 7], [00 9], [0 10], [0 24], [100] - [0128], [148] - [0159], [181], [234]; example 5 -----	1,2, 9-13,16, 25 4,7,15
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