METHODS FOR PREPARING HUMAN MELANOCYTES FROM HUMAN PLURIPOTENT STEM CELLS

FIELD OF THE INVENTION

The present invention relates to ex vivo methods for obtaining populations of human melanocytes derived from human pluripotent stem cells.

BACKGROUND OF THE INVENTION

Melanocytes are pigment-producing cells responsible for coloration of skin, eyes, and hair. In vertebrate development, melanocytes originate from the neural crest and undergo a complex process of fate-specification, proliferation, migration, survival, and differentiation before finally residing in the epidermis. Pigmentation is achieved by the highly regulated manufacture of the melanin pigment in specialized organelles called melanosomes. Through this process called melanogenesis, melanosomes are transported along the dendrites and transferred to growing hair, or surrounding keratinocytes to play a critical role in protecting human tissues from the deleterious effects of sun's light ultraviolet (UV) radiations that cause DNA damages and tissues cancer. Over the past 10 years, several genes have been associated with pigmentary disorders involving melanocytes. Those disorders can be classified in 3 types. First, diseases affecting the development of melanocytes from the neural crest (piebaldism, Waardenburg syndrome and dyschromatosis symmetrica hereditaria), second, diseases with defects in melanin synthesis (albinism) and third, disorders of melanosome maturation or transfer (Hermansky-Pudlak syndrome, Chediak-Higashi syndrome and Griscelli syndrome) (Rose PT et al). Interestingly, although a lot of efforts have been made to better understand about the function and regulation of mature melanocytes, very little is known with regard to the molecular and cellular mechanisms involved in melanocyte development and differentiation from embryonic precursors in the Human.

Transformation of somatic cells with a set of embryonic transcription factors produces cells with the pluripotent properties of embryonic stem cells (hESCs). These induced pluripotent stem (hiPSCs) cells have the potential to differentiate into

any cell type, making them a potential source from which to produce cells as a therapeutic platform for the treatment of a wide range of diseases. As a proof of concept of the therapeutic benefits of those cells, it was demonstrated that hiPSCs-derived bone marrow cells could reverse the sickle cell anaemia phenotype in a suitable humanized mouse model.

Induction of melanocytes in vitro was first reported by Yamane et al (1999), who cocultured mouse embryonic stem cells on monolayers of the stromal cell line ST2 during 21 days in medium containing stem cells factor (SCF), endothelin 3 (EDN3) , 12-O-tetradecanoyl-phorbel 13-acetate (TPA) and dexamethasone. More recently the Herlyn laboratory (Fang et al , 2006) have shown that melanocytes can be derived from hESCs using human embryoid bodies in the absence of feeder cells using a combination of the proteins inducers named Wnt3a, endothelin-3 and SCF.

However, there is no report in the prior art of a method for generating human melanocytes from human pluripotent stem cells using a 2D protocol.

Hence, there is still a need in the art for novel methods for generating human melanocytes from human pluripotent stem cells.

SUMMARY OF THE INVENTION:

The invention relates to an ex vivo method for obtaining a population of human melanocytes derived from human pluripotent stem cells comprising the step a) consisting of co-culturing human pluripotent stem cells with cells that support ectodermal differentiation in the presence of an agent that stimulates epidermal induction and an agent that stimulates terminal differentiation of keratinocytes.

The invention also relates to an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells obtainable by the above method.

The invention also relates to a pharmaceutical composition comprising the substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells described above and optionally a pharmaceutically acceptable carrier or excipient.

In a further aspect, the invention also relates to the isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells or the pharmaceutical composition as described above for use in a method of treatment.

The invention also relates to a method of preparing a human skin substitute comprising a step consisting of providing an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells according to the invention and a consisting of providing a population of human keratinocytes.

Further, the invention relates to a human skin substitute obtainable by the method described above and to its use in a method of treatment.

In another aspect, the invention relates to a method for screening compounds for a given biological effect on human melanocytes comprising the steps of:

i) incubating an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells as described above in the presence or absence of a test compound;

ii) comparing said given biological effect in the population of melanocytes in the presence or absence of said test compound.

The invention also relates to the use of an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells or of a human skin substitute as defined above for screening compounds.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to an ex vivo method for obtaining a population of human melanocytes derived from human pluripotent stem cells comprising the step a) consisting of co-culturing human pluripotent stem cells with cells that support ectodermal differentiation in the presence of an agent that stimulates epidermal induction and an agent that stimulates terminal differentiation of keratinocytes.

The term "melanocytes" has its general meaning in the art. It refers to the pigmented melanin-secreting cells in the epithelium and in the hair that are responsible for the coloration of the skin and hair.

Advantageously, the ex vivo method of the invention is a 2D method that allows the monitoring of the different stages of differentiation of the human pluripotent stem cells into human melanocytes. The method of the invention can therefore be useful for screening compounds that interfere (positively or negatively) with this differentiation process.

The expression "cell culture surface" or "cell culture matrix" refers to every type of surface or matrix suitable for cell culture. The term "cell culture surface" includes but is not limited to tissue culture plate, dish, well or bottle. In a particular embodiment, the culture surface is plastic surface of the culture plate, dish, well or bottle. The cell culture surface is compatible with the coating of dermis fibroblasts.

As used herein, the expression "cells that support ectodermal differentiation" refers to cells that provide an appropriate substrate and which secrete appropriate factors to support the growth and the differentiation of human pluripotent stem cells. In a particular embodiment, cells that support ectodermal differentiation are selected from the group of fibroblasts, more particularly of human and mice fibroblasts and more particularly of dermis fibroblasts. In a particular embodiment, the cells that support ectodermal differentiation are mitomycin-inactivated or irradiation inactivated human dermis fibroblasts.

In one embodiment, of the invention, the cells that support ectodermal differentiation are feeder fibroblasts.

As used herein, the expression "feeder fibroblasts" refers to cells that serve as a basal layer for pluripotent stem cells and provide secreted factors, extracellular matrix, and cellular contacts for the maintenance of stem cells in the undifferentiated state without losing pluripotency. Feeder cells can be inactivated by gamma irradiation or mitomycin. According to an embodiment of the invention, the feeder fibroblasts may be from the group of fibroblasts, more particularly of human fibroblasts and more particularly of dermis fibroblasts, including dermis fibroblast cell lines. Examples of dermis fibroblast cell lines include but are not limited to CCD-1112SK (Hovatta O, et al. 2003) and 3T3-J2 (Rheinwald JG et al. 1975). In a particular embodiment, dermis fibroblasts are previously treated to stop their proliferation before to be coated in the culture surface. Therefore, dermis fibroblasts may be irradiated or treated with a cell cycle blocking agent such as mitomycin.

As used herein, the term "dermis fibroblast" refers to a population of cells that synthesizes and maintains the extracellular matrix of dermis. Specific markers of dermis fibroblasts include vimentin and FAP (fibroblast activation protein).

According to an embodiment of the invention, the cell culture surface is selected in the manner that dermis fibroblasts may naturally adhere on it. Various materials of cell culture surface may be selected. Examples of such materials include but are not limited to plastic tissue culture dishes or dishes coated with gelatine.

As used herein, the expression "agent that stimulates epidermal induction" refers to an agent that is capable of inducing the expression of epidermal markers such as keratin 8 and keratin 18. Typically an agent that stimulates epidermal induction inhibits trophoblast and mesoderm induction.

In a particular embodiment, the agent that stimulates epidermal induction is selected from the group consisting of Bone Morphogenetic Proteins (such as BMP-2, BMP-4 and BMP-7), receptor-regulated Smad proteins (such as Smad 1, Smad 5 and Smad 9) and ligands of the TGF-beta family (such as Growth and Differenciation Factor 6: GFD-6) (Moreau et al., 2004). In a preferred embodiment the agent that stimulates epidermal induction is selected from the group consisting of BMP-2, BMP-4, BMP-7, Smad1, Smad5, Smad7 and GFD-6. In a preferred embodiment, the agent that stimulates epidermal induction is BMP-4.

The term "BMP-4" refers to Bone morphogenetic protein 4. BMP-4 is a polypeptide belonging to the TGF-β superfamily of proteins. An exemplary native BMP-4 amino acid sequence is provided in GenPept database under accession number AAC72278.

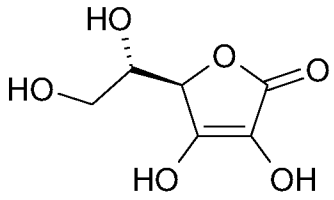
In one embodiment, the agent that stimulates epidermal induction is selected from the group consisting of BMP-2, BMP-4, BMP-7, Smad1, Smad5, Smad7 and GFD-6.

In a preferred embodiment, said agent that stimulates epidermal induction is BMP-4. The concentration of BMP-4 may vary from 0.02 nM to 77 nM or 0.3ng/mL to 1 000 ng/mL. In a particular embodiment the concentration of BMP-4 is 0.5nM.

As used herein, the expression "agent that stimulates terminal differentiation of keratinocytes" refers to an agent that stimulates the expression of keratin 5 and keratin 14. Indeed, keratin 5 and keratin 14 are markers of the basal keratinocytes which are capable of terminal differentiation in 3D culture. In one particular embodiment, the agent that stimulates terminal differentiation of keratinocytes is selected from the group consisting of ascorbic acid and retinoic acid.

In a preferred embodiment, said agent that stimulates terminal differentiation of keratinocytes is ascorbic acid.

The term “ascorbic acid” refers to (R)-3,4-dihydroxy-5-((S)- 1,2-dihydroxyethyl)furan-2(5H)-one which has the formula of :



According to an embodiment of the invention, the concentration of ascorbic acid may vary from 0.01 mM to 1 mM. In a particular embodiment the concentration of ascorbic acid is 0.3 mM.

As used herein, the term "human pluripotent stem cell" refers to any human precursor cell that has the ability to form any adult cell.

In a particular embodiment, human pluripotent stem cells include but are not limited to human embryonic stem cells (hES cells) or human induced pluripotent stem cells (hiPS cells).

In a preferred embodiment, said human pluripotent stem cells are obtained without destruction of human embryos.

As used herein, the term "human embryonic stem cells" or "hES cells" or “hESCs” refers to human precursor cells that have the ability to form any adult cell. hES cells are derived from fertilized embryos that are less than one week old.

According to an embodiment of the invention, hES cells may be selected from any hES cell lines. Examples of hES cell lines include but are not limited to, SA01, VUB-01, WA01 (H1) (Thomson JA et al 1998), and WA09 (H9) (Amit M et al. 2000). According to the invention hES cells are not previously differentiated in embryoid bodies as described in Fang et al (2006).

As used herein, the term "human induced pluripotent stem cells" or "human iPS cells" or "human iPSCs" or "hiPSCs" refers to a type of human pluripotent stem cell artificially derived from a human non-pluripotent cell (e.g. an adult somatic cell). Human induced pluripotent stem cells are identical to human embryonic stem cells in the ability to form any adult cell, but are not derived from an embryo. Typically, a human induced pluripotent stem cell may be obtained through the induced expression of Oct3/4, Sox2, Klf4, and c-Myc genes in any adult somatic cell (e.g. fibroblast). For example, human induced pluripotent stem cells may be obtained according to the protocol as described by Takahashi K. et al. (2007), by Yu et al. (2007) or else by any other protocol in which one or the other agents used for reprogramming cells in these original protocols are replaced by any gene or protein acting on or transferred to the somatic cells at the origin of the iPSC lines. Basically, adult somatic cells are transfected with viral vectors, such as retroviruses, which comprises Oct3/4, Sox2, Klf4, and c-Myc genes.

According to an embodiment of the invention human iPS cells may be selected from any human iPS cell lines. Examples of human iPS cell lines include but are not limited to clones 201B (Takahashi et al., 2007) and hiPS (Foreskin) or IMR90 (Yu et al., 2007).

Alternatively, hES cells or human iPS cells may be selected from master cell banks that may be constituted in a therapeutic purpose. In a preferred manner, hES cells or hiPS cells may be selected to avoid or limit immune rejection in a large segment of the human population. Typically hES cells or hiPS cells are HLA-homozygous for genes encoding major histocompatibility antigens A, B and DR, meaning that they have a simple genetic profile in the HLA repertory (Nakatsuji N et al, 2008 and Taylor C et al 2003). The cells could serve to create a stem cell bank as a renewable source of cells that may be suitable for preparing melanocytes for use in cell therapy of pathologies associated with melanocyte deficiencies.

In another particular embodiment, human pluripotent stem cells may carry a mutation or a plurality of mutations that are causative for a genetic disease in human, and in particular mutation that are causative for a genetic disease of the human skin.

As used herein, the expression "pathologies associated with melanocyte deficiencies", or "pigmentation disorders", refers to any pathology or disease in which there is a lack of functional melanocytes. This can be due to a deficiency in the enzymes necessary for the synthesis of melanin, to a deficiency in the development and/or proliferation and/or survival of melanocytes.

Pathologies associated with a melanocyte deficiency, or pigmentary disorders involving melanocytes, include, but are not limited to, diseases affecting the development of melanocytes from the neural crest (piebaldism, Waardenburg syndrome and dyschromatosis symmetrica hereditaria), diseases with defects in melanin synthesis (albinism), disorders of melanosome maturation or transfer (Hermansky-Pudlak syndrome, Chediak-Higashi syndrome and Griscelli syndrome) and vitiligo.

Typically, step a) is carried out in a base culture medium supplemented an agent that stimulates epidermal induction and an agent that stimulates terminal differentiation of keratinocytes. Suitable base culture medium can be for example FAD medium (3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 media), and 10% fetal calf serum, supplemented with 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 10^{-10} mol/L cholera toxin, 1.37 ng/mL recombinant epidermal growth factor.

In one embodiment, the base culture medium is free of animal-derived substances. In a preferred embodiment, the base culture medium consists essentially of synthetic compounds, compounds of human origin and water. Advantageously, said culture medium can be used for culturing cells according to good manufacturing practices (under "GMP" conditions). Typically, the serum can be replaced by N2B27 medium, as described in Ying et al., 2003, in Lowell et al., 2006 and in Liu Y et al.,

2006. N2B27 comprises DMEM/F12 and Neurobasal media in a 1/1 ratio, N2 supplement (1/100), B27 supplement (1/50) and beta-mercaptoethanol (1/1000). It is available, for example, under reference SCS-SF-NB-02 from Stem Cell Sciences UK Ltd.

In another embodiment, during step a) can be carried out in a base culture medium in the presence of an agent that stimulates epidermal induction and an agent that stimulates terminal differentiation of keratinocytes and in the presence of an agent which stimulates the differentiation of melanocytes. Typically, said agent which stimulates the differentiation of melanocytes can be selected from the group consisting of WNT family protein (such as wnt3a), stem cells factor (SCF) and endothelin 3 (EDN3) (Fang et al, 2006). These proteins are involved in the differentiation from neural crest to pigmented cells. The Wnt family of proteins induced neural cells and pigmented cells formation in mice. The absence of melanocytes in animals that are deficient in either EDN3 or its receptor suggests that this pathway is critical in the development of neural crest-derived melanocyte populations.

In vitro studies show that EDN3 promotes the proliferation, survival, and differentiation of melanocyte precursors. Mutations in *KIT* (encoding the SCF receptor) do not affect specification of melanocyte lineage but instead hamper melanoblast survival at later developmental stages. In particular, SCF/KIT signaling is essential for migration, proliferation, survival, and differentiation of the precursor melanoblasts.

Wnt3a signaling determines melanocyte fate of neural crest cells, EDN3 contributes towards cell fate, and SCF promotes proliferation/survival of the committed progenitors.

According to the invention, human pluripotent stem cells (e.g. hES cells or human iPS cells) are cultivated during step a) for a time period sufficient for allowing the apparition of clones of pigmented cell populations. Said clones of

pigmented cell population are easily identified by the skilled person by bare eye, without the use of a microscope.

According to a particular embodiment, step a) is carried out for at least 35 days, preferably at least 40 days, even more preferably at least 45, at least 50, at least 55, at least 60, at least 65, at least 70 or at least 75 days.

Typically, step a) is carried out for at most 120 days, preferably at most 100 days.

A further object of the invention relates to an isolated population of human melanocytes derived from human pluripotent stem cells obtainable by a method as above described.

As used herein, the term "isolated" refers to a cell or a population of cells which has been separated from at least some components of its natural environment.

A further object of the invention relates to an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells obtainable by a method as above described.

The term "substantially pure homogenous population", as used herein, refers to a population of cells wherein the majority (e.g., at least about 80%, preferably at least about 90%, more preferably at least about 95%) of the total number of cells have the specified characteristics of the melanocytes of interest.

In one embodiment, the isolated substantially pure homogenous population of human melanocytes according to the invention, have a few melanogenesis genesis that are differently regulated compared to adult melanocytes as assessed by Taqman array.

In one embodiment, when human iPS cells are used, the isolated substantially pure homogenous population of human melanocytes according to the invention contain a transgene (which was introduced into said cells during the reprogramming step which took place before step a)).

According to a further embodiment, step a) can be followed by the following steps:

b) isolating a pigmented cell from the population of cells obtained in step a);

c) culturing said isolated cell in an appropriate culture medium.

As used herein, the term "isolating", when referring to a cell in culture, refers to the cloning of said cell, i.e. the fact of separating said cell from other cells in culture in order to establish a novel clone. Typically, step b) can be carried out using standard techniques known to the person skilled in the art of cell culture.

In one embodiment, said isolating step b) can comprise cell dissociation using mechanical and/or enzymatic treatment. Typically, said isolation step b) can comprise a treatment with trypsin.

Typically, said isolation step b) can further comprise the seeding of the isolated pigmented cell on an appropriate cell culture surface.

As used herein, the term "appropriate culture medium" refers to a culture medium that contains nutrients necessary to support the growth, proliferation and survival of a particular cell population.

In particular, an appropriate culture medium for human melanocytes, also termed "melanocytes culture medium" is a culture medium that contains nutrients necessary to support the growth, proliferation and survival of human melanocytes. Typically, a melanocytes culture medium according to the invention may consist for example of 254CF medium supplemented with growth factors (Invitrogen).

Typically, an appropriate culture medium suitable for step c) according to the invention is devoid of BMP-4 and ascorbic acid.

The human melanocytes (or the substantially pure homogenous population of human melanocytes) of the invention can be used for several types of applications, which include, but are not limited to:

1) cell therapy of pigmentary disorders or skin lesions;

2) tissue engineering, by reconstituting human skin substitutes;

3) cellular models useful for pharmacological and toxicological studies;

4) studying the mechanisms of melanocyte development and identifying new therapeutic targets for the treatment of melanoma.

1) Cell therapy of pigmentary disorders or skin lesions

The substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells obtained according to the method of the invention may be then suitable for use in therapy.

Over the past 10 years, several genes have been associated with pigmentary disorders involving melanocytes. Those disorders can be classified in 3 types. First, diseases affecting the development of melanocytes from the neural crest (piebaldism, Waardenburg syndrome and dyschromatosis symmetrica hereditaria), second, diseases with defects in melanin synthesis (albinism) and third, disorders of melanosome maturation or transfer (Hermansky-Pudlak syndrome, Chediak-Higashi syndrome and Griscelli syndrome). Interestingly, although a lot of efforts have been made to better understand about the function and regulation of mature melanocytes, very little is known with regard to the molecular and cellular mechanisms involved in melanocyte development and differentiation from embryonic precursors in the Human.

Moreover Vitiligo is a de-pigmented patch on the skin. It occurs where the immune system has destroyed a patch of melanocytes which are the cells that produce the dark pigment melanin.

The depigmented skin becomes photosensitive on the exposed areas of the skin, leading to redness and burning on sun exposure. Depending on the type, extent, and duration of vitiligo, conventional medical therapies such as topical and systemic corticosteroids, topical immunomodulators, and phototherapy are not always successful, and repigmentation is often incomplete.

Surgical methods become important in cases where medical therapy fails to cause repigmentation or in cases of segmental vitiligo where the response to surgery is excellent. The basic principle of surgical treatment is autologous grafting of viable melanocytes from pigmented donor skin to recipient vitiliginous areas. Various grafting methods have been described including tissue grafts and cellular grafts. Stability of the disease is the most important criterion to obtain a successful outcome.

Therefore the invention relates to a pharmaceutical composition comprising a substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells of the invention and optionally a pharmaceutically acceptable carrier or excipient. In certain embodiments, a pharmaceutical composition may further comprise at least one biologically active substance or bioactive factor.

As used herein, the term “pharmaceutically acceptable carrier or excipient” refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the progenitor cells, and which is not excessively toxic to the host at the concentrations at which it is administered. Examples of suitable pharmaceutically acceptable carriers or excipients include, but are not limited to, water, salt solution (e.g., Ringer’s solution), oils, gelatines, carbohydrates (e.g., lactose, amylase or starch), fatty acid esters, hydroxymethylcellulose, and polyvinyl

pyroline. Pharmaceutical compositions may be formulated as liquids, semi-liquids (e.g., gels) or solids (e.g., matrix, lattices, scaffolds, and the like).

As used herein the term "biologically active substance or bioactive factor" refers to any molecule or compound whose presence in a pharmaceutical composition of the invention is beneficial to the subject receiving the composition. As will be acknowledged by one skilled in the art, biologically active substances or bioactive factors suitable for use in the practice of the present invention may be found in a wide variety of families of bioactive molecules and compounds. For example, a biologically active substance or bioactive factor useful in the context of the present invention may be selected from anti-inflammatory agents, anti-apoptotic agents, immunosuppressive or immunomodulatory agents, antioxidants, growth factors, and drugs.

A related aspect of the invention relates to a method for treating a subject suffering from a pathology associated with melanin deficiencies, said method comprising a step of administering to the subject an efficient amount of a substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells of the invention (or a pharmaceutical composition thereof).

As used herein, the term "subject" refers to a mammal, preferably a human being, that can suffer from pathology associated with skin damage, but may or may not have the pathology.

In the context of the invention, the term "treating" or "treatment", as used herein, refers to a method that is aimed at delaying or preventing the onset of a pathology, at reversing, alleviating, inhibiting, slowing down or stopping the progression, aggravation or deterioration of the symptoms of the pathology, at bringing about ameliorations of the symptoms of the pathology, and/or at curing the pathology.

As used herein, the term "efficient amount" refers to any amount of a substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells (or a pharmaceutical composition thereof) that is sufficient to achieve the intended purpose.

The substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells (or a pharmaceutical composition thereof) of the invention may be administered to a subject using any suitable method.

In certain embodiments, a treatment according to the present invention further comprises pharmacologically immunosuppressing the subject prior to initiating the cell-based treatment. Methods for the systemic or local immunosuppression of a subject are well known in the art.

Effective dosages and administration regimens can be readily determined by good medical practice based on the nature of the pathology of the subject, and will depend on a number of factors including, but not limited to, the extent of the symptoms of the pathology and extent of damage or degeneration of the tissue or organ of interest, and characteristics of the subject (e.g., age, body weight, gender, general health, and the like).

Typically, the human melanocytes of the invention can be used in a method for treating pigmentary disorders and pigmentary genodermatosis.

Typically, the human melanocytes of the invention can be used in a method for treating skin lesions, by establishing a melanised epidermis which resembles the human skin physiology (severely burned patients, leg ulcer such as diabetic skin ulcerations and sickle-cell anemia...).

The human melanocytes of the invention may also be used in order to provide insight onto the mechanisms involved in skin pigmentation disorders, including genetic disorders and photosensitivity.

2) Tissue engineering - reconstituting human skin substitutes

The human melanocytes derived from human pluripotent stem cells obtainable by the method as above described are able to recapitulate all morphological and functional attributes of primary human melanocytes. Indeed, the inventors demonstrated that said cells are able to produce and secrete melanin through melanosomes. When incorporated into a 3D organotypic system with human keratinocytes, they are able to participate in the formation of a melanized pluristratified epidermis.

The substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells of the invention may be also suitable for preparing human skin substitutes. Advantageously, the human skin substitutes according to the invention are melanised skin substitutes.

The substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells of the invention may be implanted alone or in combination with other cells, and/or in combination with other biologically active factors or reagents, and/or drugs. As will be appreciated by those skilled in the art, these other cells, biologically active factors, reagents, and drugs may be administered simultaneously or sequentially with the cells of the invention.

Typically, the substantially pure homogenous population of human melanocytes according to the invention may be implanted in combination with human keratinocytes in order to reconstruct a human epidermis. The skilled person in the art knows in what ratio to combine said cells.

Typically, the melanocyte to keratinocyte ratio can vary between 1:1 and 1:100, according to the desired result. In a preferred embodiment, the human melanocytes according to the invention are used in combination with keratinocytes in a ratio that varies between 1:3 and 1:20.

Keratinocytes useful for carrying out such a method can be any keratinocytes of human origin capable of reconstituting a human epidermis. They may be primary keratinocytes isolated from a human skin sample. Alternatively, they may be keratinocytes derived from human pluripotent stem cells.

Typically, the keratinocytes may be derived from human pluripotent stem cells according to the method described in Guenou et al. 2009.

Full stratification and histological differentiation of keratinocytes can be achieved by the use of three-dimensional organotypic culture methods (Doucet O, et al. 1998 ; Poumay y. et al. 2004 ; Gache Y. et al. 2004). For example, when in vitro cultures of human keratinocytes at an air-liquid interface, a highly ordered stratum corneum is formed.

As used herein, the term "organotypic culture" refers to a three-dimensional tissue culture where cultured cells are used reconstruct a tissue or organ in vitro.

In a particular embodiment, human skin substitutes according to the invention may be generated as described by Poumay, Y et al. 2004. Culture of keratinocytes may be performed on polycarbonate culture inserts. These cells may be maintained for 11 days in Epilife medium supplemented with 1.5 mM $CaCl_2$ and 50 µg/ml ascorbic acid. The cells are exposed to the air-liquid interface by removing the culture medium for 10 days.

In a particular embodiment, keratinocytes are seeded on a cell culture matrix populated with human dermis fibroblasts before providing an organotypic culture of it as above described. This particular technique allows obtaining a human skin substitute which comprises dermis and epidermis. Such a method may be performed through the protocol as described by Del Rio M. et al. (2002) or Larcher F. et al. (2007). For example, keratinocytes may be seeded on a fibrin matrix populated with live dermis fibroblasts. Organotypic cultures are then grown submerged up to

keratinocyte confluence, and finally maintained at the air–liquid interface for 7 days to enhance stratification and differentiation of the epithelium.

Typically the human skin substitute of the invention comprises a pluristratified epidermis which results from the in vitro-derived culture of the substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells as above described, together with human keratinocytes, that has stratified into melanized squamous epithelia. In a particular embodiment, the human skin substitute of the invention may comprise a melanized pluristratified epidermis as above described and a dermis.

Therefore a further aspect of the invention relates to a method of preparing a human skin substitute comprising a step consisting of providing a population of human melanocytes derived from human pluripotent stem cells as described above and providing a population of human keratinocytes.

A further object of the invention relates to a human skin substitute obtainable by the method as above described.

A further object of the invention relates to a method for grafting an animal, preferably a mammal, more preferably a mouse, with a human skin substitute as described above. In a particular embodiment said animal is an immunodeficient animal (e.g. NOD/SCID mouse). Said method may be useful to provide animal models for human skin.

In a particular embodiment, animals grafted with a human skin substitute of the invention may be generated as described by Del Rio M. et al. (2002). Briefly, animals are shaved and aseptically cleansed. Full-thickness wounds are then created on the dorsum of mice and finally grafting with the human skin substitute of the invention is performed under sterile conditions. 10-12 weeks may be then sufficient to obtain a human skin on said animal.

A further object of the invention relates to an animal model for human skin obtainable according to the method as above described.

The human skin substitutes and animal models of the present invention may have a variety of uses. These uses include, but are not limited to, use for screening compounds, substrates for culturing tumors and pathological agents (e.g., human papilloma virus), and for modelling human injuries or pathologies associated with skin damage and for pharmaco-toxicologic assays.

In particular, the skin substitutes and animal models of the present invention can be used in order to study the DNA damage induced by UV exposure and the potential effect of various drugs on DNA damage induced by UV exposure.

For example human skin substitutes and animal models of the present invention may be used for a variety of in vitro and in vivo tests. In particular but in non limiting way, the human skin substitutes and animal models of the present invention find use in the evaluation of: skin care products, drug metabolism, cellular responses to test compounds, wound healing, phototoxicity, dermal irritation, dermal inflammation, skin corrosivity, and cell damage. Typically, for animal models of the invention, the product may be administered topically on the human skin, or may be administered through an oral, sublingual, subcutaneous, intramuscular, intravenous, and transdermal route.

The present invention encompasses a variety of screening assays. In some embodiments, the screening method comprises providing a human skin substitute or an animal model of the present invention and at least one test compound or product (e.g., a skin care product such as a moisturizer, cosmetic, dye, or fragrance; the products can be in any from, including, but not limited to, creams, lotions, liquids and sprays), applying the product or test compound to said human skin substitute or animal model , and assaying the effect of the product or test compound on the human skin substitute or animal model. Typically, for animal models of the invention, the test compound or product may be administered topically on the human skin, or may

be administered through an oral, sublingual, subcutaneous, intramuscular, intravenous, and transdermal route. A wide variety of assays may be used to determine the effect of the product or test compound on the human skin substitute or animal model. The assays may be directed to the toxicity, potency, or efficacy of the compound or product. Additionally, the effect of the compound or product on growth, barrier function, or tissue strength can be tested.

In other preferred embodiments, the human skin substitutes or animal models of the invention find use for screening the efficacy of drug introduction across the skin.

In a particular embodiment, the human skin substitutes or animal models of the present invention are also useful for the culture and study of tumours that occur naturally in the skin as well as for the culture and study of pathogens that affect the skin. Accordingly, in some embodiments, it is contemplated that the human skin substitutes or animal models of the present invention are seeded with malignant cells. These reconstructed human skin substitutes or animal models can then be used to screen compounds or other treatment strategies (e.g., radiation or tomotherapy) for efficacy against the tumour in its natural environment. In some embodiments of the present invention provide methods comprising providing a reconstructed human skin substitute or animal model infected with a pathogen of interest and at least one test compound or treatment and treating the skin substitute or animal model with the test compound or treatment.

In another particular embodiment, the human skin substitutes or animal models of the present invention are also useful for modelling human injuries or pathologies associated with skin damage. For example, the human skin substitutes and animal models of the present invention may provide both in vitro and in vivo models for modelling wounds, burns (e.g. fire burns, sunburns…), or lesions caused by irradiations, pathogens…, irritations caused by chemical products or environment conditions, degenerative diseases and genetic diseases. In certain embodiments, pathologies of interest are genodermatosis such as Epidemolysis bullosa, Xeroderma

pigmentosum, ichthyosis, ectodermal dysplasia, kindler syndrome and others. Typically, the human skin substitutes or animal models of the present invention may be generated form pluripotent stem cells that may carry a mutation or a plurality of mutations that are causative for a genetic disease of the human skin. Both in vitro and in vivo models as described above may have particular interests for medical research or may be useful for screening compounds for the treatment or the prevention of said injuries and pathologies. In particular, the present invention contemplates the use of the human skin substitutes and animal models according to the invention for screening of compounds from libraries, in particular combinatorial libraries, using e.g. high throughput or high content techniques. Typically, for animal models of the invention, the test compound or product may be administered topically on the human skin, or may be administered through an oral, sublingual, subcutaneous, intramuscular, intravenous, and transdermal route.

In a further aspect of the invention, the human skin substitutes of the present invention may be used for the treatment of a pathology associated with skin damage.

Therefore the present invention relates to a method for the treatment of a pathology associated skin damage comprising a step consisting of grafting a patient in need thereof with a human skin substitute of the invention.

For example, the human skin substitutes of the present invention find use in wound closure and burn treatment applications. The use of grafts for the treatment of burns and wound closure is described U.S. Pat. Nos. 5,693,332; 5,658,331; and 6,039,760. Accordingly, the present invention provides methods for wound closure, including wounds caused by burns, comprising providing a human skin substitute according to the present invention and a patient suffering from a wound and grafting the patient with the human skin substitute under conditions such that the wound is closed.

3) Cellular models useful for pharmacological and toxicological studies

The isolated substantially pure population of human melanocytes of the present invention can provide an important source of material for various screening tests. They can therefore be used in high content screening (HCS) and high throughput screening (HTS) assays. For instance, these assays can serve to identify therapeutic targets for disorders associated with melanocytes. Other assays can be performed in order to identify cosmetically active substances or compounds which modulate skin pigmentation.

The isolated substantially pure population of human melanocytes of the present invention can be a useful alternative to animal experimentation in order to obtain predictive data in human before carrying out clinical trials.

Advantageously, the isolated substantially pure population of human melanocytes of the present invention can be obtained from a variety of different human pluripotent stem cells originating from a variety of human subjects, be they healthy or diseased. In particular, they can contain different genotypes, reflecting different ethnic origins, different genetic disorders or different phenotypes reflecting the skin sensitivity to UV radiation. Isolated substantially pure populations of human melanocytes can therefore be obtained for different phototypes. Skin phototype (SPT) is a classification system based on a person's sensitivity to sunlight. People with skin types I and II are at the highest risk for photoaging effects including wrinkles and skin cancer. However, sun effects including wrinkles and skin cancer can occur in any skin type.

SPT I - Always burns, never tans

SPT II - Burns easily, tans minimally

SPT III - Burns moderately, tans gradually to light brown

SPT IV - Burns minimally, always tans well to moderately brown

SPT V - Rarely burns, tans profusely to dark

SPT VI - Never burns, deeply pigmented

The human melanocytes of the invention can be used to study cutaneous irritation and corrosivity, in vitro phototoxicity, and response to UV radiations.

In one aspect, the invention therefore relates to a method for screening compounds for a given biological effect on human melanocytes comprising the steps of:

i) incubating an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells as described above in the presence or absence of a test compound;

ii) comparing said biological effect in the population of melanocytes in the presence or absence of said test compound.

Typically, the expression "biological effect" as used herein, can refer either to a desired (for example, stimulation of melanin synthesis) or an undesirable effect (such as toxicity) of a compound which is being screened for a dermatological and/or cosmetic purpose. The skilled person in the art will understand what type of biological effect is to be assayed, according to the specific goal of the screening method.

In another aspect, the invention relates to the use of the human melanocytes of the invention as an in vitro cellular model for screening compounds, in particular in cosmetology or in clinical studies of sunburns.

4) Studying the mechanisms of melanocyte development and identifying new therapeutic targets for the treatment of melanoma

The human melanocytes of the present invention can also be used in order to elucidate the mechanisms and the signalling pathways involved in the physiological and pathological development of melanocytes.

The human melanocytes of the present invention can also be used in order to identify new therapeutic targets for the treatment of melanoma.

The invention therefore also relates to the use of the human melanocytes as defined above as a cellular model for studying the development of melanocytes.

Embryonic development of the melanocyte initiates with cell fate specification in the neural crest, which is then followed by cell migration and niche localization. Many genes involved in melanocyte development have also been implicated in the development of melanoma, an aggressive and fatal form of skin cancer that originates in the melanocyte. Although early stage melanomas that have not spread to the lymph nodes can be excised with little risk of recurrence, patients diagnosed with metastatic melanoma have a high mortality rate due to the resistance of most tumors to radiotherapy and chemotherapy. Transformed melanocytes that develop into melanomas proliferate abnormally and often begin to grow radially in the skin. Vertical growth can then follow this radial growth, leading to an invasion through the basement membrane into the underlying dermis and subsequent metastasis. It is still unclear, however, how a normal melanocyte becomes a melanoma cell, and how melanoma utilizes the properties of the normal melanocyte and its progenitors in its progression.

Melanoma also utilizes many regulatory signals and pathways required during ontogeny and regeneration.

Uong et al in their studies described above (2010) indicate that melanoma formation shares many characteristics with melanocyte development and regeneration, though there are still a number of questions to be answered. Furthermore understanding of the normal regulation and behaviors of melanocytes and melanocyte stem cells will allow development of better strategies for cancer treatment.

The invention will be further illustrated by the following examples. However, these examples should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE: METHOD FOR PREPARING A POPULATION OF MELANOCYTES AND A HUMAN SKIN SUBSTITUTE FROM HUMAN PLURIPOTENT STEM CELLS

In this report, the inventors demonstrate that iPS reprogrammed cells (hiPSC) and hESC are able to generate pure and functional populations of melanocytes. These cells were obtained following a directed 2D protocol of epidermal induction on feeder cells in a FAD medium, supplemented with BMP4 and ascorbic acid. These melanocytes derived from pluripotent stem cells express all major markers of melanocytes, develop melanosomes and synthesize melanin capable to migrate into keratinocytes

Material & Methods

hESC culture and melanocyte differentiation

hESC from two cell lines, SA01 (Cellartis, Götenborg Sweden) and WA09 (H9) (Wicell, Madison WI) and hiPSC derived using retroviruses Yamanaka factors (Takahashi et al.) and lentivirus Thomsom factors (Thomson et al.) were grown on STO mouse fibroblasts, inactivated with 10 mg/mL mitomycin C, seeded at 30 000/cm² and grown as previously described (Guenou et al., 2009).

For differentiation, hESC and hiPSC clumps were seeded onto mitomycin C-treated 3T3 fibroblasts in FAD medium composed of 2/3 DMEM, 1/3 HAM:F12 and 10% of fetal calf serum (FCII, Hyclone, Logan, UT, USA) supplemented with 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 10^{-10}M cholera toxin, 1.37 ng/mL triodothyronin, 24 µg/mL adenine and 10 ng/mL recombinant human EGF. Three independent experiments were performed using each pluripotent cell lines. Induction of ectodermal differentiation was realized using 0.5 nM of human recombinant BMP-4 (R&D, UK) and 0.3 mM ascorbic acid (Sigma-Aldrich). Cells were grown in the same medium until clones of pigmented populations were observed and isolated. After isolation pigmented cells were dissociated using trypsin 0.05% (Invitrogen) and seeded as single cells in melanocytes specific medium 254CF supplemented with

growth factors (Invitrogen). After 1 week of culture in these conditions, cells presenting a morphology similar to melanocytes were mechanically isolated based on their morphology and amplified separately in the same medium during at least 12 passages.

Keratinocytes and melanocytes culture

As a control, primary human keratinocytes (HK) were cultured on mitomycin C treated 3T3 fibroblasts in FAD medium and primary human epidermal melanocytes (HEM) in 254CF medium supplemented with growth factors (Invitrogen).

Quantitative RT-PCR (Q-PCR).

Total RNA was isolated from hESC, HEM and melanocytes derived from hiPSC (mel-hiPSC) and from hESC (mel-hESC) using RNeasy Mini extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. An on-column DNase I digestion was performed to avoid genomic DNA amplification. RNA level and quality were checked using the Nanodrop technology. A total of 500 ng of RNA was used for reverse transcription using the Superscript III reverse transcription kit (Invitrogen). Q-PCR analysis was performed using a LightCycler 480 system (Roche, Basel Switzerland) and SYBR Green PCR Master Mix (Roche) following the manufacturer's instructions. Quantification of gene expression was based on the DeltaCt Method and normalized on 18S expression. Melting curve and electrophoresis analysis were performed to control PCR products specificities and exclude non-specific amplification. Q-PCR arrays were prepared by loading of primer mixes (1 μM) in duplicate, in 96 well plates. Q-PCR was performed as above after addition of SYBR Green PCR Master Mix and 12.5 ng cDNA.

Immunocytochemistry.

Cells were fixed in 4% paraformaldehyde (15 minutes, room temperature) before permeabilization and blocking in PBS supplemented with 0.4% Triton X-100 and 5% BSA (Sigma-Aldrich). Primary antibodies were incubated overnight at 4°C in blocking buffer. Antibodies included mouse anti-MITF (DAKO), mouse anti-

TRP1 (Abcam), rabbit anti-tyrosinase (Abcam), mouse anti-Rab27 (BD pharmingen), mouse anti-SSEA3/4 (R&D), mouse anti-TRA1-81 (eBioscience), mouse anti-RPE 65 (Abcam), rabbit anti-K14 (Novacastra) rabbit anti-PAX6 (Covance) and mouse anti-PAX3 (Santa Cruz). Cells were stained with the species specific fluorophore-conjugated secondary antibody (Invitrogen) (1 hour, room temperature); nuclei were visualized with DAPI. Three independent experiments were performed using each hESC line. Pictures were taken using a Zeiss microscope equipped with epifluorescence illumination.

FACS analyses

Cells were detached from culture plates using Trypsin-EDTA (Invitrogen) and fixed in 2% paraformaldehyde (15 minutes, room temperature). After PBS wash, cells were either permeabilized with 0.1% Saponin (Sigma-Aldrich) or not. Primary antibodies diluted at 1:100 were incubated (one hour, room temperature) in PBS containing 0.1% FCS. Controls were made with isotype specific or no primary antibody. Species specific secondary antibodies were added (1 hour, room temperature) and cells analyzed on a FACScalibur using CellQuest software (BD Biosciences). The number of events analysed for each experiment was 10,000. Three independent experiments were performed for each cell line.

Melanosome transfer quantification

Human keratinocytes (HK) and melanocytes from primary culture or derived from pluripotent stem cells were cocultured at 1:3 ratio for three days in epilife medium supplemented with growth factors. 3 days later, immunostaining was performed with anti-TRP1 antibody to visualize melanosomes and with anti-keratin-14 antibody which is specific to keratinocytes. Fluorescence was then observed with a Zeiss fluorescence microscope. The number of melanized keratinocytes was quantified using ArrayScan (Cellomics) by the detection of keratinocytes expressing the keratin 14 and presenting TRP1 positive vesicles in their cytoplasm.

Results

Establishment of a pigmented lineage

Previously we developed a protocol from hESC that respected the chronobiology of epidermis formation during human ontogenesis to generate a homogenous population of cells exhibiting all phenotypic characteristics of basal keratinocytes (Guenou et al). In this process at 40 days of differentiation only 60 % of the cells are keratinocytes (Guenou et al), allowing the establishment of other ectodermal-derived cell types. iPS reprogramming cells using Thomsom and Yamanaka factors (IMR90 and PolyF) and undifferentiated hESC (SA01 and WA09 (H9)) and were seeded on mitomycin-treated 3T3-J2 (feeder cells in FAD medium, supplemented with BMP4 (0.5 nM) and ascorbic acid (0.3 nM) following the protocol of epidermal induction for more than 40 days.

Interestingly, a gradual pigmentation was detectable a few weeks later in the areas surrounding keratinocytes derived from hiPSC and hESC. To confirm that these pluripotent stem cell-derived pigmented populations contain cells committed to the melanocyte lineage, we performed a molecular characterization all along the differentiation process by quantitative PCR (qPCR). For this, expression profiles were analyzed in hiPSC and hESC samples at different time points such as undifferentiated state, before and after pigmentation and purified pigmented cells. First, time course qPCR analysis demonstrated a decrease in the transcription of pluripotency gene markers *OCT4, NANOG* and *SOX2* that rapidly reached an undetectable level, comparable to the one of human adult melanocytes (HEM). Moreover, genes encoding the regulators of melanin synthesis such as *TRP1, TYROSINASE* and *MITF* increased progressively during pigmented cells enrichment in culture. Interestingly, in parallel of the increase of the levels of neural crest-derived cells markers, such as *SOX10* and *PAX3,* and we also observed an increase of neural tube-derived cell marker PAX6.

These data demonstrate the formation of several pigmented phenotypes derived from the ectodermal layer including neural crest-derived pigmented cells and neural tube-derived pigmented cells.

Characterization of a homogenous and pure population of melanocytes derived from pluripotent stem cells.

The pigmented population exhibited, after isolation and two passages, similar morphology to human adult melanocytes (HEM). These cells were named mel-iPSC for melanocytes derived from iPSCand mel-hESC for melanocytes derived from hESC. qPCR analysis of mel-iPSC and mel-hESC showed that expression of key genes involved in melanocyte biology as SOX10, PAX3, MITF-M isoform, TRP1 and TYR pointed similar expression patterns to HEM. Indeed we observed a decrease in the expression of all genes characteristic of pluripotency and self-renewal (OCT4, NANOG, SOX2) or in the expression of PAX6 and OTX2, confirming that this pigmented cell population was not retinal pigmentary epithelium . Immunostaining analysis confirmed the absence of OCT4, TRA1-81 and PAX6 expression in the mel-iPSC and mel-hESC nucleus as well as a correct nuclear localization of PAX3 and MITF and a cytoplasmic expression of TRP1, TYROSINASE (TYR) and Rab27.

Furthermore, FACS analysis after four passages revealed an absence of SSEA4 and TRA1-81 expression in mel-iPSC and mel-hESC compared to the control undifferentiated mel-iPS and mel-hESC. This analysis also showed that more than 80% of mel-iPSC and mel-hESC were TRP1- and MITF-positive just like the control culture of adult melanocytes.

Under those culture conditions, cells proliferated actively up to twelve passages and could be frozen and thawed at will, maintaining their morphology and their phenotype.

Melanocytes derived from pluripotent stem cells are functional.

To demonstrate their ability to produce and secrete melanin, melanosome transfer was evaluated by coculturing mel-iPSC or HEM with human adult keratinocytes (HK). After three days of coculture, melanosome transfers were detected into keratinocytes by a coimmunostaining of the protein TRP1 (expressed at the melanosomal membrane) and the keratin 14 (specifically expressed in keratinocytes). Arrayscan analysis showed that melanosomes were detectable in 20%

of keratinocytes cultivated with mel-iPSC and 45% with HEM, whereas no TRP1 staining was detectable in HK cultivated without melanocytes.

Interestingly, after their transfer, melanosomes localization was in the peri-nuclear compartment of keratinocytes as it is expected to protect their DNA from ultraviolet radiations. Similar results were obtained with mel-hESC where 20% of melanized keratinocytes were detectable after three days of coculture.

Using a 3D organotypic system allowing the generation of melanized pluristratified epidermis we evaluated the functionality of either iPSC-derived or hESC-derived melanocytes in an in vitro physiological-like context. Thus we showed that the coculture of 90% keratinocytes (HK) and 10% melanocytes (mel-iPSC, mel-hESC and HEM) led to formation of a functional and melanized epidermis with correct homing of melanocytes in the basal layer and melanization of the upper layers of the epidermis composed by differentiated keratinocytes.

Further functional evaluation of melanocytes derived from pluripotent stem cells was sought using the three-dimensional reconstruction of a pluristratified epidermis in vitro, by mixing melanocytes with adult basal keratinocytes seeded as a monolayer on a matrix, at the medium-air interface. After development of a fully pluristratified epidermis, macroscopic observation revealed a light pigmentation in the reconstructed tissue containing melanocytes derived from either hESC or iPSC. Fontana Masson staining confirmed the presence of melanin-containing cells in the basal layer of epidermis. TRP1 immunostaining confirmed this correct homing of pluripotent stem cell-derived melanocytes in the basal layer. In upper layers of the epidermis, melanin-containing processes were surrounded by keratinocytes Treatment of melanised epidermis with alpha-melanocyte stimulating hormone (αMSH), a physiological agent that controls skin pigmentation, activated the production of melanin in the tissue.

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CLAIMS:

1. An ex vivo method for obtaining a population of human melanocytes derived from human pluripotent stem cells comprising the step a) consisting of co-culturing human pluripotent stem cells with cells that support ectodermal differentiation in the presence of an agent that stimulates epidermal induction and an agent that stimulates terminal differentiation of keratinocytes.

2. The ex vivo method according to claim 1, wherein said cells that support ectodermal differentiation are feeder fibroblasts.

3. The ex vivo method according to claim 1 or 2, wherein said agent that stimulates epidermal induction is selected from the group consisting of BMP-2, BMP-4, BMP-7, Smad1, Smad5, Smad7 and GFD-6.

4. The ex vivo method according to any one of claims 1 to 3, wherein said agent that stimulates terminal differentiation of keratinocytes is selected from the group consisting of ascorbic acid and retinoic acid.

5. The ex vivo method according to any one of the preceding claims, wherein said human pluripotent stem cells are human embryonic stem cells (hES cells) or human induced pluripotent stem cells (hiPS cells).

6. The ex vivo method according to any one of the preceding claims, wherein said step a) is carried out for at least 35 days, preferably at least 40 days, even more preferably at least 45, at least 50, at least 55, at least 60, at least 65, at least 70 or at least 75 days.

7. The ex vivo method according to any one of the preceding claims, further comprising, after step a) the following steps:

b) isolating a pigmented cell from the population of cells obtained in step a);

c) culturing said isolated cell in an appropriate culture medium.

8. An isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells obtainable by the method according to any one of claims 1 to 7.

9. A pharmaceutical composition comprising the substantially pure homogenous population of melanocytes derived from human pluripotent stem cells of claim 8 and optionally a pharmaceutically acceptable carrier or excipient.

10. The isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells according to claim 8 or the pharmaceutical composition of claim 9 for use in a method of treatment.

11. A method of preparing a human skin substitute comprising a step consisting of providing an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells according to claim 8 and a step consisting of providing a population of human keratinocytes.

12. A human skin substitute obtainable by the method according to claim 11.

13. A human skin substitute according to claim 12 for use in a method of treatment.

14. A method for screening compounds for a given biological effect on human melanocytes comprising the steps of:

i) incubating an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells according to claim 8 in the presence or absence of a test compound;

ii) comparing said biological effect in the population of melanocytes in the presence or absence of said test compound.

15. Use of an isolated substantially pure homogenous population of human melanocytes derived from human pluripotent stem cells according to claim 8 or a human skin substitute according to claim 12 for screening compounds.