

Stem Cell Conditioned Medium Compositions

The present invention relates to compositions for use in pharmaceutical, cosmetic and cosmeceutical applications, particularly wound healing, including the treatment of lesions and burns.

5 Stem cells are of great interest in numerous therapeutic, cosmetic and cosmeceutical areas because of their capacity to form cells of multiple types. See for example EP 0980270. Additionally, culture media used to grow cells, including stem cells, have been described for therapeutic, cosmetic and cosmeceutical uses arising from the secretion by the growing cells of proteins and other factors into the media. See for example US7,118,746; US7,160,726 and WO2008/020815. It remains desirable to
10 identify alternative compositions for therapeutic, cosmetic and cosmeceutical purposes. It is particularly desirable to identify methods that produce conditioned media which are not contaminated with proteinaceous material employed to support growth of the stem cells. Further, it would be particularly desirable to identify effective, scalable methods for the manufacture of conditioned media.

15 According to a first aspect of the present invention, there is provided a pharmaceutical composition comprising conditioned cell culture medium obtained by a) culturing differentiated human cells retaining stem cell potential selected from the group consisting of dermal sheath cells, dermal fibroblast cells or dermal papilla cells in a growth medium; and b) separating the culture medium from the cells.

20 Growth media which can be employed in the first aspect of the present invention are culture media sufficient for growth of the cells. Cell culture procedures and culture media are well known in the art, and include basal media supplemented with serum, serum-free media, protein-free media or chemically defined growth media. Growth media typically include essential amino acids, sugars, salts, vitamins, minerals/inorganic salts,
25 trace metals, lipids and nucleosides, and are supplemented with a variety of additional components essential to support cell proliferation such as serum, proteins (for example insulin, transferrin, growth factors and other hormones), antibiotics (for example gentamycin, streptomycin, penicillins), attachment factors (for example fibronectin, collagens, laminins). Supplements can be added in combination, such as in the case of
30 serum, or individually. Growth media provide cells with components necessary to meet the particular cell type's nutritional needs to grow in a controlled in-vitro environment.

In one embodiment, the cell culture process is operated in one culture vessel, the cells are inoculated directly into the culture vessel containing microcarriers, the cells are propagated until the desired cell density is reached. In other embodiments, the cell
35 culture process is operated in at least two distinct cell culture vessels/systems, such as one or more seed expansion vessels followed by the cell production vessel. This multiple seed expansion process preferably employs culture vessels of increasing size until a sufficient number of cells is obtained for the inoculation of the final production cell culture vessel. The seed expansion culture vessels can be of the same type (e.g. tissue culture

flasks, shake flasks, roller bottles, spinner flasks, wave bioreactors, stirred tank bioreactors) but increasing in size as the seed expansion progresses or can be a mixture of culture systems increasing in size as the seed culture is expanded in readiness for transfer to the production bioreactor (for example tissue culture flasks to shake flasks to spinner flasks to stirred tank bioreactor systems).

The in-vitro environment is typically controlled to maintain optimum growth temperature, dissolved oxygen, carbon dioxide, pH and osmolality. Many cell culture medium formulations are known in the art or can be obtained readily from commercial sources. It is known to those skilled in the art that conditioned cell culture medium can be produced by seeding cells in growth medium that permits growth of the cells over the period of cell culture. At the end of the cell culture or at a selected point during the culture the cells are removed and the conditioned medium is harvested. The conditioned medium will contain many of the components of the original cell culture growth medium but in addition will also contain cellular metabolites and additional proteins secreted by the cells. Secreted proteins may be biologically active growth factors, cytokines, proteases and other extracellular proteins and peptides. In many embodiments, the composition according to the first aspect of the present invention comprises one or more of Gro- α , I-309, IL-6, IL-8, IL-13, MIF, PAI-1, SDF-1 and TGF- β proteins, especially TGF- β 1.

According to a second aspect of the present invention, there is provided a process for preparing a conditioned cell culture medium comprising:

- a) culturing eukaryotic cells in a growth medium having a composition effective to support cell growth;
- b) separating the cultured cells from the growth medium;
- c) maintaining the cultured cells in a basal medium having a composition suitable to maintain cell viability, but not to support substantial cell growth.

Eukaryotic cells which can be employed in the second aspect of the present invention are described in 'Basic Cell Culture' Oxford University Press (2002) Ed. J. M. Davis; and 'Animal Cell Culture' Oxford University Press (2000) Ed. John. R. W. Masters; both of which are incorporated herein in their entirety by reference. The term "stem cells" describes cells that can give rise to cells of multiple tissue types. Stem cells are cells from the embryo, fetus or adult which have the capacity to become different cell types when presented with specific signaling complexes that provide the directions to do so. There are different types of stems cells. A single totipotent cell is formed when a sperm fertilizes an egg, and has thereby has the capacity to form an entire organism. In the first hours after fertilization, this cell divides into identical totipotent cells. Approximately four days after fertilization and after several cycles of cell division, these totipotent stem cells begin to specialize. When totipotent cells become more specialized, they are then termed "pluripotent." Pluripotent cells can be differentiated to every cell type in the body, but do not give rise to the placenta, or supporting tissues necessary for foetal development. Because the potential for differentiation of pluripotent cells is not "total," such cells are not

termed "totipotent" and they are not embryos. Pluripotent stem cells undergo further specialization into multipotent stem cells, which are committed to differentiate to cells of a particular lineage specialized for a particular function. Multipotent cells can be differentiated to the cell types found in the tissue from which they were derived; for example multipotent (adult) stem cells such as mesenchymal stem cells, such as dermal sheath, dermal papilla and dermal fibroblast cells.

Cells may be derived from adult, neonatal or foetal tissue and may be autologous or allogenic. The cells may be genetically modified using methods well established in the art. The genetic modification may be used to alter the concentration of one or more component secreted into the cell growth conditioned cell culture medium or the conditioned basal cell culture medium such as, for example, to up or down-regulate a protein, to introduce a new protein, or to regulate ion concentration.

In certain embodiments, the cells are grown as a co-culture. Co-cultured cells are a mixture of two or more different kinds of cells that are grown together.

Cells suitable for use in the process of the second aspect can be obtained by methods known in the art. In particular cells can be isolated from tissues, expanded from cell previously established cell stocks, passaged and cultured to produce the cell growth conditioned cell culture medium or the conditioned basal cell culture medium. The cell growth conditioned cell culture medium or the conditioned basal cell culture medium may be produced using un-differentiated or differentiated cells.

Cells employed in the process of the second aspect of the invention are preferably differentiated human cells retaining stem cell potential selected from the group consisting of dermal sheath cells, dermal fibroblast cells or dermal papilla cells.

Growth media which can be employed in the second aspect of the present invention are as described above in respect of the first aspect. Cells are cultured in growth medium until the desired cell density is achieved.

Basal media employed in the second aspect of the present invention have a composition suitable to maintain cell viability, for example a pH and osmolality to avoid cell lysis, but not to support substantial cell growth, and preferably no cell growth. Basal media comprise basic constituents such as inorganic salts, amino acids, vitamins and an energy source, including sugars, but are not supplemented with components such as serum, proteins, hormones and attachment factors. A preferred energy source comprises glutamine. Basal media are protein-free prior to introduction of the cultured cells, and no protein supplements are added to the basal media after introduction of the cultured cells.

The composition of the basal media are selected so as to maintain the viability of the cultured cells to allow export of cellular metabolites and secretions into the basal media. Examples of basal media which may be employed include Ames Medium, Basal Medium Eagle's, Click's Medium, Dulbecco's Modified Eagle's Medium, Ham's Nutrient mixture F-12, Glasgow Minimum Essential Medium, Iscove's Modified Dulbecco's Medium, Minimum Essential Medium Eagle and RPMI-1640 Medium.

In many preferred embodiments of the second aspect of the present invention, the cells are washed after separation from the growth medium, and prior to introduction into the basal medium. Examples of suitable wash solutions for cells are well known in the art, and include buffers, such as phosphate-buffered saline. In some preferred embodiments, the wash solution employed is a basal medium, such as those described above, and commonly the same basal medium in the cells are to be subsequently maintained.

The introduction of cultured cells into the basal medium can be at the same cell concentration achieved at the end of cell growth period or more preferably at a higher concentration to increase the concentration of secreted components into the basal medium. Where the cells are grown in 2D culture, cells are commonly grown to yield a highly confluent monolayer. Such cell concentrations are typically from 1×10^4 to 1×10^5 cells per cm^2 , preferably from 2×10^4 to 5×10^4 cells per cm^2 . Such highly confluent monolayers of cells are also employed where the contact with the basal medium is conducted in 2D mode. Where the cells are grown in 3D culture, such as attached to microcarriers, cells are commonly grown to concentrations in the range of from 1×10^7 to 1×10^{12} cells per litre, preferably from 1×10^8 to 1×10^{10} cells per litre. In many embodiments, in either 2D or 3D cultures, the volume of basal medium employed is up to 15, commonly from 2 to 10, preferably from 4 to 6, such as about 5, times, lower than the volume of medium employed to support the growth of the cells.

The cultured cells are commonly maintained in the basal medium until the medium has the desired composition, commonly for a period of greater than 12 hours, typically from 18 to 26 hours, such as about 24 hours. At the end of this re-incubation period the cells are removed to generate cell-free conditioned basal cell culture medium. The conditioned basal cell culture medium will contain cellular metabolites and secreted proteins. Secreted proteins may be biologically active growth factors, cytokines, proteases and other extracellular proteins and peptides.

Various terms are used to describe cells in culture. 'Cell culture' generally refers to cells taken from a living organism and grown under controlled conditions. A primary cell culture is a culture of cells, tissues or organs taken directly from organisms before the first subculture. Cells are expanded in culture when they are placed in a growth medium under conditions that facilitate growth and/or division, resulting in a larger population of cells. A cell line is a population of cells formed by one or more sub-cultivations of a primary cell culture. Each round of sub-culturing is referred to as a passage. It will be understood by those skilled in the art that there may be many population doublings during the period of passaging.

Anchorage dependant or attachment dependant cells are cells that need to attach to a surface for propagation and growth in tissue culture. In some embodiments, the cells used in carrying out the invention are capable of growing in suspension cultures. As used herein, suspension-competent cells are those that can grow in suspension without making large, firm aggregates, i.e., cells that are mono-disperse or grow in loose aggregates with

only a few cells per aggregate. Suspension-competent cells include, without limitation, cells that grow in suspension without adaptation or manipulation and cells that have been made suspension-competent by gradual adaptation of attachment-dependent cells to suspension growth. If such cells are used, the propagation of cells may be done in suspension, thus microcarriers may be used only in the final propagation phase in the production bioreactor itself and in the production phase. In case of suspension-adapted cells, the microcarriers used are typically macroporous carriers wherein the cells are attached by means of physical entrapment inside the internal structure of the carriers.

As used herein, the terms "microcarrier" mean small, discrete particles suitable for cell attachment and growth. Often, although not always, microcarriers are porous beads which are formed from polymers. Microcarriers may also have a dense surface with dents. Usually, cells attach to and grow on the outer surfaces of such beads.

The process of the present invention is carried out by cultivating the cells under conditions conducive to the growth of the cells. Culture conditions, such as temperature, pH, dissolved oxygen (including hypoxic low oxygen conditions) and the like, are those known to be optimal for the particular cell and will be apparent to the skilled person or artisan within this field (see, e.g., *Animal Cell Culture: A Practical Approach* 2nd Ed., Rickwood, D. and Hames, B. D., eds., Oxford University Press, New York (1992)).

In the first and second aspects of the present invention, the cells are advantageously cultivated attached to a solid support medium. Options for large scale production include tissue culture flasks, roller bottles, perfusion based systems (e.g. hollow fibre bioreactors, internal and external spin filters, acoustic cell retention devices, filtration based cell retention devices) single, multi-plate or stacked-plate cell culture systems, cell cubes, and microcarriers. Cells may also be cultivated using a three dimensional scaffold composed of any material and or shape that allows cells to attach to it and allows cells to grow in one than one layer. The structure of the framework can include a mesh, a sponge or can be formed from a hydrogel. One suitable three dimensional framework is IntegraTM Dermal Regeneration Template (Integra Life Sciences). The cells may be cultivated directly on the three dimensional scaffold or may be harvested from tissue culture flasks, roller bottles, hollow fibre systems, single, multi-plate or stacked-plate cell culture systems, cell cubes and microcarriers prior to being re-seeded onto the three dimensional scaffold to produce cell growth conditioned cell culture medium or conditioned basal cell culture medium. Cells maybe also be cultivated using perfusion cell culture. In perfusion cell culture the cells are retained in the bioreactor using a cell retention device such as a filter (e.g. internal or external spin filters), cell retaining mesh, cell settler, acoustic device, etc. Cell culture growth medium is fed continuously or periodically to the bioreactor and cell free 'spent' medium is removed continuously or periodically.

In certain preferred embodiments, the cells are attached to the surface of solid microcarriers or attached to, or attached by physical entrapment inside, the internal

structure of macroporous microcarriers wherein the microcarrier is a gelatin (hydrolysed collagen) microcarrier. Such microcarriers can comprise gelatin particles, cross linked gelatin particles or gelatin used as a coating on carrier materials such as polystyrene or glass particles. Gelatin can be from a natural source or recombinantly or synthetically produced.

In some embodiments, the cell culture process is operated in one culture vessel. The cells are inoculated directly into the culture vessel containing microcarriers, and the cells are propagated until the desired cell density is reached. The microcarriers containing the propagated cells are aseptically harvested and washed. The washed microcarriers are then resuspended in basal medium and incubated under optimum conditions to maintain cell viability for a period of time (typically 24 hours). The conditioned medium is then harvested. The wash step may be carried out once or multiple times.

In other embodiments, the cell culture process is operated in at least two distinct cell culture vessels/systems, such as one or more seed expansion vessels followed by the cell production vessel. This multiple seed expansion process preferably employs culture vessels of increasing size until a sufficient number of cells is obtained for the inoculation of the final production cell culture vessel. The seed expansion culture vessels can be of the same type (e.g. tissue culture flasks, shake flasks, roller bottles, spinner flasks, wave bioreactors, stirred tank bioreactors) but increasing in size as the seed expansion progresses or can be a mixture of culture systems increasing in size as the seed culture is expanded in readiness for transfer to the production bioreactor (for example tissue culture flasks to shake flasks to spinner flasks to stirred tank bioreactor systems).

Medium exchange can be performed if desired by allowing the microcarriers to settle to the bottom of the cell culture vessel, after which a selected percentage, up to and including all, of the growth medium volume is removed, the microcarrier is optionally washed, and a corresponding percentage of fresh cell culture growth medium is added to the cell culture vessel. The microcarriers are then re-suspended in the medium and culturing continued. This process of medium removal and replacement can be repeated until the desired cell density is achieved.

Gelatin microcarriers which can be employed in the method of the present invention are typically roughly spherical but can have other shapes and can be either porous or solid. Both porous and solid types of microcarriers are commercially available from suppliers. Macroporous gelatin microcarriers are available commercially for example "Cultispher" microcarriers available from Percell Biolytica AB, Sweden. Gelatin macroporous microcarriers are characterised in that the particles are based on a highly cross linked gelatin matrix, particle size of 10-500µm and consist of a polymer matrix enclosing a large number of cavities having a diameter of 1-50µm. The use of microcarriers for cell attachment facilitates the use of stirred tank and related bioreactors for the growth of anchorage dependant cells. The cells generally attach to the suspended

particles. The desirability of suspensions typically limits the physical parameters of the microcarriers that can be used. Microcarrier particle size range is commonly selected to be large enough to accommodate the anchorage dependant cell type while small enough to form suspensions with properties suitable for use in cell culture bioreactors such as shake flasks, roller bottles, spinner flasks, wave bioreactors and stirred tank bioreactor systems. Gelatins or collagens can be cross-linked via the amine groups of lysine, via carboxyl groups glutamic acid or aspartic acid, or a combination thereof.

Cells are separated from the media in which they have been grown or maintained by methods known in the art for example using, cell settling and decant, batch or continuous centrifugation and/or microfiltration. The cell-free media obtained may be further processed to concentrate or reduce one or more factors or components, for example using ultrafiltration, diafiltration or chromatographic purification.

The conditioned medium produced in the process of the second aspect of the present invention is preferably employed as a pharmaceutical composition. Accordingly, such pharmaceutical compositions form a third aspect of the present invention. The pharmaceutical compositions, especially those derived from dermal sheath cells, dermal fibroblast cells or dermal papilla cells are commonly employed useful in wound and lesion healing. The compositions may also be used for other applications for which the components of the medium are known to be effective.

Preferred compositions comprise one or more of IL-6, Gro- α , SDF-1, FGF-2, SPARC, PAI-1, IL-8, Collagen, Fibronectin, I-309, IL-13, MIF and SDF-1 and TGF- β proteins, especially TGF- β 1. Especially preferred compositions comprise one or more of the proteins listed in Tables 2, 3 or 4.

The compositions of the first and third aspects of the present invention can be employed as pharmaceuticals as liquids or may be frozen, lyophilized, formed into films or dried into a powder. The compositions may be diluted, concentrated, mixed with other components, or be partially or completely purified. The compositions may be delivered to the human or animal body by any suitable means. The conditioned media may be formulated with a pharmaceutically acceptable carrier as a vehicle for internal administration, applied directly to wound/lesion, formulated with a salve or ointment for topical applications, or, for example, made into or added to or dispersed in a biodegradable polymer or hydrogel to create wound dressings, implantable compositions and coatings for medical devices. One advantage of dispersion into a biodegradable polymer is that the system can be used for slow-release delivery systems. This is particularly advantageous to the delivery of bioactive components from the polymer to chronic wounds which must be resistant to rapid degradation from the wounds proteolytic environment and have sustained release of bioactive components. It will be evident to the skilled person that the delivery method will depend on the particular in-vivo application to which the conditioned medium is to be delivered and the skilled person will be able to determine which means to employ accordingly.

Tissues may be regenerated or repaired through the enhancement of endogenous tissue repair by applying secretions from cells instead of or in-addition to the cells. The present invention is based on the premise that multiple complex processes involving the differential expression/secretion of multiple proteins are necessary for optimal tissue repair and re-modelling. The conditioned media produced in the present invention contain many of the regulatory proteins believed to be important in tissue repair, re-modelling and wound healing and which have been shown to be depleted for example in, in-vivo models of wound healing. Examples of such proteins include TGF- β , IL-6, Gro- α , SDF-1, FGF-2, SPARC, PAI-1, IL-8, Collagen, Fibronectin, I-309, IL-13, MIF and SDF-1.

TGF- β 1 is the predominant TGF- β protein in cutaneous wound healing. In wound healing TGF- β 1 is important in inflammation, angiogenesis, re-epithelialisation and connective tissue regeneration. It is shown to have increased expression with the onset of injury (Kopecki Z, Luchetti MM, Adams DH, Strudwick X, Mantamadiotis T, Stoppacciaro A, Gabrielli A, Ramsay RG, Cowin AJ, *J Pathol* 2007;211:351-61. Kane CJ, Hebda PA, Mansbridge JN, Hanawalt PC, *J Cell Physiol* 1999;148:157-73.) In-vitro studies have shown that TGF- β 1 helps initiate granulation formation by increasing the expression of genes associated with extracellular matrix (ECM) formation including fibronectin, fibronectin receptor, and collagen and protease inhibitors (White L A; Mitchell T I; Brinckerhoff C E, *Biochimica et biophysica acta*, 2000;1490 (3):259-68. Mauviel A, Chung KY, Agarwal A, Tamai K, Uitto J, *J Biol Chem* 1996;271:10917-23. Papakonstantinou E, Aletra AJ, Roth M, Tamm M, Karakiulakis G, *Cytokine* 2003, 24: 25-35. Zeng G, McCue HM, Mastrangelo L, Mills AJ, *Exp Cell Res* 1996; 228:271-6). Further in-vitro studies have shown TGF- β 1 playing a role in wound contraction by facilitating fibroblast contraction of the collagen matrix (Meckmongkol TT, Harmon R, McKeown-Longo P, Van De Water L, *Biochem Biophys Res Commun* 2007;360:709-14). In the matrix formation and re-modelling phase of wound healing, TGF- β 1 is involved in collagen production, particularly type I and II (Papakonstantinou E, Aletra AJ, Roth M, Tamm M, Karakiulakis G, *Cytokine* 2003;24:25-35). When over-expressed, TGF- β 1 has been shown to stimulate connective tissue growth factor (CTGF) also known to play an important role in the development of hypertrophic and keloid scars (Colwell AS, Phan TT, Kong W, Longaker MT, Lorenz PH, *Plast Reconstr Aesthet Surg* 2005;116:1387-90).

IL-6 has been shown to be important in initiating the wound healing response and expression is increased after wounding, tending to persist in older wounds (Sogabe Y, Abe M, Yokoyama Y, Ishikawa, O, *Wound Repair Regen* 2006;14:457-62. Grellner W, Georg T, Wilske J, *Forensic Sci Int* 2000;113:251-64. Finnerty CC, Herndon DN, Przkora R, Pereira CT, Oliveira HM, Queiroz DM, Rocha AM, Jeschke MG, *Shock* 2006;26:13-9). IL-6 has a mitogenic (Randle M Gallucci, Dusti K Sloan, Julie M Heck, Anne R Murray and Sijy J O'Dell, *Journal of Investigative Dermatology* (2004) 122, 764-772) and proliferative (Sato M, Sawamura D, Ina S, Yaguchi T, Hanada K, Hashimoto I, *Arch Dermatol Res* 1999;291:400-4. Peschen M, Grenz H, Brand-Saberi B, Bunaes M, Simon JC, Schopf E,

Vanscheidt W, *Arch Dermatol Res* 1998;290:291-7) effect on keratinocytes and is chemoattractive to neutrophils.

5 Gro- α (CXCL1) chemokine is a member of the CXC family and is a potent regulator of neutrophil chemotaxis and is upregulated in the acute wound. In-vitro studies suggest a role in re-epithelialisation by promoting keratinocyte migration (Englehardt E, Toksoy A, Goebeler M, Debus S, Bocker EB, Gillitzer R, *Am J Pathol* 1998;153:1849-60. Christopherson K II, Hromas R, *Stem Cells* 2001;19:388-96).

10 SDF-1 (CXCL12) plays a role in the inflammatory response by recruiting lymphocytes to the wound and promoting angiogenesis. When homeostasis is disturbed in an acute wound, SDF1 is seen at increased levels at the wound margin (Toksoy A, Muller V, Gillitzer R, Goebeler M, *Br J Dermatol* 2007;157:1148-54). SDF-1 promotes proliferation and migration of epithelial cells (Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ, *Am J Pathol* 1999;154:1125-35). SDF-1 may also enhance keratinocyte proliferation therefore contributing to re-epithelialisation (Florin L, Maas-Szabowski N, Werner S, Szabowski A, Angel P, *J Cell Sci* 2005;118(Pt9):1981-9).

20 FGF-2 (bFGF) regulates the synthesis and deposition of various ECM components, increases keratinocyte motility during re-epithelialisation (Sogabe Y, Abe M, Yokoyama Y, Ishikawa, O, *Wound Repair Regen* 2006;14:457-62. Grellner W, Georg T, Wilske J, *Forensic Sci Int* 2000;113:251-64. Di Vita G, Patti R, D'Agostino P, Caruso G, Arcara M, Buscemi S, Bonventre S, Ferlazzo V, Arcoleo F, Cillari E, *Wound Repair Regen* 2006;14:259-64) and promotes migration of fibroblasts and stimulates them to produce collagenase (Sasaki T, *J Dermatol.* 1992 Nov;19(11):664-6).

25 SPARC (Secreted Protein Acidic and Rich in Cysteine) is expressed in different tissues during re-modelling and repair, such as healing cutaneous wounds (Reed MJ, Puolakkainen P, Lane TF, Dickerson D, Bornstein P, Sage EH, *J Histochem Cytochem* 1993, 41:1467-1477) suggesting a function in regeneration (Louise H. Jørgensen, Stine J. Petersson, Jeeva Sellathurai, Ditte C. Andersen, Susanne Thayssen, Dorte J. Sant, Charlotte H. Jensen and Henrik D. Schrøder, *Journal of Histochemistry and Cytochemistry*, Volume 57 (1): 29-39, 2009). A number of matricellular proteins show increased expression in response to injury (Bradshaw AD, Sage EH, *J Clin Invest* 2001, 107:1049-1054). SPARC is a matricellular glycoprotein and modulates the interaction of cells with the ECM. Accelerated cutaneous wound closure and altered deposition of collagen have been reported in SPARC-null mice (Bradshaw AD, Reed MJ, Sage EH, *J Histochem Cytochem* 2002, 50:1-10). From expression patterns at the wound site and in-vitro studies, SPARC has been implicated in the control of wound healing (Basu A, Kligman LH, Samulewicz SJ, Howe CC, *BMC Cell Biol.* 2001;2:15. Epub 2001 Aug 7).

40 PAI-1 (SerpineE1) is an important physiological regulator for the generation of plasmin. While PAI-1 is not normally expressed by keratinocytes in the epidermis, it has been shown to be increased in expression following in-vitro and in-vivo wound injury

(Romer J, Lund LR, Eriksen J, Ralfkiaer E, Zeheb R, Gelehrter TD, Dano K, Kristensen P, *J Invest Dermatol* 1991, 97:803-811. Staiano-Coico I, Carano K, Allan VM, Steiner MG, Pagan-Charry I, Bailey BB, Babaar P, Rigas B, Higgins PJ, *Exp Cell Res* 1996, 227:123-134). A study supporting a role for PAI-1 in wound healing indicates that a loss of PAI-1 function results in accelerated wound healing (Joyce C.Y. Chan, Danielle A. Duszczyszyn, Francis J. Castellino and Victoria A Ploplis, *American Journal of Pathology*. 2001;159:1681-1688). Studies have indicated that uPA and PAI-1 are regulated in their expression, both spatially and temporally, during the migration of keratinocytes and connective tissue cells during re-epithelialization, and tissue remodelling associated with wound healing (Romer J, Lund LR, Eriksen J, Ralfkiaer E, Zeheb R, Gelehrter TD, Dano K, Kristensen P, *J Invest Dermatol* 1991, 97:803-811).

IL-8 expression is increased in acute wounds (E. Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R, *Am J Pathol* 1998;153:1849-60) and has been shown to play a role in re-epithelialisation by increasing keratinocyte migration and proliferation (Michel G, Kemeny L, Peter RU, Beetz A, Reid C, Arenberger P, Ruzicka T, *FEBS Lett* 1992;305:241-3. Tuschil A, Lam C, Haslberger A, Lindley I, *J Invest Dermatol*. 1992 Sep;99(3):294-8) It also induces the expression of MMPs in leukocytes, stimulating tissue remodelling (Englehardt E, Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R, *Am J Pathol* 1998;153:1849-60). It is a strong chemoattractant for neutrophils, thus participating in the inflammatory response (Rennekampff HO, Hansbrough JF, Kiessig V, Doré C, Sticherling M, Schröder JM, *J Surg Res*. 2000 Sep;93(1):41-54). Furthermore, addition of IL-8 in high levels decreases keratinocyte proliferation and collagen lattice contraction by fibroblasts (Iocono JA, Colleran KR, Remick DG, Gillespie BW, Ehrlich HP, Garner WL, *Wound Repair Regen*. 2000 May-Jun;8(3):216-25).

Collagen and Fibronectin - the proliferative phase of wound healing is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction (Midwood K.S., Williams L.V., and Schwarzbauer J.E. 2004, *The International Journal of Biochemistry & Cell Biology* 36 (6): 1031–1037). In fibroplasia and granulation tissue formation, fibroblasts grow and form a new ECM by excreting collagen and fibronectin (Midwood K.S., Williams L.V., and Schwarzbauer J.E. 2004, *The International Journal of Biochemistry & Cell Biology* 36 (6): 1031–1037). Fibroblasts begin entering the wound site two to five days after wounding as the inflammatory phase is ending, and their numbers peak at one to two weeks post-wounding (de la Torre J., Sholar A. (2006), Wound healing: Chronic wounds. Emedicine.com, accessed January 20, 2008). By the end of the first week, fibroblasts are the main cells in the wound (Stadelmann W.K., Digenis A.G. and Tobin G.R. (1998), *The American Journal of Surgery* 176 (2): 26S-38S). Fibroplasia ends two to four weeks after wounding. In the first two or three days after injury, fibroblasts mainly proliferate and migrate, while later, they are the primary cells that lay down the collagen matrix in the wound site (Stadelmann W.K., Digenis A.G. and Tobin G.R. (1998), *The American Journal*

of *Surgery* 176 (2): 26S-38S). Fibroblasts from normal tissue migrate into the wound area from its margins. Initially fibroblasts use the fibrin scab formed in the inflammatory phase to migrate across, adhering to fibronectin (Romo T. and Pearson J.M. 2005, Wound Healing, Skin. Emedicine.com, accessed December 27, 2006). Fibroblasts then deposit ground substance into the wound bed, and later collagen, which they can adhere to for migration (Rosenberg L., de la Torre J. (2006), Wound Healing, Growth Factors. Emedicine.com, accessed January 20, 2008). Collagen deposition is considered important because it increases the strength of the wound; before it is laid down, the fibrin-fibronectin clot holds the wound closed (Greenhalgh D.G. (1998), *The International Journal of Biochemistry & Cell Biology* 30 (9): 1019-1030). Also, cells involved in inflammation, angiogenesis, and connective tissue construction attach to, grow and differentiate on the collagen matrix laid down by fibroblasts (Ruszczak Z. 2003, *Advanced Drug Delivery Reviews*, 55(12): 1595-1611).

Human cytokine I-309 is a small glycoprotein, structurally related to a number of inflammatory cytokines, that specifically stimulates human monocytes during angiogenesis (Miller MD, Krangel MS, *Proc Natl Acad Sci USA* 1992b 89:2950-2954).

In general it is thought desirable in the treatment of wounds to enhance the supply of growth factors by the direct addition of these factors. With this approach the present issues associated with cell based therapy such as but not limited to immune compatibility and tumorigenicity will be eliminated. The cell growth conditioned cell culture medium and the conditioned basal cell culture medium of the present invention is also useful in the treatment of other types of tissue damage wherein the repair and/or regeneration of tissue or damage is desired since many of the array of factors known to be required are found in the applicants' cell growth conditioned cell culture medium and the conditioned basal cell culture medium.

The present invention is illustrated without limitation by the following examples.

Establishment of Cell Lines

Hair follicle mesenchymal cells were isolated essentially as described in EP980270 with the modifications described below. Human skin tissue samples were washed 3 times with Minimal Essential Medium (MEM, Sigma M4655) containing 1 µg/ml amphotericin and 10 µg /ml gentamycin. Under a dissecting microscope, anagen 'end bulbs' were dissected using fine surgical scissors and placed into small volumes (typically 100-200 µl) of MEM. The end bulbs were inverted using needles, and the papilla dissected and the sheath extracted. The papillae and sheaths were then transferred separately to 4 well cell culture plates (Nunc). Ten papillae and 10 sheath were transferred per well in 1ml of MEM supplemented with 20% foetal bovine serum (FBS), 0.5 µg/ml amphotericin and 5 µg /ml gentamycin. The four well cell culture plates were incubated under sterile and standard

conditions (37°C, 5% carbon dioxide). After 10 days cell growth, cells were detached from each well (using standard methods well established in the art) and transferred separately to a 35mm diameter cell culture dish (Nunc). When cell growth was confluent, the dermal sheath (hereinafter referred to as 'AVDS') and dermal papilla (hereinafter referred to as 'AVDP') cell lines were detached as previously indicated and transferred to T25 cell culture flasks (Nunc) for further expansion under the conditions described above.

Dermal fibroblast (hereinafter referred to as 'AVDF') cell lines were established from the same human skin tissue samples described above. The papillary dermis was separated from the reticular dermis and adipose layer and then dissected under a microscope into pieces of approximately 2-3mm² surface area. Dissected tissue was transferred to a T25 cell culture flask (Nunc) containing MEM supplemented as described for the dermal sheath and dermal papilla cell lines. The T25 cell culture flasks containing dermal fibroblast (AVDF) cell lines were incubated under sterile and standard conditions (as described previously). The dermal fibroblast (AVDF) cell lines were then further expanded using the same conditions when the cultures had reached confluency.

AVDS, AVDP and AVDF cell lines were established from a number of different human tissue samples. A summary of these cell lines which are described in the following examples is provided in Table 1 below.

20

Table 1: Summary of Cell Lines

Cell Line Designation	Type
AVDP2	Dermal papilla
AVDS4	Dermal sheath
AVDS6	Dermal sheath
AVDF3	Dermal fibroblast
AVDF4	Dermal fibroblast
AVDP3	Dermal Papilla

Example 1

AVDF4 and AVDS4 cells grown in static culture in MEM + 10% FBS were harvested (using standard methods well established in the art). The cells were used to seed 225cm²

25

flasks at 5×10^5 cells per flask in 50ml MEM + 10% Foetal Bovine Serum (FBS) and incubated at 37°C, 5% CO₂ for 8 days, with a fresh medium change (MEM + 10% FBS) on day 4. The conditioned medium was harvested on day 8, filtered (0.2µm) and stored frozen at -20°C prior to analysis.

5 Conditioned medium from AVDF4 and AVDS4, was analysed using a human cytokine array "panel A" kit (R&D Systems ARY005) following the method provided with the kit. MEM + 10% FBS was also analysed as a control since AVDF4 and AVDS4 growth medium contained 10% FBS. The results obtained are presented in Figure 1. The spots identified with increased intensity relative to the MEM + 10% FBS control were quantified
10 using methods well described in the art. The data were normalised with respect to the positive controls on each membrane and with respect to the corresponding cytokine spots on the MEM+10% FBS control membrane. Results presented in Figure 2 present the relative levels of each cytokine identified in AVDS4 and AVDF4 conditioned medium from duplicate samples. Surprisingly amongst the cytokines identified (limited to those included
15 in the "panel A" kit used) cytokines Groα, I-309, IL6, IL-8 PAI-1 were detected in the conditioned medium produced by both AVDS4 and AVDF4 cell lines. These have been established in the art as being important in facilitating the wound healing process.

Example 2

20 Serum free conditioned medium was prepared from dermal fibroblasts, dermal sheath and dermal papilla cell lines. AVDF4, AVDS4 and AVDP2 cells were grown in static culture in MEM + 10% FBS cell culture growth medium at 37°C, 5% CO₂ for 6 days. The cells were harvested (using standard methods well established in the art) and used to separately seed 75cm² flasks at 2×10^6 cells per flask in 15ml of MEM + 10% Foetal
25 Bovine Serum (FBS). The flasks were incubated for 24 hours at 37°C, 5% CO₂. After this incubation time the growth medium was removed from each flask and discarded. Cell monolayers were carefully washed three times with 20ml phosphate buffered saline (PBS) and then a further three times with 20ml MEM alone (no FBS). Fresh MEM (no FBS, 6 ml) supplemented with 2mM glutamine was then added to each flask and the flasks
30 incubated for 24 hours at 37°C, 5% CO₂. The conditioned basal medium was harvested from each flask, filtered (0.2µm) and stored frozen at -20°C prior to analysis. A sample of MEM growth medium (no FBS) was also included as a control. Samples (8ml) were thawed and concentrated to 250µl using Amicon Ultra 15 Centriprep devices (Millipore) at 3200 RCF, 45 min, room temperature. 200µl of each sample was then concentrated a
35 further 2-fold using a Speedvac. 10µl of each of the four samples were then analysed

using a 4-20% NuPage (Invitrogen) reducing SDS-PAGE gel and stained using EZBlue (Sigma). The SDS-PAGE gel is presented in Figure 3. Each lane (1-4) of the SDS-PAGE gel was cut with a clean scalpel blade into 10 bands, proteins in each of the 40 bands were digested with trypsin using standard procedures for a ProGest digestion robot. 5µl of each of the digests were analysed using a Thermo LTQ XL Orbitrap Electrospray mass spectrometer using LC-MS-MS as is well established in the art. MS-MS spectra were searched against a current version of the database Swissprot using Mascot (Matrixscience) and Sequest (Thermo) search engines and the ProteomeDiscoverer (Thermo) Interface. Search parameters were set very stringently (FDR < 1%). The proteins identified are presented in Tables 2, 3 and 4 for conditioned medium from AVDP2, AVDF4 and AVDS4 respectively.

Table 2: Proteins identified in AVDP2 conditioned basal cell culture medium

Identified proteins		
45 kDa calcium-binding protein	Filamin-A	Phosphoglycerate kinase 1
6-phosphogluconolactonase	Filamin-B	Phosphoglycerate mutase 1
72 kDa type IV collagenase	Filamin-C	Phosphoserine aminotransferase
Abhydrolase domain-containing protein 14B	FK506-binding protein 9	Pigment epithelium-derived factor
Adipocyte enhancer-binding protein 1	Fructose-bisphosphate aldolase A	Plasminogen activator inhibitor 1
Alcohol dehydrogenase [NADP+]	Galectin-1	Plectin-1
Aldose reductase	Galectin-3-binding protein	Polypeptide N-acetylgalactosaminyltransferase 10
Alpha-2-macroglobulin	Gamma-glutamyl hydrolase	Polypeptide N-acetylgalactosaminyltransferase 2
Alpha-actinin-4	Gelsolin	Polypeptide N-acetylgalactosaminyltransferase 5
Alpha-enolase	Glia-derived nexin	Pregnancy-specific beta-1-glycoprotein 5
Annexin A1	Glucose-6-phosphate isomerase	Procollagen C-endopeptidase enhancer 1
Aspartate aminotransferase, cytoplasmic	Glutathione S-transferase P	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1

Table 2 (Continued)

ATP synthase subunit beta, mitochondrial	Glutathione transferase omega-1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2
Basement membrane-specific heparan sulfate proteoglycan core protein	Glyceraldehyde-3-phosphate dehydrogenase	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3
Bifunctional aspartokinase/homoserine dehydrogenase 1	Glycyl-tRNA synthetase	Profilin-1
Biglycan	Heat shock 70 kDa protein 6	Protein disulfide-isomerase A6
Biliverdin reductase A	Heat shock protein beta-1	Protein S100-A6
Cadherin-11	Heme-binding protein 2	Purine nucleoside phosphorylase
Calsyntenin-1	HLA class I histocompatibility antigen, alpha chain G	Putative heterogeneous nuclear ribonucleoprotein A1-like protein 3
Calumenin	Hyaluronan and proteoglycan link protein 1	Putative nucleoside diphosphate kinase
Carbonyl reductase [NADPH] 1	Inactive serine protease RAMP	Putative quinone oxidoreductase
Cartilage oligomeric matrix protein	Insulin-like growth factor-binding protein 2	Pyruvate kinase isozymes M1/M2
Cathepsin B	Insulin-like growth factor-binding protein 3	Quinone oxidoreductase
Cathepsin Z	Insulin-like growth factor-binding protein 4	Rab GDP dissociation inhibitor alpha
CD109 antigen	Insulin-like growth factor-binding protein 6	Rab GDP dissociation inhibitor beta
CD166 antigen	Insulin-like growth factor-binding protein 7	Ras GTPase-activating-like protein IQGAP1
Chloride intracellular channel protein 1	Integrin beta-like protein 1	Reticulocalbin-1
Chondroitin sulfate proteoglycan 4	Interstitial collagenase	Rho GDP-dissociation inhibitor 1

Table 2 (Continued)

Clusterin	Keratin, type I cytoskeletal 9	Scavenger receptor cysteine-rich domain-containing protein LOC284297
Collagen alpha-1(I) chain	Kinesin-like protein KIF7	Semaphorin-7A
Collagen alpha-1(III) chain	Lactoylglutathione lyase	Serine protease HTRA1
Collagen alpha-1(IV) chain	Lamin-A/C	Serpin B6
Collagen alpha-1(V) chain	Laminin subunit beta-1	Serpin B7
Collagen alpha-1(VI) chain	Latent-transforming growth factor beta-binding protein 2	Serum albumin
Collagen alpha-1(VII) chain	Latent-transforming growth factor beta-binding protein, isoform 1S	S-formylglutathione hydrolase
Collagen alpha-1(XI) chain	Leucine-rich repeat-containing protein 15	Stanniocalcin-2
Collagen alpha-1(XII) chain	Leucine-rich repeat-containing protein 40	Stromelysin-1
Collagen alpha-2(I) chain	L-lactate dehydrogenase A chain	Sulfhydryl oxidase 1
Collagen alpha-2(IV) chain	L-lactate dehydrogenase B chain	Target of Nesh-SH3
Collagen alpha-2(V) chain	Lumican	Thioredoxin domain-containing protein 5
Collagen alpha-2(VI) chain	Lysyl oxidase homolog 2	Thioredoxin-dependent peroxide reductase, mitochondrial
Collagen alpha-3(VI) chain	Malate dehydrogenase, cytoplasmic	Thrombospondin-1
Complement C1q tumor necrosis factor-related protein 5	Malate dehydrogenase, mitochondrial	Transaldolase
C-type lectin domain family 11 member A	Mammalian ependymin-related protein 1	Transforming growth factor-beta-induced protein ig-h3
Cystatin-C	Matrix-remodeling-associated protein 8	Transketolase
Dickkopf-related protein 3	Metalloproteinase inhibitor 1	Trypsin-3
Dihydropyrimidinase-related protein 2	Mitochondrial fission factor homolog A	Tryptophanyl-tRNA synthetase, cytoplasmic
DNA-(apurinic or apyrimidinic site) lyase	Moesin	Ubiquinone biosynthesis protein COQ7 homolog
EGF-containing fibulin-like extracellular matrix protein 2	Neuropilin-1	Ubiquitin carboxyl-terminal hydrolase isozyme L1

Table 2 (Continued)

Endoplasmic reticulum aminopeptidase 1	Nucleobindin-1	Ubiquitin-like modifier-activating enzyme 1
Endoplasmic reticulum protein ERp29	Olfactomedin-like protein 2B	Uncharacterized metallophosphoesterase CSTP1
Endosialin	Olfactomedin-like protein 3	Urokinase-type plasminogen activator
Extracellular matrix protein 1	Out at first protein homolog	Vasorin
F-actin-capping protein subunit alpha-1	Peptidyl-prolyl cis-trans isomerase B	Versican core protein
Fascin	Peroxidasin homolog	Vimentin
Fibrillin-1	Peroxiredoxin-1	Vinculin
Fibronectin	Peroxiredoxin-4	WD repeat-containing protein 1
Fibulin-1	Peroxiredoxin-6	Xaa-Pro dipeptidase
Filaggrin-2	Phosphatidylethanolamine-binding protein 1	Zinc finger protein 276

Table 3: Proteins identified in AVDF4 conditioned basal cell culture medium

Identified proteins		
2',3'-cyclic-nucleotide 2'-phosphodiesterase	Fibronectin	Plasminogen activator inhibitor 1
45 kDa calcium-binding protein	Fibulin-1	Plastin-3
60 kDa heat shock protein, mitochondrial	Filamin-A	Pregnancy-specific beta-1-glycoprotein 5
72 kDa type IV collagenase	Filamin-B	Procollagen C-endopeptidase enhancer 1
Aggrecan core protein	Filamin-C	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1
Aldose reductase	FK506-binding protein 10	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2
Alpha-2-macroglobulin	Fructose-bisphosphate aldolase A	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3
Alpha-actinin-4	Galectin-3-binding protein	Profilin-1

Table 3 (Continued)

Alpha-enolase	Gamma-glutamyl hydrolase	Prolyl 3-hydroxylase 1
Arylsulfatase B	Gelsolin	Protein BCCIP homolog
Aspartate aminotransferase, mitochondrial	Glucose-6-phosphate isomerase	Protein disulfide-isomerase A6
ATPase family AAA domain-containing protein 5	Heat shock protein beta-1	Protein ZNF750
Band 3 anion transport protein	Immunoglobulin superfamily containing leucine-rich repeat protein	Putative heterogeneous nuclear ribonucleoprotein A1-like protein 3
Basement membrane-specific heparan sulfate proteoglycan core protein	Inactive serine protease RAMP	Putative nucleoside diphosphate kinase
Biglycan	Insulin-like growth factor-binding protein 4	Pyruvate kinase isozymes M1/M2
Biotinidase	Insulin-like growth factor-binding protein 6	Rab GDP dissociation inhibitor alpha
Calsyntenin-1	Insulin-like growth factor-binding protein 7	Rab GDP dissociation inhibitor beta
Calumenin	Integrin beta-like protein 1	Ras GTPase-activating-like protein IQGAP1
Cartilage oligomeric matrix protein	Interstitial collagenase	Reticulocalbin-1
Cathepsin B	Laminin subunit alpha-4	Semaphorin-7A
Cathepsin Z	Laminin subunit beta-1	Serine protease HTRA1
CD109 antigen	Laminin subunit gamma-1	Serpin B6
Collagen alpha-1(I) chain	Latent-transforming growth factor beta-binding protein 2	Serpin B7
Collagen alpha-1(III) chain	Latent-transforming growth factor beta-binding protein, isoform 1S	Stanniocalcin-2
Collagen alpha-1(IV) chain	L-lactate dehydrogenase A chain	Stromelysin-1
Collagen alpha-1(V) chain	Lumican	Sulfhydryl oxidase 1
Collagen alpha-1(VI) chain	Lysyl oxidase homolog 2	Testican-1
Collagen alpha-1(XII) chain	Macrophage metalloelastase	Thioredoxin domain-containing protein 5

Table 3 (Continued)

Collagen alpha-2(I) chain	Malate dehydrogenase, cytoplasmic	Thrombospondin-1
Collagen alpha-2(V) chain	Matrix-remodeling-associated protein 5	Thrombospondin-2
Collagen alpha-2(VI) chain	Matrix-remodeling-associated protein 8	Transaldolase
Collagen alpha-3(IV) chain	Metalloproteinase inhibitor 1	Transforming growth factor-beta-induced protein ig-h3
Collagen alpha-3(VI) chain	Moesin	Transgelin
Collagen triple helix repeat-containing protein 1	Nidogen-1	Transketolase
Complement C1r subcomponent	Olfactomedin-like protein 3	Tryptophanyl-tRNA synthetase, cytoplasmic
Complement C1s subcomponent	Olfactory receptor 5C1	Tyrosine-protein kinase receptor UFO
Dickkopf-related protein 3	Out at first protein homolog	Ubiquitin carboxyl-terminal hydrolase isozyme L1
Dihydrolipoyl dehydrogenase, mitochondrial	Peptidyl-prolyl cis-trans isomerase B	Ubiquitin-like modifier-activating enzyme 1
Dihydropyrimidinase-related protein 2	Periostin	Vasorin
EGF-containing fibulin-like extracellular matrix protein 2	Peroxiredoxin-1	Vimentin
Endoplasmic reticulum protein ERp29	Phosphoglycerate kinase 1	Vinculin
Extracellular matrix protein 1	Phosphoglycerate mutase 1	Vitronectin
Fascin	Phosphoserine aminotransferase	WD repeat-containing protein 1
Fibrillin-1	Pigment epithelium-derived factor	

Table 4: Proteins identified in AVDS4 conditioned basal cell culture medium

Identified proteins		
45 kDa calcium-binding protein	Extracellular matrix protein 1	Nucleobindin-2
60 kDa heat shock protein, mitochondrial	Ezrin	Olfactomedin-like protein 3
72 kDa type IV collagenase	Fascin	Peptidyl-prolyl cis-trans isomerase B

Table 4 (Continued)

78 kDa glucose-regulated protein	Fibrillin-1	Periostin
Adenylyl cyclase-associated protein 1	Fibrillin-2	Peroxidasin homolog
Aldose reductase	Fibronectin	Peroxiredoxin-1
Alpha-2-macroglobulin	Fibulin-1	Peroxiredoxin-6
Alpha-actinin-4	Filamin-A	Phosphoglycerate kinase 1
Alpha-enolase	Filamin-B	Phosphoglycerate mutase 1
Alpha-N-acetylglucosaminidase	Filamin-C	Phosphoserine aminotransferase
Aspartate aminotransferase, cytoplasmic	Fructose-bisphosphate aldolase A	Pigment epithelium-derived factor
Aspartate aminotransferase, mitochondrial	Fructose-bisphosphate aldolase C	Plasminogen activator inhibitor 1
Band 3 anion transport protein	Galectin-1	Platelet-derived growth factor D
Basement membrane-specific heparan sulfate proteoglycan core protein	Galectin-3-binding protein	Plectin-1
Beta-1,4-galactosyltransferase 5	Gamma-glutamyl hydrolase	Polypeptide N-acetylgalactosaminyltransferase 10
Biglycan	Gelsolin	Polypeptide N-acetylgalactosaminyltransferase 2
Biotinidase	Glia-derived nexin	Procollagen C-endopeptidase enhancer 1
Cadherin-11	Glucose-6-phosphate isomerase	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1
Cadherin-2	Glutathione S-transferase P	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2
Calsyntenin-1	Glutathione transferase omega-1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3
Calumenin	Glyceraldehyde-3-phosphate dehydrogenase	Profilin-1
Cartilage oligomeric matrix protein	Glycyl-tRNA synthetase	Prolyl 3-hydroxylase 1
Cathepsin B	G-protein coupled receptor 143	Protein disulfide-isomerase A6

Table 4 (Continued)

Cathepsin D	Heat shock 70 kDa protein 6	Protein ZNF750
Cathepsin Z	Heat shock protein beta-1	Putative heterogeneous nuclear ribonucleoprotein A1-like protein 3
CD109 antigen	Hyaluronan and proteoglycan link protein 1	Putative nucleoside diphosphate kinase
CD166 antigen	Ig gamma-1 chain C region	Pyruvate kinase isozymes M1/M2
CD44 antigen	Immunoglobulin superfamily containing leucine-rich repeat protein	Rab GDP dissociation inhibitor alpha
Chloride intracellular channel protein 1	Inhibin beta A chain	Rab GDP dissociation inhibitor beta
Collagen alpha-1(I) chain	Insulin-like growth factor-binding protein 4	Ras GTPase-activating-like protein IQGAP1
Collagen alpha-1(III) chain	Insulin-like growth factor-binding protein 6	Reticulocalbin-1
Collagen alpha-1(IV) chain	Insulin-like growth factor-binding protein 7	Semaphorin-7A
Collagen alpha-1(V) chain	Integrin beta-like protein 1	Serine protease HTRA1
Collagen alpha-1(VI) chain	Interstitial collagenase	Serpin B6
Collagen alpha-1(VII) chain	Lamin-A/C	Serpin B7
Collagen alpha-1(XI) chain	Latent-transforming growth factor beta-binding protein 2	Stanniocalcin-2
Collagen alpha-1(XII) chain	Latent-transforming growth factor beta-binding protein, isoform 1S	Stromelysin-1
Collagen alpha-2(I) chain	Legumain	Sulfhydryl oxidase 1
Collagen alpha-2(IV) chain	Leucine-rich repeat-containing protein 15	Testican-1
Collagen alpha-2(V) chain	L-lactate dehydrogenase A chain	Thioredoxin domain-containing protein 5
Collagen alpha-2(VI) chain	Lumican	Thrombospondin-1
Collagen alpha-3(VI) chain	Lysyl oxidase homolog 2	Thrombospondin-2
Complement C1r subcomponent	Macrophage mannose receptor 2	Transaldolase
C-type lectin domain family 11 member A	Macrophage migration inhibitory factor	Transforming growth factor-beta-induced protein ig-h3

Table 4 (Continued)

Cyclin-A2	Malate dehydrogenase, cytoplasmic	Transgelin
Cystatin-C	Malate dehydrogenase, mitochondrial	Transketolase
Decorin	Matrix-remodeling-associated protein 5	Tryptophanyl-tRNA synthetase, cytoplasmic
Dickkopf-related protein 3	Metalloproteinase inhibitor 1	Tyrosine-protein kinase receptor UFO
Dihydrolipoyl dehydrogenase, mitochondrial	Microfibrillar-associated protein 2	Ubiquitin carboxyl-terminal hydrolase isozyme L1
Dipeptidyl-peptidase 3	Microfibrillar-associated protein 5	Ubiquitin-like modifier-activating enzyme 1
DnaJ homolog subfamily A member 4	Moesin	Vasorin
Dystroglycan	Moesin/ezrin/radixin homolog 1	Vimentin
EGF-containing fibulin-like extracellular matrix protein 2	Neuropilin-1	Vinculin
Elongation factor 1-gamma	Nidogen-1	Vitronectin
Endoplasmic reticulum protein ERp29	Nuclear receptor coactivator 5	WD repeat-containing protein 1
Endosialin	Nucleobindin-1	

Surprisingly a total of 177 different human proteins were identified in conditioned basal cell culture medium from cell line AVDP2, 131 different human proteins in conditioned basal medium from cell line AVDF4 and 167 different human proteins in conditioned basal medium from cell line AVDS4. This was particularly un-expected given the relatively short incubation period and basal cell culture medium used to produce the conditioned basal cell culture medium. The cells described in this document can therefore be used as sources of any or all of these proteins, or any proteins or other molecules which are secreted or expressed by them. Those skilled in the art will appreciate the analysis indicates the presence of proteins well established in the art as important for wound healing and will appreciate that new proteins have been identified. It will be evident to those skilled in the art how the differences between the three cell lines could be used to produce different physical embodiments of the conditioned basal cell culture medium or how the conditioned basal cell culture medium may be further processed to concentrate or reduce one or more factors or components, for example using ultrafiltration, diafiltration or chromatographic purification.

Example 3

A 225cm² cell culture flask (Nunc) of dermal fibroblast cells AVDF3 grown in static culture conditions were detached and cell number determined using methods well described in the art. 2.3 x 10⁶ cells were used to seed a 1.5L cell culture spinner flask containing 1.5g/L CultiSpher S microcarriers (prepared as described by the manufacturer) in a total volume of 330ml of serum free growth medium supplemented with 2mM glutamine (Sigma). The headspace of the spinner flask was equilibrated with 5% CO₂, 2% O₂ gas. The spinner flask was transferred to a cell culture incubator at 37°C and agitated at 35rpm using a magnetic stirrer base. After 4 days incubation under the conditions described 35ml of cell culture supernatant was removed from the spinner flask and replaced with fresh serum free growth medium (as described above). On day 5 and day 7 incubation, 50ml of culture supernatant was removed and replaced with fresh serum free medium as described above. On day 8, 80ml of culture supernatant was removed and replaced with fresh serum free medium. After a total of 10 days in culture the AVDF3 cells were detached from the microcarriers using methods well established in the art. 1.35 x 10⁷ cells were used to inoculate a glass cell culture bioreactor (Applikon) in a total volume of 2L of serum free growth medium supplemented with 2mM glutamine, 0.2% Pluronic F-68 and 1.5g/L CultiSpher S microcarriers (prepared as described previously). The bioreactor was cultured at a temperature of 36.5°C, pH 7.0 (manual control by carbon dioxide gas sparging and/or addition of sodium hydroxide), dissolved oxygen tension 5.0% (air saturation) and an agitator speed of 40rpm which was increased gradually to 60rpm over the course of the culture. The dissolved oxygen level in the cell culture was maintained using CO₂ and N₂ gas sparging. Emulsion C antifoam agent (Sigma) was added to the bioreactor when foaming was observed. After 4 days incubation under the conditions described, 200ml of culture supernatant was removed from the bioreactor and replaced with fresh serum free growth medium (as described above). A further 200ml of culture supernatant was removed and replaced with fresh serum free growth medium (as described above) on days 6, 8, 10,11,13, 15, 17 and 21. After 17 days of growth, 200ml of culture (medium and microcarriers with cells attached) was harvested aseptically. The harvested culture was aliquoted equally into four 50ml conical sample tubes and the microcarriers with cells attached were allowed to sediment to the base of the sample tube under gravity. Microcarrier free culture medium was carefully removed and the sedimented microcarriers with cells attached were washed first 3x with PBS and a further 3x with MEM (no FBS) to remove traces of spent growth medium from the original cell culture. The microcarriers with cells attached were pooled into a final volume of 45ml of

MEM (no FBS) + 2mM glutamine. This suspension was used to seed 3 x E125 shake flasks, with approximately 2×10^6 cells per flask. The headspace of the flask was equilibrated with 5% CO₂, 2% O₂ gas and transferred to an orbital shaker for 24 hours at 37°C, 60rpm. The conditioned basal medium was harvested from each flask, filtered
5 using a 0.2µm filter, and stored at -20°C prior to analysis.

Example 4

Two 225cm² cell culture flasks (Nunc) of dermal fibroblast cells AVDP3 grown in static culture conditions were detached and counted using methods well described in the art.
10 2.4×10^6 cells were used to seed a 1.5L cell culture spinner flask containing 1.5g/L CultiSpher S microcarriers (prepared as described previously) in a total volume of 300ml of MesenPro growth medium (low serum, Invitrogen) supplemented with 4mM glutamine. The headspace of the spinner flask was equilibrated with 5% CO₂, 2% O₂ gas. The spinner flask was transferred to a cell culture incubator at 37°C and agitated at 35rpm
15 using a magnetic stirrer base. After 3 days incubation under the conditions described 80ml of cell culture supernatant was removed from the spinner flask and replaced with fresh growth medium (as described above). After a total of 9 days incubation under the conditions described, a 10ml sample was taken from the spinner flask and cell number and cell viability determined using standard methods well described in the art. The cell
20 number and cell viability was used to estimate the total viable cell number retained in the spinner flask attached to the microcarriers. The microcarriers with cells attached from the spinner flask were washed using PBS as described previously then suspended in 75ml MEM (no FBS) and used to seed one 250ml shake flask with approximately 2.5×10^7 cells attached to microcarriers. The headspace of the flask was equilibrated with 5% CO₂, 2%
25 O₂ gas and transferred to an orbital shaker for 24 hours at 37°C, 60rpm. The conditioned basal cell culture medium was harvested from the flask, filtered using a 0.2µm filter, and stored at -20°C prior to analysis.

Two 225cm² cell culture flasks (Nunc) of dermal fibroblast cells AVDS6 grown in static culture conditions were detached and counted using methods well described in the art.
30 2.4×10^6 cells were used to seed a 1.5L cell culture spinner flask containing 1.5g/L CultiSpher S microcarriers (prepared as described previously) in a total volume of 300ml of MesenPro growth medium (Invitrogen) supplemented with 4mM glutamine. The headspace of the flask was equilibrated with 5% CO₂, 2% O₂ gas. The spinner flask was transferred to a cell culture incubator at 37°C and agitated at 35rpm using a magnetic
35 stirrer base. After 3 days incubation under the conditions described 80ml of cell culture

supernatant was removed from the spinner flask and replaced with fresh growth medium (as described above). After a total of 9 days incubation under the conditions described, a 10ml sample was taken from the spinner flask and cell number and cell viability determined using standard methods well described in the art. The cell number and cell viability was used to estimate the total viable cell number retained in the spinner flask. The microcarriers with cells attached were washed using PBS as described previously then pooled in a final volume of 54ml MEM (no FBS) and used to seed one 250ml shake flask with approximately 1.8×10^7 cells attached to microcarriers. The headspace of the flask was equilibrated with 5% CO₂, 2% O₂ gas and transferred to an orbital shaker for 24 hours at 37°C, 60rpm. The conditioned basal medium was harvested from the flask, filtered using a 0.2µm filter, and stored at -20°C prior to analysis.

Example 5

Conditioned medium from AVDF4, AVDS4 and AVDP2 (prepared as described in Examples 1 and 2), AVDF3 (prepared as described in Example 3) and AVDS6 and AVDP3 (prepared as described in Example 4) were analysed using SearchLight Array Technology (Aushon Biosystems Inc.). MEM and MEM + 10% FBS growth media were included as controls. Aushon SearchLight Protein Array Technology is a multiplexing sandwich-ELISA system based on chemiluminescent or fluorescent detection of analytes whose respective capture-antibodies are spotted in arrays within each well of a 96-well microplate. Up to 16 analytes (4 x 4 array in each well) can be measured per well, thus 16 cytokines or other biomarkers can be assayed simultaneously with each sample (50µl). Samples were assayed for TGFβ-1, IL-6, IL-8 and PAI-1 levels. The concentrations of each protein identified in the conditioned medium/conditioned basal medium samples are presented in Figures 4, 5, 6 and 7. Samples are designated numbers 1 to 10 and the identity of these samples is shown in Table 5.

Table 5: Sample Identities

Sample number	Cell line designation	Conditioned medium production method
1	MEM control	-
2	AVDF3	Example 3
3	AVDS6	Example 4
4	AVDP3	Example 4
5	AVDS4	Example 1
6	AVDF4	Example 1
7	AVDP2	Example 2
8	AVDS4	Example 2
9	AVDF4	Example 2
10	MEM + 10% FBS control	-

- 5 The data presented in Figures 4-7 exemplify that key proteins involved in wound healing (TGF β -1, IL-6, IL-8 and PAI-1) can be detected and quantified in conditioned media from the three novel cell types AVDS, AVDP and AVDF. The levels of proteins in the conditioned cell culture medium or conditioned basal cell culture medium can be varied by adjusting the cell concentration used and/or the growth medium composition and/or the
- 10 cell culture system. It will be also be evident to those with skill in the art how further development of the cell culture growth conditions, cell line used can be carried out to increase cell number attached to the microcarriers and how this will influence secretion of proteins when producing conditioned basal cell culture medium. It will be evident to those skilled in the art that the data generated using the 2L stirred tank cell culture bioreactor
- 15 using cells attached to microcarriers demonstrates production of conditioned basal medium that is a scaleable and economic manufacturing system for the large scale production of the physical embodiments of the present invention. It will be apparent to those skilled in the art that on completion of a microcarrier based bioreactor process to expand cells that microcarriers can be sedimented, conditioned medium harvested and/or
- 20 cells attached to microcarriers washed in-situ and incubated with basal cell culture medium. The conditioned basal medium can be easily be harvested by in-situ sedimentation of the microcarriers (gravity sedimentation) and decanting of cell free conditioned basal cell culture medium.

Example 6

Conditioned medium from AVDF4, AVDS4 and AVDP2 (prepared as described in Examples 1 and 2), AVDF3 (prepared as described in Example 3) and AVDS6 and AVDP3 (prepared as described in Example 4) were analysed by western blotting as is well established in the art. Briefly, samples were reduced and run on 4-12% BisTris gels (Invitrogen) with SeeBlue molecular weight marker (Invitrogen) using MES running buffer. The samples were then transferred to PVDF membranes. After blotting, the membrane was incubated in 15ml of blocking buffer (PBS + 1% BSA) on a rocking platform for 1 hour at room temperature. The blocking buffer was decanted and the membrane was incubated in 8ml of 1/2000 dilution of mouse monoclonal anti-SPARC antibody (Sigma WH0006678M2) at 4°C overnight. The membrane was then washed three times in 15ml PBS + 0.05% Tween 20 each for 5 minutes at room temperature on a rocking platform. 8ml of 1/10,000 dilution of rabbit anti-mouse IgG (whole molecule)-peroxidase (Sigma A9044) was added and incubated at room temperature on a rocking platform for 1 hour. The membrane was then washed three times in 15ml PBS + 0.05% Tween 20 each for 5 minutes at room temperature on a rocking platform. The membrane was developed by incubating in SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma D4418) dissolved in 15ml of water for 15 minutes at room temperature on a rocking platform. The results of the western blot is presented in Figure 8.

Conditioned media from all three cell lines AVDS, AVDP and AVDF expanded using cell culture flasks, spinner flask and bioreactor indicate the secretion and accumulation of SPARC protein.

Example 7

Conditioned media from AVDF4, AVDS4 and AVDP2 (prepared as described in Examples 1 and 2), AVDF3 (prepared as described in Example 3) and AVDS6 and AVDP3 (prepared as described in Example 4) were analysed by dot blot (as is well established in the art) for secretion and accumulation of fibronectin and collagen proteins. The PVDF membranes were wetted with methanol and then soaked in PBS, 10µl of each sample and controls were spotted onto on the membranes. 50ng and 5ng of standard (fibronectin – Sigma F1141 or collagen – Sigma C8919) was also spotted onto the membrane and all spots were allowed to air dry for 1 hour. The membranes were then wetted again in methanol, rinsed in PBS and then incubated overnight in 8ml of blocking buffer (PBS + 1% BSA) at 4°C. The blocking buffer was decanted and the membranes were incubated in 4ml of 1/200 dilution of either mouse monoclonal anti-fibronectin antibody (Sigma F7387)

or mouse monoclonal anti-collagen antibody (Sigma C2456) for 2 hours at room temperature on a rocking platform. The membranes were then washed three times in 8ml PBS + 0.05% Tween 20 each for 5 minutes at room temperature on a rocking platform. 4ml of 1/2000 dilution of rabbit anti-mouse IgG (whole molecule)–peroxidase (Sigma A9044) was added and incubated at room temperature on a rocking platform for 1 hour. The membranes were then washed three times in 8ml PBS + 0.05% Tween 20 each for 5 minutes at room temperature on a rocking platform. The membranes were developed by incubating in SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma D4418) dissolved in 15ml of water for 15 minutes at room temperature on a rocking platform. The results of the dot blots are presented in Table 6.

Conditioned media from all three cell lines AVDS, AVDP and AVDF expanded using cell culture flasks, spinner flask and bioreactor indicate the secretion and accumulation of collagen protein.

Table 6: Analysis of cell growth conditioned cell culture medium and conditioned basal cell culture medium for the presence of collagen and fibronectin

Sample	Conditioned medium preparation	Spot intensity relative to 50ng standard (+++)	
		Collagen	Fibronectin
50ng standard	-	+++	+++
MEM control	-	ND	ND
AVDS4	Example 1	+	+++
AVDF4	Example 1	+	++++
AVDP2	Example 1	++	++++
AVDS4	Example 2	(1)	++
AVDF4	Example 2	+	++++
AVDF3	Example 3	++++	++++
AVDS6	Example 4	++	+++
AVDP3	Example 4	+++	+++
MEM + 10% FBS		ND	+

ND= not detected

(1) = dot masked by FBS in sample. LC-MS-MS analysis (Example 2) indicates secretion and accumulation of collagen.

Claims

1. A process for preparing a conditioned cell culture medium comprising:
 - a) culturing eukaryotic cells in a growth medium having a composition effective to support cell growth;
 - b) separating the cultured cells from the growth medium;
 - c) maintaining the cultured cells in a basal medium having a composition suitable to maintain cell viability, but not to support substantial cell growth.
2. A process according to claim 1, where the cells are dermal sheath, dermal papilla or dermal fibroblast cells.
3. A process according to either preceding claim, wherein the basal medium is protein-free prior to introduction of the cultured cells.
4. A process according to any preceding claim, wherein the cells are cultured attached to a microcarrier.
5. A process according to any preceding claim, wherein the cells are washed after separation from the growth medium and prior to introduction into the basal medium.
6. A process according to any preceding claim, wherein the product of step c) is subsequently subjected to one or more of the following processes:
 - a) partial or complete purification;
 - b) freezing;
 - c) lyophilisation; and
 - d) drying.
7. A composition produced by a process according to any preceding claim.
8. A pharmaceutical composition produced by a process according to any one of claims 1 to 6.

9. A composition according to claim 8 which comprises one or more of IL-6, Gro- α , SDF-1, FGF-2, SPARC, PAI-1, IL-8, Collagen, Fibronectin, I-309, IL-13, MIF and SDF-1 and TGF- β proteins.
- 5 10. Use of a pharmaceutical composition according to claim 8 or 9 for wound healing.
11. A pharmaceutical composition comprising conditioned cell culture medium obtained by a) culturing differentiated human cells retaining stem cell potential selected from the group consisting of dermal sheath cells, dermal fibroblast cells or
10 dermal papilla cells in a growth medium; and b) separating the culture medium from the cells.
12. A composition according to claim 11 which comprises one or more of Gro- α , I-309, IL-6, IL-8, IL-13, MIF, PAI-1, SDF-1 and TGF- β proteins.
15
13. A composition according to claims 11 or 12, which has been further subjected to one or more of the following processes:
a) partial or complete purification;
b) freezing;
20 c) lyophilisation; and
d) drying.
14. Use of a composition according to any one of claims 11 to 13 for wound healing.
- 25 15. Use of a composition according to any one of claims 8, 9, 11, 12 or 13 as a medicament.