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(54) Title: EXPANSION METHOD

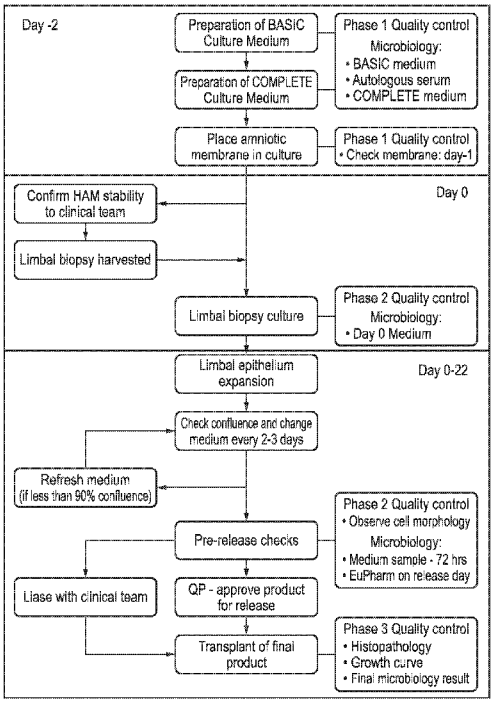

FIG. 1

(57) Abstract: The invention provides a method for expansion of limbal stem cells, comprising:(i) providing a limbal biopsy on a growth support;(ii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and(iii) substituting the epithelial culture medium of step ii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period.

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Expansion method

Background

The cornea is the clear dome-shaped window at the front of the eye, and its clarity and regular surface is vital for the transmission and focusing of light onto the retina, allowing accurate visual perception. Corneal disease represents the second most common cause of world blindness after cataract [Chee KY et al., Clin Exp Opthalmol (2006); 34:64–73]. The cornea is covered by a stratified squamous epithelium which serves the dual function of protection of the ocular surface and also contributing to high quality vision. The corneal epithelium is continuous with that of the surrounding conjunctiva, the transition between the two epithelia being formed by the limbal epithelium. There is now a substantial body of evidence pointing to the basal layer of the limbal epithelium as the location for corneal epithelial stem cells, also known as limbal stem cells (LSCs) [Ahmad S et al., Regen Med (2006); 1:29-44; Chee KY et al., Clin Experiment Ophthalmol (2006); 34:64-73].

The population of LSCs are believed to be responsible for the regenerative function allowing the maintenance of the corneal epithelium and for the barrier function against the migration of conjunctival cells onto the cornea [Dua et al., Surv Ophthalmol (2000);44:415-425]. Significant loss of or damage to LSCs or their niche/stromal microenvironment leads to limbal stem cell deficiency (LSCD). LSCD is characterised by recurrent breakdown of the epithelium, vascularisation, destruction of the corneal epithelium basement membrane, corneal scar formation, chronic inflammation and conjunctivalisation of the corneal surface, which eventually leads to significant/persistent ocular pain and blindness [Osei-Bempong et al, Bioessays 365: 211-219 (2012)]. Corneal vascularisation and opacity have been estimated to cause blindness in eight million people worldwide each year (10% of total blindness), with various forms of LSCD contributing to this total.

Following diagnosis of LSCD, the treatment options depend on both the extent of the LSCD (i.e. partial or total) and whether the condition is unilateral or bilateral. In partial LSCD, functioning LSCs are still present in limited numbers, and when the visual axis is covered with normal corneal epithelium and the patient is relatively asymptomatic, with good vision, LSCD is typically managed using medical treatment only. However, in partial LSCD where there is central corneal involvement, with consequent decreased

vision, significant irritation and persistent epithelial defect then surgical management, including sequential epithelial removal combined with amniotic membrane transplantation, may be required. In total LSCD, where there is no evidence of functioning LSCs, the only available treatment is surgical and involves a stem cell therapy that allows for replacement of the damaged or absent LSC population.

The standard treatment for unilateral total LSCD involves transplanting large, whole tissue limbal grafts from the patient's healthy fellow eye [Kenyon and Tseng, Opthalmology (1989); 96;709-723]. However, this treatment method puts the patient's healthy donor eye at potential risk of developing iatrogenic LSCD and limits the scope for obtaining additional grafts following any complications with the transplantation procedure, including failure. Additionally, this treatment option is not available in the subset of patients experiencing bilateral LSCD. Although obtaining limbal allografts from living related or cadaveric donors is possible, it necessitates the use of high dose, systemic immunosuppression along with its associated health risks and long-term graft survival has been demonstrated to be poor (Shortt et al, Stem Cells Trans Med. (2014) 3: 1-11).

Recently, successful treatment of unilateral LSCD and partial bilateral LSCD (i.e. at least one eye is not totally LSCD) has been achieved by the transplantation of *ex vivo* expanded autologous LSCs taken from a small limbal biopsy from the healthy contraleteral eye [Pellegrini et al., Lancet (1997); 349:990-993]. The requirement of much smaller amounts of tissue for the *ex vivo* expansion process results in four main advantages to patients. Firstly, the small amount of tissue required for *ex vivo* expansion is much less likely to damage the LSC population of the healthy donor eye than previously used autologous whole tissue grafts that required large quantities of limbal tissue for direct transplantation [Jenkins et al., Eye (1993); 7:629-633]. Secondly, the small biopsies needed can be taken from the fellow eye of the patient with LSCD, even if the disease is bilateral to an extent, providing that there are remaining areas of healthy limbus in one eye [Sangwan et al., Cornea (2003); 22:478-481]. Additionally, using autologous donor tissue eliminates the requirement of systemic immunosuppression, compared to previously used whole tissue allografts [Holland et al., Opthalmology (2003); 110:125-130]. Thirdly, if a biopsy does not grow in culture, it is safe and acceptable to harvest another biopsy from the fellow (donor) eye.

The *ex vivo* expansion of LSCs prior to transplantation typically uses heterologous, non-human animal-derived cells and products, such as a mouse 3T3 fibroblast cell feeder layer for co-culture and fetal calf serum (FCS) in the growth medium. The use of these non-human animal-derived products in LSC expansion has several drawbacks. Firstly, such a transplant would potentially be a xenograft and, as such, the patient may require immunosuppression to prevent rejection of the tissue. Secondly, the use of non-human animal-derived products in tissue destined for human transplantation has the potential to result in interspecies pathogen transfer. This latter risk would be further augmented on a background of immunosuppression.

One potential solution to these problems is the *ex vivo* expansion of LSCs using a completely non-human animal product-free system. Successful transplantation of cells cultured in the presence of human autologous serum (HAS) as a replacement for FCS has been reported [Kolli et al., Stem Cells (2009); 28:597-610].

There remains a need for an improved *ex vivo* LSC expansion method that provides cells suitable for direct transplantation into a patient and which does not suffer from the disadvantages of the prior art.

Brief summary of the disclosure

In a first aspect the invention provides a method for expansion of limbal stem cells, comprising:

i) providing a limbal biopsy on a growth support;

ii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and

iii) substituting the epithelial culture medium of step ii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period.

Preferably the method is free from non-human animal derived products.

In one embodiment the growth support is human amniotic membrane. Preferably, the human amniotic membrane is intact membrane. Preferably, a stromal surface of the limbal biopsy is in contact with the growth support.

In one embodiment, the culture medium comprises at least one growth factor. Preferably, the at least one growth factor comprises insulin, hydrocortisone, tri-iodothyronine, adenine, epidermal growth factor.

In one embodiment, the epithelial culture medium comprises cholera toxin.

In one embodiment, the one or more antibiotics comprises two antibiotics, preferably penicillin and streptomycin.

In one embodiment, the serum is derived from the same donor as the limbal biopsy.

In one embodiment, the expanded limbal stem cells form an epithelial sheet on the growth support.Preferably, the limbal stem cells are expanded for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support. Preferably, the epithelial sheet is at least (90% of 1.5cm^2), and preferably from about (90% of 1.5cm^2) to about (90% of 3.5cm^2).

In a further aspect the invention provides a method of treating limbal stem cell deficiency comprising:

i) isolating a limbal biopsy from a patient in need of treatment;
ii) placing the limbal biopsy on a growth support;
iii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and
iv) substituting the epithelial culture medium of step iii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support; and
v) transplanting the composite structure onto an eye of the patient.

In a further aspect the invention provides a method for expansion of limbal stem cells, comprising:

i) providing a limbal biopsy on a growth support;
ii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium; and

iii) substituting the epithelial culture medium of step ii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period.

In a still further aspect the invention provides a method of treating limbal stem cell deficiency comprising:
i) isolating a limbal biopsy from a patient in need of treatment;
ii) placing the limbal biopsy on a growth support;
iii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium; and
iv) substituting the epithelial culture medium of step iii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support; and
v) transplanting the composite structure onto an eye of the patient.

In a still further aspect the invention provides a composite structure comprising an epithelial sheet upon the growth support obtainable by any one of the aforementioned methods.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of them mean “including but not limited to”, and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Various aspects of the invention are described in further detail below.

Brief summary of the drawings

Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

Figure 1 provides a schematic representation of a Manufacturing method.

Figure 2 provides a schematic diagram to represent the limbal explant with human amniotic membrane (HAM) co-culture technique. (a) The limbal explant is placed stromal side down on the intact HAM. (b) According to current understanding, the limbal epithelium would be expected to proliferate and spread onto the HAM. The HAM would support the continuing proliferation and maintenance of the progenitor characteristics of the expanded limbal epithelium.

Figure 3 illustrates human limbal explant cultures on human amniotic membrane. A. Macroscopic picture of culture showing two inner rings indicating previous days growths and the present outgrowth as the outer ring. B. Phase contrast micrograph of the culture showing the explant in the upper right corner and the epithelial outgrowth on the amniotic membrane. Scale-bar = 200 µm.

Figure 4 illustrates explant outgrowths from limbal explants on human amniotic membrane using foetal calf serum and human serum. The outgrowth areas at weekly intervals were measured. The day of outgrowth is shown on the x-axis and the outgrowth area is shown on the y-axis.

Figure 5 illustrates outgrowth areas of four explant cultures.

Figure 6 illustrates outgrowth areas of explant cultures grown in medium previously stored at either 4°C or -20°C.

Figure 7 illustrates the effect of 4°C storage for 3 hours on explant outgrowth area.

Figure 8 provides a photograph of the limbal explant with 3T3 co-culture technique to show gross appearance (day 7). A photograph of the culture well with the explant culture is shown in the left panel. The corresponding greyscale image has been labelled to show the position of the explant (E) and the edge of the explant outgrowth (arrows) and the pre-plated 3T3 fibroblasts (3T3).

Figure 9 illustrates the microscopic appearance of limbal cultures using explant technique with inactivated 3T3 mouse fibroblast co-culture. The left column shows low magnification images of a single field on day 4 (appearance of definitive colonies) to day 16 (full confluence reaching edge of culture well)- Scale bar= 500µm. The right column shows a corresponding high magnification view of the culture during the same time period illustrating the maintenance of small tightly packed regular cells- Scale bar= 200 µm. The arrows indicate the edge of the outgrowth.

Figure 10 illustrates the macroscopic and microscopic appearances of explant culture on HAM. (a) Photograph of limbal epithelial outgrowths on HAM to show gross appearance (day 8). (b) The corresponding greyscale image has been labelled to show the edges of the outgrowths (indicated by red arrows). (c) Low power photomicrograph of limbal epithelial outgrowth. The edge of the outgrowth is indicated by the arrows. Scale bar=200µm (d) High power photomicrograph. Note that the regular tightly spaced round cells with little cytoplasm.

Figure 11 illustrates the fate of ex vivo expanded limbal epithelium using a limbal explant and HAM co-culture technique (in the absence of mouse 3T3 cells). The top panel is a low power montage showing a cross section of a whole culture system including explant, HAM and outgrowth. 3 sections of the montage are shown at higher magnification below. (i) The surface of the limbal stroma is covered in a stratified epithelium as expected. However, this epithelium has proliferated to travel down the sides of the explant and onto the surface of the HAM. The movement of the expanded epithelium is shown by the arrow. (ii) The majority of the HAM is covered by a uniform layer of small undifferentiated cells with high p63 expression. (iii) At the extreme periphery of the culture, the epithelium has begun to stratify and differentiate as seen by the lack of p63 staining in the superficial layers.

Figure 12 provides a panel showing the light microscopic and immunohistochemistry appearances for ex vivo expanded epithelium of patient 1. H & E staining reveals an epithelium with a basal layer of tightly packed cuboidal cells which express high levels of p63, ABCG2, Vimentin & Ki67. Conversely, the basal layer does not express high levels of CK3.

Figure 13 illustrates TEM of cultured epithelium from patient 1. (a) The ex vivo cultured epithelium (EE) sits on the HAM. The basal cells are much smaller and cuboidal (outer (larger) outline) with high N/C ratios (inner (smaller) outline) compared with the large

columnar cells of adult corneal epithelium. Areas of the expanded epithelium (shown by the dotted rectangles) were viewed at higher magnifications to reveal (b) the presence of microplicae (MP) on the superficial epithelial cells (c) the presence of desmosomes connecting the basal cells together and (d) hemidesmosomes connecting the basal cells to the basement membrane.

Figure 14 illustrates the impact of cholera toxin addition to the culture media. Two different explant pairs (R31 and R32 and R35 and R36) from one donor corneal ring were cultured in the absence (R31 and R35) and presence (R32 and R36) of cholera toxin. No significant changes were found in growth rate.

Detailed description

The inventors have investigated alternative methods for *ex vivo* expansion of LSCs.

The inventors have found a method for improving the quality and/or success and /or safety of a limbal stem cell transplant for direct transplantation into a patient. The inventors have identified the minimal requirements necessary for successful *ex vivo* expansion of LSCs, enabling direct transplantation of the resultant cells into the eye of a patient. Advantageously, the culture media and methods of the invention comprise minimal (preferably no) constituents that are detrimental to the overall success of subsequent transplantation procedure, or compromise safety of the product.

In one embodiment, the inventors have developed a novel method that comprises culturing a limbal biopsy for a first time period in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and substituting the epithelial culture medium with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period. The method of the invention therefore comprises at least one time period in which antibiotics are present in the culture medium and at least another (subsequent) time period in which the antibiotics are omitted from the culture medium.

The standard use of antibiotics in the culture of LSC has been accepted due to the risk of contamination with pathogens present in the non-sterile biopsy environment. However, the culture of LSCs in the presence of antibiotics may mask the detection of pathogen contamination by conventional screening techniques, leading to a false-

negative screening result, prior to transplantation. This poses a risk to the transplant recipient, as pathogens may be transferred to them during the transplantation procedure.

Rinsing a limbal stem cell in antibiotic-supplemented medium prior to being placed in culture in medium which is free from antibiotics has been reported [Zakaria et al., Tissue Engineering (2010); 16:921-927]. However, rinsing the limbal biopsy in antibiotic-supplemented medium may not be sufficient to kill or inactivate any pathogens present in the biopsy. The residual presence of contaminating pathogens, if detected, would render the resulting graft unsuitable for medical use and result in the wastage of valuable transplant material and the requirement for further biopsy collection, which has cost implications, presents a risk to the patient, and may not be possible in cases of severe bilateral LSCD. Grafts produced by such methods would be unlikely to comply with the requirements of regulatory products to gain clinical approval.

The method of the invention provides an improved *ex vivo* LSC expansion method that provides cells suitable for direct transplantation into a patient and which does not suffer from the disadvantages of the prior art. Culturing of the limbal biopsy for a first time period in the presence of epithelial culture medium supplemented with serum and one or more antibiotics removes the risk of contamination with pathogens present in the non-sterile biopsy environment, whereas substituting the epithelial culture medium with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period ensures that any (residual or new) pathogen contamination is not masked and can be detected by conventional screening techniques prior to transplantation.

Graft Production

The invention provides a method for expansion of limbal stem cells, comprising: providing a limbal biopsy on a growth support; culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and substituting the epithelial culture medium with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period.

As used herein, the terms "culture" and "cell culture" are used interchangeably and refer to the process whereby cells, preferably stem cells, are grown (e.g. divide) under controlled conditions, preferably *in vitro* or *ex vivo*.

The term "stem cell" is used herein to refer to immature cells having the capacity to self-renew and to differentiate into more mature cells. “Progenitor cells” also have the capacity to self-renew and to differentiate into more mature cells, but are committed to a lineage (e.g., limbal progenitors are committed to the ocular lineage), whereas stem cells are not necessarily so limited. For the purposes of this disclosure, progenitor cells can be interchangeably described as "stem cells" throughout the specification.

As used herein, the term “biopsy” refers to the isolation of cells from a piece or pieces of tissue. The term “biopsy” is used interchangeably with the term “explant” or “donor tissue”. In biopsy culture, tissue pieces themselves are cultured, where cells are left in their surrounding extracellular matrix to more accurately mimic the *in vivo* environment. Preferably the biopsy is from healthy donor tissue, and most preferably histocompatible with the recipient for the graft. The explant is preferably from an adult. The explant may be obtained from an animal (xenogenic cells). The explant may be from a living donor or cadaver (allogenic cells). Preferably the explant is from the proper patient / recipient and the method thus uses autologous cells as the explant. The use of autologous explants is free from some of the disadvantages of the other methods of cell therapy, such as the lack of donors, need of immunosuppresive treatment to avoid rejection by the patient, as well as the ethical considerations linked to the use of embryonic cells. Preferably the limbal biopsy is provided immersed in sodium hyaluronate, e.g. 14 mg/mL.

Preferably the donor tissue is mammalian, most preferably human.

The biopsy is placed on a growth support. As used herein, the term “growth support” refers to any material that allows for attachment and growth of cells. "Attachment", "attach" or "attaches" as used herein, refers to cells that adhere directly or indirectly to a substrate (e.g. a growth support) as well as to cells that adhere to other cells. The growth support may be a two dimensional support or a three dimensional support. The support may comprise any natural, recombinant or synthetic material which acts to support the viability, and /or proliferation the limbal stem cells of the biopsy. In one embodiment the support may be naturally derived and contain cell derived materials e.g. extracellular matrix, (e.g.one or more of Matrigel, laminin, Amgel, Humatrix, polylactic-polyglycolic acid sponges, Dexon sponges, sea sponges, fibrin, fibronectin, vitronectin, laminin, collagen). Alternatively, the support may be synthetic, such as a polymeric

scaffold, for example an electrospun scaffold or temperature responsive plastic, a hydrogel or contact lens. Alternatively, the support may be a corneal disc, such as a thin denuded posterior corneal layer, taken from the patient or a donor from the eye bank.

Most preferably, the growth support is an amniotic membrane, preferably wherein the amniotic membrane has an extracellular matrix. The amniotic membrane may be a denuded membrane or an intact membrane. Preferably the amniotic membrane is derived from the amniotic membrane of a pregnant mammal, preferably a human (HAM). Preferably the amniotic membrane has been maintained under sterile conditions and has been determined to be virus free, e.g., free from the hepatitis-B and C viruses and human immunodeficiency virus, and free from bacterial contamination.

As used herein the terms "medium", “culture medium”, “culture media” and "media" are used interchangeably. Preferably, the cells are cultured in a defined epithelial culture media containing the minimum essential elements necessary to maintain the growth of mammalian stem cells, wherein the components of the media are both known and controlled. Such defined minimum essential media for epithelial culture are known in the art.

Chemically defined culture media for mammalian epithelial cell culture have been extensively developed and published over the last several decades. All components of defined media are well characterized.

Defined media typically consist of roughly fifty chemical entities at known concentrations in water. The chemical components of the media fall into four broad categories: amino acids, vitamins, inorganic salts, trace elements.

The trace elements consist of a variety of inorganic salts included at micromolar or lower levels. The four most commonly included trace elements present in almost all defined media are iron, zinc, selenium and copper. Iron (ferrous or ferric salts) and zinc are typically added in micromolar concentrations, while the others are usually at nanomolar concentrations. The numerous less common trace elements are usually added at nanomolar concentrations.

Defined epithelial culture media comprising minimum essential elements necessary to maintain the growth of epithelial cells are well known in the art and include, by way of

example only Minimum Essential Medium Eagle, Minimum Essential Medium Dulbecco, ADC-I, LPM (Bovine Serum Albumin-free), FIO(HAM), F12 (HAM), DCCMl, DCCM2, RPMI 1640, BGJ Medium (with and without Fitton-Jackson Modification), Basal Medium Eagle (BME-with the addition of Earle's salt base), Dulbecco's Modified Eagle Medium (DMEM- without serum), Yamane, IMEM-20, Glasgow Modification Eagle Medium (GMEM), Leibovitz L-15 Medium, McCoy's 5A Medium, Medium M199 (M199E- with Earle's sale base), Medium M 199 (M 199H- with Hank's salt base), Minimum Essential Medium Eagle (MEM-E-with Earle's salt base), Minimum Essential Medium Eagle (MEM-H-with Hank's salt base) and Minimum Essential Medium Eagle (MEM-NAA with non essential amino acids), among numerous others, including medium 199, CMRL 1415, CMRL 1969, CMRL 1066, NCTC 135, MB 75261, MAB 8713, DM 145, Williams' E, Williams' G, Neuman & Tytell, Higuchi, MCDB 301, MCDB 202, MCDB 501, MCDB 401, MCDB 411 and MDBC 153.

Preferably the defined epithelial culture media comprises D-MEM + GlutaMAX™ -1+ 1 g/L D-Glucose +Pyruvate (75%) and F-12 + GlutaMAX™ -1 Nutrient mixture (25%).

The defined epithelial culture media may be supplemented with additional supplementary components at the beginning of the culture process or at a time or times subsequent to the beginning of the culture process. In certain embodiments, supplementary components may be added to the initial cell culture. In certain embodiments, supplementary components may be added after the beginning of the cell culture.

In one embodiment, the defined epithelial culture media is supplemented with serum. Preferably the serum is autologous and from the same donor as the tissue biopsy. Autologous human serum, if desired, can be subject to a conventional treatment, for example, thermal treatment, with the aim to inactivate complement. The human autologous serum (HAS) can be obtained from the same patient object of the subsequent implant of the graft by means of any conventional treatment, for example, from blood samples of the patient or either, preferably, by means of the carrying-out of plasmapheresis to the mentioned patient. Alternatively, the serum is any biologically-acceptable or biologically equivalent serum derivative or synthetic substitute thereof.

Alternatively, the biopsy is cultured in the absence of serum, i.e. in a culture medium that is essentially free from serum, or not supplemented with serum.

Additionally or alternatively, the defined epithelial culture media may also be supplemented with one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements, or any mixture and/or combination thereof.

Additionally or alternatively, the defined epithelial culture media may also be supplemented with one or more growth factors.

As used herein, the terms “growth factor” or “growth factors” refer to any substance capable of maintaining or stimulating cellular growth, proliferation and/or cellular differentiation, including cytokines, steroids and hormones. Exemplary growth factors include, but are not limited to IGFs, such as IGF-I and IGF-II, VEGF, PDGF, EGF, fibroblast growth factor, bFGF, osteopontin, thrombospondin-1, tenascin-C, PAI-1, plasminogen, fibrinogen, fibrin, transferrin, Adenine, Adrenomedullin, Angiopoietin, Autocrine motility factor, Bone morphogenetic proteins, Brain-derived neurotrophic factor, Epidermal growth factor, Erythropoietin, Fibroblast growth factor, Glial cell line-derived neurotrophic factor, Granulocyte colony-stimulating factor, Granulocyte macrophage colony-stimulating factor, Growth differentiation factor-9, Hepatocyte growth factor, Hepatoma-derived growth factor, Hydrocortisone, Insulin, Insulin-like growth factor, L-glutamine, Migration-stimulating factor, Myostatin, Nerve growth factor and other neurotrophins, Platelet-derived growth factor, Transferrin, Thrombopoietin, Transforming growth factor alpha, Transforming growth factor beta, Tri-iodothryonine, Tumor necrosis factor-alpha, Vascular endothelial growth factor, Wnt Signaling Pathway, placental growth factor, Foetal Bovine Somatotrophin, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, or any biological equivalent, derivative or combination thereof.

Additionally or alternatively, the culture is supplemented with insulin, preferably from about 0.0001 to 0.1 M insulin, preferably from about 0.001 to 0.01 M insulin, most preferably about 0.008 M insulin.

Additionally or alternatively, the culture is supplemented with hydrocortisone, preferably from about 0.01 to 2 M hydrocortisone, preferably from about 0.1 to 1 M hydrocortisone, most preferably about 0.82 M hydrocortisone.

Additionally or alternatively, the culture is supplemented with tri-iodothyronine, preferably, from about 10 nM to 10 mM tri-iodothyronine, preferably from about 100 nM to 1 mM tri-iodothyronine, most preferably about 233 nM tri-iodothyronine.

Additionally or alternatively, the culture is supplemented with adenine, preferably, from about 0.01 to 10 mM adenine, preferably from about 0.10 to 1 mM adenine, most preferably about 0.13 mM adenine.

Additionally or alternatively, the culture is supplemented with a growth factor, such as epidermal growth factor, preferably from about 0.01 to 100 nM epidermal growth factor, preferably from about 0.1 to 10 nM epidermal growth factor, most preferably about 1.5nM epidermal growth factor.

Additionally or alternatively, the defined epithelial culture media may also be supplemented with one or more antibiotics. As used herein, the term “antibiotic” or “antibiotics” refers to any natural or synthetic substance that inhibits the growth of or destroys microorganisms. For example, the antibiotic may inhibit cell wall synthesis, protein synthesis, nucleic acid synthesis, or alter cell membrane function. Examples of antibiotics include amoxycillin, ampicillin, penicillin, clavulanic acid, aztreonam, imipenem, streptomycin, gentamicin, vancomycin, clindamycin, ephalothin, erythromycin, polymyxin, bacitracin, amphotericin, nystatin, rifampicin, teracycline, coxycycline, chloramphenicol and zithromycin, or any mixture or combination thereof. Preferably, the media is supplemented with penicillin and/or streptomycin. Preferably the antibiotic supplemented culture medium comprises from about 0.5 to about 1.5 % (v/v) antibiotic, more preferably about 1% (v/v) antibiotic.

In an alternative embodiment, the defined epithelial culture media does not comprise, i.e. is free from antibiotics.

Additionally or alternatively the defined epithelial culture media may be supplemented with a protein having ADP-riobosylation activity, for example a bacterial toxin. Suitable bacterial toxins include cholera toxin (CT), shiga toxin (ST1, ST2, etc.), heat-labile toxin (LT, LT-IIa, LT-IIb, etc.) from *E. coli*. In some preferred embodiments, the toxins are modified to be non-toxigenic while remaining potent immunostimulatory molecules. It is believed that both shiga toxin and heat-labile toxin are structurally similar or analogous

to cholera toxin. Preferably, the culture is supplemented with from about 0.15 to 0.05 mM of cholera toxin, preferably about 0.096 mM of cholera toxin.

In an alternative embodiment, the culture does not comprise, i.e. is free from a protein having ADP-riobosylation activity.

In a preferred embodiment, the method is essentially free from non-human cells.

The method comprises culturing for a first time period the limbal biopsy in the presence of the above described epithelial culture medium supplemented with serum and one or more antibiotics. The epithelial culture medium supplemented with serum and one or more antibiotics may be refreshed (i.e. removed and replaced with fresh epithelial culture medium supplemented with serum and one or more antibiotics) during the first time period. Preferably, the first time period is for at least a sufficient time for the limbal cells to undergo at least one division, for example the first time period is at least about 12 hours, more preferably at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 days.

The method additionally comprises substituting (i.e. removing and replacing) the epithelial culture medium used in the first time period with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period. The epithelial culture medium supplemented with serum and free from antibiotics may be refreshed (i.e. removed and replaced with fresh epithelial culture medium supplemented with serum and free from antibiotics) during the second time period. Preferably, the second time period is for at least a sufficient time for the limbal cells to expand to form an epithelial sheet, for example the second time period is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 days.

As used herein the terms “expand” or “expanded” are used interchangeably to refer to increasing the cell number, e.g. the number of limbal stem cells, by cell division. The cells may be expanded about 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 21-fold, 22-fold, 23-fold, 24-fold or 25-fold, or more compared to a suitable control, for example an unexpanded biopsy tissue.

Alternatively the second time period is for at least a time sufficient for the limbal stem cells to expand to form a defined area or size, for example, cells are cultured for a time sufficient so as to form an epithelial sheet that has an approximate area of about 0.5, 1, 1.5, 2, 2.5, 3, or 3.5 cm^2, or any range thereinbetween.

Preferably the second time provides that the cells are cultured for a time sufficient to achieve at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% confluence (or any range thereinbetween), most preferably 90% confluence on a defined surface of the growth support. In a preferred embodiment the epithelial sheet is at least 90% confluent over a defined surface having an area of 1.5cm^2, and most preferably at least 90% confluent over a defined surface having an area of from about 1.5cm^2 to about 90% of 3.5cm^2.

The method expands the limbal stem cells to form an epithelial sheet upon the growth support, resulting in a composite structure. As used herein, "composite structure" refers to a structure comprising two or more distinct layers, a support layer and an epithelial layer. Preferably the individual components remain separate and distinct within the composite structure. The epithelial layer may comprise TAC (transient amplifying cells), TDC (terminally differentiated cells) and LSC (Limbal stem cells).

Alternatively or additionally the method includes a step of histochemical staining or impression cytology to determine if the limbal stem cells have been expanded to the desired end point, i.e. to analyze the number and quality of cells in the epithelial sheet. As used herein, the term "impression cytology" refers to the application of cellulose acetate filter material to the surface of the epithelial sheet, so as to remove the superficial layers of the epithelium for analysis. For example, Impression cytology may be carried out to confirm the nature of LSCD (total or partial) and the presence of conjunctival cells on the central cornea. For example a combination of CK13/CK12 or a combination of CK7/CK12 may be used as markers of the conjunctiva. Alternatively MUC5AC may be used as a marker of goblet cells in the conjunctiva. The histological analysis is done to confirm both the multi-layered nature of the epithelium we are going to transplant/or have transplanted and confirm the location of stem cells in the basal layer and differentiated cells on the superficial layers.

As used herein, the term "histochemical staining" refers to the identification of chemical compounds or biological molecules within and between biological cells using histological techniques. Histological techniques include (but are not limited to) immunocytochemistry, immunofluorescence, immunohistochemistry and histology. The term "immunohistochemistry" refers to the process of detecting antigens in cells of a tissue section through the use of antibodies binding to said antigens. The detection or visualisation of said antibody-antigen interactions can be accomplished using multiple techniques, for example conjugating an enzyme, such as peroxidase, to an antibody or tagging an antibody with a fluorophore.

Preferably, the cells are cultured for a time sufficient to achieve an epithelial sheet that comprises at least about 2%, 3%, 4%, 5% or 10% limbal stem cells.

Therapeutic uses

The invention provides a method of treating limbal stem cell deficiency comprising:

i) isolating a limbal biopsy from a patient in need of treatment;
ii) placing the limbal biopsy on a growth support;
iii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and
iv) substituting the epithelial culture medium of step iii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support; and
v) transplanting the composite structure onto an eye of the patient.

The expanded epithelial stem cells of the invention are of particular use in various therapeutic settings. In particular, the expanded cells are of particular use in transplantation and engraftment, for example to treat disease, injury or wounding. The grafts may be used to treat unilateral ocular disease, wounds or injury or bilateral ocular disease, wounds or injury.

Following expansion, the limbal stem cells may be used as grafts. The expanded cells may be retained on a growth support (e.g. an amniotic membrane) and transplanted as a composite structure into a patient's eye. Alternatively, the cells may be separated from the growth support (e.g. amniotic membrane) and transplanted onto a fresh support,

suitable for implantation into an eye, to obtain a surgical graft. The cultured or cultivated epithelial cells on the support are then transplanted to a patient's eye.

Accordingly, the invention provides the use of the expanded epithelial stem cells of the invention as a medicament.

The invention also provides a method of treating an ocular disease, wounds or injury comprising implanting a limbal stem cell graft of the invention into the eye of a mammalian subject in need thereof.

Also provided is a method of corneal replacement, comprising implanting an limbal stem cell graft of the invention into the eye of a mammalian subject in need thereof. There is also provided a limbal stem cell graft of the invention for use in corneal replacement.

The invention provides a limbal stem cell graft according to the invention for use in the treatment of ocular injury or ocular disease. Also provided is a method of treating ocular injury or ocular disease, comprising administering an limbal stem cell graft of the invention into the eye of a mammalian subject in need thereof. As used herein, the term "ocular injury" refers to conditions resulting in an insufficient stromal micro-environment to support stem cell function, for example aniridia, keratitis, neurotrophic keratopathy, and chronic limbitis; or conditions that destroy limbal stem cells such as Partial limbal stem cell deficiency, Total stem cell deficiency, chemical or thermal injuries, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, contact lens wear, or microbial infection. The grafts of the invention are of particular use in the treatment of thermal or chemical injury's, such as burns. The grafts of the invention are also of particular use in the treatment of ocular injury resulting from genetic disease.

The grafts are of particular use for the reconstruction of the ocular surface in patients with limbal stem cells deficiency, for reconstruction of the ocular surface in patients with corneal persistent epithelial defects and for the treatment of total and partial epithelial stem cell deficiency.

Preferably, said limbal stem cell graft is derived from autologous cells, i.e. said cells are derived from the individual to be treated.

As used herein the terms “wound” and “wounding” relate to damaged tissues, preferably damaged cornea, where the integrity of the cornea or tissue is disrupted as a result from i.e. external force, bad health status, aging, exposure to sunlight, heat or chemical reaction or as a result from damage by internal physiological processes.

Examples

Aim: To evaluate the safety and effectiveness of human *ex vivo* expanded autologous limbal stem cells for the treatment of unilateral total limbal stem cell deficiency.

Antibiotics removed from day 3 to reduce the risk of not detecting contamination organisms by BacT/ALERT testing Changes to timing of microbiology testing as agreed with QP.

Contents

1.0 Glossary

3T3s	Murine fibroblasts
ABCG2	ATP-binding cassette sub-family G member 2
ALSC	Autologous Limbal Stem Cells
Anti-HBc	Hepatitis B Core Antibody
Anti-HBs	Hepatitis B Surface Antibody
AS	Human Autologous Serum
BMF-HS	Biomanufacturing Facilities-Haematology Science
BMR	Batch Manufacturing Record
BSS	Balanced Salt Solution
CESC	Corneal Epithelial Stem Cell
CF	Counting Fingers
CFE	Colony Forming Efficiency
CK	Cytokeratin
DMEM	Dulbecco Modified Eagle Medium
FCS	Foetal Calf Serum
HAM	Human Amniotic Membrane
HBsAg	Surface Antigen of Hepatitis B Virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HM	Hand Movements
HTA	Human Tissue Authority
HTact	Human Tissue Act
HTLV	Human T-Lymphotropic Virus
GMP	Good Manufacturing Practice
HS	Human Serum
IMP	Investigational Medicinal Product
Ki67	Cell Proliferation Marker
LSC	Limbal Stem Cells
LSCD	Limbal Stem Cell Deficiency
MHRA	Medicine and Healthcare Products Regulatory Agency
MRC	Medical Research Council
MSC	Mesenchymal Stem Cell
NBCTF	The Newcastle Biomedicine Cellular Therapies Facility

NBS	National Blood Service
NHS-BT	NHS Blood and Transplant
P63	Transcription factor
PCR	Polymerase Chain Reaction
PKP	Penetrating Keratoplasty
QA	Quality Assurance
QC	Quality Control
rtPCR	Real Time Polymerase Chain Reaction
RVI	Royal Victoria Infirmary
SAERS	Serious Adverse Event or Reactions
SOP	Standard Operating Procedure
SC	Stem Cells
SLA	Service Level Agreement
TAC	Transient Amplifying Cell
TEM	Transmission Electron Microscope
TPA	Third Party Agreement

2.0 Specifications and Analytical Methods

2.1 IMP Definition

The *ex vivo* expansion of autologous limbal stem cells (ALSC) of limbal epithelium is a method which involves taking a small biopsy of limbal epithelium and culturing it according to good manufacturing practice (GMP) to produce epithelial stem cells for transplantation to cure unilateral limbal stem cell deficiency (LSCD).

Autologous limbal stem cells are thus prepared from a small biopsy of limbal tissue over a feeder cell layer of human amniotic membrane (HAM). It is cultured for 12-22 days or until the outgrowth measures approximately 2 cm diameter. The cells are then ready for transplantation.

This final product is a sheet of autologous epithelial progenitor cells (ALSC), together with additional epithelial cells, such as TAC and TDC, which remains attached to the amniotic membrane and the composite structure is used for transplantation to the contralateral limbal stem cell deficient eye. The final product is for immediate, autologous, single use and not stored or quarantined. The minimum number of SC must equal or more than 3% of the total number of epithelial cells present in *ex vivo* expanded culture system

2.2 Background

The corneal epithelium is maintained by a population of stem cells known as limbal stem cells (LSCs) due to their location in the basal layer of the outer border of the cornea known as the limbus. Significant loss or damage to LSCs leads to limbal stem cell deficiency (LSCD) characterised by breakdown of the epithelium and conjunctivalisation of the corneal surface, which eventually leads to significant ocular pain and blindness (Ahmad et al., 2006).

The standard treatment of unilateral total LSCD involves transplanting large whole tissue limbal grafts from the patient's healthy fellow eye (putting that eye at potential risk of developing LSCD) or from living related or cadaveric donors (requiring high dose systemic immunosuppression with its associated health risks) (Kenyon and Tseng, 1989). Recently, successful treatment of this condition has been achieved, while avoiding those risks mentioned, by the transplantation of *ex vivo* expanded LSCs taken from a small autologous limbal biopsy (Pellegrini et al., 1997). The current protocol described uses smaller amounts of tissue for the *ex vivo* expansion and this process has three main

advantages. Firstly, the small amounts of tissue required for *ex vivo* expansion are much less likely to damage the LSC population of the healthy donor eye than previously used autologous whole tissue grafts that required large quantities of limbal tissue for direct transplantation (Jenkins et al., 1993). Secondly, the small biopsies needed can be taken from the fellow eye of the patient with LSCD, even if the disease is bilateral to an extent, providing there are remaining areas of healthy limbus in one eye (Sangwan et al., 2003a). Thirdly, if a biopsy does not grow in culture, it is safe and acceptable to harvest another biopsy from the fellow (donor) eye. This means the tissue is autologous and eliminates the requirement of systemic immune suppression compared to previously used whole tissue allografts (Holland et al., 2003). More recently, *ex vivo* expansion of oral mucosa epithelium has been used successfully to transplant onto the ocular surface of rabbits and subsequently in humans with total LSCD, which will potentially provide treatment for autologous stem cell therapies for patients with total bilateral LSCD (Hayashida et al., 2005; Nakamura et al., 2003; Clinch et al., 1992).

These stem cell therapies are relatively new and as such, specific national and/or international protocols/guidance have yet to be established. Due to the lack of such specific guidance, the inventor has sought to minimise any risk to the patient by adopting certain modifications to the research methodologies in use at present. These include first, the replacement of all non-human animal products from the culture system and second, the conversion of all production procedures to current Good Manufacturing Practice (GMP) standards. In addition, for the first time, a strictly defined uniform group of patients with total unilateral LSCD and no other significant ocular conditions has been used to allow the success or failure of treating LSCD to be attributable directly to the proposed stem cell therapy (Kolli et al., 2010).

2.3 Pre-clinical and Clinical Data

The successful use of regenerative stem cell therapy in the context of LSCD can only be achieved if healthy limbal epithelium (which houses the LSCs) can be successfully expanded. The inventors studied the various techniques available for this purpose in detail (Baylis et al. 2011; Osei-Bempong et al., 2009). Ultimately, the ability to translate these techniques into the clinical arena depends on them being reproducible, reliable and safe.

Pre-clinical data were extensively developed using various *in vitro* studies which culminated in 2 PhD theses and has also been extensively peer-reviewed in a number of publications (Kolli et al., 2010; Kolli et al., 2009; Ahmad et al., 2008a; Ahmad et al.,

2008b; Kolli et al., 2008; Ahmad et al., 2007). The validation studies are shown in the Annexes. The intended Clinical Trial Protocol has been approved and funded by the MRC UK, and also peer-reviewed by the Newcastle Clinical Trial Unit, Newcastle University and the Joint Research Office (Research & Development) for the Newcastle upon Tyne Hospitals NHS Foundation Trust, as Sponsors for the study (EudraCT Number: 2011-000608-16).

The conventional culture of limbal epithelium requires the use of animal cells (mouse 3T3 fibroblast feeder cells) or animal products (foetal calf serum) in the culture system (Daya et al., 2005; Ramaesh and Dhillon, 2003; Koizumi et al., 2001). However, there are two main concerns regarding the use of animal ingredients in the culture of biological materials destined for human purposes: one is the potential interspecies pathogen transfer and the second is the possible increased immune response that may require systemic immune suppression.

In order to eliminate these risks, the studies outlined in the annex eliminated animal ingredients in the culture of human limbal epithelium.

Below are tables summarising the culture results and clinical outcomes.

Table 1. Summary of Limbal Explants Cultures

Initials	Serum Prep[n]	Complete medium Prep[n]	Date harvest	Days in culture	Microbiology	Medium prep[n] to release	Date of transplant
PRo	04/04/2006	04/04/2006	06/04/2006	No-growth	Negative	Stem cell deficiency due to chemical injury in fellow eye not identified by impression cytology method used[1]	
PRi	04/04/2006	04/04/2006	06/04/2006	14	Negative	16 days	20/04/2006
AB	26/06/2006	28/07/2006	03/08/2006	14	Negative	19 days	16/08/2006
KG	20/11/2006	20/11/2006	23/11/2006	13	Negative	16 days	06/12/2006
RT	20/11/2006	20/11/2006	23/11/2006	14	Negative	17 days	07/12/2006
JR	13/08/2007	13/08/2007	16/08/2007	13	Negative	16 days	29/08/2007
DH	13/08/2007	13/08/2007	16/08/2007	14	Negative	17 days	30/08/2007
GM	11/09/2007	11/09/2007	13/09/2007	14	Negative	16 days	27/09/2007
RG	19/02/2008	19/02/2008	21/02/2008	14	Negative	14 days	04/03/2008
GT	09/06/2008	09/06/2008	10/06/2008	12	Negative	Technical failure due to repeated detachment of amniotic membrane.[1]	
MD	05/01/2009	05/01/2009	08/01/2009	No-growth	Negative	Stem cell deficiency also present in donor eye, due to contact lens wear and previous limbal tissue donation[1]	
MC	05/01/2009	05/01/2009	08/01/2009	19	Negative	22 days	27/01/2009

Notes 1. In published series of limbal stem cell cultures, there is a recognised rate of no-growth (Meller et al., 2002).

Table 2. Safety Outcome of Clinical Studies with Autologous Cultured Limbal Cells

Patient		Primary objective outcome: Impression cytology		Primary objective outcome: Epithelial defect		Secondary objective outcomes: visual acuity		Corneal vascularisation (central 8mm)		Corneal opacity	
		Pre-op	**Post-op**	**Pre-op**	**Post-op**	**Pre-op**	**Post-op**	**Pre-op**	**Post-op**	Pre-op	Post-op
1	PRi	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.01 *6/600*	0.17 *6/36*	Present	Absent	++++	+++ (0 post PKP)
2	AB	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.33 *6/18*	0.5 *6/12*	Present	Absent	++	+
3	KG	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.02 *CF: 6/300*	0.02 *CF: 6/300*	Present	Absent	++++	+++
4	RT	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.33 *6/18*	0.67 *6/9*	Present	Absent	+++	+
5	JR	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.02 *CF: 6/300*	0.5 *6/12*	Present	Absent	+++	++
6	DH	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.02 *CF: 6/300*	0.67 *6/9*	Present	Absent	+++	+
7	GM	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.33 *6/18*	0.67 *6/9*	Present	Absent	++	+
8	RG	CK19 +ve Conjunctival	CK 19-ve Corneal	Present	Absent	0.1 *6/60*	0.17 *6/36*	Present	Absent	+++	++ (0 post PKP)
9	MC	CK19 +ve Conjunctival	CK 19-ve Corneal	Absent	Absent	<0.02 *HM*	<0.02 *HM*	Present	Absent	++++	++++

Notes on Table 2.

1. Impression cytology involves using a small filter paper to lift off the most superficial cells from the cornea. These are then stained to look for cytokeratin 19 (CK19). This is a marker on the cells which is specific to conjunctiva as opposed to corneal epithelium. Being CK19 positive is a hallmark of limbal stem cell deficiency, whereas normal cornea is CK19 negative (Sacchetti et al., 2005).

2. Epithelial defects occur in limbal stem cell deficiency. They lead to pain, inflammation and reduced vision. Improving this parameter is one of the main outcomes.

3. Visual acuity. The lower number in italics is the reading taken from a Snellen-type eye chart. [6/18 means that the patient can read at 6 metres what a patient with normal vision could be expected to see at 18 metres. 6/6 is defined as normal vision.) When the vision is poor, it is defined as CF (counting fingers) or HM (hand movements only). The upper number is the decimal equivalent. The larger the number the better vision (1.0 is normal vision).

4. Corneal vascularisation is another hallmark of limbal stem cell deficiency. Normal corneal epithelium contains no blood vessels.

5. Corneal opacity occurs in stem cell deficiency due to scarring and is related to vision. Opacity can occur in either the surface (epithelium) and/or deeper layers (stroma). It is difficult to appreciate the depth of scarring by normal examination and a simple plus score is recorded. Epithelial opacity will be removed by a successful limbal stem cell transplant, although deep stromal opacity will remain. However, if successful, a subsequent corneal graft can be performed to remove residual opacity. This was done for patients 1 and 2 with removal of opacity and marked vision improvement.

2.4 Safety of the Use of *ex vivo* Expanded ALSC

In 1997, Pellegrini and co-workers were the first to show that small amounts of healthy human limbal epithelium could be expanded *ex vivo* in culture to form epithelial sheets, which could then be successfully used for transplantation in cases of LSCD (Pellegrini et al., 1997). As is the case with previous techniques, healthy donor tissue for culture can be obtained from the patient's other eye (if healthy), a living related healthy eye, or the eye of a cadaveric donor. The requirement of much smaller amounts of tissue with consequently less risk to the donor eye means that patients with unilateral LSCD are more likely to be willing to use their other healthy eye as the donor eye, with the added benefit of eliminating the requirement of systemic immunosuppression. Subsequent to this original publication, few other studies have been conducted with promising results (Sangwan et al., 2003b; Rama et al., 2001; reviewed by Baylis et al. 2011).).

The safety and efficacy of this treatment modality is however impossible to establish due to the heterogeneity both within and between the published studies, as well as the small number of cases often reported. In previous studies, there are differences in the types of limbal stem cell deficiency treated (both partial and total), the causes of limbal stem cell deficiency (both congenital and acquired), the source of the donor tissue (both autologous and allogeneic), the types of culture method used (which mostly include the use of mouse 3T3 fibroblasts), and the outcome measures used to assess the effectiveness of the treatment (Baylis et al., 2011). Transplantation of cultured limbal epithelium can therefore not be established as a recognised clinical treatment option for eyes with LSCD, enabling National Health Service funding, until its safety and efficacy is established by a formally structured clinical trial.

Most of the published culture techniques utilise animal products to a varying degree, whether it be mouse fibroblasts feeder layers or foetal calf serum (reviewed by Shortt et al. 2007) (Shortt et al., 2007), (Baylis et al., 2011). There is an increasing need to substitute animal based products during the *ex vivo* expansion of limbal epithelial cells. Human amniotic membrane (HAM) is non-immunogenic and has been extensively transplanted by the inventors and others in acute chemical eye burns amongst other conditions for many years without the requirement of immunosuppression (Meller et al., 2000). Recently human amniotic epithelial cells have been put forward as a suitable feeder layer for the culture of limbal stem cells; however an established cell line is not available as yet (Chen et al., 2007). To that effect, the inventors have developed over the last 8 years a culture system for expanding human limbal epithelium without using any animal cells or products

which involves the culturing of a small piece of human limbal tissue on HAM using autologous human serum.

Good Manufacturing Practice (GMP) protocols have been set up for the culture technique of the invention and the process has been successfully validated in the BMF-HS. A number of methods including colony forming efficiency (data not shown), clonal analysis, quantitative RT-PCR (data not shown), immunocytochemistry and flow cytometry (data not shown) for putative LSC markers such as p63α and ABCG2 as well as terminally differentiated cell markers such as CK3/12 were carried out to ensure that explants grown under GMP conditions showed the expected growth characteristics and colony phenotype to the ones observed previously in the lab (This validation data is shown in the Annexes). Subsequent to this, the inventors have successfully transplanted 9 patients with unilateral severe LSCD (7 chemical eye burns, 1 thermal eye burn and 1 local radiotherapy) using this technique at the Department of Ophthalmology, Royal Victoria Infirmary, Newcastle University, in Newcastle upon Tyne.

In all 9 successful cases, symptom severity significantly declined ($p<0.002$) (both pain and visual impairment from the LSCD). In 7 out of 9 patients there was also a significant increase in Snellen visual acuity. In addition, corneal impression cytology at 6 months post-procedure has confirmed the reversal of conjunctival isation (Table 2).

The above findings from the inventors Phase I study essentially demonstrate primary safety and efficacy results of this technique.

2.5 Starting Material

Table 3

Compendium Materials	Supplier
1.4% Sodium Hyaluronate gel (Healon GV)	Abbott Medical Optics (AMO), UK

Table 4

Non-Compendium Materials	Supplier
DMEM Low Glucose Culture Medium	Invitrogen, UK
Ham's F12 Culture Medium	Invitrogen, UK
Hydrocortisone	Sigma, UK
Insulin	Sigma, UK
Tri-iodothyronine	Sigma, UK
Adenine	Sigma, UK
Cholera Toxin	Sigma, UK
Epidermal growth factor	Sigma, UK
1 N Sodium Hydroxide	Sigma, UK
1 N Hydrochloric Acid	Sigma, UK
Penicillin/Streptomycin	Invitrogen, UK
Gentamicin	Invitrogen, UK
Sterile Water	Sigma, UK
Dulbecco's Phosphate Buffered Saline	Sigma, UK

Some materials are divided into aliquots for single production use. Details of aliquot production and usage are retained. Records of all materials, aliquots and disposable items used for production, along with lot numbers and expiry dates are kept in individual batch manufacturing records.

2.5.1. Human Limbal Stem cells

The consented patient will be selected as an appropriate candidate for this procedure according to the criteria described in the clinical protocol (see Clinical Trial Protocol) and cells harvested for autologous use only and in accordance with the Human Tissues Act (HTact) and the Tissues and Cells Directive 2004/23/EC.

A 1.5 mm x 1.5 mm partial thickness (i.e. 100µm depth) limbal biopsy will be taken from the unaffected eye of an individual patient with total unilateral LSCD. The limbal biopsy is obtained as a surgical procedure, if possible under local anaesthesia, in the ophthalmology theatre, at the Royal Victoria Infirmary (RVI) according to local current procedure.

This starting material will only be accepted into the Newcastle Biomedicine Cellular Therapies Facility (NBCTF) once certain criteria are met:

Acceptance criteria at BMF-HS: Patient is subject to informed consent; Patient must be negative for mandatory infectious markers, i.e. HBsAg, anti-HBc/HBs, anti-HCV, anti-HTLV, syphilis and Anti-HIV1&2; The limbal tissue must be between 1-2mm x 1-2mm; The limbal tissue must be suspended/protected in a drop of 1.4% hyaluronic acid; the upper surface (epithelial side) of the limbal biopsy must be marked with a gentian violet surgical pen to indicate the orientation of the biopsy.

2.5.2 Autologous Peripheral Blood Serum

Human autologous serum (AS) is a component of the epithelial growth medium used in the culture of the ALSC.

AS has been used and validated in the inventors laboratory and clinical studies (see Annex).

Blood is taken by venesection (no anticoagulant) from the patient during the work up and assessment visits following consent to the procedure. The protocol specifies a volume of 60mls.

Procurement of blood and limbal tissue is under informed consent and patients are screened prior to limbal donation for the mandatory infectious markers as set out above in accordance with Directive 2006/17/EC.

Acceptance criteria: The tubes must be labelled with at least three points of identification of the patient (i.e. patient name, date of birth and RVI number); There should be a minimum of 30ml volume and the blood should show no signs of haemolysis.

2.5.3. Human Amniotic Membrane (HAM)

HAM for clinical transplantation is obtained under a Third Party Agreement (TPA) from NHS BT Tissue Services, Liverpool (see Clinical Trial Protocol). The amniotic membrane is prepared for culture using patient-specific growth medium 24-48 hours prior to the procurement of the limbal tissue to establish a stable base for culture of the limbal stem cells.

The HAM is supplied frozen (-80ºC), attached to a nitrocellulose paper stored in nutrient medium in a sterile glass vial within sterile packaging.

The tissue must remain frozen (below -40°C) until required. Once thawed at ambient temperature 14-22°C, if not used immediately, must be kept at 4°C and used within 24 hours.

Acceptance criteria: At delivery the tissue vial must be intact, held securely within its primary packaging and still be frozen; Supplied tissue size is 3 cm x 3 cm; On thawing, the nutrient medium containing the tissue must show no sign of contamination i.e. be cloudy or opaque.

2.6 Final Product

2.6.1 Autologous Limbal Stem Cells (ALSC)

The final product is a sheet of autologous epithelial progenitor cells (ALSC), which remains attached to the amniotic membrane and the composite structure is used for transplantation to the contralateral limbal stem cell deficient eye of the same patient.

The final product is for immediate single use and not stored or quarantined.

2.6.2 ALSC Tissue Release from the BMF-HS

The Qualified Person will release the tissue after ensuring that it has met the specific release criteria. The final product is released for issue but remains in the GMP facility (in 37°C incubator) until the preparatory work on the patient is underway (same day). The theatre team will liaise in a timely manner (i.e. 30 minutes in advance) with the GMP laboratory for the delivery of the tissue.

Release Criteria: ALSC must be shown to be at least 90% confluent by visual assessment; ALSC must be morphologically sound (an evenly distributed layer of epithelial phenotype i.e. small and regular with primitive cytoplasm and large nuclei) both macro and microscopically; The donor must have negative results for infectious disease screening from a blood sample taken on the day of limbal biopsy. Day 0 medium must be negative for aerobes and anaerobes after 5 days BacT/ALERT culture. Day 0 medium mycology screen must be negative. A medium sample taken within 72 hours of release must be negative after 48 hours BacT/ALERT culture.

3.0 Manufacturing Methods

See Figure 1.

Product is manufactured in the Biomanufacturing Facility, Haematology Science, Royal Victoria Infirmary (BMF-HS).All of the processes, including incubation are 'open' procedures

and as such are undertaken within the Class II Grade A safety cabinet in a Grade B clean room; Sterile disposable filtered pipettes and calibrated automatic pipettes with sterile filtered tips are used throughout. All disposable consumables are sterilised by irradiation; All the surgical instruments, i.e. forceps, are sterile for disposable single use only and are used for manipulating the amniotic membrane and tissue biopsy.

This applies to all stages of production.

3.1 Starting Materials

Starting materials consist of:

a) Human autologous serum;
b) Epithelial growth media;
c) Human amniotic membrane;
d) Human autologous limbal stem cells explant.

a) Human Autologous Serum (AS)

Process Description

AS is a component of the epithelial growth medium (described below) used in the culture of the ALSC (see Annex 2 for validation). 40-60 mls of autologous blood is collected into sterile universal tubes [Sterilin] in the Department Ophthalmology and transported to BMF-HS by a member of staff from either Ophthalmology or BMF-HS.

Manufacturing Methods: Serum preparation

The serum is prepared after it has been left to clot for a minimum of 2 hours. Blood is centrifuged at 1600g for 10mins [Centra CL2 Bench top Centrifuge; Thermo Scientific, UK] and returned to the Class II cabinet. The serum is removed using a sterile pipette and transferred to new sterile tube(s). The serum is centrifuged again at 1600g for 10mins. On return to the Class II cabinet the supernatant serum is transferred to sterile 20ml tubes and labelled with the patient's demographics and Trial No. If not used immediately for medium preparation then it is stored at -20°C in a controlled freezer.

b) Epithelial Growth Medium Process Description

Basic and Complete Epithelial Growth Media are prepared according to current procedures. They are prepared 24-48hrs prior to the harvesting of the limbal tissue. All supplements are added according to the concentrations described in table 5, having

been prepared according to manufacturer's instructions. The media is stored at 2-8°C in a monitored refrigerator.

Manufacturing Methods: Preparation of Medium

i. Basic culture medium is prepared and filtered [0.22µm filter sterilising unit - Nalgene] using supplements and growth factors that will support the growth of limbal epithelial tissue in culture (see Table 5). It is then labelled with a Batch No; date of preparation and expiry date (22 days from date of preparation - see validation).

ii. Complete medium is then prepared using 90% basic medium with the addition of 10% autologous serum (therefore specific to the patient tissue undergoing culture). This medium is filtered [0.22µm filter sterilising unit [Nalgene] and sufficient aliquots prepared in 15ml centrifuge tubes [TPP] to allow for single use at each medium change during the course of the cell expansion. Two 10ml aliquots are used on day -2 or -1 for plating the amniotic membrane and on day 0 of the culture to add the limbal explant. To these aliquots only, penicillin and streptomycin (or gentamicin in the case of penicillin allergy) is added in the concentrations below. Other 6ml aliquots without antibiotic are used for subsequent feeds from day 3 (thus removing antibiotic from the culture system). A 2ml and 1 ml sample respectively is taken for sterility testing into a blood culture bottle [BacT/ALERT®PF Plus, Biomerieux] and mycology screen and sent to the Dept Microbiology, Freeman Hospital, Newcastle Hospitals NHS Foundation Trust.

Basic Epithelial Growth Medium		
Reagent	**Composition**	**Supplier**
Low glucose DMEM	375 ml	Invitrogen
Ham's F12 medium	125 ml	Invitrogen
Hydrocortisone	0.4 µg/ml	Sigma
Insulin	5 µg/ml	Sigma
Tri-iodothyronine	1.4 ng/ml	Sigma
Adenine	24 µg/ml	Sigma
Cholera toxin	8.4 ng/ml	Sigma
Epidermal growth factor	10 ng/ml	Sigma
Complete Epithelial Growth Medium		
Reagent	**Composition**	**Supplier**
Basic Epithelial Medium	90ml	As above
Autologous serum	10ml	Patient
Penicillin & Streptomycin1	50 units & 50 µg/ml	Invitrogen

1. Pen/Strep (or gentamicin) is added to 2 aliquots only used for plating HAM and limbal explants.

c) Human Amniotic Membrane (HAM)

Process description

The amniotic membrane is prepared 24-48hrs prior to the harvesting of the limbal tissue to establish a stable base for culture of the limbal stem cells. This process produces a biological substrate for the growth of normal donor epithelial cells for clinical use.

Manufacturing methods: preparation of HAM prior to limbal cell culture

The HAM is supplied attached to nitrocellulose paper within a nutrient medium in a glass vial in sterile packaging and is delivered on dry ice. After acceptance of the product at BMF-HS (checking Specification, Batch No. and expiry date) the HAM is stored in a -80°C controlled freezer if not used on the day of delivery.

The HAM is defrosted at ambient temperature 14-22°C immediately prior to use. Subsequently, it is washed three times by submersing the tissue twice in 2mls of Dulbecco's Phosphate Buffered Saline [Sigma, UK] with 1% Penicillin / streptomycin and then in

2mls Complete culture medium containing antibiotic. It is then placed on the lid of a six-well plate [TPP, Switzerland] to provide a sterile platform for manipulating the tissue.

Two 24mm x 24mm glass coverslips [VWR, USA], packaged and sterilised by autoclaving (Sterile Services department, Royal Victoria Infirmary) are washed three times as above and placed adjacent to the HAM.

The HAM is peeled from the nitrocellulose paper using sterile forceps and is placed over one sterile glass coverslip ensuring that the coverslip is fully covered and that there is an equal overhang of HAM at each side of the coverslip, trimming excess as necessary. Care is taken to ensure that the stromal side (sticky side) remains in contact with the coverslip and the epithelial side is facing up. The overhanging HAM is folded over the underside of the coverslip and secured by placing the second sterile coverslip underneath as a sandwich. This procedure ensures that the HAM remains in situ throughout the culture process.

The HAM plus coverslips is then placed in a sterile 9.6 cm^2 culture well of a 6 well plate [TPP, Switzerland] and covered with 2 ml of the patient specific Complete culture medium with antibiotics. The plate is labelled with patient demographics according to current procedure and must only be used for that patient. It is then placed in the tissue culture incubator at 37°C in a humidified atmosphere containing 5% CO_2.

The prepared HAM is checked 24 hours later to ensure that it remains securely held on the coverslips and that the medium remains clear, negative for any bacterial contamination via visual inspection prior to commencing the limbal epithelial culture.

d) Human autologous limbal stem cells explant

Process Description

The Biomanufacturing Facility at the RVI (BMF-HS) provides a kit containing a receptacle for the tissue, labels, packaging material and documentation. When production is initiated, a member of BMF-HS will ensure a kit is received by ophthalmology theatre staff prior to limbal cell biopsy. Once the limbal biopsy has been retrieved by the Ophthalmic surgeon, a member of theatre staff, following the current procedures immediately transfers the tissue to the BMF-HS.On arrival at the BMF-HS receipt of the tissue is documented accordingly using established procedures. The limbal biopsy material is transferred into the BMF-HS clean room by receiving staff.

Manufacturing Methods:

3.2 Culture and Expansion of Limbal Stem Cells

This process produces a single batch of ALSC sufficient to treat one individual once only.

The culture is initiated using the amniotic membrane that has been prepared specifically for that patient as it contains, in the culture medium, the patient's autologous serum.

The epithelium culture medium is removed from the culture well containing the HAM. Using sterile forceps, the limbal biopsy is then removed from the hyaluronic acid in which it has been suspended and placed on the centre of the HAM and gently pressed downward to promote attachment. 1.3 ml of epithelial medium is gently added to the culture well very slowly to ensure that the limbal biopsy does not become detached from the underlying HAM. Subsequently, the lid of the plate is replaced. The culture is then incubated at 37°C, 5% CO_2 in a humidified atmosphere.

The culture medium is replaced every 2-3 days in the Class II cabinet by carefully removing most of it with a sterile filtered pipette tip and replacing it with fresh medium, increasing the volume added to a minimum of 1.5ml.

The morphology of the tissue is observed at the same interval using the inverted microscope as described in section 2 and assessment is recorded on the worksheet. The area of explant outgrowth is marked on the underside of the culture well at the time of each medium change to allow subsequent measurement of growth rate.

Phase 2 QC for sterility is taken at day 0 and within 72 hours of the transplant date, to allow for a 48 hour result to be available.

3.3 Final Product

Process Description

When outgrowing cells cover at least 90% of the total area of the HAM, the product is ready for transplant.

Manufacturing Methods

The ALSC product is transported to theatre (Ophthalmology Dept., RVI) in the plate in which it was cultured. The culture plate with label attached is removed from the incubator and placed

in a sterile overwrap and sealed. The approved copy label is attached to this before leaving the cleanroom. Procedures are in place for packing and transporting the tissue at ambient temperature.

Appropriate documentation accompanies the product to the Ophthalmology theatre. Receipt of the ALCS product is documented.

Any adverse reactions must be recorded by the clinician and reported back to the GMP facility.

4.0 In-Process Testing & Methods

Use of automated blood culture system for the detection of aerobic and anaerobic organisms has been established for other cellular therapy products, especially haematopoietic progenitor cells, for many years. Previous studies have shown that these methods are suitable for detecting microbial contamination providing on-site validation is performed (Khuu et al., 2006).

The BMF-HS have over twenty years experience of using these methods for assessing sterility of haematopoietic progenitor cell products. The inventors have found that using BacT/ALERT [bioMerieux] paediatric blood culture bottles we are likely to detect relevant organisms when testing small sample volumes i.e. 1 ml.

There is some evidence that BacT/ALERT testing may be less effective in the presence of antibiotics in the medium (Flayhart et al., 2007). However, since the limbal biopsy is taken from a non-sterile site and may contain commensal organisms, it is necessary to include antibiotic in the culture medium initially. Therefore penicillin/streptomycin (or gentamicin) will be present in the medium on day -2/-1 for plating the amniotic membrane and on day 0, when the limbal culture is commenced. From the first feed on day 2 or 3 and subsequently, there will be no antibiotic in the added medium. Thus, the pre-release microbiology assessment using BacT/ALERT culture system will be entirely valid.

4.1 Quality Control

For this product the quality control is divided into 3 phases.

4.1.1 Phase 1 QC

i. Preparation of media

A 2ml sample is taken from the prepared basic culture medium and transferred to a blood culture bottle [BacT/ALERTOPF Plus, bioMerieux] and sent to the Department of Microbiology, Newcastle Hospitals NHS Foundation Trust.

A 1 ml sample is taken from the prepared autologous serum and tested as above.

A 1 ml sample is taken from the prepared 'complete' culture medium containing autologous serum and tested as above.

All of the above must be negative for both aerobic and anaerobic bacteria.

ii. Preparation of membrane

The prepared HAM is checked after 24 hours to ensure that it remains securely held on the coverslips and that the medium remains clear, negative for any bacterial contamination via visual inspection prior to commencing the limbal epithelial culture.

4.1.2 Phase 2 QC

i. Cell culture

On day 0, the plated amniotic membrane is removed from the incubator and the medium is removed before placing the limbal explant. 0.5ml of the medium is collected in a cryo tube for mycology screening. The remaining medium is put into a BacT/ALERT®PF Plus [bioMerieux] culture bottle for culture. Both samples are sent to the Department of Microbiology, Newcastle Hospitals NHS Foundation Trust for testing.

Release criteria requires that the results for the mycology screen and aerobic and anaerobic bacteria culture are negative.

Within 72 hours prior to tissue release, 1 ml of the culture medium from the well containing the limbal cell culture is put into a BacT/ALERT®PF Plus [bioMerieux] culture bottle for testing. As this is nearing the end of the culture/production period only the 48hr report of blood culture results will be available when the tissue is due to be released. The result 'no growth at 48hr' is acceptable for the release criteria

ii. 24hrs before transplant

A macroscopic and microscopic inspection of the culture will be performed prior to the expected transplant date. This will ensure that the product meets the release criteria and also that there is no visual evidence of infection, such as cloudy medium.

Tissue failing to meet the criteria for release will be discarded or used for Quality Assurance. In certain circumstances Concessionary release may be appropriate if safety criteria are met.

iii. Day of transplant

A further 1 ml sample is removed from the media taken from the final product immediately prior to release for retrospective QA. This is sent to an external contract laboratory (Stockton Quality Control Laboratory, University Hospital of North Tees, Hardwick Stockton on Tees, TS19 8PE) for Eu.Ph sterility testing.

4.1.3 Phase 3 QC

i. Follow up After Transplant

Following transplantation, a biopsy of residual IMP material not transplanted (removed when trimming the graft to size) will be sent for further testing to an external laboratory (Sheffield). The cells are tested using immunohistochemical analysis. They are required to demonstrate:

i) High expression of the putative LSC markers p63, ABCG2 & vimentin;
ii) Low expression of the CK 3 differentiation marker;
iii) High expression of the cell proliferation marker Ki67.

When reports are available, they will be reviewed and copies will be entered into the batch manufacturing record (BMR).

ii. Record of growth curve

Following transplantation, using the six well plate, accurate measurement of the growth area at each time point will be performed. This data will be plotted as a growth curve and retained in the BMR.

iii. Record of outstanding microbiological testing

As part of phase 3 QC, all microbiology and external testing results will be reviewed and recorded in the BMR. This includes the 5 day results from the samples taken 72 hours pre-release and the Eu.Ph sterility testing performed at the Stockton Quality Control Laboratory.

4.2 Monitoring of Culture Conditions

CO_2 and temperature logs during the production period are recorded by a wireless monitoring system (Monitherm).

4.3 Prevention of Cross Contamination

Although a single batch is produced for one patient it is beneficial for growth comparison purpose to have two limbal expansion cultures from different patients running concurrently.

Necessary controls are in place to minimise any risk of cross contamination or compromising the integrity of each of the cultures being undertaken e.g. use of specific product labels; use of separate shelves in the incubator, processed separately in the Class II cabinet, use of patient specific culture medium and worksheet for each patient's LSC expansion culture.

4.4 Storage of Materials

Reagents and worksheets for each patient's LSC expansion culture are prepared in separate trays for transfer to the laboratory.

Autologous culture medium for each patient will be stored in separate racks in the refrigerator. No more than two ALSC cultures will be undertaken at any one time.

Once the ALSC product has been released for transplantation any unused patient specific medium will be discarded.

4.5 Processing

Only one set of patient materials are placed in the Class II Cabinet at a time. The cabinet will be cleaned and sanitised according to local protocols between each patient culture.

On removing the culture plate from the incubator for examination or medium change, personnel must ensure that the patient I.D. on the culture plate corresponds with the label on the growth medium and confirmation of this is recorded on the worksheet.

5.0 Approved Copy Label

Product will be on a glass coverslip with 2 outer wrappers as shown in diagram below. Each layer will contain a label to ensure all patient checks are possible at each stage of the procedure with the approved copy label attached to the second (sterile) bag such that the surgeon may make final patient demographic checks in theatre. All unused labels will be

returned to the BMFHS for label reconciliation. Details will be kept in the batch manufacturing record.

6.0 Clinical Trial Protocol

Clinical trial end points include:

1. Corneal Impression Cytology

The cornea is anaesthetised using topical oxybuprocaine hydrochloride 0.4% eye drops [Minims, Chauvin Pharmaceuticals]. Corneal impressions are obtained using a single sterile Biopore membrane which is provided stretched over a short plastic tube [Millicell-CM 0.4µm, 13mm diameter, Millipore]. The patient is asked to look at a target placed in front of them and the lids are held apart. The Biopore membrane is pressed gently on the central cornea for up to 5 seconds to obtain a corneal impression. It is vital at this stage to ensure accurate placement of the membrane so that only a central corneal impression is made without inadvertent conjunctival contact which would produce a false positive result. The area of impression is easily identified as the white membrane becomes transparent when moist. This area is marked immediately so that the area of interest can be identified when dry. The membranes can then be stained using histology or immunocytochemistry techniques. Finally, the areas of impression are cut out and mounted for light or fluorescent microscopy. One impression is taken from each cornea and is sent to an Ophthalmic Pathologist at the Royal Hallamshire Hospital for independent assessment using PAS staining and cytokeratin markers.

2. Best Corrected Visual Acuity

Corrected visual acuity (in Snellen equivalents) are measured for each eye using a logarithmic visual acuity chart for testing at 4 metres. The results from the manifest refraction may be placed in a trial frame and visual acuities measured. The chart should be set at approximately eye level to the average height of a seated patient. A mark will be made on the floor (e.g., with tape) that is 4 metres away from the chart. If the patient reads no letters at 4 metres, the chart is then moved to 1 metre. The test distance and illumination for the chart must be kept constant throughout the study. Begin by testing the right eye and the left eye must be covered. The patient is asked to read each letter, line by line, left to right, beginning with line 1 at the top. The patient should be told that the chart has letters only, no numbers. If the patient reads a number, he or she should be reminded that the chart contains no numbers, and the examiner should then request a letter in lieu of the number. The patient is not to proceed to the next letter until s/he has given a finite answer.

If the patient changes a response (e.g., that was a “C” not an “O”) before s/he has read aloud the next letter, then the change must be accepted. If the patient changes a response, after having read the next letter, then the change is not accepted. The examiner must not point to specific letters on the chart during the test.

When the letters become difficult to read, or if the patient identifies a letter as one or two letters, he/she should be asked to choose one letter and, if necessary, to guess. Record the visual acuity as the lowest line read with one or no mistakes. Record the acuity in Snellen equivalent units (e.g., 6/9). Repeat for the left eye.

The pinhole method for measuring visual acuity is not acceptable.

3. Pain Score
It measures a patient’s pain intensity or other features. The pain score is based on self-report. The patient marks on a horizontal scale from 0 (No pain/discomfort) to 10 (severe pain).

8.0 Stability Data
The intent is to transplant the final product when cells are more than 90% confluent, over most of the area of the amniotic membrane on the 2.4cm x 2.4cm coverslip. They should not be allowed to remain in culture at full confluence otherwise epithelial cells will start to differentiate into corneal epithelial cells, forming cell layers and consequently lose progenitor cell capacity/stem cell phenotype.

- Transplant of the limbal epithelium is scheduled to coincide with optimal growth of the limbal stem cells. The ophthalmology theatre is provisionally booked for the transplant to take place 12-14 days after the initial limbal biopsy collection.
- Progress of the growth is monitored throughout the culture period to predict the time when the tissue will have reached the release criteria. BMF-HS staff will liaise with CI and his surgical team to confirm if the culture is on target for the scheduled date. It is usually possible to do this by day 7-9.
- If the cells appear to be growing too fast (area of explant outgrowth >50m m^2 at day 6 and >150mm^2 at day 9) then it may be prudent to bring forward the transplant by a day or so to prevent over growth of the tissue.
- Conversely if the cells are growing slowly then the transplant procedure may be deferred to allow more time for optimal growth to be achieved.

- The limbal tissue remains in culture at 37°C until release procedure has been completed but is only sent to the ophthalmology theatre once the preparatory work on the patient's LSCD eye is almost completed as this may take 1-2 hours depending on the condition of the affected eye.

9.0 Storage and Shipment Conditions

Transportation from BMF-HS to Ophthalmology operating theatre:

- It is a short distance of only a few hundred metres from the BMF-HS to the Ophthalmology theatre therefore the transit time is relatively short and predictable.
- To further minimise the risk of compromising the integrity of the tissue it is released and transported in the well of the plate in which it was cultured. The medium in the culture well remains to keep the tissue hydrated.
- The plate containing the product (with the primary packaging label affixed) is transferred to a sterile plastic bag. A secondary packaging annex 13 compliant label is attached to the outer bag.
- After transfer from the clean room, the product is placed into another transparent bag with a further secondary packaging annex 13 compliant label before being placed flat in suitable/dedicated transport box using suitable packing material, e.g. 'bubble wrap' material to fill the space between the plate and box so that it will not move during transportation.
- The box is taken by foot to the eye theatre (Ophthalmology Dept., RVI) by competent laboratory or theatre staff ensuring it is kept upright at all times. In the theatre, the outer bag is opened before passing the product to the sterile area.
- Acceptance of the product is documented and received by personnel who sign and date the initial release form.
- The end point for stability assessment is that the product is stable at room temperature for about 24 hours.

Annex 1 - Human Amniotic membrane (HAM). Product description

Amniotic membrane dissected from placentas donated during elective caesarean section deliveries. Processed in-house, in licensed pharmaceutical grade cleanrooms [minimum GMP classification C). The amnion is washed in isotonic citrate solution to remove blood and treated with an antibiotic cocktail to reduce microbial load. The antibiotic solution contains gentamicin sulphate (4g/l), imipenem (0.2g/l), nystatin (2.5x106 U/l), polymyxin B sulphate (0.2g/l), and vancomycin hydrochloride (0.05g/l), prepared in medium 199 supplemented with 25mM HEPES.

Frozen in the final packaging with 50% glycerol/Hanks solution. Stored at -80°C whilst on site at NHS Blood and Transplant (NHSBT) premises. Quarantined for 180 days to retest the donor for serological markers, or tested at time of donation by PCR technology for hepatitis B & C and HIV, in addition to the routine serology. Supplied as individual units measurements approximately 3x3cm squares, mounted stromal side on to nitrocellulose paper.

Quality and Safety

Tissue is sourced from UK donors in compliance with rigorous ethical and clinical standards. The consent process is approved by the Human Tissue Authority. In house experts on tissue donor selection and medical history influence the standard across all donation programmes (blood, tissue and organ). The standard is written by UK blood services in compliance with MSBTO (advisory committee in the Microbiological Safety of Blood, Tissues and Organs). Much of the standard is above and beyond the minimum required by European/UK legislation and regulation. Tissue Services was previously licensed by the MHRA (Medicines and Healthcare product

Regulatory Authority) under the UK code of practice and now holds establishment licences under the HTA (Human Tissue Authority). The services and facilities comply with Good Manufacturing Practice.

Hospitals donating placentas are carefully selected to ensure quality and continued proficiency. Theatre staff are trained in the donation procedure. Donation sites are audited every year. All microbiology testing is performed by in-house accredited laboratories specialising in donation screening. Final donor assessment and selection is undertaken by in-house senior nurse specialists in tissue donation. Donations are tracked by barcode including automated test result transfer to the database (the same database used for blood

donation, processing and supply). This database has automated controls to prevent release of non-conforming tissue. Tissue is stored at -80°C to ensure continued storage below the required -40°C with full audit trail for stock location. Final product release is undertaken as an independent function by specialist NBS Quality Assurance personnel. Containers and transport boxes are validated in-house by the Tissue Development Laboratory.

All activity is regularly reviewed against practice considered best by international standards, with professional links to the British, European and American Tissue Banking Associations.

Labelling and Packaging

This product is attached stromal side to nitrocellulose paper contained within a 7ml translucent polypropylene sterile bijou. The secondary package is heat sealed in low density polyethylene bags compliant with EC Commission Directive 2002/72/EC. The outer bag is labelled with graft type, unique donation number, expiry date, size, and storage requirements. Donation number, product type, status and expiry date are ISBT 128 barcoded. Enclosed within the packaging is a transplant reporting form with a freepost envelope, which can be used for feedback. If an adverse event or reaction is suspected, telephone the tissue bank immediately.

Delivery

Transportation protocols are validated to ensure that grafts arrive with the customer undamaged and in perfect condition. Packaging materials are validated to ensure that the integrity of the graft is maintained up the point of use. Delivery is in a disposable transport box containing dry ice (solid carbon dioxide) validated to keep the graft frozen until the time written on the box. It is delivered by either NHSBT Transport or via a courier, usually direct to the point of use e.g. eye ward/theatre at the RVI. Next working day delivery is included in the product price. More urgent delivery e.g. same day or by specified time can be arranged at additional cost. Where an operation is graft critical, the patient must not be taken to theatre before the graft has arrived and its condition checked.

Storage

This product must be stored below -40°C if it is not to be used within 48 hours. Freezers need to be designated for clinical use with 24/7 alarms and monitoring. Once thawed, this product must be used within 24hrs.

Annex 2 - Validation of Culture Conditions.

Pre clinical studies

Pre clinical studies have demonstrated the successful culture of human limbal epithelium on human amniotic membrane (HAM). The inventors have shown, as have many others, the use of HAM is a suitable alternative to 3T3 fibroblasts (Burman et al., 2004; Meller et al., 2000). Details of the use of HAM are shown in section 3 and in Annex 1. The HAM for the clinical study was obtained from NHS BT Tissue Services Liverpool under a Third Party agreement (TPA). HAM provides a physical carrier for which to transplant the cells, even prior to culture confluence or sheet formation **(Figure 2)**. It is also non-immunogenic, eliminating the requirement of recipient immunosuppression (Kruse et al., 2000). Both explant and suspension cultures of human limbal epithelium can be established on HAM, although explant cultures grow more quickly which is clearly beneficial **(Figure 3).** However, the sole problem with the limbal epithelium and HAM co-culture system is the use of FCS in the epithelial medium, which is sub-optimal for human transplantation purposes.

Elimination of Foetal Calf Serum Use in the Culture of Human Limbal Epithelium.

The studies using human serum (HS) containing epithelial medium show that this is a viable alternative to the use of FCS containing medium for the culture of human limbal epithelium. The co-cultures of human limbal epithelium with either 3T3 fibroblasts or HAM can both be established using FCS and HS containing medium. However, cell expansion was greater in the HS containing culture, as shown by the larger explant outgrowth **(Figure 4).**

Development of an Animal Free Culture System for Human Limbal Epithelium Suitable for Transplantation.

The combination of HAM and HS containing medium for the culture of human limbal epithelium results in the production of a culture system without non-human animal products and cells. It overcomes the following problems encountered with the other culture systems investigated in this chapter:

1. Use of animal cells or products,
2. Use of immunogenic tissue, and
3. Requirement of culture confluence and sheet formation.

Development of the animal free culture system of the invention was vital for translation of this research to human patients with unilateral total LSCD.

Pre-clinical data have shown that exponential growth of cells is achieved by days 8 and 9 post-culture on human amniotic membrane, and by day 12-19 sufficient cells have expanded *ex vivo* for transplantation.

A prospective well-designed clinical translation study (Phase I) was carried out by the inventors in a strictly defined uniform group of patients with total unilateral LSCD and no other significant co-existing ocular pathology so that success or failure of reversal of total LSCD could be attributed solely to the LSC therapy (Kolli et al., 2010). Twelve consecutive patients with unilateral total LSCD were offered treatment with *ex vivo* expanded autologous LSCs on human amniotic membrane (HAM). Nine of twelve patients had successful limbal stem cell cultures and went on to successful transplants with a mean follow-up of 19 months (Table 1). This failure rate in 3 cultures is in line with published data (Meller et al., 2002) where the majority of failures are due to underlying/undetected stem cell deficiency. Postoperatively, clinical and histological reversal of LSCD with the establishment of stable corneal epithelium was obtained in all 9 eyes (100%) with significant improvements in both vision impairment and pain scores (Table 2). This study demonstrates that transplantation of autologous limbal epithelial stem cells cultured on HAM without the use of non-human animal cells or products, is a safe and efficaceous method of successfully treating patients with unilateral total LSCD. The full study has been recently published in the journal Stem Cells (Kolli et al., 2010).

Assessment of Potential Effects of Using -20°C Stored Medium as Compared to 4°C Stored Medium on the Outgrowth Areas.

1. The culture of human limbal explants on human amniotic membrane under good manufacturing practice as per Validation Plan.
2. The effects of using -20°C stored medium as compared to 4°C medium.
3. The effects of altering the culture conditions (i.e. temperature) on the growth of cultures.

Methods

1. 4 limbal explants (2 from the same limbal ring and the other 2 from different rings) were cultured as per standard operating procedures for preparation of culture medium and the ex vivo expansion of limbal epithelium.

2. Microbiological assessment of all 4 limbal expansion experiments was performed as described in the validation plan. Samples for detection of bacteria and fungus were tested by Dept Microbiology, RVI and Mycoplasma testing performed in-house (Academic Haematology) by ELISA technique.
3. Functional assessment in the form of explant outgrowth area was performed on all 4 cultures.
4. In the 2 explants from the same limbal ring, 1 explant was cultured with -20°C stored medium and 1 with 4°C stored medium.
5. 1 explant was cultured under standard conditions in a tissue culture incubator (at 37°C with 5% CO_2 and 95% humidity) until the 5th day. The culture was then placed at 4°C for 3 hours under standard atmospheric conditions, and then back into the tissue culture incubator for the remainder of culture growth.

Results

Microbiological assessment

Limbal Culture medium

All samples of medium assessed (at initiation of culture) displayed no signs of contamination when examined microscopically all samples were negative for bacterial or fungal contamination.

Cell Culture

All cultures were assessed on the 10th day of growth for bacteria and fungus by plating tissue culture medium on appropriate agar plates (Dept. Microbiology) All cultures were negative for both bacteria and fungus.

Mycoplasma testing on medium taken from 12 day old cultures was also performed. All cultures were negative for Mycoplasma arginini, Mycoplasma hyorhinis and Mycoplasma orale.

Explant outgrowths

All cultures grew well under good manufacturing practice. All 4 cultures had achieved >1 cm outgrowth diameter by the 13th day of culture (day of culture termination). The outgrowth areas are shown in Figure 5.

Effect of medium storage

There were no deleterious effects of using -20°C stored medium as compared to 4°C stored medium on the outgrowth areas (Figure 6).

Effect of culture environment

Incubation of the culture at 4°C for 3 hours had no deleterious effects on subsequent outgrowth (Figure 7).

Conclusions

1. The cultures grew well and as expected (from non GMP validation studies) under good manufacturing practice conditions.
2. All 4 cultures were negative for bacteria, fungus and Mycoplasma as assessed by testing of growth medium.
3. By the 13th day of culture, the epithelial outgrowth from all the explants was sufficient for corneal and limbal coverage if transplantation were to be performed.
4. There was no significant difference between using 4°C or -20°C stored growth medium on the outgrowth areas. As -20°C allows storage of medium for longer periods of time, medium may be stored at -20°C prior to use.
5. There was no deleterious effect on outgrowth area of altering the culture conditions to 4°C for 3 hours. It may therefore be concluded that the period of removing the culture from tissue culture incubator conditions to the period of any subsequent transplantation will not have a significant effect on the viability of cultured cells.

The validation results passed the acceptance criteria for culturing ALSC for transplant use.

Annex 3 - Validation Studies on ex vivo Expansion of Human Limbal Epithelium and Characterisation of Limbal Epithelia Cultures

Only a tiny portion of the total limbus was used in the explant technique. Since the distribution of LSCs at the limbus was not uniform but concentrated at the upper limbus), we cultured four separate 1-2 mm^2 explants per limbal ring (one explant per quadrant to achieve an average) and used three organ culture stored rings in total. The time to observe epithelial outgrowths and time taken for the outgrowth to reach the edge of a single well of a Tis 5200 6 well culture plate [Iwaki, UK, or equivalent] was recorded and the average values taken. The morphology of the outgrowth was also assessed and photographed. (see Figure 8 & Fig 9).

Successful outgrowths were achieved in each of the 4 cases. The average time for the appearance of the first outgrowths from the explant was 4.08 days (±1.00, range 3-6 days) and reached the edge of the well at 17.33 days (±2.57, range 14-21). The cells continued to proliferate until the outgrowths reached the edge of the culture well and stratification was observed.

The microscopic examination of the expanded tissue shows a stem cell like phenotype with small round tightly packed cells with large nuclei and little cytoplasm.

To show the detailed relationship between the limbal explant, HAM and proliferating cells, the culture shown in Figure 10 was placed in formalin after 14 days in culture and then paraffin embedded and used for immunohistochemistry. Antibody against human p63 (which is a putative marker of SC cells and early TACs) was used to allow easy visualisation of the proliferating cells. Since a horse radish peroxidise (HRP) visualisation system was used, the progenitor/TAC cells would be expected to have nuclei which stained dark brown whereas differentiated cells (lacking p63 expression) would show no brown staining of their nuclei. The results of this analysis showed an arrangement in close agreement to this model and are shown in Figure 11. That is, the progenitor cells of the basal layer of the epithelium covering the explant proliferated and covered the sides of the explant and then continued to expand on to the surface of the HAM. The cells on the HAM close to the explant showed heavy staining with p63 whereas those distant from the explant are seen to begin to stratify, differentiate and lose p63 staining.

Annex 4 - *Ex vivo* Expansion and Transplantation of Autologous Human Limbal Epithelium for the Treatment of Unilateral Total Limbal Stem Cell Deficiency.

Although the use of animal products has been used for certain epithelial cultures such as epidermal cultures for burns patients the ratio of benefit versus risk must be considered (Banks-Schlegel et al., 1981). In the case of life threatening burns, the risks associated with cultures containing animal products may be acceptable.

The successful culture of LSCs on alternative feeder layers to mouse 3T3 fibroblasts has been established on extracellular matrix components including collagen IV coated shields, laminin and fibronectin and on human limbal fibroblasts (Ahmadiankia et al., 2009; Nakagawa et al., 1990).

As previously discussed, successful culture of LSCs has been established on HAM for clinical transplantation by several other workers (Koizumi et al., 2001; Meller et al., 2000; Tsai et al., 2000). HAM has been consistently and successfully used as a co-culture substrate in the expansion of LSCs in studies by the inventors. HAM is the preferred choice for co-culture substrate on which to grow LSCs for several reasons:

1. HAM allows the 3T3 mouse fibroblast layer to be totally excluded;
2. HAM provides excellent and consistent expansion of LSC numbers;
3. HAM has a physically resilient basement membrane on which the LSCs can grow and allows easy handling at surgery;
4. HAM contains many factors conducive to epithelial cell growth and may mimic the natural LSC niche and;
5. HAM does not elicit an immune response (Dua et al., 2004).

The main animal product other than murine fibroblasts used in the laboratory culture of LSCs is foetal calf serum (FCS) used in the epithelial growth medium. Approaches to resolving this issue would include using a serum free epithelial growth medium or replacing the FCS with human (autologous) serum (HAS). HAS has been successfully used to replace the need for FCS in epithelial growth medium for the culture of a variety of epithelial cell types including skin, oral mucosa and cornea (Mazlyzam et al., 2008; Nakamura et al., 2006a; Nakamura et al., 2006b). HAS has been used therapeutically for some time in the treatment of ocular surface disorders due to its many favourable properties, including support of epithelial growth (Rauz and Saw, 2010; Tsubota et al., 1999). Therefore, the use

of HAS to replace FCS in epithelial growth medium has many additional advantages as well as a means of eliminating animal products from the culture system.

The culture of human limbal epithelium has been successfully established with both 3T3 fibroblast co-culture and culture on HAM with epithelial growth medium containing HS by the inventors. Epithelial outgrowths using an explant culture technique on HAM using epithelial growth medium supplemented with either FCS or HS were established under otherwise identical culture conditions. The results consistently showed the equivalence or superiority of HS compared with FCS in terms of faster and greater epithelial outgrowths. The consistent and reproducible growth of limbal epithelium using an explant technique on HAM relies on the specific and stable arrangement of the HAM being stretched flat across a coverslip while bathed in the HS supplemented growth medium.

In summary, a completely animal product free system combining HAM and HAS for limbal epithelial culture has been established and validated by the inventors. This overcomes the problems associated with potential interspecies pathogen transfer and potential rejection of immunogenic tissue.

***Ex vivo* Expanded Tissue**

The histology and immunohistochemical analysis of the cultured epithelium confirmed that an epithelium is formed on the HAM (Figure 12). This epithelium is 2-3 cells layers thick although thicker at the edges of the cultures specimen. The basal layer of cells adjacent to the HAM showed characteristics indicating an actively expanding SC phenotype including:

i. Small cuboidal cells with undifferentiated appearance (in contrast to the tall columnar cells with large amounts of cytoplasm typical of mature corneal epithelium).
ii. High expression of the putative CESC markers p63, ABCG2 & vimentin.
iii. Low expression of the CK 3 differentiation marker.
iv. High expression of the cell proliferation marker Ki67.

TEM confirms the light microscopy findings demonstrating the formation of a primitive epithelium with a prominent basal layer of cuboidal cells with a high nucleus: cytoplasm ratio (Figure 13). The basal cells are attached to their basement membrane via hemidesmosomes and to each other via desmosomes. The superficial squamous

cells which are not in contact with the HAM show early differentiation demonstrating primitive microplicae formation in keeping with a corneal epithelial phenotype.

Annex 5 - Risk Assessment Table.

RISK	Inherent risk		Controls in place	Residual risk		Action planned	Target date	Initial
	Impact	Likelihood		Impact	Likelihood			
PROCUREMENT								
Starting tissues, i.e. patient material, amniotic membrane, may contain pathogens	HIGH	LOW	All samples from patient or donor material has been tested for mandatory ID markers as described in 2006/17/EU.	HIGH	LOW	All blood tissues are treated as being capable of transmitting disease.		
Loss/damage to limbal biopsy during transport, i.e. due to rough handling, etc.	HIGH	MED/ LOW	Transport is of short duration but procedures by competent staff ensure integrity of material and minimise delay to GMP facility.	HIGH	LOW	No further action.		
PROCESSING								
ALSC product may become contaminated with microbes/pathogens								
a) from reagents, growth factors, etc.	HIGH	LOW	All GMP grade materials are reconstituted according to manufacturer's instructions and culture medium filtered prior to use.	HIGH	LOW	Positive result has to be reported to QP and PI to discuss course of action.		
b) as a result of processing, i.e. culture is 'open' process	HIGH	MED/ LOW	Asceptic techniques employed by competent staff according to GMP. Culture medium is aliquoted into single use volumes to minimise risk. Microbiological testing performed prior to release of tissue.	HIGH	LOW			

BacT/ALERT testing system may not detect pathogens when there is antibiotic in the test media.	HIGH	MED.	Antibiotic will be omitted from the media following day 3 of the culture, such that the pre-release test sample will contact no antibiotic. Media is inspected at ever medium change and pre-release. Growth medium is also tested by an external control laboratory.	HIGH	LOW	No further action.		
Failure of amniotic membrane to provide stable matrix for culture of biopsy	HIGH	MED.	Experienced staff to manipulate tissue. Visual microscopic inspection to ensure membrane is satisfactory prior to limbal culture. A second AM is held in a controlled freezer.	HIGH	LOW	No further action.		
Risk of cross-contamination, i.e. using wrong culture medium	HIGH	MED	Procedures in place for segregation of materials when working on more than one patient. Staff trained in good working practice.		LOW	None.		
Substances used in processing may cause harm to patient/staff	MED	LOW	COSHH RA for all materials used. Harmful substances used at very low concentration. Staff trained and RA signed.	MED/ LOW	LOW	No further action.		
Failure of essential equipment, i.e CO_2 incubator	HIGH	LOW	Equipment checks are made prior to initiating production. A reserve CO_2 cylinder is always available. In case of major failure continuing procedures to transfer culture to BMF CfL.	HIGH	LOW	No further action.		

ISSUE								
Risk of damage to final product, i.e. rough handling during transportation to ophthalmic theatre.	HIGH		MED	Carefully packaged and placed in as insulated tissue transplant box with suitable materials to avoid movement in transit. Competent staff take the product short distances to theatre, < 10 minutes.	HIGH		LOW	No further action.

Annex 6 – Cholera toxin data

See Figure 14.

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Claims

1. A method for expansion of limbal stem cells, comprising:

i) providing a limbal biopsy on a growth support;

ii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and

iii) substituting the epithelial culture medium of step ii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period.

2. The method according to claim 1, wherein the method is free from non-human animal derived products.

3. The method according to any preceding claim, wherein the growth support is human amniotic membrane.

4. The method according any preceding claim, wherein the human amniotic membrane is intact membrane.

5. The method according to any preceding claim, wherein a stromal surface of the limbal biopsy is in contact with the growth support.

6. The method according to any preceding claim, wherein the culture medium comprises at least one growth factor.

7. The method according to claim 6, wherein the at least one growth factor comprises insulin.

8. The method according to claim 6 or 7, wherein the at least one growth factor comprises hydrocortisone.

9. The method according to any one of claims 6 to 8, wherein the at least one growth factor is tri-iodothyronine.

10. The method according to any one of claims 6 to 9, wherein the at least one growth factor is adenine.

11. The method according to any one of claims 6 to 10, wherein the at least one growth factor is epidermal growth factor.

12. The method according to any preceding claim, wherein the epithelial culture medium comprises cholera toxin.

13. The method according to any preceding claim, wherein the one or more antibiotics comprises two antibiotics, preferably penicillin and streptomycin.

14. The method according to any preceding claim, wherein the serum is derived from the same donor as the limbal biopsy.

15. The method of any preceding claim, wherein the expanded limbal stem cells form an epithelial sheet on the growth support.

16. The method according to any preceding claim, wherein the limbal stem cells are expanded for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support.

17. The method according to claim 16, wherein the epithelial sheet is at least (90% of 1.5cm^2), and preferably from about (90% of 1.5cm^2) to about (90% of 3.5cm^2).

18. A method of treating limbal stem cell deficiency comprising:

i) isolating a limbal biopsy from a patient in need of treatment;

ii) placing the limbal biopsy on a growth support;

iii) culturing for a first time period the limbal biopsy in the presence of epithelial culture medium supplemented with serum and one or more antibiotics; and

iv) substituting the epithelial culture medium of step iii) with epithelial culture medium which is supplemented with serum and which is free from antibiotics, and culturing for a second time period for a time sufficient to provide a composite structure comprising an epithelial sheet upon the growth support; and

v) transplanting the composite structure onto an eye of the patient.

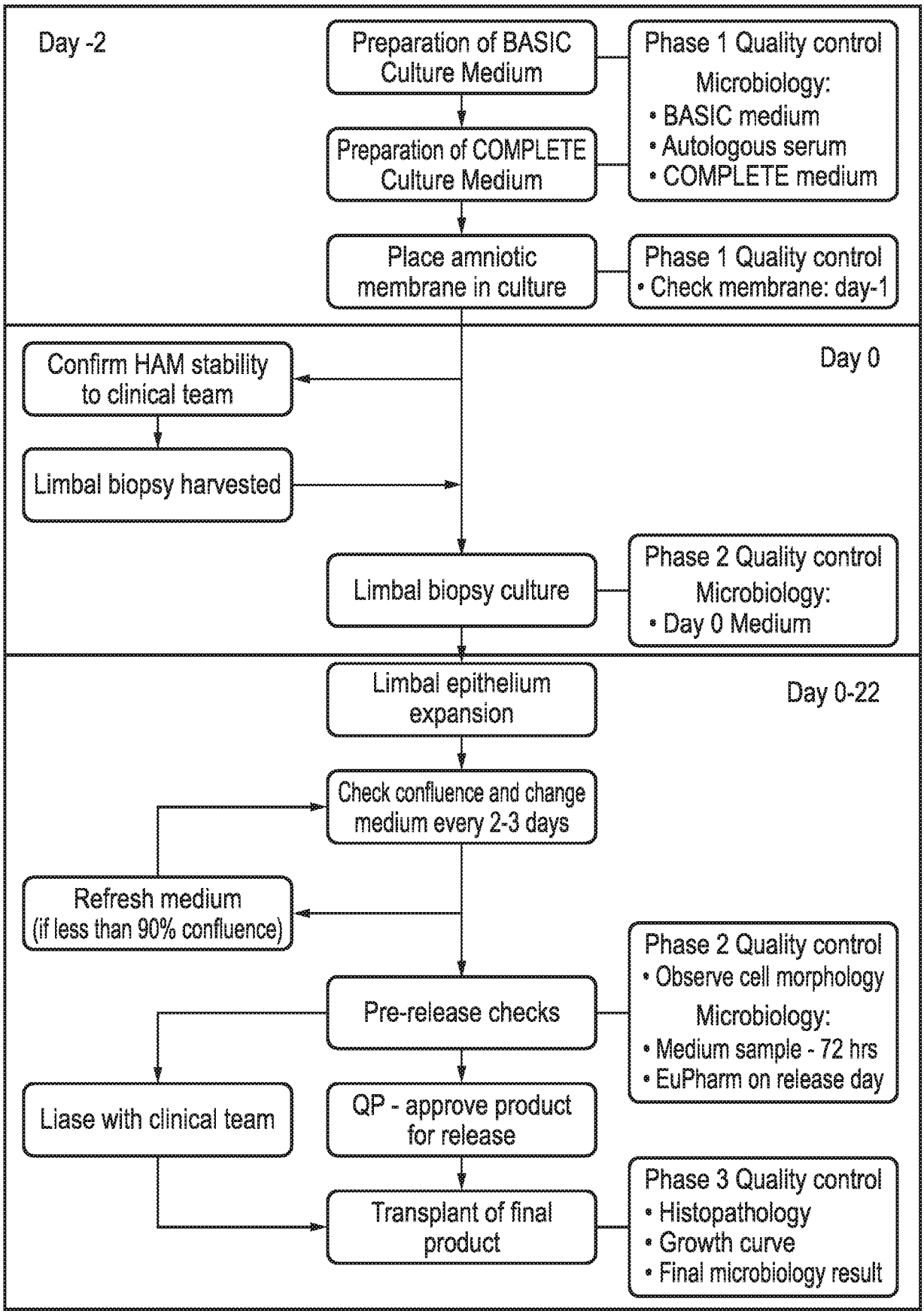
Day -2
Preparation of BASIC Culture Medium
Preparation of COMPLETE Culture Medium
Place amniotic membrane in culture
Phase 1 Quality control
Microbiology:
• BASIC medium
• Autologous serum
• COMPLETE medium
Phase 1 Quality control
• Check membrane: day-1
Day 0
Confirm HAM stability to clinical team
Limbal biopsy harvested
Limbal biopsy culture
Phase 2 Quality control
Microbiology:
• Day 0 Medium
Day 0-22
Limbal epithelium expansion
Check confluence and change medium every 2-3 days
Refresh medium (if less than 90% confluence)
Pre-release checks
Phase 2 Quality control
• Observe cell morphology
Microbiology:
• Medium sample - 72 hrs
• EuPharm on release day
Liase with clinical team
QP - approve product for release
Transplant of final product
Phase 3 Quality control
• Histopathology
• Growth curve
• Final microbiology result

FIG. 1

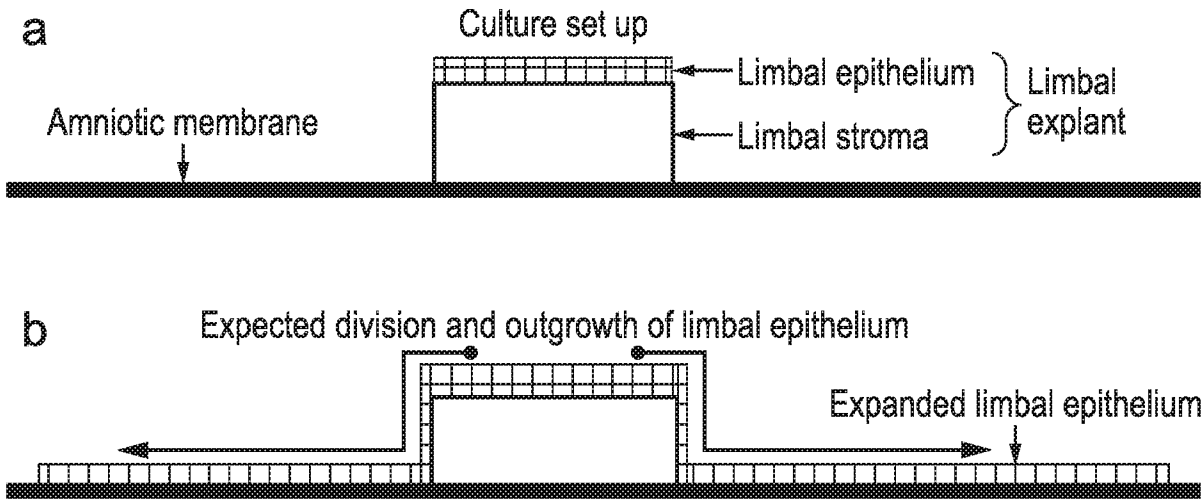
a
Culture set up
Limbal epithelium
Limbal stroma
Limbal explant
Amniotic membrane
b
Expected division and outgrowth of limbal epithelium
Expanded limbal epithelium

FIG. 2

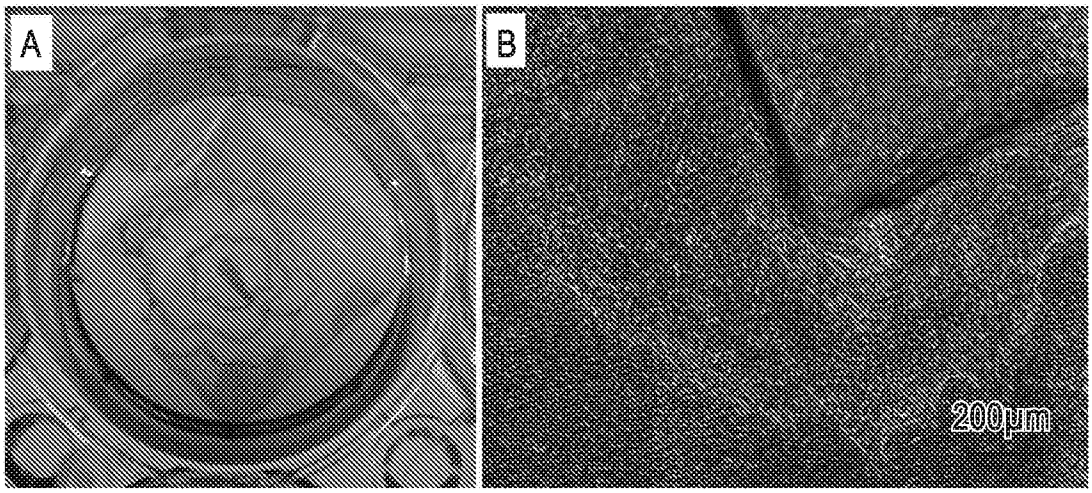

FIG. 3

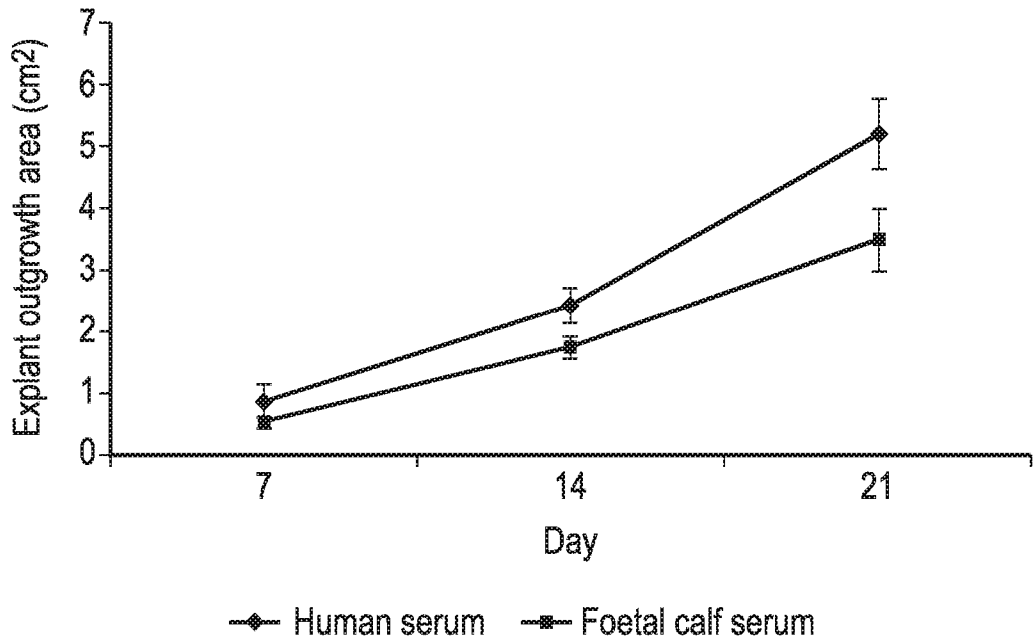
Explant outgrowth area (cm2)
0
1
2
3
4
5
6
7
7
14
21
Day
Human serum
Foetal calf serum

FIG. 4

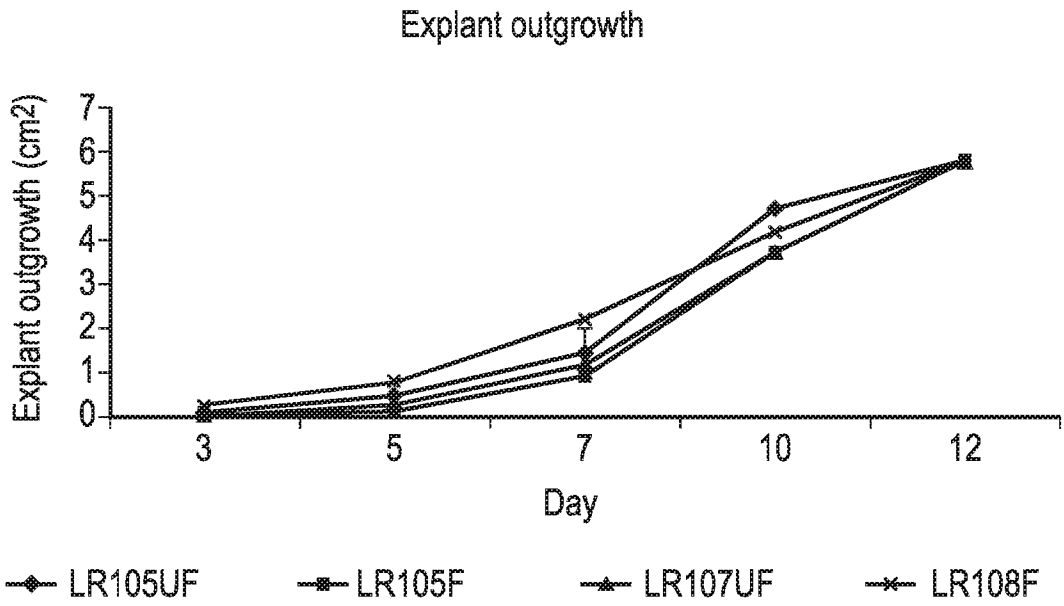
Explant outgrowth
Explant outgrowth (cm2)
0
1
2
3
4
5
6
7
3
5
7
10
12
Day
LR105UF
LR105F
LR107UF
LR108F

FIG. 5

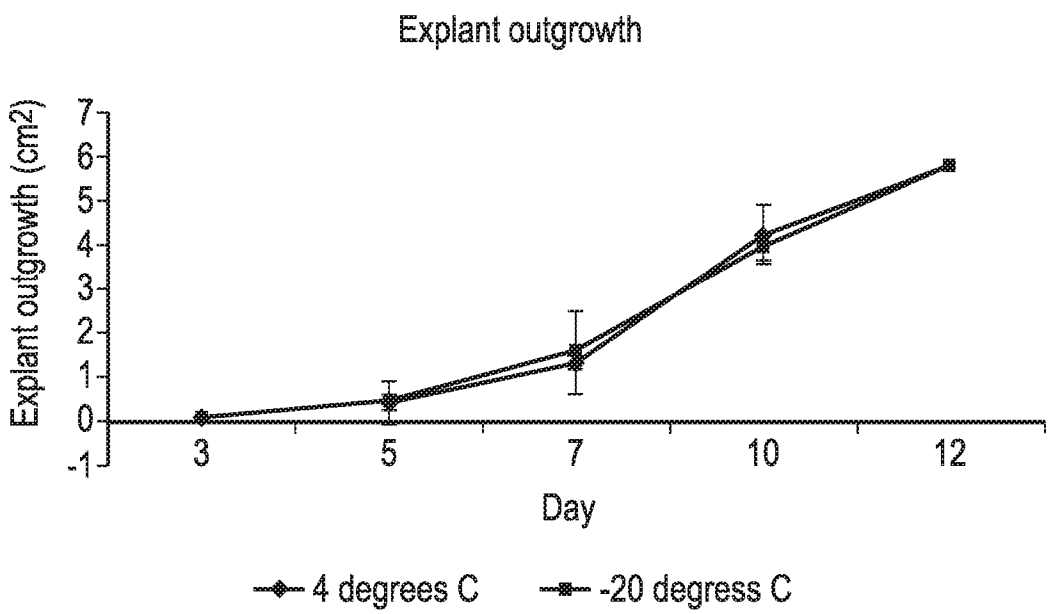
Explant outgrowth
Explant outgrowth (cm2)
7
6
5
4
3
2
1
0
-1
3
5
7
10
12
Day
4 degrees C
-20 degress C

FIG. 6

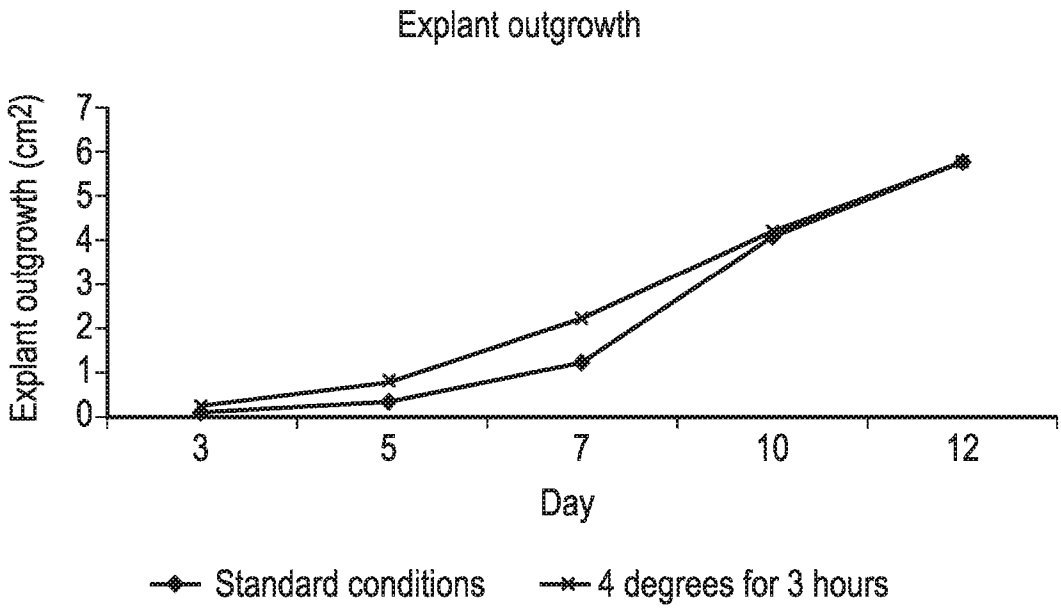
Explant outgrowth
Explant outgrowth (cm2)
0
1
2
3
4
5
6
7
3
5
7
10
12
Day
Standard conditions
4 degrees for 3 hours

FIG. 7

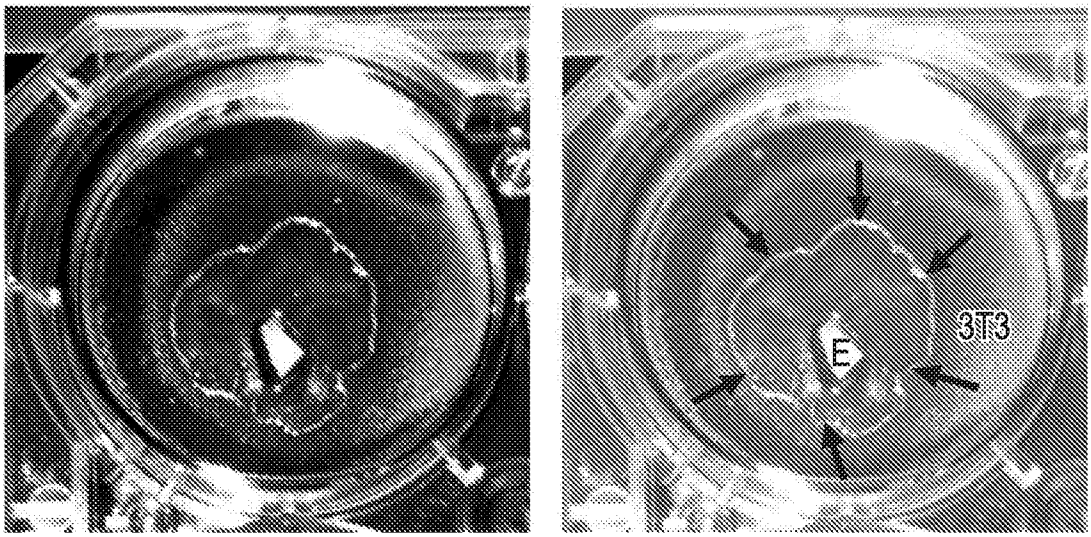

FIG. 8

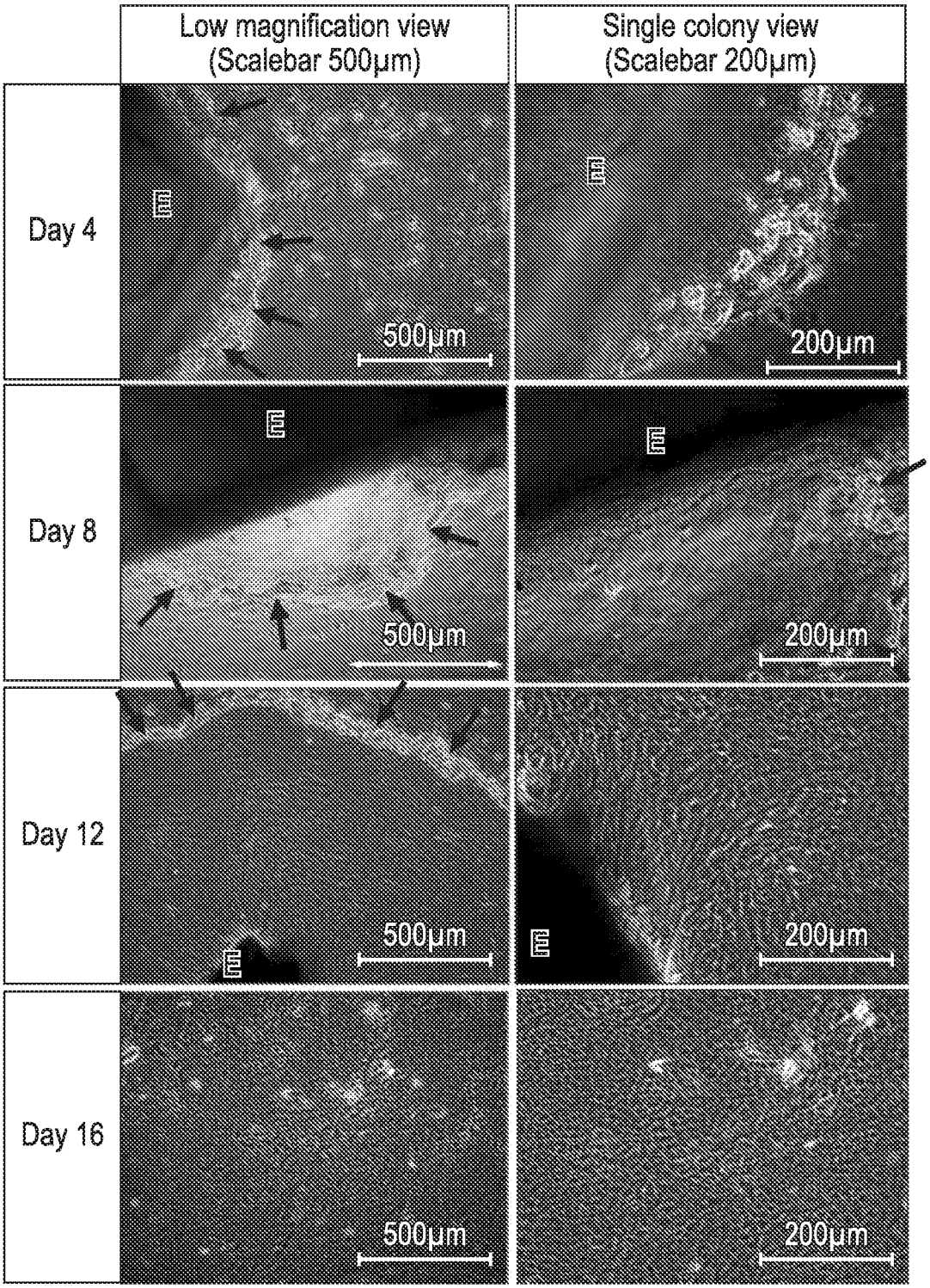
Low magnification view
(Scalebar 500µm)
Single colony view
(Scalebar 200µm)
Day 4
Day 8
Day 12
Day 16
500µm
200µm

FIG. 9

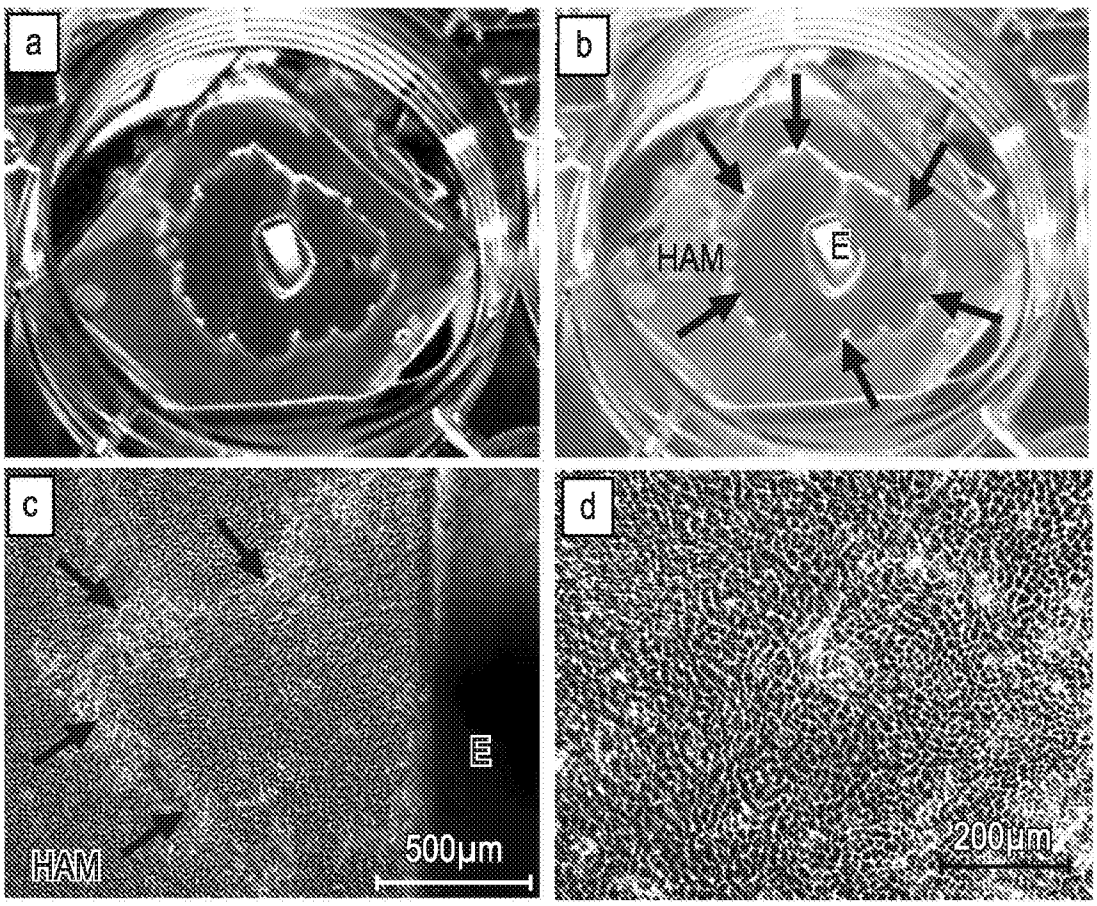

FIG. 10

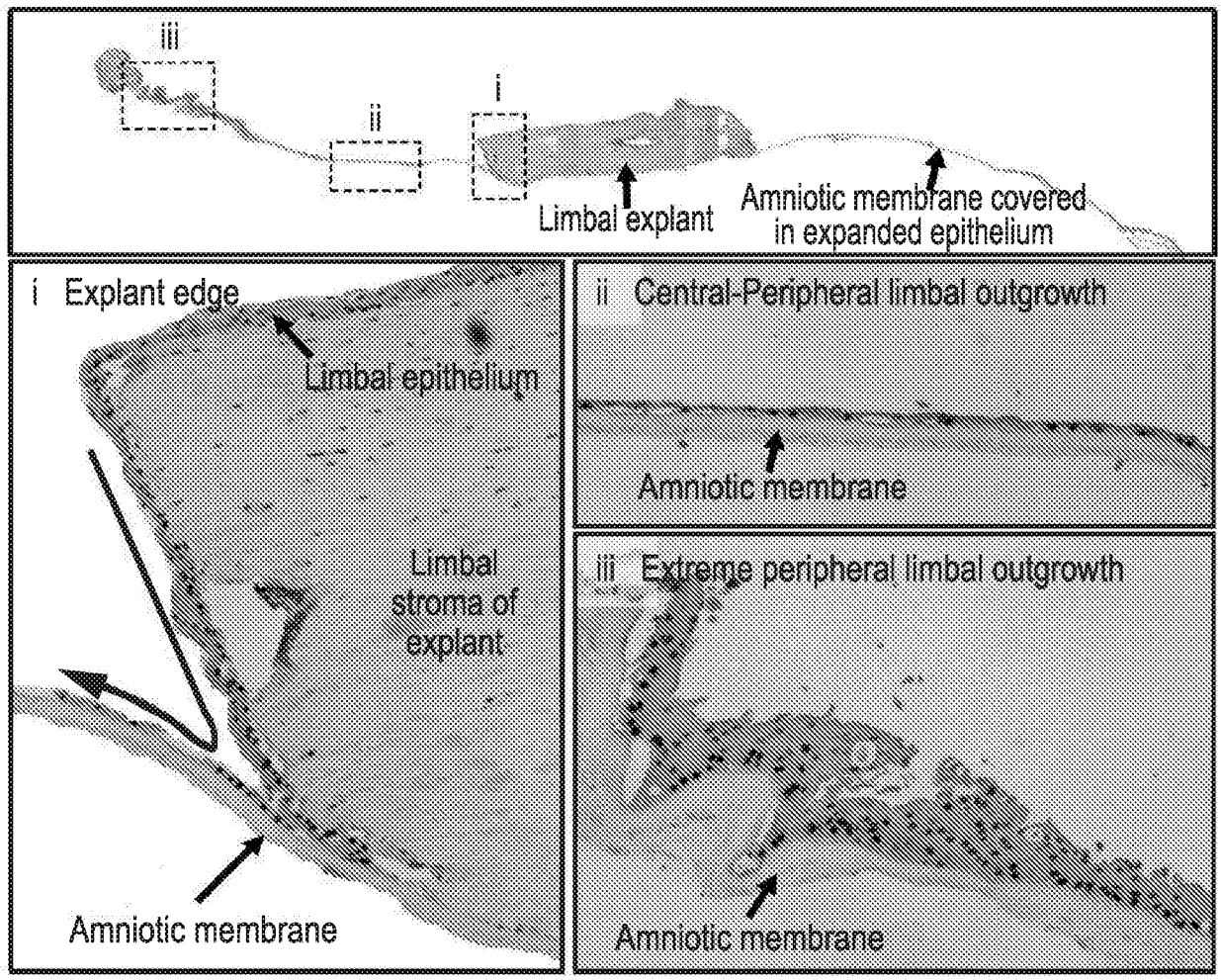
iii
ii
i
Limbal explant
Amniotic membrane covered in expanded epithelium
i Explant edge
Limbal epithelium
Limbal stroma of explant
Amniotic membrane
ii Central-Peripheral limbal outgrowth
Amniotic membrane
iii Extreme peripheral limbal outgrowth
Amniotic membrane

FIG. 11

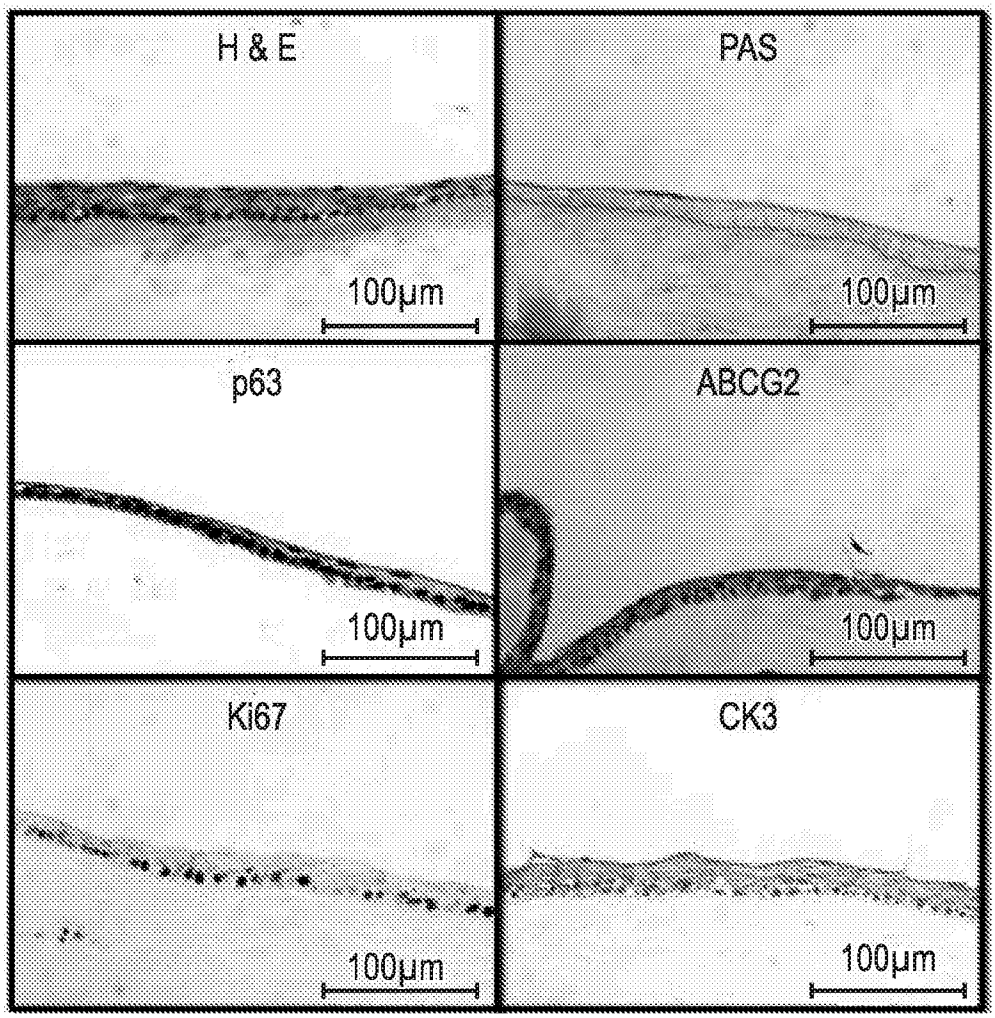
H & E
100µm
PAS
100µm
p63
100µm
ABCG2
100µm
Ki67
100µm
CK3
100µm

FIG. 12

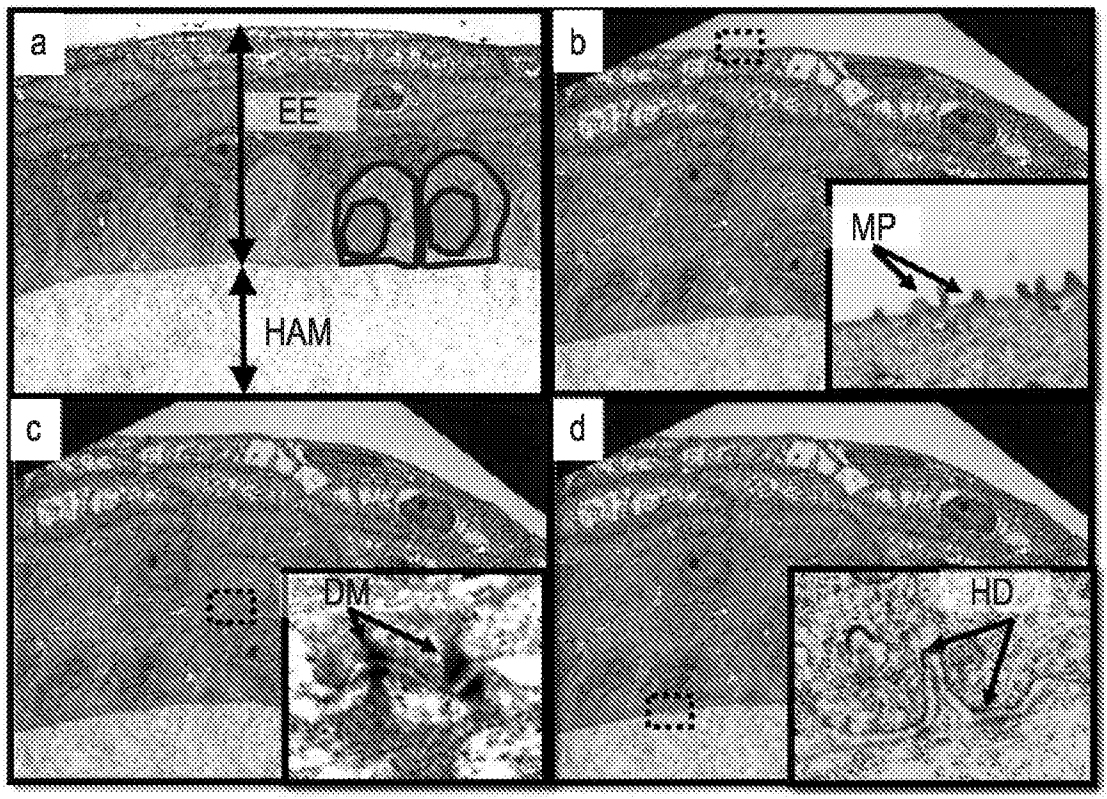

FIG. 13

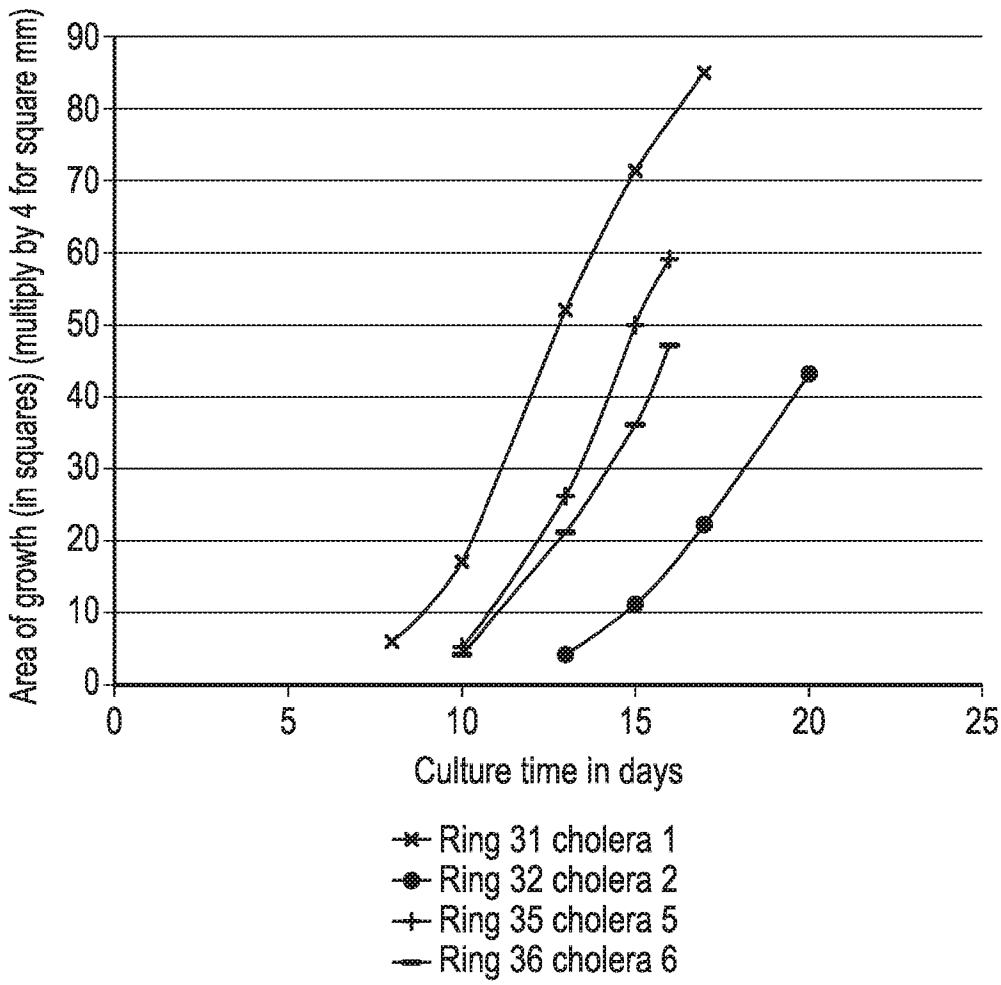
Area of growth (in squares) (multiply by 4 for square mm)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Culture time in days
Ring 31 cholera 1
Ring 32 cholera 2
Ring 35 cholera 5
Ring 36 cholera 6

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050434

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/079
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAI KOLLI ET AL: "Successful Clinical Implementation of Corneal Epithelial Stem Cell Therapy for Treatment of Unilateral Limbal Stem Cell Deficiency", STEM CELLS, 1 January 2009 (2009-01-01), pages N/A-N/A, XP055179363, ISSN: 1066-5099, DOI: 10.1002/stem.276	18
A	the whole document page 599, paragraph 5 - paragraph 6 page 606 - page 607; figures 6-7	1-17

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 2015	21/04/2015

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050434

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABOULGHASSEM SHAHDADFAR ET AL: "Ex vivo expanded autologous limbal epithelial cells on amniotic membrane using a culture medium with human serum as single supplement", EXPERIMENTAL EYE RESEARCH, vol. 97, no. 1, 1 April 2012 (2012-04-01), pages 1-9, XP055179671, ISSN: 0014-4835, DOI: 10.1016/j.exer.2012.01.013	18
A	the whole document page 7; figure 3	1-17

X	NADIA ZAKARIA ET AL: "Standardized Limbal Epithelial Stem Cell Graft Generation and Transplantation", TISSUE ENGINEERING PART C: METHODS, vol. 16, no. 5, 1 October 2010 (2010-10-01), pages 921-927, XP055153962, ISSN: 1937-3384, DOI: 10.1089/ten.tec.2009.0634	18
A	the whole document page 922, last paragraph	1-17

A	WO 2010/133853 A1 (UNIV READING [GB]; CONNON CHE [GB]) 25 November 2010 (2010-11-25) the whole document page 19 - page 20; examples 9-10	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2015/050434

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010133853 A1	25-11-2010	CA 2762362 A1	25-11-2010
		CN 102449142 A	09-05-2012
		EP 2432871 A1	28-03-2012
		GB 2470644 A	01-12-2010
		JP 2012527283 A	08-11-2012
		US 2012148543 A1	14-06-2012
		WO 2010133853 A1	25-11-2010