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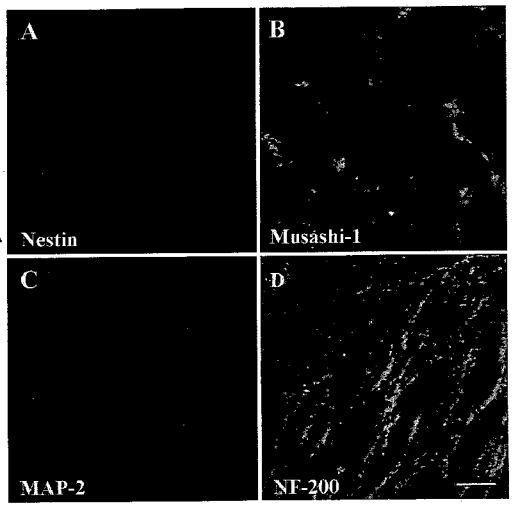
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(54) **Title**: METHODS FOR PRODUCING NEURONAL CELLS AND USES THEREOF

Figure 2



(57) **Abstract**: Methods for producing neuronal cells, methods of treating or preventing ophthalmic diseases or conditions or of promoting recovery from an ophthalmic therapy, and methods of treating or preventing neurological diseases or conditions or of promoting recovery from a neurological therapy are provided. The methods involving treating ophthalmic cells, particularly corneal cells, with insulin, epidermal growth factor and fibroblast growth factor 2 for a period sufficient to trans-differentiate the cells to neuronal cells.

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METHODS FOR PRODUCING NEURONAL CELLS AND USES THEREOF

TECHNICAL FIELD

The present invention relates to methods for producing neuronal cells, methods of treating or preventing ophthalmic diseases or conditions or of promoting recovery from an ophthalmic therapy, and methods of treating or preventing neurological diseases or conditions or of promoting recovery from a neurological therapy.

BACKGROUND OF THE INVENTION

Over the past decade there have been significant developments in stem cell research. Limited availability of stem cells due to ethical concerns or technical challenges have prompted researchers to investigate converting differentiated adult cells into other differentiated cell types through a transdifferentiation process, otherwise known as cell reprogramming.

Cell reprogramming has been reported using genetic modification. In one example four key transcription factors, Oct 3/4, Sox2, Klf4 and c-Myc are reported to play a vital role in cell differentiation and as tools for cell reprogramming (Takahashi and Yamanaka, 2006). Other work has reported that re-expression of key developmental regulators Ngn3, Pdx1 and Mafa reprogrammed adult pancreatic exocrine cells to β-cells in the mouse (Zhou 2008).

However, only limited efficacy is often observed (Zhou et al, 2008) and there are reported concerns about the oncogenic nature of these cells (Zhou et al, 2009). For review see Yu et al, 2007 and Zhou et al, 2009.

It would be desirable to have a method of directly reprogramming cells into differentiated cells without the need for genetic modification that avoids or minimises uncontrolled phenotypic changes and cell proliferation.

Accordingly, it is an object of the present invention to provide an improved or alternative method for reprogramming cells or to at least provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the present invention provides a method for producing neuronal cells comprising

(1) providing an ophthalmic tissue comprising one or more ophthalmic cells, and

(2) contacting the tissue with

(a) epidermal growth factor or a functional variant or functional fragment thereof (EGF) or an agonist of the EGF receptor, and

(b) fibroblast growth factor 2 or a functional variant or functional fragment thereof (FGF2) or an agonist of one or more of the FGF family of receptors and

(c) insulin or a functional variant or functional fragment thereof or an agonist of the insulin receptor or of the insulin-like receptor,

for a period sufficient to produce one or more neuronal cells from one or more of the one or more ophthalmic cells.

In one embodiment, the ophthalmic tissue comprises one or more ophthalmic tissues selected from the group comprising cornea, ophthalmic epithelia, ophthalmic endothelia, limbus, retina, optic nerve and sclera.

In one embodiment, the ophthalmic tissue comprises ophthalmic tissue selected from the group comprising corneal epithelia, corneal stroma, and corneal endothelia.

In one embodiment, the tissue comprises at least two populations of differentiated ophthalmic cells.

In one embodiment, substantially all of at least one of the populations of differentiated ophthalmic cells is reprogrammed to neuronal cells.

In one embodiment, the one or more ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, corneal endothelial cells, differentiated limbal cells, retinal cells, and optic nerve cells.

In one embodiment, the method additionally comprises the step of isolating the one or more reprogrammed neuronal cells from the tissue.

In various embodiments, the tissue is in contact with EGF, FGF2 and insulin for from about 0.5 days to about 20 days, about 0.5 days to about 10 days, or from about 1 day to about 5 days, or from about 6 days to about 10 days, or for at least about 3 days.

In another aspect the present invention provides a method for producing neuronal cells comprising:

(1) providing one or more isolated differentiated ophthalmic cells;

(2) contacting the one or more differentiated ophthalmic cells with EGF, insulin and FGF2 for a period sufficient to produce one or more neuronal cells from one or more of the one or more differentiated ophthalmic cells.

In one embodiment, the one or more differentiated ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, corneal endothelial cells, scleral epithelial cells, scleral endothelial cells, scleral fibroblasts, limbal epithelial cells, limbal endothelial cells, limbal fibroblasts, retinal cells, and optic nerve cells.

In one embodiment, the one or more differentiated ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, and corneal endothelial cells.

In one embodiment, the one or more differentiated ophthalmic cells are corneal keratocytes.

In various embodiments, the one or more neuronal cells are neuronal precursor cells, the one or more neuronal cells are Musashi-1 positive, the one or more neuronal cells are Nestin positive, the one or more neuronal cells are both Musashi-1 positive and Nestin positive, or the one or more neuronal cells are neurons.

In one embodiment, the one or more differentiated ophthalmic cells are in contact with EGF, insulin and FGF2 for from about 0.5 days to about 20 days, 0.5 days to about 10 days, or from about 1 day to about 5 days, or from about 6 days to about 10 days, or for at least about 3 days.

In another aspect the present invention provides a method for generating neuronal cells, the method comprising administering epidermal growth factor, insulin and fibroblast growth factor 2 to one or more ophthalmic cells in a mammalian subject in need thereof.

In one embodiment, the one or more ophthalmic cells are present in one or more ophthalmic tissues selected from the group comprising cornea, ophthalmic epithelia, ophthalmic endothelia, limbus, retina, cilary body, optic nerve, eyelid and enclosed orbicularis oculi muscle, and sclera.

In one embodiment, the one or more ophthalmic cells are present in one or more ophthalmic tissues selected from the group comprising corneal epithelia, corneal stroma, and corneal endothelia.

In one embodiment, the one or more ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, and corneal endothelial cells.

In one embodiment, the mammalian subject is to undergo or has undergone ophthalmic surgery.

In another aspect the present invention provides a method of treating or preventing an ophthalmic disease or condition or of promoting recovery from an ophthalmic therapy, the method comprising administering epidermal growth factor, insulin and fibroblast growth factor 2 to one or more ophthalmic cells in a mammalian subject in need thereof.

In one embodiment, the ophthalmic disease or condition is selected from the group comprising retinal degeneration including retinal ganglion cell loss, retinal ganglion cell loss as a result of trauma, ophthalmic trauma, ischemia including optic nerve ischemia, glaucoma, age related macular degeneration, corneal nerve dystrophies, dry eye, ophthalmic viral infection such as herpes infection including herpes simplex or herpes zoster infection, hypoesthesia, lost or damaged ophthalmic neurons, ophthalmic inflammation and neurological conditions.

In one embodiment, the ophthalmic therapy is corneal surgery, including corneal transplant.

In one embodiment, the administration is intraocular administration, such as but not limited to intraocular injection, intraocular depot injection, intraocular implant, retinal implant, or subretinal implant.

In one embodiment, the administration is topical administration, such as but not limited to administration by ocular drops or ocular gel.

In another aspect the present invention provides a method of treating or preventing a neurological disease or condition or of promoting recovery from an neurological therapy, the method comprising administering one or more neuronal cells prepared by a method of the invention to a mammalian subject in need thereof.

In one embodiment, the neurological disease or condition is selected from the group comprising neurological diseases associated with tissue trauma, neurological diseases associated with inflammation, and neurodegenerative diseases.

In another aspect the present invention provides a cell growth or differentiation media comprising

(1) epidermal growth factor at a final concentration of from about 0.1ng/mL to about 50ng/mL, or

(2) fibroblast growth factor 2 at a final concentration of from about 0.25ng/mL to about 50ng/mL, or

(3) insulin at a final concentration of from about 1μg/mL to about 50μg/mL, or

(4) any combination of two or more of (1) to (3) (i.e., both (1) and (2); both (2) and (3); both (1) and (3); or each of (1), (2) and (3)).

In one embodiment the cell growth or differentiation media comprises epidermal growth factor at a final concentration of from about 0.1ng/mL to about 50ng/mL, and fibroblast growth factor 2 at a final concentration of from about 0.25ng/mL to about 50ng/mL, and insulin at a final concentration of from about 1μg /mL to about 50μg/mL.

In one embodiment the cell growth or differentiation media comprises

(1) epidermal growth factor at a final concentration of from about 0.1ng/mL to about 9.5ng/mL, or

(2) fibroblast growth factor 2 at a final concentration of from about 0.25ng/mL to about 25ng/mL, or

(3) insulin at a final concentration of from about 1μg /mL to about 25μg /mL, or

(4) any combination of two or more of (1) to (3).

In one embodiment the cell growth or differentiation media comprises epidermal growth factor at a final concentration of from about 0.1ng/mL to about 9.5ng/mL, and fibroblast growth factor 2 at a final concentration of from about 0.25ng/mL to about 25ng/mL, and insulin at a final concentration of from about 1μg /mL to about 25μg /mL.

In one embodiment, the growth media additionally comprises one or more of Heparin, L-glutamine, an antibiotic, and an antimycotic.

In another aspect the present invention provides a pharmaceutical composition comprising epidermal growth factor, insulin and fibroblast growth factor 2, wherein the composition is formulated for administration to the mammalian eye.

In one embodiment, the composition is formulated for topical administration.

In one embodiment, the composition is formulated for intraocular administration.

In one embodiment, the composition is formulated for retrobulbar administration.

In another aspect the present invention provides epidermal growth factor, insulin and fibroblast growth factor 2 for use in therapy.

In one embodiment the invention provides epidermal growth factor, insulin and fibroblast growth factor 2 for use in treating or preventing an ophthalmic disease or condition or in promoting recovery from an ophthalmic therapy.

In another aspect the present invention provides for the use of epidermal growth factor, insulin and fibroblast growth factor 2 in the preparation of a medicament suitable for treating or preventing an ophthalmic disease or condition in a mammalian subject in need thereof.

In another aspect the present invention provides for the use of epidermal growth factor, insulin and fibroblast growth factor 2 in the preparation of a medicament suitable for promoting recovery from an ophthalmic therapy in a mammalian subject in need thereof.

In still another aspect, the present invention provides a product containing epidermal growth factor, insulin and fibroblast growth factor 2 as a combined preparation for simultaneous, separate or sequential use in therapy.

The embodiments set forth herein including the following may relate to any of the above aspects.

In various embodiments, the one or more neuronal cells are neuronal precursor cells or neurons.

In various embodiments, the one or more neuronal cells are one or more of the following: Musashi-1 positive, Nestin positive, MAP2 positive, Neurofilament-200 positive, SMI32 positive, Doublecortin positive, beta III Tubulin positive, Neuronal-N positive, or any combination of two or more thereof.

In various embodiments, the EGF is present at a final concentration of below about 50ng/mL, or at a final concentration of from about 0.1ng/mL to about 50ng/mL.

In various embodiments, the FGF2 is present at a final concentration of below about 50ng/mL, or at a final concentration of from about 0.1ng/mL to about 50ng/mL.

In various embodiments, the insulin is present at a final concentration of below about 50μg/mL, or at a final concentration of from about 1μg /mL to about 50μg /mL.

In one embodiment, the insulin is replaced with one or more other agonists of the insulin receptor or insulin-like receptor, including, for example, IGF1.

In one embodiment, the EGF is replaced with one or more other agonists of the EGF receptor, including, for example, TGF alpha.

In one embodiment, the FGF2 is replaced with one or more other agonists of one or more of the FGF family of receptors, including, for example, FGF1, FGF4, FGF6, FGF7, or FGF9.

In one embodiment, the insulin is replaced with one or more other agonists of the insulin receptor or of the insulin-like receptor, including, for example, IGF1, and the EGF is replaced with one or more other agonists of the EGF receptor, including, for example, TGF alpha.

In various embodiments, the media, composition, medicament or product is for use in treating or preventing an ophthalmic disease or condition or in promoting recovery from an ophthalmic therapy.

In various embodiments, the media, composition, medicament or product is for use in producing one or more neuronal cells.

In various embodiments, the media, composition, medicament or product comprises sufficient EGF, FGF2, and insulin (or any functional variants, functional equivalents, or agonists of their respective receptor(s) to provide one or more of the following:

EGF at a final concentration of below about 50ng/mL, or at a final concentration of from about 0.1ng/mL to about 50ng/mL,

FGF2 at a final concentration of below about 50ng/mL, or at a final concentration of from about 0.1ng/mL to about 50ng/mL,

insulin at a final concentration of below about 50μg/mL, or at a final concentration of from about 1μg/mL to about 50μg /mL.

For example, the media, composition, medicament or product comprises sufficient EGF, FGF2, and insulin (or any functional variants, functional equivalents, or agonists of their respective receptor(s) to provide one or more of the following:

EGF at a final concentration of below 10ng/mL, or at a final concentration of from about 0.1ng/mL to about 9.5ng/mL,

FGF2 at a final concentration of below 25ng/mL, or at a final concentration of from about 0.25ng/mL to about 25ng/mL,

insulin at a final concentration of below 25μg/mL, or at a final concentration of from about 1μg /mL to about 25μg /mL.

The term "comprising" as used in this specification means "consisting at least in part of". When interpreting each statement in this specification that includes the term "comprising", features

other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner.

As described above, in this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a depiction of the interactome of representative members of the FGF family of factors and the FGF receptors 1–4, adapted from STRING data base (http://string-db.org)

Figure 2 presents confocal microscope images of cultured corneal slices showing (A) Nestin and (B) Musashi-1 immunohistochemical labelling after 3 days in culture, and (C) MAP-2 and (D) Neurofilament-200 labelling after 8 days in culture in the reprogramming medium. The entire stromal keratocyte population has differentiated into a neuronal marker expressing phenotype, and shows a morphological change from the stromal fibroblast cell shape at day 3 into a neuronal phenotype at day 8. Scale bar, 40μm.

Figure 3 presents micrographs showing combined DAPI nuclear marker with Nestin imunnohistochemical label after 8 days in culture. (A) A view from the epithelial surface showing the neuronal morphology that the stromal cells have taken on. The presence of nuclei in these cells indicates that the neurons are not part of the cornea's anterior nerve net which has its cell bodies in the trigeminal ganglion. Scale bar, 30μm. (B) A side on view of the cornea showing that the reprogrammed cells at this stage remain constrained within the collagen layers of the corneal stroma. The epithelium is at the top. Scale bar, 40μm

DETAILED DESCRIPTION OF THE INVENTION

The present invention recognises for the first time that keratocytes present in the corneal stroma of adult humans can be reprogrammed to another cell type or to express phenotypic characteristics of another cell type.

The invention provides methods of reprogramming differentiated cells to express one or more phenotypic markers of another cell type. One such method is performed on isolated differentiated cells, and broadly comprises the steps of providing a sample comprising one or more ophthalmic cells, and contacting the one or more ophthalmic cells with EGF, insulin and FGF2 or functional variants or functional fragments thereof. Another such method is performed on a population of differentiated cells present in situ in a tissue sample from the mammal, and typically comprises the steps of providing an ophthalmic tissue comprising one or more ophthalmic cells, and contacting the sample with EGF, insulin and FGF2 or functional variants or functional fragments thereof.

One of the major applications of the present invention is in the repair of damaged tissue, for example, in the repair of damaged corneal tissue or damaged retinal tissue. The invention accordingly provides methods for in vivo cell implantation, tissue implantation, and tissue therapy. The invention also provides methods broadly comprising administering one or more growth factors to a tissue in vivo, so as to produce one or more neuronal cells or one or more neuronal precursor cells. For example, one such method comprises contacting one or more ophthalmic tissues in vivo with one or more growth factors – EGF, insuin or FGF2 – at a therapeutically efficacious concentration.

Additionally, the invention is directed towards a growth media used in reprogramming differentiated cells, such as stromal keratocytes, and particularly a defined growth media comprising the growth factors EGF, insulin and FGF2, as well as pharmaceutical compositions comprising EGF, insulin and FGF2 or functional variants or functional fragments thereof, suitable for administration to the mammalian eye.

Importantly, the methods of the present invention do not rely on genetic modification of the cells to achieve reprogramming to another cell type. In the present application, the phrase "genetic modification", including for example genetic modification of one or more cells, refers to any mechanism or technique of modulating or effecting gene expression in the cells through the use of introduced nucleic acid, such as, for example, the addition of new genetic material by DNA transfection, viral transduction, anti-sense RNA addition, siRNA, etc.

The methods of the present invention are reliant on the use of selected growth factors to produce neuronal cells from other differentiated cells. The phrase "the use of growth factors" and equivalents thereof refers to the addition of those factors either during culturing of the cells or tissue

samples, after implantation of the cells into a host, or administration of the factors in vivo, as the case may be.

1. The tissues of the mammalian eye

The human eye is a significant sense organ, allowing humans to perceive and visualise light and depth, and differentiate colour. The eye consists of three concentric layers, each comprising various tissues. The outer fibrous layer comprises the sclera and cornea; the middle vascular layer comprises the choroid, ciliary body and iris; and the inner nerve layer comprises the retina.

1.1 Cornea

The cornea is an avascular and transparent tissue, which covers the front of the eye and acts as a wndow to the outer world. It provides the eye with a clear refractive interface, tensile strength, and protection from external factors. Its transparency and regularity, together with the lens, allow formation of an optic image on the light sensitive retina at the back of the eye.

In humans, the adult cornea has a diameter of about 11mm and a thickness of 0.52mm at its centre (Ehlers & Hjortdal, 2006). The cornea is comprised of five main layers: epithelium (most superior), Bowman's layer, stroma, Descemet's membrance, and endothelium (most inferior).

1.1.1 Corneal Epithelium

The corneal epithelium is a stratified, squamous, non-keratinising epithelium, which is constantly maintained and renewed. The corneal epithelium is continuous with the conjunctival epithelium at the edges of the cornea, a region known as the limbus. Corneal epithelium comprises 5-7 cell layers.

1.1.2 Bowman's Layer

The Bowman's layer is located directly beneath the basement membrane of the corneal epithelium. It is acellular and transparent with a thickmess of 8-14μm, and consists of randomly oriented small collagen fibrils.

1.1.3 Stroma

The corneal stroma comprises about 90% of the entire corneal thickness and is continuous with the sclera at the limbus. The main cellular constituent of the stroma is the keratocyte, sparsely populated along with highly organised collagen fibres running parallel to the corneal epithelium. The stroma is composed of water (78%), collagen (15%) and other proteins, such as proteoglycans (7%). Turnover is slow (up to several months) (Ehlers & Hjortdal, 2006).

1.1.4 Descemet's Membrane

The Descemet's membrane is similar to the Bowman's layer, being a thin acellular layer composed of collagen fibres. Unlike other basement membranes, it is reasonably thick and lies between the stroma and the endothelium. As a basement membrane of the endothelium, it is secreted by the monolayer of endothelial cells and increases in thickness with age.

1.1.5 Endothelium

The endothelium is the innermost layer of the cornea, and consists of a single layer of polygonal cells. It is avascular and essential in keeping the cornea transparent. The endothelium has no or very little proliferative ability, which results in age-related decrease in cell density throughout life.

1.2 Limbus

The limbus is a transitional zone, between the cornea and the conjunctiva and sclera, where the clear cornea continues into the opaque sclera. A line connecting the termination of Bowman's layer and Descemet's membrane can be defined as the anterior boundary of the limbus and the posterior boundary is where the tissue changes from transparent to opaque. The structure of the limbal epithelium is similar to the corneal epithelium but has less regularity and more cell layers with 7-10 cell layers in humans. The radially-oriented crests in the limbus, known as the palisades of Vogt, increase the surface area of the basal cells for the absorbance of nutrients and exchange of metabolites. A number of researchers have reported that the basal layer of the limbal epithelium contains stem cells.

1.3 Sclera

The sclera, more commonly known as the white of the eye, is an opaque, fibrous, protective outer layer of the eye that contains collagen and elastin fibres. It is derived from the neural crest, and is continuous with the cornea.

1.4 Retina

The retina comprises 7 layers of nucleated cells, lining the back of the eye. The retina comprises millions of photoreceptors that convert captured light rays into electric impulses, which travel along the optic nerve before being translated into images. The neural retina consists of six major classes of neurons; photoreceptors, bipolar cells, horizontal cells, amacrine cells, ganglion cells and the mullerian glia.

1.5 Optic Nerve

The optic nerve comprises the axons from the retinal ganglion cells to the visual cortex, and their support cells including astrocytes and oligodendrocytes.

2. Cells to be reprogrammed

The methods of the present invention are directed to the production in one or more differentiated cells of one or more phenotypes characteristic of another differentiated or differentiating cell type. A person skilled in the art will realise that cell type can be determined in a number of ways, both morphologically and biochemically, for example by determining the presence or expression of a cell surface marker, or determining a gene expression profile characteristic of a particular cell type. Those skilled in the art will also recognise that the heterogenous nature of cell populations within tissue means that not all cells of a particular cell type will necessarily exhibit the phenotypic marker characteristic of that cell type at the time a determination is made. Accordingly, it is sometimes advantageous to identify or characterise a cell population with a variety of markers to ensure accurate determinations are made.

Furthermore, as cells progress along a differentiation pathway to become more specialised, their phenotypic character, such as their protein expression or gene expression profile, typically changes. Those skilled in the art will realise that this can allow both the cell type and the stage of differentiation to be determined.

2.1 Opthalmic Epithelial cells

Ophthalmic epithelial cells include corneal epithelial cells, limbal epithelial cells, and scleral epithelial cells. This distinction is primarily due to location within the eye, rather than gross differences in morphology or biochemistry.

Each of the above opthalmic cell types is an example of stratified squamous epithelia, comprising several layers of cells. Epithelial tissue is readily identified by those skilled in the art, both morphologically and by way of biological markers. Cytokeratin 3/12 is one such recognised marker for corneal cell differentiation. Cytokeratin 3 is expressed in corneal epithelium. Collagen type I is abundantly expressed and localised to the striated fibrils within the corneal stroma.

2.2 Keratocytes

Stromal keratocytes, which are derived from the neural crest, are fibroblastic cells localized in the corneal stroma of the mammalian eye. The keratocytes lie predominantly between the collagen layers.

For the sake of clarity, the phrase "corneal keratocyte" is to be read as referring to a keratocyte located in (whether presently or originally) or isolated from corneal tissue, for example,

the corneal stroma of the mammalian eye. Grammatical equivalents, components or derivatives thereof are to be read likewise. Accordingly, the phrase "corneal stromal keratocyte" refers to a fibroblastic cell in or from the corneal stroma of the mammalian eye.

Corneal keratocytes have flattened cell bodies and contain processes that extend from the cell body to link cells, primarily within the same laminar plane but also between lamella planes.

2.3 Opthalmic Endothelial Cells

Ophthalmic endothelial cells include corneal endothelial cells, limbal endothelial cells, and scleral endothelial cells. Again, those skilled in the art are readily able to identify ophthalmic endothelial cells.

2.4 Limbal Stem Cells

Limbal stem cells (LSCs) are said to be distinct subpopulation of cells which are undifferentiated or poorly differentiated, slow cycling, small in size and high in proliferative ability Boulton and Albon, 2004; Chee et al., 2006; Dua and Azuara-Blanco, 2000; Romano et al., 2003). They principally reside in the basal layer of the highly specialised and protected limbal niche and are influenced by the microenviroment to maintain their "stemness" or undifferentiated state Dua et al., 2005; Shanmuganathan et al., 2007). They serve as an ultimate source for corneal epithelial renewal and undergo asymmetric cell division rarely under normal conditions, allowing one daughter stem cell to remain while the other daughter transient amplifying (TA) cell continues with differentiation. This low mitotic activity of LSCs minimises DNA replication-related errors to conserve their proliferative potential. The TA cell has been reported with limited proliferative capacity and restricted differentiation potential and its main role is to ensure high numbers of differentiated cells produced by each stem cell division Hall and Watt, 1989; Schermer et al., 1986). The existence of epithelial stem cells in the limbus was first proposed in 1971 by Davanger and Evensen who observed pigmented epithelial migration lines moving from the limbus towards the central cornea Davanger and Evensen, 1971) and the position of LSCs suggests a centripetal movement of cells from the limbus toward the central cornea Collinson et al., 2002; Nagasaki and Zhao, 2003). Combining with the previously discussed "X, Y, Z" hypothesis of corneal maintenance, a natural turnover of human corneal epithelial cells takes place wherein superficial cells are shed from the corneal surface by constant desquamation (Z component) and replaced from a population of stem cells that reside in the basal limbal region and continue to cycle slowly throughout life. Their daughter cells, the TA cells, migrate centripetally (Y component) into the basal layer of the corneal epithelium and differentiate into upper layers of the corneal epithelium (X component) to become post-mitotic cells. This combined belief has been widely accepted in both corneal homeostasis and wound healing conditions.

3. Target cells:

3.1 Neuronal cells

As used herein, the term "neuronal cell" refers to a cell exhibiting one or more phenotypic characteristics of a neuron or a cell capable of differentiating to form a neuron, but either is not yet fully differentiated or does not yet express one or more genes or proteins expressed by a fully differentiated neuron. Neurons, including cholinergic neurons, GABAergic neurons, glutamatergic neurons, dopaminergic neurons, and serotonergic neurons, are well characterised both morphologically and biochemically.

As those skilled in the art will recognise, the neuronal cells described herein refer to all cells along the neuronal differentiation pathway through to a fully differentiated dendritic neuron, such as those with fully functional axons or neurotransmitter release.

Neuronal cells may be identified as those expressing one or more markers associated with neurons or neuronal progenitor cells, including, for example, one or more of Nestin (a class VI intermediate filament protein that is routinely used as a CNS stem cell or neural progenitor marker), Musashi-1 (an intermediate filament that is expressed in CNS cells during CNS development), doublecortin, MAP-2 (a major microtubule associated protein of brain tissue and a marker of mature neuronal cells known to interact with neurofilaments, actin and other elements of the cytoskeleton), ABCG2 (ATP-binding cassette, sub-family G, member 2, reported to be a marker for pluripotent stem cells), Neurofilament-200, SMI32, Neuronal class III tubulin, Oct4, synaptophysin, various ligands, effectors and receptors in the Notch/Delta signalling pathway including Jagged 1, Jagged 2, Delta-like 1, Notch 1 receptor, and the Notch effectors Hes1 and Hes5, Neurofilament H, and tyrosine hydroxylase.

Neuronal cells representative of early stages of reprogramming and exemplified in the Examples presented herein include cells which are positive for both Musashi-1 and Nestin, as well as cells which are Musashi-1 positive, and cells which are Nestin positive. Neuronal cells representative of the late stages of reprogramming and exemplified in the Examples presented herein include cells which are positive for one or more of Nestin, Neurofilament-200, SMI32, MAP2, Neuronal-N, beta III Tubulin, and Doublecortin. As is shown herein, cells representative of the late stages of reprogramming are frequently positive for two, three, or four or more of these markers, and in some cases may be positive for each marker.

The methods of the present invention, when applied to tissue comprising corneal keratocytes or isolated corneal keratocytes, was observed to result in morphological changes in the corneal keratocytes where the cell processes become more axon like, appearing thinner and elongated and primarily within the lamella planes of the stroma. This adoption of a morphological phenotype characteristic of a neuronal cell was accompanied by the expression of one or more of the neuronal

cell markers Musashi-1, Nestin, Neurofilament-200, SMI32, MAP2, Neuronal-N, beta III Tubulin and Doublecortin,.

4. Growth factors

4.1 Neuronal factors

Human Epidermal Growth Factor (EGF, accession numbers NP_001954 and P01133, also known as Urogastrone (URG)) is a polypeptide reportedly involved in the regulation of cell growth, proliferation and differentiation. A wide variety of mammalian orthologues have been described and are useful herein, including murine EGF (accession number Q01279). The EGF precursor is believed to exist as a membrane-bound molecule which is cleaved to generate a 53 amino acid polypeptide. The 53 amino acid polypeptide is preferred for use as described herein. Recombinant EGF (6.2kDa, 53 amino acids) is readily available from commercial sources (for example, Catalogue #100-15, PeproTech Inc., NJ, USA), and is suitable for use in the present invention.

As will be appreciated by those skilled in the art, agonists of the EGF receptor capable of initiating substantially similar biological effect(s) to that initiated by EGF binding to the EGF receptor may be substituted for EGF or a functional fragment or a functional variant thereof, and such agonists are useful herein. For example, TGF alpha is reported to agonise the EGF receptor and initiate substantially similar biological effect(s) as does EGF, and is representative of agonists of the EGF receptor (other than EGF) that are useful herein.

Human Transforming growth factor alpha (TGFalpha, accession numbers NP_003227 and P01135, also known as protransforming growth factor alpha) is a small 50 amino acid long polypeptide reportedly involved in the regulation of cell growth and proliferation. It is produced in macrophages, neurons and keratinocytes. TGFalpha shares approximately 30% homology with EGF and is an antagonist of EGF receptors. TGFalpha has been reported to be expressed in a number of cancers and is widely thought to play a role in wound healing and oncogenesis. Five isoforms of human TGFalpha have been identifed (TGFalpha isoforms 1-5).

Human Fibroblast Growth Factor 2 (FGF2, accession numbers NP 001997 and P09038, also known as Fibroblast growth factor-basic, bFGF, HBGF-2, Prostatropin) is a polypeptide reportedly involved in the regulation of cell growth, proliferation and differentiation. Five naturally occurring isoforms of human FGF2 have been described, including the 18 kDa (155 amino acid), 22 kDa (196 amino acid), 22.5 kDa (201 amino acid), 24 kDa (210 amino acid), and 34 kDa (288 amino acid) isoforms, all useful herein. A wide variety of mammalian orthologues have been described and are useful herein, including murine FGF2 (accession number AAP92385), feline FGF2 (accession number ABY47638), bovine FGF2 (accession number NP776481), and ovine FGF2 (accession number NP001009769). Recombinant FGF2 is readily available from commercial sources (such as,

for example, a 17.2 kDA, 154 amino acid recombinant FGF2 Catalogue #100-18B, PeproTech Inc., NJ, USA), and is suitable for use in the present invention. Of the naturally occurring isoforms, the 18 kDa isoform is preferred.

As will be appreciated by those skilled in the art, it has been reported that a high level of interaction between most members of the FGF family of growth factors, amongst all four reported members of the FGF family of receptors (FGF receptors 1 – 4), and other factors (see the interactome depicted in Figure 1) exists. Accordingly, agonists of one or more of the FGF family of receptors capable of initiating substantially similar biological effect(s) to that initiated by FGF2 binding to the same FGF receptor (FGFR) may be substituted for FGF2 or a functional fragment or a functional variant thereof, and such agonists are useful herein. For example, FGF1 is reported to agonise FGF receptor 3 (including variants IIIb and IIIc) and initiate substantially similar biological effect(s) as does FGF2, and is representative of agonists of one or more of the FGF family of receptors (other than FGF2) that are useful herein.

As used herein, the phrase "FGF family of receptors" includes FGF receptor 1 (FGFR1), FGF receptor 2 (FGFR2), FGF receptor 3 (FGFR3), FGF receptor 4 (FGFR4), and the variants, such as the IIIb and IIIc variants, thereof, including those depicted in Table 1 below and in Figure 1.

Table 1. Representative FGF Receptors and Their Ligands

Names	Mass (kDa)	Variant	Specificity	Cells expressing (partial list)
FGF-R1	150	IIIb	FGF-1	fibroblasts, endothelial, certain epithelial, flg-1, cek-1 vascular smooth muscle, lymphocytes
		IIIc	FGF-1, 2, 4	macrophages, hematopoietic progenitors, numerous tumour cells
FGF-R2 bek	135	IIIb	FGF-1, 2, 7	epithelial
		IIIc	FGF-1, 2, 4	fibroblasts, endothelial, vascular smooth muscle, oligodendroglia, astrocytes, hematopoietic progenitors, lymphocytes, macrophages, carcinoma and sarcoma
FGF-R3 cek-2	135	IIIb	FGF-1, 2	epithelial, keratinocytes
		IIIc	FGF-1, 4, 9, (2?)	fibroblasts, monocytes, vascular endothelial, hematopoietic progenitors
FGF-R4 flg-2	110		FGF-1, 2, 6	embryonic and multipotential stem

Human insulin (accession numbers NP_000198 and P01308) is a 51 amino acid peptide hormone produced in the islets of Langerhans in the pancreas, and is involved in the regulation of glucose metabolism. In addition to human insulin, a wide variety of mammalian orthologues have been successfully used in humans, and include bovine insulin and porcine insulin. Recombinant

insulin is readily available from commercial sources (such as, for example Lantus, Humalog and Novolin, Insulin Direct, Canada).

As will be appreciated by those skilled in the art, agonists of the insulin receptor capable of initiating substantially similar biological effect(s) to that initiated by insulin binding to the insulin receptor may be substituted for insulin or a functional fragment or a functional variant thereof, and such agonists are useful herein. Likewise, agonists of the insulin-like receptor capable of initiating substantially similar biological effect(s) to that initiated by insulin binding to the insulin-like receptor may be substituted for insulin or a functional fragment or a functional variant thereof, and such agonists are useful herein. For example, IGF1 is reported to agonise both the insulin receptor and the insulin-like receptor and initiate substantially similar biological effect(s) as does insulin, and is representative of agonists of the insulin receptor or of the insulin-like receptor (other than insulin) that are useful herein.

Human insulin-like growth factor 1 (IGF1, accession numbers NP_000609 and P01343, also known as somatomedin C or mechano growth factor) is a single chain polypeptide hormone of 70 amino acids. IGF-1 is primarily produced by the liver, upon stimulation by growth factor. IGF-1 primarily acts as a stimulator of cell growth and proliferation, and is a potent inhibitor of apoptosis (programmed cell death). Additionally, IGF-1 plays a role in regulating neural development, such as neurogenesis, myelination, and synaptogenesis as well as dendritic branching and neuroprotection after neuronal damage. Mediation of this activity is through binding to specific IGF-1 receptors, thus activating protein kinase signalling pathways. Two isoforms of IGF-1 have been identified (IGF-1A – P01343 and IGF-1B – P05019). Commercially available IGF-1 includes, for example, Increlex, Tercica, USA.

Those skilled in the art will recognise that the methods and compositions of the invention may in certain circumstances use human EGF, human FGF2, or human insulin or may use one or more mammalian orthologues. Functional variants or functional fragments of EGF, of FGF2, or of insulin, or combinations of functional variants and fragments are also useful herein, as are the agonists of the EGF receptor, agonists of one or more of the FGF family of receptors, and agonists of the insulin receptor or the insulin-like receptor as discussed above.

The terms "functional variant" and "functional fragment" as used herein, for example in respect of EGF, insulin or FGF2, refer to polypeptide sequences different from the specifically identified sequence(s), wherein one or more amino acid residues is deleted, substituted, or added, or a sequence comprising a fragment of the specifically identified sequence(s). Functional variants may be naturally occurring allelic variants, or non-naturally occurring variants. Functional variants may be from the same or from other species and may encompass homologues, paralogues and orthologues. Functional variants or functional fragments of the polypeptides possess one or more

of the biological activities of the specifically identified polypeptide, such as an ability to elicit one or more biological effects elicited by the native polypeptide.

Methods and assays to determine one of more biological effects elicited by EGF or by FGF2 are well known in the art, and such methods and assays can be used to identify or verify one or more functional variants or functional fragments of EGF, of insulin or of FGF2. For example, an assay of the ability of EGF to promote cell growth or differentiation, such as those described herein in the Examples, is amenable to identifying one or more functional variants or functional fragments of EGF.

Examples of functional fragments include polypeptide fragments that comprise amino acid sequences that are responsible for receptor binding or interaction with other polypeptides or with nucleic acids. A "fragment" of a polypeptide or a functional variant of a polypeptide as used herein is a subsequence of contiguous amino acids that is preferably at least 10 amino acids in length. The fragments of the invention preferably comprise at least 15 amino acids, at least 20 amino acids, more preferably at least 30 amino acids, more preferably at least 40 amino acids, more preferably at least 50 amino acids and when applicable most preferably at least 60 contiguous amino acids of a polypeptide used herein.

4.2 Polypeptide variants

The term "variant" with reference to polypeptides encompasses naturally occurring, recombinantly and synthetically produced polypeptides. Variant polypeptide sequences preferably exhibit at least about 50%, at least about 51%, at least about 52%, at least about 53%, at least about 54%, at least about 55%, at least about 56%, at least about 57%, at least about 58%, at least about 59%, at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to a sequence useful herein. Identity is found over a comparison window of at least about 20 amino acid positions, preferably at least 50 amino acid positions, at least 100 amino acid positions, and most preferably over the entire length of a polypeptide useful herein.

Polypeptide sequence identity can be determined in the following manner. The subject polypeptide sequence is compared to a candidate polypeptide sequence using BLASTP (from the BLAST suite of programs, version 2.2.5 [Nov 2002]) in bl2seq, which is publicly available from

NCBI (ftp://ftp.ncbi.nih.gov/blast/). The default parameters of bl2seq are utilized except that filtering of low complexity regions should be turned off.

Polypeptide sequence identity may also be calculated over the entire length of the overlap between a candidate and subject polypeptide sequence using global sequence alignment programs. EMBOSS-needle (available at http:/www.ebi.ac.uk/emboss/align/) and GAP (Huang, 1994) are also suitable global sequence alignment programs for calculating polypeptide sequence identity.

A preferred method for calculating polypeptide % sequence identity is based on aligning sequences to be compared using Clustal X (Jeanmougin et al., 1998).

Polypeptide variants useful herein also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov 2002]) from NCBI (ftp://ftp.ncbi.nih.gov/blast/). The similarity of polypeptide sequences may be examined using the following UNIX command line parameters:

bl2seq –i peptideseq1 –j peptideseq2 -F F –p blastp

Variant polypeptide sequences preferably exhibit an E value of less than 1 x 10-6more preferably less than 1 x 10-9, more preferably less than 1 x 10-12, more preferably less than 1 x 10-15, more preferably less than 1 x 10-18, more preferably less than 1 x 10-21, more preferably less than 1 x 10-30, more preferably less than 1 x 10-40, more preferably less than 1 x 10-50, more preferably less than 1 x 10-60, more preferably less than 1 x 10-70, more preferably less than 1 x 10-80, more preferably less than 1 x 10-90 and most preferably 1x10-100 when compared with any one of the specifically identified sequences.

The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. This program finds regions of similarity between the sequences and for each such region reports an "E value" which is the expected number of times one could expect to see such a match by chance in a database of a fixed reference size containing random sequences. For small E values, much less than one, this is approximately the probability of such a random match.

Conservative substitutions of one or several amino acids of a described polypeptide sequence without significantly altering its biological activity are also included in the invention. A skilled artisan will be aware of methods for making phenotypically silent amino acid substitutions (see, e.g., Bowie et al., 1990).

4.2.1 Methods for identifying variants

Physical methods

Variant polypeptides may be identified via identifying the corresponding polynculeotide using PCR-based methods (Mullis et al., 1994). Typically, the sequence of a primer, useful to amplify variants of polynucleotide molecules of the invention by PCR, may be based on a sequence encoding a conserved region of the corresponding amino acid sequence.

Alternatively library screening methods, well known to those skilled in the art, may be employed (Sambrook et al., 1987). When identifying variants of the probe sequence, hybridization and/or wash stringency will typically be reduced relatively to when exact sequence matches are sought.

Polypeptide variants may also be identified by physical methods, for example by screening expression libraries using antibodies raised against polypeptides of the invention (Sambrook et al., 1987) or by identifying polypeptides from natural sources with the aid of such antibodies.

Computer based methods

Polypeptide variants may also be identified by computer-based methods well-known to those skilled in the art, using public domain sequence alignment algorithms and sequence similarity search tools to search sequence databases (public domain databases include Genbank, EMBL, Swiss-Prot, PIR and others). See, e.g., Nucleic Acids Res. 29: 1-10 and 11-16, 2001 for examples of online resources. Similarity searches retrieve and align target sequences for comparison with a sequence to be analyzed (i.e., a query sequence). Sequence comparison algorithms use scoring matrices to assign an overall score to each of the alignments.

An exemplary family of programs useful for identifying variants in sequence databases is the BLAST suite of programs (version 2.2.5 [Nov 2002]) including BLASTN, BLASTP, BLASTX, tBLASTN and tBLASTX, which are publicly available from (ftp://ftp.ncbi.nih.gov/blast/) or from the National Centre for Biotechnology Information (NCBI), National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894 USA. The NCBI server also provides the facility to use the programs to screen a number of publicly available sequence databases. BLASTN compares a nucleotide query sequence against a nucleotide sequence database. BLASTP compares an amino acid query sequence against a protein sequence database. BLASTX compares a nucleotide query sequence translated in all reading frames against a protein sequence database. tBLASTN compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames. tBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. The BLAST programs may be used with default parameters or the parameters may be altered as required to refine the screen.

The use of the BLAST family of algorithms, including BLASTN, BLASTP, and BLASTX, is described in the publication of Altschul et al., Nucleic Acids Res. 25: 3389-3402, 1997.

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, BLASTP, BLASTX, tBLASTN, tBLASTX, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN, BLASTP, BLASTX, tBLASTN and tBLASTX algorithms also produce "Expect" values for alignments. The Expect value (E) indicates the number of hits one can "expect" to see by chance when searching a database of the same size containing random contiguous sequences. The Expect value is used as a significance threshold for determining whether the hit to a database indicates true similarity. For example, an E value of 0.1 assigned to a polynucleotide hit is interpreted as meaning that in a database of the size of the database screened, one might expect to see 0.1 matches over the aligned portion of the sequence with a similar score simply by chance. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in that database is 1% or less using the BLASTN, BLASTP, BLASTX, tBLASTN or tBLASTX algorithm.

Multiple sequence alignments of a group of related sequences can be carried out with CLUSTALW (Thompson et al., 1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680, http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/Top.html) or T-COFFEE (Notredame, C. et al., 2000) or PILEUP, which uses progressive, pairwise alignments. (Feng and Doolittle, 1987).

Pattern recognition software applications are available for finding motifs or signature sequences. For example, MEME (Multiple Em for Motif Elicitation) finds motifs and signature sequences in a set of sequences, and MAST (Motif Alignment and Search Tool) uses these motifs to identify similar or the same motifs in query sequences. The MAST results are provided as a series of alignments with appropriate statistical data and a visual overview of the motifs found. MEME and MAST were developed at the University of California, San Diego.

PROSITE (Bairoch and Bucher, 1994; Hofmann et al., 1999) is a method of identifying the functions of uncharacterized proteins translated from genomic or cDNA sequences. The PROSITE database (www.expasy.org/prosite) contains biologically significant patterns and profiles and is designed so that it can be used with appropriate computational tools to assign a new sequence to a known family of proteins or to determine which known domain(s) are present in the sequence

(Falquet et al., 2002). Prosearch is a tool that can search SWISS-PROT and EMBL databases with a given sequence pattern or signature.

4.3 Methods for isolating polypeptides

The polypeptides useful herein, including variant polypeptides, may be prepared using peptide synthesis methods well known in the art such as direct peptide synthesis using solid phase techniques (e.g. Stewart et al., 1969) or automated synthesis, for example using an Applied Biosystems 431A Peptide Synthesizer (Foster City, California). Mutated forms of the polypeptides may also be produced during such syntheses.

The polypeptides and variant polypeptides useful herein may also be purified from natural sources using a variety of techniques that are well known in the art (e.g. Deutscher, 1990).

Alternatively the polypeptides and variant polypeptides useful herein may be expressed recombinantly in suitable host cells and separated from the cells.

5. Diseases and conditions to be treated

The methods and compositions of the invention are amenable to the treatment of a wide variety of diseases and conditions associated with neuronal dysfunction including neuronal cell death. The reprogrammed cells and tissues of the present invention, being derived from various ophthalmic tissues, are particularly suited to treating diseases or conditions of the mammalian eye.

5.1 Ophthalmic conditions

Neurological diseases or conditions of the eye, or diseases or conditions afflicting the eye to which neurological dysfunction contributes, are amenable to treatment using the methods and compositions of the present invention. The following are provided as non-limiting examples of ophthalmic diseases and conditions suitable for treatment using the methods and compositions of the present invention: retinal degeneration including retinal ganglion cell loss, bipolar cell loss, photoreceptor cell loss, retinal ganglion cell loss or optic nerve cell loss as a result of trauma, ophthalmic trauma, ischemia including optic nerve ischemia, glaucoma, age related macular degeneration, retinitis pigmentosa, diabetic retinopathy, retinal detachment, multiple sclerosis, central retinal artery occlusion, tumour compression, toxic neuropathy, ruptured optic nerve (avulsion), corneal nerve dystrophies, dry eye including dry eye resulting from cataract surgery, ophthalmic viral infection such as herpes infection including herpes simplex or herpes zoster infection, hypoesthesia, lost or damaged ophthalmic neurons, ophthalmic inflammation, and ptosis due to loss of nerve function.

5.2 Neurological conditions

A wide variety of neurological diseases or conditions afflicting other parts of the mammalian body may also be treated using the methods and compositions of the present invention, for example cell or tissue implantation. The following are provided as non-limiting examples of the diseases and conditions suitable for treatment using the methods and compositions of the present invention: acute and chronic neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's chorea, multiple sclerosis, and various dementias, acute neurological conditions including stroke, impact injury or trauma, and drug and alcohol induced neuronal cell death.

6. Methods of administration and implantation

6.1 Administration of therapeutic agents

Topical administration of ophthalmic therapeutic agents is the most common method for treating conditions that affect the exterior parts of the eye, in large part due to the accessibility of the eye surface. The ease of administration is, however, offset somewhat by the difficulties the anatomy and physiology of the eye present in achieving an effective concentration at the target site. Efficient and targeted delivery of therapeutic agents past the protective ocular barriers remains a significant challenge.

Despite this, conventional eye drops account for more than 90% of the marketed ophthalmic formulations. Topical administration can be effective, particularly for quick acting agents or those requiring or benefiting from frequent dosing.

Corneal absorption is considered to be the major penetration pathway for topically applied drugs. There are two mechanisms proposed for absorption across the corneal epithelium, transcellular and paracellular diffusion. Lipophilic drugs reportedly prefer the transcellular route while hydrophilic drugs are believed to penetrate primarily via the paracellular route.

A number of non-topical modes of administration of therapeutic agents to the eye are available and are helpfully discussed by Del Amo and Urtti, 2008.

In particular, modes of administration that achieve prolonged delivery of therapeutic agent, such as EGF, insulin, or FGF2, are suitable for use in the methods of the present invention. Such modes of administration may utilise retrobulbar injection, intraocular implants, microparticles, nanoparticles, liposomal delivery systems, niosomal or discomal delivery systems, micellular delivery systems, micro- and submicro emulsions, and in situ gelling systems.

Retrobulbar injection involves administration of therapeutic agents behind the medulla oblongata, or eyeball.

Intraocular implants allow prolonged administration and controlled release of therapeutic agents, and avoid a number of the disadvantages of topical administration. Both biodegradable and

non-biodegradable implants are available, the former having the advantage of not requiring surgery for removal at the expense of reportedly less accurate control of therapeutic agent release. Non-biodegradable Hydron pellets of approximately 1.5 mm diameter containing both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been implanted intravitreally to induce angiogenesis (Erb, MH, et al., 2002). Examples of intraocular implants in common use include Vitrasert (ganciclovir in an EVA/PVA polymer, Bausch & Lomb, USA), Retisert (fluocinolone acetonide in a PVA and silicone laminate, Bausch & Lomb, USA), and the Medidur (Alimera Sciences, USA and pSivida Inc., USA) injectable implant. Such implants can provide delivery of the therapeutic agent for from 8 months to approximately 3 years.

Episcleral implant systems, such as the Surodex and Posurdex (Allergan, USA) poly lactic-co-glycolic acid (PLGA) implants, are in clinical development. Similarly, semi-solid bioerodible implant materials have been proposed, and enable delivery of soft implants with a needle and syringe.

Another strategy to achieve long duration, controlled release is to encapsulate or formulate the therapeutic agent in microparticles or nanoparticles. Microparicles and nanoparticles are defined as micron and submicron-sized polymeric colloidal particles, respectively, in which the therapeutic agent is dissolved, entrapped, encapsulated or adsorbed. Depending on the method of preparation, microspheres, nanospheres, microcapsules or nanocapsules can be obtained. Both microspheres and nanospheres have a matrix-like structure in which the therapeutic agent may be adsorbed on the surface of the particle or dispersed throughout the matrix. Microcapsules and nanocapsules typically consist of a polymer shell and a core, where the drug can either be dissolved in the inner core or adsorbed onto the surface. Again, biodegradable and biocompatible polymers, such as polylactide and PLGA, are typically used. Injected intravitreally, the delivery of therapeutic agent can be sustained for a number of weeks.

In-situ gelling systems utilise a viscous polymer-based liquid that exhibits a sol-to-gel phase transition on the ocular surface. This phase transition is induced by a change in a physico-chemical parameter, such as for example, ionic strength, temperature, pH or solvent exchange. As with other liquid topical ophthalmic solutions, in-situ gelling systems are easily administered, but have the advantage of extended delivery of therapeutic agent due to the prolonged residence time of the gel.

A physician or skilled person will be able to determine the dose of EGF, insulin and FGF2 that will be most suitable for an individual patient – a dose that may vary with age, weight, sex and response of the particular patient to be treated. A dose of 0.001 to 1000 mg/kg/day is an exemplary dose and can, of course, be varied in individual cases. Those skilled in the art will appreciate that in certain embodiments, dosages suitable to achieve the concentrations of EGF, FGF2, or insulin described herein are administered.

6.2 Cell and Tissue Implantation

The methods of the invention provide cells and tissue suitable for implantation into a mammalian subject to be treated. Methods for cell or tissue implantation at a wide variety of sites within the mammalian body are well known in the art. The site of implantation will largely be dictated by the nature of the disease or condition to be treated.

The number of cells or amount of tissue that is implanted into a subject to give a therapeutic effect can vary depending on the age and weight of the subject as well as the interior dimensions of the site of implantation in the body. Typically, if the cells or tissue(s) are to be implanted intraocularly, for example, subretinally, from about 1, 10, 50, or 100 to about 100,000 cells may be implanted. Clearly, when implanted at other sites of the body, such as when non-ophthalmic conditions or diseases are to be treated, the physical or physiological characteristics of the implantation site may determine the number of cells or the amount of tissue that can be implanted.

In any event, a physician, or skilled person, will be able to determine the actual dose which will be most suitable for an individual patient – a dose that may vary with age, weight, sex and response of the particular patient to be treated. The above mentioned doses are exemplary of the average case and can, of course, be varied in individual cases.

Implantation of the cells or tissue can be achieved by way of routine techniques, for example, by suspending neuronal cells or neuronal precusor cells in a suitable buffer or one or more pharmaceutically acceptable carriers or diluents followed by injection or infusion into a suitable body site. Likewise, the implantation of intact tissue may be performed by art-standard techniques. For example, intraocular implantation of the cells, tissues or compositions of the invention requires access to the internal tissues of the eye of the recipient. Surgical techniques to gain access to, for example, the retina or other ocular tissues are well known. Techniques for the surgical approach to the human retina, for example, are known in the art, and such techniques are suitable for subretinal implantation of tissue or cells of the present invention (Vulger et al., 2007).

As used herein the terms "administering", "introducing", "implanting" and "transplanting" when used with reference to cells or tissue are used interchangeably and refer to the placement of the cells or tissue, for example, the reprogrammed neuronal cells or neuronal precursor cells or tissue comprising same into a subject, particularly a homogeneic subject, by a method or route which results in localization of the cells or tissue at a desired site. The cells can be administered to a subject by any appropriate route which results in delivery of the cells to a desired location in the subject where at least a portion of the cells remain viable. It is preferred that at least about 5%, preferably at least about 10%, more preferably at least about 20%, yet more preferably at least about 30%, still more preferably at least about 40%, and most preferably at least about 50% or more of the cells remain viable after administration into a subject. The period of viability of the cells after

administration to a subject can be as short as a few days, to as long as a few weeks to months. Methods of administering, introducing and implanting cells, tissues or compositions for use in the invention are well-known in the art.

It is also contemplated that the present invention will be useful in combination with traditional ophthalmic treatment regimens, such as the intraocular administration of anti-angiogenics or anti-VEGF (anti-Vascular Endothelial Growth Factor) agents in the treatment of macular degeneration. However, it is expected that a significant improvement in neuronal cell function would be observed in patients who received the reprogrammed neuronal cells, tissues or compositions of the invention.

7. Sample preparation

As will be apparent to persons skilled in the art, samples suitable for use in the methods of the present invention may be obtained from ophthalmic tissues as convenient, for example, from the mammalian subject to be treated. Samples may also be obtained from tissue banks or other tissue repositories, while ophthalmic cells, including cell lines derived from ophthalmic cells, may readily be obtained from culture collections such as the American Type Culture Collection (ATCC, see www.atcc.org).

Conveniently, tissue samples may be obtained using standard techniques such as cell scrapings or biopsy techniques. For example, cell or tissue samples may be obtained by intraocular. Similarly, blood sampling is routinely performed, for example for pathogen testing, and methods for taking such tissue or cell samples are well known in the art. Likewise, methods for storing and processing ophthalmic tissue samples are well known in the art. For example, tissue samples may be frozen until tested if required. In addition, one of skill in the art would realise that certain of the methods of the present invention are practised on one or more isolated cells, for example, an essentially homogenous population of differentiated ophthalmic cells, and thus will typically require a fractionation or purification procedure, for example, a cell isolation procedure such as that described herein in the Examples.

As used herein, the term "isolated" refers to cells or tissues which have been separated from their natural environment. This term includes gross physical separation from the natural environment, e.g., removal from a donor subject, and alteration of the cells' or tissues' relationship with the neighbouring cells or tissues with which they are in direct contact by, for example, dissociation.

7.1 Cell and tissue culturing

Certain embodiments of the methods of the present invention are performed on isolated cells or tissue in vitro. Those skilled in the art are well aware of the general considerations required to maintain isolated cells or tissue in vitro, including standard cell culturing or tissue culturing

considerations such as maintaining sterile conditions, providing a suitable atmosphere and temperature, and the like. Examplary methods for maintaining isolated cells or tissue for use in the present invention are provided herein in the Examples.

In various embodiments of the in vitro and in situ methods of the present invention, EGF or a functional variant or a functional fragment thereof is present at a concentration of from about 0.001 ng/mL to about 100 ng/mL, and any subranges may be selected from this range (for example, from about 0.001 to about 90 ng/mL, 0.001 to about 80 ng/mL, 0.001 to about 70 ng/mL, 0.001 to about 60 ng/mL, 0.001 to about 50 ng/mL, 0.001 to about 40 ng/mL, 0.001 to about 30 ng/mL, 0.001 to about 20 ng/mL, about 0.01 to about 100 ng/mL, about 0.01 to about 90 ng/mL, about 0.01 to about 80 ng/mL, about 0.01 to about 70 ng/mL, about 0.01 to about 60 ng/mL, about 0.01 to about 50 ng/mL, about 0.01 to about 40 ng/mL, about 0.01 to about 30 ng/mL, about 0.01 to about 20 ng/mL, about 0.1 to about 100 ng/mL, about 0.1 to about 90 ng/mL, about 0.1 to about 80 ng/mL, about 0.1 to about 70 ng/mL, about 0.1 to about 60 ng/mL, about 0.1 to about 50 ng/mL, about 0.1 to about 40 ng/mL, about 0.1 to about 30 ng/mL, about 0.1 to about 20 ng/mL, about 1 to about 100 ng/mL, about 1 to about 90 ng/mL, about 1 to about 80 ng/mL, about 1 to about 70 ng/mL, about 1 to about 60 ng/mL, about 1 to about 50 ng/mL, about 1 to about 40 ng/mL, about 1 to about 30 ng/mL, about 1 to about 20 ng/mL, about 1 to about 10 ng/mL, about 10 to about 100 ng/mL, about 20 to about 100 ng/mL, about 30 to about 100 ng/mL, about 40 to about 100 ng/mL, about 50 to about 100 ng/mL, about 60 to about 100 ng/mL, about 70 to about 100 ng/mL, about 80 to about 100 ng/mL, or about 90 to about 100 ng/mL).

In various embodiments of the in vitro and in situ methods of the present invention, EGF or a functional variant or a functional fragment thereof is present at a concentration of about 0.001. 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 ng/mL, and ranges may be selected between any of these values (for example, about 0.001 to about 10 ng/mL, about 0.01 to about 10 ng/mL, about 0.1 to about 10 ng/mL, about 1 to about 10 ng/mL, about 2 to about 10 ng/mL, about 3 to about 10 ng/mL, about 4 to about 10 ng/mL, about 5 to about 10 ng/mL, about 6 to about 10 ng/mL, about 7 to about 10 ng/mL, about 8 to about 10 ng/mL, or about 9 to about 10 ng/mL). A preferred concentration is from about 0.1ng/mL to about 9.5ng/mL.

In various embodiments of the in vitro and in situ methods of the present invention, FGF2 or a functional variant or a functional fragment thereof is present at a concentration of from about 0.001 ng/mL to about 250 ng/mL, and any subranges may be selected from this range (for example, from about 0.001 to about 200 ng/mL, from about 0.001 to about 150 ng/mL, from about 0.001 to about 100 ng/mL, from about 0.001 to about 90 ng/mL, 0.001 to about 80 ng/mL, 0.001 to about 70 ng/mL, 0.001 to about 60 ng/mL, 0.001 to about 50 ng/mL, 0.001 to about 40 ng/mL, 0.001 to

about 30 ng/mL, 0.001 to about 20 ng/mL, about 0.01 to about 250 ng/mL, from about 0.01 to about 200 ng/mL, from about 0.01 to about 150 ng/mL, from about 0.01 to about 100 ng/mL, about 0.01 to about 90 ng/mL, about 0.01 to about 80 ng/mL, about 0.01 to about 70 ng/mL, about 0.01 to about 60 ng/mL, about 0.01 to about 50 ng/mL, about 0.01 to about 40 ng/mL, about 0.01 to about 30 ng/mL, about 0.01 to about 20 ng/mL, from about 0.1 to about 250 ng/mL, from about 0.1 to about 200 ng/mL, from about 0.1 to about 150 ng/mL, from about 0.1 to about 100 ng/mL, about 0.1 to about 90 ng/mL, about 0.1 to about 80 ng/mL, about 0.1 to about 70 ng/mL, about 0.1 to about 60 ng/mL, about 0.1 to about 50 ng/mL, about 0.1 to about 40 ng/mL, about 0.1 to about 30 ng/mL, about 0.1 to about 20 ng/mL, about 1 to about 250 ng/mL, about 1 to about 200 ng/mL, from about 1 to about 150 ng/mL, from about 1 to about 100 ng/mL, about 1 to about 90 ng/mL, about 1 to about 80 ng/mL, about 1 to about 70 ng/mL, about 1 to about 60 ng/mL, about 1 to about 50 ng/mL, about 1 to about 40 ng/mL, about 1 to about 30 ng/mL, about 1 to about 20 ng/mL, about 1 to about 10 ng/mL, about 10 to about 250 ng/mL, about 20 to about 250 ng/mL, about 30 to about 250 ng/mL, about 40 to about 250 ng/mL, about 50 to about 250 ng/mL, about 60 to about 250 ng/mL, about 70 to about 250 ng/mL, about 80 to about 250 ng/mL, or about 90 to about 250 ng/mL).

In various embodiments of the in vitro and in situ methods of the present invention, FGF2 or a functional variant or a functional fragment thereof is present at a concentration of about 0.001. 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12. 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, or 25 ng/mL, and ranges may be selected between any of these values (for example, about 0.001 to about 25 ng/mL, about 0.01 to about 25 ng/mL, about 0.1 to about 25 ng/mL, about 1 to about 25 ng/mL, about 2 to about 25 ng/mL, about 3 to about 25 ng/mL, about 4 to about 25 ng/mL, about 5 to about 25 ng/mL, about 6 to about 25 ng/mL, about 7 to about 25 ng/mL, about 8 to about 25 ng/mL, or about 9 to about 25 ng/mL). A preferred concentration is from about 0.25ng/mL to about 25ng/mL.

In various embodiments of the in vitro and in situ methods of the present invention, insulin or a functional variant or a functional fragment thereof is present at a concentration of from about 0.001 μg/mL to about 250 μg/mL, and any subranges may be selected from this range (for example, from about 0.001 to about 200 μg/mL, from about 0.001 to about 150 μg/mL, from about 0.001 to about 100 μg/mL, from about 0.001 to about 90 μg/mL, 0.001 to about 80 μg/mL, 0.001 to about 70 μg/mL, 0.001 to about 60 μg/mL, 0.001 to about 50 μg/mL, 0.001 to about 40 μg/mL, 0.001 to about 30 μg/mL, 0.001 to about 20 μg/mL, about 0.01 to about 250 μg/mL, from about 0.01 to about 200 μg/mL, from about 0.01 to about 150 μg/mL, from about 0.01 to about 100 μg/mL, about 0.01 to about 90 μg/mL, about 0.01 to about 80 μg/mL, about 0.01 to about 70 μg/mL,

about 0.01 to about 60 μg/mL, about 0.01 to about 50 μg/mL, about 0.01 to about 40 μg/mL, about 0.01 to about 30 μg/mL, about 0.01 to about 20 μg/mL, from about 0.1 to about 250 μg/mL, from about 0.1 to about 200 μg/mL, from about 0.1 to about 150 μg/mL, from about 0.1 to about 100 μg/mL, about 0.1 to about 90 μg/mL, about 0.1 to about 80 μg/mL, about 0.1 to about 70 μg/mL, about 0.1 to about 60 μg/mL, about 0.1 to about 50 μg/mL, about 0.1 to about 40 μg/mL, about 0.1 to about 30 μg/mL, about 0.1 to about 20 μg/mL, about 1 to about 250 μg/mL, about 1 to about 200 μg/mL, from about 1 to about 150 μg/mL, from about 1 to about 100 μg/mL, about 1 to about 90 μg/mL, about 1 to about 80 μg/mL, about 1 to about 70 μg/mL, about 1 to about 60 μg/mL, about 1 to about 50 μg/mL, about 1 to about 40 μg/mL, about 1 to about 30 μg/mL, about 1 to about 20 μg/mL, about 1 to about 10 μg/mL, about 10 to about 250 μg/mL, about 20 to about 250 μg/mL, about 30 to about 250 μg/mL, about 40 to about 250 μg/mL, about 50 to about 250 μg/mL, about 60 to about 250 μg/mL, about 70 to about 250 μg/mL, about 80 to about 250 μg/mL, or about 90 to about 250 μg/mL).

In various embodiments of the in vitro and in situ methods of the present invention, insulin or a functional variant or a functional fragment thereof is present at a concentration of about 0.001. 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12. 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, or 25 μg/mL, and ranges may be selected between any of these values (for example, about 0.001 to about 25 μg/mL, about 0.01 to about 25 μg/mL, about 0.1 to about 25 μg/mL, about 1 to about 25 μg/mL, about 2 to about 25 μg/mL, about 3 to about 25 μg/mL, about 4 to about 25 μg/mL, about 5 to about 25 μg/mL, about 6 to about 25 μg/mL, about 7 to about 25 μg/mL, about 8 to about 25 μg/mL, or about 9 to about 25 μg/mL). A preferred concentration is from about 0.25μg/mL to about 25μg/mL.

In various embodiments of the in vitro and in situ methods of the present invention, the sample is in contact with EGF, insulin and FGF2 for about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 days, and ranges may be selected between any of these values (for example, about 0.5 days to about 10 days, or from about 1 day to about 5 days, or from about 6 days to about 10 days). In vivo methods may be expected to require similar periods.

It will be appreciated that it is not intended to limit the invention to the above example only, many variations, which may readily occur to a person skilled in the art, being possible without departing from the scope thereof as defined in the accompanying indicative claims.

The invention consists in the foregoing and also envisages constructions of which the following gives examples only.

EXAMPLES

EXAMPLE 1 – Corneal stromal cell reprogramming *in situ*

This example describes the investigation of cell reprogramming in adult human corneal tissue slices.

Materials and methods

All research was conducted in accordance with Human Ethics Committee consent. Consent from the donor family was obtained prior to tissue harvest and use.

1. Tissue collection

Cadaveric human corneas were obtained from the New Zealand National Eye Bank. The corneas were processed as described in Chang et al. 2008. 1mm thick tissue slices were obtained from the central and rim regions of the cornea.

Alternatively, research corneas were consented for transplant and were excised for transplant with 3-5mm of sclera attached for handling. Once the cornea was sent out for transplant, the surgeon removed a 7-8mm disc of tissue from the centre of the 12 mm cornea, leaving the tissue known as the limbal rim, a ring of tissue comprised of 3-5mm of sclera and 2-3mm of peripheral cornea. Limbal rims recovered from theatre were dissected to provide 1mm thick tissue slices comprising the sclera, limbus and peripheral cornea.

2. Tissue processing

After excision, the corneal tissue slices were placed onto culture inserts for air-liquid interface culture, as described in Gahwiler et al, 1981.

Three types of growth media were used. Opti-MEM and Neurobasal-A as described in Tables 2 and 4 with or without supplements as described in Tables 3.1 and 3.2, and a third growth media, Opti-MEM supplemented with 10% Fetal Calf Serum (instead of the 2% FCS). Cell culture was maintained at 34°C with 5% CO2 for 3 or 8 days.

Table 2: Growth media

Opti-MEM I	*Neurobasal-A*
Opti-MEM I *2% Fetal Calf Serum (Gibco, Cat #10091-148)* *2 mM L-glutamine (Gibco, Cat #21051-024)* *Antibiotic-Antimycotic (100 x stock, Gibco, Cat #15240-062)*	*Neurobasal-A (1x, Gibco, Cat #10888)* *B27 (50 x stock, Gibco, Cat #17504-044)* *2.5 ng/mL FGF2 (Australian Laboratory Services, or Cat#100-18B, Peprotech Inc. NJ, USA)* *1 ng/mL EGF (Australian Laboratory Services, or Cat#100-15, Peprotech Inc. NJ, USA)* *N-2 (100 x stock, Gibco, Cat #17502-048)* *2 µg/mL Heparin* *2 mM L-glutamine* *Antibiotic-Antimycotic (100 x stock, Gibco, Cat #15240-062)*

Table 3.1: Composition of B27 Medium Supplement for Neurons

Biotin	L-carnitine	Corticosterone	Ethanolamine
D(+)-galactose	Glutathione (reduced)	Linoleic acid	Linolenic acid
Progesterone	Putrescine	Retinyl acetate	Selenium
T3 (triodo-1-thyronine)	DL-α-tocopherol (vitamin E)	DL- α-tocopherol acetate	Albumin, bovine
Catalase	insulin	Superoxide dismutase	Transferrin

Table 3.2: Composition of N2 Medium Supplement for Neurons

Human transferring (holo)	insulin recombinant full chain	Progesterone
putrescine	selenite	

Table 4: Composition of Neurobasal Medium

		mg/liter	μM
Inorganic salts	$CaCl_2$ (anhydrous)	200	1,800
	$Fe(NO_3)_3$-$9H_2O$	0.1	0.2
	KCl	400	5,360
	$MgCl_2$ (anhydrous)[b]	77.3	812
	NaCl[b]	3,000	51,300
	$NaHCO_3$[b]	2,200	26,000
	NaH_2PO_4-H_2O	125	900
Other components	D-glucose	4,500	25,000
	Phenol red[b]	8.1	23
	HEPES[B]	2,600	10,000
	Sodium pyruvate[b]	25	230
Amino acids	L-ananine[b]	2.0	20
	L-arginine-HCl	84	400
	L-asparagine-H_2O[b]	0.83	5
	L-cysteine[b]	1.21	10
	L-glutamine[a,b]	73.5	500
	L-glutamate[c]		
	Glycine	30	400
	L-histidine-HCI-H_2O	42	200
	L-isoleucine	105	800
	L-leucine	105	800
	L-lysine-HCl	146	5
	L-methionine	30	200
	L-phenylalanine	66	400
	L-proline[b]	7.76	67
	L-seribe	42	400
	L-threonine	95	800
	L-tryptophan	16	80
	L-tryosine	72	400
	L-valine	94	800

Vitamins	D-Ca pantothenate	4	8
	Choline chloride	4	28
	Folic acid	4	8
	i-Inositol	7.2	40
	Niacinamide	4	30
	Pyridoxal-HCl	4	20
	Riboflavin	0.4	1
	Thiamine-HCl	4	10
	Vitamin B12	0.34	0.2

[a]Not supplied in liquid medium; needs to be added.
[b]Changed from DMEM.
[c]Glutamate at 3.7 μg/ml (25 μM) should be added to start primary embryonic hippocampal neurons

3. Immunohistochemistry

3.1 Opti-MEM holoclone Immunohistochemistry

Following fixation in 4% Paraformaldehyde (PFA, TAAB Laboratories) and blocking in 10% Goat serum, the tissue slices were labelled with Nestin, Musashi-1, cytokeratin 3/12 or ΔNp63α (see Table 5). Primary antibodies were applied overnight at 4°C.

Secondary antibodies (Goat anti-rabbit Alexa 488 or 546 conjugated, Molecular Probes) were applied for 2.5 hours at room temperature according to the manufacturer's instructions.

Table 5: Antibodies used for in vitro Opti-MEM holoclone immunohistochemistry

Antibody against	Full Name	Target Cells	Dilution Factor	Source
Nestin	Mouse anti-nestin	NSPCs	1:500	Chemicon MAB5326
Musashi-1	Rabbit anti-Musashi-1	NSPCs	1:500	Chemicon AB5977
Cytokeratin 3/12	Mouse anti-Keratin K3/K76	Mature corneal epithelial cells	1:300	Chemicon CBL218
ΔNp63α	Rabbit anti-ΔNp63 alpha	LSCs and possibly other stratified epithelia stem and progenitor cells	1:400	PickCell Laboratories Private Order

3.2 Neurobasal holoclone Immunohistochemistry

The tissue slices were incubated in primary antibodies against isolectin β4, MAP-2, cytokeratin 3/12, ABCG2, GFAP, Nestin or Musashi-1 (see Table 6). Secondary antibodies were applied for 2.5 hours at room temperature according to the manufacturer's instructions.

Table 6: Antibodies used for in vitro Neurobasal-A holoclone immunohistochemistry

Antibody against	Full Name	Target Cells	Dilution Factor	Source
Nestin	Mouse anti-nestin	NSPCs	1:500	Chemicon MAB5326

Musashi-1	Rabbit anti-Musashi-1	NSPCs	1:500	Chemicon AB5977
Cytokeratin 3/12	Mouse anti-Keratin K3/K76	Mature corneal epithelial cells	1:300	Chemicon CBL218
ΔNp63α	Rabbit anti-ΔNp63 alpha	LSCs and possibly other stratified epithelia stem and progenitor cells	1:400	PickCell Laboratories Private Order
Isolectin β4	Isolectin β4-Alexa 488 conjugated	Microglia and perivascular cells	1:50	Molecular Probes I-21411
MAP-2	Mouse anti-microtubule associated protein- 2	Neurons	1:200	Chemicon MAB3418
ABCG2	Mouse anti-ATP Binding Cassette subtype G member 2	Most stem cells	1:20	Calbiochem OP191
GFAP	Rabbit anti-cow Glial Fibrillary Acid Protein	Astrocytes and certain neural stem cells	1:400	Dako Z0334
Collagen type I	Rabbit anti-mouse collagen type I	Many connective tissue cells	1:300	Biogenesis 2150-1410
TAT-1	Mouse anti-alpha tubulin	Microtubules of eukaryotic cells	1:5	Donated by Trevor Sherwin

4. Determination of required growth factors and media

Human corneal tissue slices from either the limbal rim or the entire cornea were cultured as described above in the following media:

1. Opti-MEM growth media (see Table 2),
2. Opti-MEM with serum,
3. Opti-MEM with neurobasal supplements B27 and N2, EGF, and FGF2,
4. Neurobasal-A growth media (as detailed in Tables 2, 4),
5. Neurobasal-A with neurobasal supplement B27 (see Table 3.1), EGF, and FGF2,
6. Neurobasal-A with neurobasal supplement N2 (see Table 3.2), EGF, and FGF2,
7. Neurobasal-A with neurobasal supplements B27 and N2, EGF, and FGF2,
8. Neurobasal A with neurobasal supplements B27 and N2, and EGF,
9. Neurobasal A with neurobasal supplements B27 and N2, and FGF2, and
10. Neurobasal-A with EGF, insulin and FGF2 only.

Results

1. Immunohistochemistry

1.1 Neurobasal-A

In Neurobasal-A medium alone no expression of any neural marker was observed in tissue slices, indicating there was no progression to a neural phenotype.

1.2 Neurobasal-A with or without B27 and N2 supplements, and with or without EGF and/or FGF2

Neurobasal-A medium plus supplements B27 and N2 and containing both EGF and FGF 2 showed cell reprogramming. Within 3 days, corneal stroma mesenchymal fibroblastic cells were en masse expressing Musahi-1 and Nestin, indicating acquisition of neural precursor properties. Expression was observed across the entire corneal stroma.

Immunohistochemical staining as depicted in Figure 2 showed that all cells in the stroma were expressing Nestin (Figure 2A) and Musahi-1 (Figure 2B) and thus were being reprogrammed and had acquired a neural precursor phenotype. There was no evidence for morphologically immature keratocytes being involved in the transformation.

Immunohistochemical staining in conjunction with DAPI nuclear staining as depicted in Figure 3A showed that after 8 days, all cells remained Nestin positive but Musahi-1 expression was lost. Also, the expression of neural markers Doublecortin, beta III Tubulin, SMI-32, MP2 and Neurofilament-200 was observed (see Figure 2C and 2D).

Reprogramming occurred across the entire stroma, including the posterior stroma which does not contain peripheral nerves in vivo (see Figure 3B). There was little evidence of Nestin or Musashi-1 expression in the epithelium, endothelium or scleral fibroblasts of the conjunctiva.

In the absence of either one of the supplements B27 or N2, reprogramming still occurred, but the removal of either EGF or FGF2 halted acquisition of the neural phenotype. In the absence of both B27 and N2 supplements, reprogramming did not occur regardless of whether either or both growth factors EGF and FGF2 were present.

No reprogramming was observed when either growth factor was absent. Similarly, no reprogramming was observed when both supplements were absent.

1.3 Neurobasal-A plus EGF, insulin and FGF2

Stromal keratocytes present in corneal tissue slices and cultured in a defined medium comprising Neurobasal-A supplemented only with the three factors EGF, FGF2, and insulin were reprogrammed into a neural phenotype.

At day 8 corneal slices cultured in this defined medium expressed neural markers such as Neurofilament-200 and MAP2, and took on a neuronal morphology. This result means that EGF, FGF2 and insulin together are capable of reprogramming cells to a neural phenotype.

1.4 Opti-MEM

No expression of any neural marker was observed in tissue slices cultured with either Opti-MEM or Opti-MEM plus serum, indicating there was no progression to the neural phenotype. However, in Opti-MEM supplemented with the two neurobasal supplements B27 and N2, EGF and FGF2 cells were reprogrammed into a neural phenotype. After 3 days, expression of Nestin was observed in corneal segments, while after 8 days, Neurofilament-200 expression was observed.

This result indicates that the reprogramming to a neural phenotype is not medium dependent.

Discussion

This work demonstrated that en masse cellular reprogramming of fibroblastic cells to neurons in situ is feasible. Redifferentiaiton occurs directly, rather than backwards via a stem or pluripotent cell phenotype.

Neural marker positive cells were observed in both Neurobasal-A and Opti-MEM cultures containing EGF, FGF 2 and supplements B27 and N2. Both of these supplements contain insulin. Neural marker positive cells were observed when cultured in defined medium comprising Neurobasal-A supplemented only with EGF, insulin and FGF 2. This indicates that the three factors EFG, FGF2 and insulin together are capable of reprogramming cells towards the mature neuronal cell phenotype. This reprogramming is independent of medium type.

Very low expression of Nestin and Musashi-1 in the limbus of the cornea indicates that the hypothesised limbal stem cells are not involved.

Expression of neuronal cell markers in the stromal cells suggested progression towards a mature neuronal cell phenotype. Expression of neuronal cell markers was co-localised with the DAPI-stained nuclei of corneal stromal keratocytes, spatially restrained within the collagen layers of the adult cornea (see Figure 3B). Thus, the cells taking on both a neuronal morphology and expressing neuronal markers are of stromal keratocyte origin.

EXAMPLE 2– Stromal cell isolation

This example describes the investigation of the neural potential of cells isolated from human corneal stroma.

Materials and Methods

All research was conducted with ethics committee approval. Consent from the donor family was obtained prior to tissue harvest and use.

1. Human limbal rim collection

Cadaveric human limbal rims were obtained from the New Zealand National Eye Bank after the corneal button had been removed for corneal transplantation surgery.

2. Stromal tissue preparation

After collection, limbal rim tissue was rinsed in Opti-MEM I (1 x, Gibco, Cat# 31985) to remove any transport medium serum. Non-stromal tissue was removed from the limbal rim tissue using a 2-step scrape and digestion in 1.2 U/mL Dispase (Gibco, Cat# 17105-041) for an initial 45 minutes followed by a further 20 minutes at 37°C.

The remaining stromal tissue was placed in a solution containing 2 mg/mL Collagenase Type II (Gibco, Cat # 17101-015) and 0.5 µg/mL Hyaluronidase Type III (Sigma, Cat # H2251) for 4 hours at 37°C, with gentle agitation. The tissue was removed and placed face down in a well containing 4 mL growth media, to allow remaining cells to grow out. This sample was labelled the Sample 1.

The Collagenase and Hyaluronidase solution was collected and centrifuged (405 x g for 7 minutes) and the supernatant removed. The remaining pellet was resuspended in a separate well with 4 mL growth media. This sample was labelled Sample 2. All samples were incubated separately at 37°C with 5% CO_2.

Each sample was grown in either Opti-MEM I or Neurobasal – A medium containing the two supplements B27 and N2 plus EGF plus FGF2.

3. Stromal cell culture

Cell culture was maintained at 34°C with 5% CO_2. Spent growth media was replaced every 3 to 4 days.

4. Immunohistochemistry and holoclone selection

Holoclones were lifted from the cell culture dish and characterised to determine their properties and potential. Antibody labelling of the holoclones was carried out using the same antibodies and reagents as described in Example 1.

4.1 Opti-MEM holoclone Immunohistochemistry

The Opti-MEM growth media was removed and the spheres were rinsed with fresh Opti-MEM with no FCS, followed by PBS. To fix the cells, 4% Paraformaldehyde (PFA, TAAB Laboratories) was applied for 1 hour. The spheres were then washed and incubated with cold methanol for 10 minutes at -20°C, blocked in 10% Goat serum for 1 hour and double-labelled with antibodies (see Table 5).

Primary antibodies were applied overnight at 4°C. Fibroblastic cells forming a sheath around the holoclone were used as a negative control. Epithelial cells of the sheath were stained as a positive control for cytokeratin 3/12 antibody. A section of rat spinal cord was used as a positive control for Nestin and Musashi-1.

Secondary antibodies were applied for 2.5 hours at room temperature according to the manufacturer's instructions. Spheres were counterstained with the nuclei marker 0.5 μg/mL DAPI (Gibco) and mounted in Citifluor AF-1 antifading reagent (Citifluor Ltd).

4.2 Neurobasal holoclone Immunohistochemistry

Spheres were picked and fixed in 4% PFA before being embedded in Optimal Cutting Temperature (OCT, Tissue Tek Inc, Japan) and frozen in liquid nitrogen. 20 μm sections were cut using a cryostat microtome (Microm HM550), mounted on Superfrost slides (Menzel Glazer) and warmed to room temperature for 3 hours before being stored at -20°C. Sections were incubated in primary antibodies (see Table 6). Sections were stained for collagen type I as a negative control or α-tubulin (TAT-1) as a positive control. Sections were not double labelled.

Results

1. Stromal cell culture

After 2 - 4 days culture in vitro, the corneal stromal cells started to proliferate to form small clusters. At 7 - 10 days post collection, sphere colonies were clearly visible.

2. Holoclone formation

Three typical sphere morphologies (holoclones, meroclones and paraclones) formed in culture, based on ratios of undifferentiated to differentiated cells, respectively.

3. Immunohistochemistry

Holoclones grown in either medium (Neurobasal-A or Opti-MEM, each supplemented with B27 and N2, EFG, and FGF2) stained very strongly for the neural stem and progenitor markers Musashi-1 and Nestin. Subsets of cells were identified that expressed only one of the two markers.

No cytokeratin 3/12 expression was detected. Positive MAP-2 but negative GFAP labelling was observed in some Neurobasal-A (supplemented with B27, N2, EFG, and FGF2) holoclones.

Discussion

This work demonstrated that Neurobasal-A holoclones may be more neuronally inclined than Opti-MEM holoclones, with more cells showing neural markers associated with a more mature phenotype. The removal of non-stromal tissue using the 2-step scrape and digestion described in

the methods above means that the holoclones generated in this system originate from stromal cells, rather than non-stromal cells.

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CLAIMS

1. A method for producing neuronal cells comprising:
   (1) providing an ophthalmic tissue comprising one or more ophthalmic cells;
   (2) contacting the tissue with
      (a) epidermal growth factor or a functional variant or functional fragment thereof (EGF) or an agonist of the EGF receptor, and
      (b) fibroblast growth factor 2 or a functional variant or functional fragment thereof (FGF2) or an agonist of one or more of the FGF family of receptors and
      (c) insulin or a functional variant or functional fragment thereof or an agonist of the insulin receptor or of the insulin-like receptor,

   for a period sufficient to produce one or more neuronal cells from one or more of the one or more ophthalmic cells.
2. The method of claim 1 wherein the ophthalmic tissue comprises one or more ophthalmic tissues selected from the group comprising cornea, ophthalmic epithelia, ophthalmic endothelia, limbus, retina, cilary body, optic nerve, eyelid and enclosed orbicularis oculi muscle, and sclera.
3. The method of claim 2 wherein the ophthalmic tissue comprises one or more of corneal epithelia, corneal stroma, and corneal endothelia.
4. The method of any one of claims 1 to 3 wherein the tissue comprises at least two populations of differentiated ophthalmic cells.
5. The method of claim 4 wherein substantially all of at least one of the populations of differentiated ophthalmic cells are reprogrammed to neuronal cells.
6. A method for producing neuronal cells comprising:
   (1) providing one or more isolated differentiated ophthalmic cells;
   (2) contacting the sample with
      (a) epidermal growth factor or a functional variant or functional fragment thereof or an agonist of the EGF receptor, and
      (b) fibroblast growth factor 2 or a functional variant or functional fragment thereof or an agonist of one or more of the FGF family of receptors and
      (c) insulin or a functional variant or functional fragment thereof or an agonist of the insulin receptor or of the insulin-like receptor,

   for a period sufficient to produce one or more neuronal cells from one or more of the one or more differentiated ophthalmic cells.
7. The method of claim 6 wherein the one or more differentiated ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, corneal endothelial

cells, scleral epithelial cells, scleral endothelial cells, scleral fibroblasts, limbal epithelial cells, limbal endothelial cells, limbal cells, and retinal cells including retinal pigment epithelial cells and retinal ganglion cells.

8. The method of claim 1 or claim 8 wherein the one or more ophthalmic cells or the one or more differentiated ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, and corneal endothelial cells.
9. The method of claim 8 wherein the one or more differentiated ophthalmic cells are corneal keratocytes.
10. The method of any one of claims 1 to 9 wherein the one or more neuronal cells are neuronal-precursor cells.
11. The method of any one of claims 1 to10 wherein the one or more neuronal cells are *Musashi-1* positive.
12. The method of any one of claims 1 to 11 wherein the one or more neuronal cells are *Nestin* positive.
13. The method of any one of claims 1 to 12 wherein the one or more neuronal cells are *MAP2* positive.
14. The method of any one of claims 1 to 13 wherein the one or more neuronal cells are *Neurofilament-200* positive.
15. The method of any one of claims 1 to 14 wherein the one or more neuronal cells are *SMI32* positive.
16. The method of any one of claims 1 to 15 wherein the one or more neuronal cells are *Doublecortin* positive.
17. The method of any one of claims 1 to 16 wherein the one or more neuronal cells are *beta III Tubulin* positive.
18. The method of any one of claims 1 to 17 wherein the one or more neuronal cells are *Neuronal-N* positive.
19. The method of any one of claims 1 to 18 wherein the one or more neuronal cells are neurons.
20. The method of any one of claims 1 to 19 wherein the one or more ophthalmic cells are in contact with EGF, insulin and FGF2 for from about 0.5 days to about 10 days.
21. The method of claim 20 wherein the one or more ophthalmic cells are in contact with EGF, insulin and FGF2 for from about 1 day to about 5 days.
22. The method of claim 20 wherein the one or more ophthalmic cells are in contact with EGF, insulin and FGF2 for from about 6 days to about 10 days.
23. The method of any one of claims 1 to 22 wherein the one or more ophthalmic cells are in contact with EGF, insulin and FGF2 for at least about 3 days.

24. A method for generating neuronal cells, the method comprising administering epidermal growth factor or a functional variant or functional fragment thereof or an agonist of the EGF receptor, and fibroblast growth factor 2 or a functional variant or functional fragment thereof or an agonist of one or more of the FGF family of receptors, and insulin or a functional variant or functional fragment thereof or an agonist of the insulin receptor or of the insulin-like receptor, to one or more ophthalmic cells in a mammalian subject in need thereof.
25. The method of claim 24 wherein the one or more ophthalmic cells are present in one or more ophthalmic tissues selected from the group comprising cornea, ophthalmic epithelia, ophthalmic endothelia, limbus, retina, and sclera.
26. The method of claim 25 wherein the one or more ophthalmic cells are present in one or more ophthalmic tissues selected from the group comprising corneal epithelia, corneal stroma, and corneal endothelia.
27. The method of claim 24 wherein the one or more ophthalmic cells are selected from the group comprising corneal keratocytes, corneal epithelial cells, and corneal endothelial cells.
28. The method of any one of claims 24 to 27 wherein the mammalian subject is to undergo or has undergone ophthalmic surgery.
29. A method of treating or preventing an ophthalmic disease or condition or of promoting recovery from an ophthalmic therapy, the method comprising administering epidermal growth factor or a functional variant or functional fragment thereof or an agonist of the EGF receptor, and fibroblast growth factor 2 or a functional variant or functional fragment thereof or an agonist of one or more of the FGF family of receptors, and insulin or a functional variant or functional fragment thereof or an agonist of the insulin receptor or of the insulin-like receptor, to one or more ophthalmic cells in a mammalian subject in need thereof.
30. The method of claim 29 wherein the ophthalmic disease or condition is selected from the group comprising retinal degeneration including retinal ganglion cell loss, bipolar cell loss, photoreceptor cell loss, retinal ganglion cell loss or optic nerve cell loss as a result of trauma, ophthalmic trauma, ischemia including optic nerve ischemia, glaucoma, age related macular degeneration, retinitis pigmentosa, diabetic retinopathy, retinal detachment, multiple sclerosis, central retinal artery occlusion, tumour compression, toxic neuropathy, ruptured optic nerve (avulsion), corneal nerve dystrophies, dry eye including dry eye resulting from cataract surgery, ophthalmic viral infection such as herpes infection including herpes simplex or herpes zoster infection, hypoesthesia, lost or damaged ophthalmic neurons, ophthalmic inflammation, ptosis due to loss of nerve function, and neurological conditions.
31. The method of claim 29 wherein the ophthalmic therapy is corneal surgery including corneal transplant.

32. The method of any one of claims 29 to 31 wherein the administration is intraocular administration.
33. The method of claim 32 wherein the administration is by intraocular injection, intraocular depot injection, intraocular implant, retinal implant, or subretinal implant.
34. The method of any one of claims 29 to 31 wherein the administration is topical administration.
35. The method of claim 34 wherein the administration is by ocular drops or ocular gel.
36. The method of any one of the preceding claims wherein the EGF is present at a final concentration of below about 50ng/mL.
37. The method of any one of the preceding claims wherein the EGF is present at a final concentration of from about 0.1ng/mL to about 25ng/mL.
38. The method of any one of the preceding claims wherein the FGF2 is present at a final concentration of below about 50ng/mL.
39. The method of any one of the preceding claims wherein the FGF2 is present at a final concentration of from about 0.25ng/mL to about 25ng/mL.
40. The method of any one of the preceding claims wherein the insulin is present at a final concentration of below about 50μg/mL.
41. The method of any one of the preceding claims wherein the insulin is present at a final concentration of from about 0.1μg/mL to about 25μg/mL.
42. A method of treating or preventing an ophthalmic disease or condition or of promoting recovery from an ophthalmic therapy, or treating or preventing a neurological disease or condition or of promoting recovery from an neurological therapy, the method comprising administering one or more neuronal cells prepared by the method of any one of claims 1 to 28 or 36 to 41 to a mammalian subject in need thereof.
43. The method of claim 42 wherein the ophthalmic disease or condition is selected from the group comprising retinal degeneration including retinal ganglion cell loss, bipolar cell loss, photoreceptor cell loss, retinal ganglion cell loss or optic nerve cell loss as a result of trauma, ophthalmic trauma, ischemia including optic nerve ischemia, glaucoma, age related macular degeneration, retinitis pigmentosa, diabetic retinopathy, retinal detachment, multiple sclerosis, central retinal artery occlusion, tumour compression, toxic neuropathy, ruptured optic nerve (avulsion), corneal nerve dystrophies, dry eye including dry eye resulting from cataract surgery, ophthalmic viral infection such as herpes infection including herpes simplex or herpes zoster infection, hypoesthesia, lost or damaged ophthalmic neurons, ophthalmic inflammation, and ptosis due to loss of nerve function.

44. The method of claim 42 wherein the neurological disease or condition is selected from the group comprising neurological diseases associated with tissue trauma, neurological diseases associated with inflammation, and neurodegenerative diseases.

45. A cell growth or differentiation media comprising

(1) epidermal growth factor or a functional variant or functional fragment thereof at a final concentration of from about 0.1ng/mL to about 50ng/mL, or

(2) fibroblast growth factor 2 or a functional variant or functional fragment thereof at a final concentration of from about 0.25ng/mL to about 50ng/mL, or

(3) insulin or a functional variant or functional fragment thereof at a final concentration of from about 0.1μg/mL to about 50μg/mL.

(4) any combination of two or more of (1) to (3).

46. A pharmaceutical composition comprising

(a) epidermal growth factor or a functional variant or functional fragment thereof, and

(b) fibroblast growth factor 2 or a functional variant or functional fragment thereof, and

(c) insulin or a functional variant or functional fragment thereof,

wherein the composition is formulated for administration to the mammalian eye.

47. The composition of claim 46 formulated for topical administration.

48. The composition of claim 46 formulated for intraocular administration.

49. The composition of claim 46 formulated for retrobulbar administration.

50. Epidermal growth factor, insulin and fibroblast growth factor 2 for use in therapy.

51. The epidermal growth factor, insulin and fibroblast growth factor 2 of claim 50 wherein the therapy is treating or preventing an ophthalmic disease or condition or promoting recovery from an ophthalmic therapy.

52. The use of epidermal growth factor, insulin and fibroblast growth factor 2 in the preparation of a medicament suitable for treating or preventing an ophthalmic disease or condition in a mammalian subject in need thereof.

53. The use of epidermal growth factor, insulin and fibroblast growth factor 2 in the preparation of a medicament suitable for promoting recovery from an ophthalmic therapy in a mammalian subject in need thereof.

54. A product containing epidermal growth factor, insulin and fibroblast growth factor 2 as a combined preparation for simultaneous, separate or sequential use in therapy.

55. The product according to claim 54 wherein the product is for use in treating or preventing an ophthalmic disease or condition or in promoting recovery from an ophthalmic therapy.

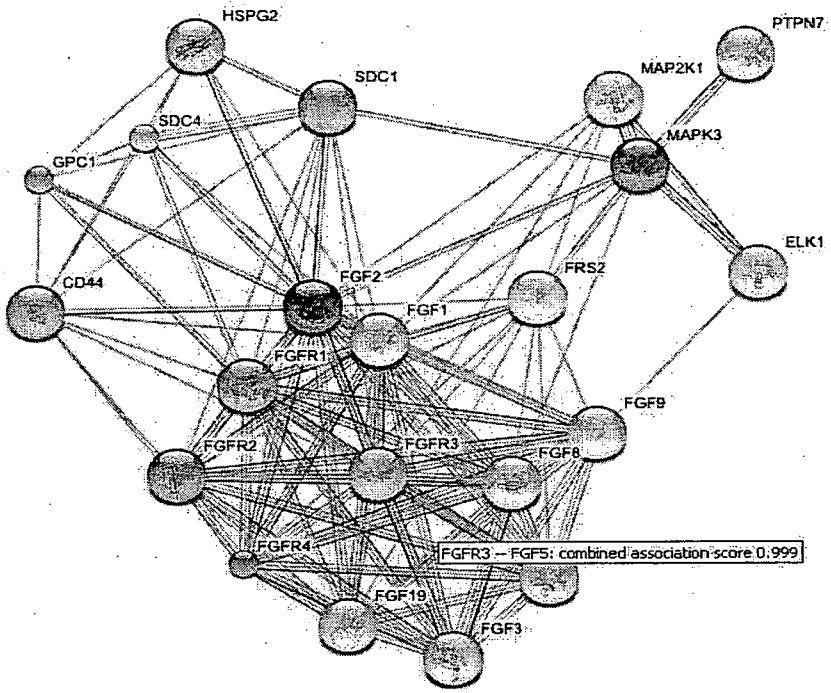

HSPG2
PTPN7
SDC1
MAP2K1
SDC4
MAPK3
GPC1
ELK1
CD44
FGF2
FRS2
FGF1
FGFR1
FGF9
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FGF8
FGFR4
FGFR3 -- FGF5: combined association score 0.999
FGF19
FGF3

Figure 1

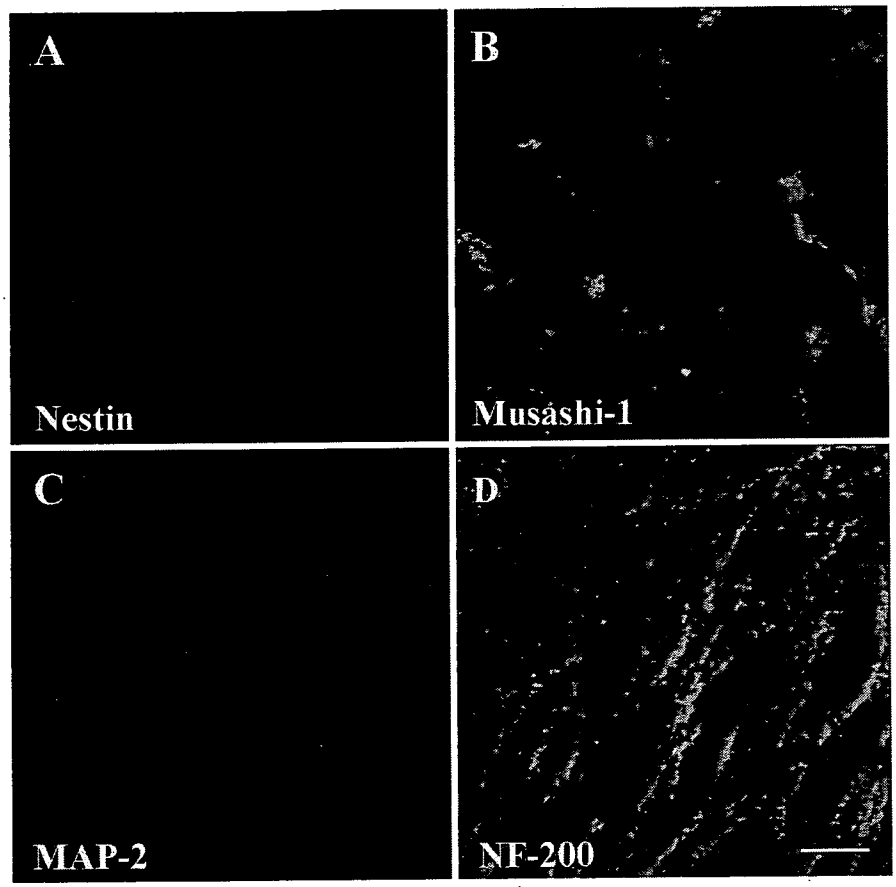


Figure 2

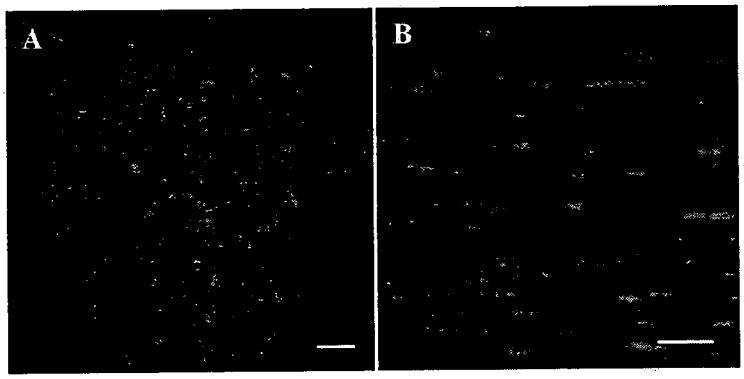


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2010/000099

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 5/071 (2010.01) *A61K 38/28* (2006.01)
A61K 38/18 (2006.01) *C12N 5/079* (2010.01)

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)

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)
WPI, EPODOC, CA, Medline, Biosis; ophthalmic, eye, iris, cornea, retina, limbus, scleral, ciliary, neuron, glial, ganglion, differentiate, program, insulin, IGF, somatomedin, mechano growth factor, fibroblast growth factor 2, FGF2, bFGF, epidermal growth factor, EGF, urogastrone and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 2005229870 A (HOYA CORP *et al*) 02 September 2005 (English abstract) in: World Patents Index [database online], Abstract Accession 2005-632772 [retrieved on 24 September 2010]. English abstract	1-55
X	Zhao X *et al*, "Derivation of Neurons with Functional Properties from Adult Limbal Epithelium: Implications in Autologous Cell Therapy for Photoreceptor Degeneration", *Stem Cells*, 2008, 26(4):939-949 pages 939 to 941; whole of document	1-5, 8-55
X	AU 2006235917 A1 (RELIANCE LIFE SCIENCES PVT LTD) 14 February 2008 abstract; pages 11, 12, 22, 33; whole of document	1-5, 8-55

[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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
24 September 2010

Date of mailing of the international search report
30 SEP 2010

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2010/000099

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1 to 44, 46 to 49, 52, 53 (completely) and 45, 50, 51, 54, 55 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Fischer AJ *et al*, "Growth factors induce neurogenesis in the ciliary body", *Developmental Biology*, 2003, 259:225-240 abstract; pages 229 to 233; whole of document	1-41, 45-55
X	WO 2000/047040 A1 (THE SCHEPENS EYE RESEARCH INSTITUTE INC) 17 August 2000 abstract; page 8; claims 19 to 21; whole of document	24-41, 45-55
X	Kohno R *et al*, "Sphere formation of ocular epithelial cells in the ciliary body is a reprogramming system for neural differentiation", *Brain Research*, 2006, 1093:54-70 ABSTRACT; pages 57, 58, 60, 61, 63, 65, 66; whole of document	1-6, 8-55
X	Cicero SA *et al*, "Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells", *Proceedings of the National Academy of Sciences*, April 21 2009, 106(16):6685-6690 & "Supporting Information" <URL: http://www.pnas.org/content/suppl/2009/04/03/0901596106.DCSupplemental/0901596106SI.pdf> [retrieved on 22 September 2010] retrieved from the internet. pages 6685, 6687; "Supporting Information" page 1; whole of documents	1-6, 8-55
L	page 6689 L – raised for Article 5 purposes.	1-55

Supplemental Box
(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

This International Searching Authority has found that there are different inventions as follows:

- Claims 1 to 44, 46 to 49, 52 and 53 are directed to methods of producing neuronal cells from ophthalmic cells, methods of treating or preventing ophthalmic disease or conditions in mammals, and compositions for treating or preventing ophthalmic disorders or promoting recovery from ophthalmic therapy wherein a combination of epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and insulin, or functional variants or fragments thereof or agonists of the receptors thereof are used to treat the ophthalmic tissue. It is considered that using the combination of EGF, FGF2 and insulin to treat ophthalmic tissue to produce neuronal cells comprises a first distinguishing feature.

- Claim 45 (partially) is directed to a cell growth or differentiation media comprising EGF or a functional variant or fragment thereof at a final concentration of 0.1 ng/L to 50 ng/L. It is considered that a cell growth or differentiation media comprising EGF comprises a second distinguishing feature.

- Claim 45 (partially) is directed to a cell growth or differentiation media comprising FGF2 or a functional variant or fragment thereof at a final concentration of 0.25 ng/L to 50 ng/L. It is considered that a cell growth or differentiation media comprising FGF2 comprises a third distinguishing feature.

- Claim 45 (partially) is directed to a cell growth or differentiation media comprising insulin or a functional variant or fragment thereof at a final concentration of 0.1 µg/L to 50 µg/L. It is considered that a cell growth or differentiation media comprising insulin comprises a fourth distinguishing feature.

- Claims 50, 51, 54 and 55 are directed to EGF, FGF2 and insulin for use in therapy, either simultaneously, separately or sequentially. It is considered that EGF, FGF2 and insulin for use in therapy comprises a fifth distinguishing feature.

Each of the aforementioned groups of claims has a different distinguishing feature and they do not share any feature which could satisfy the requirement for being a special technical feature. Because there is no common special technical feature it follows that there is no technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention *a priori*.

There is some overlap in the first and fifth invention, involving the use of the combination of EGF, FGF2 and insulin. However the first invention encompasses *in vitro* uses of the combination and is not limited to therapeutic uses of the combination, and the fifth invention includes the use of EGF, FGF2 and insulin separately and is not limited to the use of these as a combination. Therefore these are taken to be separate inventions. In addition the use of the combination of EGF, FGF2 (previously called basic FGF, bFGF) and insulin is known from documents D1 to D7 raised in Box V.

The search with respect to inventions two to five has been made for those inventions only as far as they fall within the scope of the first invention.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ2010/000099

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
JP	2005229870	NONE					
AU	2006235917	NONE					
WO	0047040	AU	28782/00	AU	73878/00	US	6541256
		US	6548059	WO	0106843		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX