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Research Article

Cells from the adult corneal stroma can be reprogrammed to a neuron-like cell using exogenous growth factors

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ABSTRACT

Cells thought to be stem cells isolated from the cornea of the eye have been shown to exhibit neurogenic potential. We set out to uncover the identity and location of these cells within the cornea and to elucidate their neuronal protein and gene expression profile during the process of switching to a neuron-like cell. Here we report that every cell of the adult human and rat corneal stroma is capable of differentiating into a neuron-like cell when treated with neurogenic differentiation specifying growth factors. Furthermore, the expression of genes regulating neurogenesis and mature neuronal structure and function was increased. The switch from a corneal stromal cell to a neuron-like cell was also shown to occur *in vivo* in intact corneas of living rats. Our results clearly indicate that lineage specifying growth factors can affect changes in the protein and gene expression profiles of adult cells, suggesting that possibly many adult cell populations can be made to switch into another type of mature cell by simply modifying the growth factor environment.

Introduction

Adult stem cells have been isolated from a variety of tissues and convention holds that they arise in embryogenesis and persist into adulthood where they contribute to steady state tissue renewal and injury induced repair. Many such cells have been identified on the basis of their expression of specific proteins and genes, though using markers to classify cells as stem cells without a full molecular profile may be misleading [21]. Emerging research suggests that stem cell identity may not, in fact, be restricted to a specific pool of cells that self-renew, but that stemness can be acquired by differentiating progenitors after tissue injury and throughout life [5]. The field of cell reprogramming has also challenged the notion of the terminally differentiated adult cell [53,56,23,58,27].

Cell reprogramming induces a major switch in gene expression, usually after introduction of specific transcription factors and most commonly allowing reversion of a mature cell to a pluripotent phenotype [62]. More recently, however, research has shown that re-expression of key developmental regulators *Ngn3*, *Pdx1* and *Mafa* reprogrammed adult pancreatic exocrine cells into β-cells in mice [61], suggesting that it is possible to convert one mature cell into another without first reverting to a pluripotent state. In another study, transplantation of differentiating mouse spermatogonia into germ cell depleted testes was sufficient to induce a reversal of differentiation after transplantation. Importantly, the differentiating

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spermatogonia could also be reprogrammed back to functional self-renewing stem cells *in vitro* in the presence of the growth factors glial derived neurotrophic factor (GDNF) and fibroblast growth factor-2 (FGF-2) [5].

The eye is a complex structure that allows us to obtain visual information from our surroundings and the cornea serves as the window into the eye. It accounts for more than two-thirds of the total refractive power of the eye. The cornea is made up of several layers, an anterior stratified epithelium which is composed of stratified squamous epithelial cells, an intermediate connective tissue stroma along with sparsely distributed cells known as keratocytes, and a posterior endothelial monolayer which consists of polygonal cells arranged in a mosaic pattern [25]. The stromal layer comprises about 90% of the corneal tissue and is composed of highly organised lamellae which are made up of tightly packed collagen fibrils made up mostly of collagen types I and V. The keratocytes are responsible for maintaining the integrity of the fibrils and the extracellular matrix by a steady turnover [19]. The keratocytes of the cornea are wholly derived from the neural crest [26]. When keratocytes are removed from the stromal tissue and cultured in a monolayer they exhibit the morphological characteristics of fibroblasts [18] and switch from a stellate shaped cell to a fusiform shaped cell.

In the cornea, the stem cells are thought to reside at the corneal limbus and may be maintained by a variety of intrinsic and extrinsic factors such as the local environment, survival factors and cytokines [14].

The formation *in vitro* of highly proliferative, compact and round colonies known as holoclones has become the culture phenotype of stem/progenitor cells [39]. This property was originally demonstrated in stem cell cultures of keratinocytes [4], followed by human hair follicles [44] and later, epithelial cells from the corneal limbus [40] and keratocytes from the corneal stroma [60].

We initially set out to uncover the origin of these sphere forming stem-like cells *in situ* by culturing human cornea in an organotypic slice culture model using a standard neuronal differentiation media routinely used for neuronal differentiation of stem cells. We investigated the potential of adult corneal stromal keratocytes to differentiate into neuronal cells using three specific neurogenic differentiation factors that we have identified. We then moved this to an animal model to demonstrate adult corneal stromal cell reprogramming *in vivo*.

Material and methods

Human tissue collection

Cadaveric whole human corneas, human limbal rims and surgeon cut DSEK caps (excess stromal tissue from Descemet's Stripping Endothelial Keratoplasty) were obtained from donors sourced through the New Zealand National Eye Bank (Auckland, New Zealand). Human limbal rims were collected after the central corneal button had been removed for corneal transplantation surgery leaving a 2 mm corneal margin from the limbal junction. Prior to the use of tissue, research ethics approval and consent were obtained from the Northern X Regional Human Ethics Committee. All tissue, until use, was stored in New Zealand Eye Bank medium 2% Fetal Calf Serum (FCS) (Gibco #10091), 2 mM GlutaMax (Gibco #35050), 1x Antibiotic-Antimycotic (Gibco # 15240) in Eagle's Minimum Essential Medium (MEM) (Gibco #12360) and transported in New Zealand Eye Bank transport medium (eye bank medium supplemented with 5% Dextran).

Animal tissue collection

Ethics approval for animal studies was obtained from the University of Auckland Animal Ethics Committee. All procedures were conducted in compliance with the ARVO Statement of Use of Animals in Ophthalmic and Vision Research. Adult Wistar male rats approximately 300 g (±50 g) were sacrificed with carbon dioxide gas. The eyes were removed and washed with povidone-Iodine (PVP-I) and sodium thiosulphate. The corneas were dissected out and stored for a minimal amount of time in phosphate buffer saline solution until use.

Organotypic slice culture and cell culture

Human and rat corneas were thin-sliced (1–2 mm) in an anteroposterior plane with surgical blades. An organotypic air-liquid interphase culture model, modified from one designed for brain slice cultures was used [50]. Sections were placed epithelium up on Millicell-CM culture plate inserts (30 mm diameter, 0.4 μm pore size, Millipore) with 4 ml of culture medium.

Limbal rims were dissected to isolate stroma from sclera. The corneal epithelium and endothelium were gently scraped off with a keratome and discarded. DSEK caps also received gentle scraping with a keratome to remove endothelium. Remaining stromal tissue was digested in 0.4% type II collagenase in HBSS at 37 °C with gentle mixing for 5 h on an orbital shaker. After tissue digestion was complete, the cells were pelleted by centrifuging at 1200 rpm for 7 min. The cells were resuspended in appropriate cell culture medium and after counting were cultured in either 12 or 24 well cluster plates (Falcon) on plastic or glass coverslips in 2–3 ml of cell culture media. Cells were kept in a humidified incubator at 37 °C with 5% CO2. Culture media was changed after 24 h then every 48 h subsequently or more frequently if required.

Culture media

The following types of growth media were used for tissue slice and cell cultures: (A) fibroblast proliferation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine and 1× Antibiotic-Antimycotic; (B) neuronal reprogramming media containing commercial supplements consisted of Neurobasal–A medium supplemented with 2 ng/ml human/rat recombinant epidermal growth factor (EGF), 1 ng/ml human/rat recombinant basic fibroblast growth factor (FGF-2), B27, N2, 2 μg/mL Heparin, 2 mM L-glutamine and 1× Antibiotic–Antimycotic; (C) neuronal reprogramming media containing defined growth factors and without commercially available supplements consisted of Neurobasal–A medium supplemented with 2 ng/ml human/rat recombinant EGF, 1 ng/ml human/rat recombinant FGF-2, 50 ng/mL human/rat Insulin-like Growth Factor-1 (IGF-1), 2 mM L-glutamine and 1× Antibiotic–Antimycotic.

Omission experiment to determine the key differentiation factors

In order to determine the key supplement(s) driving neuronal differentiation in the supplemented Neurobasal–A complex, each of the five supplements, namely EGF, FGF-2, Heparin, N2 and B27