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Rapid tissue engineering of biomimetic human corneal limbal crypts with 3D niche architecture

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ABSTRACT

Limbal epithelial stem cells are responsible for the maintenance of the human corneal epithelium and these cells reside in a specialised stem cell niche. They are located at the base of limbal crypts, in a physically protected microenvironment in close proximity to a variety of neighbouring niche cells. Design and recreation of elements of various stem cell niches have allowed researchers to simplify aspects of these complex microenvironments for further study *in vitro*. We have developed a method to rapidly and reproducibly create bioengineered limbal crypts (BLCs) in a collagen construct using a simple one-step method. Liquid is removed from collagen hydrogels using hydrophilic porous absorbers (HPAs) that have custom moulded micro-ridges on the base. The resulting topography on the surface of the thin collagen constructs resembles the dimensions of the stromal crypts of the human limbus. Human limbal epithelial cells seeded onto the surface of the constructs populate these BLCs and form numerous layers with a high proportion of the cells lining the crypts expressing putative stem cell marker, p63α. The HPAs are produced using a moulding process that is flexible and can be adapted depending on the requirements of the end user. Creation of defined topographical features using this process could be applicable to numerous tissue-engineering applications where varied 3-dimensional niche architectures are required.

1. Introduction

The cornea is the transparent window at the front of the eye and the outermost corneal epithelial layer undergoes constant regeneration throughout life. A population of limbal epithelial stem cells (LESCs), morphologically small cells with a high nucleus to cytoplasm (N/C) ratio, are thought to be responsible for the maintenance of this epithelium both in healthy homeostasis and after injury, a concept first proposed by Davanger and Everson [1]. Destruction or damage to this population of stem cells can lead to LESC deficiency and consequent absence of an intact epithelial layer, conjunctival ingrowth and vascularisation, chronic inflammation and impaired vision [2]. Many other organs in the adult body also possess a mechanism to replace cells lost through natural wear and tear or injury, which requires a life-long reservoir of stem cells that retain the ability to self-renew. Given the importance of these cells, it is perhaps unsurprising that they are often found safely hidden away in physically protective environments. These stem cell niches, first described by Schofield in 1978 [3], are frequently characterised by the presence of a diverse array of accessory cell types, which contribute to the specialised extracellular matrix and secreted factor milieu. Examples of these niche environments include the bulge of the hair follicle, the crypt of an intestinal villus, the rete ridges of the epidermis of the skin or the canals of Hering in the liver [4,5], where stem cells are typically located in physically protected positions surrounded by supporting mesenchymal cells.

The LESC stem cell population also resides in a specialised, physically protective niche at the limbus, the vascularised border between the cornea and the conjunctiva. Extensive characterisation of the specific microenvironment, including differential expression of extracellular matrix components and growth factors in the limbal niche has been completed and is well reviewed by Schlötzer-Schrehardt and Kruse [6]. Using advanced imaging techniques, our group has previously identified unique niche structures that house these cells, such as the limbal crypts (LCs) and focal stromal projections (FSPs) [7]. The stromal tissue that surrounds the LCs is highly cellular with a distinct vascular supply that is closely associated with the small epithelial cells at the base of the crypts. The LCs and FSPs are not uniformly distributed around the corneal

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circumference but are predominantly located in the superior and inferior corneal limbal quadrants, areas that are afforded extra protection from damaging ultraviolet light by the eyelids [8]. One major advantage of studying the human limbal niche is that it is readily accessible allowing detection of the LCs and FSPs using *in vivo* confocal microscopy in healthy human volunteers. In studies in patients with LESC deficiency, no LCs or FSPs could be detected [7], emphasising the importance of these structures to maintenance of normal LESC function. Confocal imaging of limbal tissue has revealed a close spatial relationship between basal LESCs of the LCs and stromal cells [9], much like the mesenchymal cells of the haematopoietic stem cell niche in the bone marrow [10]. In a tissue-engineered model of the central cornea, we have previously demonstrated how the presence of these stromal cells in close proximity to an overlying epithelial layer positively affects the organisation of that epithelium [11].

Considerable progress has been made in the design of engineered, completely artificial niches that allow researchers to simplify elements of complex stem cell niches for further study *in vitro* (for review see Ref. [12]). Elements of the niche that have been targeted for reproduction have included integral membrane proteins on support cells, localized secreted ECM components and soluble proteins such as growth factors and cytokines. In addition, conventional attempts to recreate aspects of the stem cell niche have commonly focussed on two-dimensional microenvironmental configurations, such as growth on nano-scale topography on tissue culture plastic. More recently, bioprinting technologies have been employed in this field. Creation of spatially defined patterns of immobilized proteins using inkjet-printing technology instructs progenitor cells to differentiate into disparate cells types depending on whether on-pattern or off-pattern [13]. Albrecht et al. have taken this one step further by precisely organising cells in 3-dimensional (3-D) microenvironments using electropatterning within hydrogels [14] and Nelson and Tien highlight the importance of mimicry of the microscale geometry of niche extracellular matrix for tissue engineering [15].

We have previously reported production of a model of the central cornea using plastic compressed collagen containing limbal fibroblasts as a substrate for epithelial cell growth [11] and have further developed this process to increase robustness and allow scale-up in production of engineered tissues [16]. Since then, the process has been further refined taking into consideration the requirements for compliance for production in good manufacturing practice (GMP) facilities. The new process for production is referred to as **R**eal **A**rchitecture **F**or 3D **T**issue or RAFT™. We believe that the unique physical LC structures identified in the limbus are a major contributing element to the LESC niche. We aim to create a model of the LESC niche with increased biomimicry, by engineering crypts into the surface of stromal fibroblast-containing RAFT constructs using a novel, rapid, reproducible and tunable process. We aim to show that these bioengineered limbal crypts (BLCs) are stable in culture and that human limbal epithelial (HLE) cells and cells with limbal stem cell characteristics consistently populate these crypts.

2. Materials and methods

2.1. Donor tissue

Cadaveric donor corneal rims and whole corneas with appropriate research consent were obtained from Moorfields Lions Eye Bank (London, UK) and Florida Lions Eye Bank (Miami, FL, US). Ethical permission was obtained from the Research Ethics committee (UK) (Ref No. 10/H0106/57). Corneas were stored at 4 °C in Optisol (Chiron Ophthalmics Inc. Irvine, California) after enucleation and prior to fixation or HLE cell and human limbal fibroblast (HLF) isolation.

2.2. Isolation and culture of human limbal epithelial cells

Human donor corneal rims were quartered and placed in a solution of 1.2 U ml^{-1} dispase II (Roche Diagnostics, GmbH, Manheim, Germany) for 2 h at 37 °C. HLE cells were isolated by scraping the limbus with forceps and collecting the liberated cells in corneal epithelial cell medium (CECM). CECM contained DMEM:F12 basal medium (3:1), 10% foetal bovine serum (FBS), 1% antibiotic-antimycotic, epidermal growth factor (EGF, 10 ng/ml; Life Technologies, Paisley, UK), hydrocortisone (0.4 mg/ml), insulin (5 mg/ml), adenine (0.18 mM), transferrin (5 mg/ml), T3 (2 nM) and cholera toxin (0.1 nM; Sigma–Aldrich, Dorset, UK). A single cell suspension of HLE cells was seeded onto a feeder layer of 3T3-J2 cells that had been growth arrested with mitomycin C (Movianto, Stuttgart, DE) for 2 h. Cells were maintained in a 5% CO_2 humidified incubator at 37 °C. Culture medium was changed 3 times a week and cells harvested when 80% confluent using 0.5% trypsin-EDTA (Life Technologies, Paisley, UK).

2.3. Culture of HCE-T cell line

The SV-40 immortalized human corneal epithelial cell line, HCE-T, was cultured in HCE-T medium containing DMEM:Ham's F12 (1:1), 5% FBS, insulin (5 μg/ml), cholera toxin (0.1 μg/ml) and EGF (10 ng/ml). The cell line exhibits a cobblestone appearance similar to normal corneal epithelial cells in culture [17]. HCE-T cells were used to optimize cell seeding experiments on surfaces with varied topography.

2.4. Isolation and culture of human limbal fibroblasts

After isolation of HLE cells, the remaining stromal pieces were trimmed to remove excess sclera and central cornea and placed into a solution of 2 mg/ml collagenase (Life Technologies, Paisley, UK) overnight at 37 °C. The isolated cells were then cultured in HLF medium containing DMEM-Glutamax, 10% FBS and 1% antibiotic-antimycotic (Life Technologies, Paisley, UK). Cells were maintained in a 5% CO_2 humidified incubator at 37 °C. Fibroblasts were expanded and harvested using 0.05% trypsin-EDTA and used in experiments up to passage six.

2.5. Preparation of collagen solution

A collagen solution was prepared by mixing 80% vol/vol sterile rat tail type I collagen (2 mg ml^{-1}; First Link, Birmingham, UK) with 10% vol/vol 10× Minimum Essential Medium (Life Technologies, Paisley, UK). After neutralisation with 5 M sodium hydroxide the final 10% vol/vol HLF medium containing 80,000 HLF/ml of final volume was added or HLF medium alone if acellular RAFT constructs were required.

2.6. Preparation of RAFT constructs

A volume of 2.4 ml of collagen solution (acellular or cellular) was added to the wells of 24 well plates (Greiner Bio-One, Stonehouse, UK). The well plate was placed on a plate heater (TAP Biosystems, Royston, UK) set to 37 °C for 30 min to allow the collagen solution to form a hydrogel. While still on the plate heater, 24-well hydrophilic porous absorbers (HPAs) or ridged HPAs (RHPAs) (TAP Biosystems, Royston, UK) were applied to the surface of the hydrogels. The RAFT absorption process was carried out for 15 min and the majority of the liquid in the collagen hydrogels was removed gently by capillary wicking. The absorbers were then removed and 500 μl of HLF medium was added to each well until HLE cell seeding. Subsequently, HLF medium was removed and 560,000 HLE cells were seeded onto the surface of the constructs in CECM without EGF and cells were allowed to adhere overnight before addition of EGF. Cellular RAFT constructs were maintained at 37 °C with 5% CO_2 in air in submerged culture for 14 days and then placed at the air-liquid interface using 6 well plate cell culture inserts (Millipore, West Lothian, UK) to allow stratification for a further 7 days. When using HCE-T cells, 580,000 cells were seeded on the surface of the constructs in HCE-T medium and maintained in submerged culture for 7 days before airlifting for a further 7 days.

2.7. Thickness measurements of RAFT constructs

The thickness of representative RAFT constructs made using HPAs and RHPAs was measured using optical coherence tomography (OCT) with an anterior segment lens (Spectralis, Heidelberg Engineering, Hemel Hempstead, UK). Constructs were

Table 1
Primary antibody dilution and supply details.

Antibody	Cat#	Concentration	Supplier
Cytokeratin 3	CBL218	1:500	Millipore
p63α	4892	1:100	Cell Signalling Technology
Pax6	PRB-278P	1:100	Covance
Ki67	AB9260	1:100	Millipore