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Limbal melanocytes support limbal epithelial stem cells in 2D and 3D microenvironments

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ABSTRACT

Human limbal epithelial stem cells (LESCs) are essential for the maintenance of the corneal epithelium of the ocular surface. LESCs are located within limbal crypts between the palisades of Vogt in the limbus; the interface between the peripheral cornea and conjunctiva. The limbal crypts have been proposed as a LESC niche owing to their support of epithelial cells, which can form holoclone colonies *in vitro*. Closely associated with the limbal crypts is a concentrated population of melanocytes. The anatomical location and close proximity to putative LESC suggests that melanocytes might play a role in maintenance of these stem cells in the niche. The aim of this study was to assess the ability of human limbal melanocytes (hLM) to support the expansion of human limbal epithelial cells (LECs) *in vitro* as an indicator of functional cell–cell interaction.

After observing that hLM co-localize with clusters of compact epithelial cells in the native limbal crypts, hLM were isolated from crypt-rich cadaveric limbal biopsies and used as feeders for the culture of LECs. Interestingly, LECs grown on mitotically active hLM were able to generate large epithelial colonies that contained small and compact cells with morphological stem cell characteristics. Immunocytochemistry revealed that LECs expanded on hLM were positive for the expression of the putative stem cell markers CK15, Bmi-1 and p63α and negative for the marker of terminal cell differentiation CK3. LECs and hLM were finally co-cultured on RAFT (real architecture for 3D tissue) collagen tissue equivalents. In 3D co-cultures, hLM promoted multi-layering of the epithelial sheet in which basal cells were maintained in an undifferentiated state.

Taken together, these observations suggest melanocytes could play an important role in the maintenance of LESCs in the native human limbal stem cell niche.

1. Introduction

The cornea, located at the front of the eyeball, is a highly specialized tissue that refracts and transmits light through the lens and onto the retina. Regeneration of transparent corneal epithelium is therefore essential for vision. Homeostasis of the corneal epithelium relies on a population of stem cells located within a highly vascularized area called the limbus (Graves, 1934). The limbus corresponds to a 1.5–2 mm wide ring of tissue at the interface between the peripheral cornea and the opaque conjunctiva. It has been proposed that limbal epithelial stem cells (LESCs) reside in a structure called limbal crypts that correspond to downward projections of the limbal epithelium into the limbal stroma between the palisades of Vogt (Shortt et al., 2007; Dziasko et al., 2014). LESCs have slow-cycling properties and are located within the basal layer of the epithelium (Cotsarelis et al., 1989). Morphologically, LESCs are small and circular with a high nucleus to cytoplasm ratio (Romano et al., 2003; Arpitha et al., 2005). Although no single reliable marker has yet been identified, LESCs are believed to express putative stem cell markers such as the transcription factors p63α (Pellegrini et al., 2001) and its ΔNp63α isoform (Kawasaki et al., 2006; Di Iorio et al., 2005), Bmi-1 (Umemoto et al., 2006), the ATP binding cassette ABCG2 (de Paiva et al., 2005), keratins such as CK15 (Yoshida et al., 2006) and cell adhesions molecules such as integrins α9/β1 and N-cadherin (Higa et al., 2009). Moreover, LESCs do not express markers of terminal differentiation such as CK3/CK12 (Schermer et al., 1986; Chaloin-

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Dufau et al., 1990), involucrin and connexin 43 (Matic et al., 1997). LESCs concentrate in a niche that corresponds to a highly dynamic and complex microenvironment maintaining the potency and self-renewal properties of the adult stem cell. The limbal stem cell niche consists of a distinct anatomical location (Dua, 2005; Shortt et al., 2007), specific composition of the local extra-cellular matrix (Schlötzer-Schrehardt et al., 2007; Mei et al., 2012), and interaction with underlying stromal niche cells. Interaction between stem cells and cells of the niche are important in maintaining the stem cell potency (Zhang et al., 2003). It has been reported that limbal niche cells are closely associated with putative LESC and that such association is important for clonal expansion of the epithelial progenitors *in vitro* (Chen et al., 2011; González and Deng, 2013; Higa et al., 2012; Dziasko et al., 2014; Li et al., 2014). Limbal epithelial cells (LECs) isolated from the limbal crypts are highly positive for the expression of stem cell markers, and have the greatest proliferative potential and the ability to generate holoclones *in vitro* (Shortt et al., 2007; Dziasko et al., 2014). Clinically, limbal crypts cannot be detected in patients affected by limbal stem cell deficiency (Shortt et al., 2007; Lagali et al., 2013).

Davanger and Evensen 1971, reported pigmentation of the palisades of Vogt (Davanger and Evensen, 1971). This pigmentation has been attributed to melanocytes located on the basal side of the epithelium and transfer of melanin granules into the surrounding limbal epithelial cells. Higa et al., 2005, proposed the limbal melanocytes as an anti-oxidant system in order to protect the LESCs in the limbal niche (Higa et al., 2005). Later, Hayashi et al., 2007, reported that N-cadherin positive (+ve) limbal melanocytes were co-localizing with CK15/N-cadherin +ve limbal basal epithelial cells and suggested that N-cadherin mediated cell–cell interactions would be important in maintaining potency of the epithelial stem cells in their niche (Hayashi et al., 2007). Interestingly, limbal melanocytes are not uniformly distributed around the limbus and are highly populating the limbal crypts that also concentrate LESCs (Dziasko et al., 2014). Little is known, however, about the exact function of melanocytes and their contribution to maintaining potency of the limbal epithelial progenitors.

In the present study, we aimed to demonstrate a functional role for human limbal melanocytes (hLM) in the support of limbal epithelial cells (LECs) maintaining stem cell characteristics by removing both cell types from their native niche and co-culturing them together *in vitro*. hLM were first isolated and expanded from cadaveric corneas and then used as a feeder layer for the culture of LECs. Clonal growth, epithelial layer morphology and expression of putative stem cell markers were assessed in 2D co-cultures and tissue equivalents (TEs) prepared using RAFT (Real Architecture For 3D Tissue).

2. Materials and methods

2.1. Ethics statement

Human tissue was handled according to the tenets of the declaration of Helsinki and written consent was acquired from next of kin of all deceased donors regarding eye donation for research. Research consent was obtained via the Moorfields Eye Hospital Lions Eye Bank (UK). All experiments were approved by the National Research Ethics Service, South west 3 REC, reference 10/H0106/57.

2.2. Isolation and culture of human limbal melanocytes

Highly pigmented or non-pigmented limbal biopsies were cut from human cadaveric corneas (n = 3) and transferred into a solution containing 1.2 U/mL dispase II (Roche diagnostics GmbH, Mannheim, Germany) in corneal epithelial cell culture medium (CECM) containing a 1:1 ratio of DMEM:F12, 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL Fungizone, epidermal growth factor (EGF) 10 ng/mL (Life technologies, Paisley, UK), hydrocortisone (0.4 μg/mL), insulin (5 μg/mL), adenine (0.18 mM), transferrin (5 μg/mL), T3 (2 nM), cholera toxin (0.1 nM) (Sigma–Aldrich, Dorset, UK) and incubated overnight at 4 °C. Crypt rich limbal biopsies were then gently scrapped with the points of thin forceps and cells were transferred into a T25 culture flask in 254 medium supplemented with HGMS-2 (Life technologies, Paisley, UK). Culture medium was changed every other day. After 10–12 days, melanocytes and stromal cells were mechanically separated from epithelial cells by using a solution of 0.025% trypsin-0.01% EDTA (Life technologies, Paisley, UK) and seeded into a new T25 flask in 254 medium. After reaching 75% of confluency, cultures were treated with Geneticin (Life Technologies, Paisley, UK) (0.2 mg/mL) in 254 medium for 48 h. At low concentration, geneticin has a very limited toxicity on melanocytes but is harmful for most of fibroblasts or stromal cells (Horikawa et al., 1996; Halaban and Alfano, 1984). Therefore, an enriched population of mitotically active melanocytes was finally expanded in 254 medium and used as a feeder layer at the concentration of 2×10^4 cells/cm^2.

2.3. Isolation and culture of human limbal epithelial cells (LECs)

Limbal biopsies from human cadaveric corneas (n = 3) were digested with dispase II 1.2 U/mL in CECM and limbal epithelial cells were separated with the points of thin forceps. LECs were then pre-expanded for 7 days in T25 flasks containing a feeder layer of growth-arrested 3T3s that had been seeded at a density of 1.6×10^4 cells/cm^2 and previously growth arrested with 4 μg/mL mitomycin C (Sigma–Aldrich, Dorset, UK) (Sun and Green, 1977; Lindberg et al., 1993).

After reaching 70% confluence in LECs-3T3 primary cultures, 1x TE (Life technologies, Paisley, UK) was used to detach and discard non-epithelial cells. Remaining LECs were detached with 10X TE (Life technologies, Paisley, UK) and sub-cultured onto plates at the density of 200 cells/cm^2 together with either new growth arrested 3T3s or mitotically active hLM. Co-cultures were maintained for a further 12 days in 0.5% (v/v) fetal bovine serum in CECM (0.5% FBS-CECM) before being imaged using a Nikon Eclipse TS100 inverted microscope, fixed with 4% PFA and stained with 2% rhodamine.

2.4. Flow cytometric analysis

Geneticin treated or untreated hLM were detached using 0.05% trypsin-0.02% EDTA before being fixed in 4% PFA for 10 min. Cells were washed with PBS, permeabilized with 0.5% triton-X and blocked with 10% goat serum in PBS for 30 min. Cells were then incubated with anti-MelanA primary antibody for melanocyte specific staining, diluted 1:100 (Abcam ab51061, Cambridge, UK) for 1 h at 37 °C. Cells were then incubated with the secondary antibody Alexa-594 conjugated goat anti-rabbit, diluted 1:500 (A-11032, Life Technologies, Ltd Paisley, UK) for 30 min at 37 °C before being washed and analyzed with a FACScalibur flow cytometer (Becton Dickinson).

2.5. Colony forming efficiency assay

LECs isolated from 3 different donors were expanded on either growth-arrested 3T3s or mitotically activated hLM as described above. After 12 days of culture, feeder cells were detached and removed using 0.05% trypsin-0.02% EDTA before a second solution of 0.5% trypsin-0.2% EDTA (Life Technologies, Paisley, UK) was used