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Letter to the Editor

Infant limbus: An immunohistological study

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ABSTRACT

All published literature to date has identified the human corneo-scleral limbus as the site within which stem cells of the ocular surface reside. Recently we described a unique anatomical structure at the limbus, termed the Limbal Epithelial Crypt (LEC) that has features of a putative stem cell niche. In this study we examined infant limbus tissue (donor age 4 months) for evidence of LEC and performed immunohistological comparison between infant limbus and adult LEC. No defined LEC were detected in the infant limbus. However, the entire infant limbus has characteristics resembling adult LEC. Both infant limbus and LEC demonstrated negative expression for desmoglein 3. p63 and integrin β1 expressions were located to the distal region of the infant limbus and to the basal region of the LEC. ABCG2 expression was positive throughout most of the infant limbus as was connexin 43. Infant limbus and in particular the distal region, appeared to house cells that are more "stem-like" in nature. The LEC may be a result of normal physiological developmental in order to protect and maintain stem cells at the ocular surface.

The stem cell niche is a proposed region where the surrounding microenvironment provides for and nurtures stem cells to enable them to maintain themselves under homeostatic conditions and offers protection from external stimuli (Fuchs and Segre, 2000). The theory was first introduced in 1978 (Schofield, 1978) and was popularized following studies on the *Drosophila* (Lin, 2002). Stem cells have the ability to self-renew, to produce more stem cells, and to differentiate into further specialized cells. It is important that the balance between stem cell maintenance and differentiation is maintained in order to allow for normal tissue homeostasis.

The Limbal Epithelial Crypt's were first described in 2005 (Dua et al., 2005) as a potential stem cell niche at the ocular surface. LEC consist of solid cords of epithelial cells that extend from distal ends of limbal palisades into the adjacent stroma and are few in number. Further work (Shanmuganathan et al., 2007; Yeung et al., 2008) has elaborated the immunophenotypical and morphological characteristics of the LEC and cells contained therein.

The aim of this study was to compare the immunophenotype of cells of a four-month-old infant limbus with that of the adult LEC.

Tissue samples were collected and prepared as previously described (Dua et al., 2005). In brief, 3 pairs of consented human cadaver eyes (2 adult, 1 infant) were removed within 48 h post mortem, with the approval of the local ethics committee. No evidence of any disease, desiccation or damage was noted. During preparation, a complete 360° circumferential frill of conjunctiva was retained and a corneo-scleral-conjunctival disc was then punched out using a 17-mm trephine. Each disc was divided into 8 equal parts, snap frozen in optimum temperature compound (Emitech Ltd, East Sussex, England) with liquid nitrogen, and stored at −80 °C until further use. Each block was sectioned serially at 7 μm sections with a cryostat (Leica Microsystems Ltd, Milton Keynes, England) and monitored for the presence of the LEC under the light microscope. On identification of an LEC, preceding and subsequent sections were used for immunofluorescence staining. All of the experiments on human tissue were approved by our institutional research ethics committee.

Established standard techniques were used (Yeung et al., 2008) to prepare samples. In brief, samples were air dried and fixed in acetone, followed by blocking in normal goat serum (dilution, 1:10). Samples were incubated with primary antibodies at optimal concentrations (p63; 1:100; mouse; Cat: 559951; BD Biosciences, San Jose, California, USA, ABCG2; 1:20; mouse; Cat: MAB4146; Chemicon, Billerica, Massachusetts, USA, integrin β1; 1:100; mouse; Cat: MAB2000; Chemicon, Billerica, Massachusetts, USA, desmoglein 3; 1:25; mouse; Cat: 32-6300; Zymed Laboratories, Inc., South San Francisco, California, USA, connexin 43; 1:2000; rabbit; Cat: C6219; Sigma, Dorset, UK) overnight at 4 °C. Samples were washed and incubated with secondary antibodies to Alexa Fluor 488 (anti-mouse) or Alexa Fluor 555 (anti-rabbit) (Invitrogen Ltd., Paisley, Scotland) for 1 h. Samples were counterstained with Propidium iodide or 4′,6-diamidino-2-phenylindole, mounted with glycerol mounting medium (Dako, Glostrup, Denmark), and

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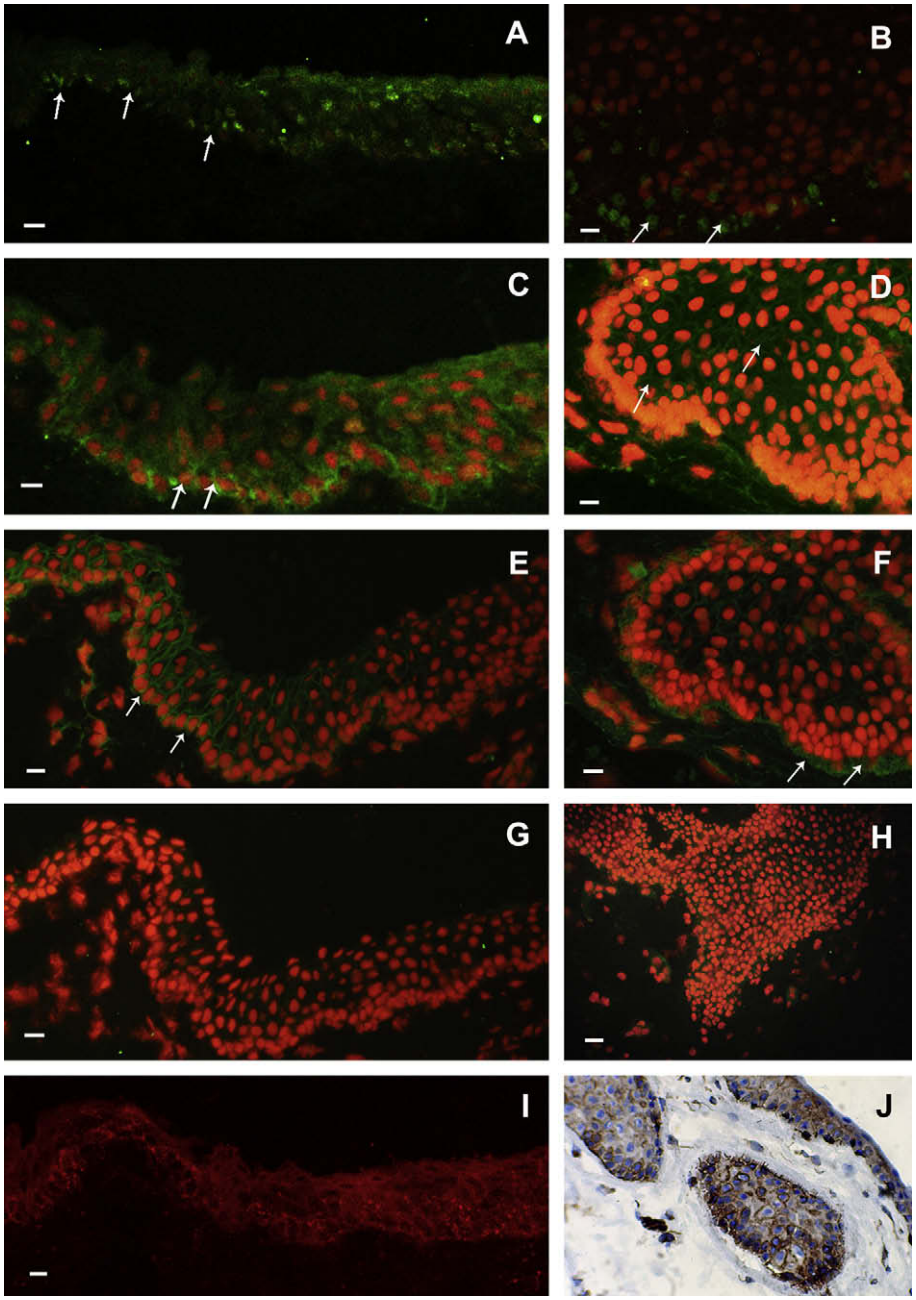

Fig. 1. Orientation of all images – the right and left side of the image represents the corneal and conjunctival aspects of the limbus respectively. A. Infant Limbus; p63, arrows show positive expression with greatest distribution in the distal region, B. LEC; p63, arrows show positive expression in the recess of the LEC, C. Infant Limbus; ABCG2, arrows show positive expression at the basement membrane and most of the limbus is positive, D. LEC; ABCG2, arrows show positive expression in the LEC, E. Infant Limbus; integrin β1, arrows show positive expression in distal portion of limbus, F. LEC; integrin β1, arrows show positive expression at LEC basement membrane, G. Infant Limbus; desmoglein 3, negative expression throughout whole limbus, H. LEC; desmoglein 3, negative expression throughout whole limbus, I. Infant Limbus; connexin 43(red) expressed throughout, J. LEC; connexin 43(brown) expression around the LEC and adjacent limbal basal epithelium. Images 1A–H counterstained with Propidium Iodide (red). Scale bar = 10 μm. Image 1J Reprinted courtesy of the British Journal of Ophthalmology (Shanmuganathan et al., 2007).

examined under an Alphaphot-2 fluorescent microscope (Nikon UK, Surrey, England). Images were captured on a D70S digital camera (Nikon UK) using Camera Control software (Nikon UK). All images were merged on Photoshop CS2 software (Adobe Systems Inc., San Jose, California).

In our study, our infant sample was limited to one pair of eyes that was retrieved from a 4-month-old who died following the complications of congenital heart disease.

The central cornea thickness of premature and full term babies compared to small children aged between 2 and 4 years of age have been known to decrease (Ehlers et al., 1976) and may be a result of eye development with rapid growth during the first year (Howland, 1982; Ronneburger et al., 2006).

Lesueur examined the structural changes in the developmental process of premature infant and children corneas (Lesueur et al., 1994) and determined that the adult structure is reached at approximately 6 months after birth. In addition, corneal keratocyte density decreases in a linear fashion with increasing age (Moller-Pedersen, 1997). Changes in Tenascin C expression at the ocular surface have been noted with greater expression in the cornea and