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Title: Differential nuclear expression of Yap in basal epithelial cells across the cornea and substrates of differing stiffness

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Abstract

Corneal epithelium is maintained throughout life by well-orchestrated proliferation of limbal epithelial stem cells, followed by migration and maturation centripetally across the ocular surface. The present study sets out to explore the role tissue stiffness (compliance) may have in directing both differentiation and centripetal migration of limbal epithelial stem cells during homeostasis. For that, we analyzed the localization of the Yes-associated protein (Yap), a transcriptional co-activator previously shown to mediate cellular response and mechanical stimuli. Using both models of ocular surface compliance and normal bovine corneas we evaluated the nuclear/cytoplasmic expression ratio of Yap. Expression levels within corneal epithelial cells were compared *in situ* between the limbus and central cornea, and *in vitro* between limbal epithelial stem cells expanded upon biomimetic collagen gels of increasing stiffness. Nuclear expression of Yap was shown to increase within the expanded cells upon substrates of increasing stiffness. Subsequently, Yap was used as a novel molecular probe to investigate the mechanical microenvironment within a normal ocular surface. The *in situ* localization of Yap was predominantly cytoplasmic within basal limbal epithelial cells and nuclear within basal central corneal epithelial cells. Furthermore, nuclear p63 expression was not co-localized with Yap in basal limbal epithelial cells. In conclusion, the current investigation provides new insights into the relationship between Yap and distinct cell populations across the ocular surface indicating that cells experience a different mechanical environment between the limbus and central cornea. A new hypothesis is put forward, in which centripetal differences in substrate stiffness drives the migration and differentiation of limbal epithelial stem cells, thus controlling corneal epithelium homeostasis.

Key words

Corneal homeostasis; mechanotransduction; Yap; migration; differentiation; LESC

The cornea is the transparent tissue at the anterior of the eye. Its function is largely dependent on the conservation of a healthy stratified epithelium which lines its external surface (Mann, 1944). Maintaining this process relies on a small population of putative limbal epithelial stem cells (LESC) that are located in the basal region at the periphery of the cornea, termed the limbus (Cotsarelis et al., 1989; Davanger and Evensen, 1971; Huang and Tseng, 1991; Lehrer et al., 1998; Schermer et al., 1986). Following migration from the edge towards the center of the cornea, LESCs undergo differentiation, stratify, and are eventually shed from the ocular surface (Collinson et al., 2002; Nagasaki and Zhao, 2003; Thoft and Friend, 1983). The mechanism underpinning this homeostatic process remains unclear; in particular what stimulates epithelial cell differentiation and migration centripetally across the corneal surface. Previously growth factors have been shown to play important roles in the maintenance, homeostasis and wound healing of the cornea and are typically supplied by the adjacent tear film (Rolando and Zierhut, 2001; Wang et al., 2013; Watanabe et al., 1987; Welge-Lüssen et al., 2001; Wilson et al., 1994), the aqueous humour (Welge-Lüssen et al., 2001), the epithelial cells (Watanabe et al., 1987), and keratocytes in the supporting stroma (Wilson et al., 1994). However, the mechanical environment to which cells are exposed has also been shown to strongly influence cell behavior including differentiation (Discher et al., 2005; Engler et al., 2006; Wang et al., 2012), attachment, and migration towards less compliant environments using a process known as durotaxis (Harland et al., 2011; Lazopoulos and Stamenović, 2008; Lo et al., 2000; Pelham and Wang, 1997). More recently, we used biomimetic collagen gels with defined elastic modulus to show how LESC differentiation can also be modulated by substrate stiffness (Chen et al., 2012; Jones et al., 2012). As such, there is increasing evidence to suggest that substrate rigidity does not only regulate LESC phenotype *in vitro* but may also contribute to the physiological maintenance of corneal phenotype and homeostasis, i.e., that the centripetal migration and differentiation of LESC across the corneal surface is at least in part driven by mechanotransduction. Mechanotransduction involves the transformation of substrate-stimulated mechanical cues into intracellular biochemical signalling enabling cells to sense and respond to their extracellular environment (Ross et al., 2013; Vogel and Sheetz, 2006). Part of this process

involves the remodelling of the cytoskeleton through contractile proteins and the activation of specific cellular pathways including PI3 kinase (Phosphatidylinositol 3-kinase) pathway, Rho, ROCK (Rho-associated protein kinases), translocation of the Yes-associated protein (Yap), and the up-regulation of focal adhesion complexes such as non-muscle myosin II (NMMII) (Dupont et al., 2011; Engler et al., 2006; Halder et al., 2012; Shewan et al., 2005). Yap is a critical downstream intracellular regulatory target in the Hippo signalling pathway but has recently been implicated as a sensor and mediator of substrate mechanical cues together with its homologue, TAZ (transcriptional co-activator with PDZ-binding motif) (Lamar et al., 2012). In particular, the retention of Yap in the cytoplasm is involved in the cellular response to mechanical stimulation on soft, compliant substrates (Dupont et al., 2011). Here we analyzed the expression ratio of nuclear/cytoplasmic Yap in response to different levels of substrate compliance to probe the LESC mechanical environment *in situ* across freshly excised bovine corneas. Our data suggests a centripetal gradient in substrate stiffness across the cornea that may drive both differentiation and centripetal migration of LESC.

Bovine eyes were obtained from a local abattoir (Chity wholesale abattoir, Guildford, UK) within 2 h of death and transported to the laboratory on ice ready for dissection. Bovine corneal tissue including the sclera and limbus was removed from the eye using a surgical blade. The excised bovine corneas were dissected into quarters and remaining scleral tissue removed. The corneal-limbal tissue was either directly embedded in optimal cutting tissue (O.C.T.) compound (Jung tissue freezing medium, Germany), snap-frozen in liquid nitrogen and stored at -80°C, or cut into several small pieces (approximately ~25 mm^2 in area) and incubated overnight in 0.02 % collagenase solution (Life Technologies, Carlsbad, USA) at 37 °C. The whole epithelium was then peeled from the enzyme-treated limbal pieces (corneal epithelium was not included), incubated with 0.05 % trypsin–ethylenediamine-tetraacetic acid (Sigma-Aldrich) for 10 min at 37 °C, and the detached cell sheets dissociated into single cells by agitation through a 22-gauge needle. The resulting suspension of LESC was placed in supplemented media (1:1 Dulbecco's Modified Eagle's Medium: Ham's F12; Life Technologies) including 5 % Fetal Bovine Serum (FBS; Biosera,

Boussens, France), 2 ng.mL^{-1} human epidermal growth factor, 5 mg.mL^{-1} insulin, and 1% penicillin-streptomycin (Life Technologies) before 1×10^{5} cells being seeded onto substrates (collagen gels) of differing stiffness. Collagen gels with predetermined levels of elastic moduli were prepared as previously described (Chen et al., 2012). Briefly, collagen gels were made by neutralizing 4 ml sterile rat-tail type I collagen (2.2 mg.ml^{-1} in 0.6 % acetic acid; First Link Ltd) in 1 ml 10× Modified Eagle's Medium (MEM; Life technologies) and 0.5 ml 1 M sodium hydroxide (Fisher, U.K.) (Jones et al., 2012). The solution was gently mixed and cast into rectangular molds (33 × 22 × 8 mm) prior to gelling at 37 °C, 5 % CO_2 for 30 min. Stiffness of the collagen gels was then increased by plastic compression under load between a layer of nylon mesh (50 μm mesh size) (Brown et al., 2005). Collagen gels compressed under a fixed load of 134 g for 5 min were termed C1 whereas collagen gels compressed under a fixed load of 64 g for 2.5 min were termed C2. Gels without subsequent compression (uncompressed) were termed UC gels. Previously, we have shown these gels to have G' values of approximately 2500, 170, and 3.0 Pa, respectively (Chen et al., 2012; Jones et al., 2012). The collagen gel substrates were then placed into Transwell culture inserts and upper surfaces were coated with laminin (1.5 μg.cm^{-2}; Life Technologies) for 2 h prior to cell seeding. Laminin-coated Transwell culture inserts alone (TW) were also used as a positive control. All cultures were incubated at 37 °C under 5 % CO_2 for 3 weeks, and the cell culture medium was replaced every 2 days.

Following culture of LESCs on substrates of differing compliance, cellular proteins were then separated according to their subcellular localization (cytoplasmic or nuclear) using the subcellular fractionation kit (Calbiochem, UK). Briefly, a step-wise sequence of extraction buffers was used for selective membrane permeabilisation and separation of cytoplasmic and nuclear protein fractions. The recovered fractions were quantified using the BCA protein assay system (Bio-Rad, UK), and analyzed by Western blotting. Briefly, 10 μg protein samples from each fraction were run by SDS-PAGE using a 10% gel, and transferred to polyvinylidine difluoride (PVDF) membranes (Thermo Scientific). Membranes were blocked with 5% non-fat milk dissolved in Tris-Buffered Saline (20 mM Tris-base, 0.14 M NaCl pH 7.6) containing 0.1% Tween-20 (TBS-T) and

then incubated with anti-Yap or anti-GAPDH primary antibody ($1mg.ml^{-1}$) diluted 1:500 in blocking buffer at 4°C overnight. Blots were washed for 45 min in TBS-T before incubation with anti-mouse conjugated secondary antibody (1:4000 dilution) for 2 h at room temperature. Proteins were imaged using the ImageQuant LAS 4000 mini system (GE healthcare, UK) using an enhanced chemiluminescence system (Millipore, Germany). Band intensity was quantified by densitometry using ImageJ gel analysis plugin normalized to GAPDH (biological n=4).

Frozen bovine corneal-limbal tissue was cryo-sectioned (7 µm thick), thaw-mounted onto poly-lysine coated slides (Thermo Scientific, UK), and air-dried for 2-3 h at room temperature. Slides were then fixed in 4 % paraformaldehyde for 15 min at room temperature and subsequently permeabilised in Phosphate-buffered saline (PBS) containing 0.1 % Triton X-100 (Fisher Scientific) for 5 min, then washed briefly in PBS and incubated in blocking solution comprising 2 % BSA, 2 % goat serum, 0.04 % sodium azide, and 0.05 % Triton X-100 in PBS for 1 h prior to overnight labeling at 4 °C with anti-Yap (Santa Cruz Technologies, USA) and anti-p63 (Abcam, UK) antibodies diluted 1:500 in blocking solution. The Alexa 488-conjugated goat anti-mouse IgG in blocking solution (1:1000) was subsequently applied for 1 h at room temperature. Controls (no primary antibodies) were run in parallel. Mounted slides (Vectashield containing DAPI; Vectorlabs, UK) were examined by fluorescence microscopy using an Axio Imager A1 (Zeiss, Germany) and TCS SP2 confocal microscope (Leica, Germany). The mean±S.D. band intensity and percentage of basal cells with nuclear Yap staining in the limbus and central cornea determined from three replicates were determined, and statistical differences evaluated using ANOVA with Bonferroni's *post-hoc* tests.

Force-distance spectroscopy was performed using a Nanosurf Easyscan 2-controlled atomic force microscope (AFM) equipped with commercial soft contact mode cantilevers (qp-SCONT, Nanosensors; nominal spring constant 0.01 N/m). The C1 and C2 samples were held in a plastic petri dish and submerged in PBS whilst 512 force curves were acquired at different positions across each sample at 2 µm/s. SPIP data analysis software (Image Metrology A/S, Denmark) was employed to analyze the force curves using the Sneddon model

(Sneddon, 1965) shown to be applicable to the analysis of force curves acquired on soft biological material (Lin and Horkay, 2008; Domke and Radmacher, 1998). The cantilever spring constant was calculated using Sader's method (Sader et al, 1999) and histogram of the elastic modulus was compiled.

Freshly-isolated bovine LESC, once expanded *ex vivo* upon the surface of collagen gels of increasing stiffness, displayed a measureable difference in the cellular localization of Yap at the protein level. Nuclear expression of Yap was most clearly seen within those cells expanded upon the stiffer C1 collagen gel (Fig 1A) whereas LESC expanded upon the less stiff C2 gel showed areas with a distinct absence of nuclear staining; for these cells, mainly cytoplasmic staining of Yap was observed (Fig 1B). The percentage of Yap nuclear staining was quantified by Western blotting from LESC expanded upon substrates of differing stiffness including uncompressed collagen (UC) gels and polycarbonate culture inserts (TW) as negative and positive controls, respectively (Fig 1C). A significant difference in the amount of nuclear Yap was measured from LESC grown on the stiff C1 collagen gel (~45%) when compared to the more compliant C2 (~15%) and uncompressed UC gels (~5%). LESC expanded upon tissue culture plastic (TW), representing infinite stiffness, gave ~70% of cells with nuclear Yap (Fig 1D). Interestingly, the 4-fold difference in stiffness between C1 and C2 collagen gels, as measured by AFM, (65.3 and 15.3 kPa, respectively; Fig. 1E) was comparable to that determined between the stoma and the Bowman's membrane in the human cornea (Last et al., 2012). This correlation suggested that Yap localization in the cornea might also be affected by the stiffness of underlying corneal tissues. Indeed, *in situ* detection of Yap across the normal bovine epithelial surface by immunohistochemistry showed remarkable differences in its cellular localization, from the conjuntiva, through the limbus and towards the central region of the cornea (Fig 1F). In particular, a prominent change in Yap localization was observed within basal cells at the interface between the limbus and central cornea. Yap was located mainly in the cytoplasm of basal limbal cells, whereas central cornea basal cells showed a predominant nuclear localization of Yap (Fig 1F, insets). Accordingly, the percentage of basal cells showing nuclear Yap staining in the limbus was significantly lower compared to the central cornea (11±4 and 89±6% of total basal cells,

respectively). This difference was further evidenced by the detection of p63, a known marker of corneal epithelial basal cells and presumptive stem cell marker in many normal epithelial tissues (Di Iorio et al., 2005). In basal limbal cells, Yap did not co-localize with p63 staining (purple staining, Fig 1G), further supporting the observation that Yap was mainly localized to the cytoplasm within basal limbal epithelial cells. In contrast, Yap and p63 strongly co-localized within the nucleus of basal epithelial cells from the central cornea (white staining, Fig 1H).

Recently we have shown that by controlling collagen gel hydration via different levels of plastic compression, substrates with defined elastic moduli can be obtained whilst retaining the same topography (Chen et al., 2012). When these collagen gels are subsequently used as a substrate for the expansion of LESCs in culture, those with a low elastic moduli (compliant) preserve LESCs in an undifferentiated state whereas stiffer collagen gels promote LESC differentiation towards a central corneal phenotype (Chen et al., 2012; Jones et al., 2012). From these studies we concluded that LESC differentiation could be modulated by substrate stiffness *in vitro*.

Previous studies have shown that stem cells are generated and renewed at the limbus (Cotsarelis et al., 1989; Schermer et al., 1986) and migrate centripetally under both wounded and normal conditions (Auran et al., 1995; Buck, 1985; Kinoshita et al., 1981; Nagasaki and Zhao, 2003). However, little is understood about the mechanisms that regulate this process. We now suggest that a possible explanation may lie with regional mechanical stimuli. Recently published work has provided evidence to show that Yap is a key regulator in cellular mechanotransduction in response to the mechanical signals exerted by substrate rigidity (Dupont et al., 2011). Accordingly, by assessing localization of Yap we can infer that *in situ* basal epithelial cells from the limbus are sensing a different mechanical environment to those basal cells outside of the limbus (i.e., from central cornea). Our results also suggest that the extracellular matrix beneath the basal limbal epithelial cells is more complaint than the matrix beneath basal central corneal epithelial cells. Centripetal differences in stiffness across the cornea from limbus to central cornea has been previously reported (Hjortdal, 1996). We speculate that this difference may be due to the underlying Bowman's membrane, which is absent at the limbus (Wiley et al., 1991) and has

been shown to be significantly stiffer than extracellular matrix beyond it (Last et al., 2012). Furthermore, x-ray diffraction studies have shown discreet changes in the ultra-structural arrangement of collagen fibres between the limbus and central cornea. The collagen fibres underlying the limbal epithelial cells are proposed to run in an aligned annulus, whereas the extracellular matrix of the central cornea is comprised of orthogonally-orientated collagen fibres running from limbus to limbus (Aghamohammadzadeh et al., 2004). Together with the different localization of Yap shown in the present study (predominately nuclear in the central cornea and cytoplasmic in the limbus), these mechanical and structural differences lend strong support to our hypothesis that the mechanical properties of the limbus and central cornea are key contributing factors to the differentiation status of cells located therein. In addition, these same mechanical properties could potentially stimulate cell migration via durotaxis. A schematic describing how this process might work along side the established *X, Y, Z* hypothesis of Thoft and Friend is shown in Figure 2.

In conclusion, we have demonstrated a centripetal ascent in the nuclear localization of Yap across the bovine cornea and shown that increasing substrate stiffness *ex vivo* (using physiologically relevant values) can drive such localization. We therefore hypothesise that known differences in the regional elasticity of the cornea contribute to the maintenance and regulation of cell phenotype across its surface. Due to the relative isolation of the cornea, and a constantly renewing tear film, centripetal chemical gradients would be difficult to maintain. It is therefore tempting to suggest that basement membrane compliance regulating Yap localisation is a robust regulator of epithelial homeostasis. With further studies these initial observations may form a significant step forward in our understanding of ocular surface homeostasis, with considerable implications for current and future corneal treatments.

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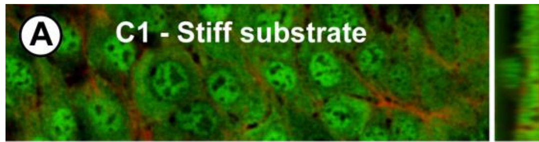
A
C1 - Stiff substrate

B
C2 - Compliant substrate

Figure 1. Localization of Yap expression in basal bovine corneal epithelial cells. A) Immunocytochemistry shows predominant localization of Yap (green) in the nuclei of limbal epithelial cells expanded (as a monolayer) upon the more stiff C1 collagen gels, whereas **B)** displays discreet areas of cytoplasmic Yap within limbal epithelia cells expanded upon the more compliant C2 compressed collagen gels (scale bar: 50 μm, depth 20 μm). **C)** The relative expression of Yap between nuclei (n) and cytoplasm (c) across a range of substrate stiffness by immunoblotting shows greater amount of Yap localized to the nuclei in all but the least stiff substrate (UC). **D)** The percentage of Yap localized to the nucleus in the stiffer collagen substrate C1 was significantly greater than either of the more compliant collagen gels C2 and UC. Substrates included compressed stiff (C1) and compliant (C2) collagen gels, least stiff uncompressed collagen gels (UC) and infinitely stiff tissue culture plastic (TW). **E)** Force-distance spectroscopy on the compressed collagen gels gave Elastic moduli (E) measurements of 65.3 (C1) and 15.3 kPa (C2). **F)** Yap expression across the intact bovine cornea by immunohistochemistry shows nuclear expression of Yap (green) is absent from basal limbal epithelia (left inset) but present in basal central epithelia (right inset). Image represents a 4.5 mm transversal view of the bovine cornea, from the limbus (left) towards the center (right). Immunohistochemistry of the bovine limbus shows that co-localization between p63 (red) and Yap (green) was not detected in the nucleus (blue) of limbal basal cells **(G)**, but strongly present within basal epithelial cells from the central cornea **(H)**. Nuclei were detected using DAPI. Scale bars: F), 300 μm (insets, 30 μm); G) and H), 30 μm.

Figure 2. Schematic showing how centripetal stiffness potentially underpins the *X, Y, Z* model of corneal homeostasis. A) Based upon our previous findings that increased stiffness increases limbal stem cell differentiation and that we now believe that limbal basal cells sense a more compliant substrate in nature we propose a centripetal stiffness gradient across the healthy cornea (blue compliant; red stiff). This has potential implications in the treatment of ocular surface disease. **B)** Basal limbal cells located on the more compliant substrate, i.e., the limbus (blue), express Yap (green) in the cytoplasm and remain undifferentiated. Attracted to the stiffer substrate (red) via durotaxis, the basal cells migrate towards the central cornea (y). Concomitantly, the stiffer substrate induces Yap translocation to the nucleus, hence modulating cell differentiation (x) via mechanotransduction. Finally, cells leave the ocular surface (z) via nonpathologic desquamation.