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Localization of the low-affinity nerve growth factor receptor p75 in human limbal epithelial cells

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Abstract

Biological effects of nerve growth factor (NGF) are mediated through receptors known as nerve growth factor receptors (NGFR), which include p75 and TrkA. This study was initiated after identifying NGFR as an up-regulated gene in the limbus by cDNA microarray analysis and we postulate that its expression may be indicative of a stem/progenitor cell phenotype. Immunohistochemistry was performed on normal human adult ($n = 5$) and foetal ($n = 3$) corneal tissue using antibodies directed against p75, TrkA, NGF, p63, ABCG2 and CK3/12. Limbal, conjunctival and pterygium tissue was obtained from patients ($n = 10$) undergoing pterygium resection and used for immunohistochemical assessment. Paraffin-embedded archival human skin specimens ($n = 4$) were also evaluated. *In vitro* expression of NGFR was determined in limbal, conjunctival and pterygium-derived epithelial cells. p75 was selectively expressed by basal epithelial cells in pterygia, conjunctiva and limbus, but was absent in the central cornea. These results were confirmed with two additional p75 specific antibodies. In contrast, TrkA was found in full-thickness pterygium, conjunctival, limbal and corneal epithelium in both adult and foetal eyes. p75 expression was identified in a small percentage, while TrkA was found on the entire population of cultured conjunctival, limbal and pterygium-derived epithelial cells. This receptor was also observed in selective regions of the human epidermis and hair follicle bulge. Our results illustrate the selective expression of p75 in basal pterygium, conjunctival and limbal epithelium, while staining was absent in adult and foetal central cornea. p75 may represent an additional ocular surface epithelial stem/progenitor cell signature gene.

Keywords: cornea • limbus • epidermis • pterygium • hair follicle

Introduction

It is widely accepted that a small percentage of basal limbal epithelial cells comprise the stem cells (LESC) that are responsible for maintaining corneal epithelial integrity [1, 2]. Causes of limbal stem cells deficiency (LSCD) include multiple surgeries, chemotherapy, chemical burns and contact lens wear. LSCD results in persistent epithelial defects including conjunctivalization, corneal vascularization and scarring [3, 4]. Kenyon and Tseng (1989) were first to show that autologous limbal grafts from a healthy eye could regenerate corneal epithelium in the contralateral diseased eye [5]. Similarly, limbal allografts have also been successfully used to restore the corneal epithelium [6]. Although these techniques introduced the concept of LESC transplantation, they are limited by a shortage of donor corneas, rejection in the case of allografts and by risk to the healthy eye in autografts. *Ex vivo* expansion of limbal epithelial cells overcomes these problems and successful transplantation of cultured limbal epithelium has been reported [7–9]. Identification of phenotypic markers for LESC may improve selection and expansion of epithelial cells and ultimately the effectiveness of these cells for transplantation. Numerous molecules have been studied as potential LESC markers [10–13]. Of these markers, cell surface molecules are of particular interest as they facilitate the selection, sorting and expansion of viable SC [12, 13].

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Nerve growth factor receptors (NGFRs) p75 and tyrosine kinase receptor A (TrkA) have emerged as important molecules in corneal epithelial physiology and pathology [14–17], functioning to modulate processes including cell survival, proliferation, differentiation and apoptosis [18, 19] after their neurotrophin ligands (NGF, BDNF, NT-3, NT-4) have bound in neuronal and non-neuronal cells. Interestingly, p75 has been identified as a SC marker in human oesophageal [20], oral [21], epidermis and hair follicle [22–24], while a small percentage of $p75^+$ cells with high proliferative potential have been identified among tumour cells from human oesophageal squamous cell carcinoma [25] and p75 expression was associated with thymus epithelial tumour proliferation [26] indicating the importance of this receptor in physiological and pathological processes.

In this study, we examined the distribution of NGF and its receptors p75 and TrkA in diseased and normal human ocular surface epithelium since in a previous investigation we identified NGFR as one of several potential LESC markers by cDNA microarray analysis [27]. We demonstrate the presence of p75 in the basal limbal epithelium as well as in a small subset of cultured ocular surface epithelial cells and show that the expression of this receptor diminishes concurrently with credible LESC markers including p63 and ABCG2 in serial generations. We therefore propose that p75 could be an additional limbal epithelial stem/progenitor cell marker.

Materials and methods

Human tissue specimens

Normal human adult whole eyes ($n = 5$) were obtained from the Lions Eye Bank (Sydney, Australia). Pre-mortem informed consent for tissue donation for research purposes was obtained. Post-mortem foetal whole eyes ($n = 3$; 18 weeks gestation) were acquired from the Diabetes Transplant Unit, Prince of Wales Hospital (Sydney, Australia). Corneas from adult and foetal eyes were fixed in 10% formalin, bisected to access the limbus and central cornea, embedded on the cut surface and sectioned. Normal human skin biopsies ($n = 4$) were obtained from the Skin and Cancer Foundation Australia.

Thirteen pterygium specimens were used in this study, 10 derived from surgical resection of either primary ($n = 8$) or recurrent ($n = 2$) lesions while three additional specimens were post-mortem. Surgical resection was performed by the same ophthalmic surgeon (Minas T Coroneo) [28]. Specimens were collected from July 2004 to August 2006 and randomly selected based on available autologous normal remnant graft tissue (*i.e.* separate conjunctiva [cj] and limbus). Patients included six males and four females (age range of 21–79 years [mean 48.9 years]) with left and right eyes equally represented (5-right and 5-left). All research protocols were approved by the UNSW Human Research Ethics Committee (HREC 04088) and carried out in accordance with the tenets of the World Medical Associations Declaration of Helsinki.

Human ocular surface epithelial cell culture

Ocular surface epithelial cells were grown from fresh cadaveric corneal rims (<12 hrs post-mortem delay) or tissue obtained from pterygium surgery [29–32]. In brief, explants derived from resected pterygium specimen or from the remnant graft tissue composed of either separate limbus or cj were placed in 6-well culture plates (Nunc, Roskilde, Denmark) until sufficient epithelial growth was noted (usually no longer than 10 days), at which time the explants were removed, adherent cells were enzymatically dispersed and subcultured in serum containing media (Eagles minimum essential medium). Epithelial cell purity was estimated with cytokeratin-specific antibodies [29–31]. Early generation cells (passage 2–5) were pelleted, formalin-fixed, paraffin-embedded, sectioned and stained for NGFRs as outlined below. Cadaveric corneal-scleral rims were cut into small segments, nurtured in CnT-20 or CnT-50 (Millipore, Billerica, MA, USA), media formulated to preserve corneal progenitors then subjected to flow cytometry as described below.

Immunohistochemical analysis

Immunohistochemistry was performed on formalin-fixed paraffin-embedded ocular and skin tissue sections. Positive control tissue included normal human small intestine, basal forebrain and pancreatic carcinoma. Antigen retrieval for both mono and polyclonal NGFR p75 was performed with a pressure cooker with Epitope Retrieval Solution™ (Novacastra, Newcastle upon Tyne, UK) for 1 min., while for TrkA, NGF, p63 and ABCG2 antigens were retrieved by microwaving for 10 min. in Epitope Retrieval Solution™ (Novacastra). Sections were blocked with 2% skim milk powder in Tris-buffered saline (TBS) then incubated for 1 hr with primary antibody (Table 1). Immunostaining was performed on a Bond-X™ automated immunostainer (Vision BioSystems) with the Bond Polymer Refine Detection System (Vision BioSystems, Mount Waverley, VIC, Australia) consisting of polymer conjugated antimouse/rabbit secondary antibody and diaminobenzidine (DAB, brown) as the chromogen. Some sections were stained manually and reactivity visualized by adding 3-amino-9-ethylcarbazole (AEC, red). For immunofluorescence, paraffin sections were treated as above and incubated with an appropriate p75 antibody (Table 1). Secondary antibodies included either biotinylated goat antimouse or goat anti-rabbit (DakoCytomation, Carpinteria, CA, USA; 1:200 dilution). Sections were subsequently incubated with Steptavidin-FITC (DakoCytomation).

Flow cytometric analysis of putative SC markers in corneal epithelial cultures

Primary cultures from adult cadaveric corneal rims were characterized by flow cytometry using antibodies targeting p75, p63 and ABCG2 (Table 1). Adherent cells were detached from culture flasks with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution and allowed to recover in complete medium at 37°C for 30 min., followed by fixation in 2% paraformaldehyde. For p63 and ABCG2 staining, the cells were permeabilized in cold 100% methanol (10 min.) and 0.1% Triton X-100/PBS (5 min.). After fixation, cells were incubated with the appropriate primary or isotype control antibody (Table 1) for 30 min. on ice, washed thrice in 2% bovine serum albumin (BSA)/phosphate-buffered saline (PBS), blocked in 20% human serum for 30 min., and then incubated with an fluorescein isothiocyanate (FITC)-conjugated goat antimouse $F(ab')_2$ fragment for 20 min. Cells were re-suspended in 1% paraformaldehyde and analysed with a FACScan flow cytometer and CellQuest Pro software (Becton Dickinson, San Jose, CA, USA).

Table 1 Primary and secondary antibodies used for immunohistochemistry and flow cytometry

Antibody/Application/ Specificity	Host	Clone	Concentration/ Dilution	Source	Catalogue No.
Anti-p75/ IHC/ (extracellular domain)	Mouse	7F10	1:100	Novacastra	NCL-NGFR
Anti-p75/ IHC/ (cytoplasmic domain)	Rabbit	9992	2 μg/ml	Promega	G323A
Anti-p75/ IHC & FC/ (extracellular domain)	Mouse	NGFR5[1]	10 μg/ml	Dako	M3507
Anti-NGF/ IHC/ (native NGF)	Rabbit	-	10 μg/ml	Chemicon	AB1526P
Anti-TrkA/ IHC/ (C-terminus)	Mouse	763	5 μg/ml	Santa Cruz	Sc-118
Anti-p63/ IHC & FC/ (all isoforms)	Mouse	4A4	40 μg/ml	Dako	M7247
Anti-ABCG2/ IHC & FC/ (internal epitope BCRP)	Mouse	BXP-21	20 μg/ml	Millipore	MAB4146
Anti-CK3/12/ IHC/ (corneal cytokeratins)	Mouse	2Q1040	10 μg/ml	USB	C9097-34M
Anti-IgG_1/ IHC & FC	Mouse	-	20 μg/ml	Dako	X0931
Anti-IgG_{2a}/ IHC & FC	Mouse	-	40 μg/ml	Dako	X0943
Antimouse IgG $F(ab')_2$ FITC-conjugated/ FC	Goat	-	40 μg/ml	Jackson	115-096-072

Antibody dilutions were made from stock solutions provided by the manufacturer. Manufacturer recommended positive control tissue and antigen retrieval was performed as required for immunohistochemistry. Some antibodies were used for both immunohistochemistry (IHC) and flow cytometry (FC). While most antibodies were supplied as affinity purified immunoglobulins, p75 (clone 7F10) was supplied as culture supernatant.
Supplier details: Novacastra, Newcastle upon Tyne, UK; Promega Corporation, Madison, WI; DakoCytomation, Glostrup, Denmark; Chemicon International, Temecula, CA; Santa Cruz Biotechnology, Santa Cruz, CA; Jackson ImmunoResearch Laboratories, West Grove, PA; Millipore Corporation, Billerica, MA; USB, United States Biological, Swampscott, MA.

Results

Localization of NGFR and NGF in ocular surface tissue

In our previous investigation, we used a cDNA microarray hybridization approach to identify NGFR as a candidate LESC marker in adult human primary cultures of limbal-derived epithelial cells [27]. The present study was initiated to corroborate those observations in human tissue specimens and to determine the expression profile of the alternative NGFR (p75). Resected pterygia, with a separate segment of normal autologous bulbar cj and limbus was selected from 10 different patients. In each of the specimens analysed, intense membrane associated antigenicity for p75 was identified on basal pterygium epithelium (Fig. 1A), while progressive loss of reactivity was noted on suprabasal cells (Fig. 1A, arrows) suggesting that expression of this receptor decreased with differentiation. A similar staining profile was observed in basal epithelial cells from normal cj (Fig. 1D) and limbus (Fig. 1G) derived from the same donor. In this set of experiments, fresh normal cornea was not included as this tissue is not routinely harvested from patients undergoing surgery for pterygium extraction. Positive control sections from normal human small intestine displayed intense p75 reactivity in nerve bundles (Fig. 1J, arrowheads) and nerve fibres (Fig. 1J,

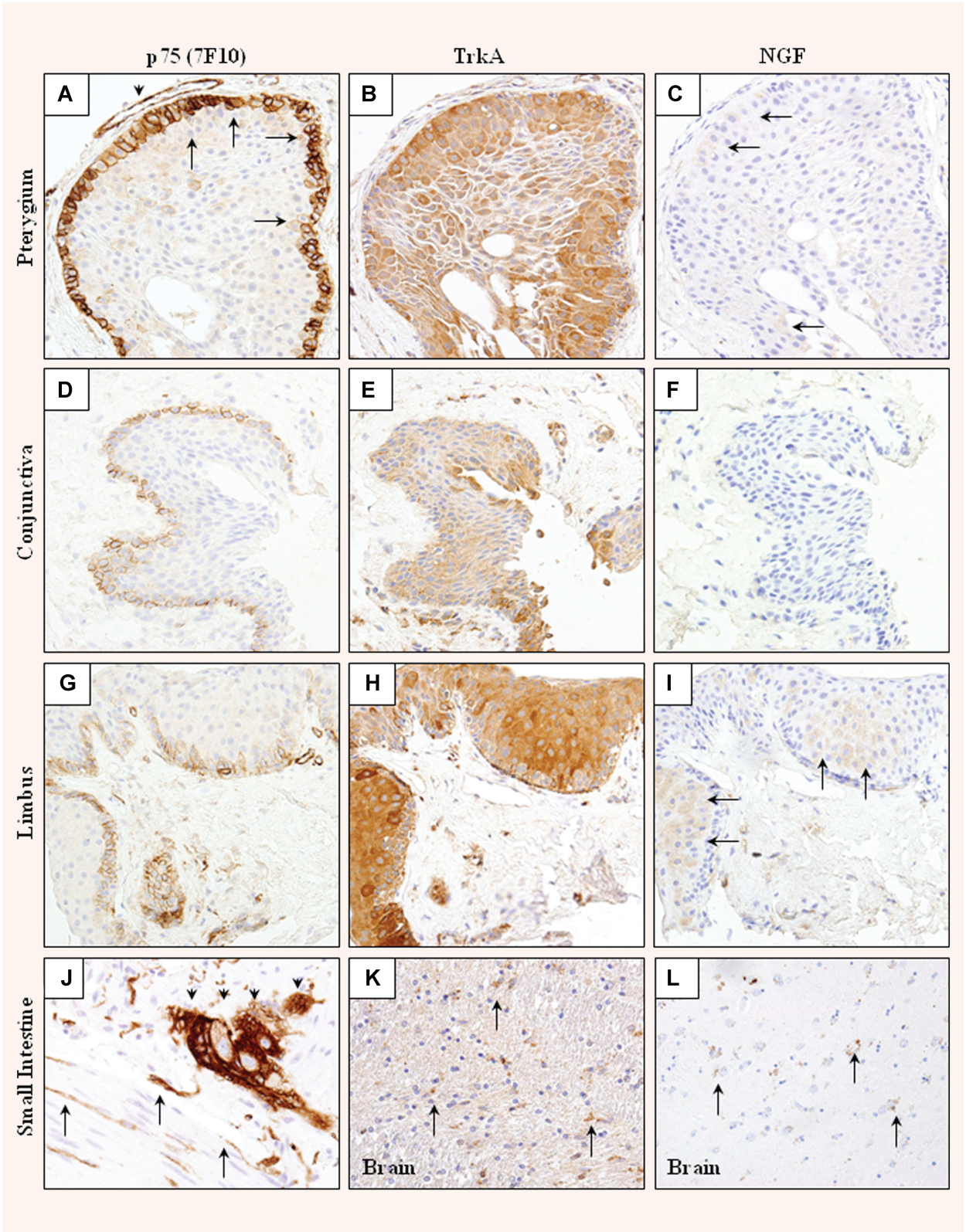

Fig. 1 Distribution of nerve growth factor receptors (NGFRs) in diseased and normal ocular surface tissue. Pterygium (**A**–**C**) and autologous normal bulbar conjunctival (**D**–**F**) and limbal (**G**–**I**) tissue was serially sectioned and immunohistochemically assessed with an anti-human p75 (clone 7F10; **A**, **D**, **G** and **J**), an anti-human TrkA (**B**, **E**, **H** and **K**), or an anti-human NGF (**C**, **F**, **I** and **L**) antibody. Sections from normal human small intestine (**J**) and forebrain (**K** and **L**) were included as positive controls. Each section was counterstained with haematoxylin to identify cell nuclei and immunoreactivity was denoted by brown cell-associated reactivity. Sections in (**A**–**I**) were derived from one patient, with similar results observed in the 10 patients tested. The arrowhead in (**A**) points to a p75 positive blood vessel and arrows identify suprabasal pterygium epithelial cells with diminished p75 expression. Arrows in (**C**) and (**I**) indicate mild NGF reactivity in basal and suprabasal pterygium and limbal epithelium, respectively. Arrowheads in (**J**) identify a large nerve bundle while arrows signify p75 positive nerve fibres. Arrows in (**K**) and (**L**) point to immunoreactive neurons in human forebrain. Original magnification ×400.

Table 2 Summary of NGFR and NGF immunohistochemical staining in ocular surface epithelium

Tissue type	Tissue/cell source	p75 (clone 7F10)	TrkA (clone 763)	NGF (clone not specified)
Pterygium	Adult	++ basal epithelium	++ full-thickness epithelium	+ basal epithelium
	Cells	++ < 1% of cells	++ entire culture	SNP
Conjunctiva	Adult	++ basal epithelium	++ full-thickness epithelium	− staining
	Foetal	− staining	++ full-thickness epithelium	− staining
	Cells	++ < 1% of cells	++ entire culture	SNP
Limbus	Adult	++ basal epithelium	++ full-thickness epithelium	+ basal epithelium
	Foetal	− staining	++ full-thickness epithelium	− staining
	Cells	++ < 1% of cells	++ entire culture	SNP
Cornea	Adult	− staining	++ full-thickness epithelium	− staining
	Foetal	− staining	++ full-thickness epithelium	− staining

Diseased and normal adult and foetal ocular surface tissue and cells (passage 5) cultured from pterygium, conjunctiva and limbus were stained with antibodies directed against human p75, TrkA and NGF and the level of immunoreactivity scored as (++) intense, (+) mild and (−) no staining; (SNP) no staining performed.

arrows). In contrast, TrkA stained full thickness pterygium (Fig. 1B), conjunctival (Fig. 1E) and limbal (Fig. 1H) epithelium. Positive control sections from human basal forebrain demonstrated immunoreactivity for TrkA in neurons (Fig. 1K, arrows). NGF was mildly expressed in pterygium (Fig. 1C, arrows) and limbus (Fig. 1I, arrows), rarely present in cj (Fig. 1F) but found in human forebrain neurons (Fig. 1L, arrows). Table 2 summarizes these immunostaining results.

NGFR p75 expression in the human limbus

Several reports [16, 17, 33, 34] are in conflict with our p75 and TrkA immunolocalization data in the human cornea. In an attempt to clarify the expression pattern, post-mortem whole eyes were bisected to incorporate an intact cornea with limbus and bulbar cj (Fig. 2A). Intense and specific p75 antigenicity was observed in the basal limbal (Fig. 2A, a^1) and conjunctival (Fig. 2A, a^2) epithelium, mild-to-moderate staining was displayed by peripheral corneal epithelium (Fig. 2A, a^3), while the central cornea was void of p75 reactive cells (Fig. 2A, a^4). Limbal vascular endothelial cells displayed p75 reactivity (Fig. 2A, arrows). Higher magnification of the limbal region identified suprabasal epithelial cells with less intense membranous p75 (clone 7F10) reactivity (Fig. 2A, a^1; arrowheads). An identical staining pattern was observed with the p75 polyclonal Ab (clone 9992), however the staining intensity was significantly reduced (compare Fig. 2A with Fig. 2B). A third p75 Ab (clone

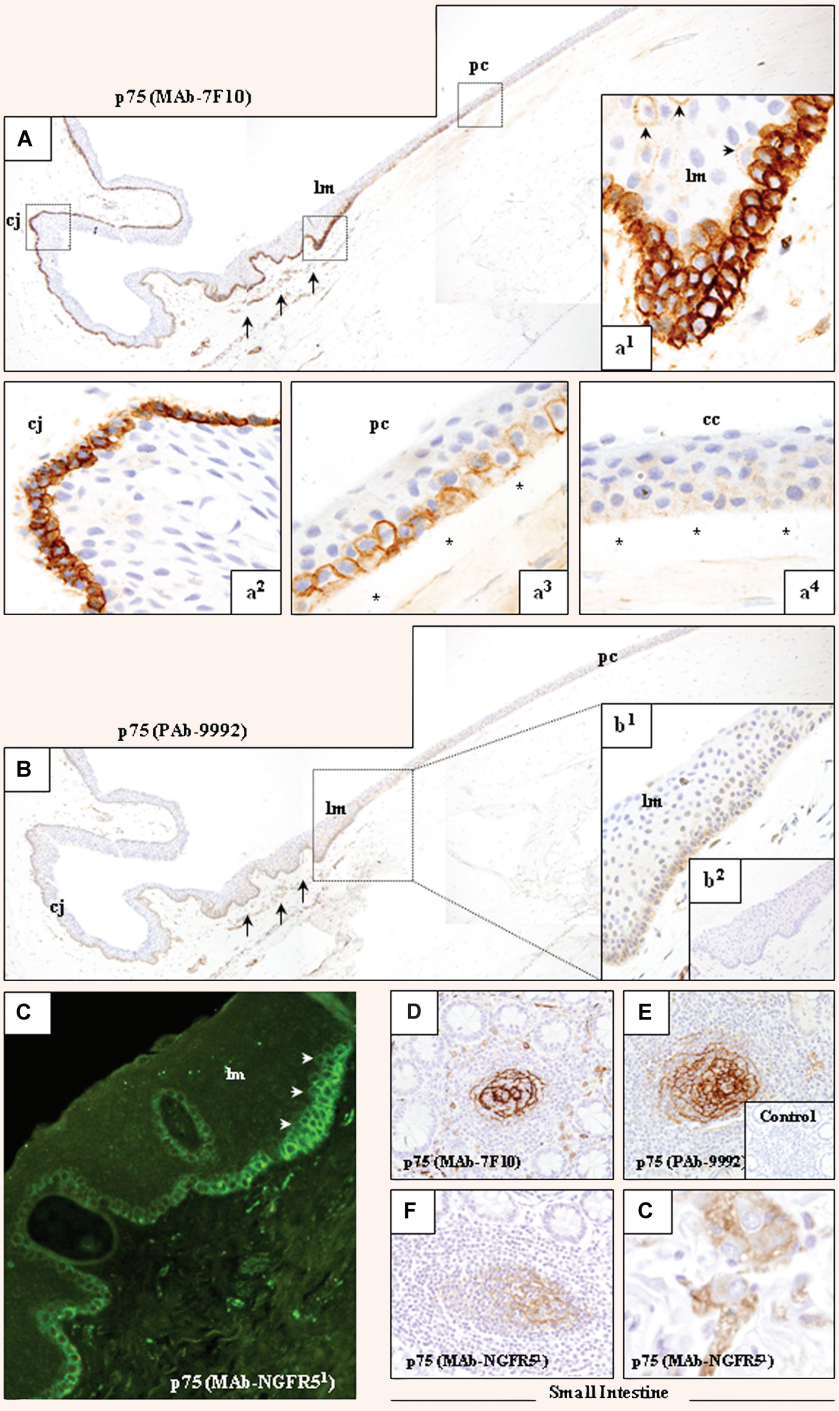

Fig. 2 p75 Expression in the Limbus. p75 expression was determined in whole adult corneal specimens (**A**–**C**) using three different antibody clones (**A**, clone 7F10; **B**, clone 9992 and **C**, clone NGFR5[1]). A similar epithelial cell staining pattern was observed in the conjunctiva (cj), limbus (lm) and peripheral (pc) and central cornea (cc) with each antibody tested. Sections (**A**, **B** and **D**–**G**) were counterstained with haematoxylin to identify cell nuclei and immunoreactivity was denoted by brown cell-associated staining. Tissue in part (**C**) was not counterstained and viewed under fluorescence microscopy. Tissue in **A**–**C** was serial sectioned and derived from one representative specimen. Two additional whole eyes were examined and similar results obtained (micrographs not shown). The area encompassed by the squares in **A** and **B**; ×40 is magnified (insets a^1–a^4 ×1000 and b^1; ×400). Original magnification of **C**–**G** is ×400. Sections incubated without a primary antibody (inset, b^2) or with an isotype control antibody (**E**; inset) displayed no immunoreactivity. Sections in **D**–**G** were derived from normal human small intestine and were incubated with an appropriate p75 antibody (see panel labels). **A** and **B** are composed of 3 contiguous micrographs. The arrows in **A** and **B** identify limbal vasculature; the arrowheads in (a^1 and **C**) point to diminished p75 expression in suprabasal cells; the asterisks in (a^3 and a^4) depict Bowman's Layer. The central cornea (a^4) was not captured in the low powered micrograph (**A** and **B**).

NGF5[1]) intensely stained basal limbal epithelium with diminishing fluorescence in suprabasal cells (Fig. 2C, arrowheads). p75 reactivity was prominent in germinal centres of lymphoid aggregates (Fig. 2D–F) and in large nerve bundles that were present in the human small intestine (Fig. 2G). Reactivity was absent in sections incubated without a primary antibody (Fig. 2B; inset b^1). Table 2 summarizes these immunostaining results.

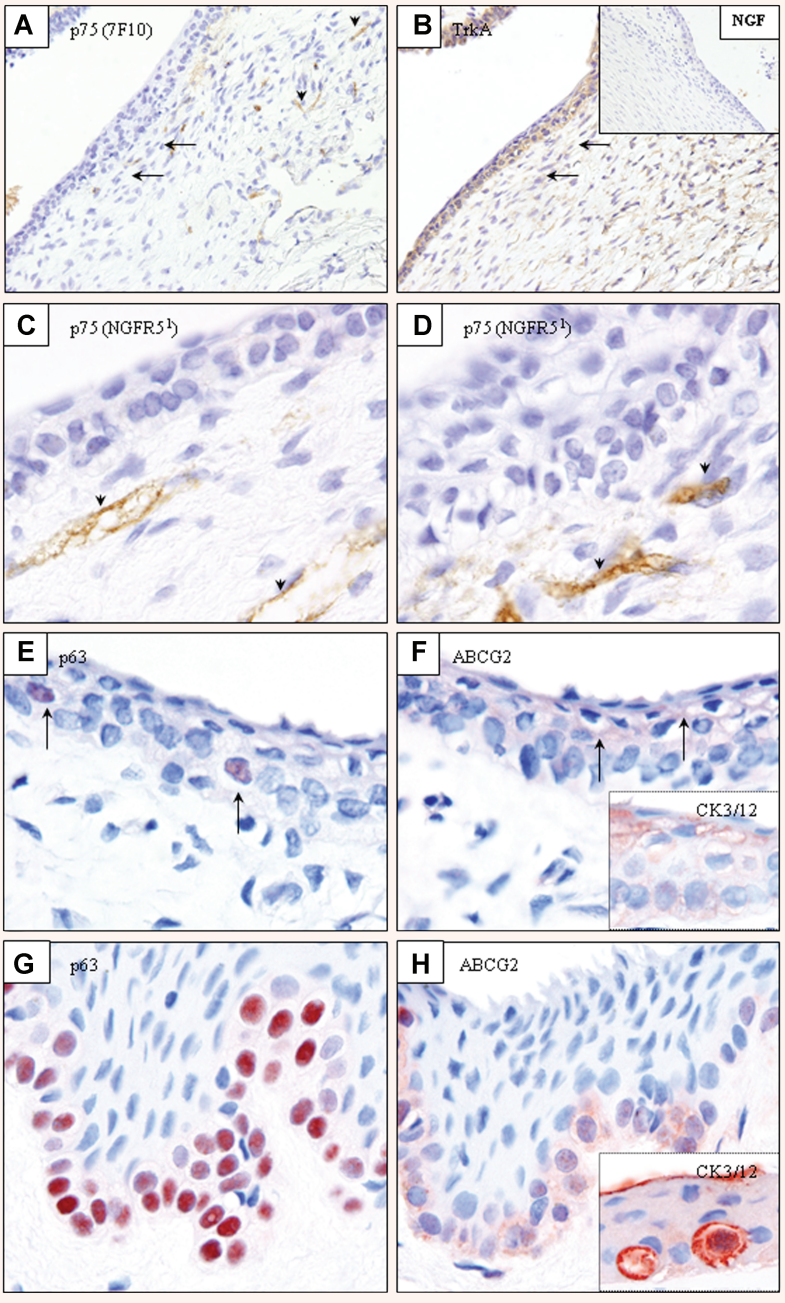

Fig. 3 Localization of NGFRs in the human foetal cornea. Human foetal (**A**–**F**) and adult (**G** and **H**) corneas were serially sectioned, incubated with anti-p75 (**A**, clone 7F10; **C** and **D**, clone NGFR5[1]), anti-TrkA (**B**), anti-NGF (**B**, inset), anti-p63 (**E** and **G**), anti-ABCG2 (**F** and **H**) or anti-CK3/12 (**F** and **H**, insets) antibodies and processes for immunochistochemistry. Arrows in (**A** and **B**) identify morphologically distinct tissue architecture corresponding to the foetal limbus. Arrowheads in (**C**) point to immunoreactive limbal blood vessels, while those in (**D**) identify nerve fibres. Arrows in (**E** and **F**) point to occasional p63 reactive and faint ABCG2 stained foetal limbal cells. All tissue was counterstained identically, but immunoreactivity developed with diverse chromogens (**A**–**D**, DAB [brown]; **E**–**H**, AEC [red]). Results are representative of all adult and foetal specimens assessed. Original magnification ×400 (**A** and **B**), and ×1000 (**C**–**H**).

NGFR and LESC marker expression in foetal corneas

If p75 is a marker of primitive, undifferentiated LESC, then one would anticipate reactivity in foetal basal limbal epithelial cells. Foetal corneas ($n = 3$) consisted of 2–3 layers of epithelial cells and the limbal region was identified by subtle changes in tissue architecture consisting of loose connective tissue (Fig. 3A and B, arrows) and the presence of a capillary network. To our surprise, p75 was absent in the foetal limbo-corneal epithelium, but was expressed by the limbal vasculature (Fig. 3C, arrowheads) and nerve fibres (Fig. 3D; arrowheads). In contrast, TrkA stained the entire foetal epithelium (Fig. 3B), while NGF was not detected in the same specimens (Fig. 3B, inset). A summary of these immunostaining results is presented in Table 2. The absence of any significant p75 staining in foetal corneas

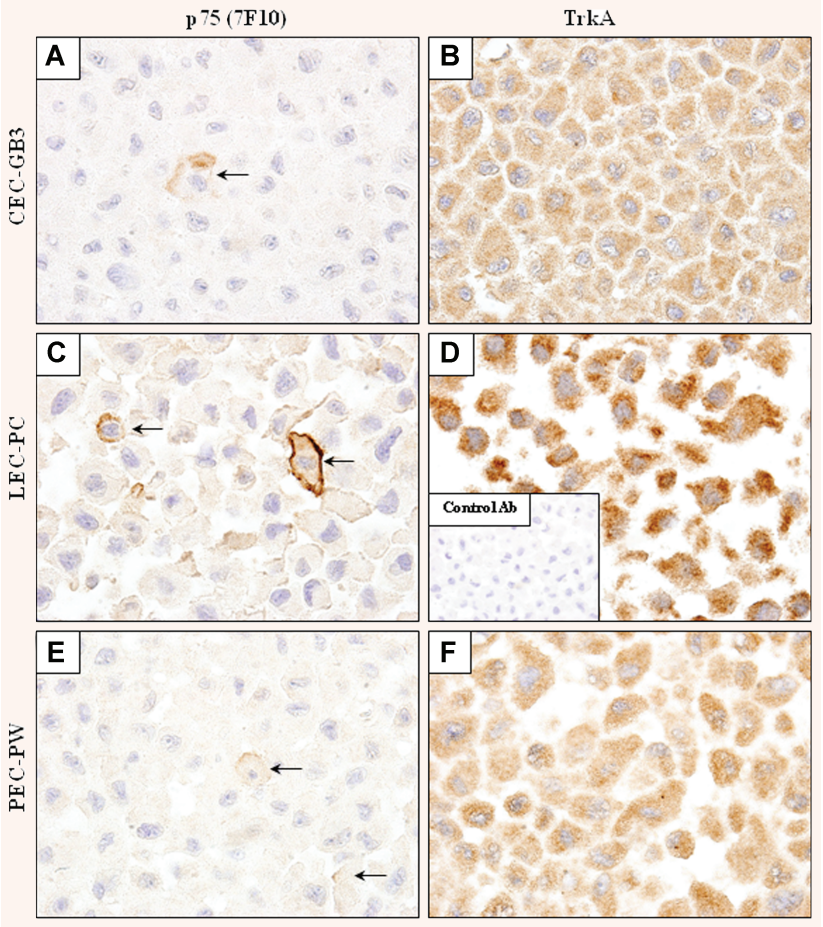

Fig. 4 Localization of NGFRs in cultured ocular surface epithelial cells. Human conjunctival (CEC-GB3, **A** and **B**), limbal (LEC-PC, **C** and **D**) and pterygium (PEC-PW, **E** and **F**) epithelial cells were subcultured, each collected at passage 5, fixed and processed in paraffin blocks. Sections from each block were incubated with anti-p75 (**A**, **C** and **E**, clone 7F10), anti-TrkA (**B**, **D** and **F**) or an isotype control antibody (**D**, inset). Immunoreactivity and counterstaining was identical to sections displayed in Figs. 1–3. Arrows (**A**, **C**, **E**) indicate intense p75 membrane-associated staining on a small percentage of cells. Original magnification ×1000.

prompted us to investigate whether the ATP binding-cassette transporter protein ABCG2 or the transcription factor p63, both credible LESC markers [35, 36], could be identified. Unlike the selective p63 (Fig. 3G) and ABCG2 (Fig. 3H) staining in discrete clusters confined to adult basal limbal epithelium, expression of both LESC markers was less pronounced in foetal corneas (Fig. 3E and 3F respectively). Foetal and adult corneas displayed abundant CK3/12 reactivity (insets Fig. 3F and H, respectively).

NGFR expression in cultured ocular surface epithelial cells

NGFR expression was investigated in three ocular-surface epithelial cell types. Intense antigenicity for p75 (clone 7F10) was found in <1% of cultured conjunctival (Fig. 4A, arrow), limbal (Fig. 4C, arrows) and pterygium (Fig. 4E, arrows) epithelium. In contrast, TrkA protein was identified on most conjunctival, limbal and pterygium cells (Fig. 4B, D and F, respectively). No reactivity developed when an isotype control antibody was used (Fig. 4D, inset). A summary of these immunostaining results is presented in Table 2.

Loss of LESC marker expression in serial generation

Serially passaged human cadaveric limbal epithelial cells were nurtured and the proportion of stem/progenitor cells estimated between passage 3 and 4 cultures by flow cytometry. Progressive loss of SC marker expression was observed in serial cultures as denoted by a left-shift in the histograms (Fig. 5, red graphs). The percentage of p75, p63 and ABCG2 positive cells at passage 3 represented 81.7%, 69.4% and 55.2% respectively, while a significant loss of expression was noted in limbal progenitors at passage 4 (15.9, 46.4 and 50.0% respectively).

Localization of p75 in human skin

The eyes and skin are the organs most likely to encounter environmental, physical and chemical assaults. Both organs must therefore undergo continuous self-renewal to replace damaged or dying surface epithelium. To substantiate our hypothesis that p75 may identify stem/progenitor cells in the ocular surface,

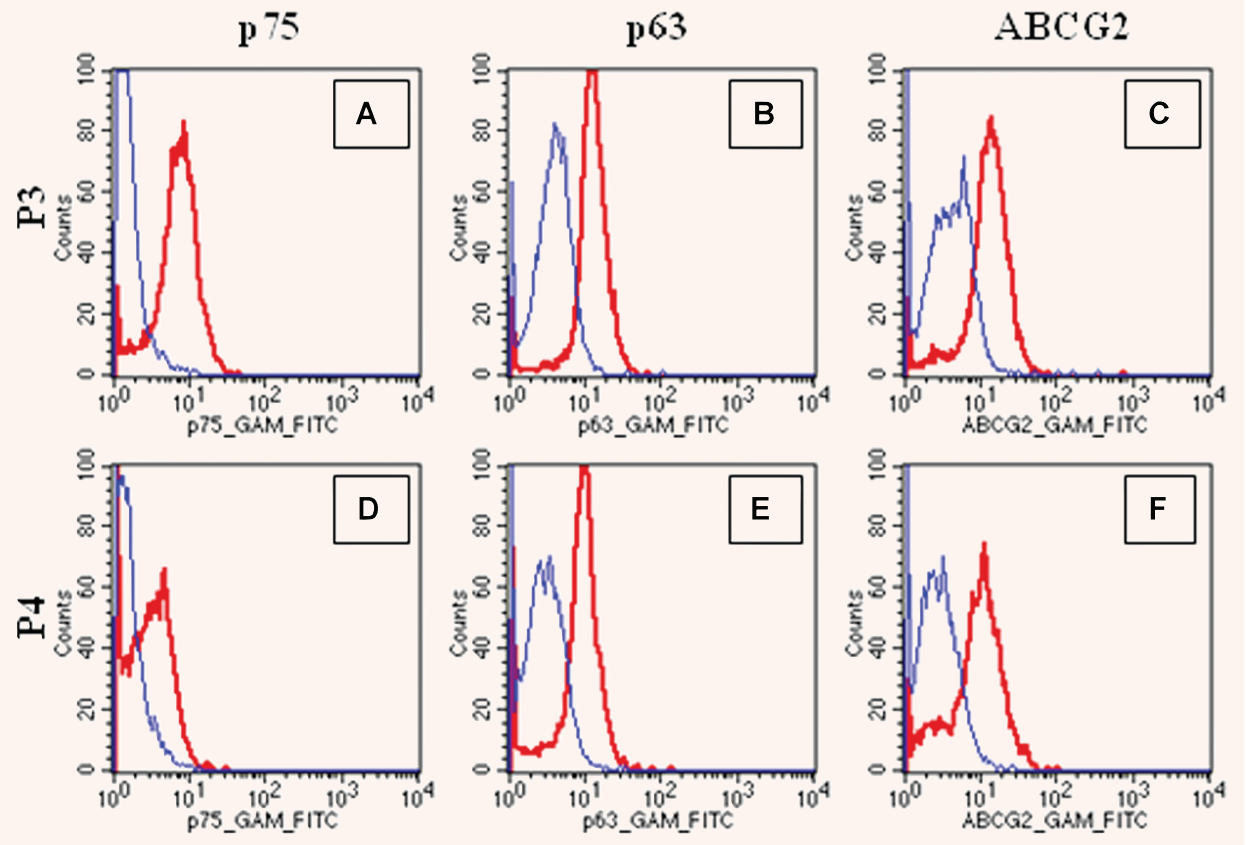

Fig. 5 Flow cytometric analysis of SC markers and p75 in cultured limbal epithelial cells. Histogram plots were generated from at least 100,000 enzymatically dissociated human corneal-limbal cells from passage 3 (P3) and passage 4 (P4) that were labelled with mouse monoclonal (p75 [**A** and **D**], p63 [**B** and **E**], ABCG2 [**C** and **F**], red histograms) or an appropriate isotype control antibody (**A**–**F**, blue histograms).

sections of normal human skin were probed for this receptor. Indeed, p75 expression intensely stained a selective cluster (10–15) of basal epithelial cells located at the tips of the dermal papillae, where the basal layer of the epidermis is closest to the skin surface (Fig. 6A–C, arrowheads). Immunoreactivity progressively diminished deeper along the rete ridge (Fig. 6B and C, red arrows). Prominent staining for p75 was also observed in subcutaneous nerve fibres (Fig. 6C, black arrows). The other known epidermal SC niche is the hair follicle. Here, BrdU-label-retaining cells (indicative of slow cycling SC) have been detected in the outer-most layer of the outer root sheath [37, 38]. Vertical sections through hair follicles disclosed p75 reactivity in precisely this layer of cells, where immunoreactivity extended continuously from the hair bulge to the dermal papillae (Fig. 6D, *asterisks and dp respectively). Transverse sections clearly defined p75 reactivity to the outermost cells of the root sheath, with progressive loss of receptor expression towards the hair shaft orifice (Fig. 6E, and inset e).

Discussion

Our results demonstrate for the first time that p75 expression is more pronounced in cells of the basal limbal epithelium where limbal epithelial stem/progenitor cells reside, while epithelial cells in the suprabasal epithelium and within the adjacent peripheral cornea showed mild-to-moderate p75 reactivity with no staining in central cornea (Fig. 2). This weaker and sparser staining in the peripheral cornea is consistent with observations that activated LESC undergo a phenotypic switch as they migrate, differentiate and are shed. Moreover, we corroborated these results *in vitro*, where $<1\%$ of cultured ocular surface epithelium demonstrated strong positive staining for p75 and receptor expression diminished in serial generations (Fig. 5). This supports the notion that within a given epithelial population, a small proportion of cells are likely to represent putative LESC or their transient amplifying cell progeny and p75 may be a phenotypic marker that identifies

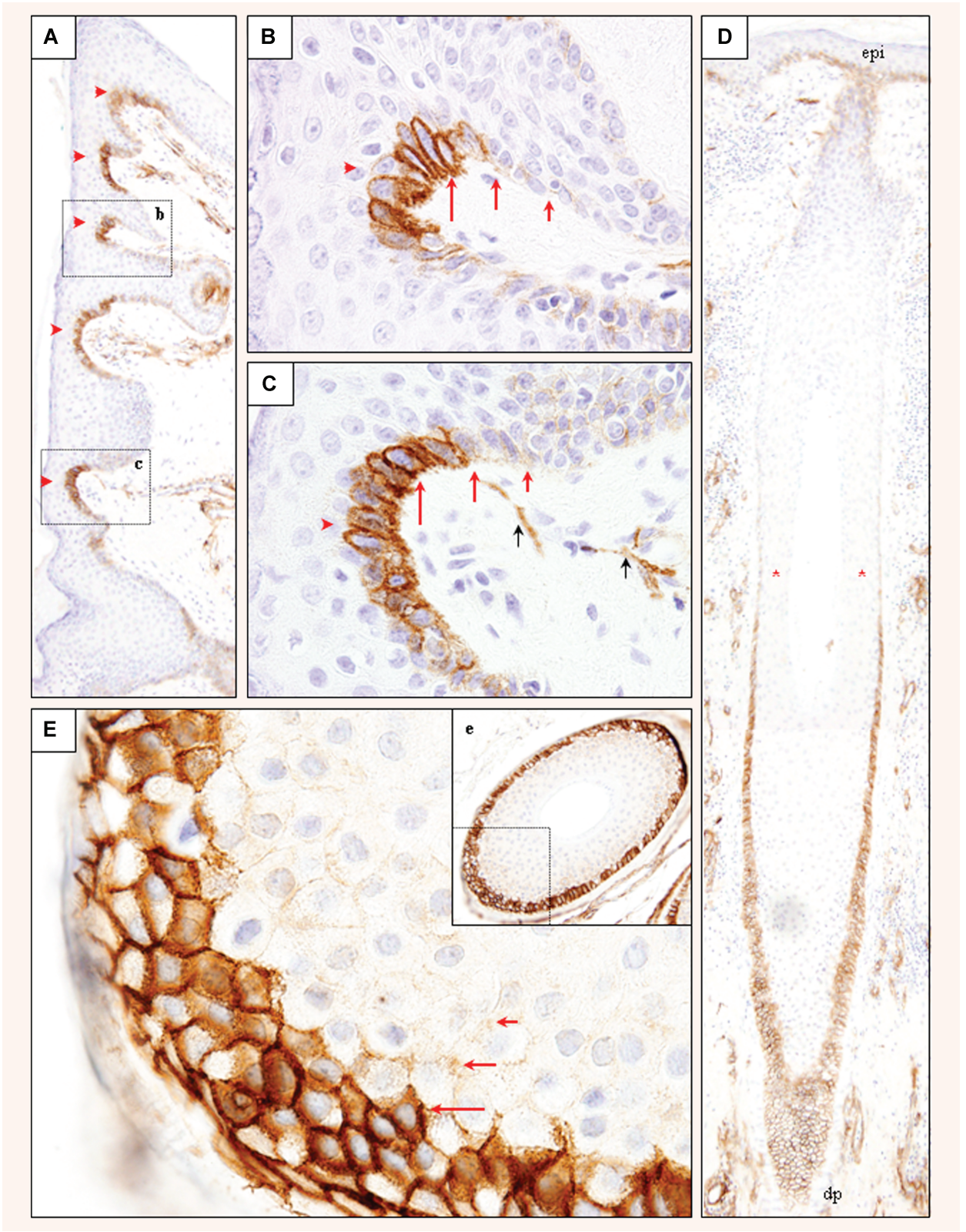

Fig. 6 Localization of p75 in the normal human epidermis. Epidermal specimens (tissue 1, **A**–**C**; tissue 2, **D**; tissue 3, **E**) were sectioned and incubated with an anti-human p75 monoclonal antibody (clone 7F10, **A**–**E**). Intense epidermal p75 staining developed in specific clusters of cells located in shallow rete ridges (**A**–**C**, arrowheads) while membraneous reactivity diminished in basal cells of the deeper rete ridges (**B** and **C**; red arrows). Black arrows in (**C**) identify subcutaneous nerve fibres. Vertical sections (**D**) through the skin identified intact hair follicles where intense reactivity for p75 was noted in the bulge (* asterisks) and dermal papillae (dp) but diminished in regions closest to the epidermis (epi). In transverse sections (**E**), p75 was highly expressed in the outer root sheath, while reactivity diminished in cells closest to the hair follicle orifice. The boxed region in (b and c) is magnified in (**B** and **C**, respectively). The area encompassed in (inset e) is magnified in (**E**). Sections were stained and counterstained as in Figs. 1–4. Original magnification (×100, **A** and **D**; ×200, inset e; ×400, **B** and **C**; ×1000, **E**).

these cells. In contrast, TrkA stained the full thickness epithelium of the normal cornea, including limbus and cj as well as pterygium. This result contrasts with studies from two independent laboratories, where TrkA was identified in the basal peripheral and central cornea and limbus and p75 was expressed in the full thickness corneal epithelium and limited to the superficial layers of the limbus [16, 17, 33, 34]. Indeed, our p75 results are highly reproducible and specific as similar data was recorded with three different antibody clones in paraffin-embedded human ocular (Figs 1–4) and cutaneous (Fig. 6) tissue. Moreover, a similar pattern of p75 reactivity has been described in frozen human skin specimens [22, 24, 39].

NGF has been immunohistochemically localized to canine basal limbal epithelium [14] as well as in rat and human corneal epithelium [16]. NGF mRNA was principally detected by RT-PCR and *in situ* hybridization in the corneal epithelium, while protein production was only found in tissue extracts by ELISA and uptake of radio-labelled NGF was assessed in organ-cultured corneas [16]. Consistent with previous studies [17], we demonstrated minimal NGF reactivity in the central cornea, although NGF expression was found at low levels in scattered basal limbal cells in normal and pterygium affected corneas (Fig. 1). These results imply that NGF signalling *via* p75 may be a mechanism by which LESC are activated to undergo proliferation and are protected from apoptosis until the shedding phase [40]. This is a likely scenario as other investigators have suggested a similar role for p75 and NGF in human oral keratinocyte SC [21] where $p75^{+}$ oral mucosal epithelial cells: (*i*) were not actively cycling (*i.e.* expressed low levels of Ki-67), (*ii*) were significantly smaller in size, (*iii*) had the highest clonogenicity, (*iv*) were abundantly present in holoclones (SC compartment) and (*v*) formed multiple, well-differentiated layers on an amniotic membrane substrate compared to $p75^{-}$ cells. Moreover, NGF stimulated $p75^{+}$ oral mucosal epithelial proliferation and rescued them from UV-induced apoptosis [21].

One puzzling observation from our foetal study, was the absence of any significant p75 reactivity in the limbal region (Fig. 3) which contrasted remarkably with the expression in the adult limbal region (Figs. 1 and 2). Interestingly, p75 staining was exclusively confined to the vascular endothelium in foetal corneas of 18 weeks gestation and we postulate that this finding may be due to an immature limbal niche. Several studies have corroborated this observation in other foetal tissue. For example, p75 was absent in foetal (13–21 weeks gestation) ovarian germ cells or by ovarian surface epithelium, although some expression was confined to adjacent ovarian stromal cells [41]. In the human foetal (16–18 weeks gestation) hair follicle bulge, p75 was more prominent in mesenchymal cells surrounding the hair follicle than within the lower portion of the bulge [22], while in mouse embryonic skin, p75 only illuminated the mesenchyme of developing hair follicles [39]. Studies of foetal corneal development demonstrate that there is a gradual transition in the morphological development of the cornea and phenotype of putative LESC in the cornea throughout foetal life. Corneal-specific keratin 3 (K3) is not found in the full-thickness foetal corneas until 36 weeks gestation, despite changes in the K3 profile beginning in the first trimester [42]. Histological examination of foetal corneas also indicate that maturity is not achieved until late in development. The emerging concept of the limbal-stromal microenvironment as a regulator of LESC fate in the cornea lends support to the notion that growth factors from the stroma may indeed regulate LESC activity [43, 44]. It is also likely that the limbal niche [12, 34, 43, 44] may play a role in the maintenance of SC phenotype as it has been demonstrated in epidermal regeneration [37, 38, 45] and in carcinogenesis [46]. The less pronounced staining for ABCG2 and p63 (two well-accepted LESC [35, 36]) in foetal corneas is further evidence that early progenitors have not committed to an adult phenotype (Fig. 3).

While the expression of p75 in the cj is intriguing, it is perhaps not so surprising, as the bulbar cj is likely to harbour epithelial progenitors [47, 48] with phenotypic features common to cells of the limbus. Intriguingly, it has recently been demonstrated that cultivated conjunctival epithelium can be successfully used for autologous corneal epithelial transplantation and that conjunctival cells are able to switch to a corneal phenotype with expression of CK3/12 [47]. Similarly, the skin is thought to house SC in several niches [37, 38, 45, 50]. The hair follicle organizes its SC as clusters of slow-cycling cells in the bulge, an area where intense p75 staining was noted (Fig. 6D and E) in our skin biopsies. Interestingly, bulge SC can be immobilized to regenerate an epidermis, viable hair follicles and sebaceous glands under wound-healing conditions [51, 52] or when placed subcutaneously in immunodeficient mice [53] highlighting their multi-potential nature. However, the precise spatial location of the epidermal SC niche remains an unsettled issue, as some investigators have demonstrated staining for SC markers (*e.g.* CK15) [50] and label-retaining cells [37] in deep rete ridges. Yet others have suggested SC reside in the shallow rete ridges (*i.e.* where the basal layer of

the epidermis is closest to the skin surface [54, 55]. Interestingly, this was precisely where intense membranous p75 staining was confined to in our specimens (Fig. 6A–C), an observation confirmed in human [23] and mouse [39] skin.

The results of the present study suggest that neurotrophic factors may play a key role in maintaining a SC-rich microenvironment on the ocular surface under physiological conditions or participate in the development of ocular surface diseases such as pterygia [56, 57]. The expression of p75 in basal pterygium epithelial cells supports a prevailing theory that pterygia may arise from altered basal LESC [58, 59]. Our data confirm the selective expression of p75 in basal limbal, conjunctival and pterygium epithelium in human adult, but not foetal eyes and suggest that this membrane-associated receptor may be an additional limbal epithelial stem/progenitor cell signature gene that could be utilised to facilitate the isolation and purification of ocular surface SC for transplantation.

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