Limbal stem cells, a review of their identification and culture for clinical use

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Abstract The surface of the eye is covered by two distinct epithelial populations, the conjunctival and corneal epithelia. The stem cell population for the corneal epithelia has been found to be located at the area known as the limbus. This is a narrow ring of tissue at the transitional zone between the cornea and conjunctiva. This stem cell population is responsible for generating transient amplifying cells which are responsible for renewing the cornea epithelia. There are currently no definitive markers for the stem cell population in the limbus. Instead using morphological features, such as small cells with a high nucleus-to-cytoplasm ratio, in conjunction with the presence of certain markers e.g. Δ NP63 α and the absence of others, e.g. the cytokeratin pair 3 & 12, are taken as being indicative of the stem cell population. Damage can occur to the corneal epithelium due to a number of causes including, Steven-Johnson syndrome, and chemical or thermal burns. This results in invasion of the cornea by the conjunctival epithelium resulting in impaired vision. In 1997 Pellegrini et al. (Lancet 349, 990) successfully used cells sheets from cultured limbal cells to successfully treat patients with corneal damage. Since then several other

groups, have successfully treated patients, using similar methods.

Keywords Limbal · Cornea · Stem cell · Cytokeratin · p63 · ABC-G2 · Integrin · Cell culture

Introduction

Stem cell populations have been identified in a number of adult tissues such as skeletal muscle, skin and intestine. The function of adult stem cells is to replenish cells lost in normal and damaged tissue. These adult stem cells are defined by a number of key features including (A) slow/long cycling time during homeostasis in vivo; (B) poorly differentiated with primitive cytoplasm; (C) high capacity to maintain a normal and stable genome during replication; (D) proliferation without maturation, and (E) generate a large number of functional differentiated progeny via transient amplifying cells (TACs) as seen during wounding or placement in culture (Potten and Loeffler 1990; Watt and Hogan 2000).

The ocular surface is covered by cells from two distinct cell lineages the conjunctival and corneal epithelia (Pellegrini et al. 1997). The conjunctival epithelium is well vascularised and consists of loosely organised cell layers populated by mosaic type cell or mucin-secreting goblet cells, which

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contribute to the maintenance of a tear film on the ocular surface (Pellegrini et al. 1997; Wolosin et al. 2004). The corneal epithelium, in order to be transparent, is an extremely flat, stratified squamous epithelium and sits on a stroma of highly organised collagen fibrils below which lies a single layer of endothelium (Nishida 1997). It is devoid of goblet cells, as well as other cell types, and is remote from a capillary network, the nearest being located in the limbus which is narrow ring of tissue at the transitional zone between the cornea and conjunctiva (Schermer et al. 1986; Nishida 1997). It is this limbal region that has been identified as the location where the corneal stem cell population resides (Schermer et al. 1986; Pellegrini et al. 1999). Adult stem cells are believed to reside in anatomical structures known as crypts or niches which protect their “stem cellness” (Potten and Loeffler 1990; Watt and Hogan 2000). This protection of “stem cellness” is due to the stem cells receiving various short and long range signals that control cell fate. While the full range of signals and the mechanisms, by which they operate, are not yet fully understood, these signals can be divided into the three broad categories of secreted factors, cell-cell interactions and cell-extracellular interactions. Within these categories certain factors recur in the process controlling stem cell fate in a wide range of tissues. For example in the case of secreted factors the Wnt signalling pathway has been found central in the stem cells of the intestinal, epidermal, and haematopoietic systems (Reya and Clevers 2005). While in cell-cell interactions the transmembrane protein Notch and its ligand Delta have been found important in stem cells of mammary gland, retina and haematopoietic systems (Lewis 1998). The stem cell population of the limbus appears to be localised to the Palisades of Vogt of the limbus (Pellegrini et al. 1999). It is here that the location of a structure, termed the limbal epithelial crypt has recently been identified, there are an estimated six such structures per human limbus (Dua et al. 2005). It is hypothesised that the limbal stem cells, give rise to TACs, which migrate centripetally towards the centre of the cornea. As these TACs migrate away from the limbus and upward from the basal layer they become terminally

differentiated. (Schermer et al. 1986; Pellegrini et al. 1999). The exact cues that trigger the differentiation process have not yet been identified, and are likely to involve a subtle combination of changes in basement membrane, soluble factors and cell-cell connections. However, in contrast to the even distribution of the conjunctival epithelium stem cells, the corneal epithelium stem cells located in the limbus are well separated from their differentiated progeny making them a useful model for studying epithelial stem cell differentiation. (Schermer et al. 1986; Pellegrini et al. 1999)

Identification of corneal–limbal stem cells

Unfortunately, there are no definitive markers for the identification of adult stem cells in general and corneal stem cells in particular. Rather, the presence and absence of various morphological features, proteins and gene expression are used in combination to attentively identify the stem cell and TAC populations from the fully differentiated cell population.

Adult epithelial stem cells are reported to be morphologically small cells with a high nucleus-to-cytoplasm ratio. Furthermore, the limited cytoplasm they do possess lacks any visible granular structures. These general morphological features have been identified for a sub-population of limbal cells, and these cells also express other markers associated with the stem cell population (Chen et al. 2004; Arpitha et al. 2005;)

Besides these morphological considerations, the expression of various proteins and genes associated with differentiated status is also used to identify and characterise the stem cell population in the limbus.

The expression of the cytoskeletal proteins, the cytokeratins (CK) are linked to the differentiation status of various epithelia. The simple epithelia which are composed of a single layer of cells and line organs such as the stomach, kidney etc. are characterised by expression of CK8 and CK18 and also occasionally CK7, CK17, and CK19. While stratified epithelia, such as the epidermis, are composed of several layers with each successive layer representing a more differ-

entiated state. Each layer of the stratified epithelia characterised by expression of a unique keratin pair (Daly et al. 1998).

Thus, the absence of expression of the cytoskeletal proteins, cytokeratins 3 and 12 (which are terminal markers of corneal epithelial differentiation) by the limbal basal epithelial cells is taken as a marker for the presence of stem cells (Schermer et al. 1986; Kasper et al. 1988). Similarly, the positive expression for cytokeratin 19 of limbal basal cells is taken as further indicator of a possible stem cell or TAC phenotype (Kasper 1988). Another potential marker for the stem cell phenotype in the limbus is the glycolytic enzyme α -enolase. Positive expression of α -enolase has been observed in the basal cells of the limbus but not in the basal cells of the central cornea (Zieske et al. 1992); however, other studies have found it expressed in the suprabasal cells of the limbus suggesting that it may be labelling the TAC population rather than the stem cell population.

The absence of the cell-cell communicating gap junction connexin 43 (Cx43) in the basal epithelial cells of the limbus and its expression in the basal cells of the cornea is also viewed as a potential marker for the stem cell phenotype (Matic et al. 1997). The exchange of small signalling molecules between cells via gap junctions is involved in co-ordinating the cell growth and differentiation of cells (Trosko 2005). The lack of Cx43 expression in limbal cells and scrape assays which show only minimal dye transfer between cells imply only a limited number of cell-cell interactions between limbal cells (Matic et al. 1997). The lack of such connections mean stem cells can exist isolated with their niches.

The expression of p63 is important in the maintenance of stem cellness and has an important role in development of stratified epithelia (Parsa et al. 1999). p63 protein is detected in the cells of limbal, peripheral and central cornea tissue, although, the cells in the limbus show significantly higher levels of expression (Arpitha et al. 2005). However of more significance, is the expression of the p63 gene splice variant, Δ Np63, of which there are three isoforms α , β and γ . The α isoform of Δ Np63 is present only in discrete clusters of cells (approximately 8% of the total cell population) in the basal layer of the limbus

(Di Iorio et al. 2005). Δ Np63 α functions as a transcriptional repressor by binding to the 14-3-3 δ promoter. During epithelial differentiation a reduction in Δ Np63 α expression correlates with increased expression of 14-3-3 δ protein (a known differentiation marker) (Westfall and Pietenpol 2004). This relationship was also reported for limbal basal epithelial cells with high expression of p63 by Arpitha et al. (2005). The β and γ isoforms are not present in significant amounts in resting cornea, however upon wounding all three isoforms, α , β , and γ , become abundant in cornea and limbal epithelium. The cultivation of limbal cells also causes an increase in expression of these isoforms (Di Iorio et al. 2005).

A number of cell surface markers for the stem cell population in the limbus have been identified. Such markers potentially allow the isolation of stem cell populations from limbus by fluorescent based cell sorting. One such set of markers are the integrin cell adhesion proteins. The integrins are a large family of heterodimeric transmembrane glycoproteins that bind to components of the extracellular matrix and to cell-cell adhesion molecules. The β 1 subunit is found expressed in both the cornea and limbal epithelia (Chen et al. 2004; Li et al. 2005); however the expression of β 1 integrin protein is highest in the limbal basal epithelial cells (Li et al. 2005). While β 1 integrin expression appears to be widespread in the cornea and limbus, a greater heterogeneity in expression is observed for the α integrin subunits. For example integrin α 9, which forms a heterodimer with integrin β 1, is expressed by a subpopulation in the limbus whereas cells of the central cornea are negative for α 9 integrin (Stepp et al. 1995).

It has been proposed that the side population observed in flow cytometry studies may be a general marker of stem cells (Zhou et al. 2001). The side population phenotype is identified by the ability to efflux the DNA dye, Hoechst 33342. This is mediated by the ABC-G2, a subtype of the ATP-Binding Cassette (ABC) family of cell surface transport proteins. This family includes more than 50 members and are involved in the transfer of a wide variety of substances across cellular membranes. Studies have shown that the ABC-G2 protein is expressed by discrete clusters of basal

limbal epithelial cells, with no expression detected in the corneal epithelium. (De Pavia et al. 2005; Wolosin et al. 2004; Budak et al. 2005).

To identify the genes important in maintenance of the stem cell phenotype and in differentiation, a number of microarray studies have been performed, comparing the gene expression profile of limbal tissue to corneal tissue. In an effort to create a preliminary database of human corneal gene expression Jun et al. (2001) performed a microarray study using a cDNA array constructed from human donor corneas. The study showed a wide range of genes being expressed including, genes for six types of collagen subunit were found to be expressed (e.g. $\alpha 1$ type IV, and $\alpha 1$ type XI) and five genes for apoptosis (e.g. TRAIL Bcl-xL, and caspase 7. Adachi and colleagues (2006) used serial analysis of gene expression (SAGE) to search for differences in gene expression between the limbal and central corneal epithelia of 6 week old rats. SAGE analysis uses total mRNA isolated from tissue to create a series of tags which provide a snapshot of gene expression in a tissue. An advantage of SAGE analysis is that it allows the identification of previously undiscovered genes. Their results showed not surprising, considerable overlap in the genes expressed between limbal and central corneal epithelium. In a microarray study Zhou et al (2006) used laser capture microdissection to isolate basal limbal and corneal epithelial cell populations directly from frozen sections, for comparison. Between the two epithelial populations, 50 genes (with a 3-fold or greater change) were identified as being differently expressed. Of the genes up-regulated in limbal or cornea epithelium, 42% could be grouped to roles in protein metabolism, 24% roles cellular transport, 13% in nucleotide regulation, intracellular signalling and transcription, 4% involved in cell surface receptor-linked signal transport and regulation of signal transduction (Zhou et al. 2006).

Cell culture and therapeutic use

Patients who suffer from a loss of the corneal–limbal epithelium are unable to maintain a stable

cornea. This leads to corneal repair by the conjunctival epithelium, which results in neovascularisation, chronic inflammation, recurrent epithelial defects and stromal scarring, causing a pronounced decrease in visual acuity and severe discomfort. Such deficiencies can be observed in a multitude of disorders, including aniridia, chemical and thermal burns, Steven-Johnson syndrome, ocular cicatricial pemphigoid, severe contact lens-induced keratopathy and can also occur as a result of multiple surgical procedures. In order to restore vision, limbal defects must be restored by transplantation of limbal grafts taken from uninjured eyes. In the case of unilateral defects limbal grafts can be taken from the uninjured eye. In bilateral disorders, stem cell allografts dissected from living tissue-matched eyes or non-matched cadaver eyes are performed. It should be noted that this procedure requires a large limbal withdrawal from the healthy eye; hence a potential serious complication arising from this procedure is limbal deficiency in the donor eye.

In (1997) Pellegrini et al., reported the first successful use of cultured limbal cell sheets to resurface the corneas of two patients with unilateral stem cell deficiency. This ground-breaking procedure allowed the use of a 1 mm diameter biopsy of limbal tissue from the patients own stem cell proficient eye, to generate an epithelial sheet (approximately 2 cm in diameter) in vitro. Progress of the culture was monitored by immunocytochemical analysis of CK 3 (a specific marker of the corneal lineage). This epithelial sheet was transplanted onto the patients' limbal deficient eye, which integrated with a high degree of clinical success. At two years post treatment both patients still possessed stable corneal epithelium, an absence of vascularisation, improved visual acuity and an improvement in the subjective parameters of pain and photophobia. Since then several reports have been made using limbal cell cultures for successful clinical treatment of limbal stem cell deficiency (Schwab et al. 2000; Koizumi et al. 2002; Nakamura et al. 2004; Daya et al. 2005). In the study by Daya and colleagues (2005) for example, 7 out of 10 patients showed improvements in visual acuity, vascularisation, and comfort. While, a study by Nakamura et al. (2004) reported that a patient had their visual

acuity improved from 2/200 to 20/20 and this was maintained 19 months post treatment.

In the clinical setting, amniotic membrane was used in combination with limbal tissue or expanded cells as a bandage. It is believed its use allows for a more rapid re-epithelisation and may help prevent infection (Daya et al. 2005). Amniotic membrane in conjunction with a feeder layer of 3T3 cells also appears to provide a good matrix for limbal cells attachment and growth. Gruererich et al. (2003) showed that both amniotic membrane and 3T3 cells help maintain cells in a less differentiated phenotype at the monolayer stage. The beneficial effect is seen when 3T3 cells are not in direct contact with the expanded epithelium, suggesting diffusible factors or cytokines are responsible. Corneal cell sheets can be generated from primary cultured explants or isolated cells. However, it has been reported that cultures derived from isolated limbal cells had a better morphology with CK3 and CK12 more prominent on the basal membrane and possess tighter cell junctions (Koizumi et al. 2002). The technique of airlifting of cell cultures was developed to make skin cell culture sheets for transplantation. It simply involves a culture that is initially submerged in medium, being exposed to air by lowering the medium level. The aim is to match the tissue culture environment to the in vivo environment to promote improved differentiation. A number of reports suggest that air lifting of limbal cell cultures results in improved differentiation within the cell sheets generated (Zieska et al. 1994; Ban et al. 2003). For example, airlifted cultures showed improved cell-cell attachment than that seen in submerged cultures (Ban et al. 2003).

Such corneal epithelial cell sheets can also be prepared from eye banked corneal limbal rings, which are normally discarded after keratoplasty (James et al. 2001). These are available in relatively large numbers and pose no risk to the contra lateral patients eye or to the eyes of healthy relatives. The study by James et al. (2001) indicated that an important variable in generating successful limbal derived stem cells cultures was donor age. In a follow up study with 10 patients using corneal cultures derived from eye banked corneal limbal rings Daya et al. (2005) reported a

successful outcome in seven patients. Interestingly, no donor DNA could be detected beyond 9 months.

Conclusion

Our knowledge of the stem cell population of the limbus and the process of differentiation is steadily increasing. With microarrays and proteomic analyses being now used to study the cornea and limbus, we can expect major in-sight into how the stem cell population differ from their differentiated progeny. This increased information on the stem cell population and its differentiation will lead to further refinements in the cell culture of corneal cell sheets for clinical usage. The findings of Daya et al. (2005) demonstrating no donor DNA in recipient patients following treatment with cell culture sheets is extremely interesting. Investigating this phenomenon will likely provide fundamental data on how adult stem cell therapies work in vivo in general as well as the cornea.

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References

- Adachi W, Ulanovsky H, Li Y, Norman B Davis J, Piatigorsky J (2006) Serial Analysis of gene expression (SAGE) in rat limbal and central corneal epithelium. *Invest Ophthalmol Vis Sci* 47:3801–3810
- Arpitha P, Prajna NV, Srinivasan M, Muthukkaruppan V (2005) High Expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: Implications on epithelial stem cells. *Invest Ophthalmol Vis Sci* 46:3631–3636
- Ban Y, Cooper LJ, Fullwood NJ, Nakamura T, Tsuzuki M, Koizumi N, Dota A, Mochida C, Kinoshita S (2003) Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting. *Exp Eye Res* 76:735–743

- Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM (2005) Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 118:1715–1724
- Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ (2004) Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 22:355–366
- Daly N, Meleady P, Walsh D, Clynes M (1998) Regulation of keratin and integrin expression in cancer and drug resistance. *Cytotechnology* 27:321–344
- Daya SM, Watson A, Sharpe JR, Giledi O, Rowe A, Martin R, James SE (2005) Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. *Ophthalmology* 112:470–477
- De Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li D-Q (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 23:63–73
- Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M (2005) Isoforms of Δ Np63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Nat Acad Sci USA* 102:9523–9528
- Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A (2005) Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 89:529–532
- Grueterich M, Espana EM, Tseng SCG (2003) Modulation of keratin and connexion expression in limbal epithelium expanded on denuded amniotic membrane with and without 3T3 fibroblast feeder layer. *Invest Ophthalmol Vis Sci* 44:4230–4236
- James SE, Rowe A, Ilari L, Daya S, Martin R (2001) The potential for eye bank limbal rings to generate cultured corneal epithelial allografts. *Cornea* 20:488–494
- Jun AS, Liu SH, Koo EH, Do DV, Stark WJ, Gottsch JD (2001) Microarray analysis of gene expression in human donor corneas. *Arch Ophthalmol* 119:1629–1634
- Kasper M, Moll R, Stosiek P, Karsten U (1988) Patterns of cytokeratin and vimentin expression in the human eye. *Histochemistry* 89:369–377
- Koizumi N, Cooper LJ, Fullwood NJ, Nakamura T, Inoki K, Tsuzuki M, Kinoshita S (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. *Invest Ophthalmol Vis Sci* 43:2114–2121
- Lewis J (1998) Notch signalling and control of cell fate choices in vertebrates. *Semin Cell Dev Biol* 9:583–589
- Li De-Q, Chen Z, Song XJ, de Paiva CS, Kim H-S, Pflugfelder SC (2005) Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. *Exp Eye Res* 80:581–590
- Matic M, Petrov IN, Chen S, Wang C, Dimirijevich SD, Wolosin JM (1997) Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation* 61:251–260
- Nakamura T, Inatomi T, Sotozono C, Koizumi N, Kinoshita S (2004) Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand* 82:468–471
- Nishida T (1997) Cornea. In: Krachmer JH, Mannis MJ, Holland EJ (eds) *Fundamentals of Cornea and external disease*. Mobsy, St. Louis
- Parsa R, Yang A, McKeon F, Green H (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* 113:1099–1105
- Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 349:990–993
- Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 145:769–782
- Potten CS, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 110:1001–1020
- Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. *Nature* 434:843–850
- Schermer A, Galvin S, Sun TT (1986) Differentiation-related expression of a major 64 K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 103:49–62
- Schwab IR, Reyes M, Isseroff RR (2000) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea* 19:421–426
- Stapp MA, Zhu L, Sheppard D, Cranfill RL (1995) Localized distribution of $\alpha 9$ integrin in the cornea and changes in expression during corneal epithelial differentiation. *J Histochem Cytochem* 43:353–362
- Trosko J (2005) The role of stem cells and gap junctions as targets for cancer chemoprevention and chemotherapy. *Biomed Pharmacother* 59:S326–S331
- Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. *Science* 287:1427–1430
- Westfall MD, Pietenpol JA (2004) p63: molecular complexity in development and cancer. *Carcinogenesis* 25:857–864
- Wolosin JM, Budak MT, Akinci MA (2004) Ocular surface epithelial and stem cell development. *Int J Dev Biol* 48:981–991
- Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sam-path J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP (2001) The ABC transporter Bcrp/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7:1028–1034
- Zhou M, Li X-M, Lavker RM (2006) Transcriptional profiling of enriched populations of stem cells *versus* transient amplifying cells. *J Biol Chem* 281:19600–19609
- Zieske JD, Bukusoglu G, Yankauckas MA, Wasson ME, Keutmann HT (1992) α -Enolase is restricted to basal cells of stratified squamous epithelium. *Dev Biol* 151:18–26
- Zieske JD, Mason VS, Wasson ME, Meunier SF, Nolte CJ, Fukai N, Olsen BR, Parenteau NL (1994) Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. *Exp Eye Res* 214:621–633