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Limbal Stem Cell Transplantation and Corneal Neovascularization

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1. Introduction

The human ocular surface spans from the conjunctiva to the cornea and plays a critical role in visual perception. Cornea, the anterior portion of the eye, is transparent and provides the eye with two-thirds of its focusing power and protection of ocular integrity. The cornea consists of five main layers, namely, corneal epithelium, Bowman's layer, corneal stroma, Descemet's membrane and corneal endothelium.

The outermost layer of the cornea, which is exposed to the external environment, is the corneal epithelium. Corneal epithelial integrity and transparency are maintained by somatic stem cells (SC) that reside in the limbus. The limbus, an anatomical structure 1-2 mm wide, circumscribes the peripheral cornea and separates it from the conjunctiva (Cotsarelis *et al.*, 1989, Davanger and Evensen, 1971) (**Figure 1**). Any damage to the ocular surface by burns, or various infections, can threaten vision. The most insidious of such damaging conditions is limbal stem cell deficiency (LSCD). Clinical signs of LSCD include corneal vascularization, chronic stromal inflammation, ingrowth of conjunctival epithelium onto the corneal surface and persistent epithelial defects (Lavker *et al.*, 2004). Primary limbal stem cell deficiency is associated with aniridia and ectodermal dysplasia. Acquired limbal stem cell deficiency has been associated with inflammatory conditions (Stevens–Johnson syndrome (SJS), ocular cicatricial pemphigoid), ocular trauma (chemical and thermal burns), contact lens wear, corneal infection, neoplasia, peripheral ulcerative corneal disease and neurotrophic keratopathy (Dua *et al.*, 2000, Jeng *et al.*, 2011). Corneal stem cells and/or their niche are known to play important anti-angiogenic and anti-inflamatory roles in maintaining a normal corneal microenvironment, the destruction of which in LSCD, tips the balance toward pro-angiogenic conditions (Lim *et al.*, 2009).

For a long time, the primary treatment for LSCD has been transplantation of healthy - keratolimbal tissue from autologous, allogenic, or cadaveric sources. In the late 1990s, cultured, autologous, limbal epithelial cell implants were used successfully to improve vision in two patients with chemical injury-induced LSCD (Pellegrini *et al.*, 1997). Since then, transplantation of cultivated epithelial (stem) cells has become a treatment of choice for numerous LSCD patients worldwide. While the outcomes are promising, the variability of methodologies used to expand the cells, points to an underlying need for better standardization of *ex vivo* cultivation-based therapies and their outcome measures (Sangwan *et al.*, 2005, Ti *et al.*, 2004, Grueterich *et al.*, 2002b, Kolli *et al.*, 2010).

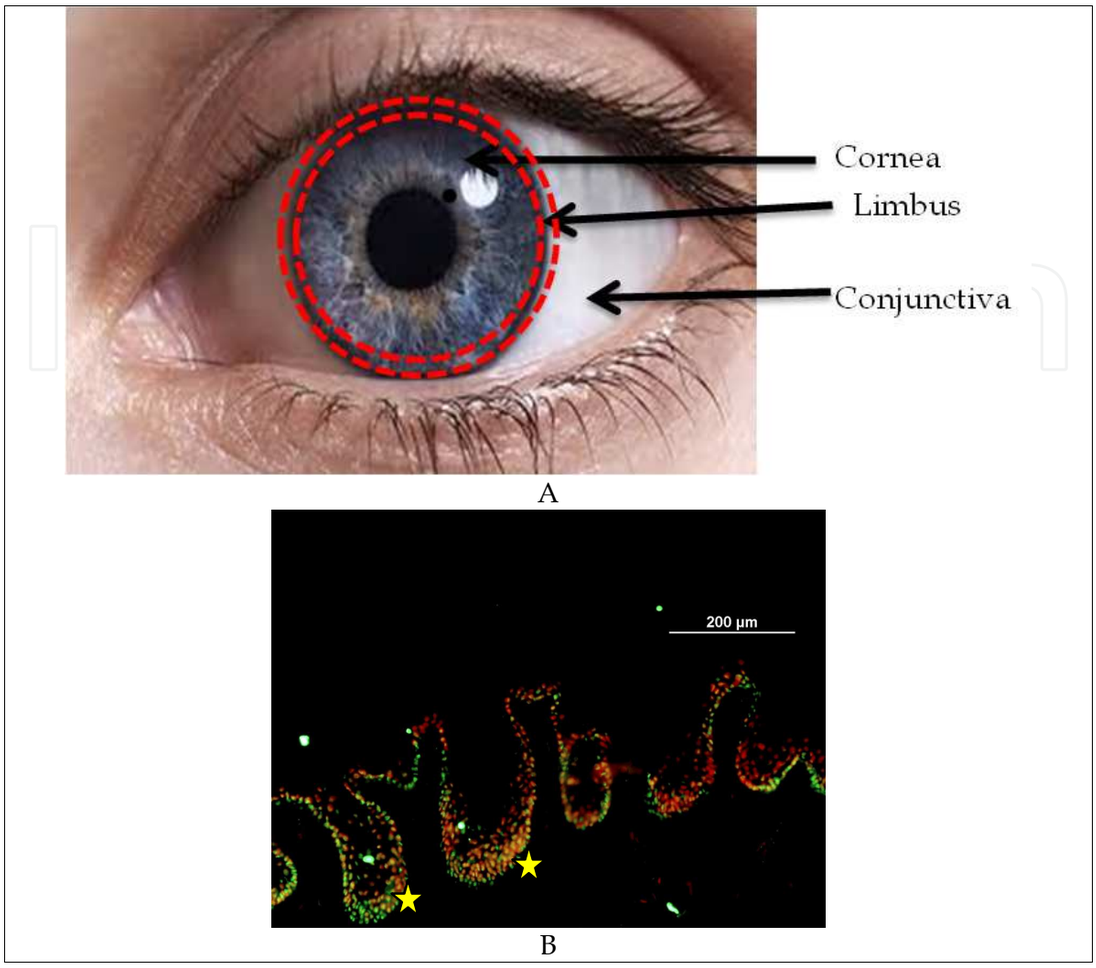

Fig. 1. (A) Photograph of the front of the human eye. The cornea, which is clear, can be seen at the front of the eye highlighted by the light reflections. The cornea is continuous with the limbus, the narrow band of tissue found encircling the cornea. The limbus is continuous with the conjunctiva and its underlying sclera, together forming the anterior portion of the eye. (B) Cross section of human limbal palisades of Vogt.

This chapter provides a general update on LSCD pathogenesis, the key role of stem cells and their niche in maintaining corneal homeostasis and avascularity and a general update on LSCD pathogenesis. An overview of different methodologies used for *ex vivo* stem cell expansion will shed some light on the future of cell-based therapy for LSCD patients.

2. What defines limbal stem cell niche

The ocular surface is anatomically composed of the entire epithelial surface of the cornea, limbus and conjunctiva, and is physically continuous with the eyelids and adenexa. The limbal epithelium, which separates the corneal epithelium from the conjunctival epithelium, is made up of a nonkeratinizing, stratified squamous epithelium, which is much thicker, up to 10 cell layers, than the central corneal epithelium. Moreover, limbal epithelium is thought to contain the source of the stem cells (SC) that serve as the source of corneal epithelial cell

renewal and provide a barrier between conjunctival and corneal epithelia. These cells are known as corneal epithelial SCs or limbal stem cells (LSCs). In addition to intrinsic factors (i.e., characteristics inherent to the stem cells), extrinsic influences from the microenvironment surrounding the stem cells may also play a role in corneal cell renewal (Barros *et al.*, 1995, Morrison *et al.*, 1997, Tseng, 1996), as discussed below.

2.1 Limbal microenvironment and stem cell properties

Within the limbus, LSCs are thought to reside in a stem cell niche, which maintains them in their undifferentiated state. This stem cell niche is anatomically denoted as the palisades of Vogt. The palisades of Vogt are radial infoldings (Figure 1 B) located at the limbo-corneal junction, extending outwards of 1–2 mm from that junction (Goldberg and Bron, 1982); a anatomical feature unique to the human eye that can be used as a clinical marker indicating the presence of corneal epithelial stem cells. Davenger and Evensen (1971) and Bron (1973) reported the significant characterizations of the palisades of Vogt in 1971 (Davanger and Evensen, 1971, Bron, 1973). The authors observed heavily pigmented epithelium migrating in lines from the limbus to the central cornea in response to corneal epithelial defects.

2.1.1 Characterization and microenvironment of the palisades of Vogt

The limbal basement membrane differs from that of the cornea that undulates, with pegs of stroma extending upward and interconnecting with anchoring fibrils that link to the basement membrane (Gipson, 1989). This structure could provide resident stem cells with an adherent niche, protecting them from injury and movement within their microenvironment. Differences in the composition of the limbal and central corneal basement membranes have been observed, and these are thought to play a role in the maintenance of their respective populations of epithelial cells. Moreover the LSC niche lies beneath a number of cell layers where oxygen tension is likely to be lower. Limbal cells are in close proximity to a blood supply and have access to survival factors such as keratinocyte growth factor (KGF), IL-6 and components of basement membrane unavailable to cells of the central cornea. Interestingly, hypoxic *ex vivo* conditions have been found to produce larger, less differentiated limbal epithelial cell colonies, suggesting that low oxygen levels may induce selective proliferation of undifferentiated cells (Miyashita *et al.*, 2007).

Photomicrographic, angiographic and histological studies have demonstrated the fibrovascular nature of the palisades and the presence of "ridges of thickened epithelium" in the interpalisade zone (Goldberg and Bron, 1982, Townsend, 1991). Confocal microscopy along with scanning electron microscopy (SEM) provides the opportunity to optically section the corneal limbus and create 3D reconstructions of the tissue (Romano *et al.*, 2003, Shortt *et al.*, 2007). This approach allows identification of previously unrecognized candidates for the LSC niche, limbal crypts (LCs) and focal stromal projections (FSPs), and has significantly advanced understanding of the structure of this adult stem cell niche.

2.1.2 Asymmetric cell division

Adult SCs exist in an optimal microenvironment or 'niche' that promotes their maintenance in an undifferentiated state (Fuchs *et al.*, 2004). When SCs undergo asymmetric division, only one of the daughter cells can re-enter the niche to replenish the SC population (**Figure 2**). The other cell loses the protection of the niche and is destined to differentiate and become a transient amplifying cell (TAC). The role of the TAC is to divide at an exponential rate in

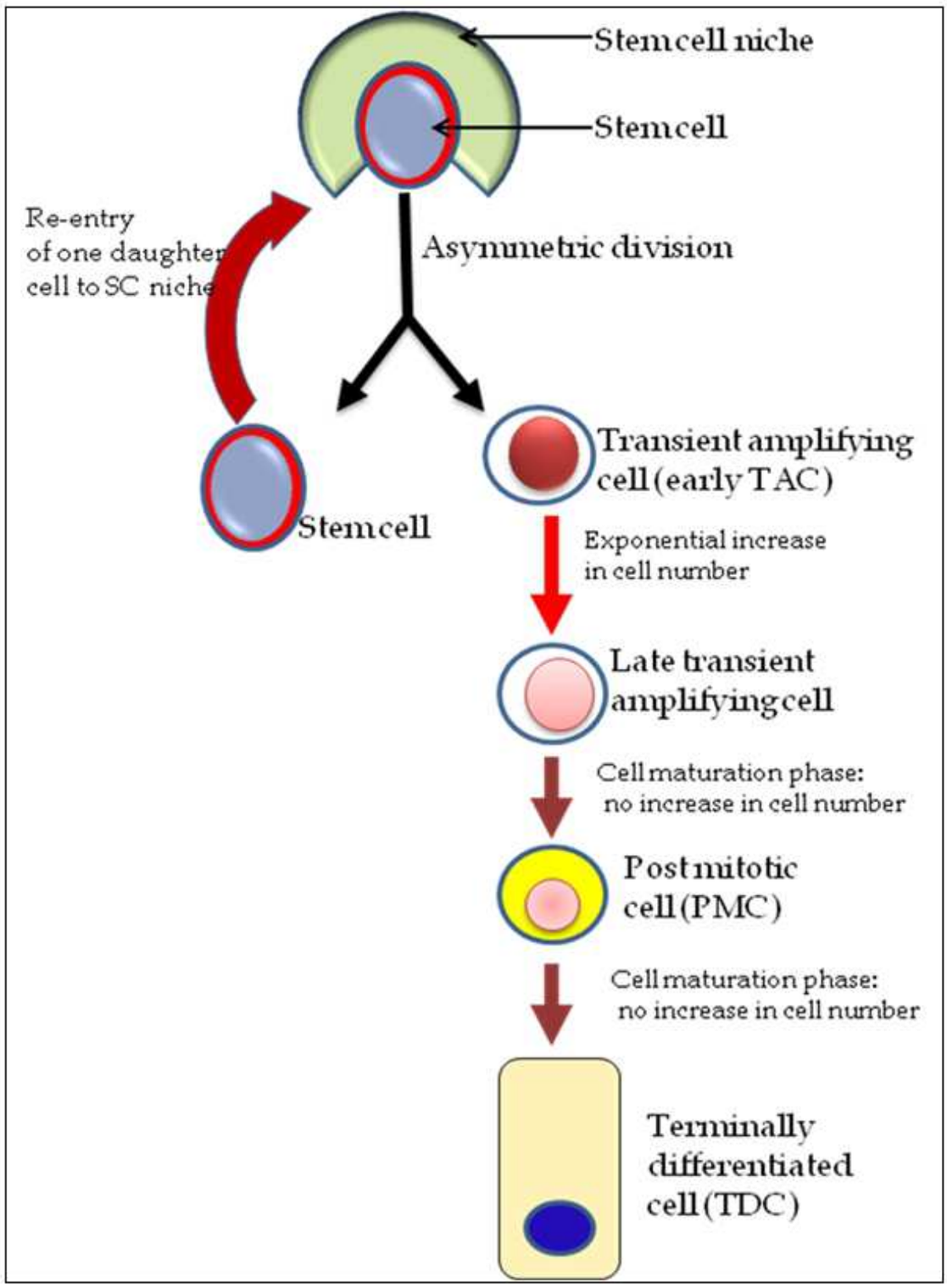

Fig. 2. Schematic diagram of the SC hierarchy. Modified from (Drug Discovery Today. Volume 15,Number 7/8.April 2010.

order to provide increased cell numbers. The ability of the TAC to multiply is limited and will eventually differentiate into a post-mitotic cell that can no longer multiply (Cotsarelis *et al.*, 1989). The PMCs are committed to cellular differentiation and mature to form terminally differentiated cells that represent the final phenotypic expression of the tissue type. These mechanisms are essential for the understanding of normal stem cell function and homeostasis. Moreover the LSC population is positioned to be influenced by a wide variety of cells. These influences include cell-to-cell contact, cell–extracellular matrix contact, and paracrine signalling factors and their receptors, and they from adjacent conjunctival epithelial cells and fibroblasts, corneal epithelium and fibroblasts, limbal blood vessels, limbal melanocytes, corneal nerves and Langerhans cells. The limbal fibroblasts in the underlying stroma are heterogeneous and express secreted protein acidic and rich in cysteine (SPARC), which may contribute to LSC adhesion (Shimmura *et al.*, 2006).

2.1.3 Cell signalling

Cytokine of the cornea and limbus implied specific differences in their microenvironments, suggesting fibroblast-mediated paracrine regulation of LSCs. For example, corneal fibroblasts release hepatocyte growth factor (HGF), whereas limbal fibroblasts secrete keratinocyte growth factor (KGF) (Sotozono *et al.*, 1994). Most importantly, epithelial cells at the basal layer of the limbus express high levels of the KGF-R, in contrast to central corneal epithelium, which expresses fewer of this receptor. KGF is a potent stimulator of proliferation in epithelial cells, and its presence in the limbus results in the proliferation of LSCs and TACs (Sotozono *et al.*, 1994). HGF is known to stimulate the migration of epithelial cells, and it has been proposed that HGF aids the migration of new TACs produced at the limbus through the action of KGF (Wilson *et al.*, 1994). The basement membrane separates the LSC from stroma and, thus modulates growth factors and cytokines involved in LSC regulation and function (Klenkler and Sheardown, 2004).

Sonic hedgehog, Wnt/β-catenin, TGF-β and Notch signalling pathways have all been implicated in niche control of stem cells; however, little is known of their potential roles in the LSC niche. Lack of Dkk2, leads to increased Wnt/β-catenin signalling in limbal stroma. This demonstrates the importance of limbal niche control over LSC differentiation during development. PAX6 expression is also lost in the corneal epithelial cells of mutant mice, suggesting the control downstream of Dkk2 (Mukhopadhyay *et al.*, 2006). PAX6 deficiency leads to aniridia, resulting in turn to impaired corneal epithelial function and eventual LSC failure, which may be due to altered niche development. A more recent study by Schlotzer-Schrehardt *et al.* found patchy immunolocalization of the laminin γ3 chain, BM40/SPARC and tenancin C, which were also found to colocalize with ABCG2/p63/K19-positive cell clusters. These factors may be involved in retaining cell stemness (Schlotzer-Schrehardt *et al.*, 2007).

3. Insult to the niche and corneal limbal stem cell deficiency

Corneal blindness is a condition currently affecting about 10 million people worldwide (Klenkler *et al.*, 2005). Corneal damage and disease can be a result of LSC deficiency, which can be heritable or acquired. Limbal stem cell deficiency (LSCD) results from the loss or dysfunction of LSC, most often because of injury or inflammation (Dua *et al.*, 2000). When

the corneal limbal stem cells are depleted below a certain threshold, the cornea becomes covered by an abnormal conjunctiva-like epithelium, a process termed "conjunctivalization." As a result of LSC deficiency, neovascularization, chronic inflammation, recurrent erosions, ulceration and stromal scarring can occur, causing painful vision loss (Holland and Schwartz, 1996, Kenyon and Tseng, 1989).

LSCD is classified as either primary or secondary. Primary LSCD is characterized by the absence of identifiable external factors and an insufficient microenvironment to support the LSCs. Primary LCSD is seen in hereditary aniridia, congenital erythrokeratoderma, keratitis with multiple endocrine deficiencies and inadequate nutrition or cytokine supply, neurotrophic keratopathy, peripheral inflammation, and sclerocornea (Puangsricharern and Tseng, 1995). Dysfunction/poor regulation of stromal microenvironment of limbal epithelial SCs results in gradual loss of the SC population or TAC generation and amplification.

Secondary LSCD occurs following the destruction of LSCs by external factors such as trauma, chemical–acid or alkali or thermal injuries (Tseng, 1985), or ultraviolet and ionizing radiation (Fujishima *et al.*, 1996); systemic conditions such as Stevens-Johnson Syndrome (SJS), or ocular cicatricial pemphigoid (OCP) (Pfister, 1994).

3.1 Clinical conditions

The corneal epithelium cannot be maintained or renewed in eyes with LSCD, and leads to chronic epithelial defects. As the condition progresses, punctate epithelial keratopathy may develop and lead to severe epithelial defect. Due to decreased healing ability, corneal epithelial defects may become persistent, which can lead to stromal scarring, ulceration, and perforation (Puangsricharern and Tseng, 1995). Conjunctivalization of the cornea may occur, where the corneal epithelium is replaced with a conjunctival epithelial phenotype (Huang and Tseng, 1991). Loose intercellular connections result in stippled or late staining with fluorescein, and a lack of normal hemidesmosomal attachments at the base of corneal epithelial cells (Dua and Forrester, 1990). The cause of the LSCD often dictates whether the disease is unilateral or bilateral (i.e., affecting one eye or both).

3.1.1 Pathology of limbal stem cell deficiency and histologic, ultrastructural studies

Currently, diagnosis of LSCD is based on clinical evaluation, although clinical signs and symptoms of LSCD are nonspecific and common to several ocular surface diseases. The disease manifests as epithelial defects, chronic inflammation, keratitis, vascularization, and fibrosis, ultimately resulting in corneal blindness. In the absence of specific markers for limbal stem cells, identification of conjunctival goblet cells on the corneal surface is used to demonstrate conjunctival epithelial ingrowth or growth of epithelia on the corneal surface and to confirm the diagnosis of LSCD (Tseng, 1989, Dua and Forrester, 1990, Pfister, 1994).

Normal limbal architecture, with rows of palisades and a perilimbal vascular arcade, is usually best defined at the superior and inferior limbus. Definition of the palisades is less distinct nasally and temporally. Delicate changes such as staining of conjunctiva-derived cells on and across the limbus, which may be associated with a persistent epithelial defect, and other changes such as scarring, vascularization, or limbal hyperaemia, indicating chronic inflammation, can be seen in the early stages. SC deficiency following mild injury, a superficial or a disease process is slowly progressive, and loss of a segment of limbal epithelium may occur without significant damage to the substratum. A sheet of

conjunctival/metaplastic epithelium without any notable vascularization consequently covers the cornea (Dua, 1998). Other pathological conditions resulting from LSCD are abnormal conjunctival/metaplastic epithelium; tags of loose epithelium filaments with mucus, and recurrent erosions, with unstable tear film that readily takes up fluorescein dye (Huang and Tseng, 1991).

In moderate to severe cases of SC deficiency, superficial and/or deep vascularization of the cornea occurs. Initially it is restricted to the segment with SC deficiency, but in later stages, the entire circumference may become involved. However, in moderate to severe cases of SC deficiency, epithelial cover of the denuded cornea is associated with encroachment of fibrovascular tissue of varying thicknesses (Kenyon and Tseng, 1989). In human eyes, ocular surface insult from chemical burns may go beyond damage to the limbal tissue, leading to long-term ocular surface damage. This damage may include distortion and dysfunction of proximal or distal bulbar conjunctiva, tarsal conjunctiva, and anterior orbital tissues, resulting in abnormalities in surface mucous secretion and wetting, cicatrizing of conjunctiva, symblepharon, and entropion (McCulley, 1990, McCulley *et al.*, 1983, Grant and Kern, 1955).

In summary, alkali burns are the commonest cause of severe LSCD, producing long-standing and persistent changes, like epithelial hyperplasia, fibrosis, and inflammation of the ocular surface. Presence of goblet cells on the cornea confirms the diagnosis of LSCD, but their absence does not rule it out, and correlates presence or absence with prolonged interval and squamous metaplasia of the entire surface in such cases (Fatima *et al.*, 2008).

3.1.2 Accompanying neovascularization

The cornea is avascular, but a wide variety of insults can cause capillary invasion from the limbal vascular plexus. This process of new blood vessel formation is termed as corneal neovascularization (NV) (Chang *et al.*, 2001, Takahashi *et al.*, 1999). The three major categories of corneal NV are superficial vascularization, fibrovascular pannus, and deep stromal vascularization. Superficial vascularization rarely causes a decrease in vision; however, the latter two types of corneal NV can lead to significant loss of vision if they involve the visual axis. The most frequent causes of corneal angiogenesis are injury and defective limbal stem cells.

4. Putative mechanisms of LSCD and corneal neovascularization

There is a delicate balance between angiogenic and anti-angiogenic factors in the cornea, which determines its vascularity or lack thereof. The normal cornea is avascular, and corneal epithelial stem cells may play a significant role in maintaining the angiogenic balance in favour of avascularity. Nevertheless, there are numerous pro-angiogenic factors that play key roles in pathologic corneal NV. A comprehensive review of all the factors was described by Lim *et.al,* and included key factors and their possible mechanisms of action (Lim *et al.*, 2009).

4.1 Angiogenic factors and anti-angiogenic factors

Vascular endothelial growth factor (VEGF) is a protein that acts as a mitogen for vascular endothelial cells, stimulating these cells to divide and multiply. The most important member

of this group is VEGF-A, which has been shown to stimulate endothelial cell proliferation and migration. It also increases microvascular permeability. In rats with surgically induced LSCD, high levels of VEGF correlated with inflammation and corneal NV. The corneal NV was suppressed with the addition of anti-VEGF antibody (Amano *et al.*, 1998). Other members of this family include VEGF-B, VEGF-C, VEGF-D, and placenta growth factor.

Inflammatory cytokines, chemokines, and cell adhesion molecules (CAMs) play a significant role in inflammatory cell infiltration and angiogenesis. The inflammatory cytokine interleukin-1 (IL-1) interacts directly with endothelial cells to stimulate migration and proliferation. IL-6 can stimulate corneal and inflammatory cells, in a paracrine manner, to secrete VEGF (Biswas *et al.*, 2006). Tumor necrosis factor-α (TNF-α) enhances leukocyte infiltration (Yoshida *et al.*, 1997). Chemokines are a family of secreted proteins that act as chemoattractants for inflammatory cells through activation of chemokine receptors. They may also enhance endothelial cell chemotaxis and proliferation (Berger *et al.*, 1999). The chemokines IL-8 (Strieter *et al.*, 1992, Koch *et al.*, 1992) and monocyte chemoattractant-1 (MCP-1) (Goede *et al.*, 1999) have been shown to induce corneal NV in rabbit corneas. Cell adhesion molecules (CAMs) are proteins located on the cell surface involved in binding with other cells or with the extracellular matrix. They facilitate inflammatory cell infiltration into the corneal stroma by mediating the rolling, adhesion, or trans-endothelial migration of leukocytes (Vaporciyan *et al.*, 1993). Fibroblast growth factors (FGF) are a family of heparin-binding peptides that are expressed in tissues during angiogenesis, cellular differentiation, mitogenesis, and wound repair. Basic fibroblast growth factor (bFGF) is an angiogenic factor that exhibits differential binding of vascular basement membranes in newly formed corneal vessels, based on degree of maturation of the vessels (Soubrane *et al.*, 1990).

Several anti-angiogenic factors are produced by corneal epithelial cells, and a variety of vascular endothelial cell inhibitors including angiostatin, endostatin, restin, neostatin, and thrombospondins. Several of these endogenous anti-angiogenic factors are derived from the proteolysis of larger extracellular matrix proteins that have no intrinsic anti-angiogenic activity. Angiostatin, for example, is derived from the proteolysis of plasminogen. Endostatin is another example, produced by the cleavage of collagen XVIII. It inhibits proliferation (Hanai *et al.*, 2002) and migration (Dhanabal *et al.*, 1999) of endothelial cells (EC), and induces EC apoptosis. It may block the activation and catalytic activity of matrix metalloproteinases (MMPs) (Kim *et al.*, 2000) and interfere with the binding of VEGF to its receptors. Arrestin, canstatin, and tumstatin, derived from collagen IV, also inhibit the proliferation and migration of ECs and induce EC apoptosis. Restin, which is related to endostatin, is produced by the proteolytic cleavage of collagen XV and is believed to inhibit EC migration (Ramchandran *et al.*, 1999). Neostatin, derived from collagen XVIII, and thrombospondin are other inhibitors of EC proliferation.

Avascular tissue-derived factors, such as pigment epithelium-derived factor (PEDF), and angiogenic factor antagonists, such as angiopoetin-2, have been found in the cornea (Karakousis *et al.*, 2001). PEDF and angiopoetin-2 inhibit angiogenesis by inhibiting endothelial cell migration and inducing EC apoptosis.

4.1.1 Role of matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases. They are commonly categorized as collagenases, gelatinases, matrilysins, or membrane-type MMPs,

and they play a role in the proteolytic processes involved in angiogenesis (Chang *et al.*, 2001). Their action is inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs). Some are involved in the cleavage process that generates the anti-angiogenic factors angiostatin and endostatin. Plasminogen activator inhibitors inhibit tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the activators of plasminogen, thereby preventing fibrinolysis and the physiologic breakdown of blood clots.

In response to corneal injury, lipid mediators are released from cell membranes. Platelet activating factor (PAF) is a lipid mediator that facilitates endothelial cell migration through the cornea via increased expression of VEGF (Tao *et al.*, 1996), MMP-9 (Tao *et al.*, 1995), and uPA (Tao *et al.*, 1996). Another important lipid mediator is 12(R)-HETrE, an arachidonic acid metabolite that binds to limbal endothelial cells and causes endothelial mitogenesis and neovascularization (Stoltz and Schwartzman, 1997).

4.1.2 Animal disease models

Development of experimental animal models to study ocular NV have been useful in understanding its mechanisms and pathogenesis, as well as to developing potential therapeutic modalities. For example, this basic research led to the discovery of new therapeutic targets for pathologic ocular NV (Barouch and Miller, 2006). In the field of neovascular research, the testing of angiogenic and anti-angiogenic substances relies heavily on the sensitivity and specificity of *in vivo* and *ex vivo* bioassays.

Several corneal angiogenesis models in the rabbit have been described, including direct intrastromal injections of substances like heparin-copper complex (Alessandri *et al.*, 1983), induced chemical or thermal injury (Deutsch and Hughes, 1979, Korey *et al.*, 1977), and intrastromal tumor implantation (Gimbrone *et al.*, 1974). The cornea suture model created in mice and rabbits simulate a clinical scenario and are not expensive. However, models inducing an inflammatory component have disadvantages that are surgery dependent and yield highly variable responses. Recently developed micropocket assay models are not expensive, less time consuming; however, they are surgery dependent and yield highly variable responses (Rogers *et al.*, 2007, Loughman *et al.*, 1996).

A conjunctivalized corneal epithelium lacks the smoothness and cohesion of normal corneal epithelium, making it optically inferior and prone to erosions. It is also heavily vascularized. To study inflammatory response, an important component of NV, Moromizato *et al.* produced burn and chemical cornea model lesions by corneal cauterization or local injection into the cornea of small amounts of acid (hydrochloric acid, 0.1 N) or alkali (sodium hydroxide, 0.1 N) (Moromizato *et al.*, 2000). Complex pathophysiology is a disadvantage of this model.

Animal models will continue to be needed for evaluation of safety and efficacy of combination therapies (Kim *et al.*, 2006). Each model has strengths and weaknesses; thus, in order to gain the most useful information about the disease process and responses to therapy, the best approach may be combination of different models. Therefore, search for better, more reliable and reproducible animal models should continue.

5. Treatment modalities

A number of therapeutic strategies have been adopted to treat LCSD. In patients with partial limbal stem cell deficiency, mechanical debridement of the conjunctivalized epithelium can

be performed. This prevents conjunctival epithelium from crossing the limbus and allows the corneal epithelium to heal the defect. A groundbreaking report by Kenyon and Tseng, describes case studies where autologous limbal stem cell transplantation proved to be a powerful and effective surgical procedure to restore severely damaged ocular surfaces (Kenyon and Tseng, 1989). However, in later studies, where donor tissue was harvested in a "blinded" fashion, two surgical risks were identified. First, if the donor limbal grafts do not contain adequate numbers of stem cells, the surgery will either fail immediately or lack longevity. Second, if too many stem cells are removed, the donor eye will develop problems associated with LSCD (Copeland and Char, 1990, Holland and Schwartz, 1996, Holland, 1996, Frucht-Pery *et al.*, 1998).

5.1 Amniotic membrane transplantation

Human amniotic membrane (HAM) has several properties that render it extremely useful as a biomaterial for surgical purposes and it promotes epithelialization, inhibits fibrosis, has anti-inflammatory, anti-angiogenic, antimicrobial and antiviral properties, and has a high hydraulic conductivity (van Herendael *et al.*, 1978, Gomes *et al.*, 2005, Fernandes *et al.*, 2005) Moreover, HAM shows low or no immunogenicity (Akle *et al.*, 1985, Akle *et al.*, 1981). Over the past decade, HAM's abilities to reduce scarring and inflammation, and enhance wound healing, as well as its epithelialization properties, has led to an increase in its use as a biomaterial in ophthalmic surgery, particularly for ocular surface reconstruction. HAM has also been used to promote healing in corneal diseases such as chemical and thermal burns, neurotrophic ulcers, persistent epithelial defects, shield ulcers, microbial keratitis, band keratopathy and bullous keratopathy (Dua *et al.*, 2004, Tosi *et al.*, 2005a, Tosi *et al.*, 2005b). Furthermore, the use of HAM as a biomaterial has not been confined to ophthalmology—it is also widely used in general surgery and wound treatment, e.g., for burned skin, bedsores, ulcers (Faulk *et al.*, 1980), and in head and neck surgery (Zohar *et al.*, 1987). More recently, HAM has been used as a culture substrate for *ex vivo* cultured LSC transplantation.

5.1.1 Corneal limbal autografts and allografts

The current treatment for LSCD is limbal transplantation using auto- or allograft limbal tissues, each of which have their associated risks and benefits. In 1989, Kenyon and Tseng, treated LSCD by transplanting healthy limbal tissue on diseased limbus, procedure termed as conjunctival limbal autograft (CLAU) and living-related conjunctival limbal autograft (lr-CLAL) (Kenyon and Tseng, 1989). The main disadvantage of taking limbal tissue from the patient's own contralateral healthy eye or from that of a living related donor is the risk of inducing LSCD in the donor eye, if a large amount of tissue is required (Jenkins *et al.*, 1993). In bilateral cases with total LSCD, or in a one-eyed patient who develops total LSCD in the seeing eye, an allograft limbal transplant utilizing donor tissue from a cadaver or a living relative is the only option. A high risk of immune rejection is associated with such transplantation because of the vascularity of the limbus, the high immunogenic stimulus of the limbal transplant relative to the abundance of Langerhan's cells and HLA-DR antigens. Hence, an effective immunosuppressant is essential indefinitely or until the graft is viable. Moreover, the dosage of immunosuppression can be increased or decreased if it proves ineffective or causes adverse effects, respectively (Tseng *et al.*, 1998, Coster *et al.*, 1995, Santos *et al.*, 2005). For this reason, transplantation of auto-or allograft limbal tissues is not often a viable treatment option.

5.1.2 *Ex vivo* expansion of corneal limbal stem cells

A novel method of transplanting limbal stem cells is *ex vivo* expansion of LSCs. This technique is based on Rheinwald and Green's pioneering work in skin (Green *et al.*, 1977). Skin epithelial cells have been grown successfully on a feeder layer of 3T3 fibroblasts that have been rendered mitotically inactive by irradiation or treatment with mitomycin C (Rheinwald, 1980). In the late 1990s, cultured autologous limbal epithelial cells were successfully used to improve vision in two patients with chemical injury-induced LSCD. From 1–2 mm^2 limbal tissue, epithelial cells were isolated and expanded in the laboratory on tissue culture plastic, in the presence of growth-arrested 3T3 mouse fibroblast feeders, before transfer to the eye on a petrolatum gauze or a soft contact lens (Pellegrini *et al.*, 1997). This technique has theoretical advantages over conventional treatments. Its proposed advantage over CLAU and lr-CLAL is that the required size of the limbal biopsy is substantially smaller. This minimizes the risk of precipitating stem cell failure in the donor eye and provides the option of taking a further biopsy if required.

Since 1997's landmark report, a variety of culture techniques have been developed to produce contiguous epithelial cell sheets for transplantation. These techniques can be broadly defined as either explant culture in which cells migrate out from limbal tissue attached to a surface (Grueterich *et al.*, 2002b, Koizumi *et al.*, 2001a, Koizumi *et al.*, 2001b, Sangwan *et al.*, 2006) or suspension culture in which cells are released from enzymatically digested extracellular matrix before culture (Daya *et al.*, 2005, Nakamura *et al.*, 2006, Pellegrini *et al.*, 1997). The aforementioned methods have been used in studies to culture limbal epithelial cells successfully, on either a growth-arrested 3T3 fibroblast feeder layer or an amniotic membrane (AM), with varying results (Kim *et al.*, 2004, Zito-Abbad *et al.*, 2006).

5.1.3 Explant culture method

The explant culture method involves plating a whole piece of limbal tissue or limbal explant, sized approximately 1 mm by 1 mm. One variation of the explant culture system uses AM, which acts as both a substrate and a carrier for the cultured cells. To attain growth from limbal explants the limbal biopsy is placed on the basement membrane surface of the amniotic membrane and allowed to adhere to it. Once attached, the biopsy and amniotic membrane are submerged in culture medium (**Figure 3**). This contains nutrients and mitogens that stimulate LSCs to proliferate and migrate out of the biopsy and cover the surface of the AM, which occurs over a period of 14 to 28 days.

Recently, exciting discussions have led to the question of whether or not AM epithelial cells are necessary to cultivate limbal epithelial cells. Several groups have cultured limbal epithelial cells on intact AM (AM with amniotic epithelium) and have reported that these limbal epithelial cells contained slow-cycling and label-retaining characteristic cells, which did not express corneal epithelial differentiation proteins cytokeratin 3 (CK3) or cytokeratin 12 (CK12) and connexin 43. The amniotic epithelial cells were killed by cryopreservation and then removed by enzymatic digestion, chemical treatment, or physical scraping of the membrane prior to use (Koizumi *et al.*, 2001a, Koizumi *et al.*, 2001b, Nakamura *et al.*, 2004b, Sangwan *et al.*, 2005). Grueterich *et al.* demonstrated that culturing LSC on amnion with intact amniotic epithelium may result in a more stem cell-like phenotype than with de-epithelialized amnion (Grueterich *et al.*, 2002b). Their study showed that, while cells on both the substrates were well attached to the AM stroma, morphologically superior, better stratified and/or differentiated limbal cells could be cultured on denuded AM compared to those cells cultured on intact AM. As a result, these studies hypothesized that denuded AM

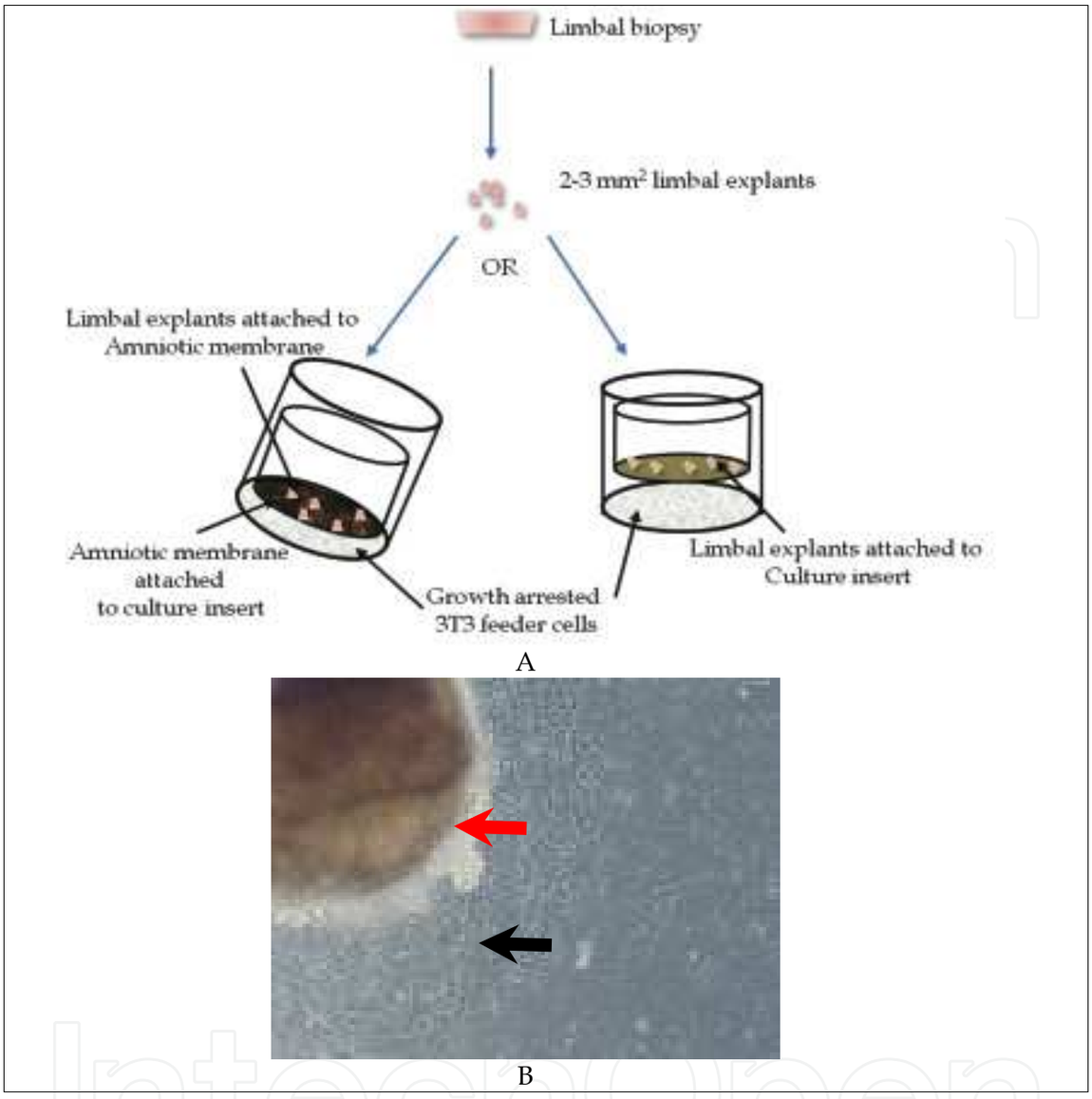

Fig. 3. The explant culture system. This method employs amniotic membrane as the carrier for cultured cells (**A**, Left); or cell culture insert (**A**-Right) as a substrate. Phase contrast micrograph reveals LSC (black arrow) migrating out of the explants (red arrow) and covering the surface of the cell culture insert over the course of 14 to 28 days (**B**).

is probably a more practical carrier for human limbal epithelial cell cultures in a cell-suspension culture system. Kolli *et al.* analyzed the outgrowths from human cadaveric limbal explants cultured on AM by dividing the explant outgrowths into three zones—inner, middle, and outer, depending on proximity to the explant. This yielded a successive decline in colony-forming efficiency (CFE), and ΔNp63α and ABCG2 expression, and an increase in expression of CK3 in zones further away from the explants. These Results support the importance of putative niche environment in maintaining the undifferentiated state of the limbal stem cells during explant outgrowth (Kolli *et al.*, 2008).

5.1.4 Suspension culture system

The cell suspension culture system employs the enzymes dispase, which digests basement membrane collagen and separates epithelial cells from the stroma, and trypsin, which separates clumps of limbal epithelial cells into a suspension of single cells (**Figure 4**). The single cells are then seeded either onto AM or onto a plastic tissue culture dish containing a feeder layer of growth-arrested 3T3 fibroblasts. Culture medium is then added, and the cells are incubated for 14 to 21 days. When confluent, the epithelial sheet is transferred to the ocular surface using either a contact lens or fibrin gel (Pellegrini *et al.*, 1997, Pellegrini *et al.*,

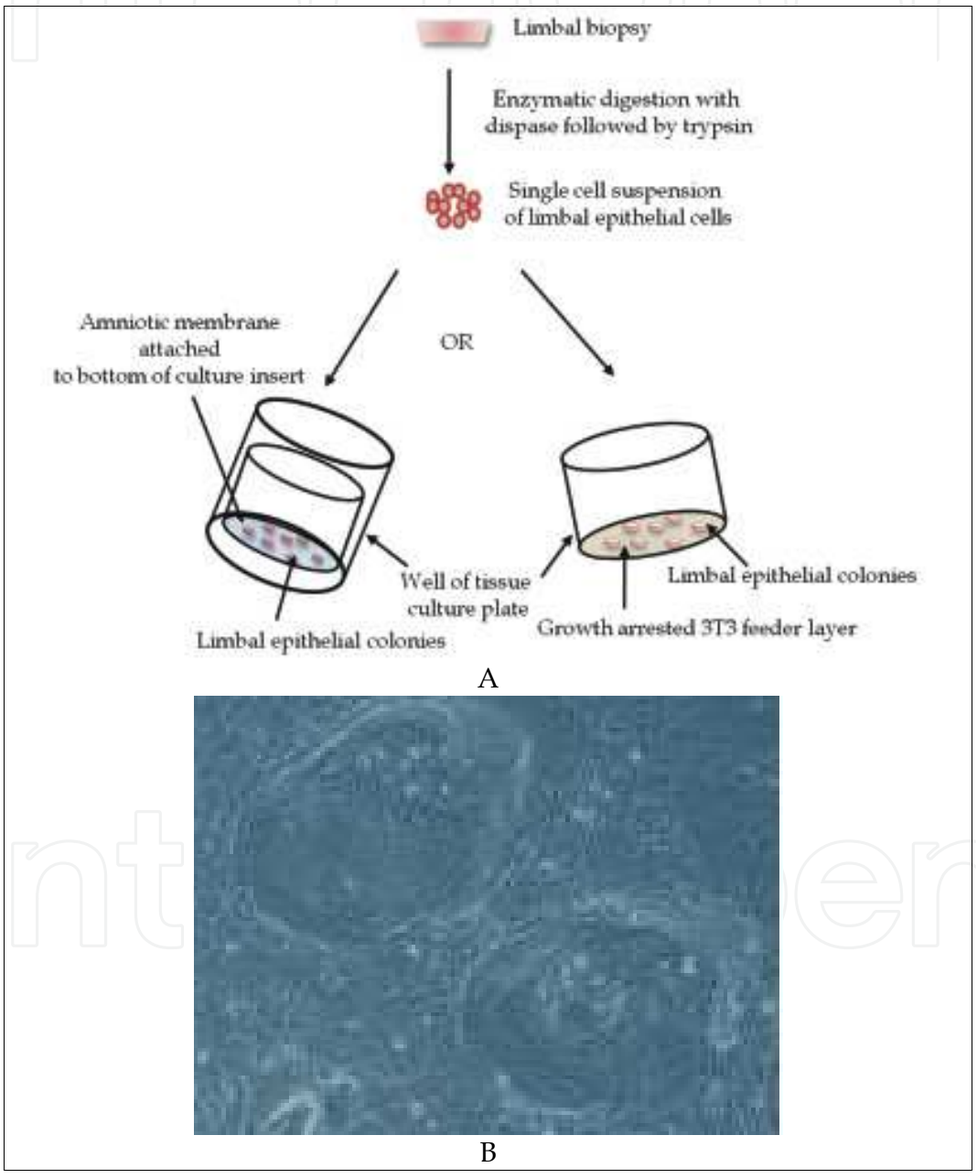

Fig. 4. The suspension culture system. This method employs the enzymes dispase and trypsin. This suspension is seeded onto either amniotic membrane (**A**, Left) or a plastic tissue culture dish that contains a feeder layer of growth-arrested 3T3 fibroblasts (**A**, Right). Phase contrast micrograph of limbal epithelial colonies on 3T3 cells (**B**).

1999). Recently, Di Girolamo *et al.* cultured limbal epithelial cells in autologous serum, on lotrafilcon A and balafilcon A contact lenses. The cells cultured on the lotrafilcon A contact lenses proliferated, migrated, showed a corneal phenotype and microvillae on the apical surface; whereas cells cultured on balafilcon A contact lenses showed no growth (Di Girolamo *et al.*, 2007).

Various substrates have been used in suspension culture systems for proliferation of limbal epithelial cells, for example, reconstituted collagen substrate membrane from HAM (Merrett *et al.*, 2008), a chitosan cross-linked collagen membrane (Auxenfans *et al.*, 2009), which has good adherence, retains epithelial morphology and supports the nontoxic nature of the membrane. Reconstituted human amniotic collagen membrane has been used to act as collagen scaffolding for the culture of limbal epithelial cells (Doillon *et al.*, 2003, Griffith *et al.*, 1999, Li *et al.*, 2005). Rama *et al.* have provided data demonstratng that the use of fibrin as a carrier supports stem cell maintenance (Rama *et al.*, 2001).

5.1.5 Variations in explant and suspension cultures

The variations in explant and cell suspension culture systems are airlifting and/or (with or without the addition) of 3T3 feeder cells. In airlifting cultures, the level of culture medium in the dish is lowered to the surface level of the epithelium, promoting stratification and differentiation of the epithelium (Ban *et al.*, 2003, Cooper *et al.*, 2004, Koizumi *et al.*, 2001a, Nakamura *et al.*, 2003b). This method can, therefore, be used for investigating the stratification and differentiation potential of cultured limbal epithelial cells with high expression of CK3 and CK12. The latter culture method, adding 3T3 feeder cells as an additional feeder layer of growth-arrested 3T3 fibroblasts in the bottom of the cell culture well is a duplex system (Miyashita *et al.*, 2008). 3T3 fibroblasts are primitive cells isolated from embryonic mice. They have a high proliferative capacity and have been used extensively in the culture of epithelial stem cells especially in skin and cornea. Both amniotic membrane and growth-arrested 3T3 fibroblasts inhibit the differentiation of corneal epithelial cells *ex vivo*, which allows the expansion of the population of LSCs (Grueterich *et al.*, 2003, Pellegrini *et al.*, 1999). Denuded AM is thought to be an excellent substrate for the corneal limbal cell culture used for ocular surface reconstruction, even though AM epithelial cells contain various growth factors with 3T3 feeder cells. This theory, however, remains to be fully investigated.

The variation in suspension culture techniques are use of novel temperature-responsive culture surfaces. Temperature-responsive polymers chemically immobilized to thin films on cell-culture surfaces facilitate cell adhesion and growth of cells in normal culture conditions, at 37°C. Temperatures below 30°C can reversibly alter cell hydration properties with respect to temperature, hydration and swelling—prompting complete detachment of adherent cells without the use of proteolytic enzymes or treatment with EDTA (Nishida *et al.*, 2004b, Nishida *et al.*, 2004a). The advantage of this system is that no enzymes are needed to free the epithelial sheet from the culture membrane; however, application of temperature-responsive polymers in a clinical setting remains questionable for safety and efficacy.

6. *Ex vivo* expansion of alternative sources of epithelium

Although adult stem cells hold considerable promise for the treatment of a number of diseases in regenerative medicine, the second major obstacle has been to obtain sufficient number of autologous or allogeneic stem cells.

6.1 Oral mucosal cells

In an attempt to overcome the problems inherent in the transplantation of allogeneic tissue, there has been recent interest in the possibility of using alternative autologous epithelial cells. There are several potential sources of non-keratinizing, stratified squamous epithelium in the adult human, including oral mucosal, conjunctival, nasal, esophageal, vaginal and rectal epithelia. The main concept is to use the smooth surface of the oral mucosa, with its stem cell properties, to reconstruct the ocular surface. Oral mucosae are thought to be at a lower stage of differentiation than epidermal keratinocytes (Collin *et al.*, 1992, Schermer *et al.*, 1986) because they divide rapidly and can be maintained in culture for prolonged periods without keratinization (Hata *et al.*, 1995). Various antimicrobial peptides (AMPs) are known to be present on the epithelial cells of ocular and oral surfaces (Haynes *et al.*, 1999). Moreover, keratin 3 is expressed by both corneal epithelium (Sangwan *et al.*, 2003b) and oral mucosa (Collin *et al.*, 1992, Juhl *et al.*, 1989), suggesting that gene expression in oral and corneal epithelium may be similar. Wet-surfaced epithelia, produce a group of highly glycosylated, protective membrane glycoproteins termed mucins (Gipson, 2007). Although the function of these mucins in the oral cavity remains to be elucidated, it is possible that they contribute to the epithelial protective mucin layer and act as receptors initiating one or more intracellular signal transduction pathways (Argueso *et al.*, 2003). At the ocular surface, at least three membrane-associated mucins (MUC1, -4, and -16) and two secreted mucins (MUC5 and -7) are expressed (Hori *et al.*, 2007).

More extensive studies have been performed to check the feasibility of using cultivated oral mucosal epithelium (COME) for this purpose, as it is readily available and could be harvested without invasive surgery. A small biopsy of the patient's oral mucosa is harvested, connective tissue is dissected and, after enzymatic digestion, an epithelial cell suspension is prepared. The oral epithelial cells are cultivated on the substrate with a feeder layer. The sheet of cultivated cells is then transplanted onto the diseased ocular surface (**Figure 5**). These studies suggest that oral mucosal epithelium is a feasible alternative for

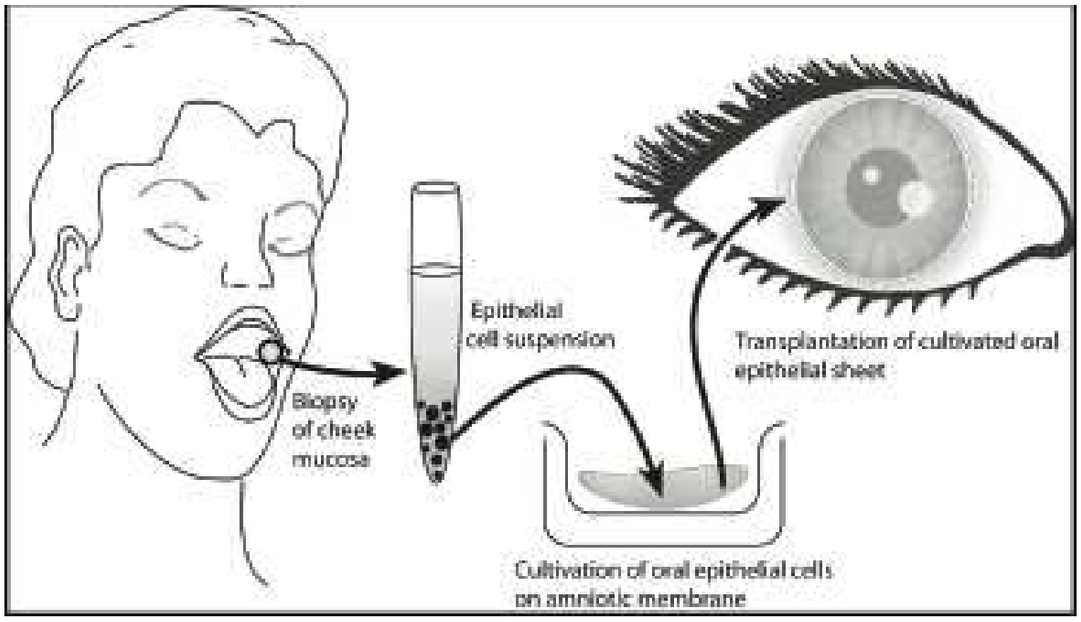

Fig. 5. Systematic representation of cultivated oral mucosal epithelium transplantation. *Adopted from (Lim et al., 2009)*.

allogenous limbal transplants (Nakamura *et al.*, 2003b, Nakamura *et al.*, 2004a, Inatomi *et al.*, 2006, Nishida *et al.*, 2004b, Hayashida *et al.*, 2005). Oral mucosal epithelium cultured on human amniotic membrane with the help of feeder cells has been characterized extensively, and has been used to reconstruct the ocular surface in rabbits (Nakamura *et al.*, 2003a) as well as humans with chemical injury and SJS (Nakamura *et al.*, 2004a). In the lengthiest study reported so far, cultured oral mucosal epithelial cells were transplanted into patients with LSCD and followed up with for up to 34 months (Inatomi *et al.*, 2006) where the followups continued until tissue became viable.

The main advantages of using oral mucosa are the absence of tissue rejection and, therefore, avoidance of immunosuppression; easy access to the oral cavity; inconspicuous location of the resultant small scar, and repeatability. This treatment method has not, however, provided satisfactory long-term results. The oral mucosal epithelial cell grafts seldom transdifferentiate to a corneal epithelium phenotype, e.g., expression of keratin 12 (Krt12) (Inatomi *et al.*, 2006). This new approach, however, provides an exciting possibility for treatment of this difficult group of patients with blindness from bilateral total LSCD and warrants further study.

6.1.2 Dental pulp stem cells

In an attempt to prevent rejection, isolated human immature dental pulp stem cells (hIDPSC) from deciduous teeth have been used as an autologous alternative epithelium source for LSCD treatment or corneal reconstruction. hIDPSCs are known to express both mesenchymal stem cell markers (SH2, SH3 and SH4) and human embryonic stem cell markers (OCT 4, NANOG, SSEA-3 and SSEA-4) (Gomes *et al.*, 2010). Furthermore, hIDPSCs have a normal karyotype and show the capacity for multilineage differentiation into neurons, smooth and skeletal muscle, cartilage, bone, and other cell types *ex vivo* and *in vivo* (Kerkis *et al.*, 2006, Lavagnolli *et al.*, 2009). Limited studies have been performed to test dental pulp stem cells as potential treatment for LSCD. Monteiro *et al.* demonstrated, using immunohistochemistry and reverse transcription polymerase chain reaction, that hIDPSCs express markers in common with LSCs, such as ABCG2, integrin β1, vimentin, p63, connexin 43 and cytokeratins 3/12. These have been shown to be capable of reconstructing the eye surface after induction of unilateral total LSCD in rabbits (Monteiro *et al.*, 2009). Further studies are required to understand the long-term outcome of LSCD treated using hIDPSCs.

6.1.3 Hair follicle stem cells

Hair has the potential to regenerate from stem cells located at the lower part of the follicle called the bulge (Oshima *et al.*, 2001). Additionally, hair follicle stem cells (HFSCs) can give rise to epidermis and sebaceous gland tissues. Recently, Blazejewska *et al.* demonstrated that murine HFSCs can transform into epithelia-like cells when cultured in limbal fibroblast-derived medium (Blazejewska *et al.*, 2009). Although, these findings need further substantiation using *in vivo* functional studies in animal models, they provide the first step towards the design of protocols that could use human autologous hair follicle stem cells to replace corneal epithelium in therapeutic applications. Due to their multipotency, easy accessibility, and high proliferation rate *ex vivo*, hair follicles are an attractive source of autologous adult stem cells and a promising therapeutic tool for ocular surface reconstruction.

6.1.4 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent (i.e., they can give rise to any cell type in the body) and are derived from the inner cell mass of a blastocyst. Differentiation of ESCs to a corneal epithelial lineage could be achieved by replication of the LSC niche environment. Transplantation of epithelial cells derived from mouse ESCs have been used to reconstruct chemically injured mouse corneas (Homma *et al.*, 2004). Moreover, human ESCs express cornea-like epithelial markers such as CK3 and CK12 when cultured in conditions similar to those of the limbal stem cell niche. *Ex vivo* replication of the LSC niche environment has been achieved by culturing hESCs on an extracellular matrix of collagen IV and fed with medium conditioned with limbal fibroblasts (Ahmad *et al.*, 2007). The differentiated human ESCs demonstrate phenotypic differences to cultured human limbal epithelial cells, such as differences in size and cilia length, which are characteristically much longer than those of limbal epithelial cells. These data suggest that some differences exist between LSCs and hESC-derived corneal progenitors (Ahmad *et al.*, 2007). The translation of this approach to human therapeutic use requires further work to overcome problems associated with functionality, complete hESC differentiation and immune rejection as well as ethical concerns.

7. Future considerations for cultivated epithelial transplants

Ex vivo expansion of limbal epithelium has been performed by several unrelated groups in a number of countries and used to treat a variety of ocular surface disorders that are thought to be the result of limbal stem cell failure. Despite a substantial number of experimental models of this technique and an ever-growing body of laboratory data on limbal epithelial stem cell biology, scientific basis for this procedure is poorly understood. Future considerations in the field of limbal epithelial cell culture and transplantation methods are discussed below.

7.1 Culture methodologies

The culture of limbal epithelium is essential for furthering our understanding of limbal stem cell biology. *Ex vivo* expansion of LSCs for the treatment of LSCD is one of the more recent stem cell treatments available in the field of regenerative medicine. Although the technique of *ex vivo* expansion of LSCs is still in its infancy, it is starting to be used by several groups for clinical purposes, and different approaches have been attempted. There are many varying techniques for culturing limbal epithelial cells, such as different media and sera; culture with 3T3 fibroblasts or amniotic membrane, or both; removal of amniotic membrane epithelial cells from the membrane, or not; use of explant or suspension methods; and airlifting of the culture, or not (Shortt *et al.*, 2007, Grueterich *et al.*, 2003). Whichever culture method is used, the end result, often after a period of weeks, is a cultivated epithelial sheet composed of LSCs. The ease of explanting culture technique could be adapted without initial dispase treatment for transplantation studies. However, further studies on the interaction between epithelial progenitor and autologous stromal cells *ex vivo,* and complete characterization of limbal cells in this culture system, including cell junction proteins, are required.

7.1.2 Autologous sources and xenobiotic-free conditions

With growing concerns regarding the potential transmission of opportunistic agents such as prions and animal viruses, it would be preferable to culture cells for human transplantation

under xenobiotic-free conditions. In limbal corneal epithelial cultures, the use of autologous human serum in the medium and/or human amniotic membrane would be appropriate as it would reduce the need for animal cells or products in the culture (Kolli *et al.*, 2010, Nakamura *et al.*, 2004a, Nakamura *et al.*, 2006). To avoid contamination from xeno-feeder layers, different human feeder layers have also been developed and analyzed (Chen *et al.*, 2007). Moreover, cells have been cultured on feeder layers during the proliferative phase and further separated using robotic technology (Schneider *et al.*, 2008). Limbal epithelial cells grown with MRC–5 human embryonic fibroblasts can sustain the stem cell phenotype (Notara *et al.*, 2007). The successful use of autologous serum–derived oral epithelial equivalent to treat severe ocular surface disease was reported in 2006 (Ang *et al.*, 2006, Nakamura *et al.*, 2006). This represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation.
In conclusion, a specific culture system that mimics natural tissue architecture and its niche closely by enabling formation of an autofeeder layer, thereby eliminating the need for xeno-feeder layers for *ex vivo* expansion of epithelial progenitor cells is important.

7.1.3 Combination techniques with anti-angiogenic treatments

Angiogenic and anti-angiogenic cytokines are involved in cell migration and differentiation in wound healing and tumor progression (even in the absence of angiogenesis). The ever-increasing use of anti-angiogenic therapy for corneal neovascularization brings great promise for the treatment of a wide variety of corneal conditions, including LSCD. TGF-β, IL-1, and VEGF are the key pro-angiogenic factors involved in the process of angiogenesis; and are targets for anti-angiogenic therapies. The ultimate goal for research on corneal angiogenesis is to discover effective treatment for pathological corneal neovascularization; however, to date, most anti-angiogenic therapies remain experimental.
Angiostatin, a 38 kDa proteolytic fragment of plasminogen, is a potent anti-angiogenic factor. Recombinant angiostatin has been used successfully to suppress tumor growth and metastasis in animal model systems (Shin *et al.*, 2000, Ambati *et al.*, 2002). Gabison and associates have demonstrated the involvement of angiostatin in corneal avascularity after wounding. They confirmed that angiostatin-like molecules are expressed in the corneal epithelium and in cultured corneal epithelial cells (Gabison *et al.*, 2004). Corneal neovascularization was observed after excimer laser keratectomy when anti-angiostatin antibodies were injected into the cornea; this was significantly higher than when plasmin B chain antibodies were injected. These studies suggest that angiostatin may contribute to the maintenance of corneal avascularity after excimer laser keratectomy.
The corneal epithelium may be the source of anti-angiogenic molecules. It has long been demonstrated that corneal epithelium inhibits angiogenesis (Kaminski and Kaminska, 1978). Ferreras and associates demonstrated the generation of endostatin-like fragments by cleaving collagen XVIII with MMP-2, -3, -9, -12, -13, or -14 (Ferreras *et al.*, 2000). Upregulation of matrix-derived anti-angiogenic factors such as endostatin (Kato *et al.*, 2003) and restin (Saika *et al.*, 2004), along with increased anti-inflammatory factor IL-1ra may play an important role in LSC and AM transplantation-mediated anti-angiogenic effect. Recently, Cursiefen *et al.* demonstrated a critical mechanism that contributed to corneal avascularity by VEGF receptor 3, which is normally present on lymphatic and proliferating blood vascular endothelium, is strongly constitutively expressed by corneal epithelium and is mechanistically responsible for suppressing inflammatory corneal angiogenesis (Cursiefen *et al.*, 2006). Knowledge gained from using epithelia–matrix interaction to regulate corneal

angiogenesis will enable us to optimize the anti-angiogenic effect of the cultivated cells like oral mucosal epithelial cells or mesehchymal stem cells for future ocular surface reconstruction.

8. Clinical trials and outcomes: present and future

LSCD by any cause may result in poor corneal epithelialization, persistent epithelial defects, corneal vascularization, corneal scarring, and so-called conjunctivalization of the cornea. These problems may in turn lead to decreased vision, ocular discomfort and pain, as well as an unstable ocular surface. Limbal stem cells may be partially or totally depleted, resulting in varying degrees of stem cell deficiency and resulting abnormalities on the corneal surface. The first use of *ex vivo* expanded LSCs for the treatment of LSCD in human subjects was described by Pellegrini and co-workers, who used a culture system of LSCs grown on mouse J2-3T3 fibroblasts with fetal calf serum (FCS) supplemented media (Pellegrini *et al*., 1997). Since then, several studies have been reported on the transplantation of *ex vivo* cultured LSCs to treat LSCD (Schwab *et al*., 2000, Tsai *et al*., 2000, Koizumi *et al*., 2001b, Grueterich *et al*., 2002a). Schwab *et al.* reported the results of treating 19 eyes of 18 patients (Schwab, 1999). In 2003, Sangwan *et al.* reported the largest study to date, which enrolled 125 patients (Sangwan *et al*., 2003b). In this study *ex vivo* cultured limbal and conjunctival epithelial cells were grown on AM. In 2006, Sangwan *et al*. published a more detailed report on clinical outcomes for 88 eyes of 86 patients (Sangwan *et al*., 2006). In 2001, Rama *et al*. reported treatment outcomes for 18 eyes of 18 patients (Rama *et al*., 2001). The same group recently reported a 10 year follow-up with permanent restoration of transparent corneal epithelium in 76.6% of eyes, with few 63 instances of inflammation out of 113 eyes treated (Rama *et al*., 2010). The outcome of cultured limbal stem cells transplants differed significantly depending on whether the transplanted cultures contained more than 3% p63-bright holoclone-forming stem cells or 3% or less — the success rates were 78% with the larger number of stem cells and 11% increase with the smaller number of p63 cells (Rama *et al*., 2001, Rama *et al*., 2010), suggesting the importance of a high percentage of p63 in cultured limbal stem cells. The remaining studies each treated between 2 and 14 patients (Kolli *et al*., 2010, Grueterich *et al*., 2002b, Nakamura *et al*., 2004a, Sangwan *et al*., 2003a).

In total, current studies reported data to enable analysis of outcomes in 506 eyes (Baylis *et al*., 2011) treated using *ex vivo*, cultured LSC transplants and 27 eyes treated with *ex vivo* cultured oral mucosal epithelium. To date, the limited periods (mean follow-up was 16.74 months, median 14 months, range 6 to 29.5 months), the use of subjective outcome measures, and the absence of a reliable method for detecting transplanted (stem) cells shows that a long term success rate has yet to be established.

Ex vivo expansion and transplantation of limbal epithelium have been performed by several unrelated groups in a number of countries, with uncertainty in the limbal stem cell cultivation, with different variables, including source of donor limbal tissue, culture method (e.g., suspension versus explant culture), culture composition (e.g., 3T3 fibroblasts, amniotic membrane and fibrin), and variation in culture time. Overall the success rate for cultured limbal epithelial transplantation is 76%. This is based on clinical restoration of the corneal epithelium. It is interesting that, despite the different methodologies employed, the success rates and number of significant clinical outcomes are remarkably high. It would, therefore, appear that, as long as viable limbal epithelial stem cells are transferred, the method used to achieve this is relatively unimportant.

The future directions in LSC transplantation involve modifications of existing technologies to allow improved safety and efficacy of techniques at hand. The studies on the molecular mechanism of limbal stem cells and their niche will enhance our knowledge of how cell therapy can be further modified to closely mimic *in vivo* conditions. Identification of key niche factors controlling limbal stem cell behaviour would allow these conditions to replicate *ex vivo* and, thus, make the process of culturing limbal stem cells safer and more efficient. Newly developed transplantation techniques using tissue-engineered epidermal adult stem cells, immature dental pulp stem cells (Gomes *et al*., 2010), and hair follicle bulge-derived stem cells (Blazejewska *et al*., 2009) were reportedly successful for the reconstruction of corneal epithelium in an animal model of severe LSCD. The recombinant human cross-linked collagen scaffold and a Food and Drug Administration-approved contact lens are also promising new techniques for successfully achieving ocular surface reconstruction (Di Girolamo *et al*., 2007). There are main challenges in improving the established techniques, in elimination of animal-derived products and minimization of allogeneic human tissue use. Since cell-based therapies are being scrutinized by ever-increasing regulatory requirements, many investigators that have been successfully performing ex vivo cultivation in the past are reformulating their methodologies to conform to these guidelines (Daniels *et al*., 2006, Kolli *et al*., 2010). The future of this methodology lies in the standardization of the stem cell preparation and cultivation techniques with clearly defined endpoints and outcome measures of treatment success.

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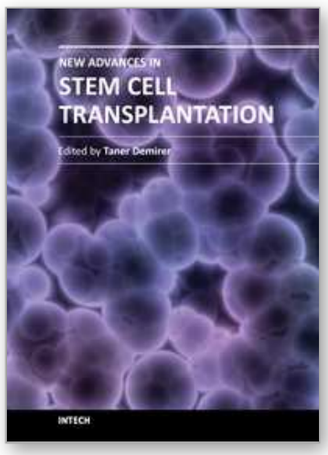

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