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Selective Expansion of Limbal Epithelial Stem Cells in Culture Using Hypoxia

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Abstract

Limbal epithelial stem cells (LESCs) maintain the corneal epithelium throughout life and are crucial for both corneal integrity and vision. In this study, LESCs were expanded in either a culture system using 3T3 feeder cells in growth medium supplemented with serum, or in a culture system without feeder cells using commercially available serum-free medium (EpiLife). Cells were maintained at an ambient oxygen concentration of 20% or at various levels of hypoxia (15%, 10%, 5%, and 2%) throughout the period of expansion. The effect of ambient oxygen concentration on growth, cell cycle, colony forming efficiency (CFE), and expression of stem cell markers ABCG2 and p63α and differentiation marker CK3 were determined at different time points. Low oxygen levels were found to maintain a stem cell phenotype with low proliferative rate, high CFE, and high expression of ABCG2 and p63α as well as low expression of CK3. The relation between degree of differentiation and ambient oxygen concentration in the culture system seems to mirror the natural environment of the limbal niche. Hypoxic culture could therefore potentially improve stem cell grafts for cultured limbal epithelial transplantation (CLET).

Keywords: Limbus cornea, Adult stem cells, Regenerative medicine, Cell hypoxia, Primary cell culture.

1.1 Introduction

Vision is important for normal quality of life and blindness is universally feared. The World Health Organization (WHO) has estimated that the prevalence of blindness is around 39 million people worldwide with up to 285 million people having impaired vision [1]. Corneal opacities accounts for 4% of blind cases and around 1% of impaired vision cases, and treatment for these causes of decreased visual acuity has traditionally relied on tissue donations for either keratoplasty or limbal tissue transplantation. Regenerative ophthalmic medicine holds great promise to deliver new treatment regimens to address current challenges such as scarcity of donor materials.

The cornea can be regarded as the window of the eye as it allows light to enter into the eye to reach the phototransducing cells of the retina. This function is crucially dependent on intact transparency of corneal tissue, which can be disturbed in a variety of clinical settings. The cornea and associated tear film are furthermore responsible for almost two-thirds of the total refractive power of the eye [2] and hold important protective properties for inner ocular structures.

The human cornea is composed of three cellular layers and two interface layers. The superficial cellular layer facing the external environment is a multilayered, non-keratinized squamous epithelium [3], that is separated from the corneal stroma by a thin acellular layer termed Bowman's membrane. The corneal stroma accounts for 90% of the total corneal thickness of 515 $\pm$ 33 μm [4], and is composed of regularly arranged collagen lamellae that promotes transparency by removal of light scatter in a process of destructive interference [5]. The innermost cellular layer of the cornea is the corneal endothelium, that is composed of a monolayer of non-regenerative cells *in vivo* that maintains corneal clarity by both a barrier function, ascribed to intercellular tight junctions, but also an active pump function using Na^+/K^+ ATPase to regulate hydration levels of the corneal stroma [6]. Corneal transparency and thereby function is dependent on the integrity of all corneal cellular layers but also health of ocular adnexa. Diseases involving one or more cellular layers of the cornea give rise to decreased visual acuity and debilitating symptoms in patients.

In recent years, the field of regenerative ophthalmic medicine has experienced great progress towards curing corneal diseases affecting one or more of the cellular layers using bioengineered tissue replacements. As mentioned below, Pellegrini and co-authors pioneered the treatment of corneal epithelial diseases by transplantation of *ex vivo* expanded epithelial stem cells [7]. Bioengineering techniques are rapidly developing that enable surgeons to perform keratoplastic procedures using artificially crafted stromal replacements [8]. Finally, corneal endothelial substitutes can treat diseases of the innermost endothelial cell layer that impede the pump function resulting in corneal edema [6].

1.2 Clinical Application of Bioengineered Corneal Epithelial Stem Cell Grafts

The corneal epithelium is a multi-layered, non-keratinized squamous epithelium that is continuously regenerated by dedicated unipotent tissue-specific stem cells. These stem cells are termed limbal epithelial stem cells (LESCs), and are located in specific stem cell niches in the corneal limbus called limbal crypts [9], limbal epithelial crypts [10] and focal stromal projections [10]. From this nurturing micro-environment, LESCs divide by symmetrical or asymmetrical cell division to give rise to a population of more committed progenitors termed transient accelerating cells (TACs) that migrate centrally and superficially to differentiate into postmitotic cells (PMCs) and ultimately to terminally differentiated cells (TDCs) that are continuously lost to the external environment (Figure 1.1). This spatially unique differentiation scheme for corneal epithelial homeostasis has been mathematically explained in the so-called X, Y, Z hypothesis of corneal epithelial regeneration [11]. Various diseases, most often grouped into acquired, hereditary, iatrogenic and idiopathic causes [12], give rise to defect or dysfunctional LESCs thereby disrupting epithelial homeostasis resulting in decreased visual acuity and pain in patients. This disease of the corneal epithelial stem cell population has been termed limbal stem cell deficiency (LSCD) and treatment requires transplantation of LESCs.

Traditional keratoplasty cannot cure these patients, as only the central button being devoid of LESCs is transplanted to the recipient. Historically, LESCs have been transplanted using whole tissue blocks from donors by homologous penetrating central limbal keratoplasty (HPCLK) [13], keratolimbal

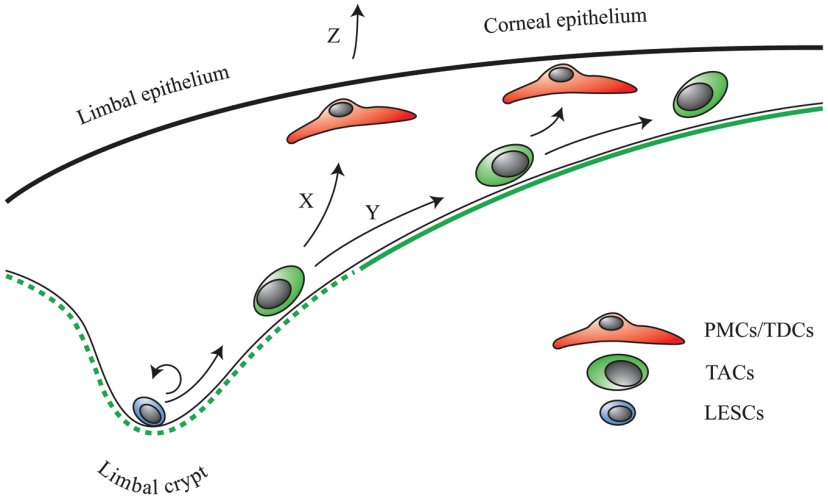

Figure 1.1 The X, Y, Z hypothesis of corneal epithelial maintenance. Stem cells (LESCs) are capable of symmetrical/asymmetrical cell division and differentiate into TACs during centripetal cell migration (Y). Transient accelerating cells mature into PMCs/TDCs during superficial migration (X) and are ultimately sloughed off to external environment (Z). Epithelial homoeostasis requires X + Y = Z. Green line represents basement membrane. LESCs, limbal epithelial stem cells; TACs, transient accelerating cells; PMCs, postmitotic cells; TDCs, terminal differentiated cells. Reproduced from [3] with permission from Wiley Blackwell.

allografts (KLAL) or conjunctival limbal autografts (CLAU) [14]. Recently, researchers have focused on curing LSCD by transplantation of bioengineered tissues containing LESCs to patients in a process termed cultured limbal epithelial transplantation (CLET) [14]. CLET was originally performed by Pellegrini and co-authors [7] using culture techniques analogous to epidermal research [15, 16]. The process of CLET is outlined in Figure 1.2. Briefly, the technique includes acquiring a small biopsy containing LESCs from either a donor eye in cases of bilateral LSCD or the contralateral eye of the recipient in cases of unilateral disease. These cells are subsequently expanded in the laboratory on a dedicated carrier like e.g. amniotic membrane. The bioengineered transplant containing LESCs is then transferred to the surgical facility, where the recipient eye is prepared by debridement of pannus. The bioengineered stem cell transplant is finally placed on the corneal wound bed to enable recreation of a stable corneal epithelium. In a subsequent surgical procedure it is possible to replace opaque stroma in more profound disease by either conventional keratoplasty or by using artificial stromal replacements [8]. CLET has theoretical advantages compared to traditional

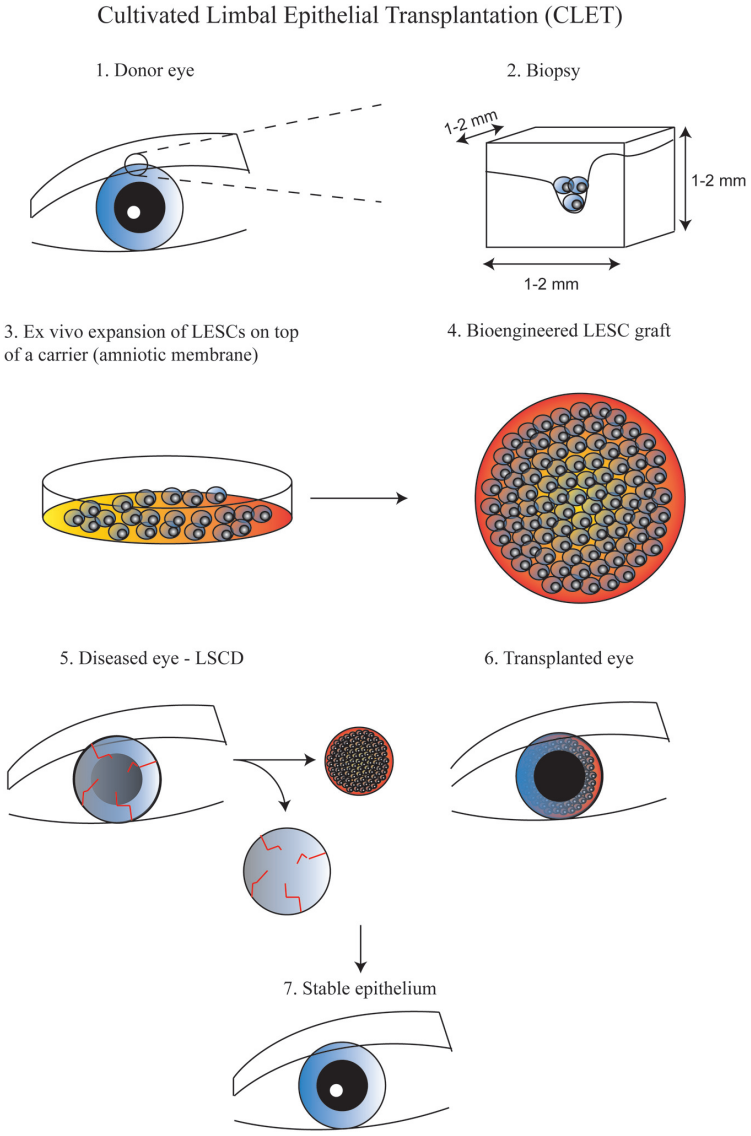

Figure 1.2 Procedure of cultivated limbal epithelial transplantation (CLET). (1) A biopsy containing LESCs is obtained from healthy corneal limbus. (2) LESCs in limbal crypts are enzymatically isolated from a small biopsy of 1–2 mm for *ex vivo* expansion. (3–4) A monolayer of limbal epithelial cells expanded *ex vivo* on a carrier constitutes a bioengineered stem cell graft. (5) Recipient eye is prepared by debridement of diseased pannus tissue. (6–7) Placing the graft on the wound bed recreates a stable surface epithelium. CLET, cultivated limbal epithelial transplantation; LESCs, limbal epithelial stem cells; LSCD, limbal stem cell deficiency.

tissue transfer techniques like KLAL or CLAU such as reduced risk of iatrogenic LSCD in the donor eye, diminished risk of rejection due to lack of Langerhans´cells within the graft and the possibility of repeating surgical procedure in case of initial failure [3]. Optimisation of culture techniques though seem important, as it has been shown that the likelihood of success after CLET is substantially increased if the cultured graft contains above 3% ΔNP63α positive cells [17], which is a widely accepted stem cell biomarker for LESCs [18].

1.3 Culture Techniques for Bioengineered Stem Cell Sheets

Currently, two major culture techniques exist for propagation of LESCs *ex vivo* prior to CLET. The explant system expands cells by placing small tissue blocks measuring 1–2 mm containing LESC niche structures on a suitable substrate allowing subsequent outgrowth of cells during the culture process. The so-called "dissociation" system enzymatically dissociates cells from a tissue sample prior to seeding cells on a suitable substrate for *ex vivo* expansion. A direct comparison of these methods for crafting bioengineered cell sheets for CLET seem to support that the dissociation system is superior to the explant system in expanding LESCs [19]. Some authors have suggested that the explant system mainly allows outgrowth of TACs, and it has also been suggested that progenitors could be lost in a process of epithelial-mesenchymal transition [20]. Optimal enzymatic dissociation of cells in the dissociation system seem to be performed using a combination of dispase II and trypsin/EDTA [21], and evidence using scanning electron microscopy supports that also LESCs located in deep niche structures are harvested by this enzymatic treatment [19]. Since the advent of CLET, much emphasis has been placed on the optimisation of various culture variables to improve outcome of surgery, but only recently has emphasis been placed on the gaseous environment of cultures [22].

1.4 Selective Expansion of LESCs Using Hypoxia

Increasing awareness of oxygen as a regulator of stem cell growth and differentiation has prompted investigators to rename traditional hypoxic culture conditions as *in situ* normoxia [23] or physiological normoxia [24], as these

gaseous conditions reflect the expected milieu encountered by stem cells in their niches. Hypoxia has been shown to maintain various stem cells in culture [25, 26], most likely attributable to a concerted action by hypoxia inducible factors (HIFs) (reviewed in [27]). Kwan and co-authors performed measurements of oxygen concentrations in various layers of the central cornea in rabbits breathing atmospheric oxygen, and were able to show that oxygen decreased from 123 ± 10 mmHg below the tearfilm to 65 mmHg at the epitheliostromal junction [28]. Several factors could easily contribute to an even lower oxygen concentration in stem cell niches at the corneal limbus such as human corneal epithelium being thicker than rabbit epithelium [29] and the limbal epithelium having more cellular layers than central corneal epithelium [30]. Furthermore, a nocturnal decrease in oxygen availability during sleep due to eye lid closure could easily increase hypoxia in the limbal epithelium [31], and as oxygen concentrations in most peripheral capillaries are 0.5–2.6%, the perilimbal vascular arcades are not expected to change the hypoxic nature of the niche.

Using a dedicated hypoxic cell culture facility (BioSpherix Xvivo facility, http://www.biospherix.com/equipment/cytocentric/systems.html), we have recently tested the effect of different oxygen concentrations of 2%, 5%, 10%, 15%, and 20% on the growth and differentiation status of limbal epithelial cells (LECs) in the dissociation culture system using two commonly used media formulations [22]. Cultures were analyzed using advanced computerized fluorescence microscopy and software analysis on whole populations of LECs after expansion in either a culture system employing γ-irradiated 3T3 cells as feeder cells and serum containing medium or in a newer system devoid of feeder cells using a commercially available serum-free semi-defined medium called EpiLife. Expanded cells exhibited a LESC phenotype in hypoxic culture conditions of 2–5% with slow growth, high colony forming efficiency, high expression of presumed stem cell markers tumor protein 63 isoform α (p63α) and ATP-binding cassette subfamily G member 2 (ABCG2) and low expression of differentiation marker cytokeratin 3 (CK3). Conversely, cells expanded in 15% O_2 exhibited slow growth, low colony forming efficiency, low expression of p63α and ABCG2, and high expression of CK3. Interestingly, 15% O_2 resembles the oxygen concentration measured by Kwan and co-authors in the differentiation compartment directly below the tear film [28]. An intermediate O_2 concentration of 10% revealed cellular phenotypes resembling transient accelerating cells with fast growth, intermediate expression levels of both stem cell and differentiation markers as well as intermediate level of colony

forming efficiency. The upregulation of particular genes related to hypoxia in the deep limbal crypts has been shown using laser capture microdissection and RNA-sequencing [32].

1.5 Future Perspectives

Being able to reproduce cellular phenotypes in culture, as they exist along their spatially defined differentiation pathway *in vivo*, by controlled atmospheric oxygen levels could have great implications for the optimization of bioengineered stem cell grafts used in the CLET procedure, as it has been shown that the success ratio of surgery is critically dependent on a high fraction of stem cells contained within the graft [17]. Novel regenerative therapies within ophthalmology using tissue replacements hold promise to circumvent current shortcomings of therapy caused by scarcity of donor materials.

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