CHAPTER 2

LITERATURE REVIEW

Cornea

Cornea is one of the important structures that accounted for obtaining a good vision. It is the most anterior portion of the eyeball, comprising one-sixth of the outermost tunic fibrosa of the eye (Figure 2.1). Cornea is the transparent avascular tissue that receives nutrients from aqueous humor which circulates from posterior to anterior chambers. Moreover, it gets oxygen directly that dissolves in the tears and then diffuses through the cornea.

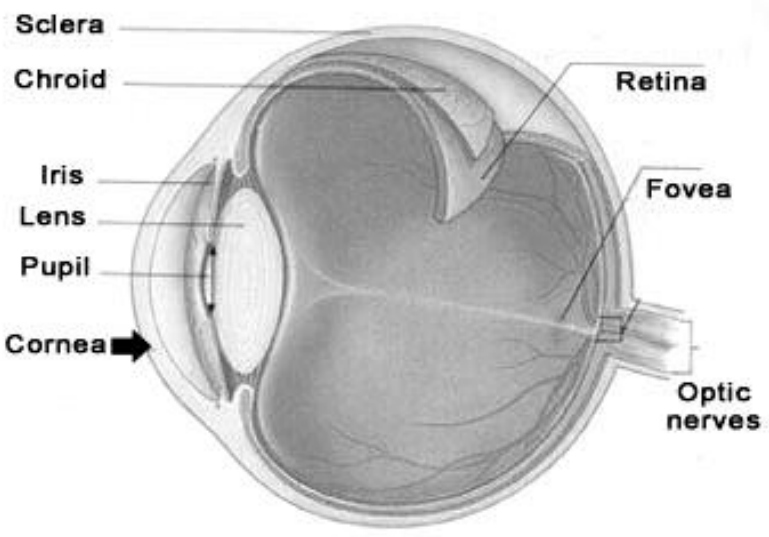

Figure 2.1 Eye in cross section.

(http://admin.harunyahya.com/arabic/books/science

/miracle_eye/miracle_eye_04.html)

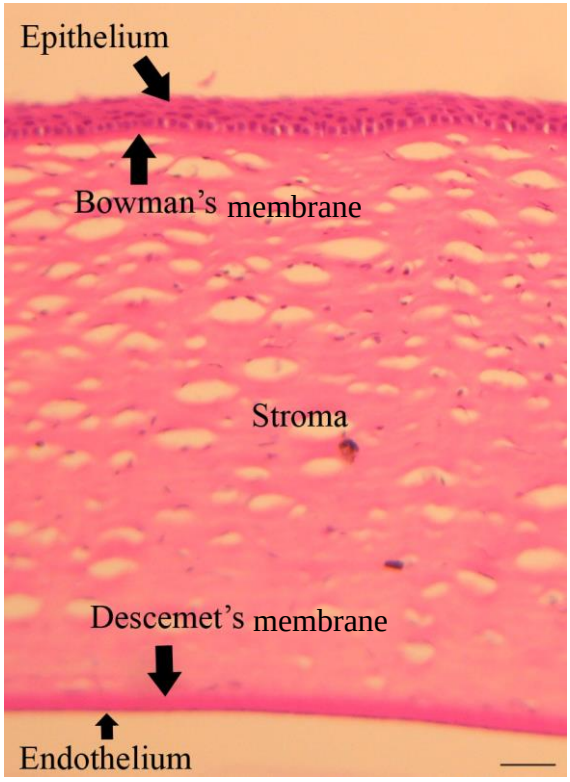

Figure 2.2 Hematoxylin and eosin staining of rabbit corneal section.

Scale bars = 50 µm.

The histological structure of cornea composes of 5 layers (Figure 2.2). First, the outermost corneal epithelium is non-keratinized stratified squamous epithelium that has 5 to 7 cell layers. At the apical surface of superficial cells, there are microvilli that called microplicae. This epithelium underlines with Bowman's membrane. The basal cells of the corneal epithelium attach to this membrane by hemidesmosome. Next to Bowman's membrane, the third layer is corneal stroma or substantia propria, a very thick layer up to 90% of the corneal thickness. This layer consists of a large amount of collagen fibers that regularly arranges parallel to the corneal surface. The collagen fibers form collagen lamellae oriented at an angle to one another. Between these lamellae, there are corneal fibroblasts or keratocytes. The forth layer is

Descemet's membrane, which is the basement membrane of the innermost corneal endothelium. Corneal endothelium is simple squamous epithelium that lines in front of anterior chamber of the eye (Kierszenbaum and Tres, 2011).

Limbus is 1-2 mm wide area between conjunctival epithelium and corneal epithelium. The basal layer of limbal epithelium that show undulating surface with stroma extending upward calls palisades of Vogt are believed to be the best niche for limbal stem cells (LSCs) by protecting them from shearing forces (Davanger and Evenson, 1971; Gipson, 1989). Moreover, this area shows heavily of melanin pigmentation that has ability in protection of resident stem cells from ultraviolet light (Cotsarelis et al., 1989). The palisades are predominantly found in the superior and inferior limbus where can be protected by the eye lids (Shortt et al., 2007). The underlying stroma in limbal region has blood supplies in order to directly provide high levels of nutrients and cytokines to cells whereas the rest of cornea is avascular (Goldberg and Bron, 1982) (Figure 2.3).

Limbal stem cell population, tritiated thymidine label-retaining cells, has only about 0.5% to 10% of total population located in limbus. They have low mitotic activity but high proliferative capacity and self-renewal. These cells are obviously importance in regeneration of corneal epithelial cells during injuries or normal cell desquamation by undergoing asymmetric division (Cotsarelis et al., 1989; Huang and Tseng, 1991; Morrison et al., 1997; Dua and Azuara-Blanco, 2000a). During asymmetric division, one of the daughter cell remains stem cell to replenish the stem cell pool while the other is differentiated into transient amplifying cells (TACs). Early TACs are located in basal layer of limbus and have similar characters as stem cells but when they migrate centripetally to peripheral cornea they become shorter life

span, rapid cycling. TAC function is to multiply the cell number until at critical point they stop mitosis and differentiate into post mitotic cells (PMCs) (Lauweryns et al., 1993). PMCs still migrate toward the central cornea in the upward and centripetal direction and finally differentiate into terminal differentiated cells (TDCs) which located at superficial layer of central cornea. These cells have no proliferative potential and limited lifespan (Figure 2.3).

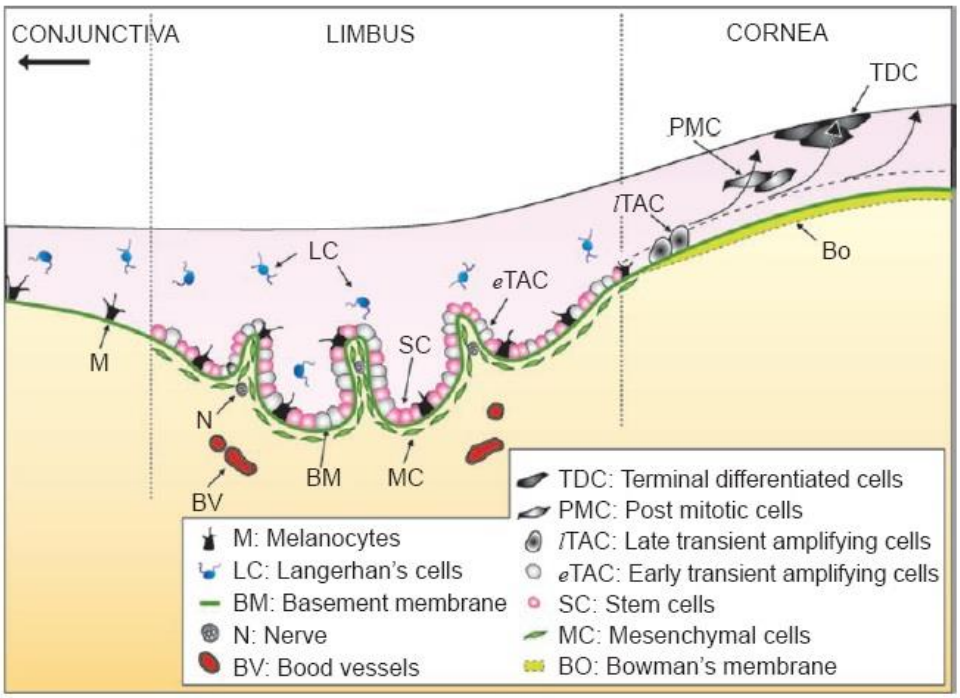

Figure 2.3 Limbal stem cell niche and model for the maintenance of the corneal epithelium.

(Li et al., 2007)

Oral mucosa

Oral mucosa consists of thick stratified squamous avascular epithelium. Type of epithelium is divided into keratinizing (gingiva, hard palate) and non-keratinizing stratified epithelia (buccal mucosa, vestibular mucosa, ventral surface of tongue, floor of mouth, soft palate). Epithelium is underlined with lamina propria that indents the epithelium with papillae. There are blood vessels in lamina propria so the avascular epithelium still alive by diffusion of nutrients via these blood vessels (Figure 2.4). Moreover, lamina propria contains collagen fibers, elastic fibers and connective tissue cells. Epithelial cells at the surface are continuously shed and the renewal rate is 12-14 days (William and Patrick, 2008).

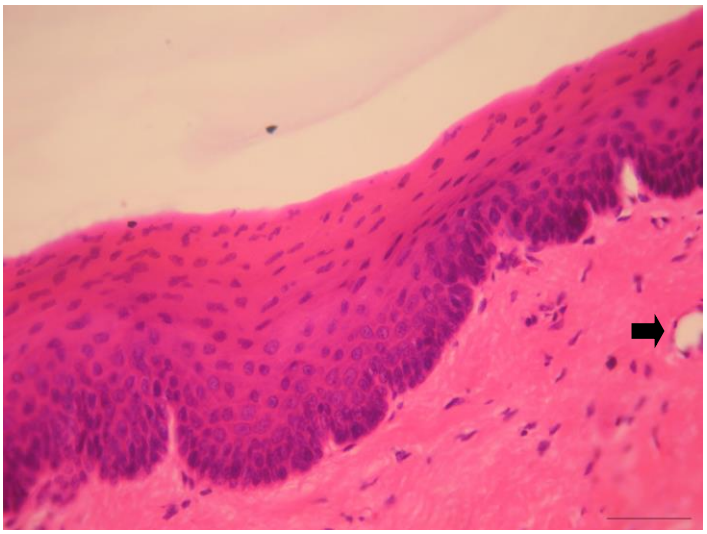

Figure 2.4 Hematoxylin and eosin staining of rabbit oral mucosal (buccal mucosa) section. The arrow indicated blood vessel. Scale bars = 50 µm.

Limbal Stem Cell Deficiency (LSCD)

The lack of limbal stem cells (LSCs) is known as Limbal Stem Cell Deficiency (LSCD) that can be occurred due to primary or secondary causes. Primary LSCD is a dysfunction of LSCs due to an altered of the limbal stem cells niche such as aniridia, neurotrophic keratopathy, keratitis associated with multiple endocrine deficiencies, pterygium or pseudopterygium. Secondary LSCD can be caused by chemical or thermal burns, Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), contact lens wear, severe microbial infection, multiple surgeries or cryotherapies. LSCD can lead to conjunctivalization to the cornea, corneal vascularization, corneal scar and persistence corneal epithelial defect (Kruse, 1994; Puangsricharern and Tseng, 1995). LSCD is also divided by its severity into partial and total. In partial LSCD, residual limbal stem cells still persist and are able to renew the corneal epithelium. In case of total LSCD, the patients suffer from the loss of vision and life discomfort due to the lack of the stem cells to regenerate the new corneal epithelial cells.

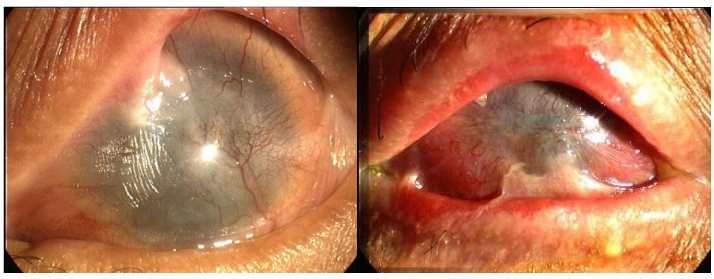

Figure 2.5 Eyes of the patients with limbal stem cell deficiency.

Treatment of LSCD

Human amniotic membrane (HAM) has been used in many types of reconstructive surgery (Trelford and Trelford, 1979). Amniotic membrane transplantation (AMT) was reported for corneal surface reconstruction by many studies and showed the improvement in both corneal surface and the vision. Amniotic membrane consists of simple cuboidal epithelium, thick basement membrane and an avascular stroma. The advantages of HAM may be due to its ability to reduce inflammation, vascularization, adhesions and scarring. It also enhances wound healing and epithelialization. Furthermore, immunological rejection after transplantation has not been occurred because the amniotic membrane expresses incomplete HLA-A, B, C, and DR antigens 2 (Akle et al., 1981; Kim and Tseng, 1995; Lee and Tseng, 1997; Tseng et al., 1998).

Nevertheless, in patient with severe and total LSCD, only AMT is not appropriate due to the inadequate or lack of the limbal stem cells to regenerate the new corneal epithelial cells. Limbal autograft transplantation is not recommended because it requires the large tissue that may induce the risk of epithelial problem in the donor eye. From the limitations mentioned above, ex vivo expansion of limbal stem cells has been developed by using tissue culture technique and shown the effective results in treatment of the LSCD. The principal of this culture technique is to increase the number of limbal stem cells and then transplanted onto the patient's corneal surface. In 1997, Pellegrini and colleagues reported the first successful in using cultivated autologous limbal stem cell transplantation for reconstructing the ocular surface of two patients with severe alkali burns. They cultivated limbal stem cells from small limbal tissue that was biopsied from the healthy eye. The patients were followed up

for 2 years. The results showed the stability of regenerated corneal epithelium and improvement of vision. The phenotype of corneal epithelium was confirmed by using biological marker which was positive for keratin 3 (K3) (Pellegrini et al., 1997).

As mentioned above about the advantages of HAM, several researchers have applied the HAM as a culture substrate. Koizumi and colleagues were first cultivated rabbit limbal epithelial cells on HAM. They took a small biopsy of rabbit corneal epithelial tissue and cultured on acellular HAM. They found that, after 14 days of cultivation, the limbal corneal epithelial cells had reached confluent and 5 days after transplantation the ocular surface has epithelialized up (Koizumi et al., 2000a). Moreover, they reported that denuded HAM provided a better culture substrate than HAM with intact epithelia. Cultivated limbal epithelial cells on denuded HAM growth rapidly, uniformly and adhered well to the underlying amniotic membrane (Koizumi et al., 2000b). Since then, there were many groups of researcher have used this culture technique and transplanted in patients (Tsai et al., 2000, Sangwan et al., 2002, Shimazaki et al., 2002). The culture technique continuously developed. Air-lifting technique was used to promote epithelial stratification. The study of Ban and colleague reported that air-lifted culture had tightly packed epithelial cells and small intercellular space (Ban et al., 2003). The 3T3 fibroblast was also used as a feeder cell as Rheinwald and Green in 1975 has been successfully grown human epidermal keratinocytes with 3T3 fibroblast (Rheinwald and Green, 1975). The 3T3 fibroblasts were treated with mitomycin C (MMC) to inactivate proliferation activity prior to use. Limbal epithelial cells cultivated with 3T3 fibroblast shows the ability in maintaining the expression of putative stem cell markers. Due to the advantages of each factors mentioned above, many researchers succeeded in cultivated limbal biopsy on denuded

HAM with MMC treated 3T3 fibroblast and air- lifting method (Koizumi et al., 2002; Sudha et al., 2008).

However, in bilateral LSCD, the patients need to use the donor tissues that cause graft rejection and problems with chronic use of immunosuppressive drugs. Therefore, alternative sources of autologous tissue are considered. In 2003, Nakamura and colleague were the first who reported the transplantation of cultivated autologous oral mucosal epithelium onto injured eye of rabbit. They were subsequently succeeded in treating the patients with LSCD in the next year. Since then, several researchers have reported the further utility of this method (Nakamura et al., 2003; Nakamura et al., 2004; Chen et al., 2009; Ma et al., 2009).

Characterization of differentiated cells and stem cells

To date, there is no specific marker to identified LSCs but there are many studies that proposed several putative stem cell markers. For this reason, in order to get more specific identifying of LSCs, it is appropriate to use the combination of marker expression. For instance, positive marker of LSCs included p63 and negative markers included keratin 3 (K3) and Connexin 43 (Cx43).

The marker p63 is a transcription factor that found in nucleus. This protein plays a critical role in epidermal morphogenesis and was proposed as a stem cell marker to identify epidermal stem cells as well as corneal epithelial stem cells. This protein is highly present in basal cell layer of limbal epithelium that interspersed with patches of p63-negative cells. A few numbers of p63 stained cells were also found in suprabasal cell layer of limbal epithelium. In corneal region, cells were negative staining for p63 (Mills et al., 1999; Pellegrini et al., 2001; Chen et al., 2004).

Keratins are one of the intermediated filaments which they acted as epithelial cell cytoskeleton that has function in maintenance of cell shape. These proteins are divided into two families, type I and type II. All epithelial cells composed of one type I and one type II in pairing pattern but have different pairs of keratin proteins depending on epithelial cell type and stage of differentiation (Presland and Dale, 2000). A marker of corneal epithelial cell differentiation, keratin 3 (K3), can be used as a negative marker for LSCs which is found in all layers of the differentiated corneal epithelium together with keratin12 (K12). In limbal epithelium, a weak staining of K3 was found in the suprabasal layer but not in the basal layer suggesting that this keratin absent in limbal stem cells and early transient amplifying cells (TACs) (Schermer et al., 1986; Chen et al., 2004).

Connexin 43 (Cx43) is a transmembrane protein. Six molecules of them forms the communicating cell to cell junction called gap junction that involved in cell growth and differentiation (Kumar and Gilula, 1996). In the corneal epithelium, Cx43 strongly express in suprabasal layer and weakly express in basal layer. In limbal region, Cx43 expression is absent in limbal basal cells while cells in superficial layer stain weakly positive. The lack of Cx43 in limbal stem cells help to maintain their stemness by protecting the cells from damage that can be affected from extracellular environment and adjacent cells (Matic et al., 1997; Chen et al., 2004).

Normally, epithelial tissues express different pairs of keratin proteins depending on epithelial cell types and stage of differentiation. In oral non-keratinized epithelium, K4 and K13 express in superficial and intermediate layers. Basal epithelial cells express K5 and K14 (Presland and Jurevic, 2002). The epithelial stem cells located in the basal layer were negative for Cx43 but highly positive for p63. K3

and Cx43 expressions were found in superficial and intermediate layer of oral mucosal epithelium (Juhl et al., 1989; Chen et al., 2009; Priya et al., 2011).

Cryopreservation

There are several cryopreservation methods but some factors needed to be concerned including the selection of cryoprotective agents (CPAs), cryopreservation methods, cooling and thawing rates as well as the cellular types to prevent ice crystal formation, osmotic stress and toxicity of CPAs that causing cell injury during freezing (Davis, 2002). The conventional method or slow-freezing method uses low concentration of CPA, in which 10% DMSO is the most common used, it has low toxicity and osmotic effect to cells but it allows the ice to form and prolong exposure to CPA that can lead to decreasing cell viability (Mazur, 1984; Frederik and Busing, 1981). On the other hand, the vitrification, one of the cryopreservation methods, uses higher concentration of CPA that can avoid the ice crystal formation, the main disruption of the successful cryopreservation, but it causes cell toxicity and shrinkage. To solve these problems, it is necessary to use the combination of CPAs and increase cooling rate (Cabrita et al., 2003; Vajta et al., 2009). Moreover, the suitable CPAs formula is also important. Dimethyl sulfoxide (DMSO) and glycerol 5 to 10% final concentration are most common CPAs in cryopreservation process. DMSO was widely used because it had higher penetration ability and allowed water in the cells to flow out during freezing (Davis, 2002). Moreover, DMSO was suggested to be one of the compositions in vitrification solution in order to decrease cell damage (Kartberg et al., 2008). By the way, the optimal condition was different in each cell types and high concentration of DMSO was found to be toxic to cells (Kaufman et al., 1966; Monica

et al., 1997; Cabrita et al., 2003; Yeh et al., 2008). Glycerol has low toxicity than DMSO but it permeates the cell slower (Tavassoli et al., 2009). Propylene glycol, also called 1,2 -propanediol or propane-1,2-diol, is one of the widely used as CPA. In 1990, Rich and Armitage studied the effect of propylene glycol on the structure and function of rabbit corneal endothelium. They suggested that propylene glycol was a potential component of a vitrification solution for corneas (Rich and Armitage, 1990).

In 2008, Yeh and colleagues generated the expanded epithelial sheets on amniotic membrane from limbal tissue outgrowth that were cultivated for 3 weeks and then these epithelial sheets were cryopreserved for 8 weeks in liquid nitrogen with different formulas of CPAs. They reported the optimal formula of CPAs for expanded limbal cell sheets was 60% Dulbecco modified Eagle medium, 30% fetal bovine serum and 10% dimethyl sulfoxide that were able to maintain the cell characters during 8 weeks of cryopreservation and the average cell viability was 53.8% ± 5.8% (Yeh et al., 2008). Qu and colleagues cryopreserved limbal stem cells that were amplified at 2-4 passages, in liquid nitrogen using a storage medium of DMEM/Ham's F12 with 10% DMSO and 25% bovine serum albumin. After 1–3 months of cryopreservation, the cells were then cultured for 12–14 days and were used in transplantation. This study reported that cryopreserved LSCs still retained the stem cell and differentiated corneal epithelial cell markers and the epithelial sheet that were generated from cryopreserved cells can repair damaged rabbit cornea (Qu et al., 2009). Bratanov and colleagues using Optisol GS^{R} medium with 10% dimethyl sulfoxide and 20% human serum albumin in cryopreserved limbal tissue from cadaver. After long time cryopreservation (up to 12 months), these tissues were still able to proliferate and generate the epithelial sheets in vitro and containing stem cell

population that positive for p63 and vimentin (Bratanov et al., 2009). To date, there is no study about cryopreservation of limbal and oral mucosa tissues by vitrification method.