Altered Versus Unaltered Amniotic Membrane as a Substrate for Limbal Epithelial Cells

Tor Paaske Utheim[1,2,3,4], Øygunn Aass Utheim[5], Panagiotis Salvanos[2], Catherine Jackson[1], Stefan Schrader[6], Gerd Geerling[6] and Amer Sehic[7*]

[1] Department of Medical Biochemistry, Oslo University Hospital, Norway
[2] Department of Ophthalmology, Drammen Hospital, Vestre Viken Hospital Trust, Norway
[3] Department of Ophthalmology, Stavanger University Hospital, Norway
[4] Department of Clinical Medicine, Faculty of Medicine, University of Bergen, Norway
[5] Department of Ophthalmology, Oslo University Hospital, Norway
[6] Department of Ophthalmology, University of Düsseldorf, Germany
[7] Department of Oral Biology, Faculty of Dentistry, University of Oslo, Norway

Running head:
Amniotic Membrane as a Substrate for LEC

Author contributions:
Tor Paaske Utheim: review of the literature, writing, final approval of manuscript
Øygunn Aass Utheim: manuscript review, writing, final approval of manuscript
Panagiotis Salvanos: manuscript review, writing, final approval of manuscript
Catherine Jackson: manuscript review, writing, final approval of manuscript
Stefan Schrader: manuscript review, writing, final approval of manuscript
Gerd Geerling: manuscript review, writing, final approval of manuscript
Amer Sehic: review of the literature, figure artwork, writing, final approval of manuscript

Key Words:
Cornea; Cell biology; Gene expression; Cell transplantation

**** Corresponding author:***
Amer Sehic PhD, Associate professor,
Department of Oral Biology, Faculty of Dentistry
University of Oslo, 0318 Oslo, Norway
E-mail: amer.sehic@odont.uio.no
Telephone: +47 95752912

Abstract

Limbal stem cell deficiency (LSCD) can result from a variety of corneal disorders, including chemical and thermal burns, infections, and autoimmune diseases. The symptoms of LSCD may include irritation, epiphora, blepharospasms, photophobia, pain, and decreased vision. There are a number of treatment options, ranging from non-surgical treatments for mild LSCD to various forms of surgery that involve different cell types cultured on various substrates. *Ex vivo* expansion of limbal epithelial cells (LEC) involves the culture of LEC harvested either from the patient, a living relative, or a cadaver on a substrate in the laboratory. Following the transfer of the cultured cell sheet onto the cornea of patients suffering from LSCD, a successful outcome can be expected in approximately 3 out of 4 patients. The phenotype of the cultured cells has proven to be a key predictor of success. The choice of culture substrate is known to affect the phenotype. Several studies have shown that amniotic membrane (AM) can be utilised as a substrate for expansion of LEC for subsequent transplantation in the treatment of LSCD. There is currently a debate over whether AM should be denuded (i.e. de-epithelialized) prior to LEC culture, or whether this substrate should remain intact. In addition, cross-linking of the AM has been used to increase the thermal and mechanical stability, optical transparency, and resistance to collagenase digestion of AM. In the present review, we discuss the rationale for using altered versus unaltered AM as a culture substrate for LEC.

Introduction

In the early 1900s, Davies was the first to report the therapeutic use of human amniotic membrane (AM) in skin transplantation to treat burned and ulcerated skin surfaces (1). A considerable decrease in pain and improved rate of skin-surface healing was reported. Subsequently, there was a lag period of more than two decades before any additional use of AM was reported in the literature. In the 1930s, AM was applied in surgical reconstruction of vaginas (2). Thereafter, AM was used following head injury to prevent meningocerebral adhesions (3), in repair of abdominal herniation (4), closure of pericardium (5), treatment of non-healing wounds in diabetic patients (6), to aid head and neck surgery (7), as a biological dressing in correction of abdominal birth defects (8), surgical repair of refractory labial adhesions (9), in wounds as a biologic dressing (10), and after total removal of the tongue (11).

In the 1940s, several authors reported the beneficial role of AM in treating a variety of ocular surface disorders (12-15). It was first used as a substitute for rabbit peritoneum in the management of chemical burns of the eye. Successful outcomes were reported with dried amniotic tissue, termed ‘amnioplastin’ (12, 13). Following these initial procedures there was no report on the use of AM in ophthalmology until the early 1990s, when AM experienced a renaissance with regard to treatment of ocular surface disorders. In 1993, Batle and Perdomo introduced AM preserved in 95% ethyl alcohol as a substitute for conjunctival membranes in fornix reconstruction and in the treatment of recurrent pterygia and alkali burns (16). Two years later, Tseng and Kim performed AM transplantation in rabbits for ocular surface reconstruction (17). Subsequently, various authors have reported the beneficial effects of human AM transplantation in ever-expanding ocular indications (18).

Numerous studies have demonstrated that AM also can be utilized as a substrate

for expanding limbal epithelial stem cells (LEC) for subsequent transplantation in the treatment of limbal stem cell deficiency (LSCD) (19). Tsai and colleagues were the first to report the use of AM to culture LEC (20). The choice of culture substrate for LEC is of key importance for growth characteristics and phenotype preservation. However, so far there is no standardized culture method for LEC on the AM. Different culture techniques on AM are employed and are differing regarding the composition of AM (e.g., AM with or without the epithelium), air-lifting prior to transplantation, and the use of an additional 3T3 feeder layer. Furthermore, there are challenges with human AM that still are undetermined, e.g. the thinness of membrane affecting the suture strength, crushing while transplanting, early detachment, and considerable dissolution of the membrane after transplantation (21). In order to improve these characteristics, the researchers have focused on different methods to alter the AM and increase the mechanical and thermal stability, optical transparency, and resistance to collagenases. It has been proposed that the devitalized epithelium on preserved AM may be of significant importance to promote expanded human LEC maintain a less differentiated phenotype compared with the limbal basal epithelium *in vivo* (22). On the other hand, studies have shown that the intact AM (with the amniotic epithelium) exhibits higher levels of growth factors comparing with epithelially denuded AM (23). The growth factors are implicated in epithelium–stroma interactions of the human ocular surface (24), therefore, the amniotic epithelium may have a substantial role in the micro-environmental niche of limbal progenitor cells. More research is warranted to explore this potential mechanism of action, in order to control LEC behaviour. Additionally, further research on alteration of AM may improve its properties of the membrane and thereby increase the therapeutic efficacies.

The present review is also timely as AM has recently got a new clinical

indication as a culture substrate for simple limbal epithelial transplantation (SLET) (25). This is a new clinical procedure for the treatment of unilateral LSCD. In SLET a small piece of limbal tissue (e.g. 2 x 2 mm) is divided into smaller pieces and distributed over an AM placed on the cornea. Although long-term results are not available, the results so far are promising. What conditions of the AM that will give the best short- and long-term clinical outcome following SLET is unknown, but laboratory and clinical data based on LEC cultured on altered and unaltered AM *ex vivo* are clearly relevant to consider when designing future SLET studies where the culture is performed *in vivo* instead of *ex vivo*.

Mechanical Properties and Possible Mechanisms of Action

The AM is the innermost layer of the foetal membranes, and is normally 0.02 to 0.5 mm in thickness (26, 27). The AM consists of five layers, from the innermost outwards: 1) epithelium, 2) basement membrane, 3) compact layer, 4) fibroblast layer, and 5) spongy layer (Figure 1) (26). The monolayer of cells in the epithelial layer varies from columnar over the placenta to cuboidal or flat away from the placenta (26). The basement membrane is a thin layer composed of reticular fibres. It adheres closely to the amniotic epithelium from which multiple processes interdigitate into it. The remaining three layers are collectively termed the stroma. The compact layer is a dense layer almost totally devoid of cells and consists mainly of a complex reticular network. The fibroblastic layer is the thickest layer of the AM and consists of fibroblasts embedded in a loose network of reticulum. The outermost spongy layer forms the interface between the AM and chorion and consists of wavy bundles of reticulum covered with mucin (27). The AM supports the homeostasis of amniotic fluid (28); however, its precise function is still elusive. During pregnancy, the amniotic epithelium

is metabolically active (28, 29). It lacks a blood supply of its own; oxygen and nutrients are derived from the amniotic fluid, surrounding chorionic fluid, and foetal surface blood vessels. It is suggested that energy is derived through an anaerobic glycolytic process due to this limited oxygen supply (30).

The AM exhibit several properties that makes it suitable for use in tissue engineering (31). Cells in the epithelial layer of the AM have significant similarities to stem cells. They express pluripotent markers of stem cells, have the ability to be differentiated into all three germ layers, and have no need for a feeder layer throughout their cultivation (31). Other important characterizations of AM, which are all crucial for use in tissue engineering are anti-tumourigenicity, anti-fibrosis, anti-inflammation, anti-microbial, anti-scaring, low immunogenicity and useful mechanical property (31).

There are, however, some challenges with the use of the AM for tissue engineering. The AM has a thin structure and exhibits technical limitations on the occasion of suturing. It has been suggested that the use of glues as a substitute for suturing may be promising (32). Furthermore, the AM shows a viscoelastic mechanical response (31). In a majority of tissues, viscoelasticity is crucial for scaffolding, e.g. stiff scaffolds of the arteries that may encourage hyperplasia and occlusion (33). It has been demonstrated that preterm AM exhibits greater mechanical integrity compared with the term AM, however, the stiffness of term AM is more applicable for a majority of protocols in tissue engineering (34). It has been suggested that this may be related to the collagen content, although there are contradictory studies showing that the content of amnion collagen decreases with gestational age (35). Moreover, it is also proposed that elastin, which is detected in the fetal amnion, provides the molecular basis for elasticity in the AM (36).

There are differences concerning AM location, i.e. samples of AM taken from

locations distal and proximal to the placental disc. It has been demonstrated that proximal human samples of AM were thicker and stronger, however, with poorer optical properties compared with distal samples (37). Furthermore, AM may be used in surgical procedures either fresh or modified through different preservation methods such as cryopreservation, freezing, or lyophilization (38). Cryopreservation, compared to freezing, seeks to reach very low temperatures without causing additional damage by the formation of ice during freezing. It has been reported that cryopreservation better preserves growth factors compared to freezing (38). When comparing cryopreserved and fresh AM it is shown that the epithelial cells do not survive the cryopreservation and that they exhibit poor proliferative capacity. No morphological differences were detected between fresh and cryopreserved AM (39). Recently, studies have shown that the combination of AM preservation and sterilization by gamma-irradiation, paracetic acid, and/or trehalose reduces the risk of infections that may be transmitted by AM (38).

The AM secretes several growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor β (TGFβ) (40, 41). EGF is a powerful mitogen for the growth of epithelial cells, and its high level of expression following transplantation may explain improved wound healing of the ocular surface (23). It has been shown that EGFs mainly are found in the amniotic epithelium (38). PDGF participates in cellular responses including proliferation, migration, survival, and the deposition of extracellular matrix and tissue remodelling factors (42). Koizumi and colleagues reported that the amniotic epithelium secretes HGF and KGF, which are generally produced by mesenchymal cells such as fibroblasts in corneal stroma (23). These growth factors in the epithelium of AM may affect wound healing of cornea through paracrine action (43, 44). It may therefore be suggested that ocular surface re-

epithelialization may be accelerated by HGF and KGF secreted by the amniotic epithelium following transplantation of AM.

Studies have also shown an anti-inflammatory effect associated with AM (19, 45, 46). Expression of IL-1α and IL-1β by human LEC was significantly suppressed when cultured on the stromal matrix of the AM, even when challenged by application of bacterial derived lipopolysaccharides (46). In a study in which the corneas of rabbits were covered by human AM after phototherapeutic keratectomy, acute inflammatory reaction was significantly reduced by apoptosis of polymorphonuclear neutrophils (47). This finding was also supported in patients with acute burns where CD20+ lymphocytes were trapped by the AM and exhibited cell death (48). Upon inoculation of rat corneas with herpes simplex virus type 1 to induce necrotising keratitis, inflammation decreased when the cornea was covered with preserved human AM (49). Chronic inflammation in the limbal region can cause LSCD. Furthermore, inflammation can negatively affect integration of transplanted conjunctival-limbal auto-grafts in the treatment of LSCD (50). Thus, the anti-inflammatory property of AM may explain its beneficial effect. Furthermore, numerous factors participate in the anti-fibrotic effect of the AM (24, 51). Tseng and colleagues have shown that it induces a down-regulation of transforming growth factor β signalling, which is responsible for activation of fibroblasts in wound healing (51).

Culture Techniques and Use of Intact and Denuded Amniotic Membrane

Currently, there is no standardised method for *ex vivo* expansion of LEC. Culture of LECs can follow the explant or cell suspension method. In the explant method, cells grow out from a small biopsy attached to the base of the culture dish. Cell suspension

means that cells are first enzymatically released from the tissue. Once attached to the base of a culture dish the single cells divide and grow to form a confluent layer. Some culture methods employ air-lifting to encourage differentiation of the superficial layer. This is achieved via lowering the medium until it is just at the level of the superficial cell layer. The use of irradiated or Myotomicin C treated mouse embryonic fibroblasts was originally developed to enable culture of skin epidermal cells (52). It is now a culture technique often employed for culture of all types of epithelial cells to supply cytokines and growth factors that promote proliferation.

In Vitro Experiments with Intact and Denuded Amniotic Membrane

The precise role of the devitalized amniotic epithelium is not yet fully understood. It is suggested that the devitalized epithelium covering the amniotic basement membrane may be important to help expanded human LEC assume a less differentiated epithelial phenotype (22). A native, intact AM has been found to comprise higher levels of growth factors compared to a denuded AM (23), suggesting that these growth factors are primarily present in the amniotic epithelium. These growth factors are believed to be involved in epithelium–stroma interactions of the human ocular surface (24).

Several studies have shown that LEC cultured on an intact AM maintain a more stem cell-like phenotype compared with LEC cultured on a denuded AM (22, 53, 54). Expression of slow cycling and label-retaining cells that do not express the differentiation-associated markers K3, K12 (22, 55) or Cx43(22) has been demonstrated in limbal epithelial sheets cultured on intact AM. Krishnan *et al*. compared the expression of ΔNp63α, a marker for non-differentiated cells, in LEC cultured on intact human AM with denuded human AM (56). Interestingly, only LEC cultured on intact AM gave rise to ΔNp63α expression (56). The expression of p63-

isotypes ΔNp63 (57) and ΔNp63α (58) has been confirmed in other studies in which LEC has been cultured on intact AM.

The nerve growth factor (NGF) signalling pathway, which is known to be involved in stem cell survival, was preserved in the intact AM culture system (22). Furthermore, cultured LEC on intact human AM has been found to maintain high proliferative potential when compared to denuded human AM (56). However, contrary results have also been demonstrated (22, 59). Koizumi and colleagues showed that LEC cultured on a denuded AM formed a more stratified and differentiated epithelium and exhibited a higher number of desmosomes and hemi-desmosomes compared to culture on intact AM (59, 60). The authors concluded that for purposes of transplantation of differentiated epithelial sheets, denuded AM is probably the more suitable carrier for human LEC cultures when using the cell-suspension culture system. However, denuded AM did not improve the structural integrity of cultured human LEC following one week of eye bank storage (61). Moreover, the highest levels of K3 and Cx43 were observed when denuded AM was used without an additional 3T3 feeder layer (fibroblasts synthesizing the extracellular layer and collagen) (22). Addition of a 3T3 feeder layer to denuded AM increased the level of Cx43 but decreased that of Cx50, reflecting a less differentiated phenotype compared with denuded AM without 3T3 fibroblasts.

Clinical Studies Using Intact and Denuded Amniotic Membrane

Only seven clinical studies (sub-studies excluded) involving transplantation of *ex vivo* cultured LEC have applied intact AM (20, 62-68) (Table 1) as a culture substrate, whereas 29 clinical studies used denuded AM to culture LEC (69-97) (Table 2).

Tsai and colleagues were the first to report the use of intact AM to culture LEC to treat patients with unilateral partial or total LSCD (20). The authors utilised

autologous limbal tissue obtained from a biopsy of the contralateral eye for explant cultures on cryopreserved intact AM. The results showed a success rate of 83% with regard to visual acuity and a 100% success rate regarding reconstruction of a stable ocular surface. During the follow-up time of 15 months, no conjunctivalization was observed in the treated eyes (Table 1). The remaining six studies all performed transplantation of *ex vivo* cultured limbal epithelium on intact AM without the use of a 3T3 fibroblast feeder layer or air-lifting (Table 1). With a mean follow-up time of 22 months (range: 14 (67) to 48 (68) months), visual acuity improved, ranging from 56% (68) to 83% (20, 67). Immunosuppression was used in four studies (62, 64, 65, 68) and conjunctivalization was reported in one study (68).

The first clinical trial using denuded AM as a culture substrate for LEC in treating LSCD was published in 2000 by Schwab and colleagues (90). LEC were expanded on an inactivated 3T3 fibroblast feeder layer and subsequently seeded onto denuded AM. Ten of 14 patients with allogeneic and 6 of 10 patients with autologous transplants maintained a stable corneal surface after a follow-up period of between six and 19 months. A year later, two cases of acute Stevens–Johnson syndrome with large persistent epithelial defects were treated with the same technique (79). The authors expanded allogeneic limbal tissue from donor corneal buttons on denuded AM, taking advantage of an inactivated 3T3 fibroblast feeder layer. The renewed epithelium was stable and without defects after a follow-up time of six months. Koizumi and colleagues thereafter used the same approach to treat 13 patients with total LSCD. Ten of 13 eyes exhibited visual improvement and a stable ocular surface without epithelial breakdown after a mean follow-up period of 11.2 months (78).

In 2002, Shimazaki and colleagues, using denuded AM, reported on the transplantation of *ex vivo* expanded LEC from allogeneic (n = 7) and living related (n

= 7) donors to 13 eyes with total LSCD (93). They showed that corneal epithelial restoration was achieved in 46.2% of cases. One eye did not show epithelialization at all, five eyes failed with recurrent conjunctivalization, and one eye failed with dermal epithelialization. Following transplantation of cultivated allogeneic LEC on AM, improved visual acuity was observed in 77% of patients.

The remaining studies using denuded AM as a culture substrate for LEC utilised both allogeneic (69, 74, 77, 80, 82, 86, 91, 92, 95-97) and autologous (70-73, 75-77, 80, 81, 83-87, 89-92, 94-97) explants, with and without the use of a 3T3 fibroblast feeder layer or air-lifting (Table 2). Immunosuppression was used in all studies using allogeneic limbal explants except for one (88), and in some studies using autologous explants (Table 2). The reported follow-up period was up to 66 months. Following transplantation of cultured LEC on denuded AM visual acuity ranged from 53% to 100%. Moreover, 100% clinical success was reported in seven of 29 studies (Table 2).

Cross-Linking of Amniotic Membrane

The topography of the underlying substrates affects the cells, and it has been shown that physical cues control cell morphology, migration, and embryonic development (98). Studies using photolithography showed that surfaces with single 5-µm-tall steps was sufficient to selectively slow the migration rate of baby hamster kidney and fibroblast cell types, but not of neutrophils (99). Microarray analysis of cells seeded onto substrates with hexagonal pits compared with flat surfaces demonstrated significant changes in expression of hundreds of genes that were associated with extracellular matrix protein production and regulation of cell-cycle (100). These results clearly show how small features can exhibit an important impact on development, regulation, and homeostasis of cells and tissues.

It is known that structural changes in the molecules that are the constituents of the matrix will likely result in changes in cell signaling (101). Collagen undergoes many post-translational modifications that are important for its structural and mechanical properties, and the interruption with some of these processes leads to severe dysfunction of the cells. The final steps in the formation of the collagen include the cleavage of the N and C pro-peptides, self-assembly of the resulting collagen molecules into fibrils, and formation of covalent crosslinks (102). Optimal cross-linking of collagen is essential for the collagen binding to its receptors, however, it is also important for regulation of the availability of growth factors and for the mechanical characteristics of the extracellular matrix (103). Previous studies have shown that the inhibition of collagen cross-linking in the mouse pre-osteoblast cell line weakens the osteogenic program (104). Furthermore, impairing the cross-linking of collagen is associated with exposure of cryptic nucleation sites, resulting in enhanced mineralization (105). Insufficient collagen cross-linking makes the collagen more prone to proteolytic degradation (106).

Collagen nanofibers, an essential structural component of the AM, exhibit significant degradation after being exposed to endogenous collagenases *in vivo*. The collagenase activity is enhanced in many diseases affecting the cornea and may therefore lead to accelerated degradation of AM transplants (107). Spoerl *et al*. demonstrated that insufficient biological stability of an AM graft may be a significant cause of early AM detachment during corneal wound healing (108). As enzymatic degradation of the AM matrix is considered a major cause for failures after surgical transplantation, the development of strategies for improvement of the molecular biostability of AM is warranted. Since it is desirable that the collagen in the AM serves as a limbal stem cell niche, several researchers have tried to modify it to a cross-linked

molecular biopolymer chain network. Different cross-linking strategies have been used in order to increase the stability of AM for culture of LEC, including glutaraldehyde- (108-111), carbodiimide- (112-117), radiation- (111), photo- (118), and $Al_2(SO_4)_3$- (21) cross-linking (Table 3).

Glutaraldehyde Cross-Linking

Glutaraldehyde is a widely utilized, highly effective, chemical cross-linking substrate used for the stabilization of collagenous biomaterials. Fujisato and colleagues have demonstrated that glutaraldehyde cross-linked AM is more resistant to degradation from collagenases (111). It has also been demonstrated that the effect of glutaraldehyde cross-linking on the nanostructure of AM material is critical to maintenance of LEC stemness (109). Furthermore, glutaraldehyde cross-linking of collagenous materials affects corneal epithelial characteristics of stem cell culture (109). After modification with glutaraldehyde using a variable cross-linking activation time, the AM samples were investigated by determining the degree of cross-linking, nanofibrous structure, *in vitro* degradability, cytocompatibility, anti-inflammatory activity, and stemness gene expression. After a six-hour reaction time, the cross-linking degree and *in vitro* degradability of glutaraldehyde treated samples were much lower than those of the carbodiimide cross-linked counterparts. Furthermore, the increased biostability of collagen within cross-linked AM was positively correlated with the amount of cross-linker in the reaction system. Nevertheless, a method involving chemical modification of AM with glutaraldehyde likely reduces the level of safety, especially when the extent of cross-linking reaches high levels (119). Various studies have reported that using glutaraldehyde as a cross-linking agent is not advisable due to its toxic nature (120, 121).

Carbodiimide Cross-Linking

The modification of AM with carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) does not introduce foreign structures into the biomaterial network and is therefore considered a more biocompatible technique (122). The EDC/NHS carbodiimide method of cross-linking has been previously used for the development of chemically cross-linked AM materials (117). However, with carbodiimide treatment for a longer duration (i.e. four hours), the AM samples showed significant weight loss after four weeks of incubation with matrix metalloproteinases, suggesting low cross-linking efficiency of biological tissues (115). With an optimum concentration of 0.05 mmol EDC/NHS per mg AM, chemical cross-linking can significantly enhance mechanical stability and retard enzymatic degradation (117). It is expected that the increased stability introduced by cross-linking could be useful in an inflammatory wound. However, *in vitro* cell culture studies demonstrate that EDC cross-linked AM can support human LEC proliferation and reserve epithelial progenitor cells *in vivo* and *in vitro* (117). Enhanced expression of p63 and ABCG2 and increased LEC growth were also significantly associated with the greater cross-linking degree of AM samples (115). The expression of K3 and ABCG2 suggests that both differentiated and progenitor phenotype can be preserved by cross-linking AM.

Radiation and Photo Cross-Linking

In a study by Lai *et al*. it was demonstrated that UV radiation physically cross-links AM (118). Results of cross-linking density measurements and *in vitro* degradation tests showed that the bio-stability of these biological tissues strongly depended on the number of the cross-linked structures, which was affected by the duration of exposure to UV radiation. The number of cross-links per unit mass of photo-cross-linked AM

played an important role in determination of matrix permeability. *In vitro* biocompatibility studies, including cell viability and pro-inflammatory gene expression analyses, demonstrated that the physically cross-linked biological materials did not cause harm to the corneal epithelial cells, irrespective of UV radiation time. It was found that undifferentiated precursor cell phenotype was significantly improved with an increase in cross-linking density (123). Therefore, both duration of UV radiation and riboflavin may be important for the generation of AM matrices for cultivation of LEC.

Aluminum Sulfate Cross-Linking

A recent study showed that aluminum sulfate ($Al_2 (SO_4)_{3)}$ may be utilized as a cross-linking agent to improve the mechanical properties of AM. Crosslinking with $Al_2(SO_4)_3$ supported improved attachment and proliferation of corneal LEC (21). Using infrared spectroscopy to confirm the cross-linking of AM with $Al_2(SO_4)_3$ it has been demonstrated that there is an approximate 125% increase in tensile strength in the cross-linked AM. Importantly, the cross-linked AM was found to be sterile for up to one year and the morphology of confluent sheets of epithelial cells resembled in vivo morphological features of LEC. Based on these results, the $Al_2(SO_4)_3$ cross-linked AM should be further investigated as a candidate substrate for ocular surface reconstruction.

Cross-Linking and the Limbal Stem Cell Niche

Stability and biocompatibility are both important factors that need to be taken into consideration when studying biomaterial cross-linking and its applications. Using l-lysine as an additional amino acid bridge the stabilization of an EDC/NHS cross-linked AM collagen matrix for potential use as a limbal stem cell niche was investigated (114). The results showed that the number of positively charged amino acid residues

incorporated into the tissue collagen nanofibers was highly correlated with the l-lysine-pretreatment concentration, thereby influencing the cross-linked structure and hydrophilicity of the resulting scaffold. The variation in thermal and biological stability was correlated with the number of cross-links per unit mass of AM. It is noteworthy that the samples prepared using a relatively high l-lysine-pretreated concentration (i.e. 30 mM) appeared to have decreased light transmittance and cell viability. This was likely due to the effects of an increase in nanofiber size and subsequent higher charge density. However, in the 1–30 mM range of l-lysine pretreatment, expression of p63 and ABCG2 in LECs were upregulated. This corresponded with an increased number of amino acid bridges in the chemically cross-linked AM scaffolds. Therefore, mild to moderate l-lysine pretreatment appears to be a useful strategy to assist in the construction of a stable LEC niche using EDC/NHS cross-linked AM.

Future Perspectives and Conclusions

Data from *in vitro* experiments indicate that intact AM supports expansion of cells with a partly undifferentiated limbal phenotype, while the denuded AM culture system encourages differentiation. The results obtained so far suggest that the addition of 3T3 feeder cells decreases but do not prevent differentiation of LEC on denuded AM.

Currently, the progenitor cell marker p63 is the only known predictor of clinical outcome following transplantation in the treatment of LSCD (124). Rama and colleagues showed that successful transplantation was achieved in 78% of patients when using cell cultures in which p63-bright cells constituted more than 3% of the total number of clonogenic cells. In contrast, successful transplantation was only seen in 11% of patients when p63-bright cells made up 3% or less of the total number of cells.

Quantitative expression of p63 in cultured LEC on intact and denuded AM has been rarely reported. Expression of p63 (54, 125) and ΔNp63α (56) has been found to be higher following culture of LEC on intact AM compared to denuded AM. In light of the seminal work by Rama et al. (124), it can be speculated that the use of intact AM may be more effective than denuded AM in treating LSCD. However, prospective clinical studies comparing the use of cultured LEC on intact and denuded AM are warranted before a conclusion can be reached. More studies to quantify phenotypic data of cultured LEC would be of high value to advance regenerative medicine in the cornea.

Cross-linking of AM has been investigated as a method of increasing the thermal and mechanical stability, optical transparency and resistance to collagenase digestion of AM following transplantation. It has been shown that the addition of l-lysine molecules to the cross-linking system can increase cross-linking efficiency (114). Further research should be directed towards more fully exploring the role of lysine concentration on stabilization of the cross-linked AM. Moreover, quantification of phenotypic data with particular emphasis on stemness-associated markers of LEC cultured on AM using various cross-linking systems should be given emphasis. At present, the routine of using intact, non-crosslinked AM remains the standard for treating patients with SLET and for *in vitro* culture of LEC on amnion.

Acknowledgments

The authors would like to thank Astrid Østerud, Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway. Funding received from Department of Oral Biology, Faculty of Dentistry, University of Oslo and Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Marques PI, Fonseca F, Sousa T et al. Adaptive Evolution Favoring KLK4 Downregulation in East Asians. Mol Biol Evol 2016;33(1):93-108.
2. Burger K. Artificial vaginal reconstruction with the help of amnios. Zantralblatt Fur Gynakol 1937;2437-2440.
3. Chao YC, Humphreys S, Penfield W. A New Method of preventing Adhesions. The Use of Amnioplastin after Craniotomy. Br Med J 1940;1(4134):517-538.
4. Gharib M, Ure BM, Klose M. Use of amniotic grafts in the repair of gastroschisis. Pediatr Surg Int 1996;11(2-3):96-99.
5. Muralidharan S, Gu J, Laub GW et al. A new biological membrane for pericardial closure. J Biomed Mater Res 1991;25(10):1201-1209.
6. Troensegaard-Hansen E. Amnion implantation in peripheral vascular disease. Br Med J 1956;2(4987):262-268.
7. Zohar Y, Talmi YP, Finkelstein Y et al. Use of human amniotic membrane in otolaryngologic practice. Laryngoscope 1987;97(8 Pt 1):978-980.
8. Seashore JH, MacNaughton RJ, Talbert JL. Treatment of gastroschisis and omphalocele with biological dressings. J Pediatr Surg 1975;10(1):9-17.
9. Lin YH, Hwang JL, Huang LW et al. Amniotic membrane grafting to treat refractory labial adhesions postpartum. A case report. J Reprod Med 2002;47(3):235-237.
10. Trelford JD, Hanson FW, Anderson DG. Amniotic membrane as a living surgical dressing in human patients. Oncology 1973;28(4):358-364.
11. Kothary PM. Total glossectomy and repair with amniotic membrane. J Indian Med Assoc 1974;62(3):87-88.
12. Sorsby A, Haythorne J, Reed H. Further Experience with Amniotic Membrane Grafts in Caustic Burns of the Eye. Br J Ophthalmol 1947;31(7):409-418.
13. Sorsby A, Symons HM. Amniotic membrane grafts in caustic burns of the eye (burns of the second degree). Br J Ophthalmol 1946;30:337-345.
14. FS L. Lime burn of conjunctiva and cornea treated with amnioplastin graft. Trans Ophthalmol Soc UK 1946;66.
15. DeRoth A. Plastic repair of conjunctival defects with fetal membrane. Arch Opthalmol 1940;23:522-525.
16. Batle JF, Perdomo PF. Placental membranes as a conjunctival substitute. Ophthalmology 1993;100:A107.
17. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 1995;14(5):473-484.
18. John T. Human amniotic membrane transplantation: past, present, and future. Ophthalmol Clin North Am 2003;16(1):43-65.
19. Grueterich M, Espana EM, Tseng SC. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 2003;48(6):631-646.
20. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 2000;343(2):86-93.
21. Sekar S, Sasirekha K, Krishnakumar S et al. A novel cross-linked human amniotic membrane for corneal implantations. Proc Inst Mech Eng H 2013;227(3):221-228.

22. Grueterich M, Espana E, Tseng SC. Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. Invest Ophthalmol Vis Sci 2002;43(1):63-71.
23. Koizumi NJ, Inatomi TJ, Sotozono CJ et al. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 2000;20(3):173-177.
24. Li DQ, Tseng SC. Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. J Cell Physiol 1995;163(1):61-79.
25. Sangwan VS, Sharp JAH. Simple limbal epithelial transplantation. Curr Opin Ophthalmol 2017;28(4):382-386.
26. Bourne GL. The microscopic anatomy of the human amnion and chorion. Am J Obstet Gynecol 1960;79:1070-1073.
27. Danforth D, Hull RW. The microscopic anatomy of the fetal membranes with particular reference to the detailed structure of the amnion. Am J Obstet Gynecol 1958;75(3):536-547.
28. van Herendael BJ, Oberti C, Brosens I. Microanatomy of the human amniotic membranes. A light microscopic, transmission, and scanning electron microscopic study. Am J Obstet Gynecol 1978;131(8):872-880.
29. Weser H, Kaufmann P. [Lightmicroscopical and histochemical studies on the chorionic plate of the mature human placenta (author's transl)]. Arch Gynakol 1978;225(1):15-30.
30. Benedetti WL SM, Alvarez H. Histochemical demonstration of enzymes in the umbilical cord and membranes of human term pregnancy. Eur J Obstet Gynecol Reprod Biol 1973;3:185-189.
31. Niknejad H, Peirovi H, Jorjani M et al. Properties of the amniotic membrane for potential use in tissue engineering. Eur Cell Mater 2008;15:88-99.
32. Szurman P, Warga M, Grisanti S et al. Sutureless amniotic membrane fixation using fibrin glue for ocular surface reconstruction in a rabbit model. Cornea 2006;25(4):460-466.
33. Sarkar S, Sales KM, Hamilton G et al. Addressing thrombogenicity in vascular graft construction. J Biomed Mater Res B Appl Biomater 2007;82(1):100-108.
34. Wilshaw SP, Kearney JN, Fisher J et al. Production of an acellular amniotic membrane matrix for use in tissue engineering. Tissue Eng 2006;12(8):2117-2129.
35. Benson-Martin J, Zammaretti P, Bilic G et al. The Young's modulus of fetal preterm and term amniotic membranes. Eur J Obstet Gynecol Reprod Biol 2006;128(1-2):103-107.
36. Hieber AD, Corcino D, Motosue J et al. Detection of elastin in the human fetal membranes: proposed molecular basis for elasticity. Placenta 1997;18(4):301-312.
37. Massie I, Kureshi AK, Schrader S et al. Optimization of optical and mechanical properties of real architecture for 3-dimensional tissue equivalents: Towards treatment of limbal epithelial stem cell deficiency. Acta Biomater 2015;24:241-250.
38. Riau AK, Beuerman RW, Lim LS et al. Preservation, sterilization and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. Biomaterials 2010;31(2):216-225.
39. Kruse FE, Joussen AM, Rohrschneider K et al. Cryopreserved human amniotic membrane for ocular surface reconstruction. Graefes Arch Clin Exp Ophthalmol 2000;238(1):68-75.
40. Shimazaki J, Shinozaki N, Tsubota K. Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. Br J Ophthalmol 1998;82(3):235-240.

41. Sato H SJ, Shimazaki N. Role of growth factors for ocular surface reconstruction after amniotic membrane transplantation. Invest Ophthalmol Vis Sci 1998;39:S428.
42. Hoch RV, Soriano P. Roles of PDGF in animal development. Development 2003;130(20):4769-4784.
43. Sotozono C, Kinoshita S, Kita M et al. Paracrine role of keratinocyte growth factor in rabbit corneal epithelial cell growth. Exp Eye Res 1994;59(4):385-391.
44. Wilson SE, He YG, Weng J et al. Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. Exp Eye Res 1994;59(6):665-678.
45. Dua HS, Azuara-Blanco A. Amniotic membrane transplantation. Br J Ophthalmol 1999;83(6):748-752.
46. Solomon A, Rosenblatt M, Monroy D et al. Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br J Ophthalmol 2001;85(4):444-449.
47. Park WC, Tseng SC. Modulation of acute inflammation and keratocyte death by suturing, blood, and amniotic membrane in PRK. Invest Ophthalmol Vis Sci 2000;41(10):2906-2914.
48. Shimmura S, Shimazaki J, Ohashi Y et al. Antiinflammatory effects of amniotic membrane transplantation in ocular surface disorders. Cornea 2001;20(4):408-413.
49. Heiligenhaus A, Bauer D, Meller D et al. Improvement of HSV-1 necrotizing keratitis with amniotic membrane transplantation. Invest Ophthalmol Vis Sci 2001;42(9):1969-1974.
50. Tsai RJ, Tseng SC. Effect of stromal inflammation on the outcome of limbal transplantation for corneal surface reconstruction. Cornea 1995;14(5):439-449.
51. Tseng SCG, LD-Q, Ma X. Down-regulation of TGF-β1, β2, β3, and TGG-β receptor II expression in human corneal fibroblasts by amniotic membrane. Invest Ophthalmol Vis Sci 1998;39:S428.
52. Llames S, Garcia-Perez E, Meana A et al. Feeder Layer Cell Actions and Applications. Tissue Eng Part B Rev 2015;21(4):345-353.
53. Grueterich M, Espana EM, Tseng SC. Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without a 3T3 fibroblast feeder layer. Invest Ophthalmol Vis Sci 2003;44(10):4230-4236.
54. Sudha B, Sitalakshmi G, Iyer GK et al. Putative stem cell markers in limbal epithelial cells cultured on intact & denuded human amniotic membrane. Indian J Med Res 2008;128(2):149-156.
55. Meller D, Pires RT, Tseng SC. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. Br J Ophthalmol 2002;86(4):463-471.
56. Krishnan S, Sudha B, Krishnakumar S. Isoforms of p63 in corneal stem cells cultured on human amniotic membrane. Biologicals 2010;38(5):570-576.
57. Hernandez Galindo EE, Theiss C, Steuhl KP et al. Expression of Delta Np63 in response to phorbol ester in human limbal epithelial cells expanded on intact human amniotic membrane. Invest Ophthalmol Vis Sci 2003;44(7):2959-2965.
58. Utheim O, Islam R, Lyberg T et al. Serum-free and xenobiotic-free preservation of cultured human limbal epithelial cells. PloS one 2015;10(3):e0118517.
59. Koizumi N, Fullwood NJ, Bairaktaris G et al. Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. Invest Ophthalmol Vis Sci 2000;41(9):2506-2513.

60. Koizumi N, Rigby H, Fullwood NJ et al. Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells. Graefes Arch Clin Exp Ophthalmol 2007;245(1):123-134.
61. Raeder S, Utheim TP, Messelt E et al. The impact of de-epithelialization of the amniotic membrane matrix on morphology of cultured human limbal epithelial cells subject to eye bank storage. Cornea 2010;29(4):439-445.
62. Fatima A, Vemuganti GK, Iftekhar G et al. In vivo survival and stratification of cultured limbal epithelium. Clin Experiment Ophthalmol 2007;35(1):96-98.
63. Grueterich M, Espana EM, Touhami A et al. Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency. Ophthalmology 2002;109(8):1547-1552.
64. Kolli S, Ahmad S, Lako M et al. Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells 2010;28(3):597-610.
65. Pauklin M, Fuchsluger TA, Westekemper H et al. Midterm results of cultivated autologous and allogeneic limbal epithelial transplantation in limbal stem cell deficiency. Dev Ophthalmol 2010;45:57-70.
66. Schwab IR. Cultured corneal epithelia for ocular surface disease. Trans Am Ophthalmol Soc 1999;97:891-986.
67. Tseng SC, Meller D, Anderson DF et al. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane for treating corneal diseases with total limbal stem cell deficiency. Adv Exp Med Biol 2002;506(Pt B):1323-1334.
68. Pathak M, Cholidis S, Haug K et al. Clinical transplantation of ex vivo expanded autologous limbal epithelial cells using a culture medium with human serum as single supplement: a retrospective case series. Acta Ophthalmol 2013;91(8):769-775.
69. Ang LP, Sotozono C, Koizumi N et al. A comparison between cultivated and conventional limbal stem cell transplantation for Stevens-Johnson syndrome. Am J Ophthalmol 2007;143(1):178-180.
70. Baradaran-Rafii A, Ebrahimi M, Kanavi MR et al. Midterm outcomes of autologous cultivated limbal stem cell transplantation with or without penetrating keratoplasty. Cornea 2010;29(5):502-9.
71. Basu S, Ali H, Sangwan VS. Clinical outcomes of repeat autologous cultivated limbal epithelial transplantation for ocular surface burns. Am J Ophthalmol 2012;153(4):643-650.
72. Dobrowolski D, Wylegala E, Orzechowska-Wylegala B et al. Application of autologous cultivated corneal epithelium for corneal limbal stem cell insufficiency-short-term results. Klin Oczna 2011;113(10-12):346-351.
73. Fatima A, Matalia HP, Vemuganti GK et al. Pseudoepitheliomatous hyperplasia mimicking ocular surface squamous neoplasia following cultivated limbal epithelium transplantation. Clin Experiment Ophthalmol 2006;34(9):889-891.
74. Gomes JA, Pazos HSB, Silva AB et al. Transplante de celulas-tronco epiteliais limbicas alogenas expandidas ex vivo sobre membrana amniotica: relato de caso. Arq Bras Oftalmol 2009;72:254–256.
75. Guarnieri A, Moreno-Montanes J, Alfonso-Bartolozzi B et al. Quantification of corneal neovascularization after ex vivo limbal epithelial stem cell therapy. Int J Ophthalmol 2014;7(6):988-995.
76. Harkin DG, Barnard Z, Gillies P et al. Analysis of p63 and cytokeratin expression in a cultivated limbal autograft used in the treatment of limbal stem cell deficiency. Br J Ophthalmol 2004;88(9):1154-1158.

77. Kawashima M, Kawakita T, Satake Y et al. Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. Arch Ophthalmol 2007;125(10):1337-1344.
78. Koizumi N, Inatomi T, Suzuki T et al. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 2001;108(9):1569-1574.
79. Koizumi N, Inatomi T, Suzuki T et al. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. Arch Ophthalmol 2001;119(2):298-300.
80. Nakamura T, Inatomi T, Sotozono C et al. Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 2006;113(10):1765-1772.
81. Nakamura T, Inatomi T, Sotozono C et al. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. Acta Ophthalmol Scand 2004;82(4):468-471.
82. Nakamura T, Koizumi N, Tsuzuki M et al. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. Cornea 2003;22(1):70-71.
83. Sahu SK, Das S, Sachdeva V, Sangwan VS. Alcaligenes xylosoxidans keratitis after autologous cultivated limbal epithelium transplant. Can J Ophthalmol 2009;44(3):336-337.
84. Sangwan VS, Basu S, Vemuganti GK et al. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. Br J Ophthalmol 2011;95(11):1525-1529.
85. Sangwan VS, Matalia HP, Vemuganti GK et al. Clinical outcome of autologous cultivated limbal epithelium transplantation. Indian J Ophthalmol 2006;54(1):29-34.
86. Sangwan VS, Matalia HP, Vemuganti GK et al. Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. Arch Ophthalmol 2005;123(3):334-340.
87. Sangwan VS, Murthy SI, Vemuganti GK et al. Cultivated corneal epithelial transplantation for severe ocular surface disease in vernal keratoconjunctivitis. Cornea 2005;24(4):426-430.
88. Sangwan VS, Vemuganti GK, Singh S et al. Successful reconstruction of damaged ocular outer surface in humans using limbal and conjuctival stem cell culture methods. Biosci Rep 2003;23(4):169-174.
89. Satake Y, Shimmura S, Shimazaki J. Cultivated autologous limbal epithelial transplantation for symptomatic bullous keratopathy. BMJ Case Rep 2009; doi:10.1136/bcr.11.2008.1239.
90. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 2000;19(4):421-426.
91. Sharma S, Tandon R, Mohanty S et al. Culture of corneal limbal epithelial stem cells: experience from benchtop to bedside in a tertiary care hospital in India. Cornea 2011;30(11):1223-1232.
92. Shigeyasu C, Shimazaki J. Ocular surface reconstruction after exposure to high concentrations of antiseptic solutions. Cornea 2012;31(1):59-65.
93. Shimazaki J, Aiba M, Goto E et al. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. Ophthalmology. 2002;109(7):1285-1290.

94. Shimazaki J, Higa K, Morito F et al. Factors influencing outcomes in cultivated limbal epithelial transplantation for chronic cicatricial ocular surface disorders. Am J Ophthalmol 2007;143(6):945-953.
95. Shortt AJ, Secker GA, Rajan MS et al. Ex vivo expansion and transplantation of limbal epithelial stem cells. Ophthalmology 2008;115(11):1989-1997.
96. Vazirani J, Basu S, Kenia H et al. Unilateral partial limbal stem cell deficiency: contralateral versus ipsilateral autologous cultivated limbal epithelial transplantation. Am J Ophthalmol 2014;157(3):584-590.
97. Zakaria N, Possemiers T, Dhubhghaill SN et al. Results of a phase I/II clinical trial: standardized, non-xenogenic, cultivated limbal stem cell transplantation. J Transl Med 2014;12:58.
98. Gasiorowski JZ, Murphy CJ, Nealey PF. Biophysical cues and cell behavior: the big impact of little things. Annu Rev Biomed Eng 2013;15:155-176.
99. Clark P, Connolly P, Curtis AS et al. Topographical control of cell behaviour. I. Simple step cues. Development 1987;99(3):439-448.
100. Gasiorowski JZ, Liliensiek SJ, Russell P et al. Alterations in gene expression of human vascular endothelial cells associated with nanotopographic cues. Biomaterials 2010;31(34):8882-8888.
101. Stevens MM, George JH. Exploring and engineering the cell surface interface. Science 2005;310(5751):1135-1138.
102. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004;20(1):33-43.
103. Gelse K, Poschl E, Aigner T. Collagens--structure, function, and biosynthesis. Adv Drug Deliv Rev 2003;55(12):1531-1546.
104. Fernandes H, Dechering K, Van Someren E et al. The role of collagen crosslinking in differentiation of human mesenchymal stem cells and MC3T3-E1 cells. Tissue Eng Part A 2009;15(12):3857-3867.
105. Ottani V, Martini D, Franchi M et al. Hierarchical structures in fibrillar collagens. Micron 2002;33(7-8):587-596.
106. Hong HH, Pischon N, Santana RB et al. A role for lysyl oxidase regulation in the control of normal collagen deposition in differentiating osteoblast cultures. J Cell Physiol 2004;200(1):53-62.
107. Slansky HH, Dohlman CH. Collagenase and the cornea. Surv Ophthalmol 1970;14(5):402-415.
108. Spoerl E, Wollensak G, Reber F et al. Cross-linking of human amniotic membrane by glutaraldehyde. Ophthalmic research 2004;36(2):71-77.
109. Lai JY, Ma DH. Glutaraldehyde cross-linking of amniotic membranes affects their nanofibrous structures and limbal epithelial cell culture characteristics. Int J Nanomed 2013;8:4157-4168.
110. Kitagawa K, Okabe M, Yanagisawa S et al. Use of a hyperdried cross-linked amniotic membrane as initial therapy for corneal perforations. Jpn J Ophthalmol 2011;55(1):16-21.
111. Fujisato T, Tomihata K, Tabata Y et al. Cross-linking of amniotic membranes. J Biomater Sci Polym Ed 1999;10(11):1171-1181.
112. Ma DH, Chen HC, Ma KS et al. Preservation of human limbal epithelial progenitor cells on carbodiimide cross-linked amniotic membrane via integrin-linked kinase-mediated Wnt activation. Acta Biomater 2015;31:144-155.
113. Lai JY. Carbodiimide cross-linking of amniotic membranes in the presence of amino acid bridges. Mater Sci Eng C Mater Biol Appl 2015;51:28-36.

114. Lai JY, Wang PR, Luo LJ et al. Stabilization of collagen nanofibers with L-lysine improves the ability of carbodiimide cross-linked amniotic membranes to preserve limbal epithelial progenitor cells. Int J Nanomed 2014;9:5117-5130.
115. Lai JY, Lue SJ, Cheng HY et al. Effect of matrix nanostructure on the functionality of carbodiimide cross-linked amniotic membranes as limbal epithelial cell scaffolds. J Biomed Nanotechnol 2013;9(12):2048-2062.
116. Tanaka Y, Kubota A, Yokokura S et al. Optical mechanical refinement of human amniotic membrane by dehydration and cross-linking. J Tissue Eng Regen Med 2012;6(9):731-737.
117. Ma DH, Lai JY, Cheng HY et al. Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. Biomaterials 2010;31(25):6647-6658.
118. Lai JY. Photo-cross-linking of amniotic membranes for limbal epithelial cell cultivation. Mater Sci Eng C Mater Biol Appl 2014;45:313-319.
119. Lai JY. Interrelationship between cross-linking structure, molecular stability, and cytocompatibility of amniotic membranes cross-linked with glutaraldehyde of varying concentrations. RSC Adv 2014;4(36):18871-18880.
120. Chang Y, Tsai CC, Liang HC et al. In vivo evaluation of cellular and acellular bovine pericardia fixed with a naturally occurring crosslinking agent (genipin). Biomaterials 2002;23(12):2447-2457.
121. Jin J, Song M, Hourston DJ. Novel chitosan-based films cross-linked by genipin with improved physical properties. Biomacromolecules 2004;5(1):162-168.
122. Lai JY, Li YT. Functional assessment of cross-linked porous gelatin hydrogels for bioengineered cell sheet carriers. Biomacromolecules 2010;11(5):1387-1397.
123. Lai JY LL. Effect of riboflavin concentration on the development of photo-cross-linked amniotic membranes for cultivation of limbal epithelial cells. RSC Adv 2015;5(5):3425-3434.
124. Rama P, Matuska S, Paganoni G et al. Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med 2010;363(2):147-155.
125. Li W, He H, Kuo CL et al. Basement membrane dissolution and reassembly by limbal corneal epithelial cells expanded on amniotic membrane. Invest Ophthalmol Vis Sci 2006;47(6):2381-2389.

Figure 1. Schematic Representation of the 5-layered Human Amniotic Membrane