The conjunctival extracellular matrix, related disorders and development of substrates for conjunctival restoration

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Abstract

The conjunctiva is an essential component of the ocular surface. It can be damaged by numerous pathological processes leading to scarring, loss of tissue and dysfunction. Depending on the extent of the damage, restoration of function may require a conjunctival graft. A wide variety of biological substrates have been tested in the search for optimal conditions for the *ex vivo* culture of conjunctival epithelial cells as a route toward tissue grafts for transplantation. Each substrate has specific advantages but also disadvantages related to their unique physical and biological characteristics and identification and development of an improved substrate remains a priority. To achieve the goal of mimicking and restoring a biological material, requires information from the material itself. Specifically, the extracellular matrix (ECM) derived from the conjunctiva tissue. Knowledge of the composition and structure of the native ECM and identifying the contribution of individual components to its function would enable using or mimicking those components to develop improved biological substrates. ECM is comprised of two components: the basement membrane secreted predominantly by the epithelial cells containing laminins and type IV collagens, which directly support epithelial and goblet cell adhesion, differentiation and growth and, the interstitial matrix secreted by the fibroblasts in the lamina propria, which provides mechanical and structural support. This review presents the current knowledge on the anatomy, composition of the conjunctival ECM and related disorders that affect the conjunctiva. The requirements of potential substrates for conjunctival tissue engineering and transplantation are discussed. Biological and synthetic substrates and their components are described in an accompanying review.

Introduction

The conjunctiva is a translucent mucous membrane covering the ocular surface around the cornea (1). It protects the eye from external insults by acting as a physical and an immunological barrier thereby promoting innate and adaptive immunity (2)(3). Despite the existence of multiple defence mechanisms, the conjunctiva is prone to injury and entry of pathogens, or to damage as a result of systemic autoimmune diseases (4)(5). This damage may lead to anatomical and functional impairment (6), with a loss of homeostasis of the ocular surface including the limbus and the cornea, and an associated loss of vision (7). Importantly, limbal and corneal transplantation fail in the absence of a functioning conjunctival epithelium, underscoring the importance of this component of the ocular surface (8)(9).

The current ideal conjunctival replacement is a conjunctival autograft (6). The availability, however, of this tissue is a major limitation in bilateral disease or when there is insufficient uninvolved tissue in the fellow eye (10). Although allogeneic grafts may be an option, they carry risks of graft rejection and microbial disease transmission (11). An alternative to auto- or allogeneic grafts is a functional *ex vivo* tissue construct that can be transplanted to restore the anatomical structure and function of the original tissue. The success of an *ex vivo* expanded tissue construct depends on three factors: the source of cells and presence of stem cells, the carrier substrate, and the presence of growth factors (12). Here we will focus on the substrate, which provides the initial support for the cultured cells, physical and biological cues for the growth of cells, support for the delivery of cultured cells to the host and provide a three-dimensional scaffold for the formation of new tissue (13).

Numerous biological and synthetic substrates have been investigated in the search for the optimal substrate for the *ex vivo* culture of conjunctival epithelial cells. Biological substrates based on extracellular matrix proteins will be discussed herein. Other biological substrates and synthetic

substrates will be described in a second review. Biological substrates are generally biodegradable and contain biologically active domains that support cellular functions; however, they can be fragile, difficult to handle, and may elicit an immune reaction. Therefore, there is scope for the development of improved biological substrates for conjunctival tissue transplantation.

The extracellular matrix (ECM), the non-cellular component of tissues consisting of biomolecules secreted by the resident cells, regulates the growth and differentiation of the cells by providing specific biological and physical cues (14)(15). The native conjunctival ECM has been tailored throughout evolution to support conjunctival function. Therefore, understanding the composition and function of the native ECM and specifically identifying those components that aid conjunctival cell culture provides a valuable rationale for the development of improved biological substrates.

1 Anatomy of the conjunctiva

The conjunctiva extends from the limbus to the posterior surfaces of the eyelids and ends at the muco-cutaneous junction thus covering most of the ocular surface area (1). Histologically, the conjunctiva is divided into the epithelium, the basement membrane (BM) and the lamina propria (**Figure 1**).

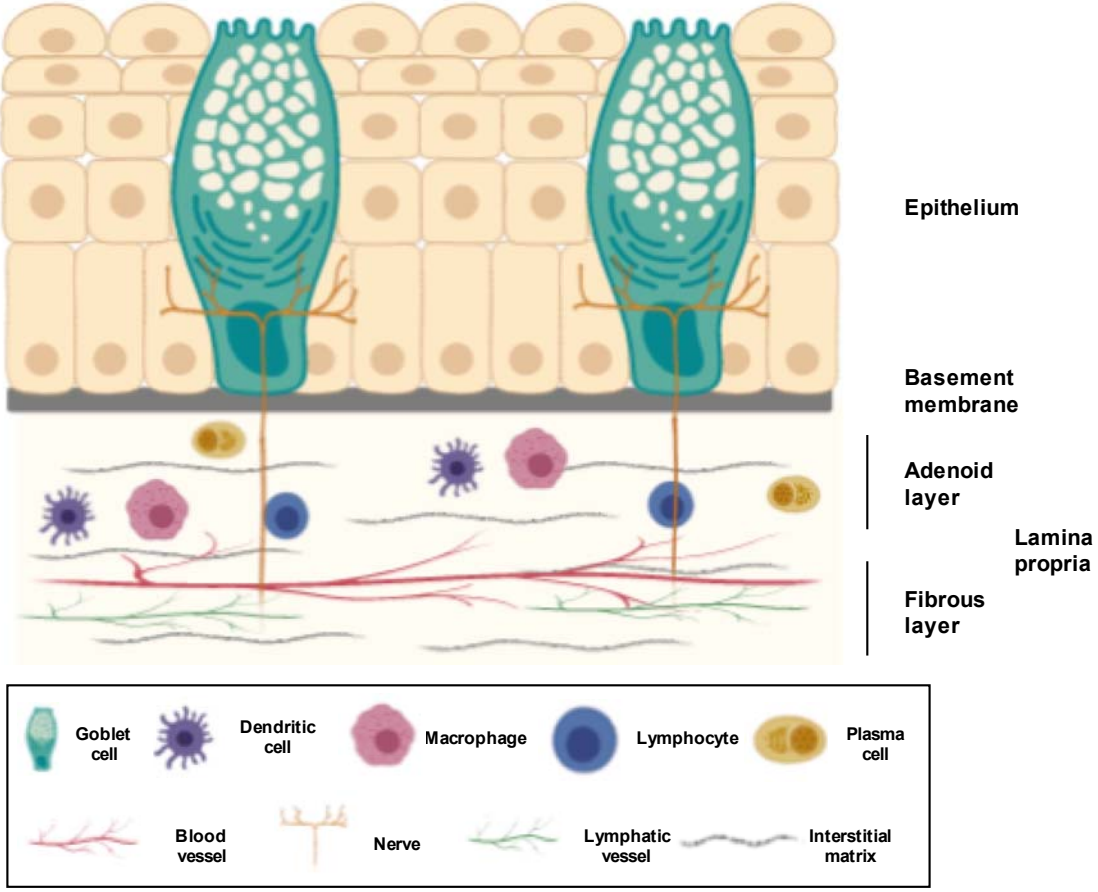

Figure 1: Schematic diagram of the histology of the conjunctiva (not to scale). The conjunctival epithelium consists of stratified epithelial cells (superficial, intermediate and basal cells) and goblet cells, supported bya basement membrane. The underlying lamina propria consists of the interstitial matrix containing collagen fibrils, elastic fibrils, immune cells (dendritic cells, macrophages, lymphocytes and plasma cells), and blood vessels, lymphatic vessels and nerves. Note that in human beings the goblet cells do not quite reach the basement membrane. (Created with BioRender.com)

The epithelium is non-keratinised, stratified , and approximately six cell-layers thick at the limbus and up to twelve cell-layers at the fornices (1). The cells of the epithelium are arranged into superficial, intermediate and basal cell layers, with the superficial cells having microplicae that support the glycocalyx of the tear film (16). In addition, the conjunctival epithelium contains mucin-

secreting goblet cells, which contact the BM in rats and mice, but only extend to the middle of the epithelium in human beings (17). Stratified squamous and goblet cells are the major epithelial cell types in the conjunctiva. Stratified squamous cells are flat, multi-layered cells that surround the goblet cells localized either singly or in clusters. Together these two types of cells form a very leaky epithelium that allows penetration of electrolytes, water, small molecules, and antigens into the lamina propria (17)(18). The conjunctival epithelium also consists of melanocytes, intraepithelial dendritic cells and lymphocytes and contains its own pool of stem cells supporting stratified squamous and goblet cell renewal (3)(19). A successful conjunctival replacement needs to support and maintain each of these disparate cell types and their functions.

One of the primary functions of the conjunctival epithelium is to help maintain the tear film. The stratified squamous cells secrete electrolytes and water into the tear film under the control of sympathomimetic and purinergic agonists (17). The goblet cells also secrete electrolytes and water, but this has not been directly studied. Stratified squamous cells traffic membrane-spanning mucins MUC 1, 4, 16 and 20 on to the apical membranes where they interact with the glycocalyx and the tear film (20). The goblet cells synthesize and secrete the large gel-forming mucin MUC5AC into the tear film (21). Secretion of this mucin is under neural control stimulated by parasympathomimetic agonists and growth factors such as epidermal growth factor and nerve growth factor (22)(23)(24). These nerves via a complex neural reflex stimulate secretion in response to changes in the external environment to protect the ocular surface from pathogens, pollutants, antigens, chemicals, and mechanical trauma. It is particularly important for goblet cells to be present in the conjunctiva as their absence leads to ocular surface disease (25).

The lamina propria is a layer of loose fibrovascular connective tissue (16). It consists of interstitial matrix predominantly made up of fibrillar collagen types I and III secreted by the resident fibroblasts (26). The lamina propria is divided into a superficial adenoid and an inferior fibrous layer (27). The

adenoid layer contains conjunctiva-associated lymphoid tissue, a type of mucosa associated lymphoid tissue, consisting of immune cells including T and B lymphocytes, plasma cells, macrophages and dendritic cells, all of which serve a role in the immune defence system of the ocular surface (1). The fibrous layer is highly vascularised receiving branches from the anterior ciliary and palpebral arteries and their accompanying veins (1)(16). The lymph from the medial and lateral aspects of the conjunctiva drain into submandibular and superficial pre-auricular nodes, respectively (1). Innervation is from the ophthalmic and maxillary divisions of the trigeminal nerve and consists of afferent sensory nerves. The efferent parasympathetic and sympathetic nerves are from the sphenopalatine and superior cervical ganglia, respectively (1)(16)(28). All these cells and structures play an important role in wound healing, tear secretion and protecting the eye and the ocular surface in response to external insults.

2 Extracellular matrix

The ECM is a non-cellular component of tissues and consists of biomolecules secreted by the resident cells (15)(29). The ECM provides anchorage sites for cells by presenting ligands to cell receptors (29)(30). Individual proteins have multiple binding sites for cell surface receptors that initiate intracellular signalling pathways influencing a multitude of cellular processes, including those critical for cell migration, proliferation and lineage-specific differentiation (15)(30)(31). The ECM further contributes to the mechanical properties of tissues by defining tensile strength and stiffness (29)(32). Specific proteins within the ECM are responsible or required for self-renewal, maintenance and activation of stem cells (31). The ECM also provides a reservoir of growth factors and cytokines and coordinates their release to influence the behaviour of cells in physiological or pathological states (15)(30).

In terms of the conjunctiva, the ECM can be considered as two linked but distinct structures: the BM and the interstitial matrix (15). In general, BMs, also known as basal laminas, as observed by

transmission electron microscopy are electron-dense, sheet-like structures containing two layers, the lamina rara (lamina lucida) and lamina densa. These BM layers are located basolateral to sheets of cells, thereby simultaneously separating and linking the cell layers from and to the underlying connective tissue (29)(30). BMs are found in all tissues; on the basal side of epithelial cells, adjacent to vascular endothelial cells and Schwann cells of peripheral nerves, and encircling smooth, cardiac and skeletal muscle fibres (33). Based on transmission electron microscopy, the BM thickness is between 50 nm and 100 nm (16)(30). Studies using atomic force microscopy, however, suggest that in a hydrated state the BM may be two-fold thicker (33). These matrix structures are formed during the embryonic period where they help to establish polarity in the epiblast (34). There are no published articles on the embryonic development of the conjunctival BM. In human beings, however, the corneal BM is first detected during gestation weeks eight and nine, and the epithelium separates from the underlying lamina propria as early as week sixteen (35). Structurally, each BM consists of large insoluble protein networks that are cross-linked into network structures by undergoing self-assembly guided by cellular receptors, and then further connected by a series of smaller linker proteins (29).

The interstitial matrix in most tissues, including the conjunctiva, is a comparatively less structured three-dimensional amorphous ‘gel’ found beneath BMs. This gel surrounds cells primarily of mesenchymal origin, such as adipocytes, chondrocytes, myoblasts and osteoblasts and acts as the ‘glue’ that holds the cells in place (15)(36). Interstitial matrices, in general, are less dense, more porous and contain more collagen, elastin and fibronectin compared to the highly cross-linked, sheet-like BMs (15). The interstitial matrix, plays an important role in the functioning of tissues. It contributes to the mechanical properties that are dictated by the amount and orientation of collagen fibrils synthesised and secreted by the fibroblasts. This matrix also contains the immune cells and vascular tissue, thus playing a role in the defence mechanisms and nutrition of the tissue.

The conjunctiva consists of both these forms of ECM: the epithelial cells that sit on and secrete the proteins of the BM, as well as the fibroblasts in the lamina propria that secrete fibrillar collagens. In tissue engineering, it is the function of the BM that is targeted as a substrate for the culture of epithelial cells. It is the components of this part of the matrix that directly guide cellular functions via outside-in signalling pathways, whereas the interstitial matrix, in general, models the bulk of the mechanical properties of the substrate.

2.1 Composition of basement membranes

The composition of BMs is diverse, tissue-specific and dynamic (30). Although all human BMs appear broadly similar when viewed by electron microscopy, which proteins and isoforms are present is diverse and specific to each type of tissue. Indeed BM composition provides one of the primary determinants of tissue specificity by dictating individual tissue and cell functions within an organ (29)(30). At the core of each BM are two networks of structural proteins: type IV collagens and laminins (**Figure 2**).

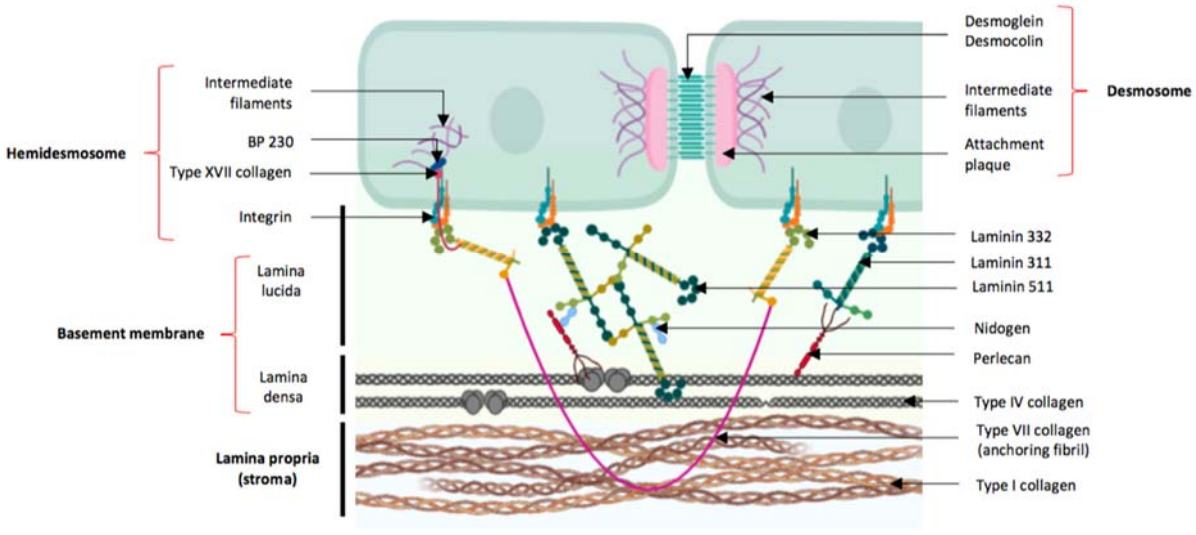

Figure 2: Schematic diagram of the basement membrane components (not to scale). (Created with BioRender.com)

These networks are cross-linked by additional proteins, with heparin sulphate proteoglycans, nidogens and perlecan being ubiquitous. Expression of different isoforms of type IV collagen and the relative abundance of the different members of the laminin family account for major structural specificity between BMs of different tissues. Especially in the case of the laminins, the isoforms present are important mediators of lineage specification (30)(37). The specific composition of the other minor proteins adds further to tissue specificity and heterogeneity (30)(38), as does the structural assembly and patterning of the components (38). Studying the unique composition of ECMs of different tissues and their structure can uncover information regarding the effect of cellular microenvironments on the regulation of the functionality of the resident cells (29)(31)(39)(40). For example, this information on the specificity of laminin function has been exploited, with numerous studies detailing improved cell culture outcome when the contextually appropriate laminin family member is used as a coating (41)(42)(43).

In addition to variation in composition between the different types of tissues, the ECM is a dynamic structure in which the composition and assembly of proteins, and the biomechanical properties differ depending on the physiological state. Changes also occur throughout development and ageing, and as a consequence of numerous pathological states including infection, wound repair and fibrosis (30)(33). For example, the corneal BM is known to have differential expression of proteins during development, including the expression of collagen and laminin isoforms (44). One notable effect of remodelling of the ECM is the exposure of cryptic binding sites on the proteins, allowing cells to interact with different domains of the same molecule giving rise to distinct cellular functions (29).

2.2 Basement membrane components of the conjunctiva and the ocular surface

Numerous studies have investigated the BM components of the ocular surface using immunohistochemical approaches to detail the distribution of the structural protein components in adult tissue (**Table 1** and **Figure 3**).

Table 1: Summary of the structural proteins present in the basement membrane of human ocular surface tissues (+ present; - absent)

ECM component		Cornea	Limbus	Conjunctiva	References
Collagens	**Type IV α_1**	-	+	+	
	Type IV α_2	-	+	+	
	Type IV α_3	+	+/-	-	
	Type IV α_4				
	Type IV α_5	+	+	+	
	Type IV α_6	+	+	+	
	Type V	+	-	-	
	Type VII	+	+	+	
	Type XV	+/-	+	+	
	Type XVII	+	+	+	
	Type XVIII	+	+	+	
Glycoproteins	**Laminin α_1**	+/-	+	+/-	
	Laminin α_2	-	+	+	
	Laminin α_3	+	+	+	
	Laminin α_4				
	Laminin α_5	+/-	+	+	
	Laminin β_1	+/-	+	+	
	Laminin β_2	-	+	+	
	Laminin β_3	+	+	+	
	Laminin γ_1	+/-	+	+	
	Laminin γ_2	+	+	+	
	Laminin γ_3	-	+	-/focal	
	Nidogen 1 + 2	+	+	+	
	Fibronectin	+	+	+	
	Vitronectin	-/weak	Sub-BM	-	
	Fibulin 2	-/+	+	-	
	Fibrillin 1	+	-	-	
	Fibrillin 2	+	+/-	-	
	SPARC	-	+	-	
	Tenascin C	-	+	-/focal	
	Matrilin 2 + 4	+	+	+	
	Thrombospondin 1	+	-	-	
	Thrombospondin 4	-/focal	Focal	Focal	
Proteoglycans	**Perlecan**	+	+	+	
	Agrin	-/weak	+	+/weak	

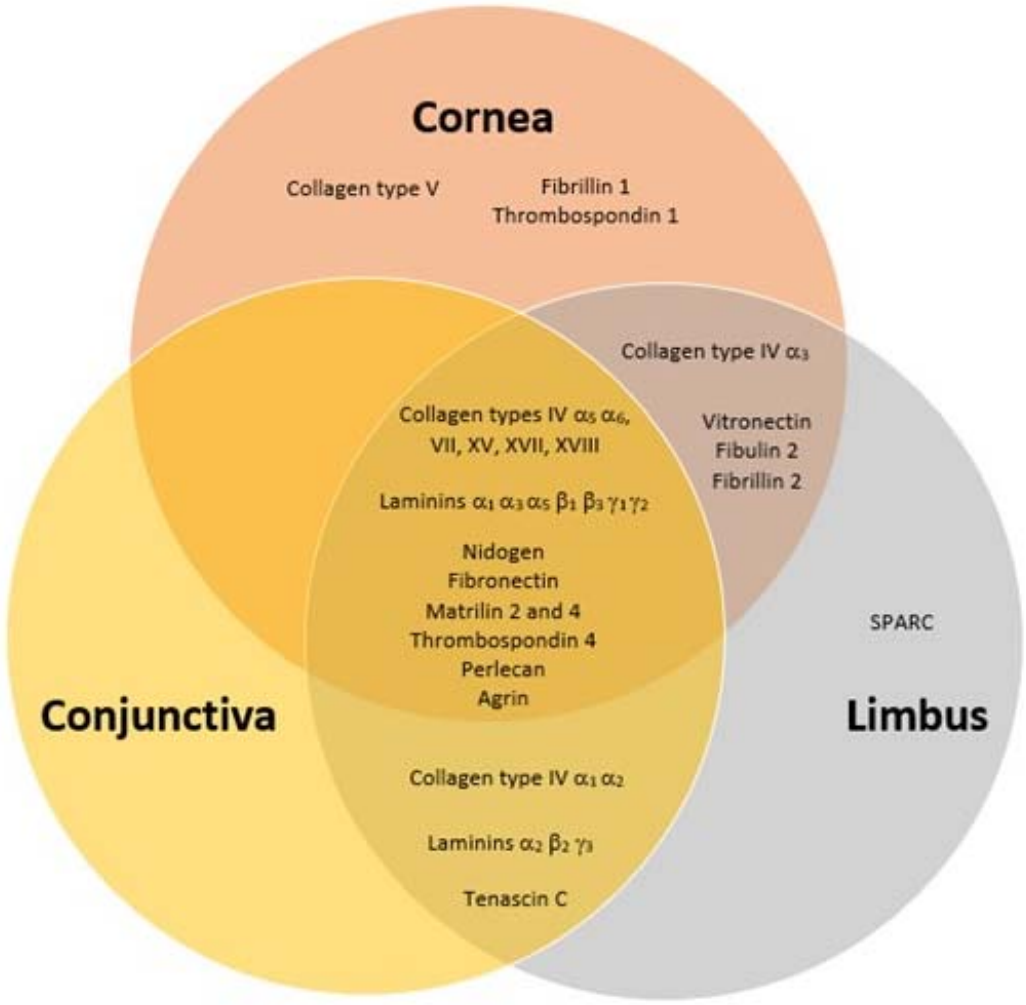

Figure 3: Distribution of structural proteins present in the basement membrane of human ocular surface tissues.

2.2.1 Type IV collagens

Type IV collagen is found in the BMs throughout the ocular surface with variations in the distribution of its isoforms (31). This collagen is the most abundant component of the BM and forms a network-like structure that binds other BM components. Type IV collagens consists of α chains and there are six α chain isoforms that can form at least three different heterotrimeric triple-helical forms (45). The α_1 and α_2 chains were identified within limbal and conjunctival BMs, whereas the α_3 chain was enriched in the corneal BM with weaker immunoreactivity in the limbal BM. The α_4 chain was not detected in the BMs of the ocular surface and the α_5 and α_6 chains were found throughout the

ocular surface; however, the intensity was lower in the conjunctiva compared to the rest of the ocular surface (31).

Other collagens found throughout the ocular surface include type VII (anchoring fibrils), the hemidesmosome-associated transmembrane type XVII collagen (Bullous pemphigoid antigen 2) and BM-associated type XVIII collagen, which can be cleaved to form endostatin (31). Type XV collagen has also been identified (31); however, one study described it being absent from the BM of normal corneas (46). Type V collagen was found only in the corneal BM, whereas type XVI collagen was observed as a sub-epithelial band in association with the BM in the corneo-limbal transition zone (31).

2.2.2 Laminins

Each laminin is a heterotrimer comprising α, β, and γ chains (37)(47). In human beings there are five α, four β, and three γ genes, and in addition the *LAMA3* gene produces two major isoforms, a short α3A form and a longer α3B form. There are 72 potential $\alpha\beta\gamma$ combinations but only 16 laminin trimers have been detected *in vivo*. The assembled trimers are named based on their chain composition. For example, laminin α3, β3 and γ2 subunit is termed laminin (LM) 332 (formerly known as laminin-5).

LM332 generally displays a uniform distribution throughout the ocular surface, as well as in most surface epithelia including the skin and tongue (31). One study, however, showed staining for the laminin β3 chain in only 7% and γ2 chain in only 34% of normal conjunctival samples (38). LM 521 has also been found in the limbal and conjunctival BM, but generally absent within the BM of the corneal epithelium (31)(48). The laminin α1 chain has been reported in the conjunctival BM in two studies (38)(49), but not in one (31). Laminin α4 was not detected in the ocular surface epithelium, which is not surprising as it is usually associated with BMs of vascular tissue (30)(31). In addition to the main laminin isoforms, the laminin superfamily member laminin N terminus (LaNt) α31, an alternative splice isoform from LAMA3, has also been described throughout the ocular surface, but is

enriched in the limbal region (50). LaNt α31 is smaller than laminin subunits and does not contain many of the domains responsible for laminin functions; however, can influence laminin organisation and has been implicated in regulating tissue remodelling and wound repair. The differential distribution of laminin chains between studies, may be due to differences in the sites of tissue harvesting and the specificity of antibodies used particularly in terms of cross-reactivity against multiple chains (38). Additional studies of the heterotrimeric laminins are needed in the conjunctiva.

2.2.3 Other glycoproteins and proteoglycans

Other glycoproteins found throughout the ocular surface BMs include nidogen 1 and 2, fibronectin, matrilin 2 and 4 and perlecan, while agrin is comparatively enriched in the limbal BM compared to the rest of the ocular surface (22). Fibronectin immunoreactivity has been described as thicker and more intense in the limbal and conjunctival BMs compared to the corneal epithelial BM (31)(51). The matricellular protein thrombospondin 4 has also been observed in a patchy distribution throughout the ocular surface, with higher intensity staining in the conjunctiva compared to the cornea (31). The glycoproteins secreted protein acidic and rich in cysteine (SPARC) and tenascin C, that were initially thought to be limbus-specific, have also been detected in the conjunctiva with some focal staining of the latter protein (31). The corneo-limbal transition zone contains fibulin 2, fibrillin 2 and vitronectin, the latter mainly found as a sub-epithelial band in association with the BM (31). Cornea specific components include fibrillin 1 and thrombospondin 1 (31). Corneal epithelial cells, especially the basal cells, express thrombospondin 1 mRNA and protein at a much higher level compared to the conjunctiva (52)(53). The presence of thrombospondin 1, an anti-angiogenic protein, likely contributes to the maintenance of the avascular cornea along with a plethora of other anti-angiogenic proteins (52).

The BM is a dynamic structure the composition of which changes not only during development, but also with age and following repair of injury. Although not measured *in vivo*, *in vitro* studies using a human immortalized conjunctival cell line (HCjE) showed changes to the composition of the ECM

over a twenty-eight day culture period (54). Using mass spectrometry, the whole proteome of the ECM was studied and proteins such as α-2-HS-glycoprotein were identified in the conjunctival ECM. The proportion of fibronectin and α-2-HS-glycoprotein were high in the early ECM compared to the late ECM preparations, whereas the proportion of growth factors and cytokines decreased and proteases increased over the culture period (54).

2.3 Disorders of the basement membrane

The importance of BM function in the conjunctiva is exemplified by the numerous disease processes that arise due to defective function. Extracellular matrix proteins bind the epithelium to the underlying lamina propria forming cell–matrix adhesions (hemidesmosomes) and adjacent cell-cell adhesions (desmosomes). Using this anatomical concept, the major groups of BM disorders affect the basal epithelial cells, the dermal-epidermal junction, or the underlying reticular tissue. These include pemphigus and pemphigoid diseases, epidermolysis bullosa and dermatitis herpetiformis (55)(56). Other disease entities that do not fall within this grouping system also exist, including paraneoplastic cicatrising conjunctivitis, drug-induced cicatrising conjunctivitis, atopic keratoconjunctivitis, acne rosacea, graft-versus-host disease and Alport syndrome. The rarity (0.2 - 1.5 per million population) (57)(58)(59) and clinical similarities of the conjunctival phenotype in these disorders lead to diagnostic delays (57)(60)(61)(62). Alternative classifications categorise patients into those with blinding scarring disorders, ocular surface squamous neoplasia (OSSN), and conjunctival scarring disorders (60). Understanding the components of the BM is fundamental to understanding the pathology of disease, clinical manifestations and to develop targeted therapy for these progressive, blinding, disorders (**Table 2**).

Table 2: Molecular features of ocular basement membrane disorders

Disease	Aetiology	Description	Targeted Protein	Location of BMZ antigen/Function	References
Pemphigus disorders					
Pemphigus vulgaris	Autoimmune immunobullous	Intercellular IgG and C3	Dsg 3, Dsg 1	Desmosome	
Pemphigus foliaceus	Autoimmune immunobullous	Intercellular IgG and C3	Dsg 1	Desmosome	
Paraneoplastic pemphigus	Autoimmune immunobullous	IgG and C3 intercellularly and at the dermal–epidermal junction	Dsg 3, Dsg 1, plakines	Desmosome	
IgA pemphigus	Autoimmune immunobullous	Intercellular IgA and C3	Dsc1, Dsg 3	Desmosome	
Pemphigoid disorders					
Bullous pemphigoid	Autoimmune immunobullous	Linear C3 and IgG at the dermal–epidermal junction	Type XVII Collagen, BP230	Hemidesmosome	
Pemphigoid gestationi	Autoimmune immunobullous	Linear C3 at the dermal--epidermal junction	Type XVII Collagen, BP230	Hemidesmosome	
Mucous Membrane Pemphigoid	Autoimmune immunobullous	HLA-DQB*0301 Linear IgG, IgA and C3 at the dermal–epidermal junction	LM α3 Type XVII Collagen BP230 α6 integrin β4 integrin Laminin 332 Collagen VII 45kDa protein 168kDa protein	Basement membrane Hemidesmosome Hemidesmosome Hemidesmosome Hemidesmosome Basement membrane Fibro-reticularis Implicated in pure ocular disease Implicated in pure oral disease	
Mucous Membrane Pemphigoid Linear IgA variant	Autoimmune immunobullous	Linear IgA (and C3) at the dermal–epidermal junction	LAD-1 Type VII collagen	Fibro-reticularis	

Antilaminin γ-1 (p200) pemphigoid	Autoimmune immunobullous		3	Laminin γ1 200kDa protein	Basement membrane	
Epidermolysis bullosa disorders						
Epidermolysis bullosa simplex	Genetic: Autosomal Dominant	KRT5, KRT14, PLEC, KLH24		Keratin 5, Keratin 14, Plectin, Kelch like member 24	Hemidesmosome (intraepidermal)	
	Genetic: Autosomal Recessive	KRT5, KRT14, DST, EXPH5, PLEC, CD151		Keratin 5, Keratin 14, BP230, Exophilin 5, Plectin, Tetraspanin	Hemidesomosome (intraepidermal)	
Junctional epidermolysis bullosa	Genetic: Autosomal Recessive	4	LAMA3, LAMB3, LAMC2, COL17A1	Laminin 332, Type XVII Collagen Integrin $\alpha6\beta4$, integrin $\alpha3$	Hemidesomosome (Junctional)	
		5	ITGA6, ITGB4, ITGA3			
Dystrophic epidermolysis bullosa	Genetic: Autosomal Dominant	COL7A1		Type VII Collagen	Hemidesomosome (fibro-reticularis)	
	Genetic: Autosomal Recessive	COL7A1		Type VII Collagen	Hemidesomosome (fibro-reticularis)	
Kindler epidermolysis bullosa	Genetic: Autosomal Recessive	FERMT1		Kindlin-1	Fibro-reticularis: conserved focal adhesion and integrin activation through binding of β-integrin tail	
*Epidermolysis bullosa acquisita**	Autoimmune immunobullous	Linear IgG, IgA and C3 at the dermal–epidermal junction		Type VII collagen	Fibro-reticularis (hemidesmosomal instability)	
Other disorders						
Dermatitis herpetiformis	Autoimmune	Granular IgA deposits in the dermal papillae		Transglutaminase	Fibro-reticularis: subepidermal abscess formation	
Alport Syndrome	Autoimmune	COL4A5		Type IV Collagen	Fibro-reticularis	

Abbreviations: BP, Bullous Pemphigoid Antigen; Dsg, desmoglein; †Dsc, desmo, *sometimes categorised under pemphigoid

The main and most common disorders are as follows.

2.3.1 Inherited disorders: Epidermolysis bullosa

Epidermolysis bullosa is a group of genetic disorders where patients present with skin fragility ranging from classical epidermolysis bullosa to other epithelial fragility syndromes including peeling skin and erosive disorders (56)(63)(64). Also included is skin fragility associated with hyperkeratotic and connective tissue disorders. Defects arise due to loss of function any of the components of the hemidesmosome-LM-anchoring fibril complex. The point of epithelial tissue separation depends on where the mutations arise, with EB often sub-categorised based on plane of splitting. Molecular findings enable a more accurate sub-classification. Genotype-phenotype correlation can be complex as mutations in the same gene can lead to inheritance in autosomal dominant or recessive manner depending on the location and mutation type. Specifically, mutations in keratins *(KRT)5* and *KRT14* are usually dominant and lead to splitting within cells*,* whereas mutations to the core hemidesmosome proteins plectin *(PLEC1),* type XVII collagen *(COL17A1),* integrin α6 *(ITGA6)* and β4 *(ITGB4*) split at the dermal-epidermal junction and can particularly severe and manifest in multiple additional defects including muscular dystrophy for plectin or pyloric atresia for the integrins. A mild form of skin peeling has also associated with mutations affecting BP230 *(*BPAG1e, derived from the dystonin gene *DST).* Recessive mutations in any of the three chains of LM332 (*LAMA3*, *LAMB3*, *LAMC2*) leads to separation along the plane of the BM, with a hotspot nonsense mutation in *LAMB3* being particularly prevalent. Defects to type VII anchoring fibrils (*COL7A1*), which can be dominant or recessive, leads to dystrophic form of EB. Similar phenotypes can also arise due to mutations in exophilin *(EXPH)5*, kelch-like42 *(KLHL24*) or kindlin-1 (*FERMT1)* and integrin α3 (*ITGA3*), latter two of which are involved in focal adhesion cell-to-matrix adhesions but also leads to defects in cell adhesion due to cross-talk between focal adhesions and hemidesmosomes (63)(65).

Reported frequencies of ocular complications are dependent on the disease subtype varying from 4-51% with manifestations ranging from corneal erosions and blister formation, corneal scarring, along with symblepharon, blepharitis, lacrimal duct obstruction and impaired vision (66)(67). The activity and damage indices are scored according to the Epidermolysis Bullosa Disease Activity and Scarring Index (EBDASI) (68)(69).

2.3.2 Autoimmune blistering diseases.

Autoimmune blistering diseases comprise a group of rare immunobullous diseases frequently resulting in progressive conjunctival scarring including mucous membrane pemphigoid, pemphigus, epidermolysis bullosa acquista.

Mucous membrane pemphigoid is the most common of these disorder that is characterised by a bilateral, sight-threatening conjunctival inflammation leading to ocular surface failure and blinding keratopathy as a result of chronic limbitis, progressive conjunctival scarring, and limbal epithelial stem cell (LESC) failure. The molecular processes of conjunctival scarring and chronic inflammation disrupt not only the intricate immunoprotective ocular surface microenvironment, but also the protective action of the eyelids and tear film, leading to dry eye and ocular surface damage. The compromised ocular surface is predisposed to corneal ulceration, complicated by infectious keratitis and corneal scarring. Both the surface changes and the inflammation result in pain which is often unremitting and severe.

The disease develops due to loss of tolerance to epithelial BM proteins secondary to circulating autoreactive T cells in genetically predisposed individuals expressing HLA-DQB*0301 (70)(71). Autoreactive T cells interact with autoreactive B cells in regional lymph nodes leading to generation of plasma cells that produce IgG or IgA circulating autoantibodies. Antibodies may also be produced by local plasma cells found in the lesional tissue. The antibodies recognise antigens in a number of

BM proteins including LM332, or the type XVII collagen (BP180 or BPAG2), BP230 (BPAG1e) and α6β4 integrin components of hemidesmosomes (72)(73). Antibodies binding to the BM proteins in the conjunctival mucosa results in complement fixation and separation of the epithelial-stromal (dermal) junction. Precipitation of the complement cascade leads to the 'injury and inflammation' phase of the disease causing recruitment of inflammatory effector cells (neutrophils, dendritic cells, mast cells, eosinophils, macrophages and T cells) and production of the cytokines interleukin (IL) IL-2, IL-5, IL-13 and growth factors interferon (IFN) γ and tumour necrosis factor (TNF) α that perpetuate, often severe, inflammation (74)(75)(76)(77)(78)(79). This is followed by a fibrosis and modelling phase characterised by progressive conjunctival scarring driven by low grade inflammation and endogenous aldehyde dehydrogenase/retinoic acid (ALDH/RA) that maintains a probiotic state in clinically quiescent eyes (80)(81).

Similarly, epidermolysis bullosa acquisita (EBA) is an acquired autoimmune blistering disease where patients develop autoantibodies targeting type VII collagen anchoring fibrils. Deposition of IgG induces complement and neutrophil recruitment that release reactive oxygen species resulting in splitting of the dermal-epidermal junction subsequently leading to two key phenotypes: mechanobullous and bullous pemphigoid-like forms characterised conjunctival fibrosis in 60%, cicatrising conjunctivitis and trichiasis-associated mechanical irritation(82).

By contrast, Pemphigus is a group of prototypical, organ-specific autoimmune diseases characterised and caused by autoantibodies targeting desmosomal adhesion molecules, desmoglein (Dsg) 1 and Dsg3. The destabilised desmosomal structural adhesions lead to keratinocyte acantholysis, induction of signal transduction and inflammatory cascades that subsequently manifest as epithelial barrier disruption, blistering and painful erosions (83)(84). Anti-Dsg1 antigen interactions lead to intraepidermal blistering with pathology restricted to skin causing pemphigus foliaceus (PF), while anti-Dsg3 antigen interactions are limited to mucosal sites including the ocular surface (pemphigus

vulgaris (PV)). The presence of both anti-Dsg1 and anti-Dsg3 leads to mucocutaneous PV affecting both dermal and mucosal sites (84)(85)(86). Common presentations include irritation and features of dry eye disease associated with a bilateral conjunctivitis, lid margin ulceration, meibomian gland dysfunction, fornix foreshortening, symblepharon, ankyloblepharon, and entropion (87)(88).

Dermatitis herpetiformis can coexist with a number of other autoimmune BMZ diseases including ocular pemphigus and pemphigoid (89). Immune reactivity against tissue transglutaminase 2 in coeliac disease induces cross-reactive IgA autoantibody formation against epidermal transglutaminase (eTG) produced by keratinocytes (90) giving rise to dermatitis hepetiformis. Formation of immunogenic immune complexes triggers complement activation, fibrin deposition and neutrophil recruitment leading to sub-epithelial, fluid-filled neutrophilic abscesses.

Epithelial fragility in each of these disorders highlights the importance that should be placed on the ability of a substrate to support a fully functioning hemidesmosome complex and highlights the critical function of cell-matrix and cell-cell adhesive processes. Any transplanted ocular surface epithelium will invariably put under shear stress through the blinking action and any weakness in cell-matrix adhesion will result in epithelial detachment. In addition, the immunobullous disorders underscore the importance of avoiding an immune reaction with any transplanted or bioengineered tissue or device, despite the often-reported immune privilege status of the eye.

3.0 Development of an ideal substrate for conjunctival transplantation on biological and synthetic substrates

Advancing toward a goal of an ideal substrate requires an appreciation of the requirements of that substrate. While substantive advances can be made through semi-empirical and through derivative research, identifying the definitive features and challenges is essential in focusing our collective efforts.

3.1Support of stratified squamous, goblet, and undifferentiated cell growth

A clearly essential feature of the substrate is its ability to support the function of multiple cell types: stratified squamous, goblet, and undifferentiated epithelial cells and stem cells. Importantly, the ECM proteins and substrate mechanical properties that regulate the optimal growth of each of these cell types is likely to be different and is not fully identified.

Although undoubtedly the most obvious requirement, the growth of the appropriate cell types is still the most challenging aspect of conjunctival replacement. The problem lies not only in identifying substrates but conditions that will work for each cell type on their own. Methodically working through media formulations, supplement strategies and cell selection procedures have led to a series of effective protocols to grow each cell type individually (82)(83)(84). Of particular importance are the goblet cells as these have historically been the most challenging to maintain. Goblet cells are found throughout the conjunctiva, however, higher densities are found in the medial canthus and inferior fornices (85). This localised difference may provide an opportunity; it may be possible to compare the protein profiles of the ECM in these areas compared to the rest of the conjunctiva to identify components that promote goblet cell growth, then use this information to target substrate modification. Similarly, stem cells in the conjunctiva are essential for renewal of epithelial and goblet cells and therefore the long-term survival of the transplant. In the corneal limbus, the location of the corneal stem cell niche, specific proteins have been identified that are enriched in stem-cell rich areas and have been exploited *ex vivo* to improve limbal stem cell cultures. Similar proteins or a similar approach may be enriched in the BM of the conjunctiva where stem cells are abundant. Constructing a substrate that supports one cell type or another is obtainable. The challenge now is how to identify conditions to support *all* the relevant cell types which not only retain their function but also grow at appropriate relative densities and locations to achieve correct tissue function. Moreover, cells should maintain those densities over time once transplanted. Clearly, this will be a challenge, but can we achieve the equivalent of the native environment using an artificial substrate? An answer could take two approaches.

The first option is to mimic the mature conjunctival microenvironment as closely as possible. This will likely require substrate patterning; presenting the ideal protein or peptide ligands for the different cell types in defined densities and orientations to support the final mixed cell population. For this option, the cells applied could be expanded *ex vivo* and applied either fully differentiated, i.e. as conjunctival stratified squamous or goblet cells, or could be applied lineage-committed but with the final differentiation occurring *in situ*. Using pre-differentiated cells may present the quickest route forward. In this situation, the patterned substrates would not necessarily have to mimic the native environment completely. Instead, the substrates could be designed to select for the different cell populations based on the relative expression levels of specific cell surface receptors between the goblet and stratified squamous cells. Through presenting selective ligands in an appropriate patterning, the specific cell types could be differentially selected. Once attached, the cells would elaborate their own matrix assuming the bulk properties of the substrate supported protein binding. (Figure 6). The alternative approach is patterning the substrate with matrix components that are usually associated with each cell type. Ostensibly, this appears the more straightforward process. As most matrix components can be bound by multiple receptors, however, the ability to achieve differential patterning through more generic components may not be successful. In both of these "pre-patterned" approaches, work will be required to identify which matrix components support each cell type, especially their relative affinities and the relative expression of cell surface receptors.

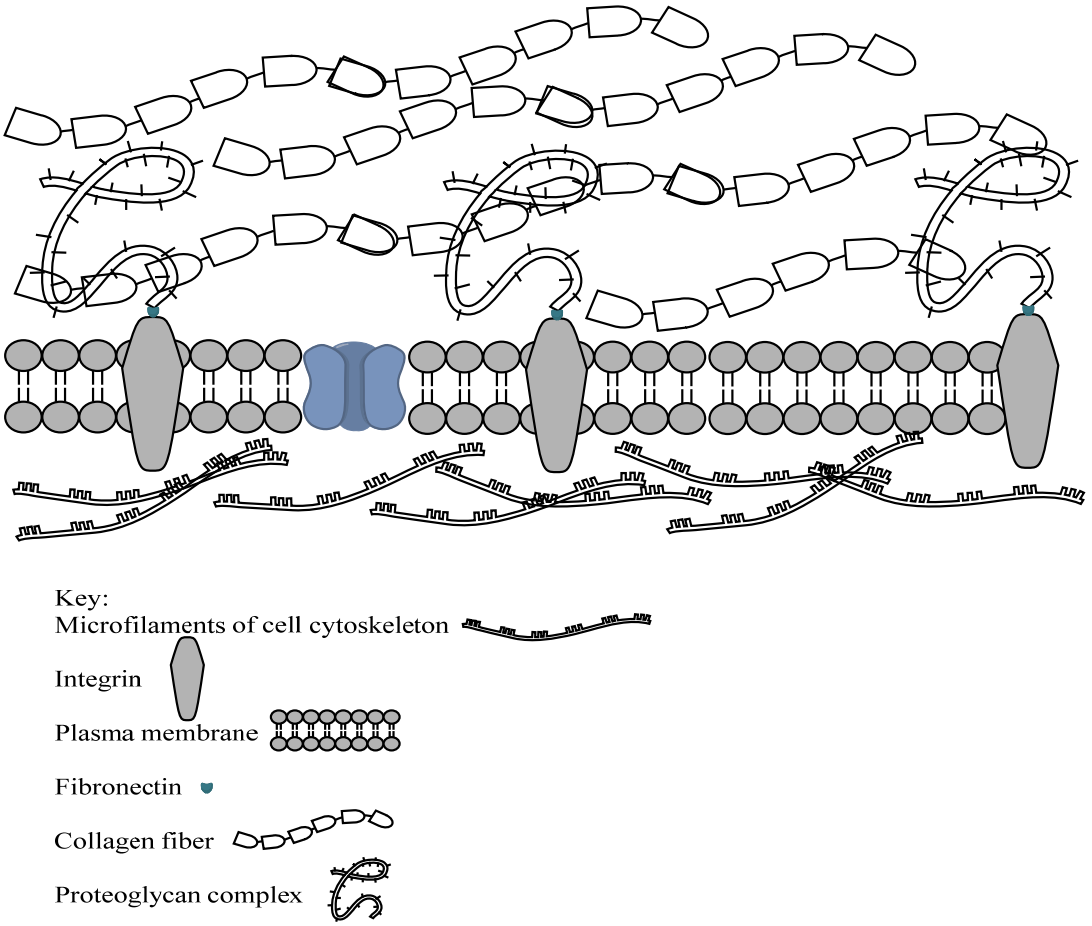

Figure 3: Representation of the components of the cellular ECM (ChemDraw)

A second option, which is undoubtedly more complicated in the short term but may be better in the longer-term, is to create an environment that mimics the conjunctiva during development. The cells would then establish their own niches, essentially completing the developmental process *ex vivo* by modifying their own substrate. Realistically, to artificially mimic the *entire* development process would be unfeasible not only from a complexity point of view but also when considering the costs that would be required for the time to prepare a substrate for therapy. Therefore, such a "developmental precursor approach" would likely entail seeding an appropriate stem cell population on a substrate that mimics not the most primitive environment, but instead something closer to the final structure. Culture conditions and additives would then be controlled to drive the cells down appropriate final stages, where they would orient and pattern their niche. The advantage of this approach is that a less complicated starting substrate would be required and likely the final output

would be more “real”. With those advantages comes a potentially major disadvantage of how complex the following steps would need to be and how long they would take. A deep understanding of the native processes, as provided through discovery science projects, is likely to be the most effective way forward in this area.

In terms of patterning, most approaches described thus far involve direct conjugation of ligands. This is not, however, the only option. The substrates could act as reservoirs for appropriate cytokines. In essence, this would be mimicking a role that the native BM plays. BMs sequester growth factors and control their release through targeted modification. How tightly the artificial substrate will need to mimic this native situation of controlled release is not yet known; however, the ability of conjunctival cells to modify their surroundings could be exploited. For example, a substrate containing cleavage sites for matrix modifying enzymes, most likely matrix metalloproteinases, will gradually be digested and release stored active molecules. This approach could allow gradual replacement of the artificial substrate over time as the material matures. Understanding more of how the native ECM and particularly the BM is remodelled could yield new targets and mechanisms to achieve this goal.

As the BM and the ECM are reservoirs for growth factors, the synthetic substrates must also be able to bind proteins such as growth factors and be able to release them into the microenvironment in a controlled manner. Growth factors need to be chosen to support the differentiation of stem or progenitor cells and both stratified squamous and goblet cells must be produced. In addition, the substrate must be thin and porous to allow interaction between the graft and the underlying host tissue. Growth factors also need to be added to the substrate to encourage growth of afferent and efferent nerves into the epithelium as well as to encourage vascularization of the transplanted tissue. Nerves will regulate function of the epithelial cells and blood vessels provide growth factors and other compounds to the cells. Vascularization will also provide rapid exchange of nutrients and metabolic waste products in and out cells, respectively. For these reasons it is important, that there

is an adequate, functioning innervation within the epithelium and vascularization subjacent to the transplanted area once the diseased conjunctival tissue is excised and the tissue construct placed. For both pre-patterned and developmental precursor approaches, the *ex vivo* steps taken before transplantation procedures are likely to require stratification rather than application of single layer cultures. Conjunctival epithelial cells stratify easily in culture, unlike corneal epithelial cells, even on glass coverslips. It is likely that substrates that support cell growth will also support cell stratification. Therefore, the measures needed for the formation of a fully stratified, transplantable corneal epithelium are unlikely to be needed for the conjunctiva. It will be important, however, to investigate when the BM matures and the cell-to-matrix adhesive structures, the hemidesmosomes, assemble and mature. Modifications that aid adhesion maturation may improve the ability of the cell sheet to survive surgical handling. Achieving a goal of a functional conjunctiva will also require cross-talk between stromal cells and the epithelial cells. As such, the ideal substrate will need to allow interaction to occur, preferably, through direct contact. *Ex vivo* stratification would likely progress to a more effective overall structure with embedded fibroblasts, although fibroblasts may have to be treated to prevent them from proliferating. To achieve this, the substrate could be degradable or porous or both; all of which present potential for development.

3.2Controlled growth of cells and minimizing inflammation

As with all transplanted tissue equivalents, the largest challenge to success comes with immune-mediated rejection. In patients who require conjunctival transplantation the surface being restored is unhealthy and inflamed. Although pre-transplantation treatment will “quieten down” the immune response, any interventional approach runs a risk of re-stimulating inflammatory responses. In addition, surgical intervention will stimulate a scarring response from local fibroblasts with negative functional implications. The easiest solution to prevent inflammation and subsequent fibrosis may be the use of an inert substrate, either biological or synthetic, that isn’t recognised as a foreign body. This substrate, however, must support restoration of a functional epithelial layer, especially a functional basement membrane ultimately lessening fibrosis.

A more complex solution for an ideal substrate would be one that not only acts passively to ensure epithelial cell growth, but actively suppresses the immune and fibrotic responses. Surface conjugation with anti-immune peptides would be a first step. The ideal substrate would not only provide contact dependent suppression of immune responses within the substrate, but also act at a distance into the stroma and perhaps the draining lymph nodes. For controlled release of growth promoting factors, an artificial substrate could also act as a reservoir for anti-immune and anti-fibrotic factors. The disease milieu could be exploited designing the substrates by identifying matrix-modifying proteases that are enriched in the diseased conjunctiva, but not expressed in healthy conditions. When inflammation is increased, the substrate could be designed to release its sequestered cargo that inhibits the matrix-metalloproteases thus returning the substrate, its epithelium and surrounding stroma back to health. Drugs that control cell growth on the substrate and prevent inflammation could be delivered to the transplant in the conjunctiva using nanoparticles. Areas that prevent normal topical treatments from being effective are anatomical and physical barriers in the ocular surface, drug degradation by the ocular surface epithelia and the tear film, and the rapid removal of the drug by the tears and the lacrimal drainage system. Nanoparticles can circumvent many of these issues and are particularly suited to synthetic substrates. As nanoparticles have very large surface area: volume ratio, this enables them to have a small volume, but provide a large surface area for potential drug-target interaction. Nanoparticles are also able to persist longer on the surface of the eye by being trapped in the mucus layer of the tear film, allowing them to interact with the surface of the newly grown epithelium and perhaps being taken up by the cells. For example, PLGA nanoparticles encapsulated with 6-coumarin were readily taken up by cultured primary rabbit conjunctival epithelial cells. This strategy could potentially be used to maintain homeostasis, induce growth, or prevent inflammation of transplanted conjunctival cells (86)(87).

Reducing or minimising the host inflammatory response and the fibrotic response is an important step in transplantation biology. In the conjunctiva this is particularly important when reconstructing

damaged or diseased forniceal conjunctival tissue. The higher the local inflammatory process, the more likely is the scar tissue formation. Low *in vivo* shrinkage is important to prevent contracture of the conjunctiva and potential adhesions between lids and the globe.

3.3Ensuring biocompatibility and non-deleterious biodegradability

Another important characteristic for the transplantable conjunctival equivalent is that the substrate must be biocompatible *in vivo*. That is the substrate must be able to integrate into the host tissue without causing inflammation or rejection by the host's immune system once transplanted. Many biological substrates may be biocompatible; however, this might not be the case for the synthetic substrates. Substrates that are optimal for cell growth need to be tested for biocompatibility. Biodegradability of the ideal substrate is desired but not essential. If the substrate is biodegradable, it is essential that the degradation products are not toxic and that the process occurs in a controlled manner. In the latter case, the rate of degradation should be tailored to ensure that the substrate remains maintains functionality until the transplanted tissue incorporates into the surrounding tissue of the host by secreting ECM proteins. If the substrate breaks down too quickly the transplanted cells will also be lost prior to them forming strong adhesions with the underlying tissue, thus the scaffolds would lose their cell carrier function (88)(89)(90).

Naturally-derived substrates, including autologous and allogeneic grafts, and more processed biologics, such as amniotic membrane and vitrified collagens gels, were assessed in various *in vivo* models, and all deemed suitable to support epithelialisation. Unfortunately, little discussion focuses on their degradation. Authors usually allude to these substrates being degraded in relatively short time-frames, typically ranging between two to four weeks (2). In contrast, silk fibroin, a naturally occurring biopolymer, is a long-term degrading material that breaks down following phagocytosis but remains intact 145 days post-subcutaneous implantation in rats (91). In all three studies assessments were made qualitatively from histological tissue sections and sometimes from gross images only. Ideally, a more thorough histological and immunohistochemical analysis with comparison to appropriate control groups needs to be conducted. More thorough and quantitative

types of analysis that will provide clear evidence of the local inflammatory response and remodelling of these grafts with the surrounding tissue over time are needed (92).

Monitoring the degradation of a synthetic compared to a biological substrate *in vivo* is an easier task as the substrate can often be identified *in situ* from histological sectioning. Visual representation will only provide information about the scaffold's structural changes. It is possible, however, to obtain additional, quantitative data regarding the physical breakdown of the polymeric chains by measuring the change in molecular weight. For this measurement scaffolds may be excised from the tissue, excess tissue digested by collagenase (for example) and the remaining structure dissolved in solvent and passed through a Gel Permeation Chromatographer (93)(94). Currently, however, analysis stretching beyond histology and immunohistochemistry has not been applied for assessment of conjunctival grafts, only for other types of tissue. For example, histological analysis of tissue engineered nanofiber scaffolds following subcutaneous implantation into nude mice for four weeks was performed and new tissue formation and scaffolds appeared less compact and dense (95).

Poly(L-lactic) acid and poly(ε-caprolactone) are slow degrading, biocompatible polyesters that typically remain in excess of one year depending on structure and local tissue environment. These synthetic substrates undergo hydrolytic attack of their ester groups principally within the materials' bulk, with lactic acid and capronic acid being the main breakdown components, respectively (96)(97)(98)(99). Despite being acidic in nature, these breakdown products are not a cause for concern due in part to the slow degradation of the bulk material, but also because of continual removal of the by-products by surrounding tissue fluid, where they are subsequently metabolised through the tricarboxylic acid cycle (100)(101). Yet, to gain a deeper understanding of these silk fibroin and poly(L-lactic-*co*-ε-caprolactone) composite scaffolds, a longer period of *in vivo* investigation is needed as all components are slow degrading materials.

Two other factors, in addition to biodegradability, need to be considered when developing synthetic substrates for implantation: substrate design and intended site of implantation. With regard to

substrate design, the structure, topography and surface area to volume ratio of the material all need to be explored. For example, scaffolds with a greater exposed surface compared to its bulk provide shorter diffusion pathways and hence faster rates of degradation (102)(103)(104). Anatomical location can also influence the rate of degradation with detrimental impact on local tissue environment. For example, tissues with slow matrix turnover or low fluid flow can result in accelerated degradation of a substrate caused by the accumulation of acidic breakdown products prompting autocatalysis, as well as changes to the local pH causing cell and tissue necrosis (105)(106).

Overall, most studies have concluded that the presence of a biomaterial is essential to prevent the formation of scarred and contracted tissue, which is otherwise likely to result in further complications requiring intervention (2)(3)(75)(107). Yet, there are no definitive degradation properties available to engineer the optimal graft. Further investigation is required to gain a complete picture of a graft's impact *in vivo* – for both large and small defects, with greater emphasis on tracking tissue remodelling long-term, including inflammatory response, and determining the method and rate of its degradation**.**

3.4Mechanical/physical properties of biological and synthetic substrates used for conjunctival transplantation

The mechanical attributes of the substrate are important to consider when developing substrates for growing epithelial cells for transplant. Parameters, such as elasticity and topography affect the microenvironment of the BM or ECM, which in turn influence the tension of the actin cytoskeleton, and stiffness signalling pathways, with subsequent influences on cellular processes including differentiation and proliferation (108). Similarly, the resident cells can modulate the mechanical properties by affecting the types and amounts of proteins secreted into the ECM or BM. For example, as the elastic modulus of polyacrylamide hydrogels covalently coupled with type I collagen increased, epidermal stem cell spreading increased and differentiation into keratinocytes

decreased (108). The optimal mechanical properties required for maintenance of stem cells and the differentiation of epithelial stratified squamous and goblet cells of the conjunctiva have not been studied. Investigation of this research area will provide important information for the field of tissue engineering.

Mechanical properties also need to be given due consideration, especially their handle-ability, including surgical handling of transplant, placement of transplant *in situ,* and ability of transplant to be secured by sutures or glue. For the conjunctiva, the data suggest that the ideal substrate is elastic, meaning any physical distortion caused by loading is completely recovered once that load has been removed. Materials that are unable to return to their original length are said to have gone past their yield point of elasticity and entered 'plastic deformation' (109). Elasticity of the conjunctiva is required to support eye movement and blinking, which can be considered a stretch in the tensile direction, although the forces experienced are low (63). Another property to consider is tensile strength. Ultimate tensile strength is commonly reported, and this is taken as the maximum stress the material has reached before failure occurs. This is likely, however, to have extended beyond the material's elastic limit and the material is experiencing plastic deformation. Determining the material's maximum strength without transitioning to permanent deformation, the yield stress, is needed for successful use. A recent review of tissue mechanics and graphical representation of stress-strain curves (110) provides a useful resource for further information. Mechanical strength of human conjunctiva is low; values vary within the literature, with reports of 3.9 MPa and 1.1 MPa (63)(64). Differences in sample dimensions and conjunctival location, and application of non-standardised testing set-ups indicate that this variation is not unexpected. Consideration should also be given to wet versus dry tensile testing as hydrated tissues are known to exhibit different mechanical properties than dry tissues (111)(112). Tensile properties of substrates intended for support and repair of conjunctival injury are regularly reported. For example, decellularised conjunctival substrates demonstrated similar mechanical properties (elasticity and strength) when compared to their cell-containing equivalents, but the decellularized

substrates remain delicate to handle and can be easily damaged (63)(64). Tensile strength of human amnion can be described as stronger and stiffer compared to conjunctiva, with one study reporting a 20-fold and 2-fold increase in strength and stiffness, respectively (63). It is possible to tailor the mechanical properties of other biological substrates that undergo variable processing parameters, such as vitrified collagen. In this case, the temperature applied during vitrification had a direct impact on the degree of collagen cross-linking resulting in ultimate tensile strength ranging between 0.7-8.7 MPa (88). Silk fibroin is an incredibly strong natural material (*Bombyx mori*) with tensile strength of 300-740 MPa, a Young's modulus ranging between 10-17 GPa and strain at break 4-24 % (65). Synthetic polymers similarly possess substantially greater tensile properties compared to the conjunctiva; with modulus, strength and strain for poly(ε-caprolactone) being ~700 MPa, 4-28 MPa and 700-1000% and poly(propylene fumarate), 2000-3000 MPa, 3-35 MPa and 20%, respectively (69). These bulk material properties, however, can be tailored depending on the final architecture and composition of the substrate required. For example, a scaffold with greater porosity or pore size will be naturally weaker than a non-porous scaffold (89), and an aligned (anisotropic) fibrous scaffold will yield greater stiffness and strength (in the direction of the fibres) compared to a randomly-organised fibre network (isotropic) material (113). Mechanical properties of both biological and synthetic substrates with epithelial cell layers should be measured and the resultant success of the transplant determined so that only substrates with the optimal mechanical properties will be considered for conjunctival transplantation.

The microarchitecture, or topography, of substrates can be purposefully controlled to elicit favourable mechanical properties but also to influence cell response directly. Surfaces with micro or nano-features that convey contact guidance cues and thus induce changes in cell phenotype include grooves, pillars, pores, pits and fibres (114). Similar to substrate stiffness, cells are able to sense their local environment via focal adhesions, which can directly impact their migration, proliferation and differentiation (90)(115). Not only is a substrate's microarchitecture important for controlling cell response, but it also plays a useful role in terms of diffusion pathways for both gas exchange and

also nutrient and waste removal. To allow for adequate diffusion and to support cell migration and viability throughout, scaffolds need to possess an interconnected porous network. Overall dimensions of the scaffold also need to be considered, as thick scaffolds will struggle to provide adequate rates of diffusion without appropriate porosity or pore size. In this case, scaffolds with a porosity gradient should be considered, which will accommodate necessary diffusion (including vascularisation) and cell and tissue ingrowth without compromising mechanical stability (89).
The design of a biological or synthetic substrate for any tissue requiring repair and regeneration, such as the conjunctiva, needs to consider several criteria: topography, surface chemistry, mechanical properties, inter-connectivity and degradation; all of which require optimisation in order for its long-term success following implantation *in vivo*.

3.5 Substrate biocompatibility and biodegradability

Biocompatibility of the core material and tolerated degradation are primary concerns for all transplanted material and are of particular relevance to synthetics. The term "biocompatible", first coined in the 1970s, has undergone several changes in its definition driven by the rapid evolution in the biomaterials field. A deeper understanding of biological response to materials has led to an updated definition "*the ability of a material to locally trigger and guide non-fibrotic wound healing, reconstruction and tissue integration*" (11). A more precise definition is provided in the guidelines provided by the International Organisation for Standardisation (ISO 10993 Biological evaluation of medical devices) (12). These regularly updated guidelines provide a series of tests to determine if the developed implantable device is acceptable prior to clinical testing.

Biodegradation as an over-arching term can be subdivided depending on the form of degradation: biodegradable, where macromolecular breakdown occurs but elimination *in vivo* has not been proved; bioresorbable, where breakdown products are naturally metabolised and eliminated; bioabsorbable, where the material dissolves in bodily fluids without loss in molecular mass and bioerodible, where degradation is localised to the material surface (13). A more detailed description

of the degradation of synthetic and natural biomaterials can be found in a review by Nair and Laurencin (14). Not all biomaterials, however, are, or need to be biodegradable. Depending on the application a more permanent substrate may be required, e.g. intraocular lens made from polyHEMA. In these cases, the materials are classed as biostable (15). This does not necessarily mean the material is non-degradable, but the rate of its degradation and local impact are negligible (16). Similar to all tissues regenerating via support of a degradable substrate, the rate of new tissue formation should match that of material breakdown and a mismatch may result in failure of the graft and function of the neo-tissue (17). For the conjunctiva, the optimal degradation time of a substrate, irrespective of material, has not been fully determined but almost certainly requires epithelialisation of the substrate to have completed before its removal or complete breakdown *in vivo* (2)(18)(19).

Substrate Biom5. Conclusion

The conjunctiva plays an important role in the maintenance of homeostasis of the ocular surface by providing immunological defence and secreting protective mucins that contribute to the tear film. The conjunctiva may be damaged secondary to trauma or inflammatory pathology that may lead to scarring requiring replacement. This review highlights the numerous ways that the ECM and their proteins can be utilised as a substrate for conjunctival tissue engineering. Insight into the composition, microstructure and functions of the ECM proteins *in vivo* aids in the development of substrates. In turn, the substrates provide tissue-specific biological and physical cues and are biocompatible due to the lack of antigenic molecules. The long-term goal is to develop the optimum ECM peptides and underlying substrate to enhance the culture of multiple types of conjunctival epithelial cells (stratified squamous and goblet cells), as well as undifferentiated stem cells forming a transplantable tissue with mechanical and physical properties similar to native conjunctiva for ease in transplantation. An accompanying review considers other biological substrates and synthetic substrates for conjunctival regeneration and replacement. Substantial discovery and developmental

work, however, is needed to develop a substrate optimal for conjunctival epithelial cell growth and transplantation into the conjunctiva.

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