Heinrich Heine Universität Düsseldorf

Decellularised Human and Porcine Conjunctiva for Ocular Surface Reconstruction

Dezellularisierte humane und porcine Bindehaut als Matrix für die Augenoberflächenrekonstruktion

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Content

Abbreviations

ABCG2	ATP-Binding Cassette super-family G member 2
ALI	Air Liquid Interface
AM	Amniotic Membrane
ANOVA	Analysis Of Variance
ARVO	Association for Research in Vision and Ophthalmology
ATMP	Advanced Therapy Medicinal Products
CALT	Conjunctiva Associated Lymphoid Tissue
CECM	Conjunctival Epithelial Cell Medium
CK	Cytokeratin
COMET	Cultivated Oral Mucosal Epithelial Cell Transplantation
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ds	double stranded
e.g.	exempli gratia
ECM	Extracellular Matrix
EDC	1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
EMA	European Medicines Agency
Emod	Elastic Modulus
ePTFE	Poly(Tetrafluoroethylene)
et al.	et alibi
FBS	Fetal Bovine Serum
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
h	hour
HE	Haematoxylin/Eosin
HC	Human Conjunctiva
HCEC	Human Conjunctival Epithelial Cells
HCEx	Human Conjunctival Explant
HCF	Bulbar Subconjunctival Fibroblasts

HDC	Human Decellularised Conjunctiva
HLA	Human Leukocyte Antigen
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz
LFU	Lacrimal Functional Unit
LG	Lacrimal Gland
M	Molar
MG	Masson's Goldner
MHC	Major Histocompatibility Complex
MUC5AC	Mucin 5AC
NaCl	Sodium Chloride
NaDC	Sodium Deoxycholate Monohydrate
o/n	over night
ocMMP	ocular Mucous Membrane Pemphigoid
p.	page
P/S	Penicillin/Streptomycin
PAS	Periodic Acid-Schiff's
PBS	Phosphat Buffered Saline
PCC	Plastic Compressed Collagen
PCL	Poly-E-Caprolactone
PDC	Porcine Decellularised Conjunctiva
PEI	Paul-Ehrlich-Institut
PERV	Porcine Endogenous Retrovirus
PFA	Paraformaldehyde
PLCL	Poly(L-Lactic Acid-Co-3-Caprolactone)
PLGA	Poly (Lactide-Co-Glycolide)
SD	Standart Deviation
SDS	Sodium Dodecyl Sulfate
SJS	Stevens-Johnson Syndrome
T3	Triiodo-L-Thyronine
TE	Trypsin/Ethylenediaminetetraacetic Acid
TEM	Transmission Electron Microscopy
TFF	Trefoil Factor
UTS	Ultimate Tensile Strength
α-Gal	Galactose-Alpha-1,3-Galactose

List of Figures

Abstract

The ocular surface is the interface between the eye and our environment, and its homeostasis is maintained by the interaction of its various anatomical structures, including the conjunctiva. The conjunctiva is a specialized mucous membrane that plays an important role in defending against pathogens and environmental influences and in moisturizing the ocular surface. Various disorders can damage the conjunctiva and its epithelium, leading to chronic inflammation, tear film disorders, scarring and, secondarily to keratinisation of the cornea, visual impairment or even blindness. Especially in cases of severe ocular surface damage, the long-term success of visual rehabilitation often requires surgical reconstruction of the conjunctiva with a suitable substitute tissue. Various tissues are used clinically for this purpose, but they also have some disadvantages, such as the lack of secretory goblet cells – essential for the tear film –, limited availability, and lack of stability or strong tissue heterogeneity.

This dissertation investigated decellularised conjunctiva as a promising novel substitute tissue for conjunctival reconstruction. Decellularised tissue has the decisive advantage of having a low antigenic potential that could lead to a rejection reaction in the recipient. The articles published in the framework of this cumulative dissertation reveal that human decellularised conjunctiva and porcine (pig-derived) decellularised conjunctiva show no disadvantages in terms of stability, elasticity, tissue integration or biocompatibility, neither in vitro nor in vivo after transplantation in an animal model, compared to amniotic membrane, the most commonly used graft used in the surgical treatment of ocular surface diseases.

Since severe ocular surface damage can lead to a loss of conjunctival epithelial stem cells, long-term visual rehabilitation requires the renewal of the conjunctival epithelial progenitor cell population. This work shows that the decellularised conjunctiva can be successfully recellularised in vitro with human conjunctival epithelial progenitor cells. For this purpose, different cultivation methods for the formation of a stable, stratified, goblet and progenitor cell-rich epithelium were evaluated. Subsequently, reepithelialised conjunctiva was transplanted into a conjunctival defect in a rabbit model and examined

in vivo. The transplanted conjunctiva maintained good stratification, equivalent to a healthy human conjunctiva, showed high progenitor cell maintenance and increased goblet cell numbers.

Porcine decellularised conjunctiva showed no disadvantage compared to human decellularised conjunctiva in our experiments and is a readily available tissue source. Based on the present results, porcine decellularised conjunctiva thus shows a great potential as an alternative conjunctival substitute tissue as well as a carrier for human conjunctival progenitor cells for surgical rehabilitation of the ocular surface.

Zusammenfassung

Die Augenoberfläche ist die Schnittstelle zwischen dem Auge und unserer Umwelt, und ihre Homöostase wird durch das Zusammenspiel ihrer verschiedenen anatomischen Strukturen - einschließlich der Bindehaut - aufrechterhalten. Die Bindehaut ist eine spezialisierte Schleimhaut, die eine wichtige Rolle bei der Abwehr vor Pathogenen und Umwelteinflüssen sowie dem Befeuchten der Augenoberfläche spielt. Verschiedene Störungen können die Bindehaut und ihr Epithel schädigen und zu chronischen Entzündungen, Störungen des Tränenfilms, Narbenbildung und sekundär zu Sehbehinderung oder Blindheit durch Verhornung der Kornea führen. Vor allem bei schweren Augenoberflächenschäden erfordert ein langfristiger Erfolg bezüglich visueller Rehabilitation oft eine chirurgische Rekonstruktion der Bindehaut mit einem geeigneten Ersatzgewebe. Verschiedene Gewebe kommen hierbei klinisch zum Einsatz, weisen jedoch auch einige Nachteile auf, wie z.B. das Fehlen von - für den Tränenfilm essentiellen - sekretorischen Becherzellen, eine limitierte Verfügbarkeit, mangelnde Stabilität oder eine starke Gewebeheterogenität.

In dieser Dissertation wird dezellularisierte Bindehaut als ein vielversprechendes, neuartiges Ersatzgewebe für die Bindehautrekonstruktion untersucht. Dezellularisierte Gewebe haben den entscheidenden Vorteil, nur in geringem Maß immunogene Faktoren aufzuweisen, die im Empfänger zu einer Abstoßungsreaktion führen könnten. Die im Rahmen dieser kumulativen Dissertation veröffentlichten Artikel zeigen, dass humane dezellularisierte Konjunktiva und vom Schwein stammende (=porzine) dezellularisierte Konjunktiva im Vergleich zur Amnionmembran, dem am häufigsten verwendeten Transplantat zur Augenoberflächenrekonstruktion, weder in vitro noch nach Transplantation im Tiermodell in vivo, Nachteile hinsichtlich Stabilität, Elastizität, Gewebeintegration oder Biokompatibilität aufweisen.

Da eine schwere Schädigung der Augenoberfläche zu einem vollständigen Verlust von konjunktivalen, epithelialen Vorläuferzellen führen kann, erfordert eine langfristige visuelle Rehabilitation in solchen Fällen die Erneuerung der konjunktivalen epithelialen Vorläuferzellpopulation. Im Rahmen dieser Arbeit konnte gezeigt werden, dass sich die

dezellularisierte Konjunktiva in vitro erfolgreich mit humanen konjunktivalen Vorläuferepithelzellen rezellularisieren lässt. Hierzu wurden in vitro verschiedene Kultivierungsmethoden zur Bildung eines stabilen, stratifizierten, Becher- und Progenitorzell-reichem Epithels evaluiert. Im weiteren Verlauf wurde die reepithelialisierte Konjunktiva in einen Bindehautdefekt in ein Kaninchenmodell transplantiert und in vivo untersucht. Die transplantierte Konjunktiva wies weiterhin eine, einer gesunden, humanen Konjunktiva äquivalenten Stratifizierung auf und zeigte einen hohen Anteil an Progenitorzellen sowie eine erhöhte Becherzellzahl im Vergleich zu in vitro kultivierten Bindehaut-Konstrukten.

Porzine dezellularisierte Konjunktiva zeigte in unseren Versuchen gleichwertige Ergebnisse im Vergleich zu humaner dezellularisierter Bindehaut und stellt zusätzlich eine leicht verfügbare Gewebequelle dar. Basierend auf den vorliegenden Ergebnissen, weist porzine dezellularisierte Konjunktiva somit ein großes Potential als alternatives Bindehautersatzgewebe zur chirurgischen Rehabilitation der Augenoberfläche auf.

Preface

Parts of the Section "Conjunctival Reconstruction" of the review article "Eye Socket Regeneration and Reconstruction" [1] that was written during the doctoral period are included in this dissertation and were used as part of the introduction (with permission of Current Eye Research, Taylor and Francis Online). Specifically, sections 1.5, 1.7.1, 1.7.2, 1.7.3, and 1.8.2. of this dissertation were adapted from the sections "Indications for Conjunctival Reconstruction", "Conjunctival Autograft", "Oral and Nasal Mucosa", "Isolated Amniotic Membrane" and "Synthetic Polymer-based Scaffolds", respectively. Further information to the original article is given on page 20.

Chapter 1

1 Introduction

1.1 The Ocular Surface – Anterior Border of the Eye

The human eye is a specialised organ, which is directly exposed to the environment and must deal with several external factors such as air pollution, pathogens and changes of air humidity and temperature [2]. The anterior border of the eye, named ocular surface, is a complex system composed of different anatomical structures, such as conjunctiva, cornea and its epithelia (Figure 1). Besides these directly related structures, the ocular surface system includes also the tear film produced by the main lacrimal gland and, the accessory lacrimal glands (Krause`s Glands, Wolfring`s gland and Popov´s glands) and the meibomian glands, located in the eyelids [3]. Hence, the drainage system and the eyelids itself take also part in keeping the eye moistured by spreading the tear film over the ocular surface. Each of these structures fulfils a distinct role, but all together, supported by nerval innervation and the immune-/ endocrine system, form the lacrimal functional unit (LFU) that prevents infections and maintains corneal clarity by regulating hydration and epithelial integrity [4].

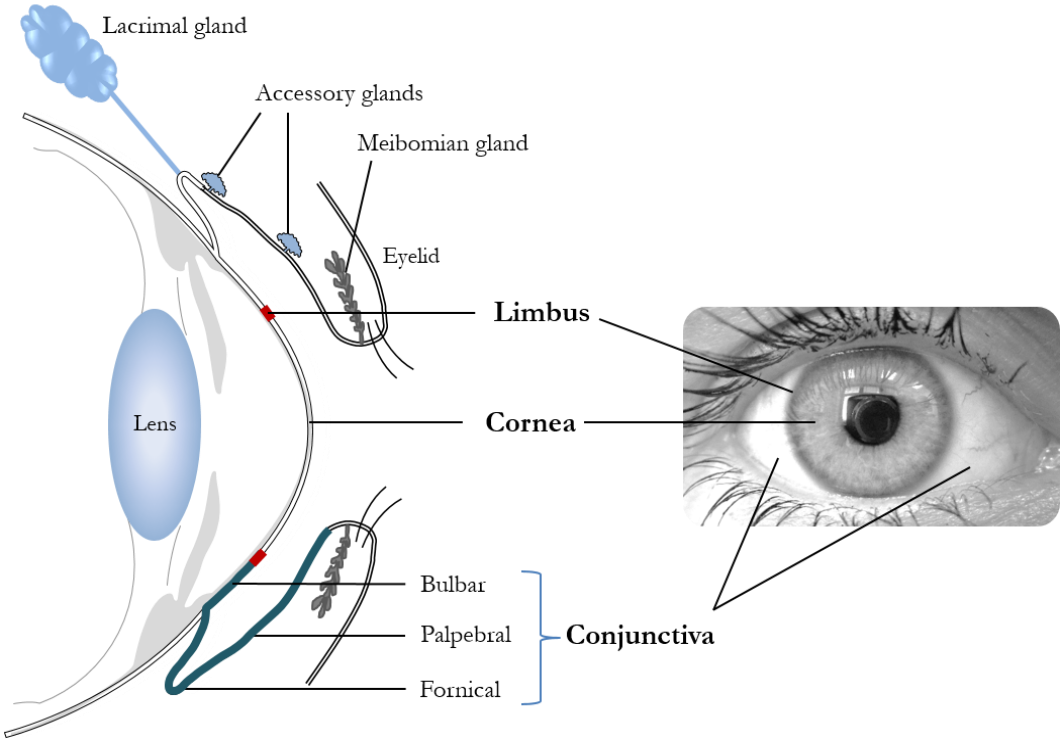

Figure 1: Overview of the ocular surface and the associated glands. The ocular surface consists of the corneal and conjunctival (blue line) surfaces and epithelia, the tear film, the lacrimal drainage apparatus, associated eyelid structures (including the meibomian glands) and several glands (e.g. lacrimal gland). The conjunctiva begins at the transition zone to the cornea, named limbus (red line), extends over the eyeball up to the fornix and covers the inner eyelid.

1.2 The Tear Film

The corneal and conjunctival epithelium is covered by the tear film, which allows smooth movement of the bulbus and protects from external factors. The tear film is a complex mixture of components, growth factors and cytokines, which are essential for epithelial integrity and regeneration. It is maintained through secretion by the main lacrimal gland, the accessory lacrimal glands, conjunctival goblet cells and the meibomian glands. The correct fluid composition is crucial, for instance, to allow the tear film to adhere to the epithelial surface and to ensure the moisture and nutrition of the corneal and conjunctival epithelium.

According to a classic model by Wolff - although very simplified -, the tear film can be divided into three layers: A mucous, an aqueous and a lipid layer [5, 6]. The innermost layer of the tear film is the 2.5 to 5-µm-thick mucous layer, which mainly consists of secreted, highly glycosylated glycoproteins, such as the gel-forming Mucin 5AC

(MUC5AC) that is produced by the conjunctival goblet cells and is particularly important to stabilize the tear film by increasing the viscosity. The interface between the tear film and the corneal and conjunctival epithelia is composed of membrane-spanning mucins which form the glycocalyx [7]. The approximately 4-µm-thick, aqueous layer contains water, electrolytes, antimicrobial proteins, immunoglobulins and several nutrients to nurse the ocular epithelium [8]. The main contributor to this layer is the lacrimal gland with a continuous secretion of an average of 1.2 µl/minute [9]. The aqueous phase provides nutrients and oxygen to the avascular cornea and cleans the surface from foreign bodies and cell debris. The meibomian glands, located in the eyelids, secrete the lipid component, which forms the outer layer of the tear film. This only 0.015 to 0.160-µm-thick layer creates a barrier at the air-liquid interface and retards the evaporation of the tear film from the ocular surface [10]. Moreover, it reduces the surface tension of the tear film and supports the tear film respreading after blinking [11].

1.3 The Cornea and the Limbus

The cornea is a highly specialised and innervated optical tissue, that due to its transparent properties and avascularity, is often referred to as the window of the eye and it enables light transmission and focus into the inner eye. The cornea is dependent on nutrients and growth factors provided by the tear film and the diffusion of aqueous humour, as corneal vessels are exclusively restricted to the limbal arcade to ensure transparency of the central cornea. Its main functions are to protect the eye from environmental factors such as pathogens and mechanical damages while allowing focusing the light into the posterior segment.

According to the current knowledge, the cornea consists of six different layers. The corneal epithelium is the outermost layer made of non-keratinized squamous epithelial cells, which provide a barrier against pathogens and environmental factors. The epithelium undergoes continuous regeneration by ectoderm-derived stem cells, which are located in a 1-2 mm wide transition zone between cornea, conjunctiva and sclera named the corneal limbus. The limbal microanatomy reveals an unique, blood vessel containing, stromal architecture with radially-oriented ridges known as the Palisades of Vogt or limbal epithelial crypts [12, 13]. The Bowman's membrane is part of the anterior corneal stroma, although differently organized and forms the subepithelial basement

membrane. The underlying corneal stroma (substantia propria) plays several important roles within the eye. Optically, it has the highest refractive index and mechanically, it has to be extremely tough to protect the posterior eye. The stroma comprises about 80-90% of the total corneal thickness and consists of highly regular arranged sheets of collagen fibrils called lamellae that are arranged orthogonally throughout the stroma. The precise arrangement and spacing of lamellae and fibrils are crucial for the transparency of the corneal stroma. Sparsely distributed within the stroma are quiescent, mesenchyme-derived cells named keratocytes [14]. In 2013, Dua et al. introduced a new term for a thin but highly pressure-resistant anatomical structure posterior of the stroma (Dua's layer) [15]. The underlying Descemet's membrane is a basement membrane, which is secreted by the posterior endothelium and is composed of different types of collagen than the corneal stroma. The corneal endothelium is a monolayer of hexagonal cells with limited regenerative potential in humans [16]. Endothelial cell loss during lifetime has to be compensated by migration and expansion (polymegathism) of the neighbouring cells in order to maintain a continuous cell layer [17, 18]. This reduces the overall cell density of the endothelium, affecting fluid regulation. If the endothelium is no longer able to maintain the fluid balance, stromal swelling can occur, resulting in transparency loss, corneal edema and visual impairment.

1.4 The Conjunctiva

The conjunctiva is a transparent mucous membrane and covers over 90% of the ocular surface. It plays an important role in immune surveillance, prevention of microbial entrance into the eye and lubrication of the ocular surface by production of the mucin component of the tear film, thus maintaining the integrity of the entire eye [19].

Anatomically, the conjunctiva is divided into three regions: The bulbar, fornical and palpebral/tarsal conjunctiva (Figure 1). The bulbar conjunctiva covers the anterior sclera and is loosely attached to the episcleral tissue except from a 3 mm wide zone near the corneal limbus where it is tightly adherent. At the medial canthus, the conjunctiva forms a semilunar fold (plica semilunaris conjunctivae), a vestigial remnant of the nictitating membrane or "third eyelid". The palpebral (or tarsal) conjunctiva lines the inside of the eyelids up to the fornix, and is tightly adhered to the tarsal plate of the eyelids. Its surface contains crypt-like infoldings know as crypts of Henle [20]. The fornical conjunctiva

forms the junction between bulbar and palpebral conjunctiva, is loose, elastic, and thrown into folds to form the conjunctival sac and to enlarge the surface. Accessory lacrimal glands named Krause's glands are embedded in the fornical conjunctiva. A deep fornix plays an essential role in maintaining the ocular surface integrity by providing a tear reservoir and the ocular motility [21].

1.4.1 The Conjunctival Stroma

The conjunctival stroma or substantia (lamina) propria is in contrast to the cornea - a vascularized connective tissue with a loose structure that allows a movement of the epithelium without causing too much friction to the underlying sclera during eye movements and blinking (Figure 2). The lamina propria contains lymphoid tissue known as conjunctiva associated lymphoid tissue (CALT), several resident fibroblasts and abundant immune cells such as lymphocytes, mast cells, dendritic cells, granulocytes, and macrophages [22]. The triggering of an inflammatory reaction by external factors or pathogens results in a dilatation of the conjunctival blood vessels, increase of the vascular permeability and allows immune cells to enter into the conjunctival stroma with subsequent activation of the resident fibroblasts.

1.4.2 The Conjunctival Epithelium

The conjunctival epithelium is important in contributing to the production and stability of the tear film and in providing a barrier to injury and infection by synthesis of antimicrobial peptides (e.g. defensins) that can be released against pathogens [23]. It consists of non-keratinized, stratified columnar epithelial cells with numerous interspersed secretory active goblet cells (Figure 2). Depending on the site of the conjunctiva, the epithelial thickness varies from 2 to 10 cell layers [24].

The apical cells of the squamous conjunctival epithelium show a lot of microvilli and harbour several membrane-associated mucins such as Mucin 1, 4, and 16, which extend from their cell surface into the tear-film-interface to form the glycocalyx [25]. Goblet cells can be found in the entire conjunctival epithelium, with a range from 1,000 to 56,000 per square millimetre depending on the area. Studies have shown that the inferior conjunctiva harbours a higher density of goblet cells compared to the superior

conjunctiva, and the nasal area contains a higher count than the temporal conjunctiva. Likewise, the fornices contain a higher portion of goblet cells than the tarsal or bulbar conjunctiva and inferior is a higher density than superior. In addition to MUC5AC, goblet cells also produce peptides (trefoil family peptides, TFF1 and 3), which promote epithelial cell migration [26, 27].

Both cell populations, squamous epithelial and goblet cells, derive from the same bipotent progenitor [28]. In contrast to corneal stem cells, which are known to be located exclusively in the limbus, the location of human conjunctival epithelial stem cells has been discussed more controversially [29, 30]. By analysing epithelial colonies cultured from different parts of the human conjunctiva, Pellegrini et al. were the first to suggest that the epithelial stem cells are distributed uniformly throughout the whole conjunctival surface [28]. According to current studies, it is assumed that putative stem cells are indeed present on the whole conjunctival surface, but with different distribution [31]. For example, Steward et al. showed that the medial canthal and inferior fornical areas contain higher proportion of putative conjunctival stem cells with clonogenic ability than the limbus-near areas [32].

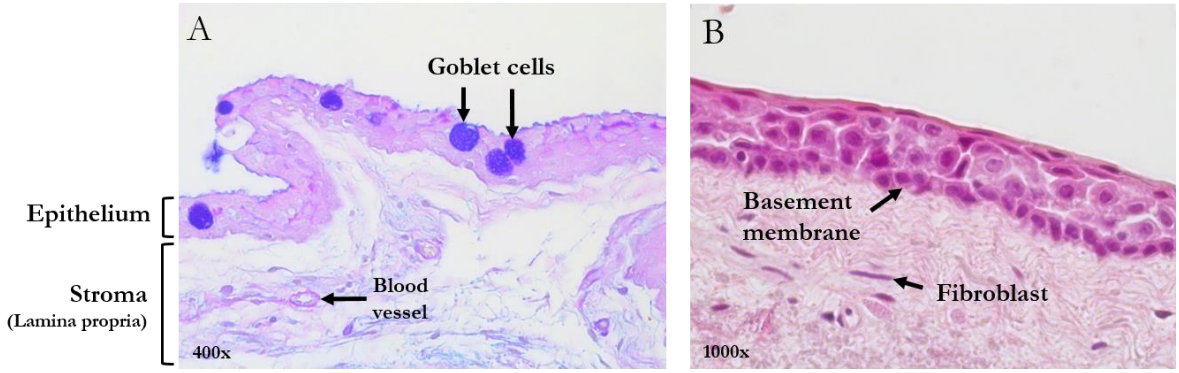

Figure 2: Histology of the conjunctival epithelium.
Microanatomy of the conjunctival epithelium A: Periodic Acid-Schiff's (PAS) stained cross section of a human conjunctiva. The conjunctival epithelium lays over a vascularized connective tissue, the lamina propria. Secretory cells, named goblet cells, are embedded into a squamous, non-keratinised epithelium. B: Haematoxylin/Eosin (HE) stained cross section. In a higher magnification, cuboidal basal cells laying on a basement membrane, intermediate wing cells and superficial flattened squamous cells are visible within the conjunctival epithelium. Embedded into the stroma, fibroblasts are visible.

1.5 Severe Ocular Surface Disorders (modified from [1], section "Indications for Conjunctival Reconstruction")

Severe ocular surface damages can be caused by a variety of diseases or injuries. Immunologic diseases such as mucous membrane pemphigoid with ocular involvement (ocMMP), Stevens-Johnson syndrome (SJS) or graft-versus-host disease, infections, trauma, chemical or thermal burns, as well as surgical complications can cause severe functional impairment and scarring of the ocular surface and thus, the conjunctiva. This can result in further complications, such as dry eye disease, symblephera[1], epithelial erosions or ulcers, loss of goblet and stem cells and potentially keratinisation of the entire ocular surface epithelium [1]. Isolated loss of goblet cell function and the resulting mucous deficiency is a mild form, while end stage ankyloblepharon[2] and the contracted socket syndrome[3] are the most advanced variants of conjunctiva loss. Consequently, patients suffer from pain, discomfort, secondary infection, and further destruction of the ocular surface resulting in corneal opacity and finally loss of vision [1, 33]. The effective treatment is often highly challenging, complex and depends on the patients pre-existing conditions and the damage of the individual ocular surface structures.

1.6 Conjunctival Reconstruction

In most of severe ocular surface injuries or diseases, both cornea and conjunctiva are affected. In some cases, irreversible damage and scarring occurs such that conventional medical strategies alone may be insufficient to enable the regeneration of healthy ocular surface epithelia. If the cornea cannot be saved due to severe damage, a possible surgical treatment option is an allogenic corneal transplant (keratoplasty) or, if the corneal stem cells are depleted, an allogenic or autologous limbal transplant. However, before any surgical intervention, the stabilization of the ocular surface homeostasis including reduction of inflammation, systemic treatment in autoimmune involvement and restoration of the tear film should be ensured. The next step includes the reconstruction of the conjunctiva, generating deep fornices and the restoration of the eyelids (if necessary). Poor conjunctival integrity and complications such as dry eye

[1] Symblephera - adhesions of the tarsal conjunctiva of the eyelid to the bulbar conjunctiva of the eyeball
[2] Ankyloblepharon - partial or complete fusion of the eyelids by webs of skin
[3] Contracted socket syndrome - contraction of the socket after enucleation

disease or adnexal deformities such as trichiasis[4] or ectropion[5] are associated with a higher risk for corneal transplant failure leading to poor outcomes in these patients [34]. Thus, it is important to consider the entire surface, not just the individual components, when restoring the ocular surface.

1.7 Tissue Substitutes for Conjunctival Reconstruction

The choice of the ideal conjunctival substitute tissue or material depends on the individual patient's situation, pre-existing diseases, extend of conjunctival defect or goblet cell function loss, and if postoperative complications have occurred. Several surgical approaches, including excision of the scar tissue following application of a substitute tissue have been described to reconstruct the conjunctiva and the fornices [1, 35, 36] They include autologous tissues such as conjunctival autografts and nasal/buccal mucosa or allogeneic materials such as amniotic membrane.

1.7.1 Conjunctival Autografts (modified from [1], section "Conjunctival Autograft")

Healthy autologous conjunctiva has ideal properties as a substitute, as it is morphologically and functionally identical to the affected tissue and does not elicit an immune reaction. Conjunctival autografts are frequently used to cover conjunctival defects following pterygium[6] excision because they reduce wound contracture and the risk of recurrence significantly [37-39]. Since the graft is usually small in size, the donor site heals spontaneously or can be closed with a conjunctival flap [40, 41]. However, there are several limitations. Conjunctival autograft transplantation is unsuitable for the repair of larger defects including fornix reconstruction or extended or bilateral damages due to insufficient tissue availability. Since previous severe infection, other forms of inflammation, repeated surgical interventions and corneal or conjunctival melting processes could cause scarring and long-term damage to the conjunctiva and the adjacent tissue, there is often insufficient tissue for conjunctival reconstruction. In addition,

[4] Trichiasis - eyelid abnormality with misdirected eyelashes towards the ocular surface, touching the cornea or conjunctiva
[5] Ectropion - outward turning of the eyelid margin. Patients may suffer from dry eye due to ocular exposure and inadequate lubrication.
[6] Pterygium - wing-shaped fibrovascular lesion that extends from the conjunctiva onto the cornea

incising the conjunctiva to obtain the conjunctival autograft is counterproductive in autoimmune diseases as it can lead to further inflammation and scarring [1].

1.7.2 Oral and Nasal Mucosa (modified from [1], section "Oral and Nasal Mucosa")

To overcome the limitations of conjunctival autografts, mucous membranes from other areas were considered as substitutes. Oral (buccal) mucosa membrane grafts have been widely used for conjunctival reconstruction in the past and are currently the 'gold standard' for larger conjunctival defects [42]. In patients with a shallow fornix, mucosal grafts are usually used in combination with fornix-deepening sutures, which are placed through the eyelid at the level of the new, deep fornix. Split-thickness oral mucosa can serve as an alternative for bulbar conjunctiva but is less suitable for fornix reconstruction as it contracts more than full-thickness mucosa. Mucosa harvested from the hard palate is the stiffest graft and contracts the least but is more difficult to obtain compared to buccal or labial mucosa. It may be an option if there is not enough other oral mucosa (labial/buccal) available or to reconstruct the tarsal conjunctiva, especially if the lower lid needs to be lifted [1, 42]. The labial mucosa, including the minor salivary glands, can be harvested to improve tear film volume [43]. However, extensive damage to the ocular surface (e.g. after severe burns) can lead to extensive loss of the goblet cells. Since the oral mucosa does not contain goblet cells, an alternative graft is required that can compensate for a mucus deficit by carrying healthy goblet cells. In these cases, the use of a nasal mucosa graft can be considered and it has shown satisfactory results in the past [44, 45]. A nasal turbinate mucosal graft features the highest number of goblet cells and can therefore alleviate symptoms in severe conjunctival damages or dry eye condition. Nasal septal cartilage grafts contain fewer goblet cells and are stiffer, but may be an option for posterior lamellar reconstruction if no hard palate is present [46]. Disadvantages of nasal mucosal grafts are that they are more complex to harvest, and that donor site morbidity cannot be excluded. In recent years, the use of a combination of different graft types such as amniotic membrane and oral or nasal mucosa graft in the same eye has led to satisfactory results [47].

1.7.3 Amniotic Membrane Transplantation (modified from [1], section "Isolated Amniotic Membrane")

The human amniotic membrane (AM), the innermost layer of the placenta is collected under sterile conditions from placentas after elective caesarean delivery from healthy donors and is currently one of the most frequently applied substitutes for conjunctival reconstruction [48, 49]. It has been successfully used as a graft in the treatment of mild chemical injuries [50], symblepharon [48, 49] and SJS [51].

The AM has several positive properties: First, it does not express – or only to a very limited extend before cryopreservation – Human Leukocyte Antigens (HLA) class Ia and b, hence immunological rejections does not occur after AM grafting [52-54]. Second, AM contains several cytokines and growth factors, is able to promote cell growth, migration and inhibits inflammation and fibrosis [55-57]. However, despite the positive effects and the several successful clinical applications, it should be noted that AM, in contrast to oral or nasal mucosa, is a 'substrate graft' and does not carry any viable epithelial cells [58]. Thus, AM graft has to be epithelialised from the host stem cell pool. In an ocular surface without any healthy proliferative active conjunctival epithelial cells, AM grafting alone is insufficient as it cannot be reepithelialised [46]. Another drawback of AM in general is that properties and growth factor composition can vary significantly between individual donors, sampling location and processing [59-62]. This, and a non-standardized processing procedure, might also be a reason for inconsistent clinical outcomes in severe ocular surface damages with inflammatory involvement such as in acute ocular burns [63, 64]. Another issue to be mentioned is the risk of disease transmission by the donor. Although the potential for transmission of infectious diseases is very low in Germany, it is a serious problem in countries where fresh, non-quarantined membranes are used, which are sometimes not tested for dangerous infectious pathogens such as HIV and hepatitis [64]. The given variabilities in tissue quality that can lead to inconsistency in clinical outcomes, and the dependence on human donor material, which requires expensive screenings, point out the need for new, reproducible conjunctival substitutes.

1.8 Tissue Engineering – A Tool for the Development of Tissue Substitutes

Tissue Engineering utilises synthetic materials, biomaterials, cells and biochemical features to generate tissue-like-structures. One goal of Tissue Engineering is to create tissue substitutes that can maintain, restore or improve damaged or lost tissues [65]. In recent years, researchers have investigated different matrices for tissue engineered conjunctival substitutes, ranging from artificial synthetic polymers to materials of natural origin (=biomaterials). Most of them were initially suggested for corneal surface repair and were then adapted for conjunctival reconstruction [33, 66].

Some general mechanical and biological properties should be addressed when designing and developing a conjunctival substitute tissue. An ideal conjunctival substitute

- needs appropriate mechanical properties,
- must be stable enough to allow surgical handling,
- should be elastic to enable eye movement and lid closure [67],
- must not be cytotoxic,
- needs to integrate into the host conjunctiva without causing inflammation or allergies,
- should mimic the host microenvironment.

Biocompatibility is a dynamic two-way process that involves the effects of the recipient tissue on a scaffold and the other way around. The material itself should not be negatively affected by the host (e.g. due to too fast degradation) and the host should not be negatively affected by the implanted scaffold. A scaffold needs to mimic the host microenvironment and properties of the tissue since a specific cell niche has been shown to influence the cell identity, proliferation, differentiation and stemness [68-71]. Compared to the corneal epithelium, the conjunctival epithelium adds extra considerations when designing a scaffold to facilitate its expansion before transplantation: The substrate must promote the growth of both squamous epithelial cells and goblet cells. For the treatment of patients with extensive conjunctival damage or complete ocular surface failure, the graft should ideally be covered with a previously in vitro cultured, self-renewing conjunctival epithelium with progenitor and goblet cells.

1.8.1 AM-based Substitutes

While numerous studies have shown AM (or denuded AM) to be a good carrier for corneal epithelial cells or oral mucosal epithelial cells, its utility as a carrier for conjunctival epithelial cells has been less addressed. Kinoshita and colleagues were first who used denuded (=amniotic epithelial cells removed) AM for in vitro expansion of autologous oral mucosal epithelial cells (COMET = cultivated oral mucosal epithelial cell transplantation) [72]. Their approach was primarily developed for corneal surface reconstruction but was also successfully used to restore the conjunctiva including fornix [73-75]. However, a major drawback of this technique remains the absence of conjunctival, in particular goblet cells, which can contribute to mucin secretion. Denuded AM has also been shown to be a good substrate for conjunctival epithelial cells in previous studies in vitro and in a rabbit model [76, 77]. However, data on percentages of goblet cell numbers and their density on re-cultivated AM is sparse in literature [78-80].

1.8.2 Synthetic Polymer-based Scaffolds (modified from [1], section "Synthetic Polymer-based Scaffolds")

A main advantage of synthetic polymers is that they can be produced with tuneable material properties and are free of disease transmission risk [81]. Several synthetic polymers such as poly-D-lysine, poly(acrylic acid), poly(vinyl alcohol), or poly-ε-caprolactone (PCL) have been tested for conjunctival reconstruction in the past [82]. He et al. reported that there was outgrowth of conjunctival epithelial cells on all substrates although on PCL only 3.6 ± 2.2% of cells were viable [82]. In vivo studies in rabbits showed that a modified porous poly (lactide-co-glycolide) (PLGA) matrix had a positive effect on cell growth and cell adhesion and that it significantly prevented conjunctival contracture and scarring when sewed into a 7-mm-diameter bulbar conjunctival defect in a rabbit [83]. However, the main disadvantage of these grafts are their inferior biomechanics (lack of sufficient elasticity) and that synthetic tissue substitutes are often based on non-degradable materials resulting in poor host tissue integration, increased inflammatory host response and scarring [67].

The working group of Rachel Williams modified a poly(tetrafluoroethylene) (ePTFE) surgical membrane that has been used before by Kim et al. in recurrent pterygium to separate the scar tissues [84, 85]. To overcome the hydrophobicity of ePTFE and thus enabling cell growth, they used an ammonia gas plasma [86]. They found a good growth of primary conjunctival cells on ePTFE by day 14 and about 15% of the cells were goblet cells. However, after longer time periods in vitro the total cell number decreased [87]. Although many of these approaches seem encouraging, there are no publications on the successful use of synthetic polymers in conjunctival reconstruction in patients to date.

1.8.3 Natural Polymers: Silk and Keratin

Silk from the cocoons of the Bombyx mori silkworm has been widely studied for Tissue Engineering applications. For medical applications, a glue-like glycoprotein that covers the fibroin fibres must first be removed [88]. After this, the fibroin can be dissolved into an aqueous solution that can be further processed into many materials. The effect of modified silk films such as arginine-glycine-aspartic acid-modified silk and poly-D-lysine coated silk on conjunctival epithelial cells has already been shown with good results regarding cell expansion and viability [82]. However, the cells were not investigated for mucin production, e.g. by staining against MUC5AC. Yao et al. were able to generate a hybrid scaffold composed of silk fibroin and poly(L-lactic acid-co-3-caprolactone) (PLCL) using electrospinning. Electrospinning is a fabrication process in which fibres can be produced from polymer solutions or molten polymers using a strong electric field. The directed deposition of the fibres permits the generation of three-dimensional fibrous scaffolds. Several synthetic and natural materials have been successfully electrospun in recent years [89].

Another approach is the production of films made of human hair keratin [90]. Borrelli et al. showed that keratin films have a high transparency [91] and good biocompatibility when implanted into corneal stroma pockets of rabbits. However, their use as conjunctival tissue substitutes and their effect on the proliferation and differentiation of conjunctival cells still needs to be investigated.

1.8.4 Collagen Gels

Collagen is ubiquitously found in connective tissue in all mammals and is a key protein to maintain the structural integrity and extracellular matrix (ECM) strength of an organ [92]. For this reason, it is one of the most frequently used substrates for corneal substitutes and gained attention for conjunctival reconstruction as it features high biocompatibility and degradability. Nevertheless, a main drawback of hydrated collagen gels is their lacking mechanical stability and structural weakness due to a high-water content. One method to enhance the biomechanical properties of collagen gels is chemical cross-linking using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [93] but the substances might impact cell-induced scaffold remodelling [94]. Another approach to overcome this issue was reported 2005 by Brown et al., who improved the mechanical properties of collagen hydrogels through controlled removal of the fluid, a process termed plastic compression [95]. Our group was able to show that plastic compressed collagen (PCC) gels offer good biomechanical properties and that conjunctival epithelial cells cultured on PCC gels are able to form a confluent and tightly connected epithelial cell layer with cells expressing putative progenitor cell markers [96]. Recently, in own experiments using an in vivo conjunctival defect model, PCC gels showed high biocompatibility without relevant signs of scarring such as myofibroblast persistence or fornix shortening at the implantation site [97]. A high stability as well as a good integration into the host tissue with formation of a multi-layered epithelium including secretory active goblet cells were shown in this study. These results and the findings of a case report from our group in which a PCC gel has already been successfully used in humans as a corneal surface graft without causing inflammation or scarring [98], suggest that PCC is also a promising candidate as a novel conjunctival substitute for clinical use. Own research on PCC gels published during this doctoral period can be found in this document on pages 68 “A Additional Publications, A.2”.

1.8.5 Decellularised Tissues

Studies have shown that the ECM composition and the tissue-specific biomechanics are unique for every tissue [13]. In the past decade, substantial progress in the development of ECM-based biomaterials has been made. However, until now,

researchers are not able to design an ECM that fully mimics the complexity of the biochemistry and the architecture of the human conjunctiva.

A promising approach to overcome this obstacle is the use of decellularised human or even xenogeneic tissue as the ECM proteins of a tissue are highly conserved between vertebrates [99]. During decellularisation, donor cells, cellular components and major histocompatibility complex (MHC) antigens are removed while the ECM remains [81]. Simultaneously, it minimizes or ideally prevents an immunological rejection risk, thus enabling the use of allogeneic and even xenogeneic donor tissue (e.g. pig tissue due to a removal of galactose-alpha-l,3-galactose during decellularisation). The main benefit of decellularisation is that it preserves these tissue-specific, unique ECM as it has been shown that the specific protein composition and 3D architecture of the ECM enhance cell adhesion and proliferation and determine organ specific biomechanical properties [92, 100]. The ECM can interact over cell surface receptors like integrins with cells to trigger intracellular signalling pathways to regulate e.g. apoptosis or cell survival [101]. This responsivity of cells to mechanical stimuli is termed mechanosensing. Therefore, it is crucial to avoid a disruption of the ECM during decellularisation. Many approaches have been tested to achieve effective decellularisation including chemical, enzymatic and physical treatments [102]. Chemical solutions often include detergents (e.g. Triton X-100, sodium dodecyl sulphate or sodium deoxycholate), solvents, acids and alcohols [103, 104]. Enzymatic agents most used are dispases and nucleases whereas physical processes include freeze/thaw cycles, pressure and agitation). The ideal choice for the best result depends on the specific tissue properties such as source, size, cell and matrix density, fat and lipid content as well as the number of cells [103, 105]. Most protocols published combine some of the above-mentioned treatments.

Decellularised matrices are already applied clinically (e.g. heart valves [106, 107], skin [108], oesophagus [109], trachea [110], or urethra [111]) and the decellularisation techniques are continuously improved, not only for planar tissues but also for whole organs using perfusion decellularisation through the veins. Using this technique, not only the ECM can be preserved but also the vascular tree. The decellularised tissue can be then cultivated in specifically designed bioreactors that mimic the physiological environment such as the application of pressure during recellularization of veins with

patient derived microvascular endothelial cells. Preliminary studies using decellularised and subsequent recellularised vascularised organs have been tested in animal studies for the heart, lung, liver, and pancreas [112].

In the field of ophthalmology, decellularised tissues have already been used clinically for indications such as the reconstruction of extended defects in eyelid surgery [113, 114] or tendon elongation in strabismus [115]. Tissues such as decellularised human or porcine dermis [108, 114, 116-118] and decellularised bovine pericardium [115, 119] were used for such cases. Moreover, there are already good approaches for the use of decellularised tissues to reconstruct the ocular surface, such as the cornea [120-123], the conjunctiva [124] and the lacrimal gland [125-127]. A co-authored publication characterising the human limbus after decellularisation can be found in "Additional Publications, A.2" on page 69. Parallel to our work, two groups worked on the establishment of a protocol for the decellularisation of conjunctivas. Kasbekar et al. were able to decellularise human conjunctiva using a slightly different protocol than ours, but their decellularisation efficiency was also satisfactory and the published results highly encouraging [124]. However, they examined neither porcine conjunctiva nor its recellularisation with conjunctival epithelial cells. The second group published results on decellularisation of porcine conjunctiva, with comparably efficiency in cell and DNA removal [123]. They cultivated corneal epithelial cells on the decellularised conjunctiva with the intention to use it as corneal graft in casas of limbal stem cell deficiency. However, none of these two research groups compared human to porcine decellularised conjunctiva or tested a recellularised graft for conjunctival reconstruction in vivo.

Concluding, the rehabilitation of severe ocular surface damages, including extensive conjunctival scarring, remains surgically challenging. Clinically, various autologous (e.g. oral mucosa) and allogenic (e.g. AM) substitutes are used to reconstruct the conjunctiva [1, 36]. However, none of these clinically applied grafts is free of limitations and therefore, there is a substantial need for the development of new, reproducible, biocompatible and easily available conjunctival substitutes. Several tissue-engineered biomaterials or synthetic scaffolds have been described in recent years, but none of them are routinely used in clinical practice [67, 85]. Most of these substitutes have not yet been tested in vivo or need to be improved with regard to the stability required for

transplantation [36]. One- and two-component materials such as collagen gels can only mimic the complexity of the natural niche of highly specialized conjunctival cells to a limited extent. Decellularised conjunctiva has a potential for a biocompatible substitute tissue that could imitate the niche of the conjunctival epithelial cell population and thus, may promote the stemness of the conjunctival epithelial cell population and the formation of goblet cells. The use of porcine decellularised conjunctiva, a xenogeneic tissue source, could be an easily available and donor tissue independent option to compensate for the lack of human donor material.

1.9 Aims of This Work

Aim of this study was to develop a conjunctival tissue substitute based on human decellularised conjunctiva (HDC) and porcine (pig derived) decellularised conjunctiva (PDC). As every tissue decellularisation inevitably leads to an impact on the ECM structure to some degree, the first step was the establishment of an adequate protocol to generate a cell-free conjunctiva with as little destruction of the ECM as possible (Figure 3 A, B). The removal of all conjunctival cells including epithelial and stromal cells had to be proven histologically. As the DNA content is directly correlated to adverse host reactions and can cause undesired transplant rejection, the efficient reduction was confirmed. Besides the verification of the effective cell and DNA removal, the effects of decellularisation on the ECM and the integrity of the collagen fibres are of interest as well as changes in biomechanical properties. Therefore, quantitative collagen content measurements, transmission electron microscopic pictures and strength tests using a material testing machine were performed. Additionally, to prove a sufficient washing and a lack of toxicity due to remaining decellularisation reagents in the ECM, cytotoxicity assays of supernatants of the decellularised conjunctivas on fibroblasts and human conjunctival epithelial cells were examined.

In order to take a further step towards clinical application, another aim was to test the surgical manageability, a potential inflammatory response or rejection reaction of the recipient conjunctiva to PDC and HDC, the degradability of the graft, the host tissue integration and a possible consecutive scar formation. Therefore, conjunctival hyperaemia, granulocytic infiltration, wound healing, epithelialisation and fornical

scarring were examined after transplantation of PDC and HDC in a bulbar conjunctival defect in a rabbit model (Figure 3 C).

The aims of the second part of the study were to compare two different methods of human epithelial cell expansion on PDC for the development of a stratified, progenitor cell-rich, conjunctival epithelium (Figure 3 D). Therefore, the optimal cultivation duration for transplantation of the preferred construct (high amount of progenitor cell markers but stable stratified epithelium, goblet cell growth) was investigated. The preferred construct was evaluated in a conjunctival defect rabbit model (Figure 3 E). In vivo, the aim was to assess the biocompatibility of the tissue, the survival and the proliferation of the transplanted cells, the maintenance of the transplanted progenitor cells, and a possible maturation of the epithelial cells.

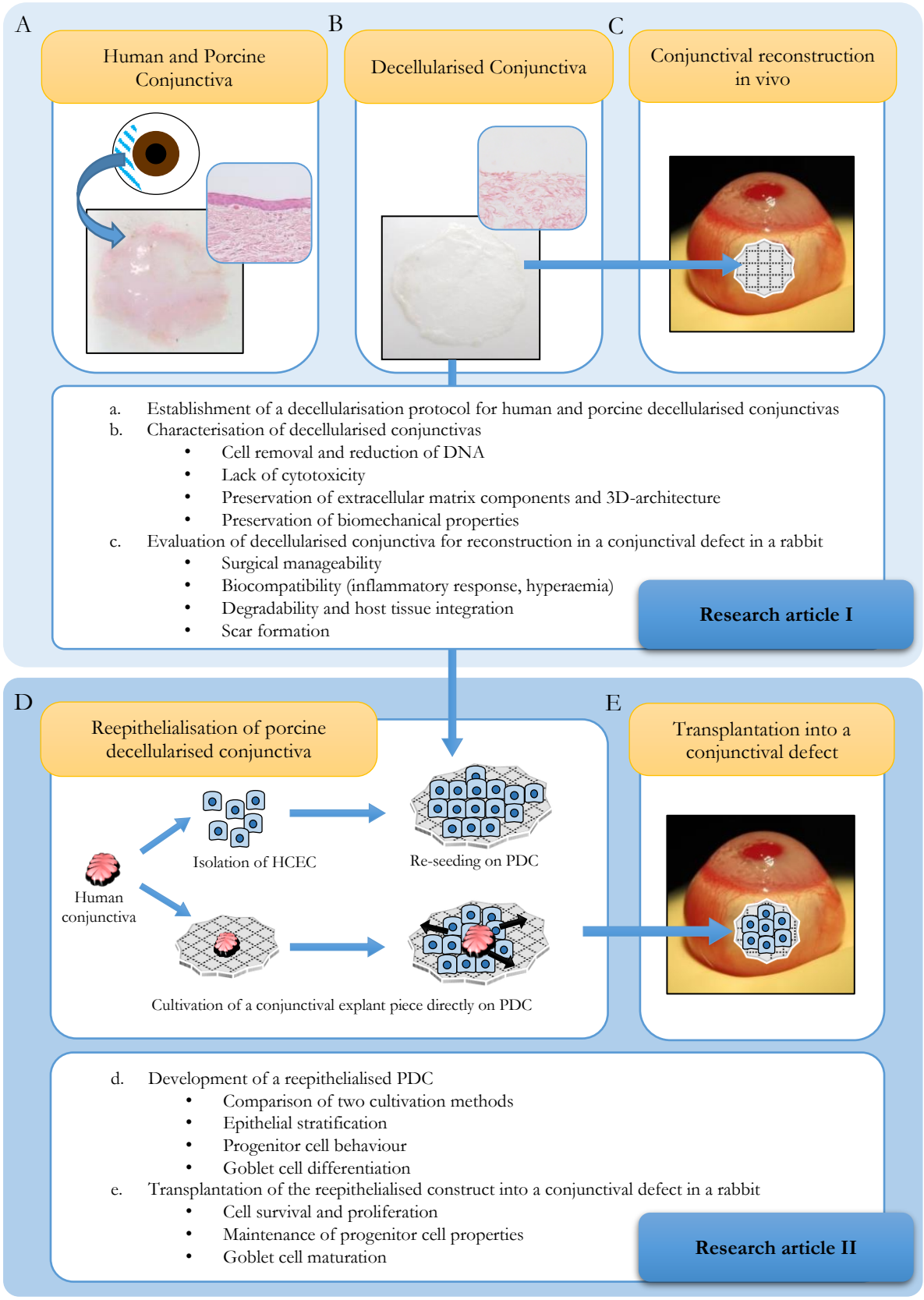

Figure 3: Schematic workflow and project overview

Chapter 2

2 Publications

2.1 Review Article

Eye Socket Regeneration and Reconstruction

Borrelli M, Geerling G, Spaniol K, **Witt J*** (2020)
*Corresponding author

Journal: Current Eye Research, Taylor & Francis Group

Impact factor: 1.754

Abstract

When an eye has become irreversibly blind or painful it is removed by enucleation or evisceration. The resulting anophthalmic socket usually receives a volume replacing implant and is subsequently fitted with a prosthetic shell for adequate cosmesis. Trauma, tumour or immunological pathomechanisms can induce loss of bone, orbital soft tissue volume, and conjunctival contraction or implant exposure, which result in difficult or impossible prosthesis wear. In this situation as well as in numerous diseases limited to the conjunctiva (e.g. Pterygium, or cicatrizing conjunctivitis) strategies to substitute the lost tissue are required.
A review of the literature search using various electronic databases (PubMed and MEDLINE) was performed on indications, surgical techniques and materials used to restore the ocular socket.
Amniotic membrane and oral mucosa are still the most commonly used substitutes for the reconstruction of larger conjunctival defects and ocular socket reconstruction. However, due to limitations of clinical available grafts, synthetic scaffolds, biomaterials or tissue-engineered grafts have been described in preclinical studies but most of them have not been investigated adequately in clinical studies yet. In orbital volume

replacement, porous and nonporous spheres are used and both show acceptable results. However, more clinical studies are required that directly compare the outcomes in patients with similar conditions. Dermofat graft remains a good option in case of sockets with significant orbital volume and conjunctival surface loss.
Beyond established techniques using autologous or allogeneic tissue, various approaches of engineering tissue based on scaffolds and stem cell expansion techniques are currently under investigation and may become alternatives in socket reconstruction in the not too far future.

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Link to publication:

https://www.tandfonline.com/eprint/MKSJV7GUJHG5TQFTAEUK/full?target=10.1080/02713683.2020.1712423

Contribution: Literature review, manuscript writing (Section 1 "Conjunctival reconstruction"), figure preparation, manuscript reviewing

Approximated total share of contribution: 40 %

2.2 Research Articles

I. Decellularised Conjunctiva for Ocular Surface Reconstruction

Witt J, Mertsch S, Borrelli M, Dietrich J, Geerling G, Schrader S, Spaniol K* (2018)
*Corresponding author

Journal: Acta Biomaterialia, Elsevier

Impact factor: 7.242

Abstract

Conjunctival reconstruction is an integral component of ocular surface restoration. Decellularised tissues are frequently used clinically for tissue engineering. This study identifies porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC) as promising substitutes for conjunctival reconstruction. PDC and HDC were nearly DNA-free, structurally intact and showed no cytotoxic effects in vitro, which was confirmed by DNA quantification, histology, transmission electron microscopy, collagen quantification and cytotoxicity assay. Comparing the biomechanical properties to amniotic membrane (AM), the most frequently applied matrix for ocular surface reconstruction today, the decellularised conjunctiva was more extensible and elastic but exhibited less tensile strength. The in vivo application in a rabbit model proofed significantly enhanced transplant stability and less suture losses comparing PDC and HDC to AM while none of the matrices induced considerable inflammation. Ten days after implantation, all PDC, 4 of 6 HDC but none of the AM transplants were completely integrated into the recipient conjunctiva with a partially multi-layered epithelium. Altogether, decellularised conjunctivas of porcine and human origin were superior to AM for conjunctival reconstruction after xenogeneic application in vivo.

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Contribution: Study conception and design, data collection, analysis and interpretation, manuscript writing, figure preparation, manuscript reviewing

Approximated total share of contribution: 60 %

Acta Biomaterialia 67 (2018) 259–269

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Full length article

Decellularised conjunctiva for ocular surface reconstruction

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ABSTRACT

Conjunctival reconstruction is an integral component of ocular surface restoration. Decellularised tissues are frequently used clinically for tissue engineering. This study identifies porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC) as promising substitutes for conjunctival reconstruction. PDC and HDC were nearly DNA-free, structurally intact and showed no cytotoxic effects *in vitro*, which was confirmed by DNA quantification, histology, transmission electron microscopy, collagen quantification and cytotoxicity assay. Comparing the biomechanical properties to amniotic membrane (AM), the most frequently applied matrix for ocular surface reconstruction today, the decellularised conjunctiva was more extensible and elastic but exhibited less tensile strength. The *in vivo* application in a rabbit model proofed significantly enhanced transplant stability and less suture losses comparing PDC and HDC to AM while none of the matrices induced considerable inflammation. Ten days after implantation, all PDC, 4 of 6 HDC but none of the AM transplants were completely integrated into the recipient conjunctiva with a partially multi-layered epithelium. Altogether, decellularised conjunctivas of porcine and human origin were superior to AM for conjunctival reconstruction after xenogeneic application *in vivo*.

Statement of Significance

Conjunctival integrity is essential for a healthy ocular surface and clear vision. Its reconstruction is required in case of immunological diseases, after trauma, chemical or thermal burns or surgery involving the conjunctiva. Due to limitations of currently used substitute tissues such as amniotic membrane, there is a need for the development of new matrices for conjunctival reconstruction. Decellularised tissues are frequently applied clinically for tissue engineering. The present study identifies porcine and human decellularised conjunctiva as biocompatible and well tolerated scaffolds with superior integration into the recipient conjunctiva compared to amniotic membrane. Decellularised conjunctiva depicts a promising substitute for conjunctival reconstruction in ophthalmology.

1. Introduction

The ocular surface environment is a complex system consisting of interacting structures such as cornea, conjunctiva, eyelids and tear film. A healthy, stable and physiologic conjunctiva is important to keep this system in balance.

The conjunctiva is a thin mucous membrane that covers the ocular surface from the corneal limbus to the posterior surface of the lids. Histologically, it is composed of a stratified and non-keratinized epithelium with secretory cells (goblet cells) and underlying vascularized connective tissue (substantia propria).

Immunologic diseases as ocular cicatricial pemphigoid or Stevens–Johnson syndrome, trauma as chemical or thermal burns, and surgery involving the conjunctiva as for pterygium or glaucoma can cause severe conjunctival damage leading to cicatrisation, fibrous tissue growth and fornix shortening. These complications can result in dry eye, epithelial erosions, loss of goblet cells and potentially keratinisation of the entire ocular surface epithelium [1]. As a consequence patients suffer from discomfort, pain and poor vision up to blindness.

A substitute for conjunctival reconstruction must be biocompatible, stable and elastic. Additionally, applied biomaterials should mimic the properties of the native tissue since it is known that tissue development and cell differentiation depend on the

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environmental niche. Autologous tissue substitutes of conjunctiva [2,3], nasal [4,5] or buccal [6] mucosa are used clinically but reveal certain disadvantages. A major problem is the limited availability of autologous tissue, especially in patients suffering from bilateral diseases / injuries or systemic autoimmune involvement.

Amniotic membrane (AM) is currently the most frequently applied substitute for conjunctival reconstruction [7,8]. AM contains various cytokines and growth factors and is able to inhibit inflammation [9–11]. However, some of the cytokines and growth factors in AM may also act contradictory by inducing scar tissue formation (reviewed in [12]). Denuded AM has been shown to be a good substrate for conjunctival epithelia cells in previous studies [13,14] and shows better results as a carrier with cultivated limbal corneal epithelial cells than intact AM alone [15]. Although the AM is a useful adjunct in the ocular surface reconstruction and most clinical outcomes are positive, it reveals some disadvantages. AM can degrade quickly in an inflammatory environment leading to a decreased chance of epithelialisation, which limits its usability in an inflamed surrounding. In addition, thickness and quality of AM vary between individual donors and depend on the sampling location [16,17].

In the recent years, several biomaterials have been developed as collagen-based substitutes like plastic compressed collagen [18], vitrified collagen-based conjunctival equivalent [19], porous collagen-glycosaminoglycan [20], or keratin-based scaffolds [21,22]. Major problems of existing synthetic tissue-engineered scaffolds are inferior biocompatibility, surgical manageability and mechanical strength *in vivo*.

The use of decellularised xenogeneic tissue, in which donor cells, DNA and antigens are completely removed, represents a promising alternative [23–25]. Main advantages of decellularised scaffolds are their tissue specific biomechanics and biocompatibility. Further, numerous studies have shown that the extracellular matrix (ECM) of an organ-specific tissue promotes cellular migration, proliferation and differentiation [26–29]. The ECM protein composition, its specific surface structure and the growth factor composition were found to contribute to this effect [30].

Porcine tissue shows a huge potential as xenograft source as it is easily available and can be obtained at low costs. Pigs share high sequence homology for ECM proteins with humans and good biocompatibility, which enables the xenogeneic transplantation of decellularised porcine scaffolds. Decellularised porcine scaffolds have successfully been used in animal studies and clinical applications for numerous different tissue types, such as dermis, intestine and heart valves [31–33]. The decellularisation of porcine tissues has already been explored for ocular structures as cornea [34,35] or lacrimal gland [36].

This study investigates the use of porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC) as an alternative conjunctival substitute *in vitro* and *in vivo*.

2. Materials and methods

2.1. Decellularisation of human and porcine conjunctiva

Human conjunctivas and AM were obtained from the Lions Cornea Bank North Rhine–Westphalia (Department of Ophthalmology, University Hospital Düsseldorf, Heinrich-Heine-University, Germany) after informed consent for research purposes. AM was obtained from human placentas after caesarean deliveries. Placenta was first washed with PBS to remove blood clots and amniotic membrane was separated from the chorion under sterile conditions. Amniotic membrane was spanned onto a nylon carrier paper (Roti-Nylon 0.2 μm, Roth, Karlsruhe), cryopreserved and stored at −80 °C in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies) and glycerol (Sigma-Aldrich, Schnelldorf, Germany) at the ratio of 1:1 (vol/vol). Immediately prior to use the AM was thawed and washed three times with sterile PBS. The study was approved by the local ethics committee (number 4722).

Porcine eyeballs were obtained from a local slaughter and transported in phosphate buffered saline solution (PBS, Sigma-Aldrich, Schnelldorf, Germany) containing 5% penicillin/streptomycin. The bulbar conjunctiva was dissected within 2–3 h after slaughtering.

For decellularisation, human and porcine conjunctivas were washed for 2 h in cold PBS containing 5% Penicillin/Streptomycin (P/S). Then the tissue was incubated in 2% sodium deoxycholate monohydrate solution (NaDC, Sigma-Aldrich) for 3 h and afterwards in fresh NaDC solution at 4 °C for additional 24 h. After each step conjunctivas were washed thoroughly with PBS. Conjunctivas were transferred into DNase solution (670 U/ml, Worthington, Lakewood, USA) and incubated for 3 h at 37 °C followed by a washing step in cold PBS (+5% P/S) over night (o/n). All incubation steps were performed on an orbital shaker (MiniShaker, VWR, Radnor, USA) at 200 rpm.

All decellularised conjunctivas were sterilized by γ-irradiation with 25 kGy (BBF Sterilization-Service GmbH, Rommelshausen, Germany) and spanned on a nylon carrier paper (Roti-Nylon 0.2 μm, Roth, Karlsruhe).

2.1.1. Histology of decellularised conjunctivas

Native porcine conjunctiva, native human conjunctiva, PDC and HDC were fixed in 4% paraformaldehyde (Roti Histofix, Roth) for one hour. For Haematoxylin/Eosin (H/E) staining and Feulgen reaction the tissue was embedded in paraffin and cut into 4 to 5 μm slides with a microtome (Leica RM2255).

2.1.2. Transmission electron microscopy (TEM)

PDC and HDC were fixed in 2.5% (v/v) glutaraldehyd with 4% (v/v) PFA in 0.1 M cacodylate buffer (pH 7.4), embedded in Spurr media (Modified SPURR Embedding Kit, Serva, Heidelberg, Germany) and slices were analysed using a Hitachi H600 transmission electron microscope.

2.1.3. DNA extraction and quantification

Native and decellularised conjunctivas from both species were lyophilized for 2 h in a vacuum concentrator (Eppendorf concentrator plus, Eppendorf, Hamburg), cut into small pieces and incubated at 56 °C o/n in lysis buffer (10 mM Tris pH 8; 100 mM NaCl; 10 mM EDTA pH 8; 0.5% SDS) containing 0.4 mg/ml Proteinase K. 1 vol Phenol/Chlorophorm/Isoamyalcohol (25:24:1; Roth) was added and mixed well. After separation of the phases through centrifugation, the upper aqueous phase was transferred to a fresh tube. The DNA was precipitated by addition of 1/20 vol NaCl and 1 vol 100% Ethanol and the pellet was washed with 70% Ethanol followed by 100% Ethanol. The DNA pellet was dried and dissolved for 10 min at 68 °C in TE buffer (10 mM Tris, pH 8; 1 mM EDTA). DNA from four samples of native and decellularised conjunctiva was quantified using Fluorescent DNA Quantitation Kit (Biorad, Hercules, USA). A range of dilutions of calf thymus DNA was used for a standard curve to determine the content of DNA per mg of dried tissue.

2.1.4. Determination of collagen content

To determine the extracellular matrix collagen content of PDC and HDC a colorimetric assay was used (Sircol collagen assay, Biocolor, UK). Dried conjunctivas were incubated at 4 °C for 48 h in a pepsin solution and collagen was extracted according to the manufacturer's protocol. Samples were measured at 550 nm (Viktor X multilabel plate reader, PerkinElmer, Waltham, MA, USA). The collagen content referred to the dry weight was determined using a

standard curve of a collagen mix standard delivered by the manufacturer.

2.1.5. Cytotoxicity assay

To determine a potential cytotoxicity of the decellularised tissue a mouse fibroblast cell line (3T3/CCL-92, ATCC) and primary human conjunctival epithelia cells (HCECs) were incubated with extracts of PDC and HDC. HCECs were isolated and cultured as described previously [18,37,38]. Briefly, human bulbar conjunctiva (about 5 × 5 mm) was cut into small pieces and transferred into a T25 cell flask seeded with growth-arrested 3 T3 fibroblasts. HCECs were cultivated to 70–80% confluence at 37 °C and 5% CO_2 in conjunctival epithelial cell medium (CECM) containing DMEM mixed 1:1 with Ham's F 12 (DMEM/F12 with Glutamax; GIBCO, Life Technologies), fetal bovine serum (FBS, 10% v/v; Biochrom), hydrocortisone (0.4 μg/ml; Sigma), cholera toxin (0.01 nM; Sigma), sodium bicarbonate (0.075% w/v; Life Technologies), adenine (0.18 mM; Sigma), triiodo-L-thyronine (T3, 2 nM; Sigma), transferrin (5 μg/ml; Sigma), insulin (5 μg/ml; Sigma), antibiotic–antimycotic solution (1% v/v; Life Technologies) and epidermal growth factor (10 ng/ml; Life Technologies). Medium was changed every two days. 3 T3 feeder cells were removed using 0.5% trypsin (1x Trypsin-EDTA solution, Sigma) followed by 5% trypsin (10x Trypsin-EDTA solution, Sigma) to detach the human conjunctival epithelial cells.

For the generation of PDC and HDC extracts, pieces with a diameter of 8 mm were incubated in 1.5 ml media (DMEM + 10%FCS for 3 T3 and CECM for HCECs) at 37 °C for 48 h. 3 T3 mouse fibroblasts and HCECs were seeded at a density of $3.5x10^3$ per well in a 96-well plate and cultured with either extracts or the respective media. Cell viability was determined after 0, 24 and 48 h by an MTT assay.

2.1.6. Biomechanical testing

Biomechanical properties were examined by a stretch–stress test [18] using 5 × 10 mm conjunctival piece. After clamping into a Zwicki-line Z 0.5 static materials testing machine with a 10 N load cell the samples were pulled vertically with a uniaxial stretching device until broken. The test speed was set to 5 mm/min for elastic modulus (Emod) determination and 25 mm/min for tensile strength testing. Ultimate tensile strength (UTS), elongation at break and Emod were calculated from the stress-strain curves obtained using the testXpert® II software (Zwick, Ulm, Germany).

2.2. ***In vivo evaluation of PDC and HDC in the rabbit***

2.2.1. Surgical procedure

The experiments were performed with 24 female New Zealand white rabbits with a weight of 2.5 kg (Charles River Laboratories). All experiments were conducted in accordance with the ARVO statement for the use of animals in ophthalmic research. Rabbits were anaesthetised using 5 mg/kg ketamine (Ketaset®, Zoetis) and 5 mg/kg xylazin (Rompun®; Bayer). Oxybuprocain (Conjuncain EDO, Bausch-Lomb, Rochester, USA) eye drops were additionally applied for topical anaesthesia.

A bulbar conjunctival defect of 5 mm diameter in the upper temporal part of the right eye at a distance of 2 mm from the limbus was performed using a trephine (Fig. 1). The conjunctiva was removed including Tenon's capsule down to the level of bare sclera to create a stromal defect model. Rabbits were divided into four groups, each group with 6 rabbits. Defects were either closed with PDC (1st group), HDC (2nd group), amniotic membrane (1st control) or remained ungrafted (2nd control). All transplants (5 mm diameter) were sutured with eight single stich sutures using Vicryl 9-0.

For post-operative treatment, 4 mg/kg Carprofen (Rimadyl, Pfizer, NY, USA) was injected subcutaneously once a day for three days. Additionally, topical steroidal eye drops (1% Prednisosolon, Predni-POS, Ursapharm, Saarbrücken) and antibiotic eye drops (3 mg/ml Ofloxacin, Floxal EDO, Bausch-Lomb, Rochester, USA) were applied twice a day for duration of one week.

2.2.2. Clinical examination

Intraoperative ruptures of the transplant and tearing of sutures were documented and counted. Suturing time was measured with a stopwatch during surgery. Rabbit eyes were daily examined macroscopically for signs of inflammation (redness, discharge, and swelling). Follow-up controls were performed in anaesthesia at day 3 and 10 postoperatively. Transplants were observed with a microscope (Ophtamic 900 S, Möller-Wedel, Wedel) and remaining sutures were counted. Conjunctival hyperaemia was graded using a scoring system: grade 0, no hyperaemia; grade 1, slight hyperaemia; grade 2, moderate hyperaemia; grade 3, severe hyperaemia. Conjunctival, temporal fornix depth was measured with a measuring spatula (Geuder, Heidelberg) to exclude an inflammation-induced, consecutive fornix shortening and a strong contraction of the transplant. Epithelialisation of the transplants or the ungrafted defect was assessed by measuring the lissamine green (Lissamine Green Ophthalmic Strips, 1.5 mg Lissamine per strip, Optitech) stained area in documentary photographs taken during surgery and after 3 and 10 days. Herein, staining with lissamine green indicated lacking or damaged epithelium. The lissamine green stained area was assessed using ImageJ freehand tool.

2.3. Histological evaluation

Ten days after surgery, rabbits were euthanised and whole intact rabbit eyes including eyelids were excised and fixed in 4% paraformaldehyde (Roti Histofix, Roth) for 6 h. Vitreous body and lens were removed and the eyes were fixed for additional 24 h. Samples were embedded in paraffin and sections of 4.5 μm were cut.

For evaluation of epithelialisation and lymphocytic infiltration slides were stained with H/E. Periodic acid-Schiff's (PAS)/Alizarin staining was performed to determine secretory goblet cells. The number of goblet cells in ten high-power fields (400x magnification) with a total of six slides per rabbit were counted to determine the goblet cell distribution. The goblet cells were counted on the transplant beginning at the fornical edge of the transplant where the first goblet cells were seen.

Masson's Goldner (MG) staining was performed to visualize the connective tissue of the grafted conjunctiva.

The neutrophilic immune cell infiltration of PDC, HDC, AM and untreated eyes was assessed in H/E stained slides. Neutrophilic granulocytes were quantitated by counting ten high-power microscopic fields per slide (with a total of six slides per eye, 400 x magnification).

2 4. Statistical analysis

All values are expressed as mean value ± standard deviation. Statistical analysis was performed using student's *t*-test or ANOVA with Tukey correction for multiple testing (Prism 6.0, GraphPad Software). All experiments were performed in triplicate if not further indicated. A value of $p < .05$ was considered as statistically significant. The animal number (N) was calculated a priori with G*Power 3.1.9.2. with a type-1 error (α) of 0.05 and type-2 error (β) of max. 0.2 (power level min. 0.8) and validated by the State Agency for Nature, Environment and Consumer Protection (LANUV) of the German state of North Rhine-Westphalia.

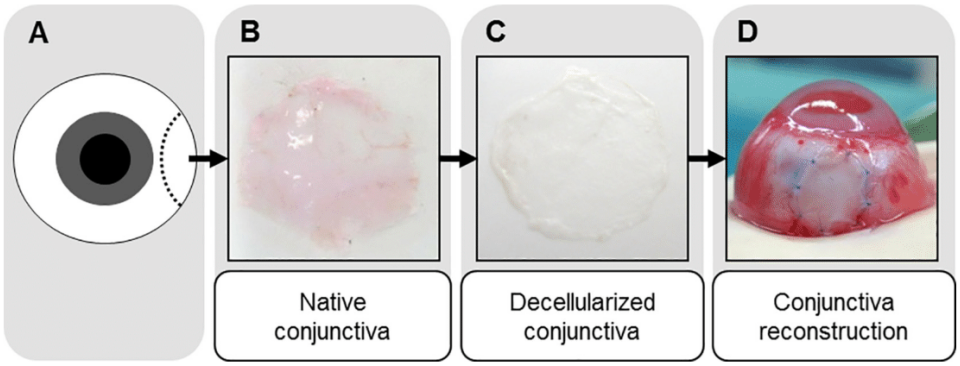

Fig. 1. Conjunctival reconstruction with decellularised porcine and human conjunctiva (PDC and HDC). A: Bulbar conjunctiva was dissected from porcine or human eyes with a distance of 2 mm from the corneal limbus. B: Untreated conjunctiva had a pink colour and particular vascular structures were visible (arrowhead). C: After the decellularisation the tissue appeared whitish and still revealed a stable structure. Former orientation (epithelium upwards, stroma downwards) was maintained during decellularisation. The tissue was spanned on a carrier paper for surgical application. D: Conjunctival bare sclera defects of 5 mm diameter were recovered either with PDC, HDC or amniotic membrane graft. Untransplanted eyes served as controls (n = 6 each group). Scale bar: 2 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. *In vitro evaluation of PDC and HDC*

3.1.1. *Efficiency of decellularisation*

Native porcine and human conjunctivas showed a stratified epithelium with an underlying vascularized stroma in which numerous intrastromal fibroblasts were embedded. These cells were absent in PDC and HDC (Fig. 2A and B). Feulgen reaction indicated absence of DNA (Fig. 2D). A fluorescent DNA Quantitation Assay was used to quantify the content of residual DNA. Before decellularisation, the porcine conjunctiva contained 1.74 ± 0.48 μg DNA per mg dry weight, which was reduced to 0.002 ± 0.002 μg/mg after decellularisation ($p = .0004$) (Fig. 3A). This was similarly true for HDC (human conjunctiva: 3.75 ± 2.187 μg/mg; HDC: 0.0019 ± 0.00178 μg/mg, $p = .014$).

3.1.2. *Preservation of extracellular matrix*

A histological evaluation after H/E staining revealed only minimal disruption of the conjunctival histoarchitecture. As before decellularisation, the stroma contained still visible lamellar fibres and vascular structures. The ultrastructural analysis by transmission electron microscopy showed intact, regularly arranged collagen fibrils without disruption. No remaining cells or cell components were detected (Fig. 2E). The collagen quantification confirmed that the collagen content of PDC and HDC was not affected during the decellularisation process compared to the native tissue (untreated human conjunctiva: 8.88 ± 2.25 μg/mg compared to HDC 10.46 ± 0.75 μg/mg; $p = .3$; untreated porcine conjunctiva: 56.38 ± 1.62 μg/mg compared to PDC 70.23 ± 24.85 μg/mg; $p = .4$) (Fig. 3B).

3.1.3. *Cytotoxicity*

3 T3 mouse fibroblasts (Fig. 3C) and HCECs (Fig. 3D) were cultured with conditioned medium from PDC and HDC to determine a possible cytotoxicity of the decellularised matrices. Neither PDC nor HDC conveyed cytotoxic effects on the cell viability of 3T3 mouse fibroblasts or HCEC after 24 or 48 h compared to the medium control. The cell count of 3 T3 fibroblasts incubated with conditioned medium from PDC and HDC increased from 100% (day 0) to 470.74 ± 20.64% (PDC) and 484.91 ± 19.36% (HDC) after 48 h of incubation. Conditioned medium from both matrices did not

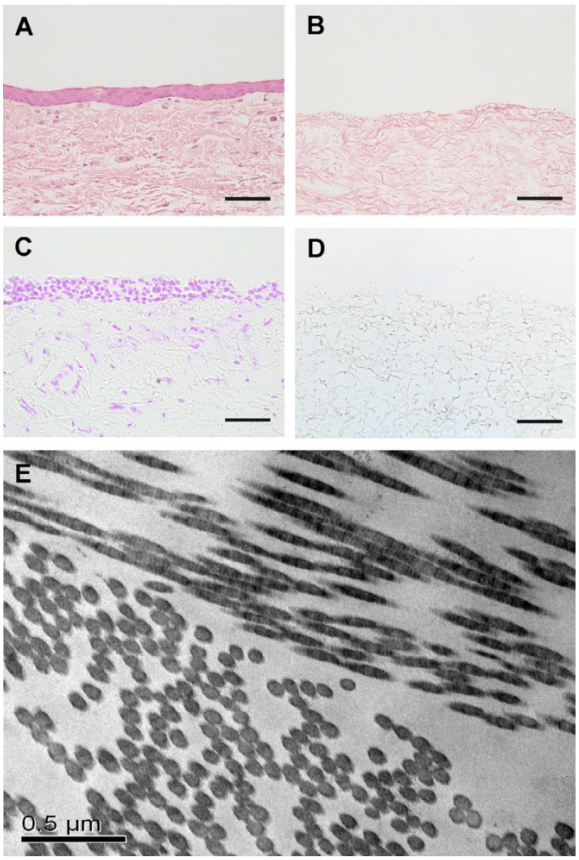

Fig. 2. Histological and ultrastructural analysis of untreated porcine (A, C) and decellularised porcine (B, D) conjunctiva. A, B: Haematoxylin/Eosin staining exhibited complete removal of the multi-layered conjunctival epithelium and the stromal fibroblasts after decellularisation. C, D: Feulgen staining showed no remaining nuclear components in the conjunctiva after decellularisation. E: Transmission electron microscopy images of porcine conjunctiva showed regular transverse and longitudinal sections of intact collagen fibrils after decellularisation. N = 4. Scale bar A-D: 50 μm.

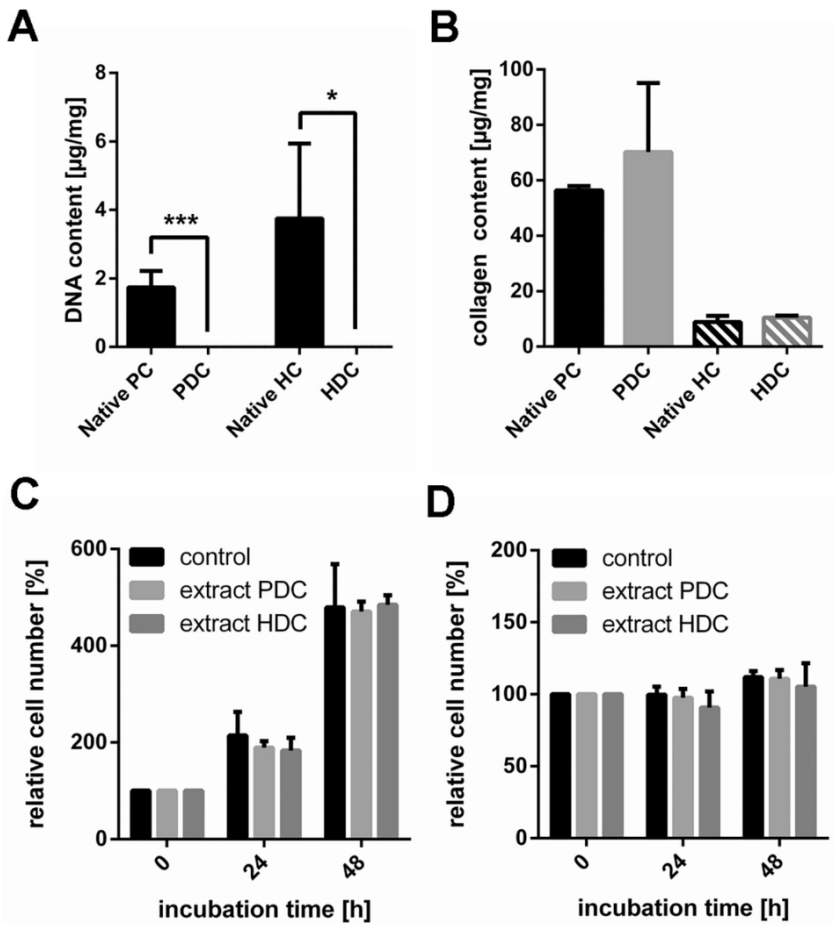

Fig. 3. Comparison of native and decellularised porcine and human conjunctiva regarding DNA content, collagen content and cytotoxic effects. A: DNA content of human conjunctiva and porcine conjunctiva was significantly reduced after decellularisation. B: Collagen I-V content of human and porcine conjunctiva was not affected after decellularisation. C, D: Extracts derived from decellularised human and porcine conjunctiva did not show a cytotoxic effect on 3T3 mouse fibroblasts or HCEC compared to media control. The differing time-dependent increase of cell viability of the 3T3 mouse fibroblasts and HCEC is due to a different cell proliferation rate of both cell types. N = 3. All Data represented as mean value ± SD.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$.

influence the cell viability of 3 T3 fibroblasts significantly compared to the medium control (479.14 ± 89.85%, PDC: p = .96; HDC: p = .98). The cell viability of HCEC increased to 110.79 ± 5.99% (incubated with PDC extract) and 105.29 ± 16.37% (incubated with HDC extract) after 48 h incubation, which was not significantly different compared to the medium control (PDC: p = .98; HDC: p = .55).

3.1.4 Biomechanical properties

The UTS, the elongation at break and the Emod of AM, native porcine conjunctiva (PC), PDC, native human conjunctiva (HC) and HDC were evaluated. AM showed significant higher UTS than PC, PDC, HC or HDC (AM: 0.02354 ± 0.0148 N/mm^2; PC: 0.004 ± 0.002 N/mm^2, p < .0001; PDC: 0.005 ± 0.0037 N/mm^2, p < .0001; HC: 0.001 ± 0.0005 N/mm^2, p < .0001; HDC: 0.0003 ± 0.0002 N/mm^2, p < .0001). In contrast, the maximal elongation at break of AM (20.23 ± 4.25%) was significantly less compared to PC (99.70 ± 34.22%, p = .0013), PDC (98.02 ± 25.88%, p = .0017), HC (85.75 ± 15.85%, p = .037) and HDC (75.49 ± 38.73%), p = .035). AM could be stretched about 20% of the initial length, PC about 100%, PDC about 98%, HC about 86% and HDC about 76% before the tissue ruptured. Additionally, PC, PDC, HC and HDC showed a lower Emod compared to AM (PC: 2109 ± 1235 kPa, p = .0002; PDC: 2326 ± 853 kPa, p = .0007; HC: 1136 ± 553 kPa, p < .0001; HDC: 1259 ± 575 kPa, p < .0001; AM: 4973 ± 1533 kPa). These data indicate a higher form stability of AM but a 4- to 5-fold higher extensibility and 2- to 4-fold higher elasticity of PC, PDC, HC and HDC compared to AM (Fig. 4). The decellularisation process did not affect the UTS (PC vs. PDC: p = .99; HC vs. HDC: p > .99), the elongation at break (PC vs. PDC: p > .99; HC vs. HDC: p = .98) or the Emod (PC vs. PDC: p = .99; HC vs. HDC: p < .99) of porcine or human conjunctiva, respectively.

3.2. In vivo evaluation of PDC and HDC in the rabbit

The time needed to suture either a PDC, HDC or AM into a conjunctival defect was not significantly different (PDC: 26 ± 6 min; HDC: 25 ± 5 min; AM: 28 ± 13 min) (Supplementary data 1). No significant difference of suture tearing during surgery between PDC (8.00 ± 0.0 intact sutures; p = .97) and HDC (7.833 ± 0.408 intact sutures; p > .99) was observed compared to AM (7.83 ± 0.41 intact sutures). Complete ruptures of the transplants did not occur in any group.

3.2 1. Stability of PDC and HDC on the ocular surface

In the AM group all 6 transplants were present after 3 days, while after 10 days 3 of 6 AM were lost. The PDC and HDC transplants were present in all 6 animals after 3 and 10 days. The AM

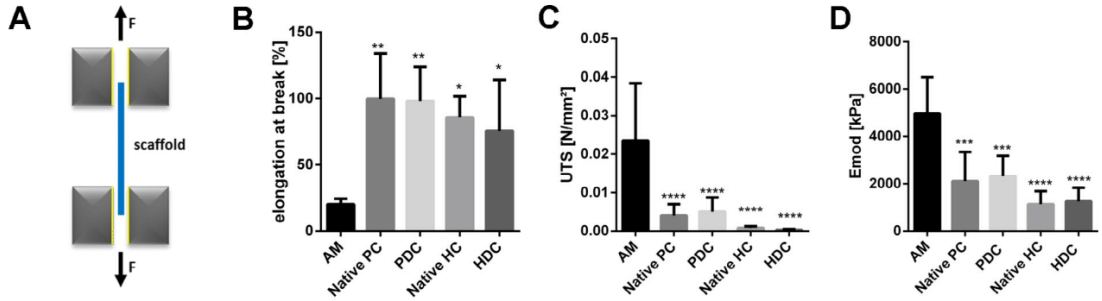

Fig. 4. Measurement of the mechanical properties of native porcine conjunctiva (PC), porcine decellularised (PDC), native human conjunctiva (HC) and human decellularised conjunctiva (HDC). A: Schematic illustration of the test set-up. The scaffold was clamped into a material testing machine and pulled with a defined force (F) until it broke. B: The maximum elongation at break of PC, PDC, HC and HDC was significantly higher compared to the amniotic membrane (AM). C: The ultimate tensile strength (UTS) of PC, PDC, HC and HDC was significantly less than AM. D: The elastic modulus (Emod) of PC, PDC, HC and HDC showed a decreased Emod compared to AM, indicating higher elasticity. No significant differences in the biomechanical properties could be measured between native and decellularised conjunctivas, respectively. N = 4–10. Data represented as mean value ±SD.*$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.005$.

group revealed a significantly higher amount of suture loss after 10 days compared to PDC (6.33 ± 0.52, p = .0011) and HDC (6.83 ± 0.75, p = .0002) (Supplementary data 2).

3.2.2. Inflammatory response

Clinically, no adverse inflammatory response occurred after surgery. In all groups the grade of bulbar hyperaemia was normal (0) to mild (1) directly after surgery and normal (0) to moderate (2) three days after surgery. After 10 days, hyperaemia decreased again to 0-1 (Fig. 5A). Histological sections were examined to determine the grade of neutrophil immune cell infiltration for PDC, HDC, AM and uncovered sclera (control) (Fig. 5B). The amount of neutrophil granulocytes was lowest in the control group (73.9 ± 11.8 granulocytes per mm^2) and differs significantly to PDC (p = .0042), HDC (p = .0142) and AM (p = .0068). Between the groups PDC, HDC and AM the amount of neutrophil immune cell infiltration did not significantly differ (PDC: 447.4 ± 187.7; HDC 393.3 ± 203.8; AM: 504.8 ± 155.9 granulocytes per mm^2, p > .05 for all group comparisons).

3.2.3. Fornix involvement

Fornix depth of all eyes in all groups was assessed as an indicator for consecutive conjunctival scarring and wound contraction (Fig. 5C). The fornix depth after 3 and 10 days compared to the preoperative value (100%) was not significantly reduced in any of the groups (PDC day 3: 96.70 ± 21.85%, p = .473; day 10: 91.78 ± 7.06%, p = .134; HDC day 3: 96.78 ± 20.37%, p = .873; day 10: 82.83 ± 16.67%, p = .086; AM day 3: 90.62 ± 9.23%, p = .323; day 10: 87.90 ± 11.30%, p = .225; control day 3: 96.70 ± 21.85%, p = .867; day 10: 91.78 ± 7.06%, p = .418).

3.2.4. Epithelialisation and integration of the transplant

Three days postoperatively, only the PDC and the HDC group revealed a slightly significant reduction of the defect size compared to the defect size immediately after surgery (PDC: 73.94 ± 21.90%, p = .0495; HDC: 70.79 ± 30.25%, p = .0203) while the defect size of the AM group and the control was not significantly reduced after 3 days (AM: 93.40 ± 27.72%, p > .99; control: 94.39 ± 23.57%). After 10 days, all groups showed a highly significant defect size reduction compared to the initial measurement (PDC: 46.43 ± 7.29%, p < .0001; HDC: 33.14 ± 9.67%, p < .0001; AM: 27.72 ± 20.05%, p < .0001; control: 22.51 ± 18.68%) (Fig. 5D).

Although the defect size indicated by Lissamine staining revealed no difference between PDC, HDC and AM after 10 days, further histological evaluation showed stratified epithelium containing cuboidal and squamous epithelial cells only on PDC and HDC. Such stratified epithelium covering the transplant was observed in none of the eyes grafted with AM. AM was abundant, coiled or folded and an integration of the AM with the recipient conjunctiva was not found (Fig. 6). The reduction of the defect area in AM grafted eyes was rather mediated due to contraction of the conjunctival wound edges and growth of fibrous scar tissue and

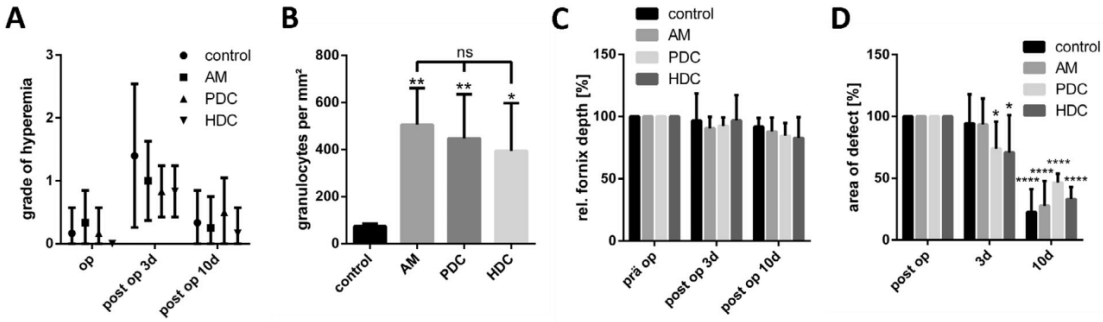

Fig. 5. Fornix depth, inflammatory response and defect closure of porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC). A: Clinical grading of bulbar hyperaemia before surgery and after three and ten days revealed no significant difference compared to the amniotic membrane control (AM) and the control. B: Infiltration by neutrophilic granulocytes per mm^2 was not significantly different between PDC, HDC and AM but increased compared to control. C: Fornix depth of PDC and HDC did not differ significantly compared to AM or the control. D: Clinically, the defect size was not significantly different between PDC, HDC, AM and control after 10 days. Data represented as mean value ± SD.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$.

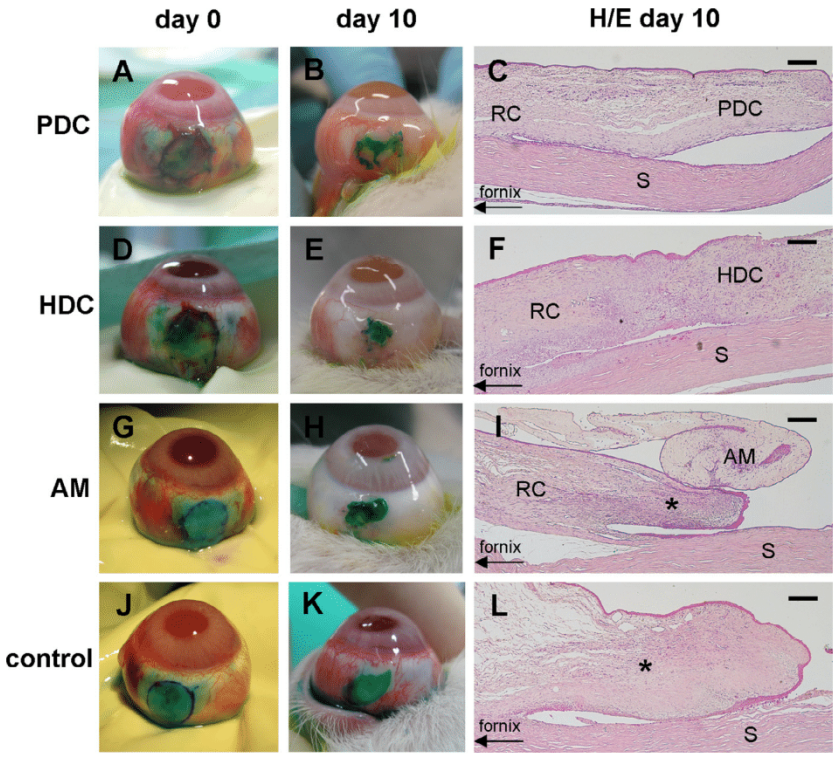

Fig. 6. Representative clinical and histological findings of porcine decellularised conjunctiva (PDC), human decellularised conjunctiva (HDC) and amniotic membrane (AM) transplanted in a conjunctival defect of the rabbit. The control group remained ungrafted. Pictures of lissamine stained conjunctiva taken immediately after suturing of the transplant (A, D, G, J) and after 10 days (B, E, H, K). The histological analysis of cross sections (Haematoxylin/Eosin staining) showed a complete integration of PDC (C) and HDC (F) into the recipient conjunctiva (RC). In contrast, AM was rolled up or folded and not adapted to the recipient conjunctiva in the remaining 3 cases (I). The reduction of wound size in AM grafted animals was mediated by wound contraction and fibrous scar tissue growth under the transplant (asterisk). The ungrafted control exhibited strong synthesis of fibrotic scar tissue with dense collagen fibres. The arrow indicates the direction to the fornix. S: Sclera. Scale bar: 200 μm.

not due to epithelialization of the transplant. In contrast, all PDC (6 of 6) and 4 of 6 HDC grafted eyes showed a good integration of the decellularised extracellular matrix with the collagenous stroma of the recipient rabbit conjunctiva without extensive growth of fibrous scar tissue. Integration of the extracellular matrix at the fornical edge of the transplant was completed after 10 days, whereas the border of the corneal edge of the transplant was still clearly visible. Masson's Goldner staining exhibited a loose network of collagen fibres in the PDC and HDC grafted conjunctivas with absence of dense collagen fibrils, which are characteristic for scar tissue. Vascular structures containing red blood cells were observed in PDC and HDC but not in AM after 10 days. The histological evaluation of PAS/Alizarin staining revealed goblet cells embedded in the epithelium at the fornical end of PDC and HDC (Fig. 7, indicated by an asterisk). No goblet cells were found at the corneal edge and in the central transplant. Quatitative, no significant changes in bulbar and fornical goblet cell amount of PDC, HDC and AM group could be measured compared to the control (PDC: 9.89 ± 5.86, p = .28; HDC: 9.88 ± 5.75, p = .23; AM: 9.67 ± 5.14 goblet cells/mm^2, p = .15) (Supplementary data 3).

4. Discussion

The conjunctiva is a main functional part of the ocular surface as it protects and moistens the cornea and contributes to the normal function of the tear film and to the homeostasis of the entire ocular surface. Clinically, conjunctival reconstruction is required frequently after extensive thermal or chemical burns, after radiation therapy or after surgically induced conjunctival damage (pterygium / tumour surgery, filtrating glaucoma surgery) [39–43].

An autologous conjunctival transplant from the patient provides an ideal tissue for small conjunctival defects. However, autologous grafting is not possible in case of extensive conjunctival damage, bilateral involvement or when autoimmune diseases destroy the conjunctival integrity.

Amniotic membrane, which is widely used for ocular surface reconstruction today, has some considerable disadvantages as limited availability, heterogeneity regarding donor, sampling-side, and preparation, a potential infectious risk, and accelerated degradation in an inflamed surrounding so that an alternative matrix is desirable [12,16,17,44].

Here, we report the development of PDC and HDC as tissue substitutes for conjunctival reconstruction. The benefit of using decellularised matrices for organ reconstruction or replacement is that each organ comprises an unique ECM with specific molecules and a particular arrangement of structural proteins, which enhance cell adhesion and proliferation and determine organ specific biomechanical properties [45,46]. Such matrices are already frequently applied clinically (e.g. heart valves [47,48] , skin repair [31] , repair of tracheal [49], or urologic defects [50]). Ophthalmological indications include scleral or corneal patching, tendon elongation in strabismus surgery [51] or grafting of extended defects in lid surgery [52]. Matrices used in such cases are e.g. decellularised bovine pericardium [51], decellularised dermal allograft [53], bovine sclera [54] or fascia lata [55,56]. However, a conjunctival

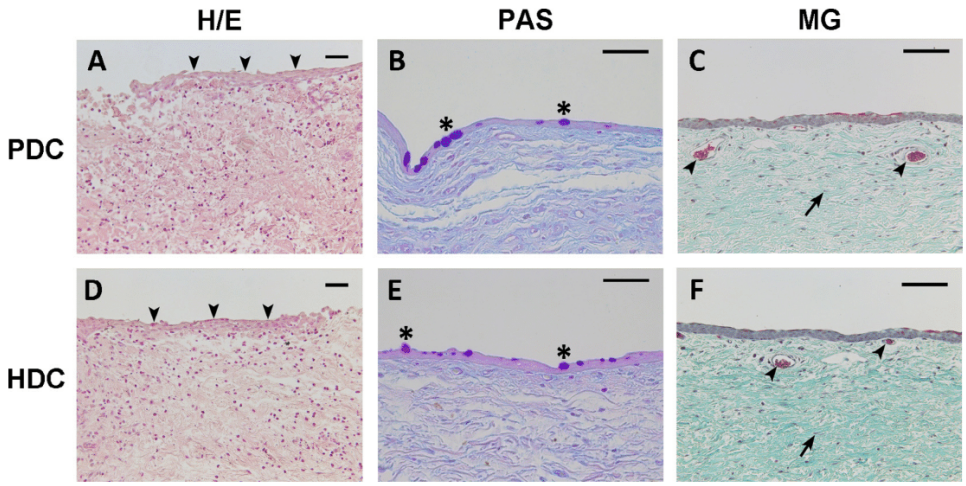

Fig. 7. Histological findings after transplantation of porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC) into conjunctival defects of rabbit eyes. A, D: Ten days after transplantation PDC and HDC were partially covered with a multi-layered epithelium (arrowhead). B, E: Goblet cells (asterisk) were embedded into the epithelium (stained with Periodic acid-Schiff/Alizarin, PAS). C, F: Regarding PDC and HDC, collagen fibres (green) appeared loosely packed and fibroblasts evident (arrow). Blood vessels filled with red blood cells were visible (arrowhead), indicating perfusion ten days after transplantation (stained with Massons' Goldner, MG). Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substitute is not available so far. Kasbekar et al. decellularised human conjunctiva to analyse it's usability as a substrate for conjunctival cell growth *in vitro* and could show that conjunctival epithelia cells were able to form a stratified epithelium on cell-free human conjunctiva [57]. Recently, Zhao et al. had proven decellularised porcine conjunctiva as a substitute for corneal reconstruction in the rabbit with encouraging results [58]. However, an *in vitro* and *in vivo* comparison of human and porcine conjunctiva and AM for conjunctival reconstruction has not been performed yet.

This study shows that decellularisation of porcine and human conjunctiva generates cell free, stable and extensible matrices, which are biocompatible and exhibit superior tissue integration compared to AM *in vivo*.

Histologically, PDC and HDC contained an intact extracellular matrix in which vascular, tube-like structures were still visible. To investigate the ECM more closely, we performed transmission electron microscopy, which is an established method to proof the ultrastructural integrity of collagen fibrils in decellularised tissues [59]. Collagen is ubiquitously found in connective tissue in all mammals and a key protein to maintain the structural integrity and ECM strength of an organ [45]. Electron microscopy revealed that the decellularisation process did not disrupt the collagen fibrils, which is a prerequisite to generate a "conjunctival-like" tissue. In line with the structural findings, collagen quantification revealed that the collagen content was not significantly reduced after decellularisation. Interestingly, the native porcine conjunctiva showed a 5- to 6-fold higher collagen content than the human tissue, which might be due to donor age and differences in tissue processing. Human donors had an average age of 79 years whereas the pigs were about 8 months old. It is known that connective tissue degenerates with age, which explains the different collagen amounts [60,61]. Moreover, porcine tissue was processed within 2–3 h after euthanasia, while human donor blood had to undergo serological testing before laboratory processing started, which took 9–13 days. This long-term storage in culture medium could have induced a partial collagen-degradation. Nevertheless, these findings also indicate that PDC might be a more supportive conjunctival substitute than HDC due to the higher collagen amount. Pigs may represent a tissue source with a more consistent conjunctival tissue quality as they are equal in age and raised under standardised conditions.

To evaluate the biomechanical properties of PC, PDC, HC and HDC, mechanical testing was performed. The decellularisation process did not affect the tissue strength, extensibility or elasticity of porcine or human conjunctiva. The force needed to rupture AM was significantly higher compared to PC, PDC, HC and HDC, indicating a "stronger" ECM of AM. However, the Emod measurement of AM displayed high standard deviations due to the restricted sample number and the heterogeneity of the AM. Interestingly, the maximal elongation at break and Emod of PC, PDC, HC and HDC was significantly superior compared to AM indicating superior extensibility and elasticity of the native and decellularised conjunctiva.

Regarding AM, tensile strength is a main quality criterion as it needs to carry the neonate and prevent preterm rupture of the fetal membranes [62]. The conjunctiva does not require such tensile strength but needs to possess good elastic properties as the eye moves into different directions of gaze and the lids close and open continuously all day. Due to preservation of the specific conjunctival ECM in PDC and HDC, the decellularised matrices provide these particular biomechanical properties.

As the collagen content of PDC was about 6-fold higher compared to HDC, a significant greater Emod difference would be expected between these groups. A reason for the relatively low Emod of PDC might be differences in elastic fibre composition and different degrees of collagen cross-linking. The human donor average age in our study was about 79 years while the pigs were about six month old. Elastic fibre network disorganisation and gradual fragmentation is known as a consequence of aging and as a result of environmental factors such as ultraviolet light [63,64]. Fibrillin and elastin rich tissues display a lower Emod [63,65,66]. A higher elastic fibre amount of younger donor tissues might be causal for the relatively low Emod of the PDC. Elastic fibre composition and collagen cross-linking of human and porcine conjunctiva have to be evaluated in future studies.

Lack of tissue toxicity is essential prior to any *in vivo* testing. Regarding decellularised tissues, lack of donor DNA is crucial as the amount of donor DNA is directly correlated to adverse host reactions [67]. To proof lack of significant residual DNA amounts,

DNA quantification needs to be performed in decellularised tissues. The acceptable amounts of residual DNA were found to be <50 ng dsDNA per mg dry weight [67,68]. Both, PDC and HDC fulfil these criteria. Further, *in vitro* toxicity testing with conditioned medium from PDC and HDC on a fibroblast cell line and on the more susceptible primary human conjunctival epithelial cells proofed that the decellularisation process did not leave toxic substances in the cell free matrices as proliferation of cells cultured with the conditioned medium was similar to the proliferation of cells cultured with standard culture medium.

To investigate the *in vivo* applicability of decellularised conjunctiva, conjunctival defects in rabbits were closed with PDC or HDC in comparison to AM, the most frequently used matrix for ocular surface reconstruction. In the clinical context, handling and surgical manageability are of main importance. AM, PDC and HDC are delivered on a nylon carrier mash, which enables easy handling, orientation of the matrix and trepanning a tissue sample of the desired size. Intraoperatively, both decellularised conjunctival matrices showed similar surgical manageability as AM. In all three groups the transplants did not tear and the suturing time as well as the amount of sutures required to adapt the transplant to the host conjunctiva did not differ significantly between the three matrices.

Histologically, PDC and HDC were well integrated into the host conjunctiva after ten days. H/E staining showed that the transplant edges were barely visible and the collagen fibrils of the transplant were connected to or woven into the host collagen (Fig. 6). Such integration into the host ECM with degradation of the decellularised tissue and replacement by the donor ECM is well known for decellularised matrices [69]. Importantly, remodelling of decellularised (xenogeneic) transplants by the recipient occurs without necrosis or scar tissue development [45]. This was also true for PDC and HDC. Clinically, it is described that AM grafted defects show good wound healing and low scaring potential [70]. However, in this study AM transplanted eyes histologically showed contraction of the conjunctival wound edges and growth of fibrous scar tissue. Such healing pattern is characteristic for spontaneous conjunctival wound healing, in which wound contraction occurs due to fibrous tissue growth of collagen synthesized in the wound bed (Fig. 6) [71]. Apart from the well-known fact that AM depicts strong intra- and inter-donor variations concerning tissue quality and thickness, these histological findings may explain the inconsistent clinical outcomes after AM transplantation [16,17]. This study showed that PDC and HDC consistently permitted superior wound healing by better integration into the recipient tissue compared to AM.

The degradation of transplants is mediated by infiltration of host immune cells (leukocytes and monocytes), which progressively invade the transplant three to 14 days after transplantation [45]. Immune cell invasion was similar for PDC, HDC and AM, indicating a similar immune response to all matrices. Probably, the tissue-foreign ECM composition of AM impeded its degradation by the host immune cells and thus a scar free integration. A scar free healing is essential for successful conjunctival reconstruction as scar formation causes fornix shortening and induces reduced mobility of the eye and the lids, which eventually leads to ocular surface disturbances, lid closure defects, cosmetic disfiguration and pain. PDC and HDC could therefore be supportive especially in large, fornix destroying defects, although this has not been tested here.

Both, PDC and HDC showed better mechanical stability on the ocular surface compared to AM: Half of the AM transplants were not present after 10 days whereas all PDC and HDC were well integrated into the rabbit conjunctiva. Apart from the better integration of PDC and HDC into the host conjunctiva, which induces higher stability, differences in the biomechanical properties could account for the accelerated tissue loss of AM. Mechanical stress is known to influence ECM remodelling [45]. Probably, the more expandable PDC and HDC could withstand the movement of the lids over the transplant better than the more rigid AM.

In line with the fact that decellularised tissues show early neovascularisation after transplantation, we observed stromal, blood filled vessels in PDC and HDC after 10 days but not in AM grafts [45]. The tube-like, vascular formations, which were evident within PDC and HDC, might have served as guiding structures to induce vessel formation. Vascularisation is essential for the long-term survival of transplants and an important indicator for a potential successful clinical application. Previous studies with decellularised matrices of different origin like decellularised bovine pericardium showed limitations in building of stratified epithelium because of a dense collagen array which might slow down the nutrition transport needed for re-epithelialization [72]. However, like untreated conjunctiva, PDC and HDC showed a loose network of collagen fibrils in the reconstructed conjunctival stroma and blood filled vessels after transplantation. These anatomical preconditions allow nutrition by diffusion and direct oxygen delivery. In accordance, PDC and HDC exhibited a stratified epithelium with cuboidal and squamous cells ten days after transplantation.

Different studies showed that natural ECM from decellularised tissues guides differentiation into the specific cell types from which the ECM was derived [30]. Yang et al. [73] reported that homogenates of decellularised human conjunctivas promote the differentiation of human epithelial progenitor cells into mature conjunctival epithelial cells and MUC5AC expressing secretory cells by mimicking the *in vivo* microenvironment [73]. Decellularised conjunctiva may therefore exhibit a supportive niche for conjunctival cells to promote differentiation into secretory competent cells. In our study we observed differentiated goblet cells on PDC and HDC at the fornical edge of the transplant, which indicates early differentiation into functionally competent cells, although the centre of the transplants did not contain secretory cells after ten days *in vivo* application.

Pigs show a huge potential as source for xenographic transplantation as they can be breed well in captivity, mature quickly and are already used as food source [74]. Moreover, extracellular matrix proteins are evolutionary preserved among humans and pigs indicating biological similarity [75]. Decellularised porcine scaffolds are successfully used in animal studies and clinical applications for different tissues [32,76–79]. Although there is concern about the risk of bacterial, viral (e.g. porcine endogenous retrovirus) or prion transmission from porcine xenografts, various studies proofed the safety of decellularised porcine matrix scaffolds after implantation [33,80].

Human tissue is scarcely available because of the lack of donor organs. This study revealed that decellularised conjunctiva of porcine origin is not inferior to human tissue regarding structural integrity, biomechanical properties, toxicity and *in vivo* compatibility. Hence, PDC may represent an easily available conjunctival substitute with clinical and economic benefits.

Denuded AM displays good clinical results as a carrier for ocular epithelial cells. Therefore the potential of PDC and HDC as a conjunctival epithelial cell carrier and potential benefits compared to AM will be evaluated in future studies.

Although this study extensively investigated decellularised conjunctiva *in vitro* and after a ten day follow up *in vivo,* it has certain limitations. First, a higher animal number per group might have increased the study result significance. Secondly, a long term-follow up to further evaluate epithelialisation, secretory capacity and potential scar formation *in vivo* is required and in progress. The conjunctival defect was induced by a surgical intervention, relatively small and restricted to a distinct area of the conjunctiva. Clinical cases often come along with other complications, such as

chronic inflammation with elevated matrix-metalloproteinase (MMP)-9 levels, dry eye or extensive fornix scarring. A potential superiority of decellularised conjunctiva in an inflammatory environment, in case of preoperative fornical scarring or extensive conjunctival defects needs further investigations *in vivo*.

5. Conclusions

Here we show that both, decellularised conjunctiva of porcine and human origin are promising matrices for conjunctival reconstruction with superior wound healing capacity compared to AM in the rabbit model. As PDC was similarly biocompatible and stable on the ocular surface as HDC it might be an easily available, ethically legitimate alternative matrix for conjunctival reconstruction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2017.11.054.

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II. Decellularized Porcine Conjunctiva as an Alternative Substrate for Tissue-Engineered Epithelialized Conjunctiva

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Abstract

Purpose: The long-term success of visual rehabilitation in patients with severe conjunctival scarring is reliant on the reconstruction of the conjunctiva with a suitable substitute. The purpose of this study is the development and investigation of a reepithelialized conjunctival substitute based on porcine decellularized conjunctiva (PDC).

Methods: PDC was reepithelialized either with pre-expanded human conjunctival epithelial cells (PDC+HCEC) or with a human conjunctival explant placed directly on PDC (PDC+HCEx). Histology and immunohistochemistry were performed to evaluate epithelial thickness, proliferation (Ki67), apoptosis (Caspase 3), goblet cells (MUC5AC), and progenitor cells (CK15, ΔNp63, ABCG2). The superior construct (PDC+HCEx) was transplanted into a conjunctival defect of a rabbit (n=6). Lissamine green staining verified the epithelialization in vivo. Orbital tissue was exenterated on day 10 and processed for histological and immunohistochemical analysis to examine the engrafted PDC+HCEx. A human-specific antibody was used to detect the transplanted cells.

Results: From day-14 in vitro onward, a significantly thicker epithelium and greater number of cells expressing Ki67, CK15, ΔNp63, and ABCG2 were noted for PDC+HCEx versus PDC+HCEC. MUC5AC-positive cells were found only in PDC+HCEx. The PDC+HCEx-grafted rabbit conjunctivas were lissamine-negative during the evaluation period, indicating epithelial integrity. Engrafted PDC+HCEx

showed preserved progenitor cell properties and an increased number of goblet cells comparable to those of native conjunctiva.
Conclusion: Placing and culturing a human conjunctival explant directly on PDC (PDC+HCEx) enables the generation of a stable, stratified, goblet cell-rich construct that could provide a promising alternative conjunctival substitute for patients with extensive conjunctival stem and goblet cell loss.

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High Impact Original Research

Decellularized porcine conjunctiva as an alternative substrate for tissue-engineered epithelialized conjunctiva

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ABSTRACT

Purpose: The long-term success of visual rehabilitation in patients with severe conjunctival scarring is reliant on the reconstruction of the conjunctiva with a suitable substitute. The purpose of this study is the development and investigation of a re-epithelialized conjunctival substitute based on porcine decellularized conjunctiva (PDC).
Methods: PDC was re-epithelialized either with pre-expanded human conjunctival epithelial cells (PDC + HCEC) or with a human conjunctival explant placed directly on PDC (PDC + HCEx). Histology and immunohistochemistry were performed to evaluate epithelial thickness, proliferation (Ki67), apoptosis (Caspase 3), goblet cells (MUC5AC), and progenitor cells (CK15, ΔNp63, ABCG2). The superior construct (PDC + HCEx) was transplanted into a conjunctival defect of a rabbit (n = 6). Lissamine green staining verified the epithelialization in vivo. Orbital tissue was exenterated on day 10 and processed for histological and immunohistochemical analysis to examine the engrafted PDC + HCEx. A human-specific antibody was used to detect the transplanted cells.
Results: From day-14 in vitro onward, a significantly thicker epithelium and greater number of cells expressing Ki67, CK15, ΔNp63, and ABCG2 were noted for PDC + HCEx versus PDC + HCEC. MUC5AC-positive cells were found only in PDC + HCEx. The PDC + HCEx-grafted rabbit conjunctivas were lissamine-negative during the evaluation period, indicating epithelial integrity. Engrafted PDC + HCEx showed preserved progenitor cell properties and an increased number of goblet cells comparable to those of native conjunctiva.
Conclusion: Placing and culturing a human conjunctival explant directly on PDC (PDC + HCEx) enables the generation of a stable, stratified, goblet cell-rich construct that could provide a promising alternative conjunctival substitute for patients with extensive conjunctival stem and goblet cell loss.

1. Introduction

In the healthy human eye, conjunctival stem cells, which are omnipresent throughout the entire conjunctival surface, are essential for repopulation of both epithelial cells and mucin-producing goblet cells throughout one's entire lifespan [1]. Severe damage to the ocular surface has several significant consequences. Firstly, trauma (e.g. thermal or chemical burns, tumor surgery) or systemic autoimmune disease (e.g. cicatrizing mucous membrane pemphigoid, Stevens–Johnson Syndrome) can induce extensive damage to the ocular surface and its mature cell population. Although the injured conjunctiva is capable of spontaneous re-epithelialization, concomitant fibrosis can cause fornix shortening and even ankyloblepharon, with subsequent dry eye, and/or corneal disease, ultimately resulting in loss of vision. Removal of scar tissue and reconstruction of the conjunctiva are critical steps to maintain or reestablish ocular surface homeostasis [2,3]. Secondly, severe ocular surface damage often results in a massive or complete loss of conjunctival epithelial stem cells. In this situation, long-term visual rehabilitation requires renewal of the (i) conjunctival epithelial progenitor cell population and matrix with supportive niche features in order to ensure the survival of the conjunctival graft. This will also replenish (ii) the goblet cell population, which is crucial for maintenance of the tear film's mucous layer [4–7].

While autologous tissue substitutes—e.g. healthy conjunctiva from unaffected ocular sites [8], nasal mucosa [9], and oral mucous membranes [10]—have been employed clinically, they have significant

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drawbacks, including limited tissue availability and risk of disease exacerbation (e.g. in autoimmune disorders), donor site morbidity or paucity of goblet cells and undesirable cosmesis (e. g. in oral mucosa) [4,8,9,11,12]. Consequently, there have been numerous efforts to develop alternative substrates for conjunctival reconstruction [2,13,14]. Owing to its growth-supporting and anti-inflammatory properties, amniotic membrane (AM) is frequently used as a matrix for the expansion of autologous conjunctival epithelial cells. While this approach has yielded encouraging results, it has several disadvantages, including limited availability, costly donor screening, time-consuming preparation, and inconsistent tissue properties such as thickness, biomechanical instability, and/or variable growth factor content [15,16]. Furthermore, the protocols for preparation of AM may vary and/or AM grafts can rapidly degrade in inflammatory environments, resulting in decreased epithelialization [17,18].

Keratin [19,20], fibrin [14], collagen [21–23] and various synthetic polymers, including poly (lactide-*co*-glycoside) (PLGA) and poly (epsilon-caprolactone) (PCL) [13,24] have been used as alternative substrates for engineering the conjunctiva. In vivo studies of cultured epithelial sheets [25,26], porous collagen-glycosaminoglycan [22], vitrified collagen [21], and PLGA [13] have yielded promising results in terms of the re-epithelialization and inhibition of scar formation. However, most of these materials investigated in vivo have poor mechanical strength/elasticity, varying thickness, and paucity of goblet cells; they also degrade either too fast or not all [2,4,22]. Three-dimensional (3D)-printed gelatin-based membranes, as well as compressed collagen-based constructs are among the most promising candidates, as they are stable and easy to handle surgically [27–29]. However, the potential of 3D-printed gelatin-based membranes as carriers for conjunctival epithelial cells and their capacity for goblet cell formation have not yet been established.

Several studies have investigated decellularized cornea [30], limbus [31], and lacrimal gland [32] for their utility as conjunctival-substitute matrices. The properties of decellularized scaffolds are ideal and include an organ-specific and biomechanically stable three-dimensional structure, with distinct extracellular matrix arrangements [33]. The extracellular matrix is a structurally and functionally complex system comprising proteins, peptides, and polysaccharides, among other components [34,35]. The composition and organization of these essential molecules is highly tissue-specific and varies between microenvironments [35,36]. Furthermore, many studies have shown that the basement membrane and extracellular matrices from decellularized tissues promote differentiation in specific cell types from which the tissue was derived [37,38]. Porcine decellularized conjunctiva (PDC) not only possesses ideal biomechanical properties, including exceptional elasticity and extensibility, but also has excellent biocompatibility in vivo [39]. Furthermore, PDC elicits neither inflammatory reactions nor scarring following xenogeneic transplantation, and has a better wound healing capacity than AM [39].

Epithelial cell proliferation, differentiation, and viability are dependent upon a complex system of interactions between cells and the stromal extracellular matrix of their immediate environment [40,41]. Stem cell populations that are located close to their niche environment maintain their undifferentiated states longer than those located further away [42]. The proliferative capacity of epithelial cells on plastic substrates having neither matrix nor fibroblast support is limited [43]. To avoid the loss of progenitor cell properties and to preserve the proliferative potential in vitro, epithelial cells are commonly expanded in co-culture with growth-arrested 3T3 murine feeder cells [4,43–45]. However, since such xenobiotic components present risks for graft rejection and transmission of infections, they are unfavorable for clinical use.

The aims of the present study were to (i) compare two different methods of cell expansion on PDC for the development of a stratified, progenitor cell-rich, conjunctival epithelium and (ii) evaluate the superior construct in a conjunctival defect animal (rabbit) model in vivo.

2. Materials and methods

2.1. Generation of PDC

PDC was produced as previously described [39]. Porcine eyes with the attached conjunctiva were obtained from a local slaughterhouse and transported to the lab in a phosphate-buffered saline (PBS) solution (Sigma-Aldrich, Schnelldorf, Germany) containing 5% penicillin/streptomycin (P/S). The bulbar conjunctiva was excised within 2–3 h of death. Porcine conjunctivas were washed for 2 h in PBS+5% P/S, after which they were incubated in 2% sodium deoxycholate monohydrate (NaDC, Sigma-Aldrich) at 4 °C for 3 h, followed by the addition of fresh NaDC solution and incubation at 4 °C for 24 h. Between each step, conjunctivas were washed thoroughly with PBS. Deoxyribonuclease (Dnase) I, Grade II, from bovine pancreas (Worthington Biochemical Corp., Lakewood, NJ, USA), was reconstituted and diluted with PBS to a concentration of 670 Units/ml. The tissue was transferred into the DNase solution, incubated at 37 °C for 3 h, washed in PBS at 4 °C overnight, sterilized by 25 kGy of γ-irradiation (BBF Sterilization-Service GmbH, Rommelshausen, Germany) and placed onto 0.2 μm nylon carrier papers (Roti-Nylon, Roth, Karlsruhe). Every batch of PDC was analyzed by DNA quantification and histological cross sections as described in a previous study [39] to verify effective decellularization.

2.2. Culturing of PDC + HCEC and PDC + HCEx in vitro

Two different methods for re-epithelialization of PDC (PDC + HCEC versus PDC + HCEx) were compared with respect to stratification and organization of the epithelium, goblet cell differentiation and maintenance of progenitor cell properties. For both methods, human donor conjunctivas were used as epithelial cell source. The human conjunctivas were obtained from the Lions Eye Bank of North Rhine–Westphalia of the Department of Ophthalmology, University Hospital Duesseldorf, Heinrich-Heine-University, Germany, after obtaining informed consent for research use. The study was approved by the local ethics committee (No. 4722). Human conjunctivas with a size of about 1 cm diameter were cut up into smaller (1 mm diameter) sizes and cryopreserved in conjunctival epithelial cell medium (CECM) supplemented with 10% dimethylsulphoxide (DMSO) and 20% fetal bovine serum (FBS Superior, S0615, Biochrom, Gründau, Germany) in liquid nitrogen for up to 6 months. CECM composition is listed in Supplementary Table 1.

2.2.1. PDC + HCEC

For the cultivation method PDC + HCEC, HCEC were expanded in co-culture with growth-arrested feeder cells (3T3-Swiss albino mouse fibroblasts/CCL-92, LGC, Wesel, Germany) as described previously [6,23,46] (Fig. 1). Therefore, an 80–90% confluent T75 cell culture flask of 3T3 fibroblasts were incubated for 2 h in 4 μg/ml mitomycin C (Sigma-Aldrich) in 10 ml cell culture media. Next, the 3T3 cells were washed with PBS, detached with 0.05% trypsin and 0.02% Ethylenediaminetetraacetic acid (EDTA) in Hanks' Balanced Salt Solution (Sigma-Aldrich), and seeded at a cell density of 2.4×10^4 cells/cm^2 in a T75 cell culture flask (TC, Sarstedt, Nümbrecht, Germany). The 3T3 cells were allowed to adhere for at least 4 h.

The cryopreserved conjunctival pieces were thawed in a 37 °C water bath, diluted in 10 ml prewarmed CECM and washed twice in CECM. After this, the conjunctival pieces were transferred into the cell flasks seeded with the previously prepared, growth-arrested 3T3 fibroblasts. The cells were cultivated in CECM under 5% CO_2 at 37 °C and the medium was changed every 2 days. When the outgrown HCEC reached a confluence of 70–80%, the feeder cells were removed with 0.05% trypsin and 0.02% EDTA in Hanks' Balanced Salt Solution (Sigma-Aldrich), followed by a change to 10-fold concentrated trypsin-EDTA solution (0.5% trypsin and 0.2% EDTA in Hanks' Balanced Salt Solution, Sigma-Aldrich) to detach the HCEC. Next, 3.5×10^5 HCEC were

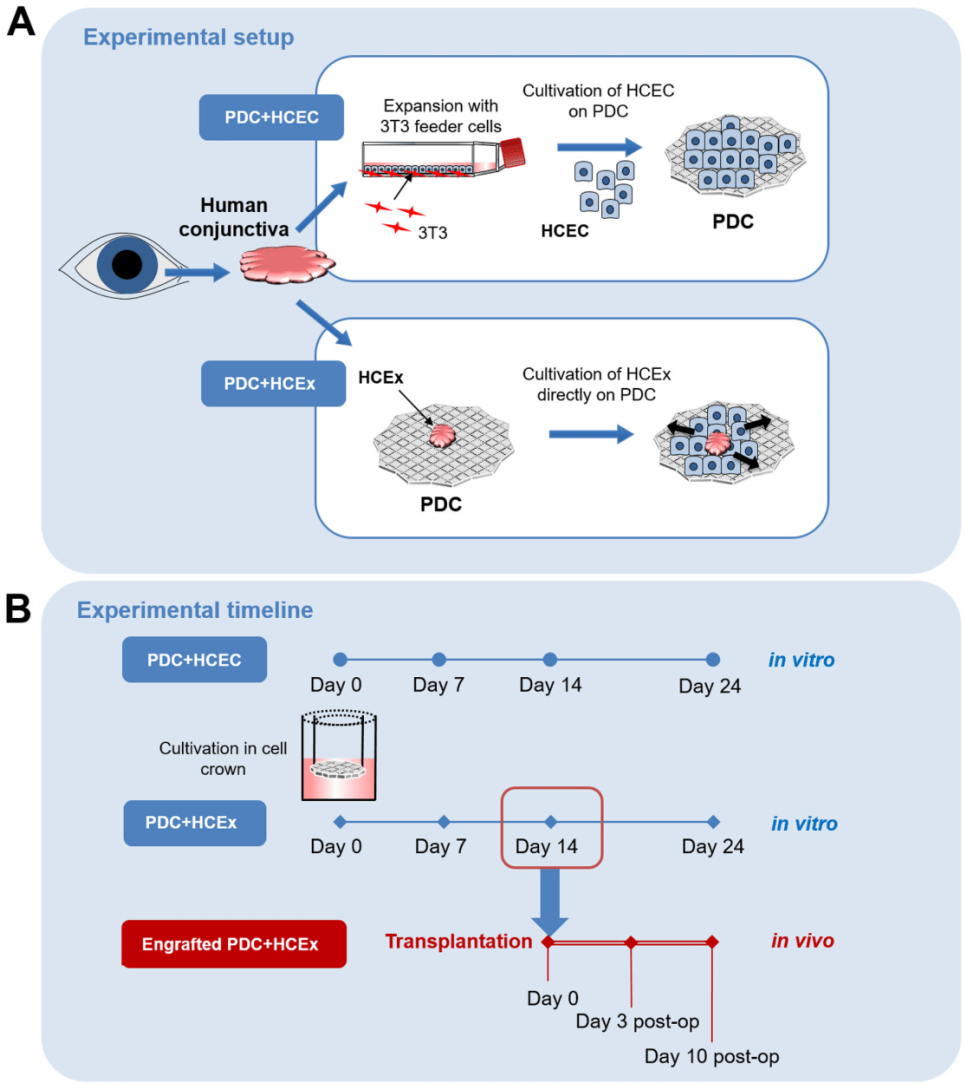

Fig. 1. Experimental setup and timeline of PDC re-epithelialization A: Human conjunctiva was either cultured in cell culture flasks containing growth-arrested 3T3-fibroblasts (PDC + HCEC) or cultured directly on PDC (PDC + HCEx). In PDC + HCEC, the HCEC were isolated after expansion, separated from the 3T3-fibroblasts, and seeded onto the PDC placed in a cell crown. In PDC + HCEx, a conjunctival piece was placed directly on top of PDC for further expansion. B: Both, PDC + HCEC and PDC + HCEx were cultured submersely for 7 days and then air-lifted for the remaining incubation period by removing the apical medium. After 7, 10, 14, 21 and 24 days, PDC + HCEC and PDC + HCEx were fixed for histological examinations. After determining the ideal cultivation method and duration, PDC + HCEx was transplanted into a conjunctival defect of a rabbit for 10 more days (=engrafted PDC + HCEx).

seeded onto PDC that have been placed in a cell crown (Polyether ether ketone, custom-made, inner diameter 0.8 cm, Fraunhofer IGB, Würzburg, Germany). The cell crowns were inserted into 24-well culture plates and PDC + HCEC was cultured while immersed in medium for the first 7 days. The culture medium was changed daily. Upon achieving confluence (day 7), the apical portion of medium (inside the cell crown) was removed, leaving the top of the HCEC layer exposed to the air while the basal cells were still in contact with medium to allow further stratification and differentiation (air-liquid interface culture). From this point of time, the concentration of EGF in the CECM was reduced to 0.5 ng/ml and the culture medium was changed daily. PDC + HCEC was cultured until a maximum of day 24.

2.2.2. *PDC + HCEx*

For the cultivation method PDC + HCEx the cryopreserved conjunctival pieces were thawed and washed as described above. Then, a 1 mm diameter conjunctival piece was placed directly onto a PDC that was placed in a cell crown. The conjunctival piece was allowed to attach to PDC for 5 min before culture medium was added. Further cultivation was performed as described above for PDC + HCEC.

2.3. *Histological and immunohistological analysis of in vitro cultured PDC + HCEC and PDC + HCEx*

On days 7, 10, 14, 17, 21, and 24 of culture, PDC + HCEC und PDC + HCEx were fixed in 4% paraformaldehyde (Roti Histofix®, Roth, Karlsruhe, Germany) for 2 h. The samples were then dehydrated in a graded ethanol series and embedded in paraffin. Sections of 4.5 µm thickness were cut on a Leica RM2255 microtome and stained with either hematoxylin and eosin (HE) or a combined Periodic acid-Schiff/

Alcian blue (PAS/AB) staining. Epithelial thickness was measured in HE-stained cross-sections using ImageJ. A total of 18 measurements, six points in three images per tissue construct, and time-point were made (n = 4). To compare the epithelium grown in PDC + HCEC and PDC + HCEx with a normal conjunctival epithelium, human conjunctiva from healthy donors were processed simultaneously as described above.

Immunohistological analysis was performed on day 7, 14, and 24. Following deparaffinization and rehydration of sections, antigen retrieval was accomplished in sodium citrate buffer, pH 6.0, in a steamer for 20 min. Nonspecific binding sites were blocked using 5% donkey serum (Sigma-Aldrich) in PBS or 5% donkey serum combined with 0.25% bovine serum albumin (VWR, Radnor, USA) in PBS for 45 or 60 min at room temperature. Slides were incubated overnight at 4 °C in primary antibody solution (listed in Supplementary Table 2). Next, the samples were washed in PBS and incubated with a secondary antibody solution of Alexa Fluor 594 donkey anti-mouse (Jackson ImmunoResearch Biotechnology Co, West Grave, PA, USA), dilution 1:500, or Alexa Fluor 488 donkey anti-mouse (Jackson ImmunoResearch), dilution 1:500 in PBS. The slides were washed with PBS and mounted with Mowiol (Sigma-Aldrich), containing 4′,6-diamidino-2-phenylindole (DAPI, Merck, Darmstadt, Germany). Images were viewed and taken with a Leica DM 4000 B microscope (Leica Microsystems, Wetzlar, Germany). Stained cells were counted by one individual, who was blinded to the identity of the group. The percentage of cells stained positively in relation to the total number of cells with DAPI-stained nuclei in 10×high power fields (HPF, 400 × magnification) was calculated per donor (n = 4).

2.4. Evaluation of engrafted PDC + HCEx constructs in vivo

PDC + HCEx yielded a more stratified epithelium and expressed a higher concentration of putative progenitor cell markers than PDC + HCEC. Moreover, a 14-day cultivation period was found to be optimal with regard to the ratio of epithelial thickness and goblet cell differentiation to the preservation of the progenitor cell properties. Consequently, 14-day-incubated PDC + HCEx were used for all further in vivo experiments. Conjunctival explant pieces of PDC + HCEx were left in place and were not removed before the in vivo experiment.

All animal experiments were conducted in accordance with the ARVO statement for the use of animals in ophthalmic research. The experimental animals comprised 12 female New Zealand white rabbits (Charles River Laboratories, Wilmington, DE, USA)—6 for engrafted PDC + HCEx and 6 for engrafted PDC (non-epithelialized as a control)—weighing ~2.5 kg each. Rabbits were immunosuppressed with daily intramuscular injections of 10 mg/kg/day cyclosporine A (Sandimmun, Novartis Pharmaceuticals, East Hanover, NJ, USA) for 3 days pre-operatively to 10 days post-operatively. This dosage has been reported to effectively prevent graft rejection in rabbits [47,48]. The rabbits were anaesthetized with 5 mg/kg ketamine (Ketavet, Zoetis, Parsippany, NJ, USA) in combination with 5 mg/kg xylazin (Rompun, Bayer Animal Health, Leverkusen, Germany). Oxybuprocain-containing eye drops (Conjuncain EDO, Bausch-Lomb, Rochester, NY, USA) were applied topically for pain prevention.

Prior to transplantation, the formation of an intact epithelium on PDC + HCEx was confirmed by vital staining with lissamine green. A single lissamine green strip (1.5 mg lissamine green per strip, Lissamine Green Ophthalmic Strips, Optitech, Allahabad, India) was incubated for 30 s in 500 µL 0.9% sodium chloride, after which 250 µL of the solution was added to PDC + HCEx or PDC. After another 30 s, the implants were carefully washed with 1 ml 0.9% sodium chloride. Positive lissamine green staining indicated an absent or damaged epithelium as lissamine green stains collagenous stroma and non-intact, membrane-damaged epithelial cells, but it does not stain healthy, in vitro cultivated, or in vivo grown epithelia [49]. Using a 5-mm diameter trephine, a defect was created in the upper temporal bulbar conjunctiva of the rabbit's right eye, after which the conjunctiva—including Tenon's capsule—was removed. PDC + HCEx or PDC with 5-mm diameter was placed into the defect and fixed with eight single 9–0 vicryl sutures.

For analgesia, 4 mg/kg/day carprofen (Rimadyl, Pfizer, New York, NY, USA) was administered subcutaneously for 3 days. Steroidal (1% prednisolone, Predni-POS®, Ursapharm, Saarbrücken, Germany) and antibiotic (3 mg/ml ofloxacin, Floxal EDO®, Bausch & Lomb, Rochester, NY, USA) eye drops were applied topically twice a day for one week. Rabbits were anaesthetized on post-operative days 3 and 10 and the eyes were examined with an operating microscope (Ophtamic 900 S®, Moeller-Wedel, Wedel, Germany). Conjunctivas were stained with lissamine green to evaluate the epithelium as described above. On day 10 postoperatively, rabbits were anaesthetized as described above and euthanized with an intracardiac injection of 6 ml sodium pentobarbital (Narcoren, Boehringer Ingelheim, Germany).

2.5. Histological and immunohistological analysis of in vivo engrafted PDC + HCEx

All orbital tissues were exenterated including whole rabbit eyes and eyelids and were fixed in 4% paraformaldehyde (Roti Histofix, Roth, Karlsruhe, Germany) for 6 h. After removal of the vitreous body and lens, the remaining tissue was fixed for an additional 24 h. Following dehydration through a graded ethanol series and embedding in paraffin, 4.5 µm thick sections were cut. The sections were then deparaffinized, hydrated, and stained with (i) HE for histological and morphological examination or (ii) PAS/AB for the detection of mucin. Measurements of the epithelial thickness and immunohistochemistry was performed as described above. Endogenous immunoglobulin was blocked using IgG Fab Fragments (AffiniPure Fab Fragment Donkey Anti-Rabbit IgG, Jackson ImmunoResearch). For detection of engrafted human epithelial cells, a human-specific, anti-mitochondria antibody (Supplementary Table 1) in 2% donkey serum was used.

2.6. Statistical analyses

Statistical analysis was performed by using analysis of variance (ANOVA) and Tukey's multiple correction testing (Prism 6.0, GraphPad, La Jolla, CA, USA). All values are expressed as mean ± SEM. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Epithelialization, stratification and goblet cell formation

3.1.1. PDC + HCEC versus PDC + HCEx (in vitro)

Within 7 days, both PDC + HCEC and PDC + HCEx formed a confluent, mono- or bi-layered epithelium with thicknesses of 4.29 ± 0.66 µm and 8.09 ± 0.64 µm, respectively (Fig. 2 A and B). From day 7 onward, the epithelial thickness of PDC + HCEC increased steadily until day 24, but in comparison, the epithelial thickness of PDC + HCEx increased more rapidly. PDC + HCEx reached a thickness of 23.38 ± 3.54 µm on day 14, with no further significant change until day 24 ($p = 0.89$), whereas PDC + HCEC only reached 15.15 ± 0.44 µm. At day 10, the difference in epithelial thickness of PDC + HCEx compared to PDC + HCEC was significant: 16.15 ± 3.69 versus 7.22 ± 0.12 µm ($p = 0.013$). From day 14 onward, the differences in epithelial thickness between both groups were highly significant (all p-values <0.0001). PDC + HCEx achieved an epithelial thickness comparable to that of a healthy human conjunctiva within 21 days (27.64 ± 1.70 µm versus 33.21 ± 0.57 µm, $p = 0.299$), whereas PDC + HCEC reached only about half the thickness (15.15 ± 0.44 µm) within the entire cultivation period. With regard to epithelial organization, both culture methods produced a stratified epithelium after 14 days. However, the epithelium of PDC + HCEx exhibited superior organization and stratification with cuboidal basal cells, flattened superficial cells, and a healthy intermediate wing cell layer compared to that of PDC + HCEC.

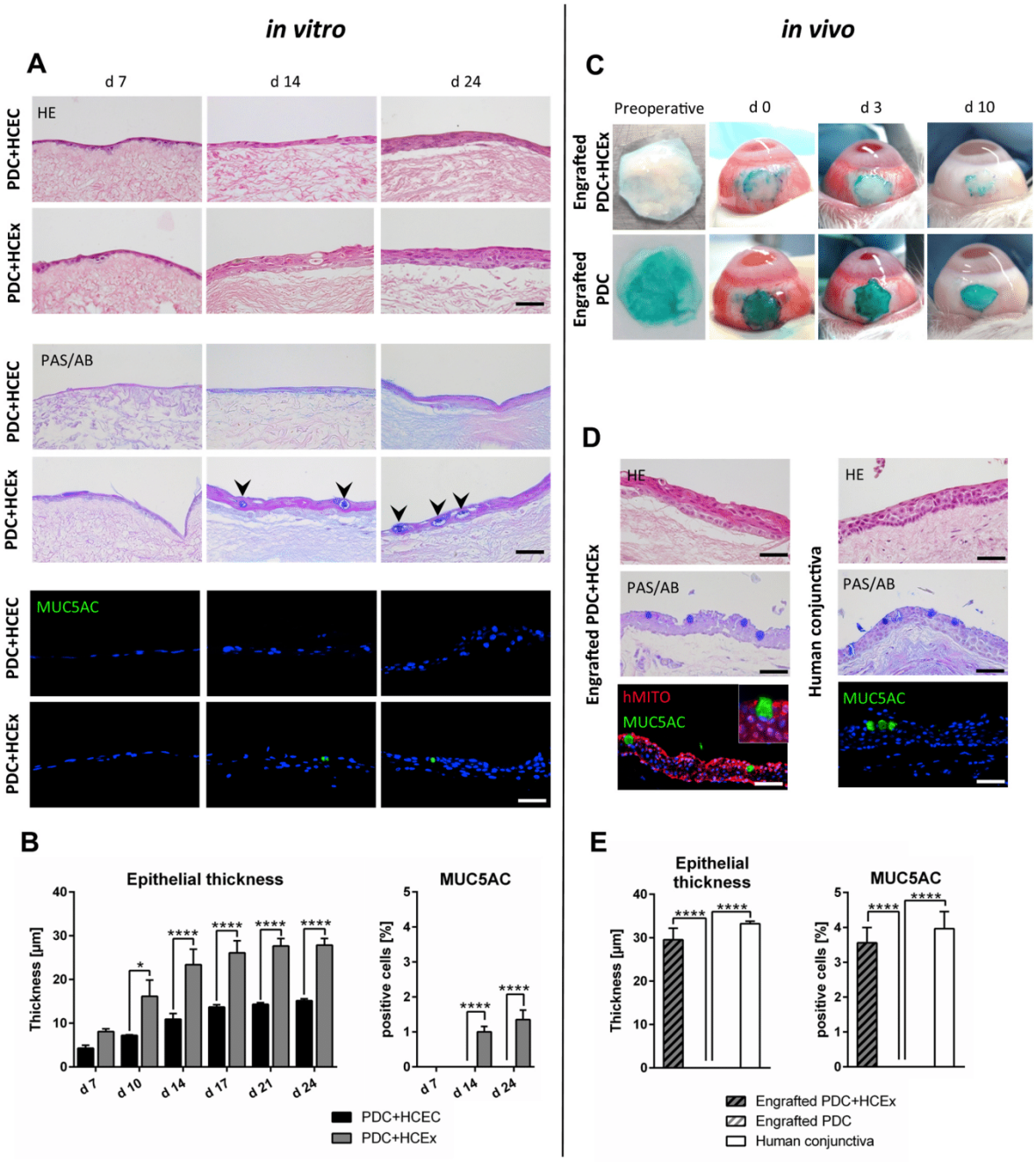

Fig. 2. Epithelialization, stratification and goblet cell formation of PDC + HCEC, PDC + HCEx and engrafted PDC + HCEx. **A**: HE-, PAS/AB- and *anti*-MUC5AC-stained cross-sections of PDC + HCEC and PDC + HCEx (in vitro). PDC + HCEx yielded a thicker and more organized epithelium from day 10 onward than PDC + HCEC. PAS/AB-positive cells (arrowheads) and MUC5AC-positive cells (green) were detected in the outgrown epithelium in PDC + HCEx from day 14 onward but not in PDC + HCEC. **B:** Corresponding epithelial thicknesses and percentages of MUC5AC-positive cells of PDC + HCEC versus PDC + HCEx. **C**: Lissamine green stained engrafted PDC + HCEx and engrafted PDC (in vivo). Engrafted PDC + HCEx showed no lissamine green staining immediately before the surgery, indicating an intact epithelium. After the transplantation (d 0) and after 3 days postoperatively, the graft showed no lissamine staining, except for a slight staining at the graft edges. However, this continued to decrease until day 10. Engrafted PDC without cells (control) showed a strong green staining before and after the surgery, which was still detectable after 3 and 10 days postoperatively, indicating a lack of epithelialization. **D**: HE-, PAS/AB- and *anti*-MUC5AC-stained cross-sections of engrafted PDC + HCEx and human conjunctiva. Engrafted PDC + HCEx showed a well-organized epithelium with interspersed PAS/AB-positive goblet cells with a similar thickness and number of MUC5AC-filled cells (green) compared to human conjunctiva. Inset: Higher magnification of a MUC5AC-positive goblet cell. All engrafted PDC + HCEx showed a positive staining for the human-specific anti-mitochondria antibody (hMITO, red), confirming the human origin of the cells. No MUC5AC-positive cells of human origin were detected in the engrafted PDC control group because no epithelium was present on the engrafted PDC. **E:** Corresponding epithelial thicknesses and percentages of MUC5AC-positive cells of engrafted PDC + HCEx, engrafted PDC and human conjunctiva. N = 4, *p ≤ 0.05, ****p ≤ 0.0001. All microscopic pictures were captured at 400 × magnification with the scale bar representing 50 µm.

No goblet cell-like cells could be discerned at any time with PAS/AB-staining in PDC + HCEC. In contrast, the outgrown epithelium of PDC + HCEx showed a few, goblet cell-like cells from day 14 onward, which, although they increased in number up to day 24, did not appear to be completely mucin-filled (Fig. 2A, arrowheads). To check whether these cells could produce any soluble mucin, immunohistochemical staining for MUC5AC was performed. PDC + HCEx revealed, analogous to the PAS/AB staining, a few small MUC5AC-positive cells on day 14 (1.00 ± 0.15%) and day 24 (1.35 ± 0.27%). In contrast, no MUC5AC-positive cells were detectable at any time in PDC + HCEC.

The cells on the conjunctival explant piece itself, that have been placed on PDC in the PDC + HCEx group before culturing, showed different MUC5AC-expressions than those of the outgrown epithelium. The number of positive cells on the explant piece itself was counted separately to those of the outgrown epithelium and are not included in the data or the shown figures. However, this data is included in the supplementary Table 3. On the explant piece itself, some MUC5AC-positive cells could still be detected at the beginning of the cultivation (3.61 ± 0.52%, day 7), but the number of positive cells reduced during the cultivation period to 0.67 ± 0.15% on day 24.

3.1.2. Engrafted PDC + HCEx (in vivo)

Compared to PDC + HCEC, PDC + HCEx resulted in a significantly thicker and better-organized epithelium with cellular properties comparable to human conjunctiva. Since the optimal cultivation period with regard to goblet cell formation, epithelial thickness and expression of progenitor cell markers was 14 days, the 14-day incubated PDC + HCEx was used for all further in vivo experiments.

Epithelialization of PDC + HCEx was verified by lissamine green staining before and after grafting into a conjunctival defect in a rabbit (Fig. 2C). Immediately after suturing, the engrafted PDC + HCEx stained only at the graft edges, where the graft had been grasped with forceps during surgery. The other area of the engrafted PDC + HCEx displayed no lissamine green staining, indicating an intact epithelium of the graft. This lack of lissamine green staining was also observed on follow-up days 3 and 10, while the staining of the graft edges was reduced by day 10. In contrast to these findings, the PDC control grafts demonstrated strong lissamine green staining of the entire transplant before and after transplantation. Although the defect area decreased slightly as a result of wound contraction, the engrafted PDC control grafts still displayed this green staining on days 3 and 10 postoperatively.

Histologically, the engrafted PDC + HCEx still revealed an epithelium with a high degrees of stratification and organization in basal, intermediate and superficial epithelial cells after 10 days (Fig. 2D and E). The epithelium was 29.54 ± 2.66 μm in thickness—not significantly different from that of a healthy human conjunctiva ($p = 0.49$). A human-specific antibody (hMITO) verified the human origin of cells in the engrafted PDC + HCEx group after transplantation (Fig. 2D, inset). The conjunctival cells in the untreated fornix region (rabbit cells) did not demonstrate reactivity (not shown). The edges of the engrafted PDC control grafts showed signs of re-epithelialization with rabbit cells, but no epithelium could be demonstrated histologically in the center of the grafts (Supplementary Table 3).

With a number of 3.56 ± 0.44% MUC5AC-positive cells, the engrafted PDC + HCEx showed no significant difference to healthy human conjunctiva ($p = 0.77$). Interestingly, the engrafted PDC + HCEx revealed a higher number of MUC5AC-positive cells compared to the solely in vitro cultivated PDC + HCEx (engrafted PDC + HCEx: 3.56 ± 0.44% versus PDC + HCEx, d 24: 1.35 ± 0.27%, $p = 0.0007$). Moreover, the goblet cells of the engrafted PDC + HCEx also contained more mucin and were more voluminous than those in the in vitro cultivated PDC + HCEx. No MUC5AC-positive cells of human origin were detected in the engrafted PDC control group because no epithelium was present on the engrafted PDC.

3.2. Apoptotic and proliferative active conjunctival epithelia cells

All slides were counterstained with CK19. Epithelial cells grown in PDC + HCEC and PDC + HCEx uniformly expressed CK19 at all measurement times in vitro, confirming their conjunctival origin (Fig. 3). CK19 was also expressed by epithelial cells on the engrafted PDC + HCEx after 10 days in vivo. Staining for corneal epithelial cell-specific CK12 was negative for all cells in vitro and in vivo (not shown).

3.2.1. PDC + HCEC versus PDC + HCEx (in vitro)

Both cultivation methods showed, with an average range of 0.6–5.4% caspase-3-positive cells, a relatively low percentage of apoptotic cells. However, the outgrown epithelium of PDC + HCEx showed less apoptotic cells during the cultivation period than that of PDC + HCEC (Fig. 3A and B). Especially at the beginning and at the end of the cultivation period, PDC + HCEC showed a significantly elevated number of caspase-3-positive cells compared to PDC + HCEx: On day 7, 5.42 ± 0.90% of epithelial cells in PDC + HCEC were caspase 3 positive, compared to 1.41 ± 0.27% of cells in PDC + HCEx. Compared to the outgrown epithelium in PDC + HCEx, an increased number of apoptotic cells was observed on the explant piece itself (Supplementary Table 3).

Staining of Ki67, on the other hand, showed that PDC + HCEx contained a significantly increased number of proliferative active cells compared to PDC + HCEC (Fig. 3A and B), especially in early phase of cultivation. After 7 days, PDC + HCEx showed a more than 14-fold higher rate of proliferative cells compared to PDC + HCEC, with percentages of 67.05 ± 5.26 and 4.68 ± 0.79, respectively. However, this high proliferation rate of PDC + HCEx decreased to 8.02 ± 0.69% by day 14 and to 2.09 ± 0.35% by day 24. In the PDC + HCEC group, the number also decreased to a very low percentage of 0.27 ± 0.15 b y day 14 and 0.21 ± 0.12 b y day 24.

3.2.2. Engrafted PDC + HCEx (in vivo)

The percentage of apoptotic cells in the engrafted PDC + HCEx after 10 days in vivo was 4.11 ± 0.32, slightly higher than in a healthy conjunctiva (0.58 ± 0.11%, Fig. 3C and D). A slightly increased number of apoptotic cells in the engrafted PDC + HCEx was also observed compared to the preoperative, 14-days in vitro cultured PDC + HCEx (0.62 ± 0.14%, $p < 0.0001$). Ki67-positive epithelial cells were found exclusively in the basal cell layer at a density of 2.7 ± 0.60%. The mean percent of Ki67-positive cells in healthy human conjunctiva was 5.67 ± 0.49% ($p = 0.0002$). No Caspase-3- or Ki67-positive cells of human origin were detected in the engrafted PDC control group.

3.3. Conjunctival epithelial progenitor cell properties: CK15, ΔNp63, and ABCG2

3.3.1. PDC + HCEC versus PDC + HCEx (in vitro)

At all points of time, the outgrown epithelium of PDC + HCEx showed a significant higher percentage of progenitor cell marker-expressing epithelial cells than that of PDC + HCEC. The percentage of CK15-positive cells was 14.84 ± 1.54, 25.88 ± 2.39, and 27.82 ± 2.42 in PDC + HCEC versus 33.04 ± 3.11, 41.11 ± 3.21, and 40.38 ± 2.12 in PDC + HCEx on day 7, 14, and 24, respectively (Fig. 4 A and B). Furthermore, the epithelium in PDC + HCEx demonstrated more specific staining of the basal cell layers on days 14 and 24 compared to those in PDC + HCEC, where the expression of CK15 was also noted in both intermediate and surface epithelial layers. The percentage of ΔNp63-positive cells in PDC + HCEC was about half as high as in PDC + HCEx on days 7 ($p = 0.0068$), 14 ($p < 0.0001$) and 24 ($p < 0.0001$). From day 7 to day 14, the number of positive cells in both PDC + HCEC and PDC + HCEx increased to 20.02 ± 1.66% and 37.70 ± 2.22%, respectively. After 24 days, the numbers of ΔNp63-positive cells were equal to those of day 14 in both groups ($p > 0.6$). The number of ABCG2-positive cells was also significantly lower in PDC + HCEC compared to PDC + HCEx: 18.67 ± 2.33, 37.60 ± 3.39, and 36.19 ± 3.15% versus 44.10 ± 3.28,

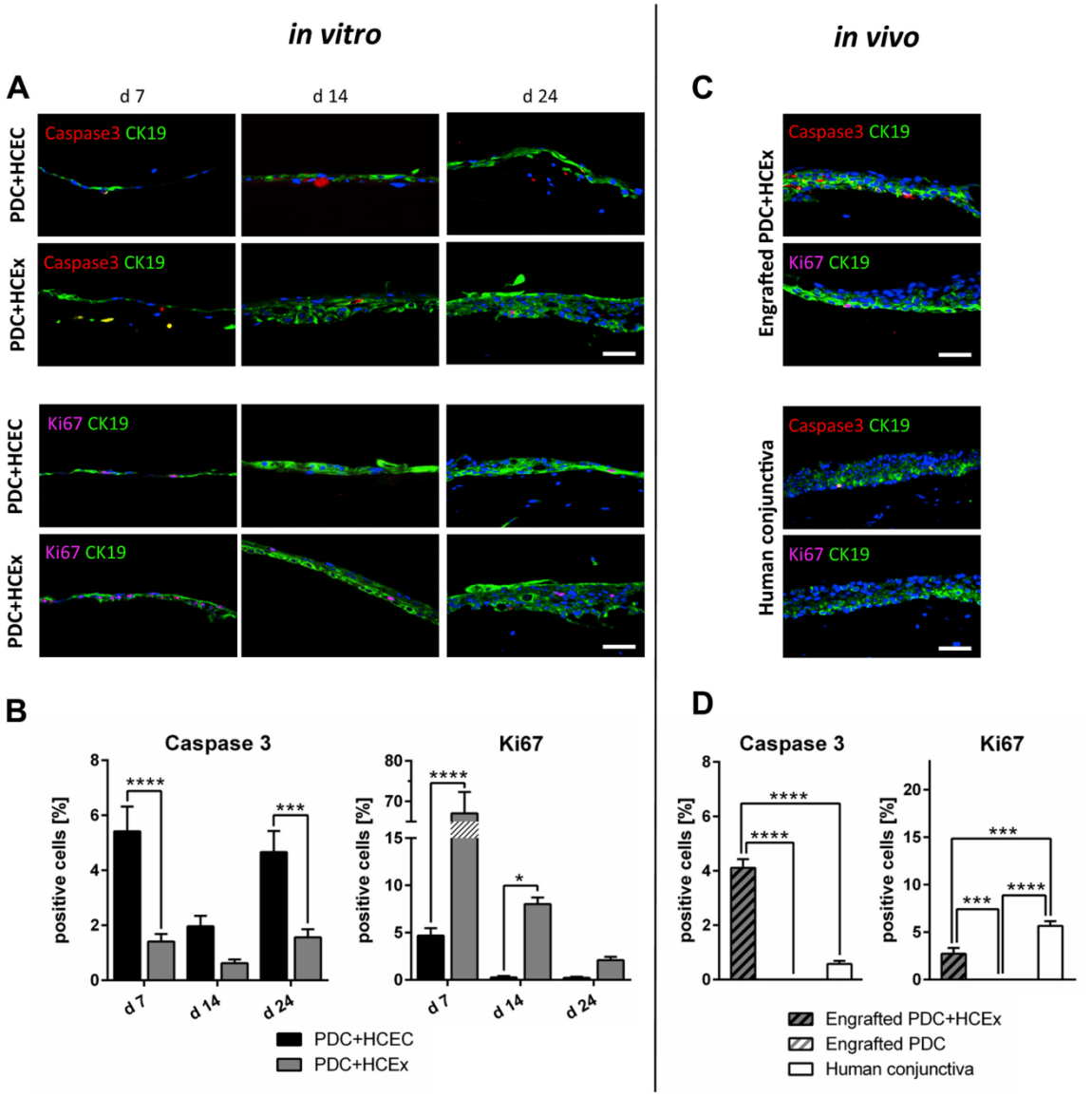

Fig. 3. Apoptosis and proliferation of PDC + HCEC, PDC + HCEx and engrafted PDC + HCEx. **A:** Caspase 3 (red) and Ki67 (magenta) expression in immunohistochemically stained cross sections of PDC + HCEC and PDC + HCEx (in vitro). The outgrown epithelium of PDC + HCEx showed a smaller number of apoptotic cells on days 7, 14 and 24 and a greater number of proliferative active cells on days 7 and 14 than PDC + HCEC. **B:** Corresponding percentages of Caspase 3- and Ki67-positive cells of PDC + HCEC versus PDC + HCEx. **C:** Caspase 3 (red) and Ki67 (magenta) expression in immunohistochemically stained cross sections of engrafted PDC + HCEx (in vivo) and human conjunctiva. Engrafted PDC + HCEx showed a greater number of apoptotic cells and a slightly smaller number of proliferative cells compared to healthy human conjunctiva. The cells on engrafted PDC + HCEx were verified for their human origin as shown in Fig. 2. All sections were counterstained with the conjunctival epithelial cell marker CK 19 (green). No Caspase 3- and Ki67-positive cells of human origin were detected in the engrafted PDC control group because no epithelium was present on the engrafted PDC. **D:**Corresponding percentages of Caspase 3- and Ki67-positive cells of engrafted PDC + HCEx, engrafted PDC and human conjunctiva. N = 4, *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001. All microscopic pictures were captured at 400 × magnification with the scale bar representing 50 μm.

54.49 ± 2.78, and 50.70 ± 2.22%, on days 7, 14, and 24, respectively.

3.3.2. *Engrafted PDC + HCEx (in vivo)*

Expressions of CK15, ABCG2, and ΔNp63 in engrafted PDC + HCEx (Fig. 4C and D) did not differ significantly from either in vitro cultured PDC + HCEx or human conjunctiva (all $p > 0.08$). ABCG2-and ΔNp63-positive cells were found predominantly in the basal cell layer of engrafted PDC + HCEx as well as of human conjunctiva. In contrast, CK15 was expressed in both the basal and intermediate layers. No CK15-, ABCG2-, or ΔNp63-positive cells of human origin were detected in the engrafted PDC control group.

4. Discussion

Many ocular disorders and injuries cause conjunctival scarring and loss of conjunctival cells. Suitable substitute matrices for conjunctival reconstruction must be endowed with inherent stability, elasticity, surgical manipulability, and capability for assimilation into the recipient

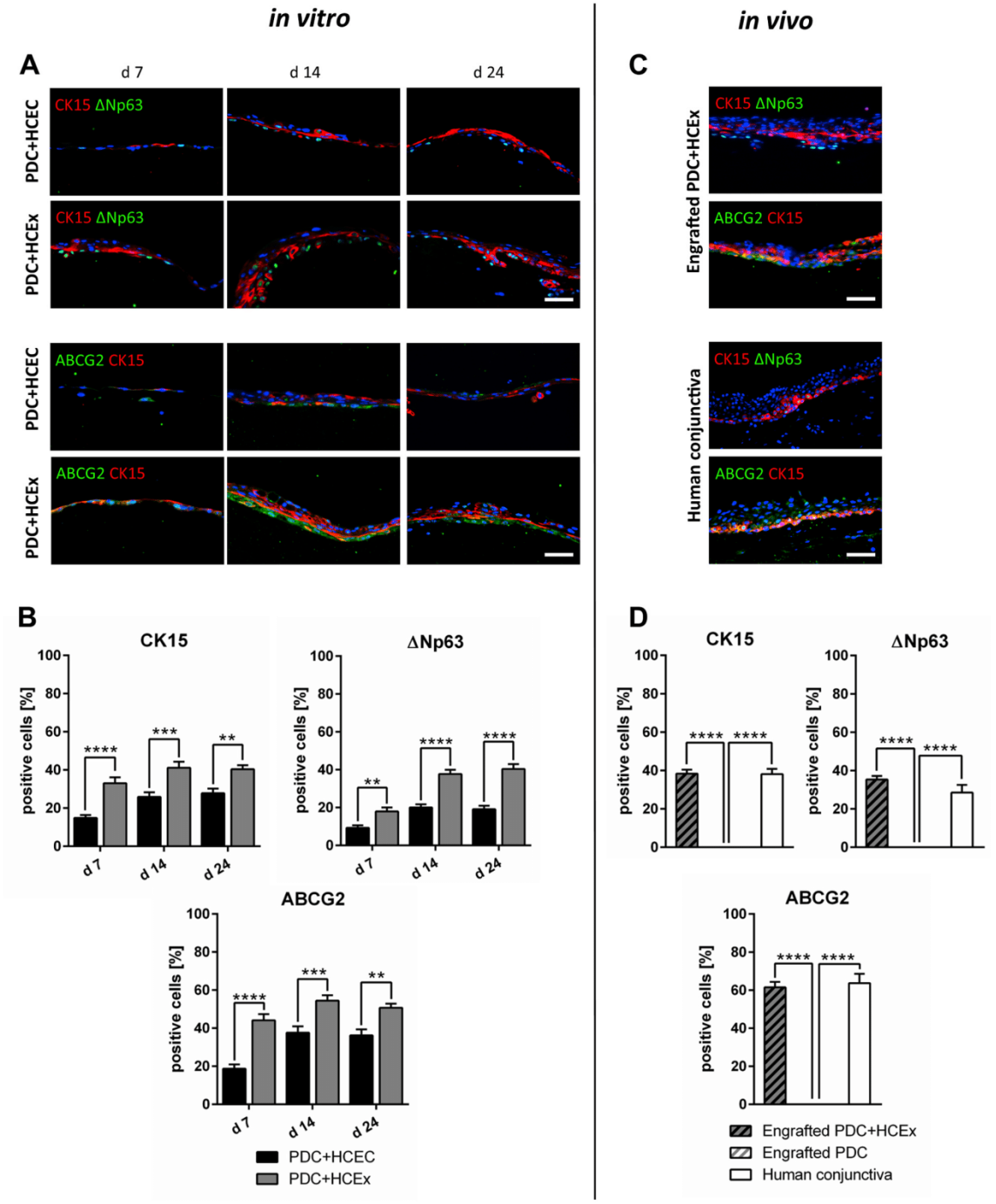

Fig. 4. Conjunctival progenitor cell properties of PDC + HCEC, PDC + HCEx and engrafted PDC + HCEx. A: CK15 (red), ΔNp63 (green), and ABCG2 (green) expression in immunohistochemically stained cross sections of PDC + HCEC and PDC + HCEx (in vitro). The outgrown epithelium of PDC + HCEx showed a more specific progenitor cell marker expression to the basal and intermediate cells and significantly more positive cells than in PDC + HCEC at all points of time. B: Corresponding percentages of CK15-, ΔNp63-and ABCG2-positive cells of PDC + HCEC versus PDC + HCEx. C: CK15 (red), ΔNp63 (green) and ABCG2 (green) expression in immunohistochemically stained cross sections of engrafted PDC + HCEx (in vivo) and human conjunctiva. Engrafted PDC + HCEx showed a high preservation of the progenitor cell markers with a comparable number and expression pattern to human conjunctiva. The cells on engrafted PDC + HCEx were verified for their human origin as shown in Fig. 2. No CK15-, ΔNp63-, and ABCG2-positive cells of human origin were detected in the engrafted PDC control group because no epithelium was present on the engrafted PDC. D: Corresponding percentages of CK15-, ΔNp63-, and ABCG2-positive cells of engrafted PDC + HCEx, engrafted PDC and human conjunctiva. N = 4, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. All microscopic pictures were captured at 400 × magnification with the scale bar representing 50 μm.

ocular surface tissue without eliciting any inflammatory reaction or pathological scarring [3]. Although several of the existing substitutes, such as autologous conjunctiva or oral mucous membranes, have demonstrated encouraging results in clinical studies [8,11,12], their more comprehensive use is limited for a variety of reasons—absence of goblet cells, cosmesis, lack of availability of unaffected conjunctiva in autoimmune diseases, and/or bilateral damage [4,7,15,17].

While numerous studies have shown AM (or denuded AM) to be a good carrier for corneal epithelial cells, its utility as a carrier for conjunctival epithelial cells has been less addressed. Meller et al. [50] reported that devitalized AM epithelium impairs conjunctival epithelial cell growth and adhesion. Regardless of whether AM with or without epithelium is used, several studies have shown that HCEC forms a multilayered, goblet cell-containing epithelium on AM [50,51]. However, there is sparse data on goblet cell density on cultivated AM [7,14,38], with no reports on the percentages of cell numbers.

Based on these limitations, many recent efforts to develop alternative sources for conjunctival reconstruction have been undertaken. However, the newly developed tissue-engineered substitutes also have some major inherent drawbacks, including inferior biocompatibility, lack of surgical manageability, and/or poor mechanical strength in vivo [2,4,52,53]. A promising approach is that of using a decellularized extracellular matrix as scaffolds. Our group recently demonstrated that the tissue properties of PDC, in comparison to those of AM, are not only equivalent to those of human decellularized conjunctiva but also display high elasticity, exceptional stability, and capability for integration into the recipient conjunctiva [39].

The pig is increasingly being used as a xenogeneic tissue source. Not only is porcine tissue readily and quickly available, but its extracellular matrix proteins also share a common sequence homology with that of humans, thereby allowing successful transplantation of decellularized porcine tissue. Decellularized porcine scaffolds are often used in animal studies and for clinical applications in humans such as the heart valve, intestine, and dermis [54–56]. The technique for the decellularization of porcine tissue has already been established for ocular structures—the cornea [57,58] and the lacrimal gland [32].

In this study, we analyzed and compared conjunctival epithelium formation and stratification, goblet cell formation, and epithelial progenitor cell behavior between two methods for culturing conjunctival epithelial cells. Since both the proliferative capacity and preservation of conjunctival epithelial progenitor cells are restricted in the absence of any matrix or fibroblast support on most substrates in vitro [5,7,46], many studies expand HCECs by co-culturing them with feeder cells prior to their cultivation on a presumptive tissue substitute [4,43–45]. Hence, we investigated a cultivation method (PDC + HCEC) with prior expansion of HCEC on 3T3 mouse fibroblasts following seeding of the outgrown HCEC on PDC. For clinical applications, however, the epithelial progenitor cell population needs preservation during expansion, with strict avoidance of xenobiotic components. Consequently, we further investigated a direct cultivation method in which a small human conjunctival explant piece was cultivated onto PDC (PDC + HCEx), with no prior expansion on xenogeneic feeder cells. The main advantage of this method is that it requires only an exceedingly small piece (about 1 mm) of healthy conjunctiva and not, as in autologous conjunctival transplantation, a piece that is precisely the size of the defect, which must be covered for protection after transplantation. Using re-epithelialized PDC, it is, therefore, possible to overlay a larger defect area, while requiring only a minimal incision into the patient's healthy conjunctiva. The removal of a small-sized segment, as used in our cultivation method, can be considered unproblematic in terms of scarring at the donor site.

PDC + HCEx formed a stratified epithelium more quickly and with significantly higher proliferative activity during the first week of cultivation compared to PDC + HCEC. Using the direct cultivation method (PDC + HCEx), epithelial thicknesses on the PDC reached a thickness similar to that of normal human bulbar conjunctiva after 21 days. Moreover, the outgrown epithelium demonstrated superior stratification and organization, with low caspase 3 activity. There were more epithelial cells expressing CK15, ΔNp63, and ABCG2, in PDC + HCEx indicating greater preservation of conjunctival progenitor cells (including putative stem cells). It has to be mentioned that the markers used in this study—CK15, ABCG2 and ΔNp63—are not specific to conjunctival stem cells. As all of them can detect putative stem cells and transient amplifying cells, they may, therefore, also be regarded as being progenitor cell markers. So far, a specific conjunctival stem cell marker has remained elusive. However, CK15, a minor cytoskeletal component, is expressed in conjunctival basal cells and has been suggested to be a putative conjunctival stem cell marker. Highly differentiated progenitor cells may also express CK15 [6]. ABCG2, a transmembrane transporter, is present in many adult stem cells and has been found in those human conjunctival basal epithelial cells that have epithelial stem cell features, such as clonogenic capacity, slow cycling, and resistance to phorbol-induced differentiation [59,60]. Similar to limbal epithelial stem cells, conjunctival epithelial cells possess a stem cell-rich side population (when sorted after incubation with Hoechst dye), which is attributed to the ABCG2 transporter function. This is believed to be a mechanism by which long-lived stem cells are able to diminish the potential for genomic damage over their lifespan [61]. The transcription factor p63 is expressed by limbal stem cells and transient amplifying cells [62,63]. Among the various existing p63 isoforms, the ΔNp63α isoform is the most prevalent in those epithelial cells that have stem cell-like phenotypes [64]. Our results regarding the ΔNp63 expression were similar to those of Tanioka el al., who cultivated HCEC on AM for 14–20 days and observed increased expressions of ABCG2 and p63 in basal cells. However, that group did not quantify positively stained cells [60]. Other studies of tissue-engineered conjunctival equivalents reported similar expression patterns, but neither did they quantify the number of cells nor the expressions of ABCG2 and p63 [23,65].

Goblet cell formation and density are of particular importance when developing conjunctival tissue substitutes, because goblet cells maintain the stability of the tear film mucin — this is essential for the lubrication and integrity of the ocular surface [66]. Following ocular injury and subsequent spontaneous re-epithelialization, goblet cell density in the healing epithelium often gets reduced, resulting in a reduction of the tear film mucin [21,22,27]. In the present study, mucin-filled goblet cells were detected in vitro with in PDC + HCEx from day 14 onward. With PDC + HCEC, no goblet cells were detected at any time point. One explanation for this might be conservation of the cell niche through preservation of the explant piece — i.e. fibroblasts in the explant piece can support epithelial growth through the secretion of growth factors and cytokines. As a result, the putative stem cells are less manipulated and not detached from their cell niche, but can grow directly from their original niche into a new, similar niche – namely PDC. Yet another explanation can be attributed to the fact that the cultivation time of PDC + HCEC was too short to allow any differentiation in goblet cells. This reasoning is based on the observation that the stratification of the epithelium was considerably slower in PDC + HCEC than in PDC + HCEx. Furthermore, the HCEC were pre-cultured in the PDC + HCEC group and passaged once before being seeded onto PDC. It has been reported that passaging of primary conjunctival epithelial cells leads to a reduction in the colony-forming efficacy [46]. Kasbekar et al. noted that conjunctival explants were superior to previously expanded HCEC on human decellularized conjunctiva in vitro [65]. This group detected CK7-positive cells after 28 days of cultivation on human decellularized conjunctiva, thereby suggesting the presence of immature goblet cells. In that study, mature MUC5AC-positive cells were sparse and not quantifiable. Till date, no techniques have been described for culturing HCEC on decellularized porcine tissue.

The epithelium grown in the PDC + HCEx group exhibited superior stratification compared to that grown in the PDC + HCEC group at any time point. Since 14-day PDC + HCEx conjunctival epithelial cells revealed the highest expression of putative progenitor cell markers, we

chose this method and time point for the in vivo application. The engrafted PDC + HCEx showed an intact epithelium over the entire in vivo evaluation period, whereas the PDC control graft showed virtually no epithelialization. Human cells that survived on the engrafted PDC + HCEx after transplantation formed an organized stratified epithelium, which had slightly more apoptotic cells than the surrounding conjunctiva. This is perhaps owing to an immune response of the rabbit to the xenogeneic human cells, despite prior treatment with cyclosporine A. In fact, immune suppression, itself, might also account for the delayed wound closure in the control group. Our previous study using PDC in rabbits without immune suppression revealed that the wound closure was complete by day 10 [39]. In the clinical context, however, a systemic immunosuppression would not be necessary since, as already shown in our previous study [39], PDC is not expected to cause an immune response after transplantation because of the lack of immunogeneic factors. In the animal model used here, immunosuppressive treatment had to be administered because of the use of human conjunctival cells on PDC. In a clinical application, a small biopsy of the patient's autologous conjunctiva would be used for re-epithelialization—systemic immunosuppression would therefore be redundant.

We detected proliferating cells in the engrafted PDC + HCEx, albeit to a lesser extent than in the healthy human conjunctiva. Notably, progenitor cell properties were preserved even after 10 days and were similar to those of human conjunctiva. Thus, PDC + HCEx seems able to maintain cells that are not only replete with their progenitor properties, but also have the potential to survive and proliferate after transplantation.

Interestingly, there was a significantly greater number of completely mucin-filled goblet cells after 14 days in vitro and the subsequent 10 days post implantation in vivo (engrafted PDC + HCEx) than after 24 days of in vitro cultivation (PDC + HCEx, day 24) alone. Such an increase might be due to in vivo maturation, which can only be emulated partially in vitro. Meller et al. cultured conjunctival epithelium on AM for 14 days, after which it was implanted subcutaneously into mice for 11 more days in vivo [51]. After this in vivo period, the resulting conjunctival epithelium revealed PAS/AB- and MUC5AC-positive cells. Thus, it is likely that the in vivo environment promotes goblet cell maturation, which is necessary for effective mucin production. In patients with larger conjunctival defects resulting in impairment of the tear film layer, transplanted goblet cells appear to be valuable for their presumptive contribution to the tear film mucin.

5. Conclusion

We report the development of a re-epithelialized conjunctival substitute on a base of PDC. When human conjunctival explants were cultivated by placing them directly onto PDC, the developing conjunctival epithelial cells formed a confluent, stratified, and organized epithelium, complete with mucin-containing goblet cells and a significant number of progenitor cells, all within 14 days in vitro. The epithelia cells were still detectable after being transplanted into a conjunctival defect in an animal (rabbit) model, and maintained both organization and progenitor cell properties even after an additional 10 days in vivo. The number of goblet cells on PDC increased in vivo to a number that is comparable to those in the healthy human conjunctiva.

PDC, which is easily obtainable, closely approximates the three-dimensional native tissue-microenvironment of the human conjunctiva, whose matrix can hardly be replicated synthetically. Our results suggest that PDC is a suitable cell carrier for autologous human conjunctival epithelium, as it allows the preservation of self-renewal and goblet cell formation, even after transplantation. Therefore, the re-epithelialized PDC offers a promising option for patients with extensive conjunctival damage, including conjunctival stem cell and goblet cell deficiency.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jtos.2020.08.009.

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Chapter 3

3 Results and General Discussion

The conjunctiva is an essential functional part of the anterior eye segment as it contributes to the normal function of the tear film and to the homeostasis of the entire ocular surface. Surgical reconstruction of the conjunctiva and the fornices is frequently required in different pathologies, such as after extensive thermal or chemical burns, after radiation therapy or after surgically induced conjunctival damage caused by e.g. pterygium/tumour surgery or filtrating glaucoma surgery [128-132]. A surgical procedure usually includes the excision of scar tissue with subsequent application of an appropriate substitute tissue. Currently clinically applied substitutes include autologous conjunctiva, nasal and buccal mucosa and allografts such as the AM. However, the choice of conjunctival substitutes for surgical repair is limited and none of the currently used substitutes if free of limitations [66, 67]. These limitations include insufficient tissue availability, intra- and inter-donor-specific variations in quality and thickness and – especially important for a conjunctival substitute – the lack of goblet cells [36, 85].

Based on these limitations, many recent efforts to develop alternative sources for conjunctival reconstruction that mimic the physiological cell niche have been undertaken. The most encouraging approaches have been done in the field of Tissue Engineering using specific scaffolds and cell sources. The proposed scaffolds can be divided into two main types: biomaterials and synthetic materials. Both have distinct advantages and disadvantages. Synthetic materials can be consistently manufactured and therefore show only minimal variability in quality. Moreover, the material properties are tuneable to some extent as single manufacturing steps can be changed whereas

biomaterials derived from a human or xenogeneic source show greater variability. On the other side, a main disadvantage of synthetic tissue substitutes is that they are often based on non-degradable materials, which can be associated with inflammatory host responses or enhanced scarring and inferior biomechanics [133]. Currently, many efforts are being undertaken to improve these limitations - with encouraging results - but this requires further investigations. Until now, none of the synthetic conjunctival substitutes has reached clinical application [1, 85].

The most investigated biomaterial is collagen, as it is the most abundant ECM component of the ocular surface. Significant steps have been made, from a fragile collagen gel to a suturable collagen construct, using techniques such as cross-linking and plastic compression [134, 135]. Methods such as the use of Real Architecture For 3D Tissue (RAFT™) absorbers for plastic compression instead of weights lead to high reproducibility in the manufacturing process, better thickness constancy and better applicability [136-138]. Although many approaches for tissue engineered applications have been intensively investigated, there are few satisfactory in vivo studies regarding elasticity and stability in a conjunctival defect [66, 67, 139]. To date, researchers have not yet succeeded in designing a complex ECM framework that fully mimics the 3D-architecture, composition and biochemistry of the human conjunctival ECM.

3.1 Decellularised Tissues as Alternative Tissue Source

Decellularised tissues, in which donor cells and antigens are completely removed, represent promising approaches in the field of Tissue Engineering [133, 140-147]. In recent years, researches made substantial progress in the establishment and investigation of decellularisation techniques for the preservation of the ECM composition and ultrastructure [103, 133]. Main advantages of decellularised tissues are their biocompatibility and tissue specific biomechanics. Further, numerous studies have proven that the organ-specific ECM of a tissue promotes proliferation, differentiation, cellular migration and adhesion of the tissue specific cells [68, 69, 103, 148, 149]. The ECM protein composition, its specific surface structure and the growth factor composition were found to contribute to this effect [71]. Many studies have shown that cells can not only respond to chemical signals such as growth factors, chemokines or signalling molecules, but also to mechanical signals [71, 133, 150-152]. Because of these

positive properties, decellularised tissues are already applied clinically. Ophthalmological indications include tendon elongation in strabismus surgery [115], corneal and scleral patching, or grafting of extended defects in lid surgery [113]. Decellularised tissues used in such cases are fascia lata [153, 154], bovine pericardium [115], bovine sclera [155], or dermal allograft [118].

Porcine (pig derived) tissues are being increasingly used as a xenogeneic source for transplants and show huge potential in this field. Pigs can be breed well in captivity, mature quickly, can be raised under sterile conditions and are already used as food source [156]. Hence, porcine tissue is readily and quickly available, and the most important point is that its ECM proteins share a common sequence homology with that of humans. Decellularised porcine scaffolds enable a successful transplantation without risk of xenogeneic graft rejection and have been successfully used not only in animal studies but also for certain time for clinical applications such as heart valve, intestine, and dermis [108, 157, 158]. Decellularisation of porcine tissue has already been established for ocular structures, for example for the cornea [159, 160] and the lacrimal gland [125].

3.2 Decellularisation of Human and Porcine Conjunctivas

The aim of the first part of this project (Research Article I, p.22) was the development of a decellularised human and porcine conjunctival tissue substitute and the testing of its application in a conjunctival defect in a rabbit model. Decellularised tissue substitutes have to fulfil several criteria: they have to be cell free, stable, biocompatible and non-toxic. In case of the conjunctiva, further requirements are necessary; also after decellularisation. For example, the decellularised conjunctiva must remain elastic and extensible and should still allow or promote squamous epithelial and goblet cell growth. To fulfil these criteria, a decellularisation protocol for human and porcine conjunctivas was established in this doctoral thesis.

3.2.1 Efficiency of Decellularisation

The effective cell removal is a critical step to render the decellularised product immunologically inert. Major histocompatibility complexes (MHC) on allogeneic cells and xenoantigens such as galactose-alpha-l,3-galactose (α-Gal) that are expressed on the

surface of porcine cells can lead to hyperacute immune and rejection reactions if decellularisation is incomplete [161, 162]. In the present study, HE-stained cross-sections exhibited the complete removal of the conjunctival epithelium and the stromal fibroblasts after decellularisation. Neither intact nor dead cells nor cell debris could be observed.

The absence of donor DNA in decellularised tissues is crucial as the amount of residual donor DNA contributes to the lack of cytocompatibility and is directly correlated to adverse host reactions [163]. Crapo et al. proposed that the amounts of residual DNA should be less than 50 ng dsDNA per mg dry weight and that visible nuclear material in tissue sections should be absent [102]. Importantly, Feulgen reaction on tissue slides showed no remaining nuclear components in PDC and HDC. In addition, the quantified DNA content measured with a fluorescence-based Quantitation Assay was reduced by 99.9% in both, PDC and HDC.

3.2.2 Cytotoxicity of Porcine and Human Conjunctiva after Decellularisation

Effective washing of chemicals and detergents residues that have remained in the ECM after decellularisation is a step of great importance. Otherwise, remaining detergents could possibly leak from the ECM during a subsequent cultivation or in vivo testing. Cytotoxicity is possible even at reduced detergent concentrations and might inhibit or negate the benefits of a decellularised ECM scaffold [102, 164, 165]. If necessary, the decellularisation protocol have to be adapted and additional washing steps with higher agitation frequency have to be added to ensure that no cytotoxic substances remain in the tissue.

In this study, toxicity testing with supernatants from decellularised conjunctivas was performed. 3T3 mouse fibroblasts and HCEC were cultured with conditioned medium from PDC and HDC to determine a possible cytotoxicity or inhibition of cell growth. Neither supernatants from PDC nor HDC conveyed cytotoxic effects on the viability or proliferation capacity of fibroblasts or HCEC compared to cells cultured with standard culture medium. Our results proofed that the decellularisation process did not leave any toxic substances in the remaining ECM that might impair a following epithelialisation.

3.2.3 Preservation of the Extracellular Matrix and its Biomechanical Properties

A major goal of decellularisation is to preserve the unique ECM structure and biomechanical properties of the tissue. The use of too harsh decellularisation solutions and a too long decellularisation time can lead to a destruction of the ECM structural proteins and an impairment of their mechanical properties [133, 166, 167]. Damage to the structural fibres and the associated loss of elasticity and tensile strength must be avoided or kept as low as possible.

HE-stained cross sections of PDC and HDC revealed an intact and histologically normal ECM. Cell-free, vascular structures (tube-like holes in the ECM) were still visible after decellularisation. Collagen is a key protein for maintaining the structural integrity and ECM strength of most mammal organs and is also a major component of the conjunctiva [92]. To investigate the ECM and the collagen fibrils more closely, we performed transmission electron microscopy, which is an established method to proof the ultrastructural integrity of decellularised tissues [168]. Electron microscopy showed no disruption of the collagen fibrils that are essential for the structure, and the mechanics of decellularised tissues. In line with these findings, the decellularisation process did not significantly reduce the quantified collagen content.

Changes in scaffold elasticity can influence cell fate, especially in stem or progenitor cells whose differentiation is affected by mechanotransduction [151, 169]. The effect of decellularisation on the mechanics of a scaffold is tissue dependent. Studies show that in some tissues the measured elasticity decreases after decellularisation, indicating stiffening [145, 170], while in others the stiffness decreased [146] or remained unchanged [171]. To evaluate changes in biomechanical properties after decellularisation, mechanical stress tests were performed with decellularised and non-decellularised conjunctivas. The decellularisation process did not affect the ultimate tensile strength, maximal extensibility or elasticity of porcine or human conjunctiva. The force needed to rupture the porcine and human conjunctiva and its decellularised equivalents PDC and HDC was significantly lower compared to the force required to rupture AM, indicating a "stronger" ECM of AM. Interestingly, the extensibility (maximal elongation at break) and the elasticity (elastic modulus) of decellularised and untreated conjunctival specimens were significantly superior compared to AM. The elastic modulus or Young's

modulus describes the stiffness of a material. The tested material undergoes elastic deformation when a load is applied to the material during extension. Elastic deformation is reversible (the tested material returns to its initial shape after the load or force is removed). Regarding AM, its tensile strength is an important factor as it needs to carry the neonate and prevent preterm rupture of the fetal membranes [172]. In the eye, such high tensile strength is not required; instead, the conjunctiva needs to possess high elasticity and extensibility to enable eye movement to different directions and continuously lid closure during lifetime. Thus, HDC und PDC seem to be superior to AM with regard to their biomechanical properties for conjunctival surface reconstruction.

3.3 Conjunctival Reconstruction using Decellularised Porcine and Human Conjunctiva in a Rabbit Model

To investigate the applicability, biocompatibility and performance of decellularised conjunctiva in vivo, conjunctival defects in rabbits were covered with either PDC, HDC or AM, the most frequently used matrix for ocular surface reconstruction.

3.3.1 Surgical Manageability

In the clinical context, handling and surgical manageability are of great importance. Both decellularised conjunctival transplants, PDC and HDC, showed similar surgical manageability as AM during surgery. In all groups, the transplants did not tear and the suturing time as well as the number of sutures required to adapt the transplant to the host conjunctiva did not differ significantly between the groups.

3.3.2 Inflammatory Response

The hosts' immune response to decellularised scaffolds is affected by several variables including the tissue source, the decellularisation processing and the transplantation site [173]. No adverse inflammatory response occurred after transplantation of the decellularised conjunctivas into a conjunctival defect in the rabbit, neither human nor porcine. In all groups, including AM, the grade of bulbar hyperaemia was normal to mild directly after surgery and normal to moderate three days after surgery. After 10 days,

hyperaemia decreased again to a normal level. Wound healing and transplant remodelling or degradation are mediated by infiltration of host immune cells, which progressively invade the transplant 3 to 14 days after transplantation [92]. Histologically, immune cell invasion was not significant different between PDC, HDC and AM, indicating a similar immune response to all transplants.

3.3.3 Stability and Integration of the Transplant

Both, PDC and HDC showed better mechanical stability on the ocular surface compared to AM. The AM group revealed a significantly higher amount of suture loss after 10 days and half of the AM transplants were not detectable after 10 days whereas all PDC and HDC were well integrated into the rabbit conjunctiva. In the remaining AM transplants, folded or rolled up edges with poor adaption to the conjunctival wound edges were observed. Histologically, a growth of fibrous scar tissue at the wound edges was observed in these eyes. Such healing pattern is characteristic for spontaneous conjunctival wound healing, in which wound contraction occurs due to fibrous tissue growth of collagen synthesized in the wound bed [83]. The poor outcome of the AM group was unexpected as the AM is the most frequently applied allogeneic substitute tissue in conjunctival reconstruction. Several studies have reported that the AM is able to improve wound healing and to reduce scarring processes due to growth factor activity [56, 57]. However, a characterization of the growth factor content in AM and though, the variation between different AM is not determined before surgery. Several studies showed that the AM depicts strong intra- and inter-donor variations concerning tissue quality, growth factor content and stromal thickness [62, 174]. Hopkinson et al. demonstrated that the content of TGF-β, a major modulator of wound healing, ECM remodelling and scarring, highly depends on the donor and the handling of the AM [174]. The thickness of AM reported in several studies varies greatly and depends on the method of processing, handling and the sample site and it has been reported that inter-donor variations in the physical structure of the AM can be related to age, maternal health, or gestational age [62, 175, 176]. Alternating thickness and the above described quality variations of AM might have led to the unsatisfactory stability of AM in our study and may also explain the inconsistency in clinical outcomes [175]. This is supported by own results showing the strong variance in the thickness of AM [138]. In this study (see

"A Additional Publications, A.1" on page 68), the thickness of AM showed a high variance and ranged between about 7 to 58 µm. Another reason for the early loss of the sutures and/or the AM transplant itself could be a possible enzymatic digestion of AM that may have led to the unsatisfactory stability in the conjunctival defect and may also explain the controversial outcomes in clinical cases with strong inflammatory involvement [50, 63, 177]. First in vitro studies (unpublished data) in which we tested the influence of enzymatic activity on different potential conjunctival substitutes, indicate a higher resistance of decellularised conjunctiva to enzymatic digestion compared to AM.

In the groups transplanted with PDC and HDC, the transplant edges were histologically barely visible, and the collagen fibrils of the transplants were connected to the host collagen. Such integration into the host ECM with degradation of the decellularised tissue and replacement by the donor ECM is well known for decellularised matrices and termed "constructive remodelling" [103, 178, 179]. Constructive remodelling is characterized by gradual, partial or total degradation and replacement of the decellularised scaffold with site typical, functional tissue. Importantly, the remodelling of the decellularised transplants, even though they were of xenogeneic origin, occurred without scar tissue development [92, 180]. This is in particular of importance, as extensive conjunctival scarring can cause fornix shortening or further ocular surface disturbances such as pain, reduced eye or lid motility and cosmetic issues.

Apart from the better integration of PDC and HDC into the host conjunctiva, which induces higher stability, the differences in the mechanical properties previously measured in vitro could account for the accelerated tissue loss of AM. Mechanical stress is known to influence ECM remodelling [92]. Probably, the more expandable PDC and HDC could withstand the movement of the lids over the transplant better than the more rigid AM.

3.3.4 Vascularisation

In line with studies that have shown that decellularised tissues (e.g. skin) show early neovascularisation after transplantation [181], we observed stromal, blood filled vessels after 10 days in PDC and HDC but not in AM grafts [92]. The tube-like, vascular

formations, which were evident within PDC and HDC, might have served as guiding structures to induce vessel formation. Vascularisation is essential to allow nutrition and oxygen delivery and important for the long-term survival of transplants too. Hence, these results could be an important indicator for a potential successful clinical application.

3.3.5 Epithelialisation and Goblet Cell Formation

In this in vivo study, the previous in vitro findings were confirmed, as the rabbit conjunctival epithelial cells were able to form a continuous layer on PDC and HDC, Histologically, a stratified, well-organized epithelium was observed on both PDC and HDC, closing the conjunctival defect. After 10 days, already 33-46% of the transplant was epithelialised in both groups. In contrast, only one out of three detectable AM was covered with a mono-layered epithelium. In contradiction to these results, AM has been reported in several studies to promote conjunctival epithelialisation [77, 182]. Most likely, the poor epithelialisation seen in our study was caused by the poor stability and integration of AM into the wound bed.

Studies reported that the tissue-specific ECM of decellularised tissues is capable to guide cell fate to the cell type from which the ECM was derived by mimicking the in vivo microenvironment [71]. In accordance to that, Yang et al. demonstrated that homogenates of decellularised human conjunctivas promote the differentiation of human epithelial progenitor cells into mature conjunctival epithelial cells and goblet cells, the hallmark of conjunctival epithelium [79]. A decellularised conjunctival scaffold may therefore exhibit a supportive niche for the patient's conjunctival progenitor cells to promote differentiation into goblet cells. In our in vivo experiment, we observed mucin-filled goblet cells on PDC and HDC at the fornical edge of the transplant, indicating early differentiation into functionally competent cells, although the centre of the transplants did not contain secretory cells after ten days postoperative. In contrast, goblet cells were not found at any site on the AM grafts, presumably because the AM was either absent or not in place and insufficiently adapted to the recipient's conjunctiva to allow successful epithelialisation and thus, goblet cell growth. However, a long-term follow-up (unpublished data) showed that the distribution and number of goblet cells

normalized in all groups after 28 days, and a continuous epithelium was visible at the grafting site in all groups. It should be noted that we induced a conjunctival defect by a relatively small surgical incision in a healthy eye of a young rabbit. Clinical cases often come along with chronic inflammation, dry eye symptoms, extensive scarring, eyelid deformities or other complications. In our study, we have chosen this type of defect model to evaluate clearly the surgical handling, the area of epithelialization, and the biocompatibility of the graft. A potential superiority of PDC or HDC in a disease model with preoperative severe scarring or more extended conjunctival damage in an inflammatory environment has to be further investigated.

3.4 Development of a Recellularised Conjunctival Substitute

In the healthy human eye, conjunctival stem cells are essential for re-population of both epithelial cells and mucin-producing goblet cells throughout an entire lifespan [183]. Trauma, repeated surgical interventions or systemic autoimmune diseases might induce damage to the ocular surface and its mature cell population. Although the injured conjunctiva is capable of spontaneous reepithelialisation, severe ocular surface damage often results in massive or complete loss of conjunctival epithelial stem cells. In this situation, long-term visual rehabilitation requires a matrix with supportive niche features and a conjunctival epithelial population containing goblet cells or a progenitor cell population that is capable to differentiate into the latter. Therefore, the second milestone of this project (Research Article II, p.34) was to develop a recellularised tissue substitute for conjunctival reconstruction covered with a stable, stratified and organized conjunctival epithelium that can resist a transplantation and can then support the ocular surface in severely affected eyes.

Our previous study showed no disadvantages of porcine compared to human decellularised conjunctiva [184]. Therefore, we decided to focus on the development of a reepithelialised conjunctival substitute based on porcine decellularised conjunctiva. The main advantage of porcine tissue is that it is readily and quickly available. Another point is the donor age. Pigs for slaughtering are about eight month old, whereas human donors are much older, e.g. our human donors are on average 79 years old. It is known that extracellular matrix components such as elastic fibres change significantly with age and that the senescence of the extracellular matrix has an impact on the stem cell niche

and its cells [185-188]. Pigs may therefore represent a tissue source with a more consistent conjunctival tissue quality because they are the same age and raised under standardised conditions.

3.4.1 Cultivation and Characterization of Human Conjunctival Progenitor Cells

The main challenge of conjunctival epithelial recellularisation – in comparison to the cornea – is to enable the growth of both cell types, normal squamous epithelial cells and goblet cells. Many efforts have concentrated on the cultivation and differentiation of conjunctival progenitor cells. Because the proliferative capacity and conjunctival progenitor cell properties are restricted in the absence of matrix or fibroblast support on most substrates in vitro [78, 189, 190], many studies describe an expansion of HCEC by co-culturing them with 3T3 feeder cells prior to their cultivation on a presumptive tissue substitute [28, 66, 191, 192]. The co-culture with murine 3T3 fibroblast feeder cells provides satisfactory support for cell growth and maintenance of the progenitor cell properties but does – due to the xenobiotic component causing immune rejection and transmission of unknown viruses and zoonotic pathogens – not comply with Good Manufacturing Practice (GMP), and is thus not desired for transplantation. This aspect is discussed in more detail in section 3.6.1 – "Xenogen- and Serum-free Cultivation of Conjunctival Epithelial Cells" on page 62.

So far, a specific marker for the identification of conjunctival stem cells has not been found. The most accepted markers Cytokeratin (CK) 15, ΔNp63 and ABCG2 are able to detect putative stem cells and transient amplifying cells. They may therefore also be regarded as being progenitor cell markers. Because of their different patterns of expression, cytokeratins have been widely used to distinguish the different epithelial cells of conjunctival, limbal and corneal epithelium [193]. Whereas differentiated corneal epithelial cells show positivity for CK 12 and 3, the conjunctival cells lack these cytokeratins and express CK 19 and 13 instead [194]. Many studies showed that CK 15 is expressed in conjunctival basal cells suggesting being a putative conjunctival stem cell marker. However, some studies reported also that more differentiated progenitor cells may also express CK 15 [195]. Importantly, all studies consistently show that CK 15 is absent in the central corneal epithelium [196]. The transcription factor p63 is expressed

by limbal stem cells and transient amplifying cells [197, 198]. Among the various existing p63 isoforms, the ΔNp63α isoform is the most prevalent in epithelial cells that possess stem cell-like phenotypes [199]. A significant expression of ABCG2, a transmembrane transporter, has been found in human conjunctival basal epithelial cells that exhibit epithelial stem cell features such as slow cycling, clonogenic capacity, and resistance to phorbol-induced differentiation [200, 201]. Conjunctival epithelial cells possess a stem cell-rich side population, which is attributed to the ABCG2 transporter function [202]. This is believed to be a mechanism by which long-lived stem cells are able to diminish the potential for genomic damage over their lifespan.

3.4.2 Reepithelialisation of Decellularised Conjunctiva In Vitro

Besides an adequate cell source, recellularisation of decellularised tissues requires the appropriate culture method adapted and optimised to the physiological function and origin of these cells. In this thesis, two different methods for culturing HCEC were compared for conjunctival epithelium formation and stratification, goblet cell formation, and epithelial progenitor cell behaviour. The first method (method A, described in section 3.4.1) has been widely used for expansion of HCEC in cell culture laboratories. In this technique, the HCEC are expanded in co-culture with a mouse fibroblastic feeder cell line to obtain the epithelial progenitor phenotype [28, 66, 191, 192]. After expansion, the cells can be seeded on PDC or HDC for further cultivation and stratification. The main advantage of this technique is that a large number of cells can be expanded in cell culture flasks and the cells can be reseeded on a scaffold with a defined cell density. However, even when feeder cells are used, the proliferation capacity in vitro is limited and the progenitor cell properties decrease with every passage [190]. Furthermore, xenobiotic components should be avoided during expansion of human donor cell that are intended for transplantation.

Therefore, we investigated a second method to generate an epithelialised conjunctival substitute: In this direct cultivation method (method B), a small, engrafted human conjunctival explant biopsy is placed onto PDC without prior expansion with feeder cells. A big advantage of this method is that it requires only an exceedingly small piece of donor conjunctiva and not, as in autologous conjunctival transplantation, a piece

that is precisely the size of the defect, which must be covered for protection after transplantation. Using reepithelialised PDC, thus, it is possible to cover a larger defect area, while requiring only a minimal incision into the patient's healthy conjunctiva. The removal of such a small-sized segment can be considered unproblematic in terms of scarring at the donor site.

Both culture methods included a cultivation period in an air liquid interface (ALI). It was reported that air lifted conjunctival epithelial cells show higher proliferation rates, exhibit more cell layers and have a superior epithelial polarity and orientation than those cultured submerged during the entire cultivation period [182]. Furthermore, basal HCEC in air lifted cultures show a more natural, cuboidal shape while the superficial epithelial cells show a more flattened morphology. Moreover, studies have proven that goblet cell differentiation and MUC5AC secretion increases in airlifted conjunctival epithelial cell cultures [203, 204].

In our study, method B formed a stratified epithelium more quickly and with significantly higher proliferative activity during the first week of cultivation compared to the pre-expansion method (method A). Using direct cultivation, epithelial thicknesses on PDC reached a thickness similar to that of normal human bulbar conjunctiva after 21 days. Moreover, the outgrown epithelium demonstrated superior stratification and organization, with a low number of apoptotic cells. There were more epithelial cells expressing CK 15, ΔNp63, and ABCG2, in method B than method A, indicating a greater preservation of conjunctival progenitor cells. Our results regarding the progenitor cell marker expression were similar to other studies of tissue-engineered conjunctival equivalents. Unfortunately, most of these studies did not report the number or percentage of ABCG2 or p63-positive cells [96, 201, 205].

When developing conjunctival tissue substitutes in vitro, special attention should be paid to the goblet cell formation, because they contribute to the mucous layer of the tear film, which is essential for the lubrication and stability of the ocular surface [24]. Following ocular injury and subsequent spontaneous reepithelialisation, goblet cell density is often reduced, resulting in a reduction of the mucous tear film layer [139, 206, 207]. With culture method B, we detected mucin-filled goblet cells from day 14 on. In contrast, no goblet cells were detected at any time point with culture method A. There

are several possible reasons why culture method B has achieved better results. The first could be the preservation of the cell niche of the human explant biopsy. Fibroblasts embedded in the explant piece might be able to support epithelial growth by secreting growth factors and cytokines. Another reason could be that the epithelial cells in method B were able to migrate from their cell niche (the explant piece) directly into a structure that is presumably similar to this niche (PDC). In contrast, the cells of method A were pre-cultured on plastic and passaged once before being seeded onto PDC. It has been reported that passaging of primary conjunctival epithelial cells leads to a reduction in the colony-forming efficacy of such cultures [190].

3.5 Conjunctival Reconstruction with Reepithelialised PDC in a Rabbit Model

To evaluate cell survival and proliferation, the maintenance of the progenitor cells, and a possible maturation of the human epithelial cells in vivo, the reepithelialised PDC was transplanted into a conjunctival defect in immunosuppressed rabbits. Un-epithelialised PDC were transplanted as controls. Since our previous in vitro results have shown that the cultivation of a conjunctival explant piece on PDC generated an epithelium with superior stratification compared to a prior expansion in cell culture flasks, the explant method was preferred for the following in vivo experiments. The 14 days-cultivation with method B revealed the highest expression of putative progenitor cell markers in the epithelium, hence, this time point was set for the transplantation.

The in vitro reepithelialised PDC showed an intact epithelium over the entire in vivo evaluation period, whereas the PDC control graft showed virtually no epithelialisation until day 10. Human cells that survived on the construct after transplantation formed an organized stratified epithelium, which had only slightly more apoptotic cells than the surrounding conjunctiva. Proliferating cells in the epithelium of the transplanted construct were still present after 10 days, albeit to a lesser extent than in normal conjunctiva. Notably, progenitor cell properties were preserved even after 10 days and their expression was comparable to that of normal conjunctiva. Thus, the reepithelialised PDC seemed able to maintain cells that have the potential to survive and proliferate even after transplantation.

It is to mention that the PDC control group in this study did not show an epithelialization by day 10 whereas our previous study using PDC in rabbits revealed that the wound closure was complete by day 10 [184]. This difference might be due to the prior treatment with cyclosporine A in our second study to suppress immunological reaction to the transplanted human cells on PDC+HCEx. The immune suppression itself might account for the delayed wound closure in the PDC control group in our second study, whereas in the first study, the rabbits were not treated systemically with an immunosuppressive drug. It has been shown that systemically applied Cyclosporine A negatively affects wound healing in rats [208]. Another study in rabbits showed that a Cyclosporine A dose that effectively leads to immunosuppression also has a high level of toxicity that causes discomfort and weight loss in rabbits, which can have a secondary negative effect on wound healing [209]. In the clinical context, however, a systemic immunosuppression would not be necessary since, PDC is not expected to cause an immune response after transplantation because of the lack of immunogenic factors and a small biopsy of the patient's autologous conjunctiva would be used for reepithelialization.

A very interesting observation in our study was that the number of mucin-filled goblet cells on PDC+HCEx increased during the in vivo period. After 14 days in vitro and the following 10 days after implantation in vivo, a significantly higher number of goblet cells was detected than after 24 days in vitro cultivation alone. This increase in goblet cells could be due to possible in vivo maturation, which can only be partially emulated in vitro. A similar observation was made in a study in which conjunctival epithelium was first cultivated on AM for 14 days and then implanted in mice for further 11 days [77]. The resulting conjunctival epithelium showed an increase in MUC5AC-positive cells during this in vivo phase, too. It can be presumed that the in vivo environment promotes the maturation of goblet cells and thus mucin production. To induce maturation of goblet cells and mucin production already in vitro, cultivation methods, growth media and media supplements should be optimized in the future. This could be achieved in particular by a better understanding of the individual media components and signalling pathways as well as the targeted use of suitable growth factors for the regulation of goblet cells. For patients suffering from larger conjunctival defects

with resulting impairment of the tear film layer, a higher number of transplanted goblet cells would be valuable for their presumptive contribution to tear film mucin.

3.6 Towards Clinical Application of Decellularised Conjunctiva

The field of Tissue Engineering is rapidly progressing and pushing forward to clinical application. Although substantial progress in the development of biomaterials for ocular surface reconstruction have been made in the past years too, many hurdles remain before clinical translation. First, greater knowledge and more data on substitute tissue degradation during matrix remodelling and disease-associated turnover are needed. In our study, the conjunctival defect was induced by a surgical incision in a healthy rabbit eye. This defect model was chosen to evaluate clearly the surgical manageability, the epithelialisation area and the inflammatory reaction. However, clinical cases often come along with dry eye, chronic inflammation, extensive scarring or other complications. The performance of a graft may vary with disease or severe ocular surface damage, especially when disease-associated proteolytic enzymes are involved. A potential superiority of decellularised conjunctiva in a disease model with more extended conjunctival damage or scarring needs therefore to be investigated in future studies. In addition, a better understanding of the ECM as a whole (matrisome), its components and the interactions with cells, especially with progenitor cells and resident stem cells is required. Although the understanding of the ECM of tissues increases continuously in all medical fields, the release of ECM-associated cell signals and cryptic peptides and their influence on tissue substitutes and host remodelling is still not fully understood to date [133].

3.6.1 Xenogen- and Serum-free Cultivation of Conjunctival Epithelial Cells

In most literature describing conjunctival epithelial cell cultivation, the growth media used is supplied with growth factors, hormones and the addition of fetal calf serum to a total of 10 % of the media. Disadvantages of the use of xenogeneic growth factors and serum might lay in inconsistent quality and the reproducibility (variabilities in batches cannot be excluded) as well as animal welfare concerns [210]. Studies reported that it is possible to cultivate HCEC under serum-free culture conditions using 3T3 feeder cells and that goblet cells could be observed [211]. In contrast, a cultivation of goblet cells

under feeder cell-free conditions seems more challenging: Risse Marsh et al. have shown that it is possible to cultivate HCEC without 3T3 feeder cells in serum free media. However, goblet cells were not observed in their culture system [212].

Since the use of 3T3 is not ideally suited for clinical application given the risk of transfer of xenobiotic components, there have been attempts to replace 3T3 cells with human cells such as bulbar subconjunctival fibroblasts (HCF). Under these culture conditions, HCEC showed similar progenitor cell preservation and a significantly higher colony-forming efficiency compared to a cultivation with 3T3 cells. However, goblet cells were significantly less common under HCEC-HCF conditions than under HCEC-3T3 conditions. In contrast, another study showed that cultivation of HCEC on collagen gels containing HCF led to higher numbers of goblet cells than on collagen gels casted with 3T3 cells [191]. Summarising the current data, cultivating goblet cells without feeder cells, serum or ECM components such as collagen, still seems difficult. Hence, further research in how progenitor and goblet cells are influenced under different culture conditions, especially under serum- and xenogeneic-free culture conditions in order to improve the reproducibility, safety and quality of tissue engineered conjunctival substitutes, is needed.

3.6.2 Storage of Decellularised Conjunctivas

Another point of interest in the step towards a clinical application is the evaluation of the storage conditions of decellularised tissues in future studies. For the storage of decellularised tissues, different methods are described in the literature. Most literature uses PBS for the storage of decellularised tissues [102, 213], but freeze-drying and storage conditions at -80 °C in cryoprotection solutions are also described [214, 215]. The effects of the different methods on the decellularised constructs vary greatly between the different tissue types. Since the ECM composition is highly tissue-specific, the optimal processing and storage condition that preserves the ultrastructure of the conjunctival ECM and retains the recellularization capacity will be evaluated in future studies in our research group.

3.6.3 Medical Product Safety and Regulatory Routes

The evaluation of biocompatibility and immunogenicity of utmost importance for a possible approval and clinical application. As mentioned in Section 3.2.1, effective removal of MHC or α-Gal from the tissue is essential to prevent the human recipient from rejecting HDC or, respectively, PDC after transplantation. Although it seems legitimate to assume, that a tissue that does not contain cells after decellularisation, does also not contain cell surface complexes, this is a point that should be confirmed before transplantation into humans. Several clinical studies have shown in the past that inadequate and incomplete examination of decellularised tissue can lead to complications upon transplantation [144, 216, 217]. One of the steps to ensure the maximum achievable safety should be the treatment of PDC with α-galactosidase that removes any remaining α-Gal molecules prior to applications in humans [218]. Another promising approach to reduce immune reactions is the use of genetically modified Galactosyl-transferase knockout pigs [219, 220]. Another concern while using pig tissue is a possible transmission of porcine viruses such as porcine endogenous retrovirus (PERV) or hepatitis E virus [221]. However, several studies proofed the safety of decellularised porcine tissues and reported the effective elimination of virus DNA such as from PERV after decellularisation [112, 222, 223].

To ensure high reproducibility of a decellularised product, standardised protocols, GLP (Good Laboratory Practice) and GMP (Good Manufacturing Practice) compliant processes should be established, even at an early stage of the development. The safety of medical products is of particular importance, which is why their approval and manufacture is under strict regulatory control. Medicinal products for the use in humans based on genes, tissues or cells are categorized as Advanced Therapy Medicinal Products (ATMPs). This includes also tissue engineered medicinal products because they are defined as: "[…] a biological medicinal product that contains bioengineered cells or tissues or consists of the latter. It serves the regeneration, restoration, or replacement of human tissue" [224]. In Germany, the Paul-Ehrlich-Institut (PEI) is the competent authority for this group of medicines. At European level, the European Medicines Agency (EMA) is responsible. From a clinical point of view, tissue engineered substitutes are still a new field: According to the PEI, only ten tissue engineered medicinal products

currently have a valid marketing authorization (status 25.11.2020). In contrast to recellularised scaffolds, decellularised tissues alone can be categorized under non-advanced therapy medicinal products. This is true for e.g. decellularised skin scaffolds, which are the most frequent used acellular ECM matrices in clinical applications (e.g. AlloDerm, LifeCell; FlexHD, Ethicon; Allopatch HD, Conmed). The EMA decided that even transgenic decellularised porcine dermal matrix does not fall within the definition of an ATMP, because the product "does not contain viable tissue or cells and does not contain or consist of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, adding or deleting a genetic sequence" [225].

ATMPs undergo a different regulatory process from non-ATMPs. The authorisation and manufacturing procedure for non-ATMPs is simpler than for ATMPs, which are among the most strictly regulated medical products. This might be one reason why there are so few ATMPs with a valid marketing authorization. The application of engineered substitute tissues containing autologous or allogeneic cells in clinical routine will therefore probably take some time. Nevertheless, the currently available decellularised tissues can help to pave the way for recellularised scaffolds by familiarising physicians and patients with the concept of a decellularised product.

4 Conclusion

Our first study (Research Article I) identified porcine decellularised conjunctiva (PDC) and human decellularised conjunctiva (HDC) as promising substitutes for conjunctival reconstruction. In the first part of this study we were able to generate PDC and HDC that

- were nearly DNA-free, structurally and ultrastructurally intact and showed no cytotoxic effects in vitro,
- showed biomechanical properties equal to the respective untreated conjunctiva,
- were more extensible and elastic but exhibited less tensile strength than AM,
- showed good surgical manageability,
- did not induce considerable inflammation in an in vivo rabbit model,
- revealed significantly enhanced transplant stability, less suture losses and better integration into the host conjunctiva compared to AM in vivo,
- showed superior wound healing and epithelialisation capacity compared to AM in the rabbit model

Furthermore, our study showed that

- PDC was not inferior to HDC regarding structural integrity, biomechanical properties, toxicity, in vivo biocompatibility and stability on the ocular surface
- PDC may represent an easily available, ethically legitimate conjunctival substitute with clinical and economic benefits

In the second part of this project, (Research Article II) we reported the reepithelialisation and use of a conjunctival substitute on a PDC-base.

In this study we could showed that

- in vitro cultured human conjunctival epithelial cells were able to grow on it and to build a stratified epithelium,

- the use of a direct explant method (PDC+HCEx) was superior compared to a prior expansion and subsequent reseeding of HCEC onto PDC (PDC+HCEC) (From day 14 on, a significantly thicker and more organized epithelium with higher numbers of putative progenitor cells were noted for PDC+HCEx versus PDC+HCEC in vitro. Goblet cells were only present with the PDC+HCEx cultivation method),
- the in vitro cultivated epithelial cells of PDC+HCEx were still detectable, viable and maintained both organization and progenitor cell properties even after a transplantation into a conjunctival defect in the rabbit and additional 10 days in vivo,
- the number of goblet cells on transplanted PDC+HCEx increased in vivo to a number comparable to that of normal human conjunctiva.

The current literature suggests that recent advances in Tissue Engineering offer many potential therapeutic substitute tissues for ocular surface reconstruction. Decellularised human and porcine conjunctiva is very similar to the three-dimensional native tissue microenvironment of the human conjunctiva and could therefore be an alternative substitute for conjunctival reconstruction in the future. Further, our results suggest PDC to be a suitable cell carrier for autologous human conjunctival epithelial cells, as it allows for preservation of the potential for self-renewal and goblet cell formation, even after transplantation. Thus, reepithelialised PDC might offer a promising option for patients with extensive conjunctival damage, including conjunctival stem cell and goblet cell deficiency. The performance of decellularised and recellularised conjunctivas in a pathological condition needs further investigation in future studies.

Appendices

A Additional Publications

A.1 Evaluation of Plastic Compressed Collagen for Conjunctival Repair in a Rabbit Model

Witt J*, Borrelli M, Mertsch S, Geerling G, Spaniol K, Schrader S (2019)
*Corresponding author

Journal: Tissue Engineering Part A, Mary Ann Liebert Inc.

Impact factor: 3.776

Abstract

Conjunctival integrity is crucial for a healthy ocular surface and visual acuity. In severe cases of inflammatory surface disorders or after trauma, thermal or chemical burns as well as after ocular surgery, a surgical reconstruction using conjunctival substitutes is required. Due to limitations of currently used substitutes, such as the amniotic membrane, there is a need for the development of new scaffolds of consistent quality for conjunctival reconstruction. This study explored the biocompatibility and surgical usability of plastic-compressed collagen as an alternative conjunctival substitute in a rabbit model.

doi: 10.1089/ten.TEA.2018.0190.

Link to publication:
https://www.liebertpub.com/doi/full/10.1089/ten.TEA.2018.0190?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed

Contribution: Study conception and design, data collection, analysis and interpretation, manuscript writing, figure preparation, manuscript reviewing

Approximated total share of contribution: 65%

A.2 Generation and Characterisation of Decellularised Human Corneal Limbus

Spaniol K*, **Witt J**, Mertsch S, Borrelli M, Geerling G, Schrader S (2018)
*Corresponding author

Journal: Graefe's Archive for Clinical and Experimental Ophthalmology, Springer

Impact factor: 2.396

Abstract

Limbal epithelial stem cells (LESC) reside in a niche in the corneo-scleral transition zone. Deficiency leads to pain, corneal opacity, and eventually blindness. LESC transplantation of ex-vivo expanded human LESC on a carrier such as human amniotic membrane is a current treatment option. We evaluated decellularised human limbus (DHL) as a potential carrier matrix for the transplantation of LESC. Human corneas were obtained from the local eye bank. The limbal tissue was decellularised by sodium desoxychelate and DNase solution and sterilised by γ-irradiation. Native limbus- and DHL-surface structures were assessed by scanning electron microscopy and collagen ultrastructure using transmission electron microscopy. Presence and preservation of limbal basement membrane proteins in native limbus and DHL were analysed immunohistochemically. Absence of DNA after decellularisation was assessed by Feulgen staining and DNA quantification. Presence of immune cells was explored by CD45 staining, and potential cytotoxicity was tested using a cell viability assay. In the DHL, the DNA content was reduced from 1.5 ± 0.3 µg/mg to 0.15 ± 0.01 µg/mg; the three-dimensional structure and the arrangement of the collagen fibrils were preserved. Main basement membrane proteins such as collagen IV, laminin, and fibronectin were still present after decellularisation and γ-irradiation. CD45-expressing cells were evident neither in the native limbus nor in the DHL. DHL did not convey cytotoxicity. The extracellular matrix (ECM) of the limbus provides a tissue specific morphology and three-dimensionality consisting of particular ECM proteins. It therefore represents a substantial component of the stem cell niche. The DHL provides a specific limbal niche surrounding, and might serve as an easily producible carrier matrix for LESC transplantation.

doi: 10.1007/s00417-018-3904-1

Link to publication: https://link.springer.com/article/10.1007%2Fs00417-018-3904-1

Contribution: Data collection, analysis and interpretation, manuscript writing, figure preparation, manuscript reviewing

Approximated total share of contribution: 15%

A.3 Plastic Compressed Collagen Transplantation - a New Option for Corneal Surface Reconstruction?

Schrader S*, **Witt J**, Geerling G (2019)
*Corresponding author

Letter to the Editor – Case Report

Journal: Acta Ophthalmologica, Wiley

Impact factor: 3.362

doi: 10.1111/aos.13649.

Link to publication: https://onlinelibrary.wiley.com/doi/full/10.1111/aos.13649

Contribution: Data collection, analysis and interpretation, manuscript writing and reviewing

Approximated total share of contribution: 20%

A.4 MSC Transplantation Improves Lacrimal Gland Regeneration after Surgically Induced Dry Eye Disease in Mice.

Dietrich J*, Ott L, Roth M, **Witt J**, Geerling G, Mertsch S, Schrader S (2019)
*Corresponding author

Journal: Scientific Reports, Nature Publishing

Impact factor: 3.998

Abstract

Dry eye disease (DED) is a multifactorial disease characterized by a disrupted tear film homeostasis and inflammation leading to visual impairments and pain in patients. Aqueous-deficient dry eye (ADDE) causes the most severe progressions and depends mainly on the loss of functional lacrimal gland (LG) tissue. Despite a high prevalence, therapies remain palliative. Therefore, it is of great interest to develop new approaches to curatively treat ADDE. Mesenchymal stem/stromal cells (MSC) have been shown to induce tissue regeneration and cease inflammation. Moreover, an increasing amount of MSC was found in the regenerating LG of mice. Therefore, this study investigated the therapeutic effect of MSC transplantation on damaged LGs using duct ligation induced ADDE in mice. Due to the transplantation of sex-mismatched and eGFP-expressing MSC, MSC could be identified and detected until day 21. MSC transplantation significantly improved LG regeneration, as the amount of vital acinar structures was significantly increased above the intrinsic regeneration capacity of control. Additionally, MSC transplantation modulated the immune reaction as macrophage infiltration was delayed and TNFα expression decreased, accompanied by an increased IL-6 expression. Thus, the application of MSC appears to be a promising therapeutic approach to induce LG regeneration in patients suffering from severe DED/ADDE.

doi: 10.1038/s41598-019-54840-5.

Link to publication: https://www.nature.com/articles/s41598-019-54840-5

Contribution: Data collection, manuscript reviewing

Approximated total share of contribution: 5%

A.5 Comparative Analysis on the Dynamic of Lacrimal Gland Damage and Regeneration after Interleukin-1α or Duct Ligation Induced Dry Eye Disease in Mice.

Dietrich J*, Schlegel C, Roth M, **Witt J**, Geerling G, Mertsch S, Schrader S (2018)
*Corresponding author

Journal: Experimental Eye Research, Elsevier

Impact factor: 3.011

Abstract

The loss of functional lacrimal gland (LG) tissue causes quantitative tear deficiency and is the most common reason for the development of severe dry eye disease (DED). The induction of LG regeneration in situ would be a promising approach to curatively treat DED, but underlying mechanisms are mainly unclear. Therefore, this study aims to comparatively evaluate the dynamic of LG damage and regeneration in two mouse models in order to study mechanisms of LG regeneration. Male C57BL/6 J mice were used to induce damage to the right extraorbital LG either by a single interleukin (IL) 1α injection or a ligation of the secretory duct for 7 days. Fluorescein staining (FL) and LG wet weight were assessed. In addition, the dynamic of damage and regeneration of acini structures as well as inflammation and the appearance of progenitor cells were (immuno-) histologically evaluated on day 1, 2, 3, 5, 7 after IL-1α injection and day 3, 7, 14, 21, 28 after duct ligation (DL). While LG weight was only slightly affected after IL-1α injection, DL led to a significant decrease at day 7 followed by an increase after re-opening. Additionally, DL resulted in a more pronounced inflammatory reaction than IL-1α injection. After DL the infiltration with CD3+ T cells, CD138 + plasma cells and CD68 + macrophages increased, while IL-1α injection only caused an infiltration with CD68 + macrophages. Furthermore, the damage of LG structures was significantly higher after DL than after IL-1α injection. Accordingly, regeneration of LG was prolonged and only partial at day 28 after DL, whilst 5 days after IL-1α injection a complete LG completely regeneration was achieved. We also found a significantly increased number of nestin + mesenchymal stem cells in both models during injury phase. Our results showed that

both models induce LG damage followed by a spontaneous regeneration of acini structures. IL-1α injection caused an immediate inflammation with a transient period of slight tissue damage. However, DL caused a more distinct tissue damage followed by a prolonged period of regeneration, which might make it appear more attractive to study regenerative therapies and their effects on LG regeneration.

doi: 10.1016/j.exer.2018.03.026.

Link to publication:
https://www.sciencedirect.com/science/article/pii/S0014483517308369?via%3Dihub

Contribution: Data collection, manuscript reviewing

Approximated total share of contribution: 5%

B Poster Presentations

- **Witt, J.**, Geerling, G., Schrader, S., Reichl, S., & Spaniol, K. (2020) *Collagenase resistance of alternative biomaterials for ocular surface reconstruction.* Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Baltimore, U.S.A. (accepted, Congress cancelled)
- Dietrich, J., Ott, L., Roth, M., **Witt, J.**, Geerling, G., Mertsch, S., Schrader, S. (2019) *Transplantation von mesenchymalen Stammzellen zur Regeneration der Tränendrüse im Mausmodell.* Kongress der Deutschen Ophthalmologischen Gesellschaft (DOG), Berlin
- **Witt, J.**, Borrelli, M., Geerling, G., Spaniol, K., Mertsch, S. & Schrader, S. (2018) *In vivo Evaluation von plastisch komprimierten Kollagen-Gelen zur Bindehautrekonstruktion* Kongress der Deutschen Ophthalmologischen Gesellschaft (DOG), Bonn.
- **Witt, J.**, Mertsch, S., Geerling, G., Schrader, S. & Spaniol, K. (2018) *Untersuchung der Explantkulturmethode zur Herstellung eines Bindehautersatzgewebes auf der Basis dezellularisierter Schweinebindehaut.* Kongress der Deutschen Ophthalmologischen Gesellschaft (DOG), Bonn.
- **Witt, J.**, Borrelli, M., Mertsch, S., Geerling, G., Spaniol, K., & Schrader, S. (2018) *Plastic compressed collagen for ocular surface reconstruction: Evaluation of the biocompatibility in vivo.* Annual Meeting of The Association for Research in Vision and Ophthalmology (ARVO), Honolulu, Hawaii, USA.
- **Witt, J.**, Geerling, G., Mertsch, S., Schrader, S., & Spaniol, K. (2017). *Decellularized porcine conjunctiva for conjunctival reconstruction.* Investigative Ophthalmology & Visual Science, 2017. 58(8): p. 4362-4362 (abstract). Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Baltimore, U.S.A.
- **Witt, J.**, Geerling, G., Mertsch, S., Borrelli, M., Schrader, S., & Spaniol, K. (2017) *Entwicklung eines Ersatzgewebes zur Bindehautrekonstruktion auf der Basis dezellularisierter porziner Konjunktiva.* Kongress der Deutschen Ophthalmologischen Gesellschaft (DOG), Berlin.

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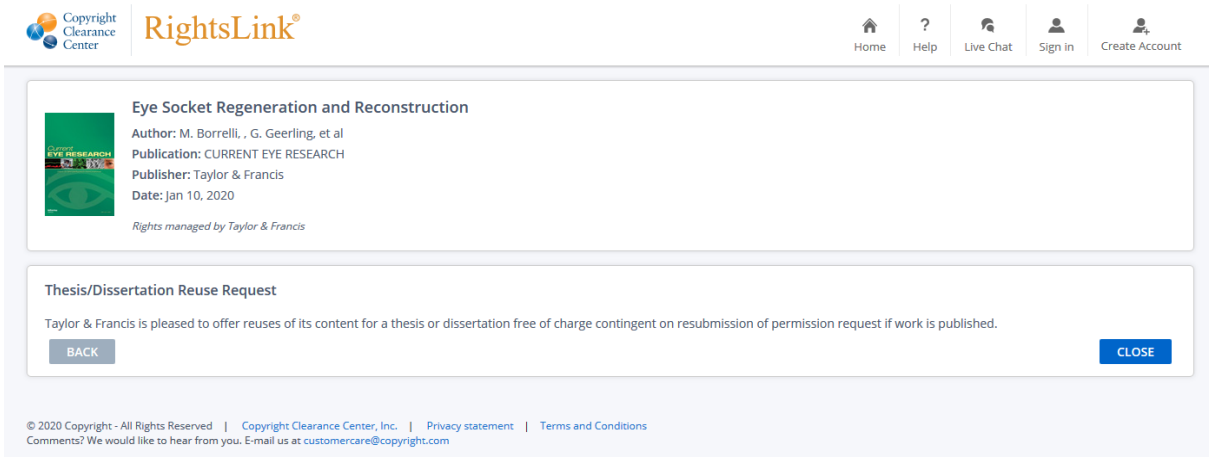

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Eye Socket Regeneration and Reconstruction

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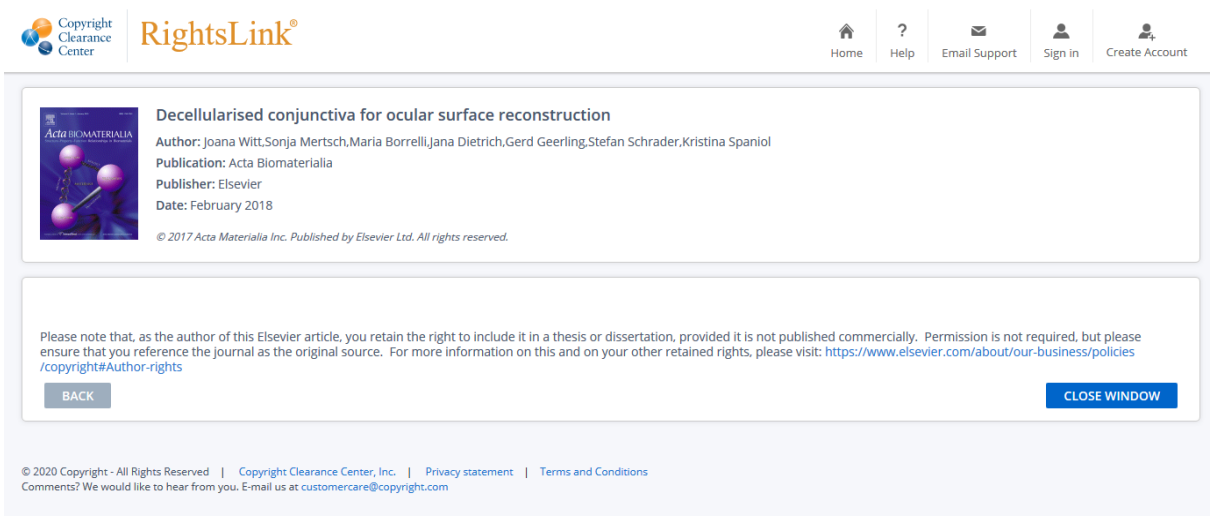

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Decellularised conjunctiva for ocular surface reconstruction

Author: Joana Witt,Sonja Mertsch,Maria Borrelli,Jana Dietrich,Gerd Geerling,Stefan Schrader,Kristina Spaniol
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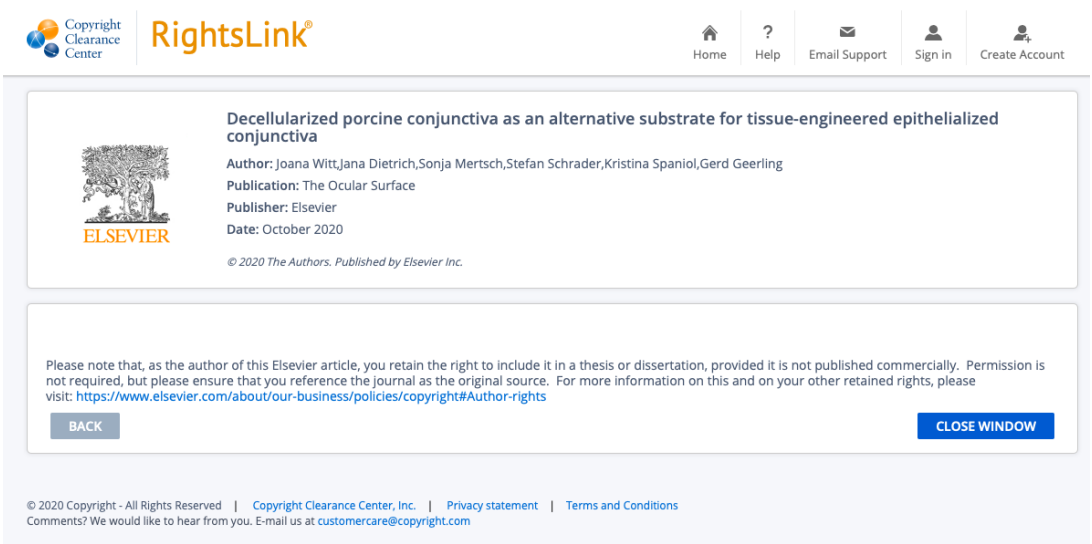

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Decellularized porcine conjunctiva as an alternative substrate for tissue-engineered epithelialized conjunctiva

Author: Joana Witt,Jana Dietrich,Sonja Mertsch,Stefan Schrader,Kristina Spaniol,Gerd Geerling
Publication: The Ocular Surface
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Declaration of authorship - Eidesstattliche Erklärung

Ich, Joana Marie Witt, versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Diese Dissertation wurde keiner anderen Fakultät vorgelegt, noch wurde bisher ein erfolgloser Promotionsversuch unternommen.

Ort, Datum Joana Marie Witt