Aus der Augenklink

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Keratin Based Biopolymer Films

As Substrate For Ocular Surface Reconstruction In Vitro

Dissertation

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SUMMARY

BACKGROUND: Severe ocular surface disease can result in blindness intractable for surgical cure by means of corneal transplantation. Limbal stem cell transplantation which uses human amniotic membrane (AM) as cell carrier is the most widely used therapeutic modality to reconstruct the corneal or conjunctival surface. However, AM has downsides including potential disease transmission, variable quality and limited transparency. Hence it is necessary to find standardized alternative substrates for ocular surface reconstruction.

MATERIALS AND METHODS: My study investigated the cell growth behavior of human corneal epithelial cell line (HCE-T) on a new, transparent keratin film (KF) based on human hair and compared this with human denuded AM and plastic as control in vitro. The cultivation was carried out in 24-well culture plates with 5×10^3 or 1×10^4 cells/well seeded onto KF, AM and plastic for 72 h to detect cell proliferation by an MTT-assay. In addition, cell migration was observed by a scratch-wound healing assay for 48 h and cell attachment behavior was investigated for 30 min with an adhesion assay. Cells cultured on KF and AM at an air-liquid interface for 2 weeks were stained with hematoxylin and eosin for histology.

RESULTS: The group seeded with 5×10^3 cells/well indicated that there was no difference between AM and KF after proliferating 24 h and 72 h ($p = 0.582$ and $p = 0.066$, respectively) while proliferation was higher on AM than KF after 48 h ($p = 0.005$). Cells cultivated on plastic showed significantly superior proliferation behavior than on AM and KF ($P < 0.05$) after 72 h. Another group seeded with 1×10^4 cells/well demonstrated superior proliferation on AM than KF after 48 h and 72 h ($p = 0.001$ and $p = 0.003$, respectively). After 24 h proliferation on AM was similar to KF ($p = 0.252$). However, proliferation was significantly better on plastic than on AM and KF ($p = 0.002$ and $p = 0.001$, respectively) after 24 h and still better compared to on AM and KF ($p = 0.002$ and $p = 0.000$, respectively) after 48 h, while similar proliferation was

observed on plastic and AM after 72 h ($p = 0.145$); The results of the scratch-wound healing assay demonstrated a significant superior cell migration on KF than on AM over 48 h ($p < 0.05$). However cell migrated on plastic still better than on KF and AM ($p < 0.05$). Moreover, there were significantly more cells attached to AM compared to plastic and KF ($p = 0.032$ and $p = 0.001$, respectively) and no significant difference was observed between KF and plastic ($p = 0.147$) after 30 min. In summary, cell proliferation and migration was best on plastic, proliferation better on AM compared to KF and migration faster on KF than AM. While adhesion was best on AM and it was similar on KF and plastic (see table). Histology demonstrated HCE-T cells cultured on KF and AM form a multilayered epithelium at an air-liqiud interface which is similar to the normal human corneal epithelium.

	Proliferation	Migration	Adhesion
KF	+	++	+
AM	++	+	+++
Plastic	+++	+++	+

"+ ~ +++" indicate progressive increase in cell growth behavior on the substrates. (KF: keratin film – AM: amniotic membrane)

CONCLUSION: Transparent biopolymer keratin films based on human hair support cell proliferation, migration, adhesion and differentiation of HCE-T cells in vitro. Therefore, it could be a promising candidate as a cell substrate for ocular surface reconstruction.

ZUSAMMENFASSUNG

HINTERGRUND: Eine schwere Augenoberflächenerkrankung kann zu einer chirurgisch therapieresistenten Erblindung führen. Die Transplantation von Limbus-Stammzellen auf Amnion-Membran (AM) als Zellträger ist die am häufigsten verwendete therapeutische Modalität zur Rekonstruktion der Augenoberfläche. Aber die AM hat einige Nachteile, einschließlich der mögliche Übertragung von Infektionen, eine variable Qualität und eine geringe Transparenz. Es ist daher notwendig, standardisierte alternative Substrate zu finden.

MATERIAL UND METHODEN: Das Zellwachstumsverhalten einer menschlichen kornealen Epithelzelllinie (HCE-T) wurde auf einem neuen, transparenten Keratin-Film (KF) auf Basis von menschlichen Haaren untersucht und mit AM und Kunststoff in vitro verglichen. 5×10^3 oder 1×10^4 Zellen/Loch wurden auf KF, AM und Kunststoff für 72 h ausgesät, um die Zellproliferation durch MTT-Assay zu bestimmen. Die Zellmigration wurde mit einem Kratz-Wundheilungsassay für 48 h beobachtet und die Zellanheftung nach 30 min durch einen Adhäsionstest untersucht. Für die Histologie wurden Zellen auf KF und AM an einer Luft-Flüssigkeitsgrenzfläche für 2 Wochen kultiviert und mit Hämatoxylin und Eosin gefärbt.

ERGEBNISSE: Proliferation und Migration waren am besten auf Kunststoff. Eine bessere Proliferation fand sich auf AM als auf KF und eine schnellere Migration auf KF gegenüber AM. Die Adhäsion war am besten auf AM und schlechter auf KF und Kunststoff (siehe Tabelle). HCE-T-Zellen kultiviert auf KF und AM an einer Luft-Flüssigkeitsgrenzschicht zeigten ein mehrschichtiges Epithel ähnlich der normalen menschlichen Hornhaut.

	Proliferation	Migration	Adhäsion
KF	+	++	+
AM	++	+	+++
Kunststoff	+++	+++	+

"+ ~ +++" zeigen progressive Zunahme des Zellwachstumsverhalten auf den Substraten. (KF: Keratin-Film – AM: Amnion-Membran)

SCHLUSSFOLGERUNG: Transparente Biopolymer-Keratin-Filme auf Basis menschlichen Haars unterstützen Zellproliferation, Migration, Adhäsion und Differenzierung von Hornhaut-Epithelzellen in vitro. Daher könnte es ein vielversprechender Kandidat als Substrat für eine Augenoberflächenrekonstruktion sein.

ABBREVIATIONS

°C	degree Celsius
%	percent
µL	microliter
µm	micrometer
µM	micromole
AM	amniotic membrane
ANOVA	analysis of variance
BCVA	best corrected visual acuity
bFGF	basic fibroblast growth factor
BSS	balanced salt solution
CO_2	carboxic oxide
Da	dalton
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
g	gram
GC	gelatin-chitosan
h	hour
HCE-T	human corneal epithelial cell line
KF	keratin film
KGF	keratinocyte growth factor
LESC	limbal epithelial stem cell
LSCD	limbal stem cell deficiency
M	mole
min	minute
mL	milliliter
mM	millimole
MTT	3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
OS	ocular surface
PBS	phosphate-buffered saline
PCL	Poly (ε-caprolactone)
PET	polyethylene terephthalate
PH	potential of hydrogen
SCID	severe combined immuno-deficiency disease
SD	standard deviation
SDS	sodium dodecyl sulfate
SJS	Stevens-Johnson syndrome
SPSS	statistical product and service solutions
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

TABLE OF CONTENTS

1 INTRODUCTION

1.1 THE OCULAR SURFACE

1.1.1 Anatomy of the ocular surface

The ocular surface (OS) is the outermost layer of the eye and is composed of cornea and conjunctiva, which includes bulbar, palpebral and forniceal conjunctiva. The normal anterior surface is covered by stratified epithelia which can protect the ocular surface from pathogens and avoid infection by producing antimicrobial peptides (McDermott, **2004**).

The cornea – a main refractive element of the eye – consists of five layers which are epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. Due to the uniform structure, avascularity and deturgescence, the cornea is transparent which is important for maintaining clear vision. Aging or damaged corneal epithelial cells can be replaced by the limbal epithelial stem cells (LESC) – which reside in the peripheral corneal limbus and have superior proliferative capacity compared to the central corneal epithelial cells. LESC can migrate from the limbus into the center of the cornea and differentiate into mature corneal epithelial cells to regenerate corneal epithelium (Sun and Green, **1977**; Schermer, Galvin et al. **1986;** Pellegrini, Golisano et al. **1999;** Dua and Azuara-Blanco, **2000;** Sun and Lavker, **2004**). (Fig. 1).

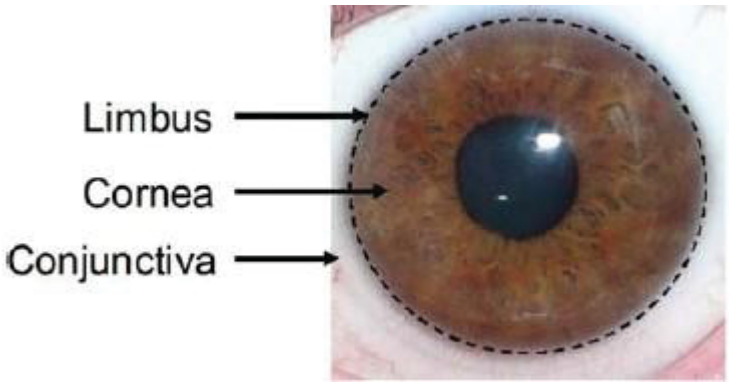

Figure 1. Location of the limbus, which is the transition zone between peripheral cornea and conjunctiva. *From O'Callaghan and Daniels, 2011.

The conjunctiva, a thin and transparent mucous membrane covering the sclera, can secrete mucus and tears to help lubricate the eye. It has three parts – bulbar, forniceal and palpebral conjunctiva, and it is composed of a stratified epithelium and vascularized stroma histologically. The conjunctival epithelium includes goblet cells, which are responsible for producing the conjunctival mucin component of the tear film. The conjunctival stroma includes the accessory lacrimal glands of Krause and Wolfring, which can secrete the baseline tear. When the LESC are seriously injured, the conjunctival epithelium can migrate onto the corneal surface and result in corneal neovascularisation and opacity (Kinoshita, Friend et al. **1983**; Danjo, Friend et al. **1987**).

The corneal and conjunctival epithelia express different cytokeratins (CK), which exist in almost all the invertebrates epithelial cells. There are two subfamilies of cytokeratins, which are type I (acidic, including CK9 to CK20) and type Ⅱ (neutral/basic, including CK1 to CK8). The type of cytokeratin expression can be used to evaluate the phenotype of the ocular surface epithelial cells, because different epithelial cells have different patterns of cytokeratins. It has been shown that keratins of the corneal epithelium are composed of a major cytokeratin pair, basic keratin CK3 and acid keratin CK12, and they have been used as a corneal type differentiation marker (Moll, Franke et al. **1982;** Meller and Tseng, **1999**). In the normal human conjunctival epithelium, the cytokeratin

pair – CK4 and CK13 – have been used as conjunctival differentiation marker (Krenzer and Freddo, **1997**). Therefore, evaluating the cytokeratin pattern is useful for the epithelial cells culture in vitro to investigate if the cells maintain the phenotype, which potentially would be important for ensuring a good epithelial graft for transplantation in vivo.

1.1.2 Ocular surface diseases

Etiologically, ocular surface diseases can be classified as inflammatory, infectious, congenital, hereditary, traumatic, toxic, nutrient, iatrogenic, immunological as well as degenerative and they can lead to discomfort, superficial punctate keratopathy and loss of corneal transparency. Advanced disorders of the ocular surface, Stevens-Johnson syndrome (SJS), severe keratitis, chemical or thermal burns, ocular cicatricial pemphigoid and aniridia, can result in severe discomfort, ulceration, severe impairment of vision and even blindness (Shapiro, Friend et al. **1981**; Dua and Forrester, **1990**).

Cornea-related disease is the second main blindness cause all over the world reported by the World Health Organization. There are about 50 million people who suffer from bilateral blindness and at least 150 million people who have impaired vision in both eyes mainly as a result of corneal disease. Moreover, patients with corneal blindness are increasing by 1.5 million to 2 million every year worldwide (Whitcher, Srinivasan et al. **2001**; Foster, **2003**). Therefore, the treatment of ocular surface diseases is a great challenge for the ophthalmologists to reduce global blindness.

1.2 LIMBAL STEM CELL DEFICIENCY

1.2.1 Introduction of the limbal stem cell deficiency

Corneal epithelial stem cells are located at the limbus which is the transition zone between the conjunctiva and the peripheral cornea. The limbal epithelial stem cells are the source of the corneal epithelial renewal and they reconstruct the aging or the damaged corneal epithelium to maintain the corneal integrity (Sun and Green, **1977**). During homeostasis or after injury of the corneal epithelium, the limbal stem cells migrate into the center of the cornea from the limbus and produce transient amplifying cells to replace the lost corneal epithelial cells and transform them into normal multilayer corneal epithelium gradually (Cotsarelis, Cheng et al. **1989**; Lavker, Dong et al. **1991**; Pellegrini, Golisano et al. **1999**) (Fig. 2). However, this can not occur if the limbal stem cells are depleted.

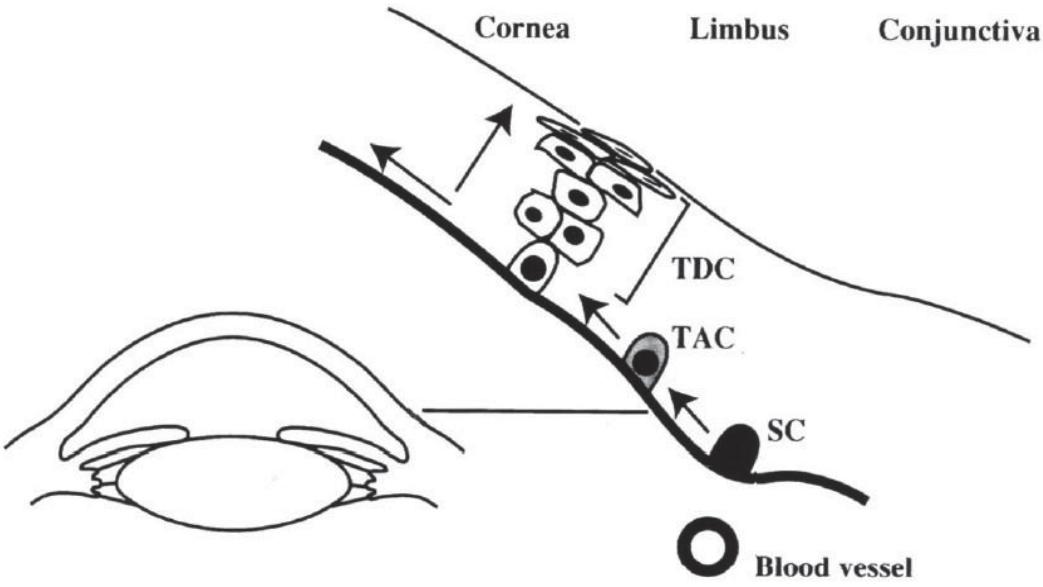

Figure 2. **Schematic diagram of the limbal stem cells transform into central corneal epithelial cells. Limbal stem cells (SC) which locate at the limbus migrate to the center of the cornea, differentiate into transient amplifying cells (TAC) and finally transform into terminally differentiated cells (TDC) to replace the lost corneal epithelium and become mature normal corneal epithelial cells. *From Holland, 1996.**

According to the causes, limbal stem cell deficiency (LSCD) can be classified as primary and secondary. The primary LSCD is related to an insufficient stromal microenvironment which can not support limbal stem cell function, such as aniridia, neurotrophic keratopathy and chronic limbitis. However, secondary LSCD is more common and associated with external factors that damage the limbal stem cells, such as thermal or chemical burn, multiple surgeries, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, severe microbial infection and contact lens-induced keratopathy. In LSCD patients, the neighbouring conjunctival epithelial cells migrate across the limbus onto the cornea surface. This leads to corneal opacity and loss of vision. Meanwhile, these patients suffer severe discomfort accompanied by chronic inflammation, neovascularization, persistent corneal epithelial defects, ulceration and stromal scar (Shapiro, Friend et al. **1981**; Puangsricharern and Tseng, **1995**; Daniels, Dart et al. **2001**; O'Callaghan and Daniels, **2011**).

1.2.2 Conventional therapy for ocular surface reconstruction

Because medical therapy often fails, surgical treatments such as penetrating or lamellar corneal transplantation are necessary to restore vision. The most widely applied treatment is full-thickness replacement of damaged cornea which is penetrating keratoplasty. If the corneal endothelium is normal, lamellar keratoplasty is used for ocular surface reconstruction which maintains the recipient's endothelium (Geerling and Seitz, **2005**). However, patients who suffer from limbal stem cell deficiency are poor candidates for conventional corneal transplantation, as grafting the central cornea does not restore limbal stem cell population. Therefore, the vision progression of these patients is limited (Williams, Roder et al. **1992**; Kuckelkorn, Keller et al. **2001**). In addition, long-term healing of the grafted epithelial surface is plagued with recurrent and persistent epithelial defects which often lead to reopacification, revascularization of the graft as well as persistent photophobia of the patients. For treating persistent corneal epithelial defects, human amniotic membrane transplantation has been widely used to

support cell repair and has also been used as cell substrate for expansion of cell sheets (Dua, Gomes et al. **2004**).

At present, LESC transplantation is being performed with cadaveric, living related-donor or autologous limbus from the contralateral healthy eye for treating patients who suffer from severe limbal stem cell deficiency. However, living-related or autologous LESC transplantation requires to take a large limbal graft from a healthy eye and thus carries the risk of inducing ocular surface disease of the allogeneic donor eye or the contralateral healthy donor eye (Chen and Tseng, **1991;** Holland, **1996**; Pellegrini, Traverso et al. **1997**; Dua and Azuara-Blanco, **2000**). Transplantation from allogeneic donors may induce rejection and requires the use of long-term systemic immunosuppressant therapy which can have significant side effects (Daya, Bell et al. **2000**; Kim, Tchah et al. **2003**).

1.2.3 Tissue engineering for ocular surface reconstruction

In recent years, methods of tissue engineering for OS reconstruction have received increased interest. Epithelial cell sheets have been expanded in vitro from small autologous limbal biopsies, which can reduce the risk of inducing ocular surface disease of the donor. To date, different cell carriers for cell sheets expansion have been proposed for ocular surface reconstruction (summarized in Table 1).

Table 1. Cell substrates for ocular surface reconstruction

Substrates	References		
	In vitro experiments (cultured cells in vitro)	**In vivo experiments**	
		Animals	**Humans (n eyes)**
Amniotic membrane	Meller et al.1999 (Rabbit conjunctival epithelial cells)	Koizumi et al.2000 (Rabbits)	Tsai et al.2000(n=6) Shimazaki et al.2002(n=13) Nakamura et al.2003(n=13)
	Koizumi et al.2000 (Rabbit corneal epithelial cells)	Wan et al.2011 (Rabbits)	Nakamura et al.2004(n=6) Shortt et al.2008(n=10) Basu et al.2012(n=50)
	Nakamura et al.2004 (Human limbal epithelial cells)		
Collagen gels	Geggel et al.1985 (Rabbit corneal epithelial cells)	Shimmura et al.2003 (Rabbits)	——
	He et al.1991 (Human limbal epithelial cells)	Merrett et al.2008 (Minipigs)	
	Dravida et al.2008 (Human limbal epithelial cells)	Liu et al.2008 (Minipigs)	
	McIntosh et al.2009 (Human limbal epithelial cells and bovine keratocytes)	Lagali et al.2008 (Minipigs)	
	Mi et al.2010 (Bovine limbal epithelial cells)		
	Levis et al.2010 (Human limbal epithelial cells)		
Fibrin gels	Han et al.2002 (Human limbal epithelial cells)	Talbot et al.2006 (Rabbits)	Rama et al.2001(n=18) Rama et al.2010(n=113)
Silicone hydrogel Contact lenses	Di Girolamo et al.2007 (Human limbal epithelial cells)	——	Di Girolamo et al.2009 (n=3)

Substrates	References		
	In vitro experiments (cultured cells in vitro)	In vivo experiments	
		Animals	Humans (n eyes)
Poly(ε-caprolactone) membranes	Ang et al.2006 (Rabbit conjunctival epithelial cells) Sharma et al.2011 (Human corneal epithelial cell line: HCE-T and human limbal epithelial cells)	Ang et al.2006 (Mice)	—
Gelatin-chitosan membranes	Zhu et al.2006 (Rabbit conjunctival epithelial cells)	—	—
Silk fibroin films	Higa et al.2008 (Murine and human limbal epithelial cells) Chirila et al.2008 (Human limbal epithelial cells) Higa et al.2010 (Rabbit limbal epithelial cells) Bray et al.2011 (Human limbal epithelial cells)	—	—
Human anterior lens capsules	Galal et al.2007 (Human limbal epithelial cells)	—	—
Keratin films	Reichl et al.2011 (Human corneal epithelial cell line: HCE-T)	—	—

"—" indicates no animal experiment in vivo or clinical result is available.

Human Amniotic Membranes

Human amniotic membrane (AM), the innermost layer of the placenta, consists of three layers – epithelium, basement membrane and avascular stroma – and has been successfully used as carrier for expansion of cell sheets in vitro and further transplanted to the ocular surface in vivo.

Several studies have shown that human AM can support proliferation, migration, differentiation and adhesion of epithelial cells in vitro (Meller and Tseng, **1999**; Schwab, **1999**; Koizumi, Inatomi et al. **2000**) and it has also been found to encourage healing of epithelial wounds and as carrier for transplantation of limbal stem cells, oral mucosal epithelial cells and conjunctival epithelial cells in vivo. Expansion of autologous epithelial cells on a carrier for transplantation clearly reduces the risk of donor site morbidity and avoids the use of long-term systemic immunosuppression (Kenyon and Tseng, **1989**; Tsai and Tseng, **1994**; Tseng, Prabhasawat et al. **1998**; Nakamura, Inatomi et al. **2004;** Ang, Tan et al. **2005**).

In this emerging clinical field and in the absence of any statistics, no standard technique for ocular surface reconstruction with bioengineered tissue has been defined, although it maybe likely that based on the numbers of clinical reports today and because AM stimulates cell growth and has antiinflammatory and antiangiogenic properties, human AM is probably the most commonly used cell carrier for tissue engineering, particularly in OS reconstruction (Tsai, Li et al. **2000**; Nakamura, Koizumi et al. **2003**; Nakamura, Inatomi et al. **2004**; Kruse and Cursiefen, **2008**; Wan, Wang et al. **2011**; Basu, Ali et al. **2012**). On the downsides, human AM has variable quality (differences in protein expression which is dependent on donor age and length of pregnancy influence clinical results after transplantation) (Hopkinson, McIntosh et al. **2006**; Gicquel, Dua et al. **2009**; López-Valladares, Teresa Rodríguez-Ares et al. **2010**), carries the risk of disease transmission from donor to recipient, induces potential immunological reactions and has

a limited transparency (Conno, Doutch et al. **2010;** Dua, Rahman et al. **2010**). Therefore, it is not surprising that clinical results also vary, that vision remains limited and that AM grafts are frequently lost in the early postoperative period (Ijiri, Kobayashi et al. **2007**; Shortt, Secker et al. **2008**). To date, a number of alternative carriers have been used as well as potential carriers proposed for ocular surface reconstruction.

Collagen Gels

Since the human corneal major component is collagen, the choice of collagen as substrate for corneal epithelial cell expansion appears obvious. Collagen has a low immunogenicity, is naturally biocompatible and relatively not expensive to isolate. In addition, collagen gels could easily be prepared from pepsin solubilized bovine dermal collagen (Elsdale and Bard, **1972**; Fagerholm, Lagali et al. **2009**).

Some investigations revealed that collagen gels support the growth of human and rabbit corneal epithelial cells in vitro (Geggel, Frienid et al. **1985**; He and McCulley, **1991**; McIntosh Ambrose, Salahuddin et al. **2009**) and of rabbits in vivo (Shimmura, Doillon et al. **2003**). However, conventional collagen hydrogels are inherently weak because of their high water content (Bell, Ivarsson et al. **1979**; Freed, Guilak et al. **2006**). Their mechanical properties can be improved by chemical crosslinking or plastic compression.

Cross-linked collagen gels can be fabricated by mixing collagen with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) and cast to produce a recombinant cross-linked collagen scaffold. These cross-linked gels supported primary human limbal epithelium growth in vitro (Dravida, Gaddipati et al. **2008**) and the findings were confirmed by transplanting the constructs with expanded corneal epithelial cells into corneas of mini-pigs in vivo (Lagali, Griffith et al. **2008**; Liu, Merrett et al. **2008**; Merrett, Fagerholm et al. **2008**). Moreover, these cross-linked

collagen grafts could be used to substitute corneal grafts in patients with keratoconus or corneal central scar (Fagerholm, Lagali et al. **2010**).

Plastic compressed collagen gels can be produced by sandwiching the collagen hydrogels between layers of nylon and metal mesh for 5 min in order to increase the mechanical strength by reducing the water content. Human limbal epithelial stem cells cultured on these gels can form multilayered epithelium which were very similar to the human central corneal epithelium in vitro (Levis, Brown et al. **2010**; Mi, Chen et al. **2010**). There is no report about plastic compressed collagen gels for OS reconstruction in vivo so far.

Fibrin Gels

Fibrin gels can be prepared by mixing fibrinogen with thrombin to induce the last step of the natural coagulation cascade. Both components – fibrinogen and thrombin – can be harvested from human plasma. Hence, fibrin gels can be produced from a totally autologous material.

The fibrin gels have successfully been used as substrate for epithelial cell expansion and differentiation in vitro and to repair corneal ulcers in humans (Lagoutte, Gauthier et al. **1989**; Duchesne, Hassan et al. **2001**; Han, Schwab et al. **2002;** Talbot, Carrier et al. **2006**). In 2001, Rama et al. cultured autologous limbal stem cells on the fibrin gels and grafted them to 18 eyes of 18 patients who suffered from unilateral limbal stem cell deficiency. After a follow-up of 12 – 27 months, 14 eyes were considered successful as evaluated by symptoms, best corrected visual acuity (BCVA), corneal transparency and impression cytology (Rama, Bonini et al. **2001**). Recently, the same group reported result of more patients (113 eyes of 112 patients who had severe limbal stem cells deficiency) showing that 76.6 % of these grafts were successful by evaluating symptoms, BCVA, corneal transparency and impression cytology after a follow-up of 1

– 10 years (Rama, Matuska et al. **2010**). Therefore, the fibrin gel is a clinically viable alternative to human AM. However, the components used for fibrin gels fabrication are harvested from plasma. This could carry the risk of disease transmission and infection.

Table 2. Fibrin gels used as cell carrier for human eyes in vivo. (LP: light perception – CF: counting fingers – HM: hand movement)

Author	Year of publication	Indications	N (eyes)	Follow-up	Success	BCVA improvement
Rama et al	2001	LSCD	18	12-27 months	77.8 % eyes	from LP or CF to 0.1– 0.8
Rama et al	2010	LSCD	113	1-10 years	76.6 % eyes	from HM or CF to 0.3-0.9

Silicone Hydrogel Contact Lenses

Human limbal epithelial stem cells expanded on silicone hydrogel contact lenses showed that contact lenses can sustain proliferation and migration from human limbal tissue to confluency with a corneal phenotype which was confirmed by immunohistochemistry in vitro (Di Girolamo, Chui et al. **2007**). So far, these silicone hydrogel contact lenses have been used for human ocular surface reconstruction in three human eyes with limbal stem cell deficiency. With a follow-up of 8 – 13 months, transparent corneal epithelium, significant improvement of symptoms and best corrected visual acuity and no corneal conjunctivalization or neovascularization were observed (Di Girolamo, Bosch et al. **2009**). Further investigations with more patients are necessary in order to confirm if they are suitable alternative carriers to human AM for ocular surface reconstruction.

Table 3. Silicone hydrogel contact lenses used as cell carrier for human eyes in vivo.

Author	Year of publication	Indications	N (eyes)	Follow-up	Morphological outcome	BCVA improvement
Di Girolamo et al	2009	- Aniridia -Conjunctival melanoma	3	8-13 months	No conjunctivalization or vascularization.	From CF / 0.03 to 0.1- 0.5

Poly (ε-Caprolactone) Membranes

Poly (ε-caprolactone) (PCL) is a material for pharmaceutical products and wound dressings, and can be prepared into highly flexible and strong membranes by dissolving PCL solution in trifluoroethanol via an electrospinning process.

Rabbit conjunctival epithelial cells which were cultured on the PCL membranes maintained their conjunctival phenotype, showed cell proliferation and formed 8 to 10 epithelial cell layers after xenografting onto dorsal subcutaneous tissue of severe combined immunedeficient (SCID) mice (Ang, Cheng et al. **2006**). Moreover, human corneal epithelial cells line (HCE-T) expanded on the PCL films retained a normal corneal phenotype and limbal epithelial stem cells grown on PCL films showed similar morphology compared with glass coverslips and human AM in vitro (Sharma, Mohanty et al. **2011**). However, reports of clinical applications for ocular surface reconstruction in humans are still missing.

Gelatin-chitosan Membranes

Chitosan is a major constituent of cornea and dermis, and as such known to play a critical role in wound healing in vitro and in vivo. It can be fabricated into gels by dissolving deacetylated chitosan into acetic acid solution and cast onto tissue culture plates until it is dry (Kratz, Arnander et al. **1997**; Sechriest, Miao et al. **2000**; Suh and Matthew, **2000**).

Pure chitosan membranes are too stiff for application to the curved ocular surface. Integration with gelatin – a soft, elastic natural material – can reduce the membrane's stiffness while further improving its mechanical strength by increasing molecular interactions (Berger, Reist et al. **2004**). Rabbit conjunctival epithelial cells cultured for

14 days on a gelatin-chitosan (GC) hydrogel grew to a confluent multilayer expressing cytokeratin CK 4, which indicated that the gelatin-chitosan membrane supported normal differentiation of conjunctival epithelial cells (Zhu, Beuerman et al. **2006)**. To date, no clinical data on the use of gelatin-chitosan hydrogels for ocular surface reconstruction have been published.

Silk Fibroin Films

Silk fibroin is a structural protein obtained from the cocoon of the silkworm Bombyx mori. It is non-immunogenic, degradable in vivo, has excellent mechanical strength and is – compared with human AM – transparent. It recently has been used as suture material and for regeneration of bone and cartilage (Kim, Jeong et al. **2005**; Lee, Baek et al. **2007**; Shangkai, Naohide et al. **2007**).

Silk fibroin can be processed as a thin transparent film by dissolving the silk fibroin by formic acid and a subsequent electrospinning process. This film can be used as biological carrier for the expansion of murine corneal limbal stem cells which maintained clonal growth characteristics and cell differentiation (Chirila, Barnard et al. **2008**; Higa and Shimazaki, **2008**; Lawrence, Cronin-Golomb et al. **2008**). In recent years, rabbit limbal epithelial stem cells and human limbal epithelial stem cells cultured on silk fibroin films in vitro were found to form stratified cells with maintained corneal phenotype (Higa, Takeshima et al. **2010**; Bray, George et al. **2011**). However, on the downsides are the considerably higher costs compared with synthetic materials. Still no clinical report about this carrier for ocular surface reconstruction so far.

Human Anterior Lens Capsules

Millions of human anterior lens capsule are removed and discarded during cataract surgery annually from cataract patients. The material is transparent and to some extent elastic. At birth, the human anterior lens capsule is approximately 4 µm thick. It consists of a dense, outer layer of collagen IV, laminin and heparin sulfate proteoglycans. Throughout life the thickness of the anterior lens capsule increases continuously by deposition of new lamellae to 28 – 33 µm at the age of 70 to 80 years (Krag, Olsen et al. **1997**; Danysh, Czymmek et al. **2008**).

Autologous and allogeneic limbal epithelial stem cells were expanded ex vivo on human anterior lens capsule obtained from 30 patients at the age of 59 to 75 years during routine cataract surgery. An identical cell density and morphology of limbal epithelial stem cells was observed compared to controls on plastic for up to 2 weeks (Galal, Perez-Santonja et al. **2007**). Further research will be necessary to evaluate whether limbal epithelial stem cells cultured on the anterior lens capsule can form a multilayered epithelium, maintain corneal phenotype and is a viable option in vivo.

The properties, advantages and disadvantages of all the biomaterials used as cell carriers are summarized in Table 4 and 5.

Table 4. Properties of biometerials as cell carriers for ocular surface reconstruction

Properties	Carriers							
	AM	Collagen gels	Fibrin gels	Silicone hydrogel contact lenses	Poly (ε-caprolactone) membranes	Gelatin-chitosan membranes	Silk fibroin films	Human anterior lens capsules
Mechanical strength	++	+ ~ ++	++	++ ?	++ ~ +++	++ ~ +++	++	++
Elasticity	+++	++~+++	+++	++ ?	++	++ ~ +++	+++	++ ~ +++
Trans-parency	+	+++	+++ (Talbot et al.2006) + (Han et al. 2002)	+++	+++ (Ang et al. 2006) + (Sharma et al. 2011)	+	+++	+++
Support for OS epithelial cell growth (in vitro)	+++	+++	+++	+++	++ ~ +++	++ ~ +++	+++	+++
Clinical success (in vivo)	++	——	++	+++	——	——	——	——
References	Nakamura et al. 2003 Shortt et al. 2008 Wan et al. 2011 Basu et al. 2012	Merrett et al. 2008 Liu et al. 2008 Dravida et al.2008 McIntosh et al.2009	Han et al. 2002 Talbot et al. 2006 Rama et al.2001 Rama et al.2010	Di Girolamo et al.2007 Di Girolamo et al.2009	Ang et al. 2006 Sharma et al. 2011	Berger et al. 2004 Zhu et al. 2006	Chirila et al. 2008 Higa et al. 2010 Bray et al. 2011	Krag et al. 1997 Galal et al. 2007

" ? " indicates clinical impression, no experimental data is available.

"——" indicates no clinical results are available.

Table 5. Advantages and disadvantages of cell carriers for ocular surface reconstruction

Carriers	Advantages	Disadvantages
AM	anti-inflammation, anti-angiogenesis, good elasticity	limited transparency, risk of disease transmission, variable quality, limited mechanical strength
Collagen gels	good transparency, good biocompatibility	limited mechanical strength
Fibrin gels	good transparency, good for tissue regeneration, degradable in vivo	risk of disease transmission
Silicone hydrogel contact lenses	good transparency, therapeutic bandage for wound healing	——
Poly(ε-caprolactone) membranes	good transparency, high mechanical strength, good biocompatibility	limited elasticity
Gelatin-chitosan membranes	good for wound healing	limited transparency, limited elasticity
Silk fibroin films	good transparency, good biocompatibility	high cost of source materials
Human anterior lens capsules	good transparency, large number of donors	variable quality (due to donor's age), risk of disease transmission, limited mechanical strength

1.2.4 Keratin films as cell carriers for ocular surface reconstruction

Keratins, cysteine-rich proteins, belong to a group of structural proteins which are formed in the epithelial cells of higher vertebrates. These proteins have high mechanical strength because of a large number of disulfide bonds. Hard or filamentous structures such as hair, wool, nails, horns, hoofs and feathers – which can be obtained from hair salons and from the food industry as unused protein-rich waste – can be used as sources for these proteins.

In recent years, coatings, films and scaffolds based on keratin from wool or hair have been established, and their practical usefulness as a growth substrate has been investigated. Mouse cell line L929 fibroblasts cultured on keratin extracted from wool demonstrated improved growth and attachment compared to collagen and glass (Yamauchi, Maniwa et al. **1998**; Tachibana, Furuta et al. **2002**).

In a previous study, the usefulness of 50 µm thick human hair keratin coated substrates – obtained by casting keratin dialysate into a plastic plate – for cell cultivation in vitro has been reported (Reichl, **2009**). The biomechanical strength and elastic modulus of the keratin films (KF) could be modified by mixing the keratin dialysate with an alkaline dialysate at different ratios and subsequent curing temperatures between 70 °C and 110 °C. Substrates with biomechanical stability and improved transparency compared with human AM could be produced. Subsequent investigation showed that the proliferation of HCE-T cells on the transparent KF in vitro were similar to those observed on human AM (Reichl, Borrelli et al. **2011**). A correlation between composition of the keratin films and their biomechanical parameters has been reported recently by our group in a surgical ex vivo feasibility assessment on enucleated porcine eyes, which showed 90/10 keratin films (90 % aqueous keratin dialysate mixed with 10 % alkaline keratin dialysate) cured at 100 °C to offer the best compromise between mechanical strength and flexibility (Borrelli, Reichl et al. **2012**).

1.3 PURPOSE OF THE INVESTIGATION

In my study I cultivated the well-established human corneal epithelial cell line (HCE-T cells) on the transparent and transferable 90/10 keratin films cured at 100 °C which were fabricated from human hair (Reichl, Borrelli et al. **2011;** Borrelli, Reichl et al. **2012**) in vitro to investigate if these films are a suitable cell substrate for ocular surface reconstruction and compared their ability to support cell proliferation, migration, attachment and morphology with denuded human amniotic membrane and commercially available plastic culture plates in vitro by using MTT, scratch-wound healing, adhesion assays as well as routine histology.

2 MATERIALS AND METHODS

2.1 MATERIALS

Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (at a ratio of 1:1), Hank's balanced salt solution (BSS), penicillin/streptomycin, phosphate-buffered saline (PBS), amphotericin B and trypsin-EDTA were purchased from PAA (Cölbe, Germany). Fetal bovine serum (FBS), insulin, epidermal growth factor (EGF) and cell scrapers were obtained from Biochrom (Berlin, Germany). 0.9 % sodium chloride (NaCl), urea, thiourea, sodium dodecyl sulfate (SDS), 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), sodium hydroxide (NaOH), ethanol, 2-mercaptoethanol, dimethyl sulfoxide (DMSO), hematoxylin, eosin, Mayer's hemalaun, xylene and methanol were purchased from Carl Roth (Karlsruhe, Germany). Spectra/Por® 1 dialysis membrane (MWCO 6-8000 Da) was purchased from Spectrum (Rancho Dominguez, US). Vivaspin® was purchased from Sartorius (Goettingen, Germany) and Polyethylene terephthalate (PET) foil was obtained from LTS Lohmann (Andernach, Germany). Dispase II and trypan blue solution were obtained from Invitrogen (Karlsruhe, Germany). Hydroxyurea, glycerol and sucrose were purchased from Sigma (Steinheim, Germany). Technovit® 8100 kit were obtained from Heraeus Kulzer (Hanau, Germany). Aceton was purchased from Otto Fischar (Saarbrücken, Germany). Coverslips were obtained from Thermo (Braunschweig, Germany). Hemacytometer was purchased from A.Hartenstein (Würzburg, Germany). Polystyrene 24-well cell culture plates were obtained from TPP (Trasadingen, Switzerland). Polystyrene 96-well cell culture plates were purchased from Greiner Bio-one (Frickenhausen, Germany). Metal rings and plastic rings were obtained from Hummer & Rieß (Nürnberg, Germany). The CellTiter 96® Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Mannheim, Germany).

2.2 METHODS

2.2.1 Keratin film fabrication

Keratin films based on human hair were prepared by the Institute of Pharmaceutical Technology of the university of Braunschweig according to a protocol previously established there (Reichl, Borrelli et al. **2011**). For this, human hair was obtained from a local hair salon, intensively washed with water containing 0.5 % sodium dodecyl sulfate (SDS), then rinsed with fresh water and air-dried. Human hair keratin was extracted according to the Shindai method (Nakamura, Arimoto et al. **2002**). In brief, the hair (20 g) was mixed with extraction medium, which is an aqueous solution (400 mL, pH 8.5) containing 25 mM Tris, 2.6 M thiourea, 5 M urea and 5 % 2-mercaptoethanol and incubated at 50 ℃ for 72 h. The mixture was centrifuged at 4500 g for 15 minutes and the supernatant filtered using filter paper with a pore size of 2.5 µm. The filtrate, denoted as "Shindai extract", was stored at –20 ℃ and thawed when required. The Shindai extract was exhaustively dialysed against demineralized water (100 mL extract in 5000 mL water) using a dialysis cellulose membrane (6-8000 Da) at 20 ℃ for 24 h. The procedure was repeated several times until no 2-mercaptoethanol was detected in the dialysis fluid. The keratin dialysate, denoted as "aqueous keratin dialysate", was centrifuged at 10,000 g for 30 minutes to remove coarse aggregates and immediately used to fabricate the keratin films. In a second phase of dialysis, the same amount of Shindai extract was dialysed against a 0.25 M sodium hydroxide (NaOH) solution in the same method as described above at 4 ℃. This NaOH dialysate was concentrated by means of ultrafiltration using Vivaspin® 20 concentrators. The filtrates were discarded and the supernatant was diluted several times with 0.05 µM NaOH to generate the "alkaline keratin dialysate".

For my studies I used keratin films with an aqueous/alkaline keratin dialysate at a ratio of 90:10 cured at 100 ℃ to evaluate proliferation, migration and adhesion of HCE-T cells and compared this with denuded human AM and plastic in vitro. Briefly, the keratin films were produced by mixing the aqueous keratin dialysate with alkaline keratin dialysate at the given ratio and 1 % glycerol was added as a softening agent. The mixture was cast on hydrophobic coated PET sheets and then allowed to dry overnight. Thereafter, they were cured at 100 °C for 2 h to form transparent and transferable keratin films. All the steps for preparing the films were performed under aseptic conditions. The keratin films were found to be chemically and mechanically stable for at least six months at room temperature (Reichl, Borrelli et al. **2011**). This 90/10 keratin film cured at 100 °C was found to offer the best compromise between mechanical strength and flexibility in an ex vivo experiment by Borrelli et al (Borrelli, Reichl et al. **2012**).

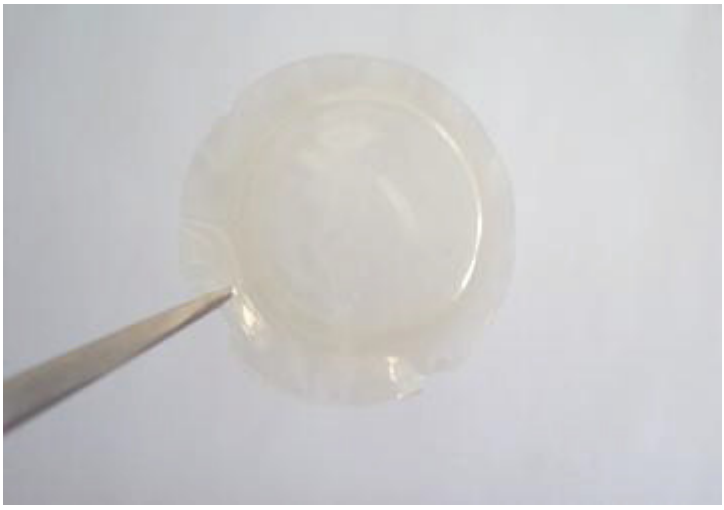

Figure 3. The keratin films (KF) based on human hair can be handled with forceps and exhibit good transparency.

2.2.2 Human amniotic membrane preparation

According to the tenets of the Declaration of Helsinki for research, human AM was obtained from an elective caesarean section after informed consent. The intact amniotic membrane was stripped from the chorion by blunt dissection, washed with sterile 0.9 % sodium chloride (NaCl) containing 1 % penicillin/streptomycin and 1 % amphotericin B to remove blood clots, and sutured onto 7.5 × 7.5 cm sterile sponge sheets (Katena, New Jersey, US), with the epithelium facing upwards. All steps were performed under aseptic conditions. Then as previously described the membranes were cryopreserved at –80 ℃ in DMEM and glycerol at a ratio of 1:1 and only thawed prior to use (Tseng, Prabhasawat et al. **1997**).

Figure 4. Human amniotic membrane (AM) stripped from the chorion and sutured on sterile sponge sheets. The AM exhibits semi-transparency.

2.2.3 Cell culture

HCE-T cells (human corneal epithelial cell line) stem from human corneal epithelial cells (Riken Cell Bank, Ibaraki, Japan) and were immortalized by Sasaki et al. by infection with a recombinant SV40-adenovirus vector in 1995. These cells retain the properties of normal corneal epithelial cells in that they are able to form a multilayered epithelium if cultured at an air-liquid interface indicating that they maintain the programmed gene for differentiation (Araki-Sasaki, Ohashi et al. **1995**). At present, HCE-T cells are the most frequently used human corneal epithelial cell line and represent a standard tool for bioavailability, toxicity and drug permeation assessments (Toropainen, Ranta et al. **2001**; Becker, Ehrhardt et al. **2007**; Reichl, **2008**).

The HCE-T cells I used for my studies were provided by Dr. K. Araki-Sasaki (Kagoshima, Japan). The cells were cultivated in 24-well cell culture plates with 500 µL culture medium per well under standard cell culture conditions in a humidified atmosphere containing 5 % CO_2 and 95 % air at 37 ℃. The cell culture media consist of 1:1 DMEM/Ham's F12 medium supplemented with 10 ng/mL EGF, 5 % FBS, 5 mg/mL insulin, 1 % penicillin/streptomycin and 0.5 % DMSO solution. The media were changed every 2 days.

Before starting the cell culture experiments, the KF were intensively equlibrated in sterile distilled water containing 1 % penicillin/streptomycin and 1 % amphotericin B under constant gentle shaking. The equilibration solution was changed twice daily for seven days. Unsuspended KF tended to show an irregular wrinkled surface which would interfere with visual cell assessment and – quite likely – also with cell migration and proliferation. In order to achieve an even seeding area and fix the substrate on the bottom of the cell culture well, KF were punched in circles (diameter were 11 mm) and

clamped between a metal ring and a plastic ring of suitable size during cell culture. The self-constructed ring arrangement also provided a standardized growth area (0.283 cm^2) for cell culture (Fig. 5-7). Before cell expansion, they were incubated with culture medium overnight.

Before using the human AM, it was thawed in a 37 ℃ water bath, washed at least three times with sterile PBS to remove the storage medium and then incubated with Dispase II (1.2 U/mL) at 37 ℃ for 2 h to loosen cellular adhesion, followed by gentle scraping to remove the remaining amniotic epithelial cells with a cell scraper to prepare denuded AM (Riau, Beuerman et al. **2010**). Thereafter, the denuded AM was intensively washed in PBS to remove the dispase, clamped within the same ring devices and incubated with culture medium overnight before cell culture. As control, cells were seeded on a plastic substrate. Empty ring arrangements were placed on the bottom of the culture wells to obtain the same seeding area for the cells as with KF and AM.

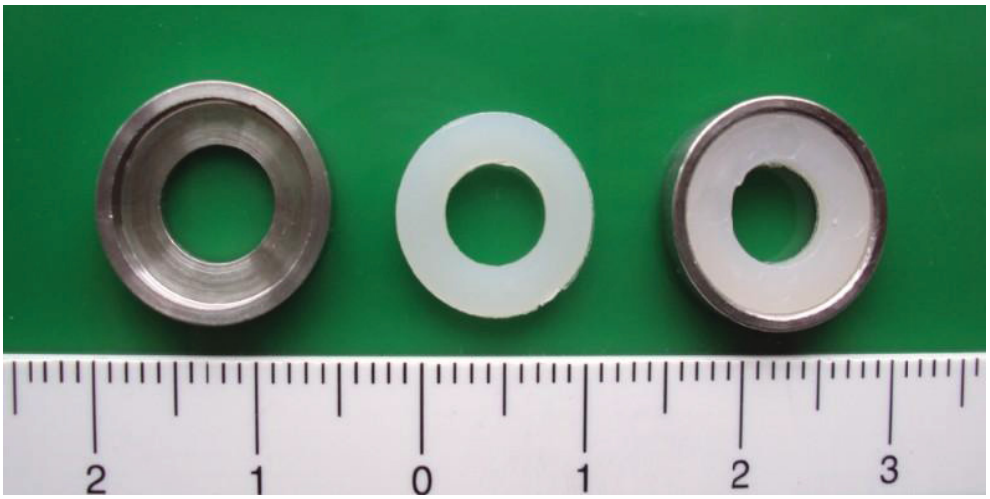

Figure 5. **The self-constructed ring arrangements used in the cell culture experiments to achieve an even and defined growth area. The metal ring, plastic ring and the constructed two-ring arrangement are shown from left to right respectively.**

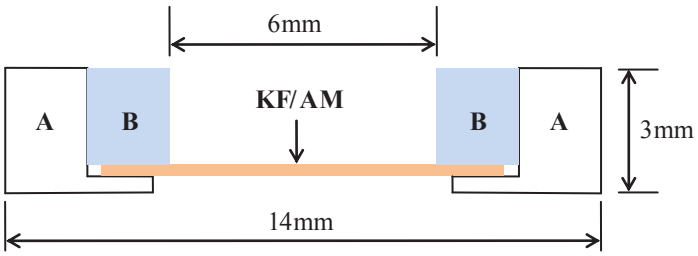

Figure 6. Schematic diagram of the ring arrangements used for cell culture. The substrates – KF or AM – were fixed between the metal ring (A) and plastic ring (B).

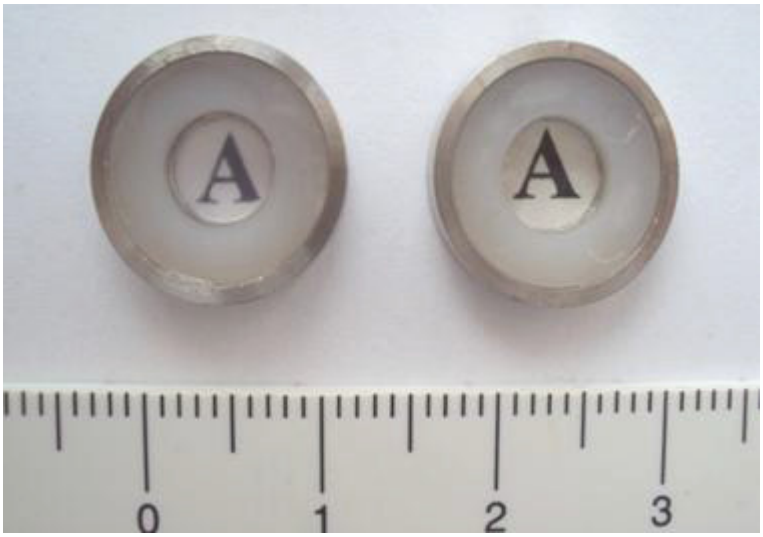

Figure 7. Optical transparency of human AM (left) and KF (right). AM and KF are clamped within the ring arrangements. KF exhibits higher transparency than the AM indicated by the clarity of the "A" underneath.

2.2.4 Cell proliferation: MTT-assay

Cell proliferation analysis on cell-carriers is an essential step for evaluating biocompatibility of biomaterials. In this study, proliferation of HCE-T cells on KF, AM and plastic was evaluated by determining the mitochondrial function of cells using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT). Living cells can convert the MTT into blue formazan and the absorbance of the solution can be analysed directly by a spectrophotometer to determine viable cell numbers (Mosmann.**1983**; Uludag and Sefton, **1990**).

My proliferation studies were performed in 24-well polystyrene plates using the ring devices described above. KF, AM and plastic were used as growth substrates. The experiments were repeated three times and each assay was performed in triplicate. The assay was carried out according to the manufacture's protocol. In brief, 5×10^3 cells/well and 1×10^4 cells/well were seeded on each substrate and incubated for 24 h, 48 h and 72 h, respectively in 5 % CO_2 and 95 % air at 37 ℃. After the respective culture period, 30 µL of MTT dye solution was added to each well and further incubated for 1 h. After that, the formed formazan crystals were dissolved by adding 200 µL solubilization solution to each well and the culture plates were gently shaked for 10 min. At last, 215 µL solution of each well was transfered into 96-well polystyrene plates and the absorbance was measured with a microplate reader (BMG Labtech, FLUOstar OPTIMA, Germany) at 570 nm. Absorbance in the wells which only the culture medium had been added served as control for zero setting.

2.2.5 Cell migration: scratch-wound healing assay

Cell migration is key to a number of therapeutically important biological responses, including angiogenesis. A commonly used method to assess cell migration is the scratch-wound healing assay, which is simple, low-cost and mimics cell migration during wound healing in vivo. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration until the wounds closed and comparing the images to quantify the wound width. It is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration (Rodriguez, Wu et al. **2005**; Liang, Park et al. **2007**).

The scratch-wound healing assays were carried out in 24-well polystyrene plates using the ring devices as previously described (Wang, Teh et al. **2010**; Zheng, Mohan et al. **2011**). KF, AM and plastic were used as seeding substrates and triplicate experiments with triplicate samples were performed. Before culturing the cells, coverslips were put underneath the KF and AM for scratching more easily. Briefly, 4×10^4 cells/well of HCE-T cells were seeded onto each substrate and incubated with culture medium for 24 h to subconfluence (about 90 % confluence). To inhibit cell proliferation, cells were treated with 5 mM hydroxyurea for 24 h until cells became confluent. Thereafter, a single linear scratch was made carefully in the center of the cell sheet with a sterile 200 µl yellow plastic pipette tip to create an 800 – 900 µm cell free wound. The cell sheet was washed three times with PBS to remove floating cells. To identify the edge of the wound, the cells on each substrate were fixed with 90 % methanol for 10 min and stained with Mayer's hemalaun solution for 30 min at room temperature. Then the width of the denuded space was measured in ten different positions randomly and the average was calculated. This was done in separate cultures after 0 h, 12 h, 24 h, 36 h and 48 h of migration. The images were photographed and the width of the wound measured under 100 × magnification using a Nikon digital camera (COOLPIX 4500) which was mounted onto the inverted microscope Eclipse TS100 (Nikon, Japan).

2.2.6 Cell attachment: adhesion assay

Adhesion capacity of HCE-T cells was analysed with an adhesion assay as previously described (Tang, Huang et al. **2011**; Germano, Kennedy et al. **2012**). The experiments were repeated three times and each assay was performed in triplicate. Assays were performed in 24-well culture plates. 5×10^4 cells/well were seeded on KF, AM and plastic with culture medium. After incubating for 30 min in 5 % CO_2 and 95 % air at 37 ℃, unattached cells were removed by washing three times with PBS. The adherent cells were fixed with 90 % methanol for 10 min, stained with Mayer's hemalaun solution for 30 min at the room temperature and washed with PBS three times. For each triplicate, the number of adherent cells in five randomly selected high power fields (200 × magnification) was counted by using the inverted microscope Eclipse TS100 (Nikon, Japan) and the counts were averaged.

2.2.7 Histology (H&E stain)

In order to observe whether HCE-T cells cultured on KF and human AM can form a multilayered epithelium as the normal corneal epithelium, HCE-T cells were seeded onto KF and denuded human AM which were fixed in the two-ring devices in 24-well culture plates. In brief, 1×10^4 cells/well HCE-T cells were seeded on the KF and AM for 7 days in submerged culture until they were confluent and cultivated further 14 days at an air-liquid interface. Thereafter, the KF and AM were punched out and a newly developed histo-Technique system (Technovit) was used for the histology. This histo-system processes the tissue at a low temperatue (4 ℃) to guarantee genuine testing and uses resins for embedding to perform the cutting procedures easily and achieve optimal stretching of specimens. Briefly, the specimens were fixed in 4 % formalin for 4 hours at 4 ℃ and then washed overnight in 6.8 % sucrose at 4 ℃. Before being dehydrated in 100 % aceton for 1 hour at 4 ℃, specimens were embedded in resin which was performed in a specific Teflon embedding mould overnight at 4 ℃.

At last, the specimens were cut into 4 μm sections by a rotary microtome (Leica RM2255, Nussloch, Germany) and stained with hematoxylin and eosin (H&E stain).

2.2.8 Statistical analysis

Values are given as the mean value ± standard deviation (SD). All Statistical analysis was carried out using SPSS 19.0 software (IBM Corporation, USA). Differences between KF, AM and plastic of HCE-T cell proliferation, migration and adhesion were assessed by one-way analysis of variance (ANOVA). Two-tailed value of less than 0.05 ($P < 0.05$) were considered to be statistically significant.

3 RESULTS

3.1 CELL PROLIFERATION IN VITRO

The data of the cell proliferation studies are listed in Table 6 and 7 (shown in the appendix) as the mean ± SD of three wells per substrate for KF, AM and plastic. At a seeding density of 5×10^3 cells/well, HCE-T cells cultivated on plastic always showed significantly superior proliferation behavior compared to on AM and KF ($P < 0.05$) during 72 h, whereas there was no difference between AM and KF detected after 24 h and 72 h ($P = 0.582$ and $P = 0.066$, respectively). However, higher proliferation on AM was observed compared to on KF after 48 h ($P = 0.005$) (shown in Fig. 8);

At a seeding density of 1×10^4 cells/well, plastic again supported cell proliferation significantly better compared to AM and KF ($P = 0.002$ and $P = 0.001$, respectively) after 24 h and still better than AM and KF ($P = 0.002$ and $P = 0.000$, respectively) after 48 h, while no difference was detected between on plastic and AM after 72 h ($P = 0.145$). Compared with KF, HCE-T cells showed superior proliferation on AM after 48 h and 72 h ($P = 0.001$ and $P = 0.003$, respectively) but not after 24 h ($P = 0.252$) (shown in Fig. 9).

In general, HCE-T cell proliferation on plastic was always best compared to on AM and KF, and higher proliferation on AM was detected compared to on KF in vitro.

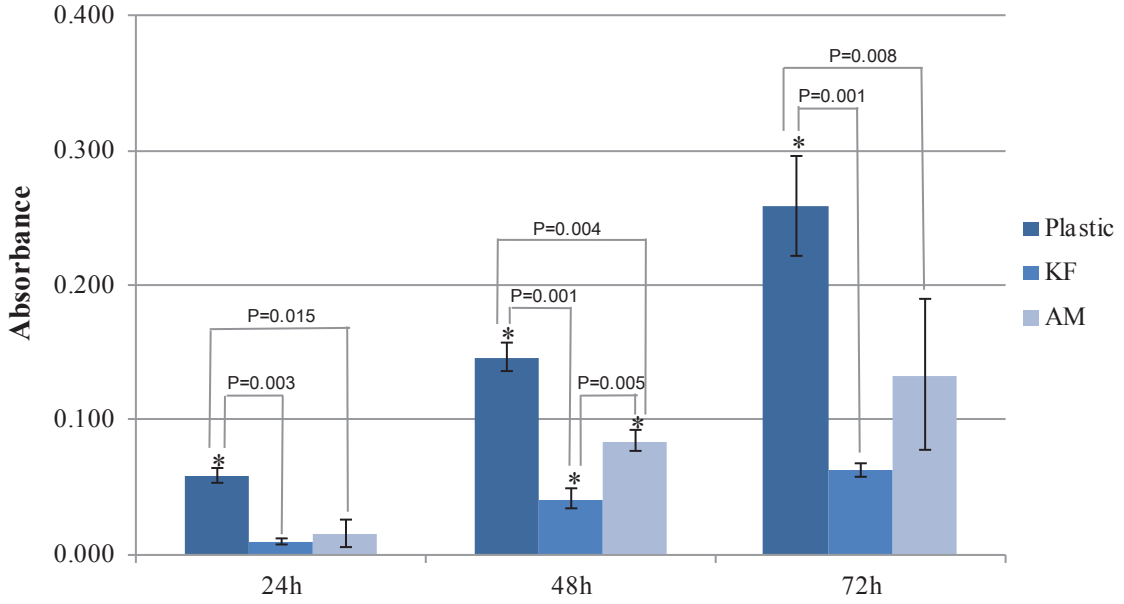

Figure 8. MTT-assay results after seeding 5×10^3 cells/well of HCE-T cells on plastic, KF and AM for 24 h, 48 h and 72 h, Mean ± SD (n=3). Bars indicate standard deviation (SD). P < 0.05 (*) were considered as significant difference. The experiments were performed in triplicate.

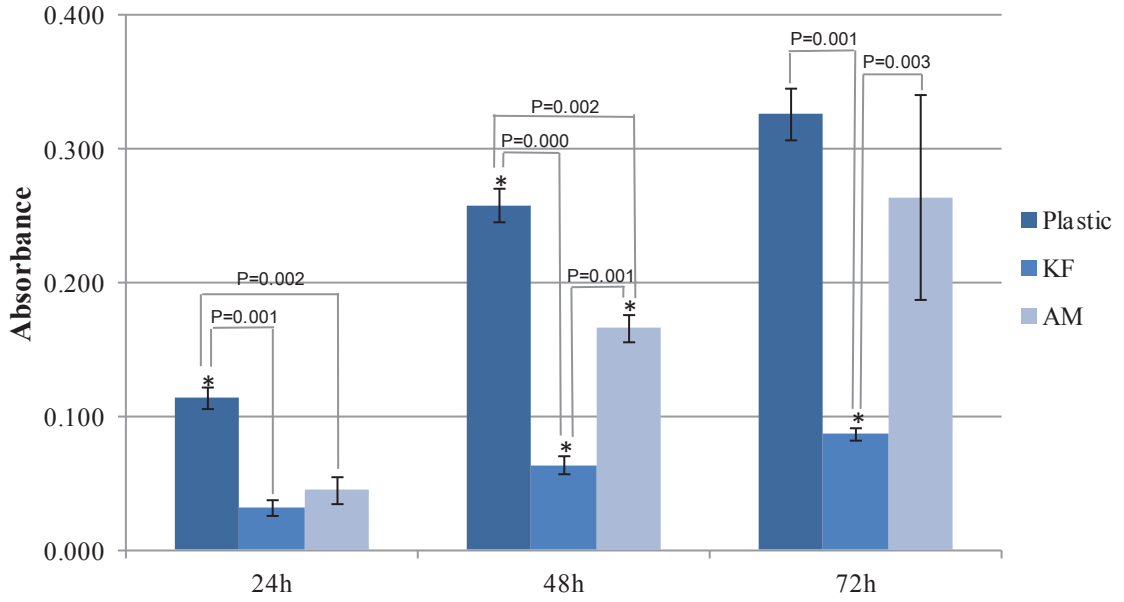

Figure 9. MTT-assay results after seeding 1×10^4 cells/well of HCE-T cells on plastic, KF and AM for 24 h, 48 h and 72 h, Mean ± SD (n=3). Bars indicate standard deviation (SD). P < 0.05 (*) were considered as significant difference. The experiments were performed in triplicate.

3.2 CELL MIGRATION IN VITRO

The representative images of the scratch-wound healing assay at different time points (0 h, 12 h, 24 h, 36 h and 48 h) on the three substrates (plastic, KF and AM) are shown in Fig. 10. The data of the wound width are listed in Table 8 (shown in the appendix), which are expressed as the mean ± SD of three wells per substrate comparing between KF, AM and plastic. The results showed that at 0 h after scratching, there was no significant difference of the wound width between plastic and KF (P = 0.935), plastic and AM (P = 0.882), KF and AM (P = 0.946).

After 12 h, the fastest migration of the HCE-T cells was found on plastic compared to on KF and AM (P = 0.005 and P = 0.000, respectively) and better migration on KF compared to on AM (P = 0.031). *After 24 h*, no difference in migration was observed between plastic and KF (P = 0.069). However, migration on plastic and KF was significantly faster compared to on AM (P = 0.000 and P = 0.000, respectively). *After 36 h*, a significantly higher cell migration was observed on plastic compared to on KF and AM (P = 0.005 and P = 0.000, respectively), whereas cell cultured on KF showed a significantly faster migration compared to on AM (P = 0.005). All wounds were healed after 48 h (shown in Fig. 11).

In brief, the results of the scratch-wound healing assay demonstrated significant better cell migration on plastic than on KF which in turn was better than on AM.

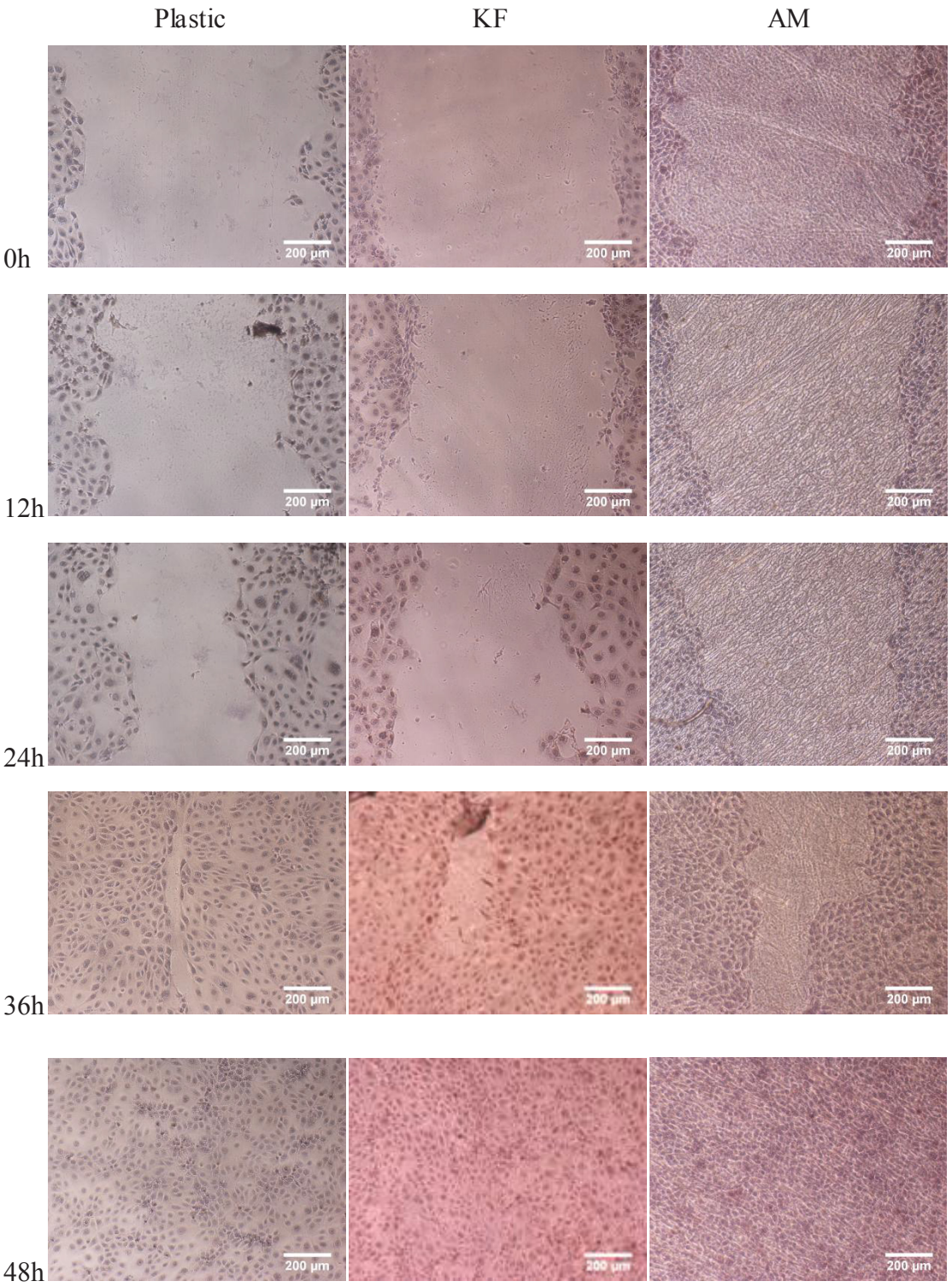

Figure 10. **Scratch-wound healing assay: migration behavior of HCE-T cells on plastic, KF and AM in vitro. Representative images taken at the different time points post wounding (at 0 h, 12 h, 24 h, 36 h, 48 h) under × 100 magnification. The experiments were performed in triplicate.**

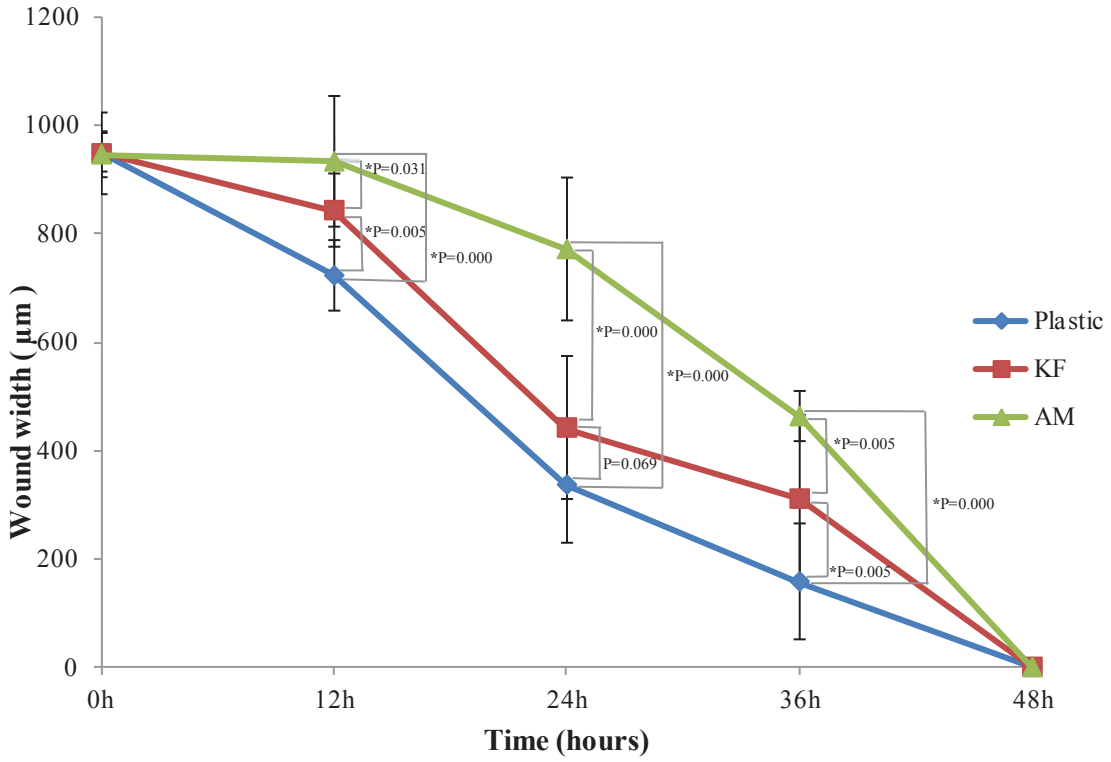

Figure 11. In vitro scratch-wound healing assay of HCE-T cells. The curve shows the wound width over 48 h and bars indicate standard deviation (SD). The experiments were performed in triplicate. P < 0.05 (*) were considered as significant difference.

3.3 CELL ADHESION IN VITRO

The data of the attached cell numbers (Table 9 in the appendix) are expressed as the mean ± SD of three wells per substrate comparing between KF, AM and plastic. After 30 min, there were significantly more HCE-T cells attached to the AM compared to plastic and KF (P = 0.032 and P = 0.001, respectively). However, no significant difference in adherence was observed between KF and plastic substrates (P = 0.147) (Fig. 12).

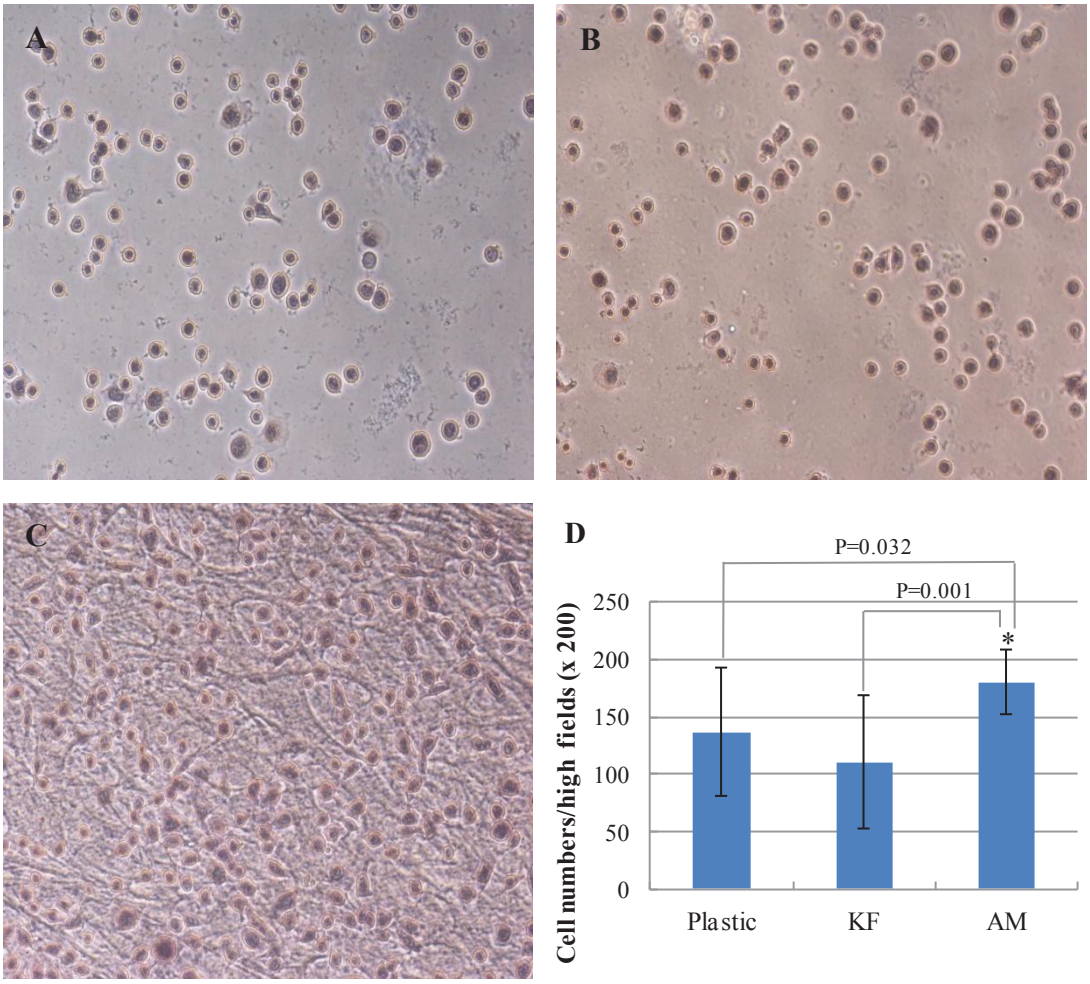

Figure 12. **Cell adhesion assay in vitro on plastic (A), KF (B) and AM (C) (under × 200 magnification). (D) Adherent cells on the three substrates expressed as mean ± standard deviation (SD) (n=3). Bars indicate standard deviation (SD). P <0.05 (*) were considered as significant difference between plastic, KF and AM.**

3.4 HISTOLOGY (H&E STAIN)

After cultivating HCE-T cells on KF and denuded AM at an air-liquid interface for 14 days, a multilayered epithelium was observed on the KF and AM similar to normal human corneal epithelium (Fig. 13).

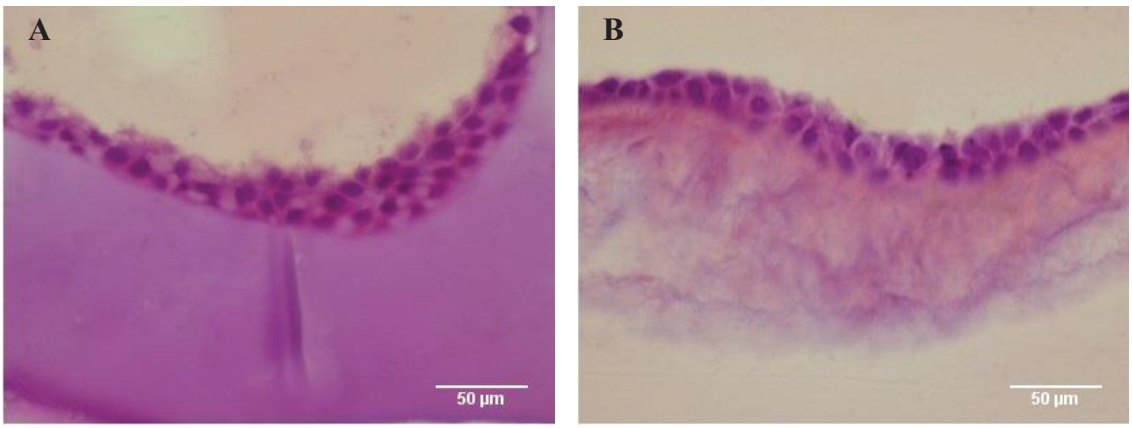

Figure 13. Histological cross sections of HCE-T cells grown on KF (A) and AM (B) after staining with hematoxylin and eosin (H&E) (under × 400 magnification). HCE-T cells cultured on KF and AM can form a multilayered epithelium of 3 to 4 layers which are similar to the normal human corneal epithelium.

4 DISCUSSION

Corneal disease is a main cause of blindness with the amount of patients affected continuously increasing by approximately 1.5 million to 2 million annually worldwide (Whitcher, Srinivasan et al. **2001**). These severe corneal disorders such as thermal or chemical burn, trauma, severe keratitis or Stevens-Johnson syndrome can lead to limbal stem cell deficiency which can not be cured by medical therapy or conventional corneal transplantation. To date, methods of tissue engineering, based on using natural or synthetic polymer scaffolds which support cell growth and regenerate tissues, for ocular surface reconstruction have received increasing interest. Choosing a suitable biomaterial is critical for successful tissue engineering application.

4.1 AMNIOTIC MEMBRANE AS CELL CARRIER FOR OCULAR SURFACE RECONSTRUCTION

The human amniotic membrane is an avascular membrane which consists of a single epithelium, a basement membrane and collagenous stroma (Van Herendael, Oberti et al. **1978**; King, **1978**). It is about 20 – 50 μm thick (Bourne, **1960**) and has been widely used as a substrate to expand limbal epithelial cells for transplantation to restore the ocular surface (Tsai, Li et al. **2000**; Shimazaki, Aiba et al. **2002;** Nakamura, Koizumi et al. **2003**; Kruse and Cursiefen, **2008**; Wan, Wang et al. **2011**). Human amniotic membrane can release a number of growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), keratinocyte growth factor (KGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) which can stimulate cell growth and promote corneal re-epithelialization, suppress inflammation, fibrosis and angiogenesis as well as support cell migration and adhesion (Meller, Tseng et al. **1999**; Schwab. **1999**; Hao, Ma et al. **2000**; Koizumi, Inatomi et al.

2000; Tsai, Li et al. **2000**; Solomon, Rosenblatt et al. **2001**; Nakamura, Koizumi et al. **2003**; Nakamura, Inatomi et al. **2004**; Mehta, Beuerman et al. **2007**; Kruse and Cursiefen, **2008**; Wan, Wang et al. **2011**; Basu, Ali et al. **2012**). However, human amniotic membrane is an allogeneic biologic material which carries the potential risk of transmitting infectious diseases such as human immunodeficiency virus (HIV), hepatitis B and C virus from donor to recipient. Moreover, it is semi-transparent and far from ideal optically which was investigated by Ijiri et al. They used amniotic membrane clipped into a dual polymethyl methacrylate ring set which was used to deliver a temporary amniotic membrane patch without sutures and put them on the corneal surface of six normal human eyes (six patients, from 23 to 30 years old) to evaluate if amniotic membrane may affect vision in normal human eyes. After 30 min, mean distant visual acuities of these patients decreased from –0.22 ± 0.06 to 0.92 ± 0.45 logarithmic minimum angle of resolution (logMAR) and near visual acuities decreased from –0.14 ± 0.07 to worse than 1.0 logMAR (the largest optotype of the near vision chart). However, after removing the amniotic membrane, both distant and near visual acuities recovered immediately to –0.19 ± 0.09 logMAR and –0.11 ± 0.10 logMAR, respectively (Ijiri, Kobayashi et al. **2007**). In addition, amniotic membrane has variable quality such as different composition and thickness depending on the donor's age and length of pregnancy (Hopkinson, McIntosh et al. **2006**; Gicquel, Dua et al. **2009**; López-Valladares, Teresa Rodríguez-Ares et al. **2010**). Amniotic membrane is usually preserved at – 80 °C. This cryostorage can lead to morphology changes such as epithelial vacuolar degeneration and stromal edema in amniotic membrane (Kruse, Joussen et al. **2000**; Rama, Giannini et al. **2001**). Moreover, Burman et al. showed that the presence of an amniotic epithelium may hinder the uniform expansion of explants cultured on the membrane and also may delay the formation of strong hemidesmosomal attachments (Burman, Tejwani et al. **2004**). Removing the superficial epithelial cells to expose the amniotic basement membrane can thus promote more uniform cell outgrowth, higher cell proliferation, attachment, mitosis and cell differentiation (Koizumi, Fullwood et al. **2000**; Grueterich, Espana et al. **2002**; Koizumi, Rigby et al.

2006). Therefore, denuded amniotic membrane has been more frequently used for experiments in vitro and clinical application in vivo to repair the defect and as cell carrier for transplantation.

To date, denuded amniotic membrane is the most commonly used cell carrier for human ocular surface reconstruction. In 2000, Tsai et al. transplanted autologous limbal epithelial cells on amniotic membrane to reconstruct the ocular surface of six eyes of six patients who suffered from limbal stem cell deficiency due to chemical burns, pseudopterygium, congenital pterygium or phlyctenular disease. After a follow-up of 13 – 17 months, complete corneal reepithelialization was observed within two to four days in all six eyes. The clarity of the corneas also was improved. In five of the six eyes, mean best corrected visual acuity improved from 20/112 to 20/45. In one eye with total opacification of the cornea due to chemical burn, best corrected visual acuity improved from count fingers at 40 cm to 20/200. No recurrent neovascularization or inflammation was observed (Tsai, Li et al. **2000**).

Another group used autologous oral epithelial cells cultivated on amniotic membrane to treat six eyes of four patients with Stevens-Johnson syndrome (three eyes) or chemical burns (three eyes). After a follow-up of 11 – 17 months, all six eyes were free of corneal epithelial defects and best corrected visual acuity improved from hand motion (HM) to 4/200 – 20/30. The corneal surface remained stable, although all eyes showed mild peripheral neovascularisation (Nakamura, Inatomi et al. **2004**).

Shimazaki et al. transplanted allogeneic limbal epithelial cells grown on amniotic membrane to treat thirteen eyes of thirteen patients with limbal stem cell deficiency due to Stevens-Johnson syndrome (eight eyes), chemical burns (two eyes) and ocular cicatricial pemphigoid (three eyes). After a follow-up of 12 – 28 days, corneal epithelialization was achieved in six eyes (46.2 %). Conjunctivalization was observed in

five, corneal perforation in four and infectious keratitis in two eyes. The best corrected visual acuity did not change in three eyes and a slight improvement from light perception (LP) – 20/2000 to hand motion (HM) – 20/50 was found in ten eyes (Shimazaki, Aiba et al. **2002**).

Recently, fifty patients (fifty eyes) who suffered from unilateral limbal stem cell deficiency due to ocular surface burns with failure of primary surgery were reported by Basu et al. They underwent repeated transplantation of autologous limbal epithelial cells expanded on amniotic membrane to restore the ocular surface. After a follow-up of 1 – 7.5 years, 33 of the 50 recipient eyes (66 %) maintained a completely epithelialized, avascular, and clinically stable corneal surface. Best corrected visual acuity was improved in 38 of the 50 eyes (76 %). None of the donor eyes showed clinical signs of ocular surface disease, conjunctival overgrowth on the cornea or decrease in vision (Basu, Ali et al. **2012**). In summary, the clinical results of ocular surface reconstruction with amniotic membrane as cell substrate vary and vision remains limited. This is likely to be associated with the variable and unstable quality and low transparency of amniotic membrane.

To overcome the disadvantages of human amniotic membrane, it would be desirable to develop a synthetic membrane which could be produced according to a standardized operating protocol and has stable quality to replace the amniotic membrane as substrate for ocular surface reconstruction. So far, different promising alternative carriers have been proposed and investigated.

4.2 ALTERNATIVE CELL CARRIERS FOR OCULAR SURFACE RECONSTRUCTION

The various options of cell substrates for ocular surface reconstruction were discussed in detail in the introduction. In general, all cell substrates – collagen gels, fibrin gels, silicone hydrogel contact lenses, poly (ε-caprolactone) membranes, gelatin-chitosan

membranes, silk fibroin films, human anterior lens capsules and keratin films – support human or rabbit corneal or conjunctival epithelial cell growth in vitro. However, so far – to the best of my knowledge – no clinical results have been reported for applying these cell substrates for human ocular surface reconstruction except the silicone hydrogel contact lenses and fibrin gels.

4.2.1 Contact lens as cell carrier for ocular surface reconstruction

Contact lenses have been commonly used for protecting the ocular surface and facilitating healing of corneal epithelial defects, but experience with contact lenses as cell carriers for human ocular surface reconstruction so far is still very limited. Human autologous limbal epithelial stem cells expanded on silicone hydrogel contact lenses were transplanted to three human eyes of three patients with limbal stem cell deficiency due to aniridia (one eye) and recurrent conjunctival melanoma (two eyes). After a follow-up of 8 – 13 months, transparent corneal epithelium, significant improvement of symptoms as well as absence of corneal conjunctivalization or vascularization were observed. Best corrected visual acuity was improved from counting fingers (CF) – 0.03 to 0.1 – 0.5 (Di Girolamo, Bosch et al. **2009**). However, further investigations with more cases are necessary in order to confirm if they are a viable alternatives to amniotic membrane for human ocular surface reconstruction.

4.2.2 Fibrin gel as cell carrier for ocular surface reconstruction

Fibrin gels have been investigated by Rama et al. in a large group of 113 eyes of 112 patients (Rama, Matuska et al. **2010**) who had severe limbal stem cell deficiency due to chemical or thermal burns and most of these eyes (84 %) had been undergone unsuccessful keratoplasties. They expanded autologous limbal epithelial stem cells which were obtained from the contralateral healthy eyes on the fibrin gels and transplanted them to the patients' corneal surface. After a follow-up of 1 – 10 years, failures of the transplantation occurred within the first year. 76.6 % of these grafts were

considered successful by evaluating symptoms, corneal reepithelialization and transparency, best corrected visual acuity and impression cytology. Therefore, it seems that the fibrin gel is a clinically viable alternative cell carrier for restoring the ocular surface. However, the fibrin gels are fabricated by combining fibrinogen and thrombin which are harvested from plasma. If a large number of fibrin gels are required to be produced for tissue engineering application with a standardized procedure, it is impossible to fabricate totally autologous bioengineered membranes. The use of allogeneic source material however could result in allogeneic rejection and disease transmission just like human amniotic membrane.

In general, an optimal candidate of human amniotic membrane alternative for ocular surface reconstruction should be optically transparent, biocompatible, produced with low cost and support cell growth of ocular surface epithelial cells as well as have sufficient biomechanical strength for suturing. Hence, a new cell carrier material – keratin – was proposed and investigated.

4.3 NEW CELL CARRIER – KERATIN FILM – FOR OCULAR SURFACE RECONSTRUCTION

Keratins are a family of structural proteins of epithelial origin, which are formed in the epithelial cells of higher vertebrates. They can be isolated in large quantities from hair, nails, horns, hoofs, wool and feathers. Moreover, these fibrous structural proteins highly contain cysteine, exhibit high mechanical strength and water-insolubility due to a large number of disulfide bonds. In the past, keratin attracted few interest as a biomaterial for tissue engineering investigation because of its water-insolubility. Whereas in recent years, chemical or microbial reduction of the disulfide bonds by chemical modification – methylation or oxidation – to obtain water-soluble keratins were reported. This resulted in an increasing research interest in keratins for tissue engineering (Schrooyen, Dijkstra et al. **2000**; Schrooyen, Dijkstra et al. **2001**; Hill, Brantley et al. **2010**). To date,

films or scaffolds based on keratins from wool and human hair have been proposed and investigated for cell culture and tissue engineering applications.

In 1998, Yamauchi et al. demonstrated that proliferation and attachment of mouse cell line L929 fibroblasts on substrates coated with keratin based on Corriedale wool were better than on collagen and glass (Yamauchi, Maniwa et al. **1998**). Later, another group found mouse fibroblasts cultured on wool keratin sponges exhibited similar proliferation behavior compared with plastic (Tachibana, Furuta et al. **2002**). Also a hybrid of keratin sponges with hydroxyapatite supported growth and differentiation of osteoblasts (Tachibana, Kaneko et al. **2005**). In 2008, Fujii et al. found that keratin obtained from human hair and nail proteins incubated with mast cells inhibited histamine release. This suggests that films based on human hair and nail keratins could be a useful material for antiallergic applications (Fujii, Murai et al. **2008**). Moreover, keratin extracted from human hair increased proliferation and attachment of rat Schwann cells indicating that keratin facilitates the regeneration of peripheral nerves (Sierpinski, Garrett et al. **2008**).

Keratin can be extracted from human hair which can be easily obtained from hair salon and can be fabricated into films in a large quantities in a standardized method with low cost. Recently, a technique to prepare a transparent and stable film based on human hair keratin which is approximately 50 µm thick with a novel multi-step process including keratin extraction, dialysis, drying and a curing was described (Reichl, Borrelli et al. **2011**). This film based on human hair keratin exhibits higher light transmission and significantly higher biomechanical strength than human amniotic membrane. Moreover, ex vivo surgery on enucleated porcine eyes showed that this keratin film can be surgically handled. While suture placement on the keratin film was more difficult due to a higher rate of suture loosening, resulting in poor adaptation to the ocular surface compared to human amniotic membrane. However, such disadvantages can be overcome by material modification of keratin film – an option that is not easily

available for amniotic membrane (Reichl, Borrelli et al. **2011;** Borrelli, Reichl et al. **2012**).

Analysis of cell proliferation, migration and adhesion on cell-carrier construct is an essential step for evaluating the biocompatibility of a substrate. My study investigated the biocompatibility of these keratin film for human corneal epithelial cells by comparing cell proliferation, migration and attachment behavior of HCE-T cells on the keratin film and human amniotic membrane in vitro. As control, plastic which is the conventional cell substrate for cultivation was used. My findings demonstrated that these films based on human hair keratin in general supported cell proliferation, migration and adhesion of HCE-T cells in vitro. Similar results have been reported for keratin from wool or human hair with mouse fibroblasts (Yamauchi, Maniwa et al. **1998**; Tachibana, Furuta et al. **2002;** Tanabe, Okitsu et al. **2002;** Verma, Ray et al. **2008**) and rat Schwann cells from peripheral nerves (Sierpinski, Garrett et al. **2008**).

4.3.1 Cell proliferation in vitro

My study showed that lower cell proliferation behavior on KF compared to AM over 72h ($P < 0.05$). However, HCE-T cells exhibited a slightly extended lag-phase on KF compared to AM after seeding investigated by Reichl et al. over 14 d (Reichl, Borrelli et al. **2011**). Therefore, in order to further evaluate cell proliferation behavior on KF and AM comprehensively, a longer culture period is required. Interestingly, HCE-T cells showed significantly higher proliferation behavior on plastic than on amniotic membrane and keratin film during 72 h ($P < 0.05$). This is in contrast to Verma et al. who showed that mouse fibroblasts cultured on keratin sponges based on human hair exhibit slightly higher proliferation than on plastic (Verma, Ray et al. **2008).** Also mouse fibroblasts cultured on wool keratin sponges exhibited similar proliferation behavior compared with plastic (Tachibana, Furuta et al. **2002**). The differing results of my investigation can be explained by different keratin film production procedure, which may influence cell growth behavior. Also porcine vascular endothelial cells and rabbit

chondrocytes seeded on denuded amniotic membrane are shown to have superior proliferation than on plastic (Tsai, Liu et al. **2007**; Krishnamurithy, Shilpa et al. **2011**), which are different from my results. These variations could be explained by the variable and unstable quality of amniotic membrane. Investigations demonstrated that variations of growth factors protein such as epidermal growth factor (EGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), transforming growth factor (TFG) and nerve growth factor (NGF) were detected in amniotic membrane. Lower growth factors levels in amniotic membrane with higher gestational ages and donor ages (Hopkinson, McIntosh et al. **2006**; López-Valladares, Teresa Rodríguez-Ares et al. **2010**). In addition, Gicquel et al. found that there is a considerable variation of EGF content in amniotic membrane, which plays a key role in supporting corneal epithelial cell expansion. EGF content varied between membranes (inter-donor) and at different sites within the same membrane (intra-donor). The highest EGF protein concentration was detected in the amniotic membrane apical and mid-region epithelium. Significant EGF protein lost after handling (Gicquel, Dua et al. **2009**). Therefore, these variations could affect the cell growth on amniotic membrane and clinical results of tissue constructs for transplantation.

4.3.2 Cell migration in vitro

The wound-healing assay which mimics cell migration during wound closure in vivo showed a significantly higher migration of HCE-T cells on plastic compared to cells cultivated on keratin film and amniotic membrane ($P = 0.005$ and $P = 0.000$, respectively). Also cells cultured on keratin film showed a significantly faster migration compared to cells cultivated on amniotic membrane ($P < 0.05$) over a 48 h period. The result demonstrated that the keratin film facilitates corneal epithelial wound healing in vitro, which is important for OS reconstruction. Further animal experiments are necessary to investigate whether KF can promote corneal wound healing in vivo. As far as I know, no investigation comparing cell migration on keratin, human amniotic membrane and plastic has been published. However, Sierpinski et al. showed that

keratin extracted from human hair added into cell culture medium enhances Schwann cell migration compared with the basal medium without keratin (Sierpinski, Garrett et al. **2008**).

4.3.3 Cell adhesion in vitro and histology

The adhesion assay I performed demonstrated that significantly more HCE-T cells attached to denuded human amniotic membrane compared to plastic and keratin film ($P = 0.032$ and $P = 0.001$, respectively). No significant difference in adhesion was observed between keratin film and plastic substrates ($P = 0.147$) after 30 min. Previous studies showed that human limbal epithelial cells, porcine arterial endothelial cells and rabbit chondrocytes attach better to AM than plastic (Bray, George et al. **2011**; Tsai, Liu et al. **2007**; Krishnamurithy, Shilpa et al. **2011**). Reichl et al. found HCE-T cells on AM show higher resistance against proteolytic detachment via trypsin compared with KF (Reichl, Borrelli et al. **2011**). This may be explained by the fact that AM is a highly collagenous natural biomaterial,containing mainly collagen I and III, which are generally known to facilitate cellular adhesion (Kim, Baez et al. **2000**; Abraham, Zuena et al. **2008**). Reichl also found HCE-T and Calu-3 cells required a significantly longer trypsin exposure time (longer than 30 min) to achieve complete cell detachment from keratin coated substrates compared to plastic (Reichl, **2009**). This differs from our results and may be explained by different keratin substrates production procedure, which may affect cell attachment behavior. Furthermore, cell adhesion of my study was assessed only at 30 min. Hence it only demonstrated cell attachment behavior at an early time point rather than dynamic cell adhesion. Mouse fibroblasts show better adhesion to substrates coated with wool keratin than coated with collagen (Yamauchi, Maniwa et al. **1998**). Also rat Schwann cells adhered more on glass slides coated with human hair keratin compared to coated with fibronectin (Sierpinski, Garrett et al. **2008**). Support of cell adhesion is likely to be associated with the amino acid sequences of keratins. Keratins from human hair or wool contain amino acid sequences which are also found in other extracellular matrix proteins such as fibronectin and collagen and

these amino acid sequences contain at least one binding domain specific to the integrins expressed on many cell types thus inducing cell adhesion (Humphries, Komoriya et al. **1987**; Chevallay, Abdul-Malak et al. **2000**; Tachibana, Furuta et al. **2002**).

Histology stained with hematoxylin and eosin demonstrated that HCE-T cells cultured on keratin film and denuded amniotic membrane at an air-liquid interface form a multilayered epithelium (3 – 4 epithelial layers) which is similar to normal human corneal epithelium. This indicates that HCE-T cells cultured on keratin film and amniotic membrane maintain differentiation properties of human corneal epithelial cells.

In summary, my investigation demonstrated that this transparent biopolymer film based on human hair keratin seems to be biocompatible in vitro, because HCE-T cells can proliferate, migrate, attach and differentiate on the keratin film. Cell proliferation and migration was best on plastic. Cell migration on keratin film was faster compared to on amniotic membrane. Cell proliferation and adhesion were better on amniotic membrane compared to on keratin film which has similar cell adhesion to on plastic.

4.4 LIMITATIONS OF MY STUDY

The cells I used in this study were immortalized human corneal epithelial cell line (HCE-T cells) which is well-known and commonly used for ocular surface investigation. However, it has to be noted that its proliferative capacity is different compared to primary corneal epithelial cells. Furthermore, the HCE-T cell line shows positivity for the corneal marker CK3 but also for CK7, CK8, CK18 and CK19, which is expressed by simple epithelial cells but is not typical for corneal epithelium. Therefore, immunohistochemical analysis of keratin expression (CK3 and CK12) and electron microscopic examinations to proof more specifically the epithelial character of cultured primary corneal epithelial cells will be required and animal models are needed to evaluate the properties of keratin film as a substrate for epithelial cells in more detail

and to assess potential toxic or immunogenic effects of this new material with future work.

5 CONCLUSION

In conclusion, this transparent biopolymer keratin film based on human hair supports cell proliferation, migration, adhesion and differentiation of HCE-T cells in vitro. Therefore, it could be a promising candidate substrate for ocular surface reconstruction. Techniques for constructing epithelialized tissue substitutes – as have already been established for AM – will hence have to be modified, i.e. longer time may be required until a sufficiently stable construct is achieved. However, these disadvantages have to be balanced with the potential advantage of improved transparency of KFs, which offer ultimately potential better visual acuity. However, further studies with primary epithelial cells in vitro and as well as in vivo studies using animal models are required to evaluate its properties as a substrate for epithelial cells and assess its potential toxic or immunogenic effects on the ocular surface prior to clinical use.

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7 APPENDIX

Table 6. MTT- assay: proliferation behavior of 5×10^3 cells/well HCE-T cells on plastic, KF and AM for proliferating 24 h, 48 h and 72 h

	Absorbance			P-values		
	Plastic	KF	AM	Plastic − KF	KF − AM	Plastic − AM
24h	0.058±0.006	0.009±0.002	0.015±0.010	0.003*	0.582	0.015*
48h	0.146±0.011	0.041±0.007	0.084±0.008	0.001*	0.005*	0.004*
72h	0.258±0.037	0.062±0.005	0.133±0.056	0.001*	0.066	0.008*

Data were shown as the mean ± SD of three wells per substrate. The difference between KF, AM and plastic were demonstrated by P values at each time point and $P < 0.05$ (*) were considered as significant difference. The experiments were performed in triplicate.

Table 7. MTT- assay: proliferation behavior of 1×10^4 cells/well HCE-T cells on plastic, KF and AM for proliferating 24 h, 48 h and 72 h

	Absorbance			P-values		
	Plastic	KF	AM	Plastic − KF	KF − AM	Plastic − AM
24h	0.114±0.008	0.032±0.006	0.045±0.010	0.001*	0.252	0.002*
48h	0.258±0.013	0.064±0.007	0.166±0.010	0.000*	0.001*	0.002*
72h	0.326±0.019	0.087±0.005	0.264±0.077	0.001*	0.003*	0.145

Data were shown as the mean ± SD of three wells per substrate. The difference between KF, AM and plastic were demonstrated by P values at each time point and $P < 0.05$ (*) were considered as significant difference. The experiments were performed in triplicate.

Table 8. Scratch-wound healing assay：migration behavior of HCE-T cells on plastic, KF and AM for 0 h, 12 h, 24 h, 36 h and 48 h

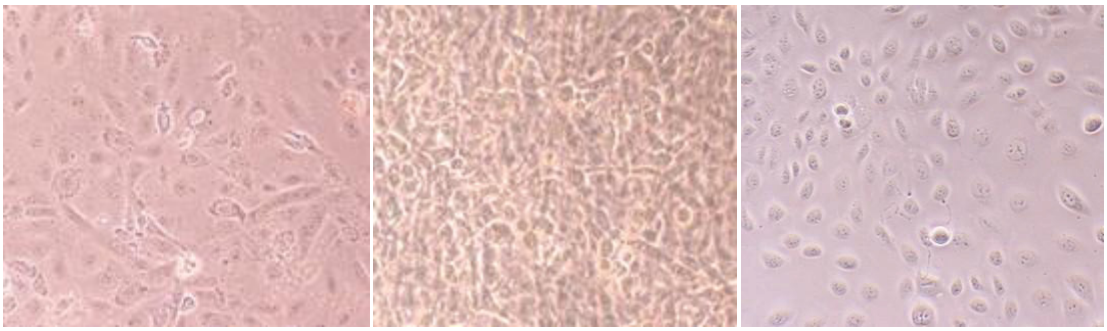
	Wound width (μm)			P-values		
	Plastic	KF	AM	Plastic − KF	KF − AM	Plastic −AM
0 h	949.5±35.6	947.6±75.4	945.9±42.3	0.935	0.946	0.882
12h	722.4±65.1	842.8±67.5	932.9±120.6	0.005*	0.031*	0.000*
24h	336.4±107.3	441.7± 131.9	771.1±131.8	0.069	0.000*	0.000*
36h	157.8±107.0	310.5±154.5	462.9±46.3	0.005*	0.005*	0.000*
48h	0	0	0	—	—	—

Data were shown as the mean ± SD of three wells per substrate. The difference between KF, AM and plastic were demonstrated by P values at each time point and $P < 0.05$ (*) were considered as significant difference. The experiments were performed in triplicate.

Table 9. Adhesion assay: attachment behavior of HCE-T cells on plastic, KF and AM for 30 min

Cell numbers			P-values		
Plastic	KF	AM	Plastic − KF	KF − AM	Plastic −AM
136.8±55.8	110.6±58.0	176.3±24.6	0.147	0.001*	0.032*

Data were shown as the mean ± SD of three wells per substrate. The difference between KF, AM and plastic were demonstrated by P values after adhering 30 min and $P < 0.05$ (*) were considered as significant difference. The experiments were performed in triplicate.



A B C

Figure 14. **Fotos of 1×10^4 cells/well HCE-T cells cultured on the KF, AM and plastic (×100 magnification). Cells cultivated on the KF (A) and AM (B) formed a confluent epithelial sheet after 5 to 7 days. However, the cell margins were less distinct because of the less transparent nature of the AM; Cells cultivated on plastic (C) formed a confluent epithelial sheet after 5 days.**

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Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbstständig und ohne unzulässige fremde Hilfe erstellt worden ist und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

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