Nonwoven-Based Gelatin/Polycaprolactone Membrane Proves Suitability in a Preclinical Assessment for Treatment of Soft Tissue Defects

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Standard preclinical assessments *in vitro* often have limitations regarding their transferability to human beings, mainly evoked by their nonhuman and tissue-different/nontissue-specific source. Here, we aimed at employing tissue-authentic simple and complex interactive fibroblast-epithelial cell systems and their *in vivo*-relevant biomarkers for preclinical *in vitro* assessment of nonwoven-based gelatin/polycaprolactone membranes (NBMs) for treatment of soft tissue defects. NBMs were composed of electrospun gelatin and polycaprolactone nanofiber nonwovens. Scanning electron microscopy in conjunction with actin/focal contact integrin fluorescence revealed successful adhesion and proper morphogenesis of keratinocytes and fibroblasts, along with cells' derived extracellular matrix deposits. The "feel-good factor" of cells under study on the NBM was substantiated by forming a confluent connective tissue entity, which was concomitant with a stratified epithelial equivalent. Immunohistochemistry proved tissue authenticity over time by abundance of the biomarker vimentin in the connective tissue entity, and chronological increase of keratins KRT1/10 and involucrin expression in epithelial equivalents. Suitability of the novel NBM as wound dressing was evidenced by an almost completion of epithelial wound closure in a pilot mini-pig study, after a surgical intervention-caused gingival dehiscence. In summary, preclinical assessment by tissue-authentic cell systems and the animal pilot study revealed the NBM as an encouraging therapeutic medical device for prospective clinical applications.

Introduction

PRECLINICAL ASSESSMENTS with regard to issues such as clinical applicability and suitability of newly developed biomaterials, in first instance, have to be evaluated by their biocompatibility, that is, the outcome of their tissue interactions.^{1,2} In reflection to this more archaic viewpoint, current biomaterial approaches integrate issues that are more closely related to the requirements of the cells from particular tissues, to be supported in the context of either augmentation, and/or regeneration after injury. Hence, in addition to minimizing harmful tissue reactions, including, for instance, inflammation, immune response and allergy, innovative biomaterials aim at addressing distinct tissue cells by specific bio-modification and/or bio-activation strategies.³ Along with chemical modifiers or molecular activators, biomechanical cues more and more run into the focus of interest in novel biomaterial development,

as exemplified in studies on cartilage repair by hyaluronic acid-based hydrogels with different biomechanical properties.⁴

While focusing on biomechanics, the elasticity of a novel biomaterial intended to be used as wound dressing in order to promote subsequent recruitment of body-own tissue cells should be adjusted in such a way that target tissue cells feel at ease with it. These "feel-good-parameters" on wound dressings are reflected by the target cells, through realising behavioral cell features, which, in response to the device, resemble their physiological tissue situation. Among others, such features are substantiated by regular cell morphogenesis, an indispensable prerequisite for expression of *in vivo* relevant tissue-specific biomarkers, which, in sum, are the cornerstones of desired tissue integrity and homeostasis. Such biomarkers integrate cell features such as differentiation, synthesis of extracellular matrix (ECM), and cell adhesion.

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With regard to differentiation, commitment of mesenchymal cells to fibroblasts is indicated by expression of the cytoskeletal intermediate filament constituting protein vimentin.⁵ While expression of this biomarker is mandatory for fibroblast function, and in a broader sense for connective tissue homeostasis, keratins (KRT) are the differentiation-indicating counterpart of intermediate filaments of keratinocytes in stratified squamous epithelia, such as skin or oral mucosa.⁶ Herein, keratin 14 (KRT14) demarcates basal epithelial keratinocytes, being in a less differentiated state; whereas KRT1 and KRT10 become more apparent in suprabasal differentiated cells. In even more progressive differentiated, that is, stages of terminal differentiation, keratinocytes exhibit expression of involucrin and filaggrin, respectively.⁷

Since connective tissue fibroblasts and epithelial keratinocytes are crucial for lifelong maintenance of tissue homeostasis of all surface covering and/or cavity lining epithelia of the human body, novel biomaterials should be capable of addressing these two cell types by being both an attractant and a conductive element. To test these self-healing power-involving properties of a novel biomaterial device, conceived as wound dressing, besides animal studies or clinical trials in humans, tissue authentic *in vitro* models render precious tools as means of choice for its preclinical validation. Ideally, with emphasis on prospective clinical application, such authentic models (i) comprise target cells of the tissue that should be reconstituted; are (ii) species specific; and (iii) employ the cells in a physiologically, that is, tissue-relevant arrangement/orientation. In the oral cavity tissue context, these requirements are fulfilled by our long-lasting expertise in complex interactive fibroblast-keratinocyte co-cultures, expressing the previously mentioned biomarkers of oral mucosa tissue homeostasis.⁷⁻⁹

Vice versa, the novel wound-dressing biomaterial device should integrate constituents that are not only suitable for attraction or conduction of cells, but also convenient for the surgeon's handling and the clinical wound indication to be addressed. These issues, on the one hand, integrate material properties such as sufficient stability in case of operative suturing, and variable size adaption, and wound healing-adjusted wound dressing resorption/degradation, on the other hand, for proper tissue-innate volume preservation. Both issues seem to be addressed adequately, when biomaterials comprise a natural biopolymer combined with an artificial partner, thereby yielding a biohybrid polymer. Using gelatin (GE) as natural constituent and polycaprolactone (PCL) as artificial constituent, *in vitro* and *in vivo* studies on either a solitary or both constituents add to the growing body of evidence that they are not only suitable for cell growth¹⁰ but also biocompatible when employed as a scaffold; for example, for periodontal stem cell grafting.¹¹ For fabrication of biodevices, electrospinning renders a powerful fabrication technique that has been described in the context of GE and PCL as well.¹¹⁻¹⁴ For example, a GE/PCL blend was electrospun onto a polyurethane wound dressing, aiming at producing a dermal analogue dual-sided fibroblast-populated device.¹⁵ Most conventional approaches toward GE electrospinning and fabrication of GE nanofiber biodevices employ toxic fluorinated solvents and require postspinning cross-linking, owing to the GE water solubility and disintegration of the devices in aqueous

medium. In our research,¹⁶ we have developed laminated collagenous nonwovens, prepared by fluorine-free electrospinning and *in situ* cross-linking. The electrospun GE nanofiber nonwovens were bonded together with PCL meshes by heating above the PCL melting temperature. The incorporation of PCL layers accounted for substantially improved mechanical stability without impairing biocompatibility and biodegradation of such modular systems for tissue repair. Here, the objective of our research was to employ tissue-authentic interactive cell systems for a preclinical evaluation of novel human-applicable nonwoven-based GE/PCL membranous biodevices. This biodevice is fabricated by fluorine-free electrospinning without post-treatment requirement, as wound dressing, which shares mutuality in convenience regarding the clinical-operative demands and the tissue cells' requirements, with the latter yielding the "feel-good factor."

Materials and Methods

Nonwoven-based membrane fabrication

For generation of the novel nonwoven-based membranes (NBMs), GE (gelatin powder EMPROVE[®], pharmaceutical grade; Merck KGAA, Darmstadt, Germany) was solubilized in a solvent mixture of acetic acid (Merck KGAA), ethyl acetate (Sigma-Aldrich, Munich, Germany), and water in the ratio of 5:3:2. The GE concentration used for NBM generation and GE layers on glass slides was 14% w/v. For *in situ* cross-linking, glyoxal (40% glyoxal solution; Sigma-Aldrich) in a ratio of 0.017 (w/w) to the GE solution was mixed with the GE solution at room temperature (RT), the GE/glyoxal mixture was filled into a syringe, mounted in a syringe pump (type: KDS100 or KDS101; KD Scientific, Holliston, MA) and the spinning process was started with a flow rate of 0.5 mL/h for 2 h, yielding a covered substrate area of 50 cm² and a deposited GE amount of 0.02 g/cm². For "jet-stream-formation," an electrical field between the cannula and the collector was established with two high-voltage power generators (Heinzinger[®] LNC 30000-2 neg. and LNC 30000-2 pos.), with field forces between 0.4 and 8 kV/cm. After depositing a sufficiently thick polymer set of layers of GE fibers on the substrate, a thin layer of LACTEL PCL (Nordmann Rassmann GmbH, Hamburg, Germany) was electrospun on the predeposited layer set of GE fibers. Subsequently, further layers comprising GE were applied. As glyoxal builds up spin dope viscosity due to linking of GE molecules, a GE nanofiber gradient was formed during electrospinning with average GE fiber diameters gradually increasing from 90 to 680 nm. During the subsequent temperature treatment (80°C; 2 h), a further cross-linking of the GE with glyoxal occurred. Furthermore, temperature treatment led to a partial melting of the PCL layer, whereas the melt punctually or laminary surrounds the GE fibers and, thus, provides for significantly improved mechanical properties of the novel NBM. For observation of periodontal cell behavior in the simple monolayer culture, GE fibers were directly deposited onto cover slips, simplifying the analysis of single cells using life cell imaging.

Before *in vitro* preclinical validation experiments, biohybrid membranes were sterilized with 70% ethanol for 20 min, and equilibrated overnight in sterile physiologic sodium-chloride solution at 4°C. Such treated membranes had a thickness of ~2 mm.

Elastic modulus measurement

The elastic modulus measurement of single GE layers and NBM was performed using a novel Bio-Nanoindenter system (CSM Instruments, Peseux, Switzerland) that was equipped with a spherical ruby indenter of a diameter of 200 μm . A loading rate of 1000 $\mu\text{N}/\text{min}$ was applied to the fleece immersed in water. The indentation curves were monitored, and the elastic modulus was calculated from the unloading curves with an assumed Poisson's ratio of 0.3 using the method introduced by Oliver and Pharr.¹⁷

Cell culture

Cultures of primary gingival fibroblasts (GF) were established by the explants technique,⁸ and tissue harvesting from patients was performed according to the Helsinki Declaration and was approved by the institutional ethics committee. For routine cell culture, cells were maintained in Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe, Germany) containing 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany) and 50 mg/mL kanamycin (Sigma-Aldrich). Passages 8–12 were used for the *in vitro* experiments, and to exclude cell-senescence-caused alterations of cell morphology and the expression of tissue-specific biomarkers, periodontal cells were previously tested on the consistent expression of respective biomarkers and native cell morphology over time.

Human gingival keratinocytes (GK) were established as an immortalized cell line, and characterization and serial cultivation was recently reviewed.⁷ During routine cell culture, GK were maintained in keratinocyte growth medium (KGM2; PromoCell, Heidelberg, Germany), including supplements and containing 50 mg/mL kanamycin (Sigma-Aldrich).

GFs and GKs monolayer cultivation on the nonwoven-based membrane

For observation of the behavior of simple periodontal monolayer cell systems on the membrane, the cover slips that were described earlier as being coated with GE fibers were used. The coated cover slips were first sterilized with 70% ethanol for 15 min, rinsed thoroughly with phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany), and equilibrated overnight in PBS. For single-cell observation, 1×10^4 cells/cm² GF and 3×10^4 cells/cm² GK were seeded separately on GE fiber-coated cover slips and cultured in their respective culture medium. The cells were maintained under standard cell-culture conditions at 37°C, 97% humidity, and 5% CO₂.

Life-cell-imaging and fluorescent microscopy of GFs and GKs on GE fibers

Imaging in the life-cell modus was initialized at 4 h after cell seeding, and specimens were transferred into a microscope-associated incubation chamber (INU-K1-F1; Tokai Hit, Shizuoka, Japan). Cell observation was conducted by phase-contrast microscopy (BZ-9000; Keyence, Neu-Isenburg, Germany) under standard cell culture conditions for 24 h, and cell migration and motility was documented every 5 min. Subsequent time-lapse microscopy data evaluation resulted in cell movies annotated as Supplementary

Movies S1 and S2 in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea).

For detection of the cytoskeleton and cell adhesion-related biomarkers, fluorescent microscopy was performed. Hence, cells seeded on GE fiber-coated cover slips were fixed after a 24 h cultivation period with 3.8% formaldehyde in PBS for 15 min and rinsed thrice with PBS. Then, the cells were permeabilized with 0.2% Triton-X-100 (Roche, Mannheim, Germany) for 5 min. and washed again thrice with PBS. For blocking unspecific binding sites, the specimens were incubated with Image-iT-FX enhancer (Invitrogen) for 30 min and rinsed thoroughly with PBS. Subsequently, for actin cytoskeleton staining, the specimens were incubated with Phalloidin-Alexa488 (wd=1:40; Invitrogen) for 45 min at RT, followed by washing thrice with PBS for 5 min, respectively. The detection of cell adhesion-related biomarkers was conducted for GFs by incubation with an integrin β 3-specific primary antibody (wd=1:50; Abcam, Cambridge, United Kingdom) and for GKs with an integrin β 1-specific primary antibody (wd=1:50; Abcam) for 1 h at RT. After washing the specimens thrice with PBS for 5 min, respectively, samples were incubated with an Alexa594-labeled secondary antibody (wd=1:200; Invitrogen) for 45 min, followed by three washing steps with PBS for 5 min, respectively. Nuclear counterstaining was performed with a 300-nM DAPI solution (Invitrogen) in PBS for 10 min, and cells were subsequently washed thrice with PBS followed by rinsing once with distilled water. Finally, the specimens were embedded in Fluoromount-G (SouthernBiotech, Birmingham, AL) and documented by fluorescent microscopy (BZ-9000; Keyence).

Scanning electron microscopy

For scanning electron microscopy (SEM), test specimens were fixed with 3.8% formaldehyde in PBS for at least 1 h and rinsed thrice with PBS. Then, the specimens were dehydrated by rinsing through graded ethanol/water mixtures (50%, 70%, 80%, 90%, and 100%; each step for 10 min at RT). Next, ethanol was slowly exchanged by liquid CO₂ (critical point dryer; Balzers CPD, Balzers Union). Finally, the samples were dried using the critical-point method,¹⁸ sputtered with a thin layer of gold of ~ 10 nm in thickness (sputter coater, Balzers SLD; Balzers Union), and documented by a Leo 435VP (Zeiss) scanning electron microscope.

Establishment of the GFs/GKs co-culture system on the nonwoven-based membrane

Biohybrid membrane was fabricated as described earlier, cut into circular pieces with a diameter of 15 mm, and placed into 24-well plates (Becton Dickinson, Heidelberg, Germany). To ensure proper cell adhesion on one side of the membrane only, 2×10^4 cells/cm² GF were seeded in a small amount of FAD medium (Ham's F12/DMEM: mixing ratio 1:3; Biochrom AG), 5% FCS (Biochrom AG), 50 mg/mL Kanamycin (Sigma-Aldrich), and supplements of KGM2 (PromoCell). After 6 h of preincubation time, the wells were filled to approximately 1 mL FAD medium, and the cells on the membrane were further incubated overnight. Next, the medium was removed, and the membrane was washed with PBS. For GK cultivation on the other side of the membrane,

the membrane was flipped upside down in the well and seeded with 1×10^5 cells/cm² GKs again in a small amount of FAD medium. After another preincubation step of 6 h, the wells were filled up again to 1 mL FAD-medium. For pre-validation experiments, the prepared membranes bearing the co-culture setup were further incubated under standard cell-culture conditions for 7 and 14 days. After the respective cultivation periods, the membrane specimens were washed several times with PBS and fixed with 3.8% paraformaldehyde in PBS for 15 min for immunohistochemical (IHC) analysis. In addition, some specimens were prepared for SEM as described earlier.

For IHC analysis, scaffolds were washed thrice with PBS. Next, the cells were permeabilized with 0.2% Triton-X-100 (Roche) for 5 min and washed thrice with PBS for 15 min, respectively. For the blocking of unspecific binding sites, the specimens were incubated with Image-iT-FX enhancer (Invitrogen) for 30 min and rinsed thoroughly with PBS. Then, the membrane sides containing either GFs or GKs were separated carefully; GF-populated sides were incubated with anti-vimentin-specific primary antibody (wd = 1:40; Abcam); and GK-covered sides were incubated with primary antibodies against cytokeratin 14 (wd = 1:100; Santa Cruz Biotechnology, Heidelberg, Germany), cytokeratin 1/10 (wd = 1:100; Abcam), and involucrin (wd = 1:50; Abcam) for 1 h, respectively. Next, specimens were washed thrice with PBS for 15 min followed by the incubation with secondary horseradish-peroxidase (HRP)-coupled antibody (wd = 1:200; Abcam) for 45 min. Then, the membranes were washed thrice with PBS for 15 min, and subsequent color development was performed with the 3,3'-diaminobenzidine (DAB) substrate kit (Abcam). The specimens were incubated with the DAB chromogen substrate for 10 min and washed thoroughly with PBS and once with distilled water. Finally, the specimens were embedded in Fluoromount-G (SouthernBiotech) and documented by bright-field microscopy (BZ-9000; Keyence). To prove specificity of the primary antibodies, negative controls with secondary HRP antibody and the substrate only revealed no visible signals on the cells.

Nonwoven-based membrane application in a pilot minipig study

For the pilot animal study, the nonwoven-based membranes with a size of about 2 cm² were generated as described earlier with pharmaceutical-grade polymers under sterile conditions. Before intervention, the membrane was first heat sterilized at 80°C for 1 h, followed by sterilization in 70% ethanol for 15 min. Next, the membrane was washed thoroughly in sterile isotonic sodium chloride solution (0.9%; Fresenius, Bad-Homburg, Germany) and left for 24 h in fresh isotonic sodium chloride solution for equilibration.

Three adult female Göttingen mini pigs (Ellegaard, Esbjerg, Denmark), at the age of 14–16 months and a weight of 20–29 kg, were used for NBM applications in intra-oral soft tissue defects, after the dental implantation procedure. The study was approved by the ethics committee for animal research at Malmö University, Sweden (vote: M 204-11.3). The animals were kept under standard animal management conditions and fed on a special soft diet for mini pigs. The complete surgical procedure was performed under general

anesthesia (10 mL of ketamine hydrochloride, Ketalar® 50 mg/mL; Pfizer, Berlin, Germany), mixed with 3 mL of midazolam (Dormicum® 5 mg/mL; Roche, Basel, Suisse). Additional local anesthetic was applied (Xylocain dental adrenalin 20 + 12.5 mg/mL; Astra AB, Södertälje, Sweden) to reduce the systemic anaesthetic dose as well as to reduce the bleeding during surgery. In the course of the healing phase of 4 weeks, the animals were monitored routinely, and further analgesia was given if necessary. To approach the prospective clinical application of primary oral soft tissue closure as close as possible, NBM pieces were trimmed in approximation to the respective wound size to cover the mandibular soft tissue dehiscence caused in preparation of the dental implantation procedure. The surgical approach was conducted with the extraction of mandibular premolars (P2, P3, P4, M1) followed by the direct application of a dental implant, replacing the first premolar, and then subsequent coverage of the remaining soft tissue dehiscence with the experimental NBM. Each hemimandibel received this direct-implantation procedure and primary soft tissue closure treatment of palatal flaps. Finally, NBM was tailored to fit the defect and cross-sutured with absorbable surgical thread (Vicryl 4-0, F2S; Ethicon, Somerville, NJ) at the flap margins. The clinical wound-healing course was followed up and conducted visually by photo documentation after 2, 3, and 4 weeks. At the end of the 4-week healing period, the animals were sacrificed after general anesthesia with an intercardiac injection of a lethal dose of sodium pentobarbital. Directly after sacrifice, the mandibles were excised and the left and right hemi-mandibles were separated with a bandsaw. Regions of interest of the hemi-mandibles were prepared in bone chambers and immersed in 4% formaldehyde solution for further histological processing.

Histology

For histological analysis of the soft tissue-healing course, mandibles were divided in smaller sections, comprising the region of interest, that is, posterior of the dental implantation site, and immersed in fresh buffered 4% formaldehyde solution. After 2 weeks, specimens were dehydrated in a graded series of ethanol and embedded in light curing resin (Technovit 7200 VCL; Kulzer, Friedrichsdorf, Germany). Sections were prepared through the longitudinal axis of each sample, enabling the detailed analysis of the soft- and hard tissue-healing course in close vicinity to NBM remnants. Forty-micrometer-thick sections were stained with toluidine blue and 1% fuchsin red. Qualitative evaluation of histological sections was performed with a light microscope (Nikon Eclipse 90i; Teknooptik AB, Huddings, Sweden).

Results

Innate features rendering the novel nonwoven-based membrane a suitable wound dressing

Cells are capable of sensing their extracellular environment at the micro- and nanometer scale.¹⁹ Besides other biological parameters, a cell's convenience, which is reflected by their behavior, is directly related to their extracellular environment.²⁰ For its suitability as wound dressing in soft tissue regeneration (STR), the gradient nonwoven-based membranes (NBMs), configured

as scaffold, should be compatible for cell adhesion and should provide extensive mechanical support, mediating the ability to withstand biomechanical forces, exerted in clinical applications. To combine both prerequisites, our novel NBM consists of *in situ* cross-linked electrospun GE nanofiber nonwovens with fiber diameter gradient varying between 90 and 680 nm, ensuring proper cell adhesion. For achieving mechanical robustness in an aqueous environment, the GE nanofiber nonwovens were laminated with layers of electrospun PCL nonwovens, which were bonded together by heating NBM above the melting temperature of PCL. The NBM concept is shown in Figure 1. Fundamental for the development of new biomaterials, applicable for clinical use in various soft tissue wound situations, are the selection of polymers and the design of gradient nanometer-scaled polymer architectures, enabling the combination of important features such as elasticity, biocompatibility, and the ability of time-directed and decelerated degradation. GE as denatured collagen is processed by electrospinning with *in situ* cross-linking and forms gradient fibrillar structures resembling those of the ECM *in vivo*. These GE gradient nanofiber nonwovens mediate cell adhesion for potential target cells in the context of STR. With regard to the synthetic polymer PCL, the NBM is fabricated such that the PCL nonwovens are imbedded by the GE nonwoven sheets (Fig. 1A), and serve as porous bioadhesive interlayers. In the course of early biomaterial characterization experiments, the NBM exhibits excellent wettability and perfusion capability which has been proved by placing a water droplet onto the fiber meshwork and the observation that this droplet was absorbed immediately. Due to our novel *in situ* GE cross-linking technique,¹⁶ which is implemented directly into the electrospinning process without requiring post treatment, the GE nanofibers maintain their structural integrity in aqueous solution and are very robust with regard to sustaining mechanical stresses (Fig. 1A).

For preclinical *in vitro* validation and animal studies, NBM was consciously sterilized and subsequently stored until use in sterile physiologic solution. Figure 1C illustrates the novel NBM ready for experimental purpose. Initially conducted elasticity measurement for the single GE layers under wet conditions revealed an elastic modulus of

35 ± 5 kPa, which represents an E-modulus, resembling epithelial tissue-innate stiffness.^{21,22} These measurements gave a first hint of how NBMs should be configured to meet the biomechanical needs of oral mucosal wound-healing situations on the basis of a target Young's modulus of oral soft tissue of about 3 MPa.²³ In this context, elongation measurement until break (45%) revealed a Young's modulus of our ready-to-use NBM of 3.9 MPa, which is in the range of the target elastic modulus of oral soft tissues.²³ Intriguingly, by changing the membrane meshwork configuration, that is, GE fiber diameter and pore size of the PCL 3D meshwork, the overall stiffness of the membrane can be adjusted in a dual fashion, thereby facilitating consideration of the demands of a potential clinical application in the first place and second, those of the desired soft tissue wound-healing situation.

Periodontal simple cell systems cultivated on the novel nonwoven-based membrane

Based on the previously described intrinsic features, we next aimed at proving the suitability of the novel NBM as wound dressing, by employing tissue-authentic cell systems for preclinical validation. As a paradigm for soft tissue, we have chosen periodontal oral mucosa, comprising soft connective tissue fibroblasts and keratinocytes.

For analyzing periodontal cell morphology, proliferation, and successful cell adhesion on GE fibers, we established a thin layer of electrospun cross-linked GE on glass cover slips. In a preclinical validation context, GF and GK were first solely seeded for direct response on biomaterial-cell appreciation, that is, as simple tissue-authentic cell systems on these surfaces, respectively. The cells were observed by phase-contrast life cell imaging for 24 h in a microscopy climate chamber. Both cell types showed adhesion to the GE fibers; whereas GF attached in minutes, GK took longer about 2 h, and both displayed proper cell morphogenesis and proliferation on the fiber meshwork (see Supplementary Movie S1 for GF and Supplementary Movie S2 for GK). In detail, life cell imaging revealed that GK exhibit increased motility while proliferating, which is an important feature for epithelial wound healing. In contrast, it should be noted that GF exhibit a pronounced slower migration rate, but

FIG. 1. Membrane meshwork fabrication. The electrospun polycaprolactone (PCL) is imbedded by electrospun gelatin (GE) meshwork sheets (A). The GE mesh exhibits in scanning electron microscopy (SEM) documentation a random but regular fiber configuration (B). The equilibrated nonwoven-based membranes (NBM) in aqueous solution illustrates the “ready to use” stage for experimental purposes (C). Color images available online at www.liebertpub.com/tea

displayed, after cell adhesion, extensive GE fiber contraction in comparison to GK over time.

For analyzing periodontal cell morphology, cells were cultivated on the biohybrid membrane for 24 h, fixed, and visualized by SEM. Documentation showed that GK and GF fairly displayed their natural morphology, and tried to overcome the GE meshwork interspaces by spreading the whole cell body or the formation of single cell extensions (Fig. 2A, B). High-resolution SEM images visualize the assumption that periodontal cells appeared to capture the GE fibers and adhered effectively, presumably with their integrins to the GE substrate (Fig. 2C, D).

Further investigations of periodontal cell behavior in the newly developed nonwoven-based membrane, using fluorescence microscopy, depicted that the cytoskeleton staining of F-actin filaments substantiated the typical morphology of GKs and GFs (Fig. 3A, B). Moreover, indirect immunofluorescence of common integrin subunits of cells under study^{24,25} clearly illustrates successful cell adhesion to the fibers, as proved by detection of the focal contact protein integrin subunit $\beta 1$ in GKs and integrin $\beta 3$ in GFs (Fig. 3C, D white arrows). The results, obtained by the tissue-authentic simple periodontal cell systems, provide evidence that our novel NBM fulfils prerequisites, which are indispensable for a prospective biomaterial-supported soft tissue wound-healing approach.

Preclinical in vitro validation by evaluation of complex interactive periodontal fibroblast-epithelial cell systems on the novel nonwoven-based membranes (NBMs)

Data derived from simple *in vitro* systems of single cell types give important information, regarding initial direct cell reactions in response to biomaterial features. However, they still suffer from a fundamental information gap in comparison to the *in vivo* context due to the lack of growth factor-mediated cell-to-cell-interactions, which represent the

physiological-relevant tissue situation. In the native context, the interplay between the mesenchymal and the epithelial compartment is crucial for epithelial tissue homeostasis and morphogenesis.²⁶ To close this gap, we established an authentic complex interactive cell system for further preclinical *in vitro* validation on the NBM, comprising GF as the mesenchymal component and GK as the epithelial counterpart of soft tissue periodontal oral mucosa.

As a prerequisite for the formation of a gingival tissue equivalent *in vitro* on the NBM, the GE fiber gradient meshwork, similar to ECM *in vivo*, should act as a biomechanical support, while concomitantly exerting fibroblast-conductive properties, to facilitate its capture by the cells. With regard to the natural GK behavior, the membrane should support their proliferation to achieve confluence and normal epithelial morphogenesis, reflected by the formation of stratified and differentiated cell layers, thereby yielding the epithelial equivalent. In this context, we were able to show in previous studies that the interplay between these two cell types is pivotal for stratification of the keratinocytes comparable to the *in vivo* situation.²⁰ Besides animal studies, such authentic tissue *in vivo*-like *in vitro* models are excellent tools that are used to obtain important data for the prospective transfer of the biomaterial approach into clinical applications.

To study the cellular behavior of GF and GK in the interactive co-culture system on the NBM, we first seeded GF (2×10^4 cells/cm²) in a small amount of culture medium to the “bottom side” of the membrane, which means that the cells were only able to adhere topically on the membrane. After 8 h, the membrane was turned upside down and GKs were seeded on the “upper side” of the membrane scaffold (1×10^5 cells/cm²). Using this technique, we aimed at mimicking the *in vivo* situation of the oral soft mucosal gingival tissue, where GFs and GKs communicate *via* soluble growth factors, as proved by our own previous studies.⁷ Based on successfully carried out preliminary experiments,

FIG. 2. SEM of a single gingival keratinocytes (GK) and gingival fibroblasts (GF) cultivated on GE fiber layers. GK (A) and GF (B) bridge between the fibers and roughly maintaining their natural morphology. Both periodontal cell types adhere properly to the GE fibers (arrows in C, D).

FIG. 3. Fluorescence microscopy of a single GK and GF cultivated on GE fiber layers. (A) F-actin staining in the GK and the GF (B) reveals complete cell spreading on the GE meshwork. Specific signals for integrin $\beta 1$ in GK (C) and for integrin $\beta 3$ in GF (D) illustrate adhesion points in the cell periphery close to the GE fibers (see arrows). The overlay of the cytoskeleton F-actin staining in green and the integrin signals in red indicate co-localization in the cell periphery (E, F). Color images available online at www.liebertpub.com/tea

characterization by detection of *in vivo*-relevant biomarkers in the interactive co-culture model was conducted with cultivation periods of 7 and 14 days to observe infiltrative capture of the membrane scaffold by GFs, and to investigate the differentiation stages of the GKs during epithelial morphogenesis.

In Figure 4, GFs as the connective tissue entity were immunohistochemically stained for the intermediate filament constituent vimentin, thereby revealing successful capture of the membrane. As exemplified for day 14, uniform vimentin distribution indicated proper cell adhesion and regular cell morphogenesis on and in between the fiber meshwork, as illustrated by the fuzzy cell layers documented *via* optical microscopy (Fig. 4A, B).

GKs, rendering the epithelial counterpart on the membrane scaffold, were stained for KRT14 as squamous epithelial structural marker, keratin 1/10 (KRT1/10) as early differentiation marker, and involucrin as late and/or terminal

epithelial differentiation marker. As illustrated in Figure 5, structural KRT14 detection showed that GKs have generally covered more of the biohybrid membrane surface, that is, were more close to confluence at day 14 (Fig. 5B) with matched day 7 (Fig. 5A). While at both culture periods, KRT14 exhibited homogenous expression, by being visible in all GKs (Fig. 5A, B), KRT1/10 (Fig. 5C, D), and involucrin as well (Fig. 5E, F), showed differential expression, revealing gradual increase in positive cells and cell clusters, respectively. Such an increase was reflected by clear extension of the patchy staining pattern at a later time point (Fig. 5D, F). This chronological gradual extension of early and late/terminal epithelial differentiation markers suggests that GKs resemble differentiation, occurring in a normal epithelium under *in vivo* conditions. This normal cellular differentiation behavior, on the one hand, is a hint of GK's appreciation of the novel membrane, and, on the other hand, adds to the body of evidence that GKs in interaction with

FIG. 4. Immunohistochemical (IHC) staining of vimentin in GFs on the NBM. (A) Overview of GFs cultivated on the membrane for 14 days, depicting the higher magnification image (B) localization in different layers visualized by the change between fuzzy and sharp cell illustrations. The inlay shows the negative control proving the specificity of the IHC vimentin staining. Color images available online at www.liebertpub.com/tea

FIG. 5. IHC staining of GKs here co-cultured with GFs on the NBM. Keratin 14 (A, B) keratin 1/10 (C, D) and involucrin (E, F) staining in GKs after 7 (A, C, E) and 14 days (B, D, F) of co-culture period. The inset shows the negative control proving the specificity of the IHC staining. Color images available online at www.liebertpub.com/tea

GFs are capable of forming a stratified epithelium, that is, *in vitro* epithelial equivalent on the prospective wound dressing device.

To further substantiate our suggestion, obtained from the observations made by optical microscopy and the IHC studies in complex interactive co-cultures, SEM imaging was conducted. At the given growth periods, SEM analysis documented efficient adhesion and morphogenesis of both periodontal oral mucosal cell types on the novel membrane scaffold after 7 days; while, though close to confluence, cells' boundaries were still visible (Fig. 6A, B). These cell boundaries suggested complete diminishment with an ongoing culture period, as visualized by SEM icons for both cell types at day 14. At this time point, a confluent cell

compartment of the respective cell type was formed, making discrimination of individual cells and cell borders hardly possible (Fig. 6C, D). In a lateral view of the specimen, it already became evident that GKs formed several cell layers (Fig. 6E), and that tightly packed GFs were covered by an overlay of ECM. This is indicated by the fine fiber-like deposits on the surface of the confluent GF compartment (Fig. 6F). The formation of layered GKs and the existence of such ECM deposits not only for GFs but also for GKs was corroborated by the high-resolution SEM icons in Figure 7, showing that epithelial keratinocytes grew over the underlying keratinocyte layer (arrow in Fig. 7A). Fibroblasts as well as keratinocytes have produced fibrous ECM components (Fig. 7B, C), which can easily be discriminated from

FIG. 6. SEM of GKs (A, C, E) co-cultured with GFs (B, D, F) on the NBM for 7 (A, B) and 14 days (C–F). While cell boundaries can be discriminated at day 7 (A, B), this is hardly possible after 14 days (C, D). Lateral view shows layered keratinocytes (E) and a confluent layer of fibroblasts with deposits of extracellular matrix (ECM) (F).

FIG. 7. High-magnification SEM of GKs (**A**, **C**) co-cultured with GFs (**B**, **D**) on the NBM for 14 days. GKs grow in layers (see arrow in **A**) and deposit ECM (**C**). In addition, GFs produce their own matrix (**B**), substantiated by the observation of fine network structures in between the GE fibers (**D**).

the huge GE fibers (Fig. 7C, D). As shown in Figure 6E, a remarkable finding was that GK morphogenesis reflected the development of a stratified epithelium with differentiated, that is, apically flattened cells, thereby paralleling the *in vivo* situation. In conjunction with the ECM deposits, this finding emphasizes the suitability of the novel NBM as soft tissue wound dressing, as preclinical validation shows the nonwoven-based membrane's support of GF and GK, regarding wound healing promoting ECM deposition, and formation of a tissue authentic oral mucosal epithelial equivalent.

Nonwoven-based membrane application in vivo after soft tissue dehiscence formation in minipigs

With regard to the planned application in humans, we would like to emphasize by excursion that state-of-the-art of surgical procedures for large periodontal and/or intraoral wound defect treatments, for example, cheek tumour resection, involves using commercial membranes or using autologous skin transplants derived from the forearm region.^{27–30} Disadvantages of both procedures are mainly arising from the facts that the currently available membranes are not suitable for large wound defects, while the autologous skin transplant approach implies an invasive and long-lasting surgical intervention for the patient.

Against the background of this still-existing clinical need, and in light of successful preclinical *in vitro* validation of our novel NBM by employing tissue-authentic cell systems, we were next interested in testing whether it is also rendered suitable for *in vivo* application. Hence, it was employed as wound dressing after minipig soft tissue dehiscence formation caused during the dental implantation procedure.

Since this was a multitasking approach for primary soft tissue closure of palatal flaps, the icons exemplarily showing the setup as well as the follow up of the *in vivo* study, in addition to our NBM, partially illustrate a dental implant. Hence, adjacent to an implant, Figure 8A shows the initial situation directly after placing the novel nonwoven-based

membrane into the soft tissue dehiscence, and inlay revealed rapid perfusion (Fig. 8A, inlay).

Next, successful membrane wound implementation became evident by stable cross-sutures, which was concomitant with its wound persistence (Fig. 8B). NBM maintenance, which was going along with wound healing, was observable after 2 weeks. In this context, healing progression revealed only slight differences between the left and the right mandible, as substantiated by marginal differences in re-epithelization (Fig. 8C, left mandible; and Fig. 8D, right mandible). Further progression with almost complete wound closure was visible after 3 (Fig. 8E, F) and 4 (Fig. 8G, H) weeks postsurgery in contrast to the wound-healing course after 2 and 3 weeks (Fig. 8I, J) without NBM due to mastication-caused NBM loss, which resulted in a clearly visible incomplete wound-healing situation (Fig. 8I, J, arrows). As already adumbrated on the macroscopic postsurgical tissue level, NBM strongly suggests promising support of soft tissue healing within 4 weeks. To corroborate this assumption, we were carrying out histology to analyze the status of wound healing on the more cellular level. In overview screens (Fig. 9A) and detailed views (Fig. 9B), histological sections after week 4 displayed PCL-membrane remnants at the alveolar bone soft connective tissue interface in conjunction with complete regeneration of gingival soft connective tissue and the corresponding epithelium too, with the latter forming its characteristic rete ridges. Moreover, sections disclose the fact that specimens were devoid of inflammation (Fig. 9A, B). By contrast, incomplete re-epithelialization after dehiscence was observed in minipigs suffering from mastication-caused NBM loss (Fig. 9C).

Discussion

For support in healing wounds and/or tissue regeneration, aiming at the achievement of reconstitution of form and function of damaged or diseased soft tissue such as the skin or the oral cavity mucosa, novel biodevices should fulfil

FIG. 8. In a pilot minipig study after soft tissue dehiscence formation, NBM was used as a wound biodevice to treat the implantologically induced soft tissue defect (**A**, see arrows) of about 2 cm. Inlay revealed rapid NBM blood perfusion. Cross-sutures were applied for NBM fixation (**B**, see arrows), and a wound-healing course was followed up after 2, 3, and 4 weeks (**C–H**). Arrows indicate NBM remnants and re-epithelialization regions. Dehiscence wound healing course without NBM **2 (I)** and **4 (J)** weeks after surgery. Arrows indicate incomplete re-epithelialization and signs of inflammation. Color images available online at www.liebertpub.com/tea

criteria, mutually considering and addressing the demands of the clinical application, and the requirements of the tissues and their cells desired for recuperation. Hence, to evaluate a modern age biomaterial as really convenient, among other criteria such as (i) biocompatibility, this issue optimally includes adjustable/manageable biodevice-innate features, such as (ii) biodegradability, (iii) mechanical stability, and (iv) targeted biomechanics with a Young's modulus of 3 MPa for oral soft tissues.²³ Moreover, for accelerating wound healing and mobilization of the bodies'

self-healing power, novel devices should take into account the pivotal aspect of perfusion, as blood supply nourishes cells by diffusible growth factors and oxygen supply.^{31,32} Regarding this clinically relevant *in vivo* aspect, our novel NBM wound dressing has behaved in a valid manner, by showing clear signs of perfusion already a few minutes after placement into the minipig dehiscence. This observed perfusion ability as a prerequisite for optimal wound healing was synergistically supported by an obvious excellent biocompatibility. In our case, this key functional requirement of

FIG. 9. Histology after a 4-week wound-healing course depicts NBM remnants at the connective tissue/alveolar bone interface (**A**, see arrows). Microscopic enlargement revealed complete re-epithelialization (**B**, see annotation and arrows) in contrast to the wound-healing course without NBM (**C**, see arrow). Color images available online at www.liebertpub.com/tea

a biomaterial³³ was indicated by the total lack of inflammatory signs, as indicated by the postoperative 4-week histology and the visual clinical photo documentation during the wound-healing course at 2, 3, and 4 weeks after surgery. By currently modifying the perspective of biocompatibility from the archaic view to a modern one, the key requirement more and more focuses on whether a medical device directly addresses or mobilizes tissue cells. This includes an important aspect that takes into account the self-healing power of body tissues in the context of the respective clinical wound-healing situation. To fulfil this central point, outstanding novel biomaterial cell adhesion affinity appears mandatory. Therefore, we have chosen GE as a natural constituent of our biohybrid polymer, as it has currently been shown *in vitro*, to enhance cell adhesion and proliferation.³⁴ In NBM, the GE nanofiber architectures reassemble the fibrillar architectures of the ECM. Regarding the novel device, the findings of fast and homogenous capture of the material by either tissue-authentic GFs or GKs in conjunction with the proper occurring cell morphogenesis, and combined actin/focal contact integrin fluorescence, strongly suggest the NBM device's cell adhesion and proliferation-assisting affinity. These findings are in line with those reported earlier. As detected by life cell imaging, the novel device enables the tissue authentic cells employed in this study to realize two additional features, observed during normally occurring wound healing. These features are reflected by keratinocyte migration, being important for wound re-epithelialization,³⁵ and fibroblast-driven wound contraction, indispensable for shortening wound margins' distance.³⁶ Interestingly, GKs exhibited increased motility on the novel device, while GFs showed extensive GE fiber contraction with matched GKs. This natural cell behavior clearly demonstrates the NBM's ability to serve as *in vivo* wound bed mimicry.

Moreover, the presence of ECM deposits detected for the GF-derived fibroblast-/connective tissue compartment as well as the GK-derived *in vitro* epithelial equivalent by SEM analysis may point to a biodevice-target cells synergism with regard to adhesion, in which context a self-perpetuating process can be hypothesized. This loop-like/circulating process includes the preference of adhesion by the novel device, concomitant with initiation as well as progression of auto-ECM production and deposition by adhered cells. This, in turn, yields accelerated and more efficient adhesion of further target cells, which are essential for wound closure and/or tissue regeneration. Such a self-recreating process of progressive cell adhesion, triggered *via* tuned ECM deposition, finally yields the creation of an extracellular microenvironment. This becomes more and more adjusted to the cell-convenient natural situation *in vivo*, with regard to its biomechanical properties. On the other hand, a favorable wound-dressing biomechanics itself, offered to the cells of a regenerating tissue particularly at the initial stages of the wound-healing situation, may appear to the cells similar to a comfortable bed, rendering a valuable kick-off point for settling, spreading, and starting the synthesis and production of biomolecules, which are required for attracting further tissue cells for wound closure. This issue can be described by the term "feel good factor," and the cell findings strongly elaborated suggest the existence of this factor holding true for the NBM. With regard to our

novel biohybrid membrane, cell effective biomechanical cues can be adjusted in a bidirectional, that is, target cell-oriented and clinical indication-specific manner, by changing the GE fiber diameter and pore size of the PCL 3D meshwork. Using this fabrication method, a device-innate elastic Young's modulus of the GE layer of 35 ± 5 kPa can be created, which is compatible with the stiffness of soft tissue oral mucosa and particularly skin in conjunction with its subcutaneous tissues, ranging from 14.0 ± 5.0 kPa to 58.8 ± 1.7 kPa.²² Having the possibility to realize this targeting with regard to the biomechanical elasticity range of native soft tissue, our nonwoven-based biohybrid membrane offers the chance to desired cells, in acting as the convenient comfortable bed mentioned earlier, rendering the initiation point for efficient wound healing. Acting as a biomechanically cell-convenient device has been successfully corroborated for the novel device with our pursuing preclinical *in vitro* validation experiments, by employing the tissue-authentic complex interactive periodontal fibroblast-epithelial cell systems. Here, completion of the development of a fibroblast-derived connective tissue compartment as well as a keratinocyte-derived epithelial compartment with expression of tissue-innate biomarkers has been observed within 14 days. In addition to the development of such tissue compartments, morphogenesis of a stratified *in vitro* gingival epithelial equivalent, which parallels the expression of native tissue biomarkers, for example, keratins and involucrin, emphasizes the novel membrane's suitability as wound dressing in biomechanical terms. This is an important point, as significance of tissue-convenient extracellular biomechanics for formation of an *in vitro* epithelial equivalent, based on fibroblast interactions, has been recently shown by our own studies.²⁰

On the other hand, the second arm of bi-directionally targeted biomechanics, represented by the demands of the clinical indication, appears likely to be also addressed adequately by the novel device, as extensive cross-suturing of the membrane has successfully been managed by the surgeon, that is, without any indications of membrane disruption. A further critical issue in the wound-healing context in addition to the device's mechanical stability is the initially mentioned biodegradability. Here, a balance between postoperative biomaterial degradation and the cell's scaffold stability till almost wound closure completion appears optimal. While long-lasting material remnants could hamper physiology of the recovered wound tissue, premature wound-dressing degradation, similar to ECM collagen deficiency in autochthonous wound defects,³⁷ could lead to incomplete tissue function, due to target tissue volume deprivation during wound healing. After *in vivo* application, postsurgical conceptual validation of our novel membrane after 4 weeks suggests that it addresses this balance adequately, as concomitant with the presence of only small PCL fiber remnants, indicating the bulk's biodegradation, soft tissue oral mucosal wound closure has almost been accomplished.

Conclusion

In this study, we employed tissue-authentic simple and complex interactive fibroblast-keratinocyte cell systems in conjunction with tissue-innate biomarkers to preclinically validate our novel nonwoven-based biohybrid polymer membrane NBM biodevices, as suitable wound dressing for

soft tissue defects, for example, oral mucosal for the first 4 weeks. The NBM, tailored to mimic the ECM, was fabricated by electrospinning of *in situ* cross-linked GE nanofiber nonwovens and subsequent lamination with electrospun PCL nanofiber nonwovens by heating the layer assemblies above the melting temperature of PCL. Gradients were introduced by varying the number and sequence of the individual GE and PCL layers, and by creating a GE nanofiber diameter gradient (90–680 nm), owing to the addition of glyoxal cross-linking agents and viscosity build-up during electrospinning. The results obtained showed clear appreciation of the novel NBM by *in vivo* relevant human target cells, as demonstrated by their proper adhesion, morphogenesis, motility, formation of respective tissue compartments, with *in vitro* epithelial equivalent formation in particular, and expression of tissue-specific biomarkers. Additional implications from our findings are that the NBM device's cell convenience and thus, "cell-addressed feel good factor" is based on a target cell/tissue-compatible biomechanics; while *in vivo* application corroborates promising guidance of soft tissue, that is, epithelial wound closure. This outcome further recommends the profound wound-dressing qualities of the membrane in prospective clinical applications, by reflecting its biocompatibility, balanced biodegradability, and mechanical operative stability.

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Disclosure Statement

The authors confirm that there are no conflict of interests.

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