# The role of mesenchymal stem cells in murine lacrimal gland regeneration *in vitro* and *in vivo*

Inaugural-Dissertation

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*Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker,*
*er steht auch vor den Naturgesetzen wie ein Kind*
*vor der Märchenwelt*
*-Marie Curie-*

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# List of Abbreviations

| | |
|---|---|
| ADDE | aqueous-deficient dry eye |
| ANOVA | analysis of variance |
| ARVO | Association for Research in Vision and Ophthalmology |
| BAC | benzalkonium chloride |
| BM | bone marrow |
| BMP7 | bone morphogenetic protein 7 |
| bp | base pair |
| BSA | bovine serum albumin |
| BW | bodyweight |
| CD | cluster of differentiation |
| cDNA | copy deoxyribonucleic acid |
| CFU | colony forming unit |
| CFU-F | colony forming unit fibroblasts |
| c-Kit | tyrosinkinase KIT |
| CM | conditioned medium |
| ConA | concanavalin A |
| cpd | cumulative population doublings |
| CT | threshold cycle |
| d | day |
| Da | Dalton |
| DAB | 3,3′-diaminobenzidine |
| DNA | deoxyribonucleic acid |
| DAPI | 4´,6-Diamidin-2-phylindol |
| DC | dendritic cells |
| DED | dry eye disease |
| DES | dry eye syndrome |
| DL | duct ligation |
| EC | epithelial cells |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| eGFP | enhanced green fluorescence protein |
| EMT | epithelial-mesenchymal transition |
| EPCP | epithelial progenitor cells |
| Eq | equation |
| Erk | extracellular signal–regulated kinases |
| EtOH | ethanol |
| EV | extracellular vesicles |

| | |
|---|---|
| Exp | explant |
| FABP4 | fatty-acid binding protein 4 |
| FACS | fluorescent-activated cell sorting |
| FBS | fetal bovine serum |
| FBS-S | fetal bovine serum superior |
| Fc receptor | fragment, crystallizable |
| FDA | Food and Drug Administration |
| FELASA | Federation of European Laboratory Animal Science Association |
| FGF | fibroblast growth factor |
| FITC | fluorescein isothiocyanate |
| FL | fluorescein staining |
| gDNA | genomic deoxyribonucleic acid |
| GFP | green fluorescence protein |
| GMP | Good Manufacturing Practice |
| GvHD | graft-versus-host-disease |
| hAM | human amniotic membrane |
| HBSS | hank´s balanced salt solution |
| HE | hematoxylin and eosin |
| HEPES | 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid |
| HGF | hepatocyte growth factor |
| HLA | human leukocyte antigen |
| HPF | high power field |
| HRP | horse-radish peroxidase |
| Hz | Hertz ($s^{-1}$) |
| i.p. | intraperitoneal |
| iCM | inflammatory conditioned medium |
| IFNγ | interferon gamma |
| IL | interleukin |
| IPCs | islet-derived precursor cells |
| iPS cells | induced pluripotent stem cells |
| JAK | Janus kinase |
| KCS | keratoconjunctivitis sicca |
| Lcn2 | lipocalin-2 |
| LFU | lacrimal functional unit |
| LG | lacrimal gland |
| M | molar |
| MAPK | mitogen-activated protein kinase |
| MHC | major histocompatibility complex |
| mRNA | messenger ribonucleic acid |
| MSC | mesenchymal stem cells |
| nCM | normal conditioned medium |
| NK cells | natural killer cells |

| | |
|---|---|
| NOD | non-obese diabetic mice strain |
| p | passage |
| PACAP | pituitary adenylate cyclase-activating polypeptide |
| PANTHER | Protein ANalysis THrough Evolutionary Relationships |
| PBL | peripheral blood lymphocytes |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pd | population doublings |
| PDGFRα | platelet-derived growth factor receptor alpha |
| PE | phycoerythrin |
| PFA | paraformaldehyde |
| PSI | pound-force per square inch |
| qPCR | real-time quantitative PCR |
| $r^2$ | coefficient of determination |
| Rac1 | Ras GTPase-activating protein-binding protein 1 |
| Rbmy | RNA-binding motif protein on Y chromosome |
| RNA | ribonucleic acid |
| RNQ | normalized relative fold change |
| RPS6 | ribosomal protein S6 |
| rt | room temperature |
| s.c. | subcutaneously |
| Sca-1 | stem cell antigen 1 |
| SD | standard deviation |
| SEM | standard error of the mean |
| SG | salivary gland |
| SP | side population cells |
| SS | Sjögren´s syndrome |
| SSDE | Sjögren´s syndrome dry eye |
| STAT1 | signal transducer and activator of transcription 1 |
| TBP | TATA-box binding protein |
| T-BUT | tear-film breakup time |
| TGF-β | transforming growth factor beta |
| TNFα | tumor necrosis factor alpha |
| TSG-6 | tumor necrosis factor α-stimulated gene 6 protein |
| u | units |
| wk | week |
| WST-1 | water soluble tetrazolium |
| x g | multiplied gravity |
| αMEM | Minimum Essential Medium Eagle - Alpha Modification |
| αSMA | alpha smooth muscle actin |

Common abbreviations, SI units and prefixes were also used.

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## Summary

Aqueous-deficient dry eye (ADDE) is a multifactorial disease mainly caused by a loss of functional lacrimal gland tissue. This subtype of dry eye disease can cause the most severe forms of the disease. Despite a high prevalence of 5 – 50%, current treatment options remain palliative. As a result, there is an increasing demand for curative therapies, which accelerates the development and validation of relevant treatment approaches. In this context, the investigation of mesenchymal stem cells (MSC) appears promising as these cells have been shown to promote tissue regeneration, possess immunomodulatory properties and have been detected within the lacrimal gland. Based on these results, this thesis studies the regenerative capacity of the lacrimal gland and the potential therapeutic efficacy of MSC for *in situ* lacrimal gland regeneration.

In this regard, the investigation of relevant mouse models can provide important insights into the dynamics of lacrimal gland damage and regeneration. In the present thesis, a suitable mouse model for the induction of ADDE was therefore established by ligation of the single excretory duct of the murine extraorbital lacrimal gland. This resulted in damage to the functional acinar cells and a severe inflammatory reaction, which was accompanied by a decrease in tear secretion and lacrimal gland weight. In addition, an increase of MSC was detected during lacrimal gland damage and regeneration, which indicates an involvement of these cells in the regeneration process. With a timeframe of 21 days, this model enables a detailed investigation of promising treatment approaches aiming for an *in situ* regeneration of the lacrimal gland. Regarding a clinical application of MSC for the management of ADDE, isolation of MSC from the lacrimal gland seems to be the most appropriate source for cell therapy (orthotopic transplantation). In the current thesis, a pure and specific MSC population with the characteristic phenotype could be isolated from the lacrimal gland both by explant culture and fluorescence-activated cell sorting (FACS). Analysis of the MSC secretome revealed potentially therapeutically relevant proteins such as Lipocalin-2, prosaposin, ras GTPase-activating protein-binding protein 1 (Rac1) and signal transducer and activator of transcription 1 (STAT1), which improved the viability of damaged lacrimal gland acinar cells *in vitro*. These proteins are promising candidates that need to be examined in future experiments regarding their impact on lacrimal gland regeneration *in vivo*. Based on the results of secretome analysis, it could be assumed that MSC secrete trophic factors that promote the regeneration of lacrimal gland acinar cells. Therefore, the therapeutic efficacy of MSC in the regeneration of damaged lacrimal gland tissue was investigated using the established duct ligation mouse model and a transplantation of extrinsic MSC isolated from the lacrimal gland. This orthotopic transplantation of MSC led to a significantly increase in the amount of vital acinar structures as well as to a reduction

of the immune response, which resulted in an improved regeneration of the lacrimal gland compared to its intrinsic regenerative capacity. Thus, the application of MSC appears to be a promising therapeutic approach to enhance lacrimal gland regeneration in a mouse model with severe ADDE.

In conclusion, the results of this doctoral thesis provide essential insights into the dynamics of lacrimal gland damage and regeneration as well as into the time-dependent involvement of MSC. In addition, this thesis presents experimental approaches based on MSC that could be successful in the treatment of ADDE by *in situ* regeneration of the lacrimal gland.

# Zusammenfassung

Das hypovolämisch trockene Auge (engl. aqueous-deficient dry eye, ADDE) ist eine multifaktorielle Erkrankung, die hauptsächlich durch den Verlust von funktionellem Gewebe der Tränendrüse verursacht wird. Dieser Subtyp des trockenen Auges führt zu den schwersten Krankheitsverläufen. Trotz einer hohen Prävalenz von 5 – 50%, sind aktuelle Behandlungsoptionen rein palliativ. Daher besteht ein wachsender Bedarf an kurativen Therapien, wodurch die Entwicklung und Validierung relevanter Behandlungsansätze vorangetrieben wird. In diesem Kontext scheint die Untersuchung von mesenchymalen Stammzellen (engl. mesenchymal stem cells, MSC) vielversprechend, da diese Zellen nachweislich die Regeneration von Gewebe fördern, immunmodulatorische Eigenschaften besitzen und in der Tränendrüse nachgewiesen wurden. Basierend auf diesen Ergebnissen untersucht die vorliegende Doktorarbeit das regenerative Potential der Tränendrüse und die therapeutische Wirksamkeit von MSC für die *in situ* Regeneration der Tränendrüse.

In diesem Zusammenhang kann die Erforschung von relevanten Mausmodellen wichtige Erkenntnisse über die Dynamik der Schädigung und Regeneration der Tränendrüse liefern. In der vorliegenden Studie wurde daher eine Ligatur des einzigen Ausführungsgangs der murinen extraorbitalen Tränendrüse durchgeführt, um ADDE zu induzieren. Dies führte zum Verlust der funktionalen Azinuszellen und zu einer schweren Entzündungsreaktion, die mit einer Reduktion der Tränensekretion und des Drüsengewichts einherging. Darüber hinaus konnte ein Anstieg der MSC während der Schädigungs- und Regenerationsphase in der Tränendrüse beobachtet werden. Dies weist auf eine Beteiligung dieser Zellen während des Regenerationsprozesses hin. Durch den zeitlichen Verlauf von 21 Tagen ermöglicht dieses Modell eine detaillierte Untersuchung vielversprechender Behandlungsansätze für die *in situ* Regeneration der Tränendrüse. Im Hinblick auf eine klinische Anwendung der MSC für die Behandlung von ADDE scheint die Isolation der MSC aus der Tränendrüse als Gewebequelle für eine Zelltherapie am besten geeignet (orthotope Transplantation). Eine reine und spezifische MSC-Population, die den charakteristischen Phänotyp aufweist, konnte in der vorliegenden Studie sowohl mittels Explantkultur als auch fluoreszenz-aktivierter Zellsortierung (engl. fluorescence-activated cell sorting, FACS) aus der Tränendrüse isoliert werden. Die Analyse des MSC-Sekretoms konnte potentiell therapeutisch relevante Proteine wie Lipocalin-2, prosaposin, ras GTPase-activating protein-binding protein 1 (Rac1) und signal transducer and activator of transcription 1 (STAT1) aufdecken, welche die Viabilität von geschädigten Azinuszellen *in vitro* verbesserten. Diese Proteine stellen aussichtsreiche Kandidaten dar, die hinsichtlich ihres Einflusses auf die Regeneration der Tränendüse in zukünftigen Experimenten *in vivo*

untersucht werden sollten. Zudem deuten die Ergebnisse der Sekretomanalyse darauf hin, dass MSC trophische Faktoren sekretieren, welche die Regeneration von Azinuszellen der Tränendrüse fördern. Daher wurde die therapeutische Wirksamkeit von MSC auf die Regeneration von geschädigtem Tränendrüsengewebe mit Hilfe des etablierten Mausmodells (Ligatur des Ausführungsgangs) sowie der Transplantation von extrinsischen MSC aus der Tränendrüse untersucht. Die orthotope Transplantation der MSC führte zu einer signifikanten Erhöhung der vitalen Azinusstrukturen sowie zu einer Reduktion der Immunreaktion. Im Vergleich zur intrinsischen Regenerationsfähigkeit wurde dadurch eine erhöhte Regeneration des funktionalen Gewebes der Tränendrüse erreicht. Daher scheint die Anwendung von MSC ein vielversprechender therapeutischer Ansatz zu sein, um die Regeneration der Tränendrüse im Mausmodell mit schwerer ADDE zu verbessern.

Zusammenfassend liefern die Ergebnisse der vorliegenden Dissertation grundlegende Erkenntnisse über die Dynamik der Schädigung und Regeneration der Tränendrüse sowie über die Beteiligung der MSC. Darüber hinaus konnten experimentelle Ansätze auf der Grundlage von MSC aufgezeigt werden, die im Hinblick auf eine kurative Behandlung von ADDE durch *in situ* Regeneration der Tränendrüse vielversprechenden sind.

# 1. Concept of the Study

## 1.1 Aims of the Study

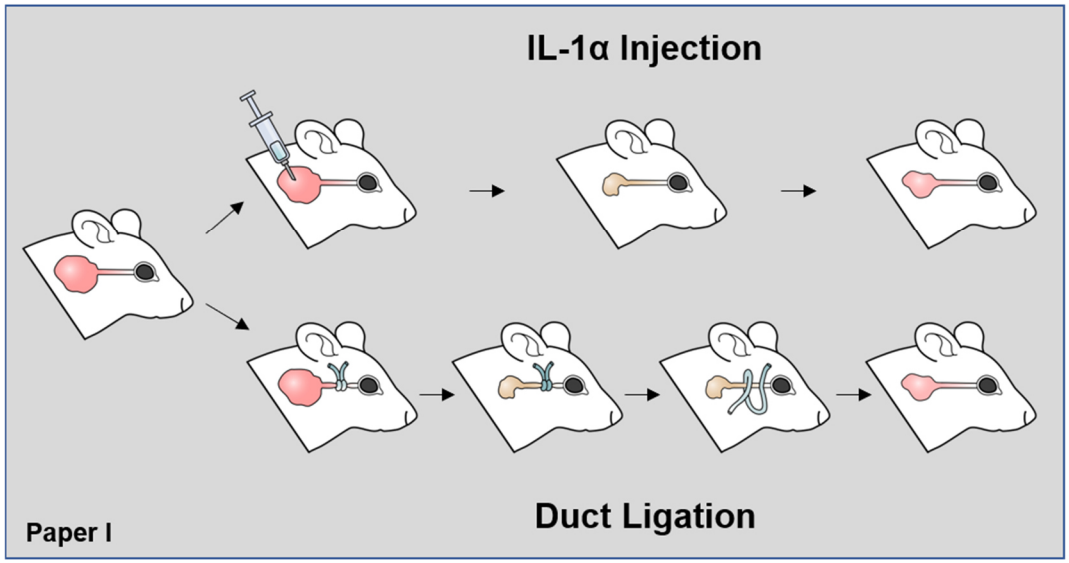
The overall aim of this study was to investigate the contribution of mesenchymal stem cells (MSC) to the regeneration of the murine lacrimal gland in order to provide starting points for new therapeutic approaches. Based on these results, fundamental insights into the therapeutic efficacy of MSC on lacrimal gland regeneration will be gained. The concept of the study was therefore organized into three important steps: (1) investigating a mouse model, (2) isolation and characterization of MSC population, (3) transplantation of MSC into the lacrimal gland.

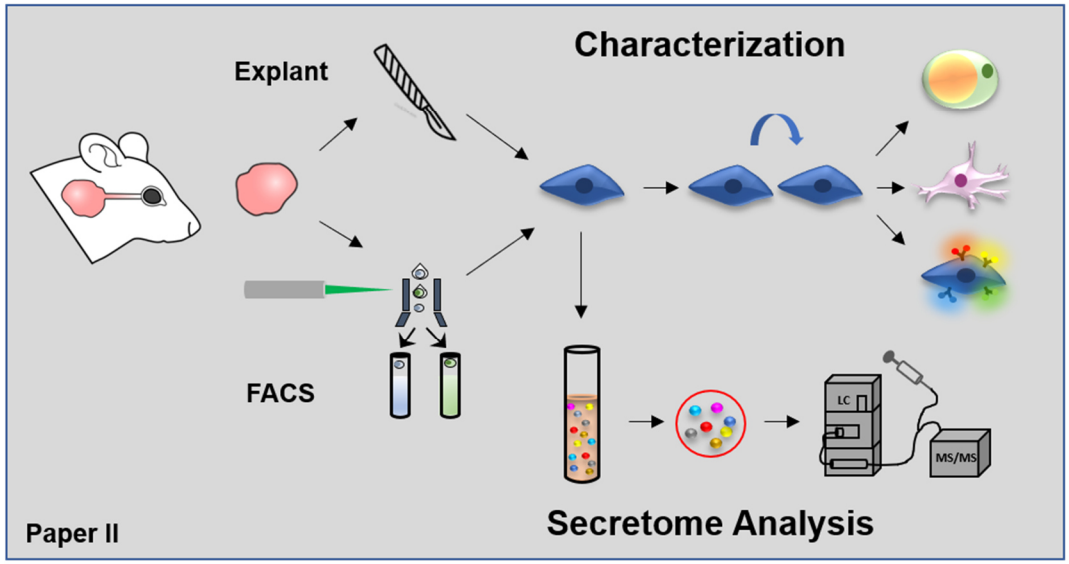
The first aim was to establish a suitable mouse model to enable the study of lacrimal gland regeneration. Aqueous-deficient dry eye was induced either by intraglandular injection of interleukin-1α or a transient ligation of single excretory duct of the extraorbital lacrimal gland. Both methods were analyzed and compared with respect to clinical signs of ocular surface damage, dynamics of damage and regeneration of functional lacrimal gland tissue, characterization of immune response as well as the contribution and localization of intrinsic mesenchymal stem cells. **Paper I**

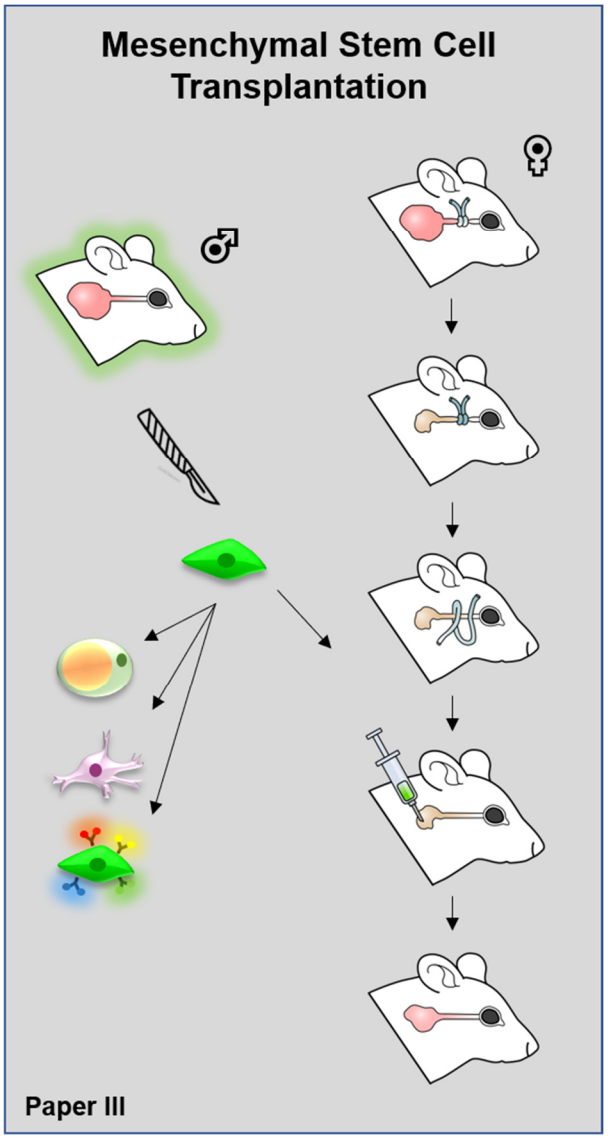
The second aim was to establish a reliable method for the isolation of a pure and specific MSC population from murine lacrimal gland for the purpose of orthotopic transplantation. The cells were isolated by explant culture and compared to isolation by fluorescence-activated cell sorting (FACS). Both MSC populations were characterized according to the criteria defined by the “International Society for Cellular Therapy”. Since it is suggested that MSC secrete trophic factors which are involved in tissue repair, the secretome of MSC was analyzed using shot-gun mass spectrometry. Proteins that might improve the regeneration of lacrimal gland acinar cells were selected and tested in appropriate *in vitro* experiments. **Paper II**

The third aim was to investigate if the transplantation of orthotopic extrinsic MSC enhance the intrinsic regenerative capacity of the murine lacrimal gland. For this purpose, lacrimal gland-derived MSC were isolated by the established and reliable method of explant culture (**Paper II**). In parallel, aqueous-deficient dry eye was induced by transient duct ligation, which was established as a suitable model (**Paper I**). Transplantation of lacrimal gland-derived MSC was performed when releasing the duct ligation. Analysis focused on the clinical signs of ocular surface damage, dynamic of lacrimal gland damage and regeneration, characterization of immune response and the contribution of intrinsic as well as extrinsic MSC. **Paper III**

## 1.2 Study Overview



# 2. Introduction

The introduction will be presented in two review articles, which describe the relationship between the lacrimal gland, dry eye disease and mesenchymal stem cells.

The review article entitled “Towards Lacrimal Gland Regeneration: Current Concepts and Experimental Approaches” provides a brief overview of the biology of the lacrimal gland and the pathology of aqueous-deficient dry eye. In detail the mechanisms of intrinsic lacrimal gland regeneration, the presence and properties of lacrimal gland resident stem cells, and concepts that might promote lacrimal gland regeneration are considered. In addition, currently investigated experimental approaches for the induction of lacrimal gland regeneration are presented and discussed in depth.
A detailed introduction to the structure and function of the lacrimal gland, the pathology and clinical management of dry eye disease, as well as the biology, behavior in the disease state and *in vitro* properties of mesenchymal stem cells can be found on page 17 – 21 as parts of the review article “Development of Causative Treatment Strategies for Lacrimal Gland Insufficiency by Tissue Engineering and Cell Therapy. Part 1: Regeneration of Lacrimal Gland Tissue: Can We Stimulate Lacrimal Gland Renewal In Vivo?”.

Both review articles were written during the period of dissertation by the doctoral candidate, one with a first and one with a shared-first authorship and approved by the supervisor (Prof. Dr. Dr. Schrader) and the mentor (Prof. Dr. von Gall) as introduction.

## 2.1 Review Article

## Towards Lacrimal Gland Regeneration: Current Concepts and Experimental Approaches

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Article contribution to the main thesis

Short introduction to dry eye disease/aqueous-deficient dry eye (DED/ADDE), lacrimal gland and mesenchymal stem cells (MSC). Detailed consideration of the necessary requirements to curatively treat ADDE and the current knowledge about processes involved in lacrimal gland regeneration, lacrimal gland resident stem cells, and deductions of possible concepts for *in situ* lacrimal gland regeneration.

(A detailed introduction of ADDE, the lacrimal gland and MSC can be found in 2.2 page 16)

Contribution to experimental design, implementation and publication

Review of the literature, manuscript structure, writing of the manuscript including preparation of all figures. The manuscript was subsequently reviewed and approved by the co-author.
Approximated total share of contribution: 85%

CURRENT EYE RESEARCH
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# Towards Lacrimal Gland Regeneration: Current Concepts and Experimental Approaches

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**ABSTRACT**

Dry eye disease (DED) is a complex and multifactorial disease resulting in a continual cycle of tear hyperosmolarity and inflammation. Patients suffering from DED experience severe pain and visual impairments leading to a reduced quality of life. Aqueous-deficient dry eye (ADDE), mainly caused through a loss of functional lacrimal gland tissue, results in the most severe forms of DED. Despite a high prevalence, the current treatments remain palliative and may be insufficient to alleviate the symptoms. Consequently, investigations on experimental approaches for *in situ* lacrimal gland regeneration are of great clinical interest. This article reviews the current knowledge about processes involved in lacrimal gland regeneration, about lacrimal gland resident stem cells, and offers deductions about possible concepts for *in situ* lacrimal gland regeneration. Promising starting points might be the utilization of therapeutic proteins, such as bone morphogenetic protein 7, mesenchymal stem cells (MSC) or MSC-based treatments such as conditioned medium, lyophilized cell extracts or adult acinar cells. This review further summarizes current experimental approaches for the treatment of ADDE in animal models and patients. Approaches investigating side population stem cells, epithelial progenitor cells and MSC showed that the transplantation of these cells had therapeutic effects on ADDE. However, the most promising and best-studied experimental approach is the use of MSC for induction/enhancement of *in situ* lacrimal gland regeneration. Their immunomodulatory effects, low immunogenicity, promotion of tissue regeneration and involvement during spontaneous lacrimal regeneration are favorable traits for clinical applications. In addition, the efficacy and safety of allogeneic MSC transplantation have already been demonstrated in a small patient cohort.

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**KEYWORDS**
Aqueous-deficient dry eye; lacrimal gland; tissue regeneration; tissue-specific stem cells; mesenchymal stem cells

## Introduction

Dry eye disease (DED) is a multifactorial disease of the ocular surface and the tear film resulting in multiple symptoms.[1] In general, DED can be divided into the evaporative dry eye (EDE) and the aqueous-deficient dry eye (ADDE).[2] While EDE is the main cause for the development of DED, patients with ADDE suffer from the most severe forms. However, during disease progression these subtypes may coexist. This article reviews the necessary requirements to curatively treat DED and the current knowledge about processes involved in lacrimal gland regeneration as well as the function of lacrimal gland resident stem cells. Furthermore, it offers deductions about possible concepts for *in situ* lacrimal gland regeneration.

### *Lacrimal gland*

The lacrimal gland is a mainly serous, tubule-acinar gland. Histologically, it is composed of acinar cells, which are arranged in individual functional units (the acini) and surrounded by ductal and myoepithelial cells (Figure 1). The acinar cells are responsible for the production and secretion of the primary lacrimal fluid[2], which is then conducted onto the ocular surface. On the ocular surface, the tear film is formed and improves the optical properties of the ocular surface by generating a smooth surface on the corneal epithelium, moistening and nourishing the epithelial cells of the conjunctiva and cornea, removing dust and debris and protecting against pathogens.[3] The lacrimal fluid with its complex composition of inorganic salts, immunoglobulin A and various proteins is important for this purpose.[4] Consequently, an interruption of lacrimal gland homeostasis resulting in a disturbance of the physiological composition and/or quantity of lacrimal fluid, may lead to the development of ADDE.

### *Aqueous-deficient dry eye*

Both primary, as well as secondary diseases, can be responsible for the development of ADDE. Primary causes are lacrimal gland ablation or congenital alacrima. More common, however, are secondary diseases, such as age-related atrophy and fibrosis of the lacrimal gland, autoimmune diseases such as Sjögren's syndrome and graft-versus-host-disease (GvHD), destruction of the lacrimal tissue, e.g. by tumors or chronic inflammations, and occlusion of the lacrimal ducts.[1] Patients describe burning eyes, foreign body sensation, itching, sensitivity to light and wind as well as visual impairments and therefore experience restrictions in their everyday life. Lacrimal gland biopsies from patients with ADDE secondary to Sjögren´s syndrome, showed a progressive lymphocytic infiltration and an increase of pro-inflammatory cytokines.[5,6]

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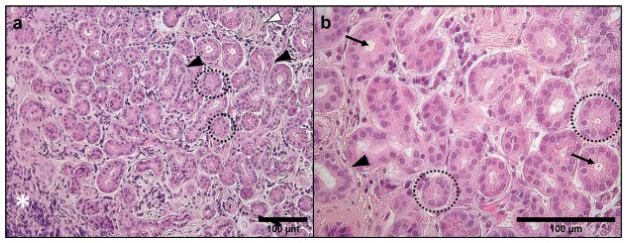


Figure 1. Human lacrimal gland. (a & b): hematoxylin and eosin (HE) staining of human lacrimal gland reveal the lacrimal gland structure with acinar cells arranged in acini (dashed line) and their lumen (black arrow), the ducts (black arrow head), blood vessels (white arrow) and a lymphocytic infiltration (white star) surrounded by loose connective tissue. Scale bar is 100 µm in 200-fold (a) and 400-fold magnification (b).

### *Routine therapy*

Although DED is well characterized, there are no causative therapies so far. Conventional therapies concentrate on the treatment of the underlying disease (secondary dry eye) and the relief of pain and discomfort by tear substitutions, closure of the *puncta lacrimalia* and the treatment of ocular surface inflammation.[7] However, in cases of severe DED, such as ADDE, these measures are often inadequate to ensure stabilization of the ocular surface homeostasis and insufficient to alleviate the symptoms. In such cases, innovative treatment options such as salivary gland transplantation have been performed.[8–10] However, this therapy does not address the underlying pathophysiology of ADDE and thus can only be considered as palliative.

## Lacrimal gland and regeneration

The lacrimal gland, like other glandular tissues, retains its regenerative capacity throughout its lifetime.[11] The capability of self-renewal of functional tissue after acute damage indicates the involvement of specialized processes as well as tissue-resident stem and progenitor cells. In extensive studies, scientists are therefore investigating the dynamics and underlying mechanisms of lacrimal gland regeneration. For this purpose, the generation of animal models should provide important insights. Indeed, a great number of different models were established to investigate DED, which further highlights the multifactorial nature of this disease (reviewed in.[12,13]) Investigations of lacrimal gland dysfunction include models of experimentally induced dry eye by interleukin 1 (IL-1) injection into the lacrimal gland,[14,15] (transient) ligation of the lacrimal excretory duct,[15–17] concanavalin A (ConA) injection into the intraorbital gland[18], and scopolamine treatment (accompanied by environmental stress).[19–24] Furthermore, different genetically modified mouse strains, including but not limited to MRL/lpr, nonobese diabetic (NOD), Id3$^{-/-}$, TSP-1$^{-/-}$, IQI/Jic mice as well as cytokine overexpressing mice were established and assessed (reviewed in.[12,13])

In general, three stages are necessary for complete tissue regeneration after injury: inflammation, new tissue formation and tissue remodeling (reviewed in.[25]) With the data obtained from the mouse models with acute damage and subsequent intrinsic regeneration, it might be feasible to assign the characteristics of lacrimal gland regeneration to these three stages of tissue repair. To address this issue, Hawley et al. contributed a complex transcriptomic study, where they investigated lacrimal gland samples at different time points after IL-1 injection induced ADDE.[26] Clusters of the inflammatory response (day 1–2), tissue repair (day 3–5) and finished repair comparable to control (day 7/14) have been found. Day 3 was discovered to be the transition between tissue inflammation (stage 1) and tissue repair (stage 2/3) in this model. Thus, it can be concluded that the dynamic of murine lacrimal gland damage and regeneration follows the classic stages of wound repair.

### *First stage of lacrimal gland repair*

Tissue inflammation – the first stage – occurs immediately after damage, and is in general characterized by an infiltration of neutrophils and macrophages. The aim of immediate early inflammation is the removal of dead and apoptotic cells as well as the response against pathogens (reviewed in.[25]) Indeed, (neutrophil) granulocyte and/or monocyte and macrophage infiltrates were found in the lacrimal gland after IL-1 injection,[15,26] duct ligation,[15] and scopolamine injection.[20,24] Additionally, apoptotic cell markers were found to be increased after IL-1 injection[15,27] and duct ligation[15,17] in the lacrimal gland. Thus, the two events of acute tissue inflammation occur concomitantly. Further, an increase of the common leukocyte marker CD45 was found,[17,19,20,26] as well as an increase of T cells after duct ligation,[15] scopolamine injection[19,20,24] and ConA injection.[18] In conclusion, the lacrimal gland displays the common signs of an immediate early immune response after acute damage as necessary to initiate tissue regeneration. In addition, depending on the model, murine lacrimal gland inflammation was similar to those found in patients with Sjögren's syndrome with regard to elevated cytokine levels and T cell invasion.[23, 24]

### *Second stage of lacrimal gland repair*

New tissue formation – the second stage – is characterized by proliferation and migration of different cell types.[25] Studies investigating lacrimal gland regeneration after acute damage detected an increase of genes and proteins associated with tissue development, epithelial plasticity/epithelial–mesenchymal transition and cell cycle.[15,17,26–28] Cell cycle processes are mainly recognized by the expression of proteins involved in proliferation, such as Ki67 and PCNA, and an increased expression of cell cycle-associated genes.[15,17,26,27] Furthermore, it was found that the bone morphogenetic protein 7 (BMP7) pathway, which is known to be essential during lacrimal gland development[29], is activated after IL-1 induced ADDE.[27] This could indicate that

a kind of repetition of developmental processes occurs during regeneration, which seems natural. Another event that is critical for physiological development is epithelial plasticity and epithelial–mesenchymal transition (EMT). EMT was described as playing an important role during branching morphogenesis, but not for acinar cytodifferentiation, in the development of salivary glands and lacrimal glands (reviewed in.[30–32]) In regenerating lacrimal glands EMT seems to be involved, as an increased expression was found for EMT-associated genes, such as snail family members and vimentin, after IL-1 and duct ligation-induced ADDE.[16,26,28] In addition to these important findings and events, it seems conceivable that the formation of new tissue may also involve the proliferation and differentiation of tissue-resident stem/progenitor cells, since their properties intrinsically serve to replace damaged tissue. The major characteristics defining stemness are the ability of self-renewal and differentiation (more or less restricted) into mature cell types. Cells expressing common stem cell markers such as nestin, musashi, nanog, Sox2, Oct4, and CD133 were found in healthy rodent, rabbit and human lacrimal glands.[33–36] Further transcriptional analysis confirmed the expression of these genes and thereby the presence of stem/progenitor cells in the lacrimal gland. Studies investigating intrinsic lacrimal gland regeneration after injury also detected stem/progenitor cells, expressing the common stem cell marker nestin. Furthermore, it was found that the number of nestin cells increased after experimentally induced ADDE by IL-1 injection and duct ligation.[15–17,27]

### *Third stage of lacrimal gland repair*

Tissue remodeling – the third stage – is characterized by the cessation of the activated processes of stage one and two, and the return to normal function. After a smooth transition into stage three, it is known to last for months.[25] In general, reorganization of the extracellular matrix (ECM) is known to be essential for tissue remodeling. In regenerating lacrimal gland, it was found that ECM remodeling was marked by an increased synthesis of collagen chains and associated processing enzymes. Further, the expression of MMPs and extracellular glycoproteins were shown to be up-regulated during the late phase of lacrimal gland repair after IL-1 induced DED.[26]

However, under certain circumstances, the intrinsic regenerative capacity of the lacrimal gland might be impaired, e.g. due to age-dependent degradation processes or chronic/autoimmune inflammation. In such cases, it would be of great interest to initiate regeneration and restore juvenile regenerative capacity. Therefore, scientists continue to investigate promising approaches in order to initiate and/or enhance lacrimal gland regeneration. The investigation of stem cells residing in the lacrimal gland appears to be promising for the development of such causative therapeutic approaches.

## Lacrimal gland resident stem cells

The presence of lacrimal gland resident stem cells has already been shown in several *in vivo* studies.[15,17,27,33,34] However, with regard to a clinical application, it might be favorable to isolate, characterize and expand these stem cells *in vitro*.

The presence of progenitor cells with potentially myoepithelial origin was suggested by Shatos *et al.* who isolated cells from rat lacrimal glands.[33] These cells undergo a spontaneous formation of spheres by day 30. Spheres formation is a well-known, albeit debated, assay to confirm stemness.[37] These spheres were further shown to express stem cell markers, such as nestin, musashi, pax6 and chx10. In addition, these spheres were positive for the proliferation marker Ki67 and thus exhibit a further stem cell property. As the investigated stem cell markers do colocalize with αSMA in lacrimal gland sections, the author concluded a myoepithelial origin or at least a myoepithelial niche for these cells *in vivo*. It was further shown that these cells were able to differentiate into neural, epithelial and myoepithelial cells.[33]

Evidence for the presence of epithelial stem/progenitor cells within the lacrimal gland was provided by studies isolating cells from mouse, pig, and rabbit lacrimal glands.[16,36,38,39] The cells isolated from new-born mouse lacrimal gland demonstrated primary and secondary sphere formation as well as expression of cytokeratin 14, as epithelial cell marker.[39] However, no further examinations proved the stem/progenitor character of these cells. Studies on pig and rabbit cells verified the epithelial cell type by pan-cytokeratin expression and an epithelial morphology. Stemness was shown in 3D by the formation of spheres[16,36] or in 2D by formation of colonies,[38] which is a similar assay as sphere formation to confirm stemness. In addition, the spheres were shown to express common stem cell marker such as nestin, ABCG2, ΔNp63[16,36] and 2D cells were shown to contain progenitor cells detected by ALDH-1 and Hoechst staining.[38] Further, the 2D cells, as well as the spheres, were able to form duct- and acinar structures in 3D matrices, such as decellularized lacrimal gland. Another study identified that the prospective isolation of c-kit$^+$dim/EpCAM$^+$/CD45$^-$/CD34$^-$/Sca-1$^-$ cells from mice using cell sorting (FACS) resulted in a putative lacrimal gland epithelial cells progenitor population.[40] These cells express Runx1, Oct4, and Pax6 as common stem cell markers and were further able to form spheres and colonies and differentiate into ductal and secretory compartments in the 3D cultures.

First clues that a stem/progenitor cell population resides within the lacrimal gland with a mesenchymal origin appeared during investigations of IL-1 damaged lacrimal glands.[27] Here, nestin-positive cells were found to increase during tissue repair and some, but not all, cells also expressed αSMA, suggesting a shared stem cell type for myoepithelial and acinar cells. However, profound characterization of these cells is necessary, which is challenging as several assays are required to ensure a mesenchymal stem cell/multipotent stromal cell (MSC) phenotype. To address this issue, `The International Society for Cellular Therapy´ released a panel of minimal criteria for defining MSC.[41] MSC phenotype should be characterized by the ability to adhere to plastic, to differentiate into adipocytes, osteocytes and chondrocytes *in vitro* as well as to express a panel of otherwise unspecific markers (Figure 2).

Profound characterization of isolated stem/progenitor cells, with potentially mesenchymal origin, was carried out in further studies according to the defined criteria.[42–44] These cells adhered to plastic and demonstrated a spindle-shaped and elongated morphology with a linear growth behavior. Stemness was verified

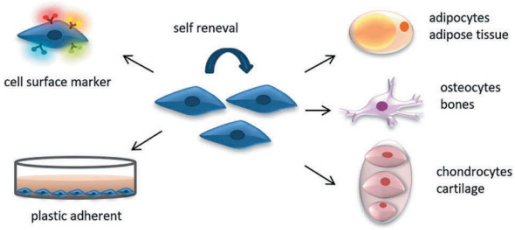


Figure 2. Mesenchymal stem cell characteristics. MSC are characterized by their ability of self-renewal, plastic adherent growth, the expression of a panel of cell surface marker and the differentiation to adipocytes, osteocytes, and chondrocytes in vitro.

by the expression of markers, such as nestin, Sca-1 and ABCG2 and the ability of colony formation. In addition, a detailed characterization of the presence/absence of the defined markers was performed to ensure MSC phenotype.[43,44] Differentiation capability toward osteogenesis and/or adipogenesis verified the multipotent stemness of the investigated cells. The studies prove the presence of MSC in the IL-1-damaged[42] and healthy lacrimal glands.[43,44]

In summary, the lacrimal gland harbors resident stem/progenitor cells, which can be isolated, and which retain their properties of stemness in culture. Detailed characterization of the isolated cells has shown that the stem/progenitor cells seem to be of myoepithelial, epithelial and mesenchymal cell origin. The results obtained from these studies can be further used to initiate and investigate experimental concepts and approaches for lacrimal gland regeneration.

## Towards lacrimal gland regeneration: concepts

The prevalence of DED ranges between 5% and 50%, depending on sex, age and ethnic group.[45] A further classification revealed a prevalence of 10–16% for pure ADDE and even 25% for mixed forms with ADDE-involvement among patients with DED, in two clinical-based patient cohorts.[46,47] Despite this high prevalence, there is a current lack of curative therapies, thus the development of new treatment strategies is clinically of great importance. Due to recent rapid development, the field of regenerative medicine offers promising concepts for the *in situ* regeneration of partially damaged tissues. In the following section, proposals and concepts for the regeneration of lacrimal gland tissue will be presented and discussed.

The requirements for a promising therapy for the regeneration of the lacrimal gland, as already described in detail in our previous review,[48] can be summarized as follows:

- restore the quantity and quality of lacrimal gland fluid
- restore a physiological arrangement of functional lacrimal gland tissue (acini formation)
- be non-immunogenic
- be feasible regarding clinical application, production, and source
- not cause adverse side effects

As reviewed in the previous sections, there are some promising starting points for the initiation or enhancement of lacrimal gland regeneration. On the one side, the experimental initiation or imitation of processes involved in the second stage of tissue repair might be promising. On the other side, the further investigation of lacrimal gland resident stem/progenitor cells could be instructive. Moreover, the utilization of these cells or related products is undoubtedly promising in terms of inducing lacrimal gland regeneration. Another starting point might be approaches derived from regeneration studies of other glandular tissues, such as salivary gland or pancreas, since the lacrimal gland shares many similarities.

### *Therapeutic proteins*

The induction of the BMP7 pathway appears to be promising as it is involved during lacrimal gland morphogenesis and lacrimal gland regeneration.[27,29] BMP7 activates receptor-regulated Smads that, upon heteromeric complex-formation, translocate to the nucleus and act as a transcription factor (reviewed in.[49]) BMP7 is able to bind to the membrane of several cell types, including MSC, and induces proliferation and differentiation among others.[49,50] In addition, BMP7 was shown to promote the proliferation of embryonic lacrimal gland mesenchyme cells *in vitro*.[29] Thus, the beneficial effects of BMP7 during lacrimal gland regeneration could be the induction of MSC proliferation, which was shown to be associated with lacrimal gland regeneration.[15–17,26] Moreover, human recombinant BMP7 is already approved for clinical application in spinal fusion and treatment of long bone fractures.[51] In addition to its role in bone regeneration, BMP7 also appears to promote liver regeneration, an organ in which BMP7 is also involved during organogenesis.[52,53] Consequently, the prospects of BMP7 administration for lacrimal gland regeneration are very promising and should be investigated in experimental studies. To the best of our knowledge, no studies have yet been carried out on this project.

Another idea is the administration of eye drops that stimulate/increase tear secretion of the remaining functional lacrimal gland tissue to circumvent complex tissue regeneration. Although this concept does not aim at the regeneration of lacrimal gland, it could be beneficial as it fulfills the most of the other postulated requirements for a promising therapy (see above) and provides interesting results. The administration of pituitary adenylate cyclase-activating polypeptide (PACAP) containing eye drops increased the tear secretion in wild type and PACAP-null mice in a time-dependent manner.[54] It was shown that PACAP induces the trafficking of aquaporin 5 from the cytosol to the apical membrane of acinar cells and thereby increase fluid secretion. When eye drops were administered twice daily for three weeks ocular surface damage was attenuated in APCAP-null mice. However, whether PACAP eye drops would also be beneficial in established ADDE mouse models needs to be investigated.

### *Mesenchymal stem cells*

Several *in vivo* studies showed an increase of nestin-positive cells during lacrimal gland regeneration after acute damage.[15–17,26] Thus, it seems promising to further investigate these cells and

make use of their properties in order to initiate/enhance lacrimal gland regeneration. Extensive *in vitro* studies on nestin positive cells suggest that they are mesenchymal stem/multipotent stromal cells (MSC).[42–44] In the course of progress in the field of regenerative medicine, MSC have emerged as a magic bullet against a variety of pathologic conditions, resulting in nearly 500 clinical trials, including 201 completed studies, that are currently listed in the US national library of medicine by the NIH.[55] Twenty-nine of these studies deal with MSC in ophthalmic research. There is a body of evidence showing that MSC can suppress inflammation and initiate endogenous repair mechanisms through trophic effects on immune cells and tissue stem cells (Figure 3). Immune modulatory effects of MSC are complex and affect the innate and the adaptive immune system (reviewed in.[56,57]) The innate immune system is modulated by the inhibition of the maturation of monocytes into dendritic cells (DC), the function of DC, the proliferation and cytotoxic activity of natural killer cells, the respiratory burst of neutrophils and their apoptosis. Effects on the adaptive immune system include inhibition of T cell and B cell proliferation and the suppression of B cell differentiation. Moreover, MSC triggers the generation and proliferation of immunosuppressive Tregs. In addition, MCS have been shown to have only a low or poor immunogenicity since they lack major histocompatibility complex (MHC)-class II expression, as confirmed by a number of studies (reviewed in.[41,48,56,58]) Furthermore, 49 completed clinical studies have reported that allogeneic MSC transplantation is effective, safe and well tolerated. Feasibility has been shown in five Phase II-III clinical trials and is currently being further investigated in 25 ongoing Phase II-III clinical trials.[55]

MSC can be isolated from a variety of different tissues such as bone marrow, umbilical cord, adipose tissue as well as glandular tissue such as lacrimal gland, salivary gland, and pancreas. This leads to a high availability of MSC for clinical applications. However, MSC from different tissues may represent subtypes of each other and may have different properties. Investigations are still needed to determine whether orthotopic transplantation is superior to ectopic transplantation. In summary, MSC exerts a variety of desirable properties for tissue regeneration, have been found in the healthy lacrimal gland and their number increases during lacrimal gland regeneration. Consequently, the transplantation of MSC appears to be a promising approach for lacrimal gland regeneration.

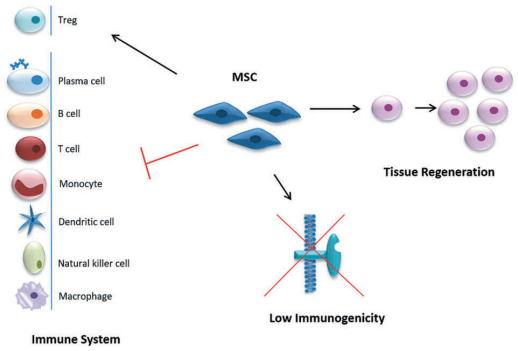


Figure 3. Favorable properties of mesenchymal stem cells for clinical application. Schematic overview of the immune modulatory effects, low immunogenicity and the support of tissue regeneration.

Various studies already investigated the transplantation of MSC, from several different tissues, for regeneration of salivary glands from rodents with Sjögren's syndrome-like disease or irradiation-induced damage (reviewed in.[59]) Summarizing, MSC therapy was successful in restoring/maintaining histological acinar/ductal salivary gland structure, acinar function, modulation of tissue inflammation and initiation of tissue repair by the induction of several genes associated with development/regeneration. Consequently, the application of MSC led to a therapeutic improvement and could also be beneficial for lacrimal gland regeneration. However, besides the application of MSC alone, recently the combination of MSC together with platelet-rich fibrin extracts was tested for the ability to regenerate permanently damaged salivary glands. It was shown that the combination of both further increased the therapeutic effect.[60] This combined therapy should be kept in mind when further improvement of lacrimal gland regeneration is necessary.

Besides the direct application of MSC, MSC-based strategies are also conceivable. Of note here is the utilization of MSC secretome as well as selected proteins thereof. Indeed, it has been shown that damaged lacrimal gland epithelial cells cultured with MSC secretome exhibited an increased regeneration potential compared to control.[43,44] Moreover, lipocalin-2, prosaposin, Rac1, and STAT1 in the secretome of MSC were identified to contribute to the improvement of lacrimal gland epithelial cell regeneration *in vitro*.[44] In studies using other glandular tissue, it was reported that the injection of MSC secretome or MSC-derived extracellular vesicles (EV) delayed the onset of type 1 diabetes after adoptive transfer from NOD/Ltj to NOD/*scid* mice and alleviated the impact of irradiation-induced salivary gland damage in mice.[61,62] In the pancreas, MSC-EV preserved the insulin-producing cells and reduced the number of CD4 T cells as effectively as a MSC transplantation.[62] In the salivary gland, the secretome has maintained the histological acinar/ductal salivary gland structure well, and preserved the function of acinar cells (mucin production, amylase activity).[61] Accordingly, MSC secrete trophic factors that retain the ability to maintain/restore glandular function, similar to MSC itself.[62,63] When using cell extracts from adipose-tissue derived MSC similar results were detected regarding the restoration of salivary gland structure and function.[64] In Conclusion, the application of MSC or MSC-based therapy seems to be very promising approaches for *in situ* regeneration of lacrimal gland tissue.

### Lyophilized cell extracts

The secretome, as well as cell extracts, have been shown to have a therapeutic impact of glandular tissue regeneration. However, the short half lifetime of proteins (*in vivo*, during storage at -80°C) may limit the effect, leading to the necessity of fresh preparation and/or multiple applications. To overcome these limitations, a recent study investigated the opportunity to use lyophilized cell extracts instead of fresh ones.[65] Lyophilization is a widely used technique for a functional preservation of proteins, DNA and RNA, which is utilized in the study as well as by commercial manufacturers. The study

showed that lyophilization had neither an impact on protein concentration, nor on the protein banding pattern. Further, salivary flow rate and acinar cell portion in irradiation-injured salivary glands were similar when treated with lyophilized or fresh bone marrow cell extracts.[65] Although the investigation of the lyophilized cell extract was designed as a proof of the concept, this study shows a refinement and improvement of the approaches currently being developed to induce/enhance the regeneration of glandular tissues.

### Adult acinar cells

In the lacrimal gland and also in the salivary gland, it is assumed that a variety of resident stem cells might be responsible for replenishing damaged tissue. Indeed, it was found that, for example, the number of MSC increases after damage to the lacrimal gland, and further that transplantation of MSC or MSC-related products can restore the structure and function of the salivary gland. However, a recent study postulated that adult acinar cells of the salivary gland itself have the ability to proliferate and replenish damaged cells *in vivo*.[66] This was shown through the labeling of individual acinar cells using the Rosa26$^{Brainbow2.1}$ mouse reporter strain in combination with an acinar-specific Cre expression (MIST1). The individual label is inherited during cell division and was initially detected in single or doublet cells randomly distributed throughout the salivary gland. After six months many acini were completely composed of labeled cells, and multicellular clones with one label were detected adjacent to each other within one acinus. In a damage/regeneration experiment, only a few labeled acinar cells remained after duct ligation. During regeneration, a large number of labeled acinar cells could be detected again. As labeling is inherited, all labeled cells originated from differentiated acinar cells by proliferation. In conclusion, this study provided evidence that differentiated, adult acinar cells were actively proliferating and served to replenish acinar cells, which were lost due to physiological turn over or pathological damage.[66] Nevertheless, it cannot be ruled out, that tissue-resident stem cells also contribute to acinar cell regeneration. Indeed, a recent study traced p63+ cells and detected that these cells function as multipotent stem/progenitor cells that contributed to and maintained all epithelial cells during morphogenesis and maintenance of the salivary gland.[67] Whether differentiated acinar cells of the lacrimal glands also actively proliferate remains to be investigated. In this instance, however, there might be a way to induce this proliferation in disease stages where this natural process has become quiet to induce lacrimal gland regeneration. Another approach could be the transplantation of *in vitro* expanded acinar cells to the damaged lacrimal gland.

## Towards lacrimal gland regeneration: experimental approaches

The previous section presented possibilities and ideas that can be utilized and/or explored in further research to initiate lacrimal gland regeneration. In the following section, promising approaches with initially experimental application in different animal models of ADDE will be presented and discussed.

### Side population stem cells

In general, side population (SP) cells were considered as a heterogenous stem cell population, as they exhibit stem cell activities and encompass embryonic stem cells populations. Mishima *et al.* isolated these cells from the lacrimal and salivary gland and verified further stem cell characteristics by the expression of ABCG2 and Sca-1[68] In addition, putative lacrimal gland epithelial stem/progenitor cells investigated by Spaniol et al. also comprised SP cells, which further indicate the stem cell character of SP cells (see also the section "Lacrimal Gland Resident Stem Cells").[38] SP cells were transplanted into irradiation damaged lacrimal glands and could restore tear secretion, which was comparable to that of healthy mice, after 8 weeks. Transplantation was performed two weeks after irradiation. In subsequent experiments, the authors postulated that clusterin contributes to the therapeutic effects, as the expression of clusterin was significantly higher in transplanted glands. Furthermore, the transplantation of SP cells from clusterin-deficient mice failed to recover the salivary gland function. However, data demonstrating that lacrimal gland function also requires clusterin are missing. In addition, detailed histological examination of functional acinar tissue needs to further confirm the benefit of SP cell transplantation for lacrimal gland regeneration. Nevertheless, the study provided a potential therapeutic approach towards lacrimal gland regeneration, which should be further investigated.

### Epithelial progenitor cells

Epithelial progenitor cells (EPCP) were prospectively isolated from murine lacrimal gland and were shown to exhibit distinct stem cell properties (as described in the section "Lacrimal Gland Resident Stem Cells"). To test the concept, these cells were transplanted into lacrimal glands after IL-1 induced ADDE.[40] It was shown that the EPCP engrafted (day 40) and were found in the ductal and acinar compartment of regenerated lacrimal glands. The efficiency of engraftment was much higher when the EPCP were applied at the beginning of the regeneration phase than during acute inflammation. However, EPCP engraftment into IL-1 damaged lacrimal glands could be further increased even during acute inflammation when blocking Panx1 and thereby reducing inflammation.[69] The TSP-1$^{-/-}$ mice represent a model with chronic DED and shares common features of Sjögren´s syndrome.[13] Transplantation of EPCP into manifested, chronic DED of TSP-1$^{-/-}$ mice was performed and engrafted cells could be detected in ductal cells and differentiated, regenerating acinar cells. Furthermore, EPCP transplantation reduced the inflammatory foci manifested in the TSP-1$^{-/-}$ lacrimal gland and improved the tear secretion.[40] In summary, the transplantation of EPCP successfully induced the regeneration of lacrimal glands from manifested, chronic DED, and is a very promising approach to curatively treat

DED. In addition, the prospective isolation of these cells could be advantageous for clinical application as it circumvents the need for *in vitro* culture and its risks.

### Mesenchymal stem cells

The largest portion of experimental approaches focusing on the regeneration of lacrimal gland, investigates the application of MSC. This seems not surprising, given that MSC modulate the immune system, are low immunogenic, encourage tissue regeneration and have been found in the regenerating lacrimal glands of mice. In the following sub-section, experimental approaches using MSC transplantation are organized according to the model used or the application in patients.

#### Mouse models

The NOD/LtJ (NOD) mouse strain is a commonly used genetically modified model to study autoimmune mediated DED in Sjögren´s syndrome. Although initially developed to study diabetes mellitus, these models also shows distinct CD4 T cell infiltrates in the lacrimal gland, a decreased tear secretion and autoantibodies (reviewed in.[12]) Aluri *et al.* investigated whether a single intraperitoneal injection of bone marrow-derived MSC (BM-MSC) alleviates the symptoms of NOD mice with manifested DED.[70] After 4 weeks, MSC transplantation resulted in a sustained improvement of tear secretion, a reduced infiltrated lacrimal gland area and a modest increase of Tregs. In addition, the expression of aquaporin 5 gene increased. However, this study provided results that promote the therapeutic effect of MSC transplantation for the treatment of chronic DED. Additionally, there are three studies investigating the therapeutic effects of MSC transplantation in NOD mice.[71–73] Although these studies examine the salivary glands, one could draw conclusions about lacrimal gland regeneration, as the MSC were administered systemically (via tail vein) and the salivary gland impairments in female NOD mice appear to be comparable to those of the lacrimal gland in male NOD mice.[74–77] The studies investigated the therapeutic effect of MSC transplantation at an early or late stage of manifested Sjögren's syndrome. When treating the mice at an early stage of Sjögren's syndrome, the salivary flow rate could be completely restored.[71,72] When treating the mice at a relatively late stage of Sjögren's syndrome, the salivary flow rate could also be improved but not restored.[71,73] In both cases, the salivary gland function could be significantly improved by the systemic transplantation of MSC. In addition, in all cases a decrease of the lymphocytic infiltration of the salivary gland could be observed. Consequently, the MSC transplantation achieved the same therapeutic effects in the salivary gland as in the lacrimal gland of NOD mice in the study of Aluri *et al.* Thus, these studies further indicate that MSC transplantation might be a desirable approach to regenerate lacrimal gland tissue.

An experimental approach to induce lacrimal gland dysfunction is the injection of ConA into the intraorbital lacrimal gland in mice to attract T cells.[18] This resulted in a reduced tear secretion, an increased ocular surface staining, and an inflammatory reaction including lymphocytic infiltrations of the lacrimal gland at day 7 after induction. Immediately after intraglandular ConA injection, either human or murine MSC were injected into the periorbital space. Transplantation resulted in a completely restored tear secretion and a reduced inflammatory reaction, including normal levels of CD4 T cells. As the MSC were injected immediately after ADDE induction, MSC may prevent ADDE development rather than induce the regeneration of damaged lacrimal gland tissue. Indeed, MSC could not be detected within the lacrimal gland. However, MSC transplantation reduced the inflammation and thus might be beneficial in treating ADDE, as inflammatory reactions are one of the key elements contributing to the pathophysiology of DED in patients (reviewed in.[78]) In addition, the authors did not mention any adverse effects in the treatment of mice with human MSC, which confirms the low immunogenicity of MSC and supports its potential clinical applicability. In conclusion, this study provided further evidence that the transplantation of MSC is beneficial to improve lacrimal gland function after experimentally induced ADDE.

#### Rabbit model

The induction of autoimmune dacryoadenitis via intravenous injection of activated peripheral blood lymphocytes (PBL) is a common model used to study DED in rabbits (reviewed in.[12]) Clinical signs, including reduced tear secretion and increased corneal fluorescein staining, were visible at day 3 after induction and manifested after 2 weeks for up to 6 months.[79] Systemic injection of MSC was performed on five subsequent time points from day 1 to day 9 after induction of DED. Transplantation resulted in an improved, but not recovered tear secretion, tear-film break up time (T-BUT) and fluorescein staining of the ocular surface.[79] Within the lacrimal gland, the amount of immunosuppressive Tregs increased while the area of lymphocytic infiltration and the expression of inflammatory mediators decreased. In conclusion, this study shows that the transplantation of MSC had therapeutic effects when treating an autoimmune-mediated DED. However, the fact that the transplantation of MSC was performed before clinical signs of DED were manifested may again lead to the conclusion that MSC therapy prevents the development of DED rather than that it caused the regeneration of lacrimal gland tissue in this experimental setup. It needs to be further investigated whether the therapeutic efficacy also occurs when MSC are administered in manifested DED in this rabbit model.

#### Canine model

Dogs, as well as humans, are prone to a spontaneous development of DED (also known as keratoconjunctivitis sicca, KCS) with a similar DED etiopathogenesis and prevalence (reviewed in.[12]) Two studies were conducted to investigate the therapeutic effect and safety of allogeneic MSC transplantation in dogs with spontaneous DED.[80,81] Bittencourt *et al.* classified the severity of DED prior to the treatment as mild-to-moderate (tear secretion of 4–14 mm/min) or severe (tear secretion <4 mm/min) compared to healthy dogs (tear secretion 15–25 mm/min).[80] Adipose-tissue MSC were injected into the periorbital space and in spatial proximity to the third eyelid lacrimal gland.[80,81] MSC transplantation resulted

in the improvement of ocular discharge, hyperemia and corneal changes, such as corneal opacity and corneal vascularization but the signs could not be resolved.[80] Tear secretion gradually increased after MSC transplantation, and remained stable after six months during long term follow-up.[80,81] Treatment of MSC resulted in a complete recovery of tear secretion in the mild-to-moderate group as the measured values were above 15 mm/min and consequently within the range of healthy dogs.[80] In dogs with severe DED, tear secretion also increased to about 11 mm/min, which was significantly increased and is now in the range of mild-to-moderate DED.[80,81] Only one severely affected eye was found to be non-responsive to MSC transplantation.[80] Further, it was shown that MSC transplantation was well tolerated by the dogs as no side effects were observed during the studies. In summary, these two studies show that the transplantation of MSC has therapeutic effects in dogs with spontaneously developed and manifested DED. Clinical signs of DED were improved and the symptoms alleviated. Whether increased tear secretion depends on regenerated lacrimal gland tissue could only be assumed as no biopsies were histologically examined. However, these studies further confirmed the therapeutic benefit and the safety of allogenic MSC transplantation for DED pathology.

### *Patients*

Only one study investigated the therapeutic effects of MSC transplantation in patients with DED secondary to GvHD.[82] In this study, 22 patients received an intravenous injection of allogeneic MSC from HLA-matched siblings. Here, Weng *et al.* identified two responder groups with 12 patients responding well to the MSC transplantation, whereas 10 did not. In the responder group, the treatment resulted in increased scores in clinical surveys of DED, improved tear secretion and an increased percentage of Tregs in peripheral blood. Additionally, the MSC transplantation modulated the cytokine profile in venous blood samples by increasing IL-2 and IFNγ and decreasing IL-10 and IL-4 concentrations. Among others, IFNγ and IL-10 are cytokines involved in DED secondary to GvHD, in Sjögren's syndrome and in chronic GvHD.[2,83] The role of IL-10 in this context appears to be contradictory, as IL-10 is generally associated with anti-inflammatory and protective properties (reviewed in.[84]) IL-4 has been described to be elevated in mice with chronic GvHD[85] and IL-2 is known to promote the development, the homeostasis and the suppressive function of Tregs (reviewed in.[86]) These could be some, albeit not all, of the immunomodulatory effects that MSC exerts upon transplantation. In addition, no adverse effects were reported during the three-month follow-up after allogeneic MSC transplantation.[82] This study confirmed the results obtained from pre-clinical studies. The transplantation of MSC resulted in improved lacrimal gland function, and could be a therapeutically effective and safe approach to curatively treat DED in patients. Further investigation is needed to determine the cause of non-responding patients, the effects of MSC on the acinar structure of lacrimal gland (regeneration) and the long-term stability of improved DED symptoms.

In conclusion, the above findings demonstrate that MSC are potentially useful for the treatment of DED as the lacrimal gland function was improved throughout all reviewed studies. In addition, the first application of MSC in patients confirmed the pre-clinical study results. Nevertheless, further studies are needed to confirm the therapeutic effects of MSC in manifested DED and to histologically determine whether functional lacrimal gland tissue can be regenerated upon transplantation.

In summary, several studies have investigated the therapeutic benefit and safety of stem cell transplantation for the treatment of DED/ADDE. Researchers utilized side population (SP) stem cells, epithelial progenitor cells (EPCP) as well as mesenchymal stem cells (MSC) to explore the restoration of lacrimal gland function (Figure 4). In conclusion, an improvement of lacrimal gland function (tear secretion) was found upon transplantation regardless of the used stem cell type. However, there was only one study (EPCP for lacrimal gland regeneration) that provided histological data to confirm the presence of regenerated acinar tissue after transplantation. Consequently, further studies are needed to determine whether the therapeutic effects of SP cells and MSC rely on regeneration of functional lacrimal gland tissue or have other causes. Nevertheless, the experimental approaches are highly encouraging and give cause to expect that the approaches could lead to the establishment of a curative therapy for patients suffering from DED/ADDE in the future.

## Conclusion and future challenges

There are several promising concepts to be investigated for lacrimal gland regeneration, such as therapeutic proteins (BMP7 and PACAP), mesenchymal stem cells (MSC), lyophilized cell extracts and the use of adult acinar cells. The first experimental approaches utilizing the proposed concepts show that lacrimal gland function benefits from stem cell therapy and that inflammation resolves. A very promising and well-studied experimental approach is the use of MSC for induction/enhancement of *in situ* lacrimal gland regeneration, as their properties are very favorable for clinical applications. MSC modulates the immune system, have low

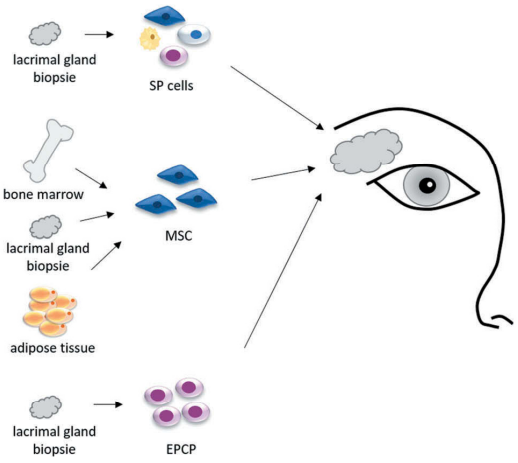


Figure 4. Schematic overview of experimental approaches for lacrimal gland regeneration. Stem cells were intensively investigated for their potential to induce lacrimal gland regeneration and improve its function. Therefore, side population (SP) cells could be isolated from lacrimal gland biopsies, mesenchymal stem cells (MSC) could be received from bone marrow aspirates, lacrimal gland biopsies, and adipose tissue and epithelial progenitor cells (EPCP) could be obtained from lacrimal gland biopsies as well.

immunogenicity, promote tissue regeneration and are involved during lacrimal gland regeneration in mice. In addition, the efficacy and safety of allogeneic MSC transplantation have already been demonstrated in a small patient cohort.

A future challenge will be to translate these promising concepts from laboratory studies to clinical application. Here it is of particular note, that the structure and location of the lacrimal gland differ between mammals.[87] Albeit the cell types and function of these cells are similar, differences exist, e.g. in the histoarchitecture. It must, therefore, be carefully assessed whether the human lacrimal gland could also benefit from treatments that are beneficial for the regeneration of the rodent lacrimal gland. However, for the understanding of lacrimal gland function and the subsequent development of experimental approaches rodent animal models remain invaluable. Where no functional lacrimal gland tissue remains, the reconstruction of the artificial lacrimal gland *in vitro* with a subsequent transplantation is encouraging. Investigations in this field represent an additional research section (reviewed.[88]) However, as long as functional tissue remains, *in situ* regeneration is more desirable as it is less invasive and retains the existing physiological conditions.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## 2.2 Review Article

## Development of Causative Treatment Strategies for Lacrimal Gland Insufficiency by Tissue Engineering and Cell Therapy. Part 1:

## Regeneration of Lacrimal Gland Tissue: Can We Stimulate Lacrimal Gland Renewal *In Vivo*?

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Article contribution to the main thesis

Detailed introduction of DED/ADDE and the lacrimal biology and function. Comprehensive description of MSC biology with discovery, definition, characterization and properties.

Contribution to experimental design, implementation and publication

Review of the literature, writing of the manuscript (lacrimal gland, structure, function and purpose, cause of quantitative DES, treatment, mesenchymal stem cells/multipotent stromal cells (MSCs), MSCs in injured and uninjured glandular tissue, MSC in cell therapies – Isolation and characterization of MSCs, parts of MSC in cell therapies – Evidence of the beneficial effect of MSCs *in vivo*, parts of conclusion and future challenges), preparation of Figure 1 A and Figure 3. The manuscript was subsequently reviewed and approved by all co-authors.
Approximated total share of contribution: 32%

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MINI-REVIEW

# Development of Causative Treatment Strategies for Lacrimal Gland Insufficiency by Tissue Engineering and Cell Therapy. Part 1: Regeneration of Lacrimal Gland Tissue: Can We Stimulate Lacrimal Gland Renewal *In Vivo*?

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**ABSTRACT**

Severe dry eye syndrome (DES) is a complex disease that is commonly caused by inflammatory and degenerative changes in the lacrimal gland, and can result in severe pain and disruption to visual acuity. In healthy subjects, the ocular surface is continually lubricated by the tear film that ensures that the ocular surface remains moist and free of debris, enabling normal vision. The lacrimal fluid, mid-layer of the tear film, is mainly produced by the lacrimal gland and if this is dysfunctional for any reason, severe DES can develop. Currently, only palliative treatments for DES exist that aim to either replace or retain tears and/or minimize inflammation. A curative approach that aims to trigger the regeneration of existing lacrimal gland tissue *in situ* may, therefore, be very beneficial to DES patients. This article reviews the different approaches that have been explored toward lacrimal gland regeneration. Progress to date *in vitro, in vivo*, and in man is described with a focus on clinical feasibility and efficacy. Promising candidates for drug-dependent treatment of DES are growth factors and cytokines, such as hepatocyte growth factor (HGF) and tumor necrosis factor α-stimulated gene 6 protein (TSG-6). Only a few studies have evaluated gene therapy for lacrimal gland deficiencies, but with promising results. However gene therapy carries a variety of risks regarding carcinogenesis and therefore a treatment in the near future using this approach seems to be unlikely. Cell therapies utilizing mesenchymal stem cells (MSCs) seem to be more applicable than those using human amniotic membrane (hAM) epithelial cells or induced pluripotent stem (iPS) cells, since MSCs combine the favorable traits of both (multipotency, capability to stimulate regeneration immunomodulatory and non-immunogenic properties).

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## Introduction

### *Lacrimal gland structure, function and purpose*

The main lacrimal gland—in humans and other mammals—is an orbital organ and lies underneath the upper temporal compartment of the orbita embedded in the lacrimal fossa.[1] In humans the lacrimal gland fluid is directly conducted onto the ocular surface by a variety of short ducts.[2] The function of the mainly serous tubuloacinar lacrimal gland, supported by accessory Krause and Wolfring glands, is to secrete lacrimal fluid—the major component of the tear film.[1,3,4] This isotonic secretion is a complex solution, which contains a variety of proteins and inorganic salts as well as immunoglobulin A. Among the proteins lactoferrin, serum albumin, lysozyme, and lipocalin are the most prominent.[5,6] The parenchyme of the lacrimal gland is structurally composed of three cell types: acinar, duct, and myoepithelial. Acinar and duct cells form distinctive tubules that are surrounded by associated myoepithelial cells[7] (see Figure 1). Acinar cells represent the main cell type with a proportion of approximately 80%, while duct cells comprise 10%–12% of the cell population. Acinar cells are the main producer of the lacrimal gland fluid, which is later modified in its composition by the duct cells (as review detailed in[8]). Between the parenchymal parts lies the interstitial space that contains several different cells types such as plasma cells, macrophages, lymphocytes, helper and suppressor T cells, and B cells, dendritic cells, and mast cells.[9]

In the classic model of the tear film, the lacrimal gland fluid is the mid-layer of the tear film, encompassed by two other layers: the internal mucous layer, mainly secreted by the goblet cells of the conjunctiva; and the external lipid layer, which is mainly derived from the meibomian glands (also known as tarsal glands).[1,3,4] In its entirety, the tear film fulfills the following functions: smoothing of the corneal epithelium and improving its optical properties; moistening of the corneal- and conjunctival epithelium; contributing to ocular surface metabolism; removing dust and other debris from the ocular surface; and protection against pathogens.

The stabilization of the tear film and its complex composition is critical and even minor variations in its quality or quantity can lead to dry eye syndrome (DES). Therefore, the lacrimal gland is tightly regulated by both parasympathetic and sympathetic nerves (as reviewed in[8]). Any impairment of one or more

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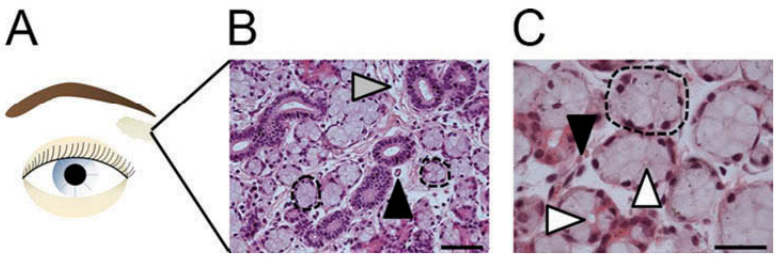


Figure 1. Lacrimal gland location and structure. A: In man, lacrimal glands are positioned lateral and superior to the eyes. B&C: H&E images of lacrimal gland tissue. The dashed black lines outline individual acini that comprise acinar epithelial cells. These cells surround a central duct (white arrowheads in C only). Blood vessels supply oxygen and nutrients (black arrowheads). Stromal tissue (gray arrowhead in B only) is found between acini. Scale bars: B = 50 μm; C = 25 μm.

components of the lacrimal functional unit (LFU) can destabilize the tear film and result in the development of DES. Patients commonly describe dryness of the eye, foreign body sensations, burning sensations, and visual disturbance.[1,3]

### Cause of quantitative DES

Quantitative tear deficiency due to lacrimal gland impairment is the most common reason for the development of severe dry eye. It can be caused by age-dependent hyposecretion, destruction of the lacrimal gland tissue by inflammatory reaction, closure of the ducts due to conjunctival inflammation and scarring, innervation dysfunction due to congenital defect or trauma, or infection (viral or bacterial). Age-dependent development of DES is characterized by a variety of structural changes, with aged lacrimal glands displaying atrophied acini, fibrosis, ductal obstruction, and an increased occurrence of lymphocytic infiltration. Furthermore, studies on aged animals also revealed functional changes, such as a decrease in stimulated protein secretion (reviewed in[10]).

Inflammation, either chronic or acute, is the major (80%) cause of severe quantitative DES.[3] In turn, the inflammation itself has many potential causes: graft versus host disease (GVHD) following transplantation; autoimmune diseases, such as Sjögren´s syndrome (SS), Sjögren´s syndrome Dry Eye (SSDE), or others, such as non-Sjögren´s syndrome Dry Eye (non-SSDE).[1,3,4,11] SS may be primary, where the lacrimal and salivary glands are the main target of the immune attack, or secondary to another autoimmune disease, such as rheumatoid arthritis.[3,12] There is no histological difference between SSDE and non-SSDE. Both are characterized by progressive lymphocytic infiltration with an ongoing destruction of the lacrimal gland throughout the disease course. Early studies of DES lacrimal gland biopsies detected abnormal arrangements of the ducts, including total diminished lobular pattern accompanied by the formation of lymph follicles, fibrosis, and acinar cell atrophy.[13] The lymphocytic infiltration consists mainly of CD4+ T cells with a smaller subset of CD8+ T cells and B cells.[14,15] Detailed immunohistochemical analysis revealed elevated lymphocyte activation markers, such as very late antigen-4, human leukocyte antigens (HLAs) and lymphocyte function associated antigen-1,[15 17] as well as a raised concentration of interferon-ɣ (IFN-ɣ).[15] In addition, expression of adhesion molecules such as vascular cellular adhesion molecule-1 and intracellular adhesion molecule-1 on acinar, duct, and epithelial cells in the lacrimal gland as well as in the conjunctiva is increased.[15,16] This upregulation is induced by pro-inflammatory cytokines, such as interleukin-1β (IL-1β), representing the initial phase of leukocyte infiltration. Similarly, the concentration of both IL-1 isoforms, IL-1α and IL-1β, was found to be significantly increased in the tear fluid of DES patients and, moreover, strongly correlates with corneal damage.[18] Production and secretion of IL-1 by endothelial cells as well as phagocytes occur in response to lipopolysaccharide contact, indicating bacterial infection.

In summary, quantitative DES is a multifactorial disorder that can occur due to trauma, degeneration, inflammation of the lacrimal gland, and the ocular surface or neurotrophic deficiency. The tear system is complex; the individual components of the LFU are interconnected and an imbalance in any single component may lead to a change in another – or even the whole system: a systemic inflammatory disease may lead to subsequent inflammation of the lacrimal gland, which in turn could result in altered lacrimal gland fluid secretion; conversely, an altered tear fluid composition or even reduced blinking frequency can lead to reduced lubrication of the ocular surface, resulting in lacrimal gland inflammation.[19,20]

### Treatment

Despite being well characterized and moderately prevalent, there is no curative treatment for DES. Current treatments remain palliative and focus on the reduction of pain and discomfort by tear substitution, tear retention, and control of ocular surface inflammation by anti-inflammatory drugs (see review[21]). One causative approach to treat severe quantitative DES is the transplantation of either partial or total submandibular salivary gland.[22 25] This procedure has shown some promise in very severe cases of DES, with some patients displaying improved symptoms (such as improved Schirmer test scores and longer tear breakup time (TBUT)). However, vision is unaffected; moreover, it is important to note that the osmolarity of saliva is very different from tears. In fact, the hypo-osmolar salivary secretions can induce microcystic corneal edema, which can in turn lead to epithelial defects.[22] Furthermore, given the high association between quantitative DES and autoimmune disease, autologous transplantation of salivary glands might often not be an option as these glands are also

regularly affected by this condition. Xenotransplantation of porcine lacrimal glands has also been considered and it has been shown that human and pig lacrimal gland architectures are similar.[26] Porcine islet cells (reviewed in[27]), corneas[28], and heart valves (into human recipients)[29] show promise, but rejection is always a possibility, as is the transmission of porcine endogenous retrovirus.[30]

## Concepts for lacrimal gland regeneration or reconstruction

Given the current lack of a curative treatment for DES, scope exists for a regenerative medicine treatment approach. There are two scenarios in which treatment could be required:

(a) the lacrimal gland is entirely nonfunctional or

(b) the lacrimal gland is partially damaged with an insufficient number of, or sub-functional, cells

The treatment approaches for these scenarios will likely differ. In the first instance, where no regenerative potential remains, *in vitro* manufacture of lacrimal gland tissue that could be transplanted into the patient to restore function may be an appropriate treatment, and this is reviewed in Part 2. However, in the second instance, triggering the regeneration of existing tissue is preferable since this is less invasive and, ultimately, the cells of the host likely hold an increased propensity for regeneration compared with anything engineered *in vitro* using current techniques, and this is discussed below.

## *In situ* regeneration of lacrimal gland

Since quantitative DES is characterized by a reduction of the tear film volume and/or altered composition, the restoration of the physiological tear volume with correct composition is the ideal result of a curative treatment. This would require the complete restoration of the native tissue features, including function. Possible approaches to trigger *in situ* lacrimal gland regeneration might be a drug or cell therapy for example, but, in either case, we suggest that ideally the regenerative therapy should:

- restore tear volume in its correct composition
- have anti-inflammatory properties since quantitative DES is often associated with inflammation – stopping the ongoing destruction of lacrimal gland cells would enable the regeneration and remodeling of the tissue and contribute to functional recovery
- be non-immunogenic (therefore, ideally autologous or HLA-matched)
- be delivered in a single application without the need for repeat intervention
- be Good Manufacturing Practice (GMP)-compliant, which is more feasible when the production is simple and quick
- be cost-effective, especially since the prevalence of quantitative DES is high (5.5%–33.7% depending on nationality[31])
- be efficient (e.g. if cells are to be administered, they should have good homing capacity or be available in large numbers)
- not result in adverse side effects

### Drugs

To date, no studies have been published using a drug for the curative treatment of quantitative DES. Only immunosuppressive/palliative drugs that reduce lacrimal gland inflammation and ease the symptoms (e.g. cyclosporine A, tacrolimus, corticosteroids, etc.) are available (for review, see[21]). In addition, further promising candidates (e.g. lacritin, lactoferrin, quercetin, and systemic pilocarpine) that improve tear flow rate are under investigation, but they cannot regenerate the tissue and thus will not cure quantitative DES.[32–38]

An injection of platelet-rich plasma adjacent to the lacrimal gland has been shown to result in improvement of lacrimal volume, increase the TBUT, and decrease the ocular staining in a small study of four patients.[39] These effects might be explained by tissue regeneration, stimulated by several growth factors and cytokines that are contained in platelet-rich plasma, as has been observed in other secretory tissues. For example, injection of an engineered form of hepatocyte growth factor (HGF) following partial hepatectomy in mice has been shown to promote liver regeneration/proliferation.[40] Also in mice, connective tissue growth factor has been shown to promote regeneration of pancreatic tissue after partial β-cell destruction.[41]

Another potential drug candidate for lacrimal gland regeneration is tumor necrosis factor α-stimulated gene/protein 6 (TSG-6), which was recently shown to improve tear production and ameliorate epithelial defects after topical administration in a murine model of DES.[42] Furthermore, recent studies evaluating the regenerative and immunomodulatory properties of MSCs in other tissues (kidney, skin, and liver) identified TSG-6 (secreted by MSCs) as one of the main cytokines responsible for their anti-inflammatory effects.[43–45] Moreover, application of recombinant TSG-6 to corneal injuries or inflammation-related murine models of DES confirm its action as a potent protective factor. Therefore, TSG-6 may form part of a regenerative therapy treatment for DES in the future.[42,46]

These studies demonstrate that the regeneration of glandular tissue simply by drug administration is feasible, although considerable further work is required before such therapies might be available clinically toward the treatment of lacrimal gland tissue regeneration. To comment as to how well a potential drug therapy might meet the criteria given above for an ideal therapy for lacrimal gland regeneration would be speculative at the moment since there is currently insufficient evidence available.

### Cell therapies

Only a few publications describe the beneficial effects of cell therapies on lacrimal gland regeneration in GVHD and SSDE patients and in animal models, but early data are promising.[47–53] Lacrimal and salivary gland tissues share a number of physiological similarities, may both be affected in SS, and only palliative treatments exist to treat their dysfunction, and so the use of cell therapies in both tissues will be discussed in the following section, since therapies that show promise in salivary gland may also prove beneficial in lacrimal gland.

### *Mesenchymal stem cells/multipotent stromal cells (MSCs)*

Several of the studies testing cell therapies exploit MSCs. MSCs are adult stem cells, residing in an undifferentiated state in adult organs and tissues among differentiated cells. These cells are characterized by their high self-renewal capacity and their ability to differentiate into mature cells.[54 56] Thus, adult stem cells *per se* serve to regenerate specialized tissues. To date, MSCs are characterized by a panel of unspecific markers (i.e. CD29, CD73, and CD105 positive; CD34 and CD45 negative, see Figure 2), which together with their properties of plastic adherence and *in vitro* differentiation capacity are currently the minimal criteria to define and verify an MSC population.[57]

Friedenstein and his colleagues were the first to describe a cell population residing in bone marrow (BM) capable of *de novo* development of osteogenic foci after heterotrophic transplantation in mice. They identified that the transplanted BM-cell population was responsible for the osteogenesis within the transplants[58] Further research on this cell population revealed their self-renewal capacity by the ability to form colony-forming unit fibroblasts (CFU-F) *in vitro*. In addition to the osteogenic lineage, fibroblastic colonies can also give rise to adipocytes, fibroblasts, and reticular cells.[59] Later, it was established that the cells discovered by Friedenstein were adult BM stem cells with a multipotent differentiation capacity toward mesenchymal lineages. A number of more recent studies have also demonstrated the maturation of neuronal,[54,55] muscular,[54] corneal epithelial,[55] salivary acinar,[60] and pancreatic[54,56] cell types from MSC. Progenitor cells with MSC characteristics have also since been found in other tissues such as umbilical cord,[61] adipose tissue,[62] and pancreas.[54,56,63 68]

MSCs are non-immunogenic as they lack major histocompatibility complex (MHC)-class II expression;[56,63,66,67,69] thus, HLA compatibility is advantageously redundant when using MSCs in therapeutic applications. Furthermore, MSCs show anti-inflammatory effects as they inhibit T-cell, B-cell, natural killer (NK) cell monocyte, macrophage and dendritic cell proliferation, modulate the maturation of dendritic cells, impair B-cell immunoglobulin production, and suppress the cytolytic activity of NK cells (reviewed in[70]), which is useful here. Each of these actions may be beneficial, as DES is often caused by inflammation. Furthermore, MSCs are also protective against hypoxia, radiation, chemicals or acidity, and mechanical damage.[49,71,72]

In summary, MSCs display a number of characteristics such as multipotency,[54] capability to stimulate regeneration,[47 53] anti-inflammatory properties (reviewed in[70]), and are non-immunogenic,[56, 66, 67, 69] which highlight their potential use in a broad range of regenerative medicine applications, and also potentially as (part of) a cure for quantitative DES.

### *MSCs in injured and uninjured glandular tissues*

MSCs have been identified in uninjured tissues. However, injury models have proven useful in identifying MSCs, as injury triggers repair and regenerative responses that increase MSC numbers.

MSC-like cells have been isolated from a rat salivary gland injury model that formed either hepatocyte- or duct-like cells when transplanted into a rat liver injury model.[73] Since normal progenitor-dependent liver regeneration relies upon bipotential cells to generate either hepatocytes or bile duct epithelial cells,[74,75] this suggests that the transplanted MSCs responded appropriately to environmental cues. MSC-like cells have also been identified in uninjured salivary gland. These cells express various progenitor markers, are multipotent, and capable of

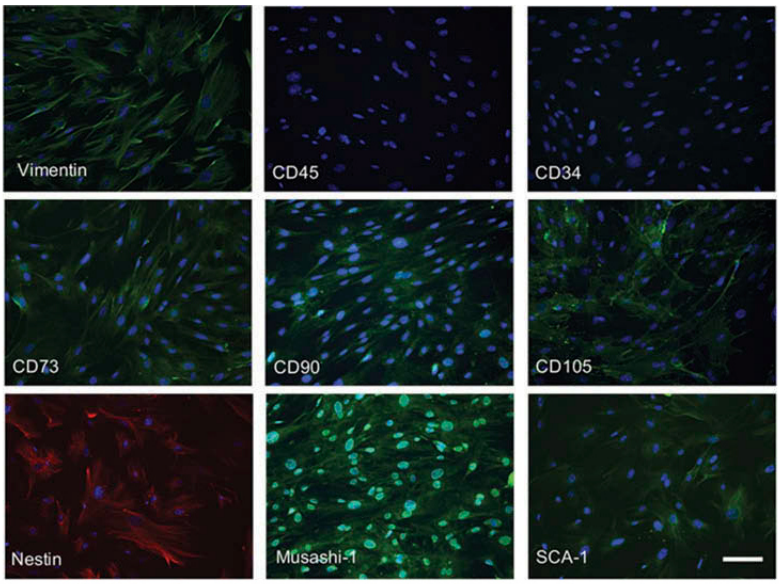


Figure 2. Mesenchymal stem cell immunocytological characterization. MSCs express vimentin denoting their mesenchyme origin. They do not express CD34 or CD45. They display positive expression of MSC markers: CD73, CD90, and CD105 along with putative stem cell markers Musashi-1, stem cell antigen-1, and nestin. Green: CD34, CD45, CD73, CD90, CD105. Red: vimentin. All nuclei are counterstained using DAPI (blue). Scale bar: 100 μm.

CFU-F.[76–79] c-kit$^+$ cells have also been isolated from the salivary gland of a single human but with a lower efficiency than from young animals,[80] highlighting a potential limitation to the use of adult human cells in therapies.

As for salivary gland, injury models have been used to observe MSCs in the lacrimal gland. Zoukhri et al. injected IL-1 directly into murine lacrimal gland, resulting in acinar cell death, a transient decrease in tears, and a subsequent regenerative response.[81] MSC-like cells, as an increase of nestin$^+$ cells, which were also positive for vimentin$^+$ and two further putative stem cell markers,[82,83] were observed. These cells also exhibited an adipogenic differentiation potential, confirming their MSC characteristics. Moreover, cells isolated from mice that had not received the IL-1 injection could not be maintained in culture beyond day 14, supporting the notion that IL-1 injection triggers a regenerative response involving MSC-like or progenitor cells.[82]

Since then, the presence of progenitor cells (co-expressing α-smooth muscle actin (SMA) and putative stem cell markers nestin, Musashi-1, ABCG2, Pax6, Chx10, Sox2, or ΔNp63) in uninjured adult rat lacrimal gland has also been demonstrated.-[55] Upon isolation, expression of nestin, Musashi-1, Pax6, and Chx10 persisted in these cells for 60 days *in vitro;* these cells also demonstrated plasticity, undergoing differentiation into neuronal, epithelial, and myoepithelial cell types. Similarly, a more recent study described the expression of Nanog, Oct4, Sox2, Pax6, and nestin in adult murine and human lacrimal gland at the mRNA level.[84] After the isolation of these lacrimal gland-derived MSCs, expression of transcription factors (C-Myc and Kruppel-like factor-4) and pluripotency markers (Nanog, nestin and Sox2) persisted at the mRNA level to passage 30. Moreover, the expression of early lineage markers for endodermal (GATA4, GATA6), ectodermal (Pax6), and mesodermal (bone morphogenic proteins-4 and -7) development was also demonstrated.

## MSC in cell therapies – Isolation and characterization of MSCs

However, to utilize the regenerative capacity of MSC and MSC-like progenitor cells in clinical practice, the isolation of a pure population is a prerequisite. Despite this, thus far only unspecific isolation methods, such as plastic adherence or tissue explant culture, are established. Ineffective purification during isolation will lead to a heterogeneous cell population, requiring costly time-consuming removal of contaminating cells with passaging.[55, 63, 69, 82, 85–93] Some groups have reported that contaminating cells persist in culture as late as passage 10.[89,92]

There is a great demand for the further development of more refined isolation and culture methods. However, this fundamental development is impaired by the lack of specific MSC markers. For this reason, researchers have attempted to establish improved isolation protocols using additional purification steps to increase the purity of the cell population generated. Ameliorated culture conditions with specific media,[60,65,68,88,90] low oxygen pressure,[94] magnetic- or[67,86,89,92,93,95] fluorescence-activated cell sorting,[87] or fibrin microbeads[91] have all been implemented.

Flow cytometric analysis of progenitors derived from glandular tissues, such as the lacrimal or salivary glands, revealed CD105, CD90, CD73, CD13, CD44, and CD29 as common cell surface markers, whilst in contrast CD34, CD45, MHC-class II, CD31, CD117, and CD133 were shown to be absent. This expression profile is consistent with that described for BM-derived stem cells with MSC characteristics and those isolated from other common tissue sources (reviewed in[96]).

A direct comparison between glandular tissue (islet-derived precursor cells (IPCs)) and BM-derived MSCs revealed similarities, regarding their immunophenotype and the expression of genes associated with stem cell characteristics and mesenchymal markers. However, IPCs had a reduced adipogenic, osteogenic, and chondrogenic but heightened endocrine differentiation capacity.[97] These results lead to the conclusion that MSC-like progenitors originating from different tissues share key features,[54,67] but also exhibit some tissue-specific properties. This might indicate that MSC-like progenitors residing in different tissues represent different subpopulations of MSCs, and so perhaps MSCs isolated from the lacrimal gland should be preferentially selected when attempting the design of a cell therapy to treat DES.

## MSC in cell therapies – Evidence of the beneficial effect of MSCs in vitro

MSCs are known to secrete growth factors such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor among others to induce cell proliferation and angiogenesis.[98,99] It is already known that wound healing, cell proliferation, and migration of epithelial cells from tissues other than lacrimal gland (lung, corneal limbus, kidney, and skin) are increased by an MSC-conditioned medium.[100–103] However, recently it was shown that MSC-conditioned medium using MSC isolated from murine lacrimal glands increases the migration and proliferation of porcine lacrimal gland epithelial cells *in vitro*.[94] While the use of conditioned medium in clinical applications may not be feasible due to its undefined nature, these studies do demonstrate the potential benefit of MSC for the regeneration of epithelial cells.

## MSC in cell therapies – Evidence of the beneficial effect of MSCs in vivo

A number of studies have described the beneficial effects of MSCs to both salivary and lacrimal glands, in both animal studies and human patients.

In murine salivary gland injury models, transplantation of both allo- and xenogeneic MSC-like cells led to an increased salivary flow rate, decreased apoptosis and lymphocytic infiltration, and preservation of the lobular structure,[60,78,104–107] and the differentiation of injected stem cells into salivary acinar and duct cells has been demonstrated.[77,106] Progenitor-like cells have also been isolated from human adult salivary gland tissues,[80] demonstrating that tissue-specific progenitor cells may be available. Together, these findings suggest that MSCs isolated from adult tissue may be used to provide autologous cell therapies.

Similar studies have been performed in lacrimal gland. In a mouse model of DES (concanavalin A injection), injection of human or mouse MSC into the periorbital space proved protective in terms of reducing inflammation both in the lacrimal gland and at the ocular surface, but importantly also offered clinical benefit: tear production and goblet cell density increased whilst IL-2, IFN-ɣ, and epithelial erosion decreased.-[49] However, it was not determined whether reducing the inflammation restored normal lacrimal gland function or normal lacrimal gland function reduced inflammation. Nevertheless, this study elegantly demonstrates the potency of MSCs as a potential cure for quantitative DES and this is encouraging even if the precise mechanism remains unknown. Similarly, in a rat model of DES induced by the topical administration of benzalkonium chloride, Beyazyildiz *et al.* applied MSC topically to the cornea.[48] Again, Schirmer test scores and TBUT increased, whilst corneal defects were reduced in the MSC-treated test group compared with the control group.

Since DES commonly occurs naturally in dogs, the canine model is considered to be superior to the small-animal models described above for the examination of topic and systemic application of MSC.[47] The safety and immunomodulatory effects (suppression of T-cell proliferation) of MSC applied either topically or via injection have been previously evaluated in canine models.[50,53] Allogeneic adipose-derived MSC implanted around lacrimal glands in dogs with naturally occurring chronic DES was shown to be beneficial with a significant increase in Schirmer test scores, whereas ocular discharge, hyperaemia, and corneal changes showed a sustained decrease over time.[51]

We could find only one small study (22 patients) describing the beneficial effects of MSC to lacrimal gland in patients with DES secondary to GVHD.[52] Human BM-derived MSCs were injected intravenously and, as a result, in 12 out of 22 patients dry eye and ocular surface disease index scores were reduced and Schirmer test scores increased during a 3-month follow-up period. However, in the remaining 10 patients, no effect was seen. Interestingly, in those patients where a clinical benefit was observed, IL-2 and IFN-γ levels were increased, contradicting the results from Lee et al. described above. This highlights the complexities of determining the relationship between administration of MSC and alterations in cytokine profiles that involve tightly controlled regulatory feedback loops, and indeed confuses the argument as to whether inflammation triggers DES or *vice versa*.

The above findings demonstrate that MSCs are potentially useful for the regeneration of lacrimal gland tissue. To this end, the results of the *in vivo* studies are particularly encouraging. The main limitation to the application of MSCs in clinical therapy is likely to be the lack of an efficient method to obtain large numbers of specific MSCs. However, expertise is rapidly advancing in this area and it seems likely that MSCs will be utilized toward lacrimal gland regeneration in the not too distant future.

### *Human amniotic membrane (hAM) epithelial cells in cell therapies*

hAM epithelial cells have also been used for the regeneration of salivary gland tissue in mouse following irradiation. hAM epithelial cells were injected into sub-mandibular glands, which restored the gland structure and increased saliva production.[108] hAM epithelial cells have previously been shown to be capable of trans-differentiation into acinar cells when cocultured with acinar cells in a double-chamber system *in vitro*[109] (presumably stimulated by secreted factors). It is likely that the more complex *in vivo* environment also supports trans-differentiation of these cells, resulting in a rescued salivary gland. Again, due to the parallels between salivary gland and lacrimal gland, this cell source may also be beneficial toward the treatment of DES; however, as there is no information on their use in this disease, the potential use of the cells in this application is unclear.

### *Induced pluripotent stem (iPS) cells in cell therapies*

iPS cells are cells isolated from adults that have been "reprogrammed" to increase potency.[110] To date, we are not aware that they have been used in any cell therapy toward the treatment of DES. However, an alternative approach is the administration of iPS cell-conditioned medium to dysfunctional murine lacrimal glands.[111] In this study, the authors injected 200 µl of the conditioned medium into the tail vein of mice 1 hour before irradiation. Administration of the conditioned medium resulted in decreased inflammation and p38 pathway activation, and maintenance of lacrimal gland tissue microarchitecture *in vivo*. However, the use of conditioned medium in clinical application is problematic since manufacturers would be required to demonstrate not only that the iPS cells and medium were safe but also that the conditioned medium produced was equivalent each time (e.g. had a similar protein profile), which significantly complicates the validation process. This, in turn, is likely to lead to increased cost and development time.

These cell therapy studies are summarized in Tables 1 and 2, according to clinical feasibility and efficacy, respectively. Owing to the absence of a regeneration-inducing drug, and the mounting evidence described above that MSCs are beneficial in regenerative applications, a cell therapy with MSCs or MSC-like cells seems to be the most promising tool for the treatment of quantitative DES. However, further work must be completed before transfer to clinic. To this end, it would be of great benefit to design protocols to isolate MSCs specifically, consistently, and rapidly under the defined conditions. Additionally, a suitable cell source with sufficient potency and availability, such as adipose tissue-derived MSCs, needs to be further investigated and established so that the large number of cells required for a successful cell therapy treatment may be delivered. Figure 3 briefly describes how such a cell therapy may be produced. In 2013, 220 clinical trials were implemented investigating the use of MSCs for immunomodulation, tissue protection, regenerative medicine, or graft enhancement (reviewed in[112]) and so it seems very likely that cell therapies exploiting the characteristics of MSCs will be available in the not too distant future for the treatment of quantitative DES.

## Gene therapy

In gene therapies, delivery of exogenous nucleic acids, such as DNA, mRNA, small interfering RNA, micro RNA, or antisense oligonucleotides, into a patient's cells is used to trigger

**Table 1.** Clinical feasibility of cell therapies for *in situ* lacrimal and salivary gland regeneration.

| Clinical feasibility | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target | Study design | Non-immunogenic | Anti-inflammatory | Efficiency | Adverse effects | GMP compliant | Cell number needed | Ref. |
| Lacrimal gland | Tail-vein injection of iPS-derived conditioned medium into mouse 1 h prior to irradiation | – | Yes | – | – | Problematic | N/A | 111 |
| | Injection of xenogeneic hMSCs into the periorbital space of an inflammation induced (concanavalin A) dry eye mouse model | Yes (MSC) | Yes | Less than 0.1% of the cells were found in the intraorbital gland (1 and 7 d post injection) | – | Yes | $1 \times 10^3$ or $1 \times 10^5$ hMSC per mouse eye | 49 |
| | Injection of allogeneic MSCs into the area surrounding the main lacrimal gland of dogs with naturally occurring DES | Yes (MSC) | – | - | – | Yes | $8 \times 10^6$ hMSC per dog eye | 51 |
| | Topical administration of autologous MSC to the corneas of rats following benzalkonium chloride to induce DES | Yes (MSC) | Yes | MSCs found in conjunctival epithelium and Meibomian gland after 1 week | – | Yes | $7 \times 10^5$ MSC per rat eye | 48 |
| | Intravenous injection of allogeneic MSC to patients with DES secondary to GVHD | Yes (MSC) | Unclear | – | – | Yes | $1 \times 10^6$ MSC/kg | 52 |
| Salivary gland | Tail-vein injection of allogeneic BMMSC into a mice model of SS | Yes (MSCs) | Yes | – | No | Yes | $1 \times 10^5$ MSC per mouse | 119 |
| | Allogeneic injection (intravenous) of umbilical cord-derived MSCs into 24 patients with primary SS | Yes (MSCs) | Yes | – | No | Yes | $1 \times 10^6$ MSC/kg (may be stored in advance) | 119 |
| | Tail-vein injection of allogeneic BMMSC ± complete Freund's adjuvant into a mice model of SS | Yes (MSCs) | Yes | – | – | Yes | $4 \times 10^7$ BMMSC per mouse | 104 |
| | Gland-directed injection of xenogeneic hSGSC into male Wistar rats | Yes (MSC-like) | – | Transplanted cells were found in the gland after 60 days | – | Yes | $5 \times 10^5$ hSGSCs per rat gland | 60 |
| | Intraglandular transplantation of allogeneic BMcMSC in mouse 24 h after irradiation | Yes (MSC) | – | - Transplanted cells were found in the gland for up to 4 wk<br>-Trans-differentiation to epithelial cells | – | Yes | $1 \times 10^5$ BMcMSC per mouse gland | 105 |
| | Intraglandular injection of xenogenic human adipose tissue- derived stem cells (hADSC) into submandibular glands of adult rats after irradiation | Yes (MSC-like) | – | - Transplanted cells were found in the gland after 24 wk<br>-Trans-differentiation to acinar and duct cells | No | Yes | $1 \times 10^6$ hADSCs per rat gland | 120 |
| | Intraglandular injection of c-kit$^+$ cells from dissociated salispheres into salivary glands of adult mice after irradiation | Yes (stem-like) | – | – | – | Yes | $3 \times 10^2$–$1 \times 10^3$ c-kit$^+$ cells per mouse gland | 78 |
| | Intraglandular injection of hAM epithelial cells into salivary glands of adult mice after irradiation | No | – | - Transplanted cells were found in the gland after 30 days | – | Yes | $1 \times 10^6$ hAM epithelial cells per mouse gland | 108 |

BMcMSC: bone marrow derived clonal mesenchymal stem cell; BMMSC: bone marrow derived mesenchymal stem cell; hADSC: human adipose tissue derived stem cells; hAM: human amniotic membrane; hMSC: human bone marrow derived mesenchymal stem cell; hSCSG: MSC-like human salivary gland stem cells; IPS: induced pluripotent stem cell; MSC: mesenchymal stem cell; SS: Sjögren's syndrome
–: not assessed.

**Table 2.** Clinical efficacy of cell therapies for *in situ* lacrimal and salivary gland regeneration.

| Clinical efficacy | | | | | |
|---|---|---|---|---|---|
| Target | Study design | Restoration of tear film or salivary output | Single treatment | Permanent cure | Ref. |
| Lacrimal gland | Tail-vein injection of iPS-derived conditioned medium into mouse 1 h prior to irradiation | Partially<br>- improved tear secretion (1 week post irradiation) | Yes | Yes<br>- restored gland structure and ultra-microstructure<br>- decrease of apoptotic cells, edema, congestion, and neutrophil infiltration<br>- upregulation of cytokine level | 111 |
| | Injection of xenogeneic hMSCs into the periorbital space of an inflammation induced (concanavalin A) dry eye mouse model | Yes<br>- restored tear secretion (1 week post concanavalin A injection)<br>- reduced T cell inflammation | Yes | Yes<br>- lower corneal staining score<br>- decreased inflammatory cytokine level<br>- decreased T cell infiltration<br>- preserved gland structure | 49 |
| | Injection of allogeneic MSCs into the area surrounding the main lacrimal gland of dogs with naturally occurring DES | Yes<br>- Schirmer test score restored to within normal range | Yes | Yes<br>- ocular discharge reduced<br>- hyperaemia reduced<br>- corneal changes reduced | 51 |
| | Topical administration of autologous MSC to the corneas of rats following benzalkonium chloride to induce DES | Yes<br>- Schirmer test score increased | No, daily injections over 1 week | Yes<br>- TBUT increased<br>- corneal defects reduced | 48 |
| Salivary gland | Tail-vein injection of allogeneic BMMSC into a mice model of SS | Yes<br>- restoration of salivary flow rate | Yes | Yes | 119 |
| | Allogeneic injection (intravenous) of umbilical cord-derived MSCs into 24 patients with primary SS | Increase of salivary flow rate | Yes | – | 119 |
| | Tail-vein injection of allogeneic BMMSC ± complete Freund's adjuvant into a mice model of SS | Yes<br>- tear quality did not change after therapy | No,<br>4 times in 2 weeks | – | 104 |
| | Gland-directed injection of xenogeneic hSGSC into male Wistar rats | Partially<br>- improved saliva flow rate (d60), but not completely restored | Yes | Yes<br>- restored tissue structures | 60 |
| | Intraglandular transplantation of allogeneic BMcMSC in mouse 24 h after irradiation | Partially<br>- improved saliva flow rate (12 wk post irradiation) | Yes | Yes<br>- restored tissue structures and gland weight | 105 |
| | Intraglandular injection of xenogenic human adipose tissue- derived stem cells (hADSC) into submandibular glands of adult rats after irradiation | Partially<br>- improved saliva flow rate (24 wk post irradiation) | Yes | Yes<br>- restored gland structure<br>- decrease of apoptotic cells<br>- paracrine effect of hADSCs | 120 |
| | Intraglandular injection of c-kit$^+$ cells from dissociated salispheres into salivary glands of adult mice after irradiation | Partially<br>- saliva flow rate 23%–70% that of non-irradiated glands (out to 120 days post-irradiation) | Yes | Yes<br>- restored gland structure | 78 |
| | Intraglandular injection of hAM epithelial cells into salivary glands of adult mice after irradiation | Partially<br>- saliva flow rate 48% that of non-irradiated glands | Yes | Yes<br>- restored gland structure | 108 |

BMcMSC: bone marrow-derived clonal mesenchymal stem cell; BMMSC: bone marrow-derived mesenchymal stem cell; hADSC: human adipose tissue-derived stem cells; hAM: human amniotic membrane; hMSC: human bone marrow-derived mesenchymal stem cell; hSCSG: MSC-like human salivary gland stem cells; iPS: induced pluripotent stem cell; MSC: mesenchymal stem cell; SS: Sjögren's Syndrome
–: not assessed.

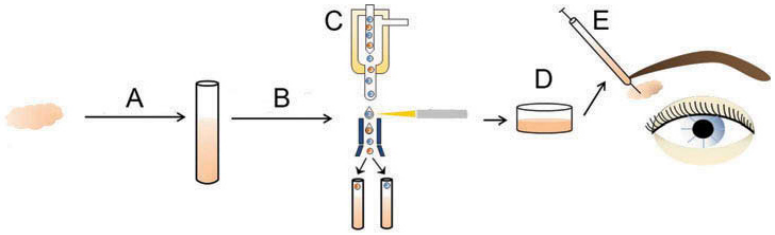


Figure 3. Schematic of potential cell therapy to regenerate lacrimal gland tissue *in situ*. A: Donated lacrimal gland tissue would undergo enzymatic and/or mechanical digestion to yield a single cell suspension. B: Cells expressing MSC markers would be labeled, and C: sorted using fluorescence-activated cell sorting to isolate a pure MSC population. D: The sorted cells would be expanded *in vitro* to achieve a sufficient cell number. E: These would be transplanted directly into the lacrimal gland of the recipient. This would reduce inflammation and regenerate damaged lacrimal gland tissue, completely restoring function.

an increase or decrease in protein expression or to correct genetic mutations in order to treat a disease. Viral or nonviral vectors are used to insert genetic information into the target cells. Viral vectors carry several risks including carcinogenesis and immunogenicity, whereas nonviral vectors have fewer safety issues, but a significantly lower delivery efficiency.[113]

The feasibility of gene transfer into lacrimal gland tissue using viral vectors has been shown both *in vitro* and *in vivo*.[114,115] In a rabbit model, the transfer of tumor necrosis factor-α inhibitor gene after induction of an autoimmune lacrimal gland inflammation resulted in the return of tear production to normal levels, improved TBUT and rose bengal scores, and a reduced infiltration of T cells and CD18+ cells.[116] In a very similar model, the vector-mediated viral (v)IL-10 gene expression led to an overall improvement in clinical symptoms and a significantly reduced number of CD18+ cells.[117]

Similarly, in salivary glands, several other genes, namely manganese superoxide dismutase-plasmid/liposome, basic fibroblast growth factor, vascular endothelial growth factor, interleukin-10, interleukin-17, vasoactive intestinal peptide, and human aquaporin-1 (which encodes a water channel membrane protein and is expected to induce fluid secretion by providing stimulated water permeability pathways in duct cells (reviewed in[118])), have been used to treat hyposalivation.

The current results of the application of gene therapy in order to treat dry eye (or dry mouth) seem very promising, but the long-term safety of gene-delivering vectors and appropriate delivery genes needs to be further identified in order to provide clinical availability.

## Conclusions and future challenges

DES is a complex, multifactorial disease for which only palliative treatments exist. We propose that regeneration of the tissue may be possible and that this may provide a curative treatment. There are three main approaches to regeneration of tissue *in situ*: drugs, cell therapies, and gene therapies.

The drugs that are proposed to be useful in regenerative treatment approaches to DES are growth factors, or similar. The regenerative potential of growth factors is unsurprising given their heavy involvement in both development and during injury. The evidence to support their beneficial role in DES is currently scant and, for this reason, considerable further work is required here before their use in clinical application becomes realistic. Gene therapies in *in vivo* models appear to be of clinical benefit and this is, of course, encouraging. However, their use is hampered due to safety concerns.

Cell therapies would appear to be the most promising approach for the treatment of DES, not least because this is the most established approach with considerably more *in vivo* studies demonstrating the efficacy of transplanted MSCs, than the approaches using either drugs or gene therapies. MSCs are multipotent, non-immunogenic, and anti-inflammatory. These properties are important since they demonstrate that MSCs can differentiate into other cell types and support existing lacrimal gland epithelial cells, may be transplanted without risk of rejection or requirement for lifelong immunosuppression, and reduce inflammation, which is often the cause of DES. MSCs have already been shown to have regeneration potential in both salivary gland and lacrimal gland in animal models.[49,60,77,104,106,119] However, their use is hampered by a lack of knowledge as to how to obtain a pure population of cells, with sufficient potency in sufficient number. Future work should seek to address this. Despite these difficulties, a number of clinical trials are under way that aim to exploit the characteristics of MSCs in various, different applications.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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# 3. Results - Publications

## 3.1 Original Research Article

### Comparative analysis on the dynamic of lacrimal gland damage and regeneration after Interleukin-1α or duct ligation induced dry eye disease in mice

Jana Dietrich*, Carlo Schlegel, Mathias Roth, Joana Witt, Gerd Geerling, Sonja Mertsch, Stefan Schrader

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Contribution to experimental design, implementation and publication

Involved in the conception and design of the experimental setup, conduction of the surgery in collaboration with CS, implementation and assembly of data concerning lacrimal gland weight and (immune-)histochemistry (except 3.1.2 and 3.3, shared 3.2.1-3.2.3), data analysis and interpretation (except 3.1.2 and 3.3, shared 3.2.1-3.2.3), manuscript writing, preparation of all figures. The manuscript was subsequently reviewed and approved by all co-authors.
Approximated total share of contribution: 43%

Data used for degrees other than this thesis

The data concerning fluorescein staining in section 3.1.2/Figure 1, dynamic of lacrimal gland damage and regeneration in section 3.2.1-3.2.3/Figure 3 and characterization of immune reaction in section 3.3/Figure 6, will be submitted to obtain the medical doctorate of Carlo Schlegel at the Heinrich-Heine-Universität Düsseldorf.

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# Comparative analysis on the dynamic of lacrimal gland damage and regeneration after Interleukin-1α or duct ligation induced dry eye disease in mice

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ABSTRACT

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

## 1. Introduction

Dry eye disease (DED) is a complex disease with an incidence between 5 and 50% depending on ethnic group, age and sex (Stapleton et al., 2017; Sullivan et al., 2017). Patients describe burning sensation, visual disturbance as well as foreign body sensation, causing severe pain and a serious restriction of their daily life. Most severe cases of DED are caused by a quantitative tear deficiency (aqueous deficient dry eye; ADDE). Common reasons for the development of ADDE, such as age-dependent hyposecretion, inflammation and occlusion of the secretory ducts, lead to a loss of functional lacrimal gland (LG) tissue. However, ADDE can also develop as consequence of a dysfunctional innervation as well as a defective aquaporin-5 distribution as described in patients with Sjögren´s syndrome, resulting in decreased tear flow, but with remaining structurally functional LG tissue (Fox and Saito, 2005; Tsubota et al., 2001).

Despite being well characterized and moderately prevalent, treatment for DED remains palliative. During disease progression tear deficiency can lead to corneal ulcers as well as conjunctival and corneal scarring, which can cause a loss of vision (Bron et al., 2017). Therefore, it is of particular importance to search for causative therapies. One possible therapeutic approach represents the regeneration of functional LG tissue. Zoukhri et al. showed that healthy LGs from young adult mice are able to spontaneously regenerate after experimentally induced

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damage (Zoukhri et al., 2007). However, the underlying mechanisms of LG regeneration are still unclear. Recent studies examine the potential of drugs, cell therapies as well as gene therapy to induce LG regeneration *in situ*, but further work is needed to overcome the current limitations (for a detailed review see (Dietrich et al., 2016)).

There are several mouse models described to study ADDE. These models can be divided into two main groups. The first group of models are using strains with a genetically modified background, developing a spontaneous ADDE, e.g. the MRL/lpr strain, the NOD mice, Id3-deficient (reviewed (Schrader et al., 2008)) and TSP-1 null mouse strains. The second type of models utilizes experimental induced ADDE in wild-type mice strains. All of these models show signs of continuous LG impairment with a mainly T cell dependent infiltration of the tissue and further characteristics of Sjögren´s syndrome, such as anti-SSA, anti-SSB or anti-α-fodrin autoantibodies (Li et al., 2004; Skarstein et al., 1995; Turpie et al., 2009; Wahren et al., 1994). Except for the Id3-deficient strain, ADDE is secondary to another autoimmune disease. Experimentally induced models include the injection of IL-1α into the LG (Zoukhri et al., 2007), application of various pharmaceuticals, such as benzalkonium chloride (BAC) or scopolamine (Dursun et al., 2002; Lin et al., 2011), ligation of the secretory duct (Liu et al., 2017) or desiccating environmental stress (Dursun et al., 2002). While the application of BAC only leads to ADDE establishment on the ocular surface, ligation of the secretory duct and IL-1α injection directly affects the LG.

The present study directly compares the dynamic of damage and regeneration in two mouse models with experimental induced ADDE, which reflects the pathophysiological situation in patients and excludes the influence of a genetically modified background with autoimmune symptoms. Therefore, we investigated (i) an inflammation based model using the injection of IL-1α and (ii) an obstructive model with the occlusion of the single secretory duct through a ligation. In these models, we analyzed and compared the dynamic of the clinical presentation (fluorescein staining), the functional anatomy (H&E staining), the immune response and the presence of progenitor cells (immunohistochemistry) in LG tissue after IL-1α injection and duct ligation (DL). The pro-inflammatory cytokine IL-1α triggers the initial inflammatory response by recruiting hematopoietic cells to the site of damage (reviewed in (Di Paolo and Shayakhmetov, 2016)). Furthermore, patients with DED exhibit significantly elevated levels of IL-1α, which strongly correlates with the observed corneal destruction and DED impact (Pflugfelder et al., 1999; Solomon et al., 2001). The experimental duct occlusion was implemented through the ligation of the secretory duct, which leads to an accumulation of LG fluid and causes a pressure-induced atrophy of functional LG tissue (Liu et al., 2017).

In summary, the aim of the study was to directly compare two mouse models of experimental induced ADDE, to study the dynamic of LG damage and regeneration and to evaluate how these models can be used to study regenerative therapies to treat ADDE.

## 2. Materials and methods

### 2.1. Mice

Male C57BL/6 J mice were purchased from Janvier labs (Le Genest-Saint-Isle, FR) and maintained under 12:12 h light:dark cycle with food and water *ad libitum*. All experiments were conducted in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research and the national ethical committee for animal experimentation (FELASA guidelines).

### 2.2. Surgery

Mice (8–10 weeks old) were anesthetized using ketamine (80 mg/kg bodyweight (BW); Zoetis, Florham Park, NJ, USA) and xylazine (7.5 mg/kg BW; Bayer, Leverkusen, Germany) for intraperitoneal (i.p.) injection. The left lacrimal gland was left untreated as a control. For inflammation induced DED the right lacrimal gland was exposed and injected with 2 µl recombinant murine IL-1α (1 µg dissolved in saline, PromoCell, Heidelberg, Germany; see video 1) or saline as control. For atrophy induced DED the right secretory duct of the lacrimal gland was exposed and ligated twice with silicon tubes (AS ONE Corporation, Osaka, Japan; see video 2) for 7 days or left untreated as control (sham operation). A second operation was implemented at day 7 to remove the DL in order to re-open the secretory duct or as a second sham operation. Right LG was exposed and the ligation removed by re-opening the silicon nodes with two fine forceps or left untreated (sham operation). In all cases, buprenorphine (0.05 mg/kg BW; Reckitt Benckiser, Slough, UK) was injected subcutaneously (s.c.) as analgesia and tramadol (Hexal, Holzkirchen, Germany) was added to the drinking water for 3 days at 1 mg/ml. Gentamicin ointment (gentamicin-POS 5 mg/g, Ursapharm, Saarbrücken, Germany) was used as an antibiotic to cover the stitched wound.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.exer.2018.03.026.

### 2.3. Experimental setup

LGs of the IL-1α (n = 6 per time point) or saline group (n = 4 per time point) were investigated on day 1, 2, 3, 5 and 7 after injection. DL (n = 6 per time point) or sham operation (n = 4 per time point) treated LGs were investigated on day 3, 7, 14, 21 and 28. The second operation, to re-open the lacrimal duct by removal of the ligation, was implemented on day 7. In total 100 mice were used for this study divided into 4 different groups treated at 5 different time points, with n = 6 for IL-1α injection, n = 6 for DL, n = 4 for NaCl injection and n = 4 for sham surgery at each investigated time point. At each day of surgery animals from control (NaCl injection or sham) as well as treatment group (IL-1α injection or DL) were handled together. At some of the investigated time points mice died during anesthesia, resulting in a reduction of group size at some of the time points, however at least n = 4 mice in the treatment groups (IL-1α injection and DL) and n = 3 mice in the control groups (NaCl injection and sham operation) were evaluated for all time points.

### 2.4. Fluorescein staining

Investigation of epithelial damage on the ocular surface was performed using fluorescein (1.7 mg/ml Fluorescein SE Thilo, Alcon, Freiburg im Breisgau, Germany) at all investigated time points. Fluorescein was directly applied to the ocular surface and surplus solution was washed out with saline. The ocular surface was examined using a slit lamp with a cobalt blue filter and punctual staining was scored according to Oxford grading in a blinded manner. Staining was graded from 0 (no staining) to 5 (severe staining).

### 2.5. Histology and hematoxylin and eosin (HE) staining

To evaluate the histopathological dynamic of LG morphology and integrity, specimens were collected after IL-1α or DL induced damage and spontaneous regeneration (see 2.3), fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. One centrally located section (4 µm) was used for hematoxylin and eosin (HE) staining. Evaluation was performed by two independent scientists in a blinded fashion.

For histological evaluation pictures of HE stained whole LG were investigated using Fiji software (Schindelin et al., 2012). To assess the parenchymal cell proportion, cell area was measured and calculated on the basis of total LG area (set as 100%). Severely affected acini structures were identified by a shrunken cell body with eosinophilia as well as edema and irregular arrangement of acini structures (due to interstitial edema). The area of severely affected acini structures was measured and the proportion calculated on the basis of total LG area (set as 100%). In addition, vital acini structures were measured and calculated

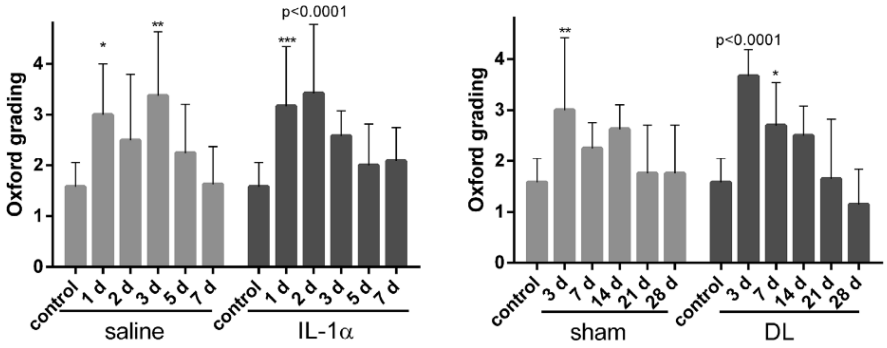


**Fig. 1.** Oxford grading of fluorescein stained ocular surface. Fluorescein staining was significantly increased in all groups for up to 3 days after surgery compared to untreated control. Only DL caused an increased staining thereafter when compared to untreated control. n = 25 for control (one quarter of untreated mice was measured before surgery as control), n = 3–4 for NaCl, n = 4 for sham, n = 4–6 for IL-1α and DL; All Data represented as mean values ± SD; * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represent $p \leq 0.001$.

as above. Inflammation was identified due to the amount of infiltrating cells in total LG area and graded as inflammatory score with 0 (no inflammatory cells visible), 1 (some sparse inflammatory cells visible, homogenously distributed), 2 (moderate number of infiltrating cells visible, homogenously distributed or condensed in an inflammatory foci) or 3 (a high number of infiltrating cells visible infiltrating the whole gland), whereby the infiltration mainly consist of granulocytes (Supplementary data 1; scale bar 100 μm).

### 2.6. Immunohistological staining

To evaluate the localization, distribution and amount of infiltrating immune cells as well as stem cells during damage and spontaneous regeneration specimens were collected as described before (see 2.3). Immunohistologcal staining was performed using a standard protocol for DAB staining. In brief, murine LG were fixed with 4% PFA and embedded in paraffin. One centrally located section (4 μm, n = 3/time point for IL-1a injection and DL treatment and n = 2/time point for sham operation and saline injection) were deparaffinized and re-hydrated using an alcohol gradient. Sections were permeabilized with 0.15% Triton-X-100 for 10 min before heat-induced antigen retrieval was performed using 10 mM citrate buffer (pH 6.0) for 35 min in a steam oven. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min before unspecific binding was blocked using 25% equine serum (Sigma Aldrich) with or without 0.25% BSA depending on the primary antibody. For mouse on mouse staining (primary antibody derived from mouse), the sections were pre-incubated with an Affini Pure Fab Fragment (1:40, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h to block Fc receptor binding of primary mouse antibodies. Primary antibodies directed against Nestin (1:500, 839801 BioLegend, San Diego, CA, USA), Ki67 (1:100, ab16667, abcam, Cambridge, UK), CD3 (1:75, ab16669, abcam), CD138 (1:100, ab34164, abcam), caspase-3 (1:100, ab4051, abcam), and CD68 (1:300, ab125212, abcam) were applied overnight at 4 °C. A biotinylated horse anti-mouse or anti-rabbit IgG (1:300, Vector laboratories, Burlingame, CA, USA) was used as second antibody. An avidin/peroxidase system was applied to the sections before staining with 3,3′-diaminobenzidine (DAB) substrate (Vector laboratories). All specimens were counterstained with hematoxylin (Carl Roth, Karlsruhe, Germany). Staining of each marker was evaluated in 20 high power fields (HPF) or, if less, in total LG area with 400× magnification using a Leica DM 4000 B (Leica, Wetzlar, Germany). For each HPF positive stained cells were manually counted and cells per $mm^2$ were calculated.

### 2.7. Statistics

Statistical data analysis was performed using GraphPad Prism 6 software (Graphpad, La Jolla, CA). Values were given in means ± standard deviation (SD). Statistical analysis was performed using ordinary one-way analysis of variance (ANOVA) with Dunnett post-hoc test. Differences with $p \leq 0.05$ were considered as significant.* represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represent $p \leq 0.001$.

## 3. Results

### 3.1. Clinical assessment of ADDE

#### 3.1.1. Protective eye closure

Mice treated with DL showed moderate symptoms of disturbance of the affected eye at day 7. The eye was protected through lid closure and repeated blinking. None of these signs were visible in IL-1α treated or control mice.

#### 3.1.2. Fluorescein staining of the ocular surface

To investigate whether LG damage, either induced by IL-1α or DL, have an influence on the integrity of the corneal epithelium, the ocular surface was examined using fluorescein staining. In control groups (saline injection or sham operation) as well as after IL-1α injection the corneal fluorescein staining significantly increased for up to day 3 after surgery (Fig. 1) compared to untreated control. Only DL led to an increased fluorescein staining beyond day 3, which was still significant at day 7 (2.7 ± 0.8, p = 0.0168) when compared to untreated control (1.6 ± 0.5).

#### 3.1.3. Lacrimal gland weight

Damage of LG was assessed by measuring the wet weight (Fig. 2) on the days of the most severe impairment (day 1 after IL-1α injection and day 7 after DL) and at the end point of the experiments. IL-1α injection led to an observable, however not significant reduction of 19.9 ± 7.7% of LG weight (10.2 ± 1.0 mg; p = 0.1525) at day 1 with a total recovery at day 7, compared to untreated control (12.75 ± 2.2 mg). In contrast, DL resulted in a drastic reduction of 72.55 ± 4.89% of LG weight (3.5 ± 0.6 mg; p < 0.0001) at day 7 and additionally exhibited morphologic signs of atrophy. During regeneration period (day 7 to day 28), LG weight significantly increased to 52.9 ± 16.3% (6.7 ± 2.1 mg; p = 0.0204) at day 28 compared to untreated control (set as 100%).

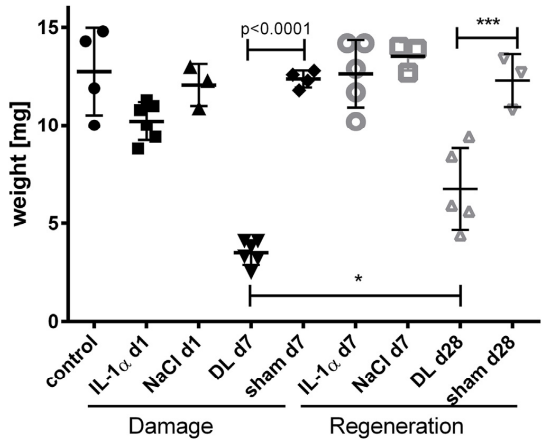


**Fig. 2.** LG weight was measured at days with severe impairment (day 1 after IL-1α, day 7 after DL) and at the investigated end points (day 7 after IL-1α, day 28 after DL). IL-1α injection resulted in a slightly decreased LG weight at day 1, which normalizes by day 7. DL caused a significant decrease of LG weight at day 7 with a significant increase to day 28. n = 3–4 for control, NaCl and sham, n = 5–6 for IL-1α and DL; All Data represented as mean values ± SD; * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represent $p \leq 0.001$.

### 3.2. Dynamic of lacrimal gland damage and regeneration

#### 3.2.1. Histologic changes in lacrimal gland structure

The dynamic of damage and regeneration of functional LG tissue after IL-1α or DL was evaluated on HE stained sections (Fig. 3A–G). Untreated LGs (control) are composed of tight acini structures with minimal connective tissue surrounding the lobules (Fig. 3A). Saline injection (Fig. 3B) as well as sham operation (Fig. 3E) caused small interstitial edema, which resulted in irregular, loosened acini structures. In contrast, IL-1α injection led to an increase of infiltrating cells and sizeable interstitial edema with damaged acini structures and an increase of connective tissue between the lobules at day 1 (Fig. 3C). At day 7 after IL-1α injection LG tissue displayed again native acini structures (Fig. 3D). Similar to the IL-1α injection, DL also resulted in a damage of acini structures by infiltrating cells, interstitial edema and an increase of connective tissue at day 7 (Fig. 3F), but the effect was more distinct. By day 28 (21 days after re-opening) acini structures were again visible with a decline of surrounding connective tissue, but interstitial edema could still be observed (Fig. 3G).

#### 3.2.2. Parenchyma cell area

The parenchymal cell area mainly consists of acini and duct structures, was only slightly affected by saline injection or sham operation (Fig. 3H). In contrast, IL-1α injection resulted in a significant decrease of cell area at day 1 (73.16 ± 8.79%, $p = 0.0001$) and day 2 (77.14 ± 7.54%, $p = 0.0037$), which started to normalize at day 3 (83.37 ± 6.56%) when compared to untreated control (91.6 ± 3.25%). However, DL led to a substantial decrease of the parenchymal cell area and an increase of fibrotic tissue (Fig. 3F) up to day 7 (47.76 ± 11.2%, $p < 0.0001$). The re-opening of the ligation at day 7 induced a constant increase of the cell area up to day 28 (78.73 ± 4.2%, p − 0.0204) accompanied by a decrease of fibrosis (Fig. 3G). Nevertheless the parenchymal cell area was still significantly reduced at day 28 when compared to untreated control.

To directly compare both DED models, the time point with most severe LG damage (day 1 after IL-1α injection and day 7 after DL) and the regeneration of LG at the investigated end point (day 7 after IL-1α injection and day 28 after DL) were considered regarding parenchymal cell area. DL induced significantly more damage to LG tissue than IL-1α injection ($p = 0.0002$). After regeneration, no significant differences were observed between the parenchymal cell area of DL (78.73 ± 1.72%) and IL-1α injection (85.19 ± 2.15%) (data not shown).

#### 3.2.3. Vital/affected acini structures

Strongly affected cells were identified due to a reduced size of the cell soma with a higher eosinophilia than vital cells, which marks the first stages of apoptosis (reviewed in (Elmore, 2007). Control treatments (saline injection and sham operation) resulted in slight, but not significant reduction of vital acini structures (Fig. 3I). However, IL-1α injection led to a significant reduction of vital acini area on day 1 (64.39 ± 10.1%) and day 2 (63.63.8 ± 16.29%) compared to untreated control (90.87 ± 3.47%). Thereafter, vital acini structures reappeared comparable to untreated control. With ongoing damage of LG tissue after DL, acini structures completely diminished and mostly edematous tissue remained with a high number small cells. Vital acini structures constantly decreased after DL up to day 7 (3.89 ± 5.17%, $p < 0.0001$). After re-opening of the ligation, the amount of vital acini structures increased, but were still significantly decreased at day 28 (54.67 ± 11.47%, $p < 0.0001$) compared to untreated control.

The direct comparison between IL-1α injection and DL at the time point of severe LG damage (day 1 after IL-1α injection and day 7 after DL) revealed a significantly lower level of vital acini structures after DL than after IL-1α injection ($p < 0.0001$). Thus DL caused a more pronounced damage of LG tissue than IL-1α injection. After regeneration (day 7 after IL-1α injection and day 28 after DL), vital acini structures in the DL group were still significantly, reduced when compared to IL-1α injection ($p = 0.0001$) (data not shown).

#### 3.2.4. Apoptosis and proliferation

In addition, apoptotic cells were counted in immunohistologic staining of capase-3 (Fig. 4F). Compared to untreated control (10.15 ± 5.12 cells/mm$^2$) neither saline, IL-1α injection nor sham operation resulted in a significant increase of apoptotic cells (Fig. 5A). Only, after DL the amount of apoptotic cells significantly increased. Most apoptotic cells were detected at day 14 (53.17 ± 35.7 cells/mm$^2$, $p < 0.0001$) after DL with a decline to control level at day 28 (13.35 ± 9.3 cells/mm$^2$). Since, regeneration of damaged tissue is accompanied by an increase of proliferating cells the number of Ki67 positive cells were evaluated (Figs. 5B and 4E). Only minor changes could be found after saline injection or sham operation, when compared to untreated control (80.95 ± 18.97 cells/mm$^2$). After IL-1α injection, the number of proliferating cells significantly increased at day 1 (670.3 ± 421 cells/mm$^2$, $p < 0.0001$), day 2 (349.9 ± 120.5 cells/mm$^2$, $p < 0.0001$) and day 5 (330 ± 120.9 cells/mm$^2$, $p < 0.0001$). At day 1 and day 2 proliferating cells appeared to be mainly immune cells, whereas at day 5 mainly acinar cells were proliferating (Supplementary data 2). DL also resulted in a significant increase of proliferating cells, which gradually increased from day 3 to day 14 (1150 ± 210.9 cells/mm$^2$, $p < 0.0001$). Thereafter the number of proliferating cells normalize by day 28 (101 ± 40.23 cells/mm$^2$, $p < 0.0001$).

### 3.3. Characterization of immune reaction

#### 3.3.1. Inflammatory score in HE staining

The overall immune reaction was graded based on the amount of infiltrating cells visible in HE stained sections (Fig. 6A). On the basis of the cell and segmented nucleus morphology in HE staining, infiltrating cells were mainly recognized as granulocytes (Supplementary data 1 insert). Untreated control LGs showed none to mild inflammation (0.8 ± 0.5). A slight increase of infiltrating cells was detected after saline or sham treatment. In contrast, IL-1α injection caused a prompt and severe inflammation (2.7 ± 0.5) at day 1, which returned to

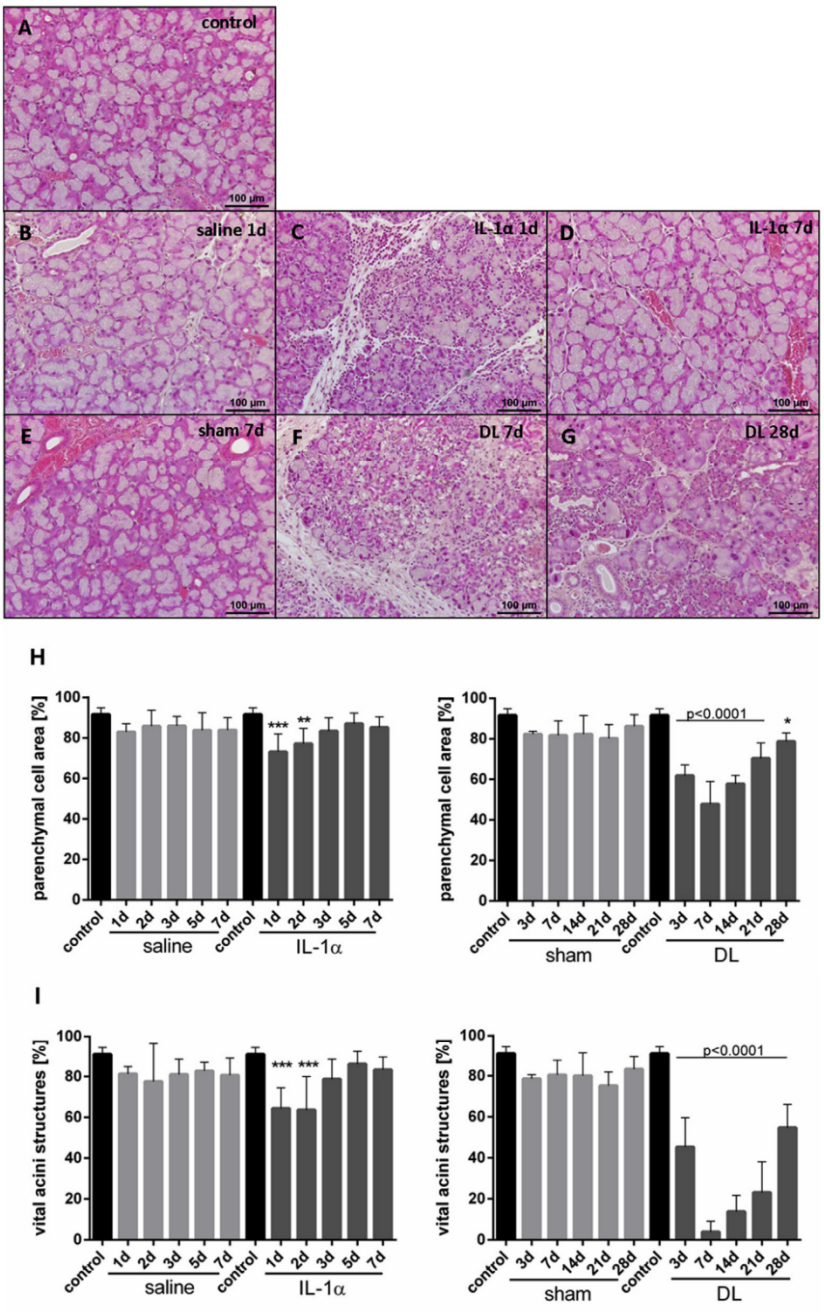


**Fig. 3.** HE staining of treated LG (A–G). Untreated LG (control) has a compact acini structure (A). Saline injection (B) and sham operation (E) led to minor changes in acini structure by interstitial edema and mild inflammation. IL-1α injection immediately resulted in a severe infiltration and interstitial edema by day 1 (C) with reappeared acini structures without any signs of inflammation by day 7 (D). DL caused a severe damage with edema, inflammation, fibrosis and apoptotic acinar cells by day 7 (F). Removal of DL led to a partial re-appearance of acinar cells as well as a decline of edema and fibrosis by day 28 (G). Scale bar 100 µm. H) Measurement of total cell area [%] within LG area revealed a significant decrease up to day 2 after IL-1α injection and up to day 21 after DL, with day 7 after DL representing the strongest decrease of cellular area. I) Measurement of vital cell area [%] within LG area show that IL-1α injection caused a significant decrease of vital cells at day 1 and day 2, which normalize by day 5. DL resulted in a gradual decline of vital cell area during ligation period (day 7) and a gradual increase thereafter, but which is still significantly decreased at day 28. n = 5 for control, n = 4 for NaCl and sham, n = 6 for IL-1α and n = 4–6 for DL; All Data represented as mean values ± SD; * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represent $p \leq 0.001$. Scale bar 100 µm; Magnification 200-fold.

baseline by day 5 (0.8 ± 0.8). However, DL resulted in a severe and prolonged inflammation from day 3 on, which slightly decreased after day 7.

To further identify infiltrating cell types, immunohistologic staining was performed and $CD3^+$ T cells, $CD68^+$ macrophages as well as CD138 + plasma cells were evaluated.

*3.3.2. $CD3^+$ T cells*

Untreated LG displayed a few, singly located $CD3^+$ T cells (7.06 ± 5.05 cells/mm$^2$). Whereby, cells were located between acini and the connective tissue (Figs. 4A and 6B). No significant changes were observed after IL-1α or saline injection, except for day 7. However, DL resulted in a significant increase of $CD3^+$ cells from day 3 (37.79 ± 14.42 cells/mm$^2$, $p < 0.0001$) up to day 14

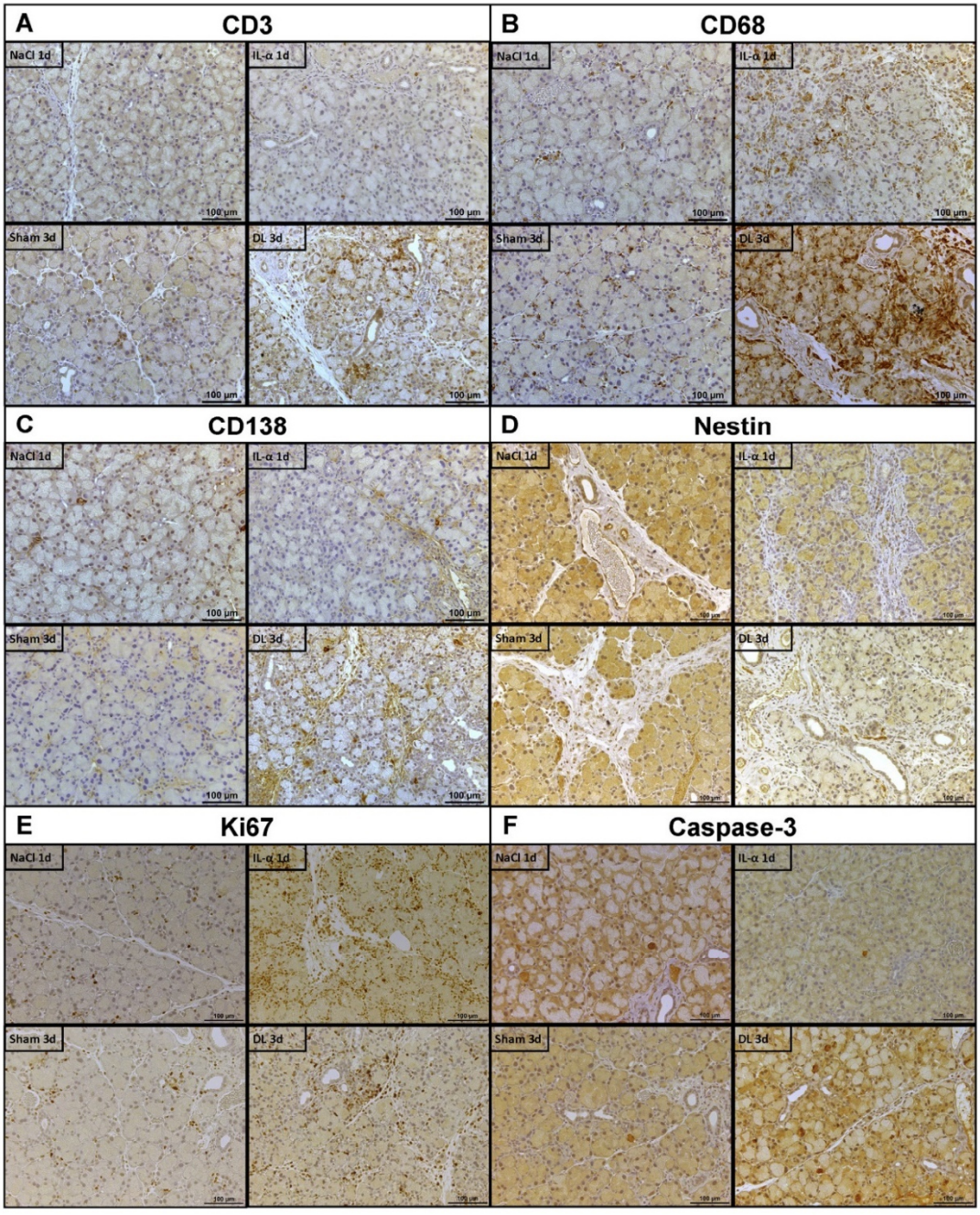


**Fig. 4.** Immunostaining at day 1 after NaCl or IL-1α injection and day 3 after sham operation or DL with antibodies against CD3 (A), CD68 (B), CD138 (C), Nestin (D), Ki67 (E), Casapase-3 (F). Scale bar 100 µm, Magnification 200-fold.

(25.27 ± 8.64 cells/mm$^2$, p = 0.0002). In these cases, CD3$^+$ cells were detected as single cells or located in small clusters with other cell types. Sham operation led to a significant increase of CD3$^+$ T cells at day 3 (33.35 ± 25.92 cells/mm$^2$, p < 0.0001) and day 28 (18.73 ± 9.27 cells/mm$^2$, p = 0.0356).

*3.3.3. CD68$^+$ macrophages*

Compared to untreated control (32.5 ± 12.2 cells/mm$^2$), saline injection as well as sham operation led to an increase of CD68$^+$ macrophages, this exhibited a varying pattern over time (Figs. 4B and 6C). Here, CD68$^+$ macrophages were homogenously

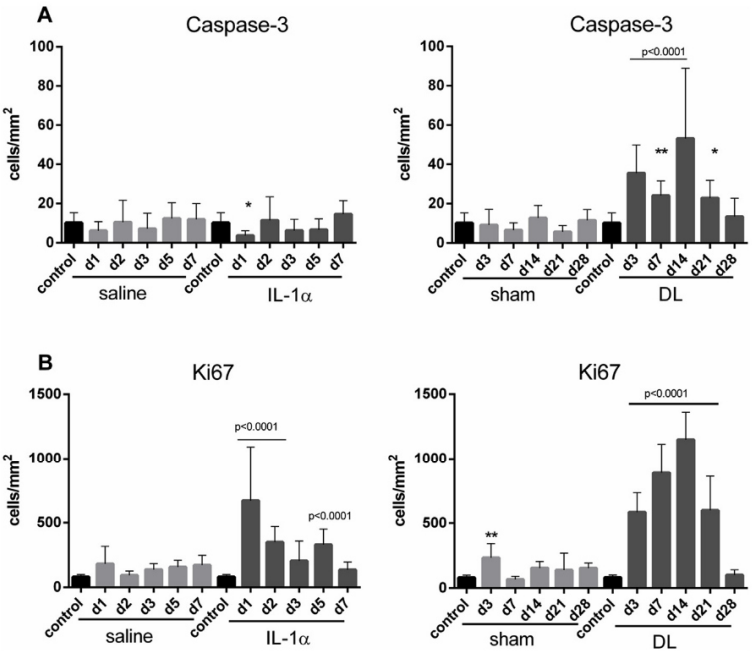


**Fig. 5.** Apoptotic and proliferating cells after IL-1α injection or DL. A) caspase-3 positive apoptotic cells were significantly increased after DL for up to day 14 with a gradual decrease to normal levels at day 28. B) Ki67 positive proliferating cells were significantly increased at day 1, 2 and day 5 after IL-1α and normalized levels at day 7. DL resulted in a significant and gradual increase of proliferating cells up to day 14 with a decline to control levels by day 28. n = 3 for control, n = 2 for NaCl and sham, n = 3 for IL-1α and DL; All Data represented as mean values ± SD; * represents p ≤ 0.05, ** represents p ≤ 0.01 and *** represent p ≤ 0.001.

distributed through the LG. However, injection of IL-1α resulted in a stronger increase, than the control groups, up to day 3 (230.01 ± 100.4 cells/mm$^2$, p < 0.0001) with a decline thereafter. Moreover, DL caused a higher increase than IL-1α injection, which was significantly elevated up to day 28 (177.6 ± 47.14 cells/mm$^2$, p < 0.0001). Nevertheless, a gradual decline from day 7 (366.1 ± 131.4 cells/mm$^2$, p < 0.0001) to day 28 after DL could be detected. In contrast to control groups, CD68$^+$ macrophages were predominately detected in areas of severely damaged acini after IL-1α injection or DL. Interestingly, during regeneration period, CD68$^+$ macrophages were again distributed throughout the LG and not predominately around severely affected acini structures.

### 3.3.4. CD138 + plasma cells

Almost no CD138 + plasma cells could be detected either in untreated (1.95 ± 2.01 cells/mm$^2$), saline, IL-1α or sham treated LGs (Figs. 4C and 6D). Only DL resulted in a significant increase of CD138 + plasma cells at day 3 (7.06 ± 7.3 cells/mm$^2$, p < 0.0001) which declines to control levels by day 28 (1.95 ± 2.01 cells/mm$^2$).

### 3.4. Mesenchymal stem and progenitor cells

Besides an increase of proliferating cells, regeneration of damaged tissue might also be accompanied by an increase of tissue resident stem cells. Since the appearance of mesenchymal stem and progenitor cells (MSC) could be detected after IL-1α induced injury (Zoukhri et al., 2008) to the LG, we aimed to determine whether MSC might be involved in the LG tissue regeneration after IL-1α injection and/or DL. Nestin was used to identify MSC, since this commonly used stem cell marker is highly expressed in isolated MSC from LG (unpublished data). The number of MSC was counted in immunohistologic staining using an anti-nestin antibody (Figs. 4D and 7). Nestin + cells were detected as elongated, spindle-shaped cells in the stroma. When compared to untreated control LG (8.96 ± 6.26 cells/mm$^2$) the saline injection as well as the sham operation led to slight, but not significant, variations in the number of nestin + MSC. But the number of nestin + MSC significantly increased at day 1 (32.14 ± 15.75 cells/mm$^2$, p < 0.0001), day 2 (23.1 ± 14.16 cells/mm$^2$, p < 0.0001) and day 5 (21.93 ± 12.24 cells/mm$^2$, p = 0.0003) after IL-1α injection. DL resulted in an even more significant increase of MSC than IL-1α injection. Here, nestin + MSC gradually increased from day 3 to day 7 (53.07 ± 22.47 cells/mm$^2$, p < 0.0001) and progressively decreased over time to control levels at day 28 (7.94 ± 4.69 cells/mm$^2$).

## 4. Discussion

The LG is responsible for the production of the aqueous part of the tear film. Therefore, any impairment of the functional LG tissue results in a quantitative or a qualitative alteration of the lacrimal fluid and can lead to a disturbance of tear film homoeostasis. This in turn may result in the development of ADDE with serious discomfort, pain and visual disturbance for those affected.

Current treatment options of ADDE remain palliative and ease the symptoms by applying artificial tear eye drops, induce retention of tears on the ocular surface through occlusion of the lacrimal puncta or by treating ocular surface inflammation (see review (Hessen and Akpek, 2014)). In very severe cases of ADDE the transplantation of the salivary gland (SG) can be performed to reduce symptoms, but due to the different composition of saliva and lacrimal fluid this option harbors some disadvantages. In particular the hypo-osmolality of the saliva can induced microcytic corneal edema, which may cause epithelial defects (Borrelli et al., 2010). Furthermore, in cases of Sjögren´s syndrome or other autoimmune diseases causing ADDE, the SG might also be affected and therefore cannot be used as a transplant. Consequently, the restoration of functional LG tissue, producing a physiologically lacrimal fluid is required for a curative treatment of ADDE. Thus, regeneration of functional LG tissue *in situ* would be a desirable approach. Promising tools to curatively treat ADDE might be the treatment with growth factors as drugs, gene therapy as well as cell therapies using stem cells such as MSC or epithelial stem cells (for a detailed review see (Dietrich et al., 2016)). In this study, we examined two mouse models of experimentally induced ADDE, to assess the dynamic of LG damage and

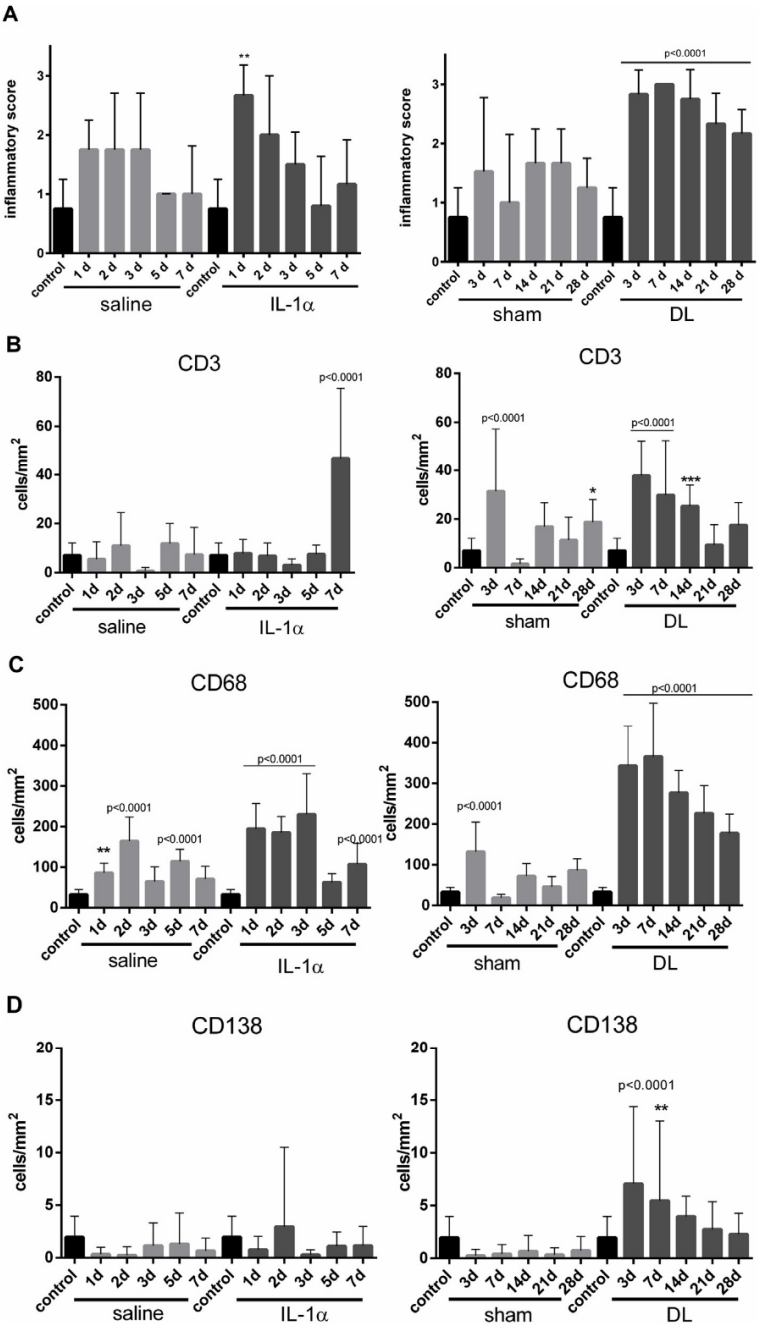


**Fig. 6.** Characterization of the inflammatory reaction in LG after IL-1α injection or DL. A) Inflammatory score graded in HE staining revealed a significant increase at day 1 after IL-1α injection with a decline to control levels by day 5. DL resulted in a severe inflammation up to day 14 with a slight decline by day 21 and day 28. B) No increase of CD3 positive T cells were detected after IL-1α injection. In contrast, DL led to a significant increase of CD3 positive T cells at day 3 with a gradual decline thereafter. C) CD68 positive macrophages were increased after all types of treatment up to day 5. However, IL-1α injection led to more pronounced increase of CD68 positive macrophages than saline injection. DL also caused a significant increase of CD68 with the most cells countable at day 7 and an ongoing decline up to day 28. D) No significant changes in the number of CD138 positive plasma cells could be detected after saline, IL-1α or sham treatment. Only, DL led to a significant increase of CD138 plasma cells by day 3 with a decline thereafter. n = 3 for control, n = 2 for NaCl and sham, n = 3 for IL-1α and DL; All Data represented as mean values ± SD; * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represent $p \leq 0.001$.

regeneration and to evaluate how these models can be used to investigate regenerative therapies to treat ADDE. We suggest that the ideal model should display the following characteristics:

- Exhibit clinical signs of ADDE
- Introduce a reproducible damage to the LG tissue
- Allow a (spontaneous) regeneration of functional LG tissue *in vivo*
- Enable a detailed investigation of damage and regeneration (time scale)
- The mechanisms of damage should mimic the pathologic signs observed in LGs of patients

The first model investigated in the current study was based on the injection of the pro-inflammatory cytokine IL-1α into the extraorbital

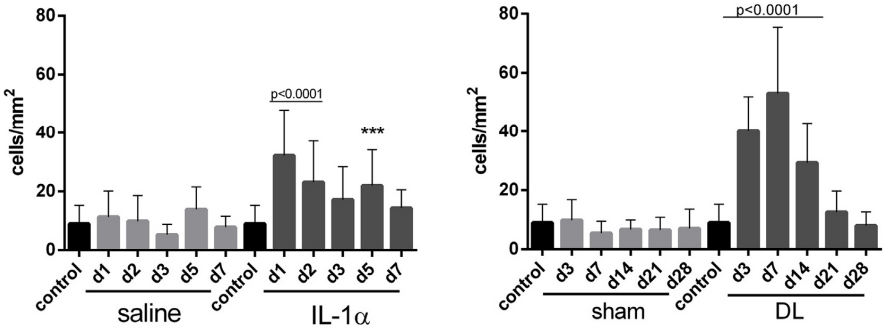


**Fig. 7.** Nestin positive Mesenchymal stem cells (MSC). IL-1α resulted in a significant increase of MSC at day 1, 2 and day 5 after injection. DL caused a significant and gradual increase of MSC from day 3 to day 7. Thereafter the number of MSC normalized to control levels by day 28. n = 3 for control, n = 2 for NaCl and sham, n = 3 for IL-1α and DL; All Data represented as mean values ± SD; * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represent $p \leq 0.001$.

LG. This resulted in an immediate but temporary inflammation of the LG with spontaneous regeneration of the tissue thereafter. These results are comparable to the findings of Zoukhri et al. (2007). It has been shown that extracellular IL-1α, such as after injection, activates tissue resident macrophages, which in turn produce a variety of cytokines that recruit myeloid cell to the site of stress. Upon reaching the IL-1α-containing milieu the IL-1α signaling triggers the myeloid cells to produce IL-1α and IL-1β. Thus, the effects of IL-1α is prolonged and further myeloid cells are recruited (Di Paolo and Shayakhmetov, 2016). In our study, this resulted in a significant increase of the inflammatory score in HE staining at day 1. The acute inflammation led to further proliferation of infiltrating cells and a loss of functional LG tissue, which was detected by a decrease of vital acinar structures at day 1 and day 2. It has been shown before that IL-1α leads to fibrosis through the mitogenic effect on fibroblasts and smooth muscle cells (Raines et al., 1989; Yamamoto et al., 2000). In this study an increase of fibrotic tissue and a consecutive decrease of parenchyma cell area were detected at day 1 and 2. The number of caspase-3 positive cells did not increase at day 1 and 2 after IL-1α injection, which implies that the cells already passed the apoptotic process or underwent necrosis. However, dying or dead cells will be engulfed by specialized phagocytes (macrophages and monocytic phagocytes) leading to the clearance of damaged tissue and recovery of physiological homeostasis (Krysko et al., 2006). Consistent with this, a significant increase of $CD68^+$ (macrophages and monocytic phagocytes) cells was detected up to day 3 after IL-1α injection. After the acute inflammatory response at day 1 and 2, signs of regeneration such as an increase of parenchymal cell amount and vital acinar structures as well as a decreased inflammation were detected at day 3 and 5. By day 5, IL-1 α induced LG damage disappeared as all values normalized to that of untreated control. The short progression time of approximately 2 days of acute destructive damage in our model is somehow shorter than in comparable studies, where the recovery after IL-1α injection took 13 days in female C57BL/6 mice (Zoukhri et al., 2007). Since sex differences are known to play an important role in the characteristics of DED in patients (Sullivan et al., 2017) as well as in mice (Cyr et al., 2016; Toda et al., 1999), the differences found between the models could be gender-specific. In addition, the injection of human instead of murine IL-1α could have led to the observed stronger effects in the present study. Furthermore, in the study of Zoukhri et al. both LG were treated with IL-1α, while in the present experimental setup only the right LG received an injection. Altogether, these differences might contribute to the moderate progression of LG damage in the current study compared to the already published one by Zoukhri et al. (2007). However, histological findings of LG tissue damage are similar, although not that severe as in the study of Zoukhri et al. In contrast to our findings, IL-1α injection caused an increase of TUNEL positive LG epithelial cells in female BALB/c mice and the cleavage of PARP-1 (Zoukhri et al., 2008) which might be due to strain differences. Consistent with mass cytometer (CyTOF) immuno-phenotyping of IL-1α injected LG of BALB/c (Hawley et al., 2017), the main infiltrating immune cells were characterized as granulocytes and $CD68^+$ macrophages and monocytic phagocytes, with the peak inflammatory response at day 1–3. Like in comparable studies (Zoukhri et al., 2008), an increase of nestin positive MSC, was detected after IL-1α injection. This result suggests an involvement of MSC during damage and regeneration of LG.

As a second model this study investigated the ligation of the main secretory duct of the extraorbital LG. It has been shown that the ligation of secretory duct leads to a loss of functional tissue, inflammation and fibrosis in SG (Correia et al., 2008; Okumura et al., 2003; Takahashi et al., 2004; Walker and Gobé, 1987) as well as in LG (Liu et al., 2017). The results of this study revealed that the ligation of the secretory duct induce atrophic damage to the LG tissue, which is accompanied by a sustained decrease of acinar structures and an increase of fibrotic tissue (decrease of parenchyma cell area). The loss of functional LG tissue resulted in a significant reduction of LG weight, which was comparable to other studies (Liu et al., 2017). Furthermore, DL led to an increase of fluorescein stained corneal epithelium at day 7. This indicates an impact on the ocular surface, albeit the increase of staining was modest regarding the marked decrease of LG functional tissue. However, corneal surface damage shown by increased fluorescein staining is an established indicator for severe DED (Baudouin et al., 2014; Bron et al., 2017). It is well known that the loss of functional acinar structures by atrophic damage as induced by duct ligation includes apoptosis (Liu et al., 2017; Takahashi et al., 2004; Walker and Gobé, 1987). In our DL model apoptosis of acinar cells was identified by an increase of caspase-3 positive cells, with the maximum at day 14. This study further aimed to characterize the infiltrating immune cells and could show that $CD3^+$ T cells, CD138+ antibody-producing differentiated B cells (plasma cells), as well as $CD68^+$ monocytic phagocytes and macrophages were infiltrating the LG after DL. A lymphocytic infiltration with mature B cells (Li et al., 2004) and T cells (Niederkorn et al., 2006; Turpie et al., 2009) have also been reported for other DED mouse models. Consistent with that, a peak concentration of $CD3^+$ T cells and CD138 + plasma cells were observed in early stages of the immune reaction. However, $CD68^+$ cells further increased from day 3 to day 7, presumably to retain the physiological homeostasis by engulfing dead cells. Further on, an increase of progenitor cells have been detected in SG (Takahashi et al., 1998) as well as in LG (Liu et al., 2014) after duct ligation. Accordingly, an increase of nestin positive MSC were observed in damaged LG after DL with the peak concentration at day 7.

In contrast to comparable studies on SG (Correia et al., 2008; Okumura et al., 2003; Walker and Gobé, 1987) as well as LG (Liu et al., 2017), the present study further aimed to evaluate the regeneration

capacity of LG after re-opening of the DL. As seen in studies from SG, the re-opening of the ligation induced the regeneration, as newly formed acini could be detected 3 days after the re-opening (Takahashi et al., 1998, 2004). Consistent, to studies on SG (Takahashi et al., 1998), newly formed acini structures arose in our model 7 days after the re-opening and further increased and matured over time. Furthermore, regeneration was accompanied by a decline of fibrosis (increase of the parenchymal cell area) and an increase of vital acinar structures. As a consequence of the ligation, LG weight was significantly decreased, but started to increase after the re-opening. However, regeneration of the LG in our models was only partial 21 day after the re-opening in terms of the weight increase as well as vital acini structures. In contrast, SG completely regenerated as the weight reached control levels by day 7 and the histological appearance was similar to that of control at day 14 after the re-opening (Takahashi et al., 1998, 2004). Thus, 7 days of ligation with a subsequent re-opening led to a complete regeneration of SG within 14 days, whereas regeneration of LG was only partial after 21 days. This discrepancy might be explained by the different species (rat vs. mouse) used in the studies and that the damage of SG might be lesser after 7 days of ligation than in LG due to the natural associated bigger size of the tissue.

In patients with ADDE either with/without Sjögren´s syndrome the pathology is characterized through a progressive lymphocytic infiltration with an ongoing destruction of functional LG tissue. Histologically LG biopsies from patients with ADDE revealed an abnormal lobular pattern accompanied with fibrosis and atrophy of acinar epithelial cell (Williamson et al., 1973). In our study, both models (IL-1α injection and DL) exhibit similar histological pattern of the LG structure as those of ADDE patients. The infiltration of LG from patients was mainly T cell dependent and consists to a fewer extent of B cells and plasma cells (Matsumoto et al., 1996; Saito et al., 1993; Xu and Kazuo, 1997). Correspondingly an increase of T cells and to fewer extent plasma cells was also visible after DL, but not after IL-1α injection. However, the main infiltrating cells visible in our models were granulocytes as described for similar animal models (Correia et al., 2008; Hawley et al., 2017). Consequently, although both investigated mouse models with experimentally induced ADDE exhibit parallels to the pathologic conditions described in patients, they exhibit also certain limitations. First of all, in patients mainly lymphocytes constitute the immune cell infiltration, whereas in the investigated mouse models mainly granulocytes are observed. This disparity of immune reaction has to be considered, when investigating potential curative treatments for ADDE. Secondly, as the immune reaction is mainly responsible for LG damage, the pathological condition of ADDE is divergent. However, both models provide the possibility to study ADDE under defined conditions, without clinical signs of Sjögren´s syndrome and other systemic autoimmune disorders as well as excluding genetic modifications of other mouse models, such as the MRL/lpr, NOD or Id3 mouse.

Concluding the current results and comparing the outcomes with the suggested ideal model, both models exhibit advantages and disadvantages (see Table 1). Both models induced a reproducible damage to the LG followed by a spontaneous regeneration of functional LG tissue. As the injection of IL-1α mimicked an acute inflammation and the pathological conditions of ADDE in patients it seems to be a suitable *in vivo* model. However, the impact on the LG tissue was temporary and no dry eye associated ocular surface damage was detected by fluorescein staining. Especially the time course as well as the fast and complete regeneration of LG seems to make it difficult to study potential effects of a regenerative therapy. However, DL successfully mimicked LG insufficiency and resulted in detectable signs of ADDE on the ocular surface. Furthermore, DL induced a severe and prolonged damage to the LG followed by a partial regeneration during the investigated 21 days of the study. The extended time course and partial character of regeneration seems to be an advantage to determine whether an investigated therapy yields an enhanced regeneration capacity. Accordingly, the DL might be more attractive as an *in vivo* model to investigate the effect of potential regenerative therapies.

**Table 1**
Comparison of IL-1α Injection and DL according to the ideal study model.

| | IL-1α injection | Duct ligation |
|---|---|---|
| Clinical signs of ADDE | No | (Yes)[a] |
| Extent of damage to the LG tissue | Partial + | Partial + + + |
| Extent of spontaneous regeneration of functional LG tissue *in vivo* | Complete | Partial + + |
| Time scale of | | |
| damage | Short (2 day) | Long (7 days) |
| regeneration | Short (3 days) | Long (> 21 days) |
| Mimic the pathologic conditions observed in LGs of patients | | |
| Fibrosis | Yes | Yes |
| Atrophy | Yes | Yes |
| T cell infiltration | No | Yes |
| B cell infiltration | No | Yes |

[a] Only minimal manifestation of fluorescein staining on the ocular surface.

The present study on IL-1α as well as DL induced LG damage and regeneration revealed an involvement of MSC, as the number of these cells significantly increases during the period of damage. Considering that there are currently over 700 clinical trials investigating the potential of MSC to treat various diseases and pathological conditions (U.S. National Institutes of (Health)), their application seems also a promising tool to curatively treat ADDE by *in situ* induction of LG regeneration. MSC are multipotent stem cells found in a variety of adult tissues including pancreas (Karaoz et al., 2010; Zhang et al., 2005), SG (Rotter et al., 2008) and have also been found in LG (Roth et al., 2015; Shatos et al., 2012; You et al., 2011). In addition, several studies showed a correlation between the presence of MSC and inflammation/tissue repair (reviewed in (Ren et al., 2008; Shi et al., 2012)) including investigations on LG (Zoukhri et al., 2008). This study confirms that nestin + MSC were present in the LG and further imply that MSC might be involved in LG regeneration as the number significantly increased after experimentally induced ADDE by IL-1α injection and DL. Furthermore, the monitoring on the presence of nestin + MSC revealed a correlation between severity of LG damage and MSC quantity. MSC exhibit a variety of anti-inflammatory properties, which affect the innate as well as the adaptive immune system (reviewed in (Bifari et al., 2008; Shi et al., 2010)). In addition, MSC secrete a variety of soluble growth factors at the site of injury to influence cell growth, cell proliferation as well as apoptosis in order to enhance tissue repair (reviewed in (Shi et al., 2012)). Thus, an increase of MSC during acute inflammation of LG tissue after IL-1α injection and DL might indicate an involvement of MCS in LG regeneration. Recent studies already showed that the treatment of ADDE and/or Sjögren´s syndrome with stem cells, such as MSC or epithelial progenitor cells (EPCs), is useful to ameliorate the symptoms as well as the tissue lesions in LG or SG in (genetic modified) mouse models (Aluri et al., 2017; Gromova et al., 2017; Lee et al., 2015; Xu et al., 2012). Our results revealed that most nestin + MSC were present at the time of maximal damage; day 1 and 2 after IL-1α injection and day 3–14 after DL. Thus, the most promising time points for transplantation of MSC into damaged tissue seem to be at the beginning of destructive inflammatory period. Thus, a MSC cell therapy could be applied during acute phase of ADDE. In this case, the immunomodulatory and regenerative properties of MSC might prevent further LG destruction and induce/enhance LG regeneration.

## 5. Conclusion

In this study, we were able to show that both mechanisms of damage induced an inflammatory response and a loss of functional LG tissue. This was followed by a spontaneous tissue regeneration, which was accompanied by an increased appearance of MSC in the LG tissue. The damage observed in the DL model was more severe compared to

the IL-1α injection and resulted in only partial regeneration. Our study indicates that DL seems to be more suitable as a model to investigate regenerative therapies due to the prolonged time and partial nature of tissue regeneration which might enable studies on regenerative therapeutics as well as identification of involved mechanisms and cell types. Further studies have to elucidate how MSC are involved in the regenerative process and whether MSC induced effects can be used to enhance LG tissue regeneration.

## Conflict of interest statement

The authors do not have any conflict of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2018.03.026.

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## 3.2 Original Research Article

### Analysis of lacrimal gland derived mesenchymal stem cell secretome and its impact on epithelial cell survival

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Contribution to experimental design, implementation and publication

Involved in the conception and design of the experimental setup, conduction of the cell isolation by explant culture, preparation of cell suspension for FACS isolation, implementation and assembly of data concerning MSC morphology, growth behavior, in vitro differentiation, flowcytometric analysis, cell viability and gene expression analysis, preparation of secretome for LC-MS/MS assisted analysis by SK, data analysis and interpretation (in cooperation with SK for secretome analysis), manuscript writing, preparation of all figures (except figure 5). The manuscript was subsequently reviewed and approved by all co-authors.
Approximated total share of contribution: 56%

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# Analysis of lacrimal gland derived mesenchymal stem cell secretome and its impact on epithelial cell survival

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ABSTRACT

*In situ* regeneration of lacrimal gland (LG) tissue would be a promising approach to curatively treat dry eye disease (DED). Mesenchymal stem cells (MSC) exhibit therapeutic effects in a variety of pathological conditions and our group recently reported that their number increases in regenerating mouse LG. Since the therapeutic effects are suggested to arise from secreted trophic factors, the application of MSC-secreted proteins seems to be a promising approach to induce/enhance LG regeneration. Therefore, this study aims to optimize the isolation of murine LG-MSC and analyze their secretome to investigate its potential for LG epithelial cell survival *in vitro*. For optimization, LG-MSC were isolated by an explant technique or cell sorting and their secretome was investigated under normal and inflammatory conditions. Results showed that the secretome of MSC had beneficial effects on the viability of ethanol-damaged LG epithelial cells. Additional, Lipocalin-2, prosaposin, ras GTPase-activating protein-binding protein 1 (Rac1) and signal transducer and activator of transcription 1 (STAT1), proteins that were up-regulated under inflammatory conditions, further improved the cell survival of ethanol-damaged LG epithelial cells. Interestingly, recovery of cell viability was highest, when the cells were incubated with STAT1. Summarizing, this study identified promising proteins for further studies on LG regeneration.

## 1. Introduction

The lacrimal gland (LG) parenchyma is composed of acinar, ductal and myoepithelial cells, with the acinus as its smallest functional unit (Bron et al., 2017). The lacrimal fluid represents the aqueous part of the tear film, which is encompassed by a mucin and lipid layer. The tear film in its entirety is responsible for the removal of dust and debris, protection against pathogens, and lubrication of the corneal- and conjunctival epithelium (Willcox et al., 2017). Imbalances in the homeostasis of the tear film lead to the development of dry eye disease (DED). Severe cases of DED develop mainly due to a loss of functional LG tissue, which results in an aqueous-deficient dry eye (ADDE) (Craig et al., 2017b). Such changes of the tear film promote inflammation and can lead to corneal ulcers as well as conjunctival and corneal scarring, which might cause a loss of vision during disease progression (Bron et al., 2017). However, despite of the high prevalence of DED with 5–50% depending on the ethnic group, age and sex (Sullivan et al., 2017) still no curative treatments exist. Therefore, it is of interest to evaluate potential curative therapies. *In situ* regeneration of remaining functional LG tissue would be a promising approach. Indeed, current studies already examine the potential of drugs, cell as well as gene therapies to induce LG regeneration, but some limitations still hamper their application (Dietrich et al., 2016).

One promising approach to induce LG regeneration might be the use of mesenchymal stem cells (MSC). These adult stem cell populations have a multipotent differentiation potential and can be found in a variety of tissues such as bone marrow, umbilical cord, adipose tissue as well as glandular tissues like pancreas (Karaoz et al., 2010), salivary gland (SG) (Rotter et al., 2008) and LG (Roth et al., 2015). Due to their properties to modulate the immune system, cell growth, cell proliferation and apoptosis (Shi et al., 2010; Shi et al., 2012) MSC were extensively studied for their application in the field of regenerative medicine, resulting in over 400 clinical trials, which currently investigate the potential of MSC as cell therapeutics to treat various diseases and pathological conditions (U.S. National Institutes of [Health], n.d). Furthermore, a correlation between the number of MSC and inflammation/tissue repair has been suggested (Shi et al., 2012). An increase of MSC was also found after experimentally induced ADDE in murine LG (Zoukhri et al., 2008; Dietrich et al., 2018). In addition, experimental studies using MSC to treat ADDE or Sjögren's syndrome

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could already show an improvement of clinical parameters like tear secretion in mice (Aluri et al., 2017; Lee et al., 2015) and in patients (Weng et al., 2012). Thus, the application of MSC might be beneficial in the treatment of ADDE. Beside the direct application of MSC, the use of secreted proteins by MSC might also be a promising approach, as MSC secrete trophic factors that are involved in tissue repair (Shi et al., 2012; Caplan and Correa, 2011). However, since tissue-specific differences between the sub-populations have been described (Dmitrieva et al., 2012; Al-Nbaheen et al., 2013; Wagner et al., 2005), the use of LG-specific MSC may be superior to treatment with ectopic MSC for patients with ADDE.

Despite the clinical demand to investigate and utilize the regenerative capacity of MSC, the current isolation procedures are mainly unspecific, as specific markers remain still elusive. Moreover, there are no tissue-specific MSC markers, *e.g.* for LG-derived MSC. Established isolations protocols include discrimination between plastic-adherent and suspension growth as well as explant culture. Therefore, MSC need to be extensively characterized by defined criteria prior to application (Dominici et al., 2006). Various studies already reported that an ineffective purification during isolation will result in a heterogeneous cell population, which needs extensive passaging to remove unwanted cell types (Dietrich et al., 2016). Consequently, it is of particular interest to further develop an effective and prospective isolation method such as through fluorescent-activated cell sorting (FACS).

In this study, we characterize murine LG-MSC isolated by explant culture (Exp MSC) or FACS. Sorting was performed on the basis of *Houlihan* et al., *2012* using stem cell antigen 1 (Sca-1) and platelet-derived growth factor receptor alpha (PDGFRα) as positive marker and the hematopoietic cell antigens CD45 and Ter119 as negative markers (PαS MSC). Both MSC populations (Exp *vs.* PαS) were phenotypically and functionally characterized by previously defined criteria (Dominici et al., 2006). In addition, the secretome of both MSC populations were analyzed under normal (αMEM) and inflammatory conditions (α-MEM ± 50 ng/ml interleukin-1α and interferon-γ) to identify differentially-regulated proteins between both conditions and isolated cell populations. Additionally, the influence of the entire MSC secretome as well as selected secreted proteins were analyzed on their capacity to enhance epithelial cell survival after acute damage *in vitro*.

## 2. Material and methods

### 2.1. Mice

Male C57BL/6 J mice were obtained from Janvier labs and kept under 12:12 h light:dark cycle with food (ssniff, Soest, Germany) and water *ad libitum*. MSC were isolated from lacrimal glands of 8–10 weeks old mice. All experiments were conducted in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research and the national ethical committee for animal experimentation (FELASA).

### 2.2. Cell isolation from LG

Extraorbital LGs were excised after euthanasia of 10 mice for each isolation protocol and placed in cold culture medium (α-MEM, 2 mM L-glutamine, 15% FBS-Superior (all purchased from Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO)). Specimens were washed with cold PBS (Sigma-Aldrich) before mincing with a scalpel.

### 2.3. Explant culture

Minced LGs were transferred to 10 cm culture dish and allowed to attach to the surface before culture medium was added.

### 2.4. Fluorescence-activated cell sorting (FACS)

For isolation by FACS viable (propidium iodide-negative), single cells were gated for expression of PDGFRα and Sca-1, while CD45 and Ter119 expression was absent resulting in PαS MSC population.

Minced LGs were incubated with an enzyme cocktail (culture medium containing 750 u/ml collagenase type I (Worthington, Lakewood, NJ), 300 u/ml Hyaluronidase type IV-S (Sigma-Aldrich) and 40u/ml DNase I (Worthington)) for 1 h at 37 °C. Digested cells were filtered (70 µm cell strainer (BD, Heidelberg, Germany). Homogenate was centrifuged for 5 min at 500 ×g, washed twice with PBS and incubated with 0.5% Trypsin-EDTA (Sigma-Aldrich) for 3 min at 37 °C. In PBS suspended cells were layered on a 4% ficoll gradient (Sigma-Aldrich) and centrifuged at 100 ×g for 5 min. Pelleted cells were washed and pre-incubated with anti-CD16/CD32 (BD Bioscience, San Jose, CA) in HBSS + (HBSS, 2% FCS-S, 10 mM HEPES, 1% P/S) for 5 min on ice to block non-specific binding. For the PαS setup cells were incubated with PDGFRα-APC, Sca-1-FITC, CD45-PE and Ter119-PE (eBioscience, San Diego, CA)) for 30 min on ice. Cells were washed with HBSS + and propidium iodide (50 µg/ml; Sigma-Aldrich) was added to discriminate between live and dead cells. FACS was performed on a Mo Flow XDP (Beckman Coulter, Brea, CA) with a 150 µm nozzle at 25 PSI. After sorting, the cells were cultured in culture medium.

### 2.5. Cumulative population doublings

Population doublings were calculated using eq. 1, where PD is the population doubling, $N_0$ is the number of seeded cells and N is the number of harvested cells. The PD for each passage was calculated and then summed up.

Equation 1 Calculation of population doublings

$$PD = \frac{\log(N) - \log(N_0)}{\log(2)} \quad (1)$$

### 2.6. Colony forming unit (CFU) assay

Cells in passage (p) 2, p3 and p6 were plated in 6 well plate with 500 cells/well. After 2 weeks colonies were fixed with ice-cold methanol and stained with 1% rhodamine red solution (A-5533, Sigma-Aldrich) for 30 min at 37 °C. Cell aggregates (> 50 cells) were manually counted using an inverse microscope (DM IL LED, Leica, Germany). The experiment was performed in triplicates for each MSC population and time.

### 2.7. Flow cytometry

Cells (p2 and p6) were washed in HBSS + and pre-incubated for 5 min on ice with anti-CD16/CD32 (BD Bioscience). Primary antibody labeling was performed for 30 min on ice (Table S1). For intracellular staining, cells were fixed in 2% paraformaldehyde (PFA) for 30 min at room temperature (rt), washed with HBSS + and permeabilized using 0.1% Triton-X-100 for 5 min before the staining proceeded as described above. Staining with the respectively labelled isotypes served as a control. Fluorescence labeling of cells was detected on a CyAn ADP Analyzer (Beckman Coulter), recording 10.000 viable, single cells. Analysis was performed using Kaluza Software (Beckman Coulter).

### 2.8. Adipogenic and Osteogenic differentiation

Differentiation potential was assessed in p3 as described earlier (Roth et al., 2015).

After induction of adipogenesis and osteogenesis, total RNA was extracted from the pelleted cells by a combination of QIAzol Lysis

Reagent (Qiagen, Hilden, Germany) and GenElute Mammalian Total RNA Midiprep Kit (Sigma-Aldrich). Cells were homogenized with a 5 mm stainless steel beads on a TissueLyser LT (Qiagen) twice for 60s at 50 Hz. Chloroform was added to the homogenate and centrifuged for 15 min at 12000 ×*g*. The aqueous upper phase was mixed with 1 volume 70% EtOH and filtrated by the GenElute Filtration Colum. Further processing was performed according to the manufacturer's instructions. Total RNA was transcribed into cDNA (High-capacity cDNA Reverse Transcription, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

### 2.9. Preparation of conditioned medium

Conditioned medium (CM) was prepared in p2 from MSC under normal and inflammatory conditions (Fig. S1). Activation of MSC is necessary to induce secretion of trophic factors. To activate MSC an inflammatory milieu was mimicked by the addition of 50 ng/ml IL-1α and IFNγ (Peprotech, UK) to the culture medium. Since IFNγ is required for MSC activation and acts synergistically with other cytokines such as IL-1α, this combination was used to mimic the inflammatory milieu (Ren et al., 2008). IL-1α was chosen as second cytokine, because a single injection of IL-1α into the LG is an established mouse model to study DED (Dietrich et al., 2018; Zoukhri et al., 2007). To prepare the CM, $2.5 \times 10^5$ Exp or PαS MSC were seeded on a 10 $cm^2$ cell culture dish and allowed to attach overnight. Cells were washed with PBS and incubated with normal culture medium or with inflammatory culture medium (culture medium + IL-1α + IFNγ). After 24 h cells were washed first with PBS and then with serum-free αMEM. Subsequently, the cells were incubated with serum-free αMEM (to produce normal CM) or serum-free αMEM + IL-1α + IFNγ (to produce inflammatory CM). Inflammatory (i) $CM^{Ex}$/$CM^{PαS}$ as well as normal (n) $CM^{Exp}$/$CM^{PαS}$ was harvested after 24 h, centrifuged for 5 min at 700 ×*g* to remove cellular debris and the supernatant was used for further experiments. The remaining cells were harvested, counted and used for total RNA extraction using the GenElute Mammalian Total RNA Midiprep Kit (Sigma-Aldrich) and cDNA synthesis (High-capacity cDNA Reverse Transcription, Applied Biosystems).

### 2.10. Quantitative real-time PCR (qPCR)

A 96 well qPCR system (7500 Fast, Applied Biosystems) was used for amplification (50 °C for 20s, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60s, Melting curve: 95 °C for 15 s, 60 °C for 60s, 95 °C for 30s and 60 °C for 15 s). Amplification was carried out in duplicates of three biological replicates using Power SYBR Green PCR Master Mix (Applied Biosystems). No-template-samples served as controls. Ribosomal protein S6 (RPS6) and TATA-box binding protein (TBP) were used as endogenous controls to normalize the expression. Data analysis was done according to Pfaffl (Pfaffl, 2001) and expression levels were calculated as normalized relative fold change from control (RNQ, Eq. 2).

Equation 2 Calculation of normalized expression level (RNQ) according to Pfaffl (Pfaffl, 2001)

$$RNQ = \frac{E_{GOI}^{(CT_{control} - CT_{treatment})}}{E_{REF}^{(CT_{control} - CT_{treatment})}} \quad (2)$$

When using two reference genes, $E_{REF}^{(CTcontrol\text{-}CTreatment)}$ is replaced with the geometric mean. Primer sequences are listed in Table S2.

### 2.11. Secretome analysis

Secreted proteins (secretome) were identified in normal CM ($nCM^{Exp}$/$nCM^{PαS}$) as well as inflammatory CM ($iCM^{Exp}$/$iCM^{PαS}$). For total protein precipitation CM was incubated with four volumes 80% acetone at −20 °C. The solution was centrifuged for 20 min at 12000 ×*g* at 4 °C and washed twice with 80% acetone. The air-dried protein pellet was further prepared for mass spectrometry-based expression analysis with Synapt G2 Si coupled to M-Class nanoUPLC (Waters Corp.) as previously described (König et al., 2018). Briefly, proteins were reduced, alkylated, digested using trypsin and injected into the instrument at 250 ng/µl (0.5 and 1 µl injections). Proteins were identified using the UniProt database of *Mus musculus*. Statistical analysis was performed with Progenesis QI (Nonlinear Diagnostics). Selected groups were compared using the ANOVA *p*-value 0.05 as a filter and calling for two identified peptides. Proteins were classified using The Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (Thomas, 2017).

### 2.12. Viability assay

The influence of the different CM and selected proteins was assessed on the survival of porcine LG epithelial cells (EC) after injury. The LG-EC were isolated from explants and cultured as previously described (Spaniol et al., 2015). Viability of LG-EC was assess after ethanol treatment as described before (Roth et al., 2015). After ethanol incubation for 1 min, EC were washed and incubated immediately (0 h) with either CM from all four culture conditions (Exp ± cytokines, PαS ± cytokines), 10, 50, 100 and 250 nM of murine rec. Lipocalin-2 (Lcn2; R&D Systems), 1, 5, 10, 50 nM of human rec. Prosaposin (Abnova, Taipei City, Taiwan), human rec. Ras GTPase-activating protein-binding protein 1 (Rac1; SignalChem, Richmond, Canada) or human rec. Signal transducer and activator of transcription 1 (STAT1β; SignalChem). Proteins were added to the medium at the time of injury (0 h). Cell viability was measured at 0 h, 6 h, 24 h and 48 h after injury using a WST-1 assay according to manufacturer's instruction (Sigma-Aldrich).

### 2.13. Statistics

Statistical data analysis was performed using GraphPad Prism 6 software (La Jolla, CA). If not otherwise stated, values were given in means ± standard deviation (SD). Statistical analysis was performed using ANOVA with Tukey post-hoc test or student's *t*-test. Differences with $p \leq .05$ were considered as significant.

## 3. Results

### 3.1. Cell morphology and growth characteristic of LG MSC

Both MSC populations exhibited a spindle-shaped and fibroblastic morphology (Fig. 1A-B). Cumulative population doubling (cpd) was recorded from p1 to p6 to measure the proliferative capacity (Fig. 1C). The coefficient of determination ($r^2$) indicated a closely linear growth behavior with $r^2 = 0.98$ for Exp and $r^2 = 0.88$ for PαS. The overall doubling time was 1.4 ± 0.4d for Exp and 1.8 ± 0.5d for PαS. The efficiency to form colonies (CFU) revealed that both MSC populations contained single cells capable of forming new colonies. CFU efficiency was stable with 32.2 ± 5.0 and 25.8 ± 8.8 colonies in p2 and p6 for Exp and 31.6 ± 7.8 and 32.0 ± 13.2 for PαS (Fig. 1D).

### 3.2. Phenotypic analysis of LG MSC by flow cytometry

The analysis of immunophenotype revealed that Exp and PαS MSC were > 95% positive for nestin, Sca-1, PDGFRα, CD29, CD90, > 70% for CD105 and < 5% for CD34, c-Kit, CD45, Ter119 in p2 (Fig. 2) and p6 (Fig. S2). Significant differences between Exp and PαS were detected for the expression of CD44 and CD73. A significantly higher amount of Exp MSC expressed CD44 (99.3 ± 0.1%) compared to PαS (86.5 ± 5.3%). Whereas in PαS population the number of CD73 positive cells was significantly increased (29.4 ± 6.8%) compared to Exp

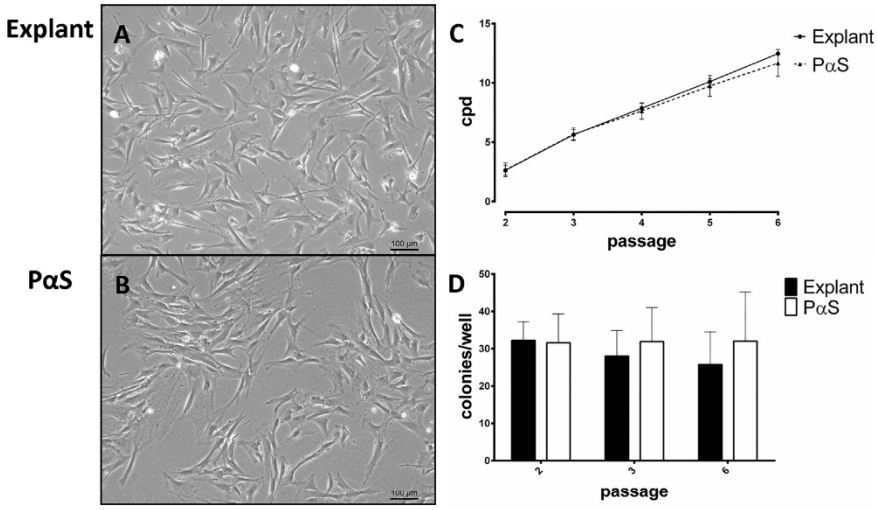


**Fig. 1.** Morphology and growth kinetics of LG-MSC.
Cells exhibit a spindle-shaped, fibroblastic morphology when isolated by explant technique (A) or FACS (B). Assessment of the cpd revealed a closely linear growth behavior of Exp and PαS MSC (C). CFU efficiency revealed no differences between Exp and PαS MSC and a stable ability to form colonies. Data are $n = 3$, mean ± SD; scale bar: 100 µm.

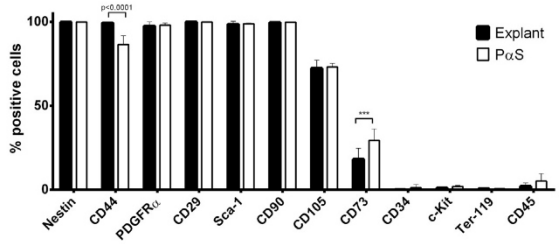


**Fig. 2.** Immunophenotyping of LG-MSC.
Flow cytometric analysis of MSC markers in p2 revealed that Exp and PαS MSC expressed typical MSC marker, while the expression of hematopoietic (stem) cells markers were absent. In the Exp population, a significantly higher number of cells expressed CD44 in p2 MSC, whereas a significantly higher number of PαS cells expressed CD73.Data are n = 3, mean ± SD; *** $p \leq .001$.

(18.2 ± 6.5%). However, statistical differences of CD44 and CD73 positive cells disappeared when analyzing cells in p6, as CD44 expression in PαS raised (98.5 ± 1.3%), while the expression of CD73 decreased in Exp (6.5 ± 5.7%) as well as in PαS (4.7 ± 2.7%).

### 3.3. Differentiation capacity of LG MSC

Induction of adipogenesis as well as osteogenesis revealed that both MSC populations were able to differentiate. The progression of adipogenesis was assessed by FABP4 expression (Fig. 3A), which is an early marker of adipogenesis. From day 7 onwards the expression of FABP4 significantly increased in Exp (2515 ± 294-fold, $p = .0005$) and PαS (2021 ± 1274-fold, $p = .0035$) MSC. In addition, formation of lipid droplets within the cytosol could be detected after Oil Red O staining at day 21 (Fig. 3B, C). Osteogenic differentiation was measured by osteopontin expression (Fig. 3D), which significantly increased at day 14 for PαS (14.6 ± 5.6-fold, $p = .0314$) and at day 21 (16.1 ± 8.9-fold, $p = .0213$) for Exp MSC. Furthermore, the mineralization of calcium-phosphate deposits was visualized by alizarin red s staining at day 21 (Fig. 3E, F).

### 3.4. Effects of CM on LG epithelial cells

To determine possible protective effects of normal CM ($nCM^{Exp}$/$nCM^{PαS}$) and inflammatory CM ($iCM^{Exp}$/$iCM^{PαS}$) on EC, cell proliferation and/or inhibition of cell death was investigated. Therefore, EC were incubated with $nCM^{Exp}$/$nCM^{PαS}$ and $iCM^{Exp}$/$iCM^{PαS}$ after ethanol injury and the viability was measured. 1 min of ethanol treatment significantly decreased the cell viability (68.8 ± 8.2%, $p < .0001$) when compared to untreated cells (100.0 ± 7.9%) (Fig. 4A). Regardless of the CM type, EC viability significantly increased after injury, when compared to αMEM control (Fig. 4B). After 24 h, a significant increase of cell viability was detected when cultured with $iCM^{Exp}$ to 72.5 ± 27.4% ($p = .0257$), with $nCM^{Exp}$ to 91.8 ± 49.2% ($p < .0001$), with $iCM^{PαS}$ to 76.0 ± 28.3% ($p = .0106$) and with $nCM^{PαS}$ to 77.3 ± 21.7% ($p = .0073$) compared to αMEM control (41.8 ± 9.9%). However, after 48 h of incubation only $nCM^{Exp}$ ($p < .0001$) and $nCM^{PαS}$ ($p = .0294$) had a beneficial effect on the viability due to a significant increase of cell viability in the αMEM control group.

### 3.5. Expression analysis of MSC Secretome

As CM showed beneficial effects on EC cell survival upon injury, the proteins within the CM (secretome) were investigated using shot-gun mass spectrometric analysis. Significantly changed proteins ($p \leq .05$, Table S3) were visualized by principal component analysis (Fig. 5A). 401 (Exp) and 321 (PαS) contributing proteins were significantly altered ($p \leq .05$, at least two peptides found) between normal (nCM) and inflammatory (iCM). Likewise, 172 proteins were significantly upregulated in $iCM^{Exp}$ compared to $nCM^{Exp}$ and 197 proteins in $iCM^{PαS}$ compared to $nCM^{PαS}$ (Table S4, S5). Since proinflammatory cytokines stimulate MSC to secrete functional proteins (Shi et al., 2012), only proteins that were significantly upregulated (at least 2-fold, $p \leq .05$) in iCM were considered for the subsequent analysis.

Using PANTHER classification system, the significantly changed proteins were assigned to biological processes (Table S3, Fig. S3A). Immunomodulatory effects of MSC depend on paracrine effects. Therefore, we further focused on proteins with binding activity. About

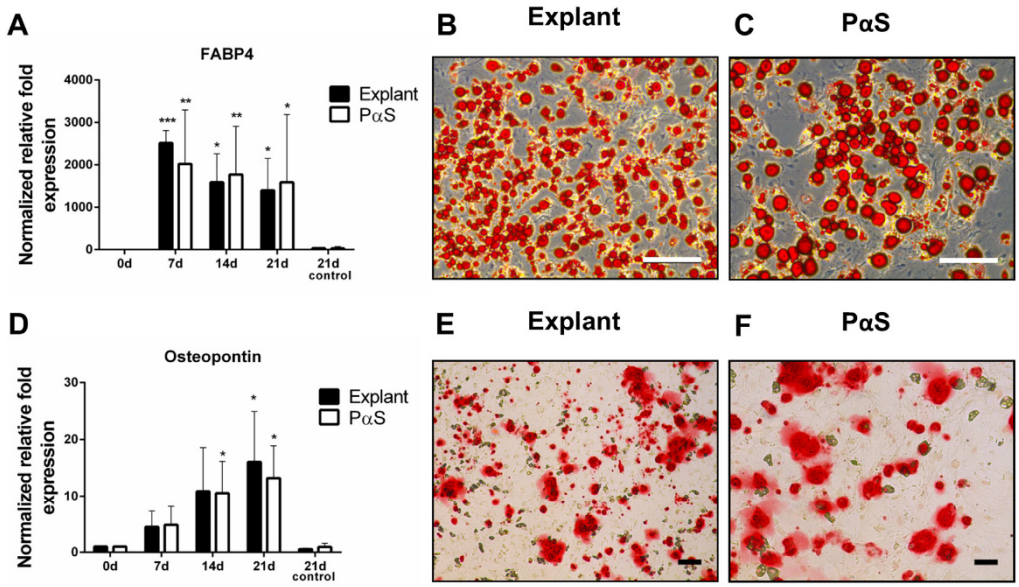


**Fig. 3.** *In vitro* Differentiation of LG-MSC.
Adipogenesis was determined by expression of FABP4 (A), which significantly increased from day 7 onwards in Exp and PαS MSC. Lipid droplets were visualized by Oil Red O staining at day 21 (B, C). Osteogenic differentiation was assessed by expression of osteopontin (D). A significantly increased expression of osteopontin was not detected until d14 in PαS and d21 in Exp. At d21 osteogenesis was visualized by Alizarin Red S staining (E, F). Data are n = 3, mean ± SD; * represents $p \leq .05$, ** represents $p \leq .01$ and *** represent $p \leq .001$; scale bar 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

one third of the proteins upregulated in $iCM^{Exp}$ and $iCM^{P\alpha S}$ showed binding activities (Fig. S3B). For further investigations on regenerative effects, proteins involved in proliferation and/or anti-apoptotic pathways were analyzed. Lipocalin-2 (Lcn2), prosaposin, signal transducer and activator of transcription 1 (STAT1) and Ras GTPase-activating protein-binding protein 1 (Rac1) were selected (Fig. 5B-G). Secretion of Lcn2 was upregulated 18-fold in $iCM^{Exp}$ and 49-fold in $iCM^{P\alpha S}$ compared to $nCM^{Exp}$ and $nCM^{P\alpha S}$, respectively. The amount of STAT1 increased about 4-fold in $iCM^{Exp}$ and $iCM^{P\alpha S}$; that of prosaposin was about 2-fold augmented in $iCM^{Exp}$ and $iCM^{P\alpha S}$. The amount of Rac1 increased about 1.3-fold in $iCM^{Exp}$ and 2-fold in $iCM^{P\alpha S}$. Only the secretion of Rac1 (Fig. 5D) was significantly higher in $iCM^{P\alpha S}$ than in $iCM^{Exp}$ ($p = .008$). The widely described immunomodulatory factors IL-6 (7-fold increase in $iCM^{Exp}$, 13-fold increase in $iCM^{P\alpha S}$) and transforming growth factor beta (TGF-β1; 3-fold increase in $iCM^{Exp}$ and $iCM^{P\alpha S}$) were also investigated.

### 3.6. Gene expression analysis of candidate proteins

To test, whether the increased secretion of Lcn2, prosaposin, Rac1, STAT1, IL-6 and TGF-β might be caused by an increase in gene expression, relative expression was analyzed using RNA from MSC after preparation of CM. Gene expression of all six candidate proteins were increased, when MSC were cultured in inflammatory milieu compared to normal milieu (Fig. 6A-F).

Expression of Lcn2 was increased in both MSC populations under inflammatory conditions (PαS 10,108 ± 6424-fold, $p = .0199$; Exp 3651 ± 1967-fold, $p = .0099$). In contrast, a significantly increased expression of prosaposin was only detected in PαS MSC (1.2 ± 0.1-fold, $p = .0437$). However, Rac1 expression increased 8.6 ± 2.0-fold in PαS ($p = .0003$) and 6.1 ± 3.2-fold in Exp ($p = .0185$). While in PαS MSC the STAT1 expression significantly increased (2.3 ± 0.4-fold, $p = .0005$), only a trend was found in Exp MSC (5.8 ± 3.5-fold, $p = .0696$). The expression of IL-6 was significantly enhanced 300 ± 204-fold in PαS ($p = .0259$) and 280 ± 122-fold in Exp MSC ($p = .0038$). In addition, a significantly elevated TGF-β1 expression was detected for PαS (1.7 ± 0.3-fold, $p = .0041$) and Exp (1.7 ± 0.4-fold, $p = .0153$).

### 3.7. Influence of candidate proteins on LG epithelial cell survival

Selected candidate proteins (Lcn2, prosaposin, Rac1 and STAT1) were further investigated regarding their influence on EC cell survival. Therefore, LG-EC were injured with ethanol and incubated with ascending concentrations of the candidate proteins (Fig. 7). Treatment with ethanol initially resulted in a decreased viability to 63.7 ± 15.3%, which is significantly reduced compared to untreated control ($p = .0001$).

Incubation with Lcn2 (≥ 50 nM) significantly improved the viability of LG-EC after 24 h compared to incubation with αMEM alone after 24 h (33.9 ± 4.5%, $p > .05$, Fig. 7A). A further incubation, up to 48 h, led to an additive improvement in viability to about 65%, which was comparable to the viability directly after ethanol damage (0 h) and were significantly higher than the viability of LG-EC incubated with αMEM alone after 48 h (43.0 ± 6.6%, $p < .0001$). Interestingly, no differences in the effect of Lcn2 could be detected between the investigated concentrations. Prosaposin treatment (Fig. 7B) recovered the cell viability after 48 h to that obtained after damage at 0 h (63.5 ± 7.4% (5 nM) and 66.1 ± 3.8% (10 nM), $p < .0001$). However, compared to αMEM control after 48 h this was a significantly improvement of cell viability. In contrast, treatment with 50 nM Rac1 (Fig. 7C) led to a significantly increased viability (60.5 ± 12.8%, $p = .007$) compared to αMEM control (45.0 ± 12.5%) already at 6 h after injury. Further incubation with Rac1 additionally improved the cell viability compared to αMEM control as detected by a positive correlation between the concentration and the cell viability. Overall,

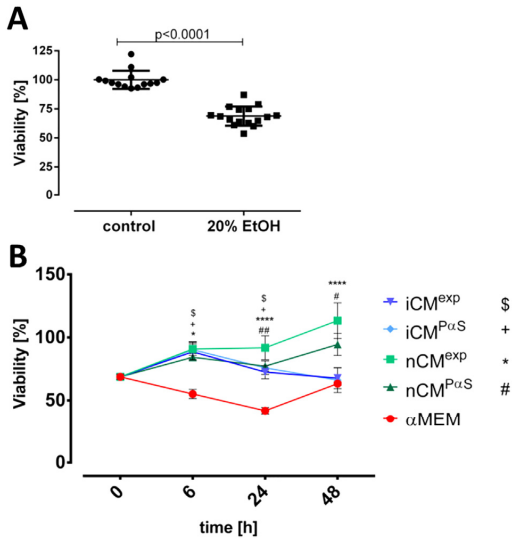


**Fig. 4.** Effect of $MSC^{CM}$ on LG epithelial cells.
Ethanol treatment significantly reduced the viability of LG epithelial cells (A). Incubation of injured LG epithelial cells with normal CM ($nCM^{Exp}$/$nCM^{PαS}$) and inflammatory CM ($iCM^{Exp}$/$iCM^{PαS}$), revealed a positive effect on the cell viability during the first 24 h. Whereas, at 48 h only $nCM^{Exp}$ and $nCM^{PαS}$ were beneficial. Data are n = 3 biological replicates for each CM and for LG-EC, mean ± SEM; $^{*}p \leq .05$, $^{****}p \leq .001$ represents significant changes for $nCM^{Exp}$ compared to control (αMEM); $^{\#}p \leq .05$, $^{\#\#}p \leq .01$ represents significant changes for $nCM^{PαS}$ compared to control (αMEM). $^{\$}p \leq .05$ represents significant changes for $iCM^{Exp}$ compared to control (αMEM); $^{+}p \leq .05$ represents significant changes for $iCM^{PαS}$ compared to control (αMEM).

incubation with 50 nM Rac1 led to 110.5 ± 10.5% cell viability, which is significantly enhanced compared to αMEM control cells with 43.0 ± 6.6% viability after 48 h ($p < .0001$). However, incubation of ethanol-treated cells with STAT1 (Fig. 7D) achieved the highest observed effect on cell viability. After 6 h, 50 nM STAT1 increased the viability to 69.1 ± 7.7%, which is significantly improved compared to αMEM control cells ($p = .0002$). In addition, cell viability further increased when treated for 24 h or 48 h with STAT1. Again, concentration and cell viability positively correlated. After 48 h all tested STAT1 concentrations resulted in significantly enhanced cell viability compared to αMEM control. Incubation with 50 nM STAT1 over 48 h, resulted in 117.2 ± 24.0% cell viability ($p < .0001$).

## 4. Discussion

Patients with ADDE suffer from severe limitations in their everyday life, but still no curative therapy exists. Experimental treatments for patients with severe ADDE comprise the application of serum eye drops and SG transplantation (Craig et al., 2017a). Serum eye drops have been shown to be more effective than conventional tear substitutes (Celebi et al., 2014; Beylerian et al., 2018). However, serum eye drops are still palliative and do not result in regeneration of LG tissue. In contrast, SG transplantation might represent a curative treatment to compensate for quantitative ADDE. But the different composition of saliva and lacrimal fluid leads to adverse symptoms, such as corneal edema (Borrelli et al., 2010). Thus, the development of an approach to induce *in situ* LG regeneration is still of great clinical interest. Therefore, this study compares two different protocols for the isolation of a pure and functional LG-specific MSC population, as we hypothesize that tissue specific MSC might be superior to ectopic MSC in term of LG regeneration. Furthermore, MSC secreted proteins were investigated for their potential to enhance LG-EC cell viability after injury, which might be useful to develop a drug-based regenerative therapy to treat ADDE.

LG-specific MSC were isolated either by explant culture or FACS. Isolating the cells by explant culture is a simple and gentle procedure, which is based on the migratory capacity of resident MSC. *Roth* et al., 2015 showed that by using explant technique, a LG-specific MSC population can be obtained. However, as the explant culture is a rather unspecific isolation method and the presence of contaminating cells was described in the past (Dietrich et al., 2016), we also investigated FACS isolation as an alternative and prospective method (PαS MSC). The obtained MSC populations were characterized using defined criteria concerning morphology, growth behavior, *in vitro* differentiation and surface antigen expression (Dominici et al., 2006). Both isolated MSC populations exhibited the characteristic fibroblastic morphology as described for MSC from bone-marrow (Al-Nbaheen et al., 2013), adipose tissue (Al-Nbaheen et al., 2013), pancreas (Seeberger et al., 2006) or LG (Roth et al., 2015). CFU efficiency and growth behavior (cpd) were comparable between Exp and PαS MSC, although slightly different to the results from Roth et al. (Roth et al., 2015). This might be due to a different set-up of the experiment (number of cells per well, manually *vs.* flowcytometric assisted plating of the cells). However, the overall population doublings per day as well as CFU efficiency were comparable to the results obtained for MSC in other studies (Dmitrieva et al., 2012; Al-Nbaheen et al., 2013; Houlihan et al., 2012), albeit differences were detected for MSC sub-populations. In our study, adipogenesis and osteogenesis was successfully induced in both MSC populations. The methods to detect differentiation and the capacity to differentiate vary widely between studies and subpopulations (Roth et al., 2015; Al-Nbaheen et al., 2013; Wagner et al., 2005). Nevertheless, characteristic staining with Oil Red O and Alizarin Red S proved the ability of the MSC to differentiate in the current study. Expression of characteristic surface markers such as CD44, CD29, CD90 and the absence of hematopoietic marker like CD34 and CD45 were comparable to other studies from LG (Roth et al., 2015), pancreas (Seeberger et al., 2006), BM and adipose tissue (Al-Nbaheen et al., 2013; Wagner et al., 2005; Houlihan et al., 2012). In addition, the expression of common stem cell markers such as Nestin (You et al., 2011; Shatos et al., 2012; Ackermann et al., 2015) and Sca-1 (Roth et al., 2015; You et al., 2011) were described for stem cells from LG and could also be detected in the current study. Although it is widely described that MSC express CD73 (Dominici et al., 2006), the MSC isolated from LG in the current study possessed only a low expression in p2, which further decreased with subsequent passaging. However, so far none of the published studies examining MSC or MSC-like stem cells from LG have verified a CD73 expression in these cells (Roth et al., 2015; You et al., 2011; Shatos et al., 2012; Ackermann et al., 2015).

The incubation with ethanol increases the permeability of cell membranes, resulting in mitochondrial damage and induction of apoptosis (Patra et al., 2006; Bonet-Ponce et al., 2015; Asai et al., 2003). A subsequent recovery of viable cells might be due to the induction of anti-apoptotic pathways and/or proliferation of the cells. Both events are essential for tissue regeneration as the second step of wound healing *in vivo* involves the formation of new tissue based on proliferation of specialized tissue cells or tissue resident stem cells. In general, mammalian tissue regeneration occurs in three stages after injury: inflammation, new tissue formation and remodeling (Gurtner et al., 2008). Therefore, we hypothesized that beneficial effects of MSC secretome and secreted proteins on EC viability might also be beneficial for LG regeneration *in vivo*. The mechanisms of MSC-mediated tissue regeneration are complex and in focus of research. It is believed that mainly two therapeutic effects, immunomodulation and secretion of trophic factors, are responsible for MSC-mediated tissue regeneration. In an inflammatory environment, MSC-secreted trophic factors that are involved in the inhibition of apoptosis and stimulation of tissue-resident (progenitor) cells (Shi et al., 2012; Caplan and Correa, 2011). In addition, recent studies could show that MSC conditioned medium (CM)

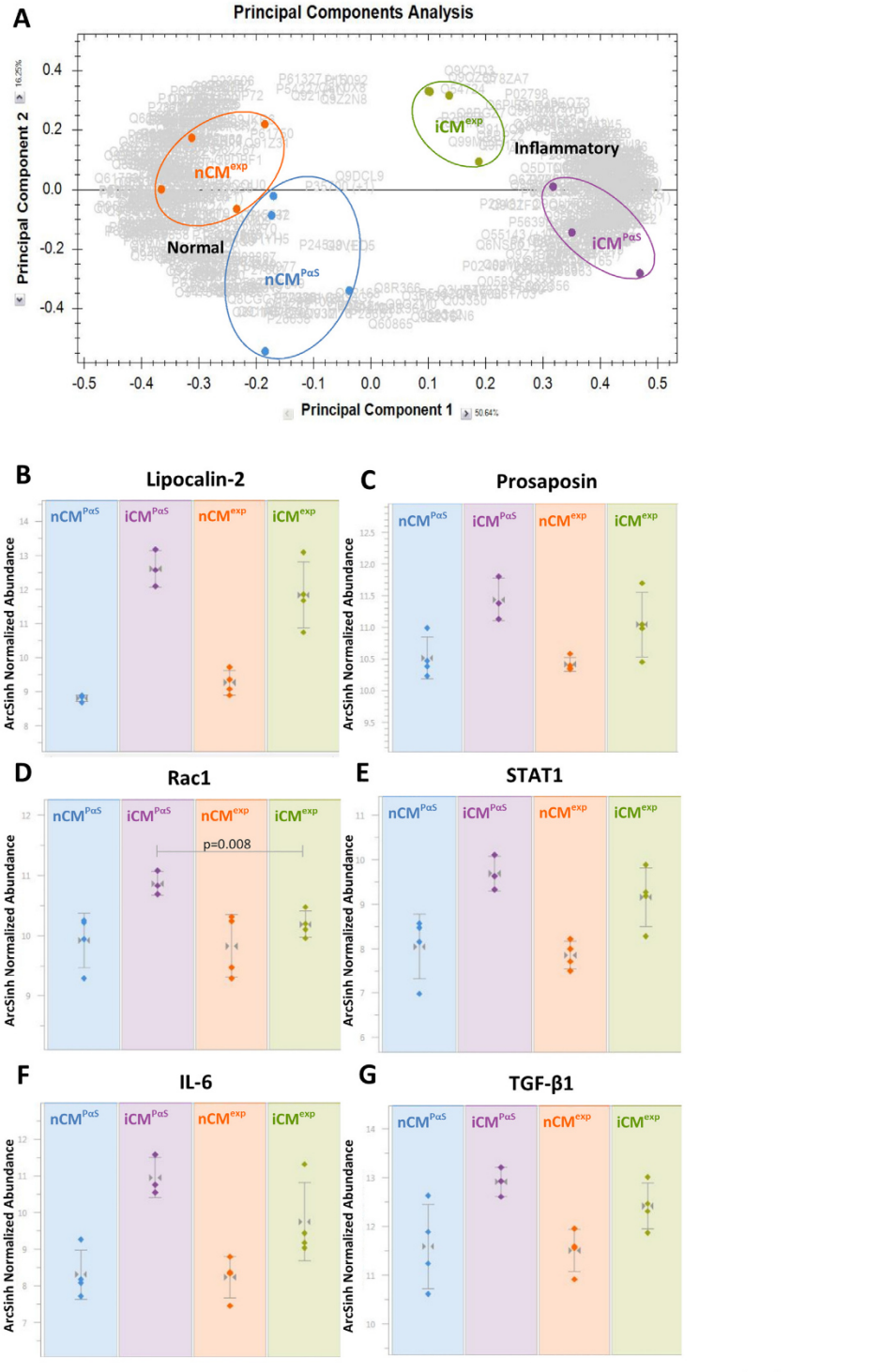


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**Fig. 5.** Expression analysis of MSC secretome.
Principal Compound Analysis (A) of secreted proteins from Exp and PαS MSC incubated under normal (nCM) or inflammatory (iCM) conditions (A, $p \leq .05$). Groups are circled according to MSC population or condition. Expression of selected proteins in the conditioned medium from explant and PαS MSC incubated under normal (nCM) or inflammatory (iCM) conditions: Lipocalin-2 (B), prosaposin (C), Rac1 (D), STAT1 (E), IL-6 (F) and TGF-β1 (G). Expression of Rac1 (D) was significantly higher in $iCM^{P\alpha S}$ than in $iCM^{explant}$ ($p = .008$). n = 3–4.

had therapeutic effects on wound healing, cell viability, cell proliferation and migration of EC from lung, corneal limbus and LG (Roth et al., 2015; Akram et al., 2013; Hu et al., 2012) as well as on irradiated SG *in vivo* (An et al., 2015). *In vitro* assays on cultured primary cells, such as viability measurements or cytotoxicity tests, are common preliminary experiments to investigate whether a component exerts regenerative effects on the underlying cell type of the complex tissue (Van Poll et al., 2008; Shabbir et al., 2015; Walter et al., 2010). Therefore, we tested the influence of CM from Exp and PαS MSC on the viability of ethanol-injured LG-EC, focusing on possible functional differences between both MSC populations. The results showed that incubation with nCM as well as iCM from both MSC populations enhanced the cell viability during the first 24 h. Between 24 and 48 h only nCM from both MSC populations was beneficial. This could be due to the bivalent effect of proteins secreted under inflammatory conditions, since we also found proteins from pro-apoptotic pathways such as Fas signaling pathway besides proteins with regenerative effects (Table S3-S5). However, results obtained from $nCM^{Exp}$ were comparable to those from $CM^{MSC\ 5\%}$ from

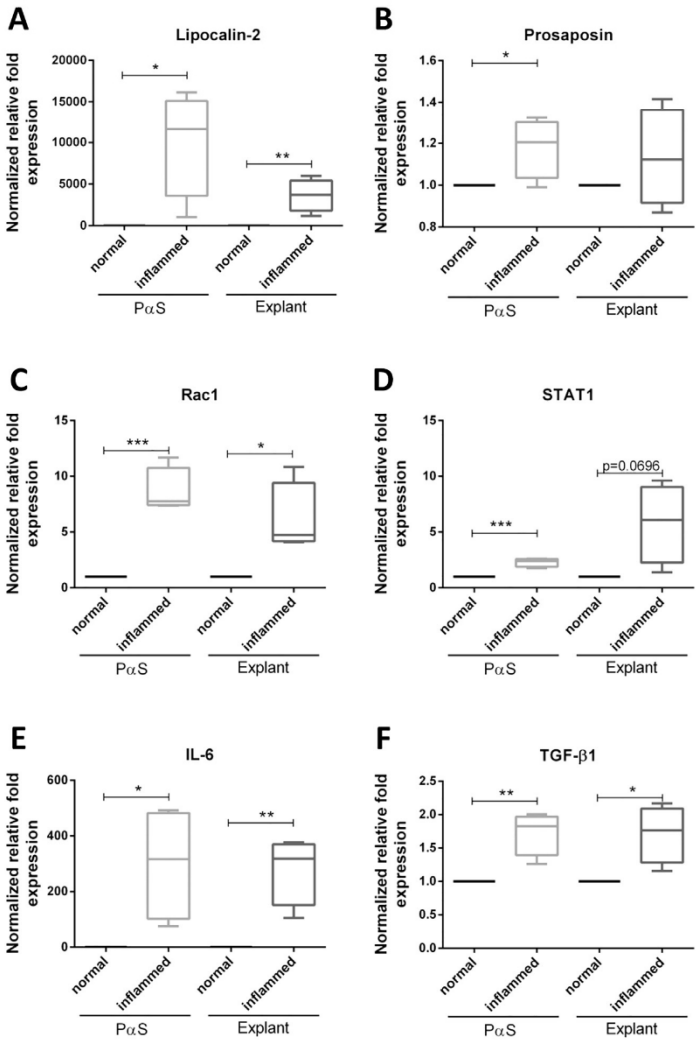


**Fig. 6.** Gene expression of selected proteins in MSC.
MSC were harvested after preparation of conditioned medium. The expression of lipocalin-2 (A), prosaposin (B), Rac1 (C), STAT1 (D), IL-6 (E) and TGF-β1 (F) was significantly increased when the MSC were cultured in inflammatory milieu, compared to normal milieu. Data are $n = 4$, Box-Plot with Min-to-Max; *$p \leq .05$, **$p \leq .01$ and ***$p \leq .001$.

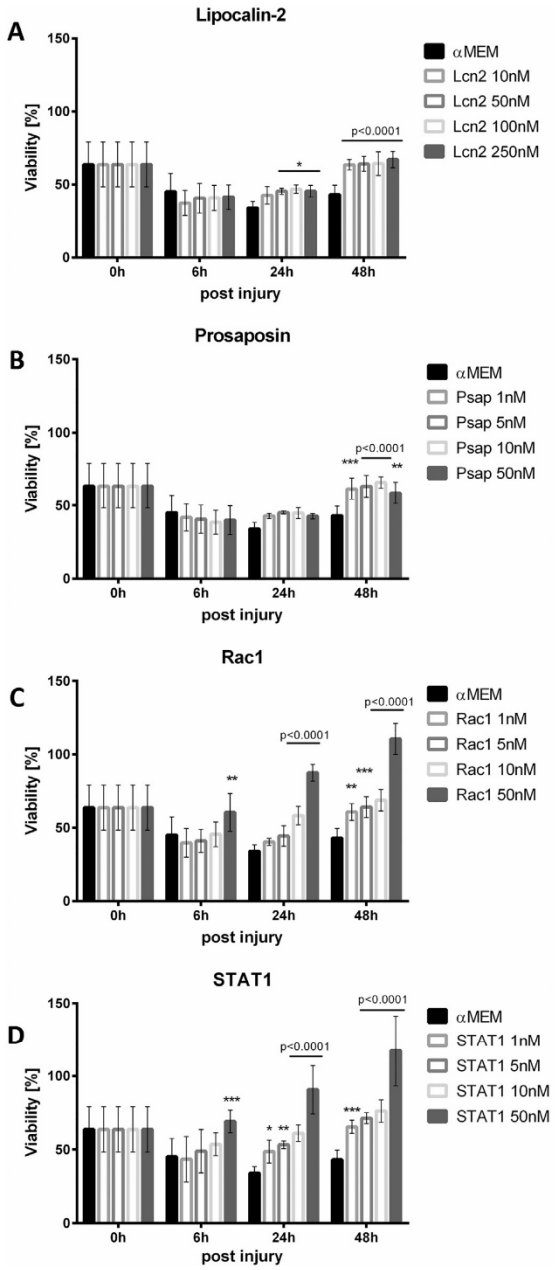


**Fig. 7.** Influence of candidate proteins on LG epithelial cell viability.
LG-EC were injured with 20% ethanol and then incubated with ascending concentrations of the candidate proteins. Incubation with lipocalin-2 (≥ 50 nM) significantly increased the cell viability after 24 h compared to αMEM control (A). Incubation with prosaposin resulted in a significant improved cell viability after 48 h (B). Significantly increased cell viability was detected already after 6 h when incubated with 50 nM Rac1. Further incubation with Rac1 resulted in additionally increased cell viability for each of the tested concentrations (C). Incubation with 50 nM STAT1β also resulted in significantly increased cell viability after 6 h, which further increased at 24 h and 48 h (D). Data are n = 3, mean ± SD; *p ≤ .05, **p ≤ .01 and ***p ≤ .001.

Roth et al. (Roth et al., 2015).

Since MSC secrete trophic factors that contribute to tissue regeneration in an inflammatory environment (Shi et al., 2012), we identified up-regulated proteins in iCM *vs.* nCM and investigated their potential to enhance LG-EC cell survival. Among others, we found Lcn2 to be upregulated in iCM. However, secretion of Lcn2 was similar from both MSC populations. Lcn2 is shown to be associated with reproduction, growth as well as immune system response (Thomas, 2017). On the one hand, Lcn2 deficient mice had a greater bacteremia and higher lethality to sepsis, indicating an important role in the anti-microbial defense of the innate immune system (Flo et al., 2004). On the other hand, Lcn2 is suggested to exhibit growth factor effects by modulating apoptosis, proliferation and differentiation (Schmidt-Ott et al., 2007). This is supported as Lcn2 expression is induced by hepatocyte-growth factor (HGF) in cultured renal EC (Gwira et al., 2005). However, HGF was not found in the current expression analysis of the LG-MSC. Further, a recent study revealed that the prospective application of a Lcn2:siderophore:iron complex caused the protection of renal EC and attenuated the loss of renal function (Mori et al., 2005). Similar to these findings, Lcn2 enhanced the cell survival of LG-EC upon injury in the current study. The association of Lcn2 with iron might further increase its regenerative effects on LG-EC as some studies described an altered and enhanced function of Lcn2 in combination with iron. However, this needs to be further elucidated. Additional to the growth factor effects on LG-EC, Lcn2 might also exert beneficial effects on MSC, which might further increase LG regeneration in *in vivo* experiments. It was recently shown that Lcn2 overexpression in MSC increased their proliferative capacity, inhibited stress-induced apoptosis and further induced expression of growth factors such as HGF and TGF-β1 (Halabian et al., 2013). This further supports the hypothesis that Lcn2 may improve tissue regeneration and may be beneficial in the treatment of DED *in vivo*. However, the effect of Lcn2 on MSC and a potential feedback loop needs to be further investigated.

Another up-regulated protein was prosaposin, which is related to cell cycle, growth and cell differentiation (Thomas, 2017). Beside its role as lysosomal precursor of the four saposins, it is described that secreted prosaposin has additional roles as a neurotrophic factor and activator of growth associated signaling cascades (Ham, 2004). Studies on neuronal PC12 cells (adrenal gland cells) as well as primary Schwann cells revealed that prosaposin induced proliferation and prevented cell death by activation of the mitogen-activated protein kinase/extracellular signal–regulated kinases (MAPK/Erk1/2) pathway (Campana et al., 1996; Hiraiwa et al., 1997; Misasi et al., 2001; Morales and Badran, 2003). In a study with prostate cells, incubation with saposin c (contains the trophic peptide-sequence of prosaposin) showed a positive effect on the proliferation of prostate stroma cells, but not on prostate EC (Koochekpour et al., 2005). Despite the consistency in the trophic sequence, the protein conformation might result in different actions of prosaposin and saposin c. However, the inhibition of apoptosis as well as the induction of proliferation could lead to the support of LG-EC survival in the current study, albeit the effect was low compared to the other investigated proteins.

Furthermore, we investigated Rac1, which is a small GTPase involved in multiple growth factor pathway, such as fibroblast growth factor (FGF). It is also associated with Wnt signaling and consequently associated with stem cell differentiation during development (Thomas, 2017). Recent *in vitro* studies revealed that Rac1 is essential for MDPC-23 cell proliferation, migration and cell adhesion (Ren et al., 2017). Studies on skin, oral mucosa and heart repair/regeneration confirmed an important role of Rac1 *in vivo*, as the elimination of Rac1 or its downstream effector led to a reduced regeneration (Liu et al., 2009; Castilho et al., 2010; Peng et al., 2016). In particular, Rac1 appears to be essential for re-epithelialization of oral mucosa after injury, as its knockout resulted in diminished epithelial precursor cell migration, proliferation and differentiation (Castilho et al., 2010). Therefore, we assumed that Rac1 might also be essential for LG regeneration. Here,

we report that the supplementation of culture medium with Rac1 resulted in an increased LG-EC survival after injury. Comparing the two MSC populations (Exp *vs.* PαS) the amount of Rac1 was significantly higher in $iCM^{P\alpha S}$ than $iCM^{Exp}$. However, as the iCM of both MSC populations did not show any differences regarding the effect on LG-EC viability, this issue might be negligible.

Interestingly, cell survival of LG-EC was highest, when the cells were incubated with STAT1. In general, STAT1 is associated with cellular defense in immune system processes as well as regulation of apoptotic pathways (Thomas, 2017). Upon activation, STAT1 isoforms α and β form homo- or heterodimers and translocate to the nucleus where it acts as a transcription factor (Adamkova et al., 2007). Signaling of STAT1 is complex and reveals somehow contrasting transcriptional functions (Ramana et al., 2000). In general, it is described that STAT1 promotes apoptosis in a variety of cell types as it upregulates the expression of caspases and the Fas receptor (Ramana et al., 2000; Stephanou and Latchman, 2005). However, despite its pro-apoptotic action, a novel role is described for STAT1 in conjunction with progenitor cells. It has been found that STAT1 stimulates proliferation of mesenchymal progenitors, both during (renal) development (Wang et al., 2010) and in adult (adipose) tissue (Song et al., 2005). Further, recent studies reported that STAT1 is required for the proliferation of premature myoblasts (Sun et al., 2007). Thus, we investigated the influence of STAT1 on epithelial progenitors of LG. As the incubation with STAT1 increased the LG-EC viability 2-fold compared to control, we could show that STAT1 is also beneficial for epithelial progenitors.

In conclusion, we report that explant culture and FACS are useful to isolate a specific and functional MSC population from murine LG as only minor differences were detected during characterization. While cell sorting might be beneficial to prepare cells on demand, explant culture is more economical in effort and cost. Furthermore, analysis on CM revealed that the secretome of MSC harbors proteins, such as Lcn2, prosaposin, Rac1 and STAT1, which positively influence the cell survival of LG-EC and could be useful to induce/enhance LG regeneration *in vivo*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101477.

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## Conflict of interest statement

The authors do not have any conflict of interest.

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## 3.3 Original Research Article

### MSC Transplantation Improves Lacrimal Gland Regeneration after Surgically Induced Dry Eye Disease in Mice

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Contribution to experimental design, implementation and publication:

Involved in the conception and design of the experimental setup, conduction of the surgery and measurement of tear secretion assisted by LO, implementation and assembly of data concerning MSC isolation and characterization, lacrimal gland analysis including (immune-) histology, gene and protein expression, data analysis and interpretation, manuscript writing, preparation of all figures (except figure 3 C, E, F). The manuscript was subsequently reviewed and approved by all co-authors.
Approximated total share of contribution: 55%

Data used for degrees other than this thesis

The data concerning fluorescein staining in Figure 3C and thickness of the corneal epithelium in Figure 3E and 3F presented as parts of section “Clinical assessment of ADDE”, will be submitted to obtain the medical doctorate of Lolita Ott at the Heinrich-Heine-Universität Düsseldorf.

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# MSC Transplantation Improves Lacrimal Gland Regeneration after Surgically Induced Dry Eye Disease in Mice

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

Dry eye disease (DED) is a multifactorial disease affecting the entire lacrimal functional unit (LFU) including the ocular surface, the lacrimal glands (LG), the meibomian glands, the nervous innervation and the lids. Collectively, the LFU produces the complex and multi-layered tear film required for maintaining a physiological ocular surface[1]. The major part of the tear film is composed of the aqueous lacrimal fluid, which is secreted by the LG. The functional tissue of the LG -the acini- consists of secretory acinar cells, duct cells and myoepithelial cells[2]. Any impairment or loss of functional LG tissue lead to an imbalance of tear film homeostasis and can result in the development of aqueous deficient dry eye (ADDE). This DED subtype causes the most severe courses of the disease. During disease progression, the imbalanced tear film results in ocular surface inflammation, which can lead to corneal ulcers, as well as conjunctival and corneal scars, and thus to impaired vision[2]. Despite a high prevalence of DED with 5–50% depending on ethnic group, age and sex[3], current treatment options remain palliative. Therefore, it is clinically of great importance to develop new approaches for a causative therapy. *In situ* regeneration of functional LG tissue has emerged to be a promising approach, and current studies are investigating drugs, gene therapy and stem cell therapy to induce/enhance LG regeneration[4]. However, further research is needed to overcome certain limitations.

One promising source for stem cell therapy to induce LG regeneration might be the use of mesenchymal stem/stromal cells (MSC), as these cells can be isolated from many different adult tissues and have already shown to exert therapeutic effects on the regeneration of glandular tissues, like pancreas, salivary gland (SG) and LG with chronic DED[5–7]. In addition, MSC have also been identified and isolated from the healthy and diseased rodent LG[8–10] and it was shown that the number of MSC increase in regenerating LGs after experimentally induced

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ADDE[11–13]. In recent years MSC have been extensively studied and it was found that MSC exert therapeutic effects in a variety of pathological conditions[14,15]. A huge body of evidence shows that the therapeutic effects of MSC rely on the ability to suppress inflammation and initiate endogenous repair mechanisms. Furthermore, it was shown that MSC secrete trophic factors that affect infiltrating immune cells as well as tissue resident stem cells[16,17].

In general, tissue inflammation and new tissue formation followed by tissue remodelling are the three stages of endogenous tissue repair initiated after acute damage[18]. Investigations on mouse models with experimentally induced ADDE revealed that the dynamic of LG damage and regeneration passes through the same three phases of tissue repair[11,19]. As the first two phases include the action of infiltrating immune cells and tissue resident stem cells, which are a target of MSC secreted factors, one could argue that the therapeutic effects of MSC are most valuable when applied directly after acute damage and during the first phase of tissue regeneration.

Ligation of the single secretory duct of the extraorbital LG was identified to induce severe ADDE in mice[11,13]. Duct ligation (DL) caused a profound loss of functional LG tissue, a severe inflammatory reaction and a reduced tear secretion. The LG, like other glandular tissues, retains the ability of self-regeneration after acute damage throughout its life-time, although it can be impaired due to chronic pathological conditions[20]. For this reason, the re-opening of the duct in the DL mouse model initiated a phase of new tissue formation/regeneration in juvenile mice, shown by the partial regeneration of vital acinar structures after 21 days by our working group[11]. This regeneration process was accompanied by an increase of intrinsic MSC.

In this study, the therapeutic efficacy of MSC transplantation was investigated regarding LG regeneration after surgically induced ADDE in mice. This will allow to assess whether the transplantation of extrinsic MSC supports the regeneration of the LG and could be useful in a clinically relevant setting. The use of tissue-specific extrinsic MSC is of great clinical relevance as the LG of patients with severe ADDE exhibit an impaired regenerative potential due to chronic pathological conditions such as persistent inflammation as well as age-dependent degeneration. Since MSC can be found in a variety of tissues and tissue-specific differences between the sub-populations have been described[21–23], the use of LG-specific MSC for the treatment of ADDE seems to be superior to treatment with ectopic MSC. Consequently, MSC were isolated from murine LG of male mice expressing eGFP and characterized according to the defined minimal criteria[24]. DL was implemented on female mice for three days and eGFP-MSC were transplanted when releasing the DL. The analysis of vital acinar structures as the functional tissue of the LG at different time points after duct re-opening (day 5 and day 21) revealed that the transplantation of extrinsic MSC led to an enhanced increase in vital tissue area compared to saline injected LGs. This study provides the first evidences of a regenerative effect of extrinsic tissue specific MSC in an ADDE mouse model.

## Results

**Characterization of eGFP-MSC.** To verify the phenotype of MSC isolated from genetically modified eGFP-mice, the cells were characterized according to the defined minimal criteria[24]. Cells emerging the LG explant (Exp) exhibited a spindle-shaped, fibroblastic morphology (Fig. 1A). The cumulative population doublings (cpd) revealed a closely linear growth behavior up to passage (p) 6 ($11.0 \pm 0.53$ cpd) as the coefficient of determination ($r^2$) was 0.9707 (Fig. 1B).

eGFP-MSC expressed the commonly analyzed markers, as the population was >95% positive for nestin, CD44, PDGFRα, CD29, CD90, Sca-1 and <5% for CD73, Ter119, CD34 and CD45 (Fig. 1C). Two CD105 populations were detected, with $59.0 \pm 2.6$% of MSC being CD105-positive.

Differentiation capacity towards osteocytes and adipocytes was determined. After induction of osteogenesis, the expression of osteopontin significantly increased over time compared to control groups ($p = 0.0187$ at d7 and $p < 0.0001$ at d14 and d21; Fig. 1D). In addition, Alizarin Red staining exposed the mineralization of calcium-phosphate deposits and confirmed the differentiation (Fig. 1E). Progression of adipogenesis was monitored by FABP4 expression, which significantly increased after induction compared to control groups ($p < 0.0001$ at all investigated time points; Fig. 1F). Furthermore, formation of lipid droplets was visualized by Oil Red O staining (Fig. 1G).

**Investigation of eGFP-MSC for transplantation.** To assess the genetical stability of eGFP-MSC concerning their stemness, we next investigated nestin, a commonly used marker for multipotent stem cells[25], and the genetically expressed eGFP, as a tracking marker. The eGFP-MSC expressed nestin, which was previously shown to be expressed by MSC isolated from wild-type (wt) B6 LG (Fig. 2A,B)[26]. In addition, nestin and eGFP expression was stable (>99% each) over 28 days (Fig. 2A). These time interval correlates with the last examination time eGFP-MSC in the transplanted LGs (7 days in culture (*in vitro*) and a maximum of 21 days after transplantation (*in vivo*)).

To investigate the influence of different solution, which can be used for transplantation in mouse surgery, on the vitality of MSC, the cells were incubated on ice for 3–5 h in either culture medium, saline, saline +2% FCS, or PBS and then treated once with a 27-gauge needle to mimic transplantation. Propidium iodide staining in flow cytometry showed that, regardless of the solution used, about 80% of the MSC remained vital (Fig. 2C).

**Clinical assessment of ADDE.** To verify the induction of ADDE after DL clinically relevant measurements were performed[27]. The major impact of ADDE is the reduced secretion of lacrimal fluid onto the ocular surface, therefore tear secretion was assessed by a Schirmer test using phenol red cotton threads (Fig. 3A). DL resulted in a tear secretion of $1.10 \pm 0.62$ mm after saline injection and $0.88 \pm 0.29$ mm after MSC transplantation (d0), which was significantly reduced compared to basal secretion of $5.04 \pm 2.36$ mm (Fig. 3B). At day 5 tear secretion was still significantly reduced in both groups ($p < 0.0001$). However, 21 days of regeneration led to a significant increase with $5.55 \pm 3.28$ mm secreted tears after saline injection and $5.23 \pm 2.32$ mm after MSC transplantation, which was comparable to basal secretion.

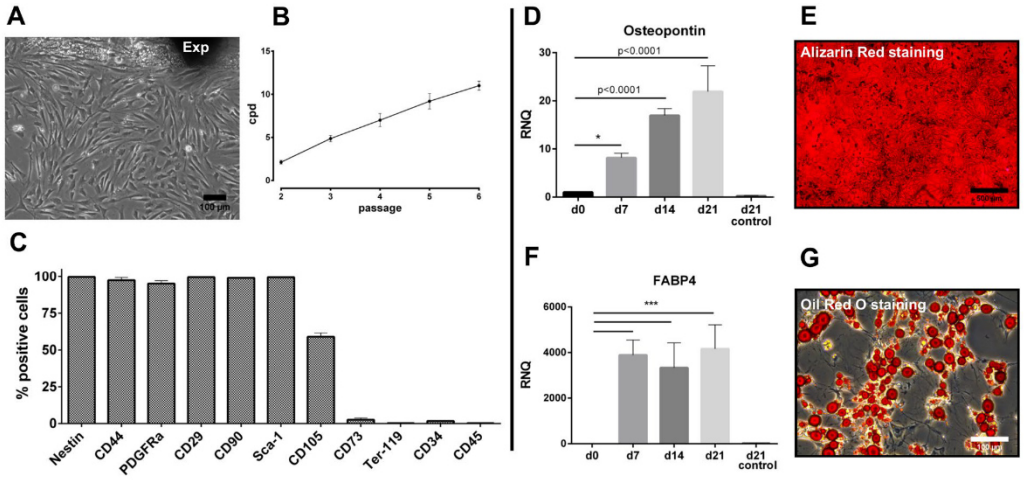


**Figure 1.** Characterization of eGFP-MSC. (**A**,**B**) eGFP MSC emerging from the explant (Exp) exhibited a spindle-shaped, fibroblastic morphology with a closely linear growth behavior, as detected by the assessment of cpd. (**C**) Immunophenotyping in p2 revealed the expression of typical MSC markers, while common hematopoietic (stem) cell markers were absent. Two distinct populations were detected for CD105. (**D**,**E**) The ability to differentiate towards osteocytes was verified by osteopontin expression and Alizarin Red staining. (**F**,**G**) Adipogenic differentiation was confirmed by FABP4 expression and Oil Red O staining. The quantity of positive cells [%] after isotype control staining are provided in the supplementary data file as Fig. S1. Data are n = 3, mean ± SD; scale bar: 100 µm (**A**,**G**) scale bar: 500 µm. (**E**) *Represents $p \leq 0.05$ and ***represent $p \leq 0.001$ compared to d0 control.

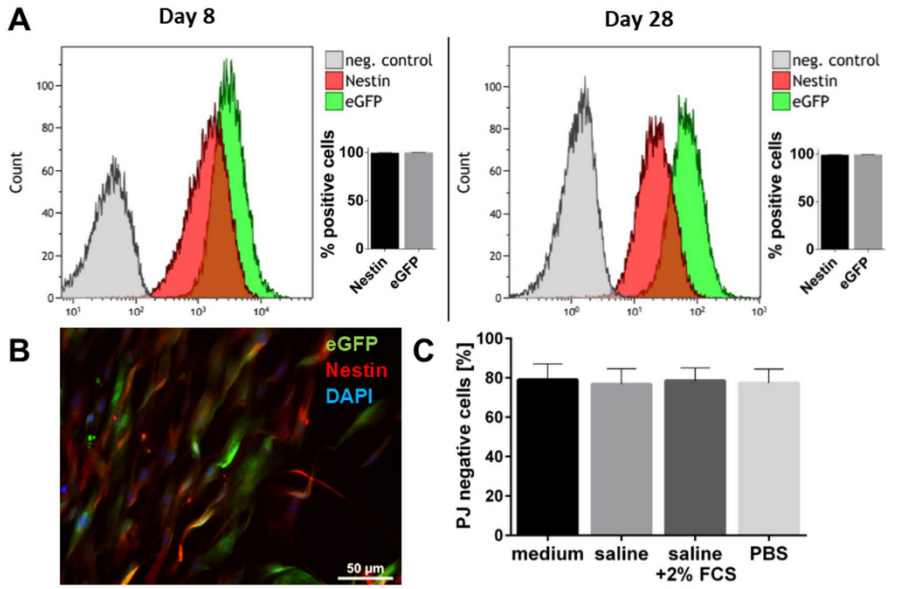


**Figure 2.** Evaluation of eGFP-MSC for Transplantation. (**A**,**B**) Nestin expression in eGFP-MSC was detected by flow cytometry and immunostaining. (**A**) A stable expression of nestin and eGFP was verified over 28 days. (**C**) Incubation of eGFP-MSC in culture medium, saline, saline +2%FCS or PBS for 3–5 hours on ice followed by treatment with a 27-gauge needle showed that approx. 80% of eGFP-MSC remained vital (PI negative). Data are n = 3, mean ± SD; scale bar: 50 µm.

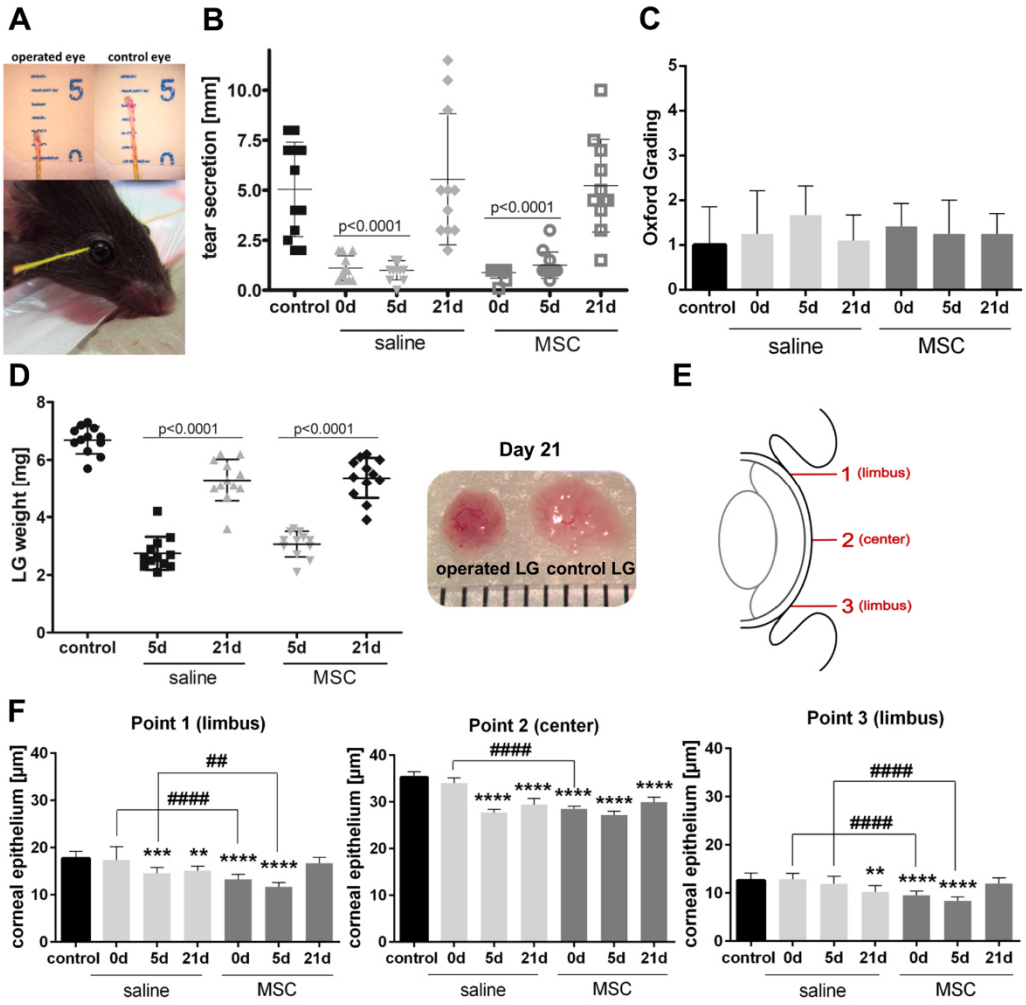


**Figure 3.** Clinical Assessment of ADDE. (**A**) Phenol red cotton threads were applied to the lateral canthus of each eye and the wetted distance was measured. (**B**) Tear secretion was significant reduced after DL (0d) but recovered to control levels by d21. (**C**) Fluorescein staining showed no differences at any time after DL. (**D**) LG weight was significantly reduced at d5 after DL and saline or MSC injection. From d5 to d21 LG weight significantly increased in both groups. (**E**) Measuring points were defined to cover the whole cornea (**F**) Thickness of corneal epithelium was significantly decreased after DL at almost any time and in any area of the ocular surface after saline and MSC injection compared to control. Data are n = 12 (**B–D**), n = 6 (**F**), mean ± SD; **represents $p \leq 0.01$, ***represent $p \leq 0.001$ and ****represent $p \leq 0.0001$ compared to control; ##represents $p \leq 0.01$ and ####represent $p \leq 0.0001$ compared between saline and MSC groups.

The presence of fluorescein staining of the ocular surface is a well-established clinical method to measure severity of DED[2,27], as fluorescein stains the corneal stroma when the epithelium is disrupted. Therefore, the integrity of the corneal epithelium was investigated by fluorescein staining (Fig. 3C). The evaluation showed no differences neither between control and treatment groups, nor between MSC and saline injection.

Damage and regeneration of LGs were further investigated by measuring the LG weight after excision (Fig. 3D). 5 days after re-opening of DL the LG weight was significantly reduced to 2.75 ± 0.57 mg in saline injected and to 3.07 ± 0.44 mg in MSC transplanted LGs compared to control (6.68 ± 0.47 mg). Regeneration of LG up to day 21 led to a significant increase in weight in saline and MSC injected LGs, respectively ($p < 0.0001$). This resulted in a LG weight comparable to control.

To further determine whether DL had an impact on the ocular surface, thickness of the corneal epithelium was assessed (Figs. 3E,F and S2). Thickness of corneal epithelium decreased significantly at almost any time and in any area after saline and MSC injection compared to control. In the limbal area, only on day 0 after saline

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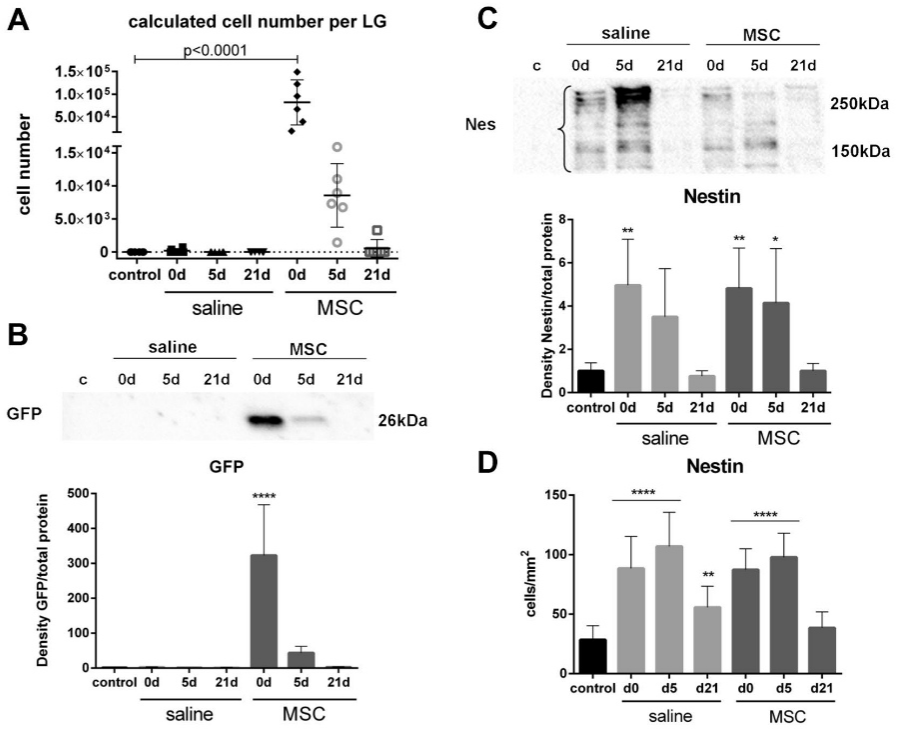


**Figure 4.** Assessment of MSC after Transplantation. (**A**) The number of transplanted cells was calculated according to the presence of male gDNA in female recipient gDNA, by *Rbmy* expression in qPCR. Five min (d0) after transplantation the number of MSC were significantly increased and then gradually returned to control. (**B**) Representative pictures of eGFP detection by western blot. Quantification revealed a significant increase 5 min (d0) after MSC transplantation, which decreased to control levels by d21. (**C**) Representative pictures of nestin detection by western blot. Nestin was significantly increased in saline and MSC injected LG at d0 and d5 compared to control. (**D**) Immunohistochemical staining detected nestin cells, which were significantly increased in saline and MSC injected LG at d0, which further increased at d5. At d21 the number of nestin cells was still significantly increased in saline, but not in MSC group. For western blot analysis the samples ($n = 42$) were run on four blots, which were processed in parallel. Full blots are provided in the supplementary data file. Data are $n = 6$, mean ± SD; *represents $p \leq 0.05$, **represents $p \leq 0.01$ and ****represent $p \leq 0.0001$ compared to control.

injection and on day 21 after MSC transplantation, values comparable to control were observed. Additionally, in the limbal region the thickness of the corneal epithelium was significantly lower after MSC transplantation than after saline injection on day 0 and day 5.

**Assessment of MSC after transplantation.** To track the MSC within the injured LGs, transplantation of MSC from male eGFP-mice into LGs of female recipients (both mouse strains had the BL/6J background) was performed. The number of MSC present in LG was calculated based on male gDNA (MSC) in female gDNA (recipient mice) using qPCR amplification of a male-specific sequence (*Rmby*). Five min after transplantation $8.19 \times 10^4 \pm 4.98 \times 10^4$ MSC were detected, which gradually decreased to $5.51 \times 10^2 \pm 1.33 \times 10^3$ MSC at day 21 (Fig. 4A).

To confirm the presence of MSC, intrinsic eGFP was quantified. The results showed that the amount of eGFP protein was significantly increased ($p < 0.0001$) 5 min after transplantation, relative to control and saline injection (Fig. 4B). During regeneration the amount of eGFP decreased and was comparable to that of control at day 21 (relative, normalized density eGFP: $2.12 \pm 1.76$, control: $1.0 \pm 0.51$). In immunohistochemical staining, eGFP cells were detected in the stroma adjacent to acinar structures and could be found 5 min, 5 days and 21 days after transplantation (Fig. S3).

To investigate the presence of intrinsic and transplanted MSC during regeneration, nestin expressing cells were studied. Due to DL (d0) the amount of nestin was significantly increased ($p = 0.004$ for saline and $p = 0.006$ for MSC) relative to control (Fig. 4C). At day 5 after re-opening of DL and injection of saline, the amount of nestin was still, albeit not significantly, increased. Whereas, at day 5 after MSC transplantation the amount of nestin was still significantly increased ($p = 0.037$) relative to control. By day 21 the amount of nestin was comparable

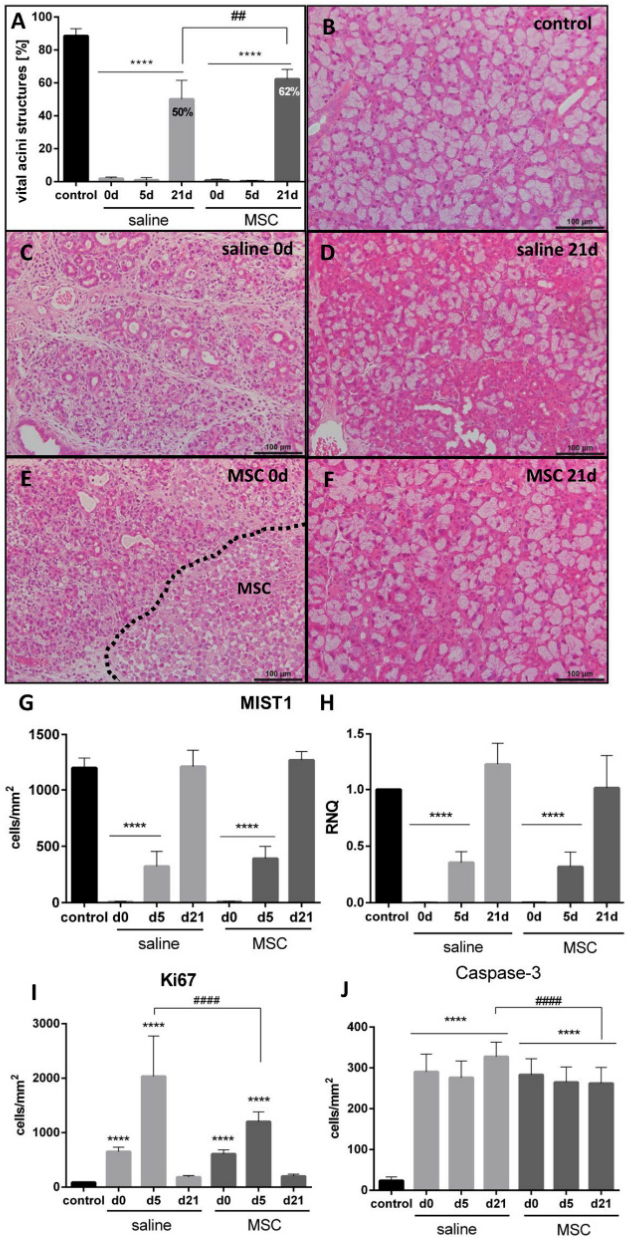


**Figure 5.** Dynamic of LG damage and regeneration. (**A**) Area of vital acinar structures calculated in HE stained LG sections. At d21 vital acinar structures recovered to significant higher extent after MSC than saline injection. (**B**) Control LG had tightly arranged acini, which were organized in lobules and surrounded by few connective tissue. (**C**,**E**) Due to DL, LG structure was damaged by interstitial edema, infiltrating cells and shrunken acinar cells with higher eosinophilia. (**E**) Transplanted MSC were detected in the stroma adjacent to acinar structures (dashed line). (**D**,**F**) After re-opening of DL and regeneration, LG structure recovered, and acinar structures re-appeared. (**G**) The number of MIST1-positive cells in immunohistochemical staining significantly decreased after DL (d0), but gradually increased thereafter and was comparable to control by d21. (**H**) MIST1 gene expression displayed comparable results to MIST1 immunohistochemical staining. (**I**) The number of Ki67-positive cells in immunohistochemical staining gradually increased up to d5 and normalized comparable to control at d21. At d5 the number of Ki67-positive cells were significantly higher after saline injection than after MSC injection. (**J**) The number of caspase-3-positive cells in immunohistochemical staining was significantly

elevated at all time points in both groups. The number of caspase-3 positive cells at d21 was significantly elevated in saline injected LGs compared to MSC injected LGs. Data are n = 6, mean ± SD; scale bar: 100 µm. ****Represent $p \leq 0.0001$ compared to control; ## represents $p \leq 0.01$ and #### represent $p \leq 0.0001$ compared between saline and MSC groups.

to control in both groups. In immunostaining, nestin expressing cells appeared as elongated, fibroblastic cells in the stroma of (damaged) LGs (Fig. S4). Number of nestin-positive cells were significantly increased after DL with 88.4 ± 26.88 cells/mm$^2$ in saline and 87.33 ± 17.64 cells/mm$^2$ in MSC injected LGs compared to control (28.4 ± 11.87 cells/mm$^2$; Fig. 4D). The number of nestin positive cells increased to 106.9 ± 28.6 cells/mm$^2$ in saline and 97.76 ± 20.18 cells/mm$^2$ in MSC injected LGs at day 5. Although the number of nestin cells decreased by day 21, it was still significantly increased when saline, but not MSC, was injected compared to control. These findings were additional confirmed by analysis of nestin expression in qPCR (Fig. S5).

**Dynamic of LG damage and regeneration.** LG damage and regeneration were investigated in HE stains, and vital acinar structures were identified and measured (Fig. 5A–F). In control LG, the acinar structures are tightly arranged, and only sparse connective tissue was found between the lobules (Fig. 5B). DL resulted in interstitial edema, infiltrating cells and shrunken acinar cell body with increased eosinophilia (Fig. 5C,E). Transplanted MSC could be detected in the stroma adjacent to the acinar structures (Fig. 5E dashed line, Fig. S4). Re-opening of the DL resulted in the reappearance of acinar structures and a decline of interstitial edema (Fig. 5D,F). Analysis revealed that vital acinar structures significantly decreased from 88.58 ± 4.38% in control to 1.86 ± 0.96% and 0.81 ± 0.69% in saline and MSC injected LGs, respectively (d0, Fig. 5A). Five days after saline injection and MCS transplantation the area of vital acinar structures was still significantly decreased ($p < 0.0001$). During 21 days of regeneration, vital acinar structures increased to 62.32 ± 5.88% after MSC transplantation, which was significantly enhanced compared to saline injection (50.11 ± 11.45%, $p = 0.0039$).

Analysis of MIST1, an acinus specific transcription factor, confirmed the loss of acinar cells during DL (Figs. 5G and S6A). The number of MIST1-positive cells decreased from 1205 ± 87.55 cells/mm$^2$ in control to 6.27 ± 5.42 and 6.93 ± 6.32 cells/mm$^2$ in saline and MSC injected LGs, respectively. MIST1-positive cells increased gradually and were comparable to control by day 21 with 1216 ± 145.1 and 1272 ± 77.21 cells/mm$^2$ after saline injection and MSC transplantation, respectively. The absence of MIST1 expression after DL was further confirmed by qPCR (Fig. 5H).

Damage and regeneration were further investigated by proliferating (Ki67) and apoptotic (caspase-3) cells. The number of Ki67-positive cells gradually increased after DL, up to day 5 to 2033 ± 742 cells/mm$^2$ after saline injection and to 1201 ± 180 cells/mm$^2$ after MSC transplantation (Figs. 5I and S6B), which was significantly more Ki67-positive cells in saline than in MSC injected LGs ($p < 0.0001$). The number of Ki67-positive cells normalized to control levels by day 21. The number of caspase-3-positive cells was significantly increased at all investigated time points and in both groups ($p < 0.0001$, Figs. 5J and S6C). However, at day 21 the number of caspase-3 cells was significantly lower after MSC transplantation than after saline injection ($p < 0.0001$).

**Characterization of immune reaction.** To investigate the immune reaction after DL, we performed immunostaining and western blot analysis of commonly infiltrating cell types and pro-inflammatory cytokines. Number of Ly6G-positive cells, a marker for (neutrophil) granulocytes, was significantly increased from 4.1 ± 4.8 cells/mm$^2$ in control to 186.9 ± 36.8 cells/mm$^2$ and to 157.7 ± 43.2 cells/mm$^2$ after saline and MSC injection (d0), respectively (Figs. 6A and S6D). Number of infiltrating Ly6G-positive cells remained high at day 5 in both groups ($p < 0.0001$, respectively), but decreased to control levels at day 21.

Number of CD68-positive cells, a marker for monocytes and macrophages, was significantly increased at all investigated time points, both in saline and MSC injected LGs, compared to control ($p < 0.0001$, Figs. 6B and S6E). The number of CD68-positive cells increased from 43.1 ± 16.5 cells/mm$^2$ in control to 268.3 ± 42.8 cells/mm$^2$ after saline injection and to 228.2 ± 72.3 cells/mm$^2$ after MSC transplantation (d0), which was significantly different when comparing the two treatment groups ($p = 0.0073$). Infiltration of CD68-positive cells further increased at day 5 to 826.3 ± 72.3 cells/mm$^2$ and to 739.7 ± 89.2 cells/mm$^2$ after saline and MSC injection, respectively. At day 5 the number of CD68-positive cells was significantly lower in MSC than in saline injected LG ($p = 0.0005$). By day 21 the number of CD68-positive cells significantly decreased in both groups compared to day 5 but was still significantly higher than in control.

Gene expression of TNFα raised 56.7 ± 18.6-fold after saline injection and 26.95 ± 8.3-fold after MSC transplantation, which was significantly up-regulated compared to control (Fig. 6C). Expression of TNFα was significantly higher after saline injection than after MSC transplantation ($p < 0.0001$). During regeneration, the expression of TNFα decreased and reached control levels by day 21. Although the expression of TNFα increased, no TNFα protein could be detected (Fig. 6D). Expression of IL-6, a widely described immune-modulatory cytokine secreted by MSC, was significantly increased at day 0 after MSC transplantation, but not after saline injection when compared to control ($p = 0.0007$; Fig. 6E). Thus, the expression of IL-6 was significantly higher in MSC than in saline group ($p = 0.0037$). However, no IL-6 protein could be detected (Fig. 6F).

**Influence of MSC.** In a previous study, we identified lipocalin-2 (Lcn2) and STAT1 in the secretome of MSC, contributing to the improvement of LG epithelial cell survival *in vitro*[26]. Therefore, we analyzed whether transplanted MSC also secrete Lcn2 and/or STAT1 *in vivo*. Expression of Lcn2 was significantly increased 5 min (d0) after saline and MSC injection compared to control ($p < 0.0001$) and gradually decreased to control levels by d21 (Fig. 7A). Investigation on Lcn2 protein level also revealed a significant increase at d0 ($p < 0.0001$ saline injection

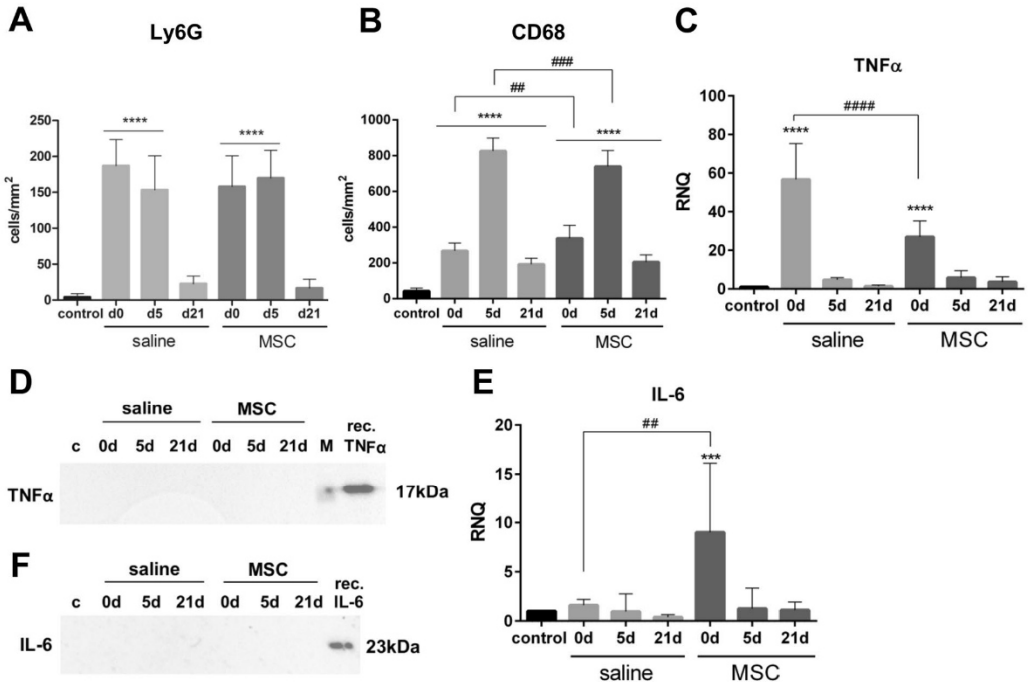


**Figure 6.** Characterization of immune reaction in LG. (**A**) Number of Ly6G cells in IHC significantly increased after DL in saline and MSC injected LGs at d0 and d5 but normalized by d21. At d0 the number of Ly6G cells tended to be higher after saline injection than MSC injection. (**B**) Number of CD68 cells in IHC was significantly increased at all time points compared to control. The number of CD68 cells differed significantly between saline injection and MSC transplantation at d0 and d5. (**C**) TNFα expression in the LG was significantly increased after DL (d0) and was significantly higher after saline injection than after MSC injection. (**D**) TNFα could not be detected in western blot. (**E**) Expression of IL-6 in the LG was increased only at d0 after MSC injection, which was significantly different compared to saline injection. (**F**) IL-6 could not be detected in western blot; for western blot analysis the samples (n = 42) were run on four blots, which were processed in parallel. Full blots are provided in the supplementary data file. Data are n = 6, mean + SD. ***Represent $p \leq 0.001$ and ****represent $p \leq 0.0001$ compared to control; $^{\#\#}$represents $p \leq 0.01$, $^{\#\#\#}$represent $p \leq 0.001$ and $^{\#\#\#\#}$represent $p \leq 0.0001$ compared between saline and MSC groups.

and p = 0.0004 after MSC transplantation) with a return to baseline by day 21 (Fig. 7B,C). Expression of STAT1 increased at d0 in both groups and gradually declined over time (Fig. 7E). Protein levels of STAT1α as well as STAT1β were significantly increased at d0 in saline and MSC injected LGs (STAT1α: p = 0.0127 saline, p = 0.0552 MSC; STAT1β: p = 0.0316 saline, p = 0.021 MSC) compared to control (Fig. 7D,F,G). In saline injected LG the level of STAT1α and STAT1β decreased thereafter. In MSC injected LG the amount of STAT1α and STAT1β declined by d5 and was again significantly increased at d21 compared to control (STAT1α: p = 0.0302, STAT1β: p = 0.0241).

## Discussion

ADDE, due to a loss of functional LG tissue, causes the most severe forms of DED[28]. Current therapies remain palliative and even advanced therapies, like the transplantation of the SG can only ease the symptoms, but are insufficient to restore the physiologic composition of the tear film and to address the underlying loss of LG tissue[29]. Therefore, this study evaluated a curative approach by investigating the therapeutic potential of LG-specific, extrinsic MSC transplantation to enhance LG regeneration in an experimental model of ADDE.

In the current study, MSC for transplantation were isolated by explant culture from mice that express eGFP in a constitutive and ubiquitous manner (eGFP-MSC). Recently, explant culture was identified as a suitable method to isolate a pure, specific and functional MSC population from the murine LG[8,26]. The eGFP expression allowed the identification and tracking of transplanted MSC throughout the study. Nevertheless, genetic modification and ectopic expression of a transgene (eGFP) might alter the physiology of the cells. Therefore, eGFP-MSC were characterized and compared to results obtained from wildtype (wt) mice in previous studies[8,26]. eGFP-MSC exhibited the characteristic fibroblastic morphology and growth behaviors comparable to that of wt-MSC[22,26,30]. In addition, phenotypic characterization and differentiation capacity was comparable to that of MSC from other tissues[22,31] and to that of wt-MSC[8,26]. The genetic stability of eGFP-MSC was shown by a constant expression of nestin

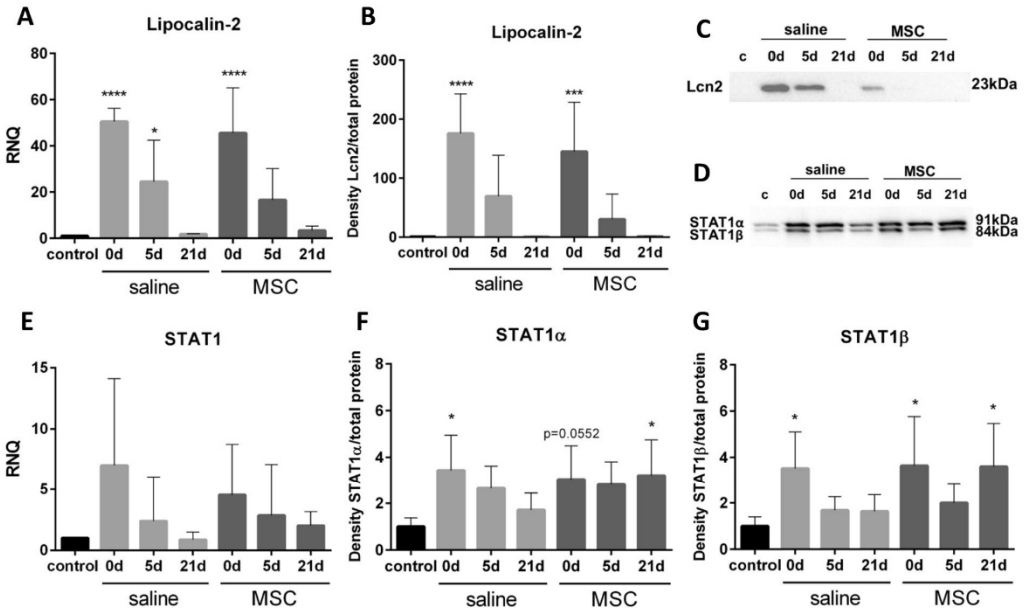


**Figure 7.** Influence of MSC. (**A**) Expression of Lipocalin-2 (Lcn2) significantly increased after DL (d0) and then gradually decreased to control level by d21 in both groups. (**B**,**C**) Western blot analysis of Lcn2. (**B**) Quantification of Lcn2 revealed a significant increase 5 min (d0), which decreased to control levels by d21 after saline and MSC injection. (**C**) Representative pictures of Lcn2 detection by western blot. (**D**) Representative pictures of STAT1α/β detection by western blot. (**E**) Expression of STAT1 slightly increased at d0 and d5. (**F**) Quantification of STAT1α revealed a slight increase at all time points after saline and MSC injection. (**G**) Quantification of STAT1β revealed a slight increase at all time points after saline and MSC injection. For western blot analysis the samples (n = 42) were run on four blots, which were processed in parallel. Full blots are provided in the supplementary data file. Data are n = 6, mean ± SD. *Represent $p \leq 0.05$, ***represent $p \leq 0.001$ and ****represent $p \leq 0.0001$ compared to control.

and eGFP over the entire timespan of the study (28 days). Overall, the results of the current study confirm that eGFP-MSC have the same properties than wt-MSC, can be tracked by a stable eGFP expression over 28 days and are therefore suitable for transplantation experiments.

A well-established model was used to induce severe ADDE in mice by ligation of the secretory duct of the LG, which has been shown to cause a profound loss of functional tissue in mice and rabbits, mimicking the tissue damage in patients with ADDE[11,13,32]. The key feature of ADDE in patients, is the abated tear secretion[2,27,33]. In the current study, a significant decrease of tear secretion confirmed the successful induction of ADDE, which was further proven by a loss of LG weight. In the course of ADDE, the reduced tear secretion causes damage to the ocular surface, which can be determined by a variety of dyes and a decreased thickness of corneal epithelium[2,27,34,35]. Although DL had no macroscopic impact on the ocular surface in the current study, visualized by fluorescein staining, the influence on the ocular surface was demonstrated by a decreased thickness of the corneal epithelium. Presumably, the period of reduced tear secretion in the mouse model was too short to cause a severe damage to the integrity of the corneal epithelium. In summary, the three-day DL resulted in clinical signs of acute ADDE with impaired functional LG tissue and tear physiology with minor impact on the ocular surface and can therefore be used as a model to study *in situ* LG regeneration.

Transplantation of MSC has emerged as a promising approach to induce regeneration of a variety of tissues and a lot of clinical trials were implemented[14,15,36]. A huge body of evidence indicates that MSC secrete trophic factors responsible for their induction of tissue repair. However, in addition to the secretion of trophic factors that affect tissue-resident progenitors or specialized tissue cells, it might be possible that MSC differentiate into the cells of the injured tissue and thereby replenish lost tissue[14]. In both cases, however, the engraftment of the MSC in the tissue of interest is a prerequisite for the successful induction of regeneration[16,37].

One of the main influences on the homing efficiency of MSC in the tissue of interest is the site of MSC delivery. In murine LG studies bone marrow MSC were applied either systemically[5] or periorbital[38] and both resulted in improved LG function. Nevertheless, it is well known that cells can get trapped, e.g. in the lung, upon systemic transplantation[14,37]. Therefore, the present study performed a locally intra-glandular injection and verified the presence of MSC within the LG. An additional major advantage of the current study was the use of traceable MSC, which was achieved by sex-mismatched transplantation of MSC isolated from male eGFP-expressing mice. This allows the detection of engrafted MSC by both their male DNA and their eGFP expression[39]. Moreover, the

detection of a male specific sequence, such as *Rbmy*, can be used to calculate the absolute number of engrafted MSC[40]. In the current study, tracking and calculation of MSC via *Rbmy* expression was done and their presence was further confirmed by the detection of eGFP in immunostaining. Accordingly, two independent tracking methods confirmed the presence of the transplanted, extrinsic MSC within the LG. The results showed that the number of MSC was high after transplantation and gradually decreased thereafter. Thus, transplanted MSC were present through acute inflammation of the LG and the initial phase of tissue repair.

The LG is composed of acinar, ductal and myoepithelial cells, which assemble into functional units (the acini). With approximately 80% of the cells, acinar cells represent the most important cell type of LG. Acinar cells produce and secrete the primary tear fluid which is a complex composition of inorganic salts, immunoglobulin A and various proteins such as lactoferrin, serum albumin, lysozyme and lipocalin[2,41]. The loss of vital acinar structures therefore leads to reduced quality and quantity of tear secretion and results in the development of ADDE. In this study we were able to prove that tissue specific MSC transplantation significantly improves the regenerative capacity of damaged acinar structures. MSC transplantation resulted in the recovery of vital acinar structures to 62% of total LG tissue, which is an increase of 25% compared to spontaneous regeneration after saline injection. The demonstrated improvement in regenerative capacity is of great clinical relevance as age-related degradation processes and chronic inflammation seems to affect the intrinsic LG regenerative capacity and therefore the initiation and restoration of LG regenerative capacity is highly desirable.

The enhanced amount of vital acinar structures after MSC transplantation confirms the high therapeutic potential of extrinsic LG-MSC and suggests that a therapeutic benefit can be achieved if the intrinsic regeneration potential is not sufficient to reinstate LG function. Our results are in line with recently published data, who showed a sustained improvement of tear secretion and a reduced infiltrated LG area after systemically bone-marrow MSC transplantation in NOD mice with autoimmune-mediated chronic DED[5].

The investigation of the intrinsic and extrinsic MSC detected by their nestin expression showed a high number on day 0 and 5, but only a low number on day 21. Differences were observed between the treatment groups, as the number of nestin positive cells on day 21 was still significantly increased after saline injection but not after MSC transplantation. Since MSC exert their therapeutic effects mainly in the initial phase of tissue regeneration, the differences in MSC count in the groups indicate that the transplantation of extrinsic MSC leads to a shortening of the initial phase of regeneration. These findings further support that MSC transplantation is beneficial for LG regeneration.

One of the main underlying causes leading to the loss of functional LG tissue and thus to the development of ADDE, is inflammation[29]. A variety of studies showed that DL resulted in an acute inflammatory reaction in SG[42,43] and LG[11,13]. In line with this, in our study a severe inflammatory reaction was also detected in form of a massive infiltration of neutrophils and macrophages at day 0 and day 5 and decreased at day 21. During this severe inflammatory reaction, the proportion of vital acinar structures is diminished and starts to regenerate when the inflammation decreases. Comparing the two treatment groups, the number of macrophages was higher at day 0 and lower at day 5 after MSC transplantation than after saline injection. This is presumably a result of the immune modulatory properties of transplanted MSC, since a variety of studies showed that MSC inhibit monocyte maturation and macrophage proliferation[16]. In general, it is assumed that modulation of the immune system is one of the key elements of MSC-mediated tissue repair[17]. One of the immunoregulatory factors secreted by MSC is IL-6, whose expression was highly upregulated upon MSC transplantation in this study. This indicates that the immunomodulation of transplanted MSC in this ADDE model also included the action of IL-6. Moreover, it was described that MSC inhibit the production/secretion of the TNFα[44,45]. This is in line with the findings of the current study, where a decreased TNFα expression was detected after MSC transplantation. Overall, the results show that the transplantation of MSC resulted in a modulation of the inflammatory reaction in the LG after DL.

The gradual loss of MSC over time in conjunction with the enhanced tissue regeneration indicates that the therapeutic effects of MSC rather rely on the secretion of trophic factors than on differentiation towards LG acinar cells. Indeed, studies of glandular tissues, others than the LG, detected that MSC-secretome or MSC-derived extracellular vesicles had a similar therapeutic effect, on the ability to maintain/restore glandular function, than the MSC itself[46–48]. Moreover, in recent studies it was shown that MSC conditioned medium had beneficial effects on injured LG epithelial cells *in vitro*[8,26]. Among others, Lcn2 and STAT1 could be identified in the secretome of MSC and were found to positively affect the viability of injured LG epithelial cells *in vitro*[26]. Therefore, the presence of these proteins was investigated in the current study.

Lcn2 expression and protein level were highly upregulated at day 0 and day 5 to comparable levels in saline and MSC injected LGs. As neutrophils also express Lcn2, it might be possible that the detected Lcn2 rather originated from the immune cells than from the implanted MSC[49]. This would coincide with the high numbers of neutrophils detected at day 0 in the current study and which were also comparable in both groups. This hypothesis is further supported by the results obtained from IL-1 induced ADDE, where Lcn2 was the most up-regulated gene at day 1 and day 2 and was accompanied by a massive neutrophil infiltration, whereas the number of MSC peaked at day 3[12,19]. Nevertheless, Lcn2 could have contributed to the regeneration of the LG as an overexpression in MSC was shown to increase their proliferative capacity, inhibit stress-induced apoptosis and induce expression of growth factors, such as HGF[50]. Consequently, Lcn2 could, in parallel to its direct effect on LG epithelial cells, influence the MSC by enhancing their regenerative effects and thus contribute to LG regeneration. Since there was a higher number of MSC in the LG directly after transplantation, the effects of Lcn2 on the MSC could have a greater impact on the MSC transplanted LG than on the saline injected LG. Consequently, Lcn2 might contribute to the improved LG regeneration after MSC transplantation.

The only difference in the protein level of STAT1 between saline and MSC injected LGs was found at day 21, where STAT1α/β was increased in MSC, but not saline injected LGs. In general, STAT1 signaling is complex and reveals somehow contrasting functions, which could be due to the large number of activating cytokines and receptors that signals through the JAK/STAT pathway as well as inducer-independent transcriptional activity of

STAT1[51,52]. Known STAT1 functions include the promotion of apoptosis, regulation of tissue remodeling, but also the stimulation of progenitor cells proliferation[51,53–55]. Tissue remodeling after acute injury is complex and involves apoptosis of various cell types, and extracellular matrix (ECM) remodeling[18]. It has been shown that PDGF induced tissue remodeling, e.g. stimulated fibroblast proliferation, collagen secretion and increased ECM synthesis involve the activity of STAT1[56,57]. The elevated STAT1 protein level on day 21 after MSC transplantation might therefore indicate that the MSC-injected LGs may enter the third phase of tissue repair - tissue remodeling. However, further studies have to confirm whether tissue remodeling occurs in the regenerating LG as soon as day 21.

In conclusion, this study revealed that the transplantation of LG-specific MSC significantly improved the regenerative capacity of LG in an ADDE mouse model. The significantly improved and accelerated regeneration after MSC transplantation compared to saline injection was demonstrated by a significant increase of vital acinar structures, a shortened presence of MSC in the LG, earlier decline of apoptotic cells, a modulated macrophage invasion and a lower number of proliferating cells during acute inflammation, a lower expression of TNFα and an increased expression of IL-6. Thus, the use of extrinsic MSC appears to be a promising approach for the curative treatment of patients with severe DED/ADDE with impaired intrinsic LG regenerative capacity.

## Methods

**Mice.** For transplantation experiments female C57BL/6J mice were obtained from Janvier labs (Le Genest-Saint-Isle, France). For the isolation of MSC, C57BL/6-Tg(CAG-EGFP)1Osb/J (eGFP) were purchased from the Jackson Laboratory (Sacramento, CA) and further bred in the Central Animal Facility (Heinrich-Heine-University, Duesseldorf, Germany). Mice were kept under 12:12 h light:dark cycle with food and water *ad libitum*. All experiments were implemented in accordance with the "Association for Research in Vision and Ophthalmology" Statement for the use of animals in ophthalmic and vision research, the national ethical committee for animal experimentation (FELASA guidelines) and were approved by the "Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen" (LANUV, IRB No. 84-02.04.2013.A268).

**Mesenchymal stromal/stem cell isolation.** MSC were isolated from male eGFP-mice (8–12 wk). After euthanasia extraorbital LGs were placed in cold culture medium (α-MEM, 2 mM L-glutamine, 15% FBS-S (all purchased from Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO)). Before mincing with a scalpel, LGs were washed thrice with PBS (Sigma-Aldrich). Minced LGs were transferred to 10 cm culture dish and allowed to attach to the surface before culture medium was added.

**Characterization of mesenchymal stromal/stem cells.** MSC were characterized according to the defined criteria[24]. LGs of 10 male eGFP-mice were used for each experiment.

Growth behavior and morphology were evaluated by inverse microscope observation and assessment of the cumulative population doubling (cpd) up to passage (p) 6.

Immunophenotyping was performed in p2 by using a CyAn ADP Flowcytometer (Beckman Coulter, Brea, CA), recording 10,000 viable single cells. In brief, detached cells were pre-incubated with anti-CD16/CD32 (BD Bioscience, San Jose, CA) for 5 min on ice. Thereafter, primary antibodies (see Table S1) were added and incubated for 30 min on ice. If intracellular staining was necessary, cells were fixed in 2% PFA for 30 min and permeabilized with 0.1% Triton-X-100 for 5 min and then proceeded as described above. Staining with the respectively labelled isotypes served as a control. Kaluza Software (Beckman Coulter) was used for analysis.

*In vitro* differentiation toward adipocytes and osteocytes was investigated in p3. In brief, 2500 cells/cm$^2$ were plated onto a 6 cm culture dish. Differentiation was induced when cells reached 70% confluency (osteogenesis) or 90% confluency (adipogenesis) by replacing the culture medium with osteogenic or adipogenic induction medium (see Roth *et al.*[8]). Differentiation was assessed after 7, 14 and 21 days by investigating the expression of osteopontin for osteogenesis or fatty-acid binding protein 4 (FABP4) for adipogenesis through qPCR. In addition, differentiation was visualized by staining with Alizarin Red S (Sigma-Aldrich) for osteogenesis and Oil-Red O (Sigma-Aldrich) for adipogenesis at day 21. Staining was performed according to manufacturer's instructions. Cells grown in culture medium served as control.

**Surgery.** Mice (8–10 wk) were anesthetizes using ketamine (80 mg/kg bodyweight (BW); Zoetis, Florham Park, NJ) and xylazine (7.5 mg/kg BW; Bayer, Leverkusen, Germany). Right extraorbital LG was exposed and the associated duct was ligated twice using a silicon tube (AS ONE Corporation, Osaka, Japan; see video 2 in Dietrich *et al.*[11]). After 3 days, duct ligation (DL) was removed by reopening the silicon nodes and $2.5 \times 10^5$ MSC in 2 µl saline were injected into the LG using a 27-gauge needle (Hamilton syringe). Injection of saline (2 µl) served as control. For transplantation experiments MSC were harvested 7 days after isolation. For analgesia, buprenorphine (0.05 mg/kg BW; Reckitt Benckiser, Slough, UK) was injected subcutaneously and tramadol (Hexal, Holzkirchen, Germany) added to the drinking water for 3 days at 1 mg/ml. The stitched wound was covered with Gentamicin ointment (5 mg/g, Ursapharm, Saarbrücken, Germany).

**Experimental setup.** Samples were collected 5 min, 5 days and 21 days after MSC transplantation or saline injection. Fluorescein staining and tear production was measured on the three time points (n = 12/time). For histological analysis, mouse eyes and LGs were excised (n = 6/time) and fixed with 4% PFA. For molecular biologic assessment, LGs (n = 6/time) were snap frozen in liquid nitrogen and RNA, DNA and protein purification was performed using the AllPrep DNA/RNA/Protein Kit (Qiagen, Hilden, Germany) according to manufacturer's manual.

**Fluorescein staining of the ocular surface.** Fluorescein staining was performed by applying 5 µl fluorescein (1.7 mg/ml Fluorescein SE, Alcon, Freiburg, Germany) to the lateral cantus of each eye. Excess solution was removed with PBS (Sigma-Aldrich). Eyes were examined using a slit lamp with a cobalt blue filter (PSL Classic, Keeler, Windsor, UK) and the defects were classified according to the Oxford Grading System.

**Tear production.** Tear production was measured using phenol-impregnated cotton threads (Zone-Quick, FCI Ophthalmics, Pembroke, MA). The threads were applied to the lateral canthus of both eyes for 60 s using forceps. In contact with tears the threads turned red and tear production was measured in millimeters under a dissecting microscope (Carl Zeiss, Oberkochen, Germany; Fig. 3A).

**HE staining.** To evaluate the thickness of the corneal epithelium, paraffin embedded sections were stained with hematoxylin and eosin (HE). Three slides, and three sections per slide, of the middle eye at a distance of 10 slides were used. Sections were photographed with a microscope (DM4000B, Leica Microsystems, Wetzlar, Germany), merged for an overview image and measured using Fiji software[58]. To compare different areas of the cornea, three measuring points were defined: upper limbus (1), lower limbus (3) and the measured center between the two points (3, Fig. 3E).

For evaluation of LG structure and acini arrangement, pictures of HE stained whole LG were investigated using Fiji software[58] and analyzed as previously described[11]. In brief, vital and severely affected acini structures were measured, and each proportion calculated based on total LG area (set as 100%). Severely affected acini were identified by a shrunken cell body with eosinophilia and irregular arrangement of acini structures.

**Immunohistochemistry.** One central section (4 µm) was used for DAB staining as described before[11]. In brief, deparaffinized and rehydrated sections were subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) for 35 min in a steam oven. Thereafter, sections were permeabilized with 0.15% Triton-X-100 for 10 min, followed by endogenous peroxidase quenching with 3% hydrogen peroxide for 30 min. Unspecific binding was blocked using 25% equine serum (Sigma Aldrich) with/without 0.25% BSA. For mouse-on-mouse staining, the sections were pre-incubated with Fab-Fragment for 1 h (Table S1). Primary antibodies were applied overnight at 4 °C (Table S1). Sections were then incubated with the corresponding biotinylated secondary antibody (Table S1) before immunoreactivity was detected using an avidin/peroxidase system with 3,3′-diaminobenzidine (DAB) substrate. Positive stained cells were counted in 20-fields of view using a graticule (grid 10 × 10 mm) in 400-fold magnification using a microscope (DM4000B, Leica) and cells per $mm^2$ were calculated.

**Immunocytochemistry.** MSC grown on slides were fixed using 4% PFA for 15 min, permeabilized with 0.1% Triton-X-100 for 5 min before unspecific binding was blocked with 0.5% BSA. Primary antibodies were applied overnight at 4 °C (Table S1). Cells were washed with PBS and incubated with the corresponding fluorescence-labelled secondary antibody (Table S1).

**Quantitative real-time PCR (qPCR).** Amplification, including melting curve, was performed on a 96 well qPCR system (7500 Fast, Applied Biosystems). Duplicates of the six biological replicates were introduced for amplification with Power SYBR Green Master Mix (Applied Biosystems). No-template-samples served as controls. Expression was normalized to ribosomal protein S6 (RPS6) as endogenous control. Data analysis was performed according to Pfaffl and calculated as normalized relative fold expression level (RNQ)[59]. Primer sequences are listed in the Supplementary Table S2.

For the assessment of MSC engraftment male genomic (g)DNA was analyzed in a 20 µl real-time PCR. Primer pairs against TATA-box binding protein (TBP) were used as endogenous control and against RNA-binding motif protein on Y chromosome (*Rbmy*) to detect male-specific DNA (Table S2). Male mouse gDNA standards were generated adding 100, 50, 1, 0.5, 0.1, or 0.05 ng male gDNA to 100 ng female gDNA and were run along with the samples. To generate the standard curve, delta threshold cycles (CT) of standards were plotted against the concentration. Calculation of male gDNA per 100 ng introduced gDNA was performed according to Hong *et al.*[40]. In brief, it is assumed that a mouse genome contains $3.4 \times 10^9$ base pairs (bp), leading to $6.8 \times 10^9$ bp for a diploid genome. In addition, a double-stranded DNA bp has a molecular weight of 645 Daltons (D), and 1 D is equal to $1.65 \times 10^{-24}$ g. Consequently, the DNA content of a diploid mouse cell is 7.2 pg ($6.8 \times 10^9$ bp × 645 D × $1.65 \times 10^{-24}$ g).

**Western blot.** Protein samples (15 µg) were denatured and separated on sodium dodecyl sulfate-polyacrylamide gels, followed by semi-dry transfer to nitrocellulose membranes (Amersham, GE Healthcare, Chicago, IL). Membranes were blocked with 5% skim milk powder or 5% BSA in Tris-buffered saline. Primary antibodies were diluted in blocking buffer and incubate at 4 °C overnight (Table S1). Horse-radish peroxidase (HRP) conjugated secondary antibodies were incubated for 1 h at room temperature. Chemiluminescence was developed using WesternBright Sirius HRP substrate (advansta, San Jose, CA) according to the manufacture's instruction and visualized using the ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA). For positive controls 40 ng recombinant murine IL-6 or TNFα (Peprotech, Rocky Hill, NJ) were run along with the samples. Total protein was stained with SYPRO Ruby according to the manufacturer's instructions (Invitrogen, Waltham, MA) and used for normalization. Analysis was performed using ImageLab 6.0.1 (Bio-Rad, Hercules, CA). Images of all blots can be found in Supplemental Material.

**Statistics.** GraphPad Prism 6 software (La Jolla, CA) was used for statistical data analysis. Values were given in means ± standard deviation (SD). Statistical analysis was performed using ANOVA with Tukey or Dunnet post-hoc test. Differences with $p \leq 0.05$ were considered as significant.

## Data availability

All data generated or analyzed during this study are included in this article, its supplementary information file or are available from the corresponding author on reasonable request.

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## Author contributions

J.D., M.R., S.M. and S.S. conception and design; J.D., L.O. and J.W. experiments and collection of data; J.D., L.O. and J.W. preparation of the manuscript; J.D. manuscript writing; S.S. financial support; S.S. and G.G. final approval of the manuscript; All authors reviewed the manuscript.

## Competing interests

J.D., L.O., M.R., J.W., G.G., S.M. and S.S. declare no financial or non-financial competing interests relevant to this article.

## Additional information

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# 4. General Discussion

The impairment of lacrimal gland function is the major cause of the development of aqueous-deficient dry eye (ADDE), which can result in the most severe disease form of dry eye disease (DED) in patients [1]. Despite the high prevalence and burden of the patients, treatment options remain palliative [2-4]. Therefore, the current thesis aimed to gain fundamental insights into lacrimal gland regeneration and the therapeutic efficacy of lacrimal gland-derived MSC as a potential curative treatment approach for *in situ* lacrimal gland regeneration.

The lacrimal gland, like other glandular tissues, retains its regenerative capacity throughout its lifetime and the functional tissue can completely recover [5-8]. However, under chronic pathological conditions or due to age-dependent degeneration, the intrinsic regeneration capacity can be impaired. As a result, experimental approaches that aim to induce *in situ* lacrimal gland regeneration and re-instate lacrimal gland function may offer a very promising therapeutic strategy.

## 4.1 Aqueous-deficient dry eye mouse models

For the understanding of lacrimal gland function, impairment and regeneration a suitable model is essential. For the development and investigation of experimental approaches addressing the underlying pathology of lacrimal dysfunction, a model that reflects the clinical appearance of ADDE is required. The first step towards lacrimal gland regeneration was to establish a mouse model demonstrating the proposed requirements (Paper I [6]):

- Exhibit clinical signs of ADDE
- Introduce a reproducible damage to the lacrimal gland tissue
- Allow a (spontaneous) regeneration of functional lacrimal gland tissue *in vivo*
- Enable a detailed investigation of damage and regeneration (time scale)
- The mechanisms of damage should mimic the pathologic signs observed in lacrimal glands in disease state

Two mouse models with experimentally induced ADDE, the IL-1α injection model and the duct ligation model, were investigated and compared for their ability to address the requirements.

The intraglandular injection of IL-1α was established as a mouse model of experimentally induced ADDE by Zoukhri and colleagues [8]. Upon binding to its signaling receptor IL-1R1, the proinflammatory cytokine IL-1α activates the expression of several genes that are involved in inflammation and causes tissue destruction [9, 10]. IL-1α also activates tissue resident macrophages to produce cytokines that lead to the recruitment of myeloid cells.

After reaching the site of injury, the myeloid cells are stimulated to produce IL-1α and IL-1β by an IL-1α-dependent mechanism, which sustains and amplifies the effect of IL-1α [9]. In the lacrimal gland the injection of IL-1α resulted in the inhibition of neural and agonist-induced secretion as well as an inflammatory reaction [11, 12]. Injection of IL-1α into the lacrimal gland induces severe, but reversible inflammation of the lacrimal gland consisting mainly of monocytes and neutrophil granulocytes [8, 13]. This is in line with our results, in which the injection of IL-1α into the lacrimal gland of male C57BL/6J mice also caused an acute, but short inflammatory reaction consisting mainly of granulocytes and macrophages (Paper I [6]). As a result, a moderate destruction of acinar cells was found, without the detection of apoptotic markers. This is different to the findings of Zoukhri and colleagues who discovered a massive destruction of acinar cells by apoptosis and autophagy [14]. Altogether, the pathological changes in the lacrimal gland led to a reduction of tear secretion in female C57BL/6J and BALB/c mice [8]. In contrast, no clinical signs such as increased fluorescein staining of the cornea or lacrimal gland weight loss were detected in IL-1α induced ADDE in male C57BL/6J mice (Paper I [6]). Overall, the damage induced in the lacrimal gland was only moderate and resulted in a complete regeneration within five days, which was less pronounced in our model compared to other studies [8, 13, 14]. This was also reflected in the shortened regeneration period. Reasons for the weak progression in our model might be differences in sex and mouse strain (compared to other studies), the application of murine instead of human IL-1α as well as the unilateral instead of bilateral treatment of the lacrimal gland [2, 15-17].

The model of duct ligation was initially established in the salivary gland. Here, the ligation of the duct induced a pronounced damage to the gland structure and function [18-22]. Since composition and function of lacrimal and salivary gland are similar, it is reasonable to assume that the duct ligation causes comparable damage to the lacrimal gland. This was confirmed by Liu *et al.* in a mouse model and by Lin *et al.* in a rabbit model [7, 23]. Analysis revealed a destruction of acinar cells and a severe inflammatory reaction, which was accompanied by a loss of tears and lacrimal gland weight [7, 23]. Throughout the current study, duct ligation was performed on male and female C57BL/6J mice to induce ADDE by atrophic damage of the lacrimal gland (Paper I [6], Paper III). The evaluation revealed a massive damage of acinar structures associated with an increase of apoptosis. Tissue destruction was accompanied by a severe inflammatory reaction consisting of infiltrating neutrophils, macrophages and, to smaller extent, of T cells (Paper I [6], Paper III). Moreover, duct ligation resulted in a loss of lacrimal gland weight and a reduced tear secretion. The impairments of the ocular surface, detected by fluorescein staining and changes in the thickness of the corneal epithelium, were minor. Overall, the evaluation of the duct ligation

model in the lacrimal gland showed that the impact on structure and function are similar to those observed in the salivary gland and in previous studies on lacrimal gland [20, 22, 23].

The re-opening of the duct ligation of the salivary gland after seven days led to a recovery of the acinar cells and a decrease in inflammation within the following 14 days [20]. This is presumably due to the ability of the salivary gland to regenerate, which it retains throughout its lifetime. In our studies, the re-opening of the duct resulted in a partial recovery of vital acinar structures, a reduction of inflammation and an increase in tear secretion within 21 days (Paper I [6], Paper III). The re-opening was performed after three days for female mice and after seven days for male mice. The spontaneous regenerative capacity was high and resulted in a recovery of vital acinar structures to about 50% of total lacrimal gland area. For comparison: in untreated lacrimal glands about 90% of the total area consists of vital acinar structures (Paper I [6], Paper III).

In patients, the pathology of ADDE can be further divided into two groups either with or without Sjögren´s syndrome. However, in terms of clinical signs and symptoms, both subtypes are similar [24]. In either case, the progression of ADDE is chronic and patients suffer from burning and itching sensation, blurred vision and ocular surface inflammation [25]. Lacrimal gland pathology is characterized by a progressive lymphocytic infiltration with mainly T cells and, to a smaller extent, with B cells as well as a destruction of lacrimal gland tissue presented by an abnormal lobular structure, fibrosis and atrophy of acinar cells [26-29]. A comparison of lacrimal gland structure between patients with ADDE and the IL-1α model shows that the destruction of the lacrimal gland tissue is similar, but less pronounced in the mouse model. The most important difference is the time response and duration of effects. While the tissue destruction in patients with ADDE is progressive, in the IL-1α mouse model it is reversible and only short-term. The inflammatory reaction was different as neither T cells nor B cells were detected in the IL-1α injected lacrimal gland (Paper I [6]). Overall, the pathology of IL-1α injection into the lacrimal gland seems to represent an acute inflammation as in dacryoadenitis rather than a chronic inflammation as in ADDE. When comparing the lacrimal gland pathology between patients with ADDE and the duct ligation model, the results regarding the lymphocytic infiltration and destruction of lacrimal gland tissue were comparable. Hence, the lymphocytic infiltration of duct ligated lacrimal glands consisted of mainly T cells and a few plasma cells. However, (neutrophil) granulocytes and macrophages were the main inflammatory cell types in the duct ligation model, which are not reported in lacrimal glands of patients with ADDE. These two cell types are associated with an acute inflammation [30]. This may be due to the fact that an acute inflammatory reaction also occurs initially after duct ligation and that only a few weeks were considered in this study. In contrast, the examined lacrimal glands of the patients have an inflammation

that has persisted already for months or years. Overall, the comparison between patients with ADDE and the established mouse models leads to the conclusion that the duct ligation model is better suited to represent the lacrimal gland pathology of patients with ADDE.

During the studies on the duct ligation model (Paper I [6], Paper III) profound differences in the (patho-)physiology of the lacrimal gland between male and female C57BL/6J mice were detected. Remarkably, the weight of the healthy lacrimal gland in male mice was about twice that of female mice (same age, strain and housing). Similar results were obtained from sex-related reports showing that male specimens had a higher lacrimal gland weight in humans [31] and a larger acinar area in humans and rodents [32]. In contrast, the body weight was only about 25% higher in male mice than in female mice (data not shown). Interestingly, the measured tear secretion of female mice was comparable to that reported of male C57BL/6J mice in an equivalent setup [23]. In humans, also no differences in tear secretion between men and women have been reported so far, and the clinical definition of a low secretion (Schirmer test ≤ 5 mm/5 min) applies to both sexes [2, 33].
Female mice were more susceptible to the damage caused by duct ligation, resulting in a more severe outcome (Paper I [6], Paper III). This is consistent with epidemiological studies that report a higher prevalence of DED in women [2, 4]. Reasons could include differences in the anatomy and physiology of the lacrimal gland, meibomian gland as well as the cornea [2]. For the lacrimal gland histopathologic changes like diffuse fibrosis and atrophy were observed more frequently in older woman than in older men [34]. In addition, greater expression of asialoglycoprotein receptor 1 was found in the lacrimal gland of female mice, which is associated with the uptake of hepatitis C virus [35, 36]. In fact, DED is a common accompanying symptom in patients with chronic hepatitis C virus infection and is characterized by a reduced tear secretion and a decreased tear lactoferrin concentration [37, 38]. In the current thesis, sex differences became visible, for example, from the results of duct ligation. After three days, the remaining vital acinar structures in female mice represented only 5% of the total lacrimal gland area, as opposed to about 45% in male mice. Moreover, macrophage infiltration and presence of apoptotic markers were raised in female mice. The more pronounced loss of vital acinar structures may cause the elevated inflammation and apoptotic reaction, and this may be due to the observed differences in lacrimal gland weight. Since the tear secretion is similar, the accumulation of tear fluid after duct ligation should also be comparable. This would lead to an increased pressure atrophy-related damage in female mice due to their smaller gland. Other influences contributing to the susceptibility of women include sexual hormones such as androgens, whose decreased concentration as well as presence of unusual metabolites in the plasma are considered as key biomarkers for DED [2, 39, 40]. Severe changes in structure and function, genes expression and protein levels as well as secretory activity upon androgen exposure were

reported for the lacrimal gland [2]. In turn, androgen deficiency resulted in lacrimal gland dysfunction and the development of ADDE. It seems likely that androgens also influence the prevalence of ADDE in mice, as it has been shown that androgen deficiency or exposure affects lacrimal gland function and secretion [41, 42]. Moreover, it was reported that some of the androgen-induced changes were sex-specific, suggesting an association with sex-specific susceptibility. In conclusion, female mice showed a higher severity of lacrimal gland damage after duct ligation than male mice. For this reason, this model might also be suitable for investigating sex-specific response to different therapies.

In summary, the IL-1α model showed few clinical signs of ADDE, had slight similarities of ADDE pathology to patients, and was difficult to investigate in detail due to the short lacrimal gland damage and regeneration time frames. However, the induced damage was reproducible and allowed a subsequent spontaneous regeneration. Although the IL-1α model appears to be more physiological than duct ligation, laboratory suitability (period of possible investigation) must be considered. Overall, the IL-1α model seemed to display signs of an acute dacryoadenitis rather than a manifested DED. In summary, the duct ligation model showed some of the clinical signs of ADDE, introduced a reproducible damage, allowed a subsequent spontaneous regeneration, enabled a detailed investigation of damage and regeneration and showed similarities to ADDE pathology in patients including female susceptibility, resulting in a robust and highly eligible model for such types of diseases.
Consequently, the duct ligation model is considered more appropriate to the proposed model requirements and seems to be suitable for investigating experimental approaches for *in situ* lacrimal gland regeneration. Another advantage of this model is that the severity of lacrimal gland damage can be controlled by the selectable day of duct re-opening, which allows for further variations and modifications.

## 4.2 Mesenchymal stem cells residing in the lacrimal gland

Friedenstein and colleagues were the first to describe a stromal stem cell population residing in the bone marrow capable of self-renewing and differentiation into mesenchymal lineages [43, 44]. Investigations on these cells revealed their presence in a variety of adult tissues and their multipotent differentiation potential, including differentiation towards cardiomyocytes [45, 46], neuron-like cells [47, 48] and also to secretory salivary gland cells [49-51]. In the course of time, these cells were considered to be mesenchymal stem cells (MSC).

MSC exhibit a variety of properties that appear to be favorable in regard to clinical application, including (described in Review I [52], Review II [53]):

- Multipotency
- Low immunogenicity (facilitating an allogenous transplantation)
- High availability

The therapeutic relevance of MSC has been confirmed in various pathologic conditions [54, 55] and as a result, over 700 clinical trials using MSC are currently listed in the U.S. National Library of Medicine by the National Institute of Health (NIH) [56].

Investigations revealed that a stem/progenitor cell population with mesenchymal origin also resides within the lacrimal gland [7, 14, 23]. Beside the presence of these cells in healthy lacrimal glands, it could be detected that their number increased after experimentally induced damage [7, 14, 23]. These cells were detected by their nestin expression, a commonly used marker for multipotent stem cells, and their fibroblastic morphology [43, 57]. Overall, these results suggest that MSC contribute to lacrimal gland physiology and are promising for the treatment of lacrimal gland damage.

The presence of this presumed MSC population was investigated in the damaged and regenerating lacrimal gland after IL-1α injection or duct ligation (Paper I [6], Paper III). A population of stem/progenitor cells was detected as nestin-expressing, spindle-shaped cells located in the stroma adjacent to lacrimal gland lobules. These results are in accordance with previous studies. In addition, the results support the hypothesis that MSC contribute to the regeneration of the lacrimal gland as their number increased after IL-1α injection or duct ligation induced damage. Since tissue-specific differences between MSC sub-populations have been described, the application of lacrimal gland-derived MSC seems to be the most promising source (orthotopic transplantation) to determine the therapeutic efficacy of MSC for *in situ* lacrimal gland regeneration [58-60]. This would address the clinical demand to develop a curative approach to treat ADDE.

## 4.3 Isolation of mesenchymal stem cells from the lacrimal gland

The presence of MSC in the lacrimal gland and their increased number associated with lacrimal gland regeneration, reinforce the assumption that MSC may be relevant in the treatment of lacrimal gland damage as described for other tissues [61, 62]. The investigation of MSC as a therapeutic approach requires the isolation of a pure and specific population of these cells from the lacrimal gland. Therefore, two reliable isolation protocols, explant culture and fluorescence-activated cell sorting (FACS), were established and directly compared concerning the purity of MSC population.

Despite the multitude of (pre-)clinical investigations that have been conducted, there is no marker to define the MSC phenotype. It is still necessary to characterize these cells by

several *in vitro* assays. To ensure consistency in the characterization of MSC the "International Society for Cellular Therapy" has defined certain minimal criteria [63]:

- Plastic-adherent growth
- Expression of specific cell surface marker such as CD90, CD105 and CD44
- Multipotent differentiation capacity *in vitro* into adipocytes, osteoblasts and chondroblasts

The isolation of putative stem/progenitor cells from healthy and injured lacrimal glands was previously performed by explant culture or enzymatic digestion [64-67]. Due to the extensive characterization according to the defined criteria [63], these cells were considered to be MSC. In the current study, MSC were isolated from healthy lacrimal glands by FACS and compared to the established explant culture (Paper II [68]). Prospective FACS isolation was established to overcome the limitation of possible ineffective purification after explant culture isolation, which was previously described to result in a heterogenous cell population [65-67, 69]. For FACS-assisted isolation platelet-derived growth factor receptor alpha (PDGFRα) and stem cell antigen 1 (Sca-1) were used as positive markers. The hematopoietic proteins CD45 and Ter119 were used as negative markers. This resulted in the PαS MSC population as previously described for bone marrow-derived MSC by Houlihan and colleagues [70]. In the current study, isolated MSC exhibited a spindle-shaped fibroblastic morphology and a linear growth behavior over the investigated six passages that were characteristic for MSC [43, 59, 64]. Stemness was verified by the expression of markers, such as nestin and Sca-1 as described before for lacrimal gland specific MSC [64-66, 71]. Stemness was also confirmed by colony formation assay. The MSC phenotype was characterized by the presence or absence of defined markers, which was similar in both investigated populations (explant vs. PαS) and comparable to other studies from lacrimal gland [64], pancreas [72] as well as bone marrow and adipose tissue [59, 60]. Moreover, *in vitro* differentiation towards adipocytes and osteocytes was assessed and confirmed by specific staining protocols and marker gene expression in the current study.
In summary, a pure and specific MSC population with the characteristic phenotype could be isolated from the lacrimal gland both by explant culture and FACS.

For pre-clinical applications, the use of genetically modified MSC, which express a marker that enables monitoring of the transplanted cells throughout a study, could be of interest. Nevertheless, the genetical modification might alter the behavior or function of the engineered cells. Therefore, MSC isolated from lacrimal glands of eGFP-expressing mice (eGFP-MSC) were characterized in the current study (Paper III) and compared to previous results from wildtype MSC (Paper II [68]). eGFP-MSC showed the fibroblastic morphology, a linear growth behavior, the expression and absence of the characteristic marker and the

ability of adipose and osteocyte differentiation *in vitro*. Overall, the results obtained from eGFP-MSC were comparable to those obtained from wildtype-MSC from the lacrimal gland in previous studies [64, 65, 68] and to MSC from other tissues [59, 72]. In addition, it was shown that the expression of eGFP was stable over the investigated time period of 28 days. Thus, the traceable eGFP-MSC can be used for pre-clinical transplantation experiments, which is a major advantage compared to other studies.

From an ethical point of view, the use of MSC for clinical application seems to be favorable, as these cells can be isolated from a variety of adult tissues, such as bone marrow, adipose tissue [59, 60] and pancreas [47, 73]. The wide distribution of the MSC in the adult human body implies a high availability of these cells through some easily accessible tissue sources, compared to other stem cells. Especially, the isolation from adipose tissue appears to be a promising cell source for clinical application, as it is easily available from liposuction, which is one of the most performed cosmetic surgical procedure [74, 75]. The application of adipose tissue-derived MSC could also overcome the potential risks of allogeneic transplantation, as autologous tissue is easily accessible. This would be desirable, although only low immunogenicity of MSC is described due to a lack of major histocompatibility complex (MHC)-class II expression (Review II [53]). Nevertheless, other MSC sources are also of high clinical interest and GMP-compliant MSC biobanks have already been established. This seems to be particularly important as MSC, isolated from different tissues, appear to be sub-populations of each other. Differences between sub-population were described concerning their phenotype [59, 60, 76], but also their therapeutic potential [77, 78]. Therefore, it is suggested that the application of orthotopic MSC seems to be superior to the application of ectopic MSC. However, with regard to the low availability of human lacrimal gland tissue, the use of lacrimal gland-derived MSC seems to be challenging and the therapeutic relevance of ectopic MSC needs to be determined for lacrimal gland regeneration in future experiments.

In summary, isolated putative stem/progenitor cells from murine lacrimal gland by explant culture or FACS revealed the MSC characteristic phenotype without any detectable differences between both populations. Accordingly, MSC could be isolated “on-demand” for clinical application by FACS isolation or for pre-clinical investigations by the gentler and more economic explant culture. Another achievement was the isolation of traceable MSC isolated from the lacrimal gland of eGFP expressing mice, allowing to easily monitor these cells in pre-clinical studies. The application of MSC in clinically relevant settings, seems to be very promising due to their favorable properties.

## 4.4 Mesenchymal stem cells for lacrimal gland regeneration

Investigations throughout the current study revealed that MSC reside in the lacrimal gland, increase in number during lacrimal gland damage/regeneration and can be easily isolated (Paper I [6], Paper II [68], Paper III). Collectively, this indicates that MSC contribute to the regeneration of the lacrimal gland. Many clinical studies have already demonstrated the therapeutic efficacy of MSC from other tissue sources [54]. In addition, it has been shown that glandular tissues, other than the lacrimal gland, benefit from MSC treatment (reviewed in [79, 80]). Therefore, transplantation of MSC into injured lacrimal glands appears to be a promising approach to enhance the *in situ* regenerative capacity and re-instate lacrimal gland function. The properties of MSC that are likely to promote regeneration of injured tissue include, but are not limited to:

- Secretion of trophic factors
- (Trans-)differentiation into specialized tissue cells
- Modulation of the immune system

In order to test the hypothesis, that MSC transplantation enhance lacrimal gland regeneration and re-instate lacrimal gland function, the previously gained knowledge regarding the ADDE mouse models and MSC isolation protocols were combined.

Duct ligation was performed for three days in female C57BL/6J mice and eGFP-MSC, isolated from the lacrimal gland of male mice, were injected immediately after releasing the ligation. Analysis revealed that the amount of vital acinar structures was significantly increased compared to spontaneous regeneration without MSC injection (Paper III). The shortened presence of MSC (intrinsic and extrinsic) in the lacrimal gland and the earlier decrease of apoptotic cells indicate an accelerated and more effective initiation of the regeneration period. In addition, macrophage invasion and the number of proliferating cells during acute inflammation were reduced after MSC transplantation. Overall, the transplantation of MSC had a therapeutic effect on lacrimal gland regeneration in this surgically induced ADDE mouse model. The results obtained are in line with previous studies that described improved tear secretion and alleviation of the symptoms of chronic DED in mice and dogs [81-83]. Since the etiopathogenesis and prevalence of DED is comparable in dogs and humans, the proof of therapeutic efficacy of MSC transplantation into the lacrimal gland of dogs is of great clinical importance [82-84]. In mice, the therapeutic efficacy of MSC on diseased lacrimal glands was demonstrated by intraperitoneal injection of bone marrow MSC (BM-MSC) into a mouse model of Sjögren´s syndrome [81, 84]. In this setup the treated lacrimal glands were histologically analyzed. A reduction of inflammation, an increase of regulatory T cells (Tregs) and an elevation of aquaporin 5 gene expression, which is essential for fluid secretion, were observed [81]. In particular, the

effects on tissue inflammation are of clinical interest, as inflammatory reactions are one of the key mechanisms contributing to lacrimal gland pathology [25]. Furthermore, Weng and colleagues were able to improve the symptoms of patients with DED secondary to graft-versus-host-disease (GvHD) through the intravenous injection of allogeneic BM-MSC without the detection of any adverse events [85]. Some studies also reported a protective effect of MSC transplantation on lacrimal gland function in mice and rabbits [86, 87]. In these two studies, an immune reaction was experimentally induced and treated with MSC isolated from adipose tissue or bone marrow. The transplantation of the MSC was carried out immediately or shortly after the induced damage and resulted in improved clinical and histological signs [86, 87]. Since the MSC were injected before clinical signs manifested, the results indicate that MSC prevented the development of immune-mediated ADDE. This could be of particular interest as a prophylactic application if ADDE is expected to develop as a secondary disease, e.g. after allogeneic hematopoietic stem cell transplantation (GvHD risk) or following irradiation therapy [4, 88]. A protective effect of MSC transplantation was also described for salivary gland function after irradiation [49, 50].

In summary, our study supports the previous results that MSC have a high relevance for the treatment of ADDE. A major advantage of our study is the use of tissue specific lacrimal gland-derived MSC as this seems to be the most promising tissue source and superior to the application of ectopic MSC. In addition, through the detailed investigation of the functional lacrimal gland area, the fate of acinar cells and the dynamic of regeneration was exposed. Reviewing the results, MSC transplantation has been shown to prevent the development of ADDE, eliminate clinical signs of acute and chronic DED and reduce inflammation of the lacrimal gland

To analyze the survival, distribution and functional effects of transplanted cells in different tissues, the tracking and detection of these cells is important. Several methods are used with variable success (reviewed in [89]). On the one hand, the use of GFP-expressing cells resulted in a reliable method for monitoring transplanted cells in several experimental settings [90-92]. On the other hand, the use of male cells allows for the detection of male-specific DNA sequence (RNA-binding motif protein on Y chromosome; Rbmy) in sex-mismatched transplantation experiments [93]. The use of male cells also provides the possibility to analyze and calculate the absolute number of transplanted cells in tissue by qPCR. In the current study detection and tracking of transplanted MSC was enabled by the use of MSC from male eGFP-expressing mice (Paper III). The results obtained were able to verify that the detection of eGFP-MSC was possible by immunohistologic staining and western blot. In addition, the detection of Rbmy by qPCR revealed that the number of transplanted MSC gradually decreased over time. In the majority of mice, only three to five cells could be detected in the lacrimal gland 21 days after transplantation (one outlier).

Using the two independent methods of eGFP and Rbmy detection, it could be verified that the MSC were successfully implanted into the murine lacrimal gland. Many studies suggest that the therapeutic effects of MSC are based on the secretion of trophic factors affecting both infiltrating immune cells and tissue-resident stem cells [94, 95]. This is consistent with our results, which also indicate that the MSC do not differentiate into epithelial acinar cells, as the male gDNA diminished over time.

Recent studies have shown that the use of MSC-secretome or MSC-derived extracellular vesicles had a similar therapeutic effect on glandular tissue regeneration as the application of MSC itself [96, 97]. Consequently, besides the direct transplantation of MSC it might also be sufficient to use the MSC-secretome or relevant proteins thereof to induce lacrimal gland regeneration. From a clinical point of view, this would be advantageous as it overcomes the potential risks associated with (mesenchymal) stem cell therapy such as immunogenicity or ectopic tissue formation by the injected cells [98, 99]. Purified therapeutics derived from cells would be a major advance for clinical application. One such possible therapeutic agent is bone morphogenic protein 7 (BMP7), which might be also beneficial for the treatment of lacrimal gland impairment, as it was shown to be involved during lacrimal gland morphogenesis and regeneration [14, 100]. Originally, discovered due to its ability to induce new bone formation as decalcified diaphyseal bone homogenate in an experimental setting in 1965, its recombinant form was approved in 2001 by the Food and Drug Administration (FDA) for the treatment of spinal fusion in patients with long bone fractures [101, 102]. The use of BMP7 is a prime example for the successful implementation of experimental approaches derived from cells into clinical practice. This pathway of development would also be conceivable for proteins of the MSC secretome, if they prove the be advantageous e.g. for damaged lacrimal glands.

In the current thesis, the secretome of lacrimal gland-derived MSC was examined by functional analysis and the protein composition was identified by shot-gun mass spectrometric analysis (Paper II [68]). Proteins present in the secretome were investigated for their potential to regenerate lacrimal gland epithelial acinar cells *in vitro*.
Conditioned medium, containing the secretome, was produced from MSC isolated by explant or FACS (Paper II [68]). The MSC were activated to secrete relevant trophic factors by establishing an inflammatory environment [61, 62, 103]. Analysis focused on proteins that might have therapeutic effects on lacrimal gland regeneration as they are involved in proliferation and/or anti-apoptotic pathways [104-112]. Lipocalin-2 (Lcn2), prosaposin, signal transducer and activator of transcription 1 (STAT1) and Ras GTPase-activating protein-binding protein 1 (Rac1) were selected to investigate their protective capacity. All six conditions, the four proteins and the conditioned medium, positively affected the survival

of damaged lacrimal gland acinar cells. Nevertheless, differences were detected in the extent and timing of protection.

The highest effect on lacrimal gland acinar cell survival was observed by adding STAT1. Signaling of STAT1 is complex and involves contrary functions such as pro-apoptotic and regenerative effects [111, 113-115]. The current results are consistent with other reports that have identified a stimulatory effect of STAT1 on the proliferation of progenitor cells [111, 112, 114]. In addition, a positive correlation between STAT1 concentration and acinar cell viability was found. This indicates that the acinar cell survival might be further improved by increasing the STAT1 concentration. Analysis of STAT1 within the lacrimal gland after duct ligation revealed an increased protein level during acute damage, which gradually decreased over time (Paper III). In contrast, the STAT1 level increased again on day 21 after MSC transplantation, which might due to the involvement of STAT1 in tissue remodeling [116, 117]. Taken together the results reinforces the assumption that STAT1 contribute to lacrimal gland regeneration.

Lcn2 exhibits a dual action associated with anti-microbial defense and growth factor effects [105, 118]. Our results further support the protective effects of Lcn2 on lacrimal gland epithelial cells (acinar cells), as described for renal epithelial cells [106, 119]. For lacrimal gland regeneration *in vivo,* it might be also beneficial that Lcn2 induce epithelial cell proliferation in quiescent cells as well as it attenuates the loss of renal function after ischemic renal injury [106, 119]. Besides the positive influence on epithelial cells, Lcn2 is also described to promote MSC survival and function [120, 121]. It was shown that a higher number of transplanted MSC could be detected in the injured kidney, when the MSC were engineered to over-express Lcn2 [121]. Consequently, the regenerative potential of engineered MSC was superior to that of normal MSC and could further enhance the renal function. This achievement could also be useful to support the therapeutic efficacy of MSC in other pathological conditions, such as ADDE.

Rac1, a small GTPase, was selected to study its effects on lacrimal gland regeneration as it is activated by many growth factors as well as being involved in stem cell associated signaling cascades such as Wnt-signaling [104]. Rac1 has been shown to promote cell proliferation and coordinated cell migration of epithelial cells from the skin [122, 123] and fetal dental papilla [124], which is essential for the re-epithelialization of wounds. *In vivo* studies on skin, oral mucosa and heart further confirmed the important role of Rac1 for regeneration, as the elimination of Rac1 or its downstream effector led an impaired wound healing [109, 110, 122, 125]. In summary, these results provide evidence that Rac1 has a regenerative effect on epithelial cells from different tissues and could therefore also promote the regeneration of lacrimal gland epithelial acinar cells. This hypothesis could be confirmed

by our result, since the addition of Rac1 to the culture medium of damaged lacrimal gland acinar cells increased their viability (Paper II [68]).

Prosaposin is the precursor of the four saposins, but also acts autonomously as secreted glycoprotein, as found in the secretome of the MSC in the current thesis (Paper II [68], [107]). Prosaposin is associated with signaling cascades of cell cycle, growth and differentiation and was also shown to act as a neurotrophic factor [104, 107, 126]. In neuronal cells prosaposin activated the mitogen-activated protein kinase/extracellular signal–regulated kinases (MAPK/Erk1/2) pathway, prevented the cells from apoptosis and induced proliferation [108, 127-129]. As prosaposin is also secreted into milk an appearance in glandular tissue is suggested [130]. Indeed, Islam and colleagues have recently been able to detect the expression of prosaposin and its receptors in the salivary and lacrimal glands of rats, indicating a role in the lacrimal gland [131, 132]. Our results support and combine the two intended functions of prosaposin, as a trophic factor and important protein in glandular tissue.

In conclusion, all four proteins investigated had a positive effect on damaged lacrimal gland acinar cells *in vitro* and could improve cell viability, suggesting their involvement in regeneration. Overall, STAT1 appears to be the most promising candidate for lacrimal gland regeneration as it increases the cell viability the most and this effect could probably be further enhanced by increasing the STAT1 concentration. Future investigations into these proteins in *in vivo* experiments must be carried out in order to elucidate their therapeutic potential for the regeneration of the lacrimal gland as well as potential toxic side effects.

Tissue repair generally consists of three successive stages 1) acute tissue inflammation, 2) new tissue regeneration and 3) tissue remodeling, that involves several specialized cell signaling pathways [30]. The overall results on the dynamics of lacrimal gland damage and regeneration after duct ligation and MSC transplantation indicate that the first two stages of tissue repair – acute tissue inflammation and new tissue formation – were accelerated compared to spontaneous regeneration. This results in an increased amount of regenerated and functional acinar structures. Consequently, the third phase of tissue regeneration - tissue remodeling – might be initiated earlier when compared to spontaneous regeneration (Paper III). This hypothesis is further supported by the increased level of STAT1 at day 21, which is known to be involved in tissue remodeling [116, 117]. In order to obtain further information on the activated signaling pathways involved in the initiation of tissue remodeling, a transcriptome analysis could be performed. Relevant targets that could possibly be induced by the external addition of an activator and thus further increase the regenerative capacity of the lacrimal gland, could be discovered in this way. Transcriptome analysis could also provide important insights into the dynamics of regeneration and the underlying pathways as well as into the role of MSC during lacrimal gland regeneration.

Collectively, transcriptome analysis could identify further relevant targets/proteins that might be advantageous for *in situ* regeneration of the lacrimal gland.

In summary, this thesis demonstrated that the transplantation of lacrimal gland-derived MSC enhance the regenerative capacity of epithelial acinar cells after duct ligation induced ADDE in mice. Therapeutic effects were seen on both, lacrimal gland structure and inflammation. In addition, proteins were discovered in the MSC secretome that had a positive effect on the survival of damaged lacrimal gland acinar cells *in vitro* and could also be detected in the damaged lacrimal gland *in vivo*. Through the detailed investigation of the functional lacrimal gland tissue, the fate of transplanted MSC over time and the involvement of proteins detected in the MSC secretome, our study was able to support and expand the knowledge on the therapeutic relevance of MSC for the treatment of ADDE. In conclusion, this thesis reinforces the hypothesis that MSC or MSC-based therapies are very promising approaches for the curative treatment of ADDE.

## 4.5 Future perspectives

The results of this thesis indicate that MSC-based therapies may result in new therapeutic approaches for patients suffering from ADDE by induction of *in situ* lacrimal gland regeneration. This is particularly important as the increase in the ageing world population increases the number of patients with DED/ADDE and age is one of the most consistent risk factors [4]. Consequently, DED is going to be a widespread disease. This implies two major challenges for the future with regard to clinical application, namely the transfer of results from young mice to older mice and also from mice to humans.

The current results in the literature and this thesis indicate that MSC-based therapies are efficient in restoring the structure and function of young lacrimal glands of mice after acute injury [68, 81]. However, ageing is associated with ongoing degeneration, inflammation and destruction of the lacrimal gland and also a decline of regenerative capacity [133]. Therefore, it is of interest to investigate the impact of MSC-based therapies on aged lacrimal glands. Two recent studies, investigating middle-aged to old dogs with a spontaneously developed DED, indicate that aged lacrimal glands might also benefit from a MSC treatment [82, 83]. Nevertheless, it is important to directly compare the therapeutic effects of MSC on young and aged lacrimal glands in a consistent experimental setting that reflects the conditions of ADDE in patients. Furthermore, it is important to elucidate the underlying mechanisms in more detail, in order conduct further experimental approaches. In addition, ageing is described to affect the proliferative capacity, morphology, differentiation potential, transcriptome and secretome of MSC and to impair their regenerative function [134, 135]. Accordingly, it could be assumed that the therapeutic effect of aged MSC for lacrimal gland

regeneration is diminished or even completely absent. Consequently, the use of young MSC donors seems to be a premise for clinical application. In cases of an autologous transplantation this would imply that only young patients can be curatively treated. In this context it should be noted that the prevalence of DED increases with age and the treatment of older patients is of high clinical relevance [4]. In this case, an allogeneic transplantation would overcome this limitation, as it enables the preparation of the MSC from young donors. As described above, allogeneic MSC appear to be safe which is currently being investigated in 144 clinical trials, including 49 completed trials [56]. The isolation of allogeneic orthotopic MSC from young donors however appears difficult as this requires surgical intervention. Another approach could be the use of ectopic MSC e.g. from bone marrow or adipose tissue. For this reason, it seems to be of particular interest to investigate the regeneration capacity of damaged lacrimal glands treated with allogeneic and ectopic MSC. In this setup it should be considered that the lacrimal gland in mice and humans differs in its histoarchitecture with regards to the structure and organization of acini lumen and stroma, the cellular proportion of the stroma as well as distribution of growth factors and cytokines [136]. As a result, the data obtained from mice needs to be carefully reviewed prior to translation to human lacrimal glands. One clinical trial currently being designed for this purpose investigates the safety and feasibility of allogeneic adipose tissue-derived MSC injected into the lacrimal gland of patients with ADDE (identification of clinical study: NCT03878628). The underlying findings on which this clinical study is based are two studies in dogs that reported a significant increase in tear production and no observed adverse events [137]. Whether the improvements are based on the regeneration of the lacrimal gland has not been reported, nor have the underlying mechanisms.

In view of a possible clinical application, the use of GMP-compliant human MSC would be highly desirable, as these could be directly introduced into first-in-man studies. The therapeutic effects of inter-species MSC transplantation are controversially discussed and failures as well as successes have already been reported [138, 139]. Whether human MSC are as efficient as murine MSC with regard to the regeneration of the murine lacrimal gland needs to be evaluated in further experiments. Collectively and with regard to a clinical application there are several requirements that need to be investigated for:

1. MSC transplantation:
- Verify if aged lacrimal gland tissue also profits from MSC treatment
- Verify if ectopic MSC are beneficial for lacrimal gland tissue regeneration
- Prove the safety of allogeneic transplantation

2. MSC-derived proteins (secretome):

- Verify the regeneration of complex 3D lacrimal gland cultures *in vitro* (spheres)
- Verify lacrimal gland regeneration *in vivo*

## 4.6 Conclusion

In this thesis, three consecutive aims were defined and achieved to provide a detailed insight into the relation between lacrimal gland regeneration and MSC after experimentally induced ADDE.

A reliable model suitable for the evaluation of lacrimal gland damage and regeneration was established by ligation of the single excretory duct of the extraorbital lacrimal gland. This model enables the detailed investigation of experimental approaches for the treatment of ADDE. In this context, the involvement of MSC in the initiation of lacrimal gland regeneration could be demonstrated by their increased number. Consequently, MSC-based therapy appears to be a promising experimental approach for *in situ* lacrimal gland regeneration. A protocol to isolate lacrimal gland specific MSC was established by FACS as well as explant culture. The gained cells were characterized in detail and revealed their MSC phenotype. The analysis of the MSC secretome discovered proteins with therapeutic effects on lacrimal gland acinar cells. As a result, STAT1 was discovered as a highly potential target that might be advantageous for *in situ* regeneration of the lacrimal gland that needs to be further analyzed in the future.
In addition, this study demonstrated that MSC transplantation could be a therapeutically relevant approach for the treatment of ADDE. Transplantation of lacrimal gland specific MSC into lacrimal glands damaged by duct ligation was able to efficiently improve the regenerative capacity of functional tissue and restore both structure and function.

Collectively, these results provide fundamental insights in the dynamic of lacrimal gland damage and regeneration as well as the time-dependent involvement of MSC. Furthermore, this study expanded our knowledge on the therapeutic efficacy of MSC for *in situ* regeneration of the lacrimal gland and the restoration of lacrimal gland function. Overall, the results reinforced the therapeutic relevance of MSC or MSC-based therapies for the curative treatment of patients with ADDE.

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# Additional Publications

## 1. Review Article

### Development of Causative Treatment Strategies for Lacrimal Gland Insufficiency by Tissue Engineering and Cell Therapy. Part 2:

### Reconstruction of Lacrimal Gland Tissue: What Has Been Achieved So Far and What Are the Remaining Challenges?

Isobel Massie*, Jana Dietrich, Mathias Roth, Gerd Geerling, Sonja Mertsch, Stefan Schrader

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Contribution of Jana Dietrich

Review of the literature, writing of the manuscript in parts

Approximated total share of contribution: 10 %

## 2. Original Research Article

### Decellularised conjunctiva for ocular surface reconstruction

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

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Contribution of Jana Dietrich

Collection and assembly of data, manuscript review

Approximated total share of contribution: 5 %

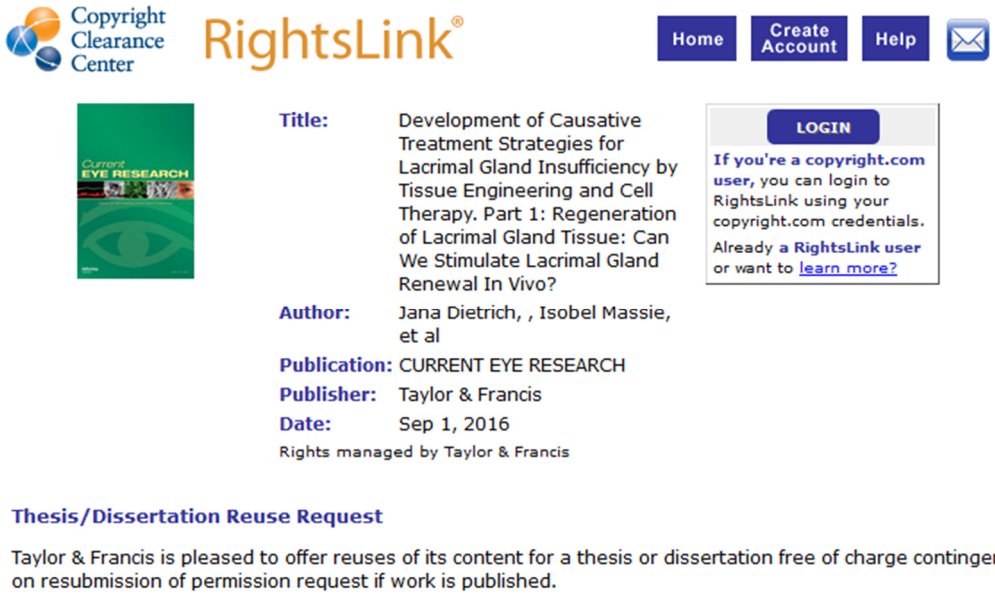
## Oral and Poster Presentations

1. Association for Research in Vision and Ophthalmology (ARVO),
   Annual Meeting 2017, Baltimore, USA, Poster Presentation
   **Evaluation of the regenerative potential of murine lacrimal gland tissue in two different models of lacrimal gland impairment**
   J Dietrich, C Schlegel, M Roth, G Geerling, S Mertsch, S Schrader
2. Biologisch-Medizinisches Forschungszentrum (BMFZ) Meeting 2017,
   Bergisch Gladbach, Oral Presentation
   **Evaluation of the regenerative potential of murine lacrimal gland tissue in two different models of dry eye syndrome**
   J Dietrich, C Schlegel, M Roth, G Geerling, S Mertsch, S Schrader
3. „Deutsche Ophthalmologische Gesellschaft" (DOG),
   Annual Meeting 2017, Berlin, Poster Presentation
   **Evaluation zweier Schädigungsmodelle zur Untersuchung des Regenerationspotentials von murinem Tränendrüsengewebe *in vivo***
   J Dietrich, C Schlegel, M Roth, G Geerling, S Mertsch, S Schrader
4. „Deutsche Ophthalmologische Gesellschaft" (DOG),
   Annual Meeting 2017, Berlin, Oral Presentation of the current project concept as part of the Sicca Advancement Award
   **Stammzell-basierte Therapieansätze zur Behandlung von trockenem Auge**
   J Dietrich, S Schrader
5. "Rheinisch-Westfälischer Augenärzte" (RWA)
   Annual Meeting 2018, Düsseldorf, Oral Presentation
   **Untersuchung des Regenerationspotentials der murinen Tränendrüse nach experimentell induzierter Schädigung in zwei verschiedenen Mausmodellen**
   J Dietrich, C Schlegel, M Roth, G Geerling, S Mertsch, S Schrader
6. Association for Research in Vision and Ophthalmology (ARVO),
   Annual Meeting 2018, Honolulu, HI, USA, Poster Presentation
   **Evaluation of the regenerative potential of the secretome of lacrimal gland derived mesenchymal stem cells isolated by explant technique and cell sorting**
   J Dietrich, M Roth, G Geerling, S Mertsch, S Schrader
7. "Deutsche Ophthalmologische Gesellschaft" (DOG),
   Annual Meeting 2018, Bonn, Poster Presentation
   **Vergleichende Untersuchung der regenerativen Eigenschaften mesenchymaler Stammzellen aus der murinen Tränendrüse isoliert über Explantkultur oder Zellsortierung**
   J Dietrich, M Roth, G Geerling, S Mertsch, S Schrader
8. "Deutsche Ophthalmologische Gesellschaft" (DOG),
   Annual Meeting 2018, Bonn, Oral Presentation of the current project concept as part of the Sicca Advancement Award
   **Experimentelle Untersuchung zur Transplantation muriner mesenchymaler Stammzellen aus der Tränendrüse zur Behandlung von trockenem Auge im Mausmodell**
   J Dietrich, S Schrader
9. Association for Research in Vision and Ophthalmology (ARVO),
   Annual Meeting 2019, Vancouver, BC, Canada, Poster Presentation
   **Transplantation of mesenchymal stem cells for lacrimal gland regeneration in a dry eye mouse model**
   J Dietrich, L Ott, M Roth, J Witt, G Geerling, S Mertsch, S Schrader

# Journal Permissions

1. Review (Review I)


2. Review (Review II)



3. Research Article (Paper I)



4. Research Article (Paper II)

5. Research Article (Paper III)

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**- DANKE -**

## Eidesstattliche Erklärung

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

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

Düsseldorf Jana Dietrich