Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

Shubha Tiwari

Under the Supervision of

Prof. Geeta K. Vemuganti

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

2015

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

CERTIFICATE

This is to certify that the thesis entitled "***Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application"*** and submitted by ***Shubha Tiwari*** ID No ***2009PHXF011P*** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

Date:

Prof Geeta K. Vemuganti
Dean, School of Medical Sciences
University of Hyderabad
Hyderabad

Acknowledgement

"***If I see any further today, it is because I stand on the shoulder of giants."***

…….Sir Issac Newton

The acknowledgement section of the thesis is probably one of the most difficult sections to write. How do I convey a sincere thanks to all those people who have been my support system… Who have inspired me, taught me, encouraged me, who have cushioned my failures and celebrated my success? To add to that feeling of inadequacy, is the fact that I probably have no words left to express any more ideas… having used all of it in discussing the experimental results!!

There have been so many individuals- so many friends, acquaintances, seniors and colleagues who have shaped my work, my thought process and my personality that I probably cannot do justice to their contribution here. Without these supporters, especially the select few that I am going to mention, I probably would not have been where I am today, at least not sanely. I hope that during the course of my PhD I have conveyed my sense of gratitude and respect to each and every one of you and that you take as much joy in reading my thesis as I did in writing it.

First and foremost I would like to express my deep sense of gratitude to my mentor and guide, ***Prof Geeta K Vemuganti, formerly Director, Ophthalmic Pathology Service, LVPEI and currently the Dean, School of Medical Sciences, UoH,*** who has been a constant source of ideas, knowledge, support and encouragement. It has been such a wonderful and enriching experience to have worked under her mentorship. Dr Geeta has taught me not just how to be a better professional but also a better human being. Her sense of purpose, commitment and ownership of work has made a deep impact on me. She has always been someone I could go to … even with negative results and botched experiments without fear of being judged and reprimanded. Her keen sense of observation and the ability to fit the pieces of experimental jig saw together are exceptional. From you Ma'am, I have learnt, actually am still learning, the art of asking the right questions at the right time and that chance favors a prepared mind. But this is not the length and breadth of the relationship that I have shared with you. There have been times when you have been my staunchest supporter, even on the

personal front. You have taught me that while it is good to have a sense of ownership it is sometimes better to let go. It's the fine line that decides your fate. Thank you for all that you have taught me and I am sure this learning curve will never plateau!

I would like to extend my sincere gratitude to ***Prof. D. Balasubramanian***, Director of Research, L. V Prasad Eye Institute. Dr Balu has been a great source of inspiration and knowledge. Sir, your depth of knowledge about everything under the sun and your zest for life is awe inspiring. It has been such an honor to have had the opportunity to be your student and to have interacted with you. Thank you so much for giving me an opportunity to be a part of the research wing, for taking a keen interest in my work and for always believing in me.

I extend my gratitude to ***Dr. G.N. Rao***, Chairman and founder Director, LV Prasad Eye Institute. Dr Rao is a true visionary whose commitment to raising the standard of eye care in India to international levels and implementing the concept of 'excellence, efficiency and equity' at every level of eye care is exemplary. It gives me immense pleasure and pride to be a part of his team, learning every step of the way and contributing in whatever little manner I did.

I take this opportunity to thank the clinicians that I have had the opportunity to interact and work with. This work would not have been possible without their support, help and inputs. Thank you very much ***Javed, Vishram, Sayan, Swati, Tarjani, Surbhi, Fairooz, Dr. Sangwan, Dr Milind, Dr Santosh, Dr Vijay Anand, Dr Gorakh, Dr Manjual, Dr Shilpa, Dr Ruchi, Dr Kaustabh and Dr Dilip. Javed*** you have been an extremely supportive colleague and a wonderful friend who took as much responsibility for my work as I did. Sometimes it was just your dedication to the work that made me do an extra set of experiments or collect additional data! Thank you for everything and I still remember what you once told me, that even though we meet very infrequently and interact only when required yet we wish the very best for each other! ***Dr Sangwan,*** I will always have the fondest memories of our varied discussions, both professional and micro/macrocosmic and I wish that at some point of time in life I would be able to adopt your cheerful and go-getter attitude! ***Dr MN***, thank you so much for believing in me at a time when I had no faith in my own abilities. It is because of your constant reassurances and humor that I am finally submitting the thesis!!

I would like to acknowledge the faculty members of Brien Holden Research Center, L V Prasad Eye Institute. Thank you ***Dr Shrikant, Dr Charanya Dr Indumathi, Dr Chitra, Dr Subho, Dr Savitri, Dr Inderjeet and Dr Usha Gopinathan*** for all your valuable suggestions and ideas. ***Charan***, thank you for all the wonderful times and the scientific discussions. Your ideas and suggestions have been truly valuable and have helped me circumvent quite a few experimental/ technical issues. ***Shrikant,*** you have been an amazing friend, teacher, counsellor and my go-to person with all my crazy ideas. One of the best things about you is that your ideas are sometimes crazier than mine! Thank you Shri for encouraging the nature of scientific curiosity in me- whether in terms of how to analyze a graph, or experimental data or how to create a 3D image, you have taught me so much. You have been instrumental in teaching me the concept of data analysis, finer nuances of scientific writing and in getting my first manuscript published. I will always have the fondest memories of our G3 club get-togethers and the discussions that always followed. You are an asset to be around and a friend for life!

I extend my sincere thanks to ***Mr. Chenchu Naidu***, ***Shridhar, Rambabu, Elizabeth*** and ***Arshya*** from Dept. of Ophthalmic Pathology Laboratory, LVPEI for the unconditional help they offered to me during the times of need. It would have been impossible for me to finish my bench-work without their help. Special thanks to ***Sridhar*** for all the help you extended in grossing and immunohistochemistry studies. Your expertise in the techniques of histological studies is truly remarkable. You have gone out of your way a number of times to help and I take this opportunity to thank you for every one of those times!

I thank the staff from various departments of L V Prasad Eye Institute. ***Mr. Srinivas, Mr. Aravind, Mr. Srinivas*** from Ramayamma International Eye Bank for providing the amniotic-membranes for research, ***Mr. Venkatesh*** from Jhaveri Microbiology Centre for being extremely helpful whenever needed, ***Ms. Banu, Mr. Venkat and M Sudhakar*** from the Library for all the help they provided in getting articles and photocopying, ***Mr Kishor Babu*** from MRD for help in accessing medical records ***Mr.Srikanth, Mr. Shiva, Mr. Mahendra*** from the Information Systems Dept., for round the clock uninterrupted internet service; ***Ms. Aparajitha, Mr. Ali*** (Stores) and ***Mr. Jagdish*** (Purchase) for their help with procurement of chemicals and reagents.

I thank ***Hasnat*** from the Biosatistics Dept., for his help with statistical analysis.

I would also like to extend my warm thanks to ***Sarika, …. and Karthik*** from the Communications Dept. for all the help in making posters and for all the fun times we had together. ***Sarika,*** thank you once again for all the interesting talks and the good times we shared!

I take this opportunity to sincerely acknowledge the help extended by ***Jai Ganesh, Moddin and Elena*** in matters relating to my fellowship and PhD tenure. Had it not been for ***Jai Ganesh and Moddin*** I don't think I would have ever met any fellowship or application deadlines! Thank you very much for your support and cheerful manner in which you accommodated every one of my last minute requests!

I would like to convey my sincere gratitude to ***Dr. Krishnamurthy*** (NCBS, Banglore) for his help and suggestions with flow cytometry experiments; ***Dr. Paresh Jain*** and ***Peter*** (BD Biosciences) for help with flow cytometry related matters.

I would also like to thank Dr. Robert P Coppes (University of Groningen) and Dr. David A. Sullivan (Schepens Eye Research Institute) for their helpful suggestions and ideas.

I would like to take this opportunity to thank the faculty at the School of Medical Sciences, UoH: ***Dr. Rishi, Dr. Shammana, Dr. Siddique, Dr. Mahadev, Rupa and Varalakshmi*** for their help and support. I would also like to thank ***Shiva, Mrs. Adhilakshmi, Jayant and Mrs. Shobha*** for their help. I would like to extend my sincere thanks to ***Dr. K. Chatti*** (Institute of Life Sciences), ***Vamshi, Prof P. Redanna (UoH), Prof. P. Babu (UoH), Suraj, Roy, Geetika*** for their help with tissue culture lab and facilities.

I extend my sincere thanks to ***Dr. Navin, Dr. Hemanth Jadav, Mr. Dinesh Kumar, Ms. Monica Sharma, doctoral committee members and the staff of RCD,*** BITS Pilani for their help and co-operation. I would also like to thank the **Dr Jayati Ray Dutta**, **DAC members- Prof Vidya Rajesh and Dr Kumar Pranav Narayan; the DRC members-Prof Suman Kapoor and Dr Palash Mandal** for their insightful

comments on my draft thesis and for facilitating the entire process of thesis submission.

Even though I am mentioning these names at the end of the section, yet these were the people who have contributed the most towards my work; whether in terms of professional advice, personal support or by just being who they are. Thank you very much ***Saritha, Rachna, Rohini, Sagar, Purush, Pulla Rao, Anusha, JayLakshmi, Rahul, Praseeda, Sindhura, Guru, Subhash, Ganeshwar, Soumya, Savitri, Venu, Kiranmai, Subba Rao, Neeraja, Nageshwar, Kiran, Surya and Sandhya. Saritha and Rachna,*** more than half the credit for this thesis goes to you guys! Had it not been for you I would have long abandoned post and given up trying to get any useful information out of the seemingly senseless data that I was generating. It was your constant prodding and belief in me which kept me going. Words fail me when I try to articulate what you guys mean to me…..thank you for everything and I hope I never find you more than a phone call away! ***Rohini,*** you have been more than a junior to me…. more like a family member. Your constant support, not just emotional, but actually stepping in and taking care of things for me, has kept me going on numerous occasions. The scientific discussions with you as well as our seemingly senseless talks have actually resulted in some good experimental results! Your sense of honesty and purpose and your zest for learning will definitely take you to great heights! Thank you for all that you have done for me, both personally and professionally, and no matter where I am I will always have a room for you in my house! ***Sagar,*** thank you for all the help with flow cytometry and for patiently listening to my experimental ideas. You are a great teacher and a good friend who could never let me feel low for more than ten minutes. Your moonwalks and comic routines were a great stress busters and I will always have the fondest memories of those times.

I would also like to thank my friends ***Ankur, Rekha, Vaishali, Vaibhav, Sargam and Nitin*** for all their help in times of need and for always being there to remind me that there is more to life than the cold walls of tissue culture lab. ***Ankur, Rekha and Vaishali***, thank you very much for stepping up and taking care of Raghav as and when required and for keeping me motivated and focused.

I would also like to thank the funding agencies ***International Atomic Energy Agency (IAEA), Champalimaud Foundation, Hyderabad Eye Research Foundation***

(HERF), Department of Biotechnology (DBT), Department of Science and Technology (DST)-Purse, Council for Scientific and Industrial Research (CSIR) and the Tear Film and Ocular Surface Society (TFOS).

I would like to acknowledge my ***parents, parents-in-law, Bhawesh, Sudhi and Gaurav***. Thank you so much for being such an amazing support system. There was never a time in my life when I have felt truly alone. Ma and Papa, I don't think I can ever articulate what you mean to me. It is because of your value systems, love and unconditional support that I have become the person that I am today. If I can ever become half as good a professional and human being that you are, then I would consider myself successful!

Lastly, but most importantly I would like to acknowledge my husband ***Sumit*** and our son ***Raghav***. You guys have been my anchors, my life support and my reason to smile even on difficult days. Sumit, had it not been for your support, I would never have been able to achieve this dream. Your love and support have always kept me going. You bring out the best in me (and the worst at times!) and make me believe in my own potential. We make a great team! Thank you for being there and for putting up with my idiosyncrasies. I hope the submission of this thesis gives you the same hope for a saner spouse as it does to me!!

ABSTRACT

The human lacrimal gland, one of the exocrine glands in the body, plays an important role in lubricating and hydrating the ocular surface epithelium. The susceptibility of the gland to immune mediated insults, radiation induced damage and age related atrophic changes, which cannot be satisfactorily managed with the current line of therapeutics, causes high incidences of dry eye related morbidity in the population (7-33%). Though the tear film is contributed by three different glands and tissue, our focus has been towards lacrimal gland since, aqueous deficiency contributes to nearly quarter of cases of DES. The current management options provide only temporary and short term relief to the patients. This raises a need for alternate and long-term management options like cell replacement therapy with functionally competent cells. The present study tries to establish a successful method of isolating, culturing and characterizing the human lacrimal gland cells and providing proof of concept of preserved function and presence of 'stem-like cells'.

The present study was approved by the Intuitional Review Board (IRB) of the L V Prasad Eye Institute, Hyderabad. Fresh human lacrimal gland was harvested after obtaining written informed consent from patients undergoing exenteration surgery for therapeutic indications. A total of 30 normal lacrimal glands and 3 post-radiated lacrimal glands were used in the study. Human lacrimal gland cultures could be established from the normal tissue as adherent monolayer on matrices like denuded human amniotic membrane, collagen I and Matrigel™; with formation of spherules on the monolayers and the attempt to form duct-like connections between them. These

cultures could be maintained in-vitro for 30-35 days, passaged for 3-4 passages, cryopreserved as well as revived with a revival efficiency of 60% for use at a later date. Epithelial cultures could not be established from the post-radiated tissue under our culture conditions.

We could also establish 3 D spherule under serum free conditions with enriched population of stem-like cells, as well as differentiated cells with secretory capacity. These were termed as 'lacrispheres' similar to the term coined for exocrine salivary gland- salispheres. Though not evaluated for their in-vivo function after transplantation, we speculate that they have a higher propensity to restore 3D structure and function.

Immunophenotyping of the cultured cells (with normal human lacrimal gland as control) indicates the presence of polymorphous population of cells- epithelial (acinar and ductal), myoepithelial as well as mesenchymal, similar to that present in the native gland, suggesting that all these cells could be proliferated in-vitro. It was also observed that the percentages of epithelial and mesenchymal cells tend to change with prolonged in-vitro culture. The percentages of epithelial cells reduce from 14.8±3.45% to 2.7± 1.7% (EpCAM positivity by FACS); while on the other hand the proportion of mesenchymal cells increases from 2.9±.91% to 13.3±10.2% (CD90 positivity). These observations, we believe, would be useful in establishing enriched population of epithelial cells vs mesenchymal cells.

Since our long term goal is to explore the possibility of a replacement therapy, the next logical step in in-vitro culture of lacrimal gland cells was to evaluate the synthesis and secretory potential of these cells. Under the present culture condition of

Matrigel™ coating as ECM and EGF supplemented HepatoSTIM media we provide the first evidence that the human lacrimal gland cells could maintain their synthesis and secretory potential in the culture system. The mRNA for the three tear proteins considered in the present study (scIgA, lysozyme and lactoferrin) as well as mucin and aquaporin 5 were found to be present in the cultured cells indicating the potential of these cells to synthesize and secrete the proteins. The proteins were also detected and quantified (by sandwich ELISA) in the conditioned media indicating the potential of the cultured cells to secrete it into the culture media (conditioned media). The results indicate that the secretion of these proteins ranged from 47.43 to 61.56 ng/ml of scIgA, 24.36 to 144.74 ng/ml of lysozyme and 32.45 to 40.31ng/ml of lactoferrin on Matrigel™ coated dishes (Table 3.6) in day 7 cultures. These cultures retained their secretory ability till day 21. The amount of protein secretion increased from day 7, peaks at day 14 of in-vitro culture and then declined by day 21.

To address the question whether the human lacrimal gland and the cultured cells would have stem-like cells, we explored the possibility using CD117 as a stem cell marker, ALDEFLUOR™ assay, label retaining studies, clonal assay and cell cycle analysis. While the presence of such cells is known in breast, pancreas and salivary gland such information is not forthcoming in human lacrimal gland. In view of this our results documenting the presence of stem cells in human lacrimal gland cells is an important contribution. The results show that the freshly isolated cells of the native human lacrimal gland, when analyzed by flow cytometry, show 6.7±2.0% of the cells to be positive for the stem cell marker CD117. CD117 was seen in both acinar and ductal compartment with varying pattern of basal and baso-lateral staining.

However our attempts to localize them in-situ were inconclusive from the limited study of the histology and immunophenotyping. The cultures, in addition to showing differentiated cells (epithelial, myoepithelial and mesenchymal) also showed the presence of stem-like cells constituting 0.2±0.05% of total cells by DIV 14. The proof of stem-like cells was also substantiated by label retaining studies, clonal assay and ALDEFLUOR™ studies. The proportion of stem-like cells reduces to 0.13±0.03% CD117 by DIV 21. This declining percentage of stem cell population under prolonged culture conditions prompted us to attempt optimizing the in-vitro conditions further. Modification of the culture conditions and serum withdrawal led to generation of 3D lacrispheres. These lacrispheres, on flow cytometric evaluation, showed higher percentage of CD117 positive cells (0.8% Vs 0.2%), higher fraction of quiescent (76.9% Vs 66.9% in the G0/G1 phase of cell cycle) cells and colony forming units (3.1%). This is a novel and promising development documenting enrichment of stem cells that warrants further exploration by in-vivo transplantation studies.

One of the important factors responsible for the development of dry eye in patients is the exposure to radiotherapy as a treatment modality for ocular malignancies and locally aggressive benign tumors. While clinical documentation of dry eye has been reported as one of the morbidities of ocular radiation, this study provides the first proof of sequential and progressive development of dry eye in patients, substantiated with histological documentation of near total ablation of glandular architecture, loss of differentiated and stem-like cells. The TEM studies also corroborate these findings. Although the mechanism of cell death was not evaluated in this study, there is evidence that post-radiated cell death could possibly be by

apoptosis.

Physiologically this destruction of lacrimal gland cells translates to reduction in the aqueous component of the tear film resulting in destabilization and hyperosmolarity of the tear film and eventual ocular surface damage. The Kaplan-Meir survival analysis showed that despite advances and refinement in the techniques and protocols of radiotherapy, nearly half of the patients (47.07%) who undergo orbital radiotherapy develop dry eye with nearly one-sixth (15.7%) developing chronic grade 4 dry eye. The analysis also showed that the average time for 50% of the patient population to develop DES ranged from 0.5 yrs (age group > 50 yrs) to 2.9yrs (age group 0-11 yrs) with female gender and >50 age group being a more susceptible population.

In conclusion, this novel study provides evidence that freshly isolated human lacrimal gland cells could be cultured successfully to expand the population of epithelial cells, with evidence of secretory function; presence of stem-like cells in the culture system and normal human lacrimal gland. These ex- vivo expanded human lacrimal gland cells have the dual potential to be used as a cell source for cell replacement therapy as well as the potential to provide source of naturally secreted products for pharmacological management of dry eye due to lacrimal gland insufficiency. This we believe is an important stepping stone towards developing cell therapy for lacrimal gland insufficiency in future.

Table of Contents

List of Tables

List of Figures

Abbreviations

3 D	3 Dimensional
3H-TdR	Tritiated Thymidine
AAAS	Aladin gene
ABCG2	ATP (Adenosine Triphosphate) Binding Cassette, Subfamily G, member 2
Ach	Acetylcholine
ACTH	AdenoCorticoTrophic Hormone
ADC	Analog-Digital Converter
ADDE	Aqueous Deficient Dry Eye
Ag-Ab	Antigen-Antibody
AIDS	Aquired Immunodeficiency Syndrome
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
ARDE	Age Related Dry Eye
BMP	Bone Morphogenetic Protein
BrdU	BromodeoxyUridine
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCL 3	Chemocine (C_C motif) Ligand 3
CCR	Chemocine (C-C Motif) Receptor
CFU	Colony Forming Units
cGy	centiGray
CK	Cytokeratin

cMyc	Cellular Myelocytomatosis Oncogene
CRL	Crown to Rump Length
CXCL	Chemokine Ligand
DAB	Diamino Benzidine
DAG	Diacyl Glycerol
DAPI	4',6-diamidino-2-phenylindole
DEAB	Diethylaminobenzaldehyde
DEWS	Dry Eye Workshop
DHT	Dihydro testosterone
DIV	Days in-vitro
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPX	P-Xylene-Bis-Pyridinium Bromide
E-Cad	Epithelial Cadherin
ECM	ExtraCellualr Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EORTC	European Organization for Research and Treatment of Cancer
EpCAM	Epithelial Cell Adhesion Molecule
ESC	Embryonic Stem Cells
FACS	Fluorescent Activated Cell Sorter
FCS	Fetal Calf Serum

FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluoresceine IsoThiocynate
FSH	Follicle Stimilating Hormone
G2	Gap 2
GFAP	Glial Fibrilic Acid Protein
GVHD	Graft Versus Host Disease
Gy	Gray
H&E	Hematoxylin and Eosin
HAM	Human Amniotic Membrane
HBSS	Hank's Balanced Salt Solution
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cells
ICAM	Intercellular Adehesion Molecule
IFN	Interferon
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IGF	Insulin Growth Factor
IgG	Immunoglobulin G
IL	Interleukin
IP3	Inositol Triphosphate
IPS	Induced Pluripotent Stem Cells
IRB	Institutional Review Board
ITS	Insulin Transferrin Selenium

KCS	Keratoconjunctivitis Sicca
Kda	KiloDaltons
KGF	Keratinocyte Growth Factor
Klf	Kruppel Like Factor
LENT	Late Effects of Normal Tissues
LFU	Lacrimal Functional Unit
LH	Luteinizing Hormone
LRC	Label Retaining Cells
LVPEI	L V Prasad Eye Institute
M	Mitosis
MAP Kinase	Mitogen Activated Protein Kinase
MGD	Meibomian Gland Disease
MHC	Major Histocompatibility Complex
MIP	Major Intrinsic Protein
mm	Millimeter
mM	milliMoles
MMC	Mitomycin C
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
Msh	Musashi
NEI	National Eye Institute
NOD	Non Obese Diabetic
NSSDE	Non-Sjogren Syndrome Dry Eye
Oct	Major Octamer Binding Protein

OD	Optical Density
PAX 6	Paired Box Protein 6
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Para Formaldehyde
PI	Propidium Iodide
PIPS	Protein Induced Pluripotent Stem Cells
RBC	Red Blood Cells
RPM	Rotations Per Minute
RPMI	Rosewell Park Memorial Institute
RTOG	Radiation Therapy Oncology Group
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
S	Synthesis
S100	Soluble 100 calcium binding protein
Sca	Stem Cell Antigen
scIgA	Secretory Component of Immunoglobulin A
shh	Sonic Hedgehog
SOMA	Subjective Objective Management Analytic
Sox	SRY (sex determining region Y)-box
Spla	Secretory Phospholipase
SPSS	Statistical Package for Social Sciences
SSDE	Sjogren Syndrome Dry Eye
TE	Trypsin- Ethylenediaminetetra acetic acid
TNF	Tumor Necrosis Factor

TSH	Thyroid Stimulating Hormone
VIP	Vasoactive Intestinal Peptide
α-MSH	α- Melanocyte Stimulating Hormone
α-SMA	α- Smooth Muscle Actin
μm	Micrometer

Chapter 1: Introduction and Review of Literature

1.1 Introduction

The eye is an integral component of the visual system, the function of which is dependent on all the layers including the ocular surface. The ocular surface is covered by a continuous sheet of non-secretory, stratified, non-keratinized epithelial cells with a turnover of about 7-10 days [1]. It can be divided into three distinct anatomic regions: the cornea, the conjunctiva and the limbus which separates the two. The stability and integrity of the ocular surface depends not only on the viability of the lining epithelial cells but also greatly on the stability of the tear film that covers the anterior surface of the eye. The tear film is a dynamic physiological secretion contributed by the lacrimal gland, meibomian gland as well as the conjunctival goblet cells. The tear film has three basic layers - the outer thin lipid layer (0.2μm) secreted by the meibomian glands, the middle bulk of aqueous layer (3-8μm) secreted by the lacrimal gland and the inner mucinous layer (1μm) secreted by the conjunctival goblet cells. Collectively, these layers of the tear film perform a number of important physiological functions: keep the cornea wet allowing gaseous exchange between the environment and the epithelium; contribute to the transparency of the cornea and determine the quality of image projected on the retina for cortical sensing. The periocular tear film is also responsible for providing nutrition to the cornea by acting as a coupling medium for the environmental oxygen; protecting the ocular surface due to its anti-microbial properties and providing physical protection against the shearing force of blinking [2].

1.2 The Human Lacrimal Gland

The human lacrimal gland is a tubulo-acinar, almond shaped exocrine gland located superior and lateral to the eye, just inside the orbital margin in the *fossa glanduloe lacrimalis* of the frontal bone [3] (Figure 1.1). Being an exocrine gland it has a system of associated ducts that drain its secretions onto the ocular surface. The lacrimal gland is divided based on the anatomic location into the main gland (orbital and palpebral portions) and the accessory glands (Glands of Wolfring and Krauss). The lacrimal gland, together with the meibomian gland, ocular surface, lids and the sensory/ motor neurons that connect them forms a system known as the ***lacrimal functional unit***, the most important function of which is to maintain the stability of the tear film, transparency of the cornea and the quality of the image projected onto the retina for cortical sensing [4].

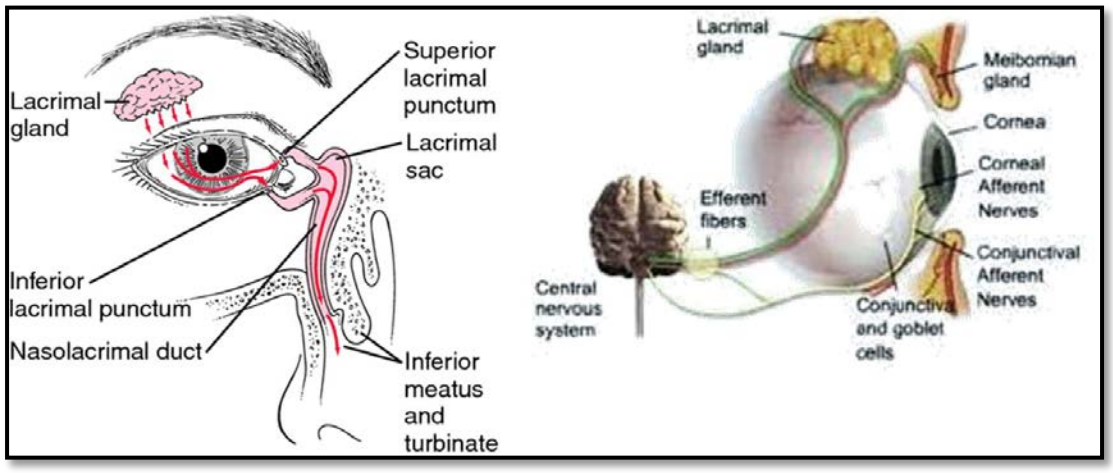

Figure 1.1: Location of the human lacrimal gland and the lacrimal functional unit *(Adapted from Perry et al., 2008 & Duane's Clinical Ophthalmology)*

1.3 Anatomy

The lacrimal gland is variable in proportion of lobes and quite a few individual variations have been noted in terms of its size. The gland is divided by the lateral horn of the aponeurosis of the levator muscle into the orbital and the palpebral lobes (Figure 1.2). The orbital lobe is the bigger of the two- almost twice the size of the palpebral lobe. It lies in the lacrimal fossa on the anterior-lateral portion of the orbit. The palpebral lobe lies below the aponeurosis of the levator muscle in contact with the superior lateral conjunctival fornix. The lacrimal gland measures 20-25 mm x 10-15 mm x 3-6mm [5]. Excretory ducts from both the orbital as well as the palpebral lobes open into the superior conjunctival fornix. Two to six secretory ducts from the orbital lobe of the lacrimal gland pass through the palpebral lobe or along its fibrous capsule, joining with ducts from the palpebral lobe to form 6 to 12 tubules that empty into the supero-lateral conjunctival fornix 4–5 mm above the tarsus [6].

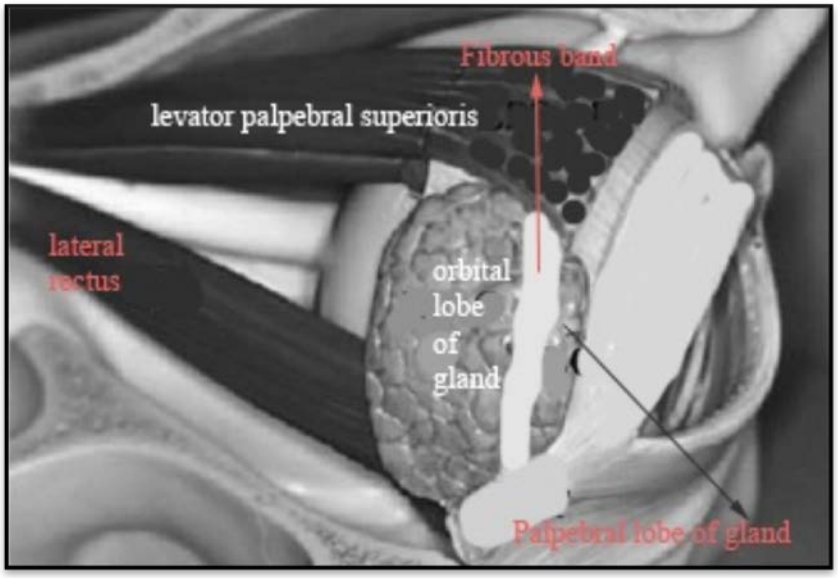

Figure 1.2: Lobes of human main lacrimal gland. *(Adapted from Gray's Anatomy)*

The accessory lacrimal glands are divided into gland of Wolfring and the gland of Krauss. Both of these are located in the lamina propria of the conjunctiva and their ducts open directly on the conjunctival surface. There are approximately 2-5 glands of Wolfring on the upper eyelid and about 1-3 on the lower lid. They are situated in the upper border of the tarsus midway between the ends of the tarsal glands. The glands of Krauss are placed deeply in the substantia propria of the upper fornix between the tarsus and the inferior lacrimal gland. There are about forty glands of Krauss on the upper eyelid and around 6-8 on the lower lid. The function of these accessory lacrimal glands is to produce and secrete tears directly onto the conjunctival surface [6].

1.4 Embryology

The development of the human lacrimal gland has been the subject of numerous studies since the early 1900s. Most of these studies, other than the one published by Tripathi and Tripathi, 1990 [7], report that the secretory gland develops from the ectoderm of the superior conjunctival fornix in human embryos with a crown to rump length of 22-24 mm [8].

The two main lacrimal gland lobes- the orbital and the palpebral lobes- originate not simultaneously but one after the other. The orbital lobe originates from the proliferation of conjunctival fornix epithelial cells in the form of five or six epithelial buds and its formation concludes by the end of the second month. This is followed by initiation of the palpebral lobe formation. The orbital and the palpebral lobes are separated by the levator muscle tendon, which forms during the third week of development.

An important study published by Caudra-Blanco *et.al.*, in 2003 [8] established a precise morphogenetic timetable for the development of the human lacrimal gland. This study divided the development of the gland in a time- wise fashion into the various O'Rahilly stages, which is an embryonic staging system initiated by George Streeter, completed by Rohan O'Rahilly and revised by O'Rahilly and Fabiola Muller [9].

This staging system divides the embryonic growth into various stages depending on the crown to rump length (CRL) of the embryos, their gestational age and their embryonic age. Described below is the morphogenetic evolution of the human lacrimal gland in the various O'Rahilly stages [8] (Figure 1.3)

O'Rahilly stage 18: During this stage only the initiation of development of the palpebral primordium has been observed without any other appreciable morphological changes.

O'Rahilly stage 19, 20 and 21: During stage 19, the superior conjunctival fornix thickens and the surrounding mesenchyme condenses with an intermediate area of very few or no cells (Figure 1.3a).

During stage 20, solid epithelial buds arise from the ectoderm of the superio-lateral conjunctival fornix, this epithelium thickens and becomes a nodular-shaped cell mass. There is condensation of mesenchyme surrounding the superior fornix epithelium, which takes on an ovoid form. This epithelial budding/ thickening and mesenchymal condensation continues till the fetus is of 21-23 mm CRL. These condensed rounded epithelial buds in the superior conjunctival fornix together with the associated mesenchyme constitute the glandular area. The early epithelial buds in the first two

months form the orbital lobe. During O'Rahilly stage 20 the palpebral primordia also get defined though the palpebral lobes originate a little later from the secondary epithelial buds (Figure 1.3 b & c).

O'Rahilly stage 22: The posterior and the medial extremes of the gland primordium, which later develops into the orbital lobe of the lacrimal gland, gets innervated at this stage with the lacrimal artery and the lacrimal nerve (Figure 1.3 d).

O'Rahilly stage 23: This stage marks the end of the embryonal period. It is marked by the appearance of lumen within the glandular epithelial buds in embryos of 28.5 mm CRL. The upper rectus muscle, surrounded by mesenchymal condensation, approaches the ocular sclera. During this stage the eyelid closure is also initiated (Figure 1.3 e & f).

9-12th weeks: In the 9th week, the levator palpebral superioris muscle formation is initiated which expands by week 10 and its aponeurotic expansion divides the lacrimal gland into the orbital and the palpebral lobes. The epithelial buds continue to invaginate from the fornix epithelium (Figure 1.3 g).

13-14th weeks: This stage of embryonic development witnesses two important events in the lacrimal gland formation: branching of the glandular parenchyma and the anastomosis between the lacrimal and the zygomatic nerve in the interior of the gland. These events are accompanied by an increase in glandular vascularization (Figure 1.3 h).

15-16th weeks: During this time, the stroma is seen to condense and the glandular lobes get organized within the presumptive glandular mass. By the end of the 16th week, each of

the lobes get vascularized. However, the formation of the epithelial buds at the conjunctival fornix continues (Figure 1.3 i).

The lacrimal excretory system is an important feature which begins its development at 7mm CRL embryo stage. During this stage the naso-optic fissure develops and by day 43 of embryonic age, the thick cord of epithelium along the floor of the naso-optic fissure, gets buried to form a rod connected to the surface epithelium at only the orbital and the nasal sides. The superior end of this rod enlarges to become the lacrimal sac which gives off two columns of cells that invaginate the eyelid margins and develop into canaliculi. By the 4^{th} month of embryonic development, canalization of this nasolacrimal ectodermal rod occurs. The central cells of the rod degenerate by necrobiosis forming a lumen closed at the superior end by the conjunctival and the canalicular epithelium and at the inferior end by the nasal and the nasolacrimal epithelium. The lacrimal gland development continues and by the 7^{th} month of embryonic age the superior membrane of the puncta gets canalized completely and the eyelids separate.

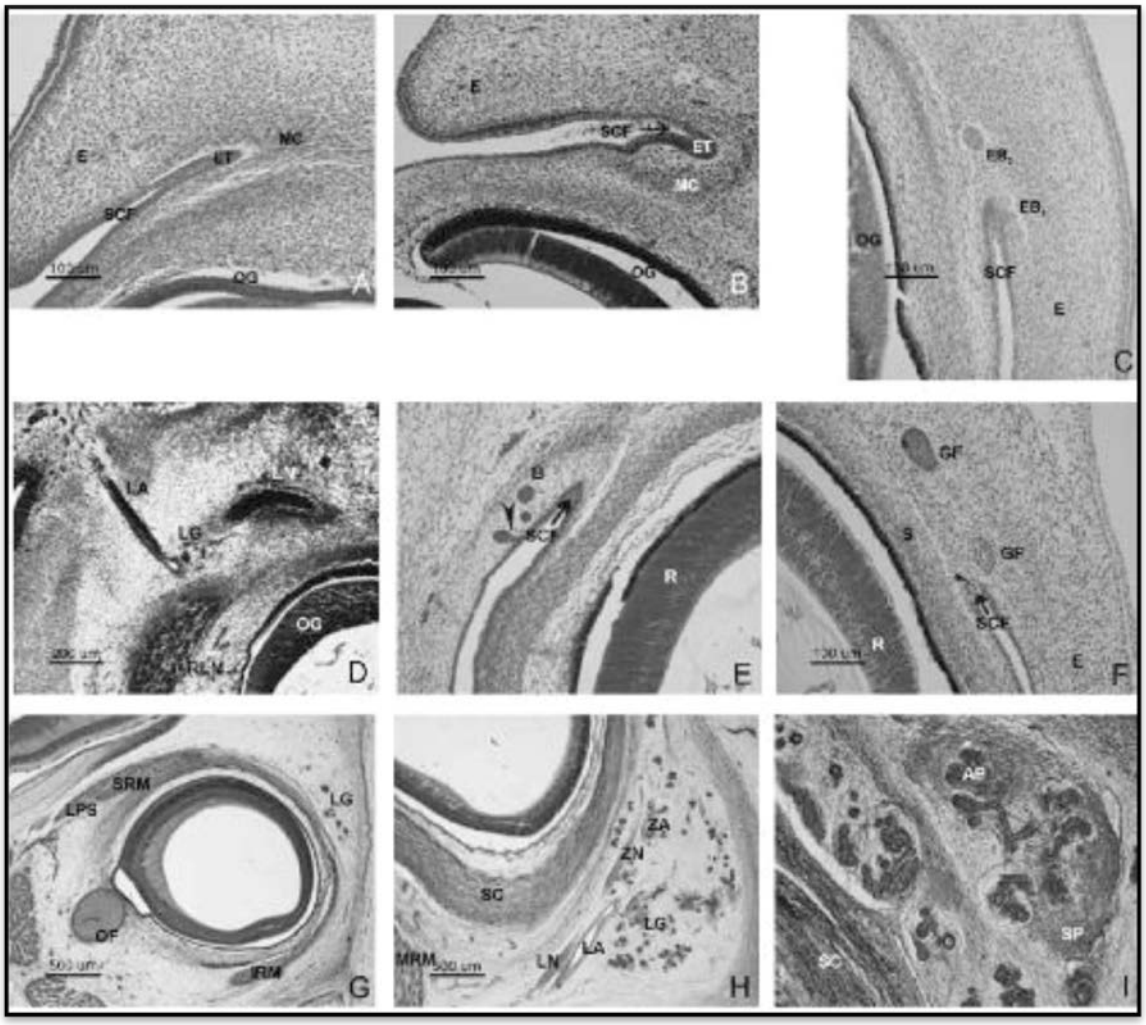

Figure 1.3: Summary of human lacrimal gland development (*Adapted from Caudra-Blanco et al., 2003) A) Human embryo O'Rahilly's stage 18;Frontal section (B) Human embryo O'Rahilly's stage 20; Oblique fronto-transverse section (C) Human embryo O'Rahilly's stage21;Frontal section (D) Human embryo O'Rahilly's stage 22;. Frontal section (×10). (E) Human embryo O'Rahilly's stage 23; Frontal section (×20). (F) Human embryo O'Rahilly's stage 23; Transverse section (×20). (G) Human fetus week 10 of development; Transverse section (×10). Fibrous expansion of the levator palpebrae superioris appears. (H) Human fetus week 13 of development; Transverse section (×10). The lacrimal and zygomatic nerves are observed to be accompanied by their respective arteries at the dorsomedial portion of the lacrimal gland (LG). (I) Human fetus week 15 of development; Transversal section (×20 magnification). The lacrimal gland, now with both a stromal portion (SP) and an acinose portion (AP), takes on an adult-like appearance.*

To summarize the three distinct phases in human lacrimal gland development (Figure 1.4):

1. Presumptive glandular stage (O'Rahilly's stages 19-20)
2. Epithelial bud stage (O'Rahilly's stages 21-23)
3. Glandular maturity stage (9th week of development onwards)

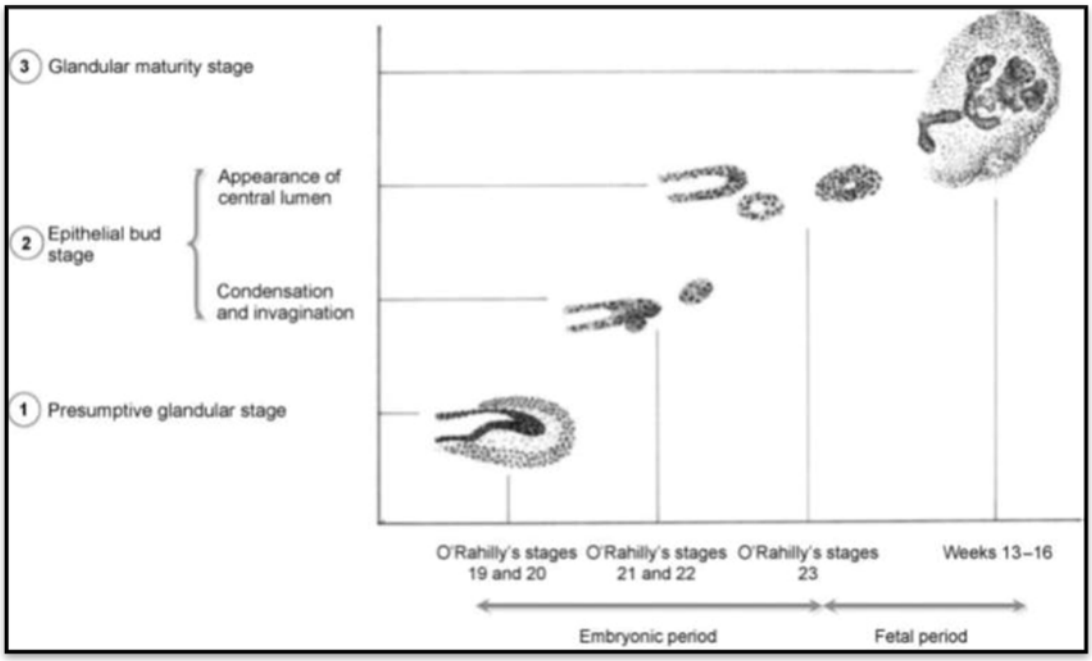

Figure 1.4: Stages in human lacrimal gland development (*Adapted from Caudra- Blanco et al., 2003)*

Table 1.1: Lacrimal gland and the lacrimal excretory system development timeline

Embryonic Development		
O'Rahilly Stage	***CRL (mm)***	***Features***
14-15	7	Naso-optic fissure develops
18	13-17	Initiation of palpebral primordium and the naso-lacrimal duct formation
19	16-18	Epithelium of superior conjunctival fornix thickens and the mesenchyme condenses
20	18-22	Mesenchyme takes on an ovoid form, upper eyelids appear
21	22-24	Epithelial buds condense and invaginate the surrounding mesenchyme in the superior conjunctival fornix
22	23-28	Lacrimal artery and the lacrimal vein enter the epithelial glandular mass
23	27-31	Lumen appear in epithelial buds, eyelid closure initiated
Fetal Development		
Week	***CRL (mm)***	***Features***
9-12	23-66	Secondary epithelial buds form the palpebral lobe, Levator palpebral superioris muscle formed which divides the gland into the orbital and the palpebral lobes, lacrimal ducts canalized
13-14	67-80	Branching of glandular parenchyma, anastomosis of lacrimal and zygomatic nerve
15-16	101-116	Glandular lobes get organized and vascularised, stroma condenses
28	376 (crown to heel)	Superior membrane of puncta canalized completely, eyelids separate

Epithelial-mesenchymal interaction has been considered by a number of authors to be responsible for morphogenesis, organogenesis, cell differentiation and growth [10, 11] . Lacrimal gland development is an example of such an interaction [12] in which the bud-like invagination of conjunctival epithelium at the fornix is the process which initiates lacrimal gland development. The mesenchymal cells surrounding the point of epithelial budding are the periocular cells of neural crest origin [13]. The tubular invaginations of the lacrimal gland extends and branches multiple times to form the lobular structure of the mature lacrimal gland.

1.5 Histology

1.5.1 Tissue architecture

Human lacrimal gland is a mixed sero-mucous tubuloacinar structure. The main secretory components of the gland are the pyramid-shaped secretory epithelial/ acinar cells, which comprise major portion of the gland. The acinar cells are secretory cuboidal epithelium with basally located nucleus and large peri-nuclear golgi apparatus [14]. Their apical portion is filled with many periodic acid Schiff's base (PAS) positive secretory granules and the base is associated with a basement membrane. This architecture is responsible for polarization of the cells and is essential for the ability of the cells to secrete water, electrolytes and proteins. The acinar cells are linked together by apical tight junctions which not only mechanically attaches the cells, but also couples them chemically and electrically. The basolateral membrane has the receptors for neuropeptides, hormones and growth factors that are involved in the secretory functions of the cell [14].

The lumen of the acinar cells come together to form the interlobular and intralobular excretory ducts lined by cuboidal epithelium (Figure 1.5). Like their acinar counterparts, the ductal cells (Figure 1.5) are also linked on their apical side by tight junction. This creates polarization and contributes to unidirectional secretion of lacrimal fluids. One of the most important functions of the ductal cells, in addition to collection and transport of lacrimal secretions, is dilution of primary lacrimal fluid by secreting electrolytes and water. These cells also synthesize proteins in their endoplasmic reticulum and Golgi complex and store the same in apical vesicles, though to a significantly lesser extent. The third important cell type that contributes to the architecture of the lacrimal gland are the myoepithelial cells (Figure 1.5). These have stellate, spindle-shaped or transitional (between the two) multiprocessed morphology and are alpha-smooth muscle actin (α-SMA) and cytokeratin 5 positive. They envelop the basal portion of the acinar and ductal cells and help them contract in order to empty their contents into the lumen or to propel the fluids forward. The myoepithelial cells have numerous G protein coupled receptors and other signaling components but their exact role in the production of lacrimal fluids still needs to be elucidated [15]

The lacrimal gland also contains a population of fibroblasts (which secrete collagen and heparin), dendritic cells, circulating lymphocytes and plasma cells. These plasma cells secrete the J chain of the IgA which couples with the secretory component synthesized by the acinar cells to form the functionally complete IgA which is involved in ocular immunity

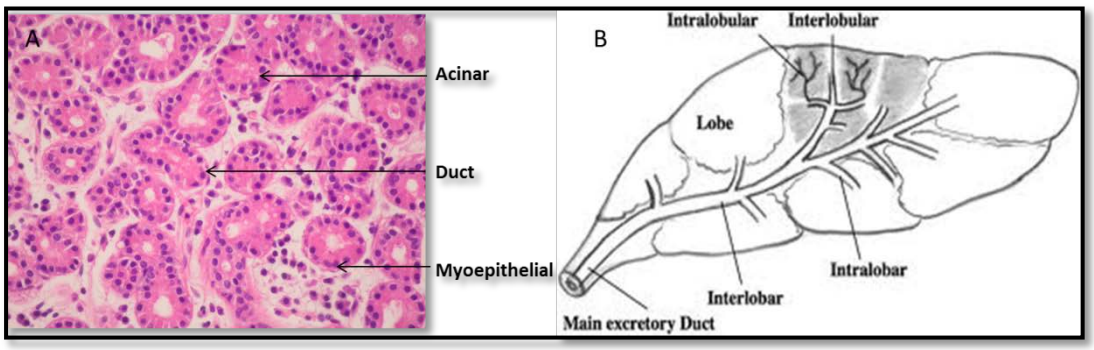

Figure 1.5: Lacrimal gland histology and duct system

A) The histoarchitecture of the gland shows the acinar, ductal and myoepithelial cells with stromal fibroblasts; B) Pictorial representation of pattern of duct system in the gland (Adapted from Dean et al., 2004)

1.5.2 Blood supply

The lacrimal gland is supplied with arterial blood through the lacrimal branch of the ophthalmic artery and sometimes from the infra-orbital branch of the maxillary artery and the recurrent meningeal artery. The venous drainage follows a similar course as the artery and drains into the superior ophthalmic vein[6] (Figure 1.6)

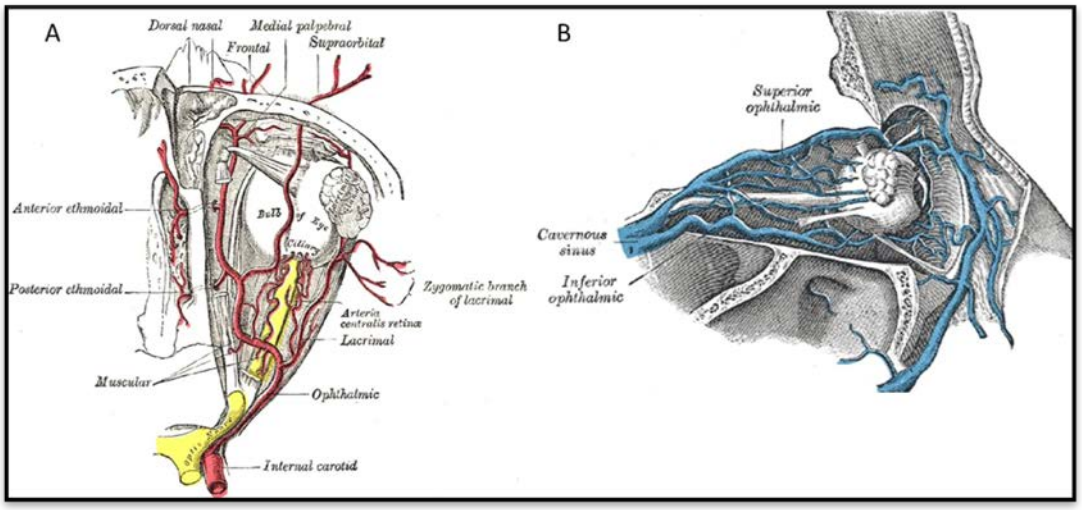

Figure1.6: Vascular supply to the lacrimal gland *(Adapted from Gray's Anatomy)*

1.5.3 Nerve supply

The lacrimal gland is innervated by the cranial nerves V and VII as well as parasympathetic and sympathetic fibres of the autonomic nervous system (Figure 1.7). However, the parasympathetic system predominates both anatomically as well as functionally.The parasympathetic innervation to the lacrimal gland is quite complex. Parasympathetic secretomotor fibers originate in the lacrimal nucleus of the pons, travel a long distance within the nervusintermedius, the greater superficial petrosal nerve, the deep petrosal nerve, and the vidian nerve to finally synapse in the pterygopalatine ganglion [16].Postganglionic parasympathetic fibers leave the pterygopalatine ganglion via the pterygopalatine nerves to innervate the lacrimal gland [17, 18]**.** In addition, some fibers may join the zygomatic nerve as it branches from the maxillary division of the trigeminal nerve and enters the orbit through the inferior orbital fissure. Branches of the zygomatic nerve may ascend and enter the posterior surface of the lacrimal gland either alone or in combination with the lacrimal nerve [6]

Sympathetic nerves come along with the lacrimal artery and parasympathetic in the zygomatic nerve. The zygomatic branch of the maxillary trigeminal nerve gives off the lacrimal branch before further divisions. This lacrimal branch anastomoses with the lacrimal nerve of the ophthalmic trigeminal nerve or travels along the periorbital region to independently enter the gland at its posterio-lateral aspect.

The sympathetic fibers originate in the superior cervical ganglion and arrive with the lacrimal artery and along with parasympathetic fibers in the zygomatic nerve to innervate the lacrimal gland [6].

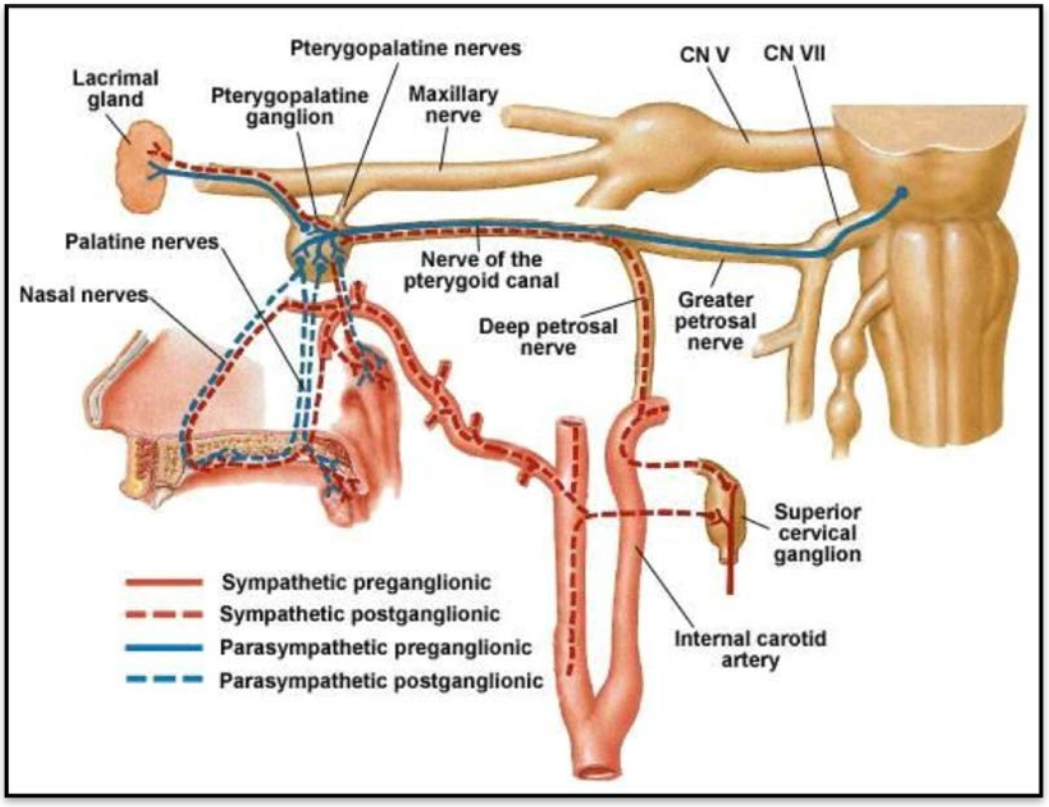

Figure 1.7: Nerve supply to the lacrimal gland *(http://www.surgicalneurologyint.com)*

1.6 Physiology

The lacrimal gland synthesizes and secretes a number of proteins and water and contributes to the aqueous component of the tear film. This synthesis and secretion of proteins and water is stimulated by neurotransmitters and neuropeptides fired by the nerves that innervate the gland (Figue 1.8). The acinar cell membrane is differentiated by junctional complexes into basolateral and apical domains. The apical domain is believed to contain water channels like aquaporin 5 and Cl^- and K^+ channels that allow the movement of solute across the epithelium. The basolateral membranes contain large numbers of Na^+ pumps, the Na^+-K^+-ATPase, which actively move K^+ into the cell and Na^+ out of the cell, maintaining the normal gradient across the membrane. The change in this gradient is the driving force behind the movement of ions and water across the epithelium. In addition, there are a number of co- transport systems or porters, which are driven by the concentration gradients created by the Na-K pump and by the HCO_3^-produced by carbonic anhydrase. One co-transport system mediates the influx of Na^+ coupled to the outward flux of H+ while a second system affects the efflux of bicarbonate ion (HCO_3^-)and associated influx of Cl^- [14, 19]**.**

The baso-lateral membranes also have ion channels, specifically for K^+, Cl^- and Ca^{2+}. The Ca^{2+} channels are involved in the process of excitation/secretion coupling and, by affecting the permeability of other ion channels, indirectly regulate the movement of water, though to a small extent. The apical membrane of the cell, however, is believed to be rich in Cl^- channels, which are Ca^{2+} sensitive. On activation of the cell, the raised

intracellular Ca^{2+} opens the Cl^-channels that allow efflux of Cl^- into the lumen. Na^+ follows this efflux across the epithelium through the junctional complexes as well as through the cation channels in the acinar cells. This osmotic movement of ions into the lumen is what drives the movement of water through the aquaporin channels into the lumen to maintain the osmotic balance [14].

The Cl^- gradient across the cell membrane and the relation of the cell membrane potential to the cell Cl^-equilibrium potential dictates the movement of Cl^- out of the apical membrane. As long as the membrane potential is below the Cl^- equilibrium potential, Cl^- moves out of the cell into the lumen and so will water. As the cell becomes depolarized, less Cl^-moves out and thus less water exits. If the membrane potential becomes equal to or depolarized above the Cl– equilibrium potential, then there is be no net outward Cl– or water movement. Because the efflux of Cl–depolarizes the membrane potential, the associated efflux of K+ in the basolateral surface and apical domains is essential to effectively provide a hyperpolarizing force required to maintain the membrane potential below the Cl^-equilibrium potential. The permeability of both Cl^- and K^+ channels therefore regulates the movement of water across the epithelium [14].

The acinar cells have receptors for acetylcholine (muscarinic M3), vasoactive intestinal peptide (VIP) (type I&II), and nor-epinephrine (α&β). Some of the cells also have receptors for peptides of proenkephalin family, neuropeptides Y, adenocorticotrophic hormone (ACTH), and α- melanocyte stimulating hormone (α-MSH). However, it is not essential that all the acinar cells of the gland possess these receptors; but since they are so

extensively and intimately coupled by gap junctions and second messengers (Ca^{+2} and inositol triphosphate), activation of any one group of cells causes secondary activation of others. As most of these receptors are G-protein coupled, the activity of these second messengers is tightly regulated by the G-proteins [14].

The muscarinic receptors are linked to G proteins, which are in turn associated with phospholipase C. This complex on activation results in increased production of inositol triphosphate (IP3) and diacyl glycerol (DAG). IP3 induces release of intracellular stores of Ca^{+2} as well as causes opening of calcium channels. This transiently increases intracellular calcium levels, which in turn causes opening of calcium dependent K^+ and Cl^- channels involved in secretion of water. DAG activates several protein kinase C iso-enzymes, which further aid secretion. Vasoactive Intestinal Peptide (VIP) receptors are coupled to G proteins that activate adenylatecyclase, which causes increased cyclic AMP levels (cAMP). This increased cAMP levels activate protein kinase A, that stimulates protein secretion. In addition, VIP also causes an increase in intracellular calcium leading to secretion of water. α- Adrenergic agonists stimulate protein secretion by activating protein kinase C, without the associated activation of IP3, calcium or cAMP. Proenkephalins have an inhibitory effect on protein secretion induced either by VIP or Acetylcholine (Ach) [14].

Protein secretion by the lacrimal acinar cells involves formation and subsequent fusion of vesicles with the apical cell membrane. This process depends on the movement of the membrane from Golgi complex to the apex. In order to conserve cell membrane

there is endocytotic process of internalization and intracellular processing of the apical membrane; while the basolateral membrane gets internalized into the cell where it is processed. Some of this membrane is used for apical secretion of scIgA complex while the rest is cycled back to the basolateral surface post intracellular processing. This basolateral membrane traffic has been implicated in a number of important processes like the means for the entry of prolactin into the secretory acinar cells and their subsequent functioning. It is also involved in the secretion of autoantigens, major histocompatibility complex II and antigen presentation which leads to immune mediated apoptosis of acinar cells and loss of physiological function as seen in conditions like Sjogren's syndrome [14].

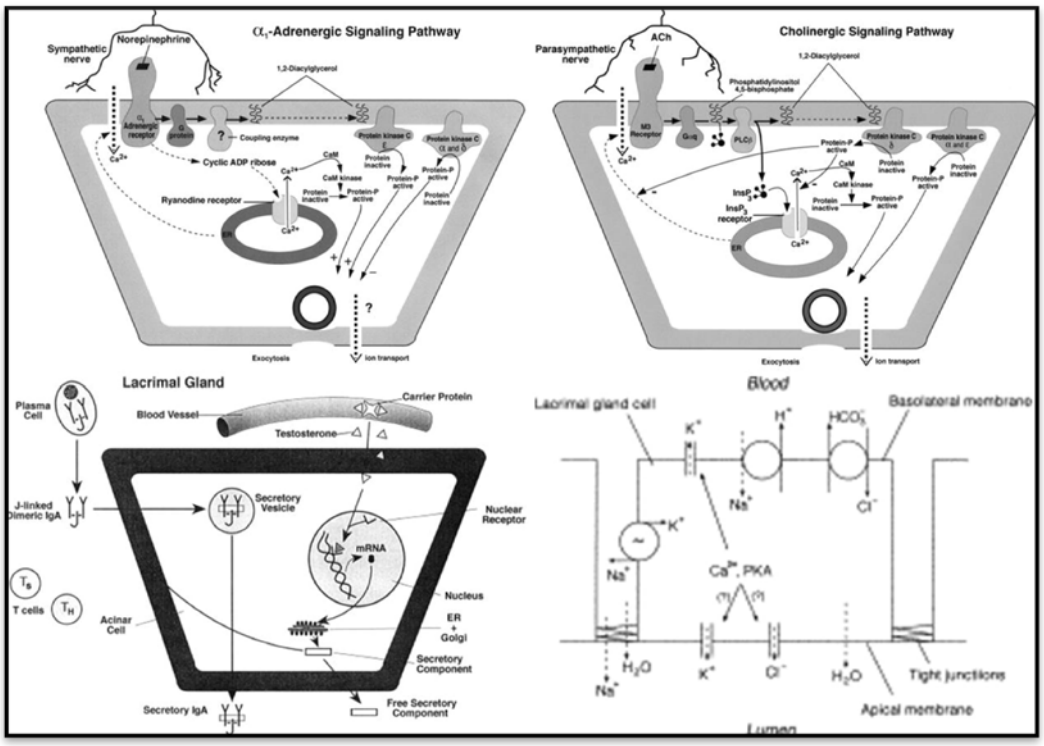

Figure 1.8: Secretion of protein, electrolytes and water by lacrimal gland
(Adapted from Dartt, 2004)

1.7 The Tear Film

The periocular tear film covers the anterior surface of the eye and contributes to the health and stability of the ocular surface. The tear film is composed of three dynamic layers: the lipid layer (0.2µ) secreted by the meibomian gland, the aqueous layer (3-8µ) secreted by the lacrimal gland and the mucin layer (1µ) secreted by the conjunctival goblet cells (Figure 1.9). The important constituents of human tear are electrolytes like sodium, potassium, calcium, magnesium, bicarbonate and chloride; and major proteins like lysozyme, lipocalin, lactoferrin, scIgA, albumin and IgG (Table 1.2). Other components of tear film include lipids like phosphatidylcholine and phosphatidylethanolamine; mucins like MUC4, MUC5AC, MUC1. Minor components like defensins, catalase and cytokines also form a part of the tear film composition [2].

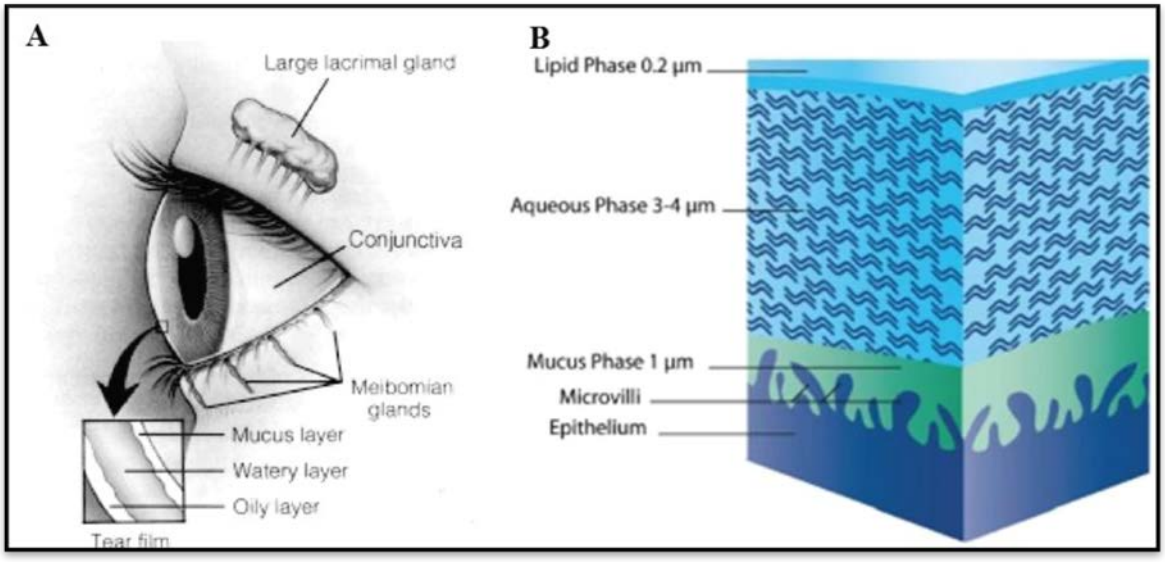

Figure 1.9:The tear film comprising of the lipid, aqueous and the mucin layer
(Adapted from http://www.clspectrum.com)

Table 1.2: Composition of human tears *(Ref: Tiffany, 2008; Paulsen 2004, Ohashi 2006)[2, 20, 21]*

Composition	**Concentration**
Electrolytes	**mmol/litre**
Sodium	128.7
Potassium	17
Calcium	0.32
Magnesium	0.35
Bicarbonate	12.4
Chloride	141.3
Major Proteins	**mg/litre**
Lysozyme	2.07
Lactoferrin	1.65
scIgA	1.93
Lipocalin	1.55
Albumin	0.04
IgG	0.004
Mucins	
MUC1	Concentration not known
MUC4	Concentration not known
MUC5AC	Concentration not known

The lacrimal secretion is controlled by the reflex arc which involves fibers from the fifth cranial nerve from the cornea, conjunctiva or the surrounding tissues. Fibers from both the parasympathetic as well as the sympathetic ganglion innervate the gland and exert positive and negative control over the secretion.

The important functions of the tear film are already mentioned in section 1.1. Briefly, the tear film provides nutrition to the ocular surface and maintains the homeostatic osmolarity

of the ocular surface, acts as a coupling medium for the environmental oxygen, provides lubrication against the friction created by blinking thereby protecting the surface epithelial cells and protects the ocular surface against microbial invasion due to the presence of antibacterial enzymes (lysozyme, lipocalin and lactoferrin) and antibodies [22].The tear film is also responsible for maintain a smooth corneal surface to enable proper image formation on the retina for cortical sensing.

1.8 Lacrimal Gland Disorders and Dysfunctions

Lacrimal gland disorder and dysfunction threatens the tear film stability and the resultant ocular surface health. Dysfunction of lacrimal gland, which is an essential component of the lacrimal functional unit, causes decreased tear secretion, hyperosmolarity of tears and results in ocular surface inflammation. Figure 1.10 enlists the major conditions affecting lacrimal gland function

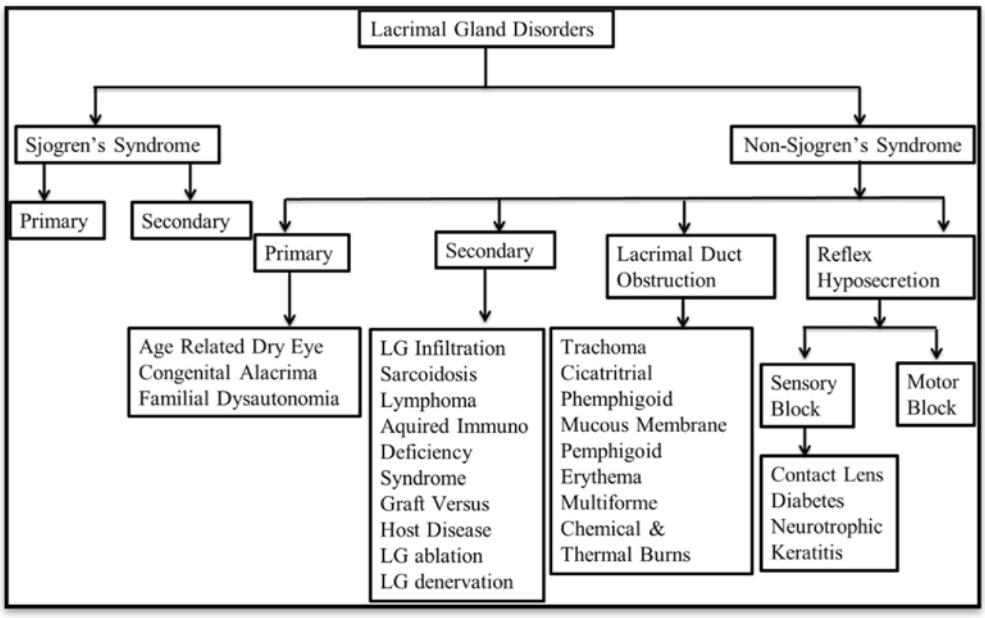

Figure 1.10: Lacrimal gland disorders

1.8.1 Sjogren syndrome

Sjogren syndrome is an autoimmune exocrinopathy in which the exocrine glands like lacrimal and salivary are attacked and destroyed by autoantibodies. The lacrimal and salivary glands are infiltrated by activated T lymphocytes leading to inflammatory activation, expression of autoantigens (eg Fodrin, Rho and La) on the cell surface and subsequent destruction of acinar and ductal cells leading to cell death and hyposecretion of tears and saliva [23]. Locally released inflammatory cytokines or circulating antibodies (like anti-M3) directed against muscarinic receptors within the gland can also cause reversible neurosecretory block amplifying the existing hyposecretory state.

Sjogren syndrome can be classified into primary Sjogren and secondary Sjogren. Primary Sjogren Syndrome is characterized by ADDE together with dry mouth (xerostomia). There is presence of autoantibodies in the blood, evidence of salivary hyposecretion with a positive focus score on minor salivary gland biopsy [24, 25]**.** Secondary Sjogren Syndrome consists of features of primary Sjogren together with autoimmune connective tissue disease like rheumatoid arthritis, systemic lupus erythematosis etc [26]

The precise trigger factors for autoimmune destruction of secretory acinar cells, as seen in SSDE, in not completely known. However certain risk factors have been positively associated like genetic predisposition profile, low androgen status and certain environmental agents like viruses, low humidity, and high wind velocity. The ocular surface dryness in SSDE is due to lacrimal hyposecretion and the accompanying

inflammatory changes in the gland. It is yet to be ascertained whether the associated conjunctival changes seen in the condition are due to autoimmune targeting of the tissue or due to the inflammatory mediatory released by the lacrimal gland in the tears.

1.8.2 Non-Sjogren's syndrome

Non-Sjogren's syndrome (NSS) lacrimal disorders are those conditions that affect the lacrimal gland but do not have the systemic autoimmune features typical of Sjogren's syndrome (DEWS 2007). The most common manifestation of NSS is age-related dry eye (ARDE) associated with increased ductal and acinar pathology like peri-ductal fibrosis, inter-acinar fibrosis, para-ductal blood vessel loss and acinar cell atrophy. These pathologies promote lacrimal dysfunction by their obstructive effects as well as reducing the secretory capacity of the acinar cells.

A number of other pathologies are also considered under NSS. The table below lists some of the important ones (Table 1.3) [4]:

Table 1.3: Non-Sjogren syndrome pathologies associated with DES

S. No.	***Disorder***	***Characteristics***
1.	Congenital alacrima	Caused due to mutations in the gene encoding the protein ALADIN, which plays a role in RNA/ protein trafficking
2.	Familial dysautonomia	Autosomal recessive disorder associated with mutations affecting the gene encoding an Iκβ kinase-associated protein.
3.	*Sarcoidosis*	Infiltration of the lacrimal gland by sarcoid granulomata
4.	Lymphoma	infiltration of the lacrimal gland by lyphomatous cells.

5.	Graft versus Host Disease	Caused due to co-localization of periductal T-lymphocytes with antigen presenting fibroblasts.
6.	LG ablation	LG ablation causes a partial or complete cessation of lacrimal secretions.
7	LG denervation	Causes a reduction in tear flow, reduced protein secretion and increased inflammatory activity within the gland
8.	*Trachoma*	Involves tarsal and conjunctival scarring, trichiasis and a cicatrizing meibomian gland obstruction
9.	Cicatricial Pemphigoid and	Muco-cutaneous disorders characterized by blistering of the mucous membrane and skin, conjunctival scarring,

1.9 The Dry Eye Syndrome

The lacrimal gland forms an integral part of the lacrimal functional unit (LFU) due to its physiological function of secreting the aqueous component of the tear film. Any perturbation in the functioning of this gland, which may be age related, drug induced, autoimmune or due to orbital radiotherapy leads to destabilization of the tear film which in turn leads to a chronic debilitating condition called the dry eye syndrome or *kerato conjunctivitis sicca* (KCS).

The International Dry Eye Workshop, 2007 [4] defined dry eye as:

"*Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of tear film and inflammation of the ocular surface.*"

The most important causative/ contributing factors for dry eye are [4]:

- Primary lacrimal gland dysfunction due to reduction in circulating androgens
- Secondary lacrimal gland dysfunction due to sarcoidosis, lymphoma etc.
- Autoimmune diseases like Sjogren's syndrome
- Reflex hyposecretion as in contact lens wear, diabetes, exposure to systemic drugs like antihistamines, beta blockers etc.
- Orbital radiotherapy for ocular malignancies
- Meibomian gland dysfunction

The patients with dry eye present with complaints of ocular itching, grittiness, increased tearing, blurred vision. The diagnosis for the condition is made based on Schirmer score values, physical eye examination and fluorescein staining (Table 1.4).

1.9.1 Panels for the classification of dry eye syndrome

To understand the phenomena of dry eye it is essential to understand the classification of this syndrome. Dry eye was classified in 1995 by the NEI/Industry workshop into evaporative and aqueous deficient dry eye. Even though this system worked well for the past nearly two decades, however it was fast becoming obsolete since it did not reflect the newly accrued knowledge about the pathophysiological mechanisms and effect on vision. To accommodate these factors the two new classification schemes were published: The Triple Classification [27, 28] and the report of the Delphi Panel [29] **.**

The Triple Classification System evolved from the reports presented at the 14th Congress of the European Society of Ophthalmology in 2003 [27]**.** An updated and

modified classification system was published in 2005, which presented three separate schemes: one based on etiopathogenesis; one based on the glands and tissues targeted in dry eye; and one based on disease severity [28]**.** Even though the concept presented here was attractive but this classification system was not formally adopted due to the lack of evidence based referencing.

The Delphi Panel was a consensus group that met in 2006 to review the existing classification of dry eye. The panel recommended a severity based grading system for dry eye, as it would form the basis for therapy. The existing and widely popular classification and grading of dry eye syndrome is based on the recommendations of both the Triple Classification System as well as the Delphi Panel.

Table 1.4: Dry eye severity grading [4] *(Adapted from DEWS Management and Therapy, 2007)*

Dry eye severity level	**1**	**2**	**3**	**4**
Discomfort- severity and frequency	Mild and/or episodic	Moderate episodic or	Severe frequent or	Severe and/or disabling and
Visual symptoms	None or episodic mild	Annoying and/or	Annoying, chronic and/	Constant and/or possibly
Conjunctival injection	None to mild	None to mild	+/–	+/–
Conjunctival staining	None to mild	Variable	Moderate to marked	Marked
Corneal staining (severity/location)	None to mild	Variable	Marked central	Severe punctate erosions

Corneal/tear signs	None to mild	Mild debris, ↓ meniscus	Filamentary keratitis,	Filamentary keratitis, mucus
Lid/meibomian glands	MGD variably present	MGD variably	Frequent	Trichiasis, keratinization,
TFBUT (sec)	Variable	≤ 10	≤ 5	Immediate
Schirmer score (mm/5 min)	Variable	≤ 10	≤ 5	≤ 2

1.9.2 Etiopathogenic classification of dry eye syndrome

The two major classes of dry eye that were proposed in 1995 NEI/ Industry workshop [30] still hold value and are widely followed. The two major categories are: Aqueous deficient dry eye caused primarily due to lacrimal gland dysfunction and evaporative dry eye (EDE) caused primarily due to meibomian gland dysfunction (Figure 1.11).

The etiopathogenic classification system of dry eye also takes into account the effect of *milieu exterieur* (surrounding external ambient environment) and the *milieu interieur* (the physiology of the individual) on the person's susceptibility to develop dry eye. The *milieu exterieur* involves the external and occupational environment, which may contribute as risk factors for the development of dry eye. Evaporative water loss from the eye may be increased in conditions like low relative humidity, high wind velocity or from occupational habits like staring at video display terminals for long periods of time.

The *milieu interieur* on the other hand, refers to the physiological conditions particular to an individual that could affect their likelihood of developing dry eye. Some of

the conditions that are known risk factors are low blink rate, wide palpebral aperture, low androgen pool and certain systemic drugs like anti-histaminics and beta-blockers. There is extensive evidence to implicate the role played by low pool of androgen and higher estrogen in the development of dry eye [31]. Biologically active androgen is known to promote the proper functioning of lacrimal and meibomian gland. With a drop in the levels of androgen (as seen in post-menopausal women and patients on anti-androgen therapy), dysfunction of lacrimal gland and meibomian gland sets in and this leads to hyposecretion of tear components and subsequent tear film instability.

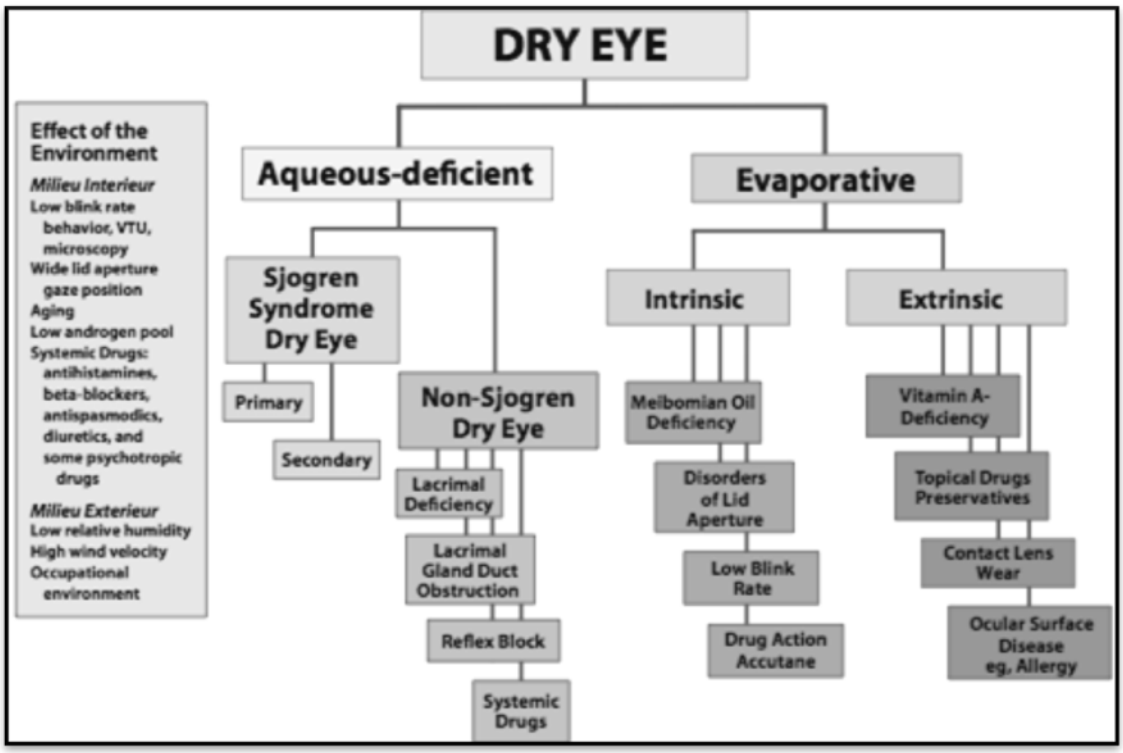

Figure 1.11: Etiological classification of dry eye *(Adapted from DEWS, 2007)*

1.9.3 Aqueous deficient dry eye

Aqueous deficient dry eye develops due to dysfunction of the lacrimal gland. The dysfunction may be due to destruction or malfunction of the secretory acinar cells leading to reduction in the aqueous component of the tear film. This, in turn, leads to hyperosmolarity of the tear film and subsequent instability. Tear film instability becomes a major contributing factor towards ocular surface hyperosmolarity initiating a cascade of inflammatory events involving MAP Kinase, NFκB signaling pathways and inflammatory mediators like interleukin- 1α, 1β, tumor necrosis factor-α (TNF-α) and matrix metalloprotein-9 (MMP-9) [32].

Aqueous deficient dry eye can be further categorized into two subclasses: *Sjogren Syndrome Dry Eye (SSDE)* and *Non-Sjogren's Syndrome Dry Eye (NSSDE)* (Figure 1.11).

The important causes for aqueous deficient dry eye are the ones that affect the normal functioning of the lacrimal gland. These factors and conditions have already been mentioned earlier under section 1.10.

1.9.4 Evaporative dry eye

Evaporative dry eye develops when there is excessive evaporation of tears from the ocular surface without there being any evidence of lacrimal hyposecretion. Evaporative dry eye may be caused due to *intrinsic* factors (like meibomian gland dysfunction, lid structure or dynamics) or *extrinsic* factors (like vitamin A deficiency, contact lens wear).

1.9.4.1 Intrinsic causes

Meibomian gland dysfunction: Meibomian gland is responsible for secreting the lipid component of the tear film. Any disturbances in the composition or volume of the lipid layer secreted leads to increased evaporation of tears from the ocular surface and results in symptoms of evaporative dry eye. Meibomian gland dysfunction may be due to posterior blepharitis, which causes obstruction of the gland secretions or due to loss of acinar density leading to hyposecretion [33-35]. The important conditions that lead to this are dermatoses, treatment of acne vulgaris with isotretinoin (causes reversible meibomian gland atrophy) and exposure to polychlorinated biphenyls [36].

Disorders of lid aperture and lid/ globe congruity: Any disorder of the lid or lid/ globe malapposition which increases the exposed evaporative surface of the eye, as in cases of proptosis, high myopia, endocrine exophthalmos, increases palpebral fissure width, leads to development of evaporative dry eye [37, 38].

Low blink rate: Low blink rate in individuals can also lead to development of dry eye. As the interval between blinks is increased there is greater evaporation from the exposed ocular surface and lesser frequent spreading of the tear film. Low blink rate may be seen in persons during performing tasks of concentration (staring at video monitors) or it may be indicative of extrapyramidal disorders like Parkinson's disease [39, 40].

1.9.4.2 Extrinsic causes

I) Ocular surface disorders

Ocular surface disorders such as those induced by vitamin A deficiency or application of topical anesthetics may lead to inadequate surface wetting, early tear film breakup and hyperosmolarity of tear film leading to dry eye.

Vitamin A deficiency: Vitamin A is required by the conjunctival goblet cells as well as the lacrimal acinar cells. Deficiency of vitamin A can cause unstable tear film due to altered composition and also hyposecretion by the lacrimal acinar cells [41, 42].

Topical drugs and preservatives: A number of studies have reported that preservatives like benzalkonium chloride, used in topical formulations have toxic effects on surface epithelial cells causing epithelial cell damage and punctate keratitis in certain cases. These conditions cause conjunctival scarring and inadequate surface wettability leading to dry eye [43].

Topical anesthetics can also induce dry eye either by reducing the blink rate or reducing the sensory input to the lacrimal gland. Chronic use of topical anesthetics has been reported to cause neurotrophic keratitis leading to corneal perforation [44, 45].

II) Contact lens wear

Contact lens wear has also been cited by in literature to be an important cause of dry eye. Even though numerous studies have published their results on this topic the exact reason behind the development of dry eye in contact lens wearers remains elusive. The most common reported mechanisms are the thinning of pre-lens tear film, reduced lipid layer

thickness, poor lens wettability and increasing the evaporation rate (in cases of hydrogel lenses) [46, 47].

III) Allergic conjunctivitis

Allergic conjunctivitis causes degranulation of IgE primed mast cells and release of inflammatory cytokines. This activates a Th2 response in the ocular surface, initially in the conjunctiva followed by the corneal epithelium causing sub-mucosal changes. There is stimulation of goblet cell secretion and loss of surface membrane mucins and surface epithelial cell death. These surface irregularities lead to tear film instability and with long standing can cause meibomian gland dysfunction. Lid swelling can aggravate the existing dry eye by increasing surface evaporation [48, 49]**.**

1.10 Hormonal Regulation and Dry Eye

Hormonal regulation of lacrimal gland secretion is an area of intense research. Although the exact mechanism behind hormonal control of tear secretion in not completely understood, yet there is no doubt that sex hormones, androgens in particular, play a very important role in this process [50]. Androgens modulate the anatomy, physiology, cellular architecture, gene expression, protein synthesis, immune activity, and secretory processes of the lacrimal gland**.** Other hormones like progesterone, luteinizing hormone [51], follicle stimulating hormone (FSH), prolactin, thyroid stimulating hormone (TSH), and estrogen also affect the functioning of the lacrimal gland [50]**.**

Receptors for various steroid hormones, androgens in particular, have been identified in the lacrimal gland. These receptor proteins have been found predominantly in the epithelial cell nuclei and have a single class of saturable, high affinity and steroid-specific binding sites. In addition to this, mRNA for androgen receptors and 5∝-reductase; the chief enzyme involved in the conversion of androgen to their biologically active derivative 5∝- dihydrotestosterone, has been found in the eye [52].

It was earlier believed that estrogen deficiency in post-menopausal women was the key reason behind the sheer number of dry eye patients in this age group. However, in clinical practice, hormone replacement therapy or eye drops containing estrogen do not benefit the patients; instead induce lacrimal gland regression, decreased metabolic function and reduced tear output [50, 53]**.**

Animal models of dry eye have shed some important light on the role played by sex hormones on the control of lacrimal secretion. Ovariectomy in rabbit models led to degeneration of the main lacrimal gland due to apoptosis of secretory acinar and interstitial cells. These effects can be prevented by the administration of androgens *(not estrogen).* Dihydrotestosterone was effective in preventing the degeneration of the lacrimal gland and could improve the metabolic functions and tear output. The mode of function of androgens here is probably their potential to stimulate production of transforming growth factor (TGF)-β, a potent immunomodulator and anti-inflammatory cytokine, and by reducing the concentration of interleukin (IL)1β and tumor necrosis factor (TNF)-α [50].

Androgens also have a potent regulatory effect on meibomian gland tear lipid secretion. Meibomian gland, like the lacrimal gland, has sex hormone receptors and 5α-reductase. The action of androgens on the secretory acinar cells is essential for the proper functioning of the gland and the resultant tear film stability. It has been clinically observed that patients on anti-androgen therapy, for urological or dermatological reasons, have significantly dysfunctional meibomian gland, which is a strong indicator for the importance of androgens in meibomian gland functioning [54-56].

1.11 Radiotherapy Induced Dry Eye

Radiation therapy is a commonly used modality in the management of ocular and oculoadnexal disorders including benign and malignant tumors like retinoblastomas, choroidal melanoma, orbital rhabdomyosarcoma, choroidal metastases from systemic cancers, nasopharyngeal carcinomas, paranasal sinus tumors, periorbital basal cell carcinoma, and Grave's ophthalmopathy. Rapid evolution in the technique of radiotherapy over the years with better delivery system, better fractionation protocols, more effective shielding techniques and introduction of megavoltage accelerators have significantly increased the therapeutic efficiency and reduced the associated complications of radiotherapy. Even with such refinements, on a clinical level a significant proportion of patients are still seen with acute and chronic ophthalmic complication like dry eye [57-59].

The effects of radiation on the eye have been known ever since the technique of radiotherapy was introduced in 1897. Ocular damage during orbital radiation therapy may

be the direct result of radiation to the intraocular tumors or it may be because the normal tissue lies in the path of radiation and does not get effectively shielded. The most common ophthalmic complications seen include corneal damage, dry eye, cataract, retinopathy and optic neuropathy. The incidence of these complications is very closely related to many factors including the total dose, fractionation schedules and use or non-use of protective measures, all of which are often tailored to the individual patient [58, 60]. This also explains the difficulty to get statistical measures of the incidence of these complications in any population. In an attempt to make the data comparable various grading systems have been proposed one of the most widely accepted being the LENT/SOMA scoring system [61].

The LENT/SOMA scoring system was formed by the collaborative efforts of European Organization Treatment of Cancer (EORTC) and Radiation Therapy Oncology Group (RTOG). The scoring system appeared as a necessity to standardize and improve the data recording and to address the question of standardized toxic effects criteria [61]. The current proposed scale is not yet validated so should be used cautiously.

Radiation doses, even at therapeutic levels, can cause atrophy of the lacrimal and meibomian glands. This leads to compositional changes in the tear film, destabilization of the ocular surface and the resultant lacrimal insufficiency- evaporative dry eye state. The current global prevalence of dry eye is estimated at around 11% to 22% [62]. A clinic based study on a cohort of patients reported that 25.4% of dry eye cases were due to lacrimal gland insufficiency [63]. In the Indian context, these numbers are estimated to be

around 18.4% to 20% and 29.25% in a hospital setting [64]. The severity varies from patient to patient and is largely determined by the duration and dose of radiation in addition to undefined host factors [60, 65].

Dry eye syndrome has been reported as a serious late consequence of orbital radiation. A number of studies have shown that lacrimal gland, similar to salivary gland, show marked radiosensitivity and undergoes atrophy at 50-60Gy total radiation dose. Doses higher than 60 Gy can induce permanent and complete loss of lacrimal secretion [66]. Karp *et al.,* in their study in 1979 published the histopathological evidence associated with therapeutic radiation-induced lacrimal gland atrophy following exenteration in a patient with chronic dry who had previously received therapeutic irradiation for paranasal sinus malignancy. In the same study, in 8 different exenteration specimens removed 5 days to 16 years following irradiation, involutional atrophy of the meibomian glands was also described [67]**.** In another long-term follow-up study of 53 patients who underwent external fractionated cobalt-60 beam irradiation of their entire orbit for extra-cranial head and neck tumors, 20 patients developed severe dry eye. A sigmoidal dose response showed a dramatic difference between incidence of injury at 30 Gy total dose (0%) and 57 Gy total dose (100%) [68]. Atrophy of the lacrimal gland has also been reported in children after treatment with fractionated doses of a cumulative 50–60 Gy over 5–6 weeks [68]**.** Parson *et.al..,* after extensive literature review and also based on their own work have suggested the tolerance dose for the lacrimal gland to be30–40 Gy (in 20 fractions) [69]**.**

Post-radiotherapy, the causation of dry eye is multifactorial:

a) Lacrimal gland atrophy leading to loss of aqueous layer

b) Meibomian gland atrophy with loss of lipid layer secretion.

c) Ocular surface damage with goblet cell loss and loss of mucin secretion

In the early post-radiotherapy phase, lacrimal gland loss has been shown to be due to apoptotic loss of acinar cells. This combined with the radiation induced fibrosis leads to loss of lacrimal function with progressive decrease in tear volume and finally dry eye [58, 66, 69].

Management of some of these ocular complications, like cataract is relatively easy, in contrast to others especially dry eye, which is remarkably difficult to manage with the patient being dependent on lifelong frequent administrations oftear replacements and lubricants. This is often a cause of great discomfort to the patient and significantly compromises the quality of life.

1.12 Etiology of Dry Eye Syndrome

There has been a considerable increase in knowledge about the etiopathogenesis of dry eye syndrome in the past few years. The pathologic features of this condition include increased epithelial proliferation, stratification and abnormal differentiation with maintenance of a basal phenotype [70]. This is accompanied by reduced expression of secretory and membrane-bound mucins by the ocular surface conjunctival epithelial cells [71] compounding the effects of existing lacrimal dysfunction.

The two most important factors that contribute to the initiation and progression of dry eye are *tear hyperosmolarity* and *tear film instability* that adversely affect the ocular surface epithelial function and differentiation [72] (Figure 1.12). Trauma to a poorly lubricated and unprotected ocular surface due to blinking or environmental factors becomes a confounding factor, which worsens the condition [30, 71].

Tear film stability, which is important in maintaining clear and sharp vision, is threatened when the interactions between stabilizing tear film constituents are compromised either by decreased tear secretion, delayed clearance or altered tear composition as is seen in xerophthalmia and allergic eye diseases. Ocular surface inflammation is secondary consequence. Reflex tear secretion in response to ocular irritation is seen as the initial compensatory mechanism, but, with time, due to severe inflammation and chronic secretory dysfunction a decrease in corneal sensation occurs which compromises the reflex response and results in even greater tear film instability. Dysfunction of the LFU is considered to play an important role in the evolution of different forms of dry eye [4].

Even though tear fluid is secreted as a hypotonic fluid, yet due to excessive evaporation from the exposed ocular surface or low rate of aqueous tear flow (or a combination of the two) tear *hyperosmolarity* may arise. This hyperosmolarity stimulates various inflammatory pathways involving the MAP kinases, inflammatory cytokines (interleukins, TNF-α) and matrix metalloproteinases (MMP9). This cascade of events attracts the circulating T cells within the lacrimal glands. Under physiological conditions,

the trafficking lymphocytes, finding no inflammation, would undergo apoptosis. However, in the presence of inflammatory signals, these lymphocytes become activated and secrete pro-inflammatory cytokines, which cause homing of additional T cells to the tissue and an increase in the level of inflammation thereby aggravating the existing condition and forming a vicious loop of inflammation [73, 74]. Any condition that results in hyper activity of the functional unit can also initiate inflammatory response within the lacrimal glands resulting in antigen presentation and cytokine secretion by the epithelial cells of the gland [75, 76]. These pro-inflammatory mediators cause epithelial cell death by apoptosis and also loss of conjunctival goblet cells – a combinatorial assault that leads to worsening of the existing condition.

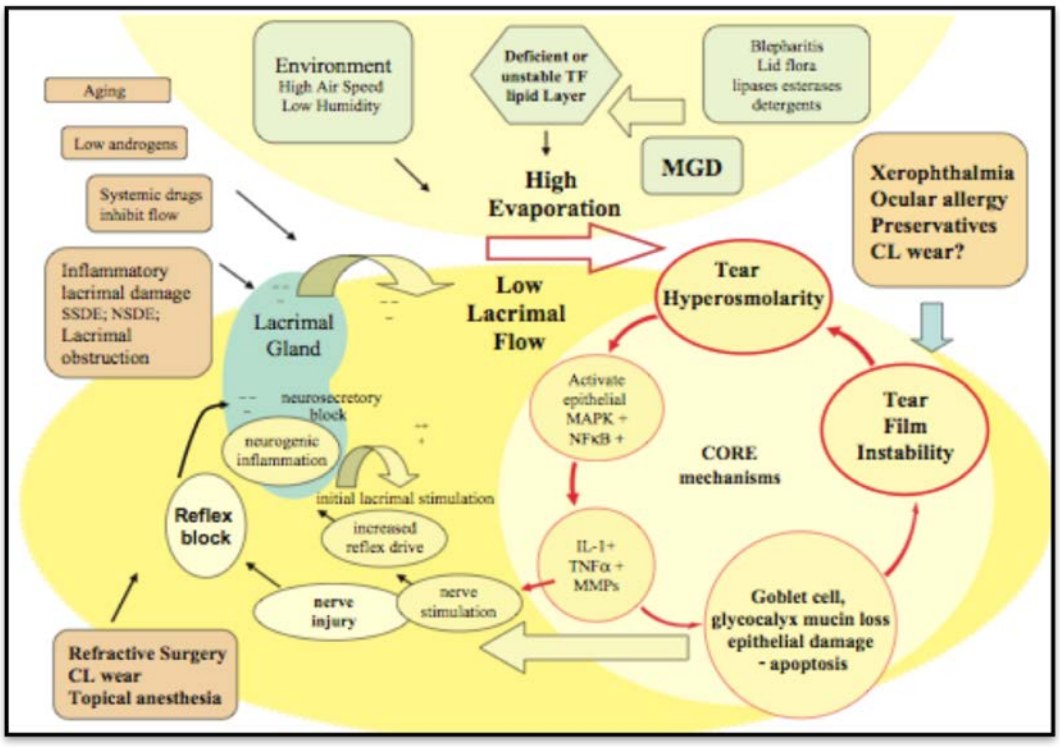

Figure 1.12: Etiology of dry eye *(Adapted from DEWS, 2007)*

1.13 Inflammation and Dry Eye

Even though there are numerous causes for the development and worsening of the dry eye condition, there is one common factor which lies at the epicenter of it all- ocular inflammation. Excessive ocular surface dryness stimulates the nerves and triggers off events involved in tissue injury/ repair. One of the important factors here is neurogenic inflammation, homing and activation of circulating T-lymphocytes and release of inflammatory cytokines into the lacrimal gland, tears and conjunctiva [4, 77, 78] **(Figure 1.9).**

The T and B lymphocytes infiltrate the gland and contribute to the dysfunction and eventual destruction of the gland. In addition to just infiltrating the lacrimal gland and conjunctival epithelium, the populating of T cells shifts predominantly from CD8+cells to CD4+cells [79]. These T cells show an increased expression of CD11a and CD23, indicating and activated phenotype. The conjunctival epithelial cells also show increased expression of immune inflammatory markers like HLA-DR, ICAM-1, CD40, CD40 ligand or CCR5 [77, 80-83]**.**

The secretory epithelial cells may also produce interleukins 1∝, 6, 8, TNF-∝, 32 and may also aggravate the local tissue inflammation [4, 32, 77]. These ocular surface and lacrimal epithelial cells may also acquire antigen-presentation capability by expression of HLA-DR class II antigens on their surface [82]. Since these cells become immunologically activated they can be targeted by the lymphocytes during cytotoxic reactions and this sets off a cascade of events wherein more and more lymphocytes get recruited to the tissue

worsening the existing inflammation. Correlation studies have revealed associations between levels of some of those cytokines and chemokines with clinical parameters**.** IL-6 tear levels in dry eye patients have been shown to correlate significantly with tear film breakup time, tear clearance, corneal surface damage, conjunctival goblet cell density and severity of irritation symptoms Additionally, IL-10, IL-8/CXCL8, MIP-1α/CCL3, IL-1α and IL-1β tear levels in dry patients correlated inversely with Schirmer's test score (indicative of tear volume); IFN-γ, IL-8/ CXCL8, MIP-1α/CCL3, IL1-α, and IL-1β correlated positively with both corneal fluoresceine and conjunctival lissamine staining scores indicative of ocular surface [4, 77, 84]**.**

More recent findings in humans suggest that IL-17 has a role in epithelial barrier disruption in human dry eye syndrome, as gene expression in conjunctiva of dry eye patients showed increased levels of the Th-17 inducers, IL-23, IL-17A and IFN-γ**.** The study also suggested MMP-9 as a possible useful biomarker of dry eye as its expression was seen increased in dry eye conjunctiva. Moreover, its level in tears could be correlated with several clinical parameters such as symptom severity score, low-contrast visual acuity, fluoresceine tear breakup time, corneal and conjunctival fluoresceine staining, topographic surface regularity index and percentage area of abnormal superficial corneal epithelia [85]. Finally, the activity of sPLA2-IIa has recently been found increased in tears from dry eye patients, indicating that sPLA2-IIa may play an important role in chronic ocular surface inflammation, especially when the ocular surface is compromised [86].

This inflammatory assault leads to gradual dysfunction and eventual destruction of the lacrimal gland and impairment of the conjunctival epithelium. In addition, the inflammatory mediators may inhibit neural signals to the lacrimal gland thus depriving the gland of the necessary trophic stimulation and leading to its progressive eventual destruction. The effect that chronically inflamed lacrimal gland has on the ocular surface stability had been shown in TGF-β1 null mice. Due to the absence of a functional TGF-β1 gene, these animals show rampant systemic inflammation. One of the very obvious symptoms that they exhibit is the development of dry, crusty eyes and presence of inflammatory lesions and high cytokine expression in their lacrimal gland [87, 88]

In addition to lacrimal gland, chronic inflammation also influences other tear components since it has a destructive influence on both the meibomian glands, which secretes tear film lipids and the conjunctival epithelial cell which secrete the mucin layer of the tear film [89].

The infiltration of inflammatory cells in the lacrimal gland and conjunctiva, the up-regulation of immune-related antigens and inflammatory cytokine, impaired apoptotic regulation of lacrimal gland and possible alternations in membrane trafficking of acinar cells, in association with squamous metaplasia and goblet cell loss are classical features of dry eye syndrome [4].

Whatever may be initial trigger for the development of dry eye it leads to epithelial lesions that, beyond a threshold, trigger local inflammatory reactions. It is hypothesized that intrinsic subclinical hormonal or autoimmune factors get aggravated by extrinsic

factors such as pollution, local infection, allergens *etc*. and result in a cascade of cellular reactions leading to inflammation and apoptosis**.** This could lead to a dysfunction and eventual breakdown of all ocular surface protective mechanisms, compositional changes in the three layers of the tear film, neural dysfunction and continuous cellular degeneration of conjunctival and corneal epithelium. Once dry eye disease has developed, inflammation becomes the key mechanism of ocular surface injury, as both the cause and consequence of cell damage. Patients with severe dry eye disease thus become entrapped in a vicious cycle of inflammation and ocular surface injury [4, 74]**.**

1.14 Current Therapies for Dry Eye Syndrome

Even though there has been significant advancement in the knowledge about lacrimal gland dysfunction and development of dry eye syndrome, yet the same cannot be said for the management of the condition. Dry eye is still a chronic debilitating condition, the treatment and management of which aims at palliation and improving the quality of life of the patient. The current treatment modalities available are lubricating agents like hydroxymethyl cellulose, solutions containing bicarbonates and potassium, hyposmotic artificial tears (Hypotears®, Novartis Ophthalmics) and artificial serum. In cases of severe dry eye, therapies such as anti-inflammatory medications (cyclosporinsA, corticosteroids), pharmacological tear stimulants like diquafosol, rebamipide, ecabet sodium, pilocarpine etc. are employed. In certain instances, where the patient does not get any relief in

symptoms by these, surgical interventions like punctal occlusion and salivary gland auto-transplantation are done to slow down the progress of the condition [90].

However, none of the therapeutic management strategies available can adequately manage the condition for a long term. Hydrating and lubricating agents are useful only so long as the condition is mild; in grade 4 (severe cases) even half hourly application proves to be inadequate. Anti-inflammatory drugs and pharmacological tear stimulants have very limited application in grade 3 and 4 dry eyes. Salivary gland auto-transplantation is a modality which can probably be used in severe cases but it has the inherent drawback of secretory components being different from tears, responding to gustatory stimulus and not being very effective on long term follow up.

On recommendation of the committee on the therapy and management of dry eye (DEWS, 2007), the treatment/ management protocol for this condition is now shifting towards employing strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce/eliminate the levels of existing inflammation (Figure 1.13). With these objectives in mind various therapeutic avenues are being explored with the inclusion of cell therapy and tissue engineering for restoring the damaged lacrimal gland.

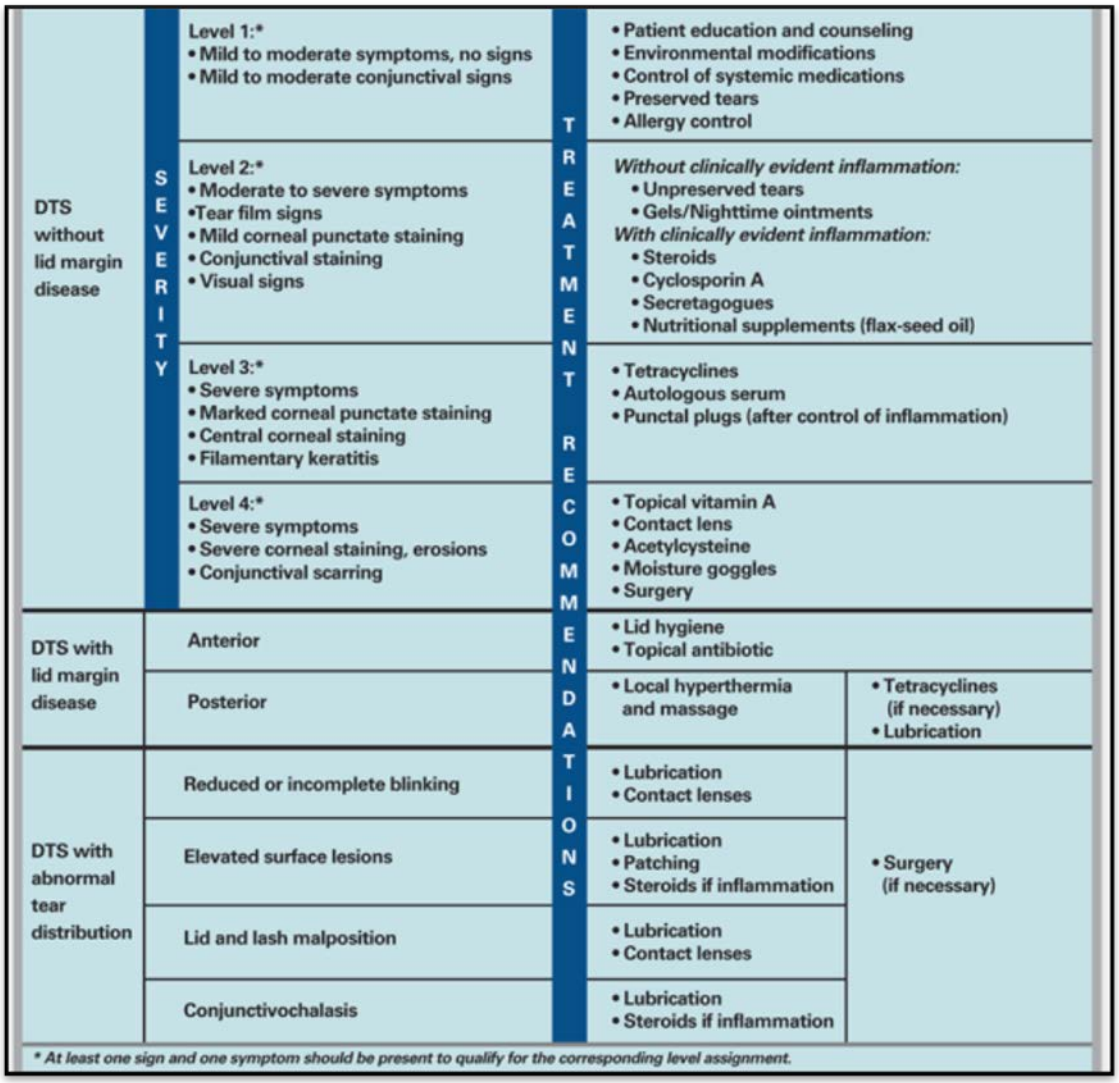

Figure 1.13: Dry eye syndrome treatment algorithm
(Adapted from Delphi Panel, Dews 2007)

1.15 Cell Therapy and Tissue Engineering

Cell therapy and tissue engineering form two important arms of Regenerative medicine *Tissue engineering* is an interdisciplinary field that applies the principles of life sciences, biomaterials and material sciences in order to artificially create biological substitutes that would restore, maintain or improve tissue function following damage/ destruction either by disease or trauma**.** Advances in this field have led to the development of a number of therapies, which are being practiced clinically. *Cell therapy*, on the other hand, has been defined by the Food and Drug Administration (FDA) as "The prevention, treatment, cure or mitigation of disease or injuries in humans by the administration of autologous, allogeneic or xenogeneic cells that have been manipulated or altered ex vivo." The goal of cell therapy, overlapping with that of regenerative medicine and tissue engineering, is to repair, replace or restore damaged tissues or organs." [91]

However, before this synergy is obtained, a number of criteria need to be fulfilled:

1. Adequate number of cells to be able to form the tissue
2. Cells should differentiate into the desired phenotype
3. Cells should be able to adopt the appropriate 3 D structure and produce ECM
4. Cells used for artificial tissue designing must be structurally and mechanically compliant with the native tissue
5. Cells should be able to integrate with the native tissue without evoking any immunological reaction
6. There should be no foreseeable associated risks

While many potential cell sources are being explored like embryonic stem cells (ESC), adult stem cells (ASC), induced pluripotent cells (iPS) etc., the ones closer to clinical application are adult stem cells. These cells can be from three important sources: *autologous*, where the patient's own body cells are used after ex-vivo proliferation/ manipulations, *allogenic*, where cells from another human donor are used or *xenogenic*, where cells from a different species are used. Even though allogenic and xenogenic sources offer a more universal and commercially viable appeal, yet autologous sources are still the best option as the technique does not suffer from the grave complication of immune reaction and rejection.

Potential cell sources can further be sub divided into adult stem cells, embryonic stem cells, totipotent stem cells like zygote and more recently induced pluripotent stem cells [71]. Stem cells because of their inherent property of self-renewal and ability to differentiate into any specific cell type make a very good candidate as cell source. Embryonic stem cells find limited applications because of the associated ethical concerns. Adult stem cells, on the other hand, have great potential for use in therapy. They are the resident population of stem cells in specialized 'niches' within an adult tissue and are responsible for maintaining tissue integrity and organ function post-injury. These adult stem cells have proliferative and self-renewal capacity, least ethical considerations, easy to harvest and they can be differentiated into specific tissue types making them very good candidates for regenerative medicine.

1.15.1 Stem cells

Stem cells are distinguished from other cell types by two important characteristics, which can also be considered as defining parameters. First, they are unspecialized cells capable of renewing themselves through asymmetric cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the skin, hair, mucosa, gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions. Recent evidence points to the presence of stem cells in brain, skeletal muscles; however, their potential for self-renewal and repair under physiological/ pathological conditions is being explored.

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing, essentially without limit, to replenish other damaged cells of the tissue. When a stem cell divides, each new cell has the potential either to remain a stem cell or become a differentiated cell with the potential to perform specialized functions (asymmetric division).

The definition of stem cell mainly takes into consideration the functional properties that the cell must possess in order to be classified as a stem cell. The properties of stem cell, enumerated by Lajtha *et.al.*,1967 [92] at the Canadian Cancer Conference are ones which has gained a lot of acceptance in the field.

So, stem cells are undifferentiated cells capable of:

1. Proliferation
2. Self-maintenance
3. Production of a large number of differentiated, functional progeny
4. Regenerating tissue after injury
5. Flexibility in the use of these options

1.15.2 Types of stem cells

Depending on its source of origin, stem cells can be classified as embryonic, fetal and adult (Figure 1.14).

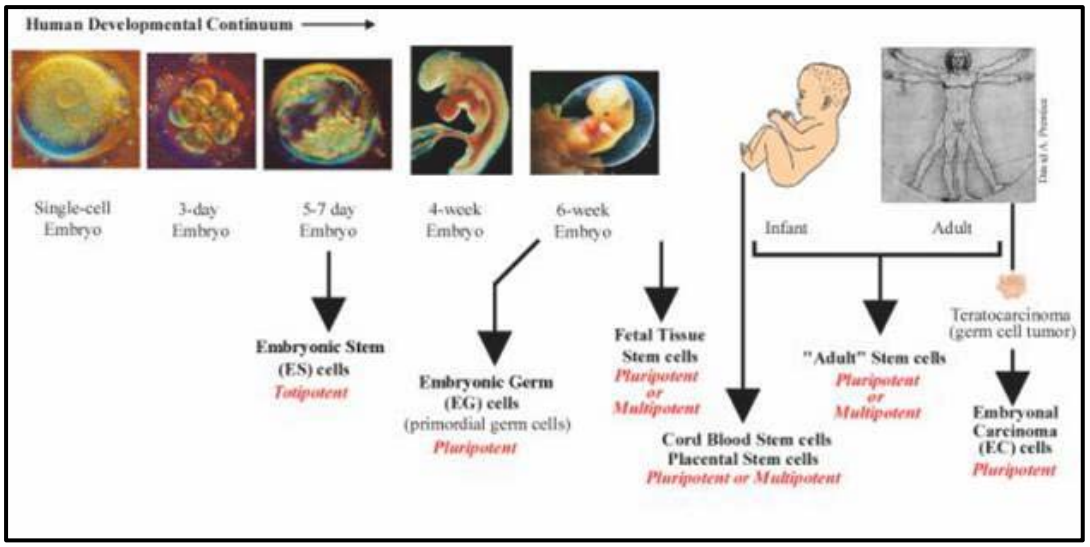

Figure 1.14: Stem cells: sources and potency *(Adapted from http://searchpp.com/stem-cells-learn-about-types-and-definition-embryonic-fetal/)*

I) Embryonic stem cell

Embryonic stem cells are derived from the inner cell mass of blastocysts. A blastocyst is an early stage embryo- four to five days old in humans and consisting of 50–150 cells. Embryonic stem cells are pluripotent and can give rise to all the derivatives of the three primary germ layers namely: ectoderm, endoderm and mesoderm. However, they do not contribute to the extra-embryonic membranes or the placenta.

II) Fetal stem cells

Fetal stem cells are found in the organs of the fetus. This category of stem cells has not been clearly defined. Fetal stem cells are sometimes clubbed together with adult stem cells.

III) Adult stem cells

Adult stem cells or somatic stem cells are self-renewing cells found in the body post-embryonic development. The potency of these cells is limited in comparison to embryonic stem cells and they are capable of generating only a few types of cells belonging to the lineage from which they originate.

IV) Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are adult cells, first reported by Yamanaka in 2006 [93] that are genetically reprogrammed to an embryonic stem cell–like state. The generation of iPS cells depends critically on the genes used for the induction. Initially, a cocktail of four transcription factors, called the Yamanaka factors, consisting of Oct4, Sox-2, Klf4 and cMyc were used to generate iPS cells. However, with more and more

research going on in the field, this cocktail of four has been reduced to two- Oct-3/4 and certain members of the Sox gene family (Sox1, Sox2, Sox3, and Sox15) as crucial transcriptional regulators involved in the induction process.

iPS cells are believed to be identical to natural pluripotent stem cells, such as embryonic stem (ES) cells in many respects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and differentiability, but the full extent of their relation to natural pluripotent stem cells is still being assessed. Although iPS cells meet the plasticity criteria of embryonic stem cells it is not known yet if there is any clinically significant difference between the properties of these two cell types. Mouse iPSCs were first reported in 2006 [93], and human iPSCs in late 2007 [94]. Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expression of stem cell markers, teratoma formation with a resident population of cells derived from all the three germ layers and the ability to differentiate into a number of cell types on transplantation.

Depending on the methods used, reprogramming of adult cells to obtain iPSCs may pose significant risks that could limit its use in humans. For example, if viruses are used to genomically alter the cells, the expression of oncogenes may potentially be triggered. However, more recently the group of Sheng Ding in La Jolla, California, has shown that the generation of iPS cells is possible without any genetic alteration of the adult cell [95, 96]: A repeated treatment of the cells with certain proteins channeled into the cells via

poly-arginine anchors is sufficient to induce pluripotency. The acronym given for these iPS cells are piPS (protein induced pluripotent stem cells).

iPSC are being investigated in the potential treatment for Parkinson's disease [97], platelet deficiency [98], spinal cord injury [99] and macular degeneration [100]. The first ever iPSC human clinical study for treatment of macular degeneration, which began active patient recruitment in August 2013, is led by "Masayo Takahashi of the Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, and being conducted in collaboration with the Institute for Biomedical Research and Innovation with support from the Kobe City Medical Center General Hospital, after approval by the Ministry of Health, Labour and Welfare, Japan. The protocol for Takahashi's new pilot study involves establishing autologous iPSCs from each of the research participants, differentiating them into retinal pigment epithelium (RPE) and transplanting these as monolayer cell sheets without the use of synthetic scaffolds or matrices. The cell sheets would be shaped into 1.3 × 3 mm grafts and transplanted into the affected site of a single eye, following excision of the damaged RPE and neovascular tissues. This pilot study follows on extensive preclinical safety and feasibility testing, including evaluations of cell morphology, physiologic activity, gene expression, immunogenicity, and tumorigenesis in rodent and non-human primate models." (RIKEN Press Release).

1.15.3 Cell therapy for organ regeneration

The FDA defines cell therapy as "The prevention, treatment, cure or mitigation of disease or injuries in humans by the administration of autologous, allogeneic or xenogeneic cells that have been manipulated or altered ex vivo". The goal of cell therapy, intertwined with that of regenerative medicine, is to repair, replace or restore damaged tissues or organs. The field emerged due to the need for reconstruction in patients whose tissue had been damaged/ destroyed by trauma, disease or congenital abnormalities. The Swiss physician Paul Niehans, is credited with invented cell therapy in 1931. During a medical emergency, Dr. Niehans injected a solution containing ground-up parathyroid cells from a calf into a patient with damaged parathyroid glands. The patient recovered, and Dr. Niehans attributed the improvement to the injection. Since then the field has gone through a lot of revolutionaly changes. Even though the field is still in its infancy, the tremendous amount of research and the large influx of accrued knowledge have pushed a lot of regenerative therapies into the clinical settings. Mentioned below are some of the important areas in which the option of cell therapy and regenerative medicine is being explored:

I) Bladder regeneration

Due to the problems associated with using gastrointestinal tissue segments for bladder replacement and repair, Dr. Anthony Atala and colleagues used the concepts of regenerative medicine to repair the organ in human patients. Urothelial and muscle cells were expanded *in-vitro* and seeded on bladder-shaped scaffold made of collagen, or a composite of collagen and polyglycolic acid (Figure 1.15a). After about 7 weeks of *ex-*

vivo expansion, these autologous bladder constructs were implanted into the patients with and without omental wrap. A follow up period ranging from 22-61 months (mean 46 months)showed that post-operatively, the mean bladder leak point pressure decrease at capacity, and the volume and compliance increase was greatest in the composite engineered bladders with an omental wrap (Figure 1.15 b) (56%, 1.58-fold, and 2.79-fold, respectively). Bowel function returned promptly after surgery. No metabolic consequences were noted, urinary calculi did not form, mucus production was normal, and renal function was preserved. The engineered bladder biopsies showed an adequate structural architecture and phenotype (Figure 1.15) [101]. The results show that it is possible to tissue engineer bladders that are anatomically and functionally similar to the normal.

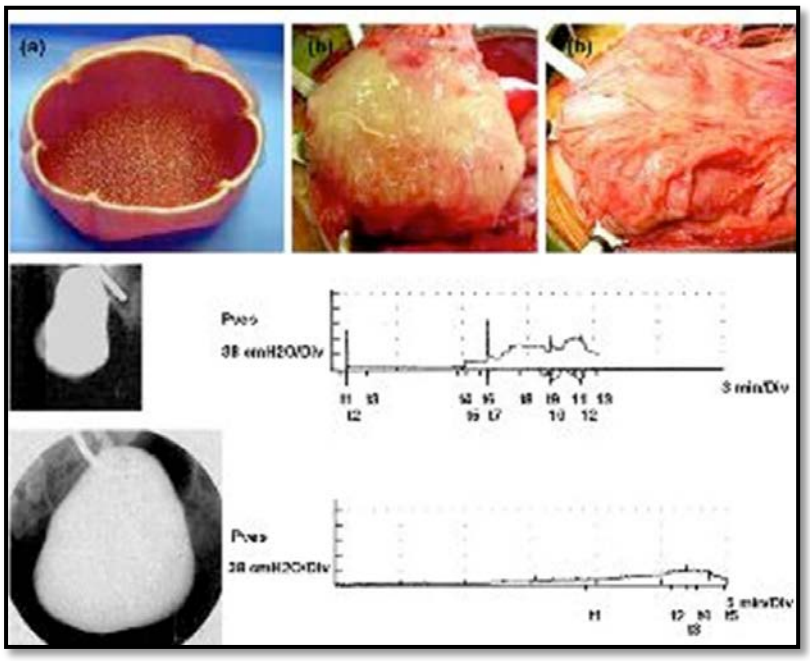

Figure 1.15: Tissue-engineered autologous bladder *(Adapted from Atala et al., 2006)*

II) Trachea

Trachea, or the wind-pipe, is an important organ of the respiratory system. It is a cartilaginous and membranous tube, extending from the lower part of the larynx (voice box), on a level with the sixth cervical vertebra, to the upper border of the fifth thoracic vertebra, where it divides into the two bronchi, one for each lung [16]. The main function of the trachea is to allow inhaled air to travel into and out of the lungs from the atmosphere. It also plays an important role in the smooth functioning of the digestive system by ensuring smooth passage of food from the mouth to the stomach. The tracheal cartilages are enclosed in an elastic fibrous membrane and are associated with non-striated longitudinal and transverse muscle fibers. The trachea also has mucous membrane which is continuous with the larynx and the bronchi. It consists of areolar and lymphoid tissue with a basement membrane, supporting a stratified, columnar ciliated epithelium [102].

Patients with malignancies and stenotic inflammatory lesions of the trachea require surgical resection of a part of the tracheal wall and subsequent reconstruction of this defect. Conventionally, these defects were reconstructed using either the end-to-end anastomosis technique or autologous tissue implantation with skin/ cartilage from the nasal septum, auricle or costal cartilage [103]. However, these are associated with the problems of limited availability of transplantable tissue as well as separation, stenosis, and infection of the reconstructed trachea. Artificial tracheas such as silicon, marlex mesh and stainless steel wire mesh have been tried but have been unsuccessful due to separation and stenosis [104-106].

More recently, the approach of tissue engineering is being applied to tracheal reconstruction. Macchiarini *et al.,* reported successful clinical implantation of tissue engineered trachea retrieved from a donor and combined with the recipient's epithelial cells and chondrocytes [107]. Omari *et al*., employed a collagen conjugated prosthesis reinforced by polypropylene mesh frame and a polypropylene ring sandwiched with porcine collagen sponge in a patient with non-circumferential tracheal resection. Even though the procedure was a success, it had the drawback of delayed epithelialization [108]. To overcome this problem, Nomoto *et al.,* incorporated fibroblast-containing collagen gel in this scaffold and were successful in accelerating the differentiation and establishment of the tracheal mucosa *in-vitro* and rat *in-vivo* studies [109, 110]. In 2011, the group of Prof Alexander Seifalian and Prof Paolo Macchiarini designed, built and transplanted a polymer based nano-composite tracheal scaffold seeded with autologous stem cells into a 36 year old male patient with promising results [111].

Though the initial studies and case reports have been very encouraging with their optimistic results, the modality of bioengineered scaffolds for tracheal regeneration needs to undergo further experimentation investigating its long-term efficacy and safety before they can come in the mainstay of therapy.

III) Pancreas

Pancreas is a glandular organ which secretes hormones like insulin, glucagon. In diabetes mellitus, the β-cells present in the pancreas, that secrete insulin either get damaged or lose their functionality. In such cases the patient is on lifelong anti-diabetic drug treatment.

However, even with the current line of good anti-diabetic drugs the patient can develop chronic systemic complications like atherosclerosis, foot disease, stroke, retinopathy, neuropathy etc. Cell therapy option of treating diabetes by generating insulin producing cells from stem cells and then transplanting them in patients is an avenue which is being investigated.

Transplanting islet cells, using the Edmonton Protocol was first performed by Dr. James Shapiro in 2000. Even though the initial success rate of this procedure was quite impressive- a year after transplant, 50 - 68% of patients did not need additional insulin, but by five years after the procedure, fewer than 10% of total patients were free of daily insulin supplementation. Other options like transplanting pancreatic β-cell precursors are also being investigated. These cells have an advantage over mature islet cells: unlike the isolated beta cells that release insulin in a monophasic, all-or-none manner, these pancreatic precursor cells can be fine-tuned *in-vitro* to produce all the cells of the islet cluster in order to generate a population of cells capable of coordinating the release of insulin in response to the blood glucose levels.

More recently, a phase 1 and 2 clinical trial is being planned to test the safety and efficacy VC-01, a stem cell-derived, encapsulated β-cell replacement therapy. This therapy involves implanting the mature β-cells in an encapsulated (usually alginate encapsulated) fashion into the body. These cells have the ability to secrete insulin and also have the advantage of being in a protective barrier within the body. The major advantage of these encapsulated β-cells is that they can assess the patient's glucose level in the blood

and secrete the required amount of insulin, while their encapsulation barrier can protect them from being destroyed by the auto-immune system. Preclinical studies have shown the effectiveness of this therapy in reducing the blood glucose levels in murine models of diabetes. However, clinical trials need to be conducted in order to determine its effectiveness and safety in humans.

IV) Salivary gland

Salivary gland destruction or dysfunction leading to hyposalivation and xerostomia (dry mouth) is seen in conditions like autoimmune disorders, head and neck cancers etc. In addition, a number of other conditions like uncontrolled diabetes mellitus, sarcoidosis and renal diseases can cause hyposalivation related xerostomia. The condition is extremely uncomfortable and debilitating and considerably compromises the quality of life of the patient. The treatment options currently available deal with managing the symptoms of the condition. Cholinergic agonists like pilocarpine and cevimeline are used to stimulate salivary secretion or artificial saliva like NeutraSal may be used. However, these provide only transient short-term relief. For a more long-term management, the option of cell therapy is being explored.

Genetic lineage tracing studies and immunophenotyping studies have been used to identify salivary gland stem/ progenitor cells that can come to the rescue of the gland post-insult. These stem/ progenitor cells reconstitute and regenerate the damaged gland and restore its function [112, 113]. However, in case of severe damage, as seen in radiotherapy induced xerostomia, the remaining stem cells are probably inadequate to regenerate and

compensate for the extensive damage. In these cases, certain interesting and novel therapeutic avenues are being explored. One of the methods is application of agents like growth factors and cytokines that stimulate the division of stem/ progenitor cells. Cytokines like EGF [114], insulin growth factor [115, 116] and bFGF [116] have been suggested to inhibit apoptosis and/or enhance proliferation. Recently, a study by Lombaert *et al.,* on the role of KGF on radiation-induced salivary gland damage in mouse submandibular gland showed that DN23-KGF treatment for 4 days prior to irradiation induced salivary gland proliferation in all cell types but especially in stem ⁄ progenitor cells, increasing their pool [117]. Even though this seems like a viable option but KGF suffers from the drawback of inducing tumorigenic proliferation of cells, thereby significantly limiting its use. Due to this, the option of transplanting adult stem cells in order to regenerate the salivary gland is also being explored. It is hypothesized that transplantation of healthy salivary glands stem ⁄ progenitor cells harvested prior to irradiation and if transplanted after treatment may reduce hyposalivation and xerostomia. Lombaert *et al.,* developed a method to isolate and culture murine submandibular gland cells as salispheres derived from putative stem cells of ductal origin [118]. These salispheres were shown to contain cells expressing stem cell markers like stem cell antigen-1(Sca-1), CD117 (c-kit) and mushashi-1(Msi-1). These spheres were able to form functional duct and acinar-like cells *in-vitro*. Interestingly, when these were transplanted as single cells (75,000) into irradiated glands they were able to restore long term submandibular gland morphology and function. Further refinement of this study showed

that c-kit positive cells are possibly the stem/ progenitor cells as transplantation of only 300 c-kit$^+$ cells into the damaged gland could regenerate it [118].

c-kit$^+$ cells have also been detected in excretory ducts from human salivary glands. Similar to mice, salispheres could be cultured from both human parotid and submandibular gland tissue and these could self-renew and differentiate into ductal structures and mucin-expressing acinar-like cells in 3D matrix cultures under *in-vitro* conditions. These results indicate that human salivary gland also contains a pool of putative stem/ progenitor cell population comparable to that found in rodents [119].

1.16 Review of Lacrimal Gland Studies

1.16.1 Developmental studies: signaling molecules in the development of the lacrimal gland

The development of lacrimal gland follows a pattern of morphogenesis that has been studied in other organ systems like limb[10] and lungs [120]. These studies have implicated a number of soluble signaling molecules in this morphogenetic development of the lacrimal gland. Some of the important signaling molecules involved are sonic hedgehog [121, 122], members of bone morphogenetic protein [123] and fibroblast growth factor families [124].

The results of a number of elegant experiments, performed by Makarenkova *et al.*, show that transcription factor PAX 6 and soluble mediator FGF10 are essential for normal lacrimal gland development [125]. PAX 6 is a soluble transcription factor expressed in the

conjunctival epithelium but not in the neural crest derived peri-ocular mesenchyme [126, 127] suggesting that the requirement of PAX 6 is inherent to the cells of the precursor conjunctival epithelium and it is likely that PAX 6 is one of the key factors which establishes competence and permits gland development from conjunctival epithelium in response to a fibroblast growth factor (FGF) ligand [125]. The Fibroblast Growth Factors (FGFs) constitute a large family of heparin-binding proteins involved in many aspects of development including cell proliferation, growth, and differentiation. They act on several cell types to regulate diverse physiologic functions including angiogenesis, cell growth, pattern formation, embryonic development, metabolic regulation, cell migration, neurotrophic effects and tissue repair. FGF family activities are mediated by receptor tyrosine kinases and are facilitated by heparan sulfate. In humans, 22 members of the FGF family have been identified, all of which are structurally related signaling molecules. Amongst these, FGF7 and FGF10 can elicit ectopic lacrimal gland development from conjunctival epithelium [128]. Between the two, FGF10 is probably the better candidate for an endogenous inducer as it is expressed with appropriate spatial pattern [125]. The response of conjunctival epithelium to FGF7 and FGF10 is probably a reflection of their high affinity for FGFR2IIIb [129], which is highly expressed in the precursor conjunctival epithelium [130]. FGFR tyrosine kinase inhibitor [131] and an antisense nucleotide specific for FGFR2IIIb mRNA [132] can prevent lacrimal gland budding. These observations, in combination with the lack of lacrimal gland development in FGF10 null

mice indicate that mesenchymal FGF10 stimulate lacrimal gland morphogenesis by directly activating FGFR2IIIb in the conjunctival epithelium [125].

Even though FGF10 appears to be an inducer of lacrimal gland development, it does not provide an instructive signal, as it is not exclusive to the lacrimal gland development. Exogenous supplementation of FGF10 leads to the growth of lacrimal tissue but without any branching morphogenesis, implicating this factor in stimulation of cell division.

1.16.2 Branching morphogenesis of lacrimal gland

The lacrimal gland is an organ that develops by branching morphogenesis [133]. The process of branching morphogenesis involves exchange of signals between the developing epithelial bud and the surrounding mesenchyme [134]. The members of bone morphogenetic (BMP) family of proteins have been implicated as the signaling molecules involved in this branching process in a number of organs like lung [135], kidney [136] and prostate gland [137]. In the context of the lacrimal gland, *Bmp7* has been shown to have a dynamic expression pattern within the mesenchyme of the gland [138] and its importance can be judged by the deficiency of lacrimal gland seen in *Bmp7*- null mice. The primary target of *Bmp7* is probably the lacrimal gland mesenchyme with indirect effect on the developing epithelium, giving rise to the lobe pattern [133]. It is also likely that other members of the BMP family, like *Bmp4*, are involved in lacrimal gland development by suppression of epithelial proliferation [133].

The role of *Bmp7* in stimulating lacrimal gland branching has been extensively studied by Dean *et al.*,[133] and they propose two hypothetical models to explain the possible role played by this signaling molecule [133]. In the first model, they suggest that *Bmp7* has a major role in facilitating and enhancing the formation of signaling centers within the developing organ (Figure 1.16). These signaling centers are, possibly, a small aggregation of closely associated cells capable of secreting factors, in the so-called community effect [139] and responding to stimuli in a manner that would stimulate development and branching. These signaling centers are proposed to include both the epithelial as well as the mesenchymal cells in the region of the developing bud tip and their shape probably influences the shape of the growing tissue [134]. In the alternative model, the authors propose that *Bmp7* may function primarily by stimulating a mesenchymal to epithelial transition [133]. This model finds support in the observations that some markers expressed at high levels in the epithelial component of the lacrimal gland like connexin 43, cadherins and smooth muscle actin, tend to get upregulated when isolated mesenchymal cells are stimulated with Bmp7. This process probably is crucial to stimulate proliferation and an epithelial to mesenchymal transition, which supplies the developing gland with the epithelial cells. However, this model is definitely a more speculative one and would require additional work in developing strategies for fate-mapping different components of the gland before it can be accepted [133].

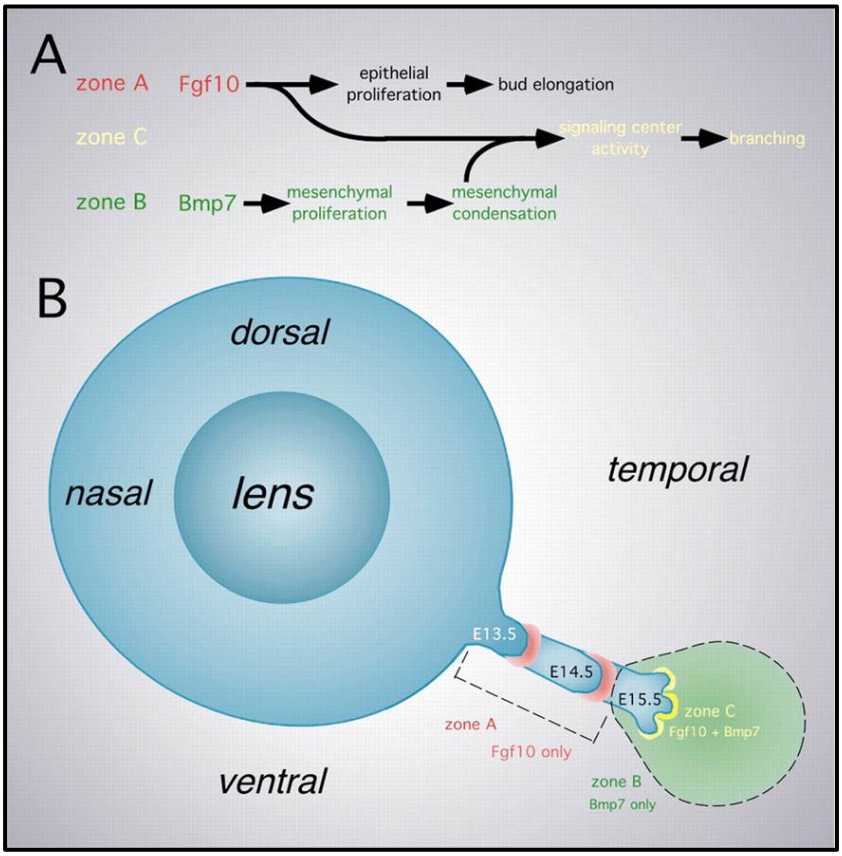

Figure 1.16: Model describing bmp7 function in lacrimal gland development.
(Adapted from Dean et al., 2004)

(A) Bmp7 promotes formation of signaling centers in the lacrimal gland, through stimulation of mesenchymal proliferation and aggregation, resulting in branching morphogenesis. (A,B) The pattern of lobe formation in the lacrimal gland can be explained in part by the combined action of Fgf10 and Bmp7 where Fgf10 is a stimulus for bud elongation and Bmp7 for branching. (A,B, zone A) During the initial 48 hours of lacrimal gland development, the primary bud extends and does not branch because the epithelium is exposed only to Fgf10. (A,B, zone C) In contrast, upon entering the defined region of Bmp7-expressing mesenchyme, epithelium will branch because it is exposed to Bmp7. It is possible that this regulation of branching is mediated not by the absolute presence or absence of Bmp7, but by different Bmp7 levels.

1.16.3 Lacrimal gland in-vivo studies

The presence of stem cells in adult tissues is a very active area of research because of the potential clinical benefits [140-142]. A number of studies have reported the self-regenerating capabilities of exocrine glands like salivary, pancreas, liver, intestine, and breast [143-147]. In particular, a lot of work has been done in investigating the regenerative capacity of the salivary glands [146-150]. It has been reported that long-term (7–21 days) ligation of the main excretory ducts of the salivary glands leads to atrophy of the glands preceded by inflammation, edema and death of acinar cells. Studies have reported that after release of the duct ligation, the salivary glands go through a phase of increased cellular proliferation and repair. It has also been shown that nestin positive cells, which are thought to be stem cells/ progenitor cells are involved in tissue regeneration of pancreas, breast and salivary gland. Since the lacrimal gland has a great deal of similarity with the salivary gland it is expected that similar results could be seen in lacrimal gland too.

Even though there are only a handful of studies that report the presence of stem-like cells in lacrimal gland, however, the last couple of years have seen an increase in the knowledge about the presence of stem-like cells in the lacrimal gland of mice [151] and rats [152]. These studies indicate the inherent potential of the gland to heal itself following an insult. The study published by You *et al.,* showed that post injection of interleukin into the mouse lacrimal gland, which destroys areas in the gland, stem-like cells migrate towards the site of injury and heal the wound. These cells can be harvested and grown

under *in-vitro* conditions too. However, the authors report minimum in-vitro growth from uninjured gland [151]. In contrast, the recent study by Shatos *et al.,* on rat lacrimal gland and our own experience with human lacrimal gland showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in-vitro* conditions [152].

The presence of stem cells in the lacrimal gland is an important finding that leads us to believe that these cells can be recruited to salvage the damaged gland and be used in cell therapy of chronic dry eye. However, before we take a leap of faith the viability, homing and functionality of these cells need to be established by more extensive *in-vitro* studies and independent animal experimentation.

1.16.4 Lacrimal gland in-vitro studies

An important area of investigation in the field is to find a common link between tear film osmolarity, tear film break up response and the resultant inflammatory stress. In order to facilitate these studies, *in-vivo* and *in-vitro* models are being developed that would greatly assist the investigation into the secretory repertoire of lacrimal gland epithelia, regulation of secretion, etiopathogenesis of lacrimal gland conditions like Sjogren's syndrome and also pave way for cell therapy.

Much of the information regarding the secretory function and pathology of human lacrimal gland has been obtained indirectly by correlating the data obtained from evaluating the tear specimen of animal models like rabbit, mice, rat and dogs or by studying the cultured animal lacrimal gland epithelia.

Procedure for *in-vitro* culturing of lacrimal gland acinar cells has been evolving for nearly two decades now. Oliver *et al.,* published one of the first reports on *in-vitro* culture of rat lacrimal gland acinar cells in 1987 wherein they described a method for culturing a dividing population of morphologically differentiated rat lacrimal acinar cells on a three-dimensional, reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules [153]. However, their culture system could only maintain the epithelial cells for 6-7 days after which fibroblast overgrowth was observed. Successful *in-vitro* culture of lacrimal acinar cells was achieved in a combination of serum free Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (1:1) containing a variety of supplements like epidermal growth factor (EGF), glutamine, sodium pyruvate and soyabean trypsin inhibitor [154, 155]. The importance of media formulation, supplement profile and extracellular matrix composition for optimal growth and functionality of these cells was first reported by Hann *et al.*,[156]and these findings were supported by a number of subsequent reports.

Guo *et al.,* (2000) described the procedure for obtaining a highly purified acinar cell preparation from rabbit lacrimal gland [157]. This population has been studied by immuno-cytochemical staining for the presence of polymeric immunoglobulin receptor, cytokeratins[158] and for the absence of rabbit thymic lymphocyte antigen [157].

A major problem faced by all these investigators was that the lacrimal acinar cells could not be induced to proliferate significantly *in-vitro.* This issue was resolved by

Schonthal *et al.,* who reported in 2000, that the i*n-vitro* proliferation of lacrimal acinar cells could be improved significantly by the combined use of EGF, dihydrotestosterone (DHT), Matrigel and HepatoStim® culture medium [159]. A number of recent publications also report the use of polyethersulfone dead-end tube [160] and denuded amniotic membrane [161] as scaffolds for successful *in-vitro* culture of rat or rabbit lacrimal glands. There have also been attempts to culture rabbit lacrimal gland as 3D spheres in rotatory culture bioreactors. However, the problem with this culture system is that the spheres could only be grown for three weeks after which they die due to progressive central apoptosis [162].

The effect of androgen on the control of secretory component output by the lacrimal gland has been well established. The effect of androgens and androgen analogues on *in-vitro* culture of lacrimal acinar cells has helped elucidate the control that the androgens exert on the synthesis and secretion of secretory component [156, 163-165] as well as other biochemical parameters related to the lacrimal secretion including the basal tear flow rate [166].

Table 1.5: In-vitro lacrimal gland research: Information matrix

Year	**Species**	***In-vitro* research**	**Reference**
1987	Rat	Established culture	[153]
1991	Rat	Importance of media & growth factors for in-vitro cultures	[156]
1994	Rabbit	Physiologically responsive *in-vitro* cultures	*[154];* [155]
2000	Rabbit	Purified acinar cell preparation	[157]
2000	Rabbit	Use of EGF, DHT, Matrigel and HepatoSTIM for culture	[159]
2000	Human	*In-vitro* culture from cadaveric tissue	[167]
1984 1990 1991	Rat	Effect of androgen on synthesis and secretion by lacrimal gland	[163, 164]; [156]; [165]
2006	Rat	*In-vitro* culture in polyethersulfone dead end tube	[160]
2007	Rabbit	*In-vitro* culture on amniotic membrane	[161]
2009	Rabbit	Rotary cell culture system	[162]
2011	Mouse	Report of mesenchymal stem cells in lacrimal gland post injury	[151]
2012	Rat	Progenitor cells in uninjured rat lacrimal gland	[168]

1.16.5 Animal models

In order to better understand the condition of dry eye, animal models have been developed which mimic the features of human dry eye syndrome. A number of mouse, rabbit, rat and a few dog models have been developed which have contributed significantly to increase the knowledge about the etiopathology of dry eye syndrome.

The important animal models that have been created are:

- *Mouse models created using scopolamine and environmental desiccating stress* show that osmolarity of tear as well as secretion of inflammatory cytokines is increased under such condition [169, 170].These models with dysfunctional lacrimal gland have allowed better characterization of epithelial cell turnover rate and ocular surface pathology in dry eye.
- Mouse model of Sjogren syndrome dry eye have also been developed. These Sjogren syndrome mouse models have revealed that ocular surface stress can induce inflammatory T cell alterations and that androgens have the potential to reduce the inflammatory response due to auto-antigen presentation [171-173].
- *Neurturin deficient mice* that develop dry eye and show elevated levels of inflammatory mediators in their tears [174].
- *Rabbit model of KCS* created using the technique of ablation, which shows that steroids like dexamethasone can be used to reverse the ocular surface damage and also to increase the low tear-film break up time [51].

Since the workshop on dry eye in 1995, a number of studies have been done with microarray analysis, showing significant changes in lacrimal gland gene expression after acute corneal injury in mice. Cytokines and other chemokines [171-173, 175], altered cholinergic function [176] and neurotransmitter release have also been identified in mouse model of Sjogren syndrome dry eye [177]. Alpha-fodrin and ICA69 have been identified as the auto-antigen in NFS mouse model of SS and NOD mouse model of SS respectively [178].

Even though it is undeniable that these animal models have indeed increased our basic understanding behind the etiopathology of dry eye, yet the fact remains that we are not completely sure of how well these animal models mimic the human condition. In order to bridge this gap in understanding the similarity/ differences between the animal form and the human form of the dry eye condition, it is imperative that studies be conducted on human tissue. Since, it is unethical to undertake such studies without sufficient background information, *in-vitro* models of human lacrimal gland become very important tool for research.

1.17 Gaps in Existing Literature

Dry eye can be an extremely debilitating condition with high incidence of associated morbidity. The need to explore cell therapy for DES can be gauged by the number of patients who present with chronic dry eye syndrome. The global prevalence of dye eye, estimated by a questionnaire based population survey was found to be to be 7% to 33%

[179]. A clinic based study on a cohort of patients reported that 25.4% of dry eye cases were due to lacrimal gland insufficiency [63]. In the Indian context, this number is about 18.4% to 20% of the population [180, 181] and 25.24% in a hospital setting [64]. The actual numbers are possibly even higher because cases of dry eye are always under reported as the patients ignore the problem. These epidemiological numbers are a good indicator of the need for research on DES.

An extensive survey of literature on lacrimal gland research shows that numerous studies have been published on the *in-vitro* culture of animal lacrimal glands like mice, rat and rabbits. The lacrimal cells have been cultured on a wide variety of matrices like HAM, collagen, Matrigel, polyethersulfone, microgravity bioreactors etc. These studies have revealed a lot of information about the gland including the mechanism of function of the gland, neural controls of secretion, effect of androgens, mechanism of Sjogren's syndrome and lymphocyte interaction. There have also been a couple of reports of the possible presence of stem-like cells in the murine and rat lacrimal gland which could be involved in healing injury. However, these studies have all been conducted on animal tissues. Though they have undoubtedly increased our store house of knowledge yet these results cannot be directly extrapolated to humans. It is imperative that the results be corroborated with human tissues. At the same time, there is no precedence on the culturing of fresh lacrimal gland from human source. The study by Yoshino *et al.,* on cadaveric lacrimal gland gives preliminary evidence that such cells can potentially be cultured *in-vitro*. The other aspect that needs extensive exploration is the potential of these cells to retain their secretory

function in-vitro as well as the possible presence of stem-like cells in the native gland that can be maintained *ex-vivo*.

The whole focus in the past for treating/ managing DES has been on standard therapeutic approaches of lubricating/ hydrating eye drops, pharmacological stimulation of tear production, punctal plugs and other palliative measures. However, now the focus is shifting towards exploring the option of cell based therapy for long term treatment of chronic DES. Though this exploratory option has been tried in salivary gland for management / treatment of radiation induced xerostomia, yet data from lacrimal gland studies is still lacking. Following the progress obtained in other exocrine glands, we hypothesize that a dry eye condition, secondary to lacrimal gland insufficiency, could potentially be corrected by the transplantation of cultured lacrimal gland cells that would survive, integrate so as to ensure adequate tear film production in the damaged eye. Towards achieving this goal we ask the following questions: 1) can human lacrimal gland cells be cultured? 2) will the cultured cells retain their function? 3) do they have stem cells that will reconstitute the functional unit under appropriate conditions? 4) is it possible to assess the damage caused to the lacrimal gland structure and function post orbital radiotherapy?

In an attempt to answer these questions, the present study has the following aims:

1. To establish *in-vitro* cultured, functionally viable lacrimal gland cells in 2 and 3 dimensional matrices which can be used to restore function of the damaged gland post transplantation.

2. To explore the presence of stem-like cells in the human lacrimal gland tissue and the established cultures.

3. To determine the radiation-induced damage caused to the lacrimal gland tissue and the effect of orbital radiotherapy on the development of dry eye syndrome.

Chapter 2: Establishing *In-vitro* Human Lacrimal Gland Cultures

2.1 Introduction

The main lacrimal gland situated in the *fossa glanduloe lacrimalis* of the frontal bone is an exocrine gland which releases its secretions onto the ocular surface through a network of 10-14 ducts. Based on its anatomic location, the main lacrimal gland is divided into the orbital and palpebral portions by the lateral horn of the aponeurosis of the levator muscle. In addition to the main lacrimal gland, the lacrimal secretions are also contributed by the accessory glands called the Gland of Wolfring and Krauss [6]. Histologically, both the main and accessory lacrimal glands are similar to other exocrine tissues like the salivary gland. There are four different types of cells present in the lacrimal gland:

The *secretory epithelial cells* or the acinar cells: the main secretory components of the gland are the pyramid-shaped secretory epithelial/ acinar cells, which comprise major portion of the gland. The acinar cells are secretory cuboidal epithelium with basally located nucleus and large peri-nuclear golgi apparatus [14]. Their apical portion is filled with many periodic acid Schiff's base (PAS) positive secretory granules and the base is associated with a basement membrane (Figure 2.1). This architecture is responsible for polarization of the cells and is essential for the ability of the cells to secrete water, electrolytes and proteins. The acinar cells are linked together by apical tight junctions that not only mechanically attach the cells, but also couple them chemically and electrically. The baso-lateral membrane has the receptors for

neuropeptides, hormones and growth factors that are involved in the secretory functions of the cell [14].

The ductal cells: the lumen of the acinar cells form the inter-lobular and intra-lobular excretory ducts lined by cuboidal epithelium (Figure 2.1). Like their acinar counterparts, the ductal cells are also linked on their apical side by tight junction. This creates polarization and contributes to unidirectional secretion of lacrimal fluids. One of the most important functions of the ductal cells, in addition to collection and transport of lacrimal secretions, is dilution of primary lacrimal fluid by secreting electrolytes and water. These cells also synthesize proteins in their endoplasmic reticulum and Golgi complex and store the same in apical vesicles, though to a significantly lesser extent [15, 19].

The myoepithelial cells: the third important cell type that contributes to the architecture of the gland are the myoepithelial cells (Figure 2.1). These have stellate, spindle-shaped or transitional multi-processed morphology and are alpha-smooth muscle actin (α-SMA) and cytokeratin 5 positive. They envelop the basal portion of the acinar and ductal cells and help them contract in order to empty their contents into the lumen or to propel the fluids forward. The myoepithelial cells have numerous G protein coupled receptors and other signaling components but their exact role in the production of lacrimal fluids still needs to be elucidated [15]

The lacrimal gland also contains a distinct population of *fibroblasts* (which secrete collagen and heparin), *dendritic cells, circulating lymphocytes and plasma cells*. These

plasma cells secrete the J chain of the IgA which couples with the secretory component synthesized by the acinar cells to form the functionally complete IgA which is involved in ocular immunity.

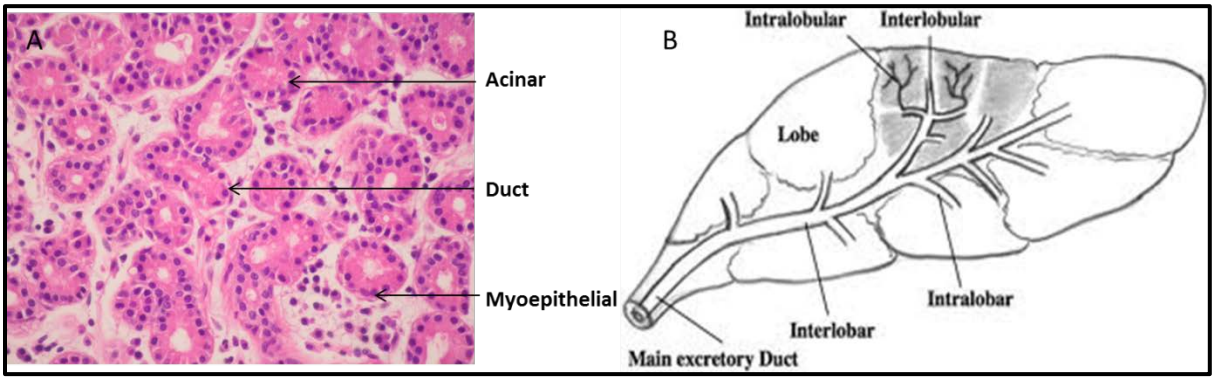

Figure 2.1: A) Hematoxylin and eosin staining of the human lacrimal gland showing the histoarchitecture B) Branching pattern in the lacrimal gland

The lacrimal gland receives its blood supply from the lacrimal branch of the ophthalmic artery, infra-orbital branch of the maxillary artery and the recurrent meningeal artery. The venous drainage follows a similar course and drains into the superior ophthalmic vein. In terms of innervation, the gland has both sympathetic as well as parasympathetic innervation- though the latter predominates both anatomically as well as functionally [6].

The lacrimal gland forms an important component of the lacrimal functional unit, comprising of lacrimal gland, meibomian gland, ocular surface, lids and the sensory/ motor neurons that connect them [182]. The secretions of the lacrimal gland form the aqueous component of the tri-layered dynamic tear film. These secretions include proteins like

lactoferrin, lysozyme, lipocalin, lacritin, water, electrolytes and mucins like MUC1 and MUC4. Any perturbations in the functioning of the lacrimal gland, either due to environmental stress or changes in body homeostasis, drugs or radiotherapy, can lead to a chronic, progressive and morbid condition called the *dry eye syndrome [4]*.

The current therapy for dry eye remains palliative, focusing mainly on need-based frequent lubrication and hydration of the ocular surface. However, this does not provide long term relief nor does it arrest the progression of the condition. In order to provide long-term relief to these patients, the option of cell therapy needs to be explored.

The success of cell therapy requires lacrimal gland cells to be grown/ expanded under *in-vitro* conditions. One of the first reports on *in-vitro* culturing of lacrimal gland was published by Oliver *et al.,* in 1987. The study described a method for culturing a dividing population of morphologically differentiated rat lacrimal gland acinar cells on a three-dimensional reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules [153]. However, their culture system could only maintain the epithelial cells for 6-7 days after which fibroblast overgrowth was observed. Successful *in-vitro* culture of lacrimal acinar cells was achieved in a combination of serum free Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (1:1) containing a variety of supplements like epidermal growth factor (EGF), glutamine, sodium pyruvate and soya-bean trypsin inhibitor [154, 155]. The importance of media formulation, supplement profile and extracellular matrix composition for

optimal growth and functionality of these cells was first reported by Hann *et al*., [156] and these findings were supported by a number of subsequent reports.

Guo *et al*., (2000) described the procedure for obtaining a highly purified acinar cell preparation from rabbit lacrimal gland [157]. This was an important milestone since the problem of fibroblast contamination could be effectively dealt with. However, a major problem faced by the investigators was the failure to induce proliferation of these cells *in-vitro*. This issue was resolved by Schonthal *et al*., who reported in 2000, that the *in-vitro* proliferation of lacrimal acinar cells could be improved significantly by the combined use of EGF, DHT (dihydrotestosterone), Matrigel™ and HepatoStim™ culture medium [159]. Recent review of literature shows the use of polyethersulfone dead-end tube [160], denuded amniotic membrane [161] as scaffolds and rotary cell culture system [162] for successful *in-vitro* culture of rat or rabbit lacrimal glands.

With respect to the human lacrimal gland *in-vitro* cultures, there is reference in literature to just one study, published by Yoshino in 1995 [167]. This study was a preliminary work with cadaveric human lacrimal gland and its main focus was to evaluate the effect of various substrates like collagen 1, Matrigel™ and plastic on the growth parameters of the *in-vitro* lacrimal epithelial cultures. The results of the study led to the conclusion that substrate on which the lacrimal gland cells were grown could significantly modulate the cell morphology, proliferative rate and tear protein (lactoferrin) production. The established

cultures were however not well characterized in terms of various cell population and functionality.

In this chapter, we describe the efforts made to establish cultures from human lacrimal gland and characterize these cultured cells, which we believe is the first step towards replacement therapy. This work would throw light on the type of substrate required to grow and maintain these cells *in-vitro,* the viability and proliferation of various sub-populations of human lacrimal gland cells and if there is any change in phenotype and function of these cells compared to the native cells present in the tissue.

2.1.1 Culture techniques for lacrimal cell isolation

Cell suspension: Studies reported in literature on the *in-vitro* culture of lacrimal gland from mice, rat, rabbit had used the cell suspension technique of culturing in which an enzyme or a cocktail of enzymes was used to isolate the cells from the tissue [153, 154, 159]. The enzymes used were trypsin, collagenase and hyaluronidase or a combination of all three. The cells were isolated from the tissue by incubating with the enzyme after which they were washed and seeded onto cell culture dishes for proliferation.

Explant culture: cultures can also be established by explant culture technique which involves chopping the tissue into very tiny explant pieces using a scalpel blade and then placing these explants onto the tissue culture dish, feeding with appropriate medium for the cells to migrate out of the explant and proliferate. However, in the case of lacrimal gland, this technique has not been used by previous groups.

2.1.2 Substrates for lacrimal cell culture

One of the important parameters to standardize in tissue culture is the substrate on which the cells would grow. A good substrate not only supports the viability of the cultured cells but also their functionality. In the case of lacrimal gland cultures, a number of substrates have been tested *in-vitro*, ranging from uncoated tissue culture dishes, collagen, Matrigel™ [153], polyethersulfone [160] to microgravity rotary bioreactors [162]. Some of these like collagen 1 and Matrigel™ seem better suited to support the viability and functionality of the cultured cells [153].

In-vitro cultures of animal lacrimal gland have provided information about the functioning of the gland, the neural and hormonal control of its secretions, insights into certain mechanisms behind lacrimal gland dysfunction [15, 164]. However, the fact remains that animal data cannot be directly extrapolated to the human condition. There is an imperative need to substantiate that data with human tissue. In order to do that the first step would be to establish an *in-vitro* culture of normal human lacrimal gland and investigate similar questions in them. The present study aims to address that need of establishing human lacrimal gland culture *in-vitro.* At the same time, the study also aimed to establish non-adherent spheres under serum free conditions, from human lacrimal gland so as to be a potential source of cells for future cell therapy in chronic DES.

In the current chapter, we describe the establishment of *in-vitro* cultures of lacrimal gland from normal human lacrimal gland tissue. Establishing *in-vitro* cultures from normal

human lacrimal gland would provide an opportunity to explore the various kinds of cells present in the tissue and would also be a source of cells for potential transplantation into severe dry eye patients in the near future.

2.2 Hypothesis: Human lacrimal gland can be cultured to expand the epithelial cell population in-vitro.

2.3 Aims and Objectives:

1. To optimize the cellular yield from fresh human lacrimal gland tissue
2. To establish human lacrimal gland adherent cultures *in-vitro* from normal tissue
3. To establish human lacrimal gland non-adherent cultures *in-vitro* from normal tissue.

2.4 Materials and Methods

2.4.1 Chemicals

HepatoSTIM culture media (BD biosciences, San Jose, CA, USA), Dulbecco's Modified Eagle's Medium (DMEM) - Ham's F12 (Sigma Aldrich, St Louis, MO,USA) Fetal calf serum (FCS) (HyClone), bovine serum albumin (BSA) (Sigma Aldrich, St Louis, Mo, USA) penicillin, streptomycin, gentamycin and amphotericin B, epidermal growth factor (Sigma Aldrich, St Louis, MO, USA), fibroblast growth factor (FGF) (Sigma Aldrich, St Louis, MO, USA) L-glutamine (2 mM) (Sigma Aldrich, St Louis, MO, USA), Matrigel® basement matrix

(BD Biosciences, San Jose, CA, USA), collagen I gel (Sigma Aldrich, St Louis, MO, USA), collagenase (Invitrogen, Carlsbad, CA, USA), hyaluronidase (Invitrogen, Carlsbad, CA,USA), Hank's Balanced Salt Solution (HBBS), treated tissue culture dishes (NUNC), ultralow attachment plates (NUNC).

2.4.2 Preparation of Chemicals

All the chemicals and culture media were prepared as described in **Appendix.**

2.4.3 Human tissue source

The study was conducted at L V Prasad Eye Institute (LVPEI), Hyderabad. The use of human tissue was approved by the Institutional Review Board (IRB) (Ref. LEC 08020) and is in accordance with the tenets of the Declaration of Helsinki.

Fresh human lacrimal gland was harvested after obtaining written informed consent from patients undergoing exenteration surgery for therapeutic indications. The glands included in the study were from those patients who had no history of undergoing orbital radiotherapy. Part of the tissue was submitted for histology and after it was evaluated to be free from any underlying pathology was used for *in-vitro* studies. The fresh gland was collected in FCS rich DMEM-Ham's F-12 media supplemented with antibiotics and transported to the lab where it was immediately taken for processing.

2.4.4 Preparation of human amniotic membrane

The human amniotic membrane was prepared by the technical staff at the Ramayamma International Eye Bank (LVPEI, Hyderabad) in accordance with the approved and established protocol and stored at -80°C, to be thawed before use.

This protocol is based on the technique published by Kim *et al.,* [183]. Following written informed consent, human placenta was obtained at the time of normal cesarean delivery at the Fernandez Hospital, Hyderabad. The placenta was transported to the Ramayamma International Eye Bank (LVPEI, Hyderabad) and processed under sterile conditions using saline/ ringer lactate solution. The amnion was separated from the chorion by peeling and attached onto sterile nitrocellulose paper and cut into 2.5x5.0 cm pieces. The amniotic membrane pieces were stored in glass vials containing DMEM and stored at -80°C. Just prior to use, it was thawed at 37°C for 30 minutes. HAM was de-epithelized by incubating with 0.25% trypsin–EDTA for 30 minutes at 37°C followed by thorough washing with PBS to remove the epithelial layer.

2.4.5 Coating of tissue culture plates and standardization of substrate

The tissue culture plates were coated with collagen 1 or Matrigel™. Collagen 1 was used in the concentration of 1mg/ml while Matrigel™ was used at a dilution of 1:50. A drop of collagen1 or Matrigel™ was added to the tissue culture dish using a micropipette and the drop

was spread evenly using the broad base of the microtip. The coated plates were incubated at 37°C for at least an hour prior to use.

The cell growth on the substrates was evaluated morphologically as well as by flow cytometry. In brief, the protocol for flow cytometry involved trypsinizing the cells growing as monolayer using 0.25% trypsin-EDTA (TE) and using them for evaluation of marker expression.

Briefly, $1x10^6$ cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with appropriate dilutions of secondary tagged primary antibody for 2 hours at room temperature. At the end of this time period, the pellet was washed with PBS, resuspended in 500µl of FACS buffer and acquired on BD FACS ARIA™ Special Order System. Appropriate controls and gating parameters were used for the experiment. A total of 20000 to 50000 events were acquired for analysis after eliminating dead cells and debris. The analysis was done using BD FACS DiVa™ software.

The antibodies used and the appropriate dilutions are summarized in Table 2.1.

Table 2.1: List of antibodies and dilutions used for flow cytometry

S. No.	**Antibody**	**Dilution**	**Company**
3.	CD90	1:200	eBiosciences
4.	EpCAM	1:100	BD Biosciences

2.4.6 Standardization of media

Various media formulations like DMEM, DMEM-Ham's F-12 and HepatoSTIM media have been used previously by different groups for culturing animal lacrimal gland [153, 157, 159]. In-order to determine the media which would optimally support the growth of human lacrimal gland cells *in-vitro* an attempt was made to culture the isolated cells in these different media formulations and observe the growth and morphology of the cells microscopically.

2.4.7 Sterility check for media:

Following filter sterilization, the sterility of media and other chemicals to be used for tissue culture was evaluated. A few drops of media/chemicals were inoculated on chocolate agar and in thioglycollate broth to screen for both aerobic and anaerobic microorganisms. The inoculated media were then incubated in a bacterial incubator at 37 °C for about 7 days before the media/ chemicals could be approved for tissue culture use.

2.5 Establishing Primary Cultures of Human Lacrimal Gland

2.5.1 Establishing adherent cultures

Fresh lacrimal gland, obtained from exenterated specimens, was washed with HBBS to remove red blood cells. The gland was chopped into small bits using a scalpel blade. The tissue mince was then incubated with the enzyme cocktail of collagenase (130 units per ml) and hyaluronidase (300 units per ml) with or without 0.25% trypsin-EDTA for 90 minutes at 37ºC with intermittent shaking. At the end of the incubation period, the suspension was

filtered through a 75 μm cell sieve and the cell pellet was obtained by centrifugation at 1500 rpm for 20 minutes. The cells were seeded on uncoated tissue culture dishes in DMEM-Ham's F12 culture media supplemented with 10% FCS and antibiotics.

The heterogeneous mix of isolated cells was separated based on their preferential adhesion to uncoated tissue culture dishes. The fibroblasts attached preferentially to the uncoated tissue culture dishes and the epithelial clumps, still in suspension at the end of about 2 hours, were aspirated and plated on Matrigel™, collagen I coated dishes and on denuded HAM. The media used for the culture of fibroblasts was DMEM- Ham's F-12 supplemented with 2mM L-glutamine, antibiotics and 10% FCS. For the culture of lacrimal acinar cells, HepatoSTIM™ culture media was used. HepatoSTIM™ is a commercially available fully defined, serum free media based on the formulation of Williams E Media [184] and contains supplements like dexamethasone, insulin-transferrin-selenium and EGF. The media was further supplemented with 2 mM L-glutamine, penicillin, streptomycin, 5ng/ml EGF and 10% FCS for the first three days. At the end of the three days/ at first media change, FCS was replaced with N2 supplement and the concentration of EGF increased to 50ng/ml. Media was changed every 3 days and the cultures passaged post-confluence using collagenase treatment. Media from day 7, 14 and day 21 were collected and stored at -80° C to be used at a later date for detecting the possible presence of secreted tear proteins.

2.5.2 Establishing non-adherent cultures

The cells were isolated as previously described under section 2.3.8.1 using a combination of collagenase and hyaluronidase. These were plated on untreated ultralow adhesion plates and fed with HepatoSTIM™ media supplemented with N2, 25ng/ml EGF, 10ng/ml bFGF, 2mM L-glutamine, penicillin and streptomycin. The media was changed every 3 days and the spheres passaged every 7-10 days.

2.5.3 Passaging in-vitro human lacrimal gland cultures

Adherent cultures: Once the cultures attain confluence they were passaged using collagenase. The cultures were incubated with 1 ml of 130units/ml collagenase till the sheet of epithelium dislodged from the plate (15-20 minutes at 37ºC). At the end of the incubation period, the cell suspension and enzyme mix was diluted with cold basal media. The cells were centrifuged at 2000 rpm for 3 minutes and the pellet resuspended in complete supplemented HepatoSTIM™ media. The cells were then plated on Matrigel™/ collagen 1 coated plate and incubated at 37ºC, 5%CO_2. Media was changed every third day.

Floating cultures: Once the spheres attained confluence, they were passaged with a split ratio of 2-3. The spheres were centrifuged at 2000 rpm for 3 minutes to obtain a pellet. The supernatant was discarded and the cell pellet resuspended in fresh supplemented media. The spheres were mechanically dissociated by repeated pipetting and then plated in 2-3 ultra-low attachment plates, fed with sufficient media and incubated at 37ºC, 5% CO_2 and allowed to grow. Media was changed every third day.

2.5.4 Cryopreservation and revival

2.5.4.1 Cryopreserving in-vitro human lacrimal gland cultures

The established human lacrimal gland cultures were cryopreserved at -196ºC in liquid nitrogen using a cryo-protectant mix consisting of DMSO (10%) and FCS (70%) and basal media (20%). The adherent cultures were enzymatically dissociated from the plate using 130 units/ml collagenase while the non-adherent spheres were collected and the cell pellet obtained after centrifugation at 2000 rpm for 3 minutes. These cells were then resuspended in cryomix and 1 ml of this was added to each cryotube and stored in isopropanol cryobox at -80ºC overnight. The tubes were then transferred to the liquid phase of nitrogen in liquid nitrogen cryotanks.

2.5.4.2 Revival of cryopreserved human lacrimal gland in-vitro cultures

The cells cryopreserved at -196ºC were thawed rapidly by immersing the cryotube in sterile water at 37ºC. Immediately on thawing, the cell suspension-cryomix was diluted by adding to 10 ml of basal media in order to dilute out the DMSO (which would otherwise prove toxic to the cells). The cell suspension was centrifuged at 1000 rpm for 3 minutes and the obtained cell pellet was resuspended in 1 ml of complete HepatoSTIM™ media. Cell count was taken using trypan blue to determine the revival efficiency. The cells were then plated on collagen 1/ Matrigel™ coated culture dishes or ultralow attachment dishes and incubated at 37ºC, 5% CO_2.

2.5.4.3 Cell counting

The cell suspension was mixed with 1:1 ratio of trypan blue. The hemocytometer was cleaned first with alcohol and then wiped dry. The coverslip was positioned carefully over both the chambers. The diluted cell suspension was mixed thoroughly and then filled into the hemocytometer chambers with the help of the micropipette. Total viable cells (trypan blue negative cells) were counted in 4 large squares (1 x 1 x 0.1 mm). The cell count (cells per ml) was determined as per the formula given below:

Cell Count/ml = (Average Cell Count/Square)* Dilution Factor*10^4

2.6 Results

After IRB approval and informed consent, 28 samples of fresh lacrimal gland tissue were harvested from exenterated specimens for orbital malignancies. Subsequent to histological confirmation of normal lacrimal gland and exclusion of any underlying pathology, 22 samples were included for the study. Cultures were attempted in 15 of the 22 samples out of which 12 samples could be successfully cultured and the remaining three were used for initial standardization experiments. Of the 22 samples, 7 were used for FACS analysis.

2.6.1 Sterility check for media

None of the media and other chemicals showed evidence of contamination as no growth was observed on chocolate agar or thioglycollate broth after 7 days.

2.6.2 Standardization of media

Of the various media formulations tested, it was observed that the growth of human lacrimal gland epithelial cells was optimal in HepatoSTIM™ media. The epithelial cells could be maintained in culture for more than 25 days with HepatoSTIM™ but not so with RPMI, DMEM or DMEM-Ham's F12 (Table 2.2).

Table 2.2: Media standardization

S. No.	Media	Cell type	Morphology maintained for
1.	RPMI1640	>50% spindle shaped cell growth	5 days
2.	DMEM	>50% spindle shaped cell growth	> 25 days
3.	DMEM: Ham's F12	>80 Epithelial cell like morphology on HAM	18-20 days
4.	HepatoSTIM™	> 80% Epithelial cell like morphology on all three matrices	> 25 days

2.6.3 Standardization of substrate

Cell growth was observed on all the three matrices- HAM, collagen 1 and Matrigel™. The cells adhere to the tissue culture dishes within 12 hours of plating. On the basis of morphology it was observed that Matrigel™ seemed optimal for epithelial growth (Figure 2.2). FACS analysis was done to determine the percentage of epithelial cells (EpCAM positive) and mesenchymal cells (CD90 positive) growing on the three substrates and the

results showed that Matrigel™ supported enhanced epithelial growth (2.2±1.70%) versus collagen (1.2±0.3%) and uncoated tissue culture dishes (0.65±.35%) (Table 2.3).

Table 2.3 FACS evaluation of cell population cultured on uncoated, collagen1coated and Matrigel™ coated dishes: DIV 14 data

S. No	Marker	Uncoated (Mean± SEM)	Collagen 1(Mean ± SEM)	Matrigel™(Mean ± SEM)
1.	EpCAM	0.65 ± 0.35	1.2 ± 0.3	2.2 ±1.70
2.	CD 90	85.1 ± 4.9	16.7 ± 0.85	13.3 ± 10.20

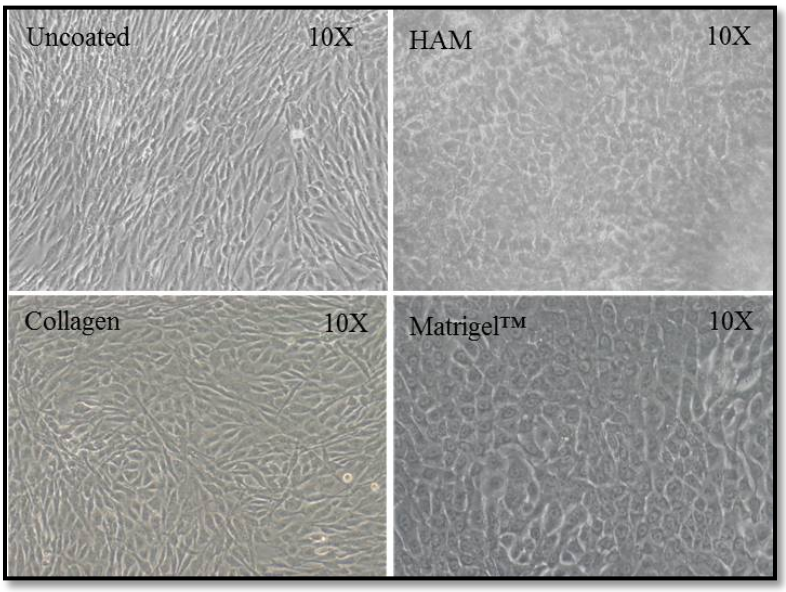

Figure 2.2: Cell growth on different substrates

Spindle shaped fibroblasts were observed on uncoated dishes; HAM supported the growth of plump cells with epithelial morphology; Collagen I supported the growth of epithelial cells mixed with fibroblasts; Matrigel™ showed epithelial cells with the characteristic morphology which could be maintained for greater than 25 days in-vitro.

2.6.4 Establishing human lacrimal gland primary cultures

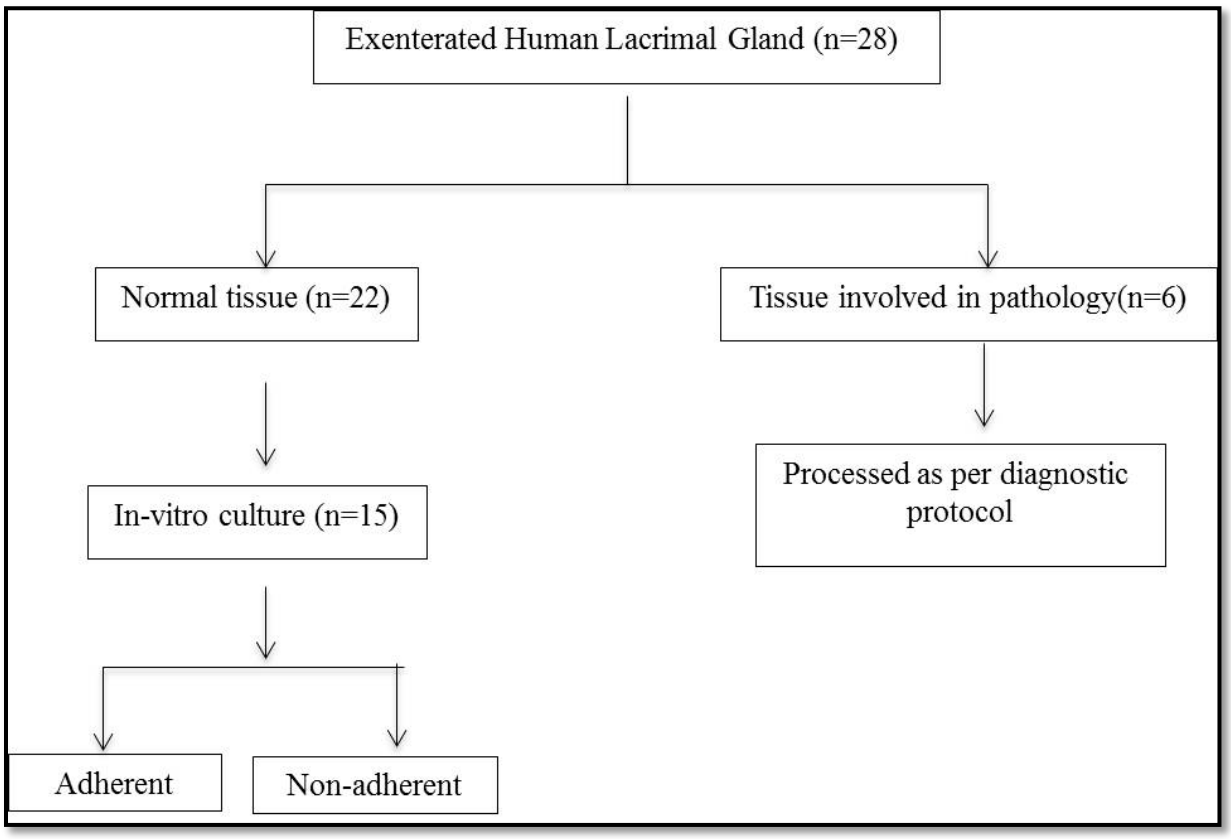

Figure 2.3: Flowchart depicting the establishment of human lacrimal gland primary cultures in-vitro

2.6.4.1 Establishing primary adherent cultures

The enzymatic digestion of the freshly harvested lacrimal gland tissue yielded a heterogeneous population of cells comprising of clumps of epithelial cells, fibroblasts, single cells of varying size and some red blood cells (Figure 2.4a).

When the cell population isolated using trypsin in the enzyme cocktail was seeded on uncoated and coated tissue culture dishes, no attachment or growth of cells was observed. Some cells that do attach showed spindle shape fibroblast morphology. No growth of epithelial cells was observed even after 3-4 days of seeding.

On seeding the cells isolated using collagenase- hyaluronidase cocktail on uncoated tissue culture dishes, some of the cells settled down and adhered to the bottom of the tissue culture dishes within 2 hours. At the end of two hours, the floating cells were aspirated and seeded on dishes coated with Matrigel™, collagen I or on denuded HAM.

The cells seeded on Matrigel™, collagen I coated dishes and denuded HAM appeared to adhere to the substrate and formed discreet epithelial islands (Figure 2.4b). Adherence and formation of epithelial islands, with optimal proliferation and morphology of the epithelial cells was seen on Matrigel™. Within 15-20 days, the epithelial islands expanded to form a monolayer on all the three substrates (Figure 2.4c). The cells showed thin cytoplasmic borders, polygonal shape, vesicular nucleus and granular cytoplasm (Figure 2.4d). The cultures on Matrigel™ showed sustained epithelial morphology for 30-35 days.

In addition, there was formation of ‘spherules’ (Figure 2.4 e, f) in all matrices by day 16-18 in 8 of the 12 cultures established. These cultures also showed development of three-dimensional thick cord-like cellular structures between two spherules which prompted us to speculate that this could be an attempt towards formation of duct-like structures (Figure 2.4 g-i).

On uncoated tissue culture dishes, there was predominance of fibroblast-like cells that form a confluent monolayer in 5-7 days. The cells were spindle shaped, with slight granularity in their cytoplasm and distinct nucleus (Figure 2.5 a). In addition, the culture also showed the presence of a third type of cellular morphology: oval and plump cells that organized themselves in whorls (Figure 2.5b). We speculated these could be myoepithelial in nature.

The cells were maintained in culture for about 30-35 days and passaged for 2-3 passages. The cells were cryopreserved and revived with a revival efficiency of 60-65%. Post revival, epithelial cells could be similarly maintained in culture.

Table 2.4: Timeline of lacrimal gland cells in-vitro growth

Time frame	**Features**
0 hr	Cells isolated by enzymatic dissociation
0-2 hr	Cells adhere to uncoated tissue culture dishes and grow with fibroblast morphology
3hr- 24hr	Cell clumps adhere to HAM/ collagen1/ Matrigel™ coated tissue culture dishes as discreet islands
24hr-15 days	Cells proliferate and form a monolayer
16-18 days	Spontaneous generation of ‘spherules’ and duct-like structures on the monolayer
Post 80% confluence (days 14-20)	Cultures passaged by enzymatic dissociation using collagenase
Post-passaging	Secondary cultures adhere to the coated tissue culture dishes within 2-6 hr and proliferate to form epithelial monolayer in 14-18 days

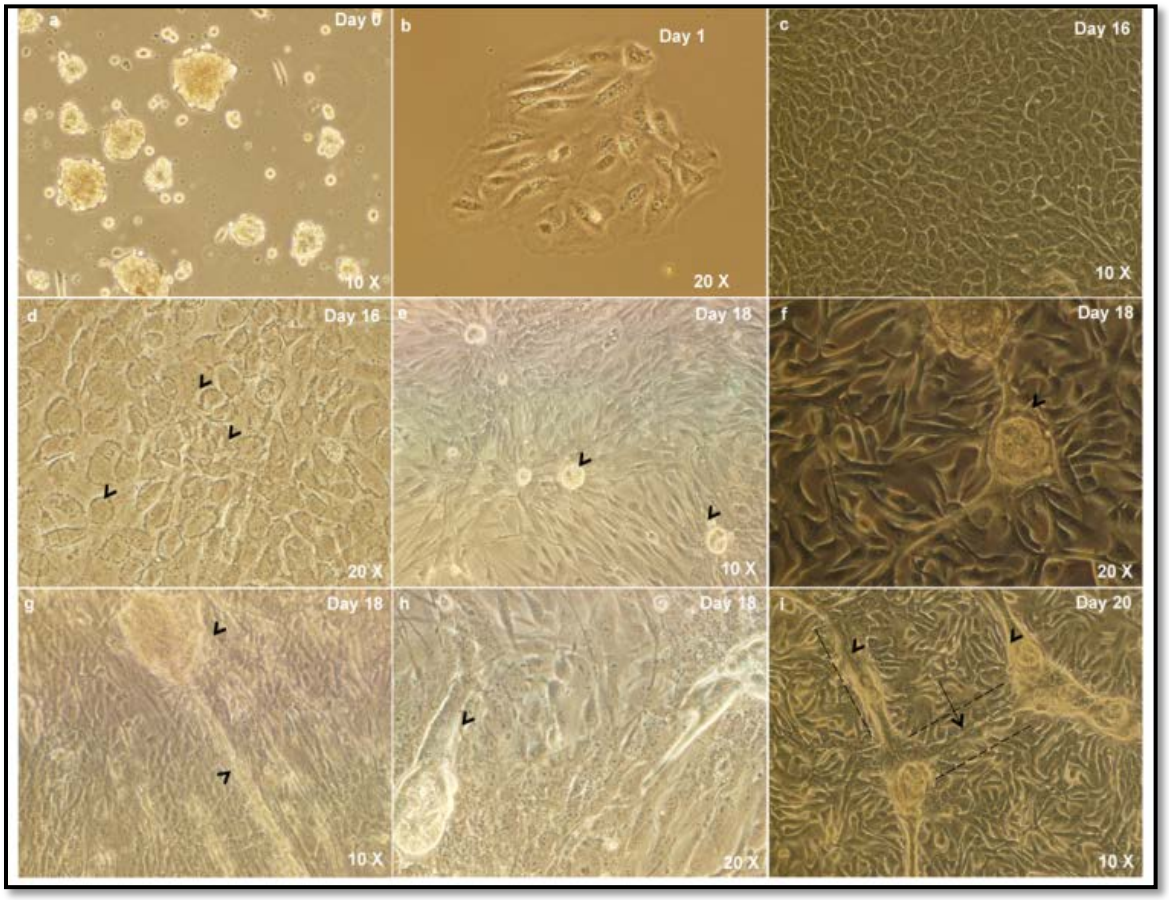

Figure 2.4: Establishment of human lacrimal gland primary cultures *(Tiwari et.al, PLOS One, 2012)[185]*

a) Heterogeneous cell population isolated after enzymatic digestion of the gland; b) Cell clumps adhere to the substrate as discrete islands c) The islands proliferate and form a confluent monolayer within 15-20 days; d)The cells in the monolayer show thin cytoplasmic border, vesicular nucleus and granularity in the cytoplasm; .e-f) Spherules are formed by day 16-18 (arrow head)g-i) Cord-like connections (arrow head) are seen to develop between the spherules.

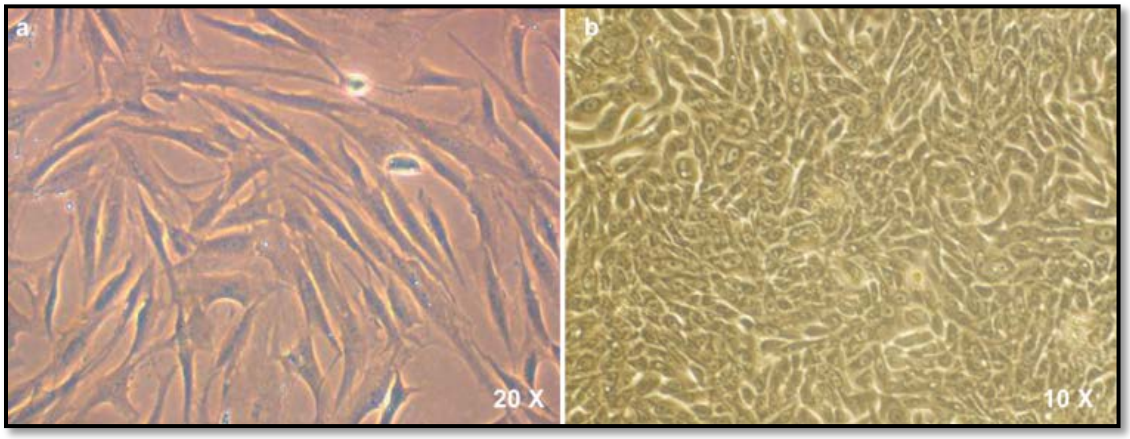

Figure 2.5: Other cell types in culture *(Tiwari et.al, PLOS One, 2012)[185]*
a) Spindle shaped cells with slight granularity in their cytoplasm and distinct nucleus, are seen on uncoated culture dishes and these attain confluence within 5-7 days. b) Oval and plump cells that organize themselves in whorls are also seen. These may be myoepithelial in nature.

2.6.4.2 Establishing serum free primary cultures

Free floating spheres or '*lacrispheres'* of human lacrimal gland cells could be generated under serum free conditions using supplemented HepatoSTIM™, N2, 25ng/ml EGF and 10ng/ml bFGF on ultralow attachment plates (Figure 2.6). These plated spheres had spheroid morphology and smooth edges with cells growing out of it as buds. They grew in size over a period of time in culture from 60.38µm on day 7 to about 200µm by day 14-16. These lacrispheres were passaged for 3-4 passages by mechanical dissociation to generate secondary spheres. The spheres were maintained in culture for more than 35 days after which some of the spheres started disintegrating while the others settled down and attached to the plate. These spheres could be cryopreserved and revived with a revival efficiency of about 50-60%.

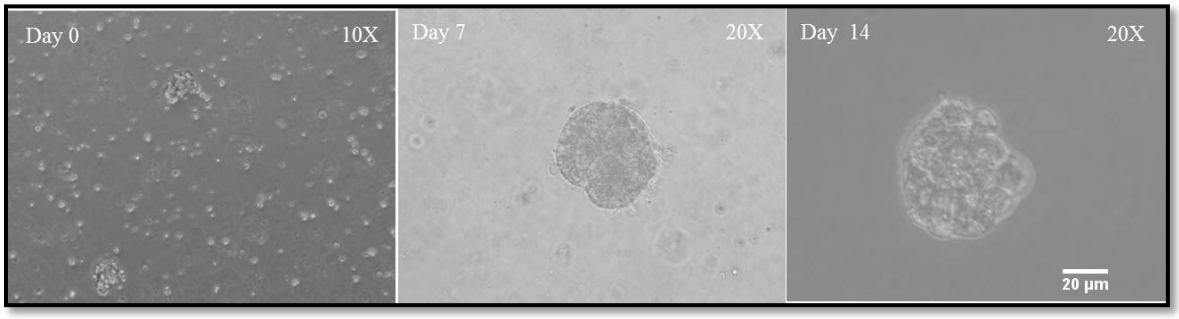

Figure 2.6: Lacrispheres cultured under serum free conditions. *The spheres grow in size over a period of time in-vitro from* 60.38µ on day 7 to about 200µ by day 14-16 *and can be maintained for 35 days*

2.7 Discussion

Similar to the role of other exocrine glands in the body, the human lacrimal gland plays an important role in lubricating and hydrating the ocular surface epithelium. The susceptibility of the gland to immune mediated insults, radiation induced damage and age related atrophic changes, which cannot be satisfactorily managed with the current line of therapeutics, causes high incidences of dry eye related morbidity in the population and raises a need for replacement therapy with functionally competent cells. The present study was aimed at establishing human lacrimal gland cultures. *The study is the first report on successful establishment of human lacrimal gland cultures from fresh tissue on HAM/ collagen 1 and Matrigel™. The study is also the first report on the establishment of non-adherent floating sphere of lacrimal cells which we propose as '**lacrisphere**.'*

One of the important pre-requisites for cell therapy is to establish a source of cells that can be transplanted to regenerate the organ; and towards this an important and sustainable source would be *in-vitro* expanded cells. While lacrimal gland cultures from rabbit [155], mice [186] and rat [187] sources are well established, similar studies from humans is lacking. Even though these animal studies have increased our understanding of the functions of the gland, the results obtained cannot be directly extrapolated to humans. It is essential that these results be corroborated with human tissue studies. To the best of our knowledge, the present study documents the first successful method of isolating and culturing functionally competent fresh human lacrimal gland cells using an enzyme cocktail of collagenase and hyaluronidase. The influence of culture conditions and substrates (collagen[156], Matrigel™[187, 188], HAM[161] and artificial matrices like polyethersulfone[160]) on cell proliferation and maintenance of function, especially in the case of lacrimal gland cultures, has been well documented in animal cultures[156, 159, 189]. Against this background, the human lacrimal gland cultures established in the present study showed the presence of heterogeneous population of cells- epithelial, fibroblasts and myoepithelial which could be maintained *in-vitro* for 30-35 days. The study with human tissues also suggests that a combination of Matrigel™/ HAM/ collagen I as substrate and EGF supplemented HepatoSTIM™ media optimally supports the growth of different sub-population of cells. However, morphological evidence, marker analysis and rate of cell proliferation seem to favor Matrigel™ as substrate even though this difference is not statistically significant. The epithelial cells in our culture

show thin cytoplasmic borders, vesicular nucleus and granularity in their cytoplasm. The cultures formed confluent monolayer within 15-20 days, could be passaged for 3-4 passages, cryopreserved as well as revived with a revival efficiency of 60%. A similar study was published by Yoshino in 1995, which used cadaveric human lacrimal gland (n=9) to establish *in-vitro* cultures on plastic, collagen and Matrigel™. These cells formed clusters in Matrigel™ with a central lumen containing secretory protein lactoferrin but with no detectable proliferative potential. When these cells were cultured in Matrigel™ along with NIH3T3 fibroblasts they formed ductal structures. On collagen I gel, the cells formed a flat monolayer but did not show secretory output. The study, however, did not explore the various sub-population of cells *in-vitro*. [190].

Hann *et al.*, have used trypsin as a component of the enzyme cocktail [156] to isolate lacrimal gland cells of mice/rat origin; our own experience with human cells shows that this enzyme, in fact, proves detrimental to the viability of cells as all the attempts to establish epithelial cultures from the cells isolated using trypsin in the enzyme cocktail were unsuccessful. Similarly trypsin treatment of the cells for sub-culturing also proved unsuitable in this study. We passaged the cultures using collagenase. The sub-cultures showed optimal viability and maintenance of epithelial morphology. The cultures could be passaged 3-4 times. One of the possible reasons for the drop in viability with trypsin could be that being a harsh enzyme it cleaves all protein bonds between the cells as well as between cell-ECM non-specifically, thereby stressing the cells [191]. On the other hand, collagenase breaks down the

bonds between the epithelial cells and the collagen of the extra-cellular matrix only, thus liberating the cells from the basement matrix and yet at the same time not severing all cell-cell contact [192]. This probably helps in retention of phenotype and viability.

An interesting feature noted in this study is morphologic evidence towards spontaneous formation of adherent spheres once the monolayer attains about 60-70% confluency by day 16-18 and development of duct/ cord-like connections between them. Our repeated attempts to separate these spheres for further characterization however were unsuccessful as any manipulation led to disintegration/ disruption of the structures. Previous reports on animal lacrimal gland cultures have not indicated the appearance of spheres or duct-like connections *in-vitro*. At the present time we do not know with certainty, but we believe that this could be indicative of the potential of certain cells in culture to reorganize into 3-dimensional organoids.

This observation of adherent spheres in the cultures and the report of floating spheres in other exocrine gland glands like salivary (salispheres) [118], prostate (prostaspheres) [193] and breast [194] prompted us to attempt culturing human lacrimal gland cells as 3D floating spheres. *Evidence of lacrisphere formation documented in this study, we believe, is the first of its kind from human lacrimal tissues.* Under the culture conditions established in the present study of serum depletion and increased EGF concentration, viable lacrispheres were generated which in turn could generate secondary spheres. These lacrispheres grew in size from 60.38µ on day 7 to about 200µ by day 14-16 and could be maintained *in-vitro* for upto 3-4 passages

(> 35 days). We believe that the novel finding of floating lacrispheres, formed under our culture conditions, could be similar to the salispheres and prostaspheres in their cellular organization which have been shown to have enriched population of stem cells (salispheres 0.6% CD117 positive cells) and the potential to generate the functional unit of the organ when appropriately stimulated and differentiated [195]. Schrader *et al.,* had reported spheroidal aggregation of rabbit lacrimal gland cells grown in microgravity environment of a rotary cell culture system, but these lacrimal spheres could only grow in the rotary bioreactors for around 28 days as they tend to die after that due to spreading central necrosis [162]. This constrain prevented the authors from exploring the functional capability of the spheres. The spheres in salivary gland were stem cell enriched, possibly solid with proven potential for function recovery after transplantation. On the other hand, even though the morphology of prostaspheres and mammospheres is similar to salispheres, the evidence points towards the presence of a central lumen in the former two. At the present time we do not know if the lacrispheres generated under our culture conditions have a central lumen or not. Further exploration of their cellular organization, stemness and differentiation status would give an indication of their functional potential.

The present study, however, is not without its set of limitations. One of the limitations of the study is the inability to dissociate the adherent spheres from the monolayers with its architecture intact. Our repeated attempts to separate these spheres from monolayers were unsuccessful as any manipulation around them led to disruption of their structure. In the

present study, we also could not ascertain if the connections that develop between these adherent spheres have a lumen or not. With respect to lacrispheres, we could not determine if these are solid spheres or if they have a central lumen. Fulfilling these goals would give an indication of the characteristics of these spheres and their associated duct/ cord-like connection.

In summary, our results prove that normal human lacrimal gland can be cultured under *in-vitro* conditions, for a considerable period of time, both as adherent as well as floating cultures. The adherent cultures form a monolayer of epithelial cells as well as subsequent spontaneous adherent spheres and duct-like connections between them possibly indicating an attempt towards generating a potentially functional lacrimal gland *in-vitro.* We propose the term ***'lacrisphere'*** for the floating spheres noted in the culture system which need to be explored for the preferential presence of stem cells within them. If these lacrispheres are found to have greater population of stem cells (as reported with salispheres and prostaspheres) then they would be a potential and easy source of cells to be transplanted into dry eye patients for regeneration of the dysfunctional gland and rescue of function in future.

Chapter 3: Characterizing Human Lacrimal Gland Tissue and *In-Vitro* Human Lacrimal Gland Cultures

3.1 Introduction

Human lacrimal gland is an exocrine gland responsible for secretion of tear proteins and the aqueous component of the tear film. With the initial progress in establishing the culture of lacrimal gland cells on HAM, collagen 1 and Matrigel™ the next logical step would be to explore the specific phenotype of all the cells present in the culture system as compared to the native gland and to investigate if they retain the secretory function of the native tissue. Unlike other exocrine glands, there is no established human culture system which has addressed these issues. Hence, the present chapter describes the efforts made to evaluate the different types of cells observed in the culture system, attempts to localize the same in the native gland and to look for the secreted proteins in the conditioned media. The tools used in this study are immunophenotyping of cells using FACS and IHC, evaluating the potential of the cells to synthesize tear protein mRNA by RT-PCR analysis and assessing the secreted proteins into the media by ELISA.

The animal lacrimal gland cultures that have been previously established have shown that it is possible to culture the lacrimal gland cells *in-vitro* with retention of their phenotype [157, 159] [154, 155]. With regard to the human tissue, the report published by Yoshino, gave a preliminary background for establishing human lacrimal gland cultures from cadaveric

tissue. However, the study did not explore the various sub population of cells or the functionality of the cultures in detail [167].

Culturing of the cells as well as ensuring that they retain their *in-vivo* phenotype and secretory potential is equally important but not concomitant. One of the major problems that earlier researchers faced with the lacrimal tissue is the inability to maintain the acinar cells in culture. These cells tended to change their morphology as well as their phenotype to fibroblasts within a few days in culture [153]. One possible reason could be that they experienced stress in culture and changed to a form more sturdy and capable of withstanding greater stress. At the same time, since the cells did not retain their basic phenotype it was redundant to look at the secretory functionality; since a cell would usually function optimally only under unstressed conditions.

With this background, the aims of the present study was to characterize the nature of cells cultured from fresh human lacrimal gland with respect to their immunophenotype and secretory capacity. In order to do that, we first aimed at characterizing the human lacrimal gland itself by immunohistochemistry by immunolocalizing the various markers in the tissue. The markers used to evaluate the nature of cells is a comprehensive list of those tested in other exocrine glands, and included epithelial markers (Cytokeratin, E-cadherin, lysozyme, scIgA), mesenchymal markers (CD90) and myoepithelial markers (Vimentin, GFAP, S-100 protein).

3.1.1 Immunophenotyping

Immunophenotyping is the analysis of heterogeneous populations of cells for the purpose of identifying the presence and proportions of the various population of interest. The markers which are used for immunophenotyping are usually functional membrane proteins (involved in cell communication, adhesion, or metabolism), intracellular proteins (cytoskeletal proteins or secretory products) or nuclear proteins.

Immunophenotyping can be done by immunohistochemistry/ immunocytochemistry and flow cytometry. In immunohistochemistry and immunocytochemistry the *in-situ* localization of the protein of interest in the tissue or cells can be determined; however this cannot be accurately quantified. Flow cytometry, on the other hand, allows for the precise quantification of the cells expressing a particular protein of interest and also allows for sorting them into various sub-populations based on this expression pattern. The proteins that are used for identification of cells can either be lineage specific wherein they identify the cells belonging to a certain lineage like epithelial, myoepithelial, mesodermal, hematopoietic *etc.* or they may be unique identifying a unique protein expressed by the cell.

In the case of lacrimal gland, the histo-architecture of the gland comprises of four essential types of cells: the acinar or the secretory epithelial cells which are responsible for the synthesis and the secretion of the tear proteins, the ductal cells which are responsible for forming the channel required for transporting these secreted proteins and water to the ocular surface; the myoepithelial cells that envelop the acinar and ductal cells and help them in

contracting in-order to expel their contents forward; and lastly the fibroblasts that secrete extracellular matrix like collagen and heparin.

For the purpose of the present study, the various markers that have been used for the identification and characterization of tissue/ cells are as below:

3.1.1.1 Epithelial markers*:* The important epithelial markers used in the present study are cytokeratin, E-cadherin and secretory proteins lysozyme and scIgA and water channel aquaporin-5.

Cytokeratins or keratins are a group of proteins, with molecular weights between 40-70 kDa that form intermediate cytoskeletal filaments in epithelial cells and are expressed in distinct patterns during epithelial development and differentiation. The family comprises of at least 20 different polypeptides, which are expressed in paired combinations of acidic and basic molecules according to type of epithelium and its state of differentiation [196]. In the human lacrimal gland, the cytokeratins are present in the secretory epithelial cells, the myoepithelial cells as well as the ductal epithelium [197].

E-Cadherin: Cadherins are a family of molecular proteins essential for the process of calcium mediated cell-cell adhesion. The cadherins divided into various sub classes and show a differential pattern of tissue expression. E-cadherin (epithelial cadherin) is a classical member of the cadherin superfamily. It is a transmembrane protein present on the surface of epithelial cells. In adults, E-cadherin is expressed in epithelial tissues, where it is constantly regenerated with a 5-hour half-life on the cell surface [198]. In the lacrimal gland, E-cadherin

is localized on the surface of the acinar and ductal cells and is a major component of the anchoring junction complex (*zonula adherens)* required to help strengthen cell-cell contact.

Lysozyme and scIgA are tear proteins which are majorly synthesized by the acinar cells of the lacrimal gland and by the ductal cells to a smaller extent. Both the proteins are present in the cytoplasm of the acinar as well as ductal cells. These are pumped out of the acinar cells into the ducts from where they are transported to the ocular surface as a part of the tear film.

Aquaporins are water channels present on the surface of cells and functions to maintain and regulate the water content of the cells thereby maintaining internal osmotic pressure. They are impermeable to charged species which is an important factor in maintaining homeostasis [199].

3.1.1.2 Myoepithelial markers: Myoepithelial cells are flat, stellate cells located surrounding the acinar cells and the cells of the intra-lobular ducts. These cells can be characterized by their immunopositivity to vimentin, Glial Fibrillary Acidic Protein (GFAP) and S-100 protein [200].

Vimentin is the major subunit protein of the intermediate filaments of mesenchymal/ myoepithelial cells involved with intracellular transport of proteins between nucleus and plasma membrane and also in anchoring the organelles in the cytosol. It is localized in the cytoplasm of the cells. With respect to the lacrimal gland, it has been reported to be localized in the cytoplasm of the myoepithelial and stromal cells [197].

Glial Fibrillary Acidic Protein (GFAP) is a class III intermediate filament closely related to vimentin. The exact function of GFAP is not clearly understood but is thought to help maintain mechanical strength, as well as the shape of cells. With respect to lacrimal gland, GFAP has been reported to be localized in the cytoplasm of myoepithelial cells [197].

*S-100 protein*s are only expressed in vertebrates and show cell specific expression pattern. Within cells, S100 proteins are involved in aspects of regulation of proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, *etc.* Some S100 proteins are secreted or released and regulate cell functions in an autocrine and paracrine manner via activation of surface receptors [201]. With reference to lacrimal gland, S100 protein has been reported to be localized in the cytoplasm of myoepithelial cells [197].

3.1.1.3 Mesenchymal marker*:* Mesenchymal cells are stromal cells, the main function of which is to support the functional cells of the organ in which they reside. They are responsible for secreting extracellular matrix proteins like collagen, heparin, and fibronectin. CD90 is expressed strongly by mesenchymal cells. It plays a role in cell–matrix and cell–cell adhesion between inflammatory mediators of the immune response [202]. In the lacrimal gland, they are present as the stromal component.

3.1.2 Flow cytometry

Immunohistochemistry and immunocytochemistry are qualitative techniques which do not give a quantitative indication of the unique population of cells present. To determine this quantitative aspect, flow cytometry is used.

Flow cytometry was developed from microscopy and as a technique has been evolving since 1947 when F.T. Gucker (1947) built the first apparatus for detecting bacteria in aerosols using a laminar sheath stream of air and an air sample stream (sheath flow principle).Since then the technique has undergone various refinements and modifications. The present applications of flow cytometry can be attributed to Van Dilla (Los Alamos Nat. Labs) and Dittrich and Göhde (Germany) who built fluorescence flow cytometers to measure cellular DNA content in the late 1960s. This was further improved upon by Len Herzenberg (1972), who developed a cell sorter that separated cells based on fluorescent molecules tagged to them. It was the Herzenberg group (Stanford University) who coined the term Fluorescence Activated Cell Sorter (FACS).

Flow cytometry uses the principle of light scattering, light excitation and emission of fluorochrome molecules to generate specific multi-parametric data from particles/ cells in the size range of 0.5-40μm diameter. The cells are hydrodynamically focused in a sheath of PBS before intercepting an optimally focused light source (laser). On intercepting the beam of laser, the fluorochromes get excited and jump to a higher energy state. This energy is subsequently released as a photon of light with specific spectral properties, unique to the tagged fluorochrome, which in turn helps in identification of cells in the sheath stream [203, 204].

The scattered and emitted light from the cells are converted to electrical pulses by optical detectors. Collimated (parallel light waveform) light is picked up by confocal lenses

focused at the intersection point of cells and light source. This collected light is sent to the photo multiplier tube (PMT) detectors by optical filters and amplified. The amplified signal is then processed by the Analog to Digital Converter (ADC) which allows the events to be plotted on a graphical scale.

There are a number of limitations and challenges too that are associated with the flow cytometric analysis of cells from solid tissues. One of the most important challenges is to ensure isolation of cells as single cell suspension before they can be analyzed. This requires harsh enzymatic treatment of cells which in turn has the inherent drawback of affecting the preservation of epitopes. With human samples, this becomes a major limitation and an aspect that requires special consideration and standardization.

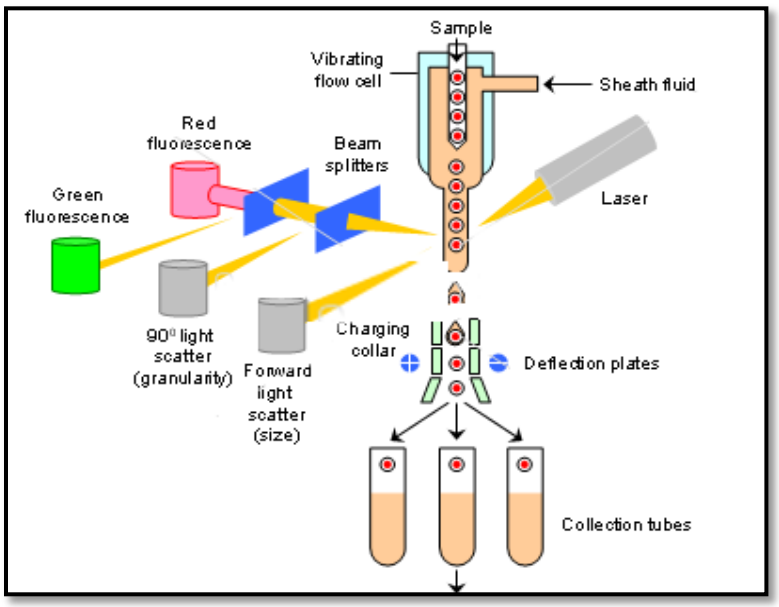

Figure 3.1: Fluorescence activated cell sorter *(Adapted from http://www.motifolio.com/6111179.html)*

3.1.3 Duct formation assay

Duct formation assay or assay for ductulogenic capacity has been used in breast and pancreatic *in-vitro* cultures to assess the potential of the cells to differentiate into duct-like structures. The assay is performed on collagen matrix, which may be floating (detached from the culture dish) or adherent. It has been reported in literature that breast as well as pancreatic cells form a network of duct-like structures under this assay condition [205, 206]. The studies have also reported these structures to have a true lumen, well-formed cell junctions, inter-digitation of plasma membranes, well-developed cytoplasmic organelles, and apical microvilli [205]. In the context of the present study, the duct formation assay was employed to assess the potential of some cells in human lacrimal gland cultures to form duct-like structures. The presence of such cells in culture would lend credence to the idea that *in-vivo* transplantation of lacrimal gland cultures would be able to restore the function of the damaged gland by supplementing not only the acinar cells but also the cells capable of duct formation.

3.1.4 Secretory function evaluation

An important aspect of the present study was to explore the inherent potential of the established human lacrimal gland cultures to secrete water (presence of water channels like aquaporin) and synthesize the mRNA for the tear proteins (lysozyme, lactoferrin, scIgA) as well as the potential to effectively pump these out into the surrounding medium. This would be the proof of concept required to ascertain that the established *in-vitro* cultures are not just phenotypically but also functionally similar to the *in-vivo* lacrimal gland.

The potential of the cultured human lacrimal gland cells to synthesize the major tear proteins and mucin was evaluated by the technique of reverse transcriptase- polymerase chain reaction (RT-PCR). The presence of mRNA for these proteins, mucin and also water channel aquaporin 5 was detected and amplified using specific primers.

The potential of the cells to synthesize and secrete the major tear proteins was assessed by sandwich enzyme linked immunosorbent assay (ELISA) method. The technique involves quantifying the antigen 'sandwiched' between two layers of antibodies (capture antibody and detection antibody). In brief, the capture antibody is coated onto the surface of ELISA plate and incubated with the sample containing the antigen to be detected. During this incubation period, specific antigen–antibody (Ag-Ab) binding occurs if there is epitope identification. This Ag-Ab complex is then tagged with another fluorescently labeled antibody (Ab*) and the concentration of this fluorescent Ab-Ag-Ab* sandwich is correlated to the absorbance spectrophotometrically.

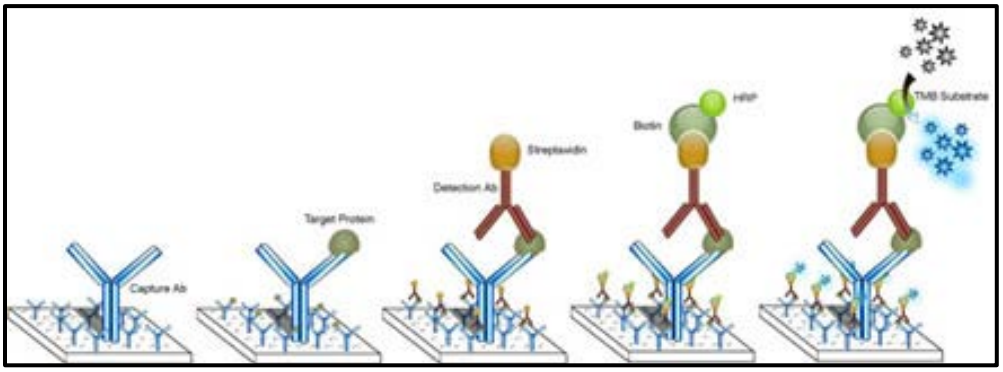

Figure 3.2: Diagrammatic representation of sandwich ELISA
(Adapted from http://www.elisa-antibody.com/ELISA-Introduction/ELISA-Principle)

3.2 Hypothesis: *Ex-vivo* expansion of human lacrimal gland could give rise to polymorphous population of cells that may represent all the cells in the tissue including the secretory cells.

3.3 Aims and Objectives

1. Characterizing the cultured cells from human lacrimal gland *in-vitro* cultures with normal human lacrimal gland as control.
2. Evaluating the secretory function of the cultured human lacrimal gland cells

3.4 Materials and Methods

Chemicals: Hank's balanced salt solution (HBSS), anti- E-cadherin antibody (Chemicon, Temecula, CA, USA), anti-ABCG2 (BD Biosciences, San Jose, CA, USA), anti c-kit (Millipore, Temecula, CA; Dako, Glostrup, Denmark), anti-p63 (Dako, Glostrup, Denmark) anti-CD133 (Miltenyi Biotech), anti-EpCAM (BD Biosciences, San Jose, CA, USA) anti-lysozyme (Abcam), anti-scIgA (Dako, Glostrup, Denmark), anti- GFAP (Dako, Glostrup, Denmark), anti-S100 protein (Dako, Glostrup, Denmark) anti CD90 (eBioscience), anti-vimentin (Dako, Glostrup, Denmark), anti-CK3/12 (Dako, Glostrup, Denmark), FlurosceinIsothiocynate (Invitrogen, Carlsbad, CA,USA), Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), Phycoerytherin (eBioscience), polymer HRP, DAB substrate, human lactoferrin ELISA kit (AssayPro, MO, USA), human lysozyme ELISA kit (AssayPro, MO,

USA), human IgA ELISA kit (Immunology Consultants Laboratory, Inc., Newberg, OR, USA).

3.5 Immunohistochemistry of Native Normal Human Lacrimal Gland

3.5.1 Tissue source

Human lacrimal gland tissue was obtained from patients who underwent exenteration of eye and orbit for a clinically indicated condition, wherein the gland itself was not involved in any pathology. Histopathology of the gland was done to confirm that is unaffected by any disease condition. Only normal tissues were taken for further evaluation.

3.5.2 Tissue processing

Part of the gland was fixed with 10% fresh formalin and embedded in paraffin. Thin 3 µm sections were taken on silane coated glass slides and used for immunostaining. Briefly, the paraffin embedded sections were de-paraffinized at around 70 ºC and then in xylene series. The sections were rehydrated in alcohol series and then in distilled water followed by 1X PBS. The endogenous peroxidase activity was blocked using methanol and hydrogen peroxide and the antigen retrieval done using Tris-EDTA buffer (pH 9). After appropriate washings with PBS and blocking with 2.5% BSA, the sections were incubated with the primary antibody in a moist chamber for 2 hours at room temperature followed by secondary antibody (polymer HRP) incubation for 30 minutes at room temperature. DAB substrate was added to the section to allow color development for 10 minutes. This was followed by counterstaining

with hematoxylin and then mounting in DPX. The sections were visualized under a light microscope.

The antibodies used and the appropriate dilutions are summarized in Table 3.1

Table 3.1: List of antibodies and dilutions used for immunohistochemistry

S. No.	**Antibody**	**Dilution**	**Company**
1.	p63	Neat	Dako
2.	∝-SMA	Neat	Dako
3.	GFAP	Neat	Dako
4.	S-100 protein	Neat	Dako
5.	Lysozyme	1:100	Abcam
6.	c-kit	1:100	Dako
7.	Pan-cytokeratin (AE1/AE3)	1:50	Dako
8.	Vimentin	1:200	Dako

3.6 Immunocytochemistry of the Cultured Cells

3.6.1 Cell source

The established *in-vitro* cultures of human lacrimal gland near confluence at day 14-18 were used for immunolocalization of the various markers for characterization.

3.6.2 Processing for immunocytochemistry

The *in-vitro* cultures of lacrimal gland at day 14-18 were immunostained for epithelial markers like cytokeratin 3/12, E-cadherin, p63; myoepithelial markers like S100, GFAP; and mesenchymal markers vimentin and CD90. In addition, the cells were also immunostained with secretory protein lysozyme and scIgA. Briefly, the cells were fixed with 4% fresh paraformaldehyde (PFA) for 10 minutes, followed by permeabilization with 50% methanol for 20 minutes for intracellular markers. The cells were then incubated with appropriate dilutions of the primary antibody for 2h at room temperature.

Secondary antibodies like Alexa Fluor 488, FITC and PE were used against the respective immunoglobulins of the primary antibody at 1:200 dilutions. The incubation time was 45 minutes at room temperature. Nuclear counter-staining was done with 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). The coverslips were mounted in 50% glycerol and the images acquired using Carl Zeiss Laser Scanning Microscope LSM 510. The antibodies used and the appropriate dilutions are summarized in Table 3.2.

Table 3.2: List of antibodies and dilutions used for immunocytochemistry

S. No.	**Antibody**	**Dilution**	**Company**
1	E- cadherin	1:100	Millipore
2.	Cytokeratin 3/12	1:100	Millipore
3.	CD90	1:200	eBiosciences
4.	Vimentin	1:100	Dako
5.	GFAP	Neat	Dako
6.	S-100 protein	Neat	Dako
7.	Lysozyme	1:100	Abcam
8.	scIgA	1:100	Dako
9.	Alexa Fluor 488	1:200	Invitrogen
10.	Phycoerythrin	1:200	eBiosciences
11.	FITC	1:200	Invitrogen

3.6.3 Processing for flow cytometry

Lacrimal gland cells, freshly isolated from the gland as well as 14-18 days and 21-25 days post *in-vitro* culture, were evaluated by flow cytometry to detect the number of cells positive for epithelial markers like E-cadherin, EpCAM and mesenchymal markers like CD90.

Cells were isolated from the human lacrimal gland by enzymatic digestion as described previously under section 2.5. Cells growing as monolayers on Matrigel™ were trypsinized using 0.25% trypsin-EDTA (TE), the cell suspension was then incubated at 37°C in a CO_2 incubator to recover from the trypsinization stress and used for evaluation of marker expression by flow cytometry.

Briefly, $1x10^6$ cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with appropriate dilutions of primary antibody for 2 hours at room temperature. The cell pellet was washed with PBS and then incubated with 1:200 dilutions of appropriate secondary antibodies for 45 minutes. At the end of this time period, the pellet was washed with PBS, resuspended in 500µl of FACS buffer and acquired on BD FACS ARIA™ Special Order System. Appropriate controls were used for the experiment. A total of 20000 to 50000 events were acquired for analysis. The analysis was done using BD FACS DiVa™ software.

The antibodies used and the appropriate dilutions are summarized in Table 3.3.

Table 3.3: List of antibodies and dilutions used for flow cytometry

S. No.	Antibody	Dilution	Company
1	ABCG2	1:100	BD Biosciences
2.	c-kit	1:100	Millipore
3.	E-cadherin	1:100	Millipore
4.	CD90	1:200	eBiosciences
5.	EpCAM	1:100	BD Biosciences
6.	CD133	1:20	Miltenyi Biotech
7.	Alexa Fluor 488	1:200	Invitrogen
8.	PE	1:200	eBiosciences
9.	FITC	1:200	Invitrogen

3.7 Duct formation assay

The duct formation assay evaluates the potential of cells in culture to differentiate into duct-lie structures in a matrix of collagen. The protocol for the assay involves mixing $1x10^3$cells/ml with 0.2% collagen I gel and plating in a well of a six well plate. The media used was DMEM with N2 supplement, 5mg/ml BSA, 10µg/ml insulin and 5µg/ml EGF. The media was changed every third day and the assay allowed to progress for 20-25 days.

3.8 Assessment of secretory function

3.8.1 Reverse- transcriptase polymerase chain reaction

Total RNA was extracted from the freshly isolated as well as cultured cells using the TRIzol® reagent according to the manufacturer's instructions. The quality of the RNA isolated was checked by visualization on agarose gel. 2µg of RNA was used for cDNA synthesis per 25µl of the reaction volume using the Superscript™ First Strand Synthesis System for RT-PCR according to the manufacturer's instructions. The primer sequences used for reverse transcriptase polymerase chain reaction are summarized in Table 3.4. PCR amplification was carried out using the Applied BiosystemsVeriti 96 well thermal cycler. The reaction was stopped after 35 PCR cycles. The amplified products were visualized on 2% agarose gel and the product size estimated.

Table 3.4: Primers used for RT-PCR

S. No.	**Name**	**Sequence**	**Product Size (bp)**
1.	Lactoferrin - F	5'-CAGACCGCAGACATGAAACT-3'	479
	Lactoferrin- R	5'-TTCAAGAATGGACGAAGTGT-3'	
2.	Lysozyme - F	5'-CTCTCATTGTTCTGGGGC-3'	350
	Lysozyme - R	5'-ACGGACAACCCTCTTTGC5-3'	
3.	scIgA - F	5'-AATGCTGACCTCCAAGTGCTAAAG-3'	242
	scIgA - R	5'-ATCACCACACTGAATGAGCCATCC-3'	
4.	Mucin-5AC- F	5'-TCCACCATATACCGCCACAGA-3'	103
	Mucin-5AC- R	5'-TGGACGGACAGTCACTGTCAAC-3'	
5.	Aquaporin-5- F	5'-CCTGTCCATTGGCCTGTCTGTCAC-3'	249
	Aquaporin-5- R	5'-GGCTCATACGTGCCTTTGATGATG-3'	
6.	GAPDH - F	5'-CAGAACATCATCCCTGCATCCACT-3'	250
	GAPDH - R	5'-GTTGCTGTTGAAGTCACAGGAGAC-3'	

3.8.2 Measurement of secretory components:

Free secretory products of lacrimal acinar cells like scIgA, lactoferrin and lysozyme were evaluated in the culture supernatant of day 6-7, day 14 and day 21 (the culture supernatant was collected at the time of media change) cultures according to the manufacturer's instructions. All the reagents used were supplied as a part of the ELISA kit. A standard curve was generated for each experiment performed (Figure 3.9 a-c).

Briefly, 50µl of the standard protein or culture supernatant was added to the wells of polypropylene U bottom ELISA plates and incubated for 2 hours at room temperature. The plates were washed at least five times with the wash buffer (supplied with the kit) ensuring complete removal of the liquid at each step. 50µl of biotinylated primary antibody (lysozyme/ lactoferrin /scIgA) (1:100 dilution) was added to each of the sample-coated wells and incubated for one hour at room temperature followed by thorough washing with the wash buffer. 50 µl of streptavidine-peroxidase conjugate was added to each well and incubated for 30 minutes. At the end of the incubation period, the wells were washed thoroughly and incubated with 50 µl/ well of the chromogen substrate for 10 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution to each well and a color change from blue to yellow is noted. The optical density (OD) was then measured immediately at 450nm on ELISA microplate reader (BioRadiMark™ Microplate Reader).

3.9 Statistical and Image Analysis

Values are expressed as mean of triplicate readings ± SEM unless otherwise indicated. The statistical test used was two-way ANOVA with post hoc Tukey test. Statistical package SPSS Version 19 was used for analysis and graphs plotted using Microsoft excel. The results were statistically compared with fresh media (as a negative control) and also with each group and were considered as statistically significant if $p \leq 0.05$.

Images were analyzed using the ImageJ software (http://imagej.nih.gov/ij/).

3.10 Results

3.10.1 Immunohistochemistry of native normal human lacrimal gland

The formalin fixed, paraffin embedded human lacrimal gland tissues, on hematoxylene and eosin staining show the typical tissue architecture of human lacrimal gland (Figure 3.3 a). These paraffin embedded sections showed immunoreactivity for pan-cytokeratin, lysozyme, vimentin, c-kit, p63, ∝-SMA, GFAP and S-100. The staining pattern reveals localization of pan-cytokeratin (Figure 3.3 b) and secretory protein lysozyme (Figure 3.3 c) mostly in the acinar cells with very few ductal cells showing faint positivity. Vimentin localized in the myoepithelial cells around the acinar cells and in the fibroblasts of the stroma. Few acinar cells also show vimentin positivity (Figure 3.3 d). c-kit expression was seen as a membrane

marker in the cell membrane of the acinar cells (Figure 3 e) while p63, GFAP, S-100 and ∝-SMA (Figure 3.3 f-i) was found in the cells enveloping the acinar cells (myoepithelial cells).

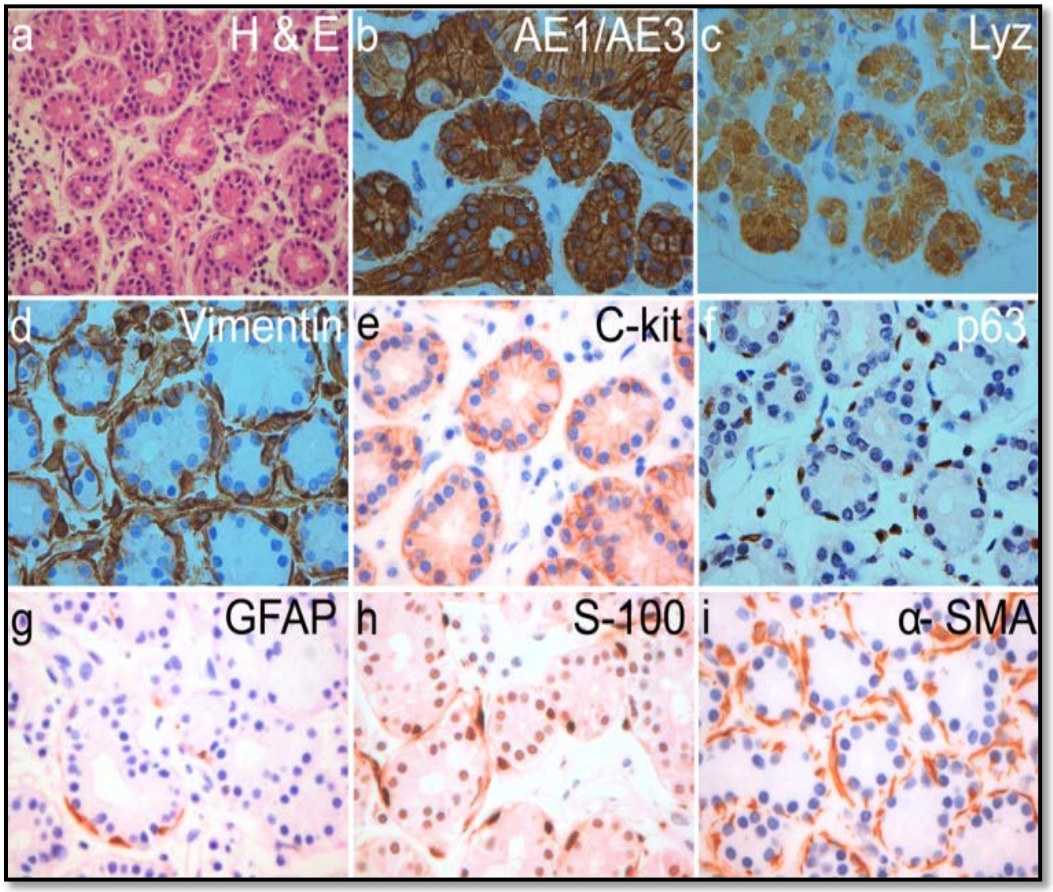

Figure 3.3: Immunohistochemistry of normal human lacrimal gland *(Tiwari et.al, 2012)[185]*

a) H&E staining shows the normal histology of the lacrimal gland. Marker staining pattern shows localization of (b) pan-cytokeratin (AE1/AE3) and (c) lysozyme (Lzy) in the cytoplasm of the acinar cells while (e) c-kit is seen in the plasma membrane of acinar cells. (d,f) p63, (g) glial fibrillary acidic protein (GFAP), (h) S-100 protein and (i) SMA localize in the myoepithelial cells enveloping the acinar cells. (d)Vimentin is seen in the myoepithelial cells and also in some of the acinar cells. All images are at 40X except H&E which is at 10X .

3.10.2 Immunophenotyping of cultured cells

3.10.2.1 Immunocytochemistry

The cultures showed a heterogeneous population of cells with immunoreactivity for epithelial, myoepithelial as well as mesenchymal markers. The cells with epithelial morphology showed positivity for CK3/12, E-cadherin and lysozyme. CK3/12 and lysozyme localized in the cytoplasm of the cells while E-cadherin was seen to localize around the plasma membrane and between the epithelial (Figure 3.4). The adherent spheres that were spontaneously generated on the confluent monolayers show positivity for tear proteins lysozyme and scIgA (Figure 3.4). The spindle shaped cells were positive for mesenchymal markers CD90 and vimentin. The oval and plump cells were immunoreactive for GFAP and S-100 protein, which may be indicative of their myoepithelial/ductal origin (Figure 3.4).

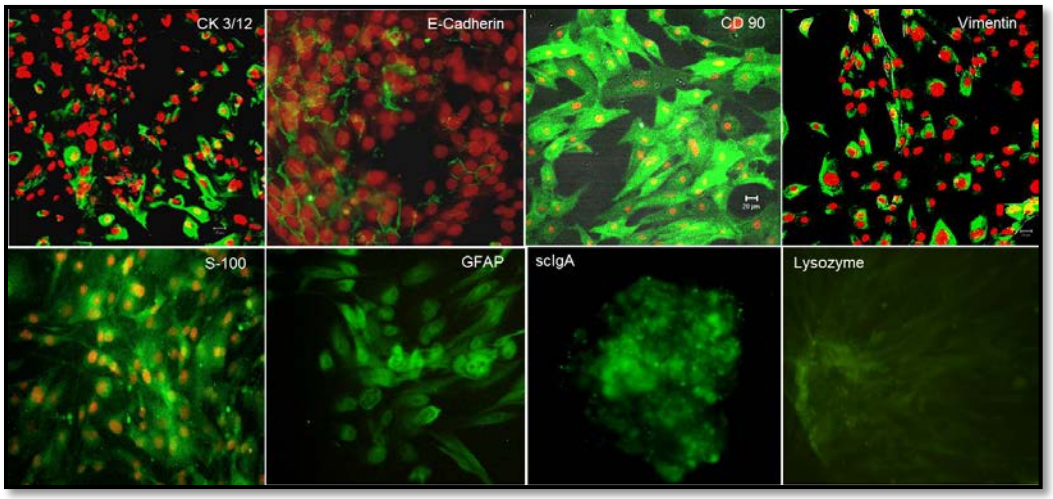

Figure 3.4: Immunocytochemistry of cultured human lacrimal gland adherent cells [185] *Cells with epithelial morphology stain positively with Ecadherin, CK3/12, lysozyme and p63; oval and plump cells stain positive for myoepithelial markers GFAP and S100 protein while the spindle shaped cells are seen to be positive for mesenchymal markers CD90 and vimentin. Some cells also show immunopositivity for ABCG2. Secondary antibody used is fluoresceine isothiocyanate (green) and the counter-stain is propidium iodide (red).*

3.10.2.2 Flow Cytometry

The FACS analysis of freshly isolated cells from the human gland showed that 14.8±3.45% of the cells were positive for epithelial marker EpCAM, 2.9±0.91% for mesenchymal marker CD90 and 3.7±0.33% positive for E-cadherin indicative of epithelial/ epithelial progenitor nature (Table 3.5) (Figure 3.5).

FACS analysis of cultured lacrimal gland cells also showed the presence of differentiated cells in day 14-18 cultures. EpCAM positivity was seen in 2.2±1.7%of the cells, 0.6 ±0.4% of the cells were positive for E-cadherin, and 13.3± 10.2 % positive for CD 90 (Table 3.5) (Figure 3.6). However, as the cultures remain under *in-vitro* conditions, these percentages tend to change. By day 21, mesenchymal marker CD90 increases to 30.25±3.35% while epithelial markers E-cadherin and EpCAM reduce to 0.45±0.25% and 0.3±0.1% respectively (Table 3.5). These results are in sync with the morphological observations that show overgrowth of fibroblast as the culture ages.

Table 3.5: Flow cytometry data: quantification of different cells in freshly isolated (t=0), day 14-18 and day 21-25 culture of human lacrimal gland.

S. No.	**Marker**	**% at t=0 (Mean± SEM)**	**% at DIV 14 (Mean± SEM)**	**% at DIV 21 (Mean± SEM)**
1.	EpCAM	14.8 ± 3.45	2.2 ±1.70	0.3 ± 0.1
2.	E-cadherin	3.7 ± 0.33	0.6 ± 0.4	0.45 ±0.25
3.	CD90	2.9 ± 0.91	13.3 ±10.20	***3.25*** 3.35

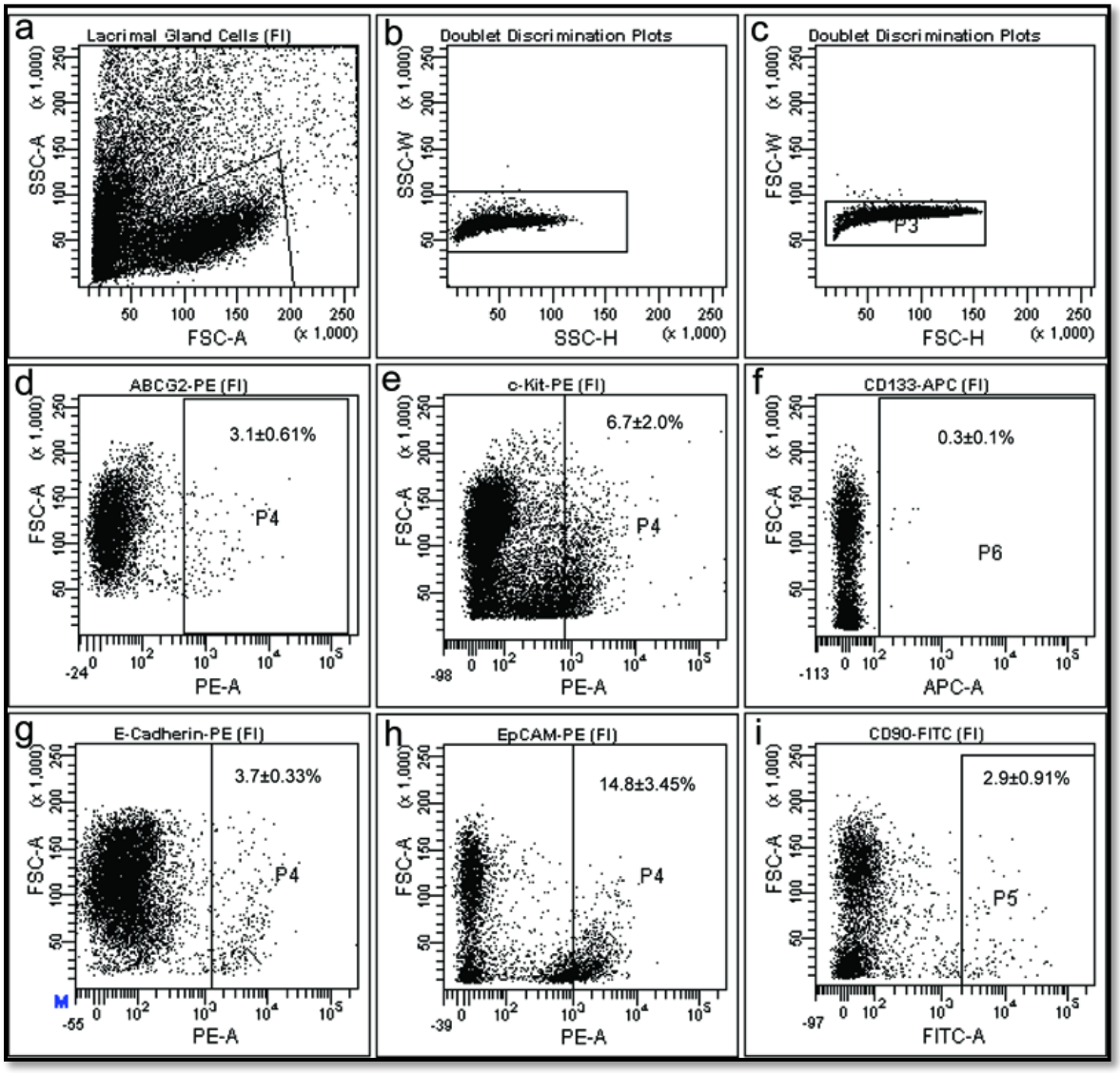

Figure 3.5: Flow cytometry data of freshly isolated (FI) cells. *The cells show positivity for ABCG2 (3.1±0.615%), CD117 (6.7±2.0%), CD133 (0.3±0.1%) (d-f); Epithelial markers E-cadherin (3.7±0.33%) and EpCAM (14.8± 3.45%)(g-h) and mesenchymal marker CD90 (2.9±0.91%) (i).*

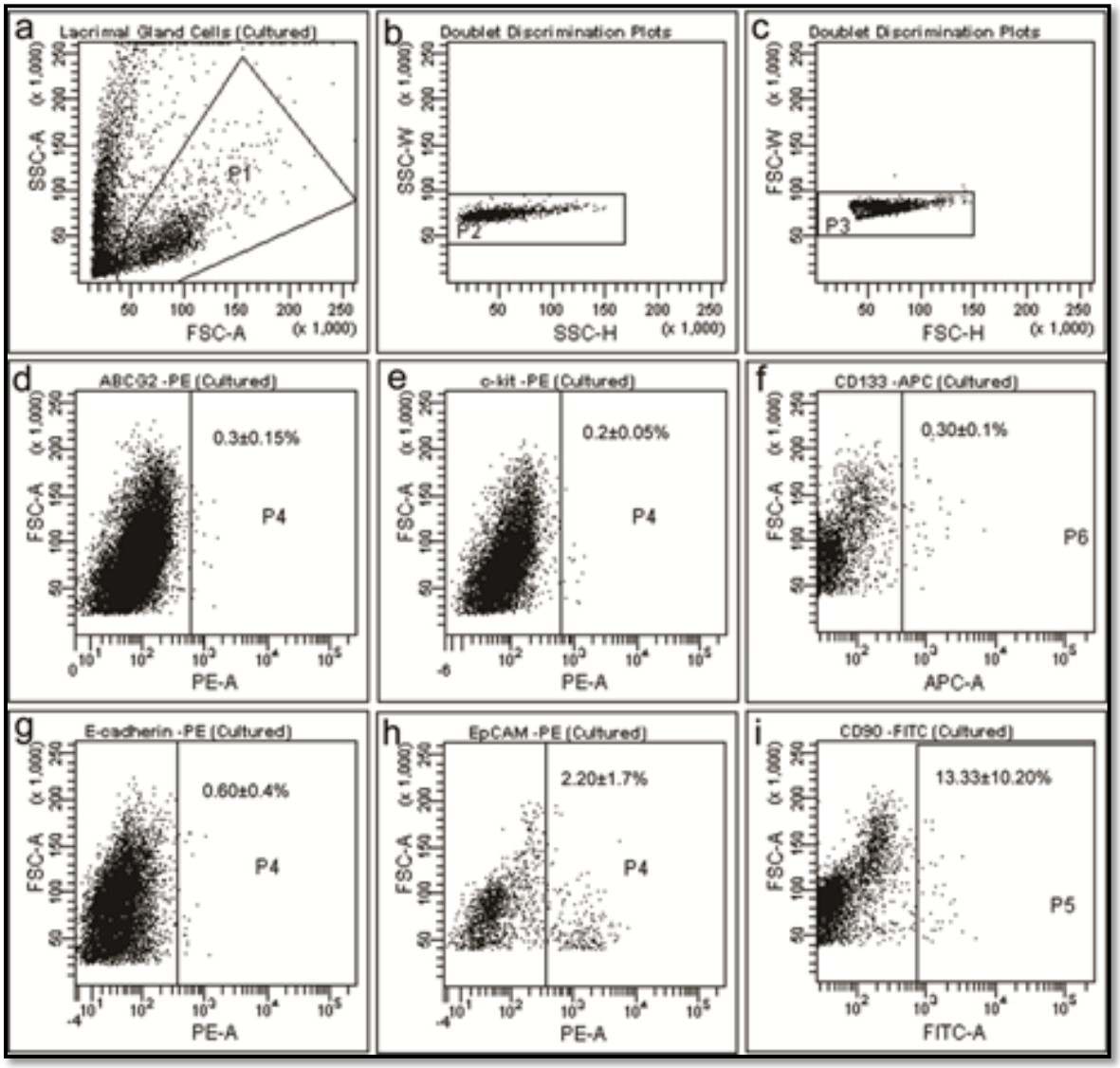

Figure 3.6: Flow cytometry data of cells 14-18 days post in-vitro culture. *The cells show positivity for ABCG2 (0.3±0.15%), CD117 (0.2±0.05%), CD133 (0.3±0.1%) (d-f); Epithelial markers E-cadherin (0.60±0.4%) and EpCAM (2.20± 1.7%)(g-h) and mesenchymal marker CD90 (13.33± 10.20%)(i).*

3.10.2.3 Duct formation assay

In order to evaluate if the duct-like connections observed *in-vitro* were indeed a potential to form ducts, an assay called the 'duct formation assay' was done on collagen. The cells were embedded in 0.2% collagen gel and supplemented with BSA, insulin and EGF. The results of the assay showed that by day 10-11, duct-like projections begin to form and elongate in size from the epithelial island. By day 15 there appeared to be a 3-D reorganization of structures and by day 19-20 a 3-D cluster or cells/ organoids with duct-like projection was seen (Figure 3.7).

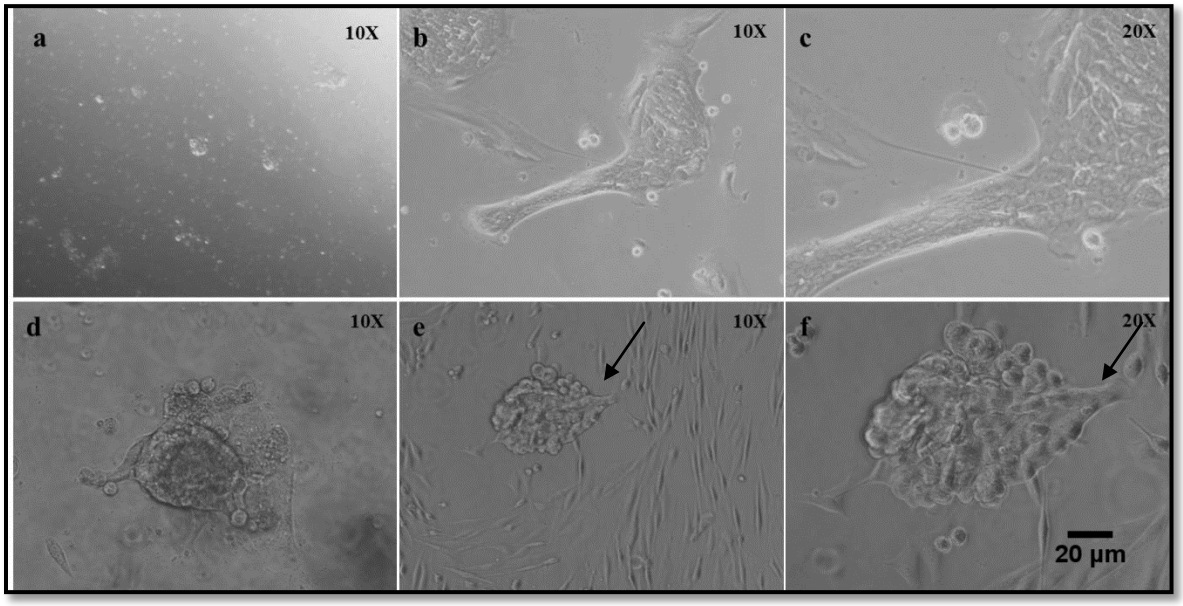

Figure 3.7: Duct formation assay *a) Cells are seeded at day=0;b-c) duct-like projections seen to develop; d-f) the cell cluster reorganizes itself with a visible duct-like projection (arrow)*

3.10.2.4 Assessment of secretory function

A) Reverse- Transcriptase Polymerase Chain Reaction

In order to confirm the presence of mRNA for the secretory proteins (lactoferrin/ lysozyme/ scIgA), water channel (aquaporin 5) and mucin-5AC in the fresh lacrimal gland tissue as well as cultured human lacrimal gland cells RT PCR was performed with specific primers. The cDNA synthesized by reverse transcription showed the expression of Aquaporin 5 (249 bp), MUC-5AC (103bp) and lysozyme (350bp) (Figure 3.8) in the cells thereby confirming that the cultured cells retained their physiological ability to synthesize the secretory proteins. RT-PCR also showed the presence of scIgA and lactoferrin mRNA in the cells cultured on Matrigel™, collagen and HAM.

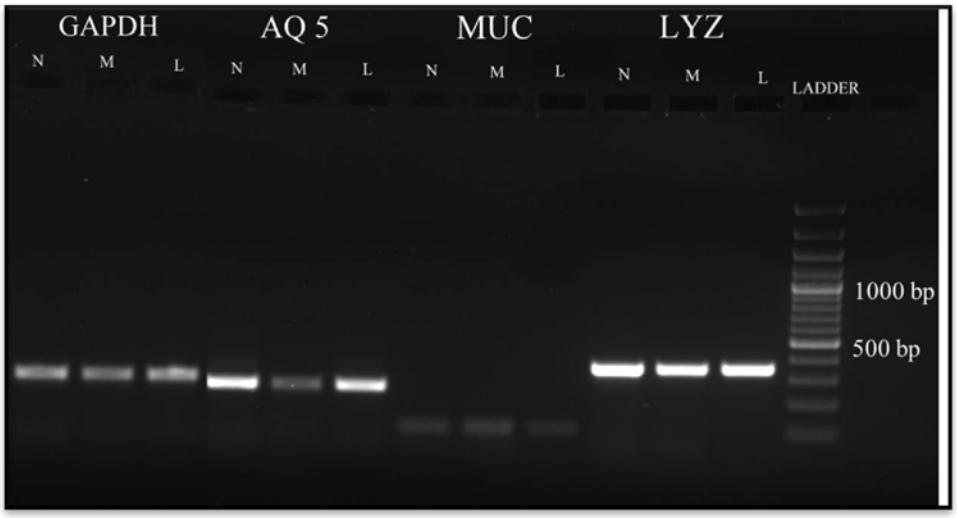

Figure 3.8: RT-PCR showing appropriate product bands for GAPDH (250bp), Aquaporin 5(AQ) (249 bp), MUC-5AC (103bp) and lysozyme (Lyz) (350bp). *Negative band showed no amplification. N: native human lacrimal gland tissue; M: cells cultured on Matrigel™; L: Lacrispheres*

B) Enzyme Linked Immunosorbent Assay

The conditioned media of day 6-7 human lacrimal gland cultures showed the presence of scIgA, lactoferrin and lysozyme secreted by the acinar cells. The protein secretion was further augmented by treatment with 100μM of carbachol for 30 min. The main effect of carbachol stimulation was statistically significant for scIgA ($F(1, 8)= 15.07$; $p< 0.01$), lysozyme ($F(1,8)= 5.86$; $p=0.02$) and lactoferrin ($F(1,8)=11.44$; $p< 0.01$) secretion.

The cells cultured on Matrigel™ showed slightly higher levels of secretory proteins in the conditioned media (Figure 3.9). However, the main effect of matrices and the interaction between matrices and carbachol stimulation was not statistically significant for scIgA ($F(2,8)=1.33$; $p=0.27$) and lactoferrin ($F(2,8)= 0.86$; $p=0.43$) secretion; but showed significance for lysozyme secretion ($F(2,8)= 5.0$; $p=0.01$). The quantity of protein secreted by the cells into the conditioned media by the cells growing on each of the matrices was calculated using the standard calibration curves. The amount of protein secreted by the cells on each of the three matrices is tabulated in Table 3.6.

Table 3.6: Secretion of tear proteins post carbachol stimulation by day 6-7 cultures

Tear Protein	HAM (ng/ml)	Collagen I (ng/ml)	Matrigel™ (ng/ml)
Lysozyme	5.78 to 33.94	0.21 to 18.34	24.36 to 144.74
Sc IgA	3.86 to 71.40	1.41 to 27.58	47.43 to 61.56
Lactoferrin	44.50 to 45.65	28.52 to 30.41	32.45 to 40.31

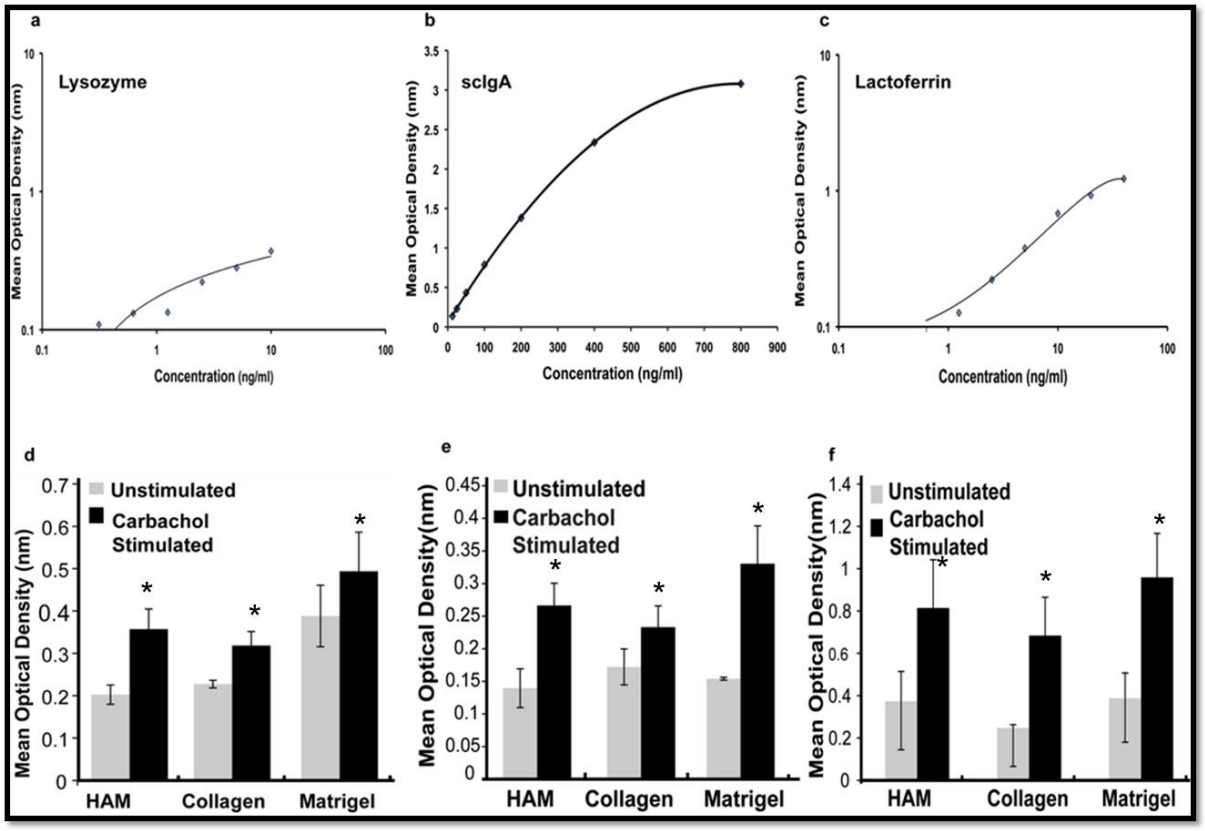

Figure 3.9: Quantification of protein secretion by cultured human lacrimal gland cells [Tiwari, 2012 #5]

a-c): Standard calibration curve for lysozyme, scIgA and lactoferrin d-f): Plot of mean optical density values for secreted proteins lysozyme, scIgA and lactoferrin on HAM, collagen and Matrigel™ pre and post carbachol stimulation

The trend in protein secretion by the cultured cells at day 7, day 14 and day 21 was also evaluated. The results indicate that *in-vitro* tear protein secretion tends to increase from day 7 to day 14 and then declines by day 21 (Figure 3.10, Table 3.7). This change over a time

period from day 7 to day 21 was statistically analysed and found to be significant: scIgA ($F(1,6)=21.92$; $p< 0.01$); lysozyme ($F(1,6)=7.45$; $p< 0.01$) and ltf ($F(1,6)=21.3$; $p< 0.01$).

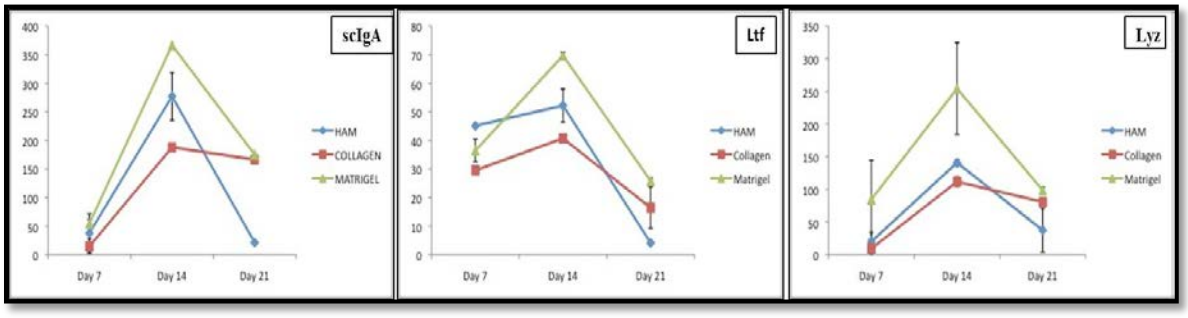

Figure 3.10: Trend in protein secretion by cultured human lacrimal cells over 21 DIV
The cells show a peak in protein production by day 14 in-vitro followed by a decline. This trend is observed across all the three matrices and was found to be statistically significant

Table 3.7: Tear protein secretion on various matrices post carbachol stimulation on day 7, day 14 and day 21 *(H: Human Amniotic Membrane; C: Collagen I; M: Matrigel™)*

DIV →	**7**			**14**			**21**		
Proteins ↓ (ng/ml)	**H**	**C**	**M**	**H**	**C**	**M**	**H**	**C**	**M**
Lysozyme	5.78 - 33.94	0.21 - 18.34	24.36 - 144.74	133.7 - 150.5	104 - 125.9	183.3 - 394.8	3.9 - 70.7	75.2 - 86.2	93.5 - 103.3
ScIgA	3.86 - 71.40	1.41 - 27.58	47.43 - 61.56	193.6- 321.2	186.5- 190.1	336.4 - 389.3	2.71 - 20.5	164.5 - 168.2	172.8 - 178.6
Lactoferrin	44.5 - 45.65	28.52 - 30.41	32.45 - 40.31	46.3 - 63.7	39.6 - 41.5	67.5 - 71.8	3.34 - 4.8	9.2 - 23.6	23.9 - 27.5

3.11 Discussion

The present chapter aimed at characterizing the heterogeneous population of cells isolated from fresh sample of human lacrimal gland as well as the cultures established from them**. *The results of the present chapter provide evidence that the human lacrimal gland cultures consist of polymorphous population of cells- epithelial cells of acinar and ductal phenotype, myoepithelial cells as well as mesenchymal cells. Under the established culture conditions, they attempt to form 2 D ductal structures and secrete the substances found in normal tears into the conditioned media***. This first of its kind evidence in human lacrimal gland, is a promising step towards exploring cell therapy in future.

The initial phase of the study involved characterizing the normal human lacrimal gland in terms of its marker expression profile. The results indicate that the human lacrimal gland tissue has four different sub-populations of cells- epithelial, myoepithelial, ductal and fibroblasts- with specific location and arrangement. The histo-architecture of the gland showed secretory epithelial cells surrounded by myoepithelial cells and a centro-acinar network of ducts. The epithelial cells as well as the ductal cells were positive for epithelial markers cytokeratin, E-cadherin, the myoepithelial cells for GFAP, S100, p63 while the fibroblasts were positive for CD90. Once the immunophenotyping of the normal native gland was established similar profiling of the established human lacrimal gland cultures was done with an intention to understand the source of these cells. The established cultures showed the presence of epithelial (CK3/12, E-cadherin,), myoepithelial (S-100, GFAP, Vimentin) and

mesenchymal markers (CD90, Vimentin) thereby indicating the presence of all three cell types *in-vitro*. Flow cytometric analysis was also done to determine the percentage of various cell populations present *immediately at isolation* as well as *in-vitro* over a time-frame of *21-25 days*. It was observed that at t=0 i.e. immediately post- isolation, the lacrimal gland had 3.7± 0.33% E-cadherin and 14.8± 3.45% EpCAM positive epithelial cells and 2.9±0.91% CD90 positive mesenchymal cells. These percentages changed in culture such that by day 14 there were 0.6±0.4% E-cadherin and 2.7± 1.7% EpCAM positive epithelial cells and 13.3±10.2% CD90 positive mesenchymal cells. These values changed even more by day 21 to 0.45±0.25% E-cadherin positive, 0.3±0.1% EpCAM positive and 30.25±3.35% CD90 positive cells. These changing percentages were also reflective of the morphological changes observed in culture. Under *in-vitro* conditions, once the cultures were passaged to generate secondary culture some of the cells were observed to change their phenotype to a fibroblastic spindle-shaped morphology. With increasing duration in culture, the epithelial cells were observed to lose their phenotype. However, even by day 35, there were epithelial cells in culture albeit their percentage was much smaller. The possible reasons for the overgrowth of spindle cells over epithelial cells, with increasing time and passages, could be that the culture conditions are not optimally conducive for epithelial expansion or the basement matrix used in the culture could be inducing differentiation. Yet another possible reason could be that, these cultures have all differentiated or transiently amplifying cells with very little or no progenitor/ stem cells. This would explain why the epithelial population seems to be reducing with

increasing time and cycles of division. The study by Yoshino et al., also reported the isolation of cytokeratin and vimentin positive cells from the cadaveric human lacrimal gland which could grow in-vitro albeit with very low proliferative potential. The authors report little or no proliferation of cells on Matrigel™ which is contrary to our observation in this study.

The spontaneously generated adherent spheres in monolayer cultures as well as the lacrispheres in culture showed positivity for tear protein lysozyme, indicating their secretory epithelial nature. The morphology of the lacrispheres studied under phase contrast microscope and after H&E staining do give some credence to the notion that these lacrispheres could be an *in-vitro* attempt to gland formation as they show what appears to be hollow central region.

The observation of spherules and duct-like connections between them prompted us to ask whether these are attempts to form duct-like structures seen in the native gland. To answer this question, we set up the duct formation assay on collagen. Conforming to our anticipation, the duct formation assay showed formation of spheres with duct-like structures projecting from one pole of the spheres. At the present time, we do not know if these duct-like projections have a true lumen or not. Though it has been previously reported in breast and pancreatic cultures that under the conditions of this assay the duct-like structures formed do indeed have a true lumen [205, 206], yet we have not confirmed the same in our study. Yoshino et al., also showed the formation of ductal structures in Matrigel™ using NIH3T3 fibroblast as a feeder layer. These ductal structures had a pattern of budding on their extremities and invaginated into the gel from several parts of the epithelial sheet. When the

HuLG cells were cultured without feeders they showed cluster of cells with 'acinar' diffentiation. In our study, though we do see the intent to duct formation we have, as yet, not made any observation of 'branching morphogenesis' *in-vitro*.

In order to fulfill the long-term goal of using the *in-vitro* expanded lacrimal cultures for rescue of function of damaged gland, it is also important that the secretory capacity of these cells be evaluated. This was an important focus of the present study. The results of the secretory protein estimation showed that *in-vitro* cultures of human lacrimal gland acinar cells can retain their functionality of secreting major tear proteins like lysozyme, scIgA and lactoferrin as well as mucin and have water channels like aquaporin 5 (Figure 3.5 & 3.8). This secretory profile was also quantified by sandwich ELISA technique using the calibration curve generated for each of the three proteins. The calibration curve was generated in two sets of experiments in which readings were taken in triplicates and since the optical density values for the remaining three sets were very similar, the same calibration curve was used to estimate the protein quantity. Our results show that the secretion of these proteins ranged from 3.86 to 71.4 ng/ml of scIgA, 5.78 to 33.94ng/ml of lysozyme and 44.5 to 45.65 ng/ml of lactoferrin on HAM, to 1.41 to 27.58ng/ml of scIgA, 0.21 to 18.34 ng/ml of lysozyme and 28.52 to 30.41 ng/ml of lactoferrin on collagen I coated dishes to 47.43 to 61.56 ng/ml of scIgA, 24.36 to 144.74 ng/ml of lysozyme and 32.45 to 40.31ng/ml of lactoferrin on Matrigel™ coated dishes (Table 3.6) in day 7 cultures. These cultures retained their secretory ability till day 21. A trend in protein secretion was also observed with the secretion peaking at day 14 *in-vitro* and

declining subsequently (Table 3.7) (Figure 3.10). The possible reasons for this reduction in secretory function could be: i) the number of secretory epithelial cells reduce with a relative increase in fibroblasts, as observed by morphology and flow cytometry studies; ii) the protein synthesis and secretory ability of the cells tends to reduce with increasing duration of culture. However, this degree of reduction is less in case of Matrigel™ which seems to preserve the secretory function comparatively. An interesting observation made here was that the relative proportion of these secreted proteins in the conditioned media is similar to that seen in natural tears i.e. Lysozyme>scIgA>Lactoferrin [2]. A wide range in the quantity of proteins secreted *in-vitro* was observed. One of the possible reasons for this could be the experimental variables like the age of donor tissue, though this aspect has not been analyzed presently. For the present study, tissue was harvested from exenterated specimens of patients with an age range of 3 years to 65 years. Since the lacrimal protein secretions tend to reduce with increasing age this could be an important factor contributing to such a wide range in quantity of protein secreted *in-vitro.* A similar study by Yoshino *et al.*, [190] on human lacrimal gland, using cadaveric tissue, showed the presence of lactoferrin protein in the central hollow of cells growing on Matrigel™ as well as in the conditioned media. However, the report was not comprehensive in terms of assessing other tear proteins and did not focus on the evaluation and quantification of secretory components of the cultured cells. In contrast, the present study, in addition to establishing cultures gave equal emphasis on evaluating the secretory potential as well as secretory profile of the cultured cells.

The present study however, is not without its set of limitations. One important limitation is the inability to fully characterize the adherent spheres and the duct-like connections that develop between them, as any manipulation around them led to breakdown of their architecture. The second limitation was the inability to differentiate between the ductal and the acinar cells in culture as both the cells showed positivity for the epithelial markers used in the study. To work around this problem, we reviewed the histology of the native gland to determine if that could throw light on a differential pattern staining of the secretory proteins. However, our observation was that the secretory proteins were seen in both acinar as well as ductal cells as both the cell types showed positive staining. This observation and the lack of literature on unique markers for the two cell types in human lacrimal gland makes us ponder if there is a gradual transition between the two cell types or if there is a common precursor to the two involving bidirectional differentiation.

In summary, the results presented here show that established cultures of human lacrimal gland have a heterogeneous population of epithelial (E-cadherin, EpCAM), myoepithelial (S-100, GFAP) and mesenchymal (CD90) cells conforming to the phenotype of the native gland with an attempt to formation of duct-like structures. The promising outcome of this study is that these cells show objective evidence of synthesis and secretion of major tear proteins (scIgA, lactoferrin and lysozyme). These results are indicative of a potential in these cells to be used for cell therapy with an important caveat that there probably exists a window period for harvesting adequate number of epithelial cells with optimal secretory

function. These results and their associated caveats should be taken into consideration for planning future preclinical and clinical studies for the treatment of chronic and debilitating aqueous deficient dry eye.

Chapter 4: Evaluating 'Stem-like' Cells in Human Lacrimal Gland Cultures

4.1 Introduction

The evolution of the stem cell concept since the early works of Regaud on spermatogenesis to the work of McCulloch and Till on bone marrow stem cells has opened up an entire avenue to explore the potential of cell based therapy for regeneration of organs[207]. As already described earlier in section 1.15.1 s***tem cells*** are capable of unlimited division during the life of the organism, giving rise to a set of progeny, one of which would self-renew while the other would enter a differentiation pathway with subsequent terminal differentiation [208]. The areas where there is a synergistic association between research and clinical trials in treating the irreversibly damaged cells or tissues in the body include: neural stem cell transplantation for brain diseases, liver cell transplantation for terminal liver failure, pancreatic islet cell transplantation for diabetes mellitus, nerve regeneration, skeletal muscle regeneration for muscular dystrophies and cardiac cell transplantation for myocardial damage [209-213].

In addition to the above mentioned areas where the research has progressed in leaps and bounds, the avenue of stem cell therapy in exocrine organs like salivary, prostate and breast are also being explored. In the case of salivary gland, *ex-vivo* expanded single functional units called 'salispheres' are being used in animal studies of radiation induced xerostomia to alleviate the symptoms of dry mouth and to reconstitute the dysfunctional gland [118, 195]. Similar is the scenario with breast and prostate [193, 214, 215]. A comparable

condition of exocrine gland dysfunction exists in the case of lacrimal gland too but there are just two reports on the possible presence of stem cells in the lacrimal gland which could potentially be used to rescue the function [151, 152]. The study published by You *et al.*, [151] showed that murine lacrimal gland had 11.98±1.84 cells/ mm^2 $BrdU^+$ cells at 2 weeks and 7.95± 1.83 cells/mm^2 $BrdU^+$ cells at 4 weeks post the initial BrdU pulse. This proportion of $BrdU^+$ cells increased 7 fold during the repair phase post interleukin-1 injection into the mouse lacrimal gland. Interestingly, BrdU positivity was seen in all the compartments- 58.2± 3.6% in the acinar, 26.4±4.1% in the myoepithelial, 0.4±0.4% in the ductal and 3.0% in the stromal cells. The stem cell marker used in the study was nestin which is a mesenchymal stem cell marker. The authors report that 2-3 days post injury during the peak of the repair phase, stem-like cells migrate towards the site of injury to heal the wound. This study indicated the inherent potential of the gland to heal itself following an insult. This was followed by a subsequent report by the same group wherein cultures were established from the murine lacrimal gland by harvesting the gland 2.5 days post IL-1 injection. The established cultures showed positivity for nestin, ABCG2 and Sca-1; thereby providing crucial evidence that murine lacrimal gland contains stem-like cells which can be harvested and maintained under *in-vitro* conditions. However, the authors noted that similar results could not be obtained from uninjured murine lacrimal gland. In contrast to this, the study by Shatos *et al.*, on rat lacrimal gland showed stem-like cells positive for nestin, Musashi1, Pax6, and CHX10 to be present

in the native, uninjured gland (these co-localized with α-SMA positive myoepithelial compartment) which could be maintained under appropriate *in-vitro* conditions [152].

One of the major drawbacks that plague stem cell research is the non-availability of a single marker or assay that can conclusively prove stemness. In order to compensate for this, a battery of markers like ABCG2, CD117, CD113, nestin etc., and functional assays like clone formation, ALDEFLUOR™ are used to evaluate and confirm stemness. In the present study we have used ABCG2 and CD117 (c-kit) as the markers to identify stem-like cells in addition to using other standard assays like label retaining studies, clone formation assay and ALDEFLUOR™ assay. A brief introduction to the techniques and markers used is given below:

4.1.1 Label retaining studies

One of the oldest techniques to define stem cell location was the Label Retaining Study. The study involved pulsing the cells/ tissue with a nucleotide analogue like BrdU or 3H-TdR, which gets incorporated into the DNA of the cells during division and label those cells. This is followed by a chase period, during which no nucleotide analogue was administered and the label gets diluted with every cell division. The less frequently dividing cells, or earlier called *quiescent cells,* retain the label, and are referred to as label-retaining cells (LRC) [216] . It was this LRC population that is thought to contain the stem cells. The proportion of LRC in most adult tissues ranges between 0-5%. In these cells, the label is retained either due to quiescence or because of asymmetric segregation of the chromosomes. However, recent

reports have shown that in a number of cases involving hematopoietic stem cells (HSC) and intestinal stem cells the label retaining cells and the most primitive stem cells were not the same [217]. Therefore, label retaining studies are currently being debated and are no longer considered as the gold standard assay for stem cells. The results obtained by LRC studies need to be further validated. In the context of the present study, we have tried to explore the possible presence of label retaining cells in culture. However, due to the limitations of the assay have also supplemented the findings using other techniques.

4.1.2 Cell cycle analysis

Cell cycle analysis studies determine the fraction of cells present in various phases of cell cycle at a point in time (Figure 4.1). Cells may be present in the G1, S or M phases of the cell cycle. In the context of stem cells, it has been reported that quiescent cells are present in the G0/G1 phase of the cell cycle. In the case of adult HSC, it has been reported that ~5-10% of the cells (termed as *activated phenotypes*) are in the active phase of the cell cycle i.e. G2/S/M phase with the remaining 90-95% in the quiescent state i.e. sub G0/ G1 stage (termed as *dormant phenotype*). These dormant cells appear to have the potential for long-term reconstitution of the organ and may represent a reservoir of stem cells kept aside in the adult organ to be called upon in cases of severe organ injury to regenerate the organ and maintain homeostasis [218]. Quiescent cells present in the G0/G1 phase of cell cycle have also been reported in muscle [219, 220], intestinal [221] and epithelial tissues [222].

One of the major limitations of the technique of cell cycle analysis is the inability to distinguish between the cells in G0 and G1 phases. True quiescent cells are in the G0 phase of the cell cycle but due to the limitation of the technique it is reported in the G0/G1 phase. Attempts have been made to differentiate between the G0 and G1 phases using loss of Ki67 expression and total nucleic acid content as possible indicators of cells in the G0 phase. These, however, are negative selection techniques and show a lot of variability in results. The latest report by Oki *et al.*, in 2014 suggests the use of mVenus-p27K$^-$, which is a defective mutant of CDK inhibitor to "identify and isolate a population of quiescent cells and to effectively visualize the G0 to G1 transition" [223].

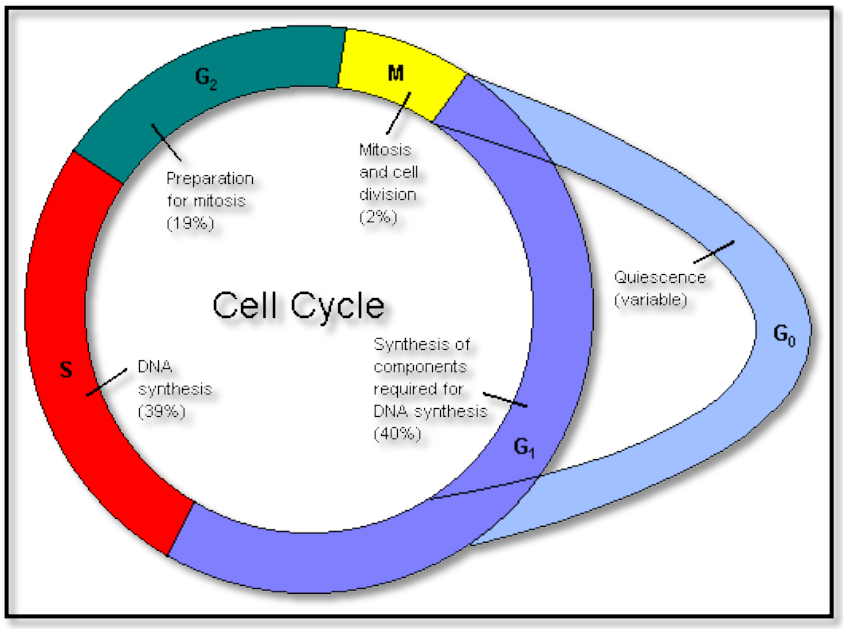

Figure 4.1: Cell cycle showing the various phases G0, G1, S, G2 and M. *Quiescent cells are believed to reside in the G0 phase.*

4.1.3 Stem cell markers

One of the most important hurdles in stem cell research is the absence of a unique and universal marker to identify stem cells. A number of markers like ABCG2, Sca-1, CD24, CD117, CD133, Musashi1 have been proposed as markers for stem cells but none of them can be called unique or exclusive. Furthermore, the expression pattern of these markers also changes based on the tissue of origin, species, potency of the cell etc. Therefore, in such a scenario, a battery of markers combined with various other accepted assays that evaluate the functionality of stem cells, are utilized to characterize the stem-like cells.

In the present study, two markers have been utilized majorly to characterize stem-like cells: ABCG2 and CD117 (c-kit). p63 and CD133 as potential stem cell markers were also screened. ABCG2 is a cell surface marker localized in the normal tissues which have secretory or barrier function like ducts and lobules of mammary tissue, islet and acinar cells of pancreas, prostate epithelium, etc. It is a xenobiotic transporter involved in multi-drug resistance of cells. In the case of stem cells, ABCG2 has been reported to be co-localized to the side population cells (which are representative of stem cells). ABCG2 high expression has also been reported in the primitive stem cells of bone marrow, the expression of which reduces sharply in response to diffentiation cues.

CD117 is a tyrosine kinase III receptor which in the recent years is being extensively used to identify the stem cells in exocrine glands. This marker has been reported to be present on the surface of HSC, germ cells and stem cells of pancreatic, mammary and salivary gland.

Specifically in the case of salivary gland, CD117+ cells have been shown to regenerate the damaged gland and restore function in animal models of radiation induced xerostomia [118]. The similarity between lacrimal gland and salivary gland in terms of the embryological origin, histoarchitecture and physiological function prompted us to select CD117 as a potential marker to identify stem like cells in the lacrimal tissue and cultures.

p63 is a transcription factor belonging to the p53 gene family. It is normally expressed in the nuclei of keratinocytes with proliferative potential, including skin, cervix, prostate and cornea. p63 is involved in the regulation of epithelial development and differentiation [224]. p63 expression was proposed as a potential stem cell indicator by Pellegrini *et al.,* who reported that holoclones of epidermal and limbal cells have the highest levels of p63 which is virtually undetectable in differentiated cells that form paraclones [225].

CD133 or prominin-1 is a member of transmembrane glycoprotein family. It was initially described to isolated $CD44^+$ stem cells of hematopoietic lineage. Subsequent studies have shown that $CD133^+$ cells have the property of self-renewal, high proliferation rate, differentiation potential and the potential to form tumors in xenografts [226]. Based on these observations and reports, CD133 has been used as a potential stem cell marker to evaluate neural progenitors and cancer stem cells [226, 227] .

4.1.4 Clone formation assay

Clone formation assay or the clonogenic assay assesses the ability of a single cells to grow into a colony of cells [228]. The technique was first proposed by Puck and Marcus in 1956 to show the radio-resistance in a subpopulation of HeLa cells [229]. Clonogenic assays have also been developed for stem cells to evaluate their potency *in-vivo*. A number of studies have reported the capacity of bone marrow derived HSC to produce colonies in the spleen of a heavily irradiated mouse and these findings have formed the basis for clinical therapy today. Similar was the case with intestinal stem cells which could regenerate radiation damaged regions of the jejunum in recipient mice [230].

Clone formation assay have also been modified to determine the clonogenic ability of the cells *in-vitro*. Under cells culture condition, the single cell capable of generating an entire colony by itself is termed as clonogenic. At the end of the assay period, the colonies are fixed using a fixative, stained with crystal violet (if required) and counted under a microscope to give an indication of the colony forming efficiency of the cells under consideration.

4.1.5 ALDEFLUOR™ assay

ALDEFLUOR™ is a fluorescent reagent system, developed by Stem Cell Technologies for the identification and isolation of stem and progenitors cells of human origin. The assay evaluates the expression levels of ALDH1 enzyme which has been reported to be elevated in

cells showing properties of stem and progenitor cells like $CD34^+$cells, $CD133^+$ cells, $CD117^+$ cells, Lineage-antigen negative (Lin^-)cells, colony-forming cells, long-term culture-initiating cells, and NOD/SCID-repopulating cells. This is a functional assay based on the cellular uptake and conversion of the ALDH substrate BODIPY® -aminoacetaldehyde (BAAA) to negatively charged fluorescent reaction product BODIPY®-aminoacetate (BAA). This product accumulates within cells as the assay buffer blocks the xenotransporter (ABC transporter) which would actively efflux it out of the cell. The fluorescent product is detected in the green channel (520-540nm) of standard flow cytometer. Higher the levels of ALDH1 in the cells, more would be the detected fluorescence and since only the cells with intact cell membrane can retain the ALDEFLUOR™ reaction product only the viable cells are detected by the assay. The principle of the assay is illustrated below in figure 4.2.

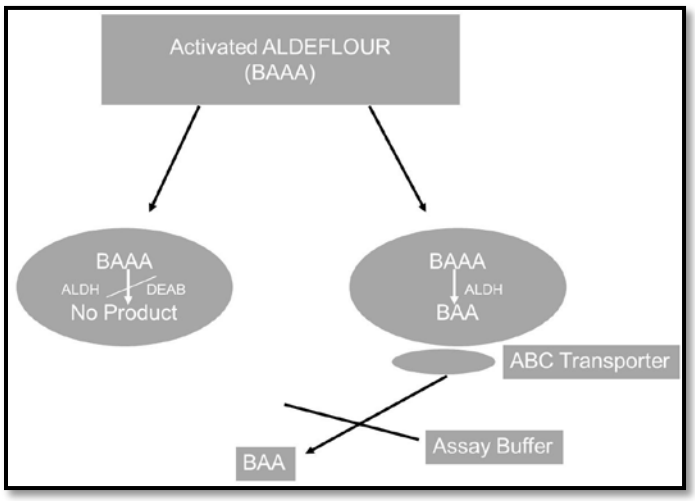

Figure 4.2: Principle of ALDEFLUOR™ assay. *The activated reagent product BAAA is converted to fluorescent reaction product BAA which is accumulated within cells with high expression of ALDH. This fluorescent product is detected in the green channel of a standard flow cytometer.*

4.2 Hypothesis: Stem-like cells are present in the human lacrimal gland tissue and in the established in-vitro human lacrimal gland cultures.

4.3 Aims and Objectives:

1. Evaluating the presence of stem-like cells in human lacrimal gland tissue

2. Evaluating the presence of stem-like cells in the established cultures of human lacrimal gland

4.4 Materials and Methods

4.4.1 Chemicals: Hank's balanced salt solution (HBSS), anti-ABCG2 (BD Biosciences, San Jose, CA, USA), anti c-kit (Millipore, Temecula, CA; Dako, Glostrup, Denmark), anti-p63 (Dako Glostrup, Denmark) anti-CD133 (Miltenyi Biotech), Fluroscein Isothiocynate (Invitrogen, Carlsbad, CA,USA), Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), Phycoerytherin (eBioscience), polymer horse radish peroxidase (HRP), DAB substrate, ALDEFLUOR™ Assay Kit (Stem Cell Technologies, Durham, NC, USA), agarose (Sigma Aldrich, St Louis, MO, USA), Propidium iodide (Sigma Aldrich, St Louis, MO, USA).

4.4.2 Tissue Source: Human lacrimal gland tissue (n=5) from post-exenterated specimens were taken for the study after informed consent. The glands included in the study were from those patients who had not undergone radiation to the head and neck region (normal tissue) and those lacrimal gland that were immunohistochemically evaluated to be free from any

underlying pathology. The fresh gland was collected in DMEM-Ham's F-12 media supplemented with antibiotics and transported to the lab where it was immediately taken for processing.

4.4.3 In-vitro human lacrimal gland cultures: Human lacrimal gland cultures that were established under in-vitro conditions as previously described under section 2.6.4 were taken for the present study. A total of 5 cultures were used for the study.

4.5 Expression of Stem Cell Marker in the Native Human Lacrimal Gland

4.5.1 Evaluation of stem cell marker expression by immunohistochemistry

CD117 (c-kit) is a stem cell marker which is known to localize in the stem-like cells of other exocrine glands like salivary [118] and prostate [231]. The localization of CD117 was evaluated in the native human lacrimal gland by immunohistochemistry.

Briefly, the gland was fixed with 10% fresh formalin and embedded in paraffin. Thin 3 µm sections were taken on silane coated glass slides and used for immunostaining. The paraffin embedded sections were de-paraffinized at around 70ºC and then in xylene series. The sections were rehydrated in alcohol series and then in distilled water followed by 1X PBS. The endogenous peroxidase activity was blocked using methanol and hydrogen peroxide and the antigen retrieval done using Tris-EDTA buffer (pH 9). After appropriate washings with PBS and blocking with 2.5% BSA, the sections were incubated with the primary antibody CD117, in a moist chamber for 2 hours at room temperature followed by secondary antibody (polymer HRP) incubation for 30 minutes at room temperature. DAB substrate was added to

the section to allow color development for 10 minutes. This was followed by counterstaining with hematoxylin, mounting in DPX. The sections were visualized under a light microscope.

4.5.2 Evaluation of stem cell marker expression by flow cytometry

The fresh human lacrimal gland was enzymatically digested using an enzyme cocktail of collagenase and hyaluronidase as previously described. The cells isolated were termed as t=0 cells and were processed for flow cytometric analysis.

Briefly, $1x10^6$ cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with 1:100 dilutions of primary antibody (Table 4.1) tagged with the fluorophore for 1 hour at room temperature. At the end of this time period, the pellet was washed thrice with PBS, resuspended in 500µl of FACS buffer and acquired on BD FACS ARIA™ Special Order System. Appropriate controls were used for the experiment. A total of 20000 to 50000 events were acquired for analysis. The analysis was done using BD FACS DiVa™ software.

Table 4.1: List of antibodies and dilutions used for flow cytometry

S. No.	**Antibody**	**Dilution**	**Company**
1	ABCG2- APC	1:100	BD Biosciences
2.	c-kit-PE	1:100	Millipore
3.	CD133-APC	1:20	Miltenyi Biotech

4.6 Expression of Stem Cell Markers in the Human Lacrimal Gland Cultures

The *in-vitro* cultures of human lacrimal gland were evaluated for the expression of stem cell markers like ABCG2, p63 and CD117 by immunocytochemistry and flow cytometry.

4.6.1 Immunocytochemistry of in-vitro cultures

Day 14 and day 21 human lacrimal gland cultures growing as monolayer were dissociated using 0.25% trypsin-EDTA and processed for immunocytochemistry. The lacrispheres were mechanically dissociated and processed similarly for immunocytochemistry.

Briefly, the cells were fixed with 4% fresh paraformaldehyde (PFA) for 10 minutes, followed by permeabilization with 50% methanol for 20 minutes for intracellular markers. The cells were then incubated with appropriate dilutions of the primary antibody for 2h at room temperature.

Secondary antibodies like Alexa Fluor 488, FITC and PE were used against the respective immunoglobulins of the primary antibody at 1:200 dilution. The incubation time was 45 min. Nuclear staining was done with 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). The coverslips were mounted in 50% glycerol and the images acquired using Carl Zeiss Laser Scanning Microscope LSM 510.

The antibodies used and the appropriate dilutions are summarized in Table 4.2.

Table 4.2: List of antibodies and dilutions used for immunocytochemistry

S. No.	Antibody	Dilution	Company
1	ABCG2- APC	1:100	BD Biosciences
2.	c-kit-PE	1:100	Millipore
3.	P63	1:20	Miltenyi Biotech
4.	Alexa Fluor 488	1:200	Invitrogen

4.6.2 Flow cytometry of in-vitro cultures

Day 14 and day 21 cultures growing as monolayers were dissociated using 0.25% trypsin-EDTA and processed for flow cytometry. The lacrispheres were mechanically dissociated and also processed similarly.

Briefly, $1x10^6$ cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with 1:100 dilutions of primary antibody (Table 4.3) tagged with the fluorophore for 1 hour at room temperature. At the end of this time period, the pellet was washed thrice with PBS, resuspended in 500µl of FACS buffer and acquired on BD FACS ARIA™ Special Order System. Appropriate controls were used for the experiment. A total of 20000 to 50000 events were acquired for analysis. The analysis was done using BD FACS DiVa™ software. The antibodies used and the appropriate dilutions are summarized in Table 4.3

Table 4.3: List of antibodies and dilutions used for flow cytometry

S. No.	Antibody	Dilution	Company
1	ABCG2- APC	1:100	BD Biosciences
2.	c-kit-PE	1:100	Millipore
3.	CD133-APC	1:20	Miltenyi Biotech

4.7 Colony Forming Assay

The lacrispheres were grown in agarose as single cells to assess their colony forming potential. The lacrispheres were collected and mechanically dissociated. A cell pellet was obtained after centrifugation. The pellet was resuspended in 1 ml of DMEM medium with 10% FCS and 2mM L-glutamine.

Two percent agarose was coated onto well of a six-well plate as a base coat, and further overlaid with cell suspension (1000 cells in 1% agarose). Plates were incubated in humidified tissue culture incubator at 37°C, 5% CO2, for 14 days. Visible colonies were counted under a phase-contrast microscope.

*Colony forming unit = (No of colonies/ no. of cells plated)*100*

4.8 BrdU Pulse Labeling and Chase of In-Vitro Human Lacrimal Gland Cultures

4.9.1 BrdU pulsing*:* On Day 7 the media of in-vitro cultures of human lacrimal gland, growing both as monolayers and as lacrispheres, was discarded and replaced with fresh media containing 10μM BrdU reagent. This BrdU pulsing was done for 24 h after which the media was changed again and replaced with fresh media without BrdU. In order to determine the number of proliferative cells, the culture was terminated and processed for anti-BrdU staining. To evaluate the number of cells that retain the label after a chase period of around 14 days (i.e., quiescent cells), the culture was allowed to grow for a further period of 14 days without BrdU and at the end of this time period was processed similarly for anti-BrdU staining.

4.9.2 Immunostaining for anti-BrdU: the cells were processed for immunostaining with anti-BrdU antibody. Briefly, the cells were fixed with 70% ice cold ethanol for 30 minutes at 4°C followed by washings with 1X PBS. DNA was denatured with 2N HCl (denaturing solution) for 30 minutes at 37°C and the conditions neutralized immediately with 0.2M borate buffer (neutralizing solution) for 10 minutes at room temperature. The cells were then blocked with 10% serum for 60 minutes at room temperature followed by incubation with 1:50 dilution of anti-BrdU antibody for 60 minutes at room temperature. Secondary antibody like FITC or PE was used against the primary BrdU Lastly the cells were washed, mounted in 50% glycerol and images were acquired using Carl Zeiss Laser Scanning Microscope LSM 510.

4.9 Cell Cycle Analysis

Cell cycle analysis was done to determine the fraction of cells present in various phases of cell cycle namely G0, G1, G2, S and M. The experimental protocol involved fixing the cells in 70% ice cold ethanol followed by incubation with 50 μg/ml propidium iodide on ice for 30minutes. The cells were then treated with treated with 0.25 mg/ml RNase A for 45 miutes at 37^0C to remove double stranded RNA. Cells were finally analyzed by flow cytometry at an excitation wavelength of 488nm.

The cells were also co-stained with CD117 (c-kit) to determine the co-localization profile. The protocol for antibody staining during cell cycle analysis differs slightly from when only cell cycle analysis is done. Briefly, the cells were fixed with 4% PFA, incubated for an hour with anti-human CD117 antibody conjugated to PE, after which the cells were washed thrice with 1X PBS and processed as mentioned above for cell cycle analysis.

4.10 ALDEFLUOR™ Assay

ALDEFLUOR™ fluorescent reagent system (Stem Cell Technologies) provides a novel method for identification of stem and progenitor cells based on their expression of enzyme aldehyde dehydrogenase1 (ALDH). The fluorescent ALDEFLUOR™ reagent diffuses freely into the cells and acts as a non-toxic substrate for ALDH. The fluorescent reaction product that accumulates in the cells can be measured in the green channel of a standard flow

cytometer and it correlates directly to the ALDH activity in the cell. With this assay, stem and progenitor cells are identified as cells with higher expression of ALDH1.

For the assay, $1x10^6$ cells/ml (freshly isolated from the lacrimal gland as well as post trypsinization from the cultured monolayer on day 14-18 and day 21-25) were taken and divided into two groups: control and test. To the control tube, 5µl of ALDH inhibitor DEAB was added. 5µl/ml of activated ALDEFLOUR™ substrate was added to the test group and immediately half of the cell suspension was transferred to the control tube. Both the test as well as the control sample was incubated at 37°C for 30 to 60 minutes. At the end of the incubation period, the supernatant was removed after centrifugation and the cells resuspended in 0.5ml of ALDEFLUOR™ Assay Buffer and the fluorescence measured in the green channel of FACS ARIA™ Special Order System**.**

4.11 Image Analysis: The images were analyzed using Image J software.

4.12 Results

4.12.1 Expression of stem cell markers in the normal human lacrimal gland tissue

4.12.1.1 Evaluation of stem cell marker expression by immunohistochemistry: The native lacrimal gland showed localization of CD117 to the cell membrane of cells of acinar (44%) and ductal phenotype ((9.8%) (Figure 4.3). These percentages have been determined using image analysis software ImageJ. Three different images were analyzed by the software to

generate these mean values. The pattern of staining was such that some of the positive cells showed intercellular and basal staining while the others showed uniform staining all over the cell membrane. None of the myoepithelial cells showed CD117 positivity.

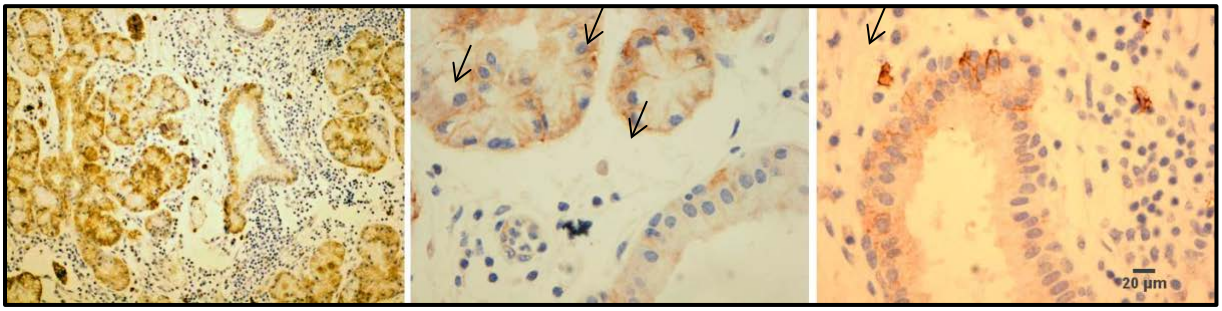

Figure 4.3: Expression of CD117 in normal human lacrimal gland. *The marker was seen to be localized in the cells of the acinar (44%) as well as cells of the ductal (9.8%) phenotype. A differential pattern of staining was observed with some cells showing uniform staining while others showed intercellular and basal staining.*

4.12.1.2 Evaluation of stem cell marker in native normal tissue by flow cytometry: Flow cytometric evaluation of cells isolated at t=0 showed that 3.1 ± 0.61% of the cells were positive for the stem cell marker ABCG2; 6.7 ± 2.0% were positive for CD117 and 0.30 ± 0.10% positive for CD113 (Table 4.4) (Figure 4.4).

Table 4.4: Flow cytometric evaluation of huLG cells for expression of stem cell markers at t=0

S. No.	**Marker**	**% Expressed at t=0 (Mean± SEM)**
1.	ABCG2	3.1 ± 0.61
2.	CD117	6.7 ± 2.0
3.	CD133	0.30 ± 0.10

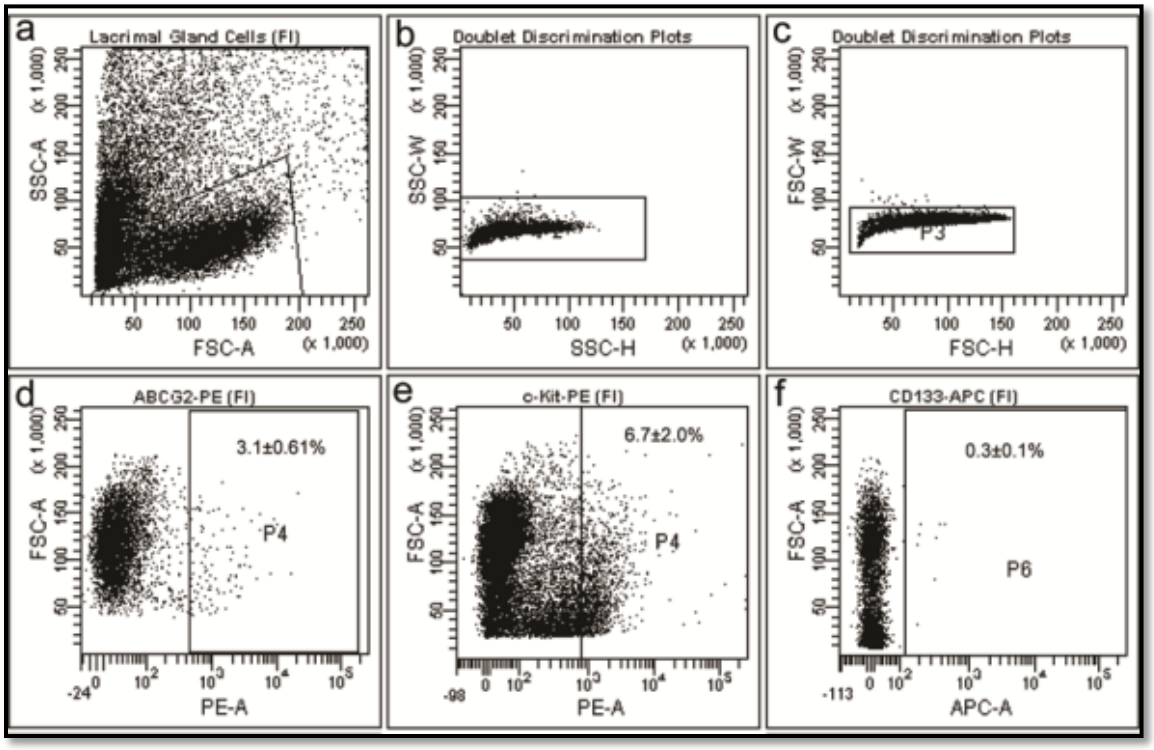

Figure 4.4: Flow cytometry data: Evaluation of stem cell marker expression at t=0. *The cells immediately at isolation showed positivity for (d) ABCG2 (3.1±0.61%), (e) C-kit (6.7±2.0%) and (f) CD133 (0.3±0.1%).*

4.12.2 *Expression of stem cell markers in the in-vitro human lacrimal gland cultures*

4.12.2.1 Immunocytochemistry of in-vitro cultures: The expression of ABCG2 and CD117 in both the adherent monolayer cultures as well as in floating lacrispheres was evaluated. The results show that these stem cell markers were seen to be localized in the epithelial cells of the monolayer (Figure 4.5).

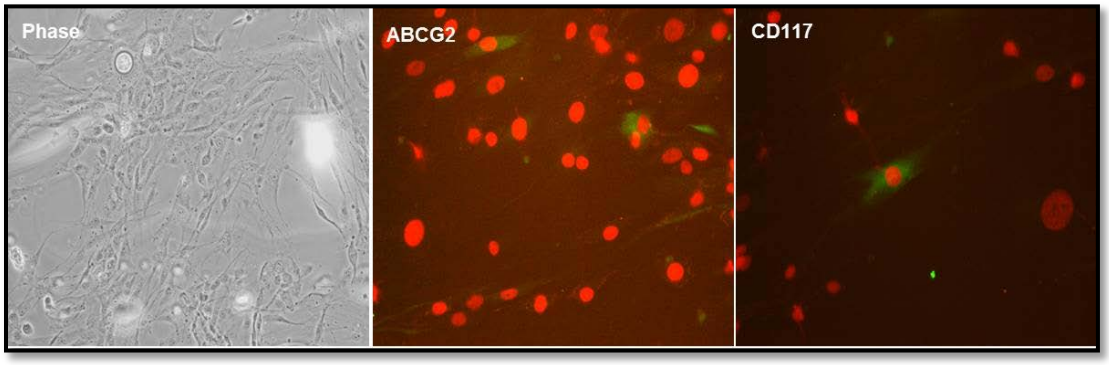

Figure 4.5: Expression of stem cell markers in adherent human lacrimal gland cultures. *ABCG2 and CD117 expression is seen in some cells of the adherent monolayer. Secondary antibody is Alexa Fluor 488 and nuclei counterstained with PE. All images are at 10X magnification.*

In the case of lacrispheres some of the cells showed CD117 positivity (Figure 4.6). However, these cells did not seem to have any specific location like central or peripheral.

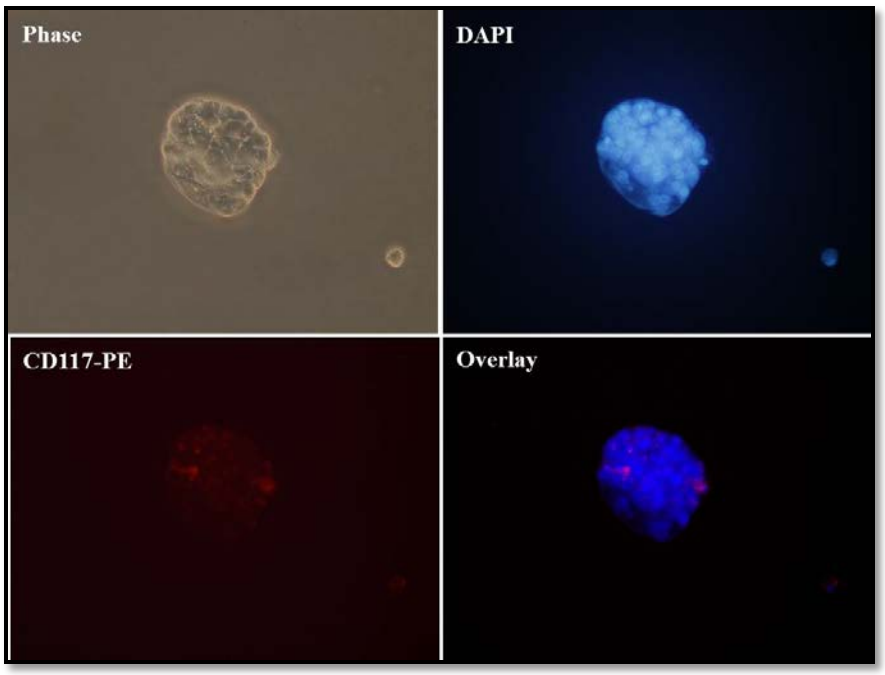

Figure 4.6: Expression of stem cell markers in cultured lacrispheres. *CD117 expression is seen in some cells of the lacrisphere. Fluorescent tag is PE and DAPI is the nuclear counter stain. The image is at 10X magnification.*

4.12.2.2 Flow cytometric evaluation for stem cell marker expression: Day 14 and day 21 cultures were also evaluated quantitatively by flow cytometry to determine the stem cell population present in them. The results show that day 14 cultures had a population of cells with positivity for stem cell markers accounting for 0.30±0.15% as ABCG2 positive and 0.20 ± 0.05% as c-kit positive. CD133 expression was seen in 0.3±0.1% of the cells (Figure 4.7). By day 21-25 of *in-vitro* culture the number of cells expressing the stem cell markers ABCG2 and c-kit had reduced to 0.2±0.13% and 0.13± 0.03% respectively (Table 4.5)

Table 4.5: Stem cell marker expression by adherent huLG cultures

S. No.	**Marker**	**% Expressed at *DIV* 14 (Mean± SEM)**	**% Expressed at *DIV* 21 (Mean± SEM)**
1.	ABCG2	0.3 ± 0.15	0.2 ±0.13
2.	CD117	0.2 ±0.05	0.13 ± 0.03
3.	CD133	0.30 ±0.10	0.25 ± 0.05

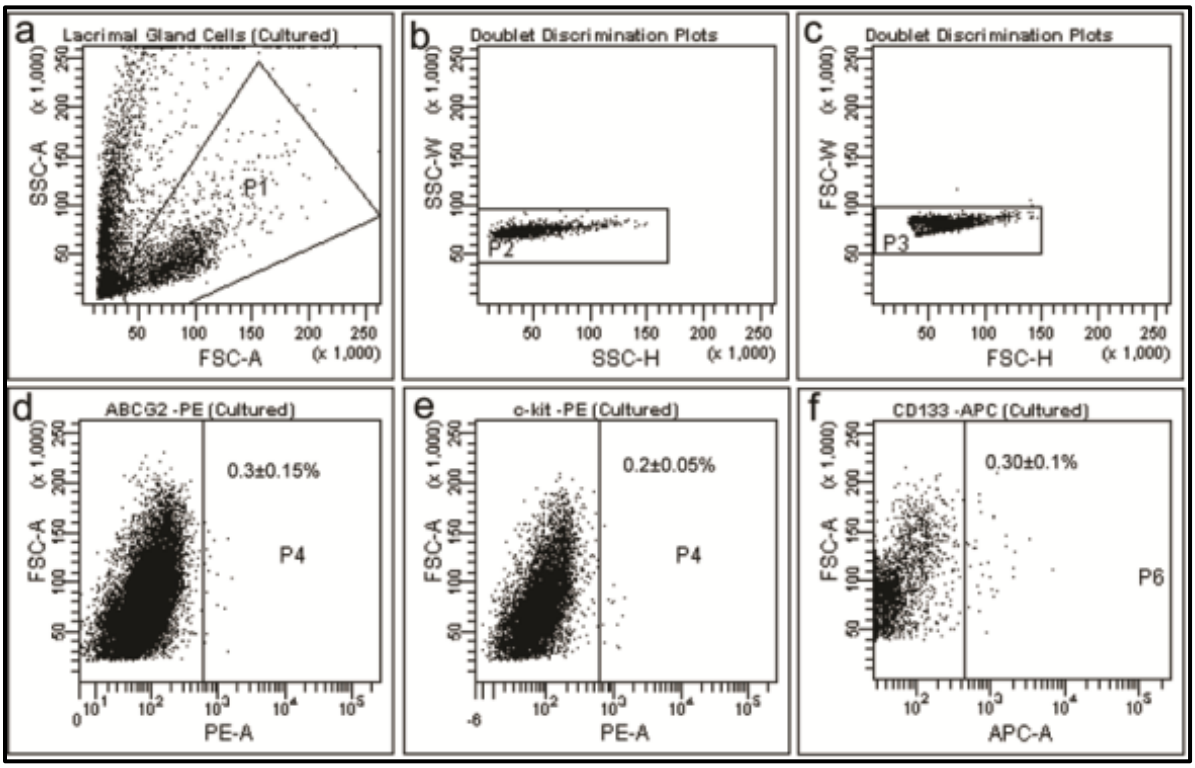

Figure 4.7: Flow cytometry data: Evaluation of stem cell marker expression at t=14. *The cells by DIV 14 showed positivity for (d) ABCG2 (0.3±0.15%), (e) C-kit (0.2±0.05%) and (f) CD133 (0.3±0.1%).*

When the lacrispheres were evaluated to determine the expression of CD117, it was observed that 0.8% of the cells were positive for CD117 indicating the presence of a higher proportion of stem-like cells (Figure 4.8).

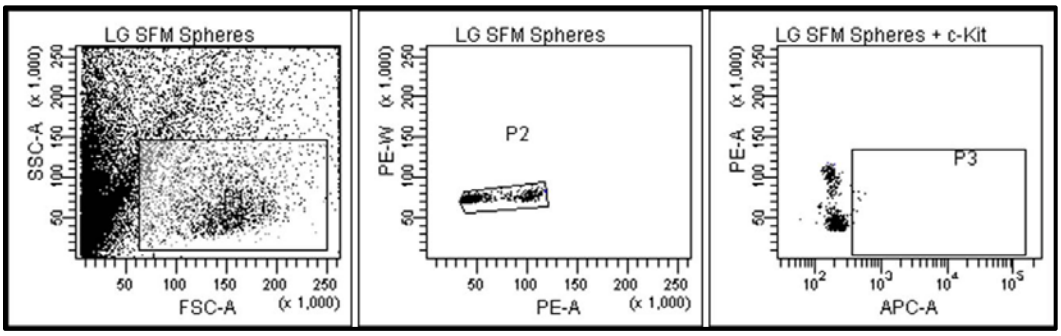

Figure 4.8: Flow cytometric evaluation of CD117 expression in lacrispheres at t=14 *showing 0.8% of the cells to be positive (P3 gate)*

4.12.3 Colony forming assay

Cells growing as lacrispheres were plated on agarose and their CFE evaluated. At the end of 14 days the number of colonies (Figure 4.9) was manually counted under the microscope. It was observed that an average of 31 colonies was generated from the 1000 cells initially plated. From this the colony forming unit (CFU) was calculated and found to be 3.1%.

Colony forming unit = (No of colonies/ no. of cells plated)*100 = (31/1000)*100 =3.1%

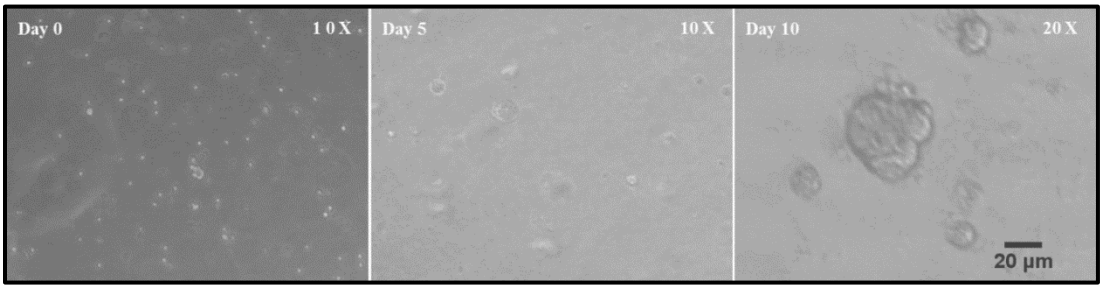

Figure 4.9: Colony formation assay on agarose. *The colony forming efficiency of the cultures was calculated to be 3.1%*

4.12.4 BrdU pulse labeling and chase of in-vitro human lacrimal gland cultures

I) BrdU pulse and chase of adherent monolayer cultures: Fixing and immunostaining of cells immediately after pulsing them with BrdU labels all the cells which are proliferating in culture. In the case of adherent cultures it was observed that a majority of the cells were labeled with BrdU (75.9±2.7%) including the cells that form duct-like connections as well as the cells in the periphery of the adherent spheres indicating proliferation. The center of the spheres showed very faint label indicating that the cells here are proliferating very slowly (Figure 4.10 b).

When the BrdU treated cells were chased for a period of 14 days, this trend gets reversed because now the label is retained by cells which proliferate very slowly or are quiescent. These cells are termed as *label retaining cells (LRCs)*. It was observed that by day 14 all the monolayer cells, cells that formed duct-like connections as well as the cells in the periphery of the adherent spheres had lost the BrdU label indicating their proliferative nature (Figure 4.10c). BrdU label was only present about 27.9±4.3% of the cells and these were localized in the center of the adherent spheres indicating the presence of label retaining slow proliferative cells (Figure 4.10 c) (Table 4.6).

II) BrdU pulse and chase of lacrispheres: When the lacrispheres were immediately fixed and immunostained with anti-BrdU antibody post treatment, a majority of cells in the sphere get labeled with BrdU (51.6±3.8%) indicating presence of proliferative cells (Figure 4.10e).

However, when these were chased for 14 days, it was found that only 9.31±0.41% of the cells retained the BrdU label (Figure 4.10f) (Table 4.6).

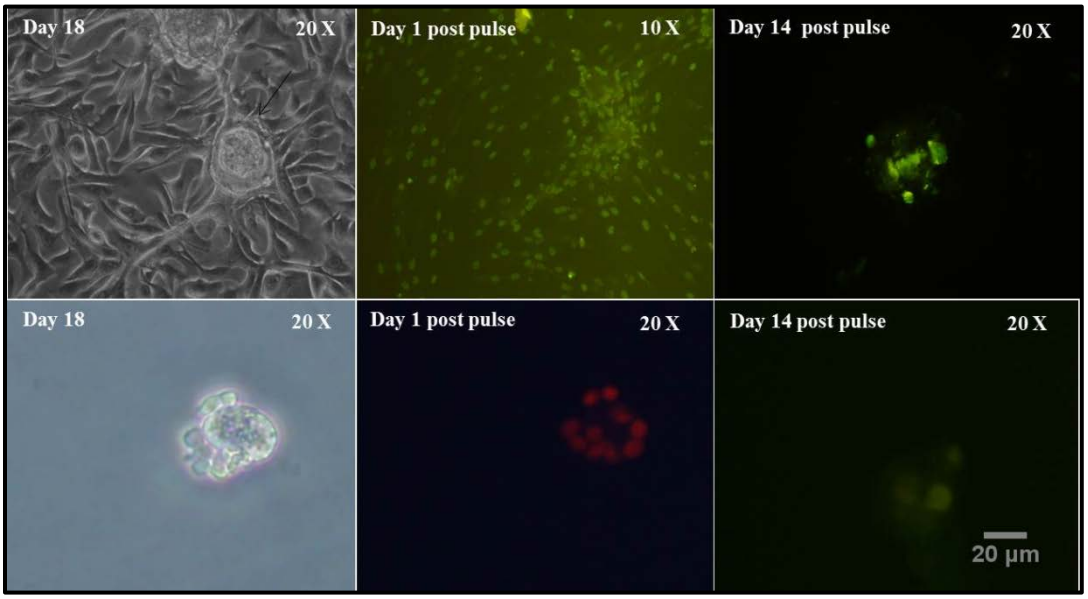

Figure 4.10: BrdU label retaining studies.
One day post pulse a majority of cells in the monolayer, periphery of adherent and lacrisphere stained positive for BrdU. After a chase period of 14 days only 27.9±4.3% cells in the adherent spheres and 9.3±0.41% cells in the lacrispheres retained the BrdU label.

Table 4.6: Label retaining cells in in-vitro cultures of human lacrimal gland

Culture	***t=0***	***t=14***
Adherent	75.9±2.7%	27.9±4.3%
Lacrisphere	51.6±3.8%	9.3±0.41

4.12.5 Cell cycle analysis

Cell cycle analysis was done on cells at t=0 (immediately after isolation) as well as t=14 (14*DIV*). It was observed that at t=0 79.9% of the cells were in G0/G1 phase and 18.9% in the G2/S/M phase (Figure 4.11) (Table 4.7). In the case of adherent monolayer day 14 cultures, 66.9% of the cells were in the G0/G1 phase and 33.9% in the G2/S/M phase (Figure 4.11) (Table 4.7).

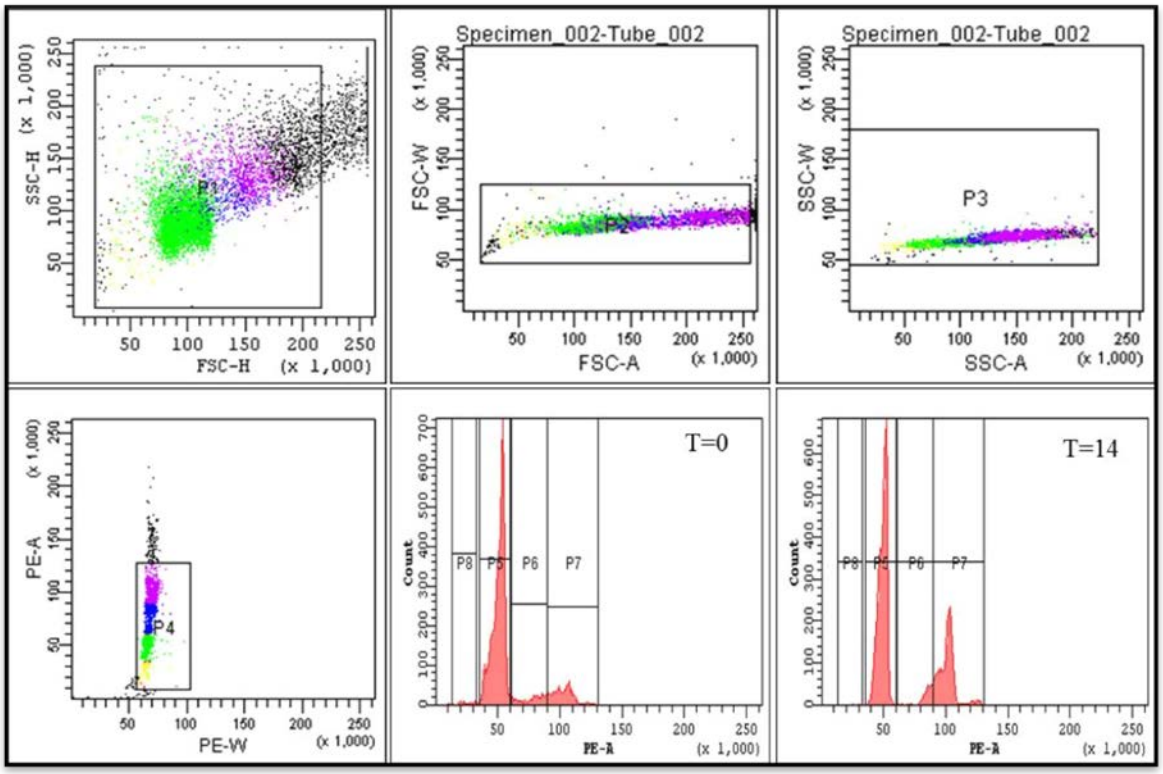

Figure 4.11: Cell cycle analysis of adherent cultures.
At day 14, 66.9% of the cells were in the G0/G1 phase and 33.9% in the G2/S/M phase

When the lacrispheres were similarly evaluated it was seen that 76.9% of the cells were in the G0/G1 phase and 22.9% in the G2/S/M phase. Of the 76.9% the cells in the G0/G1 phase about 0.3% were positive for stem cell marker CD117 (Figure 4.12) (Table 4.7).

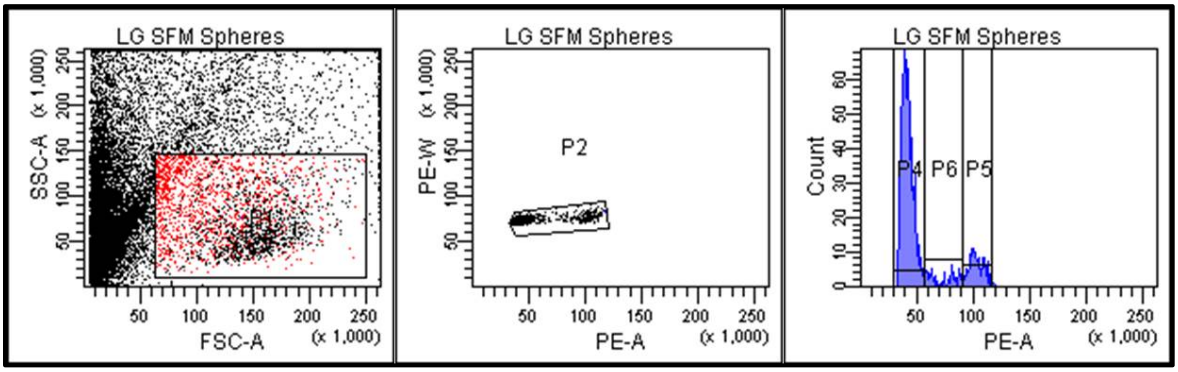

Figure 4.12: Cell cycle analysis of lacrispheres.
At day 14 76.9% of the cells were in the G0/G1 phase and 22.9% in the G2/S/M phase.

Table 4.7: Cell cycle analysis of human lacrimal gland cells

Cells/ Culture	***G0/G1***	***G2/S/M***
Freshly isolated (t=0)	79.9%	18.9%
Adherent cultures (t=14DIV)	66.9%	33.9%
Lacrispheres (t=14DIV)	76.9%	22.9%

4.12.6 ALDEFLUOR™ assay

In the freshly isolated cells**,** 2.4% to 6.3% showed high ALDH1 activity (mean: 3.8±1.26%). Co-expression of high ALDH1 and ABCG2 was seen in 0.13 ± 0.04%, and high ALDH1 and c- kit in 0.21% ± 0.02% of the cells (Figure 4.13 a-c) (Table 4.8).

The day 14-18 cultured lacrimal gland cells also showed a population with high ALDH activity accounting for 2.7± 1.5% of the total population acquired (Figure 4.13 d-f) which decreases to 1.1±0.5% by day 21. Co-expression of high ALDH1 with ABCG2/c-kit positivity was not evaluated in the cultured cells since their level of expression in the native tissue itself was low to begin with.

Table 4.8: Evaluation ALDH1 high expression at t=0 and t=14

S. No.	**Marker**	**% Expressed at t=0 (Mean± SEM)**	**% Expressed at *DIV 14* (Mean± SEM)**
1.	ALDH1 high	3.8 ±1.26	2.7± 1.54
2.	ALDH high + c-kit	0.2 ± 0.02	-
3.	ALDH high + ABCG2	0.1 ± 0.04	-

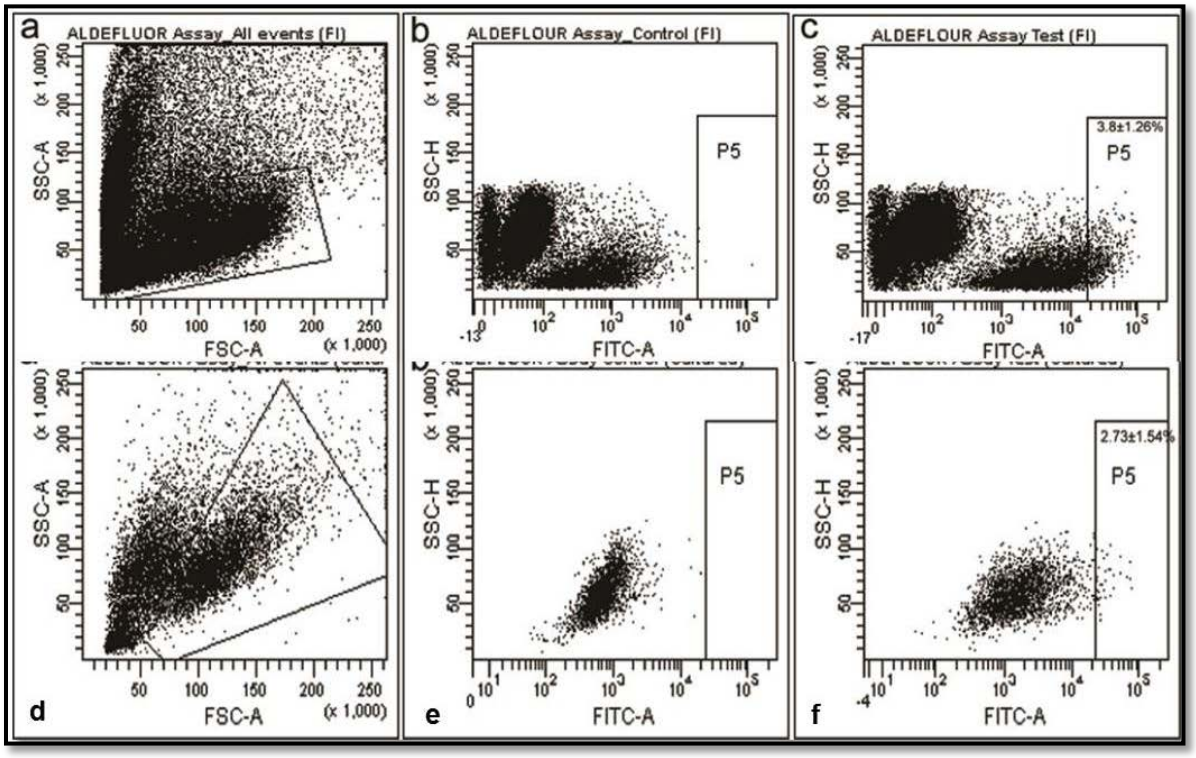

Figure 4.13: ALDEFLUOR™ Assay: Evaluation of ALDH1 high expression at t=0 (a-c) and t=14(d-f) cells. *Immediately at isolation (t=0) 3.8 ±1.26 % of the cells show ALDH1 high expression and this level reduces to 2.7± 1.54% by day 14 in-vitro.*

4.13 Discussion

The idea that stem-like cells are present in the human organs and persist even in adulthood has now gained universal acceptance. However, there still are many organ systems in which the presence of these is being explored; human lacrimal gland being one such organ. The presence of stem cells in the animal lacrimal gland has been reported very recently in the last two years [151, 152, 232]. The present study was aimed at exploring the possible presence of stem-like cells in the native human lacrimal gland tissue as well as in the *in-vitro* cultures of human lacrimal gland. *The result of this study is the first report on the presence of resident population of stem-like cells in the native human lacrimal gland as well as in the in-vitro human lacrimal gland cultures.* These findings open up the avenue of cell therapy in chronic debilitating lacrimal gland disorders.

The study of stem cells has been plagued with the fact that there is no single marker that can be counted upon as the true stem cell marker. In order to compensate for this short coming a number of strategies are used, like evaluating the expression of development and pluripotency markers, functional assays like clone formation and Aldefluor™ assay, sorting studies and gene expression signature profiling. In the present study, the presence of stem like cells was explored by evaluating the expression of stem cell markers (ABCG2, CD117), label retaining studies, ALDH1 expression levels, clonogenic potential and cell cycle analysis.

ABCG2 has been shown to label stem-like cells in a number of tissues like hematopoietic, muscle *[233-236]* while CD117 has been shown to label stem-like cells in

exocrine tissues like salivary gland, breast, prostate [118, 231, 237]. The possible presence of ABCG2 and CD117 positive cells in the normal human lacrimal gland was explored by immunohistochemistry and flow cytometry. In the present study, despite our repeated attempts, we were unable to standardize ABCG2 staining on the normal lacrimal gland tissue. CD117 localization studies show a basal and baso-lateral staining pattern on cells of acinar (44%) and ductal (9.8%) compartment. This observation contrasts what has been reported in the salivary gland and breast wherein, CD117 positive stem-like cells have been shown to reside in the terminal ductioles of the gland[118]. While CD117 staining has not been previously attempted in normal lacrimal gland, You *et al.,* had attempted to study the stem cell compartment in the murine lacrimal gland using BrdU label retaining studies. They reported BrdU label retaining cells to localize in the murine acinar (58.2±3.6%), myoepithelial (26.4±4.1%), ductal (0.4±0.4%) and stromal (15.0±3%) compartments [232]. Their findings by this method support our observation of possible presence of stem-like cells in both the acinar and ductal compartments.

Flow cytometry data of freshly isolated cells from the human lacrimal gland shows that 3.1±0.61% of the cells were $ABCG2^{+}$, 6.7±2.0% were $CD117^{+}$ and 0.3±.1% were $CD133^{+}$. The cultured cells also showed the presence of stem cells. However, they tend to undergo differentiation and their percentages fall to 0.3±0.15% ABCG2 and 0.2±0.05% CD117 by day 14 *in-vitro.* These values further reduce to 0.2±0.13% ABCG2, 0.13±0.03% CD117 and 0.25±0.05% CD133 respectively by *DIV* 21.

This important observation that number of stem-like cells reduce in culture over a period of time, while the proportion of differentiated secretory cells increases (as evidenced by the increase in secretion of tear protein over a two week period) prompts us to consider that the limitation could be in our culture system which probably does not support the proliferation of both stem cells and differentiated cells beyond 2- 3 weeks. Optimizing conditions that would sustain both these compartments would be in important step that would pave way for future clinical application.

The various techniques for maintaining and enriching stemness *in-vitro* include user of feeder cells, simulating niche conditions using bioreactors or co-culture systems or by maintaining stem-like cells as 3D spheres under serum-free conditions [118, 194, 231]. One of the methods that we explored in this study was attempting stem cell enrichment by generating 3D spheres and evaluating the cells for their clonogenic potential, label retaining capacity, quiescence, stem cell marker expression with a corresponding decrease in secretory-competent cells. In our study, by altering the EGF, FGF concentration and serum withdrawal, lacrispheres could be generated *in-vitro*. These lacrispheres were capable of surviving for greater than 35 days in culture and generating secondary spheres for 3-4 passages. Sphere generation from rabbit lacrimal gland had earlier been attempted by Schrader *et al.*, under microgravity conditions. However, their spheres could only be maintained in culture for about 3 weeks after which they died due to spreading central necrosis contrary to ours which survive and proliferate for greater than 35 days *in-vitro* [162].

The clonogenic potential of the lacrispheres was evaluated by clone formation assay. The results showed that the clonal efficiency was 3.1% indicating that they have self – renewal potential. Flow cytometric evaluation of lacrispheres showed about 0.8% of the cells to be $CD117^+$ by day 14, which is actually a four-fold increase from that seen under previous culture condition (0.2±0.05% $CD117^+$ in adherent monolayer). These lacrispheres are probably similar to the salispheres from salivary gland, which have been reported in literature, and have been shown to have 0.65% $CD117^+$cells by day 3 *in-vitro* [195].

We also attempted to investigate the presence of LRCs in the cultured human cells. Our results indicate that over a chase period of 14 days, a few cells are present in the center of both adherent spheres (27.9±4.3%) as well as lacrispheres (9.3±0.4%) that retain BrdU label. These are known as label retaining cells and are presumed to be potential stem-like cells. The other cells in culture, including the cells of duct-like connections and peripheral cells of spheres, lose the BrdU label indicating their high proliferative nature. In the cell cycle analysis we aimed at looking at the fraction of cells that would be in resting phase (G0/G1) In principle, this population would include quiescent cells (G0) as well as cells before entering into cell cycle (G1). In this study, we considered them together for logistic reasons. Cell cycle analysis showed that in the normal human lacrimal gland 79.9% of the cells are in G0/G1 or quiescent phase and 18.9% in the G2/S/M or active cell cycle phase. When these cells are cultured *in-vitro*, a higher fraction of the cells (33.9%) become active while the percentage of G0/G1 quiescent cells falls to 66.9%. However, when these cells are maintained under serum-

free conditions as lacrispheres, a more of *in-vivo* mimicry is maintained with about 76.9% of the cells being in the G0/ G1 phase and 22.9% in the G2/S/M phase. Of the 76.9% of the cells in the quiescent phase, about 0.3% are CD117 positive.

Functional assays like ALDEFLUOR™ are important because they evaluate functional properties of the stem-like cell. The ALDEFLUOR™ assesses the function of ALDH1 which is an enzyme present at high levels in the stem and progenitor cells. ALDEFLUOR™ analysis of cells at t=0 showed that 3.8± 1.26% of the cells were ALDH1 high; of which 0.13±0.04% were ABCG2 positive and 0.21±0.02% were CD117 positive. As anticipated from the results of marker studies, these ALDH1 high cell percentages fall to 2.7±1.5% and further to 1.1±0.5% by day 14 and day 21 *in-vitro.*

Some of the limitations of the present study, as has already been mentioned during the course of the discussion, is the inability to determine the ABCG2 staining pattern on the human lacrimal gland tissue and the inability to determine if the lacrispheres have a central lumen or if they are solid spheres as they could not be processed for histology and cryosection studies due to their fragile nature and presence of artifacts in the results. Another important limitation of the study was the inability to separate the cells of the G0/ G1 compartment into two distinct phases of the cell cycle. So, the results of the G0/G1 phase (79.9% at t=0, 66.9% of adherent cultures and 76.9% of lacrispheres) are indicative of not just quiescent cells but also those preparing to re-enter the cell cycle leading to mitosis. We have also been unable to correlate the percentages of stem-like cells detected using various techniques. This could

probably be because of inherent limitations of the techniques used most of which give a qualitative or semi-quantitative indication of positivity.

In summary, the present study provides *very promising, first of its kind evidence for the presence of stem-like cells and differentiated cells, in varying proportions, in native lacrimal gland and in the lacrimal gland cultures (2D monolayer and 3D lacrispheres).* The stem-like cells are being proposed based on surface markers (CD117), label retaining and quiescent studies, clonogenic potential and presence of high ALDH1 levels. We also provide evidence that the lacrispheres are possibly a better source of transplantable cells (stem cells > differentiated cells) and that 2 weeks of culture in the system established by us is probably an optimal time to harvest cells for potential cell therapy to restore function. Validating these findings and optimizing the number, mode (injection versus encapsulated transplants) and time of intervention in a preclinical model of radiation induced dry eye would take us one step closer to potential clinical application.

Chapter 5: Orbital Radiotherapy and Dry Eye Syndrome

5.1 Introduction

Radiotherapy is the medical use of ionizing radiation, as a part of the accepted modality of treatment for patients suffering from various malignancies like melanoma, lymphoma, retinoblastoma, leukemia, head and neck cancers, etc. Radiation therapy has been used in the treatment of cancer for more than a century now, beginning with the discovery of X-rays by Wilhelm Rontgen in 1895 and the pioneering discovery of radioactive isotopes radium and polonium by Madam Marie Curie in 1898.

Malignant tumors are treated with different types of radiation therapy- *external beam radiation therapy* or teletherapy which uses a high-energy x-ray machine, called a linear accelerator (*linac*), to direct radiation to the tumor; *brachytherapy* or sealed source radiation therapy where a radioactive isotope like Iodine-125, Caesium, Iridium etc. are implanted in the body near the tumor site, *and systemic radioisotope therapy* or unsealed source radiotherapy where the radioisotope is given systemically either orally or as an infusion. Radiotherapy may be curative based on the nature and location of the tumor a, or it may form a part of adjuvant therapy along with surgery and chemotherapy.

One of the major obstacles faced with radiotherapy was the toxicity to the surrounding tissue as the radiation dose delivery could only be controlled to a certain extent. The important invention of computed tomography by Godfrey Hounsfield in 1971, made possible a three dimensional planning of radiation delivery and allowed the physicians to more

accurately determine the dose distribution using axial tomographic images of the patient's anatomy. The introduction of new imaging technologies like magnetic resonance imaging (MRI) in the 1970s and positron emission tomography in the 1980s has progressed radiation therapy from 3D conformal to intensity-modulated radiation therapy (IMRT) and to image-guided radiation therapy (IGRT). These advances now allow radiation oncologists to better see and target tumors, which have resulted in better treatment outcomes, more organ preservation and fewer side effects. However, there are still a large proportion of patients who come back to the clinic for the treatment of radiotherapy induced side effects like cataract, bone marrow depression, ulcers, dry eye and dry mouth, neuropathy, retinopathy, etc.

5.1.1 Mechanism of action

The main mechanism underlying radiotherapy is DNA damage. This DNA damage may be either direct or indirect. In the case of *direct damage*, the ionizing radiation (IR) directly acts on the biomolecules (RH) causing ionization and destruction due to bond breakage and ensuing free radical induced destructive chain reaction.

$$IR+RH \rightarrow R^{\circ} + H^{\circ}$$

Both R° and H° can interact with other biomolecules like DNA, lipids and proteins and initiate chain reactions propagated by free radical formation:

$$R^{\circ} + R'H \rightarrow R'+ RH$$

It is estimated that nearly one-third of the damage due to γ radiation is caused due to direct effects.

In *indirect damage*, a major proportion of the ionizing radiation energy is absorbed by the water present in the cells and tissues. A complex cascade of reactions occurs after this called water radiolysis which leads to the formation of short lived H_2O^+ radical- cations, fast electrons and electronically excited water molecules (H_2O^+). These are unstable species and decompose within 10^{-13} seconds to form OH° and H° radicals. These free radicals which are formed, in turn, interact with biomolecules and damage them in a fashion similar to the direct effect.

$$IR + H_2O \rightarrow H_2O^+ + e^-$$

$$H_2O + H_2O^+ \rightarrow H_3O^+ + OH\bullet$$

$$IR + H_2O \rightarrow H_2O^* \rightarrow H_2O + \text{photon emitted}$$

$$e^- + H_2O \rightarrow H_2O^- \rightarrow OH^- + H\bullet$$

$$e_{aq}^- + H^+ \rightarrow H\bullet$$

$$e_{aq}^- + O_2 \rightarrow O_2$$

$$H\bullet + O_2 \rightarrow HO_2\bullet$$

5.1.2 Radiation dose

Radiation dose is deposited in a predictable way within tissue. For most malignancies the total dose used over a complete course of therapy is *30-50 Gy* for palliation, *45-55 Gy* for eradication of subclinical diseases, and *60-70 Gy* for elimination of clinically apparent disease. Therefore the dose required depends not only on therapeutic goal but also on tumor

histogenic type and radiosensitive, tumor size, tolerance of the neighboring normal tissue, further therapeutic plans (surgery/chemotherapy), and the patient's general physical conditions.

Dose is delivered in a fraction or divisions in order to enable the normal tissues to withstand the radiation toxicity and heal. The concept of dose fractionation was first introduced by French radiation oncologist Henri Coutard. The total radiation dose is delivered in several, smaller doses over a period of time. The dose fractionation calculation can be extrapolated from complex, computer-generated dose measurements or isodose distribution curves (dosimeter).

5.1.3 Tissue response to radiotherapy

Radiation treatment can cause loss of function in tissues. This loss of function may be attributed to loss of proliferative activity of stem cells in organs with high turnover index like bone marrow and mucosa. In other tissues this loss may be due to damaging effects to the underlying stroma and the associated vasculature. Traditionally, the effects of radiation are based largely on functional and histopathological endpoints and are classified into acute and chronic. Acute responses like erythema, mucositis, blepharitis, fatigue, nausea, vomiting, hair loss, xerostomia, are seen within weeks of radiotherapy; primarily in tissues where high rate of cell renewal is required to maintain function. Late responses like fibrosis and vascular damage take years to develop and manifest clinically, and are typically seen in organs with less proliferative cells like liver or kidney. In late responses, damage to the connective tissue

and vasculature of the organ also contribute to secondary cell death. Increased cytokine and chemokine levels have been reported in both acute and chronic responses. This causes excessive ECM and collagen deposition characteristic of radiation fibrosis. These elevated levels of pro-inflammatory cytokines and chemokines persist in a cyclic fashion for a sustained period of time and set up a chronic inflammatory loop in the tissue along with vascular damage, tissue hypoxia and reactive species imbalance (Figure 5.1)(IAEA handbook).

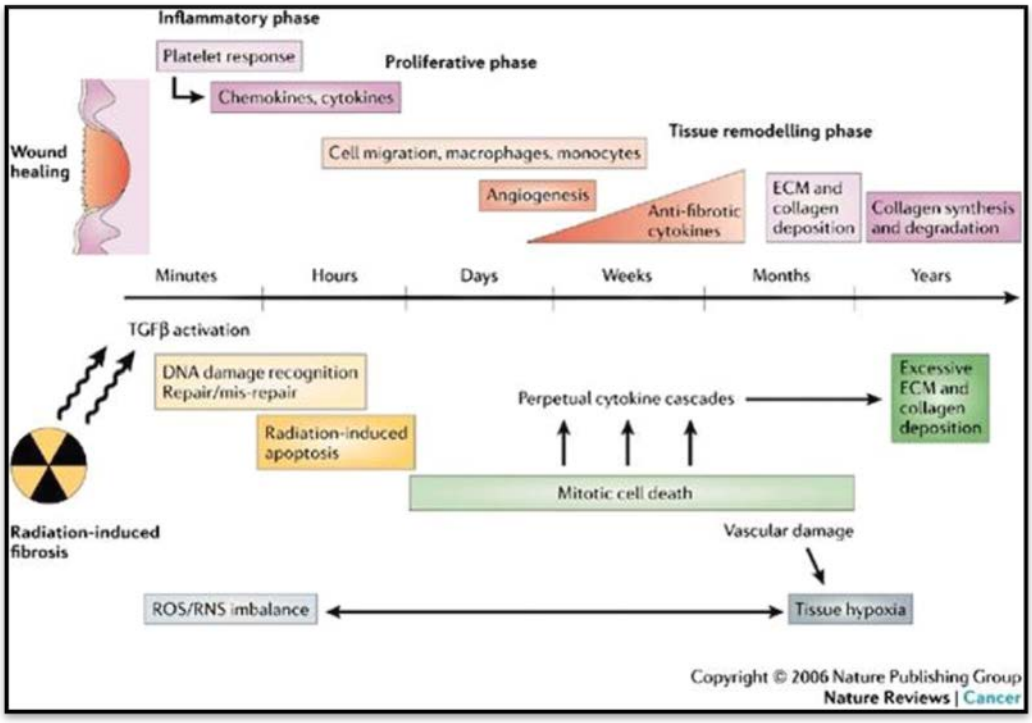

Figure 5.1: Radiation induced tissue effects. *(Adapted from Bentzen Nature Reviews) Radiation damage activates the wound healing machinery of the cell, in addition to certain unique other processes (below the timeline). The process of healing radiation induced damage involves excessive deposition of extracellular matrix (ECM) and collagen that is characteristic of radiation fibrosis, vascular damage, tissue hypoxia and reactive free radical imbalance. ROS, reactive oxygen species; RNS, reactive nitrogen species; TGFβ, transforming growth factor-β.*

Radiotherapy can cause cell death in two ways: *reproductive death* and *interphase death. Reproductive death* is associated with cell cycle factors and is caused due to the inability of the cells to repair critical levels of DNA injury. This type of cell death occurs when the cell tries to divide after a period of latency post radiotherapy. For this reason the death may occur weeks, months or years post the last insult, the timing being dependent on the cell division kinetics. *Interphase death* on the other hand, occur independent of cell division and within a matter of few hours post radiation injury. Interphase death occurs by rapid apoptosis due to the activation of killer cells which secrete cytotoxic factors inducing pycnosis and subsequent cell death [238].

5.1.4 Radiation side effects

The side effects associated with radiation therapy can be classified as acute (usually occurring within 3 months of treatment) or late (occurring many months to years after completion of treatment). Acute lesions generally affect rapidly proliferating cells, and most can be reversed by appropriate medical management. Late effects are primarily caused by permanent vascular damage and resultant ischemia. Some of the important and commonly observed side effects associated with radiation therapy are hair fall, fetal damage, secondary malignancies, infertility, fibrosis and fatigue, tiredness, sore skin in area where radiation is being given, nausea, and bone marrow depression. In external beam radiotherapy for head and neck cancers the commonly observed acute effects are mucositis dysphasia, salivary gland

toxicity and those involving the ocular anterior segment like blepharitis, conjunctivitis and keratitis. Other symptoms like *dry eye*, epiphora, ectropion, scleral-necrosis, cataract, glaucoma, optic neuropathy, and retinopathy are example of late effects which cause severe morbidity in patients [239].

5.1.5 Orbital radiotherapy

Radiation therapy to the head and neck region or to the orbit is a part of the established protocol to treat oral and ocular malignancies like salivary gland tumors, retinoblastoma, choroidal melanoma, orbital rhabdomyosarcoma, etc. The effects of radiation on the ocular tissues have been known since the late 1800s. In general, ocular damage may occur due to direct damage to the surrounding tissues or if the ocular tissues lie in the path of radiation beam. Even though there are very effective shielding techniques available today, yet the fact remains that a large proportion of these patients come back with radiation-induced side effects like cataract, retinopathy, neuropathy, dry eye, etc. Some of these are easier to manage but conditions like dry eye have a life-long associated morbidity. The only treatment available for dry eye is palliation and lubrication which does not significantly improve the patient's quality of life.

5.1.5.1 Radiotherapy induced ocular morbidity

Despite the formal implementation of the Late Effects of Normal Tissue Task Force (LENT)-Subjective, Objective, Management, Analytic (SOMA) scales or the better known LENT/SOMA toxicity grading system it is still very difficult to define and compare ocular

morbidity incidences and severity due to several biological parameters like tumor location, type, volume, latent period, patient age, adjuvant therapies and associated medical conditions [60].

Post-radiotherapy complications are classified as either acute (occurring within three months of treatment) or late (occurring many months/ years after radiotherapy). The acute complications like blepharitis, conjunctivitis, keratitis, generally involve rapidly proliferating cells and can usually be reversed/ treated by appropriate medical treatment. It is the late complications like retinopathy, cataract, dry eye, caused due to permanent vascular and tissue damage that are more difficult to manage.

Radiotherapy has profound effects on the various ocular tissues. It causes transient erythema, madarosis, palpebral conjunctivitis, telangiectasia, hyperpigmentation, lymphedema, ectropion, entropion, lid notching, and permanent eyelash loss. Conjunctiva and cornea are also affected to quite an extent leading to changes like conjunctival hyperemia, inflammation, symblepheron formation, depletion of goblet cells, punctate and filamentous keratitis, stromal edema and ulceration, corneal neurotrophic disease, etc [60]. Lacrimal gland and the Meibomian glands are another set of structures that suffer extensive tissue damage leading to alterations in the composition of the normal tear film. This in turn, has the effect of destabilizing the ocular surface and setting up a vicious inflammatory loop which worsens the condition.

5.1.6 Radiotherapy induced dry eye

Even though dry eye is a known side effect of EBRT, yet there are no studies that actually document this association. On review of the available literature only a few studies were found that dealt with the apparent link between EBRT and dry eye syndrome. A study by Parsons *et al.* showed that the "The incidence of radiation injury increases the rate of dry eye from 0% reported after doses ≤30 Gy to 100% after doses ≥57 Gy" [69]. In another study, Ozkurt *et al.*, investigated the incidence of dry eye in radiologists versus non-radiologists. Their results indicate that incidence of dry eye in the radiologist group was statistically significantly higher than the non-radiologists [240].

Gregoire *et al.*, (7) investigated if the new technology of intensity modulated radiation therapy (IMRT) for head and neck cancer could prevent/ reduce the incidence of dry eye that develop in these patients. Their results show that even though the technique of IMRT reduces the incidence of dry eye in patients significantly, yet it cannot completely alleviate it as 5.1% of the patients still present with sever grade 3 dry eye [241]. A similar study by Goyal *et. al.*, concluded that even with the advanced technique of IMRT, which gives better local control of the radiation beam, dry eye develops in the patients [242].

The study by Bhandare *et al.*, which is very similar to the present work, investigated the incidence of dry eye and lacrimal damage in patients who underwent EBRT for extra-cranial head and neck tumors. Their results indicate that orbital radiation therapy mainly injures the lacrimal gland, conjunctival goblet cells, accessory lacrimal glands, and

Meibomian gland structures. The patients who show dry eye in their case series post radiation therapy were as high as 51% [243].

The technique to radiation therapy has undergone many advancements and refinements over time. Shielding techniques, which are supposed to shield the surrounding normal tissue structures from the radiation beam, have also become much better now than they were twenty years back. Yet, despite of all this, the fact remains that a number of patients present to the clinics with symptoms of dry eye, ranging from mild to severe, post head and neck radiation. This is an observation that is made by eye health practitioners around the world. Yet there is scant literature available to support this observation. In the Indian scenario, to the best of our knowledge, there are no reports at all which document this finding. The present study is an attempt to fill the existing lacuna.

5.1.7 Diagnostic evaluation of dry eye syndrome

The following tests have been recommended by the DEWS, 2007 sub-committee to diagnose and monitor dry eye syndrome in patients:

Symptom questionnaires have been developed and are used in dry eye diagnosis, epidemiological studies and randomized control trial to assess the patient perceived symptoms of dry eye. However, these should be used in conjugation with clinical objective tests to diagnose and monitor the progress of the condition.

Grading ocular surface staining: In order to diagnose DES, the cornea and conjunctival staining is done using fluorescein and lissome green dyes or by fluorescein alone. *Fluorescein* is hydrophilic at physiologic pH and so poorly penetrates the lipid layer of the epithelium.

The dye does not stain normal corneas or pass through to the aqueous humor. Corneal and conjunctival surfaces are stained whenever there is a disruption of cell-to-cell junctions. In dry eyes fluorescein staining may also be seen on the conjunctival surface; infact it has been reported that conjunctival damage precedes corneal damage and is more severe.

Rose Bengal Staining is done to assess the damage to the ocular surface epithelium. It stains the dead and desquamated but not healthy epithelium. The association between Rose Bengal staining and dry eye has not been fully characterized yet it is still used clinically as it gives a general idea of the extent of ocular surface damage.

Evaluation of tear secretion: the tear secretion potential is measured using the Schirmer's test. It is the most widely used method to assess aqueous tear production. The test involves using a thin strip of filter paper (Whatman35) bent in to an 'L' shape and inserting into the lower fornix. Wet length of the strip gives the tear production value.

There are two types of Schirmer's test: Schirmer's I and Schirmer's II. Schirmer's I involves taking the values either with or without anesthesia and Schirmer's II involves taking the value post nasal irritation. In the present study only the Schirmer I test was employed.

Schirmer's 1(without anesthesia): The test estimates tear flow stimulated reflex (basal tearing also included) by insertions of a filter paper in to a conjunctival sac in open eye condition

Schirmer's 1(with anesthesia): A drop of local anesthetic like paracaine was instilled in the eye 2 minutes before the test. This is done to prevent reflex tearing when the filter paper is inserted in the conjunctival sac. The test estimates the basal tear secretion. The interpretation of values is given in table 5.1

Table 5.1: Interpretation of Schirmer values (Ref: DEWS, 2007)

Classification	**Schirmer Values (mm)**
Grade 1/ Normal	Variable/ 10-15
Grade 2 dry eye	≤10
Grade 3 dry eye	≤5
Grade 4 dry eye	≤2

5.2 Hypothesis:

Radiation causes significant damage to structure and function to lacrimal gland which would be reflected in the tissues and clinical outcome of patients undergoing radiotherapy.

5.3 Aims and Objectives:

1. To study the morphologic changes of radiation on lacrimal gland obtained from human subjects undergoing surgery as per the treatment protocols, status post radiation
2. To attempt establishing cultures from post radiated human lacrimal gland tissues
3. Evaluate the clinical features of DES in patients undergoing RT at various time intervals

5.4 Material and Methods

5.4.1 Chemicals: Hank's balanced salt solution (HBSS), anti c-kit (Millipore, Temecula, CA; Dako, Glostrup, Denmark), anti- lysozyme (Abcam), anti-S100 protein (Dako, Glostrup, Denmark), polymer HRP, DAB substrate, Schirmer strips, paracaine drops 1%, HepatoSTIM™ culture media (BD biosciences, San Jose, CA, USA), Fetal calf serum (FCS) (HyClone), penicillin, streptomycin, gentamycin and amphotericin B, epidermal growth factor (Sigma Aldrich, St Louis, MO, USA), L-glutamine (2 mM) (Sigma Aldrich, St Louis, MO, USA), Matrigel® basement matrix (BD Biosciences, San Jose, CA, USA), collagen I gel (Sigma Aldrich, St Louis, MO, USA), collagenase (Invitrogen, Carlsbad, CA, USA), hyaluronidase (Invitrogen, Carlsbad, CA,USA), treated tissue culture dishes (NUNC).

5.4.2 Tissue source

Human lacrimal gland was harvested from the exenteration specimens of patients who had undergone radiation therapy (as a part of their management protocol) for ocular malignancies and were undergoing the surgery as the subsequent management option. Normal tissue controls were harvested from exenterated specimens of patients who had not undergone orbital radiotherapy prior to the exenteration surgery for ocular malignancies.

Review of records of ophthalmic pathology laboratory revealed 3 such cases which fulfilled the criteria of exenteration following radiation therapy for oculo-orbital malignancies. These 3 post- radiated lacrimal gland samples were compared with 3 normal samples of lacrimal

glands. Both the test and control tissues were subjected to histology/ IHC (n=2) ultrastructure studies by TEM (n=1) and establishing *in-vitro* cultures (n=3).

5.4.3 Tissue processing for immunohistochemistry

The harvested gland was processed for histology and immunohistochemistry and studied for the tissue architecture and the expression of markers like lysozyme, S-100 and CD117. The gland was fixed with 10% fresh formalin and embedded in paraffin. Thin 3 µm sections were taken on silane coated glass slides and used for immunostaining. Briefly, the paraffin embedded sections were deparaffinized at around 70 ºC and then in xylene series. The sections were rehydrated in alcohol series and then in distilled water followed by 1X PBS. The endogenous peroxidase activity was blocked using methanol and hydrogen peroxide and the antigen retrieval done using Tris-EDTA buffer (pH 9). After appropriate washings with PBS and blocking with 2.5% BSA, the sections were incubated with the primary antibody in a moist chamber for 2 hours at room temperature followed by secondary antibody (polymer HRP) incubation for 30 minutes at room temperature. DAB substrate was added to the section to allow color development for 10 minutes. This was followed by counterstaining with hematoxylene and then mounting in DPX. The sections were visualized under a light microscope.

The antibodies used and the appropriate dilutions are summarized in Table 5.2.

Table 5.2: List of antibodies and dilutions used for immunohistochemistry

S. No.	Antibody	Dilution	Company
1.	Lysozyme	1:100	Abcam
2.	∝-SMA	Neat	Dako
3.	c-kit	1:100	Dako

5.4.4 Transmission electron microscopy (TEM)

The post exenterated human lacrimal gland tissue for TEM studies was immediately fixed in TEM fixative (recipe in Appendix). TEM studies were performed on glutaraldehyde-fixed samples at the Ruska Labs, Agriculture University, Hyderabad. Briefly, the human lacrimal gland was fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 3h at room temperature, washed with PBS, and post-fixed in 1% osmium tetroxide for 3h. Fixed specimens were then washed, dehydrated, infiltrated and embedded in Araldite resin. Ultra-thin sections (50-70 nm) were made using an ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with uranyl acetate and Reynolds lead citrate. Sections were scanned using a transmission electron microscope (JEM-2100, JEOL Ltd., Japan).

5.4.5 Establishing in-vitro cultures from post radiated tissues

Fresh lacrimal gland, obtained from exenterated specimens, was washed with HBBS to remove red blood cells. The gland was chopped into small bits using a scalpel blade. The tissue mince was then incubated with the enzyme cocktail of collagenase (130 units per ml) and hyaluronidase (300 units per ml) for 90 minutes at 37ºC with intermittent shaking. At the end of the incubation period, the suspension was filtered through a 75μm cell sieve and the cell pellet was obtained by centrifugation at 1500 rpm for 20 minutes. The cells were seeded on uncoated, collagen I and Matrigel™ coated tissue culture dishes using DMEM-Ham's F12/ HepatoSTIM™ culture media supplemented with 10% FCS and antibiotics.

5.4.6 Dry eye status evaluation in patients post radiotherapy

The dry eye status of patients who had undergone orbital radiotherapy as a part of their treatment regimen was evaluated by reviewing the medical records of the patients and determining their dry eye status from their corresponding Schirmer's score. The study was conducted at the L V Prasad Eye Institute, between2010-2012. The study was approved by the Institutional Review Board and is in accordance with the Declaration of Helsinki.

5.4.6.1 Review of medical records: Medical records of 220 patients in between January 2002 to January 2012 who were diagnosed as having ocular malignancies and aggressive benign tumors like retinoblastoma, optic nerve glioma, lymphoma, sebaceous gland carcinoma etc. and were advised EBRT were reviewed retrospectively. Of these 220 patients, 75 received

EBRT at Apollo Hospital, Jubilee Hills, Hyderabad and returned to the institute for follow-up visits.

The inclusion and exclusion criteria for the study were as follows:

Inclusion criteria:

- Advised external beam radiation therapy as part of management protocol
- Returned to LVPEI for follow-up visits post radiotherapy
- Normal Schirmer's score pre-radiotherapy (Schirmer score ≥ 10)

Exclusion criteria:

- Brachytherapy
- Preexisting DES (Schirmer score< 10)
- Patients who underwent enucleation post radiotherapy

After reviewing the records at Ophthalmic Pathology Laboratory and the Medical Records Department of LVPEI, and excluding cases with incomplete records, 51 eyes of 47 patients were included in the study.

Medical records of 28 cases whose contralateral eye had no history of ocular complaints and had not undergone any intervention were also reviewed to generate control data.

Schirmer's test: Schirmer's test is an accepted test for evaluating the dry eye status in individuals. The test involves using a thin strip of filter paper (Whatman35) bent in to an 'L' shape and inserted into the lower fornix. Wet length of the strip gives the tear production value. There are two types of Schirmer's test: Schirmer's I and Schirmer's II. Schirmer's I

involves taking the values either with or without anesthesia and Schirmer's II involves taking the value post nasal irritation. In the present study only the Schirmer I test with anesthesia was employed.

Schirmer's 1(with anesthesia): A drop of local anesthetic like 1% paracaine was instilled in the eye 2 min before the test. This is done to prevent reflex tearing when the filter paper is inserted in the conjunctival sac. The test estimates the basal tear secretion. The interpretation of values is given in Table 5.3.

Table: 5.3: Interpretation of Schirmer values (Ref: DEWS, 2007)

Classification	**Schirmer Values (mm)**
Grade1/ Normal	Variable/ 10-15
Grade 2 dry eye	≤10
Grade 3 dry eye	≤5
Grade 4 dry eye	≤2

5.4.6.2 Data collection:

Data was collected from the medical records and includes the following

- Demographics of the patient sample- mean age, sex ratio
- Common chief presenting complaints.
- Clinical diagnosis.
- First line of therapy
- The range of radiation dose given

- The main EBRT side effects
- Dry eye status post-EBRT

5.4.6.3 Data analysis:

The collected data was analyzed using R and SPSS software version 19and the graphs were plotted using the same software (s).

5.5 Results

5.5.1 Determining the effect of radiotherapy on human lacrimal gland

A total of 3 lacrimal glands were available for the present study. These glands were divided into three parts - one for histology/ IHC studies (n=2), second for ultrastructure studies (n=1) and the third part for attempting in-vitro culture establishment (n=3). 3 normal lacrimal glands were also used as controls for the study.

5.5.1.1 Histopathology of lacrimal gland, status post RT

In the study, the available glands had received an average of 4800cGy radiation dose in 25-30 fractions over a 30-45 days period. The lacrimal gland (n=3) obtained from patients who underwent RT showed disorganized and shrunken gland. The lobular architecture was lost with widespread peri-ductal and intra-lobular fibrosis and lymphocytic inundation. Very few acini were seen preserved and these showed oval nuclei. Few interlobular ducts that were seen showed bi-layered epithelium. Immunophenotyping revealed weak and focal positivity for

lysozyme and S-100 staining in the acinar cells (Figure 5.2 f & g). CD117 staining showed a similar pattern of weak positivity (figure 5.2 h).

In contrast, the normal control human lacrimal gland, H&E staining showed normal tissue architecture with intact acinar, myoepithelial and ductal cells. The ECM component appeared normal looking with a few infiltrating lymphocytes (figure 5.2 a). The expression of lysozyme, which is a secretory protein, was seen in the cytoplasm of the acinar cells and to an extent in the ductal epithelial cells too (figure 5.2b). S-100 protein was seen to be distributed in the myoepithelial cells enveloping the acinar cells (figure 5.2c). CD117 was seen to be localized in the cell membrane of some of the cells in the acinar and ductal compartment.

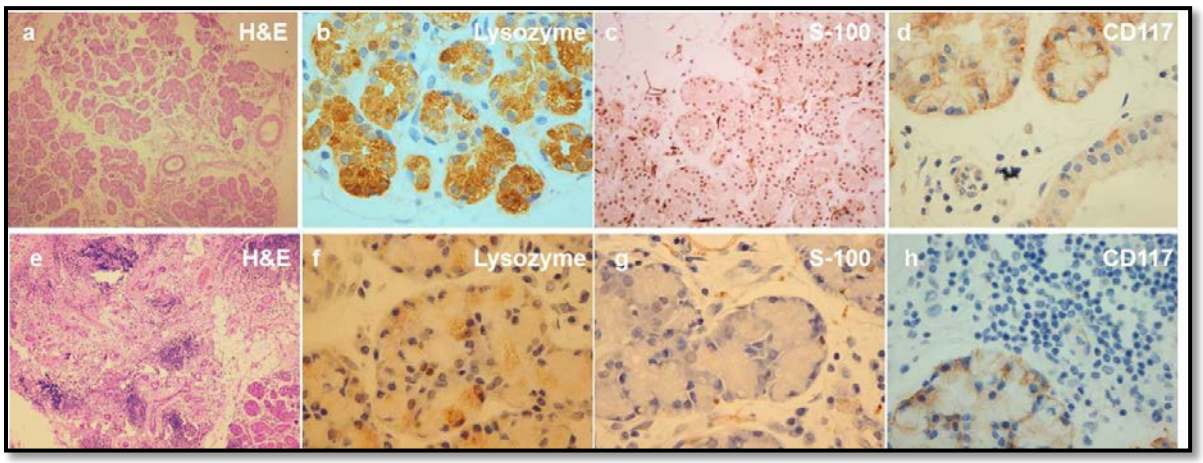

Figure 5.2: Comparison of normal (a-d) and post radiated (e-h) human lacrimal gland. *The normal lacrimal gland shows intact tissue architecture with acinar, ductal, myoepithelial as well as stromal cells. The secretory cells stain positive for lysozyme, myoepithelial cells for S-100 protein and the putative stem cells for CD117. The post- radiated tissue shows loss in lobular architecture with widespread peri-ductal and intra-lobular fibrosis and lymphocytic inundation. Immunophenotyping shows weak focal positivity for lysozyme, S100 and CD117.*

5.5.1.2 Transmission electron microscopy

Ultrastructure of the normal human lacrimal gland tissue revealed acinar cells with round nucleus, a number of secretory vesicles and junctional complexes (Figure 5.3 A-C). In contrast the post-radiated lacrimal gland showed evidence of extensive tissue damage with breaks in cell membrane and loose cell junctions, disrupted endoplasmic reticulum with dilated cisternae, condensed vesicular and varied shape mitochondria, degenerated large and fragmented nucleus, nucleolus lysis and nuclear membrane breaks (Figure 5.3 D-F).

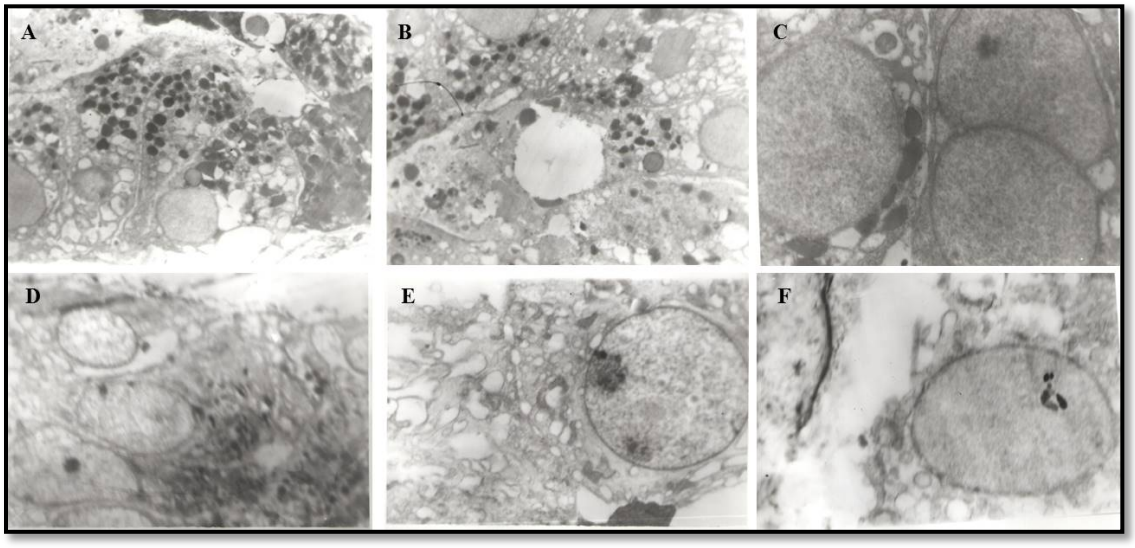

Figure 5.3: Transmission electron microscopic images of normal (A-C) and post-radiated (D-F) human lacrimal gland. *Ultrastructure of normal human lacrimal gland tissue shows acinar cells with round nucleus, a number of secretory vesicles and junctional complexes. The post-radiated gland showed evidence of tissue damage with breaks in cell membrane and loose cell junctions, disrupted endoplasmic reticulum with dilated cisternae, condensed vesicular and varied shape mitochondria, degenerated large and fragmented nucleus, nucleolus lysis and nuclear membrane breaks*

5.5.2 Establishing in-vitro cultures from post radiated tissues

The enzymatic digestion of the freshly harvested post- radiated lacrimal gland tissue yielded a heterogeneous population of cells (Figure 5.4a) with very few epithelial clumps. These cells were seeded on uncoated and collagen I/ Matrigel™ coated tissue culture dishes. No attachment or growth of cells was observed. Some cells that do attach showed spindle shape fibroblast morphology. No growth of epithelial cells was observed even after 3-4 days of seeding (Figure 5.4).

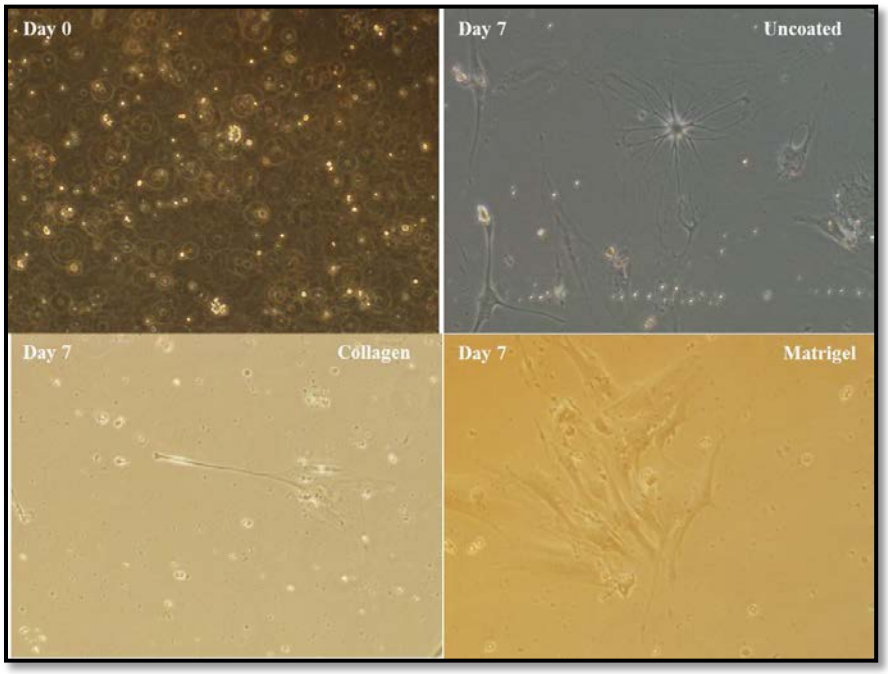

Figure 5.4: In-vitro growth of cells isolated from post radiated human lacrimal tissue

Isolated cell suspension showed very few epithelial clumps. These cells were seeded on uncoated, collagen I and Matrigel™ coated tissue culture dishes. Some cells that do attach show spindle shaped morphology. No growth of epithelial cells was observed.

5.5.3 Dry eye status evaluation in patients post radiotherapy

5.5.3.1 Demographics: Medical records of 220 patients who were diagnosed with oculo-adnexal malignancies from Oculoplasty and Ocular Oncology Services, LV Prasad Eye Institute were reviewed. Of these, 75 patients had been advised orbital radiotherapy as a part of their treatment modality, occasionally for locally aggressive benign, tumors. As per the institution policy of referral, all cases that had to receive radiotherapy were referred to Apollo Hospital, Hyderabad to a Radiation oncologist, for radiotherapy and reviews. For control, we initially included patients undergoing routine surgeries and refractory error corrections. However, since all cases did not undergo a complete dry eye work up, we considered the normal contralateral eye of the same patient as a control eye.

Of the 75 patients, those with incomplete records and those that did not match the inclusion criteria were excluded and so the final study population was 51 eyes of 47 patients (43 unilateral and 4 bilateral cases). 28 normal eyes were considered for control data.

The mean age of the patients was 39.8years (range 3-73 years) with a male: female ratio of 33:18 (1.8:1). The patients were divided into three age groups 0-11 years (Group A), 12-50 years (Group B) and> 50 years (Group C) (Table 5.3). The average follow up time following Radiation was 18.46 months (range 1.17- 120.20 months).

With regard to their radiotherapy regime, the mean radiation dose that the patient received was 4899.17cGy (4000 cGy-6600cGy); mean fraction being 25.22 (14-37) while the mean dose per fraction was 196.8cGy (163cGy-300cGy).

Medical records of 28 controls were also reviewed to establish a baseline data. The controls were similarly categorized into age groups A, B and C (Table 5.4). The average age of the control population was 41.39 years (3-73 years) with a male: female ratio of 17:11.

Table 5.4: Age group distribution

Age group	N (Controls)	N (Patients)
0-11 years	5	10
12-50 years	11	23
>50 years	12	18

The most common ocular malignancies for which the patients underwent EBRT were orbital lymphoma (61.3%) (both Hodgkin's and Non-Hodgkin's), optic nerve sheath and sphenoid wing meningioma (9.1%), rhabdomyosarcoma (6.8%), sebaceous gland carcinoma (4.5%), adenocystic carcinoma (4.5%), retinoblastoma (2.3%), *etc.* (Table 5.5)

Table 5.5: Patient data: Primary diagnosis

Diagnosis	**% of Patients**
Orbital lymphoma	61.3
Optic nerve sheath meningioma	9.1
Rhabdomyosarcoma	6.8
Adenocystic carcinoma	4.5
Optic nerve glioma	4.5
Sebaceous gland carcinoma	4.5
Lymphangioma	2.3
Plasmacytosis	2.3
Retinoblastoma	2.3
Sarcoma	2.3

5.5.3.2 Dry eye status of controls and patients: The dry eye status of the controls and patients was evaluated based on their Schirmer scores. Kaplan Meir Survival analysis curve was plotted to determine the number who remain free from dry eye during their follow up period.

For the control population: the data showed that in the age group A, there was 100% survival *i.e* no symptoms of dry eye; while in age group B it was 90.9% and 75% in age group C during the follow up period (Table 5.6).

Table 5.6: Age group distribution of dry eye

Age grp	Total N	N of Events	Censored	
			N	Percent
A	5	0	5	100.0%
B	11	1	10	90.9%
C	12	3	9	75.0%
Overall	28	4	24	85.7%

On analysis of the Kaplan Meir survival curve it was seen that dry eye is seen in age group B at 4.9 months which brings the survival percentage to 90.9%; while in age group C, 3 patients develop dry eye at 6.2 months, 17.2 months and 47.2 months respectively during the course of follow up which reduced the survival population to 75% (Figure 5.5).

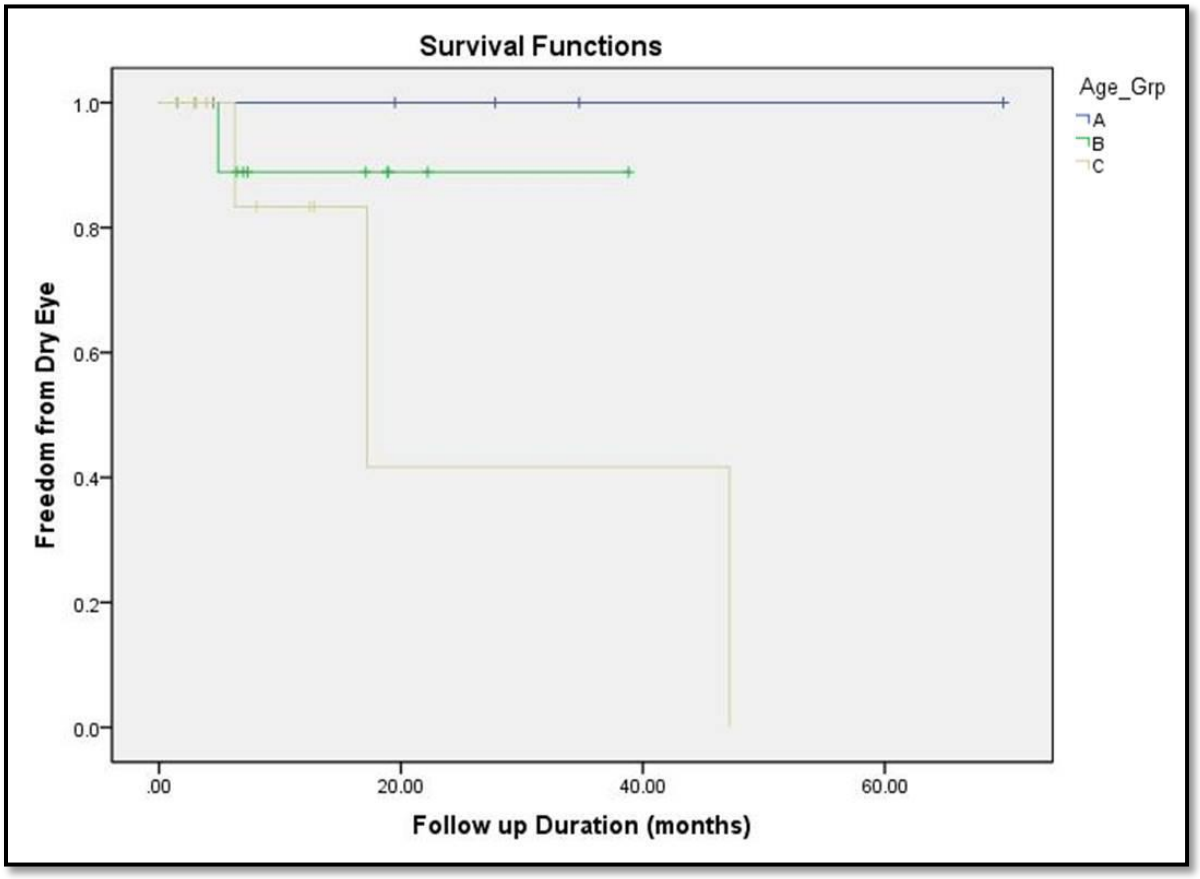

Figure 5.5: Kaplan Meir survival analysis for control population

The Kaplan Meir survival analysis showed a 100% survival rate for age group A (0-11 yr), 90.9% for age group B (12-50) and 75% for age group C (> 50yr) during the follow up period

For the patient population: The results of the survival analysis for the patient population indicate that by 6 month post last fraction of radiation therapy, nearly 23.9% of the patients develop dry eye. At the end of 18.8 months (1.6 years) nearly 50.2% of the patients develop dry eye while by the end of 47.17 months (3.9 years) about 77.2% of the patient population develop dry eye (Figure 5.6)

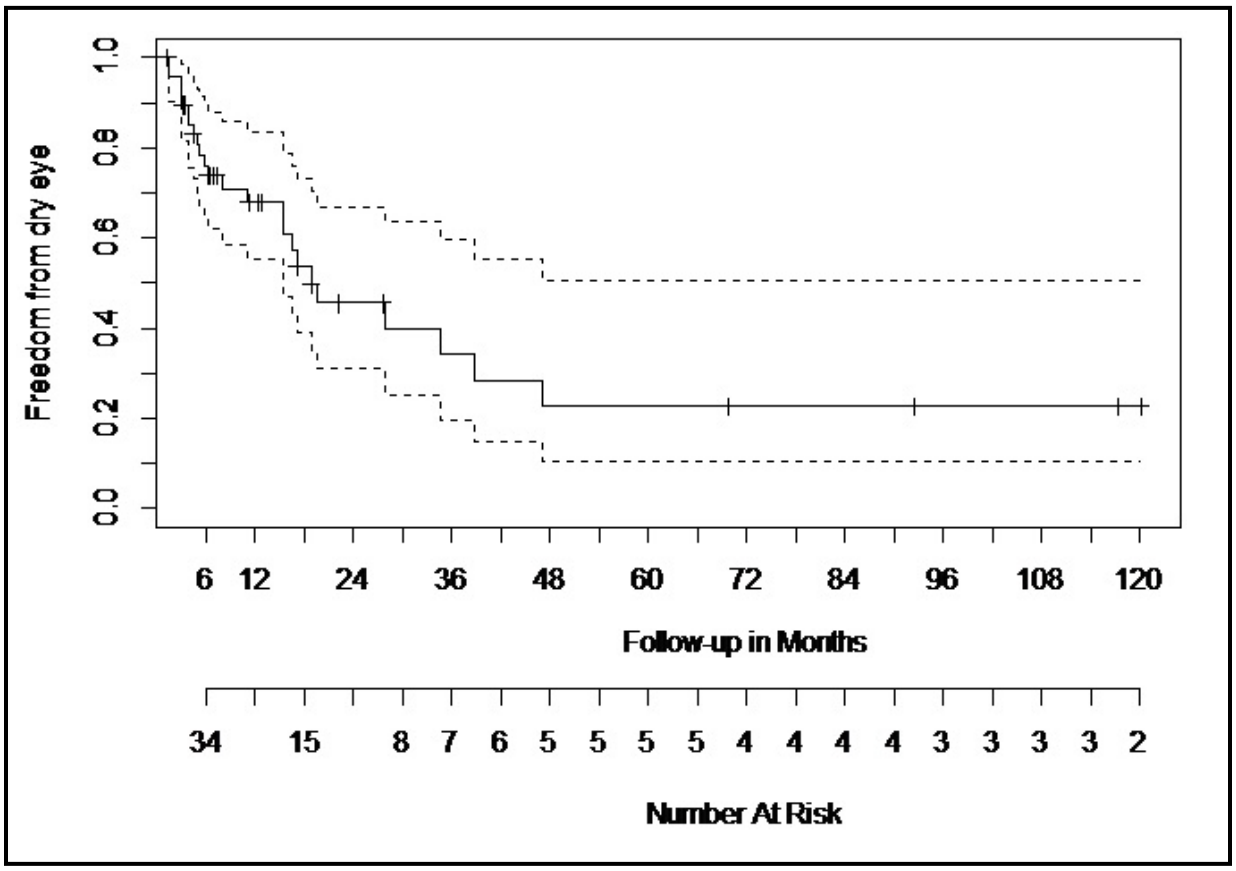

Figure: 5.6: Kaplan Meir Survival analysis for patient population.
The Kaplan Meir survival analysis showed that by 6 month post completion of radiotherapy, nearly 23.9% of the patients develop dry eye. At the end of 18.8 months (1.6 years) about 50.2% of the patients develop dry eye while by the end of 47.17 months (3.9 years) nearly 77.2% of the patient population develop dry eye. Solid line indicates the data curve while the dotted lines represent the confidence intervals.

5.3.3.3 Dry eye and age of patients: The patient population for the study was sub divided into three age groups similar to control: Group A: 0-11 yr; Group B: 12-50 yr; Group C: >50 yr The effect of therapeutic radiation dose and subsequent development of dry eye was analyzed in the three groups. The results indicate that in group A, 50% of the patients develop dry eye by 2.9 years after completion of radiotherapy; in group B, 50% of the patients develop dye eye in 1.4 years while in group C, 50% of the patients develop dry eye by 6 months post completion of their radiotherapy regime (Figure 5.7).

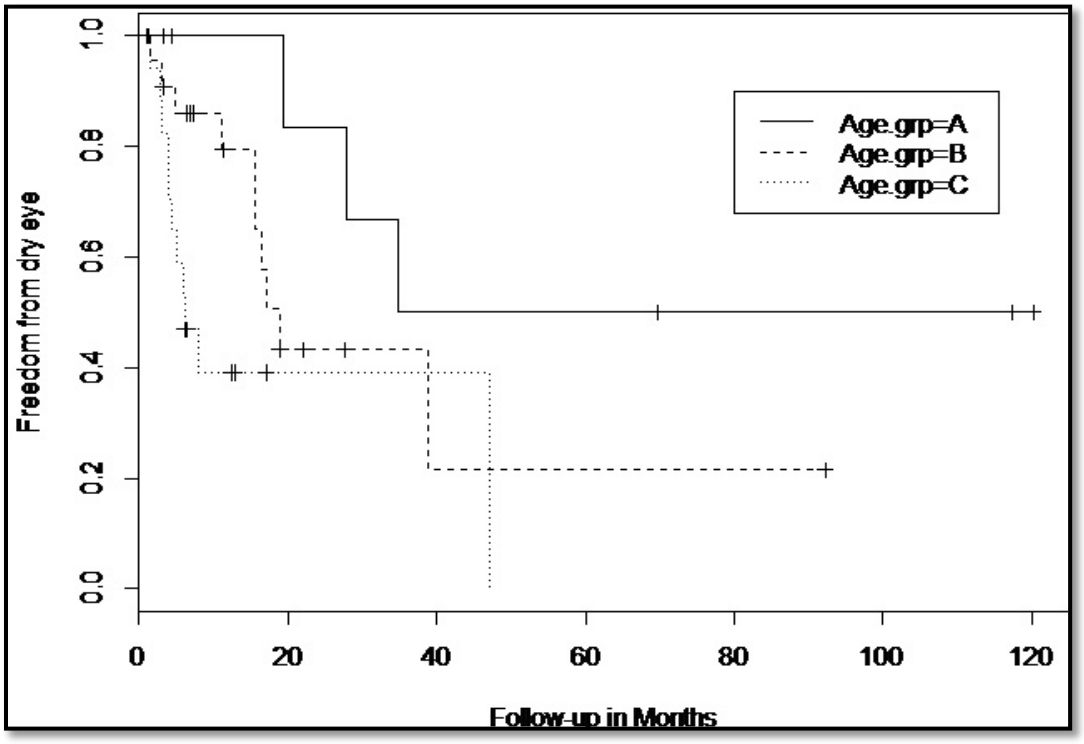

Figure 5.7: Kaplan Meir Survival Analysis for age of patient and dry eye development
The analysis showed that it takes 2.9 yr for 50% in age grp A to develop DES; 1.4 yr for those in age grp B and 0.5 yr for those in age group C to develop DES.

5.5.3.4 Dry eye grading: Of the 51 eyes of the study population, 47.07% (n=37.37) developed dry eye by 3.9 years. The dry eye status was graded into the 4 grades according to the DEWS 2007 criteria and the results indicate that 17.65% of the patients develop grade 2 dry eye, 13.73% develop grade 3 dry eye and nearly 15.69 % of the patient population develop grade 4 or severe dry eye by the end of their follow-up period (Table 5.7)

Table 5.7: Dry eye grading of patients

Grade of Dry Eye	N	% of Patients
1	27	52.94
2	9	17.65
3	7	13.73
4	8	15.69

5.5.3.5 Other ocular morbidity: the treatment plan for each patient was customized according to tumor volume, location and other anatomical parameters; however despite that side effects were noted. The majority of the side effects noted were radiation retinopathy (33.4%), radiation-induced cataract (24.9%), radiation keratopathy (20.8%), corneal scar (4.2%), radiation-induced limbal stem cell deficiency (4.2%) *etc.* (Table 5.8)

Table 5.8: Patient data: Co-morbidities observed post orbital EBRT

Diagnosis	**% of Patients**
Radiation retinopathy	33.4
Radiation-induced cataract	24.9
Radiation keratopathy	20.8
Corneal scar	4.2
Eyelid scalding	4.2
Lid symblepheron	4.2
Radiation-induced limbal stem cell deficiency	4.2
Radiation-induced periocular hyperpigmentation	4.2
Ocular and Peri-ocular burning sensation	4.2

5.6 Discussion

Radiation induced damage to the eye and ocular structures have been known for a long time. The damage to these happen either during irradiation of ocular tumors, paranasal sinus, head and neck or central nervous system malignancies or when the eye lies in the path of the radiation beam (Gordon, 1995). The present study was aimed at determining the radiation induced damage to the lacrimal gland in terms of structure, function, and presence of proliferating/stem cells that retain the capacity to grow *in-vitro*. To address this, we evaluated the lacrimal gland from exenterated specimens of patients who underwent radiotherapy in and around the area of lacrimal gland prior to the current surgery. This provided us a rare opportunity to study the structure and function of the gland post radiotherapy. For functional study, the patient data was retrospectively analyzed. The results showed that 47.07% % of patients developed dry eye within a period of 3.9yr after radiotherapy with a prediction of 50% developing it by the end of 1.6 yr. In addition, the study of the lacrimal gland provided evidence that there is loss of architecture of gland with fibrosis, loss or gross reduction of acinar structure, and failure to establish cultures *in-vitro*.

In the present study, we had an opportunity to evaluate the lacrimal gland, status post radiation, from patients who underwent EBRT followed by surgical removal of the eye and orbital tissues as part of the treatment protocols. The results of the histopathology study indicate that the tissue architecture gets nearly completely destroyed with only a small focus of preserved cellular integrity. Extensive peri-ductal and intra-lobular fibrosis and lymphocyte

inundation was also noted. The ductal cells seemed affected to the largest extent (Figure 5.2 e). Lysozyme and S-100 staining was seen to be weakly positive in the focal cells with preserved architecture (Figure 5.2 f & g). CD117 staining showed a similar pattern of weak focal area positivity. The ultrastructure studies also corroborate these findings of loss of lobular architecture, destruction of acinar as well as ductal structures, intra-lobular and interlobular peri-ductal fibrosis and inflammation. Although the mechanism of cell death was not evaluated in this study, the evidence of cell shrinkage, degenerate fragmented nucleus and nuclear membrane breaks are suggestive of apoptosis. These findings are in concert with those reported in literature wherein it has been documented that damage to the exocrine glands like lacrimal and salivary is caused due to the apoptosis of serous acinar cells. It has been reported that even at low doses, radiation causes reduction in saliva and tear flow as a consequence of rapid killing of acinar cells of the gland. The authors report that the acinar cells of the major and accessory lacrimal gland, some lacrimal duct epithelial cells as well as salivary gland epithelial cells undergo degeneration and necrosis with classical acute inflammatory response. Within 24 h post radiation neutrophilic presence was seen in the gland which later infiltrate and inundate the tissue; the nuclei were seen to be enlarged, hyperchromatic and progressed to pyknosis, karyohexis and marginated forms. The cytoplasm became homogenous and eosinophilic with loss of granularity and cytoplasmic vacuolation. Within 48 hr very few small sized acini were left intact leading to atrophy of the gland. The extent of tissue damage observed was proportional to the dose of radiation to the tissue [66].

The results presented in the previous chapters provide support to our hypothesis that there exists a store house of stem cells in the lacrimal gland which can be recruited to salvage the damaged gland. However, in extreme cases where the number of stem cells in the gland is not enough to repair this damage, then atrophy and destruction of the gland results as seen in the case of radiation damage. The histopathology observations in the present study also corroborate these beliefs. Our earlier *in-vitro* studies have also shown that lacrimal stem cells can be maintained under *in-vitro* culture conditions. With this background we attempted to culture lacrimal gland cells from post radiated gland. The objective was to expand the number of resident population of stem cells which would then be used for cell therapy, wherein they would be transplanted into the damaged gland to rescue function. However, the results of our culture attempts show that epithelial cells cultures could not be established from cells isolated from post radiated gland. One of the possible reasons could be that the number of cells that remain viable in the tissue are very low for any kind of *in-vitro* adjustment. This is also supported by the results of histopathology studies which showed extensive loss of tissue architecture with a small focal area of preserved cellular integrity.

The second objective of the present study was to document the volume of patients who develop dry eye post orbital radiation in the context of the Indian sub-population. The results of the study indicate that nearly 47.07% of the patients who undergo orbital EBRT develop dry eye by 3.9 years post the last fraction of radiation. If the patient population was further

sub-divided based on their age into three categories, the results indicate that it takes 2.9 years for 50% of the patients belonging to age group A (0-11yrs), 1.4 years for 50% of the patients belonging to age group B (12-50) and 0.5 years for 50% of the patients belonging to age group C (>50 yr) to develop DES post radiotherapy. These findings are comparable to the recent finding by Bhandare *et al*., who reported that 40 patients out of 78 (i.e 51.28%) develop dry eye post orbital radiotherapy [243]; however the authors did not find any statistical correlation between age and DES development.

In the present study, when the patients who developed radiation induced dry eye were categorized into the various grades according to the DEWS, 2007 criteria it was seen that nearly 17.65% of the patients developed grade 2 DES, 13.73% developed grade 3 DES while about 15.69% developed grade 4 or severe DES. The most common comorbidities observed were radiation retinopathy (33.4%), radiation induced cataract (24.9%) and radiation keratopathy (20.8%). Multivariate and univariate analysis of the data showed that fraction of radiation and dose of radiation per fraction were significant risk factors for developing DES with a hazard ratio of >10 for both the groups. In the analysis, male gender came up as a protective factor with a hazard ratio of < 0.001. Apart from these main factors a significant interaction was also found between the factors like age group, Schirmer's value to dose per fraction, total dose to fraction, total dose to dose per fraction and total dose to gender. Age group C (>50 yr) was a risk even though the present study could not prove it to be statistically significant. However, the interaction of radiation fraction to age group C was statistically

significant with a hazard ration < 0.001. Overall, the results also indicate age group A (0-11 yr) to be a protective factor against development of DES.

If we compare the present study with literature then the only comparable study is the one published in 2012 by Bhandare *et al*., [243] As mentioned previously, the study found that about 40 patients out of 78 develop severe dry eye post radiation leading to visual compromise. A dose-effect association was also reported with 6% of the patients who received 35.0-39.99Gy of radiation developing DES; 50% of those who received 45-49.99 Gy and 90% of those who received 60-64.99 developed DES. With a mean of 0.9 yrs (range 1month-3 yrs) latency of DES was observed to be a function of total dose and dose per fraction. Other factors like age, gender however were not found to be statistically significant. Some of these are in contrast to our findings. Univariate and multivariate analysis of our data showed number of fraction of radiation and dose of radiation per fraction were significant risk factors for developing DES (HR >10). Total dose however was not statistically significant. Male gender was a protective factor with a hazard ratio of < 0.00. Age group >50 yr was a risk factor even though the present study could not prove it to be statistically so probably because of the limited sample size. However, the interaction between this age group with the radiation fraction was a significant risk factor. There is no specific explanation we can offer to explain these differences. It could possibly be due to variation in biological parameters, spectrum of cases, therapy and work up protocol.

The present study also has its set of limitations. One of the major limitations is the lack of subjective patient responses to dry eye questionnaire. Since dry eye has both - the component of patient perceived symptoms as well as objective signs both of them need to be evaluated in order to come to a robust conclusion. However, it is logical to propose that in patients with ocular malignancies or aggressive benign tumors, the patient focus would be on these rather than the DES symptoms. Also being a retrospective study, we could only look at the objective signs of DES documented in the records. The second limitation of the study was using only one test for DES classification. Even though we accept this as a limitation yet we were constrained by the lack of data in the medical records of patients as it is not a routine practice for them to undergo other tests like fluorescein and Rose Bengal staining. The third limitation of the study was using the contralateral normal eye of the patients as control. We initially tried to recruit normal subjects undergoing routine surgeries and refractive error corrections; but were limited due to lack of DES work up data on them.

In summary, the present study documents that post orbital radiotherapy there is loss of lacrimal tissue architecture, extensive peri-ductal fibrosis and loss of acinar and ductal cells of the gland. The cells that remain viable in the gland are not sufficient for *in-vitro* epithelial culture establishment. This could be a function of loss of epithelial cells, stromal cells or niche factors. Retrospective analysis of the data showed that 47.07% of the patients who received orbital radiation develop dry eye with nearly 15.69% developed grade 4 or severe DES.

With nearly half of the patients who undergo orbital radiotherapy showing symptoms of dry eye and one-sixth of them developing grade 4 severe dry eye, one needs to look at alternate and complementary therapies to the preferred practice pattern (lubricating and hydrating eye drops, pharmacological stimulation of tear secretion, punctal plugs and salivary auto-transplantation). Extrapolating the success of tissue bio-banking in patients undergoing chemotherapy and/or radiotherapy for various malignant conditions and the cumulative evidence of culturing stem-like and differentiated cells (as 2D and 3D models) *in-vitro,* it would not be out of context to suggest adopting a similar approach for patients undergoing orbital radiotherapy for oculo-adnexal region to restore function of the gland.

Summary and Conclusion

The human lacrimal gland, one of the exocrine glands in the body, plays an important role in lubricating and hydrating the ocular surface epithelium. The susceptibility of the gland to immune mediated insults, radiation induced damage and age related atrophic changes, which cannot be satisfactorily managed with the current line of therapeutics, causes high incidences of dry eye related morbidity in the population (7-33%) [179]. This raises a need for alternate and long-term management options like cell replacement therapy with functionally competent cells. Though tear film is contributed by three different glands and tissue, our focus has been towards lacrimal gland since, aqueous deficiency contributes to nearly quarter of cases of DES [63].

One of the important pre-requisite for cell therapy is to be able to culture and enrich cells which can then be transplanted *in-vivo* to regenerate the organ and restore function. While lacrimal gland cultures from rabbit [155], mice [186] and rat [187] sources are well established similar studies from humans is lacking. The animal studies have significantly increased our understanding of the gland functioning; however, the results obtained cannot be directly extrapolated to humans. It is essential that these results be corroborated with human tissue studies. The present study tries to bridge that gap in knowledge and documents the ***first successful method*** of isolating, culturing and characterizing the human lacrimal gland cells and providing proof of concept of preserved function and presence of ‘stem-like cells’.

In the present study, the human lacrimal gland cultures could be established as adherent monolayer on matrices like HAM, collagen I and Matrigel™; with formation of spherules on the monolayers and the attempt to form duct-like connections between them (Figure 2.4 e-f). These cultures could be maintained *in-vitro* for 30-35 days, passaged for 3-4 passages, cryopreserved as well as revived with a revival efficiency of 60% for use at a later date.

This observation of spontaneous generation of spherules with duct-like connection between them prompted us to evaluate the nature of these spheres in terms of stemness and differentiation towards tubulo-acinar structure. In confirmation to our hypothesis, these 3 D spherule could be established under serum free conditions with enriched population of stem-like cells, as well as differentiated cells with secretory capacity. These were termed as 'lacrispheres' similar to the term coined for exocrine salivary gland- salispheres. Though not evaluated for their *in-vivo* function after transplantation, we speculate that they have a higher propensity to restore 3D structure and function.

Immunophenotyping of these cultured cells (with normal human lacrimal gland as control) indicates the presence of polymorphous population of cells- epithelial (acinar and ductal), myoepithelial as well as mesenchymal, similar to that present in the native gland, suggesting that all these cells could be proliferated *in-vitro*. It was also observed that the percentages of epithelial and mesenchymal cells tend to change with prolonged *in-vitro* culture. The percentages of epithelial cells reduce from 14.8±3.45% to 2.7± 1.7% (EpCAM

positivity by FACS); while on the other hand the proportion of mesenchymal cells increases from 2.9±.91% to 13.3±10.2% (CD90 positivity). These observations, we believe, would be useful in establishing enriched population of epithelial cells vs mesenchymal cells.

Since our long term goal is to explore the possibility of a replacement therapy, the next logical step in *in-vitro* culture of lacrimal gland cells was to evaluate the synthesis and secretory potential of these cells. Under the present culture condition of Matrigel™ coating as ECM and EGF supplemented HepatoSTIM media we provided the first evidence that the human lacrimal gland ***cells could maintain their synthesis and secretory potential in the culture system***. The mRNA for the three tear proteins considered in the present study (scIgA, lysozyme and lactoferrin) as well as mucin and aquaporin 5 were found to be present in the cultured cells indicating the potential of these cells to synthesize and secrete the proteins. The proteins were also detected and quantified (by sandwich ELISA) in the conditioned media indicating the potential of the cultured cells to secrete it into the culture media (conditioned media). The results indicate that the secretion of these proteins ranged from 3.86 to 71.4 ng/ml of scIgA, 5.78 to 33.94ng/ml of lysozyme and 44.5 to 45.65 ng/ml of lactoferrin on HAM, to 1.41 to 27.58ng/ml of scIgA, 0.21 to 18.34 ng/ml of lysozyme and 28.52 to 30.41 ng/ml of lactoferrin on collagen I coated dishes to 47.43 to 61.56 ng/ml of scIgA, 24.36 to 144.74 ng/ml of lysozyme and 32.45 to 40.31ng/ml of lactoferrin on Matrigel™ coated dishes (Table 3.6) in day 7 cultures. These cultures retained their secretory ability till day 21. The amount of protein secretion increased from day 7, peaks at day 14 of *in-vitro* culture and then

declined by day 21 (Table 3.7). It was noted that the level of proteins secreted by cultures growing on Matrigel™ remains the highest in all the three cases. An interesting observation made here was that the ratio of secretory products in the conditioned media was similar to that in natural tears i.e. Lysozyme>scIgA>Lactoferrin. ***The documentation of secretory products of cultured cells, similar to that of natural tears is a novel and promising finding***. This warrants further study and isolation, purification of these secreted proteins in the conditioned media, we strongly believe, would pave way for formulating natural tear preparations for potential clinical application.

To address the question whether the human lacrimal gland and the cultured cells would have stem-like cells, we explored the possibility using CD117 as a stem cell marker, Aldefluor™ assay, label retaining studies, clonal assay and cell cycle analysis. While the presence of such cells is known in breast, pancreas and salivary gland such information is not forthcoming in human lacrimal gland. In view of this ***our results documenting the presence of stem cells in human lacrimal gland cells is an important contribution***. The results show that the freshly isolated cells of the native human lacrimal gland, when analyzed by flow cytometry, show 6.7±2.0% of the cells to be positive for the stem cell marker CD117. CD117 was seen in both acinar and ductal compartment with varying pattern of basal and baso-lateral staining. However our attempts to localize them *in-situ* were inconclusive from the limited study of the histology and immunophenotyping. Complementing these with tracer studies, lineage tracer and laser capture microdissection coupled with gene expression studies would

help in localizing the stem cells and its niche in the native human lacrimal gland. This would also help us to throw light on bidirectional differentiation of acinar and ductal cells, if any. Understanding this would be an important contributory factor for planning future cell therapy by enabling fractionation of the cells as per the anatomic location. Such studies in other exocrine glands have paved way for isolation and purification of stem cells. The cultures, in addition to showing differentiated cells (epithelial, myoepithelial and mesenchymal) also showed the presence of stem-like cells constituting 0.2±0.05% of total cells by *DIV* 14. The proof of stem-like cells was also substantiated by label retaining studies, clonal assay and Aldefluor™ studies. The proportion of stem-like cells reduces to 0.13±0.03% CD117 by *DIV* 21. This declining percentage of stem cell population under prolonged culture conditions prompted us to attempt optimizing the *in-vitro* conditions further. Modification of the culture conditions and serum withdrawal led to generation of 3D spheres which we termed as 'lacrispheres' These lacrispheres, on flow cytometric evaluation, showed higher percentage of CD117 positive cells (0.8% Vs 0.2%), higher fraction of quiescent (76.9% Vs 66.9% in the G0/G1 phase of cell cycle) cells and colony forming units (3.1%). ***This is a novel and promising development documenting enrichment of stem cells that warrants further exploration by in-vivo transplantation studies.***

One of the important factors responsible for the development of dry eye in patients is the exposure to radiotherapy as a treatment modality for ocular malignancies and locally aggressive benign tumors. While clinical documentation of dry eye has been reported as one

of the morbidities of ocular radiation, ***this study provides the first proof of sequential and progressive development of dry eye in patients, substantiated with histological documentation of near total ablation of glandular architecture, loss of differentiated and stem-like cell loss.*** The TEM studies corroborate these findings of loss of lobular architecture, destruction of acinar as well as ductal structures, intra-lobular and inter-lobular, peri-ductal fibrosis and inflammation. Although the mechanism of cell death was not evaluated in this study, there is evidence that post-radiated cell death could possibly be by apoptosis.

Physiologically this destruction of lacrimal gland cells translates to reduction in the aqueous component of the tear film resulting in destabilization and hyperosmolarity of the tear film and eventual ocular surface damage. The Kaplan- Meir survival analysis showed that despite advances and refinement in the techniques and protocols of radiotherapy, nearly half of the patients (47.07%) who undergo orbital radiotherapy develop dry eye with nearly one-sixth (15.7%) developing chronic grade 4 dry eye. The analysis also showed that the average time for 50% of the patient population to develop DES ranged from 0.5 yrs (age group > 50 yrs) to 2.9yrs (age group 0-11 yrs) with female gender and >50 age group being a more susceptible population.

In conclusion, this novel study provides evidence that freshly isolated human lacrimal gland cells could be cultured successfully to expand the population of epithelial cells (acinar and ductal), with evidence of secretory function; presence of stem-like cells

in the culture system and normal human lacrimal gland. These ex- vivo expanded human lacrimal gland cells have the dual potential to be used as a cell source for cell replacement therapy as well as the potential to provide source of naturally secreted products for pharmacological management of dry eye due to lacrimal gland insufficiency. This we believe is an important stepping stone towards developing cell therapy for lacrimal gland insufficiency in future

Future Scope

The results presented here document for the first time that human lacrimal gland cells can be maintained *in-vitro* as adherent as well as non-adherent cultures with retained secretory potential. The evidence for the presence of stem cells in the native human lacrimal gland and *in-vitro* cultures prompts us to postulate that injecting/transplanting an enriched population of such cells into post- radiated damaged lacrimal tissue, wherein the evidence of damage has been proven histologically and functionally, could potentially rescue function. Towards achieving this long term goal of potential cell therapy, the following need to be explored to bridge these existing gaps:

1. While the evidence of stem cells was found in the present study, localization attempts were inconclusive. This could be explored by tracer studies coupled with laser capture microdissection and gene expression studies. The concept of a stem cell niche in the lacrimal gland has also not been addressed and needs to be explored.
2. The 3-dimensional lacrispheres reported in the study which has an enriched component of stem-like cells is another highlight of the study. The therapeutic potential of these cells to be used in injectable/ transplantable form in a radiation induced animal model of dry eye to rescue function would be an important study. The *in-vivo* tracking studies would throw light on the homing, integration and function restoration potential of these cells and would provide the proof of concept.

3. The evidence for the presence of secretory products in the conditioned media is a promising finding which can be further expanded to isolate and characterize the entire secretory repertoire of the cells, comparing it with natural tears for possible formulation of natural tear preparations.

Limitations

The work presented here is novel in many aspects and has contributed significantly in the direction towards evolution dry eye cell therapy. However, the study is not without its set of limitations. Some of the limitations of the present work are enlisted below:

1. The adherent spheres and their duct-like connections could not be separated from the monolayers with its architecture intact. Our repeated attempts to separate these spheres from monolayers were unsuccessful as any manipulation around them led to disruption of their structure.
2. We could not ascertain if the connections that develop between these adherent spheres have a lumen or not.
3. One important limitation of the marker profiling of cultured cells was the inability to differentiate between the ductal and the acinar cells in culture as both the cells showed positivity for the epithelial markers used in the study.
4. The ABCG2 staining pattern on the human lacrimal gland tissue could not be determined.
5. We were unable to determine if the lacrispheres have a central lumen or if they are solid spheres as they could not be processed for histology and cryosection studies due to their fragile nature and presence of artifacts in the results.

6. Another important limitation of the study was the inability to separate the cells of the G0/ G1 compartment into two distinct phases of the cell cycle. So, the results of the G0/G1 phase (79.9% at t=0, 66.9% of adherent cultures and 76.9% of lacrispheres) are indicative of not just quiescent cells but also those preparing to re-enter the cell cycle leading to mitosis.
7. We were unable to correlate the percentages of stem-like cells detected using various techniques. This could probably be because of inherent limitations of the techniques used most of which give a qualitative or semi-quantitative indication of positivity.
8. The study lacks data on subjective patient responses to dry eye questionnaire. Since dry eye has both -the component of patient perceived symptoms as well as objective signs-both of them need to be evaluated in order to come to a robust conclusion.
9. The study used only one criterion (Schirmer's value) for DES classification. Even though we accept this as a limitation yet we were constrained by the lack of data in the medical records of patients as it is not a routine practice for them to undergo other tests like fluorescein and Rose Bengal staining.
10. The study also has the limitation of using the contralateral normal eye of the patients as control. We initially tried to recruit normal subjects undergoing routine surgeries and refractive error corrections; but were limited due to lack of DES work up data on them.

References

1. Hanna C, Bicknell DS, O'Brien JE. Cell turnover in the adult human eye. Archives of ophthalmology. 1961 May;65:695-8. PubMed PMID: 13711260.

2. Tiffany JM. The normal tear film. Developments in ophthalmology. 2008;41:1-20. PubMed PMID: 18453758.

3. Duke Elder SW. Textbook of Ophthalmology. 1938 ed. London: Henry Kimpton; 1938.

4. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). The ocular surface. 2007 Apr;5(2):75-92. PubMed PMID: 17508116.

5. Lorber M. Gross characteristics of normal human lacrimal glands. The ocular surface. 2007 Jan;5(1):13-22. PubMed PMID: 17252162.

6. The Lacrimal System: Diagnosis, Management and Surgery. Cohen AJM, M.;Brazzo, B.G., editor: Springer; 2006.

7. Tripathi BJ, Tripathi RC. Evidence for the neuroectodermal origin of the human lacrimal gland. Invest Ophthalmol Vis Sci. 1990 Feb;31(2):393-5. PubMed PMID: 2406219. Epub 1990/02/01. eng.

8. de la Cuadra-Blanco C, Peces-Pena MD, Merida-Velasco JR. Morphogenesis of the human lacrimal gland. Journal of anatomy. 2003 Nov;203(5):531-6. PubMed PMID: 14635806. Pubmed Central PMCID: 1571187.

9. O'Rahilly R, Muller F. Developmental stages in human embryos: revised and new measurements. Cells, tissues, organs. 2010;192(2):73-84. PubMed PMID: 20185898.

10. Martin GR. The roles of FGFs in the early development of vertebrate limbs. Genes Dev. 1998 Jun 1;12(11):1571-86. PubMed PMID: 9620845. Epub 1998/06/17. eng.

11. Sanders EJ. The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem Cell Biol. 1988 Jun;66(6):530-40. PubMed PMID: 3048328. Epub 1988/06/01. eng.

12. Kammandel B, Chowdhury K, Stoykova A, Aparicio S, Brenner S, Gruss P. Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. Dev Biol. 1999 Jan 1;205(1):79-97. PubMed PMID: 9882499. Epub 1999/01/12. eng.

13. Johnston MC, Noden DM, Hazelton RD, Coulombre JL, Coulombre AJ. Origins of avian ocular and periocular tissues. Exp Eye Res. 1979 Jul;29(1):27-43. PubMed PMID: 510425. Epub 1979/07/01. eng.

14. Walcott B. The Lacrimal Gland and Its Veil of Tears. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society. 1998 Apr;13:97-103. PubMed PMID: 11390770.

15. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. Progress in retinal and eye research. 2009 May;28(3):155-77. PubMed PMID: 19376264. Pubmed Central PMCID: 3652637.

16. Gray H. Gray's Anatomy. 37 ed. Edinburgh: C. Livingstone; 1989.

17. Ruskell GL. The orbital branches of the pterygopalatine ganglion and their relationship with internal carotid nerve branches in primates. Journal of anatomy. 1970 Mar;106(Pt 2):323-39. PubMed PMID: 4986076. Pubmed Central PMCID: 1233706.

18. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibres to the lacrimal gland in monkeys. Journal of anatomy. 1971 Jul;109(Pt 2):229-42. PubMed PMID: 4997622. Pubmed Central PMCID: 1271003.

19. Mircheff AK. Lacrimal fluid and electrolyte secretion: a review. Current eye research. 1989 Jun;8(6):607-17. PubMed PMID: 2545410.

20. Ohashi Y, Dogru M, Tsubota K. Laboratory findings in tear fluid analysis. Clinica chimica acta; international journal of clinical chemistry. 2006 Jul 15;369(1):17-28. PubMed PMID: 16516878.

21. Paulsen F, Langer G, Hoffmann W, Berry M. Human lacrimal gland mucins. Cell and tissue research. 2004 May;316(2):167-77. PubMed PMID: 15052468.

22. Ariga H, Edwards J, Sullivan DA. Androgen control of autoimmune expression in lacrimal glands of MRL/Mp-lpr/lpr mice. Clinical immunology and immunopathology. 1989 Dec;53(3):499-508. PubMed PMID: 2805454.

23. Nakamura H, Kawakami A, Eguchi K. Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjogren's syndrome. Translational research : the journal of laboratory and clinical medicine. 2006 Dec;148(6):281-8. PubMed PMID: 17162248.

24. Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjogren's syndrome. Proposed criteria for classification. Arthritis and rheumatism. 1986 May;29(5):577-85. PubMed PMID: 3718551.

25. Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, et al. Preliminary criteria for the classification of Sjogren's syndrome. Results of a prospective concerted action supported by the European Community. Arthritis and rheumatism. 1993 Mar;36(3):340-7. PubMed PMID: 8452579.

26. Wiik A, Cervera R, Haass M, Kallenberg C, Khamashta M, Meroni PL, et al. European attempts to set guidelines for improving diagnostics of autoimmune rheumatic disorders. Lupus. 2006;15(7):391-6. PubMed PMID: 16898171.

27. Murube J, Benitez del Castillo JM, Chenzhuo L, Berta A, Rolando M. The Madrid triple classification of dry eye. Archivos de la Sociedad Espanola de Oftalmologia. 2003 Nov;78(11):587-93; 95-601. PubMed PMID: 14648364.

28. Murube J, Nemeth J, Hoh H, Kaynak-Hekimhan P, Horwath-Winter J, Agarwal A, et al. The triple classification of dry eye for practical clinical use. European journal of ophthalmology. 2005 Nov-Dec;15(6):660-7. PubMed PMID: 16329048.

29. Behrens A, Doyle JJ, Stern L, Chuck RS, McDonnell PJ, Azar DT, et al. Dysfunctional tear syndrome: a Delphi approach to treatment recommendations. Cornea. 2006 Sep;25(8):900-7. PubMed PMID: 17102664.

30. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. The CLAO journal : official publication of the Contact Lens Association of Ophthalmologists, Inc. 1995 Oct;21(4):221-32. PubMed PMID: 8565190.

31. Sullivan DA. Androgen deficiency & dry eye syndromes. Archivos de la Sociedad Espanola de Oftalmologia. 2004 Feb;79(2):49-50. PubMed PMID: 14988781.

32. De Paiva CS, Corrales RM, Villarreal AL, Farley WJ, Li DQ, Stern ME, et al. Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. Experimental eye research. 2006 Sep;83(3):526-35. PubMed PMID: 16643899.

33. Bron AJ, Tiffany JM. The contribution of meibomian disease to dry eye. The ocular surface. 2004 Apr;2(2):149-65. PubMed PMID: 17216085.

34. Bron AJ, Tiffany JM, Gouveia SM, Yokoi N, Voon LW. Functional aspects of the tear film lipid layer. Experimental eye research. 2004 Mar;78(3):347-60. PubMed PMID: 15106912.

35. Foulks GN, Bron AJ. Meibomian gland dysfunction: a clinical scheme for description, diagnosis, classification, and grading. The ocular surface. 2003 Jul;1(3):107-26. PubMed PMID: 17075643.

36. Mathers WD, Shields WJ, Sachdev MS, Petroll WM, Jester JV. Meibomian gland morphology and tear osmolarity: changes with Accutane therapy. Cornea. 1991 Jul;10(4):286-90. PubMed PMID: 1832371.

37. Dougherty JM, McCulley JP. Bacterial lipases and chronic blepharitis. Investigative ophthalmology & visual science. 1986 Apr;27(4):486-91. PubMed PMID: 3957566.

38. Gilbard JP, Farris RL. Ocular surface drying and tear film osmolarity in thyroid eye disease. Acta ophthalmologica. 1983 Feb;61(1):108-16. PubMed PMID: 6687972.

39. Abelson MB, Ousler GW, 3rd, Nally LA, Welch D, Krenzer K. Alternative reference values for tear film break up time in normal and dry eye populations. Advances in experimental medicine and biology. 2002;506(Pt B):1121-5. PubMed PMID: 12614039.

40. Nakamori K, Odawara M, Nakajima T, Mizutani T, Tsubota K. Blinking is controlled primarily by ocular surface conditions. American journal of ophthalmology. 1997 Jul;124(1):24-30. PubMed PMID: 9222228.

41. Sommer A, Emran N. Tear production in vitamin A-responsive xerophthalmia. American journal of ophthalmology. 1982 Jan;93(1):84-7. PubMed PMID: 7065090.

42. Tei M, Spurr-Michaud SJ, Tisdale AS, Gipson IK. Vitamin A deficiency alters the expression of mucin genes by the rat ocular surface epithelium. Investigative ophthalmology & visual science. 2000 Jan;41(1):82-8. PubMed PMID: 10634605.

43. Pisella PJ, Pouliquen P, Baudouin C. Prevalence of ocular symptoms and signs with preserved and preservative free glaucoma medication. The British journal of ophthalmology. 2002 Apr;86(4):418-23. PubMed PMID: 11914211. Pubmed Central PMCID: 1771067.

44. Chen HT, Chen KH, Hsu WM. Toxic keratopathy associated with abuse of low-dose anesthetic: a case report. Cornea. 2004 Jul;23(5):527-9. PubMed PMID: 15220742.

45. Pharmakakis NM, Katsimpris JM, Melachrinou MP, Koliopoulos JX. Corneal complications following abuse of topical anesthetics. European journal of ophthalmology. 2002 Sep-Oct;12(5):373-8. PubMed PMID: 12474918.

46. Begley CG, Caffery B, Nichols KK, Chalmers R. Responses of contact lens wearers to a dry eye survey. Optometry and vision science : official publication of the American Academy of Optometry. 2000 Jan;77(1):40-6. PubMed PMID: 10654857.

47. Nichols JJ, Mitchell GL, Nichols KK, Chalmers R, Begley C. The performance of the contact lens dry eye questionnaire as a screening survey for contact lens-related dry eye. Cornea. 2002 Jul;21(5):469-75. PubMed PMID: 12072721.

48. Fujishima H, Toda I, Shimazaki J, Tsubota K. Allergic conjunctivitis and dry eye. The British journal of ophthalmology. 1996 Nov;80(11):994-7. PubMed PMID: 8976728. Pubmed Central PMCID: 505678.

49. Kunert KS, Keane-Myers AM, Spurr-Michaud S, Tisdale AS, Gipson IK. Alteration in goblet cell numbers and mucin gene expression in a mouse model of allergic conjunctivitis. Investigative ophthalmology & visual science. 2001 Oct;42(11):2483-9. PubMed PMID: 11581187.

50. Sullivan DA. Tearful relationships? Sex, hormones, the lacrimal gland, and aqueous-deficient dry eye. The ocular surface. 2004 Apr;2(2):92-123. PubMed PMID: 17216082.

51. Nagelhout TJ, Gamache DA, Roberts L, Brady MT, Yanni JM. Preservation of tear film integrity and inhibition of corneal injury by dexamethasone in a rabbit model of lacrimal gland inflammation-induced dry eye. Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics. 2005 Apr;21(2):139-48. PubMed PMID: 15857280.

52. Rocha EM, Wickham LA, da Silveira LA, Krenzer KL, Yu FS, Toda I, et al. Identification of androgen receptor protein and 5alpha-reductase mRNA in human ocular tissues. The British journal of ophthalmology. 2000 Jan;84(1):76-84. PubMed PMID: 10611104. Pubmed Central PMCID: 1723240.

53. Azzarolo AM, Eihausen H, Schechter J. Estrogen prevention of lacrimal gland cell death and lymphocytic infiltration. Experimental eye research. 2003 Sep;77(3):347-54. PubMed PMID: 12907167.

54. Cermak JM, Krenzer KL, Sullivan RM, Dana MR, Sullivan DA. Is complete androgen insensitivity syndrome associated with alterations in the meibomian gland and ocular surface? Cornea. 2003 Aug;22(6):516-21. PubMed PMID: 12883343.

55. Perra MT, Lantini MS, Serra A, Cossu M, De Martini G, Sirigu P. Human meibomian glands: a histochemical study for androgen metabolic enzymes. Investigative ophthalmology & visual science. 1990 Apr;31(4):771-5. PubMed PMID: 2335445.

56. Steagall RJ, Yamagami H, Wickham LA, Sullivan DA. Androgen control of gene expression in the rabbit meibomian gland. Advances in experimental medicine and biology. 2002;506(Pt A):465-76. PubMed PMID: 12613947.

57. Alberti W. Acute and late side effects of radiotherapy for ocular disease: an overview. Frontiers of radiation therapy and oncology. 1997;30:281-6. PubMed PMID: 9205911.

58. Barabino S, Raghavan A, Loeffler J, Dana R. Radiotherapy-induced ocular surface disease. Cornea. 2005 Nov;24(8):909-14. PubMed PMID: 16227831.

59. Durkin SR, Roos D, Higgs B, Casson RJ, Selva D. Ophthalmic and adnexal complications of radiotherapy. Acta ophthalmologica Scandinavica. 2007 May;85(3):240-50. PubMed PMID: 17488452.

60. Gordon KB, Char DH, Sagerman RH. Late effects of radiation on the eye and ocular adnexa. International journal of radiation oncology, biology, physics. 1995 Mar 30;31(5):1123-39. PubMed PMID: 7713778.

61. LENT SOMA scales for all anatomic sites. International journal of radiation oncology, biology, physics. 1995 Mar 30;31(5):1049-91. PubMed PMID: 7713776.

62. Abelson MB, Ousler GW, 3rd, Maffei C. Dry eye in 2008. Curr Opin Ophthalmol. 2009 Jul;20(4):282-6. PubMed PMID: 19398910. Epub 2009/04/29. eng.

63. Lemp MA, Crews LA, Bron AJ, Foulks GN, Sullivan BD. Distribution of aqueous-deficient and evaporative dry eye in a clinic-based patient cohort: a retrospective study. Cornea. 2012 May;31(5):472-8. PubMed PMID: 22378109.

64. Gupta N, Prasad I, Jain R, D'Souza P. Estimating the prevalence of dry eye among Indian patients attending a tertiary ophthalmology clinic. Annals of tropical medicine and parasitology. 2010 Apr;104(3):247-55. PubMed PMID: 20507698.

65. Aristizabal S, Caldwell WL, Avila J. The relationship of time-dose fractionation factors to complications in the treatment of pituitary tumors by irradiation. International journal of radiation oncology, biology, physics. 1977 Jul-Aug;2(7-8):667-73. PubMed PMID: 197051.

66. Stephens LC, Schultheiss TE, Peters LJ, Ang KK, Gray KN. Acute radiation injury of ocular adnexa. Archives of ophthalmology. 1988 Mar;106(3):389-91. PubMed PMID: 3345155.

67. Karp LA, Streeten BW, Cogan DG. Radiation-induced atrophy of the Meibomian gland. Archives of ophthalmology. 1979 Feb;97(2):303-5. PubMed PMID: 550803.

68. Jeganathan VS, Wirth A, MacManus MP. Ocular risks from orbital and periorbital radiation therapy: a critical review. International journal of radiation oncology, biology, physics. 2011 Mar 1;79(3):650-9. PubMed PMID: 21281895.

69. Parsons JT, Bova FJ, Mendenhall WM, Million RR, Fitzgerald CR. Response of the normal eye to high dose radiotherapy. Oncology. 1996 Jun;10(6):837-47; discussion 47-8, 51-2. PubMed PMID: 8823799.

70. Jones DT, Monroy D, Ji Z, Pflugfelder SC. Alterations of ocular surface gene expression in Sjogren's syndrome. Adv Exp Med Biol. 1998;438:533-6. PubMed PMID: 9634933. Epub 1998/06/23. eng.

71. Danjo Y, Watanabe H, Tisdale AS, George M, Tsumura T, Abelson MB, et al. Alteration of mucin in human conjunctival epithelia in dry eye. Invest Ophthalmol Vis Sci. 1998 Dec;39(13):2602-9. PubMed PMID: 9856770. Epub 1998/12/18. eng.

72. Gilbard JP, Rossi SR, Heyda KG. Ophthalmic solutions, the ocular surface, and a unique therapeutic artificial tear formulation. Am J Ophthalmol. 1989 Apr 15;107(4):348-55. PubMed PMID: 2929705. Epub 1989/04/15. eng.

73. Gao J, Schwalb TA, Addeo JV, Ghosn CR, Stern ME. The role of apoptosis in the pathogenesis of canine keratoconjunctivitis sicca: the effect of topical Cyclosporin A therapy. Cornea. 1998 Nov;17(6):654-63. PubMed PMID: 9820947. Epub 1998/11/20. eng.

74. Research in dry eye: report of the Research Subcommittee of the International Dry Eye WorkShop (2007). The ocular surface. 2007 Apr;5(2):179-93. PubMed PMID: 17508121.

75. Meggs WJ. Neurogenic inflammation and sensitivity to environmental chemicals. Environ Health Perspect. 1993 Aug;101(3):234-8. PubMed PMID: 8404760. Pubmed Central PMCID: 1519776. Epub 1993/08/01. eng.

76. Mircheff AK, Gierow JP, Yang T, Zhang J, Wood RL, Azzarolo AM, et al. Sjogren's autoimmunity: how perturbation of recognition in endomembrane traffic may provoke pathological recognition at the cell surface. J Mol Recognit. 1998 Winter;11(1-6):40-8. PubMed PMID: 10076804. Epub 1999/03/17. eng.

77. Baudouin C. The pathology of dry eye. Survey of ophthalmology. 2001 Mar;45 Suppl 2:S211-20. PubMed PMID: 11587145.

78. Baudouin C. Dry eye: an unexpected inflammatory disease. Archivos de la Sociedad Espanola de Oftalmologia. 2001 Apr;76(4):205-6. PubMed PMID: 11340508.

79. Stern ME, Gao J, Schwalb TA, Ngo M, Tieu DD, Chan CC, et al. Conjunctival T-cell subpopulations in Sjogren's and non-Sjogren's patients with dry eye. Investigative ophthalmology & visual science. 2002 Aug;43(8):2609-14. PubMed PMID: 12147592.

80. Brignole F, Pisella PJ, Goldschild M, De Saint Jean M, Goguel A, Baudouin C. Flow cytometric analysis of inflammatory markers in conjunctival epithelial cells of patients with dry eyes. Investigative ophthalmology & visual science. 2000 May;41(6):1356-63. PubMed PMID: 10798650.

81. Fox PC, Speight PM. Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjogren's syndrome. Critical reviews in oral biology

and medicine : an official publication of the American Association of Oral Biologists. 1996;7(2):144-58. PubMed PMID: 8875029.

82. Tsubota K, Fujihara T, Saito K, Takeuchi T. Conjunctival epithelium expression of HLA-DR in dry eye patients. Ophthalmologica Journal international d'ophtalmologie International journal of ophthalmology Zeitschrift fur Augenheilkunde. 1999;213(1):16-9. PubMed PMID: 9838252.

83. Tsubota K, Fukagawa K, Fujihara T, Shimmura S, Saito I, Saito K, et al. Regulation of human leukocyte antigen expression in human conjunctival epithelium. Investigative ophthalmology & visual science. 1999 Jan;40(1):28-34. PubMed PMID: 9888423.

84. Choi W, Li Z, Oh HJ, Im SK, Lee SH, Park SH, et al. Expression of CCR5 and its ligands CCL3, -4, and -5 in the tear film and ocular surface of patients with dry eye disease. Current eye research. 2012 Jan;37(1):12-7. PubMed PMID: 22029739.

85. Chotikavanich S, de Paiva CS, Li de Q, Chen JJ, Bian F, Farley WJ, et al. Production and activity of matrix metalloproteinase-9 on the ocular surface increase in dysfunctional tear syndrome. Investigative ophthalmology & visual science. 2009 Jul;50(7):3203-9. PubMed PMID: 19255163. Pubmed Central PMCID: 3594995.

86. Wei Y, Epstein SP, Fukuoka S, Birmingham NP, Li XM, Asbell PA. sPLA2-IIa amplifies ocular surface inflammation in the experimental dry eye (DE) BALB/c mouse model. Investigative ophthalmology & visual science. 2011 Jun;52(7):4780-8. PubMed PMID: 21519031. Pubmed Central PMCID: 3175946.

87. McCartney-Francis NL, Mizel DE, Frazier-Jessen M, Kulkarni AB, McCarthy JB, Wahl SM. Lacrimal gland inflammation is responsible for ocular pathology in TGF-beta 1 null mice. The American journal of pathology. 1997 Nov;151(5):1281-8. PubMed PMID: 9358754. Pubmed Central PMCID: 1858095.

88. Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, et al. Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. The Journal of cell biology. 2007 Mar 12;176(6):787-93. PubMed PMID: 17353357. Pubmed Central PMCID: 2064053.

89. Argueso P, Balaram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. Investigative ophthalmology & visual science. 2002 Apr;43(4):1004-11. PubMed PMID: 11923240.

90. Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop (2007). The ocular surface. 2007 Apr;5(2):163-78. PubMed PMID: 17508120.

91. Daar AS, Greenwood HL. A proposed definition of regenerative medicine. Journal of tissue engineering and regenerative medicine. 2007 May-Jun;1(3):179-84. PubMed PMID: 18038409.

92. Lajtha LG. Stem cells and their properties. Proceedings Canadian Cancer Conference. 1967;7:31-9. PubMed PMID: 4860286.

93. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76. PubMed PMID: 16904174.

94. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov 30;131(5):861-72. PubMed PMID: 18035408.

95. Shi Y, Desponts C, Do JT, Hahm HS, Scholer HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell stem cell. 2008 Nov 6;3(5):568-74. PubMed PMID: 18983970.

96. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, et al. Generation of induced pluripotent stem cells using recombinant proteins. Cell stem cell. 2009 May 8;4(5):381-4. PubMed PMID: 19398399.

97. Kriks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature. 2011 Dec 22;480(7378):547-51. PubMed PMID: 22056989. Pubmed Central PMCID: 3245796.

98. Takayama N, Nishimura S, Nakamura S, Shimizu T, Ohnishi R, Endo H, et al. Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. The Journal of experimental medicine. 2010 Dec 20;207(13):2817-30. PubMed PMID: 21098095. Pubmed Central PMCID: 3005234.

99. Nori S, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, et al. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proceedings of the National Academy of Sciences of the United States of America. 2011 Oct 4;108(40):16825-30. PubMed PMID: 21949375. Pubmed Central PMCID: 3189018.

100. Okamoto S, Takahashi M. Induction of retinal pigment epithelial cells from monkey iPS cells. Investigative ophthalmology & visual science. 2011;52(12):8785-90. PubMed PMID: 21896853.

101. Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet. 2006 Apr 15;367(9518):1241-6. PubMed PMID: 16631879.

102. Ross MHaP, W. Histology: A Text and Atlas with Correlated Cell and Molecular Biology. 6 ed: Lippincott Williams & Wilkins; 2010 October 2010.

103. Okano W, Nomoto Y, Wada I, Kobayashi K, Miyake M, Nakamura T, et al. Bioengineered trachea with fibroblasts in a rabbit model. The Annals of otology, rhinology, and laryngology. 2009 Nov;118(11):796-804. PubMed PMID: 19999366.

104. Beall AC, Jr., Harrington OB, Greenberg SD, Morris GC, Jr., Usher FC. Tracheal replacement with heavy Marlex mesh. Circumferential replacement of the cervical trachea. Archives of surgery. 1962 Apr;84:390-6. PubMed PMID: 13866123.

105. Bucher RM, Burnett WE, Rosemond GP. Experimental reconstruction of tracheal and bronchial defects with stainless steel wire mesh. The Journal of thoracic surgery. 1951 Jun;21(6):572-83. PubMed PMID: 14841793.

106. Neville WE, Bolanowski JP, Kotia GG. Clinical experience with the silicone tracheal prosthesis. The Journal of thoracic and cardiovascular surgery. 1990 Apr;99(4):604-12; discussion 12-3. PubMed PMID: 2319780.

107. Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, et al. Clinical transplantation of a tissue-engineered airway. Lancet. 2008 Dec 13;372(9655):2023-30. PubMed PMID: 19022496.

108. Omori K, Nakamura T, Kanemaru S, Asato R, Yamashita M, Tanaka S, et al. Regenerative medicine of the trachea: the first human case. The Annals of otology, rhinology, and laryngology. 2005 Jun;114(6):429-33. PubMed PMID: 16042099.

109. Kobayashi K, Nomoto Y, Suzuki T, Tada Y, Miyake M, Hazama A, et al. Effect of fibroblasts on tracheal epithelial regeneration in vitro. Tissue engineering. 2006 Sep;12(9):2619-28. PubMed PMID: 16995795.

110. Nomoto Y, Kobayashi K, Tada Y, Wada I, Nakamura T, Omori K. Effect of fibroblasts on epithelial regeneration on the surface of a bioengineered trachea. The Annals of otology, rhinology, and laryngology. 2008 Jan;117(1):59-64. PubMed PMID: 18254373.

111. Jungebluth P, Alici E, Baiguera S, Le Blanc K, Blomberg P, Bozoky B, et al. Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a

proof-of-concept study. Lancet. 2011 Dec 10;378(9808):1997-2004. PubMed PMID: 22119609.

112. Denny PC, Denny PA. Dynamics of parenchymal cell division, differentiation, and apoptosis in the young adult female mouse submandibular gland. The Anatomical record. 1999 Mar;254(3):408-17. PubMed PMID: 10096673.

113. Lombaert IM, Brunsting JF, Wierenga PK, Kampinga HH, de Haan G, Coppes RP. Cytokine treatment improves parenchymal and vascular damage of salivary glands after irradiation. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008 Dec 1;14(23):7741-50. PubMed PMID: 19047101.

114. Ohlsson B, Jansen C, Ihse I, Axelson J. Epidermal growth factor induces cell proliferation in mouse pancreas and salivary glands. Pancreas. 1997 Jan;14(1):94-8. PubMed PMID: 8981513.

115. Limesand KH, Said S, Anderson SM. Suppression of radiation-induced salivary gland dysfunction by IGF-1. PloS one. 2009;4(3):e4663. PubMed PMID: 19252741. Pubmed Central PMCID: 2646143.

116. Thula TT, Schultz G, Tran-Son-Tay R, Batich C. Effects of EGF and bFGF on irradiated parotid glands. Annals of biomedical engineering. 2005 May;33(5):685-95. PubMed PMID: 15981868.

117. Lombaert IM, Brunsting JF, Wierenga PK, Kampinga HH, de Haan G, Coppes RP. Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool. Stem cells. 2008 Oct;26(10):2595-601. PubMed PMID: 18669914.

118. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PloS one. 2008;3(4):e2063. PubMed PMID: 18446241. Pubmed Central PMCID: 2329592.

119. Feng J, van der Zwaag M, Stokman MA, van Os R, Coppes RP. Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2009 Sep;92(3):466-71. PubMed PMID: 19625095.

120. Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. EMBO J. 1994 Jul 15;13(14):3296-301. PubMed PMID: 8045260. Pubmed Central PMCID: 395226. Epub 1994/07/15. eng.

121. Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL. Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development. 1997 Jan;124(1):53-63. PubMed PMID: 9006067. Epub 1997/01/01. eng.

122. Pepicelli CV, Lewis PM, McMahon AP. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol. 1998 Sep 24;8(19):1083-6. PubMed PMID: 9768363. Epub 1998/10/13. eng.

123. Graff JM. Embryonic patterning: to BMP or not to BMP, that is the question. Cell. 1997 Apr 18;89(2):171-4. PubMed PMID: 9108472. Epub 1997/04/18. eng.

124. Mason IJ, Fuller-Pace F, Smith R, Dickson C. FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions. Mech Dev. 1994 Jan;45(1):15-30. PubMed PMID: 8186145. Epub 1994/01/01. eng.

125. Makarenkova HP, Ito M, Govindarajan V, Faber SC, Sun L, McMahon G, et al. FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development. Development. 2000 Jun;127(12):2563-72. PubMed PMID: 10821755. Epub 2000/05/24. eng.

126. Callaerts P, Halder G, Gehring WJ. PAX-6 in development and evolution. Annu Rev Neurosci. 1997;20:483-532. PubMed PMID: 9056723. Epub 1997/01/01. eng.

127. Grindley JC, Davidson DR, Hill RE. The role of Pax-6 in eye and nasal development. Development. 1995 May;121(5):1433-42. PubMed PMID: 7789273. Epub 1995/05/01. eng.

128. Lovicu FJ, Kao WW, Overbeek PA. Ectopic gland induction by lens-specific expression of keratinocyte growth factor (FGF-7) in transgenic mice. Mech Dev. 1999 Oct;88(1):43-53. PubMed PMID: 10525187. Epub 1999/10/19. eng.

129. Orr-Urtreger A, Bedford MT, Burakova T, Arman E, Zimmer Y, Yayon A, et al. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol. 1993 Aug;158(2):475-86. PubMed PMID: 8393815. Epub 1993/08/01. eng.

130. Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science. 1989 Aug 18;245(4919):752-5. PubMed PMID: 2475908. Epub 1989/08/18. eng.

131. Mohammadi M, Froum S, Hamby JM, Schroeder MC, Panek RL, Lu GH, et al. Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. EMBO J. 1998 Oct 15;17(20):5896-904. PubMed PMID: 9774334. Pubmed Central PMCID: 1170917. Epub 1998/10/17. eng.

132. Miralles F, Czernichow P, Ozaki K, Itoh N, Scharfmann R. Signaling through fibroblast growth factor receptor 2b plays a key role in the development of the exocrine pancreas. Proc Natl Acad Sci U S A. 1999 May 25;96(11):6267-72. PubMed PMID: 10339576. Pubmed Central PMCID: 26870. Epub 1999/05/26. eng.

133. Dean C, Ito M, Makarenkova HP, Faber SC, Lang RA. Bmp7 regulates branching morphogenesis of the lacrimal gland by promoting mesenchymal proliferation and condensation. Development. 2004 Sep;131(17):4155-65. PubMed PMID: 15280212. Epub 2004/07/29. eng.

134. Hogan BL. Morphogenesis. Cell. 1999 Jan 22;96(2):225-33. PubMed PMID: 9988217. Epub 1999/02/13. eng.

135. Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development. 1996 Jun;122(6):1693-702. PubMed PMID: 8674409. Epub 1996/06/01. eng.

136. Raatikainen-Ahokas A, Hytonen M, Tenhunen A, Sainio K, Sariola H. BMP-4 affects the differentiation of metanephric mesenchyme and reveals an early anterior-posterior axis of the embryonic kidney. Dev Dyn. 2000 Feb;217(2):146-58. PubMed PMID: 10706139. Epub 2000/03/08. eng.

137. Lamm ML, Podlasek CA, Barnett DH, Lee J, Clemens JQ, Hebner CM, et al. Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate. Dev Biol. 2001 Apr 15;232(2):301-14. PubMed PMID: 11401393. Epub 2001/06/13. eng.

138. Godin RE, Takaesu NT, Robertson EJ, Dudley AT. Regulation of BMP7 expression during kidney development. Development. 1998 Sep;125(17):3473-82. PubMed PMID: 9693150. Epub 1998/08/07. eng.

139. Gurdon JB. A community effect in animal development. Nature. 1988 Dec 22-29;336(6201):772-4. PubMed PMID: 3205305. Epub 1988/12/22. eng.

140. Blanpain C, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. Cell. 2007 Feb 9;128(3):445-58. PubMed PMID: 17289566. Pubmed Central PMCID: 2408375.

141. Dor Y, Melton DA. How important are adult stem cells for tissue maintenance? Cell cycle. 2004 Sep;3(9):1104-6. PubMed PMID: 15326371.

142. Kume S. Stem-cell-based approaches for regenerative medicine. Development, growth & differentiation. 2005 Aug;47(6):393-402. PubMed PMID: 16109037.

143. Alison MR, Golding M, Sarraf CE. Liver stem cells: when the going gets tough they get going. International journal of experimental pathology. 1997 Dec;78(6):365-81. PubMed PMID: 9516869. Pubmed Central PMCID: 2694549.

144. Bjerknes M, Cheng H. Multipotential stem cells in adult mouse gastric epithelium. American journal of physiology Gastrointestinal and liver physiology. 2002 Sep;283(3):G767-77. PubMed PMID: 12181193.

145. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 2004 May 6;429(6987):41-6. PubMed PMID: 15129273.

146. Hisatomi Y, Okumura K, Nakamura K, Matsumoto S, Satoh A, Nagano K, et al. Flow cytometric isolation of endodermal progenitors from mouse salivary gland differentiate into hepatic and pancreatic lineages. Hepatology. 2004 Mar;39(3):667-75. PubMed PMID: 14999685.

147. Okumura K, Nakamura K, Hisatomi Y, Nagano K, Tanaka Y, Terada K, et al. Salivary gland progenitor cells induced by duct ligation differentiate into hepatic and pancreatic lineages. Hepatology. 2003 Jul;38(1):104-13. PubMed PMID: 12829992.

148. Kishi T, Takao T, Fujita K, Taniguchi H. Clonal proliferation of multipotent stem/progenitor cells in the neonatal and adult salivary glands. Biochemical and biophysical research communications. 2006 Feb 10;340(2):544-52. PubMed PMID: 16376857.

149. Takahashi S, Domon T, Yamamoto T, Wakita M. Regeneration of myoepithelial cells in rat submandibular glands after yttrium aluminium garnett laser irradiation. International journal of experimental pathology. 1997 Apr;78(2):91-9. PubMed PMID: 9203983. Pubmed Central PMCID: 2694527.

150. Takahashi S, Shinzato K, Domon T, Yamamoto T, Wakita M. Mitotic proliferation of myoepithelial cells during regeneration of atrophied rat submandibular glands after duct ligation. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2004 Aug;33(7):430-4. PubMed PMID: 15250836.

151. You S, Kublin CL, Avidan O, Miyasaki D, Zoukhri D. Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Investigative ophthalmology & visual science. 2011 Apr;52(5):2087-94. PubMed PMID: 21178145. Pubmed Central PMCID: 3080180.

152. Shatos MA, Haugaard-Kedstrom L, Hodges RR, Dartt DA. Isolation and characterization of progenitor cells in uninjured, adult rat lacrimal gland. Investigative

ophthalmology & visual science. 2012 May;53(6):2749-59. PubMed PMID: 22427571. Pubmed Central PMCID: 3367467.

153. Oliver C, Waters JF, Tolbert CL, Kleinman HK. Growth of exocrine acinar cells on a reconstituted basement membrane gel. In vitro cellular & developmental biology : journal of the Tissue Culture Association. 1987 Jul;23(7):465-73. PubMed PMID: 3610944.

154. Meneray MA, Fields TY, Bromberg BB, Moses RL. Morphology and physiologic responsiveness of cultured rabbit lacrimal acini. Investigative ophthalmology & visual science. 1994 Nov;35(12):4144-58. PubMed PMID: 7960597.

155. Rismondo V, Gierow JP, Lambert RW, Golchini K, Feldon SE, Mircheff AK. Rabbit lacrimal acinar cells in primary culture: morphology and acute responses to cholinergic stimulation. Investigative ophthalmology & visual science. 1994 Mar;35(3):1176-83. PubMed PMID: 8125729.

156. Hann LE, Kelleher RS, Sullivan DA. Influence of culture conditions on the androgen control of secretory component production by acinar cells from the rat lacrimal gland. Investigative ophthalmology & visual science. 1991 Aug;32(9):2610-21. PubMed PMID: 1869413.

157. Guo Z, Azzarolo AM, Schechter JE, Warren DW, Wood RL, Mircheff AK, et al. Lacrimal gland epithelial cells stimulate proliferation in autologous lymphocyte preparations. Experimental eye research. 2000 Jul;71(1):11-22. PubMed PMID: 10880272.

158. da Costa SR, Yarber FA, Zhang L, Sonee M, Hamm-Alvarez SF. Microtubules facilitate the stimulated secretion of beta-hexosaminidase in lacrimal acinar cells. J Cell Sci. 1998 May;111 (Pt 9):1267-76. PubMed PMID: 9547304. Epub 1998/06/04. eng.

159. Schonthal AH, Warren DW, Stevenson D, Schecter JE, Azzarolo AM, Mircheff AK, et al. Proliferation of lacrimal gland acinar cells in primary culture. Stimulation by extracellular matrix, EGF, and DHT. Experimental eye research. 2000 May;70(5):639-49. PubMed PMID: 10870522.

160. Long L, Liu Z, Wang T, Deng X, Yang K, Li L, et al. Polyethersulfone dead-end tube as a scaffold for artificial lacrimal glands in vitro. Journal of biomedical materials research Part B, Applied biomaterials. 2006 Aug;78(2):409-16. PubMed PMID: 16544306.

161. Schrader S, Wedel T, Kremling C, Laqua H, Geerling G. Amniotic membrane as a carrier for lacrimal gland acinar cells. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie. 2007 Nov;245(11):1699-704. PubMed PMID: 17562065.

162. Schrader S, Kremling C, Klinger M, Laqua H, Geerling G. Cultivation of lacrimal gland acinar cells in a microgravity environment. The British journal of ophthalmology. 2009 Aug;93(8):1121-5. PubMed PMID: 19416938.

163. Sullivan DA, Bloch KJ, Allansmith MR. Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. Immunology. 1984 Jun;52(2):239-46. PubMed PMID: 6735436. Pubmed Central PMCID: 1454608. Epub 1984/06/01. eng.

164. Sullivan DA, Kelleher RS, Vaerman JP, Hann LE. Androgen regulation of secretory component synthesis by lacrimal gland acinar cells in vitro. Journal of immunology. 1990 Dec 15;145(12):4238-44. PubMed PMID: 2258616.

165. Kelleher RS, Hann LE, Edwards JA, Sullivan DA. Endocrine, neural, and immune control of secretory component output by lacrimal gland acinar cells. J Immunol. 1991 May 15;146(10):3405-12. PubMed PMID: 1709194. Epub 1991/05/15. eng.

166. Azzarolo AM, Mircheff AK, Kaswan RL, Stanczyk FZ, Gentschein E, Becker L, et al. Androgen support of lacrimal gland function. Endocrine. 1997 Feb;6(1):39-45. PubMed PMID: 9225114.

167. Yoshino K. Establishment of a human lacrimal gland epithelial culture system with in vivo mimicry and its substrate modulation. Cornea. 2000 May;19(3 Suppl):S26-36. PubMed PMID: 10832719.

168. Shatos MA, Haugaard-Kedstrom L, Hodges RR, Dartt DA. Isolation and Characterization of Progenitor Cells in Uninjured, Adult Rat Lacrimal Gland. Invest Ophthalmol Vis Sci. 2012 Mar 16. PubMed PMID: 22427571. Epub 2012/03/20. Eng.

169. Gilbard JP, Rossi SR, Heyda KG. Tear film and ocular surface changes after closure of the meibomian gland orifices in the rabbit. Ophthalmology. 1989 Aug;96(8):1180-6. PubMed PMID: 2797721. Epub 1989/08/01. eng.

170. Stewart P, Chen Z, Farley W, Olmos L, Pflugfelder SC. Effect of experimental dry eye on tear sodium concentration in the mouse. Eye & contact lens. 2005 Jul;31(4):175-8. PubMed PMID: 16021005.

171. Jabs DA, Enger C, Prendergast RA. Murine models of Sjogren's syndrome. Evolution of the lacrimal gland inflammatory lesions. Investigative ophthalmology & visual science. 1991 Feb;32(2):371-80. PubMed PMID: 1993589.

172. Jabs DA, Prendergast RA, Rorer EM, Hudson AP, Whittum-Hudson JA. Cytokines in autoimmune lacrimal gland disease in MRL/MpJ mice. Investigative ophthalmology & visual science. 2001 Oct;42(11):2567-71. PubMed PMID: 11581200.

173. Sato EH, Ariga H, Sullivan DA. Impact of androgen therapy in Sjogren's syndrome: hormonal influence on lymphocyte populations and Ia expression in lacrimal glands of MRL/Mp-lpr/lpr mice. Investigative ophthalmology & visual science. 1992 Jul;33(8):2537-45. PubMed PMID: 1634351.

174. Song XJ, Li DQ, Farley W, Luo LH, Heuckeroth RO, Milbrandt J, et al. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Investigative ophthalmology & visual science. 2003 Oct;44(10):4223-9. PubMed PMID: 14507865.

175. Akpek EK, Jabs DA, Gerard HC, Prendergast RA, Hudson AP, Lee B, et al. Chemokines in autoimmune lacrimal gland disease in MRL/MpJ mice. Investigative ophthalmology & visual science. 2004 Jan;45(1):185-90. PubMed PMID: 14691172.

176. Yamamoto H, Sims NE, Macauley SP, Nguyen KH, Nakagawa Y, Humphreys-Beher MG. Alterations in the secretory response of non-obese diabetic (NOD) mice to muscarinic receptor stimulation. Clinical immunology and immunopathology. 1996 Mar;78(3):245-55. PubMed PMID: 8605700.

177. Zoukhri D, Kublin CL. Impaired neurotransmitter release from lacrimal and salivary gland nerves of a murine model of Sjogren's syndrome. Investigative ophthalmology & visual science. 2001 Apr;42(5):925-32. PubMed PMID: 11274068. Pubmed Central PMCID: 3241007.

178. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, et al. Identification of alpha-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science. 1997 Apr 25;276(5312):604-7. PubMed PMID: 9110981.

179. The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007). The ocular surface. 2007 Apr;5(2):93-107. PubMed PMID: 17508117.

180. Gupta N, Prasad I, Himashree G, D'Souza P. Prevalence of dry eye at high altitude: a case controlled comparative study. High altitude medicine & biology. 2008 Winter;9(4):327-34. PubMed PMID: 19115918.

181. Sahai A, Malik P. Dry eye: prevalence and attributable risk factors in a hospital-based population. Indian journal of ophthalmology. 2005 Jun;53(2):87-91. PubMed PMID: 15976462.

182. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pflugfelder SC. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. Cornea. 1998 Nov;17(6):584-9. PubMed PMID: 9820935.

183. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea. 1995 Sep;14(5):473-84. PubMed PMID: 8536460. Epub 1995/09/01. eng.

184. Williams GM, Gunn JM. Long-term cell culture of adult rat liver epithelial cells. Exp Cell Res. 1974 Nov;89(1):139-42. PubMed PMID: 4373256. Epub 1974/11/01. eng.

185. Tiwari S, Ali MJ, Balla MM, Naik MN, Honavar SG, Reddy VA, et al. Establishing human lacrimal gland cultures with secretory function. PloS one. 2012;7(1):e29458. PubMed PMID: 22253725. Pubmed Central PMCID: 3258235.

186. Ueda Y, Karasawa Y, Satoh Y, Nishikawa S, Imaki J, Ito M. Purification and characterization of mouse lacrimal gland epithelial cells and reconstruction of an acinarlike structure in three-dimensional culture. Invest Ophthalmol Vis Sci. 2009 May;50(5):1978-87. PubMed PMID: 19098316. Epub 2008/12/23. eng.

187. Hann LE, Tatro JB, Sullivan DA. Morphology and function of lacrimal gland acinar cells in primary culture. Invest Ophthalmol Vis Sci. 1989 Jan;30(1):145-58. PubMed PMID: 2536359. Epub 1989/01/01. eng.

188. Schechter J, Stevenson D, Chang D, Chang N, Pidgeon M, Nakamura T, et al. Growth of purified lacrimal acinar cells in Matrigel raft cultures. Exp Eye Res. 2002 Mar;74(3):349-60. PubMed PMID: 12014916. Epub 2002/05/17. eng.

189. Chen L, Glass JD, Walton SC, Laurie GW. Role of laminin-1, collagen IV, and an autocrine factor(s) in regulated secretion by lacrimal acinar cells. Am J Physiol. 1998 Jul;275(1 Pt 1):C278-84. PubMed PMID: 9688859. Epub 1998/08/05. eng.

190. Yoshino K, Tseng SC, Pflugfelder SC. Substrate modulation of morphology, growth, and tear protein production by cultured human lacrimal gland epithelial cells. Exp Cell Res. 1995 Sep;220(1):138-51. PubMed PMID: 7664830. Epub 1995/09/01. eng.

191. Buck FF, Bier M, Nord FF. Some properties of human trypsin. Archives of biochemistry and biophysics. 1962 Sep;98:528-30. PubMed PMID: 13874296.

192. Bond MD, Van Wart HE. Characterization of the individual collagenases from Clostridium histolyticum. Biochemistry. 1984 Jun 19;23(13):3085-91. PubMed PMID: 6087888.

193. Shi X, Gipp J, Bushman W. Anchorage-independent culture maintains prostate stem cells. Developmental biology. 2007 Dec 1;312(1):396-406. PubMed PMID: 17976567. Pubmed Central PMCID: 2234352.

194. Woodward WA, Chen MS, Behbod F, Rosen JM. On mammary stem cells. Journal of cell science. 2005 Aug 15;118(Pt 16):3585-94. PubMed PMID: 16105882.

195. Nanduri LS, Lombaert IM, van der Zwaag M, Faber H, Brunsting JF, van Os RP, et al. Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2013 Sep;108(3):458-63. PubMed PMID: 23769181.

196. Sun TT, Eichner R, Nelson WG, Tseng SC, Weiss RA, Jarvinen M, et al. Keratin classes: molecular markers for different types of epithelial differentiation. The Journal of investigative dermatology. 1983 Jul;81(1 Suppl):109s-15s. PubMed PMID: 6190956.

197. Kivela T. Antigenic profile of the human lacrimal gland. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 1992 May;40(5):629-42. PubMed PMID: 1374091.

198. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. Annual review of biochemistry. 1990;59:237-52. PubMed PMID: 2197976.

199. Agre P. The aquaporin water channels. Proceedings of the American Thoracic Society. 2006;3(1):5-13. PubMed PMID: 16493146. Pubmed Central PMCID: 2658677.

200. Raubenheimer EJ. The myoepithelial cell: embryology, function, and proliferative aspects. Critical reviews in clinical laboratory sciences. 1987;25(2):161-93. PubMed PMID: 3301214.

201. Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochemical and biophysical research communications. 2004 Oct 1;322(4):1111-22. PubMed PMID: 15336958.

202. Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Developmental dynamics : an official publication of the American Association of Anatomists. 2005 Jul;233(3):706-20. PubMed PMID: 15937929.

203. Brown M, Wittwer C. Flow cytometry: principles and clinical applications in hematology. Clinical chemistry. 2000 Aug;46(8 Pt 2):1221-9. PubMed PMID: 10926916.

204. Rieseberg M, Kasper C, Reardon KF, Scheper T. Flow cytometry in biotechnology. Applied microbiology and biotechnology. 2001 Aug;56(3-4):350-60. PubMed PMID: 11549001.

205. Ide H, Subbarao V, Reddy JK, Rao MS. Formation of ductular structures in vitro by rat pancreatic epithelial oval cells. Experimental cell research. 1993 Nov;209(1):38-44. PubMed PMID: 8224004.

206. Wozniak MA, Keely PJ. Use of three-dimensional collagen gels to study mechanotransduction in T47D breast epithelial cells. Biological procedures online. 2005;7:144-61. PubMed PMID: 16299584. Pubmed Central PMCID: 1285185.

207. Till JE, Mc CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961 Feb;14:213-22. PubMed PMID: 13776896. Epub 1961/02/01. eng.

208. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development. 1990 Dec;110(4):1001-20. PubMed PMID: 2100251. Epub 1990/12/01. eng.

209. Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature. 1999 Sep 23;401(6751):390-4. PubMed PMID: 10517639.

210. Hamamoto Y, Tsuura Y, Fujimoto S, Nagata M, Takeda T, Mukai E, et al. Recovery of function and mass of endogenous beta-cells in streptozotocin-induced diabetic rats treated

with islet transplantation. Biochemical and biophysical research communications. 2001 Sep 14;287(1):104-9. PubMed PMID: 11549260.

211. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. The Journal of clinical investigation. 2001 Jun;107(11):1395-402. PubMed PMID: 11390421. Pubmed Central PMCID: 209322.

212. Shen ZL, Lassner F, Becker M, Walter GF, Bader A, Berger A. Viability of cultured nerve grafts: An assessment of proliferation of Schwann cells and fibroblasts. Microsurgery. 1999;19(8):356-63. PubMed PMID: 10594909.

213. Stemple DL, Anderson DJ. Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell. 1992 Dec 11;71(6):973-85. PubMed PMID: 1458542.

214. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes & development. 2003 May 15;17(10):1253-70. PubMed PMID: 12756227. Pubmed Central PMCID: 196056.

215. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature. 2006 Feb 23;439(7079):993-7. PubMed PMID: 16395311.

216. Bickenbach JR. Identification and behavior of label-retaining cells in oral mucosa and skin. Journal of dental research. 1981 Aug;60 Spec No C:1611-20. PubMed PMID: 6943171.

217. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007 Oct 25;449(7165):1003-7. PubMed PMID: 17934449.

218. Reddy GP, Tiarks CY, Pang L, Wuu J, Hsieh CC, Quesenberry PJ. Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells. Blood. 1997 Sep 15;90(6):2293-9. PubMed PMID: 9310480.

219. Chakkalakal JV, Jones KM, Basson MA, Brack AS. The aged niche disrupts muscle stem cell quiescence. Nature. 2012 Oct 18;490(7420):355-60. PubMed PMID: 23023126. Pubmed Central PMCID: 3605795.

220. Shea KL, Xiang W, LaPorta VS, Licht JD, Keller C, Basson MA, et al. Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during

regeneration. Cell stem cell. 2010 Feb 5;6(2):117-29. PubMed PMID: 20144785. Pubmed Central PMCID: 2846417.

221. Potten CS, Hume WJ, Reid P, Cairns J. The segregation of DNA in epithelial stem cells. Cell. 1978 Nov;15(3):899-906. PubMed PMID: 728994.

222. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, et al. Defining the epithelial stem cell niche in skin. Science. 2004 Jan 16;303(5656):359-63. PubMed PMID: 14671312. Pubmed Central PMCID: 2405920.

223. Oki T, Nishimura K, Kitaura J, Togami K, Maehara A, Izawa K, et al. A novel cell-cycle-indicator, mVenus-p27K-, identifies quiescent cells and visualizes G0-G1 transition. Scientific reports. 2014;4:4012. PubMed PMID: 24500246. Pubmed Central PMCID: 3915272.

224. Di Como CJ, Urist MJ, Babayan I, Drobnjak M, Hedvat CV, Teruya-Feldstein J, et al. p63 expression profiles in human normal and tumor tissues. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002 Feb;8(2):494-501. PubMed PMID: 11839669.

225. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, et al. p63 identifies keratinocyte stem cells. Proceedings of the National Academy of Sciences of

the United States of America. 2001 Mar 13;98(6):3156-61. PubMed PMID: 11248048. Pubmed Central PMCID: 30623.

226. Irollo E, Pirozzi G. CD133: to be or not to be, is this the real question? American journal of translational research. 2013;5(6):563-81. PubMed PMID: 24093054. Pubmed Central PMCID: 3786264.

227. Peh GS, Lang RJ, Pera MF, Hawes SM. CD133 expression by neural progenitors derived from human embryonic stem cells and its use for their prospective isolation. Stem cells and development. 2009 Mar;18(2):269-82. PubMed PMID: 18651819.

228. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nature protocols. 2006;1(5):2315-9. PubMed PMID: 17406473.

229. Puck TT, Marcus PI. A Rapid Method for Viable Cell Titration and Clone Production with Hela Cells in Tissue Culture: The Use of X-Irradiated Cells to Supply Conditioning Factors. Proceedings of the National Academy of Sciences of the United States of America. 1955 Jul 15;41(7):432-7. PubMed PMID: 16589695. Pubmed Central PMCID: 528114.

230. Metcalfe C, Kljavin NM, Ybarra R, de Sauvage FJ. Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. Cell stem cell. 2014 Feb 6;14(2):149-59. PubMed PMID: 24332836.

231. Leong KG, Wang BE, Johnson L, Gao WQ. Generation of a prostate from a single adult stem cell. Nature. 2008 Dec 11;456(7223):804-8. PubMed PMID: 18946470.

232. You S, Tariq A, Kublin CL, Zoukhri D. Detection of BrdU-label retaining cells in the lacrimal gland: implications for tissue repair. Cell and tissue research. 2011 Dec;346(3):317-26. PubMed PMID: 22101331. Pubmed Central PMCID: 3245820.

233. Fatima S, Zhou S, Sorrentino BP. Abcg2 expression marks tissue-specific stem cells in multiple organs in a mouse progeny tracking model. Stem cells. 2012 Feb;30(2):210-21. PubMed PMID: 22134889.

234. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood. 2002 Jan 15;99(2):507-12. PubMed PMID: 11781231.

235. Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, Sorrentino BP. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2002 Sep 17;99(19):12339-44. PubMed PMID: 12218177. Pubmed Central PMCID: 129446.

236. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature medicine. 2001 Sep;7(9):1028-34. PubMed PMID: 11533706.

237. Kondi-Pafiti A, Arkadopoulos N, Gennatas C, Michalaki V, Frangou-Plegmenou M, Chatzipantelis P. Expression of c-kit in common benign and malignant breast lesions. Tumori. 2010 Nov-Dec;96(6):978-84. PubMed PMID: 21388062.

238. Eidus LK, Korystov Yu N, Dobrovinskaja OR, Shaposhnikova VV. The mechanism of radiation-induced interphase death of lymphoid cells: a new hypothesis. Radiation research. 1990 Jul;123(1):17-21. PubMed PMID: 2371378.

239. Finger PT. Radiation therapy for orbital tumors: concepts, current use, and ophthalmic radiation side effects. Survey of ophthalmology. 2009 Sep-Oct;54(5):545-68. PubMed PMID: 19682622.

240. Ozkurt H, Ozkurt YB, Basak M. Is dry eye syndrome a work-related disease among radiologists? Diagnostic and interventional radiology. 2006 Dec;12(4):163-5. PubMed PMID: 17160796.

241. Gregoire V, De Neve W, Eisbruch A, Lee N, Van den Weyngaert D, Van Gestel D. Intensity-modulated radiation therapy for head and neck carcinoma. The oncologist. 2007 May;12(5):555-64. PubMed PMID: 17522243.

242. Goyal S, Cohler A, Camporeale J, Narra V, Yue NJ. Intensity-modulated radiation therapy for orbital lymphoma. Radiation medicine. 2008 Dec;26(10):573-81. PubMed PMID: 19132487.

243. Bhandare N, Moiseenko V, Song WY, Morris CG, Bhatti MT, Mendenhall WM. Severe dry eye syndrome after radiotherapy for head-and-neck tumors. International journal of radiation oncology, biology, physics. 2012 Mar 15;82(4):1501-8. PubMed PMID: 21764525.

Appendix

(1) Phosphate Buffered Saline

PBS (1X) was used for washing and other purposes. The following chemicals were added to distilled water. After dissolving the chemicals completely, the pH was set to 7.2 using 0.1M NaOH or 1N HCl with pH meter and the final volume made to 100ml with distilled water.

- NaCl 0.8g
- KCl 0.02g
- KH_2PO_4 0.012g
- Na_2HPO_4 0.091g

(2) Trypsin Enzyme

Trypsin was prepared by reconstituting the lyophilized powder of trypsin in PBS, to make 0.25% trypsin solution. To this 0.1mM EDTA solution was added and filter sterilized and stored at 4^oC.

(3) Reconstitution of Growth Factors

During reconstitution, all growth factors were carefully handled at 4^oC in ice as they are heat sensitive and maintaining utmost sterility to avoid microbial contamination. The growth factors were reconstituted in sterile DMEM media as per the manufacturer's instructions for the required concentration. The growth factors are mixed gently, but thoroughly so as to make sure the entire lyophilized powder is dissolved in the media. The reconstituted growth factors are then aliquoted into 0.2ml eppendorf tubes to an appropriate volume so as to achieve the

final working concentration when mixed to 50ml of media. All throughout the procedure the reconstituted growth factors are maintained on ice. After aliquoting, the 0.5ml tubes are arranged in laxbro boxes, labeled appropriately and stored at -80^{o}C until further usage.

(4) Fetal Bovine Serum (FBS)

FBS was obtained from Sigma Aldrich. After filter sterilization aliquots of 50ml were made and stored at ‑80^{o}C.

(5) Preparation of Dulbecco's Modified Eagle's Medium (DMEM)

S.No	Ingredients	Quantity	Company
1	DMEM	13.4 gms	Sigma
2	Gentamycin	100µl(4 mg/L)	
3	Penicillin	150mg	
4	Streptomycin	100mg	
5	Amphotericin	2.50mg	
6	Sodium bicarbonate	3.7 g	Fluka

Method of Preparation: Add the first two ingredients in 500ml of Milli Q water, in asterile 1000ml flask/beaker, add sodium bicarbonate, dissolve and add the remaining ingredients. Make up the final volume to 1000ml. Ensure that the pH is 7.2 ‑ 7.5. Sterilize the medium with vacuum filter using Millipore filter membrane (0.22µ). After sterilization add a few drops of the medium to chocolate agar plates for sterility check.

(6) Washing buffer solution

The buffer consists of 2% (w/v) fetal calf serum (Sigma, USA) and 0.1% (w/v) sodium azide (Sigma, USA) in PBS. The buffer was filter sterilized and stored at 4^{o}C.

(7) 4% Paraformaldehyde Solution

For cytoplasmic antigen staining, fixation of cells is required which is carried out using 4% paraformaldehyde solution. 4gms of paraformaldehyde powder (Sigma) was added to 100ml of 1X PBS and heated to 700 in a fume hood until the paraformaldehyde dissolved in the solution. The solution was allowed to cool to room temperature (RT) i.e., 26^{o}C. The pH was adjusted to 7.4 using 0.1M NaOH or 1N HCl. This solution was filter sterilized and stored at 4^{0}C protected from light until further use.

(8)Antibody dilutions

Stock solutions were diluted to 1:10 with PBS ‑ Sodium azide buffer (working standard) and stored at 4^{o}C. From working standard, required dilution of antibody was prepared using PBS ‑ BSA

(9) Deparaffinization protocol

Slides to be deparaffinized were placed in a hot air oven set at 110^{o}C for 15-20 minutes The slides were then washed with 100% xylene for 5minutes to dissolve the melted wax. The slides were then washed in gradients of alcohol from 80%- 100% and then washed under running water and air dried until further use.

(10) RNA Isolation

Homogenization: The cells were lysed directly in a culture dish by adding 1 ml of TRIZOL LS Reagent.

Phase separation: Samples were incubated after gently homogenizing samples for 5 minutes at 15 to 30° C to permit the complete dissociation of nucleoprotein complexes by using a 1ml pipette. 200µl of chloroform was added to the 1.5ml eppendorf tube. Tubes were vigorously shaken by hand for 15 seconds and incubated them at 15 to 30° C for 15 minutes. Samples were centrifuged at 12,000 × *g* for 15 minutes at 2 to 8° C. After centrifugation, the aqueous phase with RNA is collected.

RNA precipitation: The aqueous phase is then transferred to a clean tube, and RNA is precipitated from the aqueous phase by mixing with 500 µl of isopropyl alcohol. Samples were incubated 15 to 30° C for 10 minutes and centrifuged 12,000 × *g* for 10 minutes at 2 to 8° C. RNA precipitate, after centrifugation, forms a gel-like pellet on the side and bottom of the tube.

RNA wash: The supernatant is removed. RNA pellet was washed once with 1ml of 75% ethanol, and centrifuged at 7,500 × *g* for 5 minutes at 2 to 8° C.

Re-dissolving the RNA: RNA pellet was air dried for 5-10 minutes. The pellet is then stored at -20 ^{0}C until further use.

(11) Tris- Acetate EDTA (TAE Buffer)

TAE (50X) was used as running buffer for agarose gel analysis for RT-PCR work. The following chemicals were added to distilled water. After dissolving the chemicals thoroughly the final volume made to 1000ml with distilled water.

- Tris Base 242 g
- Glacial Acetic Acid 57.1ml
- 0.5M EDTA (pH 8.0) 100ml

List of Publications and Presentations

Publications:

In Print:

1. **S Tiwari**, MJ Ali, MM Balla, MN Naik, SG Honavar, VAP Reddy, GK Vemuganti. Establishing human lacrimal gland cultures with secretory function (2012). PloS one 7 (1), e29458
2. **S Tiwari**, GK Vemuganti. Lacrimal Gland Regeneration: Progress and Promise (2013). Regenerative Medicine, 775-791
3. **S Tiwari**, MJ Ali, GK Vemuganti. Human lacrimal gland regeneration: Perspectives and review of literature. Saudi Journal of Ophthalmology 2014 Jan;28(1):12-8.

Under Preparation

1. **S Tiwari**, RM Nair, MJ Ali ,MN Naik, SG Honavar, VA P Reddy, GK Vemuganti. Stem-like cells in serum free i*n-vitro* cultures of human lacrimal gland. (Manuscript under preparation)
2. **S Tiwari**, Anusha A, Jayalakshmi N, MJ Ali, MN Naik, VA P Reddy, GK Vemuganti. Effect of Orbital Radiotherapy on the Development Dry Eye Syndrome. (Manuscript under preparation)

Presentations:

1. **Shubha Tiwari**, Md Javed Ali, M M Sagar Balla, Milind N Naik, Santosh G Honavar,Vijay Anand, P Reddy, Geeta K Vemuganti (2013). Stem-like Cells in the Serum-Free *In-vitro* Cultures of Human Lacrimal Gland. . Association for Research in Vision and Ophthalmology, Seattle, Washington, USA *(Paper)*
2. Geeta K Vemuganti, **Shubha Tiwari**, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy (2011). Establishing Human Lacrimal Gland Cultures and Evaluating their *Ex-vivo* Secretory Function. Association for Research in Vision and Ophthalmology, Fort Lauderdale, USA *(Poster)*

3. **Shubha Tiwari**, Md. Javed Ali, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy , Geeta K Vemuganti (2011). Evaluation of Human Lacrimal Gland Cultures for Secretory Function and Putative Stem Cells.Indian Eye Research Group, Hyderabad, India *(Poster)*
4. Geeta K Vemuganti, **Shubha Tiwari**, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy (2010). Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application. Association for Research in Vision and Ophthalmology, Fort Lauderdale, USA *(Poster)*
5. **Shubha Tiwari**, Md. Javed Ali, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy , Geeta K Vemuganti (2010). Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application. Indian Eye Research Group, Hyderabad, India *(Poster)*
6. **Shubha Tiwari**, Santosh G Honavar, Milind N Naik,Vijay Anand P Reddy, Geeta K Vemuganti (2009). Cultivation and Characterization of Lacrimal and Salivary Gland Cells for Potential Clinical Application. Annual conference of the Association of Radiation Oncologists of India, Hyderabad, India *(Poster)*

Awards

- 2013: Awarded the DBT Travel Fellowship to Annual Meeting ARVO, Seattle, USA
- 2013: Awarded the CSIR Travel Fellowship to Annual Meeting ARVO, Seattle, USA
- 2011: Awarded the Tear Film and Ocular Surface (TFOS) Fellowship
- 2011: Awarded the CSIR Travel Fellowship to Annual Meeting, ARVO. Fort Lauderdale. Florida. USA

OPEN ACCESS Freely available online

PLoS one

Establishing Human Lacrimal Gland Cultures with Secretory Function

Shubha Tiwari[1], Mohammad Javed Ali[2], Murali M. S. Balla[1], Milind N. Naik[2], Santosh G. Honavar[2], Vijay Anand P. Reddy[2], Geeta K. Vemuganti[1,3,4]*

1 Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory, Professor Brien Holden Eye Research Centre, L. V. Prasad Eye Institute, Hyderabad, India, **2** Ophthalmic Plastic Surgery, Orbit and Ocular Oncology, L. V. Prasad Eye Institute, Hyderabad, India, **3** Ophthalmic Pathology Services, Professor Brien Holden Eye Research Centre, L. V. Prasad Eye Institute, Hyderabad, India, **4** School of Medical Sciences, University of Hyderabad, Hyderabad, India

Abstract

Purpose: Dry eye syndrome is a multifactorial chronic disabling disease mainly caused by the functional disruptions in the lacrimal gland. The treatment involves palliation like ocular surface lubrication and rehydration. Cell therapy involving replacement of the gland is a promising alternative for providing long-term relief to patients. This study aimed to establish functionally competent lacrimal gland cultures *in–vitro* and explore the presence of stem cells in the native gland and the established *in-vitro* cultures.

Methods: Fresh human lacrimal gland from patients undergoing exenteration was harvested for cultures after IRB approval. The freshly isolated cells were evaluated by flow cytometry for expression of stem cell markers ABCG2, high ALDH1 levels and c-kit. Cultures were established on Matrigel, collagen and HAM and the cultured cells evaluated for the presence of stem cell markers and differentiating markers of epithelial (E-cadherin, EpCAM), mesenchymal (Vimentin, CD90) and myofibroblastic (α-SMA, S-100) origin by flow cytometry and immunocytochemistry. The conditioned media was tested for secretory proteins (scIgA, lactoferrin, lysozyme) post carbachol (100 μM) stimulation by ELISA.

Results: Native human lacrimal gland expressed ABCG2 (mean$\pm$SEM: 3.1$\pm$0.61%), high ALDH1 (3.8$\pm$1.26%) and c-kit (6.7$\pm$2.0%). Lacrimal gland cultures formed a monolayer, in order of preference on Matrigel, collagen and HAM within 15–20 days, containing a heterogeneous population of stem-like and differentiated cells. The epithelial cells formed 'spherules' with duct like connections, suggestive of ductal origin. The levels of scIgA (47.43 to 61.56 ng/ml), lysozyme (24.36 to 144.74 ng/ml) and lactoferrin (32.45 to 40.31 ng/ml) in the conditioned media were significantly higher than the negative controls ($p<0.05$ for all comparisons).

Conclusion: The study reports the novel finding of establishing functionally competent human lacrimal gland cultures *in-vitro*. It also provides preliminary data on the presence of stem cells and duct-like cells in the fresh and *in-vitro* cultured human lacrimal gland. These significant findings could pave way for cell therapy in future.

Citation: Tiwari S, Ali MJ, Balla MMS, Naik MN, Honavar SG, et al. (2012) Establishing Human Lacrimal Gland Cultures with Secretory Function. PLoS ONE 7(1): e29458. doi:10.1371/journal.pone.0029458

Editor: Songtao Shi, University of Southern California, United States of America

Received May 17, 2011; **Accepted** November 29, 2011; **Published** January 13, 2012

Funding: This work was supported by the International Atomic Energy Agency (IAEA), Hyderabad Eye Research Foundation (HERF), Champalimaud Translational Research Foundation(C-Tracer), Department of Biotechnology (DBT). The authors also wish to thank Council for Scientific and Industrial Research (CSIR) for a Senior Research Fellowship to Dr. Tiwari. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: deanmd@uohyd.ernet.in

Introduction

The stability and integrity of the ocular surface depends greatly on the stability of the tear film that covers the anterior surface of the eye. The tear film has three basic layers - the outer thin lipid layer secreted by the meibomian glands, the middle bulk of aqueous layer secreted by the lacrimal gland and the inner mucinous layer secreted by the conjunctival goblet cells. Collectively, these three layers of the tear film perform a number of important physiological functions [1]: it keeps the cornea wet allowing gaseous exchange between the environment and the epithelium, it provides a clear and regular optical surface for sharp image focusing on the retina, it clears the debris from the ocular surface and protects it from microbial invasion. Deficiency and loss of tear film integrity, atrophy of the lacrimal gland or apoptosis of the secretory epithelial cells due to hormonal imbalance, environmental changes, autoimmune pathologies or radiotherapy induces pathological changes in the gland and leads to a chronic disabling condition called the dry eye syndrome (DES).

The 2007 International Dry Eye Workshop (DEWS) report estimated the global prevalence of DES to be between 5% to over 35% at various ages (21 yr to >65 yr) [2]. Clinically, chronic dry eye causes a significant drop in contrast sensitivity and visual acuity leading to degraded performance in routine vision related activities like driving, reading [3,4]. The signs and symptoms include ocular dryness, grittiness, burning and foreign body

sensation, redness and blurred vision that clears on blinking [5]. Over time the loss of tear film integrity induces corneal epithelial irregularities and epithelial defects [6] with higher risks of secondary infection [7]. The pathological features of dry eye include lymphocytic infiltration of the lacrimal gland [8], reversible squamous metaplasia [8], apoptosis of secretory epithelial cells, loss of α-smooth muscle actin and tenascin C expression in the myoepithelial cells indicating loss of function [9]. Together these contribute to reduced tear secretion and result in the signs and symptoms of dry eye. Biochemically, there is hyperosmolarity of the tear film either due to reduced tear production or excessive tear evaporation from the ocular surface causing a reduction in tear film thickness from (mean ± SD) 6.0±2.4 μm in normal subjects to about 2.0±1.5 μm in dry eye patients [10].

Current treatment for dry eye primarily involves the use of lubricating eye drops or pharmacological stimulation of tears secretion [7,11]. However, these treatment modalities provide only temporary relief and have the inherent drawbacks of associated side effects and suboptimal results due to loss of secretory function of the gland [7]. In severe cases, especially in those with permanent damage to lacrimal gland, there arises a need to replace the gland and restore its functionality using appropriate cell therapy. To achieve this long-term goal it is important to establish and evaluate functionally competent *in-vitro* cell culture system.

Animal studies [12,13,14] have successfully demonstrated the establishment of *in-vitro* lacrimal gland cell cultures, using different media and scaffolds [12,15]. However, work on human *in-vitro* lacrimal gland culture is scarce [16]; possibly due to paucity of fresh tissues and the fragile nature of these cultures. Recent studies have shown the presence of stem cells in exocrine glands like salivary [17], pancreas [18,19], prostate [20] and breast [21,22]. These reports have prompted investigations on the potentials of using *in-vitro* cultured cells for regenerative therapy with promising outcomes. However in the case of lacrimal gland, there is only preliminary report on the presence of stem cells in the mouse lacrimal gland [23,24] and, to the best of our knowledge, none investigating the presence of stem cells in human lacrimal gland. With this background in mind, the present study had two important aims: 1) to establish functionally competent human lacrimal gland cultures *in-vitro* and 2) investigate the presence of stem cells in the native tissue as well as in the established *in-vitro* cultures.

Materials and Methods

Chemicals

HepatoSTIM culture media (BD biosciences, San Jose, CA, USA), Dulbecco's Modified Eagle's Medium (DMEM) - Ham's F12 (Sigma Aldrich, St Louis, MO,USA) Fetal calf serum (FCS) (Hyclone), bovine serum albumin (BSA) (Sigma Aldrich, St Louis, Mo, USA) penicillin, streptomycin, gentamysin and amphotericin B, epidermal growth factor (Sigma Aldrich, St Louis, MO, USA), L-glutamine (2 mM) (Sigma Aldrich, St Louis, MO, USA), Matrigel® basement matrix (BD Biosciences, San Jose, CA, USA), collagen I gel (Sigma Aldrich, St Louis, MO, USA), collagnease (Invitrogen, Carlsbad, CA, USA), hyaluronidase (Invitrogen, Carlsbad, CA,USA), Hank's Balanced Salt Solution (HBBS), TRIzol (Invitrogen, Carlsbad, CA,USA), Superscript first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) anti- E-cadherin antibody (Chemicon, Temecula, CA, USA), anti-ABCG2 (BD Biosciences, San Jose, CA, USA), anti c-kit (Millipore, Temecula, CA; Dako, Glostrup, Denmark), anti-p63 (Dako, Glostrup, Denmark) anti-CD133 (Miltenyi Biotech), anti-EpCAM (BD Biosciences, San Jose, CA, USA) anti- lysozyme (Abcam), anti-lactoferrin (Millipore, Temecula, CA, USA), anti-scIgA (Dako, Glostrup, Denmark), anti- GFAP (Dako, Glostrup, Denmark), anti-S100 protein (Dako, Glostrup, Denmark) anti CD90 (eBioscience), anti-vimentin (Dako, Glostrup, Denmark), anti-CK3/12 (Dako, Glostrup, Denmark), Aldefluor Assay Kit (Stem Cell Technology, Durham, NC,USA), Fluroscein Isothiocynate (Invitrogen, Carlsbad, CA,USA), Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), Phycoerytherin (eBioscience), human lactoferrin ELISA kit (Assay-Pro, MO, USA), human lysozyme ELISA kit (AssayPro, MO, USA), human IgA ELISA kit (Immunology Consultants Laboratory, Inc, Newberg, OR, USA).

Human Tissue Source

The study was conducted at the L V Prasad Eye Institute (LVPEI), Hyderabad. The use of human tissue was approved by the Institutional Review Board (IRB) and is in accordance with the tenets of the Declaration of Helsinki. Fresh human lacrimal gland was harvested, after obtaining written informed consent, from patients undergoing exenteration surgery. The lacrimal gland was immunohistochemically evaluated to be free from any underlying pathology. The fresh gland was collected in FCS rich DMEM-Ham's F-12 media supplemented with antibiotics and transported to the lab where it was immediately taken for processing.

Preparation of human amniotic membrane

The use of human amniotic membrane has been approved by the IRB of LVPEI, Hyderabad. Human amniotic membrane (HAM) was prepared according to the protocol published by Kim *et.al* [25] Following written informed consent, human placenta was obtained at the time of normal cesarean delivery at the Fernandez Hospital, Hyderabad. The placenta was transported to the Ramayamma International Eye Bank (LVPEI, Hyderabad) and processed under sterile conditions using saline/ringer lactate solution. The amnion was separated from the chorion by peeling and attached onto sterile nitrocellulose paper and cut into 2.5×5.0 cm pieces. The amniotic membrane pieces were stored in glass vials containing DMEM and stored at −70°C. Just prior to use, it was thawed at 37°C for 30 minutes. HAM was de-epithelized by incubating with 0.25% trypsin–EDTA for 30 minutes at 37°C followed by thorough washing with PBS to remove the epithelial layer [26].

Establishment of primary culture. Fresh lacrimal gland was washed with HBBS to remove red blood cells. The gland was chopped into small bits using a scalpel blade (#21). The tissue mince was then incubated with the enzyme cocktail of collagenase (130 units per ml) and hyaluronidase (300 units per ml) for 90 minutes at 37°C with intermittent shaking. At the end of the incubation period, the suspension was filtered through a 75 μm cell sieve and the cell pellet obtained by centrifugation at 1500 rpm for 20 min. The cells were seeded on uncoated tissue culture dishes in DMEM-Ham's F12 culture media supplemented with 10% FCS and antibiotics.

The heterogeneous mix of isolated cells was separated based on their preferential adhesion to uncoated tissue culture dishes. The fibroblasts attached preferentially to the uncoated tissue culture dishes and the epithelial clumps, still in suspension at the end of around 2 hours, were aspirated and plated on Matrigel™, collagen I coated dishes and on denuded HAM. The media used for the culture of fibroblasts was DMEM- Ham's F-12 supplemented with 2 mM L-glutamine, antibiotics and 10% FCS. For the culture of lacrimal acinar cells, HepatoSTIM™ culture media was used. HepatoSTIM® is a commercially available fully defined,

serum free media based on the formulation of Williams E Media [27] and contains supplements like dexamethasone, insulin-transferrin-selinium (ITS) and EGF. The media was further supplemented with 2 mM L-glutamine, penicillin, streptomycin, 5 ng/ml EGF and 10% FCS for the first three days. At the end of the three days/at first media change, FCS was completely eliminated from the media and the concentration of EGF increased to 50 ng/ml.

Characterization of native lacrimal gland and in-vitro lacrimal gland culture. The native lacrimal gland and 14–18th day *in-vitro* cultures of human lacrimal gland, growing as a monolayer on Matrigel^{TM} were characterized by immunohistochemistry, immunocytochemistry, flow cytometry, reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme linked Immunosorbent assay (ELISA).

Immunohistochemistry

Expression of markers like Pan-cytokeratin (AE1/AE3), vimentin, p63, ∝-SMA, GFAP, S-100, lysozyme and c-kit protein was evaluated in formalin fixed, paraffin embedded sections of fresh lacrimal gland. The sections were also stained with hematoxylin & eosin (H&E) as well as periodic acid schiff's (PAS) to visualize the histology of the gland.

The fresh gland was fixed with 10% fresh formalin and embedded in paraffin. Thin 3 µm sections were taken on silane coated glass slides and used for immunostaining. Briefly, the paraffin embedded sections were deparaffinized at around 70°C and then in xylene series. The sections were rehydrated in alcohol series and then in distilled water followed by 1X PBS. The endogenous peroxidase activity was blocked using methanol and hydrogen peroxide and the antigen retrieval done using Tris-EDTA buffer (pH 9). After appropriate washings with PBS and blocking with 2.5% BSA, the sections were incubated with the primary antibody in a moist chamber for 2 hours at room temperature followed by secondary antibody (polymer HRP) incubation for 30 minutes at room temperature. DAB substrate was added to the section to allow color development for 10 minutes. This was followed by counterstaining with hematoxylene and then mounting in DPX. The sections were visualized under a light microscope.

The source of the antibodies used and the appropriate dilutions are summarized in Table 1.

Immunocytochemistry

The *in-vitro* cultures of lacrimal gland at day 14–18 were immunostained for epithelial markers like cytokeratin 3/12, E-cadherin, p63; myoepithelial markers like S100, GFAP; and mesenchymal markers vimentin and CD 90. In addition, the cells were also immunostained with ABCG2 and secretory protein lysozyme. Briefly, the cells were fixed with 4% fresh paraformaldehyde (PFA) for 10 minutes, followed by permeabilization with 50% methanol for 20 minutes for intracellular markers. The cells were then incubated with appropriate dilutions of the primary antibody for 2 h at room temperature.

Secondary antibodies like Alexa Fluor 488, FITC and PE were used against the respective immunoglobulins of the primary antibody at 1:200 dilution. The incubation time was 45 min. Nuclear staining was done with 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). The coverslips were mounted in 50% glycerol and the images acquired using Carl Zeiss Laser Scanning Microscope LSM 510.

The source of the antibodies used and the appropriate dilutions are summarized in Table 2, 3 and 4.

Flow Cytometry

Lacrimal gland cells, freshly isolated from the gland as well as 14–18 days and 21–25 days post *in-vitro* culture, were evaluated by flow cytometry to detect the number of cells positive for epithelial markers like E-cadherin, EpCAM and mesenchymal markers like CD90. Putative stem cell markers like ABCG2, c-kit were also used to investigate the presence of stem cell compartment. Expression of CD133, which has recently been recognized as a stem cell marker/marker for glandular epithelium [28], was also evaluated.

Cells were isolated from the human lacrimal gland by enzymatic digestion as described previously in the study. Cells growing as monolayers on Matrigel^{TM} were trypsinized using 0.25% trypsin-EDTA (TE) and used for evaluation of marker expression by flow cytometry.

Briefly, 1×10^6 cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with appropriate dilutions of primary antibody for 2 hours at room temperature. The cell pellet was washed with PBS and then incubated with 1:200 dilutions of appropriate secondary antibodies for 45 minutes. At the end of this time period, the pellet was washed with PBS, resuspended in 500 µl of FACS buffer and acquired on BD FACS ARIA^{TM} Special Order System. Appropriate controls were used for the experiment. A total of 20000 to 50000 events were acquired for analysis. The analysis was done using BD FACS DiVa^{TM} software.

The source of the antibodies used and the appropriate dilutions are summarized in Table 2, 3 and 4.

Table 1. List of antibodies and dilutions used for immunohistochemistry.

S. No.	Antibody	Dilution	Company
1.	p63	Neat	Dako
2.	∝-SMA	Neat	Dako
3.	GFAP	Neat	Dako
4.	S-100 protein	Neat	Dako
5.	Lysozyme	1:100	Abcam
6.	c-kit	1:100	Dako
7.	Pan-cytokeratin (AE1/AE3)	1:50	Dako
8.	Vimentin	1:200	Dako

doi:10.1371/journal.pone.0029458.t001

Table 2. List of antibodies and dilutions used for immunocytochemistry.

S. No.	Antibody	Dilution	Company
1	E- cadherin	1:100	Millipore
2.	Cytokeratin 3/12	1:100	Millipore
3.	CD90	1:200	eBiosciences
4.	Vimentin	1:100	Dako
5.	GFAP	Neat	Dako
6.	S-100 protein	Neat	Dako
7.	Lysozyme	1:100	Abcam

doi:10.1371/journal.pone.0029458.t002

Table 3. List of antibodies and dilutions used for flow cytometry.

S. No.	Antibody	Dilution	Company
1	ABCG2	1:100	BD Biosciences
2.	c-kit	1:100	Millipore
3.	E-cadherin	1:100	Millipore
4.	CD90	1:200	eBiosciences
5.	EpCAM	1:100	BD Biosciences
6.	CD133	1:20	Miltenyi Biotech

doi:10.1371/journal.pone.0029458.t003

Aldefluor Assay

ALDEFLUOR® fluorescent reagent system (Stem Cell Technologies) provides a novel method for identification of stem and progenitor cells based on their expression of enzyme aldehyde dehydrogenase1 (ALDH). The fluorescent ALDEFLUOR® reagent diffuses freely into the cells and acts as a non-toxic substrate for ALDH. The fluorescent reaction product that accumulates in the cells can be measured in the green channel of a standard flow cytometer and it correlates directly to the ALDH activity in the cell. With this assay, stem and progenitor cells are identified as cells with higher expression of ALDH1.

For the assay, 1×10^6 cells/ml (freshly isolated from the lacrimal gland as well as post trypsinization from the cultured monolayer on day 14–18 and day 21–25) were taken and divided into two groups: control and test. To the control tube 5 µl of ALDH inhibitor DEAB was added. 5 µl/ml of activated ALDEFLOUR® substrate was added to the test group and immediately half of the cell suspension was transferred to the control tube. Both the test as well as the control sample was incubated at 37°C for 30 to 60 min. At the end of the incubation period the supernatant was removed after centrifugation and the cells resuspended in 0.5 ml of ALDEFLUOR® Assay Buffer and the fluorescence measured in the green channel of FACS ARIA™ Special Order System.

Reverse- Transcriptase Polymerase Chain Reaction

Total RNA was extracted from the freshly isolated as well as cultured cells using the TRIzol® reagent according to the manufacturer's instructions. The quality of the RNA isolated was checked by visualization on agarose gel. 2 µg of RNA was used for cDNA synthesis per 25 µl of the reaction volume using the Superscript™ First Strand Synthesis System for RT-PCR according to the manufacturer's instructions. The primer sequences used for reverse transcriptase polymerase chain reaction are summarized in Table 5.

PCR amplification was carried out using the Applied Biosystems Veriti 96 well thermal cycler. The reaction was stopped at 35 PCR cycles. The amplified products were visualized on agarose gel and the product size estimated.

Table 4. List of secondary antibodies and dilutions.

S. No.	Antibody	Dilution	Company
1.	Alexa Fluor 488	1:200	Invitrogen
2.	PE	1:200	eBiosciences
3.	FITC	1:200	Invitrogen

doi:10.1371/journal.pone.0029458.t004

Measurement of secretory components

Free secretory products of lacrimal acinar cells like scIgA, lactoferrin and lysozyme were detected in the culture supernatant of day 6–7, day 14 and day 21 cultures according to the manufacturer's instructions. All the reagents used were supplied as a part of the ELISA kit. A standard curve was generated for each experiment performed.

Briefly, 50 µl of the standard protein or culture supernatant was added to the wells of polypropylene U bottom ELISA plates and incubated for 2 hours at room temperature. The plates were washed at least five times with the wash buffer (supplied with the kit) ensuring complete removal of the liquid at each step. 50 µl of biotinylated primary antibody (lysozyme/lactoferrin/scIgA) (1:100 dilution) was added to each of the sample-coated wells and incubated for one hour at room temperature followed by thorough washing with the wash buffer. 50 µl of streptavidine-peroxidase conjugate was added to each well and incubated for 30 minutes. At the end of the incubation period, the wells were washed thoroughly and incubated with 50 µl/well of the chromogen substrate for 10 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution to each well and a color change from blue to yellow is noted. The optical density (OD) was then measured immediately at 450 nm on ELISA microplate reader (BioRad iMark™ Microplate Reader).

Statistical Analysis

Values are expressed as mean of triplicate readings ± SEM unless otherwise indicated. The statistical test used was two-way ANOVA with post hoc Tukey test. Statistical package SPSS Version 19 was used for analysis and graphs plotted using Microsoft excel. The results were statistically compared with fresh media (as a negative control) and also with each group and were considered as statistically significant if $p\leq0.05$.

Results

After IRB approval and informed consent, 22 samples of fresh lacrimal gland tissue were harvested from exenterated specimens for orbital malignancies. After histological confirmation of normal lacrimal gland and exclusion of any underlying pathology, 18 were included for the study. Cultures were established in 14 of the 18 samples while 4 samples were used for FACS analysis after enzymatic digestion.

Establishment of human lacrimal gland primary culture

The enzymatic digestion of the freshly harvested lacrimal gland tissue yielded a heterogeneous population of cells comprising of clumps of epithelial cells, fibroblasts, single cells of larger size and some red blood cells (Figure 1a).

On initially plating the isolated cells on uncoated tissue culture dishes, the fibroblasts settled down and adhered within 2 hours at the end of this time period, the floating cells were aspirated and seeded on dishes coated with Matrigel™, collagen I or on denuded HAM.

The epithelial cells from the suspension adhered to Matrigel™, collagen I coated dishes and denuded HAM with initiation of cell proliferation within 1–3 days (Figure 1b). Preferential adhesion of the cell clumps, optimal proliferation and morphology of the epithelial cells was seen on Matrigel™. Within 15–20 days, the epithelial islands expanded to form a monolayer in all the three substrates (Figure 1c). The cells showed thin cytoplasmic borders,

Table 5. Primer sequences used for RT-PCR.

S. No.	Name	Sequence	Product Size (bp)
1.	Lactoferrin - Forward	CAGACCGCAGACATGAAACT	479
	Lactoferrin- Reverse	TTCAAGAATGGACGAAGTGT	
2.	Lysozyme - Forward	CTCTCATTGTTCTGGGGC	350
	Lysozyme - Reverse	ACGGACAACCCTCTTTGC5	
3.	scIgA - Forward	AATGCTGACCTCCAAGTGCTAAAG	242
	scIgA - Reverse	ATCACCACACTGAATGAGCCATCC	
4.	GAPDH - Forward	CAGAACATCATCCCTGCATCCACT	250
	GAPDH - Reverse	GTTGCTGTTGAAGTCACAGGAGAC	

doi:10.1371/journal.pone.0029458.t005

polygonal shape, vesicular nucleus and granular cytoplasm (Figure 1d). The cultures on Matrigel showed sustained epithelial morphology for 30–35 days. (Panel showing day 14 epithelial cell growth on uncoated dishes, denuded HAM, collagen 1 and Matrigel^{TM} coated dishes is included as Figure S1).

In addition, there was formation of 'spherules' (Figure 1e, f) in all matrices by day 16–18. The cultures also showed development of cord-like cellular structures between two spherules suggesting an attempt towards formation of duct-like structures (Figure 1g–i).

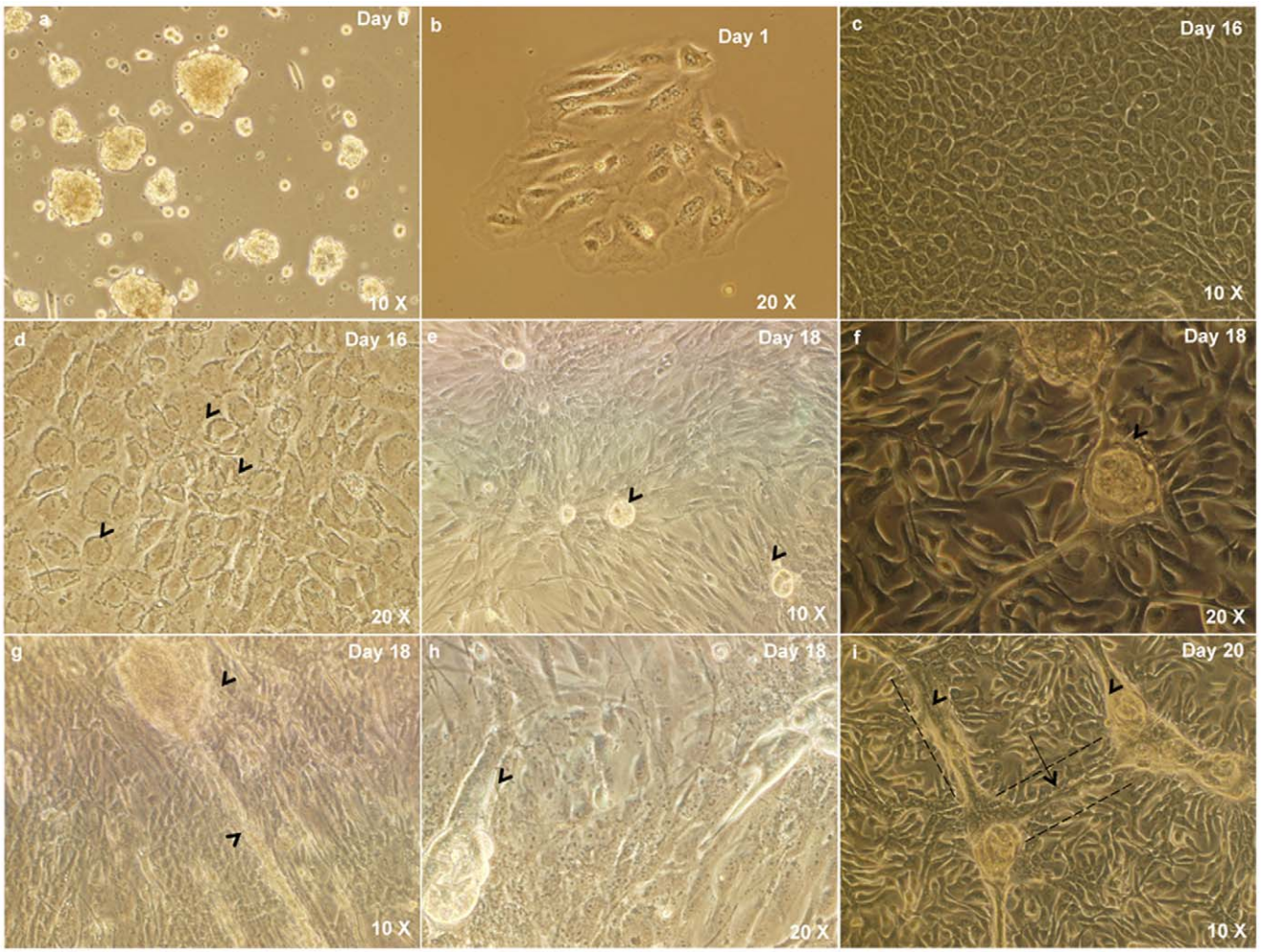

Figure 1. Establishment of human lacrimal gland primary cultures. a) Heterogenous cell population isolated after enzymatic digestion of the lacrimal gland. b) Cell clumps adhere to the substrate as discrete islands and show initiation of proliferation within 1–3 days. c) The islands proliferate and form a confluent monolayer within 15–20 days. d) The cells in the monolayer show thin cytoplasmic border, vesicular nucleus and granularity in the cytoplasm. e–f) Spherules are formed by day 16–18 (arrow head). g–i) Cord-like connections (arrow head) are seen to develop between the spherules.
doi:10.1371/journal.pone.0029458.g001

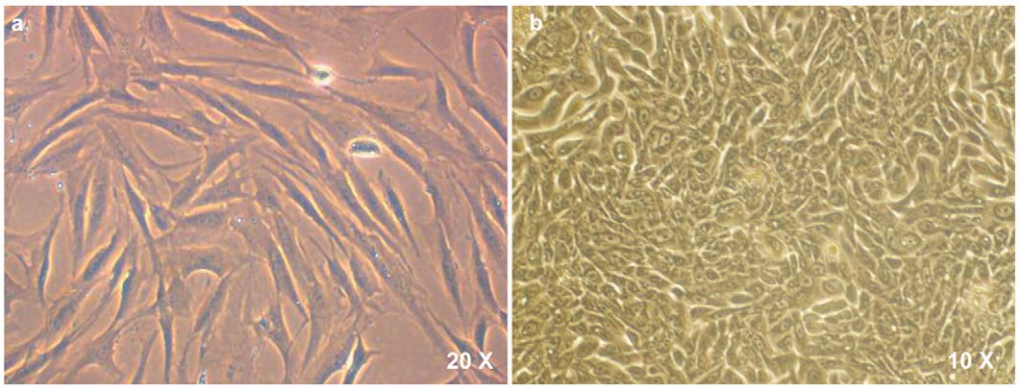

Figure 2. Other cell types in culture. a) Spindle shaped cells with slight granularity in their cytoplasm and distinct nucleus, are seen on uncoated culture dishes and these attain confluence within 5–7 days. b) Oval and plump cells that organize themselves in whorls are also seen. These may be myoepithelial in nature.
doi:10.1371/journal.pone.0029458.g002

On uncoated tissue culture dishes, there was predominance of spindle cells that form a confluent monolayer by around 5–7 days. The cells were spindle shaped, with slight granularity in their cytoplasm and distinct nucleus (Figure 2a). In addition, the culture also showed the presence of a third type of cellular morphology: oval and plump cells that organized themselves in whorls (Figure 2b). These could be myoepithelial in nature.

A flow cytometric comparison was also made of the heterogeneous cell population growing on uncoated, collagen 1 and Matrigel^{TM} coated dishes. The day 14 data shows that about 85.1±4.9% of the cells growing on uncoated tissue culture dishes are positive for mesenchymal marker CD90 while only 0.65±0.35% were positive for epithelial marker EpCAM; on collagen 1 coated dishes, 16.7±0.85% of the cells showed CD 90 positivity while 1.2±0.3% were EpCAM positive; as for Matrigel, 13.3±10.2% of the cells were mesenchymal (CD90 positive) while 2.2±1.7% were epithelial (EpCAM positive) (Table S1).

Immunophenotyping of native and cultured lacrimal gland cells

Immunohistochemistry. The formalin fixed, paraffin embedded human lacrimal gland tissues, on hematoxylene and eosin staining show the typical tissue architecture of human lacrimal gland (Figure 3a). These paraffin embedded sections showed immunoreactivity for pan-cytokeratin, lysozyme, vimentin, c-kit, p63, ∝-SMA, GFAP and S-100. The staining pattern reveals localization of pan-cytokeratin (Figure 3b) and secretory protein lysozyme (Figure 3c) mostly in the acinar cells with very few ductal cells showing faint positivity. Vimentin localized in the myoepithelial cells around the acinar cells and in the fibroblasts of the stroma. Few acinar cells also show vimentin positivity (Figure 3d). c-kit expression was seen as a membrane marker in the cell membrane of the acinar cells (Figure 3e) while p63, GFAP, S-100 and ∝-SMA (Figure 3f–i) was found in the cells enveloping the acinar cells (myoepithelial cells).

Immunocytochemistry

The cultures showed a heterogeneous population of cells with immunoreactivity for epithelial, myoepithelial as well as mesenchymal markers. The cells with epithelial morphology show positivity for CK3/12, p63, E-cadherin and lysozyme. CK3/12 and lysozyme localized in the cytoplasm of the cells, p63 in the nucleus while E-cadherin was seen to localize around the plasma membrane and between the epithelial cells. Some of the cells also showed immunopositivity for ABCG2 (Figure 4).

The spindle shaped cells were positive for mesenchymal markers CD90 and vimentin. The oval and plump cells were immunoreactive for GFAP and S-100 protein, which may be indicative of their myoepithelial/ductal origin (Figure 4).

Flow Cytometry

The FACS analysis of freshly isolated cells from the human gland showed that 14.8±3.45% of the cells were positive for epithelial marker EpCAM, 2.9±0.91% for mesenchymal marker CD90 and 3.7±0.33% positive for E-cadherin indicative of epithelial/epithelial progenitor nature. Cells in this heterogeneous mix also showed immunoreactivity towards stem cell markers like ABCG2 (3.1±0.61%) and c-kit (6.7±2.0%). CD133 positivity was seen in 0.3±0.1% of the cells (Table 6) (Figure 5i).

FACS analysis of cultured lacrimal gland cells also showed the presence of stem cells as well as differentiated cells in day 14–18 cultures. 2.2±1.7%of the cells were positive for EpCAM, 13.3±10.2% positive for CD 90. The cultures had a population of cells with positivity for stem cell markers accounting for 0.30±0.15% as ABCG2 positive and 0.20±0.05% as c-kit positive.

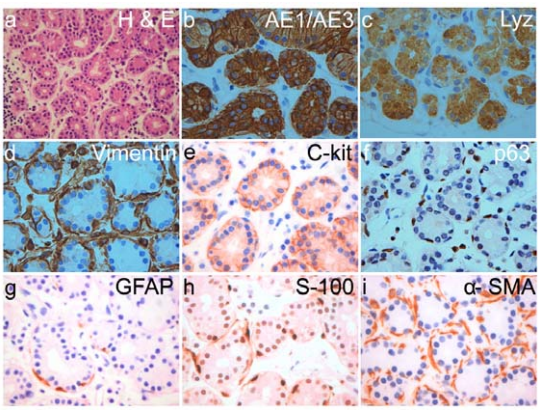

Figure 3. Immunohistochemistry on normal human lacrimal gland. H&E staining shows the normal histology of the lacrimal gland. Marker staining pattern shows localization of pan-cytokeratin (AE1/AE3) and lysozyme (Lzy) in the cytoplasm of the acinar cells while c-kit is seen in the plasma membrane of acinar cells. p63, glial fibrillary acidic protein (GFAP), S-100 protein and ∝-SMA localize in the myoepithelial cells enveloping the acinar cells. Vimentin is seen in the myoepithelial cells and also in some of the acinar cells. All images are at 40× magnification except H&E which is at 10×.
doi:10.1371/journal.pone.0029458.g003

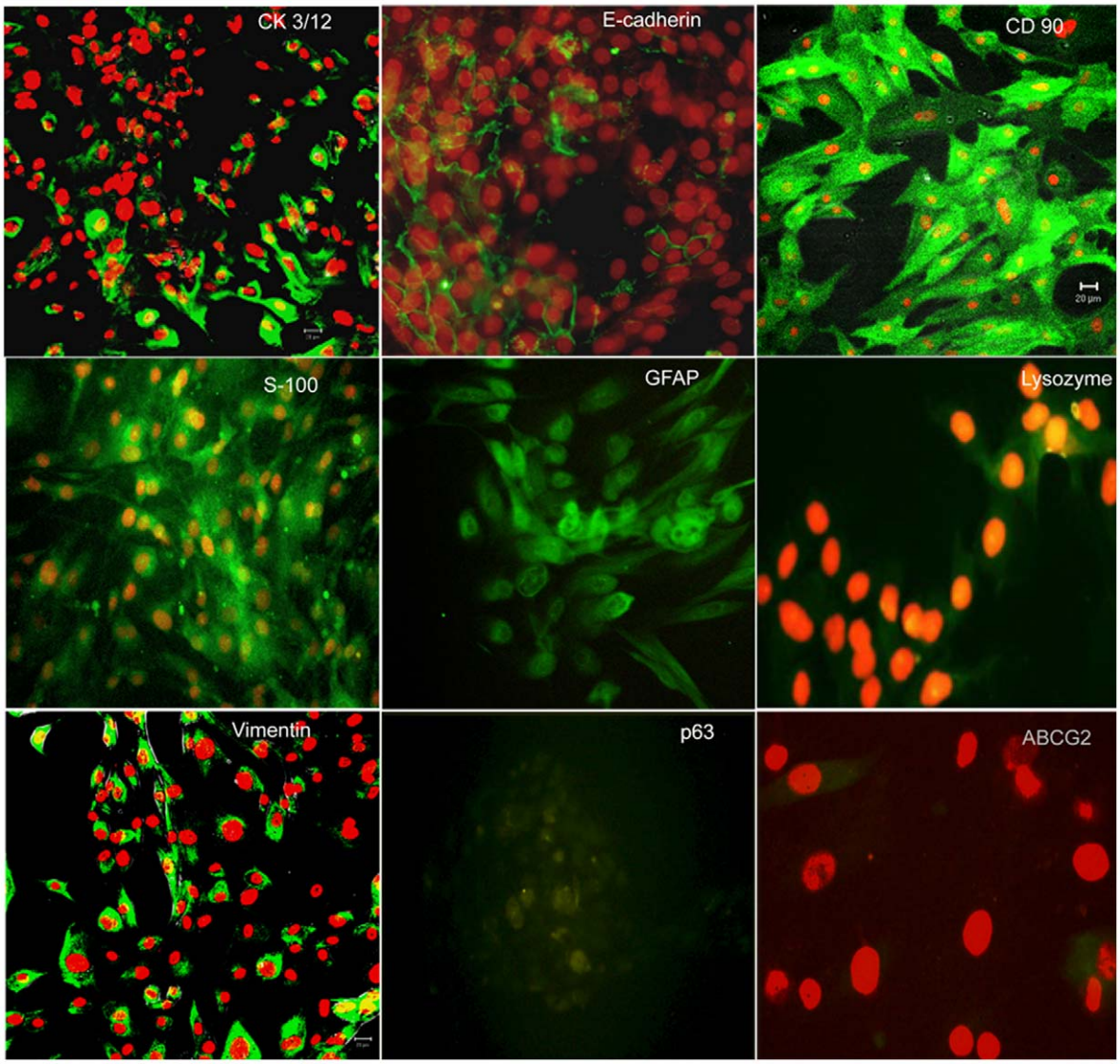

Figure 4. Immunocytochemistry on in-vitro cultured human lacrimal gland cells. Cells with epithelial morphology stain positively with E-cadherin, CK3/12, lysozyme and p63; oval and plump cells stain positive for myoepithelial markers GFAP and S100 protein while the spindle shaped cells are seen to be positive for mesenchymal markers CD90 and vimentin. Some cells also show immunopositivity for ABCG2. Secondary antibody uses is fluoresceine isothiocyanate (green) and the counter-stain is propidium iodide (red).
doi:10.1371/journal.pone.0029458.g004

CD133 expression was seen in 0.3±0.1% of the cells (Figure 5ii). By day 21–25 of *in-vitro* culture the number of cells expressing the stem cell markers ABCG2 and c-kit had reduced to 0.2±0.13% and 0.13±0.03% respectively while the expression of differentiated markers like CD90 had increased to 30.25±3.35%. This could possibly be and indication of the ongoing differentiation under *in-vitro* conditions (Table 6) (FACS plots not included for day 21–25).

ALDEFLUOR Assay

In the freshly isolated cells, 2.4% to 6.3% showed high ALDH1 activity (mean: 3.8±1.26%). Co-expression of high ALDH1 and ABCG2 was seen in 0.13±0.04%, and high ALDH1 and c- kit in 0.21%±0.02% of the cells (Table 7) (Figure 6i).

The day 14–18 cultured lacrimal gland cells also showed a population with high ALDH activity accounting for 2.7±1.5% of the total population acquired (Figure 6ii) which decreases to 1.1±0.5% by day 21. Co-expression of high ALDH1 with ABCG2/c-kit positivity was not evaluated in the cultured cells since their level of expression in the native tissue itself was low to begin with. (FACS plots not included for day 21–25).

C. Secretome Assessment

Reverse- Transcriptase Polymerase Chain Reaction. In order to confirm the presence of mRNA for the secretory proteins, lactoferrin, lysozyme and scIgA, in the cultured human lacrimal gland cells RT PCR was performed with specific primers. The cDNA synthesized by reverse transcription showed the expression of scIgA, lactoferrin and lysozyme (Figure 7) in the cells thereby confirming that the cultured cells retained their physiological ability to synthesize the secretory proteins.

Enzyme Linked Immunosorbent Assay

The conditioned media of day 6–7 human lacrimal gland cultures showed the presence of scIgA, lactoferrin and lysozyme

Table 6. Flow cytometry data: Percentage of various marker in freshly isolated, day 14–18 and day 21–25 of in-vitro culture of human lacrimal gland.

S. No.	Marker	% Expressed at t = 0 (Mean± SEM)	% Expressed at day 14 of *in-vitro* culture (Mean± SEM)	% Expressed at day 21 of *in-vitro* culture (Mean± SEM)
1.	ABCG2	3.1±0.61	0.3±0.15	0.2±0.13
2.	c-kit	6.7±2.0	0.2±0.05	0.13±0.03
3.	E-cadherin	3.7±0.33	0.6±0.4	0.45±0.25
4.	EpCAM	14.8±3.45	2.2±1.70	0.3±0.1
5.	CD90	2.9±0.91	13.3±10.20	30.25±3.35
6.	CD133	0.30±0.10	0.30±0.10	0.25±0.05

doi:10.1371/journal.pone.0029458.t006

secreted by the acinar cells. The quantity of protein secreted into the conditioned media by the cells growing on each of the matrices was calculated using the standard calibration curves (Figure 8a–c).

The protein secretion was further augmented by treatment with 100 µM of carbachol for 30 min (Figure 8d–f). The main effect of carbachol stimulation was statistically significant for sc IgA ($F(1, 8) = 15.07$; $p<0.01$), lysozyme ($F(1,8) = 5.86$; $p = 0.02$) and lactoferrin ($F(1,8) = 11.44$; $p<0.01$) secretion.

The amount of protein secreted by the cells post carbachol stimulation on each of the three matrices is tabulated in Table 8.

The cells cultured on Matrigel showed slightly higher levels of secretory proteins in the conditioned media (Figure 8d–f). However, the main effect of matrices and the interaction between matrices and carbachol stimulation was not statistically significant for scIgA ($F(2,8) = 1.33$; $p = 0.27$) and lactoferrin ($F(2,8) = 0.86$; $p = 0.43$) secretion; but showed significance for lysozyme secretion ($F(2,8) = 5.0$; $p = 0.01$).

The trend in protein secretion by the cultured cells at day 7, day 14 and day 21 was also evaluated. The results indicate that *in-vitro* tear protein secretion tends to increase from day 7 to day 14 and then declines by day 21. (Table S2).

This change over a time period from day 7 to day 21 was statistically analysed and found to be significant: scIgA ($F(1,6) = 21.92$; $p<0.01$); lysozyme ($F(1,6) = 7.45$; $p<0.01$) and lactoferrin ($F(1,6) = 21.3$; $p<0.01$).

Discussion

Similar to the role of other exocrine glands of the body, the lacrimal gland plays a major role in lubricating the ocular surface epithelium. The susceptibility of the gland to immune mediated insults, radiation induced damage and age related atrophic changes, which cannot be satisfactorily managed with the current line of therapeutics, causes high incidences of morbidity in the population and raises a need for replacement therapy with functionally competent cells.

The two important steps towards cell therapy would include establishment of functionally competent lacrimal gland cultures

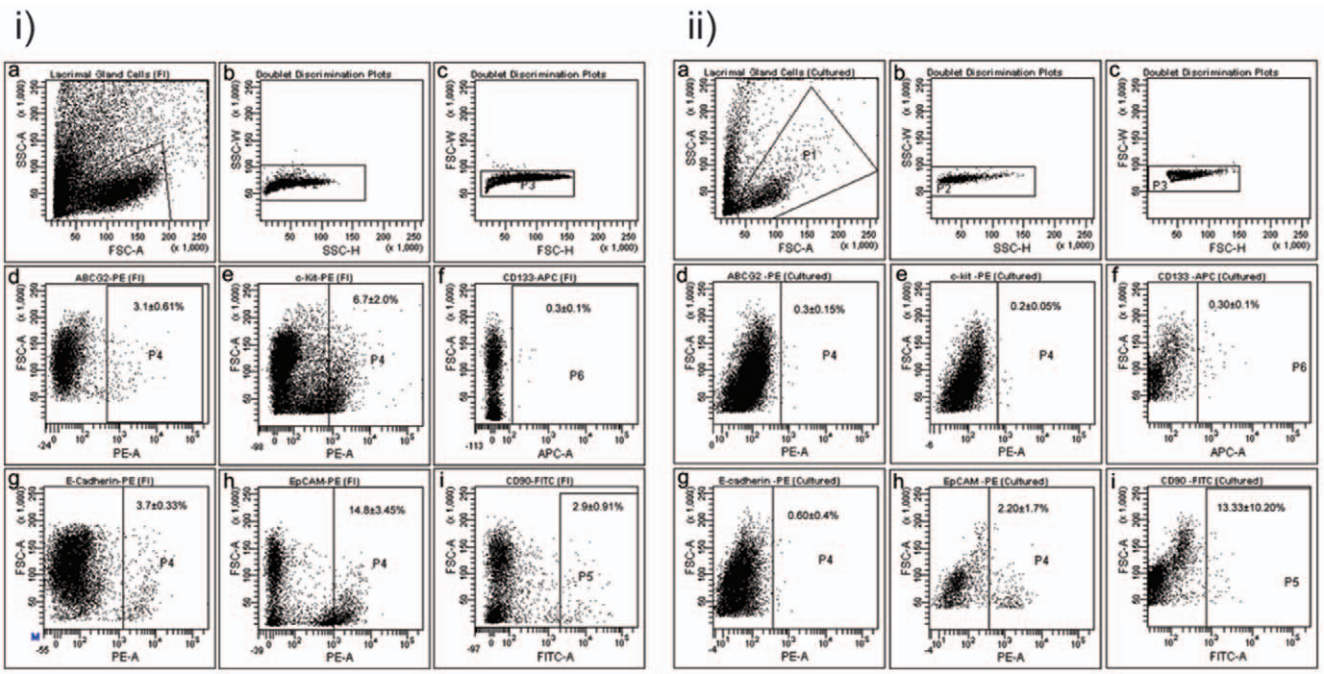

Figure 5. Flow Cytometry Data. i) Flow cytometric profile of freshly isolated (FI) cells. ii) Flow cytometric profile of cells 14–18 days post *in-vitro* culture.
doi:10.1371/journal.pone.0029458.g005

Table 7. ALDEFLUOR Assay.

S. No.	Marker	% Expressed at t = 0 (Mean± SEM)	% Expressed at day 14 of *in-vitro* culture (Mean± SEM)
1.	ALDH1 high	3.8±1.26	2.7±1.54
2.	ALDH high + c-kit	0.2±0.02	-
3.	ALDH high + ABCG2	0.1±0.04	-

doi:10.1371/journal.pone.0029458.t007

with persevered secretory function and providing evidence for the existence of stem cells in the native tissue, which can be recruited to salvage the function of the damaged gland. The present study provides evidence for both. Our results show that the human lacrimal gland cells can be cultured *in-vitro* with retained secretory function (Figure 1, Figure 7 and Figure 8). In addition to the mere presence of differentiated cells (epithelial, myoepithelial and stromal), we also show the formation of 'spherules' with attempted duct-like connections (Figure 1 e–i) and the presence of cells with stem cell-like phenotype (ABCG2 & ALDH1 high cells) (Figure 5 and Figure 6) under our culture conditions.

While lacrimal gland cultures from rabbit [13], mice [14] and rat [12] sources are well established, similar evidence from humans is limited [16]. To the best of our knowledge, this study documents the first successful method of isolating and culturing functionally competent fresh human lacrimal gland cells using an enzyme cocktail of collagenase and hyaluronidase. The established cultures can be maintained *in-vitro* for 30–35 days. This method not only gives an adequate yield of differentiated cells, but also provides early evidence for the presence of putative stem cells in the tissues as suggested by the expression of stem cell makers ABCG2 (3.1±0.61%) and high ALDH1 (3.8%±1.26%).

The influence of culture condition on cell proliferation and maintenance of function, especially in the case of lacrimal gland cultures, has been well documented [29,30,31]. It is important that the right combination of substrate, media and growth factors be

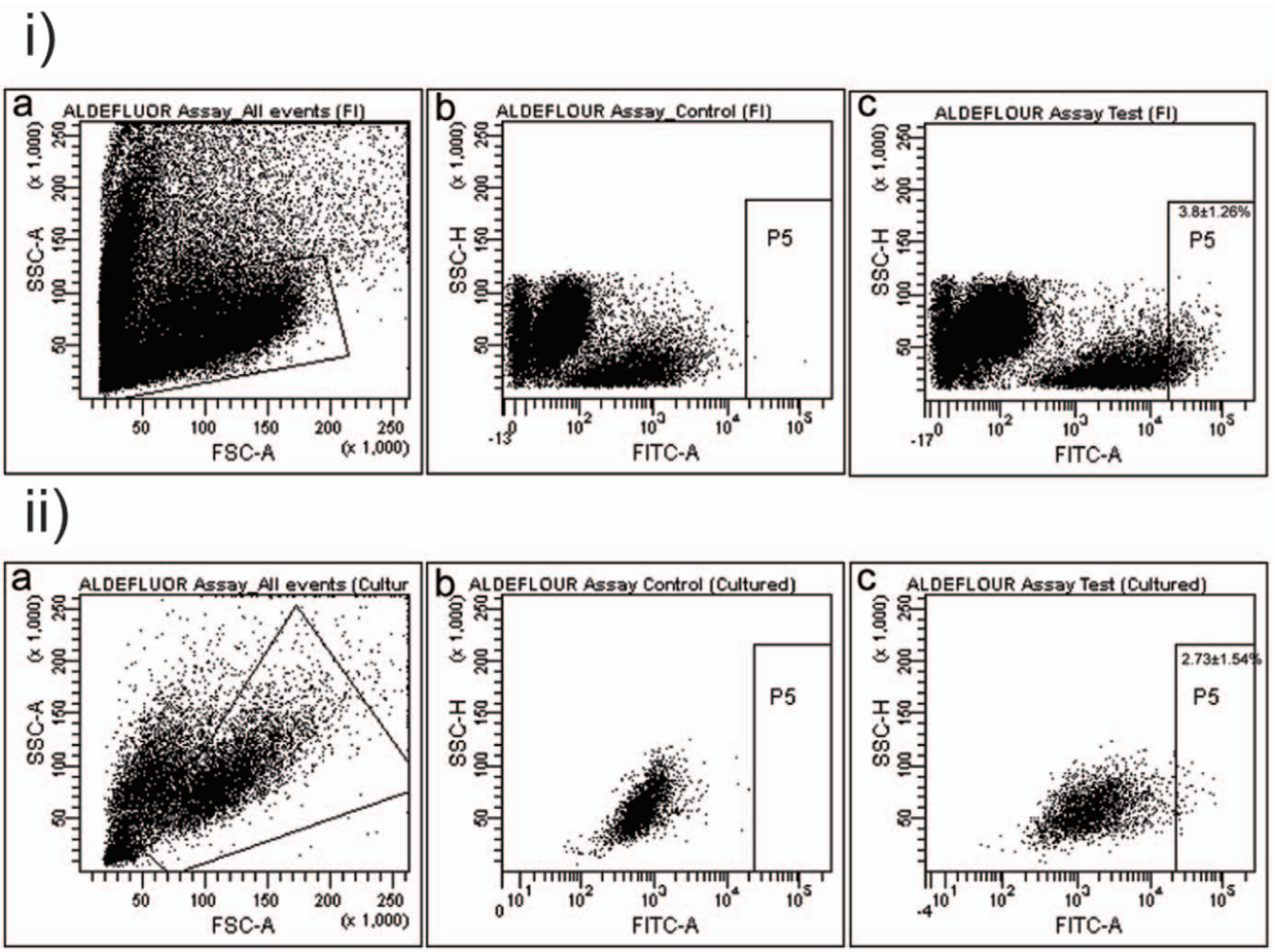

Figure 6. Aldefluor Assay. i) Flow cytometric data showing ALDH1 high cells in the freshly isolated (FI) cell population. ii) Flow cytometric data showing ALDH1 high cells in day 14–18 *in-vitro* cultures.
doi:10.1371/journal.pone.0029458.g006

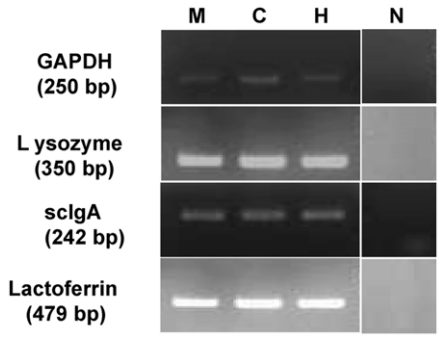

Figure 7. RT-PCR showing appropriate product bands for scIgA, lactoferrin and lysozyme in day 14 *in-vitro* cultures on all HAM (H), collagen I (C) and Matrigel^{TM}(M). Negative (N) panel shows no amplification product.
doi:10.1371/journal.pone.0029458.g007

used to simulate an *in-vivo* mimicry of function. Animal lacrimal gland cultures have been established on collagen [30], Matrigel^{TM} [12,15], HAM [32] and on artificial matrices like polyethersulfone [33]. Our study with human tissues also suggest that a combination of Matrigel^{TM}/HAM/collagen as substrate and EGF supplemented HepatoSTIM^{TM} media optimally supports the growth of different sub-populations of cells and helps them retain their characteristic marker expression (E-cadherin, CD90, S100, GFAP) as well as optimal secretory function (scIgA, lactoferrin and lysozyme secretion). However, morphological evidence and rate of cell proliferation seems to favor Matrigel^{TM} as substrate even though this difference is not statistically significant. Another interesting feature noted in this study is morphologic evidence towards formation of spherules and ductioles. The formation of spheres has been reported in salivary gland cultures (salispheres) [34], prostate cultures (prostaspheres) [35] and a single study on spheroidal aggregation of rabbit lacrimal gland cells grown in microgravity environment of a rotary cell culture system [36]. The salispheres have been shown to have stem cells and these when transplanted into animal models of radiation-induced xerostomia could rescue the function of the gland [34]. Similarly, the prostaspheres on combined transplantation with embryonic day 17 urogenital sinus (which provides the mesenchymal niche) into nude mice could give rise to tissues with glandular prostate morphology [35]. We believe that the spheres formed under our culture conditions could be similar to the salispheres and prostaspheres in their cellular organization and further investigations in this are ongoing.

In order to fulfill the long-term goal of using the *in-vitro* expanded lacrimal cultures for rescue of function of damaged gland it is important that the secretory profile of these cells be evaluated. Yoshino *et.al.* [16] reported a study on human lacrimal gland using cadaveric tissue; however, the report does not document the complete secretory profile of the cultured cells. In contrast, the present study shows that *in-vitro* cultures of human

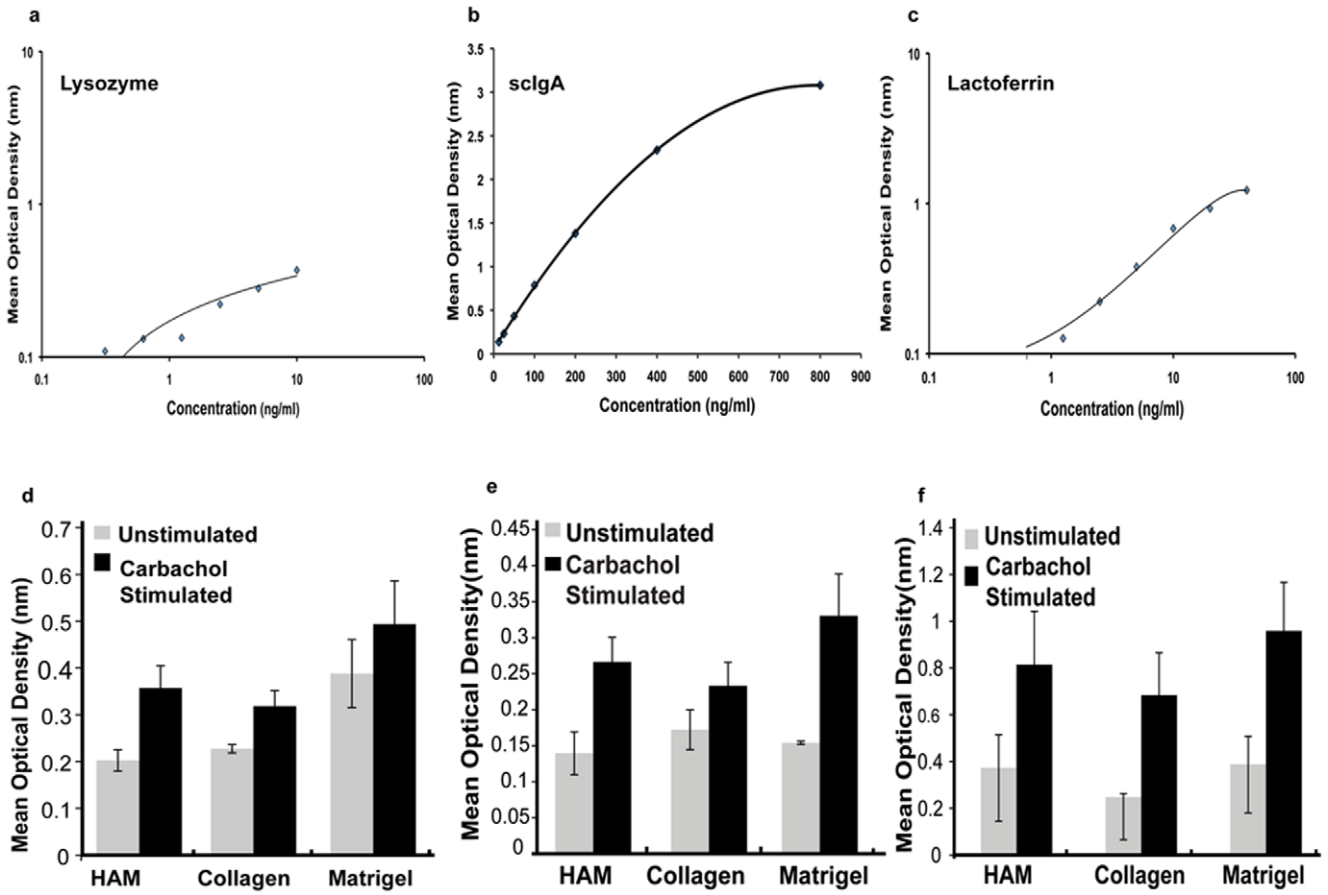

Figure 8. Secretome Assessment by ELISA. a–c): Standard calibration curve for lysozyme, scIgA and lactoferrin. d–f): Plot of mean optical density values for secreted proteins lysozyme, scIgA and lactoferrin on HAM, collagen and Matrigel^{TM} pre and post carbachol stimulation.
doi:10.1371/journal.pone.0029458.g008

Table 8. ELISA data: Secretion of tear proteins on various matrices post carbachol stimulation on day 6–7.

Tear Protein	HAM (ng/ml)	Collagen I (ng/ml)	Matrigel^{TM} (ng/ml)
Lysozyme	5.78 to 33.94	0.21 to 18.34	24.36 to 144.74
Sc IgA	3.86 to 71.40	1.41 to 27.58	47.43 to 61.56
Lactoferrin	44.50 to 45.65	28.52 to 30.41	32.45 to 40.31

doi:10.1371/journal.pone.0029458.t008

lacrimal gland acinar cells can retain their functionality of secreting major tear proteins like lysozyme, scIgA and lactoferrin (Figure 7 and 8). This secretory profile has also been quantified by sandwich ELISA technique using the calibration curve generated for each of the three proteins. The calibration curve was generated in two sets of experiments in which readings were taken in triplicates and since the optical density values for the remaining three sets were very similar, the same calibration curve was used to estimate the protein quantity. Our results show that the secretion of these proteins ranges from 3.86 to 71.4 ng/ml of scIgA, 5.78 to 33.94 ng/ml of lysozyme and 44.5 to 45.65 ng/ml of lactoferrin on HAM, to 1.41 to 27.58 ng/ml of scIgA, 0.21 to 18.34 ng/ml of lysozyme and 28.52 to 30.41 ng/ml of lactoferrin on collagen I coated dishes to 47.43 to 61.56 ng/ml of scIgA, 24.36 to 144.74 ng/ml of lysozyme and 32.45 to 40.31 ng/ml of lactoferrin on Matrigel^{TM} coated dishes (Table 8) in day 7 cultures. These cultures retain their secretory ability even till day 21. As anticipated, the amount of protein secretion tends to increase from day 7 to day 14 of *in-vitro* culture and then declines by day 21 (Table S2). The possible reason for this could be that by day 14 the cultures tend to attain confluence and so there are more number of acinar cells secreting the proteins; however post confluence the cells tend to reduce the amount of protein secretion either because on passaging these cells they acquire a more fibroblastic morphology or if they are left unpassaged they stop proliferating and hence protein synthesis also reduces. However, it was noted that the level of proteins secreted by cultures growing on Matrigel remains the highest in all the three cases. An interesting observation made here is that the *in-vitro* pattern of secretion of these proteins is the same as that seen *in-vivo* i.e. Lysozyme>scIgA>Lactoferrin [37].We do observe a wide range in the quantity of proteins secreted *in-vitro*. One of the possible reasons for this could be the experimental variables like the age of donor tissue. In the present study, tissue was harvested from exenterated specimens of patients with an age range of 3 years to 65 years. Since the lacrimal protein secretions tend to reduce with increasing age we feel this could be an important factor contributing to such a wide range in quantity of protein secreted *in-vitro*.

Both lacrimal and salivary gland acinar cells share similar developmental, morphologic and functional characteristics and show similar reduction in function when injured [38,39,40] but unlike the salivary gland where there is evidence of stem/progenitor cell compartment in the terminal ductioles [17], such evidence is restricted to one preliminary report of stem cells in the mouse lacrimal gland which can be recruited to repair damage to the gland and which can subsequently be cultured in-vitro [24]. Our results give preliminary evidence indicating that the stem-like cells are present in the lacrimal gland but their exact locations is still under investigation. The expression of these stem-like cells in the native tissue could range between 3.1±0.61% (ABCG2 expression) to 3.8%±1.26% (high ALDH1 expression) and between 0.3±0.15% (ABCG2 expression) to 2.7±1.54% (ALDH1 high expression) after 14–18 days of *in-vitro* culture. It is noteworthy that our culture system could maintain stem cells in-vitro even by day 21 though their numbers reduced to 0.2±0.13% (ABCG2) to 1.1±0.5% (ALDH1 high expression). The results of the immunohistochemistry experiments indicate that acinar cells which show strong positivity for vimentin could be the stem cells. However, for the lack of substantiating data, at the present time, no concrete inference can be drawn.

In order to substantiate the evidence of stem-like/progenitor cells in our culture system, clonal or similar stem cell assays have to be performed either with freshly isolated cells or cells obtained after sorting for the stem cell markers. While animal studies and clone forming ability would have added value to this study, the formation of spherules with 'duct-like' structures between them are important novel findings, which leads us to believe that we may be able to simulate an *in-vivo* mimicry under our culture conditions.

Even though dry eye syndrome has a global prevalence the treatment still remains palliative and conservative, with artificial tear substitutes and lubricants forming the main stay of clinical management. A similar spectrum of signs and symptoms exist in the case of dry mouth syndrome (xerostomia) caused due to damage to the salivary gland. This damage, similar to lacrimal gland, can be due to aging, hormonal imbalance or radiotherapy induced. The management strategy in such cases used to be palliative but recent reports in literature show the reversal of radiation-induced xerostomia with cell therapy in animal models [34]. The success obtained in these animal models of xerostomia has now shifted the focus towards exploring similar options, especially in conditions with irreparable damage to the lacrimal gland tissue.

In summary, this study provides the first evidence for the successful growth of fresh human lacrimal gland tissues *in-vitro* with an attempt towards duct-like formation and retained secretory function. The study also gives the first preliminary evidence for the presence of stem-like cells in the native human lacrimal gland tissue and these can be maintained under *in-vitro* condition. Further validation of this data would allow the development of a functionally competent 3 D construct for potential clinical application in severe cases of radiation induced dry eye.

Supporting Information

Figure S1 Pattern of epithelial cell growth on uncoated, HAM, collagen and Matrigel^{TM} coated dishes.
(TIF)

Table S1 Flow cytometric evaluation of cell population growing on uncoated, collagen coated and Matrigel^{TM} coated dishes.
(DOC)

Table S2 Tear protein secretion on various matrices post carbachol secretion on day 7, day 14 and day 21.
(DOC)

Acknowledgments

We wish to thank Mr Sreedhar Bowenpalli and Mr G.Chenchu Naidu for the technical assistance provided in immunohistochemistry experiments; Dr Shrikant R. Bharadwaj, Prof D Balasubramanian and Dr Robert P Coppes for their insightful comments on the manuscript.

Author Contributions

Conceived and designed the experiments: GKV ST. Performed the experiments: ST MMSB. Analyzed the data: ST GKV. Contributed reagents/materials/analysis tools: MJA MNN SGH VAPR. Wrote the paper: ST GKV.

References

1. Walcott B (1998) The Lacrimal Gland and Its Veil of Tears. News Physiol Sci 13: 97–103.
2. Dry Eye WorkShop (DEWS) (2007) The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007). Ocul Surf 5: 93–107.
3. Goto E, Yagi Y, Matsumoto Y, Tsubota K (2002) Impaired functional visual acuity of dry eye patients. Am J Ophthalmol 133: 181–186.
4. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL (2000) Reliability and validity of the Ocular Surface Disease Index. Arch Ophthalmol 118: 615–621.
5. Begley CG, Chalmers RL, Abetz L, Venkataraman K, Mertzanis P, et al. (2003) The relationship between habitual patient-reported symptoms and clinical signs among patients with dry eye of varying severity. Invest Ophthalmol Vis Sci 44: 4753–4761.
6. Tutt R, Bradley A, Begley C, Thibos LN (2000) Optical and visual impact of tear break-up in human eyes. Invest Ophthalmol Vis Sci 41: 4117–4123.
7. Calonge M (2001) The treatment of dry eye. Surv Ophthalmol 45 Suppl 2: S227–239.
8. Baudouin C (2001) The pathology of dry eye. Surv Ophthalmol 45 Suppl 2: S211–220.
9. Hakim SG, Schroder C, Geerling G, Lauer I, Wedel T, et al. (2006) Early and late immunohistochemical and ultrastructural changes associated with functional impairment of the lachrymal gland following external beam radiation. Int J Exp Pathol 87: 65–71.
10. Hosaka E, Kawamorita T, Ogasawara Y, Nakayama N, Uozato H, et al. (2011) Interferometry in the evaluation of precorneal tear film thickness in dry eye. Am J Ophthalmol 151: 18–23 e11.
11. Perry HD (2008) Dry eye disease: pathophysiology, classification, and diagnosis. Am J Manag Care 14: S79–87.
12. Hann LE, Tatro JB, Sullivan DA (1989) Morphology and function of lacrimal gland acinar cells in primary culture. Invest Ophthalmol Vis Sci 30: 145–158.
13. Rismondo V, Gierow JP, Lambert RW, Golchini K, Feldon SE, et al. (1994) Rabbit lacrimal acinar cells in primary culture: morphology and acute responses to cholinergic stimulation. Invest Ophthalmol Vis Sci 35: 1176–1183.
14. Ueda Y, Karasawa Y, Satoh Y, Nishikawa S, Imaki J, et al. (2009) Purification and characterization of mouse lacrimal gland epithelial cells and reconstruction of an acinarlike structure in three-dimensional culture. Invest Ophthalmol Vis Sci 50: 1978–1987.
15. Schechter J, Stevenson D, Chang D, Chang N, Pidgeon M, et al. (2002) Growth of purified lacrimal acinar cells in Matrigel raft cultures. Exp Eye Res 74: 349–360.
16. Yoshino K, Tseng SC, Pflugfelder SC (1995) Substrate modulation of morphology, growth, and tear protein production by cultured human lacrimal gland epithelial cells. Exp Cell Res 220: 138–151.
17. Man YG, Ball WD, Marchetti L, Hand AR (2001) Contributions of intercalated duct cells to the normal parenchyma of submandibular glands of adult rats. Anat Rec 263: 202–214.
18. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, et al. (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50: 521–533.
19. Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, et al. (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22: 1115–1124.
20. Tsujimura A, Koikawa Y, Salm S, Takao T, Coetzee S, et al. (2002) Proximal location of mouse prostate epithelial stem cells: a model of prostatic homeostasis. J Cell Biol 157: 1257–1265.
21. Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125: 1921–1930.
22. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, et al. (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17: 1253–1270.
23. Zoukhri D, Fix A, Alroy J, Kublin CL (2008) Mechanisms of murine lacrimal gland repair after experimentally induced inflammation. Invest Ophthalmol Vis Sci 49: 4399–4406.
24. You S, Kublin CL, Avidan O, Miyasaki D, Zoukhri D (2011) Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Invest Ophthalmol Vis Sci 52: 2087–2094.
25. Kim JC, Tseng SC (1995) Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14: 473–484.
26. Fatima A, Sangwan VS, Iftekhar G, Reddy P, Matalia H, et al. (2006) Technique of cultivating limbal derived corneal epithelium on human amniotic membrane for clinical transplantation. J Postgrad Med 52: 257–261.
27. Williams GM, Gunn JM (1974) Long-term cell culture of adult rat liver epithelial cells. Exp Cell Res 89: 139–142.
28. Karbanova J, Missol-Kolka E, Fonseca AV, Lorra C, Janich P, et al. (2008) The stem cell marker CD133 (Prominin-1) is expressed in various human glandular epithelia. J Histochem Cytochem 56: 977–993.
29. Chen L, Glass JD, Walton SC, Laurie GW (1998) Role of laminin-1, collagen IV, and an autocrine factor(s) in regulated secretion by lacrimal acinar cells. Am J Physiol 275: C278–284.
30. Hann LE, Kelleher RS, Sullivan DA (1991) Influence of culture conditions on the androgen control of secretory component production by acinar cells from the rat lacrimal gland. Invest Ophthalmol Vis Sci 32: 2610–2621.
31. Schonthal AH, Warren DW, Stevenson D, Schecter JE, Azzarolo AM, et al. (2000) Proliferation of lacrimal gland acinar cells in primary culture. Stimulation by extracellular matrix, EGF, and DHT. Exp Eye Res 70: 639–649.
32. Schrader S, Wedel T, Kremling C, Laqua H, Geerling G (2007) Amniotic membrane as a carrier for lacrimal gland acinar cells. Graefes Arch Clin Exp Ophthalmol 245: 1699–1704.
33. Long L, Liu Z, Wang T, Deng X, Yang K, et al. (2006) Polyethersulfone dead-end tube as a scaffold for artificial lacrimal glands in vitro. J Biomed Mater Res B Appl Biomater 78: 409–416.
34. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, et al. (2008) Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One 3: e2063.
35. Shi X, Gipp J, Bushman W (2007) Anchorage-independent culture maintains prostate stem cells. Dev Biol 312: 396–406.
36. Schrader S, Kremling C, Klinger M, Laqua H, Geerling G (2009) Cultivation of lacrimal gland acinar cells in a microgravity environment. Br J Ophthalmol 93: 1121–1125.
37. Tiffany JM (2008) The normal tear film. Dev Ophthalmol 41: 1–20.
38. Konings AW, Coppes RP, Vissink A (2005) On the mechanism of salivary gland radiosensitivity. Int J Radiat Oncol Biol Phys 62: 1187–1194.
39. Parsons JT, Bova FJ, Mendenhall WM, Million RR, Fitzgerald CR (1996) Response of the normal eye to high dose radiotherapy. Oncology (Williston Park) 10: 837–847; discussion 847–838, 851–832.
40. Stephens LC, Schultheiss TE, Price RE, Ang KK, Peters LJ (1991) Radiation apoptosis of serous acinar cells of salivary and lacrimal glands. Cancer 67: 1539–1543.

Metadata of the chapter that will be visualized online

Series Title		
Chapter Title	Lacrimal Gland Regeneration: Progress and Promise	
Chapter SubTitle		
Copyright Year	2013	
Copyright Holder	Springer Science + Business Media Dordrecht	

Corresponding Author	Family Name	Vemuganti
	Particle	
	Given Name	**Geeta K.**
	Suffix	
	Division	School of Medical Sciences
	Organization	University of Hyderabad
	Address	Prof C R Rao Road, 500 046, Gachibowli Hyderabad, India
	Email	deanmd@uohyd.ernet.in; gvmd@uohyd.ernet.in
Author	Family Name	Tiwari
	Particle	
	Given Name	**Shubha**
	Suffix	
	Division	Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory
	Organization	Prof Brien Holden Research Centre, L. V. Prasad Eye Institute
	Address	Kallam Anji Reddy Campus, L. V Prasad Marg, 500 034, Hyderabad, India
	Email	

Abstract	The lacrimal gland is a tubuloacinar gland located in the groove of the frontal bone, which is involved in synthesis and secretion of major tear proteins and other aqueous components of the trilayered tear film. Any injury to the lacrimal gland, which may be age related, drug or radiation therapy induced leads to dysfunction of the gland with a resultant hyperosmolarity of the tear film and its subsequent instability. This tear film instability leads to destabilization of the ocular surface homeostasis and to a number of morbid complications like the dry eye syndrome. The current treatment modality for chronic dry eye remains palliative, which provides only temporary symptomatic relief. One of the modalities of providing long -term benefit to these patients would be cell therapy to restore or replenish the damaged gland. This review describes the progress and promise of cell therapy for lacrimal gland regeneration for potential clinical application.

Chapter 30
Lacrimal Gland Regeneration: Progress and Promise

Shubha Tiwari and Geeta K. Vemuganti

Abstract The lacrimal gland is a tubuloacinar gland located in the groove of the frontal bone, which is involved in synthesis and secretion of major tear proteins and other aqueous components of the trilayered tear film. Any injury to the lacrimal gland, which may be age related, drug or radiation therapy induced leads to dysfunction of the gland with a resultant hyperosmolarity of the tear film and its subsequent instability. This tear film instability leads to destabilization of the ocular surface homeostasis and to a number of morbid complications like the dry eye syndrome.

The current treatment modality for chronic dry eye remains palliative, which provides only temporary symptomatic relief. One of the modalities of providing long -term benefit to these patients would be cell therapy to restore or replenish the damaged gland. This review describes the progress and promise of cell therapy for lacrimal gland regeneration for potential clinical application.

30.1 Introduction

The human lacrimal gland is a tubuloacinar, almond shaped gland located superior and lateral to the eye in the shallow depression of the frontal bone. The lacrimal gland can be divided into the main lacrimal gland (orbital and palpebral portions) and the accessory lacrimal glands (Gland of Wolfring and Krauss). The lacrimal

S. Tiwari, M.Pharm.
[AU1] Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory,
Prof Brien Holden Research Centre, L. V. Prasad Eye Institute,
Kallam Anji Reddy Campus, L. V Prasad Marg, Hyderabad 500 034, India

G.K. Vemuganti, M.D., DNB, FAMS, FICP (✉)
School of Medical Sciences, University of Hyderabad,
Prof C R Rao Road, Gachibowli, Hyderabad 500 046, India
e-mail: deanmd@uohyd.ernet.in; gvmd@uohyd.ernet.in

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient*,
DOI 10.1007/978-94-007-5690-8_30,

Fig. 30.1 Layers of tear film

gland forms an important entity of the lacrimal functional unit (LFU), which comprises of the lacrimal gland, the ocular surface (cornea, conjunctiva and the meibomian gland) and the sensory and motor nerves that connect them. The LFU controls the secretion of the major components of the tear film and is overall responsible for maintaining the stability of the tear film, transparency of the cornea and the quality of the image projected onto the retina (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007).

Tear film is constituted of the secretions of the lacrimal gland, meibomian gland and the conjunctival goblet cells. It has three basic layers: aqueous, which is 3–8 μm thick and is composed of the secretions of the lacrimal gland; lipid, which is 0.2 μm thick and is secreted by the meibomian gland; and the mucin layer, 1 μm thick, secreted mainly by the conjunctival goblet cells (Fig. 30.1). The important constituents of human tear are electrolytes like sodium, potassium, calcium, magnesium, bicarbonate and chloride; and major proteins like lysozyme, lipocalin, lactoferrin, scIgA, albumin and IgG (Table 30.1). Other components of tear film include lipids like phosphatidylcholine and phosphatidylethanolamine; mucins like MUC4, MUC5AC, MUC1. Minor components like defensins, catalase cytokines also form a part of the tear film composition (Tiffany 2008).

The tear film also contributes to the transparency of the cornea and determines the quality of image projected on the retina for cortical sensing. The periocular tear film is also responsible for providing nutrition to the cornea by acting as a coupling medium for the environmental oxygen; protecting the ocular surface due to the

Table 30.1 Composition of normal human tears

Composition	Concentration
Electrolytes	mmol/l
Sodium	128.7
Potassium	17
Calcium	0.32
Magnesium	0.35
Bicarbonate	12.4
Chloride	141.3
Major proteins	mg/l
Lysozyme	2.07
Lactoferrin	1.65
scIgA	1.93
Lipocalin	1.55
Albumin	0.04
IgG	0.004

antimicrobial properties of lysozyme, lactoferrin and lipocalin present; and also providing physical protection to the ocular surface against the shearing force of blinking due to the mucins present in it (Tiffany 2008).

30.2 Embryology and Development

The development of the human lacrimal gland has been the subject of numerous studies since the early 1900s. Most of these studies, other than the one published by Tripathi and Tripathi (1990), report that the gland develops from the ectoderm of the superior conjunctival fornix in human embryos with a crown to rump length of 22–24 mm (de la Cuadra-Blanco et al. 2003).

The two main lacrimal gland lobes – the orbital and the palpebral lobes – originate not simultaneously but one after the other. The orbital lobe originates from the proliferation of conjunctival fornix epithelial cells in the form of five or six epithelial buds and its formation concludes by the end of the second month. This is followed by initiation of the palpebral lobe formation. The orbital and the palpebral lobes are separated by the levator muscle tendon, which forms during the third week of development.

Epithelial-mesenchymal interaction, with its associated cell signaling, has been considered by a number of authors to be responsible for morphogenesis, organogenesis, cell differentiation and growth (Sanders 1988; Martin 1998; Makarenkova et al. 2000). Lacrimal gland development is an example of such an interaction in which the bud-like invagination of conjunctival epithelium at the fornix is the process, which initiates lacrimal gland development (Kammandel et al. 1999). The mesenchymal cells surrounding the point of epithelial budding are the periocular cells of

neural crest origin (Johnston et al. 1979). The tubular invaginations of the lacrimal gland extends and branches multiple times to form the lobular structure of the mature lacrimal gland.

30.3 Histology, Anatomy and Physiology

The lacrimal gland is a tuboacinar gland that consists of secretory epithelium arranged in a lobular pattern. These secretory acinar cells empty their secretions into ducts that anastomose into a larger nasolacrimal (NLD) duct which drains onto the ocular surface. Enveloping the secretory acinar cells are myoepithelial cells that contract and squeeze them facilitating the draining of the secretory components into the ducts. Between the lacrimal lobes are fibroblasts, which produce the collagen and matrix of interstitial regions, and mast cells, which secrete histamine and heparin (Fig. 30.2). In addition to this basic tissue architecture, the lacrimal gland is highly inundated with trafficking B and T lymphocytes as well as plasma cells (Walcott 1998).

The secretory acinar cells of the gland are columnar epithelium with basally located nucleus and a large perinuclear Golgi body. The ductal cells are more cuboidal in shape. The apical portion of the acinar and ductal cells has a number of vesicles and the cell base has an associated basement membrane that imparts the cells their polarity. Large junctional complex is found near the luminal pole that couples these cells electrically and chemically as well as mechanically attaches them with each other. Gap junctions like connexin 26 and 32 are also found here. The presence of a large number of junctional complexes between the epithelial cells indicate that these cells are very closely associated with each other (Walcott 1998).

The lacrimal gland is innervated by the sympathetic as well as the parasympathetic arms of the autonomic nervous system (Matsumoto et al. 1992). These nerves have a large number of cholinergic fibers and fewer adrenergic fibers. The parasympathetic

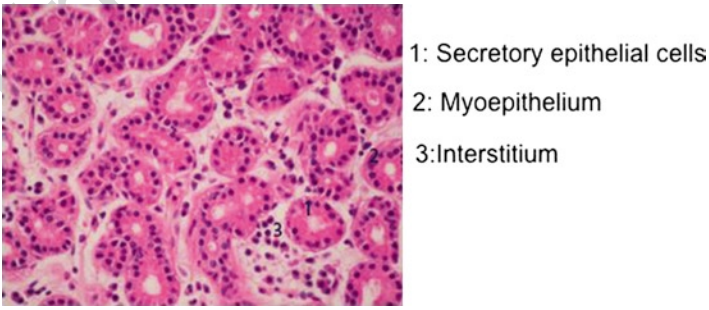

Fig. 30.2 Histology of normal human lacrimal gland *1* Secretory epithelial/acinar cells that synthesize and secrete major tear proteins. *2* Myoepithelial cells that envelope the acinar cells. *3* Interstitium that has fibroblasts that secretes collagen and other extracellular matrix

postganglionic neural cell bodies are found in the pterygopalatine (sphenopalatine) ganglion as well as the ciliary ganglion. Sympathetic fibers arise in the superior cervical ganglion. There is also some amount of sensory innervation of the gland from the trigeminal ganglia (van der Werf et al. 1996). Even though the innervation is similar across different species yet the nature and pattern of innervations as well as the pathway from these ganglia to the gland vary significantly from species to species.

The lacrimal gland secretes a number of proteins like lysozyme, lactoferrin, lipocalin, scIgA (Tiffany 2008). The secretion of these proteins is regulated by the nerves that innervate the gland and their associated nurotransmitters/neuropeptides (Walcott 1998). The important receptors present on the lacrimal gland are acetylcholine receptors like muscarinc M3 (Mauduit et al. 1993), vasoactive intestinal peptide type I and II, norepinephrine like alpha 1 and beta. Other receptors present are for neuropeptide Y, adenocorticotrophic hormone (ACTH) and alpha-melanocyte stimulating hormone. Since the epithelial cells of the gland are extensively coupled by junctional complexes, secondary messengers like inositol triphophate can easily diffuse between cells and activate the unstimulated cells too (Walcott 1998).

The muscarinic receptors in the gland are linked to G proteins, which are in turn linked to phospholipase C. This, on activation, releases inositol phosphate 3 (IP3) and diacyl glycerol (DAG) (Dartt 1989). IP3 induces the release of intracellular stores of calcium and opens calcium channels. DAG, on the other hand, activates protein kinase C isoenzymes, which further stimulates secretion. VIP receptors activate protein kinase A, which in turn causes cAMP release stimulating protein secretion (Hodges et al. 1997). Alpha adrenergic compounds cause protein secretion by activating protein kinase C (Walcott 1998).

Protein secretion in the acinar cells involves the fusion of vesicles with the apical membrane. There is also a basolateral membrane trafficking that is seen in these cells. This is responsible for the entry of molecules like prolactin into the cells. In addition, this basolateral membrane trafficking has also been implicated in antigen presentation and secretion of autoantigens (Mircheff et al. 1994) which leads to immune mediated apoptosis of acinar cells and loss of physiological function as seen in conditions like Sjogren's syndrome.

30.4 The Dry Eye Syndrome

The lacrimal gland forms an integral part of the lacrimal functional unit (LFU) due to its physiological function of secreting the aqueous component of the tear film. Any perturbation in the functioning of this gland, which may be age related, drug induced, autoimmune or due to orbital radiotherapy leads to destabilization of the tear film which in turn leads to a chronic debilating condition called the dry eye syndrome or keratoconjunctivitis sicca (KCS).

The International Dry Eye Workshop, 2007 (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007) defined dry eye as:

> Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of tear film and inflammation of the ocular surface.

The most important causative/contributing factors for dry eye are (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007):

- Primary lacrimal gland dysfunction due to reduction in circulating androgens
- Secondary lacrimal gland dysfunction due to sarcoidosis, lymphoma etc.
- Autoimmune diseases like Sjogren's syndrome
- Reflex hyposecretion as in contact lens wear, diabetes, exposure to systemic drugs like antihistamines, beta blockers etc.
- Orbital radiotherapy for ocular malignancies
- Meibomian gland dysfunction

30.5 Etiology of Dry Eye Syndrome

Dry eye may be classified as aqueous deficient dry eye, caused due to lacrimal gland dysfunction; or evaporative dry eye, caused due to meibomian gland dysfunction. In the former, there is a deficiency in tear production itself due to perturbations in lacrimal gland function; and in evaporative dry eye, the rate of evaporation of tear from the ocular surface increases due to an unstable lipid film secreted by the damaged meibomian gland (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007).

There has been a considerable increase in knowledge about the etiopathogenesis of dry eye syndrome in the past few years. The pathologic features of this condition include increased epithelial proliferation, stratification and abnormal differentiation with maintenance of a basal phenotype (Jones et al. 1998). This is accompanied by reduced expression of secretory and membrane-bound mucins by the ocular surface conjunctival epithelial cells (Danjo et al. 1998) compounding the effects of existing lacrimal dysfunction.

The two most important factors that contribute to the initiation and progression of dry eye are *tear hyperosmolarity* and *tear film instability* that adversely affects the ocular surface epithelial function and differentiation (Gilbard et al. 1989a, b). [AU2] Trauma to a poorly lubricated and unprotected ocular surface due to blinking or environmental factors becomes a confounding factor, which worsens the condition (Lemp 1995; Danjo et al. 1998). Tear film stability, which is important in maintaining clear and sharp vision, is threatened when the interactions between stabilizing

tear film constituents are compromised either by decreased tear secretion, delayed clearance, or altered tear composition as is seen in xerophthalmia and allergic eye diseases. Ocular surface inflammation is secondary consequence. Reflex tear secretion in response to ocular irritation is seen as the initial compensatory mechanism, but, with time, due to severe inflammation and chronic secretory dysfunction a decrease in corneal sensation occurs which compromises the reflex response and results in even greater tear film instability. Dysfunction of the LFU is considered to play an important role in the evolution of different forms of dry eye (The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop 2007).

Even though tear fluid is secreted as a hypotonic fluid, yet due to excessive evaporation from the exposed ocular surface or low rate of aqueous tear flow (or a combination of the two) tear hyperosmolarity may arise. This hyperosmolarity stimulates various inflammatory pathways involving the MAP kinases, inflammatory cytokines (interleukins, tumor necrosis factor alpha) and matrix metalloproteinases (MMP9). This cascade of events attracts the circulating T cells within the lacrimal glands. Under physiological conditions, the trafficking lymphocytes, finding no inflammation, would undergo apoptosis. However, in the presence of inflammatory signals, these lymphocytes become activated and secrete pro-inflammatory cytokines, which cause homing of additional T cells to the tissue and an increase in the level of inflammation thereby aggravating the existing condition and forming a vicious loop of inflammation (Fig. 30.3) (Gao et al. 1998). Any condition that results in hyper activity of the functional unit can also initiate inflammatory response within the lacrimal glands resulting in antigen presentation and cytokine secretion by the epithelial cells of the gland (Meggs 1993; Mircheff et al. 1998). These pro-inflammatory mediators cause epithelial cell death by apoptosis and also loss of conjunctival goblet cells – a combinatorial assault that leads to worsening of the existing condition.

30.6 Aqueous Deficient Dry Eye

In addition to release of inflammatory mediators, the etiology of dry eye also involves the loss of anti-inflammatory environment within the lacrimal glands, which may occur due to a drop in the levels of circulating androgens (Sullivan et al. 1984; Azzarolo et al. 1997). At the time of menopause in women, or due to various pathologic conditions, the level of circulating androgens may drop below a threshold level, thus making the tissues vulnerable for the initiation and progression of immune-based inflammation. A number of studies have shown a significant correlation between the levels of these inflammatory cytokines and the severity of ocular surface irritation symptoms, corneal fluorescein staining and the severity of conjunctival squamous metaplasia in patients (Pflugfelder et al. 1999).

Severe dry eye is also seen in patients of Sjogren's syndrome, which is an autoimmune disorder. In Sjogren's, autoantigens are expressed at the surface of the

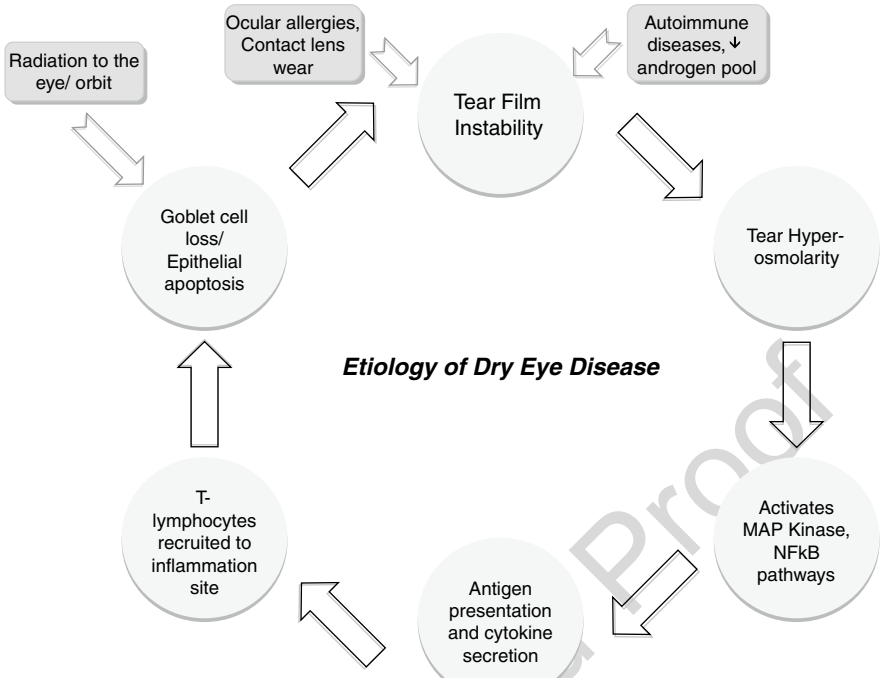

Fig. 30.3 Etiology of dry eye disease

epithelial cells which causes homing and retention of tissue specific CD4 and CD8 cells. These lymphocytes cause loss of lacrimal acinar and ductal cells due to an immune mediated attack leading to tear hyposecretion and destruction of the gland. The precise trigger factors for Sjogren's is not known but risk factors include genetic profile, low androgen pool and certain viruses.

Radiation therapy, which represents a commonly used modality in the treatment of ocular and oculoadenexal disorders including benign and malignant tumors, also contributes to the development of dry eye syndrome in patients. Despite a rapid evolution in the field of radiotherapy over the past years, a significant number of patients are still seen with acute and chronic ophthalmic complications including severe dry eye (Alberti 1997; Durkin et al. 2007). Preliminary data from our institute indicates that chronic dry eye develops in over 49 % of the patients who undergo external beam radiation therapy for ocular malignancies (unpublished data).

The causes of dry eye post-radiotherapy are multifactorial: (a) decrease in the lacrimal secretion leading to loss of aqueous layer; (b) ocular surface damage with goblet cell loss leading to loss of mucin secretion; (c) meibomian gland atrophy with loss of lipid layer secretion. In the early post-radiotherapy phase, lacrimal gland loss has been shown to be due to inflammation mediated apoptotic loss of acinar cells. In contrast, stem cells becoming sterile is proposed to be the main

cause in late phase resulting in insufficient replacement of acinar cells with resultant decrease in tear secretion. This combined with the radiation induced fibrosis leads to loss of lacrimal function with progressive decrease in tear volume and finally dry eye (Stephens et al. 1991; Parsons et al. 1996; Barabino et al. 2005; Konings et al. 2005).

Contact lens wear is yet another condition that may lead to the development of severe dry eye in long-term users. The mechanism underlying the development of dry eye in these patients is probably the reduction in corneal sensitivity and an increase in tear film osmolarity due to chronic usage. Similar arguments have been proposed for the development of dry eye post-LASIK therapy.

30.7 Current Therapies for KCS

Even though there has been significant advancement in the knowledge about lacrimal gland dysfunction and development of dry eye syndrome, yet the same cannot be said for the management of the condition. Dry eye is still a chronic debilating condition, the treatment and management of which aims at palliation and improving the quality of life of the patient. The current treatment modalities available are lubricating agents like hydroxymethylcellulose, solutions containing bicarbonates and potassium, hyposmotic artificial tears (Hypotears, Novartis Ophthalmics) and artificial serum. In cases of severe dry eye, therapies such as anti-inflammatory medications (cyclosporins A, corticosteroids), pharmacological tear stimulants like diquafosol, rebamipide, ecabet sodium, pilocarpine etc. are employed. In certain instances, where the patient does not get any relief in symptoms by these, surgical interventions like punctal occlusion and salivary gland autotransplantation are done to slow down the progress of the condition (Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop 2007).

On recommendation of the sub-committee on the therapy and management of dry eye, DEWS 2007 (Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop 2007), the treatment/management protocol for this condition is now shifting towards employing strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce/eliminate the levels of existing inflammation. With these objectives in mind various therapeutic avenues are being explored with the inclusion of cell therapy for restoring the damaged lacrimal gland.

30.8 Research on Lacrimal Gland

Given the sparsity of data on the etiopathogenesis and treatment of dry eye, it is still not clear how alteration in tear film composition can cause such a vicious cycle of tear film instability and chronic ocular inflammation. Even though a lot of research

is being directed towards profiling the proteins, lipids and other constituents present in human tear yet there is a glaring lack of comparative data between normal individuals and dry eye patients.

30.8.1 Animal Studies

30.8.1.1 *In-Vitro* Cultures

An important area of investigation in this field is to find a common link between tear film osmolarity, tear film break up response and the resultant inflammatory stress. In order to facilitate these studies, not just *in-vivo* models but also *in-vitro* models are being developed that would greatly assist the investigation into the secretory repertoire of lacrimal gland epithelia, regulation of secretion and etiopathogenesis of lacrimal gland conditions like Sjogren's syndrome.

Procedure for *in-vitro* culturing of lacrimal gland acinar cells has been evolving for nearly two decades now. Oliver et al. published one of the first reports on *in-vitro* culture of rat lacrimal gland acinar cells in 1987 wherein they described a method for culturing a dividing population of morphologically differentiated rat lacrimal acinar cells on a three-dimensional, reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules (Oliver et al. 1987). However, their culture system could only maintain the epithelial cells for 6–7 days after which fibroblast overgrowth was observed. Successful *in-vitro* culture of lacrimal acinar cells was first achieved and published by Meneray and Rismondo in two separate reports in 1994 (Meneray et al. 1994; Rismondo et al. 1994). The importance of media formulation, supplement profile and extracellular matrix composition for optimal growth and functionality of these cells was first reported by Hann et al. (1991) and these findings were supported by a number of subsequent reports.

A major problem faced by all these investigators was that the lacrimal acinar cells could not be induced to proliferate significantly *in-vitro*. This issue was resolved by Schonthal et al., who reported in 2000 that the i*n-vitro* proliferation of lacrimal acinar cells could be improved significantly by the use of EGF, dihydrotestosterone (DHT), Matrigel and HepatoStim culture medium (Schonthal et al. 2000). Recent studies report the use of polyethersulfone dead-end tube (Long et al. 2006), denuded amniotic membrane (Schrader et al. 2007) as scaffolds and rotary cell culture system (Schrader et al. 2009) for successful *in-vitro* culture of rat or rabbit lacrimal glands.

The effect of androgen on the control of secretory component output by the lacrimal gland has been well established. The effect of androgens and androgen analogues on *in-vitro* culture of lacrimal acinar cells has helped elucidate the control that the androgens exert on the synthesis and secretion of secretory component (Sullivan et al. 1984, 1990; Hann et al. 1991; Kelleher et al. 1991) as well as other

biochemical parameters related to the lacrimal secretion including the basal tear flow rate (Azzarolo et al. 1997).

The culture systems developed for the lacrimal acinar cells have also been optimized to assess the functionality of these cells. The currently employed conditions for the *in-vitro* culture of these secretory cells support the *in-viv*o mimicry of their secretion pattern as elucidated by the detection of scIgA, lactoferrin, lysozyme, lacritin and a number of other tear proteins in the culture supernatant.

The last couple of years have seen an increase in the knowledge about the presence of stem-like cells in the lacrimal gland of mice (You et al. 2011), rat (Shatos et al. 2012) and humans (Tiwari et al. 2012). These studies indicate the inherent potential of the gland to heal itself following an insult. The study published by You et al. (2011) showed that post injection of interleukin into the mouse lacrimal gland which destroys areas in the gland, stem-like cells migrate towards the site of injury and heal the wound. These cells can be harvested and grown under *in-vitro* conditions too. However, the authors report minimum *in-vitro* growth from uninjured gland. In contrast, the recent study by Shatos et al.(2012) on rat lacrimal gland and our own experience with human lacrimal gland showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in-vitro* conditions.

The presence of stem cells in the lacrimal gland is an important finding that leads us to believe that these cells can be recruited to salvage the damaged gland. However, before we take a leap of faith the viability, homing and functionality of these cells need to be established by more extensive *in-vitro* studies and independent animal experimentation.

30.8.1.2 Animal Models

In order to better understand this condition, animal models have been developed which mimic the features of human dry eye syndrome. The important animal models that have increased our knowledge of this condition are:

Mouse models created using scopolamine and environmental dessicating stress show that osmolarity of tear as well as secretion of inflammatory cytokines is increased under such condition (Gilbard et al. 1989a, b; Stewart et al. 2005).

Mouse model of Sjogren syndrome dry eye have also been developed and it has been shown in these models that androgens have the potential to reduce the inflammatory response due to autoantigen presentation.

Neurturin deficient mice that develop dry eye and show elevated levels of inflammatory mediators in their tears (Song et al. 2003).

Rabbit model of KCS created using the technique of ablation, which shows that steroids like dexamethasone can be used to reverse the ocular surface damage and also to increase the low tear film break up time (Nagelhout et al. 2005).

Even though it is undeniable that these animal models have indeed increased our basic understanding behind the etiopathology of dry eye, yet the fact remains that

t2.1 **Table 30.2** *In-vitro* lacrimal gland research: Information matrix

t2.2 Year	Species	*In-vitro* research	References
t2.3 1987	Rat	Established culture	Oliver et al. (1987)
t2.4 1991	Rat	Importance of media & growth factors for in-vitro cultures	Hann et al. (1991)
t2.6 1994	Rabbit	Physiologically responsive *in-vitro* cultures	Menerey et al. (1994) Rismondo et al. (1994)
t2.8 2000	Rabbit	Purified acinar cell preparation	Guo et al. (2000)
t2.9 2000	Rabbit	Use of EGF, DHT, Matrigel and HepatoSTIM for culture	Schonthal et al. (2000)
t2.11 2000	Human	*In-vitro* culture from cadaveric tissue	Yoshino (2000)
t2.13 1984	Rat	Effect of androgen	Sullivan et al. (1984, 1990)
t2.14 1990		on synthesis and secretion	Hann et al. (1991)
t2.15 1991		by lacrimal gland	Kelleher et al. (1991)
t2.16 2006	Rat	*In-vitro* culture in polyethersulfone dead end tube	Long et al. (2006)
t2.18 2007	Rabbit	*In-vitro* culture on amniotic membrane	Schrader et al. (2007)
t2.19 2009	Rabbit	Rotary cell culture system	Schrader et al. (2009)
t2.20 2011	Mouse	Report of mesenchymal stem cells in lacrimal gland post injury	You et al. (2011)
t2.22 2012	Human	Established functionally viable cultures from fresh tissue.	Tiwari et al. (2012)
t2.24		Preliminary report on presence of stem cells in native human lacrimal gland	
t2.27 2012	Rat	Progenitor cells in uninjured rat lacrimal gland	Shatos et al. (2012)

the extent to which these animal models mimic the human condition is not clear. In order to bridge this gap in understanding the similarity/differences between the animal form and the human form of the dry eye condition, it is imperative that studies be conducted on human tissue. Since it is unethical to undertake such studies without sufficient background information, *in-vitro* models of human lacrimal gland become very important tool for research.

30.8.2 Human Lacrimal Gland In-Vitro Cultures

In-vitro work on human lacrimal gland cultures is scarce, possibly due to the difficulty in obtaining human tissue for research. To the best of our knowledge and literature search, there is just one report published by Yoshino in 2000 (Yoshino 2000), which dealt with establishing human lacrimal cultures from cadaveric tissue. However, the study only reported the potential of these cells to secrete lactoferrin (Table 30.2). [AU3]

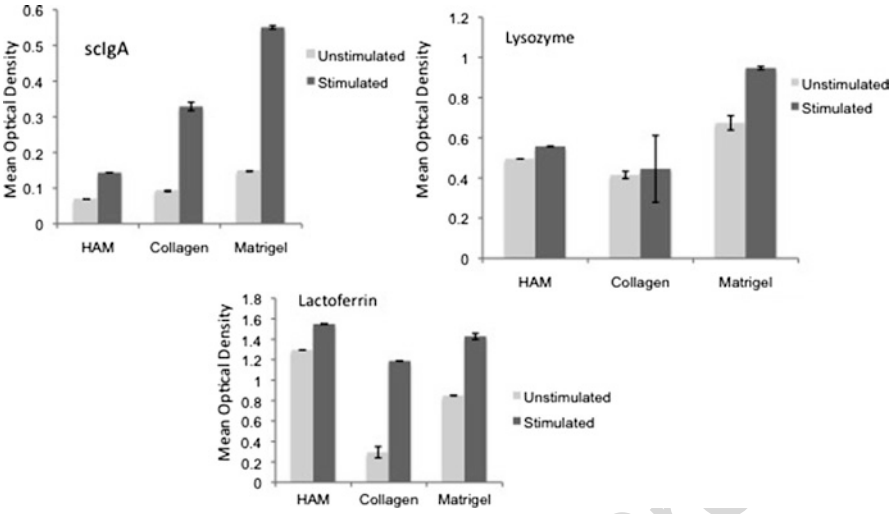

Fig. 30.4 Tear protein secretion by in-vitro human lacrimal gland cultures pre and post carbachol stimulation (Mean optical values can be correlated to protein levels)

Our group initiated work on human lacrimal gland cultures and reported the establishment of functionally viable human lacrimal gland *in-vitro* culture system from fresh exenteration specimens (Tiwari et al. 2012). The study also evaluated the growth of these cells in on three matrices: Matrigel, collagen and denuded human amniotic membrane. The cultured cells showed three distinct morphologies: the cells with cobblestone epithelial morphology showed positivity for epithelial markers like CK3/12 and E-cadherin, the spindle shaped fibroblasts were positive for CD90 and vimentin; and cells with a 'whorled' epitheloid morphology were positive for myoepithelial markers GFAP and S-100. One of the important contributions of this paper is the evaluation of the conditioned media, which provides evidence that the cultured cells synthesize and secrete quantifiable levels of major tear proteins (statistically significantly more than negative controls) like scIgA, lactoferrin and lysozyme into the culture supernatant (Fig. 30.4). These cells could be maintained *in-vitro,* with intact secretory function, for a minimum period of 21 days. The cells show maximum levels of protein secretion by day 14 and then a decline over a period of time as expected. In addition, by day 16–18, these *in-vitro* cultures show the appearance of spherules and structures that look like ductal connections between them. Though further studies are warranted, the preliminary evidence points towards *in-vitro* gland formation.

Towards the long-term goal of cell therapy in chronic dry eye condition, it would be important to evaluate if these cells could be sustained. Using FACS and immunocytochemistry, we observed presence of stem-like cells (ABCG2 positive, ALDH high) in the native human lacrimal gland as well as in our *in-vitro* cultures. The cell

suspension obtained from native gland (prior to culturing) show 3.1±0.61% the number of cells that show ABCG2 positivity by day 14 is 0.3±0.15%, which decreases slightly to 0.2±0.13% by day 21.

30.9 Conclusion

Dry eye can be an extremely debilitating condition with high incidence of associated morbidity. The current prevalence of dry eye in the world is estimated at around 11–22% (Abelson et al. 2009). In the Indian context, these numbers are estimated to be around 18.4–20% (Sahai and Malik 2005; Gupta et al. 2008). These epidemiological numbers are a good indicator of the need for research on dry eye syndrome.

Even though we have come a long way in managing the chronic dry eye patients and improving their quality of life, yet there are many existing gaps in literature and a lot more needs to be done for these patients therapeutically. Large, well-defined, staged and age-matched studies that provide further insights into pathobiology of the disease progression and pinpoint to predictive biomarkers in tears would pave way for specific treatment.

A lot of what we know of KCS today has been by correlating animal data to human scenario. In order to lend credibility to the accrued knowledge, it is essential that a comparative study be made between human and mouse/rabbit tears and ocular surface protein- lipid profiles. This would enable us to identify the common components and pathways involved in various forms of this disorder and would also give important clues about the treatment of this condition.

Finally, restoration of physiological function can possibly be achieved by replenishing the stores of damaged tissue, hence cell therapy for chronic cases of dry eye appears a promising alternative.

Acknowledgements The authors wish to thank the patients who donated their exenterated tissue for research, the surgical oncology team of Dr Santosh G. Honavar, Dr Milind N.Naik, Dr Javed Ali and Dr Vijay Anand Reddy. We also thank the Director of Research, LVPEI, Dr D. Balasubramanian, for his constant support and encouragement.

Method of literature search

A search of Pubmed database (1979–till date) was conducted. Medline, Elsevier-EMBASE and Ophthalmic literature databases was also searched. The following key words were used: *Lacrimal gland/lacrimal in-vitro studies/dry eye syndrome.*

Additional sources include review of publications cited in other articles. Google search was also used to find publication that may be missed in the above databases.

Funding Agencies: International Atomic Energy Agency (IAEA), Department of Biotechnology (DBT), Hyderabad Eye Research Foundation (HERF), Champalimaud Translational Research Centre (C- Tracer), Sudhakar and Sreekanth Ravi Brothers

Financial Disclosure: None

Referenes

Abelson MB, Ousler GW 3rd, Maffei C (2009) Dry eye in 2008. Curr Opin Ophthalmol 20(4):282–286

Alberti W (1997) Acute and late side effects of radiotherapy for ocular disease: an overview. Front Radiat Ther Oncol 30:281–286

Azzarolo AM, Mircheff AK, Kaswan RL et al (1997) Androgen support of lacrimal gland function. Endocrine 6(1):39–45

Barabino S, Raghavan A, Loeffler J et al (2005) Radiotherapy-induced ocular surface disease. Cornea 24(8):909–914

Danjo Y, Watanabe H, Tisdale AS et al (1998) Alteration of mucin in human conjunctival epithelia in dry eye. Invest Ophthalmol Vis Sci 39(13):2602–2609

Dartt DA (1989) Signal transduction and control of lacrimal gland protein secretion: a review. Curr Eye Res 8(6):619–636

de la Cuadra-Blanco C, Peces-Pena MD, Merida-Velasco JR (2003) Morphogenesis of the human lacrimal gland. J Anat 203(5):531–536

Durkin SR, Roos D, Higgs B et al (2007) Ophthalmic and adnexal complications of radiotherapy. Acta Ophthalmol Scand 85(3):240–250

Gao J, Schwalb TA, Addeo JV et al (1998) The role of apoptosis in the pathogenesis of canine keratoconjunctivitis sicca: the effect of topical Cyclosporin A therapy. Cornea 17(6):654–663

Gilbard JP, Rossi SR, Heyda KG (1989a) Ophthalmic solutions, the ocular surface, and a unique therapeutic artificial tear formulation. Am J Ophthalmol 107(4):348–355

Gilbard JP, Rossi SR, Heyda KG (1989b) Tear film and ocular surface changes after closure of the meibomian gland orifices in the rabbit. Ophthalmology 96(8):1180–1186

Guo Z, Azzarolo AM, Schechter JE et al (2000) Lacrimal gland epithelial cells stimulate proliferation in autologous lymphocyte preparations. Exp Eye Res 71(1):11–22

Gupta N, Prasad I, Himashree G et al (2008) Prevalence of dry eye at high altitude: a case controlled comparative study. High Alt Med Biol 9(4):327–334

Hann LE, Kelleher RS, Sullivan DA (1991) Influence of culture conditions on the androgen control of secretory component production by acinar cells from the rat lacrimal gland. Invest Ophthalmol Vis Sci 32(9):2610–2621

Hodges RR, Zoukhri D, Sergheraert C et al (1997) Identification of vasoactive intestinal peptide receptor subtypes in the lacrimal gland and their signal-transducing components. Invest Ophthalmol Vis Sci 38(3):610–619

Johnston MC, Noden DM, Hazelton RD et al (1979) Origins of avian ocular and periocular tissues. Exp Eye Res 29(1):27–43

Jones DT, Monroy D, Ji Z et al (1998) Alterations of ocular surface gene expression in Sjogren's syndrome. Adv Exp Med Biol 438:533–536

Kammandel B, Chowdhury K, Stoykova A et al (1999) Distinct cis-essential modules direct the time-space pattern of the Pax6 gene activity. Dev Biol 205(1):79–97

Kelleher RS, Hann LE, Edwards JA et al (1991) Endocrine, neural, and immune control of secretory component output by lacrimal gland acinar cells. J Immunol 146(10):3405–3412

Konings AW, Coppes RP, Vissink A (2005) On the mechanism of salivary gland radiosensitivity. Int J Radiat Oncol Biol Phys 62(4):1187–1194

Lemp MA (1995) Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J 21(4):221–232

Long L, Liu Z, Wang T et al (2006) Polyethersulfone dead-end tube as a scaffold for artificial lacrimal glands in vitro. J Biomed Mater Res B Appl Biomater 78(2):409–416

Makarenkova HP, Ito M, Govindarajan V et al (2000) FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development. Development 127(12):2563–2572

Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop (2007) Ocul Surf 5(2):163–178

Martin GR (1998) The roles of FGFs in the early development of vertebrate limbs. Genes Dev 12(11):1571–1586

Matsumoto Y, Tanabe T, Ueda S et al (1992) Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (Macaca fuscata). J Auton Nerv Syst 37(3):207–214

Mauduit P, Jammes H, Rossignol B (1993) M3 muscarinic acetylcholine receptor coupling to PLC in rat exorbital lacrimal acinar cells. Am J Physiol 264(6 Pt 1):C1550–C1560

Meggs WJ (1993) Neurogenic inflammation and sensitivity to environmental chemicals. Environ Health Perspect 101(3):234–238

Meneray MA, Fields TY, Bromberg BB et al (1994) Morphology and physiologic responsiveness of cultured rabbit lacrimal acini. Invest Ophthalmol Vis Sci 35(12):4144–4158

Mircheff AK, Gierow JP, Wood RL (1994) Traffic of major histocompatibility complex class II molecules in rabbit lacrimal gland acinar cells. Invest Ophthalmol Vis Sci 35(11):3943–3951

Mircheff AK, Gierow JP, Yang T et al (1998) Sjogren's autoimmunity: how perturbation of recognition in endomembrane traffic may provoke pathological recognition at the cell surface. J Mol Recognit 11(1–6):40–48

Nagelhout TJ, Gamache DA, Roberts L et al (2005) Preservation of tear film integrity and inhibition of corneal injury by dexamethasone in a rabbit model of lacrimal gland inflammation-induced dry eye. J Ocul Pharmacol Ther 21(2):139–148

Oliver C, Waters JF, Tolbert CL et al (1987) Growth of exocrine acinar cells on a reconstituted basement membrane gel. In Vitro Cell Dev Biol 23(7):465–473

Parsons JT, Bova FJ, Mendenhall WM et al (1996) Response of the normal eye to high dose radiotherapy. Oncology (Williston Park) 10(6):837–847, discussion 847–838, 851–832

Pflugfelder SC, Jones D, Ji Z et al (1999) Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjogren's syndrome keratoconjunctivitis sicca. Curr Eye Res 19(3):201–211

Rismondo V, Gierow JP, Lambert RW et al (1994) Rabbit lacrimal acinar cells in primary culture: morphology and acute responses to cholinergic stimulation. Invest Ophthalmol Vis Sci 35(3):1176–1183

Sahai A, Malik P (2005) Dry eye: prevalence and attributable risk factors in a hospital-based population. Indian J Ophthalmol 53(2):87–91

Sanders EJ (1988) The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem Cell Biol 66(6):530–540

Schonthal AH, Warren DW, Stevenson D et al (2000) Proliferation of lacrimal gland acinar cells in primary culture. Stimulation by extracellular matrix, EGF, and DHT. Exp Eye Res 70(5):639–649

Schrader S, Wedel T, Kremling C et al (2007) Amniotic membrane as a carrier for lacrimal gland acinar cells. Graefes Arch Clin Exp Ophthalmol 245(11):1699–1704

Schrader S, Kremling C, Klinger M et al (2009) Cultivation of lacrimal gland acinar cells in a microgravity environment. Br J Ophthalmol 93(8):1121–1125

[AU6] Shatos MA, Haugaard-Kedstrom L, Hodges RR et al (2012) Isolation and characterization of progenitor cells in uninjured, adult rat lacrimal gland. Invest Ophthalmol Vis Sci 53:2749–2759

Song XJ, Li DQ, Farley W et al (2003) Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 44(10):4223–4229

Stephens LC, Schultheiss TE, Price RE et al (1991) Radiation apoptosis of serous acinar cells of salivary and lacrimal glands. Cancer 67(6):1539–1543

Stewart P, Chen Z, Farley W et al (2005) Effect of experimental dry eye on tear sodium concentration in the mouse. Eye Contact Lens 31(4):175–178

Sullivan DA, Bloch KJ, Allansmith MR (1984) Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. Immunology 52(2):239–246

Sullivan DA, Kelleher RS, Vaerman JP et al (1990) Androgen regulation of secretory component synthesis by lacrimal gland acinar cells in vitro. J Immunol 145(12):4238–4244

[AU7] The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007) Ocul Surf 5(2):75–92

Tiffany JM (2008) The normal tear film. Dev Ophthalmol 41:1–20
Tiwari S, Ali MJ, Balla MM et al (2012) Establishing human lacrimal gland cultures with secretory function. PLoS One 7(1):e29458
Tripathi BJ, Tripathi RC (1990) Evidence for the neuroectodermal origin of the human lacrimal gland. Invest Ophthalmol Vis Sci 31(2):393–395
van der Werf F, Baljet B, Prins M et al (1996) Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. J Anat 188(Pt 3):591–601
Walcott B (1998) The lacrimal gland and its veil of tears. News Physiol Sci 13:97–103
Yoshino K (2000) Establishment of a human lacrimal gland epithelial culture system with in vivo mimicry and its substrate modulation. Cornea 19(3 Suppl):S26–S36
You S, Kublin CL, Avidan O et al (2011) Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Invest Ophthalmol Vis Sci 52(5):2087–2094

Author Queries

Chapter No.: 30 0001729812

Queries	Details Required	Author's Response
AU1	Please confirm the affiliation details.	Done. Confirmed
AU2	Please fix "a or b" for the Reference "Gilbard et al. (1989)" here and other occurrence.	OK as in this draft
AU3	Please check the citation for Table 30.2.	OK
AU4	Please confirm the inserted details for Reference "Shatos et al. (2012)".	OK
AU5	Please provide author name for the Reference "The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007)" and "Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop (2007)".	No authors. It is a commitee report

Saudi Journal of Ophthalmology (2014) 28, 12–18

Dacryology Update

Human lacrimal gland regeneration Perspectives and review of literature

Shubha Tiwari, M.Pharm [a]; Mohammad Javed Ali, MS, FRCS [b]; Geeta K. Vemuganti, MS, FNAMS [a,c,*]

Abstract

The human lacrimal gland is an essential component of the lacrimal functional unit (LFU). Any perturbation of this unit can lead to the debilitating morbid condition called the dry eye syndrome (DES). The current line of therapy available for dry eye remains supportive and palliative with the patient being dependent on life long and frequent administration of lubricating eye drops. Even advanced therapies like punctual plugs, cyclosporine B administration, and salivary gland auto-transplantation have led to a limited success. Under these scenarios, the option of cell based therapy needs to be explored to provide better and long term relief to these patients. This review gives an overview of the efforts in lacrimal gland regeneration and examines the past and ongoing research in cell based therapies in animals as well as human lacrimal gland cultures. The authors discuss their first of its kind functionally viable human lacrimal gland *in vitro* culture system from fresh exenteration specimens. A brief overview of research in near future and the potential implications of lacrimal gland regenerative therapies have been discussed.

Keywords: Lacrimal gland, Regeneration, Dry eye, Stem cells

http://dx.doi.org/10.1016/j.sjopt.2013.09.004

Introduction

The human lacrimal gland is an essential component of the lacrimal functional unit (LFU) which comprises of the lacrimal gland, the ocular surface (cornea, conjunctiva and the meibomian gland) and the associated sensory and motor nerves (Figure 1). The LFU controls the secretion of the major components of the tear film and is overall responsible for maintaining the stability of the tear film, transparency of the cornea and the quality of the image projected onto the retina.[1]

Any perturbation in the stability of the tear film leads to destabilization of the ocular surface which, over a period of time, can lead to the debilitating morbid condition called the dry eye syndrome (DES). The composition of the tear film can be altered due to the dysfunction of either the lacrimal gland or the meibomian glands; however for the purpose of this review we will restrict our discussion to lacrimal gland dysfunction. Lacrimal gland dysfunction and destruction is seen in cases of advancing age, autoimmune disorders, orbital radiotherapy, low androgen pool etc. This lacrimal dysfunction causes hyperosmolarity of tear film resulting in a vicious loop of ocular surface inflammation which is responsible for ocular epithelial damage leading to corneal ulceration and eventual decline in visual acuity.[1]

The current line of therapy available for dry eye remains supportive and palliative with the patient being dependent on life long, frequent administration of lubricating or hydrating eye drops. Even advanced therapies like punctal plugs, cyclosporine B administration, and salivary gland auto-transplantation have led to a limited success. Under these scenarios, the option of cell based therapy needs

Available online 30 September 2013

[a] Sudhakar and Sreekant Ravi Stem Cell Biology Laboratory, L. V. Prasad Eye Institute, Hyderabad, India
[b] Dacryology Service, L. V. Prasad Eye Institute, Hyderabad, India
[c] School of Medical Sciences, University of Hyderabad, Hyderabad, India

* Corresponding author at: School of Medical Sciences, University of Hyderabad, Hyderabad, India. Tel.: +91 4030612345.
e-mail address: gkvemuganti@gmail.com (G.K. Vemuganti).

Peer review under responsibility of Saudi Ophthalmological Society, King Saud University

Production and hosting by Elsevier

Access this article online:
www.saudiophthaljournal.com
www.sciencedirect.com

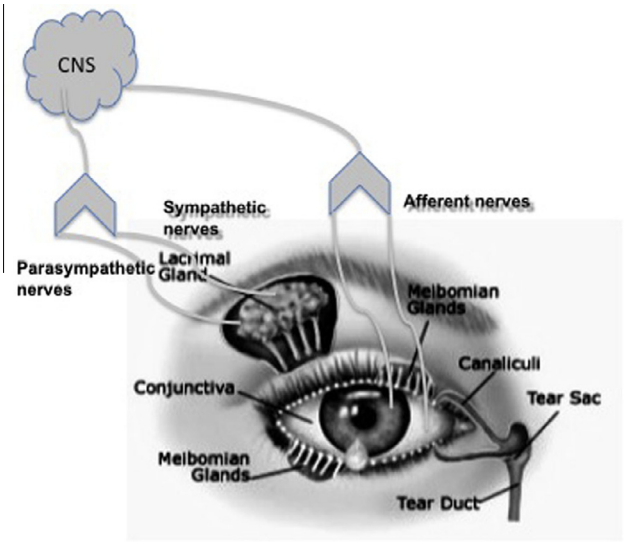

Figure 1. The lacrimal functional unit.

to be explored to provide better and long term relief to these patients.

Histology, anatomy and physiology

The lacrimal gland is a tubulo-acinar exocrine gland that consists of secretory columnar epithelium arranged in a lobular pattern. These secretory acinar cells empty their secretions into ducts that anastomose into larger excretory ducts which drain onto the ocular surface. Both the acinar and the ductal cells have numerous vesicles in their apical portion while the base is associated with a basement matrix. Enveloping the secretory acinar cells are myoepithelial cells that contract and squeeze them enabling the draining of the secretory components into the ducts. Between the lacrimal lobes are fibroblasts, which produce the collagen and matrix of interstitial spaces, and mast cells, which secrete histamine and heparin. In addition to this basic tissue architecture, the lacrimal gland is highly inundated with trafficking B and T lymphocytes as well as plasma cells.[2]

The lacrimal gland has both the sympathetic as well as parasympathetic innervation.[3] These nerves have a large number of cholinergic fibers and fewer adrenergic fibers. The parasympathetic postganglionic neural cell bodies are found in the pterygopalatine (sphenopalatine) ganglion as well as the ciliary ganglion. Sympathetic fibers arise in the superior cervical ganglion. There is also some sensory innervation of the gland from the trigeminal ganglia.[4]

The lacrimal gland secretes a number of proteins like lysozyme, lactoferrin, lipocalin, and sclgA.[5] The secretion of these proteins is regulated by the nerves and their associated neurotransmitters or neuropeptides.[2] The important receptors present on the lacrimal gland are acetylcholine receptors like muscarinic M3, vasoactive intestinal peptide types I and II and norepinephrine receptors like alpha 1 and beta.[6] Other receptors present are for interacting with neuropeptide Y, adrenocorticotrophic hormone (ACTH) and alpha- melanocyte stimulating hormone (MSH). Since the epithelial cells of the gland are extensively coupled by junctional complexes, secondary messengers like inositol triphosphate can easily diffuse between cells and activate the unstimulated cells too.[2]

The dry eye syndrome

The International Dry Eye Workshop, 2007[1] defined dry eye as:

> ''*Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of tear film and inflammation of the ocular surface.*''

Dry eye is associated with a high incidence of ocular morbidity. The current prevalence of dry eye in the world is estimated at around 11% to 22%.[7] In the Indian context, these numbers are estimated to be around 18.4–20%.[8,9] These epidemiological numbers are a good indicator of the need for research on dry eye syndrome.

The etiology of DES involves a vicious loop of tear hyperosmolarity, tear film instability and ocular surface inflammation.[10] In addition to this, there is also loss of anti-inflammatory environment within the gland which may happen in cases of low androgen pool. Severe dry eye is also seen in patients of Sjogren's syndrome in which autoantigens are expressed at the surface of the epithelial cells. These cause homing and retention of tissue specific CD4 and CD8 cells, leading to immune-mediated destruction of the acinar and ductal components.

Orbital radiation therapy, which is a commonly used modality in the treatment of oculo-adenexal disorders including malignant tumors, has also been implicated in the development of DES in patients. Despite a rapid evolution in the field of radiotherapy over the past years, a significant number of patients are still seen with acute and chronic ophthalmic complications including severe dry eye.[11,12] Preliminary data from our institute indicate that chronic dry eye develops in over 49% of the patients who undergo external beam radiation therapy for ocular malignancies (unpublished data).

Contact lens wear is yet another condition that may lead to the development of severe dry eye in long-term users. Reduced corneal sensitivity and tear film hyperosmolarity are the probable underlying mechanisms.[1]

The current treatment modalities available for DES are lubricating agents like hydroxymethyl cellulose, solutions containing bicarbonates and potassium, hyposmotic artificial tears and artificial serum. In cases of severe dry eye, therapies such as anti-inflammatory medications (cyclosporin A, corticosteroids), pharmacological tear stimulants like diquafosol, rebamipide, ecabet sodium, pilocarpine etc. have also been used. In certain severe cases, surgical interventions like punctal occlusion and salivary gland auto-transplantation are done to slow down the progress of the condition and minimize detrimental sequale.[13]

On recommendation of the committee on the therapy and management of dry eye, the treatment or management protocol for this condition is now shifting toward employing strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce or elimi-

nate the levels of existing inflammation.[13] With these objectives in mind various therapeutic avenues are being explored with the inclusion of cell therapy for restoring the function of the damaged lacrimal gland.

Lacrimal gland regeneration

Currently available data on the etiopathogenesis and treatment of dry eye are still insufficient to explain how alteration in tear film composition can cause such a vicious cycle of tear film instability and chronic ocular inflammation. Even though a lot of research is being directed toward profiling the proteins, lipids and other constituents of human tear yet there is a glaring lack of comparative data between normal individuals and dry eye patients.

Animal lacrimal gland in vitro cultures

An important area of investigation in this field is to find a common link between tear film osmolarity, tear film break up response and the resultant inflammatory stress. In order to facilitate these studies, not just *in vivo* models but also *in vitro* models are being developed that would greatly assist the investigation into the secretory repertoire of lacrimal gland epithelia, regulation of secretion and etiopathogenesis of lacrimal gland involvement in disorders like Sjogren's syndrome.

Past two decades have seen evolution in the procedure for *in vitro* culturing of lacrimal gland acinar cells. Oliver et al.[14] published one of the first reports on *in vitro* culture of rat lacrimal gland acinar cells wherein they described a method for culturing a dividing population of morphologically differentiated rat lacrimal acinar cells on a three-dimensional, reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules.[14] However, their culture system could only maintain the epithelial cells for 6–7 days after which fibroblast overgrowth was observed. Successful *in vitro* culture of lacrimal acinar cells was first achieved and published by Meneray and Rismondo in two separate reports in 1994.[15,16] Reports from Hann et al.[17] and numerous subsequent authors have emphasized the importance of media formulation, supplement profile and extracellular matrix composition for optimal growth and functionality of lacrimal acinar cells.

A major problem faced by all these investigators was that the lacrimal acinar cells could not be induced to proliferate significantly *in vitro* until Schonthal et al.[18] in 2000 improvised on the media composition by the use of epidermal growth factor (EGF), dihydrotestosterone (DHT), Matrigel™ and HepatoStim™ culture medium.[18] A number of recent publications also report the use of polyethersulfone dead-end tube[19], denuded amniotic membrane[20] as scaffolds and rotary cell culture system[21] for successful *in vitro* culture of rat or rabbit lacrimal glands. The effect of androgens and androgen analogs on *in vitro* culture of lacrimal acinar cells has helped elucidate the control that the androgens exert on the synthesis and secretion of secretory component[17,22–24] as well as other biochemical parameters related to the lacrimal secretion including the basal tear flow rate.[25]

The culture systems developed for the lacrimal acinar cells have also been optimized to assess the functionality of these cells. The currently employed conditions for the *in vitro* culture of these secretory cells support the *in vivo* mimicry of their secretion pattern as elucidated by the detection of sclg-A, lactoferrin, lysozyme, lacritin and a number of other tear proteins in the culture supernatant.

You et al.[26] showed that post injection of interleukin into the mouse lacrimal gland which destroys areas in the gland, stem-like cells migrate toward the site of injury and heal the wound. These cells could also be harvested and grown under *in vitro* conditions. However, the authors report minimum *in vitro* growth from uninjured gland. In contrast, the recent study by Shatos et al.[27] on rat lacrimal gland and our own experience with human lacrimal gland tissue[28] showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in vitro* conditions.

Human lacrimal gland in vitro cultures – our experience

In-vitro work on human lacrimal gland cultures is scarce, possibly due to the difficulty in obtaining human tissue for research. To the best of our knowledge and literature search, there is just one report published by Yoshino et al.[29] in 2000, which dealt with establishing human lacrimal cultures from cadaveric tissue. However, the study was a limited report which only partially explored the secretory potential of the cells and the various sub-populations present.

Our group has been working with human lacrimal gland cultures since 2008 and we were the first to report the establishment of functionally viable human lacrimal gland *in vitro* cultures from fresh exenteration specimens.[28] Our results show successful establishment of human lacrimal gland cultures on three matrices: Matrigel™, collagen-1 and denuded human amniotic membrane. We have extensively characterized our culture system using markers for epithelial, myoepi-

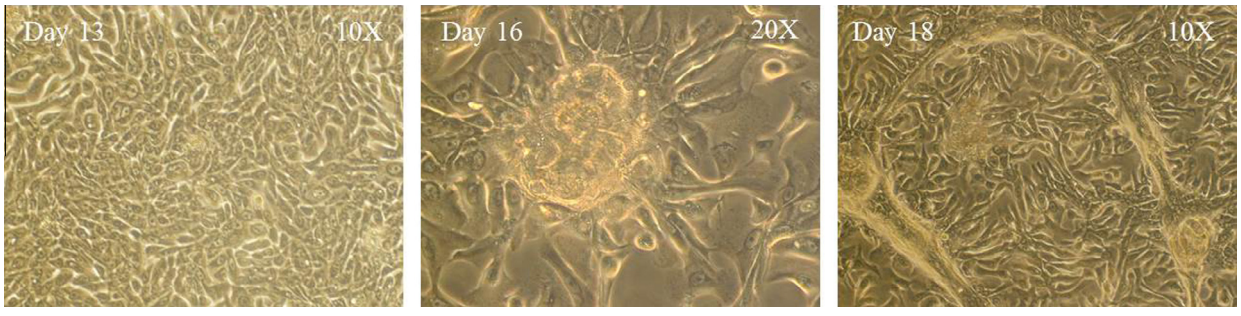

Figure 2. Human lacrimal gland *in vitro* cultures: phase contrast microscope images.

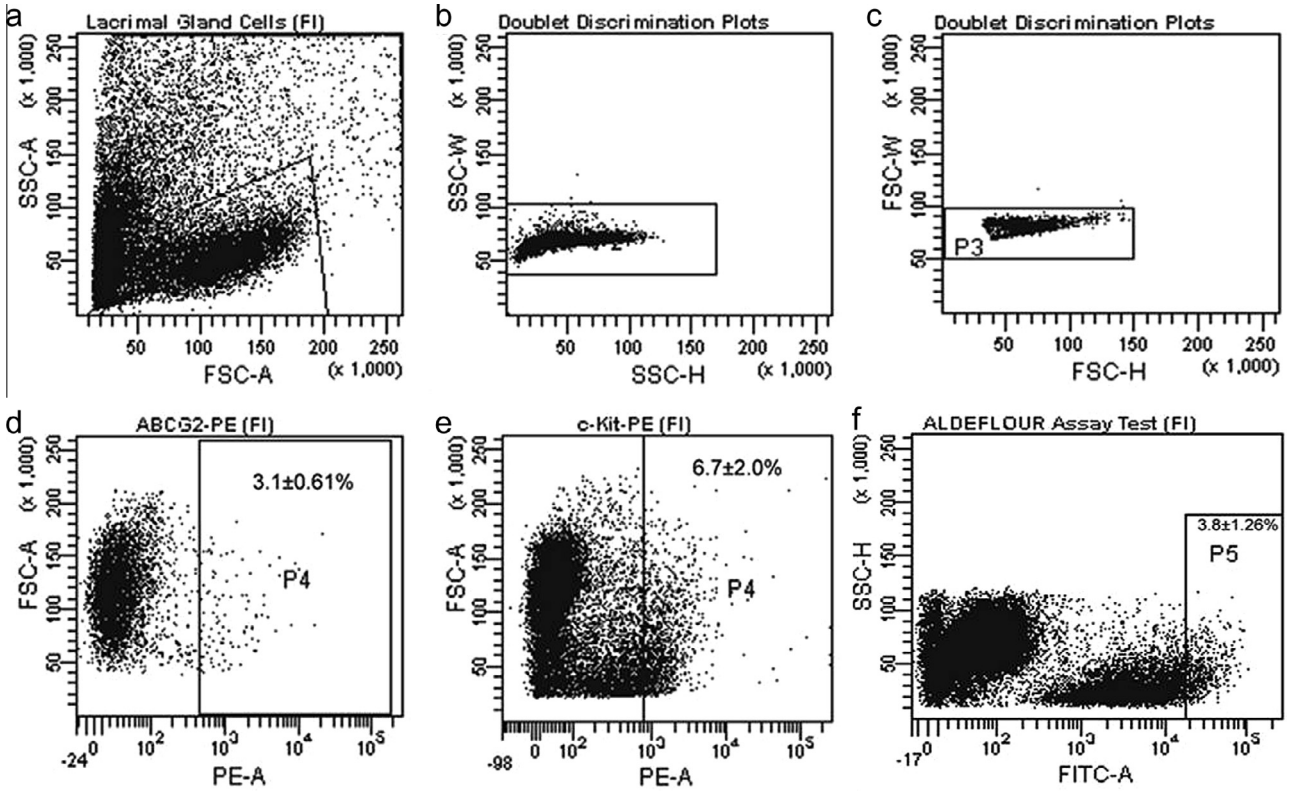

Figure 3. FACS analysis of fresh human lacrimal gland cells.

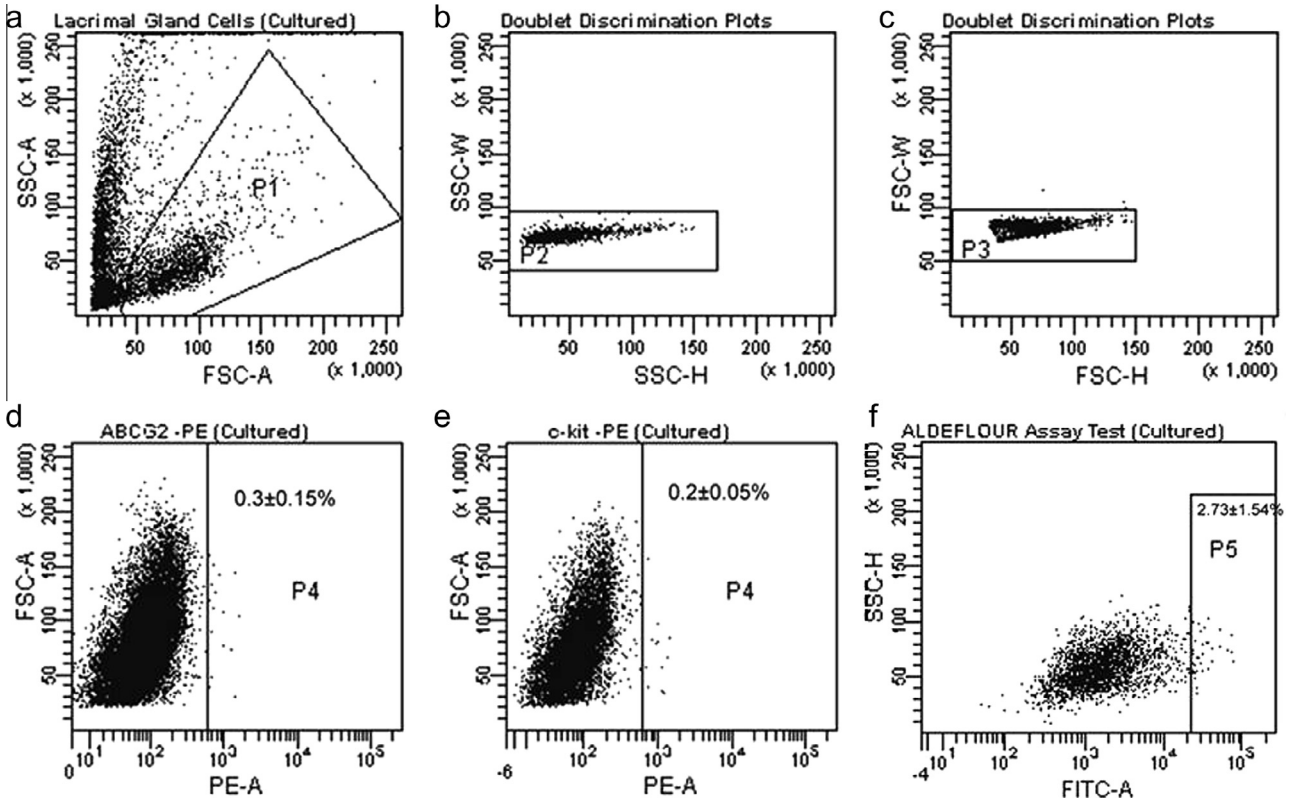

Figure 4. FACS analysis of cultured human lacrimal gland cells.

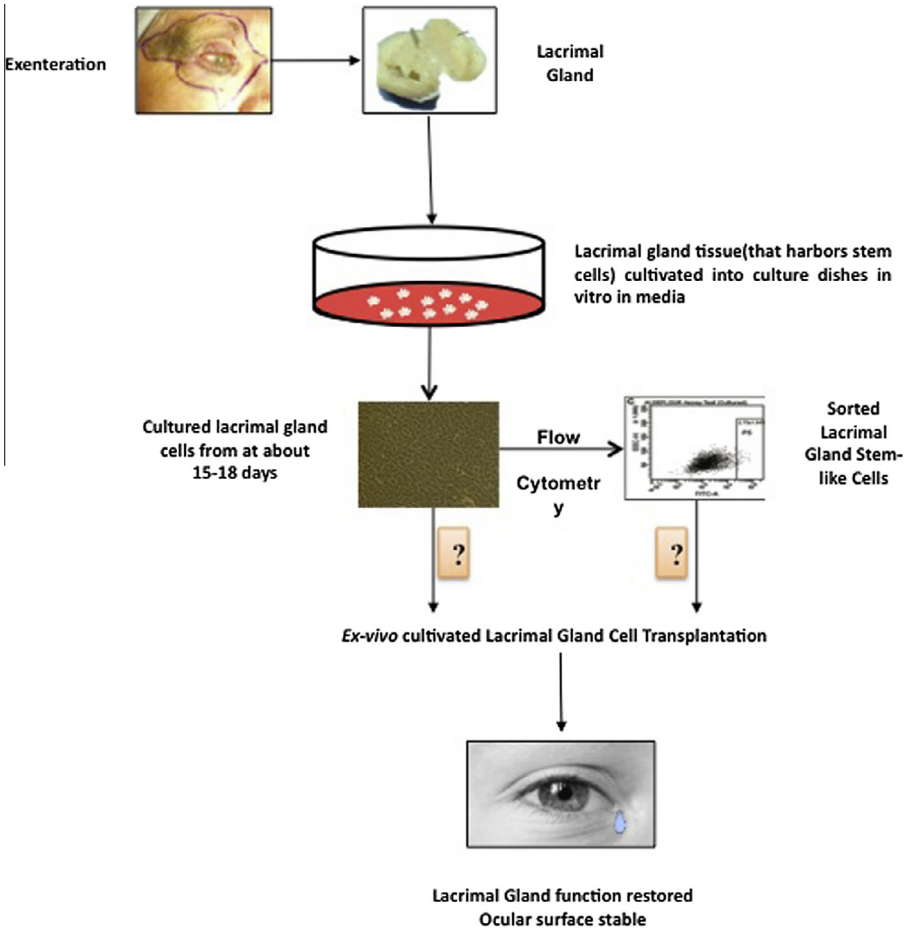

Figure 5. Potential cell therapy for aqueous deficient dry eye.

thelial and mesenchymal origin by immunohistochemistry/immunocytochemistry and flow cytometry. Our established cultures show the capacity to synthesize and secrete quantifiable levels of major tear proteins like scIgA, lactoferrin and lysozyme into the culture supernatant. We have successfully shown that these lacrimal epithelial cultures can be maintained *in vitro*, with intact secretory function, for a minimum period of 21 days. In addition, we also reported that by day 16–18, these *in vitro* cultures show the appearance of 'spherules' and structures that look like ductal connections between them (Figure 2). We believe that this indicates their potential for *in vitro* gland formation.

Toward the long-term goal of cell therapy in chronic dry eye condition, we have also looked for the presence of stem-like cells in the native human lacrimal gland as well as in the established cultures. From the criteria available, we have used ABCG2, CD117 positivity and ALDH levels, clone formation ability, quiescence and label retaining properties to indicate the presence of stem-like cells.

Using flow cytometry and immunochemistry we have shown preliminary evidence indicating the presence of stem-like cells (ABCG2 positive, ALDH high) in the native human lacrimal gland as well as in our *in vitro* cultures. In the native gland around 3.1 ± 0.61% of the cells showed ABCG2 positivity and around 3.8 ± 1.26% showed high levels of ALDH (Figure 3). In the *in vitro* cultures, the number of cells that showed ABCG2 positivity by day 14 is 0.3 ± 0.15%, which decreased slightly to 0.2 ± 0.13% by day 21. Similar is the case with ALDH. CD117, which is a stem cell marker in exocrine glands, also showed positivity: at time = 0 about 6.7 ± 2.0% of the cells were CD117 positive. This number reduced to 0.2 ± 0.05 by day 14 and 0.2 ± 0.05 by day 21 *in vitro* (Figure 4)

In order to enrich the number of stem cells *in vitro* we have also cultured these cells as free floating 'lacrispheres' which are very similar to salispheres and prostaspheres. These spheres show a fourfold increase in CD117 positive cells (0.8%), increased clone forming ability and higher percentage of label retaining cells than the adherent cultures (unpublished data).

We believe that these lacrispheres are very similar in their phenotype as well as cellular architecture to salispheres. Since the latter is being used as a potential cell therapy for restoring function in animal model of xerostomia,[30] we believe similar results could be expected from lacrispheres too.

Conclusion

Even though we have come a long way in managing the chronic dry eye patients and improving their quality of life, yet there are a number of aspects that still need to be looked at and addressed. One of the principle areas that need focused attention is to understand the mechanisms behind

the development and progression of this condition and to develop biomarker(s) for this condition. A large, well-defined, staged and age-matched study would probably be required for this.

A lot of fundamental science we know of dry eye today has been by correlating animal data with human scenario. In order to lend credibility to the accrued knowledge, it is essential that a comparative study be made between human and mouse or rabbit tears and ocular surface protein–lipid profiles. This would enable us to identify the common components and pathways involved in various forms of this disorder and would also give important clues about the treatment of this condition.

The last couple of years have seen an increase in the knowledge about the presence of stem-like cells in the lacrimal gland of mice,[26] rat[27] and humans.[28] These studies indicate the inherent potential of the gland to heal itself following an insult. In contrast, the recent study by Shatos et al.[27] on rat lacrimal gland and our own experience with human lacrimal gland showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in vitro* conditions.

Another important area that needs to be explored carefully is the potential to use cell therapy in chronic cases of dry eye. Cell therapy is being used successfully to treat a similar condition called xerostomia involving the salivary gland under preclinical settings.[30] Even though there is not enough work that has been done toward cell therapy in dry eye, yet its similarity with salivary gland leads us to believe that a similar therapeutic approach may prove beneficial to the chronic patients of dry eye too (Figure 5).

The presence of stem cells in the lacrimal gland is an important finding that leads us to believe that these cells can be recruited to salvage the damaged gland. However, before we take a leap of faith, the viability, homing and functionality of these cells need to be established by more extensive *in vitro* studies and independent animal experimentation. Subsequent use of cell based therapies to rescue and regenerate diseased lacrimal gland in humans is a promising prospect.

Funding agencies

IAEA, DBT, HERF, C-Tracer, Sudhakar and Sreekanth Ravi Brothers.

Financial disclosure

None.

Method of literature search

A search of Pubmed database (1979-till date) was conducted. Medline and Ophthalmic literature databases were also searched. The following key words were used: Lacrimal gland/lacrimal *in vitro* studies/dry eye syndrome. Additional sources include review of publications cited in other articles. Google search was also used to find publication that may be missed in the above databases.

Conflict of interest

The authors declared that there is no conflict of interest.

References

1. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007). The ocular surface 2007;5:75–92.
2. Walcott B. The lacrimal gland and its veil of tears. *News Physiol Sci* 1998;**13**:97–103.
3. Matsumoto Y, Tanabe T, Ueda S, et al. Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (*Macaca fuscata*). *J Auton Nerv Syst* 1992;**37**:207–14.
4. Van der Werf F, Baljet B, Prins M, et al. Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. *J Anat* 1996;**188**:591–601.
5. Tiffany JM. The normal tear film. *Dev Ophthalmol* 2008;**41**:1–20.
6. Mauduit P, Jammes H, Rossignol B. M3 muscarinic acetylcholine receptor coupling to PLC in rat exorbital lacrimal acinar cells. *Am J Physiol* 1993;**264**:1550–60.
7. Abelson MB, Ousler GW, Maffei C. Dry eye in 2008. *Curr Opin Ophthalmol* 2009;**20**:282–6.
8. Gupta N, Prasad I, Himashree G, et al. Prevalence of dry eye at high altitude: a case controlled comparative study. *High Alt Med Biol* 2008;**9**:327–34.
9. Sahai A, Malik P. Dry eye: prevalence and attributable risk factors in a hospital-based population. *Indian J Ophthalmol* 2005;**53**: 87–91.
10. Gilbard JP, Rossi SR, Heyda KG. Ophthalmic solutions, the ocular surface, and a unique therapeutic artificial tear formulation. *Am J Ophthalmol* 1989;**107**:348–55.
11. Alberti W. Acute and late side effects of radiotherapy for ocular disease: an overview. *Front Radiat Ther Oncol* 1997;**30**:281–6.
12. Durkin SR, Roos D, Higgs B, et al. Ophthalmic and adnexal complications of radiotherapy. *Acta Ophthalmol Scand* 2007;**85**:240–50.
13. Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye Workshop (2007). The ocular surface 2007;5:163–178.
14. Oliver C, Waters JF, Tolbert CL, et al. Growth of exocrine acinar cells on a reconstituted basement membrane gel. *In Vitro Cell Dev Biol* 1987;**23**:465–73.
15. Meneray MA, Fields TY, Bromberg BB, et al. Morphology and physiologic responsiveness of cultured rabbit lacrimal acini. *Invest Ophthalmol Vis Sci* 1994;**35**:4144–58.
16. Rismondo V, Gierow JP, Lambert RW, et al. Rabbit lacrimal acinar cells in primary culture: morphology and acute responses to cholinergic stimulation. *Invest Ophthalmol Vis Sci* 1994;**35**: 1176–83.
17. Hann LE, Kelleher RS, Sullivan DA. Influence of culture conditions on the androgen control of secretory component production by acinar cells from the rat lacrimal gland. *Invest ophthalmol Vis Sci* 1991;**32**:2610–21.
18. Schonthal AH, Warren DW, Stevenson D, et al. Proliferation of lacrimal gland acinar cells in primary culture. Stimulation by extracellular matrix, EGF, and DHT. *Exp Eye Res* 2000;**2000**(70):639–49.
19. Long L, Liu Z, Wang T, et al. Polyethersulfone dead-end tube as a scaffold for artificial lacrimal glands in vitro. *J Biomed Mater Res B Appl Biomater* 2006;**78**:409–16.
20. Schrader S, Wedel T, Kremling C, et al. Amniotic membrane as a carrier for lacrimal gland acinar cells. *Graefes Arch Clin Exp Ophthalmol* 2007;**245**:1699–704.
21. Schrader S, Kremling C, Klinger M, et al. Cultivation of lacrimal gland acinar cells in a microgravity environment. *Br J Ophthalmol* 2009;**93**:1121–5.
22. Kelleher RS, Hann LE, Edwards JA, et al. Endocrine, neural, and immune control of secretory component output by lacrimal gland acinar cells. *J Immunol* 1991;**146**:3405–12.
23. Sullivan DA, Bloch KJ, Allansmith MR. Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. *Immunology* 1984;**52**:239–46.

24. Sullivan DA, Kelleher RS, Vaerman JP, et al. Androgen regulation of secretory component synthesis by lacrimal gland acinar cells in vitro. *J Immunol* 1990;**145**:4238–44.
25. Azzarolo AM, Mircheff AK, Kaswan RL, et al. Androgen support of lacrimal gland function. *Endocrine* 1997;**6**:39–45.
26. You S, Kublin CL, Avidan O, et al. Isolation and propagation of mesenchymal stem cells from the lacrimal gland. *Invest Ophthalmol Vis Sci* 2011;**52**:2087–94.
27. Shatos MA, Haugaard-Kedstrom L, Hodges RR, et al. Isolation and characterization of progenitor cells in uninjured, adult rat lacrimal gland. *Invest Ophthalmol Vis Sci* 2012;**53**:2749–59.
28. Tiwari S, Ali MJ, Balla MM, et al. Establishing human lacrimal gland cultures with secretory function. *PloS one* 2012;**7**:e29458.
29. Yoshino K. Establishment of a human lacrimal gland epithelial culture system with in vivo mimicry and its substrate modulation. *Cornea* 2000;**19**:S26–36.
30. Lombaert IM, Brunsting JF, Wierenga PK, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. *PloS One* 2008;**3**:e2063.

Investigative Ophthalmology & Visual Science

Invest Ophthalmol Vis Sci 2013;54: E-Abstract 2191.

2191

Stem-like Cells in Serum Free In-vitro Cultures of Human Lacrimal Gland

Shubha Tiwari[1], Mohammad Ali[1], Murali Mohan Sagar Balla[1], Milind Naik[1], Santosh Honavar[1], Vijay Anand Palkonda[1] and Geeta Vemuganti[2,1]

[1] Stem Cell Biology Lab, L V Prasad Eye Institute, Hyderabad, India
[2] School of Medical Sciences, University of Hyderabad, Hyderabad, India

Commercial Relationships: Shubha Tiwari, None; **Mohammad Ali**, None; **Murali Mohan Sagar Balla**, None; **Milind Naik**, None; **Santosh Honavar**, None; **Vijay Anand Palkonda**, None; **Geeta Vemuganti**, None

Support: None

Abstract

Purpose: Tear film deficiency due to lacrimal gland dysfunction or damage is an important cause of ocular morbidity. Restoration of gland function by transplantation of autologous ex-vivo expanded stem cells located in the lacrimal gland is one of the options that could relieve this problem. We have previously reported the presence of stem-like and functionally competent differentiated cells in in-vitro cultures of human lacrimal gland. The present study focuses on the formation of 'lacrispheres' under serum-free condition and the expression of stemness in them.

Methods: Fresh human lacrimal gland tissues (n=7) from patients undergoing exenteration were harvested for cultures after IRB approval. The gland was processed by enzymatic digestion using a cocktail of collagenase and hyaluronidase. The isolated cells were plated on ultralow attachment plates as group of two-three cells and fed with HepatoStim supplemented with epidermal growth factor, fibroblast growth factor and N2. The spheres were pulse-labeled with BrDU, analyzed for the expression of CD117 by immunocytochemistry and their colony forming efficiency was assessed on Matrigel.

Results: Serum free cultures demonstrate spheres from human lacrimal gland in-vitro within 2-3 days of plating. These spheres grow in size over 3-14 days and can be serially passaged to generate secondary spheres. Anti-BrDU labeling of these spheres indicate the presence of 3.8% of high intensity cells at the periphery and about 3% dull intensity cells at the center. These also show positive labeling for CD117 and formation of clones in Matrigel (CFU 3.1%) indicating the presence of stemness.

Conclusions: This is the first report on the generation of 'lacrisphers' in human lacrimal gland cultures. It strengthens our initial reports that human lacrimal gland has a storehouse of stem cells that can be

maintained in-vitro and could possibly serve as potential source of cell therapy for the regeneration of the functionally compromised gland.

Keywords: 576 lacrimal gland • 486 cornea: tears/tear film/dry eye • 721 stem cells

Brief Biography of the Candidate

Shubha Tiwari

Stem Cell Biology Laboratory, L V Prasad Eye Institute, Kallam Anji Reddy Campus, L V Prasad Marg, Banjara Hills,

Hyderabad – 500034 India

Tel: +91-040-30612523 **Mobile:** (0) 9848248714 **Fax:** +91-040-23548271

Email:shubha@lvpei.org**,** tiwari.shubha@gmail.com_

2011 – Present: Senior Research Fellow (CSIR) (Supervisor: Prof Geeta K Vemuganti), Stem Cell Biology Laboratory, L V Prasad Eye Institute, Hyderabad, India

2009 – 2011: Research Fellow (PhD) (Supervisor: Dr Geeta K Vemuganti), Stem Cell Biology Laboratory, L V Prasad Eye Institute, Hyderabad, India

2008 – 2009: Project Fellow, Stem Cell Biology Laboratory, L V Prasad Eye Institute, Hyderabad, India

2005 – 2007: Master of Pharmaceutical Sciences (M. Pharm), Poona College of Pharmacy, BVDU, Pune

2001- 2005: Bachelor of Pharmaceutical Sciences (B. Pharm), Poona College of Pharmacy, BVDU, Pune

Awards & Honors

2013: Awarded the DBT Travel Fellowship to Annual Meeting ARVO, Seattle, USA

2013: Awarded the CSIR Travel Fellowship to Annual Meeting ARVO, Seattle, USA

2011: Awarded the Tear Film and Ocular Surface (TFOS) Fellowship

2011: Awarded the CSIR Travel Fellowship to Annual Meeting, ARVO. Fort Lauderdale. Florida. USA

2011: Qualified for CSIR Senior Research Fellow conducted by Council for Scientific and Industrial Research, Govt. of India

2006 – 2008: Qualified All India Graduate Aptitude Test in Engineering (GATE) conducted by Indian Institute of Technology (IIT)

Theses

PhD (Submitted): Cultivation and characterization of human lacrimal gland cells for potential clinical application (Registered with BITS, Pilani, India)

Masters in Pharmaceutical Sciences: Isolation and cultivation of human umbilical vein endothelial cells for ascertaining cytoprotective role of antioxidants against glucose-induced oxidative stress.

Publications

S Tiwari, RM Nair, MJ Ali ,MN Naik, SG Honavar, VA P Reddy, GK Vemuganti. Stem-like cells in serum free in-vitro cultures of human lacrimal gland. (Manuscript under preparation)

S Tiwari, Anusha A, Jayalakshmi N, MJ Ali, MN Naik, VA P Reddy, GK Vemuganti. Spectrum of dry eye in post-orbital radiotherapy. (Manuscript under preparation)

Shubha Tiwari, Mohammad Ali, Murali Mohan Sagar Balla, Milind Naik, Santosh Honavar, Vijay Anand Palkonda and Geeta Vemuganti. Stem-like Cells in Serum Free In-vitro Cultures of Human Lacrimal Gland. *Invest Ophthalmol Vis Sci* 2013;54: E-Abstract 2191

Shubha Tiwari, Mohammad Javed Ali, Murali Mohan Sagar Balla, Milind N Naik, Santosh G Honavar, Vijay Anand P Reddy, Geeta K Vemuganti. Establishing Human Lacrimal Gland Cultures with Secretory Function. *PLoS One* 2012; Volume 7, Issue 1,(Epub 2012 Jan 13)

Shubha Tiwari, Geeta K Vemuganti (2012) Lacrimal Gland Regeneration: Progress and Promise In *Regenerative Medicine*, Springer Publication.

S Tiwari, MJ Ali, GK Vemuganti. Human lacrimal gland regeneration: Perspectives and review of literature. Saudi Journal of Ophthalmology 2014 Jan;28(1):12-8.

Ujwala S Saboo, Sayan Basu, Shubha Tiwari, Ashik Mohamed, Geeta K Vemuganti, Virender S Sangwan. Clinical and Cytological Evidence of Limbal Stem Cell Deficiency in Eyes with Long-standing Vernal Keratoconjunctivitis. *Asia-Pacific Journal of Ophthalmology. 2.2 (2013): 88-93.*

Virender S Sangwan, Geeta K Vemuganti, Kunjal Sejpal, Sandhya V Subramaniam, Souvik Bandyopadhyay, Sannapaneni Krishnaiah, Subhash Gaddipati, **Shubha Tiwari**, Dorairajan Balasubramanian, Long Term Restoration of the Ocular Surface by Autologous Cultivated Limbal Epithelial Transplantation. *British Journal of Ophthalmology 2011 Nov;95(11). Page no.1525-9*

Indumathi Mariappan Savitri Maddileti Soumya Savy, **Shubha Tiwari**, Subhash Gaddipatti, Anees Fatima Virender S Sangwan Dorairajan Balasubramanian Geeta K Vemuganti (2010). *In-vitro* Culture and Expansion of Human Limbal Epithelial Cells. *Nature Protocols*. 8,1470-1479

Geeta K. Vemuganti, Murali Mohan Sagar Balla, **Shubha Tiwari** (2010). Limbal Stem Cells and Corneal Regeneration. Book Chapter In: *Applications of Flow Cytometry in Stem Cell Research and Tissue Regeneration.* 223-240

V. D'Britto, **S. Tiwari**, V. Purohit, P. P. Wadgaonkar, S. V. Bhoraskar, R. R. Bhonde and B. L. V. Prasad (2009). Composites of plasma treated poly(etherimide) films with gold nanoparticles and lysine through layer by layer assembly: a ''friendly-rough'' surface for cell adhesion and proliferation for tissue engineering applications. *Journal of Materials Chemistry*. 19, 544–550

Sachin S. Kadam, **Shubha Tiwari** & Ramesh R. Bhonde (2009). Simultaneous isolation of vascular endothelial cells and mesenchymal stem cells from the human umbilical cord. *In-vitro Cell.Dev.Biol.-Animal.* 45, 23–27

Conference presentations

Shubha Tiwari, Md Javed Ali, M M Sagar Balla, Milind N Naik, Santosh G Honavar,Vijay Anand, P Reddy, Geeta K Vemuganti (2013). Stem-like Cells in the Serum-Free *In-vitro* Cultures of Human Lacrimal Gland. . Association for Research in Vision and Ophthalmology, Seattle, Washington, USA *(Paper)*

Geeta K Vemuganti, **Shubha Tiwari**, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy.
(2010). Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application. Association for Research in Vision and Ophthalmology, Fort Lauderdale, USA *(Poster)*

Shubha Tiwari, Md. Javed Ali, Santosh G Honavar, Milind N. Naik, Vijay Anand P Reddy , Geeta K Vemuganti (2010). Cultivation and Characterization of Human Lacrimal Gland Cells for Potential Clinical Application. Indian Eye Research Group, Hyderabad, India *(Poster)*

Shubha Tiwari, Santosh G Honavar, Milind N Naik,Vijay Anand P Reddy, Geeta K Vemuganti (2009). Cultivation and Characterization of Lacrimal and Salivary Gland Cells for Potential Clinical Application. Annual conference of the Association of Radiation Oncologists of India, Hyderabad, India *(Poster)*

Kunjal Sejpal, Geeta K. Vemuganti, Anees Fatima, Subhash Gaddipati, **Shubha Tiwari**, Soumya Savy, Sannapaneni Krishnaiah, D. Balasubramanian, Virender S. Sangwan, (2009). Outcome of Autologous Cultivated Limbal Epithelial Transplantation in the Pediatric Age Group. American Academy of Ophthalmology *(Poster)*

Shubha Tiwari, Murali Mohan Sagar Balla, Virender S. Sangwan, Geeta K. Vemuganti (2009). ALDH Expression in Limbal Progenitor Cells, Asia- ARVO, Hyderabad, India *(Poster)*

Technical skills

1. Animal tissue/ cell culture
2. Basic Molecular Biology Techniques
3. *In-vitro* Cytotoxicity Studies
4. Immunohistochemistry
5. Immunocytochemistry
6. Polymerase chain reaction
7. Flow Cytometry
8. Confocal Image Analysis
9. UV-Vis Spectroscopic Analysis
10. IR Spectroscopy
11. Time-Lapse Microscopy (Real-Time Imaging)
12. Electron Microscopy

Teaching experience

20013 – Present: Ocular Pharmacology, Instructor, School of Medical Sciences, University of Hyderabad, Hyderabad.

2009 – Present: Graduate student instructor at Bausch and Lomb School of Optometry, BITS, Hyderabad.

2007 – 2008: Animal Tissue Culture, Student Instructor, Poona College of Pharmacy, Pune.

References

Prof. Geeta K Vemuganti MD, DNB, FAMS, FICP
Dean, School of Medical Sciences
University of Hyderabad
Hyderabad 500046
Tel: 91-40-23013279/23014781
Email: deanmd@uohyd.ernet.in, gkvemuganti@gmail.com

Prof. D. Balasubramanian PhD
Director of Research, L V Prasad Eye Institute,
Road #2, Banjara Hills, Hyderabad, India
Tel: +91-40-30612501
Email: dbala@lvpei.org

Prof Ramesh R Bhonde PhD
Dean, Manipal Institute of Regenerative Medicine
Bangalore. India.
Tel: +91-40-80-25356661
Email: rrbhonde@gmail.com

Brief Biography of the Supervisor

Dr Geeta K Vemuganti is a physician- pathologist by training and has contributed extensively to the field of ophthalmic pathology and translational research. In order to combat blindness due to severe ocular surface disease patients, post chemical burn, she developed a simple, cost-effective, feeder cell free method of culturing the limbal stem cells from patient's own limbal tissues through explant culture, using human amniotic membrane as a substrate. This membrane could be transplanted onto more than 700 patients so as to restore the ocular surface and thereby visual recovery. The highlights of this technique was it was simple, cost-effective, feeder cell free, xeno free and submerged technique of generating a sheet of corneal epithelium within 10-14 days of culture. For patients with more severe disease, she designed a novel method of co-culturing two types of ocular surface epithelium (peripheral conjunctiva and central limbal epithelium) on a single membrane using a self-designed ring barrier. This was useful in reconstructing the ocular surface in a single step surgery using one membrane in 50 patients, who otherwise were incurably blind. To obviate the need for immunosuppression and allogenic limbal transplantation in severe bilateral disease with no other treatment option, she developed an alternate source of epithelium using oral mucosa. The pilot study using oral epithelium showed a success with 35% of patients. Within the same culture system, she reported a novel finding of the presence of stromal cells which show a striking resemblance to mesenchymal cells derived from bone marrow. Based on the phenotype, in-situ localization and gene expression studies, she proposed that these cells could be acting as intrinsic feeder cells in the explant culture system, which possibly could be limbal niche cell. She has also explored the option of using collagen scaffolds for growing limbal cells as an alternative to human amniotic

membrane. Her current approach is to establish useful as prognostic indicators. Her work on genotype and phenotype correlation of different types of corneal dystrophies is significant.

She established the Indian Association of Ophthalmic Pathologists, and subsequently facilitated its affiliation to the International Society of Ophthalmic Pathology. She is a mentor of many ophthalmology and pathology residents and fellows and a Ph.D. guide to research scholars. Her contributions to Translational research and Ophthalmic Pathology has won her several grants, awards and honors at both national and international level, even breaching the border conflicts with Pakistan. She has also contributed to various ethics committees, Institute Review Boards, Biosafety committees and was invited to formulate guidelines for stem cell therapy in India. She has more than 198 publications to her credit, nearly 100 invited talks at international meetings and grants worth 3 million USD.

Name : Prof Geeta K Vemuganti, MD, DNB, FAMS, FICP

Address Office : Dean,
School of Medical Sciences
University of Hyderabad
Hyderabad 500046
Phone: 91-40-23013279/23014781
Email: deanmd@uohyd.ernet.in/
gvmd@uohyd.ernet.in
gkvemuganti@gmail.com

I. Posts held:

Consultant Pathologist, Medwin Hospitals, Hyderabad (March 1994-May 1998)

Head of Ophthalmic Pathology Service, L V Prasad Eye Institute (June 1998 -2004)

Director, Ophthalmic Pathology Service, L V Prasad Eye Institute (June 2004 till 23rd Dec 2010)

Head, Stem Cell Laboratory, L V Prasad Eye Institute (2001 till 23rd Dec 2010)

Adjunct Faculty, University of Rochester, US 2009 till date

Prof & Dean, School of Medical Sciences, University of Hyderabad (24th Dec 2010 till date)

II SPECIAL COURSES/TRAINING

One month training in Hemato oncology Department at Tata Memorial Hospital, Mumbai. Had training in the fields of immunophenotying, immuno-cytochemistry, PCR techniques and In-situ hybridization.

One month training in the Department of Oncopathology at Tata Memorail Hospital, Mumbai. Had training in immunohistochemisty, immunocytochemistry and electron microscopic studies. (May, June, July 1995)

Two weeks Fellowship in Ophthalmic pathology at Doheny Eye Institute, University of Southern California, Los Angeles, USA. (April- May, 1999)

Two weeks observership at Ocular Pathology services, Wills Eye Hospital, Philadelphia, PA, USA. (May, 2000)

Two weeks training at the Department of Ocular Pathology, Armed Forces Institute of Pathology, Washington DC, USA (May, 2000)

Invited as Visiting Pathologist to Eye and Ear Infirmary, University of Illinois, Chicago, IL, USA.

Observer at Centre for Disease Control, Atlanta, GA, USA.

One week course of BD FACS ARIA at San Jose, USA

III. Teaching Experience:

• DNB fellows at Medwin hospitals (1994-1998)

• Ophthalmology Fellows at LVPEI (1998- till date)

• Optometry students at BSLO (2005 till date)

Recognized Guide to PhD students at BITS Pilani, University of Hyderabad (2001- till date)

• Visiting Pathologist to University of Chicago, Illinois (2000 & 2006)

• Visiting Pathologist to University of Rochester, USA (2007 till date)

• Preceptor of Post Graduate Diploma Course in Ophthalmic Pathology under Indian College of Pathologists (2009 onwards)

• Conducted Ophthalmic Pathology course (by teleconference) to Fellows and Residents at University of Rochester (2010-2011)