IDENTIFICATION AND CHARACTERIZATION OF A CELL SOURCE TO REGENERATE SALIVARY GLANDS

OLA MARIA, D.M.D.

Faculty of Dentistry, McGill University

Montreal, Quebec, Canada

June 2010

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.) in Dental Sciences

ABSTRACT

Sjogren's syndrome and radiotherapy for head and neck cancer result in irreversible loss of functional salivary acini; known as xerostomia, for which no adequate treatment is available. Our group has been testing different biomaterials, extracellular matrix proteins, and graft cells for the development of an orally implantable tissue-engineered artificial salivary gland device. The major hurdle we faced was to find a suitable graft cell type that must establish tight junctions (TJs) and exhibit an adequate transepithelial electrical resistance (TER) in addition to being functional acinar secretory cells. Therefore, the aim of this thesis was to isolate and characterize a suitable graft cell type.

We determined the distribution of TJ proteins in human salivary tissue to serve as a reference for future studies to assess the presence of appropriate TJ proteins in the graft cells we are looking for. We have found that claudin-1 was expressed in ductal and ~25% of serous cells while claudin-2, -3, -4, JAM-A, occludin and ZO-1 were expressed in ductal and acinar cells.

We characterized two candidate graft cells; human submandibular gland (HSG) cell line and primary human salivary gland (huSG) cells cultured individually on Matrigel (a basement membrane extract). Matrigel supported their morphogenesis and cytodifferentiation into 3-D polarized functional acinar units; expressing all TJ and acinar-specific proteins and exhibiting adequate TER. Thus, on Matrigel, both candidate cells showed ideal characteristics of suitable graft. However, neither Matrigel nor HSG would be used in clinical applications. Nevertheless, they would be implanted in animal models to further examine our envisioned artificial salivary gland device.

Mesenchymal stem cells (MSCs) can differentiate into cells from all dermal lineages; carrying new promises and significant therapeutic implications. Importantly, MSCs have been isolated from different adult tissues including salivary glands, however; no reports localized such cells in

situ. We localized CD44+ and CD166+ cells in human serous and mucous acini, respectively. Therefore, huSG tissue harbors MSCs, thus, these markers would be used; either to isolate MSCs or differentiated serous/mucous acini from huSGs. Implantation of autologous functional acini would be a major achievement to treat xerostomic patients, however, this would require the generation of a great number of such acini. Autologous MSCs would be expanded in vitro and induced to differentiate into functional salivary acini.

MSCs were cocultured with huSG cells and analyzed afterward. Successfully, 40% of MSCs showed salivary acinar phenotype; expressed acinar-specific genes and proteins, exhibited a noticeable level of TER, and were able to secrete α-amylase into media. These results represent a proof-of-concept of full differentiation of MSCs guided by huSG into functional polarized acini. This supports the potential and feasibility of using MSCs in cell-based therapy. Therefore, MSCs would provide a novel alternative source of graft for an implantable artificial salivary gland device to treat xerostomic patients.

RÉSUMÉ

Le syndrome de Sjögren et la radiothérapie de la tête et du cou engendre une perte irréversible des acini salivaires fonctionnels; ce phénomène est la xérostomie, pour lequel aucun traitement adéquat n'existe actuellement. Notre groupe a testé différents biomatériaux, protéines de la matrice extracellulaire et des cellules de greffon pour le développement d'un dispositif tissulaire de glandes salivaires artificielles implantable oralement. L'obstacle majeur auquel nous avons été confrontés était de trouver un type cellulaire approprié pour la greffe qui devait établir des jonctions serrées (JSs), présenter une résistance électrique transépithéliale (RET) adéquate en plus se différencier en cellules acineuse sécrétrices fonctionnelles. Par conséquent, l'objectif de cette thèse a été d'isoler et de caractériser un type cellulaire approprié pour la greffe.

Nous avons établi le paramètre de distribution des protéines de JSs dans les tissus salivaires de l'homme comme référence pour nos études suivantes dans le but d'évaluer la présence de protéines de JSs appropriées pour les cellules potentiellement destinées à la greffe. Nous avons déterminé que la claudine-1 est exprimée par les cellules ductales et par ~ 25% des cellules séreuses alors que les claudines -2, -3, -4, occludine, JAM-A et ZO-1 sont exprimées par les cellules ductales et acineuses.

Nous avons caractérisé deux types cellulaires candidats pour la greffe: la lignée cellulaire établie à partir des glandes humaines sous-mandibulaires (HGS) et des cellules primaires de glandes salivaires humaines (huGS) cultivées individuellement sur Matrigel (une membrane basale extraite). Matrigel permet leur morphogenèse et leur cytodifférenciation en unités acineuses 3-D polarisées et fonctionnelles, exprimant toutes les JSs, les protéines spécifiques des cellules acineuses et présentant une RET adéquate. Ainsi, sur Matrigel, les deux types cellulaires candidats ont montré des caractéristiques idéales pour une greffe appropriée. Cependant, ni Matrigel ni les HGS ne peuvent être utilisés dans le cas d'applications cliniques. Néanmoins, ils

seront implantés dans des modèles animaux afin de poursuivre l'étude de notre dispositif expérimental de glandes salivaires artificielles.

Les cellules souches mésenchymateuses (CSMs) peuvent se différencier en cellules de toutes les lignées cutanées, apportant de nouvelles perspectives et un potentiel thérapeutique important. Plus particulièrement, les CSMs ont été isolés à partir de différents tissus adultes, y compris les glandes salivaires, cependant aucun rapport ne mentionne la présence de telles cellules *in situ*. Nous avons localisé CD44+ et CD166+ respectivement dans les cellules acineuses séreuses et muqueuses humaine. Par conséquent, huGS comportent des CSMs et ces marqueurs seront utilisés, soit pour isoler les CSMs ou les cellules acineuses séreuses/muqueuses différenciées à partir des cellules huGSs. L'implantation d'acini autologues fonctionnels serait une étape majeure dans le processus de traitement des patients souffrant de xérostomie. Néanmoins, cela nécessiterait la production d'un grand nombre de ces acini. Les CSMs autologues seraient amplifiées *in vitro* et induites pour se différencier en acini salivaires fonctionnels.

Les CSMs ont été co-cultivées avec des cellules huGSs et ensuite analysées. Avec succès, 40% des CMSs ont montré un phénotype de cellules acineuses salivaires, exprimant des gènes et des protéines spécifiques des cellules acineuses, présentant un niveau adéquate de RET et étant capables de sécréter de l'α-amylase dans les milieux de culture. Ces résultats représentent une preuve-de-concept de la possibilité d'induire la différenciation complète des CSMs, guidée par les cellules huGSs, en acini polarisés et fonctionnels. Cela confirme également potentiel et la faisabilité de l'utilisation de CSMs pour la thérapie cellulaire. Par conséquent, les CSMs peuvent fournir une source alternative pour de nouveaux greffons cellulaires afin de réaliser un dispositif implantable glandes salivaires artificielles dans le but de traiter les patients souffrant de xérostomie.

To Mum, Ahmed, Nehal, Osama, Mostafa and Salwa

AKNOWLEDGEMENTS

I would like to express my sincere and deepest gratitude to several individuals who have kindly contributed in helping me to achieve this endeavour. First and foremost, I would like to thank my supervisor, Dr. Simon Tran, for graciously accepting me as a member in his laboratory team and for helping me to chase my dream. He was always full of absolute support and encouragements. I would like also to express my sincere gratitude to my Ph.D. committee members who helped me in the elaboration and guidance of my PhD objectives. I would like to thank Dr. Svetlana Komarova, Dr. Monzur Murshed and Dr. Jacques Galipeau for their support and time. Many thanks to my collaborators: Dr. Bruce Baum, Dr. Eva Mezey, Dr. Anthony Zeitouni, Dr. Salwa Maria, Dr. Osama Maria, Dr. Younan Liu, Dr. Yoshinori Sumita, Dr. Hojatollah Vali, Dr. John Presley, Dr. Judith Lacoste, Dr. Olga Gologan, Mr. Saeed Khalili, Dr. Kelly Sears, Mr. Yunlin Tai, Mrs. Jeannie Mui, FEMR, CIAN at McGill University and Mr. Cory Glowinski. I would like to thank the Co-operative Human Tissue Network and Royal Victoria Hospital staff members for providing human salivary gland samples. I am so grateful to Dr. Damien Le Nihouannen for revising the French translation of my thesis abstract.

I am so grateful to the funding institutions; CIHR, NSERC, RSBO, FODQ and Egyptian Ministry of Higher Education and Scientific Research for all funding, scholarship and granted awards.

Finally, I would like to thank my Mother; words cannot say enough to thank you for everything that you have done to bestow upon me all the opportunities you so altruistically have. I would like to thank my family members for their continuous support and prayers; Mostafa, Ahmed, Nehal, Osama, and Salwa.

LIST OF FIGURES

ABBREVIATIONS

IMRT	intensity-modulated radiation therapy
AQP5	aquaporin 5
SS	Sjögren's syndrome
IgA	immunoglobulin A
EGF	epidermal growth factor
NGF	nerve growth factor
AQP1	aquaporin1
NOD	none-obese diabetic
AAVhIL 10	adeno-associated virus-mediated interleukin 10
rAAV2hVIP	recombinant adeno-associated virus encoding vasoactive intestinal peptide
PKH 26	a fluorescent linkage marker
ES	embryonic stem
HSC	hematopoietic stem cells
MSC	mesenchymal stem cell
BM	bone marrow
DNA	deoxyribonucleic acid
EMT	epithelial-mesenchymal transition
NaCl	sodium chloride
H_2O	water ions
HSG	human submandibular gland
TER	transepithelial electrical resistance
EHS	Engelbreth-Holm-Swarm
huSG	human salivary gland

TJ	tight junction
JAM-A	junctional adhesion molecule-A
ZO-1	zonula occludens-1
CLDN	claudin
CHTN	co-operative human tissue network
AEC	3-amino-9-ethyl-carbazole
DAB	di-amino-benzadine
FFPE	formalin-fixed paraffin-embedded
EDTA	ethylene-diamine-tetraacetic acid
MDCK II	Madin-Darby canine kidney II
MDCK I	Madin-Darby canine kidney I
RT-PCR	real-time polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
BME	basement membrane extract
3-D	3-dimentions
CD (44)	cluster of differentiation (number 44)
CK-18	cytokeratin-18
ECM	extracellular matrix
TEM	transmission electron microscope
DMEM	Dulbecco's modified eagle medium
FBS	fetal bovine serum
PCNA	proliferation cell nuclear antigen
PBS	phosphate buffered saline
FITC	fluorescein isothiocyanate-conjugated

RRX	Rhodamine red-x
TBST	Tris-buffered saline tween-20
HRP	horse radish peroxidase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
RNA	ribonucleic acid
AMY	α-amylase
SEM	standard error of means
ANOVA	analysis of variance
DAPI	4',6-diamidino-2-phenylindole
α-SMA	α-smooth muscle actin
CK-pan	cytokeratin-panel
CIAN	cell imaging analysis network
FEMR	facility of electron microscope research
GIT	gastrointestinal tract
RVH	royal Victoria hospital
FAM	a fluorescent (FAM)-labelled
MGB	minor groove binder
KLK-1	kallikrein-1
MUC-7	mucin-7
Msi-1	Musashi-1
ISCT	international society for cellular therapy
PRP	platelets rich plasma
PCBM	particulate cancellous bone and marrow
TMJ	temporomandibluar joint

FISH	fluorescence in-situ hybridization
FACS	fluorescence activated cell sorting
BMDC	bone marrow derived cells
G-CFA	granulocyte colony-stimulating factor
PAS	periodic acid-Schiff
ELISA	enzyme-linked immunosorbent assay
BCA	bicinchoninic
PAP	peroxidase anti-peroxidase
NKCCl	sodium potassium chloride co-transporter
TSA	tyramide signal amplification
SFR	salivary flow rate
CFA	complete Freund's adjuvant
MHC	major histocompatibility complex
LMP2	low molecular-weight protein-2
NF-κB	nuclear factor- κB
TNF-α	tumor necrosis factor-α
OCT	optimal cutting temperature

PREFACE

The aim of this thesis was to identify and characterize a suitable cell source to use as a graft in an orally implantable artificial salivary gland device to regenerate salivary glands in xerostomic patients. I have elected to present my research in a manuscript-based format. My work has been assembled in 5 first-author original papers plus 1 review paper all presented in their integrity as distinct chapters of this thesis in accordance with the guidelines for thesis preparation from the Faculty of Graduate Studies and Research at McGill University [Guidelines for Thesis Preparation].

- Chapter 2: **Ola M Maria**, Jung-Wan M Kim, Jonathan A Gerstenhaber, Bruce J Baum, Simon D Tran. Distribution of Tight Junction Proteins in Adult Human Salivary Glands, Journal of Histochemistry and Cytochemistry. 2008; 56(12):1093-1098.
- Chapter 3: **Maria OM**, Maria O, Liu Y, Komarova SV, Tran SD. Matrigel Improves Functional Properties of Human Submandibular Gland Cell Line (submitted 2010).
- Chapter 4: **Maria OM**, Zeitouni A, Gologan O, Tran SD. Matrigel Improves Functional Properties of Primary Human Salivary Gland Cells (submitted 2010).
- Chapter 5: **Maria OM**, Khosravi R, Mezey E, Tran SD. Cells from bone marrow that evolve into oral tissues and their clinical applications. Oral Diseases 2007;13(1):11-16.
- Chapter 6: **OM Maria**, SM Maria, Tran SD. Identification of Specific Population of Human Salivary Acini and their Progenitors Using Mesenchymal Stem Cell Markers (submitted 2010).
- Chapter 7: **Maria OM**, Tran SD. Human Mesenchymal Stem Cells Differentiate into Functional Salivary Acinar Cells (submitted 2010).

In addition to the work presented in this thesis, I was involved in collaborative studies with other members of the laboratory, which led to the following publications:

- Yoshinori Sumita, Younan Liu, Saeed Khalili, **Ola Maria**, Dengsheng Xia, Sharon key, Eva Mezey, Simon D Tran. Bone Marrow-Derived Cells Rescue Salivary Gland Function in Mice with Head and Neck Irradiation, (*submitted 2010*).
- Saeed Khalili, Younan Liu, Yoshinori Sumita, **Ola M. Maria**, David Blank, Sharon Key, Eva Mezey, Simon D. Tran. Bone Marrow-Derived Cells: A Source of Undifferentiated Cells to Prevent Sjögren's Syndrome and to Preserve Salivary Glands Function in the Non-Obese Diabetic Mice, (*submitted 2010*).

CONTRIBUTION OF AUTHORS

The data presented in Chapters 2-7 of this thesis has been performed by the doctoral candidate under the supervision of Dr. Simon Tran. The role the doctoral candidate performed in manuscripts is indicated prior to each manuscript.

Other individuals who have contributed to the work are listed below:

CHAPTER 2

- *Jung-Wan M Kim* is a summer student in Dr. Tran's Laboratory and provided help in writing the introduction part of this manuscript.
- *Jonathan A Gerstenhaber* is a scientist at the National Institutes of Health, USA and one of our collaborators; he shared reviewing this manuscript.
- *Bruce J Baum* is a scientist at the National Institutes of Health, USA and one of our collaborators; he shared writing and reviewing this manuscript.

CHAPTER 3

- *Osama Maria* was an MSc. student at Dr. Svetlana Komarova's laboratory; he performed the quantitative RT-PCR analysis and shared writing and reviewing this manuscript.
- *Younan Liu* is a research assistant at Dr. Simon Tran's laboratory; he performed the SDS-PAGE technical work and shared reviewing this manuscript.
- *Svetlana Komarova* is a scientist at the faculty of Dentistry, McGill University; she shared writing and reviewing this manuscript.

CHAPTER 4

- *Anthony Zeitouni* is a surgical oncologist at the Royal Victoria Hospital, Faculty of Medicine, McGill University; he provided fresh salivary gland samples and shared writing and reviewing this manuscript.

- *Olga Gologan* is a surgical pathologist at the Royal Victoria Hospital, Faculty of Medicine, McGill University; she examined the salivary gland samples, provided pathological report and shared writing and reviewing this manuscript.

CHAPTER 5

- *Roozbeh Khosravi* was an MSc. student at Dr. Simon Tran's laboratory; he shared writing and reviewing this manuscript.
- *Eva Mezey* is a scientist at the National Institutes of Health, USA and one of our collaborators; she shared writing and reviewing this manuscript.

CHAPTER 6

- *Salwa M Maria* is a Ph.D. student at Dr. Svetlana Komarova's laboratory; she took care of the rabbits as a vetrenarian and shared writing and reviewing this manuscript.

TABLE OF CONTENTS

CHAPTER- 1

GENERAL INTRODUCTION

XEROSTOMIA

Xerostomia is a condition of mouth dryness that results from irreversible salivary gland damage followed by hypofunction. Dry mouth has two main causes; either an autoimmune exocrinopathy, Sjögren's syndrome; affecting 1-4 million patients or from therapeutic irradiation of patients with head and neck cancer (30,000 new cases each year) in the United States. Moreover, some systemic diseases such as diabetes mellitus and pernicious anaemia would cause xerostomia (1-5). In addition, in 20% of xerostomic patients the cause is idiopathic (6,7). The clinical negative sequelae of salivary gland hypofunction include; an increased incidence of dental caries, periodontitis, candidiasis, mucositis, gastric and esophageal ulcers (2). Currently, the available treatment for xerostomic patients includes saliva-stimulants and artificial saliva (8,9). This symptomatic treatment is temporary as permanent curatives for such condition are not available yet. We have been working to develop a tissue-engineered artificial salivary gland device that would be implanted into a surgically created pouch inside the mouth of xerostomic patients. (10,11). The aim of thesis is to identify and characterize a graft cell type to be used in the envisioned device to treat xerostomic patients.

RADIOTHERAPY FOR HEAD AND NECK

Head and neck cancer represents around 3% of malignant tumors and around 500 000 cases diagnosed every year world-wide (12,13). These cancerous tumors are distributed on; lips, oral cavity, tongue, pharynx, larynx, nasal cavity, sinuses, ears, orbits, skull base, and salivary glands (14). Surgical dissection followed by radiotherapy is usually the standard treatment to rescue the patient and in some cases chemotherapy might be applied too. Prognosis for such kinds of cancers depends mainly on the tumor stage (12,13). Usually organ dysfunction and facial deformity are the most common results of both tumor growth and treatment. The sensitivity of

normal tissues within the radiation field might limit the radiotherapy dose. For head and neck cancers the most sensitive tissues include salivary glands, spinal cord, skin, bone, and oral mucosa. For protecting such tissues, usually radiotherapy is applied in daily bases. However, acute as well as chronic side effects follow in almost all patients. Within the first weeks, mucositis, dermatitis and xerostomia acutely develop. A progressive loss of saliva along with changes in its composition, pH and viscosity usually occurs during and after radiotherapy (15). The consequent negative sequelae include; oral pain, increased incidence of dental caries, reduced taste and smell, increased risk of oro-pharyngeal infections, difficulty in speech, chewing and swallowing (15,16). In most cases these symptoms persist for the patient's life-time (17). These symptoms severely reduce the quality of life of these patients, even when advanced radiation techniques; such as intensity- modulated radiation therapy (IMRT) are employed still 40% of the patients experience xerostomia (18). Therefore, permanent treatment of xerostomia and accompanying negative sequelae are highly valuable.

PATHOPHYSIOLOGY OF SALIVARY GLAND RADIATION-INDUCED DAMAGE

In salivary glands; serous acini are the most radio-sensitive components and are labile to severe necrosis, degranulation, cytoplasmic vacuolization, nuclear pyknosis and cell death (19-24). After heavy doses of radiotherapy, serous acini might disappear completely. To a lesser extent mucous acini react to radiotherapy. The mechanism of serous cells damage is thought to be due to the free radicals generated from metal ions contained inside the serous secretory proteins leading to DNA damage (25). Furthermore, damage to the cell membranes and disturbances in cell signaling have been reported (26,27). In addition, accompanied damage to nerve and blood supply would complicate the situation (28). The changed taste has been reported to be caused by taste cells (neuroepithelial cells) and nerve endings damage following radiotherapy (29). Salivary gland stromal adiposis and fibrosis has been described in human parotid and submandibular glands,

respectively (30). In addition, thickening of extracellular matrix components in response to high doses of IR also has been reported (31). These stromal changes following radiotherapy would diminish the diffusion of nutrients and oxygen to the parenchymal cells that would restrict future regenerative capacities within the affected gland (32), especially that the salivary tissue turnover rate is slow (~60 days) (33). Of importance, aquaporin-5 (AQP5); a water channel protein that is important for saliva secretion, and is expressed by acinar cells, has been reported to miss-distribute and its corresponding gene was down-regulated following radiotherapy for head and neck cancer (34). Consequently, gene transfer of AQP5 has been considered as a potential therapeutic approach in these xerostomic patients (35).

SJOGREN'S SYNDROME

Sjogren's syndrome (SS) is a chronic autoimmune exocrinopathy, characterized by lymphocyte infiltration of salivary and lacrimal glands, leading to glandular hypofunction that often results in characteristic symptoms of xerostomia and xerophthalmia. In addition to systemic manifestations that are either non-visceral (skin, arthralgia, myalgia) or visceral (lung, heart, kidney, gastrointestinal, endocrine, central and peripheral nervous system) (1,36). Salivary gland biopsies obtained from SS patients showed heavy lymphocytic infiltrates and up to 50% atrophy of the secretory acinar cells (37). The salivary gland pathogenesis of SS is unclear however; it was proposed to include the pro-inflammatory cytokines; interleukin-1 and tumor necrosis factor-α. These cytokines consequently act to inhibit acinar secretion (1). In addition to cytokines, there are antibodies against type-3 muscarinic receptor (38). Furthermore, in minor salivary and lacrimal glands from SS patients, abnormal distribution of AQP5 water channel was reported (39,40). SS patients experience xerostomic negative sequelae similar to those experienced by radiotherapy-induced xerostomia (1).

ADULT HUMAN SALIVARY GLAND HISTO-PHYSIOLOGY

The anatomic location and size of a salivary gland (Figure.1.1) determines the extent of their potential damage upon exposure to the radiotherapeutic beams (32). In human, there are three pairs of major salivary glands and numerous minor glands. The major glands include; the parotid that is situated just anterior and inferior to the ears and superficial to the ramus and angle of the mandible; and the sublingual and submandibular glands that are located in the floor of the mouth. The minor glands are distributed in the oral mucosa; cheeks, lips (labial mucosa), palate (posterior hard and soft), tonsillar pillars, and posterior dorsal and anterior ventral tongue.

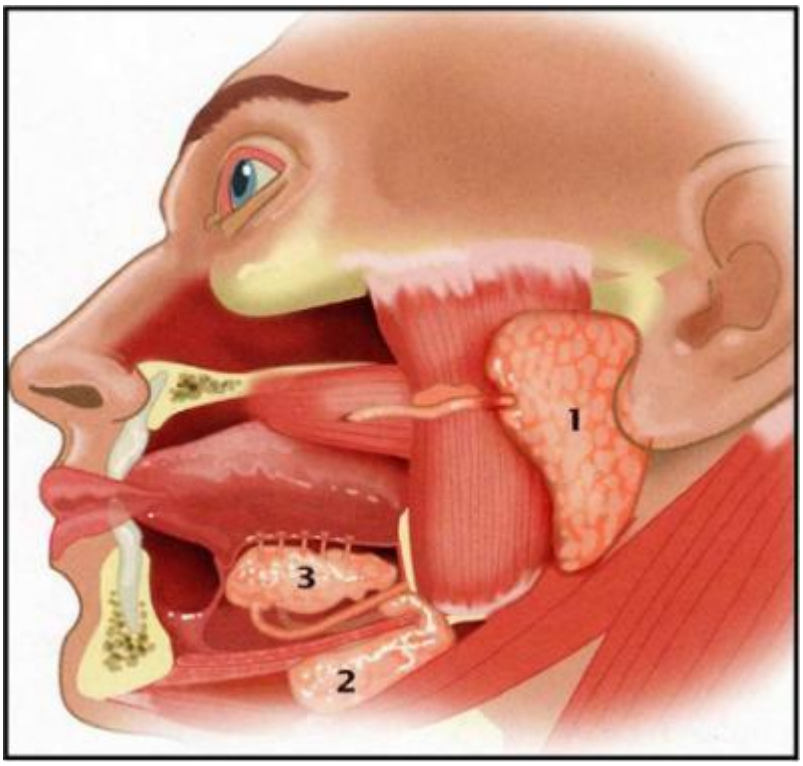
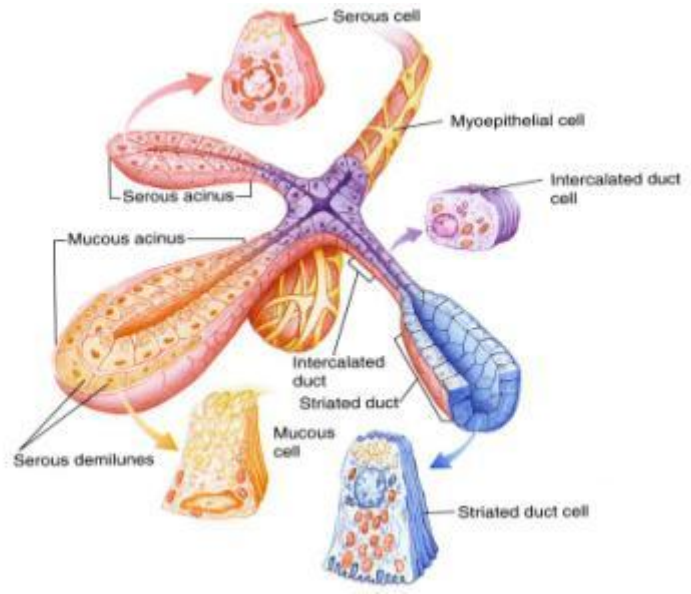


Figure.1.1: shows human major salivary glands anatomical positions; parotid (1), submandibular (2) and sublingual (3) glands. This picture is copied from reference (41)

The parotid gland is composed of serous acini and secretes watery saliva while the sublingual gland has mucous acini with serous demilunes and secretes very viscous saliva, and the submandibular gland has mostly serous acini and some mucous acini attached to serous demilunes and secretes moderate viscous saliva (42,43). Both acinar cell types (with pyramidal cell shape) drain into intercalated, striated and excretory ducts (Figure.1.2A, 1.2B). The myoepithelial cells encircle the acini and intercalated ducts to further facilitate saliva secretion (42,44). They contract rhythmically to compress the lumen with a peristalsis-like manner to further push saliva into the larger ducts. (45). The intercalated ducts which constitute 20% of salivary cell population consist of cuboidal cells arranged in a single layer (46). The striated and excretory ducts consist mainly of columnar cells have deep basolateral invaginations and intercellular interdigitations of the plasmalemma accompanied by numerous large, elongated mitochondria (47,50). The large excretory ducts consist of either stratified or pseudostratified columnar epithelium (49,50) and in both types there are basal cells around the large excretory ducts. The innervation and main blood supply enter the body of the salivary gland with the main duct (42) and further flow and divide parallel to the ductal branches to reach the acini. In the major salivary glands, the parasympathetic stimulation via cholinergic and muscarinic receptors provokes the watery saliva, while the sympathetic stimulation via α- and β-adrenergic receptors provokes the organic components (51).

A



B

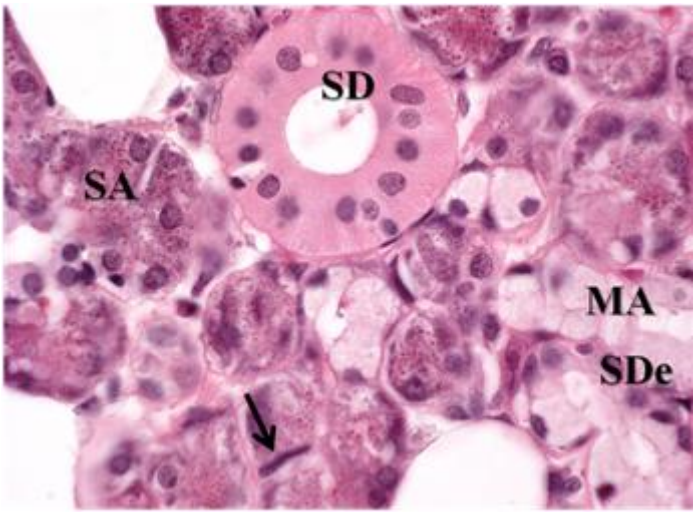


Figure.1.2: shows; (A) a diagram of salivary gland epithelial cell types, this picture is copied from reference (52). (B) a light micrograph showing the histology of human salivary gland tissue; striated duct (SD), serous acinus (SA), Mucous acinus (MA), serous demilunes (SDe) and myoepithelial cell (arrow).

Most saliva is produced by the three major glands; submandibular (~65%), parotid (~23%) and sublingual (~4%). In addition, hundreds of minor salivary glands are randomly residing in the oral mucosa and produce ~8% of the total saliva volume (53). Approximately, 1.5L of saliva is secreted by healthy persons each day; consists mainly of water, ions and proteins. However, there are some differences in regards to age (54) and gender (55) in the secreted salivary volume and composition. Saliva facilitates speech, mastication and swallowing, and initiates the digestion process of certain food types by various enzymes contained. In addition, it protects the oral mucosa by various components including; mucins, secretory IgA, histatins and agglutinin. Further protection is provided to teeth by salivary components including; proline-rich proteins, statherins, calcium, and phosphate. Moreover, saliva contains essential antibacterial; lysozyme, lactoferrin, IgA, lactoperoxidase-thiocyanate as well as buffering components; sodium, potassium, growth factors EGF and NGF that further protect and clean the oropharyngeal mucous membrane (56). Therefore, any condition that affects the composition and the volume of saliva secreted will have negative impact on the oropharyngeal field.

CURRENT MANGMENTS AND THERAPIES FOR XEROSTOMIA

Current treatment of xerostomia is symptomatic; that temporary relieves painful experiences, however; permanent curatives for xerostomia are not yet available. The available management to reduce salivary gland damage either before or following radiotherapy will be discussed in the next pages and the management of Sjogren's syndrome will follow afterward.

A- PROTECTION BEFORE RADIOTHERAPY

Owing to the weak effectiveness of available symptomatic management of xerostomic patients, certain strategies have been proposed to reduce the radiation-induced damage within limits not to interfere with tumor treatment.

1. Shielding And Cytoprotective Agents

Shielding of salivary glands could be used in case of unilateral cancers and those outside the oro-pharynx (32). In addition to the use of cytoprotecting biochemicals; such as amifostine, that act as potent scavengers against free radicals, thereby reducing radiation-induced DNA damage (57). Amifostine has shown promising results with glands receiving low to moderate doses of radiotherapy in head and neck cancer patients (58, 16). However, many patients cannot tolerate its side effects especially when radiotherapy is combined with chemotherapy (32).

2. *Stimulation Of Acini Before Each Radiation Dose*

In trials on human, pilocarpine and bethanechol were administered directly before each dose of radiotherapy and showed significant salivary tissue protection (16,59,60). The most common saliva-stimulant, pilocarpine; acts on the muscarinic receptors however it requires survival of adequate residual salivary acini in order to work properly, in addition it has some contraindications and side-effects (61) therefore, it is not applicable for all patients.

3. Intensity Modulated Radiation Therapy[IMRT]

IMRT (62) was reported to deliver accurately and specifically localized radiation dose directed to the target tumor with minimum exposure of the surrounding normal tissues (32,63). However, IMRT is not applicable for all patients including those with midline tumors and patients with contra-lateral neck lymph node metastasis (27, 32).

4. Salivary Gland Transfer Away From The Radiation Pathway

Surgical transfer of salivary glands involved in the radiotherapy field have been tried to reduce salivary gland damage upon radiation. Afterward, transferred gland would either be left at its new location or returned to its original site when radiotherapy is completed (64). However, the application of this technique is limited to patients undergoing surgical dissection before starting their radiotherapy. In addition, the connection of each transferred gland to its main duct and

blood supply should be maintained or re-established, thus the transfer distance would have some limitations. Furthermore, it is not applicable for patients with widespread head and neck tumors.

B- REGENERATION AFTER RADIOTHERAPY & FOR SJOGREN'S SYNDROME

The establishment of rigorous oral hygiene and careful follow-up to prevent and treat dental caries and oral infections are always recommended to xerostomic patients in addition to saliva-substitutes and -stimulants (65). After the completion of radiotherapy, induction of the residual salivary gland cells to regenerate glandular tissue would be applied either via gene transfer or by local infiltration of regeneration inductive factors that are involved in normal salivary gland growth and development; such as growth factors. These methods although promising, much information need to be elucidated before clinical applications.

Regeneration is a physiologic process through which the living organisms can repair their damaged tissues. The regenerative capacity differs among species as well as organs (66). In general, human tissues have limited regeneration capacity for example; the central nervous system has a very limited regenerative ability (67,68). However, the liver is known to have a great regenerative capacity and it can totally repair to normal size even after a 90% hepatectomy (69,70).

1. Gene Therapy

Gene therapy has been proposed recently. Many investigators (71,72) have started this very promising approach in rats, minipigs and nonhuman primates. Using adenoviral and nonviral vectors some genes were infused through the main duct to transfect the salivary tissue. Transfection of human aquaporin 1 (AQP1) gene (water channel associated with acinar cells) in these reports has dramatically increased saliva secretion from irradiated submandibular glands. However, for future clinical applications, gene therapy would require further studies for safe and efficient clinical outcomes. A similar study was performed on irradiated parotids of adult rhesus

monkeys however, the results were inconsistent; two out of four AQP1-treated monkeys exhibited an increased salivary flow rates (73).

In gene therapy studies of SS xerostomia, none-obese diabetic (NOD) mice are the most commonly used animals to represent a Sjogren's-like model. Interleukin 10 (IL-10) is a protein associated with wide range of immune activities including SS-related immunity. Based on the autoimmune pathogenicity of SS, a recombinant adeno-associated virus-mediated interleukin 10 (AAVhIL10) vector was infused to the submandibular salivary glands of NOD mice (74). The authors reported that the treated mice exhibited markedly higher salivary flow rates compared to controls, in addition to marked improvements in the SS-associated inflammatory reaction. Another study used a recombinant serotype 2 adeno-associated virus encoding the human vasoactive intestinal peptide (VIP) transgene (rAAV2hVIP); VIP was known initially as a gastrointestinal hormone with multifunctional capabilities which include; being a neurotransmitter, vasodilator, bronchodilator, trophic agent, secretagogue (saliva-stimulant), and an immunomodulator (66). The rAAV2hVIP was administered into the submandibular gland of NOD female mice. The results revealed immunosuppressive effect (75).

2. *The Potential Of Replacement With In Vitro Preserved Salivary Cells*

On the experimental level; Sharawy and O'Dell 1981, (77) reported some experiments using autografts from the submandibular gland cells of rats into the tongue or submandibular gland, and they reported acinar and striated ductal differentiation originated from the autograft cells. More recently, Sugito et al. 2004, (76) used cultured rat submandibular gland cells that were labeled with PKH 26 (a fluorescent linkage marker), and injected them into duct-ligated glands. However, almost all cells resided in the stroma, few if any differentiated into acinar cells.

3. *The Potential Use Of Stem Cells To Regenerate Salivary Glands*

Stem cell therapies are currently investigated for their potential to treat many clinical disorders. Stem cells are defined as clonogenic, self-renewing, and capable of generating one or more specialized cell types (78). Developmentally, stem cells are categorized either as embryonic stem cells or as post-natal stem cells (tissue-specific, adult stem cells) (79). In order for the stem cells to accomplish self-renewal; an asymmetric cell division occurs, by which a stem cell divides to generate one daughter which remains a stem cell and one progenitor cell that will further differentiate (Fig.1.3A) (80). Subsequently, the progenitor cells divides with more commitment toward mature cell lineages. However, the asymmetric cell divisions do not allow stem cells to expand. To achieve expansion, stem cells can also divide symmetrically; a stem cell gives rise to two identical daughter stem cells. The balance between symmetric and asymmetric divisions depends on the developmental stage and environmental signals in each tissue (81). Embryonic stem (ES) cells are derived from the inner cell mass of a developing blastocyst (Figure.1.3B) (80) and are considered as pluripotent cells; able to form the three fetal dermal lineages (endoderm, mesoderm and ectoderm) (82). Despite their pluripotency, many difficulties prevent their use in clinical applications. ES cell-based therapy will inevitably employ allogenic ES cells, thus will be facing a risk of immune rejection. In addition, ES cells have a tumorigenic potential where they were reported to form teratomas. Furthermore, the ethical aspects of ES cells have not been solves yet. Post-natal stem cells (derived from specific tissues or organs) are considered multipotent; able to differentiate into cells from multiple lineages to constitute an entire tissue (82).

A) B)

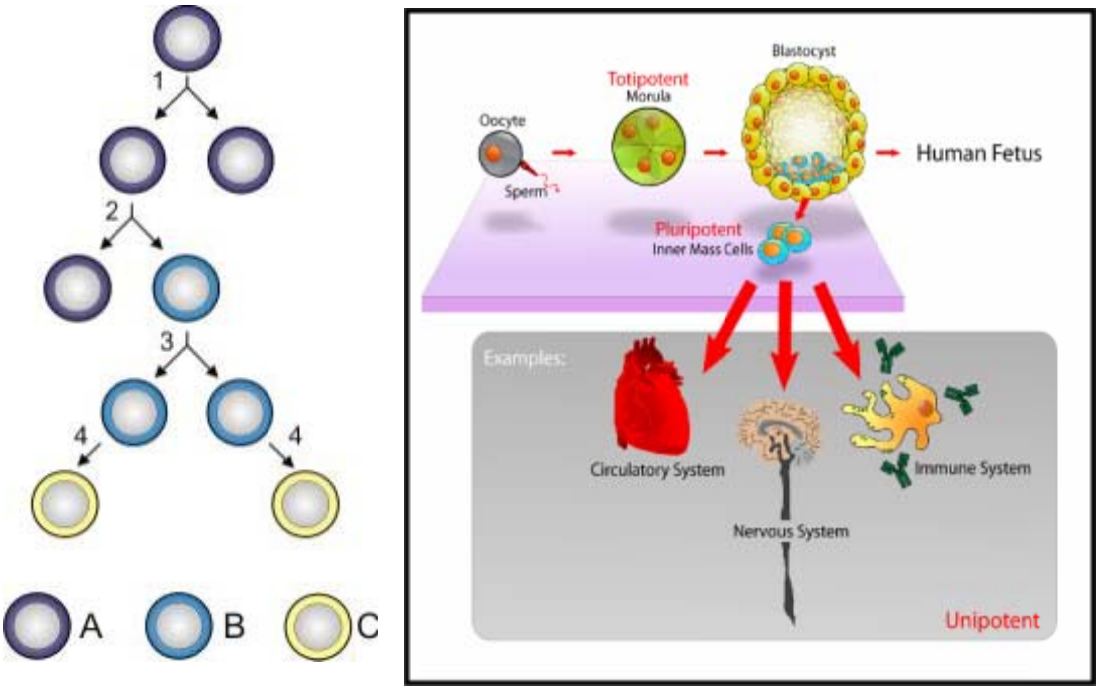


Figure.1.3: shows A); Stem cell division and differentiation diagram; 1 (symmetric stem cell division), 2 (asymmetric stem cell division); 3 (progenitor division), 4 (terminal differentiation) giving A (stem cells); B (progenitor cell), C (differentiated cell).

B); Embryonic stem cells (ES; pluripotent) derived from the inner mass cells within the blastocyst. These ES cells can form any tissue in the embryo's body except the placenta. The morula's cells are totipotent; can form the embryo's body and the placenta. Progenitor cells residing in each tissue are unipotent; can form only the cells of the specific tissue where they reside. Figure.1.3 is copied from reference (80).

In the middle of the 19th Century, a hypothesis about the origin of cells involved in tissue repair in mammals was developed by Cohnheim (83). He suggested that all cells involved in tissue repair come from the bloodstream and subsequent observations figured out the bone marrow as the origin of such cells. Although his hypothesis has been subjected to much debate, (84) most of the convincing proofs for Cohnheim's hypothesis comes from reports on animals'/patients' tissues who have received either bone marrow transplants or blood transfusion. It was reported that when females received bone marrow transplants from male donors, Y positive male cells were identified in their livers, kidneys, lungs, hearts, brains, muscles, and oral epithelia (84-104). According to our present knowledge there are two distinct populations of post-natal stem cells in the bone marrow: the hematopoietic stem cells (HSC) and the mesenchymal stem cells. HSC were recognized more than 40 years ago as they have the ability to reconstitute the hematopoietic system of a lethally irradiated host (79) since it gives rise to all blood cell lineages. Their unique ability to continuously self-renew permits HSC to sustain blood cell production throughout life. The frequency of HSC is one in 10,000-15,000 bone marrow cells (105). Under physiological conditions, quiescent HSC are interspersed with other cells within the bone marrow. However, under stressful conditions such as massive bleeding or acute bacterial infections, HSC rapidly proliferate, differentiate, and migrate from the bone marrow to circulate throughout the body (106,107). As HSC can reconstitute the entire blood system, bone marrow transplantations have long been used in the clinic to treat hematopoietic diseases (108). Mesenchymal stem cells originate from the mesodermal layer in the embryo's body and in the adult they reside in the bone marrow as well as in a variety of tissues. Mesenchymal stem cells constitute only a small number (one in 10^4-10^6) of bone marrow cells (109,110). The pivotal characteristic of mesenchymal stem cells is their ability to differentiate in vitro into several cell types based on culture conditions (110). It has been demonstrated that these cells possess a multilineage differentiation capability;

bone, cartilage, adipose, tendon, and muscle tissues (89,111). Several studies have reported that mesenchymal stem cell clones comprise a heterogeneous cell population with respect to their self-renewal characteristic (40). There are reports that bone marrow stem cells can differentiate into hepatocytes (112), skeletal myocytes (89), cardiomyocytes (113,114), neural cells (87,88), endothelial cells (118), epithelial cells (115), and pancreatic endocrine cells (116). These findings on the plasticity of post-natal stem cells carry great hope for regenerative medicine applications (117-119). Unfortunately, there are little data on salivary gland stem cells. Some reports indicate that salivary glands have the capacity to regenerate after partial extirpation and duct-ligation, (120-125) therefore; they contain stem/progenitor cells. Ligation of the main excretory duct causes total atrophy of the gland, which is characterized gradual disappearance of acini, dilation of the interlobular ducts, inflammatory cell infiltration and parenchymal fibrosis. In the rat parotid and submandibular glands; 7 days post-ligation, the total glandular weight decreased by 30-40% (123,126) and a loss of 85% in the acinar cell mass (123). Following the release of the ligature, within 7 days, an extremely high proliferation and regeneration rates of acinar cells were observed with concomitant reduction in the ducts number. Based on these studies, it was proposed that the intercalated duct cells contained the progenitors for acinar and granular convoluted tubule cells (127,128), and that striated duct cells were presumably replaced by more primitive excretory duct cells (128).

MECHANISINS OF PLASTICITY

During development, the formation of epithelia precedes the formation of mesenchyme therefore; all mesenchyme is derived from epithelia (129). The primitive mesenchyme is formed from epiblastic cells by a process known as an epithelial-mesenchymal transition (EMT). Immediately thereafter, primitive mesenchymal cells reorganize via mesenchymal-epithelial transitions to form secondary epithelial structures (130,131). Continued transitions from epithelium to

mesenchyme drive the development of various organs (132-134). At the same time during development, several studies reported that activation or de-activation of what is called “master genes” is essential for the differentiation of certain cells from one stage to another. Identification of such master genes is crucial in developmental biology as well as in understanding and experimentally controlling stem cell differentiation (135)

Plasticity is defined as the ability of post-natal stem cells to differentiate into mature and functional cells of the same or of a different germ layer (79). Four explanations for the phenomenon of plasticity in post-natal stem cells have been proposed (136-140). First, there might be persistent stem cells from embryonic development with broad developmental potentials which are maintained within the adult bone marrow (141). When transplanted into other organs, these cells are instructed to differentiate into tissue-specific cells under inductive signals from that specific tissue. A second possibility is that true precursors of post-natal stem cells with embryonic stem cell-like properties persist in adult bone marrow, such as the multipotent adult progenitor cells (142). A third explanation may be that the nuclei of the transplanted stem cells undergo reprogramming of the existing genetic information, expressing new genes and proteins that are consistent with the novel lineage, and this might be a result of de-differentiation, and re-differentiation (139,144). A final explanation is when cell fusion occurs, which is a rare phenomenon reported *in vitro* and *in vivo* in tissues where polyploidy is common, such as hepatocytes, skeletal muscle, cardiac muscle and Purkinje cells of the cerebellum (145). As a result the genetic information of both fused donor and host cells is partially changed (144, 146). Recently, the use of stem cell-based therapies has been advocated and researchers now design their studies to apply in a variety of diseases; such as myocardial infarction, Parkinsonism, Alzheimer’s disease, diabetes mellitus (type 1and 2), chronic liver failures, muscle, skin, eye and kidney disorders (146).

A TISSUE-ENGINEERED ARTIFICIAL SALIVARY GLAND DEVICE

We have been working to develop a tissue-engineered artificial salivary gland device that would be implanted into a surgically created pouch in the patient's mouth (Figure.1.4) (10,11). The standard design for such device includes; a blind-end tube fabricated from a slowly biodegradable scaffold coated with extracellular matrix on its inner (luminal) surface in order to promote attachment and polarization of epithelial cell monolayer; the graft cells that should be capable of unidirectional fluid secretion (147). The autologous graft cells (acinar cells) should be able to secret fluid towards the oral cavity. For these cells to function properly, they must be polarized and form an adequate epithelial barrier (10,11). To secrete fluid unidirectionally, cells must express a group of transport proteins (tight junctions; TJs) that generate an osmotic gradient and control the paracellular movement of water, proteins, and small solutes (148-154). Human salivary cells do express these TJ proteins at their apico-lateral membranes (155).

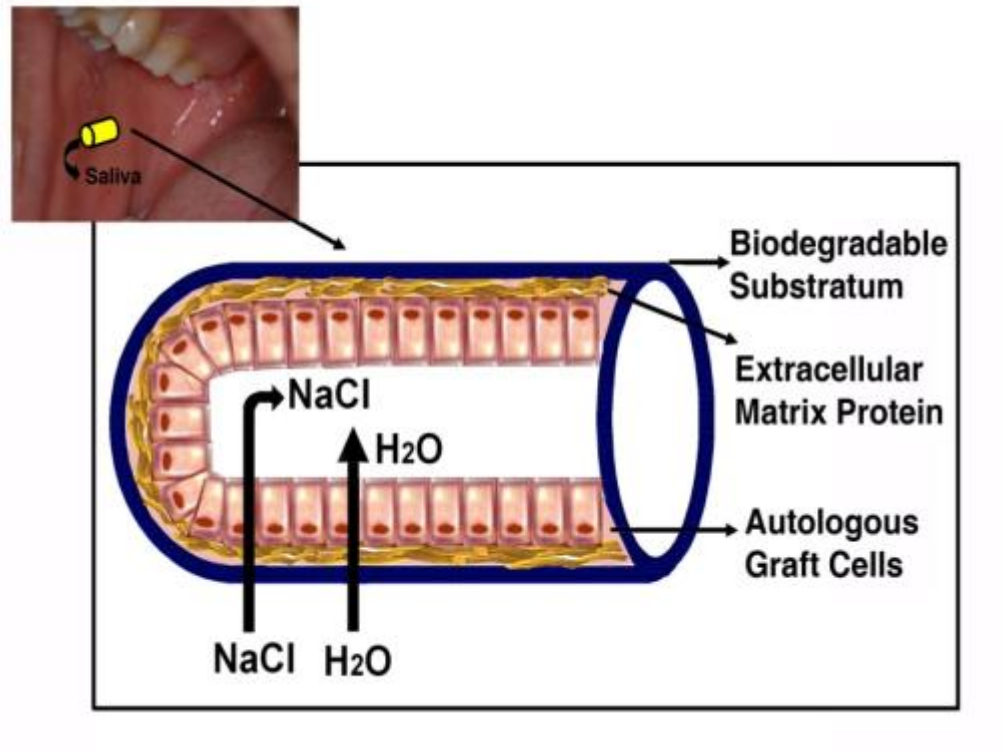


Figure.1.4: shows the design of the artificial salivary gland device as envisioned. The device is formed of a blind-end tube and composed of three essential elements: a biodegradable substratum; a coating of an extracellular matrix protein on the luminal surface of the substratum; and a polarized epithelial cell layer consisting of autologous graft cells. Graft cells would be able to generate an osmotic gradient (lumen > interstitium), shown here as unidirectional NaCl transport. Water would follow through a facilitated water-permeability pathway (water channel) existing in the plasma membrane of the cells. The device would be implanted in a surgically created pouch in the patients' buccal mucosa (inset). Figure.1.4. is copied from reference (156).

The reported scaffold biomaterials used consisted of a denuded rat tracheal preparation, poly-L-lactic acid, polyglycolic acid, chitosan and poly (ethylene glycol)- terephthalate /poly (butylene terephthalate. These polymers were pre-coated with matrix proteins; such as fibronectin and collagen I, reviewed in Kagami et al 2007, (66). However, our major hurdle has been to obtain a suitable graft cell type.

In several previous reports, we have suggested that the human submandibular gland (HSG) cell line, (147,157-159) might serve as a suitable allogeneic graft cell. However, HSG cells are not capable of forming a polarized epithelial layer as they do not express tight junctions, therefore, do not show reasonable transepithelial electrical resistance (TER) or control water movement (160). Matrigel, a basement membrane extract, (Becton Dickinson Biosciences, Bedford, MA), (161), was reported to promote complete differentiation including the expression of salivary acinar markers (162). Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells which contains collagen IV, laminin, fibronectin, entactin, perlecan, in addition to multiple angiogentic and growth factors essential for regulation of cell growth and differentiation (163,164). HSG cells grown on Matrigel were reported to form acinar structures that expressed α-amylase and cystatin proteins (165,166). Therefore, the growth of HSG cells on Matrigel can be used as a model to study salivary acinar cell formation and physiology. Also, the characterization of HSG cells cultured on Matrigel would benefit our understanding of salivary glands morphogenesis and cytodifferentiation. If HSG cells can be induced to differentiate into functional polarized acinar cells this would provide an excellent model to further study this proposed graft cell type in animal models.

We have succeeded in culturing and expanding primary human salivary gland (huSG) cells that are polarized and can provide an adequate functional epithelial barrier. However, they grow very slow; consequently do not provide adequate cells for the envisioned device. Moreover, most of

these cells have a ductal phenotype and thus, are not fluid secretory cells (11). Successful expansion of functional polarized acinar units from primary human salivary gland cells would be a great achievement toward using autologous salivary graft cells for the envisioned salivary gland device.

Salivary glands, like other parenchymal tissues contain stem/progenitor cells (167-170), but their exact source is not yet understood. The patterns of repair and regeneration in adult salivary glands suggest that they contain a stem cell-like population within the intercalated ducts (168). In addition, almost all differentiated cell types of adult glands have the ability to divide. It appears that division by both stem cells and differentiated cells contribute to the proliferation/ turnover of salivary cells (168). The identification and in vitro expansion of acinar stem/ progenitor cells that would be used as autologous graft cells in our artificial salivary gland device would be an important achievement for the treatment of xerostomic patients. However, the use of salivary gland stem cells might be difficult for clinical application if an insufficient number of stem cells are obtained from the patient's gland biopsies. Both patients with either Sjogren's syndrome or head and neck cancers are old and the gland tissues tend to be atrophic in older patients.

Another source of stem cells that have been suggested is the bone marrow that shelters two types of stem cells; hematopoietic (105) and mesenchymal (171,172). The capacity to differentiate into mesodermal (110,173), ectodermal (173) and endodermal (174) cell lineages characterizes mesenchymal stem cells (MSCs) as pluripotent cells. MSCs were reported to differentiate in vitro into several cell types based on culture conditions (110). They have a tendency to acquire tissue-specific characteristics when cocultured with specialized cell types or exposed to tissue extracts in vitro (175-177). Many reports highlighted the heterogeneity of MSCs population; therefore, a panel of key markers is used to isolate MSCs (178) as no single stem cell marker is identified yet. Many studies have successfully isolated MSCs-like populations from different tissues; adipose

tissue (171,179,180), bone marrow (180-181), synovial membrane (182), lungs (181, 183), brain, spleen, liver, kidney, large blood vessels, muscle, thymus, pancreas (181), and salivary glands (184). Interestingly, the possible use of bone marrow-derived stem cells to replace oral mucosa has been reported (86). Owing to the great expansion and differentiation potential of MSCs that are mainly present in the bone marrow (185), we decided to explore the potential of human MSCs to differentiate into huSG in Transwell-clear coculture system. Therefore, if successful differentiated MSCs would be obtained, they would be used as a graft in our envisioned artificial salivary gland device, ready for in vivo trials in animal models before clinical application.

Malfunctioning of salivary glands and consequential xerostomia is an ubiquitous and long-term complication after radiotherapy in head and neck cancer as well as in Sjogren's syndrome patients. Although new efforts led to significant progress in the prevention and treatment of xerostomia, many patients still suffer from xerostomic negative sequelae. This implies that the above described prevention and treatment strategies are not sufficient. Therefore, further research and development of novel approaches including stem cell-based therapies need to be investigated further. Two types of tissue-specific stem cells are proposed to be used in our artificial salivary gland device; MSCs isolated from the bone marrow and salivary gland stem/progenitor cells.

RATIONALE

Sjogren's syndrome and radiotherapy for head and neck cancer result in irreversible loss of functional salivary acini; known as xerostomia, for which no adequate treatment is available. Our group has been testing different biomaterials, extracellular matrix proteins, and graft cells for the development of an orally implantable tissue-engineered artificial salivary gland device. The major hurdle we faced was to find a suitable graft cell type that must establish tight junctions (TJ) and exhibit an adequate transepithelial electrical resistance in addition to being functional acinar secretory cells. The distribution of certain essential TJ proteins in human salivary tissue would serve as a reference for future studies to assess the presence of appropriate TJs in the graft cells we are looking for. In addition, characterization of two candidate graft cell types from human salivary glands; HSG cell line and primary huSG cells when cultured individually on Matrigel would be beneficial; to test salivary gland morphogenesis and cytodifferentiation and to further examine our envisioned artificial salivary gland device in animal models.

Mesenchymal stem cells (MSCs) can differentiate into cells from all dermal lineages; carrying new promises and significant therapeutic implications. Importantly, MSCs have been isolated from different adult tissues including salivary glands, however; no reports localized such cells in situ. It would be a new contribution to knowledge if we can identify and localize stem/progenitor cell population in human salivary gland tissue. Moreover, these identified cells (if any) would be isolated, expanded in vitro and used to regenerate salivary glands. In addition, this would help to answer an important question on how do salivary gland regenerate. Implantation of autologous functional acini would be a major achievement to treat xerostomic patients. However, this would require the generation of a great number of such acini. We were able to culture primary huSG cells; however, these cells grow slowly and exhibit ductal phenotype. Therefore, huSG cells are not suitable as graft in its current status. MSCs are easily expandable in vitro giving huge cell

numbers without losing their multipotency. This makes autologous MSCs a novel alternative source of graft to regenerate salivary glands if they can be induced to differentiate into functional salivary acini. Therefore, exploring the potential of MSCs to differentiate into functional huSG acini would be of interest.

HYPOTHESIS

Characterization of different candidate human salivary cells and or their stem/progenitor cells in comparison to the normal human salivary gland tissue would help in identifying the suitable graft cell type for a tissue-engineered artificial salivary gland device to treat xerostomic patients. In addition, exploring the potential of human MSCs (that are easily expandable in vitro) to differentiate into functional salivary acini would facilitate applying available autologous stem cell-based therapy to treat xerostomic patients.

AIM OF THE THESIS

The aim of this thesis is to identify and characterize a suitable graft cell type that would be used to regenerate human salivary glands of xerostomic patients.

OBJECTIVES

1. Determine the distribution of TJ proteins in human salivary tissue to serve as a reference for future studies to assess the presence of appropriate TJs in the graft cells we are testing.
2. Characterize two human salivary gland cell types; HSG cell line and huSG primary cells when cultured on Matrigel; the basement membrane extract, known to promote cellular adhesion, migration, and differentiation for further testing in animal models.
3. Identify and localize salivary gland stem/progenitor cells for further expansion.
4. Explore the potential of human mesenchymal stem cells (MSCs) from bone marrow to differentiate into functional salivary acinar cells for probability of using autologous differentiated MSCs (if any) as graft in regeneration of salivary glands.

CHAPTER 2

DISTRIBUTION OF TIGHT JUNCTION PROTEINS IN ADULT HUMAN SALIVARY GLANDS.

Published as: Ola M Maria, Jung-Wan M Kim, Jonathan A Gerstenhaber, Bruce J Baum, Simon D Tran. Distribution of Tight Junction Proteins in Adult Human Salivary Glands, *Journal of Histochemistry and Cytochemistry.* 2008; 56(12): 1093-1098.

The Ph.D. Candidate performed the following contributions in this manuscript:

- All the technical immunohistochemical staining.
- Samples examinations.
- Wrote the manuscript.

Distribution of Tight Junction Proteins in Adult Human Salivary Glands

Ola M Maria[1], Jung-Wan Martin Kim[1], Jonathan A Gerstenhaber[2], Bruce J Baum[2], Simon D Tran[1]*

[1] Faculty of Dentistry, McGill University, Montreal, Canada.

[2] National Institute of Dental and Craniofacial Research, Bethesda, MD, USA.

*_Correspondence Author_:

Simon D Tran, D.M.D., Ph.D.

Address: 3640 University Street, Room M43, Montreal, Quebec, Canada, H3A 2B2; Tel: 514 398 7203 extension 09182; Fax: 514 398 8900

E-mail: simon.tran@mcgill.ca

ABSTRACT

Tight junctions (TJs) are an essential structure of fluid-secreting cells, such as those in salivary glands. Three major families of integral membrane proteins have been identified as components of the TJ; claudins, occludin, and junctional adhesion molecules (JAMs), plus the cytosolic protein zonula occludens (ZO). We have been working to develop an orally implantable artificial salivary gland that would be suitable for treating patients lacking salivary parenchymal tissue. To date, little is known about the distribution of TJ proteins in adult human salivary cells, and thus what key molecular components might be desirable for the cellular component of an artificial salivary gland device. Therefore, the aim of this study was to determine the distribution of TJ proteins in human salivary glands. Salivary gland samples were obtained from 10 patients. Frozen and formalin-fixed paraffin-embedded sections were stained using immunohistochemical methods. Claudin-1 was expressed in ductal, endothelial and ~25% of serous cells. Claudins-2, -3, -4 and JAM-A were expressed in both ductal and acinar cells while claudin-5 was expressed only in endothelial cells. Occludin and ZO-1 were expressed in acinar, ductal, and endothelial cells. These results provide new information on TJ proteins in two major human salivary glands and should serve as a reference for future studies to assess the presence of appropriate TJ proteins in a tissue-engineered human salivary gland.

Key Words: salivary gland; tight junction; claudins; occludin; junctional adhesion molecules; zonula occludens; epithelial barriers.

INTRODUCTION

Irreversible salivary gland parenchymal damage and hypofunction result from two major causes. The first is therapeutic irradiation of patients suffering from head and neck cancer, affecting almost 30,000 new patients each year in the US. The second is an autoimmune disorder, Sjögren's syndrome, affecting about 1 million patients in the US (194, 202). In these two types of patients, both the quantity and the quality of saliva are altered to the extent that considerable morbidity occurs, such as dry mouth, dysphagia, dental caries, oropharyngeal infections, and mucositis (193).

We have been working to develop an orally implantable fluid secretory device (an artificial salivary gland) that would be suitable for treating patients lacking salivary parenchymal tissue (147, 158, 187, 186, 169). Our initial choice of an allogeneic human submandibular gland cell line (HSG) was unable to attain a polarized monolayer organization, did not express any tight junction (TJ) proteins or generate a transepithelial electrical resistance (TER) (169). We re-engineered HSG cells to express individual or a combination of key TJ proteins (claudin 1 or/and claudin 2) by establishing stable clones of transfected cells. Nonetheless, when we examined these transfectants for functional tight junctional activity (TER or fluid barrier) none was observed (169). The inability of the HSG cell line to form TJs, led to the development of a method of culturing and expanding primary human salivary gland (huSG) cells. The cultured huSG cells have been shown to possess tight and intermediate junctions between cells, a transepithelial electrical resistance and a low paracellular fluid movement, all of which are characteristics of polarized cells (11).

Epithelial cells with primary secretion functions, such as in salivary glands, must be polarized in order to achieve a unidirectional secretion. TJs are the primary structure that forms a barrier between the apical and basal region of the cell. In the apical face of the cell, the transport proteins

produce an osmotic concentration gradient, in addition to providing a facilitated water permeability pathway. TJs also promote a 'fence' function that maintains the differential composition of the basolateral and apical domains by preventing the free diffusion of lipids and proteins between these compartments (148, 149). In salivary glands, this gradient separation becomes important for the effective movement of ions, such as, sodium and chloride, required for the production of saliva. Water and solutes that will form the exocrine secretion must pass across three different barriers: the vascular endothelium, the glandular interstitium and the secretory epithelium (212). This movement in effect is regulated by tissue-specific TJs, with permissive and barrier mechanisms, to achieve the chemical specificity of saliva (190). TJs are built from almost 40 different proteins, including members from multigene families (206). Among these proteins are three types of transmembrane proteins; claudins, occludin and Junctional Adhesion Molecules (JAMs), as well as cytoplasmic proteins fulfilling roles in scaffolding, cytoskeletal attachment, cell polarity, signalling, and vesicle trafficking. The most important cytoplasmic protein is the peripheral membrane protein, zonula occludens (ZO) (216). The composition of TJs is quite complex and diverse, apparently much more than the other epithelial junctions: gap, adherens junctions, and desmosomes. These proteins have been comprehensively reviewed by Gonzalez-Mariscal et al (2003), (198).

There are few reports on the distribution of TJ proteins in human salivary glands in the literature (202, 203). Accordingly, in this study, we evaluated the presence and distribution of various TJ proteins in human submandibular and parotid glands to serve as a reference for future studies where it may be necessary to assess the presence of specific TJ proteins in a tissue-engineered human salivary gland.

MATERIALS & METHODS

Source of Human Salivary Glands: Portions of human submandibular (N = 9) and parotid (N = 1) salivary glands, from both males and females, were obtained from the Department of Pathology, University of Virginia, through the Cooperative Human Tissue Network (CHTN). For tissue use, we received an exemption from the Institutional Review Boards at McGill University and the National Institutes of Health. This report describes results obtained with glands that were excised as part of the resection of head and neck cancers. Patients, from whom samples were retained, had received no prior irradiation treatment. A pathologist at the University of Virginia (Charlottesville, VA) performed a surgical pathologic examination of all glands after their excision. We were provided with representative tissue slides after histopathologic examination by CHTN. All glandular tissues received and reported here were judged normal histo-pathologically. The salivary tissues were either frozen in liquid nitrogen or fixed in 10% buffered formalin by CHTN.

Immunohistochemistry: Frozen tissue slides were fixed in methanol over dry ice for 10 min. followed by two washing steps in phosphate-buffered saline (PBS) for 5 min each. Endogenous peroxidase and biotin activities were blocked with hydrogen peroxide 3% and Avidin D/Biotin kit (Cat.# SP-2001, Vector Labs.), respectively. All primary antibodies were obtained from Zymed Labs, (South San Francisco, CA). We used the following antibodies: rabbit anti-claudin-1 (Cat.# 51-9000), claudin-2 (Cat.# 51-6100), claudin-3 (Cat.# 34-1700), occludin (Cat.# 42-2400), JAM-A (Cat.# 36-1700) and mouse anti-ZO-1 (Cat.# 33-9100), claudin-4 (Cat.# 32-9400), and claudin-5 (Cat.# 35-2500). In addition, we used rabbit and mouse isotype control antibodies (Cat.# 08-6599 and 08-6199). These antibodies are reactive against the respective proteins from human and other species. Secondary broad spectrum antibodies (Cat.# 95-9743) were also obtained from Zymed Labs. All primary antibodies used were diluted 1:20 in PBS containing 5% goat serum (Cat.# S-1000, Vector Labs.). The slides were incubated with the primary antibodies

for 60 min. Secondary broad spectrum antibody kit was used with adding either 3-Amino 9 Ethyl-Carbazole (AEC, Cat.# 00-1111, Zymed Labs.) or Di-Amino-Benzadine (DAB, Cat.# 00-2014, Zymed Labs.) chromogen to observe the reaction color in either red or brown, respectively. Then, the slides were counterstained with Mayer's hematoxylin (Cat.# 245-653, Fisher Scientific). For formalin-fixed paraffin-embedded (FFPE) tissue slides, we used the same steps as described above, in addition to a boiling step (15 Min.) using an EDTA solution (Cat.# 00-5500, Zymed Labs. pH 8.0) for Heat Induced Epitope Retrieval. Primary antibodies were incubated overnight with the FFPE slides at 4^0C.

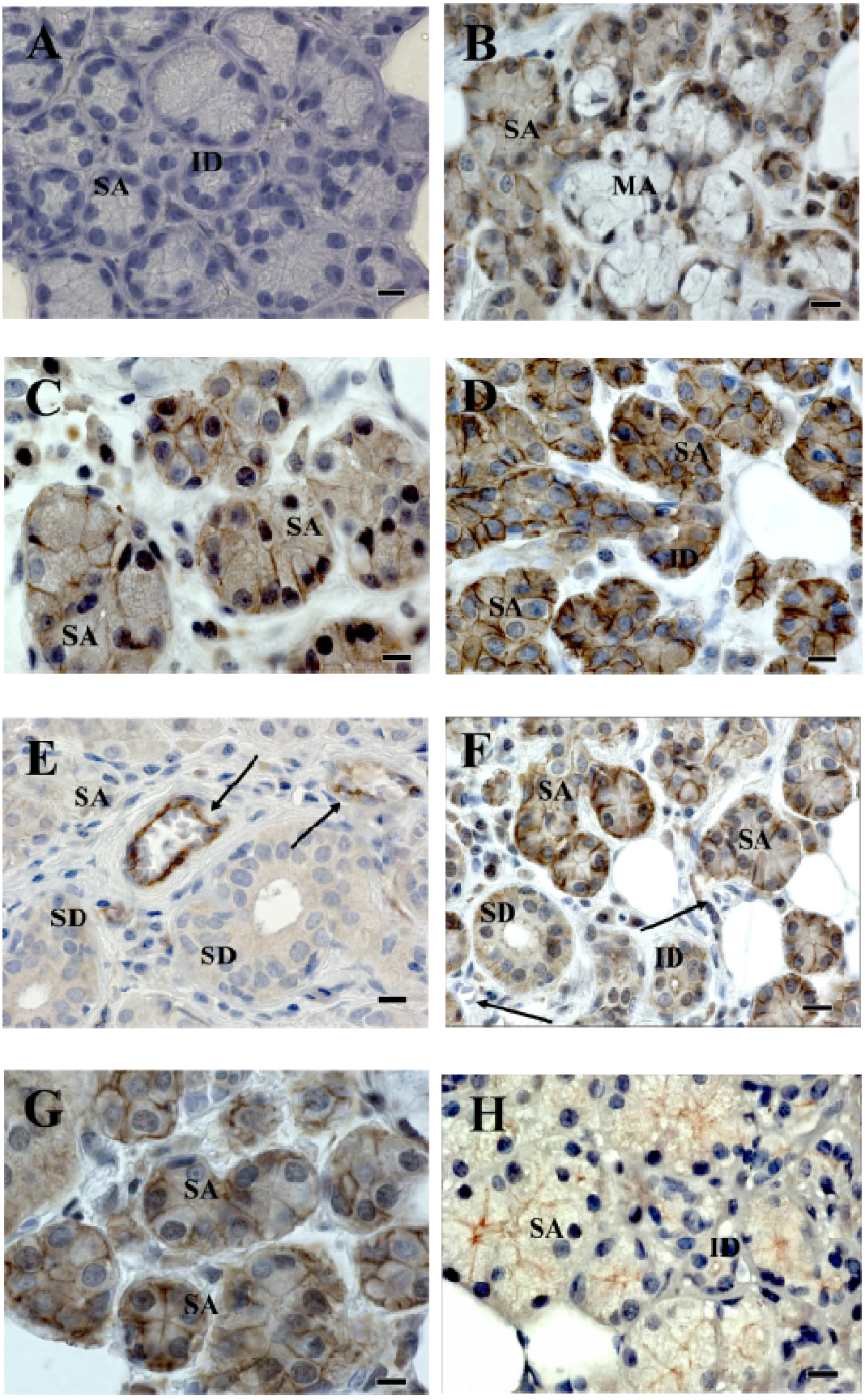
RESULTS

We examined slides of glands from both male (N = 7) and female (N = 3) patients, using both frozen and FFPE samples from each patient (approximately twenty five 5µm sections per salivary gland). Patients' age ranged between 52 and 73 years old. No differences in results were seen between the genders or in regard to patients' age. In comparison to isotype control antibodies (Fig.2.1A), our results (Table 2.1) revealed that claudin-1 was present in ductal (intercalated and striated ducts) and endothelial cells, and also in ~25% of serous acini (Fig.2.1B). Claudin-2 (Fig.2.1C), claudin-3 (not shown), and claudin-4 (Fig.2.1D) were expressed in acinar (serous and mucous), and ductal (intercalated and striated) cells, while endothelial cells were negative. Claudin-5 was detected only in endothelial cells (Fig.2.1E). JAM-A protein was detected in acinar and ductal salivary cells (Fig.2.1F). Occludin (Fig.2.1G) and ZO-1 Fig.2.1H) were detected in both ductal and acinar salivary cells, in addition to endothelial cells. None of the TJ proteins were expressed in myoepithelial cells. Results from the single parotid gland available were comparable to those from all submandibular gland samples.

Table 2.1: Summary of tight junction proteins present in human submandibular and parotid salivary glands: (+) Presence of protein, (-) Absence of protein.

ANTIBODY	DUCTS		ACINI		Myoepithelial Cells	BLOOD VESSELS
	Intercalated	Striated	Mucous	Serous		
Claudin-1	+	+	-	+ 25%	-	+
Claudin-2	+	+	+	+	-	-
Claudin-3	+	+	+	+	-	-
Claudin-4	+	+	+	+	-	-
Claudin-5	-	-	-	-	-	+
JAM-A	+	+	+	+	-	-
Occludin	+	+	+	+	-	+
ZO-1	+	+	+	+	-	+

Figure 2.1: Light micrographs of immunohistochemical staining of human salivary gland sections. (A) isotype control antibody labeling: serous acinus (SA), intercalated duct (ID) and blue nuclei stained with haematoxylin are indicated. (B) distribution of claudin-1: positive serous acinus (SA) but negative mucous acinus (MA). (C) claudin-2: positive serous acinus (SA). (D) claudin-4: strong positive intercalated duct (ID) and serous acinus (SA). (E) claudin-5: positive blood vessels (arrows) but negative striated ducts (SD) and serous acinus (SA). (F) JAM-A: positive serous acinus (SA), intercalated duct (ID), and striated duct (SD) but negative blood vessel (arrow). (G) occludin: positive serous acinus (SA), . (H) ZO-1: positive serous acinus (SA) expressed in a spider-web distribution, and intercalated duct (ID). Scale bars: 100 μm. A, C, and D are parotid gland sections, while B, E, F, G, and H are submandibular gland sections. The results observed are comparable in both submandibular and parotid glands.


A
ID
SA
B
SA
MA
C
SA
SA
D
SA
ID
SA
E
SA
SD
SD
F
SA
SA
SD
ID
G
SA
SA
H
SA
ID

DISCUSSION

In this study, we evaluated the presence of multiple TJ proteins (claudins-1, -2, -3, -4, -5, JAM-A, Occludin, and ZO-1) in human parotid and submandibular salivary glands. The summary of our results, shown in Table 2.1, provides the most detailed examination of TJ proteins in human major salivary glands reported to date (202,203), and should be a useful guide for evaluating TJ proteins present in the cellular component of any artificial salivary gland device. However, we did not examine all possible TJ proteins in these tissues for practical reasons, but rather selected the most common TJ proteins for which antibodies were readily available.

TJs, together with adherens junctions and desmosomes, form the apical junctional complex in epithelial and endothelial cellular sheets. Adherens junctions and desmosomes are responsible for the mechanical adhesion between adjacent cells, whereas TJs are essential for the tight sealing of the cellular sheets, thus controlling paracellular ion flux and therefore maintaining tissue homeostasis (216). TJs also play a crucial role in the maintenance of cell polarity by forming a fence that prevents lateral diffusion of membrane proteins and lipids, thereby maintaining the differential composition of the apical and basolateral domains. Finally, because of the ability of TJ proteins to recruit signaling proteins (205). TJs have also been hypothesized to be involved in the regulation of proliferation, differentiation, and other cellular functions. Given these cellular biological roles, the presence and function of TJ proteins in the cellular component of an artificial salivary gland is critically important.

So far, 24 members of the claudin family have been identified in mouse and human (153,206). Recent findings indicate the importance of claudin-1 in essential physiological functions as well as in the formation of barriers. For example, claudin-1 knockout mice, survive only for 1 day after birth, and also present with altered epidermal barrier properties (196). We observed claudin-1 in salivary ductal, endothelial, and in ~25% of serous acinar cells. Why claudin-1 was

apparently not detected in the remaining serous acinar, or in mucous acinar, cells is not clear. It may reflect differences in the functional state of the cells at the time of surgery, or in fact represent an example of different acinar cell sub-populations. Lourenço et al (2007), (203) reported that claudin-1 was expressed only in ductal cells of adult human minor salivary glands. The presence of claudin-1 protein may suggest that it functions as a barrier to prevent the loss of salivary tonicity through the walls of the salivary system.

We found an ubiquitous distribution of claudin-2 in salivary gland sections. The immunohistohemical staining was stronger in the acinar cells (both serous and mucous) than ductal cells. Claudin-2 has been reported to weaken the tightness of TJs, as compared to the proposed barrier function of the claudin-1 protein (214). For example, Madin-Darby Canine Kidney (MDCK) type I and II cells have similar numbers of TJs expressed, however, MDCK I cells have a tighter barrier, reflected by a higher TER, compared to MDCK II cells (214). MDCK I cells mainly express claudin-1, whereas MDCK II cells express claudin-2 in addition. Since epithelial cells of salivary glands are considered to be relatively leaky exocrine tissues (189), our observation seems to go in agreement with this organ's function. Results from human minor salivary glands indicate the absence of claudin-2 in both ductal and acinar cells (203).

In our study, claudin-3 and claudin-4 were present in both acinar and ductal cells. Similar results were reported from studies in rat (208) and in human minor salivary glands (203). Claudin-3 is not involved directly in barrier properties of epithelial cells (195, 188), while the presence of claudin-4 in TJs is known to decrease sodium permeability, causing a decreased paracellular conductance (218). When the claudin-4 in MDCK I cells was removed, there was a decrease in the number of TJ strands along with an increase in cellular permeability (213). Claudin-5 appears to have an important role in the vascular permeability of endothelial cells (207). In the present study, the detection of claudin-5 only in endothelial cells of human salivary glands is consistent

with the reported specificity of this molecule. However, Lourenço and co-workers (2007), (203) reported the expression of claudin-5 in both ductal and acinar cells. This difference might be a reflection of technical differences from our study. Herein, we used a claudin-5 monoclonal antibody while in the Lourenço et al (2007), (203) study a claudin-5 polyclonal antibody was used.

The junctional adhesion molecules (JAMs) are four glycosylated transmembrane proteins. (210), but in the present study we examined the expression of JAM-A (also known as JAM-1) protein only. JAMs were recently shown to bind directly to ZO-1 (191, 192) although the full role of JAM-related proteins still needs to be clarified. Herein, JAM-A protein was detected in ductal and acinar epithelial cells.

Occludin, a phosphoprotein, was the first transmembrane protein to be identified in TJs (197). We localized occludin in ductal, acinar, and endothelial cells of human salivary glands. These results were consistent with the results reported by Kriegs et al 2007, (202) with regard to acinar and ductal, but not for endothelial cells. The presence of occludin may provide the TJs with the ability to form seals between cells, preventing the leakage of saliva. Similarly, the expression of occludin has been reported in ductal and acinar cells of rat salivary glands (208). Occludin interacts directly with ZO proteins (ZO-1, -2, -3) (199). Also, occludin interacts with the actin cytoskeleton and JAMs proteins indirectly via ZO proteins (204). Tsukita et al (2001), (216)indicated that occludin might contribute to the electrical barrier function of TJs and possibly to the formation of aqueous pores within TJ strands, however, TJs can be present independent of occludin

There are three isoforms of Zonula Occludens (ZO) proteins: ZO-1,-2, and -3. . As ZO proteins bind to actin, they act as scaffolds to link other TJ proteins and cytoskeleton (211. We only evaluated the distribution of ZO-1 in human salivary gland tissue. Unlike other TJ proteins, ZO-1

is not a transmembrane protein; rather it is a large cytosolic phosphoprotein (215, which is critically important for claudin polymerization (217. ZO-1 was found in endothelial as well as ductal and acinar cells of human salivary glands. Gresz et al., (2004), (200) and Kriegs et al (2007), (202) reported that ZO-1 appeared to be restricted to the apical lateral region of acinar cells, presenting spider-like distribution, similar to that of aquaporin-5, in rat salivary glands and our findings are generally comparable to their results.

In summary, we have presented new data on the localization of several TJ proteins (claudins 1-5, JAM-A) in normal human submandibular and parotid glands, as well as confirmed the presence of occludin and ZO-1 in these tissues (202). In demonstrating presence and localization of these proteins, we expect that our findings will allow a more critical assessment of TJ proteins in cultured primary salivary gland cells for use in an envisioned tissue-engineered salivary gland. Further studies are required to find the exact roles and interactions of TJ proteins essential for physiological salivary gland functions.

ACKNOWLEDGEMENTS

This study was supported by Canada Research Chair and CIHR, Egyptian Cultural and Educational Department (for OMM), and by the Division of Intramural Research, NIDCR. The authors would like to thank Dr. Christopher Moskaluk for the histopathological analysis of salivary glands obtained from CHTN, and Dr. Younan Liu for technical assistance.

CHAPTER 3

Maria OM, Maria O, Liu Y, Komarova SV, Tran SD. Matrigel Improves Functional Properties of Human Submandibular Gland Cell Line (HSG). (*Submitted 2010*).

The Ph.D. Candidate performed the following contributions in this manuscript:

- All the experimental work except RT-PCR and SDS-PAGE.
- Examinations and analysis of data.
- Wrote the manuscript.

Matrigel Improves Functional Properties of Human Submandibular Salivary Gland Cell Line

MARIA OM,[1] MARIA O,[1] LIU Y,[1] KOMAROVA SV,[1] TRAN SD[1*]

[1]Faculty of Dentistry, McGill University, Montreal, Quebec, H3A 2B2, Canada.

MARIA OM, D.M.D.

Address: 3640 University Street, Room M33, Montreal, Quebec, H3A 2B2, Canada. E-mail: ola.maria@mail.mcgill.ca

MARIA O, M.D.

Address: 740 Dr. Penfield Avenue, Room 2301, Montreal, Quebec H3A 1A4, Canada. E-mail: osama.maria@mail.mcgill.ca

LIU Y, M.D.

Address: 3640 University Street, Room M36, Montreal, Quebec, H3A 2B2, Canada. E-mail: younan.liu@mcgill.ca

KOMAROVA SV, Ph.D.

Address: 740 Dr. Penfield Avenue, Room 2201, Montreal, Quebec H3A 1A4, Canada. E-mail: svetlana.komarova@mcgill.ca

[*]Correspondence Author:

TRAN SD, D.M.D., Ph.D.

Address: 3640 University Street, Room M43, Montreal, Quebec, H3A 2B2, Canada. E-mail: simon.tran@mcgill.ca, Tel: +1 514 398 7203 ext. 09182, Fax: + 1 514 398 8900

ABSTRACT

Sjogren's syndrome and radiotherapy for head and neck cancer result in irreversible loss of functional salivary tissue, for which no adequate treatment is available. Our group has been testing different biomaterials, extracellular matrix proteins, and graft cells for the development of a tissue-engineered artificial salivary gland device. Our aim is to obtain graft cells that possess essential structural features of fluid-secreting epithelial tissues. This study characterized the human submandibular gland cell line, HSG, cultured in a 3-D gel. Matrigel, a basement membrane extract (BME), promotes cellular adhesion, migration, and differentiation. HSG cells were cultured on both Matrigel-coated and non-coated plastic plates. On plastic, HSG cells did not express any of the tight junctions (TJ) proteins essential for a fluid-secreting salivary tissue. However on Matrigel, HSG cells expressed TJ (claudin-1, -2, -3, -4, occludin, JAM-1, ZO-1), acinar (AQP5, α-amylase, mucin-1), and acinar-associated adhesion proteins (CD44, CD166). Moreover, HSG formed 3-D acinar-like structures within 24h and exhibited adequate transepithelial electrical resistance (TER). Transmission electron microscopy confirmed the formation of differentiated acinar polarized cells exhibiting TJ structures, active protein synthesis (α-amylase) and numerous secretory-like granules. Surprisingly, quantitative RT-PCR analysis revealed that HSG cells express TJ genes; claudin-1and -2 while the related-proteins are not translated. Additionally, down-regulation of claudin-1, -2, CK-18, EGF, AQP5, and α-amylase genes might be linked to protein translation rather than gene transcription. Mitotic activity was significantly reduced on Matrigel where apoptotic activity was significantly increased in comparison to HSG on plastic ($P<0.01$). Our results confirmed that Matrigel provided a suitable microenvironment for morphological and functional differentiation of HSG cells into 3-D acinar cells.

Keywords: Cell morphology; Biocompatibility; Cell culture; Confocal microscopy; ECM (extracellular matrix; TEM (transmission electron microscopy); Gene expression; Growth factors.

1. INTRODUCTION

Salivary tissue is a densely packed epithelium composed mainly of acinar and ductal cells (219). Acinar cells represent the major glandular cell type that are salt-secreting and the only site of fluid movement (148, 219), while ductal cells are absorptive and relatively water-impermeable cells (148, 219). Both cell types are organized as a monolayer around an extensively branching lumen that opens directly into the oral cavity (219). Clinically, acinar secretory cells are irreversibly damaged following therapeutic irradiation for head and neck cancers or in association with the autoimmune exocrinopathy Sjogren's syndrome (220, 221). In the absence of adequate number of functioning acinar cells, the generation of salivary fluid is impossible. Additionally, the afflicted patients experience rampant dental caries, mucositis, candidiasis, dysphagia, and considerable pain and discomfort (220, 222-225). Currently, there is no satisfactory therapy for such patients. One therapeutic strategy we have been working to develop is a tissue-engineered artificial salivary gland device (147, 158, 159). The standard design for such device includes; a blind-end tube fabricated from a slowly biodegradable scaffold coated with extracellular matrix on the inner (luminal) surface of the tube in order to promote attachment and polarization of epithelial cell monolayer; the graft cells that should be capable of unidirectional fluid secretion (147). This device would be implanted in the buccal submucosa where its orifice opens in the oral cavity, mimicking the orifices of the natural salivary ducts (147, 226). In several previous reports, we have suggested that the human submandibular gland (HSG) cell line, (157). might serve as a suitable allogeneic graft cell (147, 158, 159). A critical requirement for the cellular component capable of fluid secretion is to be able to attain and maintain a polarized monolayer organization. To secrete fluid unidirectionally, cells must express a group of transport proteins that generate an

osmotic gradient and control the paracellular movement of water, proteins, and small solutes (148-151,153, 154, 158). Epithelial cells do express these proteins that are known as tight junction proteins at their apical and basolateral membranes (150, 151). However, HSG cells are not capable of forming a polarized epithelial layer as they do not express tight junctions, therefore, do not show reasonable transepithelial electrical resistance (TER) or control water movement (160). On the other hand, maintenance of the acinar phenotype in primary salivary gland cell cultures has been proved to be difficult (227). A critical factor required for maintaining an acinar phenotype in culture is the presence of an extracellular matrix (228,229). The HSG cell line we have chosen initially to first examine as a graft cell source is derived from intercalated duct cells (157). During salivary gland development, intercalated duct cells are believed to be stem cells of ductal, acinar and myoepithelial cell types (168, 230-232). HSG cells with various differentiation inducers results in generation of myoepithelial or acinar cells (233-236). Many trials have been reported using different salivary cell lines; Motegi et al 2005, (237), reported that treatment of ductal cells with 5-Aza-2'-deoxycytidine could result in the expression of the AQP5 gene, thus leading to increased water secretion. Moreover, retinoic acid induced HSG cells to adopt a keratinocyte-like phenotype expressing well-developed cytokeratin filaments (238). Matrigel, a basement membrane extract, (Becton Dickinson Biosciences, Bedford, MA) (161) is the only substrate that promotes complete differentiation including the expression of salivary acinar markers (162). Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells which contains collagen IV, laminin, fibronectin, entactin, perlecan, in addition to multiple angiogentic and growth factors essential for regulation of cell growth and differentiation (163,164). HSG cells grown on Matrigel were reported to form acinar structures that expressed α-amylase and cystatin proteins (165, 166). Therefore, the growth of HSG cells on Matrigel can be used as a model to study salivary acinar

cell formation and physiology. Also, the characterization of HSG cells cultured on Matrigel would benefit our understanding of salivary glands morphogenesis and cytodifferentiation. The aim of the current study was to develop an in vitro three-dimensional (3-D) cell culture model to evaluate and to characterize HSG cells as a candidate cell type in our artificial salivary gland device. Because of the crucial role of tight junctions in the secretion of salt, fluids, and proteins by epithelial cells, we have evaluated the ability of HSG cells to express several key TJ proteins; claudin-1,-2,-3,-4, occludin, ZO-1, JAM-A, both on Matrigel and plastic. Moreover, we tested the ability of HSG cells to provide effective paracellular barrier and to function as acinar secretory units.

Our data confirmed that undifferentiated HSG cells went through acinar morphogenesis and cytodifferentiation when cultured on Matrigel; expressing all TJ proteins tested and showing reasonable transepithelial electrical resistance. Moreover, differentiated HSG cells expressed acinar secretory markers; AQP5, α-amylase, mucin-1, and adhesion-related CD44, CD166 proteins. Certain salivary genes were down-regulated on Matrigel in association with their protein translation. Our results also showed that HSG cytodifferentiation is accompanied by a decrease of cell proliferation and an increase of apoptosis.

Portion of these data have been presented in abstract form at the AADR meeting 2010 (239).

2. MATERIALS AND METHODS

*2.1. HSG Cell Culture***:** HSG cells (a generous gift from Prof. M. Sato, Tokushuma University, Japan, (233)) were maintained in a complete Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1), containing 10% fetal bovine serum (Biofluids, Rockville, MD), 100U/ml penicillin, 100µg/ml streptomycin, and 10µg/ml gentamicin (Life Technologies Inc., Gaithersburg, MD). The cells were maintained at 37°C in a humidified 5% CO_2 and 95% air atmosphere. HSG cells were detached from confluent plates with a solution of 0.05% trypsin,

0.02% versene (Biofluids; Rockville, MD) and re-suspended ($1x10^4$ cells/ml) in fresh tissue culture media. Cells were cultured on either Matrigel-coated or non-coated 12-well, 6-well tissue culture dishes, 8-well slide chambers and 24-mm polyester filters. Matrigel (19.6mg/ml, BD Biosciences, Bedford, MA, USA) was diluted in DMEM (1:6); the final concentration used to coat the plates/slide chambers/filters was 2mg/ml. Coated filters/plates/slide chambers were incubated at 37^{o}C for 30-60 min before cell seeding. The concentration of Matrigel and seeding density were optimized to ensure reproducible 3-D formation, which occurred after 24h, (Fig. 3.1). Culture medium was changed every 2 days. The morphology of the cells was observed using phase contrast microscopy.

2.2. Measurement of TER: HSG Cells were seeded on 24-mm Transwell-Clear polyester filters that were either un-coated or Matrigel-coated and grown as mentioned above. The upper chamber containing the cells received 1.5mL of medium, and the lower chamber (no cells) received 2.6mL of medium. TER was measured after 3, 5, 7 days, (of 6 separate determinations), using a Millicell ERS epithelial volt-ohmmeter (Millipore Corp., Allen, TX) as described by the manufacturer. MDCK-II cells cultured on filters were used as positive control (MDCK-II cells have adequate TER and express TJs) (160). TER readings from filters without cells (blank) were subtracted from readings obtained from filters seeded with cells.

2.3. Evaluation of Apoptosis: An ApopTag® peroxidase in situ apoptosis detection kit (Chemicon International, MA, USA) was used to evaluate HSG cells apoptosis activity. ApopTag® is a mixed molecular and biological-histo-chemical system that allows for sensitive and specific staining of apoptotic bodies. Apoptotic reaction in three 8-well slide chambers of HSG cultured on either plastic or Matrigel at day 7 was scored at magnification of X 400. The slides were assessed by two observers in a blinded manner in ten randomly chosen fields per slide. The mean of all apoptotic cells per field was calculated.

2.4. Evaluation of Proliferation: Proliferating Cell Nuclear Antigen (PCNA) stain was performed to monitor HSG cells proliferation using Zymed kit (Invitrogen, Carlsbad, CA, USA). After endogenous peroxidase activity was blocked for 10 min., three 8-well slide chambers of HSG cells cultured on either plastic or Matrigel (at day 7) were processed with routine indirect immunoperoxidase techniques. Two examiners independently counted the number of PCNA positive cells in a blinded manner in ten randomly chosen fields per slide (X 400). The mean of all PCNA positive cells per field was calculated.

2.5. Immunofluorescence & Confocal Imaging: HSG cells cultured for 5 days were rinsed with PBS, fixed with 10% paraformaldehyde in PBS (pH 7.4) for 30 min, rinsed with PBS, and permeabilized with methanol at 20°C for 5 min followed by incubation with 0.2% Triton X-100 for 10 min at room temperature, followed by three washing steps in PBS for 5 min each. We used the following primary antibodies to characterize HSG cultured on plastic and on Matrigel; rabbit anti-claudin-1, claudin-2, claudin-3, occludin, JAM-A and mouse anti-ZO-1, claudin-4, from Zymed; Mouse anti-cytokeratin (panel), vimentin, and α-smooth muscle actin, from Invitrogen; Goat anti-aquaporin-5 (AQP-5), mouse anti-Mucin-1 from Santa Cruz; rabbit anti-α-amylase from Sigma Aldrich; goat anti-Musashi-1 and mouse multipotent mesenchymal/stromal cell marker (MSCs) antibody panel kit from R&D Systems. The panel contains a group of antibodies for the positive identification of human MSCs; anti-Stro-1, anti-CD90, anti-CD106, anti-CD105, anti-CD146, anti-CD166, anti-CD44, plus leukocytes markers; anti-CD19 and anti-CD45. In addition, we used goat (R&D Systems), rabbit and mouse (Zymed Labs) isotype control antibodies. The antibodies used are reactive against the respective proteins from human and several other species. The cells were incubated with blocking solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and 0.5% bovine serum albumin in PBS for 1h at room temperature. HSG cells were incubated with the primary antibodies (diluted 1:100

in blocking solution) overnight at 4^{o}C while incubated with either donkey anti-mouse/rabbit/goat-fluorescein isothiocyanate-conjugated (FITC) or -Rhodamine Red-X-conjugated (RRX), (Jackson ImmunoResearch Laboratories) secondary antibodies for 1h at room temperature (diluted 1:100 in blocking solution) in dark. Finally, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) was added for 3-5 minutes. Fluorescence images were taken using Zeiss LSM 510 laser scanning confocal microscope (Jena, Germany). Images shown are representative of at least three separate experiments, with multiple images taken per slide. Imaris 3D/4D image visualization and analysis software (Bitplane AG, Zurich, Switzerland; version 7.0.0), was used in the creation of 3D isosurface renderings for CLDN-1 shown in (Fig. 2).

2.6. Western Blot Analysis: Conditioned media from different wells were collected, then, HSG cells grown on either plastic or Matrigel (at 1,3,5,7 days) were washed twice with cold PBS and lysed in 200µl/well of cold RIPA buffer and kept on ice for 5 min. Lysates were collected into microcentrifuge tubes and centrifuged at 10,000 rpm for 20 minutes to pellet the cell debris and keep the supernatants for further analysis. Protein samples, 60µg each, from both supernatants and collected media were subjected to 10% SDS-PAGE on mini-gels and transferred to nitrocellulose membranes. Membranes were blocked for 1h with 5% fat-free dry milk in Tris-buffered saline [0.137M NaCl, 0.025M Tris (hydroxymethyl)- aminomethane, pH 7.4] containing 0.1% Tween-20 (TBST) and immune-blotted overnight with rabbit anti-α-amylase antibody (1:2 000 dilution; Sigma Aldrich) at 4°C in TBST containing 5% BSA and 0.02% sodium azide. After incubation with the primary antibody, membranes were washed three times for 15 min each with TBST and incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution; Santa Cruz Biotechnology) at room temperature for 1h. The membranes were washed three times for 15min each with TBST, then treated with chemiluminescence detection reagent containing 20mM Tris buffer, pH 8.5, 250mM Luminol

and 90mM coumaric acid (Sigma Aldrich) and protein bands were visualized on X-ray film. All experiments were performed in duplicate and repeated at least three separate times.

2.7. Transmission Electron Microscopy (TEM): HSG cells cultured on un-coated or Matrigel-coated 12-well plates for 1,2,3,5,7 days were fixed in 2% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2h, rinsed in 0.1M cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1h. After rinsing in cacodylate buffer, samples were dehydrated through an ethanol series and infiltrated and embedded in Epon812 (Electron Microscopy Sciences, Fort Washington, PA). Thin (70 nm) sections were cut with a diamond knife and mounted onto copper grids. Grids were stained with 3% uranyl acetate for 30 min and 2% lead citrate for 5 min, to be examined magnifications using a 100-CXII transmission electron microscopy (JEOL, Tokyo, Japan) at an accelerating voltage of 120 kV.

2.8. Quantitative Real-Time PCR Analysis: At day 3, 5, 7, 9 HSG cells grown on either plastic or Matrigel were washed in PBS, then, total RNA was isolated using the RNeasy micro kit (Qiagen Ltd, Crawly, UK) with in-column DNase digestion. The concentration of RNA was determined using Qubit (Molecular Probes). Total RNA (2 μg per sample) was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in 20 μL volume. For polymerase chain reaction (PCR) amplification, 5% of the cDNA was used with real-time PCR primers and 6-carboxy-fluorescein (FAM)-labeled minor groove binder probes (MGB). The probes and primers for claudin-1 (CLDN-1), claudin-2 (CLDN-2), α-amylase (AMY), aquaporin-5 (AQP-5), cytokeratin-18 (CK-18), epidermal growth factor (EGF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an endogenous reference) were selected from the Applied Biosystems. PCR reactions (20 μL) were performed in duplicates using TaqMan Universal Master Mix (Applied Biosystems) on a Prism Sequence Detection System 7500 (Applied Biosystems) with the default settings (50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles [95^{o}C

for 15 s, 60^{o}C for 1min]). Every culture experiment was repeated at least 3 times. Gene expression levels were calculated by normalizing the target RNA value to the value of GAPDH in the same sample. Results are expressed as fold-changes in gene expression relative to control (HSG cultured on plastic for 9 days expressing the lowest gene levels) sample.

2.9. Statistical Analysis: Data are presented as means ± SEM of results from three or more separate experiments. Our data were analyzed by Student's *t*-test and one-way *ANOVA* where *P* value < 0.05 represents significant differences between both groups at specified times.

3. RESULTS

3.1. Morphological Changes: HSG cells cultured on plastic formed monolayers; making epithelial isolated clusters (Fig. 3.1A) that proliferated continuously until confluence. On Matrigel coated surfaces (2 mg/ml), HSG cells formed both spherical 3-D acinar-like structures (20% of cells plated) within 24 h and monolayer (Fig. 3.1B). Most 3-D structures initially formed, consisted of 6–8 cells, then, the number of cells/3-D structure increased up to 12 cells at day 3 where total 3-D structures were approximately 40% of total cells attached. At day 5, some 3-D structures started to disintegrate, detached and disunited. At day 7 and 9, 20%-30% of 3-D structures were disintegrated and were seen floating in media.

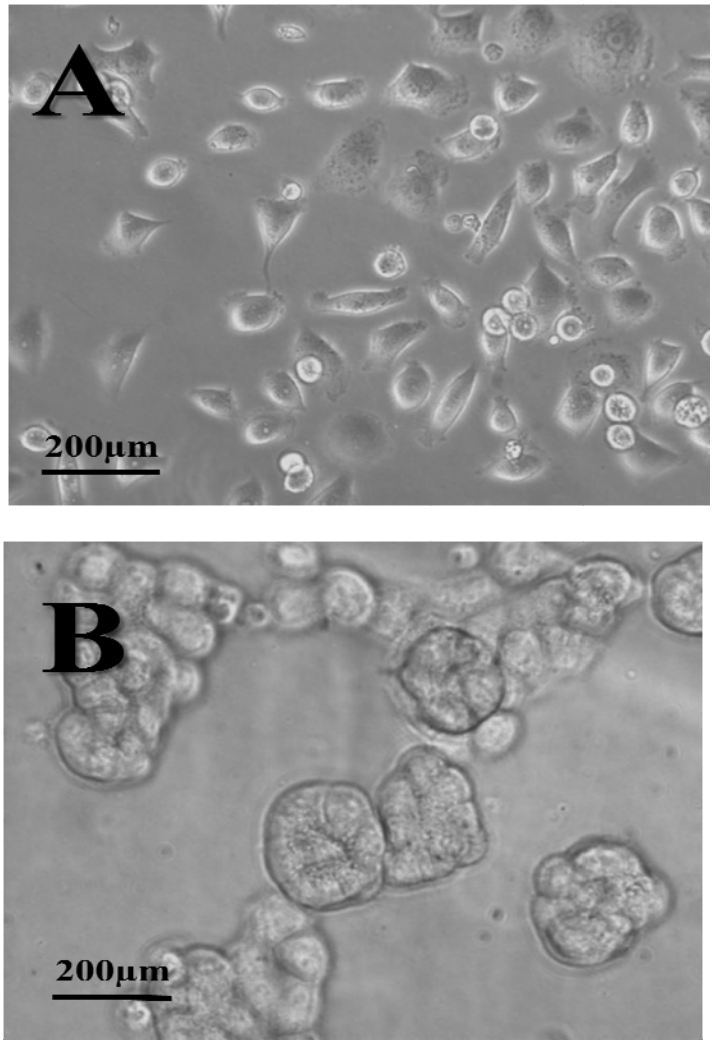


Figure 3.1: (A) HSG cells cultured on plastic formed monolayer epithelial isolated clusters. (B) HSG cells cultured on Matrigel (2 mg/ml) formed both 3-D acinar-like structures within 24 h and monolayer. [Scale bar= 200µm, X 200].

3.2. Measurement of TER: as a standard measure of TJs formation and functional development of acini, TER was assessed across HSG cells seeded on 24-mm Transwell-Clear polyester filters that were either un-coated or Matrigel-coated and grown as above. We compared TER of HSG with those obtained by MDCK-II (positive control) monolayers at day 3, 5, 7 from 6 separate determinations. HSG on un-coated filters exhibited extremely low TERs in comparison to those of MDCK-II; TER values ranged between 1 - 3 Ω.cm^2. However, HSG grown on Matrigel-coated filters exhibited TERs comparable to MDCK-II, 332 – 401 Ω.cm^2, (Fig. 3.2). TER values of MDCK-II were significantly different from both HSG culture types ($p<0.01$).

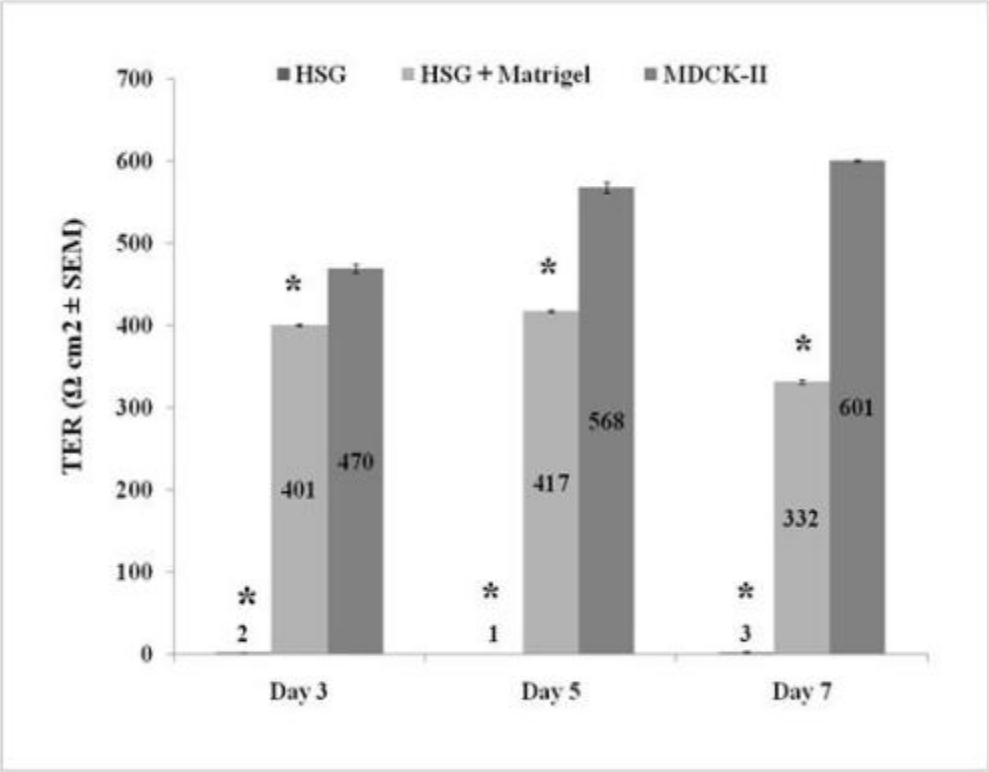


Figure 3.2: Transepithelial Electrical Resistance (TER) measurements at day 3, 5, 7. HSG on plastic exhibited extremely low TERs while HSG grown on Matrigel (2 mg/ml), exhibited TERs comparable to MDCK-II (positive control). Results represent the average ± standard error of means of 6 separate determinations for all cell types. TER values of MDCK-II were significantly different from those of HSG cultured either on plastic or Matrigel (*P<0.01).

3.3. Apoptosis versus Mitosis: Both apoptotic and mitotic activities were evaluated at day 7 for both HSG on plastic and on Matrigel. The apoptotic activity increased significantly ($p<0.01$) on Matrigel (78.46%) compared to plastic (38.36%), (Fig. 3.3). Apoptotic cells were seen mainly at the centre of intact 3-D structures where the future lumen is supposed to form and in the cell clumps forming the disintegrating 3-D (not shown). On the other hand, PCNA stain revealed a significant ($p<0.01$) decrease of the mitotic activity on Matrigel (38%) compared to plastic (90%), (Fig. 3.3).The mitotic activity was seen mainly among monolayers and fewer mitosis was evident among cells forming 3-D structures (not shown). A significant correlation (correlation coefficient = 0.99) exists between apoptosis and mitosis at day 7 on Matrigel.

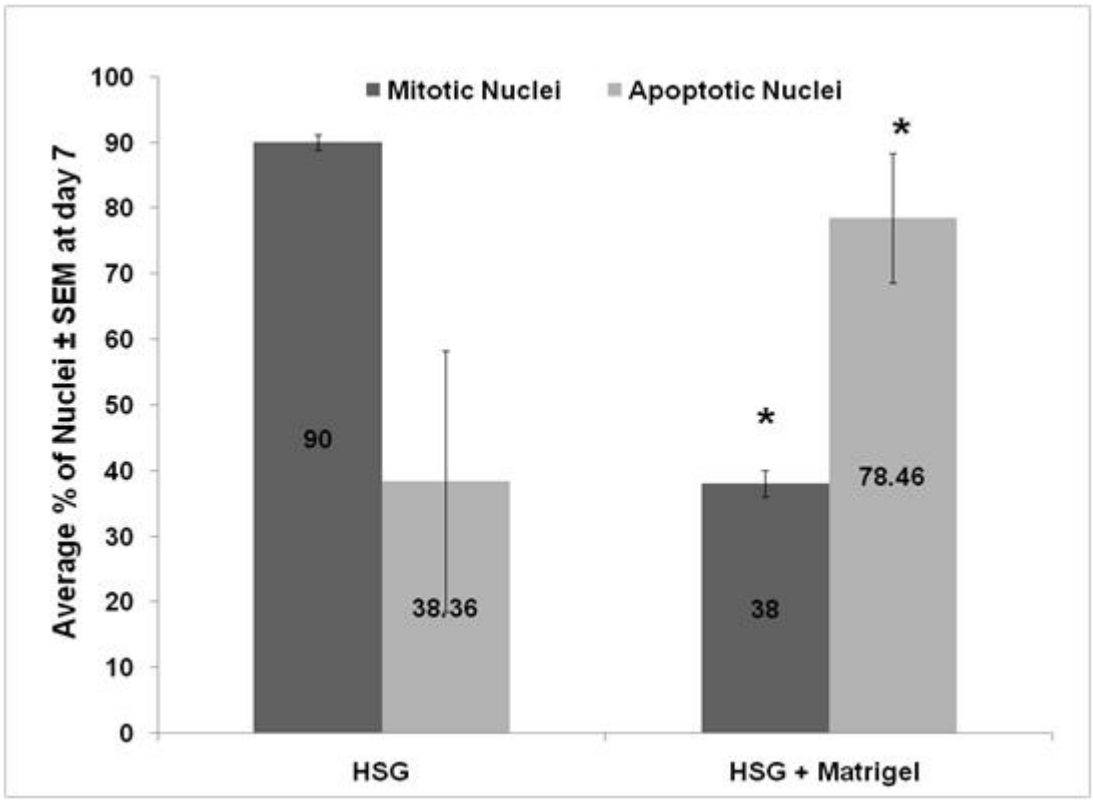
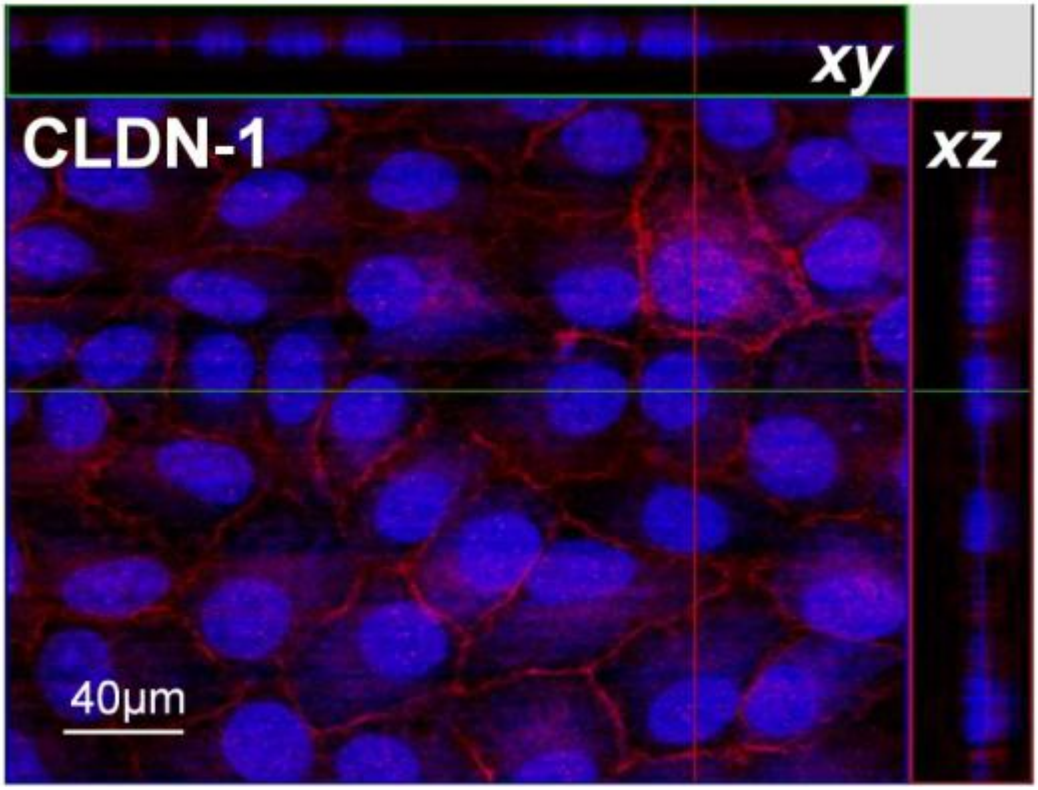


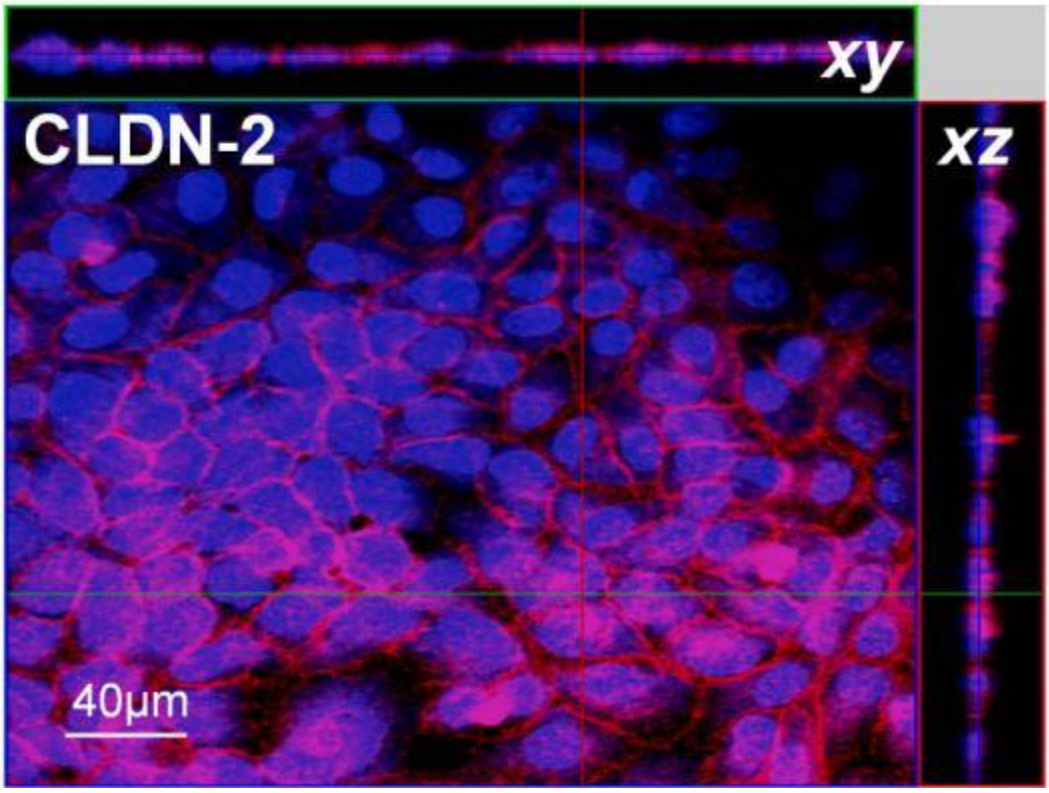
Figure 3.3: Apoptotic and Mitotic activities were evaluated at day 7 for HSG cultured on both plastic and Matrigel. The apoptotic activity (evaluated by ApopTag kit) increased significantly on Matrigel (78.46%) as compared to HSG on plastic (38.36%). The Mitotic activity (evaluated by PCNA stain) revealed a significant decrease on Matrigel (38%) compared to HSG on plastic (90%), (*P< 0.01).

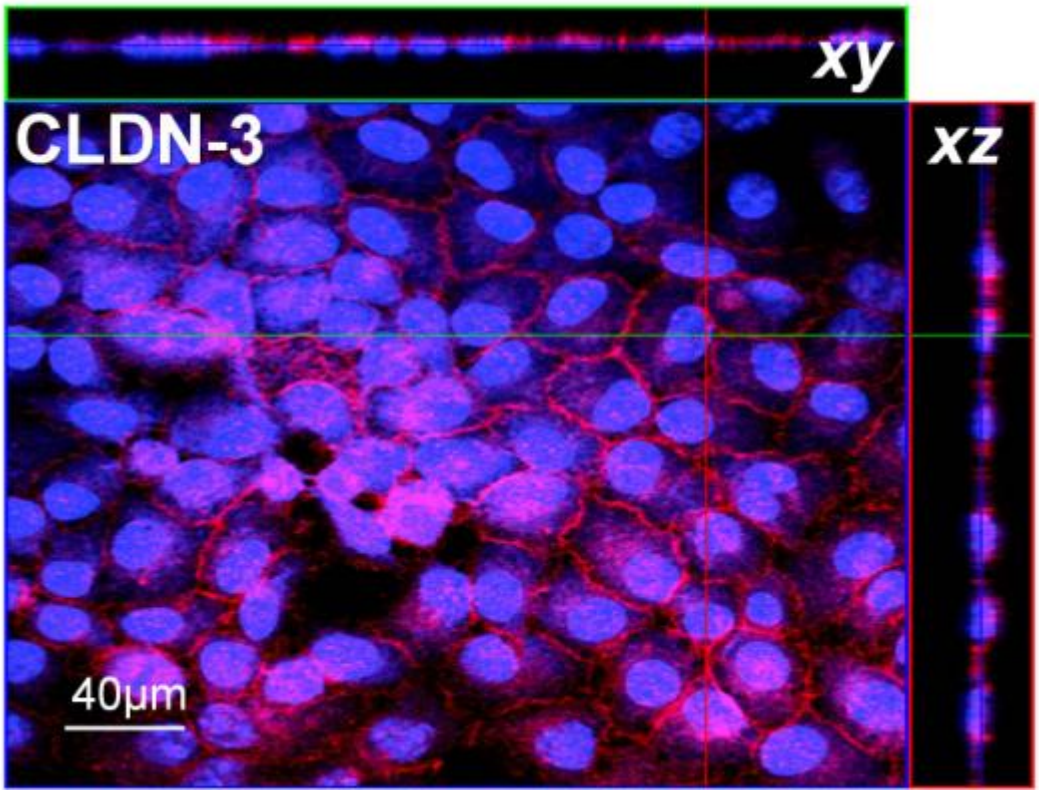
3.4. Immunofluorescence & Confocal Imaging: HSG cultured on plastic did not express any of the tight junction proteins while HSG cultured on Matrigel expressed all TJ proteins tested ; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1 (Table.3.1A). Importantly, the RRX labeled all TJ antibodies indicated that TJs are localized at the apical and basolateral sides of the cells, therefore, HSG cells are correctly polarized on Matrigel. The relative position of TJs to the medium-facing (analogous to "apical" or "lumenal," *in vivo*) and filter-facing ("basolateral") sides of the cells is shown in Fig. 3.4 (*xz* & *xy* planes). Claudin-1 protein expression was analyzed in a 3-D acinar-like structure (on Matrigel) using Imaris 3D/4D image visualization and analysis software to confirm the correct positioning of TJ structures formed. (Fig.3.4)

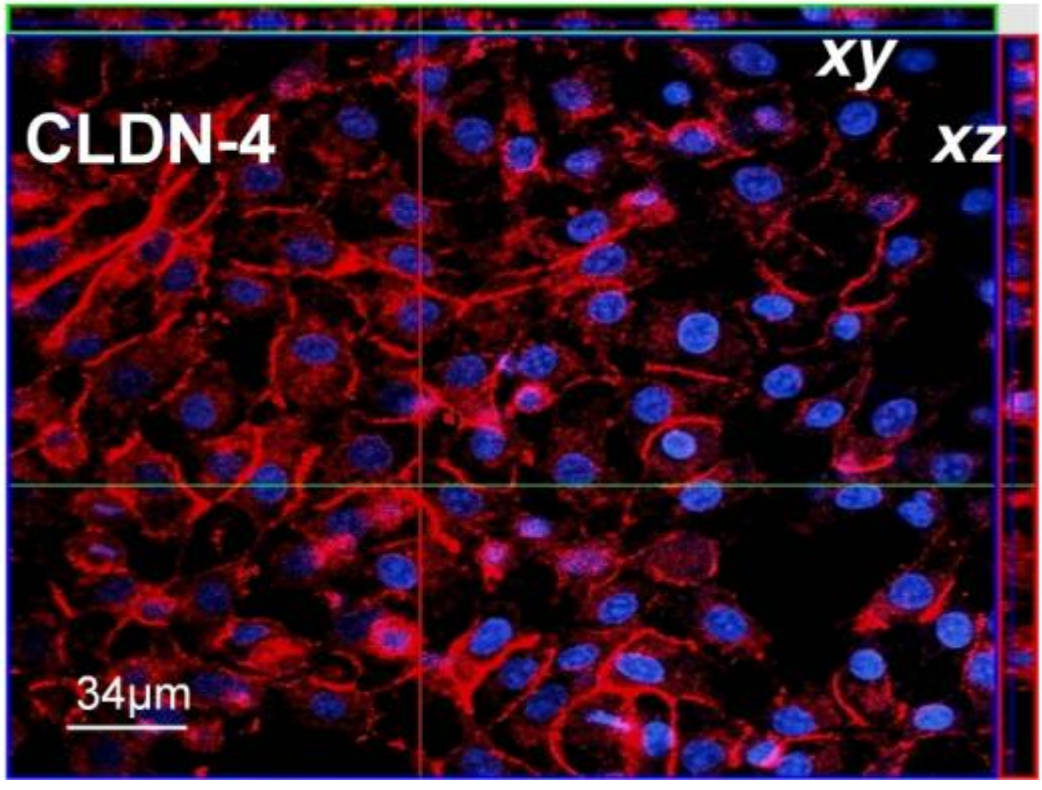
HSG cells on plastic were totally negative to cytokeratin-pan, AQP5, Mucin-1, α–smooth muscle actin, Musashi-1, CD44, CD166, CD105, CD106, CD90, CD146, Stro-1, CD45, CD19, while expressed vimentin strongly in 88.4% and α–amylase weakly in 5% of the cells (Table 3.1B, 3.1C). On Matrigel HSG cells did not express α–smooth muscle actin or Musashi-1. Both monolayers and 3-D cells expressed α–amylase (diffusely in the cytoplasm) and AQP5 (apicolateral) strongly in 98% of cells (Fig. 3.5). Vimentin, cytokeratin-pan, (Fig.3.5) and Mucin-1 were expressed by 60.5%, 95%, and 30% of the cells, respectively. Importantly, CD44 (Fig. 3.5) and CD166 were expressed by 3-D structures and monolayers (95%, 50% respectively) while CD105, CD106, CD90, CD146, Stro-1, CD45, CD19 were totally absent (Table 3.1C). In unpublished study on adult human salivary gland tissue, we have observed that CD44 and CD166 are expressed by serous and mucous salivary cells, respectively (240).

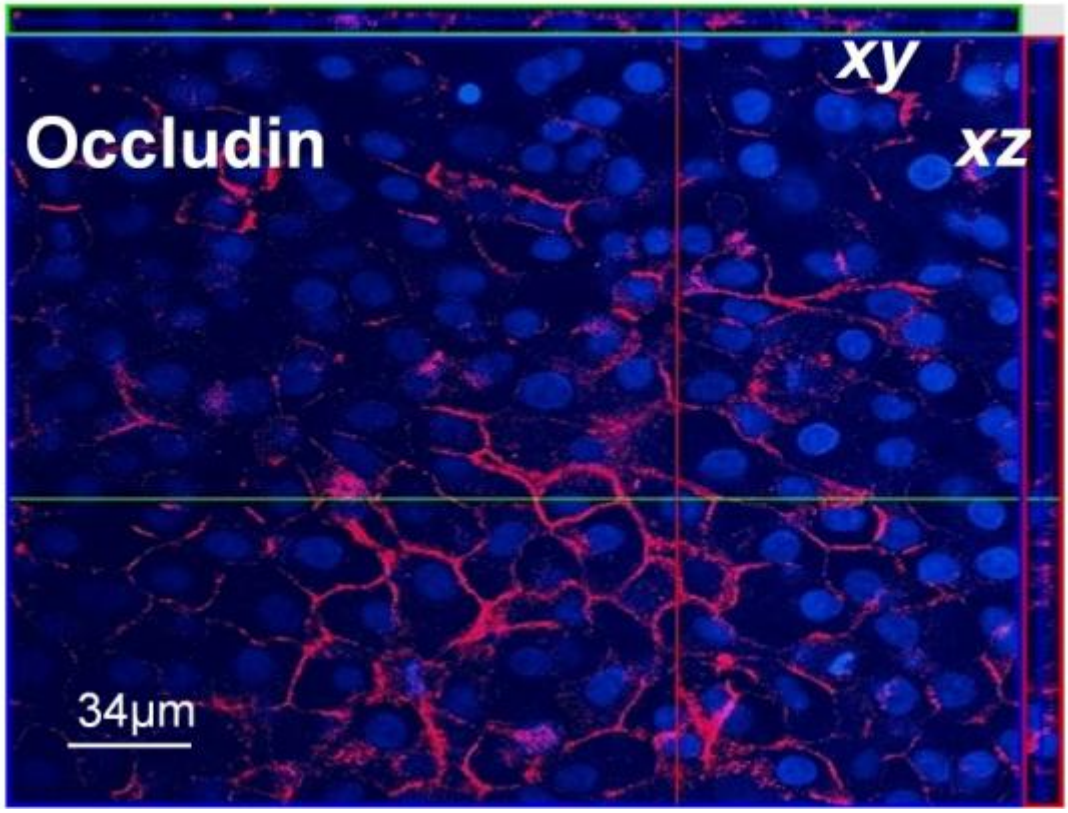
Figure 3.4: Confocal micrographs of HSG cultured on Matrigel expressed all TJ proteins tested. The *xy* plane demonstrating the presence of TJ proteins; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1, respectively (shown in red). The nuclei are stained with DAPI (shown in blue). Both *xz* and *xy* planes show TJ proteins as correctly localized at the apicolateral membranes of HSG cells cultured on Matrigel. [Scale bar= 40µm, X 630 for CLDN-1,-2,-3 & Scale bar=34µm, X 400 for CLDN-4, Occludin, JAM-A, ZO-1]. CLDN-1s shows series of confocal taken images at 1µm intervals that were used by Imaris 3D/4D image software to create isosurface for CLDN-1 protein labeled as CLDN-1iso, [Scale bar =15µm].

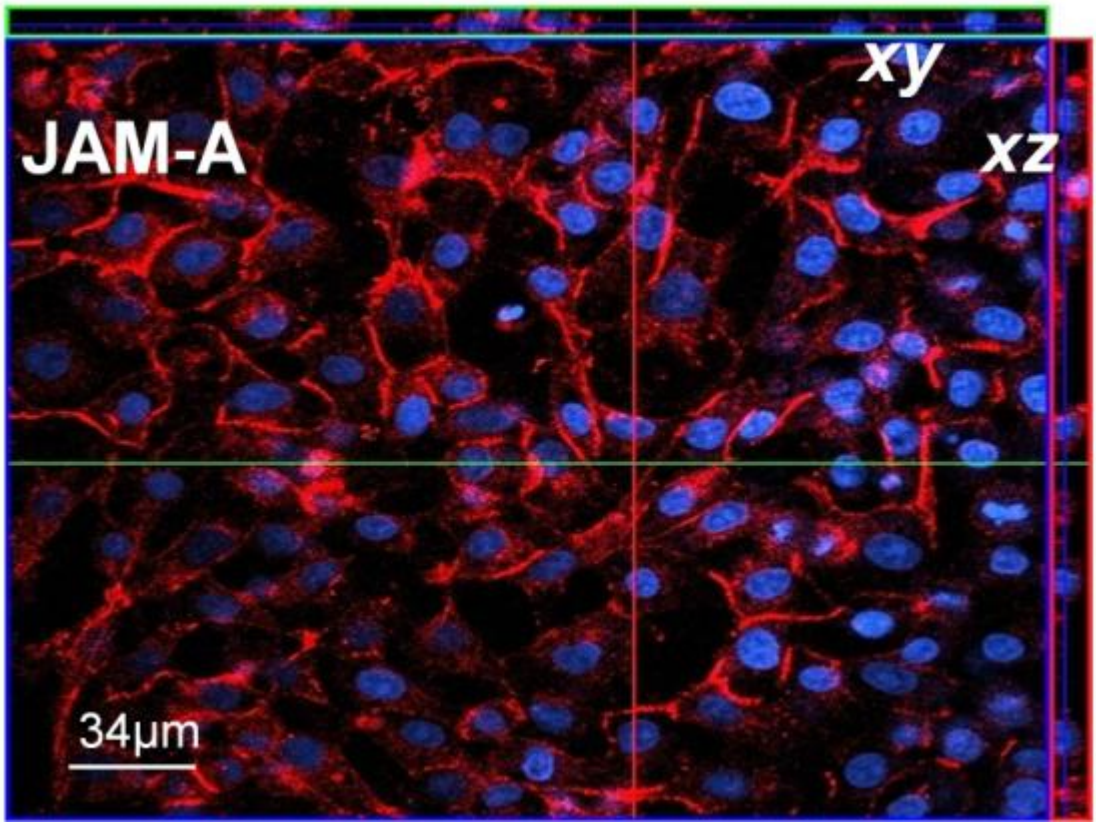

xy
CLDN-1
xz
40µm

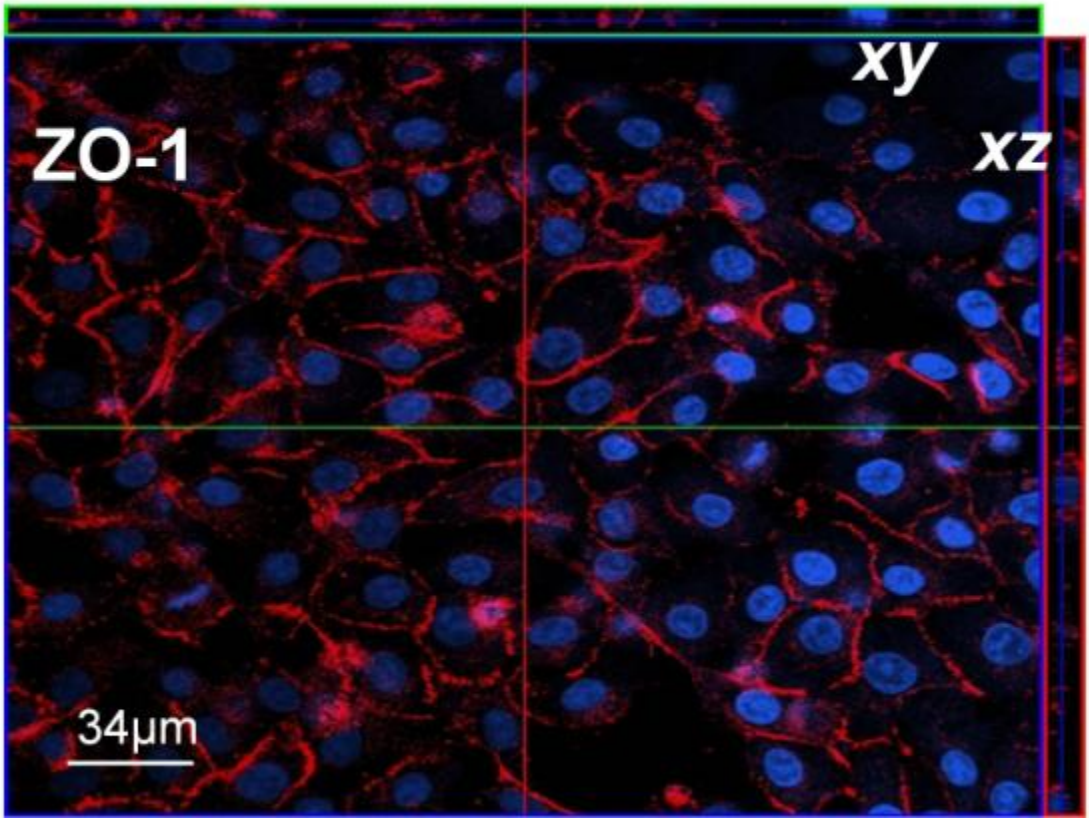

xy
CLDN-2
xz
40µm

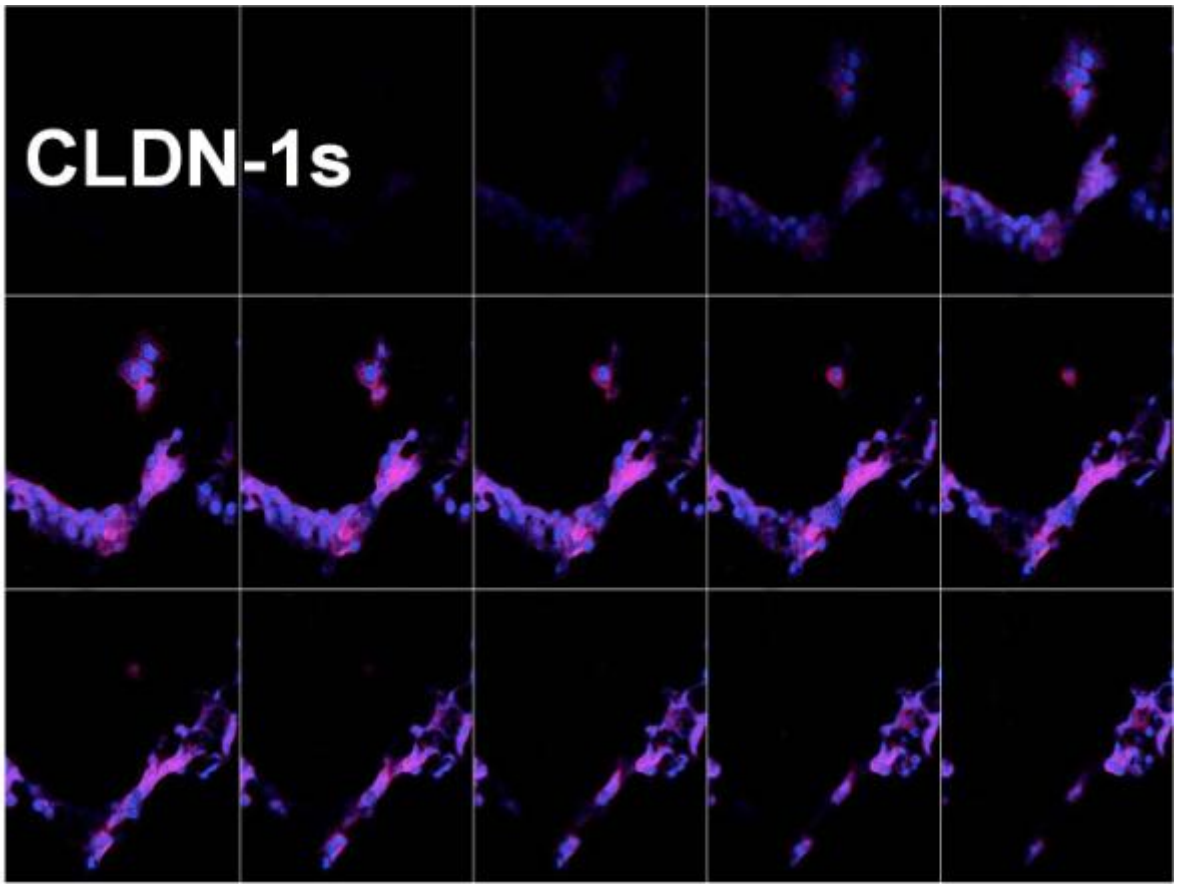

xy
CLDN-3
xz
40µm


xy
CLDN-4
xz
34µm


xy
Occludin
xz
34µm


xy
JAM-A
xz
34µm


xy
xz
ZO-1
34µm


CLDN-1s

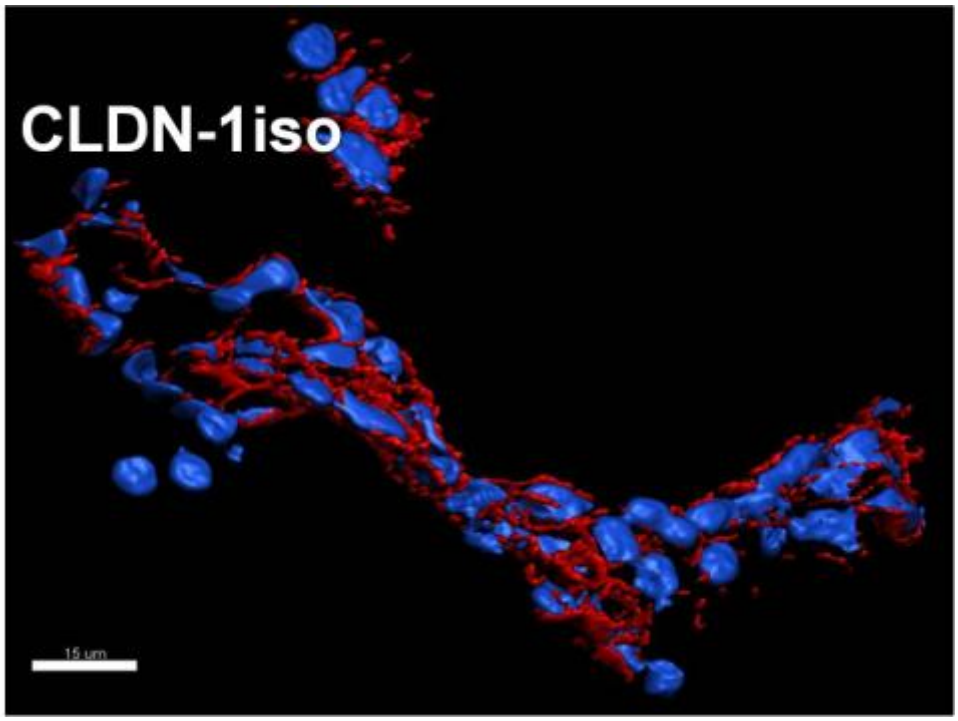

CLDN-1iso
15 um

Table 3.1: Expression of different markers/proteins in HSG cells grown on plastic and on Matrigel. Results are based on the examination of 5 slides per culture type. At least 1,000 cells were examined per slide. * The expression level of each marker/protein is indicated as percentage. All results from HSG on Matrigel were statistically significant in comparison to cells cultured on plastic, ($p<0.01$). (A) Tight junction proteins; claudin-1, 2, 3, 4, Occludin, JAM-A, and ZO-1. (B) Cytokeratin panel (epithelial), Vimentin (mesenchymal, salivary ductal, immature acinar and their progenitors), α–SMA (myoepithelial), α–amylase (salivary serous), AQP-5 (Acinar), Mucin-1 (salivary mucous). (C) Stem cell markers; Musashi-1 (Neural), Stro-1, CD44, CD166, CD105, CD106, CD90, CD146, (Mesenchymal) CD45, CD19 (Leukocyte).

[A]

Marker	Cldn-1	Cldn-2	Cldn-3	Cldn-4	Occludin	JAM-A	ZO-1
HSG + Matrigel	99%	98%	98%	98%	98%	99%	98%
HSG	0%	0%	0%	0%	0%	0%	0%

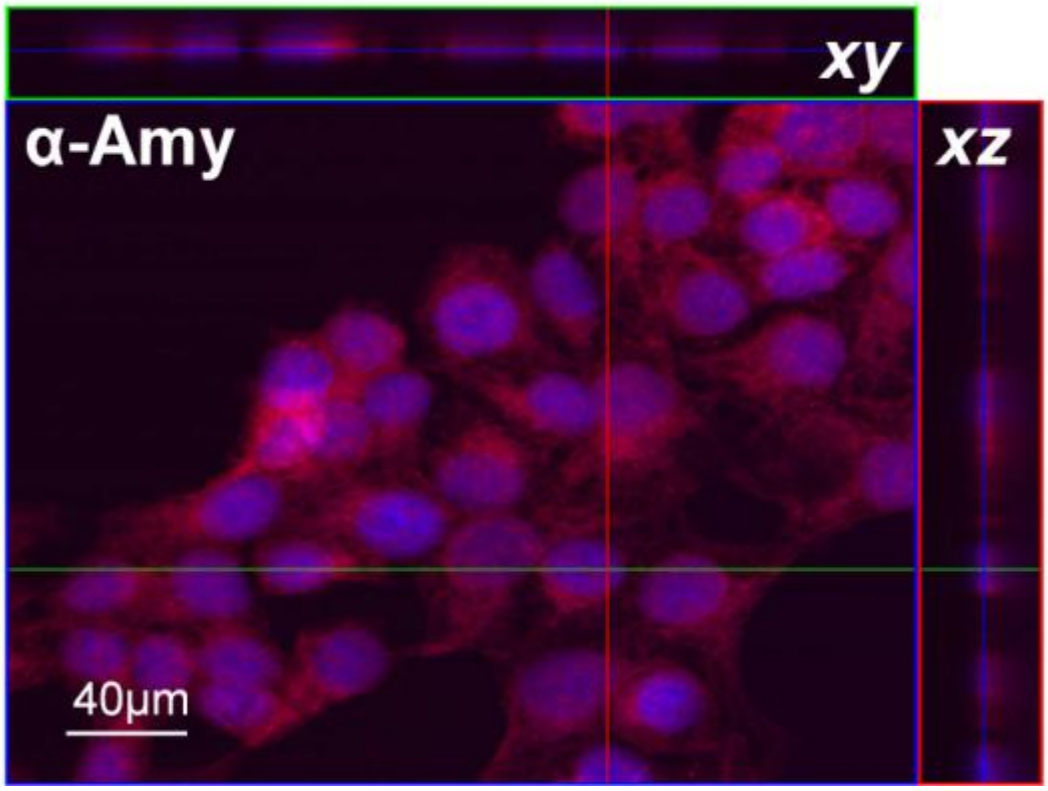
[B]

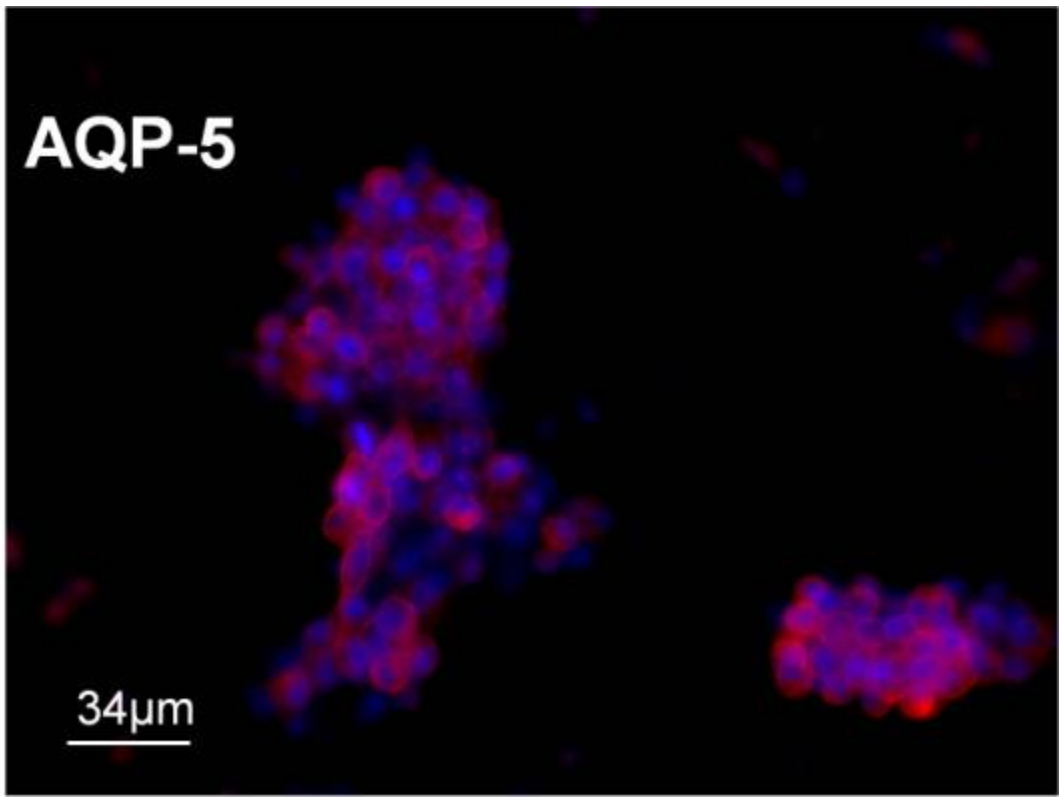
Marker	Cytokeratin	Vimentin	α–SMA	α–AMY	AQP5	Mucin-1
HSG + Matrigel	95%	60.5%	0%	98%	98%	30%
HSG	0%	88.4%	0%	5%	0%	0%

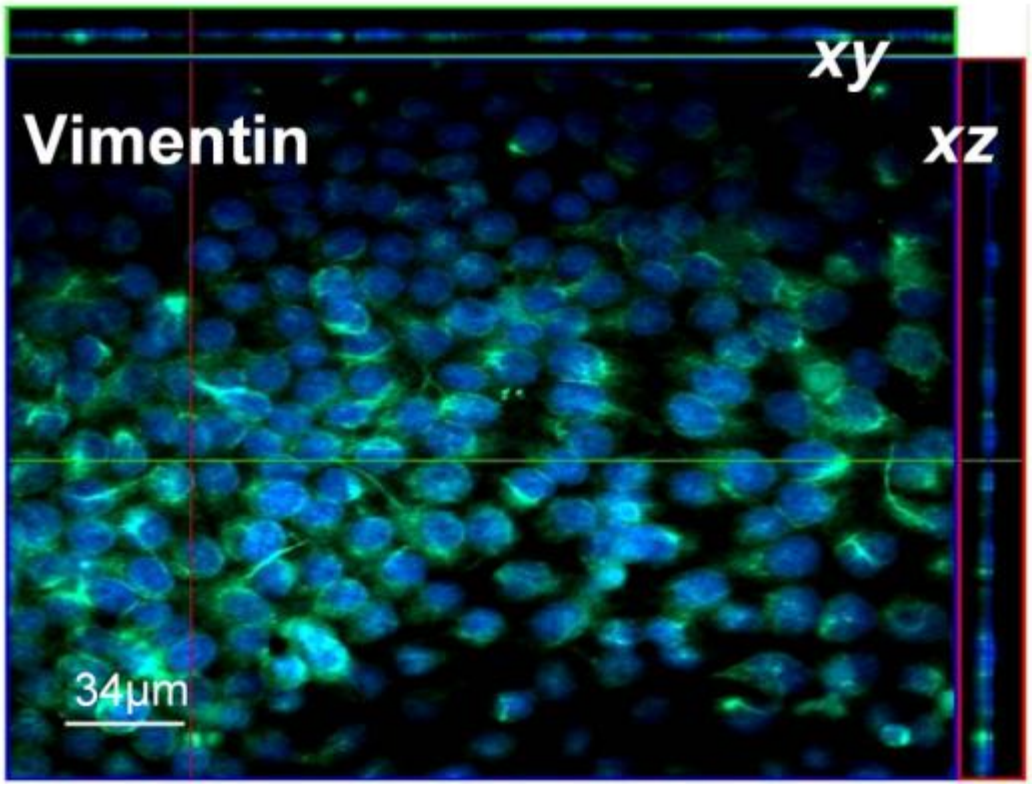
[C]

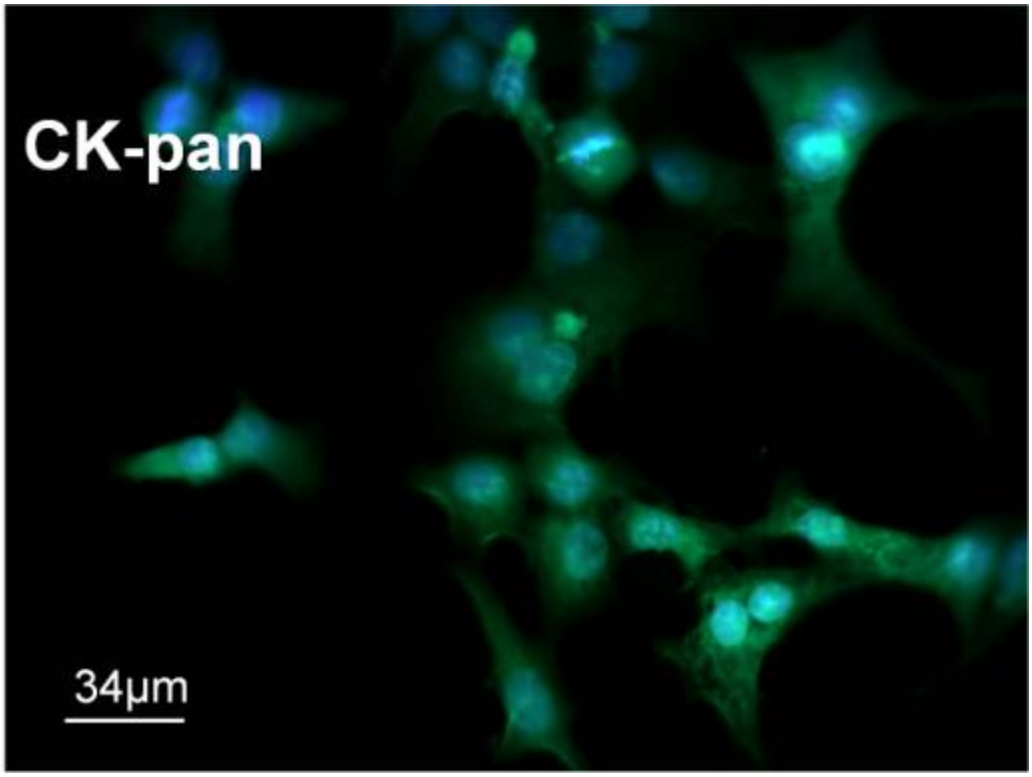
Marker	Msi-1	Stro-1	CD44	CD166	CD105	CD106	CD90	CD146	CD45	CD19
HSG + Matrigel	0%	0%	95%	50%	0%	0%	0%	0%	0%	0%
HSG	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

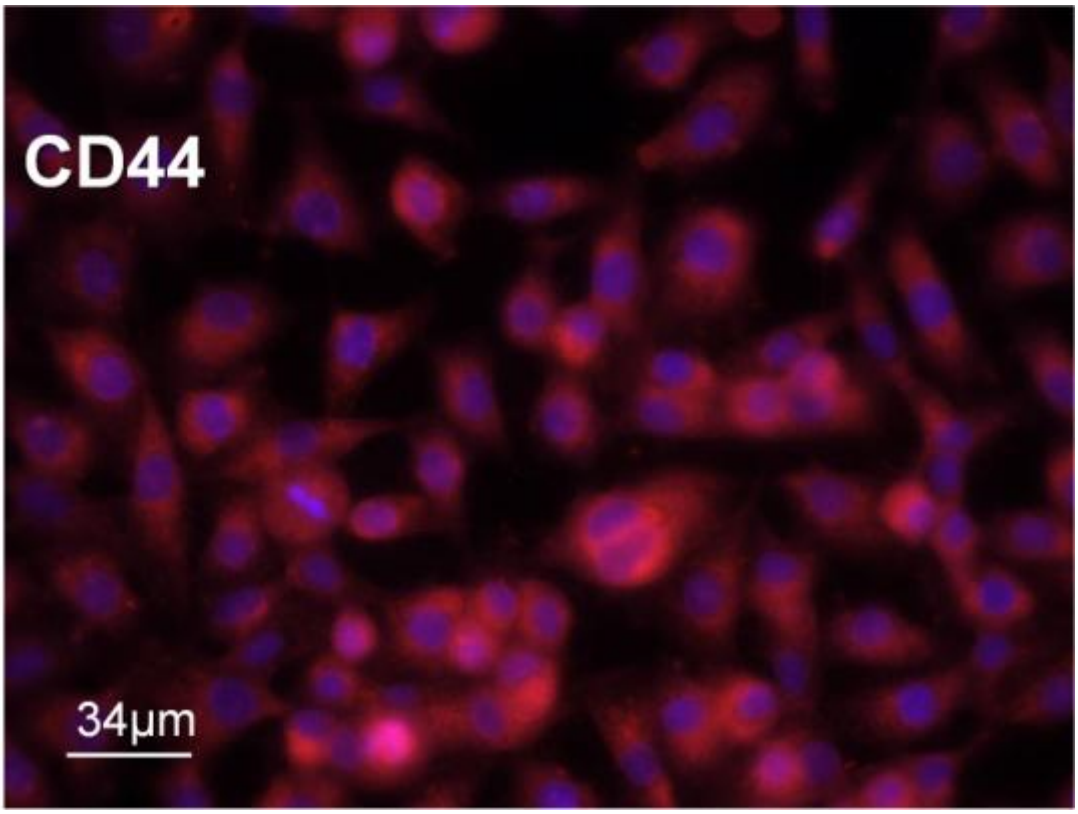
igure 3.5: Confocal micrographs of HSG cultured on Matrigel expressed acinar secretory proteins: α–amylase (α–Amy shown in red) and AQP-5 (98%, shown in green) in both monolayer and 3-D structures. Vimentin expression (60.5%, in green), cytokeratin-panel expression (95%, in green), and CD44 expression (95%, in red) in HSG cells cultured on Matrigel. [Scale bar= 40μm, X 630 for α–amylase & Scale bar=34μm, X 400 for AQP-5, Vimentin, cytokeratin-panel, CD44].


xy
α-Amy
xz
40μm


AQP-5
34μm


xy
Vimentin
xz
34µm


CK-pan
34µm


CD44
34µm

3.5. Western Blot Analysis: Conditioned media from different wells were collected, then, HSG cells grown on either plastic or Matrigel (at 1, 3, 5, 7 days) were washed with PBS, lysed, then supernatants were used for western blot analysis. We used 60μg total protein from each sample to evaluate the synthesis (in supernatants from cell lysates) and secretion (in media) of α–amylase, the most common salivary secretory protein, to test the function of HSG cells. At day 3, 5 and 7 HSG cells cultured on Matrigel could secrete α–amylase protein into media (supernatant samples showed similar results). However, HSG cells cultured on plastic did not show either synthesis or secretion of α–amylase protein until day 5 of culture. Western blot data from conditioned media presented in Fig. 3.6 indicates that Matrigel increased the synthesis and secretion of α–amylase protein to 3- and 2-folds at day 5 and 7, respectively.

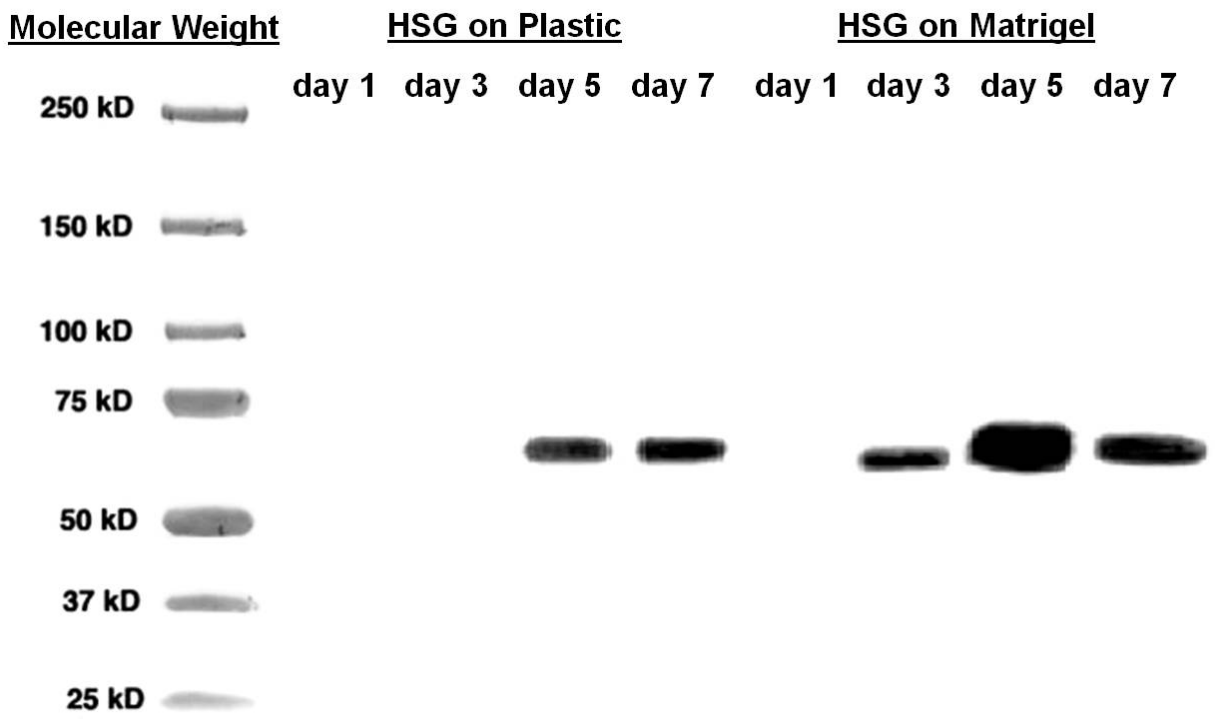
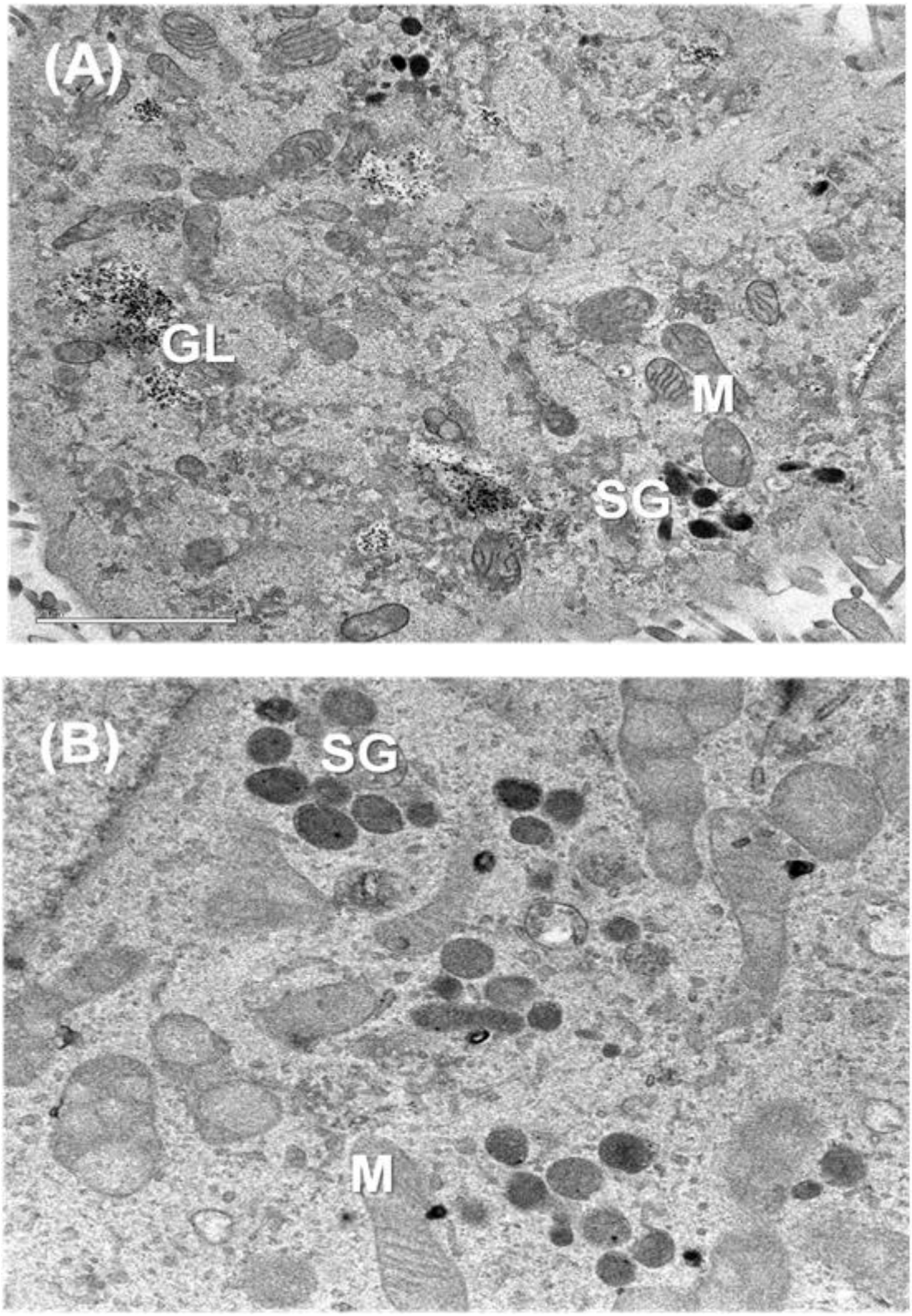


Figure 3.6: Western blot analysis of α–amylase protein (60 kDa) as evaluated in conditioned media from HSG cells (at 1,3,5,7 days). At day 3, 5, 7 HSG cells cultured on Matrigel could secrete α–amylase protein in media however HSG cells cultured on plastic did not secrete α–amylase protein until day 5. On Matrigel, HSG cells showed 3- and 2-fold increase in α–amylase secretion at day 5 and 7, respectively.

3.6. Transmission Electron Microscopy (TEM): Ultrastructural analysis by TEM revealed that HSG cells grown on plastic resembled ductal-like cells arranged in monolayers. Its cytoplasm showed tiny scarce secretory granules among the plentiful large and elongated mitochondria, small isolated islands of glycogen, and an absence of Golgi apparatus, rough endoplasmic reticulum and microvilli (Fig.3.7A). On Matrigel, many electron-dense secretory-like granules similar to the normal human salivary secretory granules (241) were clearly detected in the cytoplasm at day 3 (Fig. 3.7B). Features of active protein synthesis were obvious at day 3, 5, 7 where many Golgi saccules and rough endoplasmic reticulum were seen everywhere in the cytoplasm (Fig. 3.7C). On Matrigel, approximately 90% of HSG cells (from both monolayers and 3-D acinar-like structures) produced electron-dense secretory granules at day 5 and 7; while at day 3, 60% of these cells showed secretory granules. Tight junction structures were observed (from day 2) at the apicolateral cell membrane in polarized HSG cells grown on Matrigel (Fig. 3.7D). However, no basal lamina could be observed. On plastic, no TJ structures could be observed. On Matrigel, Membrane-bound apoptotic bodies were obvious in cells at the middle of the 3-D acinar-like structures (Fig. 3.8A). This suggests the formation of a central lumen where the surrounding cells showed well-developed microvilli, as an indication of active secretion. The microvilli resembled those of normal salivary epithelial cells. Apoptotic cells had many lysosomes in the cytoplasm where there were no signs of active protein synthesis. Moreover, the chromatin became broken and both nuclear and cellular membranes lost their integrities (Fig. 3.8B).

Figure 3.7: Transmission Electron Microscope (TEM) micrograph of HSG cells stained with lead citrate and uranyl acetate staining. (A) HSG cell grown on plastic exhibited features of ductal cells: tiny scarce secretory granules (SG) among the plentiful large elongated mitochondria (M), small isolated island of glycogen (GL) [X 9,900]. Panels B, C, D showed HSG cells grown on Matrigel. (B) HSG cell (at day 7) exhibiting multiple electron-dense secretory granules (SG) among multiple mitochondria (M) [X 16,500]. (C) HSG cell (at day 3) exhibiting features of active protein synthesis; Golgi saccules (G) and rough endoplasmic reticulum (rER), multiple mitochondria (M) and a secretory granule (SG) budding out of Golgi saccule, all swimming close to the nucleus (N) [X 16,500]. (D) Two adjacent HSG cells (at day 3) exhibiting characteristic features of salivary epithelial cells; joined by tight junction structure (TJ) at the apicolateral cell membrane, electron-dense secretory granule (SG), and microvilli (M) at the luminal cell membrane [X 43,000].


(A)
GL
M
SG
(B)
SG
M

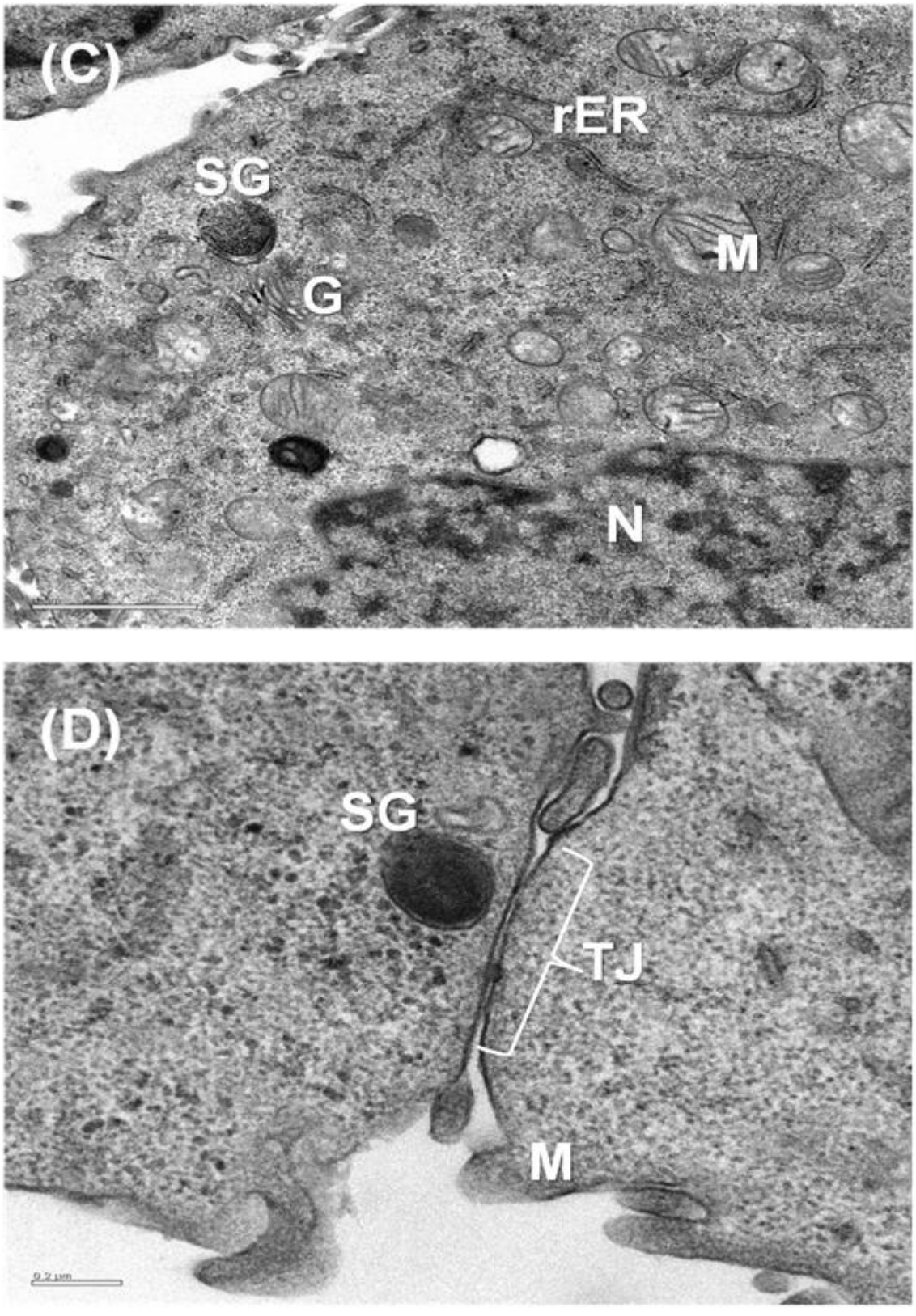
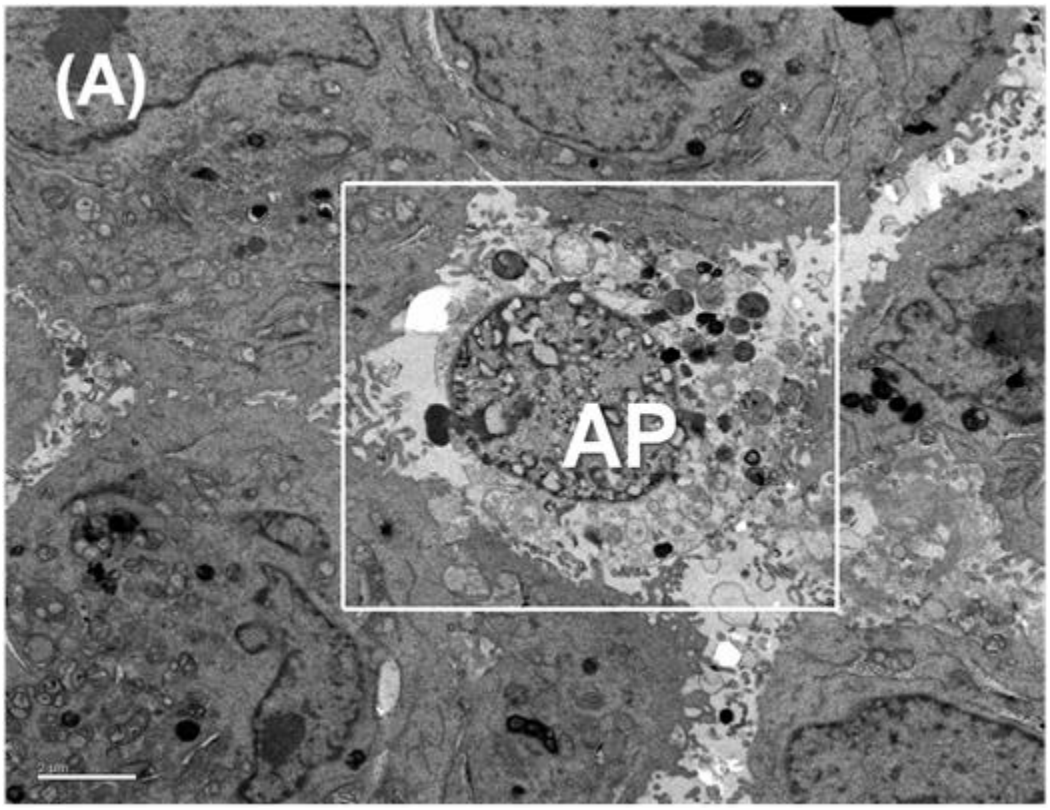
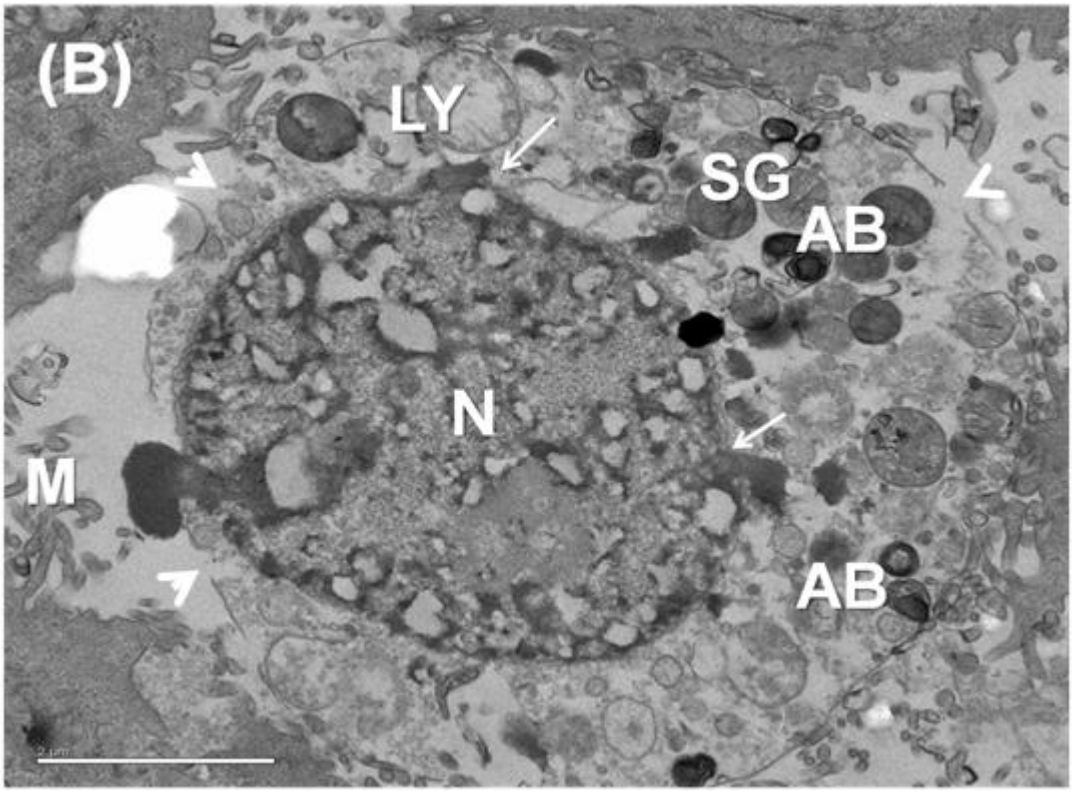

(C)
rER
SG
M
G
N
(D)
SG
TJ
M
0.2 μm

Figure 3.8: Transmission Electron Microscope (TEM) micrograph of HSG cells stained with lead citrate and uranyl acetate staining. (A) One 3-D acinar-like structure of HSG cells cultured on Matrigel (at day 3) exhibiting features of apoptosis (AP) in the middle of the 3-D structure and the formation of a central lumen [X 4,200]. (B) Inset of the apoptotic cell at a higher magnification showing membrane-bound apoptotic bodies (AB), few secretory granules (SG) among many lysosomes (LY). Apoptotic nucleus (N) with broken chromatin, the nuclear (arrows) and cellular membranes (arrow heads) lost their integrity. The surrounding cells show well-developed microvilli (M) that are an indication of active secretion. [X 9,900].


(A)
AP


(B)
LY
SG
AB
N
M
AB

3.7. Quantitative Real-Time PCR Analysis: Gene expression levels of claudin-1, claudin-2, α-amylase, AQP-5, Cytokeratin-18, EGF genes, were measured using quantitative RT-PCR. HSG cells cultured on plastic were found to express both claudin-1 and claudin-2 whereas they were not able to translate the related proteins (negative immunofluorescence and absence of TJs in TEM). When HSG cells were cultured on Matrigel, the expression of tight junctions genes; claudin-1, claudin-2, acinar-specific; α-amylase, and AQP5, and ductal-specific, cytokeratin-18 and EGF genes were decreased, (for AQP5 gene, down-regulation was statistically significant, $P < 0.01$) (Fig. 3.9). Similar trend was observed at day 3, 5 and 7 post-plating. These data suggest that the protein expression pattern associated with HSG cultured on Matrigel is achieved through regulation of translation, rather than transcription of relevant genes.

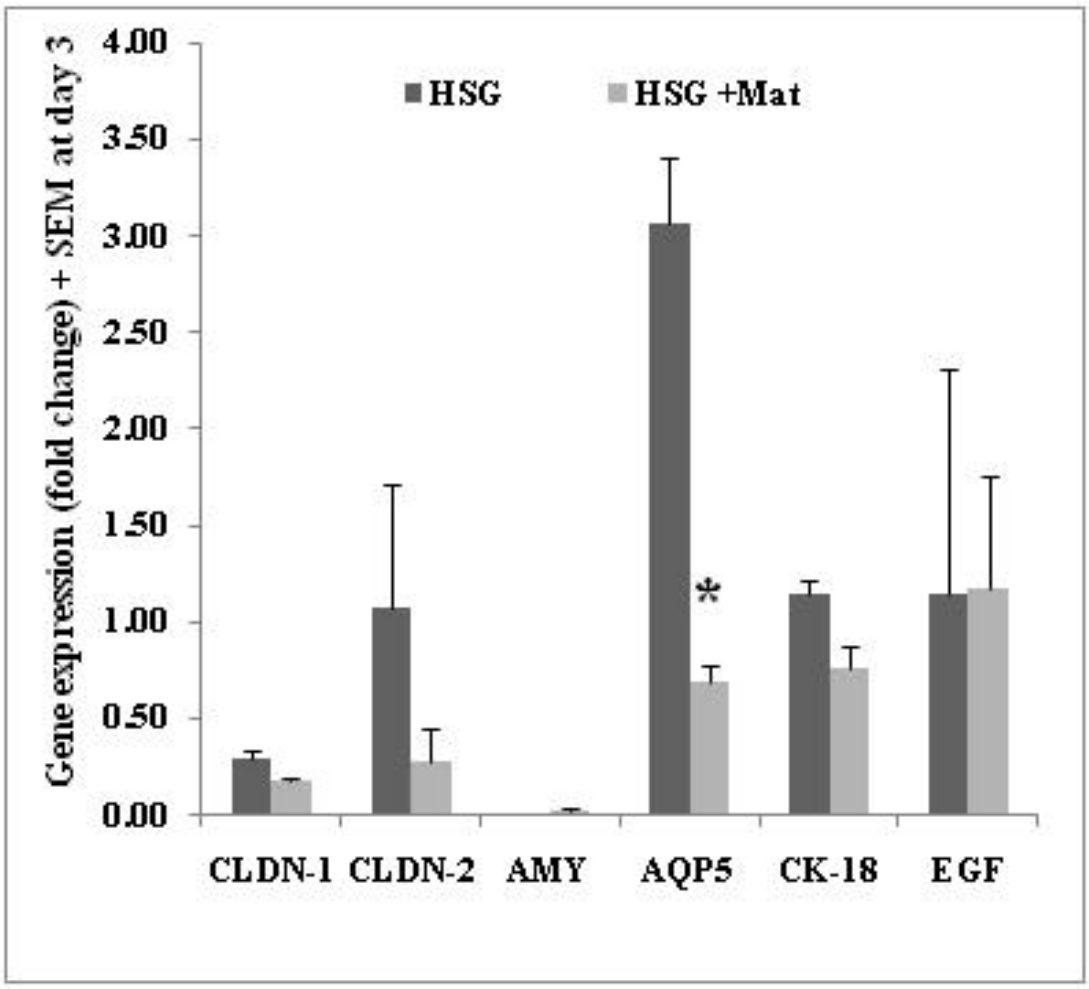


Figure 3.9: Gene expression levels (at day 3) indicated in fold change for HSG cultured on Matrigel and on plastic for; claudin-1 (CLDN-1), claudin-2 (CLDN-2), α-amylase (AMY), Aquaporin-5 (AQP-5), cytokeratin-18 (CK18) and epidermal growth factor (EGF) genes, relative to samples from cultures grown on plastic for 9 days and normalized to GAPDH levels using real-time quantitative polymerase chain reaction (at least 3 separate experiments were performed/gene). Results are reported as means ± standard error of the mean of parallel cultures, (*$P<0.01$).

4. DISCUSSION

Hereby, we report that Matrigel induced obvious morphologic changes and cytodifferentiation in HSG cells including; the formation of 3-D acinar-like polarized structures expressing TJs, showing reasonable TER and containing a well-developed Golgi apparatus, rough endoplasmic reticulum and secretory-like granules. TJ proteins; claudin-1, 2, 3, 4, occludin, JMA-A, ZO-1 as well as salivary acinar markers; α-amylase, AQP5, mucin-1, CD44, CD166 were evident by immunofluorescence technique. The α-amylase protein secretion into conditioned media was confirmed. Moreover, quantitative RT-PCR analysis revealed down-regulation of TJ, acinar and ductal-specific genes accompanied by protein translation (claudin-1, 2, α-amylase, AQP5, and cytokeratin), therefore, confirming acinar differentiation of cells grown on Matrigel. To our knowledge, this is the first report confirming that HSG cells do express claudin-1 and claudin-2 genes when cultured on plastic where the lack of TJ structures formation might be a protein translation defect. Herein, we report that the mitotic activity was significantly reduced while apoptotic activity significantly increased ($P<0.01$).

Vag et al 2007 (243). reported that on low BME concentration few acini were formed by proliferation of HSG cells, rather than migration, where cells would sink into the soft gel resulting in being less polarized compared to those cultured on higher BME concentration, therefore, forming 2-D reticular networks rather than 3-D structures. Whereas, high concentration BME is more rigid; this allows easy cell movement, differentiation and formation of 3-D structures (243). Here we used Matrigel at low concentration (2mg/ml) and were able to see 3-D acinar structures within 24h but not reticular networks. Although the physical properties of the matrix may promote acinar differentiation, the presence or absence of certain factors may also be important. Barka et al 2005 (244), described SCS-9 cell line (derived from mouse submandibular gland) cultured on Matrigel to form cellular cords and tubules and highlighted the important role

of EGF during the experiment (244). Extracellular matrix components play a major role in morphogenesis where they regulate different signal pathways across the cell membrane in addition to the physical support related to its rigidity (245).

HSG cells derived from intercalated ducts were initially reported to express markers of myoepithelial or acinar cell type after chemical induction (233). On Matrigel, HSG cells formed either reticular network with large duct-like lumens or acinar-like structures or acino-tubular structures (162166,246) while on laminin supported with growth factors, HSG cells formed acinar-like structures (165). In our study the 3-D acinar-like structures formed were similar in size, shape and number of cells/3-D structure to the normal human salivary acini, although our 3-D structures were not connected via ductal structures. Similar results were reported by Hoffman et al 1996 (248). Expression of acinar-specific markers, such as α-amylase (162,165.246) and cystatin (162), indicate that HSG cells differentiate as well, but there is no direct evaluation of secretion of these proteins into the medium to confirm functional differentiation of the HSG cells; these reports (162,165,246) analyzed proteins from cell lysates. Here we confirm that HSG on Matrigel are functional secretory acini as we detected α-amylase protein secreted into the conditioned media. Zheng et al 1998 (246), reported that both TGF-α and HGF when added to laminin are able to activate the α-amylase promoter in HSG cells, in our study these growth factors are already components of Matrigel. In addition to α-amylase acinar protein, we report that the 3-D acinar-like as well as monolayer cells expressed AQP-5 (in 98% of cells) water channel protein necessary for water secretion in human salivary glands (247, 248). Moreover, 3-D structures expressed new serous and mucous adhesion-related proteins CD44, CD166 respectively, unpublished data (240); these proteins are considered mesenchymal stem/progenitor cell markers as well.

On Matrigel, the establishment of reasonable TER essential for saliva secretion in vivo, (249) was confirmed by the distribution of TJ proteins to the apicolateral membrane of the HSG 3-D acinar-like structures and monolayer. TJs distribution in HSG cells resembled their distribution in normal human and rodent salivary glands (155,208). Although salivary gland cells have been characterized in 3-D culture (166,250-252), this is the first study to demonstrate that 3D acinar-like structures from HSG cells are capable of establishing a reasonable TER and express TJ proteins. Aframian et al 2002 (160), reported that HSG cells did not express TJ structures and therefore have low TER even after transfection with claudin-1 and claudin-2 genes the cells expressed both proteins at their membranes but did not acquire adequate TER. It seems that Matrigel drives a specific mechanism through which the translation of specific proteins as TJ proteins is triggered. However, Matrigel might not be the only required factor for translation of all proteins; here we report that α-smooth muscle actin protein was not expressed on either plastic or Matrigel. Sato et al 1985 reported that 95% of HSG cells on plastic expressed both keratin and vimentin (253). In our study, on both plastic and Matrigel, 85% and 60.5% of HSG cells expressed vimentin, respectively while cytokeratin was not expressed on plastic but expressed in 95% of the cells on Matrigel. It seems that Matrigel drove HSG cells to a keratinocyte-like direction. Normally, vimentin is expressed by the immature salivary acini and their direct progenitors, some ductal cells and myoepithelial cells as well (254).

Ultrastructural analysis confirmed the establishment of TJ structures at the apicolateral cell membrane in polarized HSG cells cultured on Matrigel, however, no basal lamina could be observed. During early development of salivary gland, the basal lamina becomes discontinuous at times of elongation and branching (255). On plastic, no TJ structures could be observed. On Matrigel, many secretory granules were detected in the cytoplasm along with features of active protein synthesis. Approximately 60-90% of HSG cells (both monolayers and 3-D structures)

were acinar (3-7 days), while on plastic HSG cells were all ductal. Membrane-bound apoptotic bodies were obvious in cells at the middle of 3-D structures for the formation of central lumen.

Ductal-specific genes reported here (CK-18 and EFG) were down-regulated on Matrigel. Acini are believed to be originally from intercalated ducts (168,230-232). CK-18 is one of the cytokeratin genes that is expressed by ductal cells, as indicated by the immunofluorescence analysis cytokeratin protein has been expressed by 95% of HSG cells when cultured on Matrigel, therefore the down-regulation of such gene might be related to the translation of its protein. EGF is known to increase DNA synthesis, cell proliferation, growth, and differentiation (256,257). We think that EGF down-regulation might confirm differentiation of HSG cells on Matrigel to acinar cells. Acinar-specific genes (α-amylase, AQP5) although were all down-regulated, their related proteins were highly expressed (98%) on Matrigel. Moreover, TEM analysis confirmed the formation of secretory granules along with TJ structures. The α–amylase gene down-regulation might be explained by the loss of 3-D acinar-like structures along with increased apoptosis activity. A feedback mechanism might cause the down-regulation of the gene to decrease α–amylase synthesis until secreted because its secretion is a slow complicated process (258). In our study 78.46% of HSG cells cultured on Matrigel were apoptotic (at day 7) mainly the disintegrating old 3-D structures; this would prevent or decrease acinar-specific gene expression. Our results coincide with those reported by Sato et al 1987, where acini differentiated by the induction of HSG cells, using 5-azacytidine, significantly reduced the cell number after 12 days (233). Vag et al. 2007 (243), observed high apoptotic activity in HSG cultured on BME for 5 days. Whereas, Szlavik et al 2008 found that after 3 days of culture in Matrigel, HSG proliferation arrested and apoptosis could be stopped by replating the cells on plastic while after 4 days on Matrigel HSG cells failed to survive, even after replating (252). Szlavik et al 2008, indicated that Matrigel alone is not sufficient for the full maturation and long-term survival of

differentiated acini (252). In our study, reduction in the mitotic activity (38%) of HSG cultured on Matrigel is consistent with other reports on the HSG cells (165, 247,252,259). Royce et al 1993 reported that Matrigel decreased HSG mitotic activity to 50% (165). In contrast, on very low concentration of either Matrigel or laminin, few acini differentiated from HSG cells that seemed to grow in similar rate to cells on plastic (166). Moreover, Lam et al 2005 reported that HSG cells cultured on either Matrigel or collagen-I had an early increase in mitotic activity in comparison to cells cultured on uncoated substrates (162). In our study, apoptosis was observed in cells that lost contact with the Matrigel as they became internalized in the 3-D acinar structures leading to central lumen formation (166, 260, 261). During salivary gland development balanced mechanisms govern the interaction between cell proliferation, apoptosis and cytodifferentiation (262).

5. CONCLUSIONS

We report here, a complete differentiation of HSG cells cultured on Matrigel including; the formation of acinar-like 3-D structures, expression of acinar proteins; AQP5, α-amylase, mucin-1, and adhesion-related proteins; CD44, CD166. Moreover, TEM confirmed an active protein synthesis along with formation of numerous secretory granules. In addition, α-amylase protein was secreted into media. Quantitative RT-PCR analysis revealed down-regulation of certain genes that is associated with the regulation of corresponding proteins translation thus confirming acinar differentiation. The mitotic activity was significantly reduced at day 7 where apoptotic activity had significantly increased ($P<0.01$). Our main important result here is the successful formation of functional polarized acinar cells attaining adequate TER and exhibiting TJs. In addition, to our knowledge this is the first study to report that HSG cells express TJ genes on plastic suggesting that a protein translation defect exists. Neither Matrigel nor HSG cell line would be used in clinical applications as both are derived from mouse sarcoma and human

adenocarcinoma, respectively. However, together they represent an excellent and easily reproducible model to further study salivary-specific gene expression, organization, morphogenesis, carcinogenesis and the mechanisms governing their biology. In addition, HSG cell line as a long-term dividing human salivary cell type would allow long-term studies in animal models. Our next step would be testing HSG (as a graft cell type) grown on Matrigel (as an extracellular matrix) carried on a scaffold designed to be implanted in animal models (mouse, rat, rabbit, monkey, etc.) to further examine our envisioned artificial salivary gland device in vivo. Certainly, these studies would help to improve the treatment of salivary glands hypofunction.

AKNOWLEDGEMENTS

We are most grateful to Mr. Cory Glowinski for the creation of 3D isosurface renderings for both CLDN-1 and DAPI channels. We are most appreciative of the technical help provided by Mr. Yunlin Tai in doing SDS-PAGE, and Mrs. Jeannie Mui and FEMR at McGill University for TEM samples preparation. The authors would like to thank Dr. John Presley, Dr. Judith Lacoste and Cell Imaging and Analysis Network (CIAN) for Confocal Microscopy use. We are also grateful to Dr. Mari Kaartinen and Dr. Monzur Murshed for helpful sharing of their laboratory equipments. The authors would like to thank the funding institutions; Canada Research Chair, CIHR, NSERC, RSBO and Egyptian Ministry of Higher Education and Research.

AUTHOR DISCLOSURE STATEMENT: No competing financial interests exist.

CHAPTER 4

Maria OM, Zeitouni A, Gologan O, Tran SD. Matrigel Improves Functional Properties of Primary Human Salivary Gland Cells. (S*ubmitted 2010*).

The Ph.D. Candidate performed the following contributions in this manuscript:

- All the experimental work.
- Examinations and analysis of data.
- Wrote the manuscript.

Matrigel Improves Functional Properties of Primary Human Salivary Gland Cells

O.M. MARIA, D.M.D.,[1] A. ZEITOUNI, M.D.,[2] O. GOLOGAN, M.D.,[3] S.D. TRAN, D.M.D., Ph.D.[1*]

[1]Faculty of Dentistry, McGill University, Montreal, Canada.

[2]Department of Otolaryngology, McGill University, Montreal, Canada.

[3]Department of Pathology, McGill University, Montreal, Canada.

O.M. MARIA, D.M.D.

Address: 3640 University Street, Room M33, Montreal, Quebec, H3A 2B2, Canada. E-mail: ola.maria@mail.mcgill.ca, Tel: +1 514 398 7203 ext. 00056, Fax: + 1 514 398 8900

A. ZEITOUNI, M.D.

Address: 687 Pine Avenue West, E4.45, Montreal, Quebec H3A 1A1, Canada. Email: anthony.zeitouni@mcgill.ca, Tel: +1 514 934-1934 ext. 34974

O. GOLOGAN, M.D.

Address: 3775 University Street, Room A5B, Montreal, QC, H3A 2B4, Canada. Email: olga.gologan@muhc.mcgill.ca, Tel: +1514 398-7192 ex. 00511, Fax: +1 514 398-2440

[*]*Correspondence Author*:

S.D. TRAN, D.M.D., Ph.D.

Address: 3640 University Street, Room M43, Montreal, Quebec, H3A 2B2, Canada. E-mail: simon.tran@mcgill.ca, Tel: +1 514 398 7203 ext. 09182, Fax: + 1 514 398 8900

ABSTRACT

Xerostomic patients are those afflicted with irreversible loss of functional salivary acini caused by Sjogren's syndrome and radiotherapy for head and neck cancer. Currently, there is no effective treatment available. A tissue-engineered artificial salivary gland device would help these patients. The cellular components (graft cells) of this device must establish tight junctions (TJs) in addition to being functional acinar secretory cells. This study analyzed a graft source from primary human salivary glands (huSG) cultured on Matrigel. Cells were isolated from parotid and submandibular glands, cultured, passaged, and plated on either Matrigel-coated (2mg/ml) or uncoated plastic. Immunohistochemistry, TEM, quantitative RT-PCR, western blot, and measurement of transepithelial electrical resistance (TER) were employed for phenotypic and functional analyses. On Matrigel, huSG cells underwent obvious morphogenesis and cytodifferentiation; forming 3-D acinar units within 24h, whereas on plastic, they formed monolayers of ductal cells. Cells expressed TJ proteins (claudin-1, -2, -3, -4, occludin, JAM-A, and ZO-1) and adequate TER in both cultures. Importantly, on Matrigel, 99% of cells expressed α-amylase and AQP5 (water channel protein) compared to only 5% and 0%, respectively on plastic. TEM confirmed acinar differentiation, showing active protein synthesis and secretory granules. Additionally, Matrigel increased the secreted α-amylase 2-5 times. Matrigel down-regulated certain salivary ductal genes and regulated the translation of acinar proteins. This 3-D cell culture system confirms the organization and differentiation of huSG cells on Matrigel and provides a useful method to culture sufficient acinar cells for implantation into our envisioned salivary gland device.

Keywords: Biomedical Engineering, 3-D Cell Culture, Cell Differentiation, Extracellular Matrix, Tissue Engineering Applications.

INTRODUCTION

Salivary epithelial tissue is composed of two main epithelial units; acinar cells which are salt, protein and water secreting units (148, 219) and ductal cells which are absorptive and relatively water-impermeable cells (148,219) The primary function of saliva is to protect and maintain the oral and the gastrointestinal tract (GIT) in addition to initial food digestion (263) Therefore, salivary gland hypofunction (xerostomia) will affect the oral and GIT mucosa. There are two major reasons for xerostomia: an autoimmune disorder called Sjogren's syndrome affecting 1-4 million patients in US and radiotherapy for head and neck cancers affecting 40,000 new patients/year in US (194,201). In addition, some medications and systemic diseases such as diabetes mellitus and pernicious anaemia might cause milder xerostomia (1-4). The main problem results from an irreversible loss of acinar secretory cells and/or their function followed by fibrosis. Consequently, the patient becomes more prone to increased incidence of dental caries, periodontal disease, candidiasis, oro-pharyngeal infections, mucositis, dysphagia and considerable pain and discomfort (2). Currently, the use of artificial saliva or saliva stimulants is not satisfactory (8,9) and a permanent treatment for xerostomia is not available yet. In order to better understand the functional capabilities of salivary acini, researchers have been working on propagating salivary cells in culture. Valuable information has been obtained from those studies which improved the understanding of salivary gland pathobiology. However, expanding acinar secretory cells in cultures was not successful yet.

In an effort to help xerostomic patients, we have been working to develop an artificial salivary gland device.(147,158, 226). This device consists of a blind-end tube constructed of a biodegradable polymer, coated on its inner (luminal) surface with an extracellular matrix protein to maintain the attachment of a polarized epithelial graft cells capable of unidirectional fluid movement (147). In order for these graft cells to function well, they should express tight

junctions (TJs), and show adequate transepithelial electrical resistance (TER) in addition to being able to secrete water, minerals and proteins like acinar cells. Therefore, for this artificial salivary gland device we need to culture and expand acinar cells which are the functional units of salivary glands. A major hurdle in the development of this salivary device has been finding the suitable graft cell part (160). Human submandibular gland (HSG) cell line derived from intercalated ducts was used initially as a proposed graft cell type (165, 166). However, HSG cells are not capable of forming a polarized epithelial layer and do not express TJs, therefore, do not show a reasonable transepithelial electrical resistance (TER), or control water movement (160). Matrigel, a basement membrane extract (BME) can induce HSG cells to differentiate into acinar-like units. (162,165,166)The advantage of the HSG differentiation model is the uniformity of the cells in the culture however their neoplastic properties might complicate the interpretation of the results (165). Additionally, it is still questionable whether a neoplastic cell line can be used to tissue-engineer an artificial salivary gland device. Therefore, we thought to investigate whether primary human salivary gland (huSG) cells would be used as a source of graft/acinar cells in our envisioned device. Moreover, to elucidate whether adult salivary glands from patients are capable of regeneration. Recently, we developed a method to obtain primary salivary epithelial cells capable of forming a polarized monolayerv (10, 11). These cells expressed TJs and showed reasonable TER but were mainly ductal cells and did not express the main water channel protein AQP5, therefore, are incapable of unidirectional fluid and protein secretion (148,249,264). Earlier studies have focused on the water secretory component (158, 186,187, 265-267) however, none of which were successful in long-term expansion of acinar secretory cells. Nevertheless, attempts to culture primary cultures of ductal/acinar cells from salivary glands have been successful (228, 229, 250, 268-272) owing to the use of extracellular matrix and/or its components in a gel form. Cells cultured on BME allow the in vitro modeling of cell behaviour,

including differentiation, apoptosis, pathophysiology and cancer growth and invasion (273). Interestingly, Matrigel has been used to stimulate and maintain differentiation of several cell types (274-279) including primary salivary cells (272). Recently, Szlavik et al 2008 could culture primary human salivary acinar cells that expressed acinar as well as TJ markers on Matrigel. Their culture model required the use of animal serum, which is known to promote fibroblasts overgrowth in epithelial cultures (272). The risk of infection increases when animal-derived components are used in culture. In this study we aimed to modify our previous huSG culture method to obtain differentiated acinar cells using serum-free Hepato-STIM medium and BME. We analyzed the primary huSG from both parotid and submandibular salivary glands. This cell culture system closely resembled the in vivo situation, in both morphological and functional differentiation into acinar-like units.

This study reports the ability of huSG cells cultured on Matrigel to differentiate into 3-D polarized acinar units and to express TJs; claudin-1, -2, -3, -4, Occludin, JAM-A, ZO-1, acinar proteins; AQP5, α-amylase, mucin-1, and adhesion-related mesenchymal stem cell markers; CD44, CD166. Moreover, the secretion of α-amylase protein increased 2-5 times into media. TEM confirmed the formation of differentiated acinar polarized cells showing active protein synthesis and numerous secretory granules. Additionally, quantitative RT-PCR analysis revealed down-regulation of certain genes along with regulation of the translation of their corresponding proteins (confirmed by immunofluorescence analysis), thus, confirming acinar differentiation on Matrigel. The mitotic activity was significantly reduced on Matrigel at day 7 while apoptosis increased significantly comparable to huSG grown on plastic ($P<0.01$).

Portion of these data have been presented in abstract form at the AADR meeting 2010 (239).

MATERIALS & METHODS

Source of huSG Tissue: Portions of human submandibular (n=9) and parotid (n=3) salivary glands, from both men (n=8) and women (n=4), were obtained from the Department of Pathology, University of Virginia, through the Cooperative Human Tissue Network (CHTN) and the Department of Pathology, Royal Victoria Hospital (RVH), McGill University. For tissue use, we received an exemption from the Institutional Review Boards at McGill University. This report describes results obtained with glands that were excised as part of the resection of head and neck cancers. Patients, from whom samples were obtained, had received no prior irradiation/chemotherapy treatment. Pathologists at the University of Virginia (Charlottesville, VA) and RVH (Montreal, Quebec) performed surgical pathologic examination of glands after excision. All glandular tissues reported here were judged as histopathologically normal. The tissue was shipped on wet ice in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1% antibiotic-antimycotic solution.

Tissue Culture: The received tissue pieces (0.5-1.5g) of huSG were washed twice in F-12 medium (GIBCO Invitrogen, Grand Island, NY) supplemented with antibiotic–antimycotic solution (200U/mL penicillin, 200µg/mL streptomycin, and 5µg/mL Fungizone; BioFluids, Rockville, MD). The pieces were mechanically minced with a pair of curved scissors until the tissue gave an appearance of a slurry thick paste. The minced tissue was transferred into 35mL of dissociation buffer and incubated for 4–5 h at 37°C incubator on a shaker, additionally, vigorous vortexing was applied every 30 min. The dissociation buffer contained Liberase Blendzyme-3 (0.2U/mL, Roche Diagnostic, Indianapolis, IN) in Dulbecco's modified Eagle's medium (DMEM; BioFluids, Rockville, MD). The cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and 20mL of cold DMEM was used to re-suspend the cell pellet. The cell suspension was again centrifuged and the supernatant was discarded. The resulting cell pellet was suspended in 5mL of culture medium: serum-free Hepato-STIM medium (BD

Biosciences Discovery Labware, Bedford, MA), supplemented with antibiotic–antimycotic solution (500U/mL penicillin, 500µg/mL streptomycin, and 12.5µg/mL Fungizone) and 1% glutamine. This cell suspension was then filtered through a cell strainer (70µm pore size; BD Biosciences Discovery Labware), plated onto a 100-mm tissue culture dish, and incubated at 37°C in 5% CO2. The culture medium was changed twice a week and cells were passaged with a mixture of 0.05% trypsin and 0.02% Versene (BioFluids) when they reached 80% confluence. For the experiments reported herein, huSG cells from passages 2 and 3 were employed. The passaged cells were cultured on either non-coated or Matrigel-coated (19.6 mg/ml, BD Biosciences, Bedford, MA) 12-well or 6-well tissue culture dishes or 8-well slide chambers. Matrigel was thawed on ice and diluted in DMEM (1:6, final concentration =2 mg/ml. Coated plates/slide chambers/polyester filters were incubated at 37^{o}C for 30-60 min before cell seeding. The concentration of Matrigel and seeding density were optimized to ensure reproducible 3-D formation, which occurred after 24h, the morphology of the cells was observed using phase contrast microscopy (Fig. 4.1). MDCK-II cells obtained from BD Biosciences Clontech (Palo Alto, CA) were maintained in DMEM/F-12 supplemented with 10% FBS (HyClone), 100U/mL penicillin, 100µg/mL streptomycin, and 2.5µg/mL Fungizone and incubated at 37°C in 5% CO2.

Measurement of TER: huSG cells seeded (1x10^{5} cells/ml) on 24-mm Transwell-Clear polyester filters (Corning Life Sciences, Acton, MA) that were either un-coated or Matrigel-coated and grown as mentioned above. The upper chamber containing the cells received 1.5mL of medium, and the lower chamber (no cells) received 2.6mL of medium. TER was measured at day 3, 5, 7 in 6 separate determinations, using a Millicell ERS epithelial volt-ohmmeter (Millipore Corp., Allen, TX) as described by the manufacturer. MDCK-II cells cultured on filters were used as positive control (MDCK-II cells have high TER and express TJs) (280). TER readings from filters without cells (blank) were subtracted from readings obtained from filters seeded with cells.

Evaluation of Apoptosis: An ApopTag® peroxidase in situ apoptosis detection kit (Chemicon International, MA) was used to evaluate huSG cells apoptosis activity. ApopTag® is a mixed molecular biological-histo-chemical system that allows for sensitive and specific staining of apoptotic bodies. Apoptotic reaction in three 8-well slide chambers of huSG grown on plastic and Matrigel at day 7 was scored at magnification of X 400. The slides were assessed by two observers in a blinded manner in ten randomly chosen fields per slide. The mean of apoptotic cells per field was calculated.

Evaluation of Proliferation: Proliferating Cell Nuclear Antigen (PCNA) stain was performed to monitor huSG cells proliferation using Zymed kit (Invitrogen, Carlsbad, CA, USA). After endogenous peroxidase activity was blocked for 10 min, three 8-well slide chambers of huSG cells grown on plastic and Matrigel (at day 7) were processed with routine indirect immunoperoxidase techniques. Two examiners independently counted the absolute number of PCNA positive cells in a blinded manner in ten randomly chosen fields per slide (X 400). The mean of PCNA positive cells per field was calculated.

Immunofluorescence Imaging: huSG cells cultured for 5 days were fixed with 10% paraformaldehyde in PBS (pH 7.4) for 30 min, rinsed with PBS, and permeabilized with methanol at 20°C for 5 min followed by incubation with 0.2% Triton X-100 for 10 min at room temperature, followed by three washing steps in PBS for 5 min each. The following primary antibodies were used to characterize huSG grown on plastic and Matrigel; rabbit anti-claudin-1, claudin-2, claudin-3, occludin, JAM-A and mouse anti-ZO-1, claudin-4 from Zymed; Mouse anti-cytokeratin (panel), vimentin, and α-smooth muscle actin, from Invitrogen; Goat anti-aquaporin-5, mouse anti-mucin-1from Santa Cruz; rabbit anti-α-amylase from Sigma Aldrich; goat anti-Musashi-1 and mouse multipotent mesenchymal/stromal cell marker (MSCs) antibody panel kit from R&D Systems. The panel contains a group of antibodies for the positive

identification of human MSCs; anti-Stro-1, anti-CD90, anti-CD106, anti-CD105, anti-CD146, anti-CD166, anti-CD44, plus leukocytes markers; anti-CD19 and anti-CD45. In addition, we used goat (R&D Systems), rabbit and mouse (Zymed Labs) isotype control antibodies. The antibodies used are reactive against the respective proteins from human and other species. The cells were incubated with blocking solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and 0.5% bovine serum albumin in PBS for 1h at room temperature. Cells were incubated with the primary antibodies (diluted 1:100 in blocking solution) overnight at 4^{o}C while incubated with either donkey anti-mouse/rabbit/goat-fluorescein isothiocyanate-conjugated (FITC) or -Rhodamine Red-X-conjugated (RRX), (Jackson ImmunoResearch Laboratories) secondary antibodies for 1h at room temperature (diluted 1:100 in blocking solution) in dark. Finally, 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) was added for 3-5 minutes. Fluorescence images were taken using a Leica DM6000 fluorescent microscope equipped with Volocity software (PerkinElmer Inc.). Images shown are representative of at least 3 separate experiments with multiple images taken per slide.

Western Blot Analysis: Conditioned media from different wells were collected, then, huSG cells grown on plastic and Matrigel (at 1,3,5,7 days) were washed twice with cold PBS and lysed in 200 µl/well of cold RIPA buffer and kept on ice for 5 minutes. Lysates were collected into microcentrifuge tubes and centrifuged at 10,000 x g for 20 minutes to pellet the cell debris and keep the supernatants for further analysis. Protein samples, 60µg each, from supernatants and collected media were subjected to 10% SDS-PAGE on mini-gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% fat-free dry milk in Tris-buffered saline [0.137 M NaCl, 0.025 M Tris (hydroxymethyl)- aminomethane, pH 7.4] containing 0.1% Tween-20 (TBST) and immune-blotted overnight with rabbit anti-α-amylase antibody (1:2000 dilution; Sigma Aldrich) at 4°C in TBST containing 5% BSA and 0.02% sodium azide. After incubation

with primary antibody, membranes were washed 3 times for 15 min each with TBST and incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The membranes were washed 3 times for 15 min each with TBST then treated with chemiluminescence detection reagent containing 20 mM Tris buffer (pH 8.5), 250 mM Luminol and 90 mM coumaric acid (Sigma Aldrich). Protein bands were visualized on X-ray film. All experiments were performed in duplicate and repeated at least 3 separate times.

TEM: huSG cells cultured on uncoated and Matrigel-coated 12-well plates were fixed (at 1, 2, 3, 5, 7) in 2% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in 0.1 M cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1 h. After rinsing in cacodylate buffer, samples were dehydrated through an ethanol series and infiltrated and embedded in Epon812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections (70 nm) were cut with a diamond knife and mounted onto copper grids. Grids were stained with 3% uranyl acetate for 30 min and 2% lead citrate for 5 min, then examined at various magnifications using a 100-CXII TEM (JEOL, Tokyo, Japan) at an accelerating voltage of 120 kV.

Quantitative Real-Time PCR Analysis: At day 3,5,7,9 huSG cells grown on plastic and Matrigel were washed in PBS, then, total RNA was isolated using the RNeasy micro kit (Qiagen Ltd, Crawly, UK) with in-column DNase digestion. The concentration of RNA was determined using Qubit (Molecular Probes). Total RNA (2 μg per sample) was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in 20 μL volume. For polymerase chain reaction (PCR) amplification, 5% of the cDNA was used with real-time PCR primers and 6-carboxy-fluorescein (FAM)-labeled minor groove binder probes (MGB). The probes and primers for claudin-1(CLDN-1), cytokeratin-18 (CK-18), epidermal growth factor (EGF), kallikrein (KLK-1), α-amylase (AMY), aquaporin-5(AQP-5), mucin-7 (MUC-7) and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an endogenous reference) were selected from the Applied Biosystems. PCR reactions (20 μL) were performed in duplicates using TaqMan Universal Master Mix (Applied Biosystems) on a Prism Sequence Detection System 7500 (Applied Biosystems) with the default settings (50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles [95^{o}C for 15 s, 60^{o}C for 1min]). Every culture experiment was repeated at least 3 times. Gene expression levels were calculated by normalizing the target RNA value to the value of GAPDH in the same sample. Results are expressed as fold-changes in gene expression relative to control (huSG sample cultured on plastic for 9 days expressing the lowest gene levels) sample.

Statistical Analysis: Data are presented as means ± SEM of results from 3 or more separate experiments. Our data were analyzed by Student's *t*-test and one-way *ANOVA* where *P* value < 0.05 represents significant differences between both groups at specified times.

RESULTS

Morphological Changes: The mechanically and enzymatically digested huSG tissue initially consisted of floating single and clumps of cells. After 24h of initial plating on plastic; 10-30% of cells attached to the dish. Small cobblestone islands (20 to 50 cells) of epithelial-like cells were observed at day 2-7. As cells proliferated, these islands enlarged until fused to form epithelial-like sheets of round, oval, and polygonal cells. The huSG cells took approximately 2-4 weeks to become 80% confluent, all huSG cells were confirmed to have striated ductal phenotype (11) and were then passaged with trypsin-versene for other experiments. The huSG cells used in this article were from passages 2 and 3 were plated as single cells. Afterward, huSG cells were seeded on 24-mm Transwell-Clear filters for TER, and on 8-well slide chambers for confocal microscopy, apoptosis and mitosis analysis, whereas other huSG cells were grown on 12-well/6-well and for TEM, western blot and quantitative RT-PCR analysis. In the current study, on plastic huSG cells formed monolayers of epithelial-like isolated clusters which increased relative to

fibroblast-like cells, owing to the serum-free medium we used (Fig. 4.1A). On Matrigel-coated surfaces (2mg/ml), huSG cells formed both 3-D acinar-like units (15% of cells plated) within 24 h and monolayers (Fig. 4.1B). Cells in close proximity to each other were able to self-assemble into acinar-like units while cells located in distant spots differentiated as single cells in situ. Most 3-D units initially formed, consisted of 6–8 cells, then, the number of cells/3-D unit increased up to 10-12 cells at day 3 where total 3-D units formed 40% of total cells attached. At day 5, some 3-D units started to disintegrate, detached and disunited. At day 7 and 9, 30-50% of 3-D units were disintegrated and were seen floating in media. We were able to culture huSG cells until passage 10 without any changes noticed in their behavior. Patients' age ranged between 20 and 73 years. No differences in results were seen between the sexes or gland type. However, in regard to age; the acinar units formed in most cultures derived from old patients (60-70 years old) appeared less organized relative to those from younger patients (20-52). In addition, younger patients' cells reached 80% confluence faster than those from older patients.

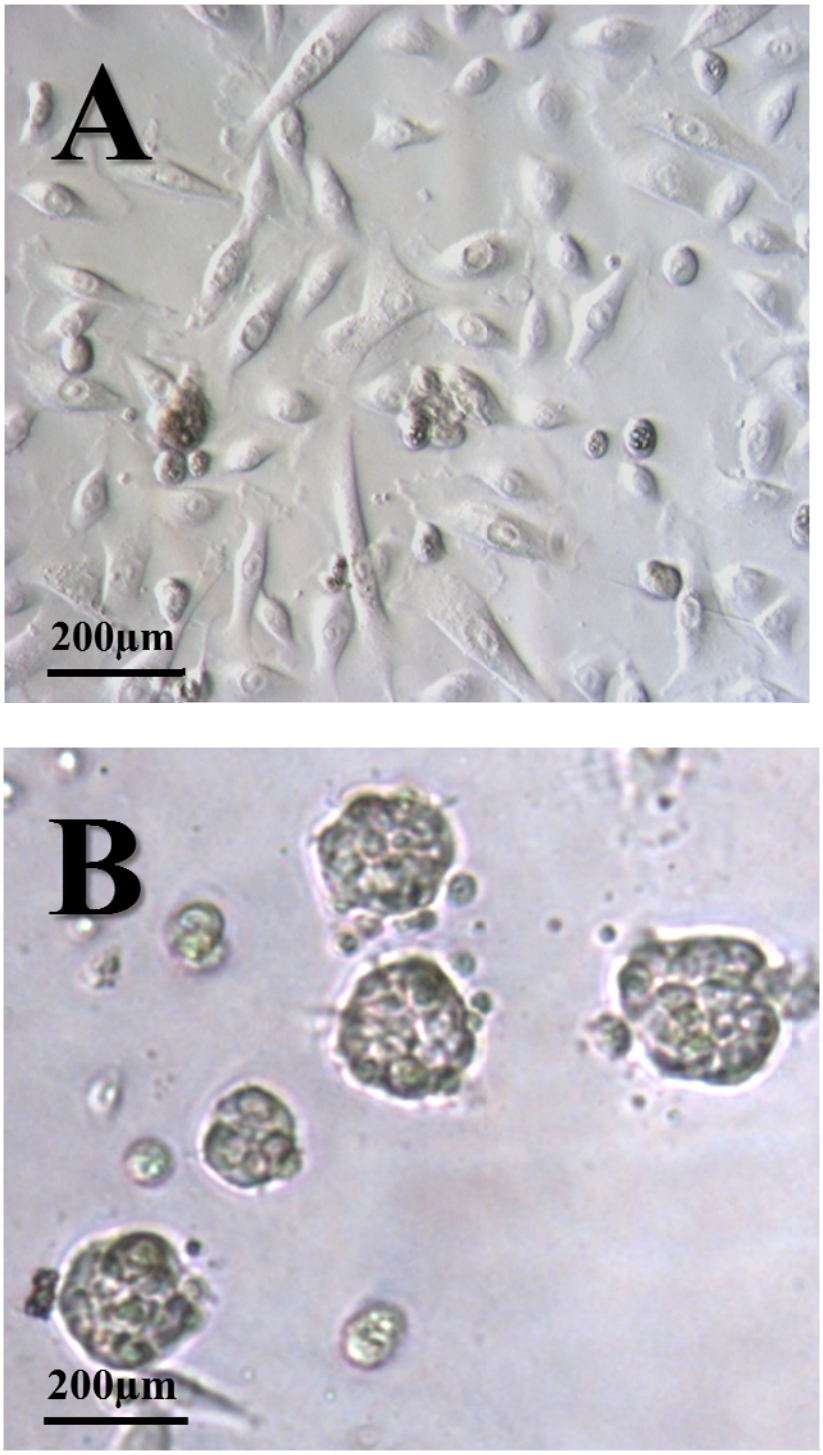


Figure 4.1: (A) huSG cells cultured on plastic formed monolayer of cobblestone-like isolated clusters. (B): huSG cells cultured on Matrigel (2 mg/ml) formed 3-D acinar-like structures within 24 h. [Scale bar= 200µm, X 200].

Measurement of TER: as a standard measure of TJs formation and functional development of acini, TER was assessed across huSG cells seeded on 24-mm Transwell-Clear polyester filters that were either un-coated or Matrigel-coated and grown as mentioned above. We compared TER of huSG with those obtained by MDCK-II (positive control) monolayers at day 3, 5, and 7 from 6 separate determinations. Both huSG culture types exhibited TERs comparable to MDCK-II (ranging from 381-415 Ω.cm^2 on plastic and 410-416 Ω.cm^2 on Matrigel). TER values of MDCK-II were significantly different from huSG cultured on plastic ($P<0.05$).

Apoptosis versus Mitosis: Both apoptotic and mitotic activities were evaluated at day 7. The apoptotic activity increased significantly ($P<0.01$) on Matrigel (26.6%) compared to plastic (10.5%), (Fig.4.2). Apoptotic cells were seen mainly at the centre of intact 3-D units where the future lumen is being formed and in the cell clumps of disintegrating 3-D (not shown). On the other hand, PCNA stain revealed a significant ($p<0.01$) decrease of the mitotic activity on Matrigel (7%) compared to plastic (89.4%), (Fig.4.2).The mitotic activity was seen mainly among monolayers while fewer mitosis was evident among cells forming 3-D units (not shown). A significant correlation (0.78) exists between apoptosis and mitosis on Matrigel.

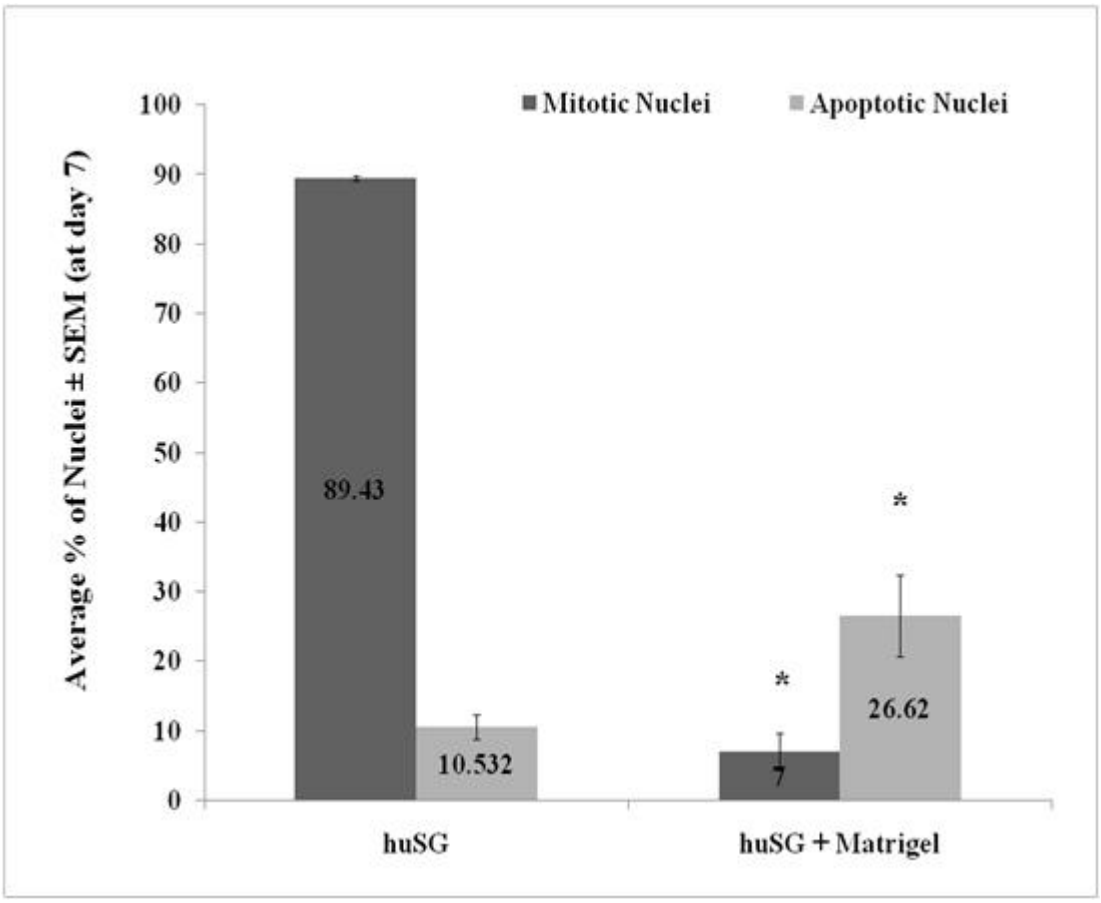


Figure 4.2: Apoptotic and Mitotic activities were evaluated at day 7 for huSG cultured on both plastic and Matrigel. The apoptotic activity (evaluated by ApopTag kit) increased significantly on Matrigel (26.6%) as compared to huSG on plastic (10.5%). The Mitotic activity (evaluated by PCNA stain) revealed a significant decrease on Matrigel (7%) compared to huSG on plastic (89.4%), (*P< 0.01). A significant correlation (0.78) exists between apoptosis and mitosis on Matrigel.

Immunofluorescence Imaging: Both huSG cultures expressed all TJ proteins tested; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1 in comparable percentage (Table 4.1A, Fig. 4.3). On Matrigel, huSG cells expressed acinar-specific; α–amylase (99%), AQP5 (%99), mucin-1 (50%), mesenchymal stem/progenitor cell-specific CD44 (99%) and CD166 (99%) shown in Fig. 4.4, and epithelial-specific cytokeratin (panel, 99%); mesenchymal-specific vimentin (99%) proteins (Table 4.1B). However were completely negative to α–smooth muscle actin, Musashi-1, CD105, CD106, CD90, CD146, Stro-1, CD45, CD19 (Table. 4.1B, 4.1C). On plastic huSG cells expressed the same proteins in fewer percentages except for being AQP5 and mucin-1 negative (Table.4.1B, 4.1C), indicating that more functional acinar cells exist on Matrigel. In unpublished study on adult human salivary gland tissue, we have observed that CD44 and CD166 are expressed by serous and mucous salivary cells, respectively (281).

Table 4.1: Expression of different markers/proteins in huSG cells grown on plastic and on Matrigel. Results are based on the examination of 5 slides per culture type. At least 800 cells were examined per slide. * The expression level of each marker/protein is indicated as percentage. (A): Tight junction proteins; claudin-1, -2, -3, -4, Occludin, JAM-A, and ZO-1. (B): Cytokeratin panel (epithelial), Vimentin (mesenchymal, salivary ductal, immature acinar and their progenitors), α–SMA (myoepithelial), α–amylase (salivary serous), AQP5 (acinar), Mucin-1 (salivary mucous). (C): Stem cell markers; Musashi-1 (Neural), Stro-1, CD44, CD166, CD105, CD106, CD90, CD146, (Mesenchymal) CD45, CD19 (Leukocyte).

(A)

Marker	Claudin-1	Claudin-2	Claudin-3	Claudin-4	Occludin	JAM-A	ZO-1
huSG + Matrigel	99%	99%	99%	98%	95%	95%	95%
huSG	92%	95%	92%	97%	92%	90%	95%

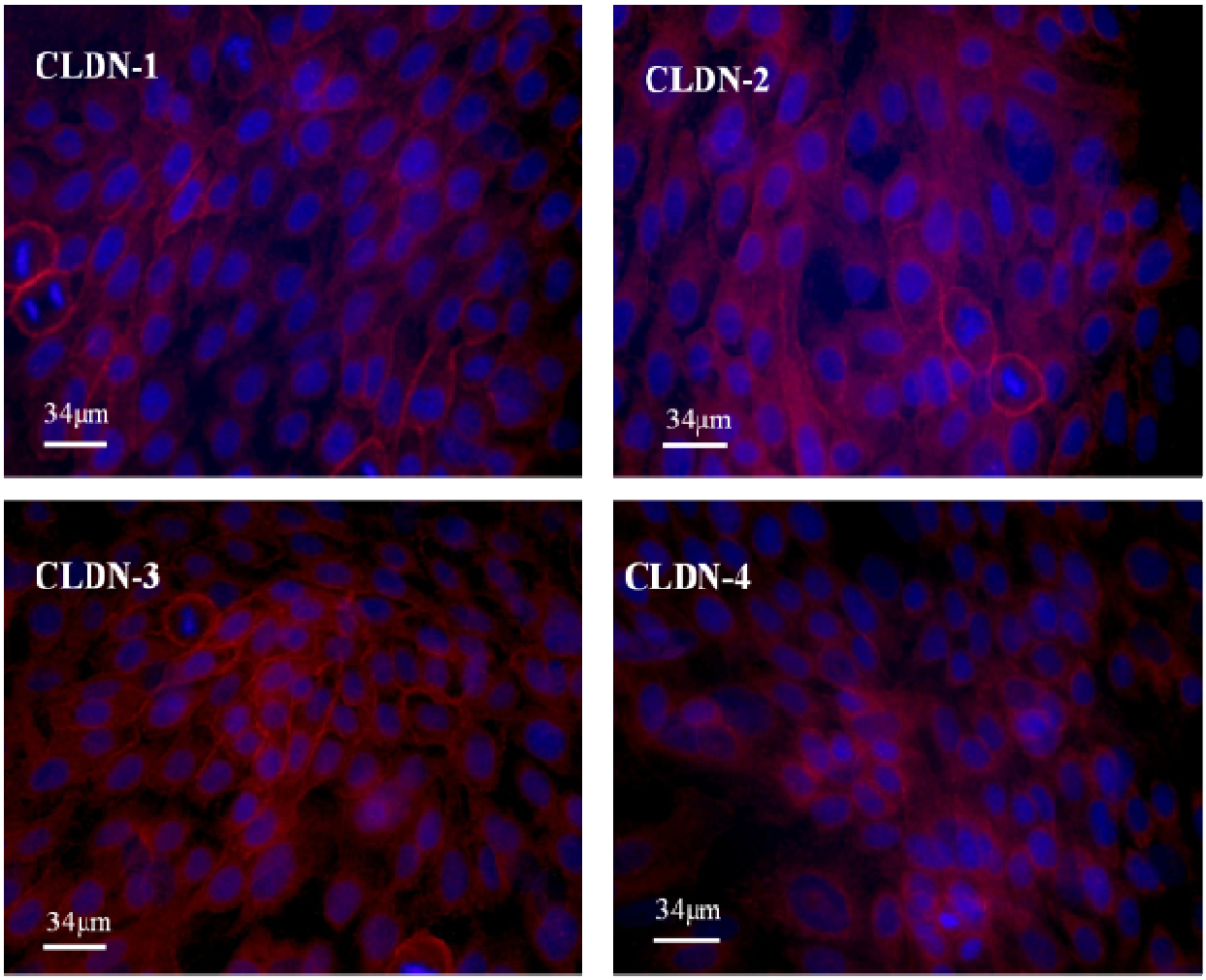
(B)

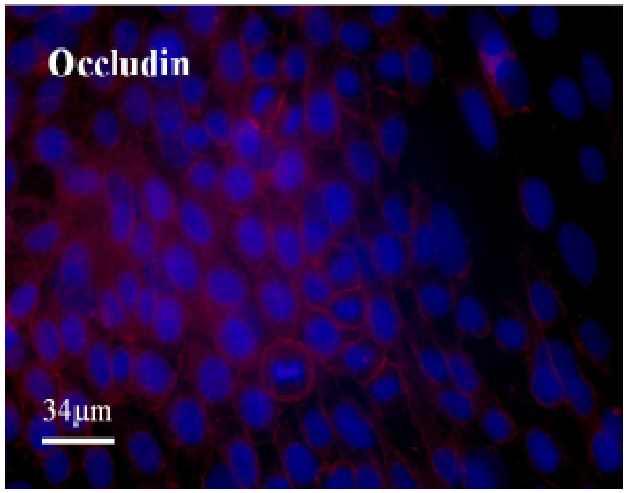
Marker	Cytokeratin	Vimentin	α–SMA	α–amylase	AQP5	Mucin-1
huSG + Matrigel	99%	99%	0%	99%	99%	50%
huSG	85%	40%	0%	5%	0%	0%

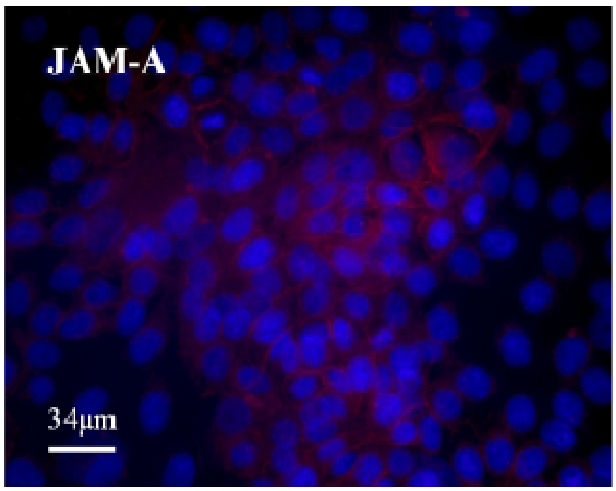
(C)

Marker	Msi-1	Stro-1	CD44	CD166	CD105	CD106	CD90	CD146	CD45	CD19
huSG + Matrigel	0%	0%	99%	99%	0%	0%	0%	0%	0%	0%
huSG	0%	0%	95%	95%	0%	0%	0%	0%	0%	0%

Figure 4.3: Immunofluorescence micrographs (Volocity software) of huSG cultured on Matrigel expressed all TJ proteins tested; claudin (CLDN)-1,-2,-3,-4, occludin, JAM-A, ZO-1, respectively (shown in red). The nuclei are stained with DAPI (shown in blue). [Scale bar= 34μm, X 400].


CLDN-1
34µm
CLDN-2
34µm
CLDN-3
34µm
CLDN-4
34µm


Occludin
34µm


JAM-A
34µm

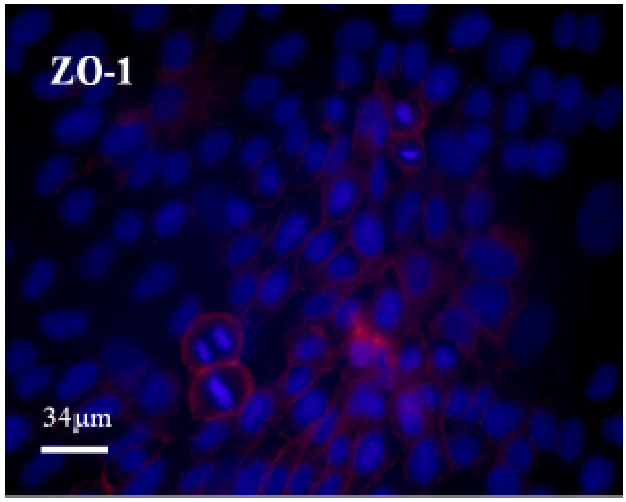
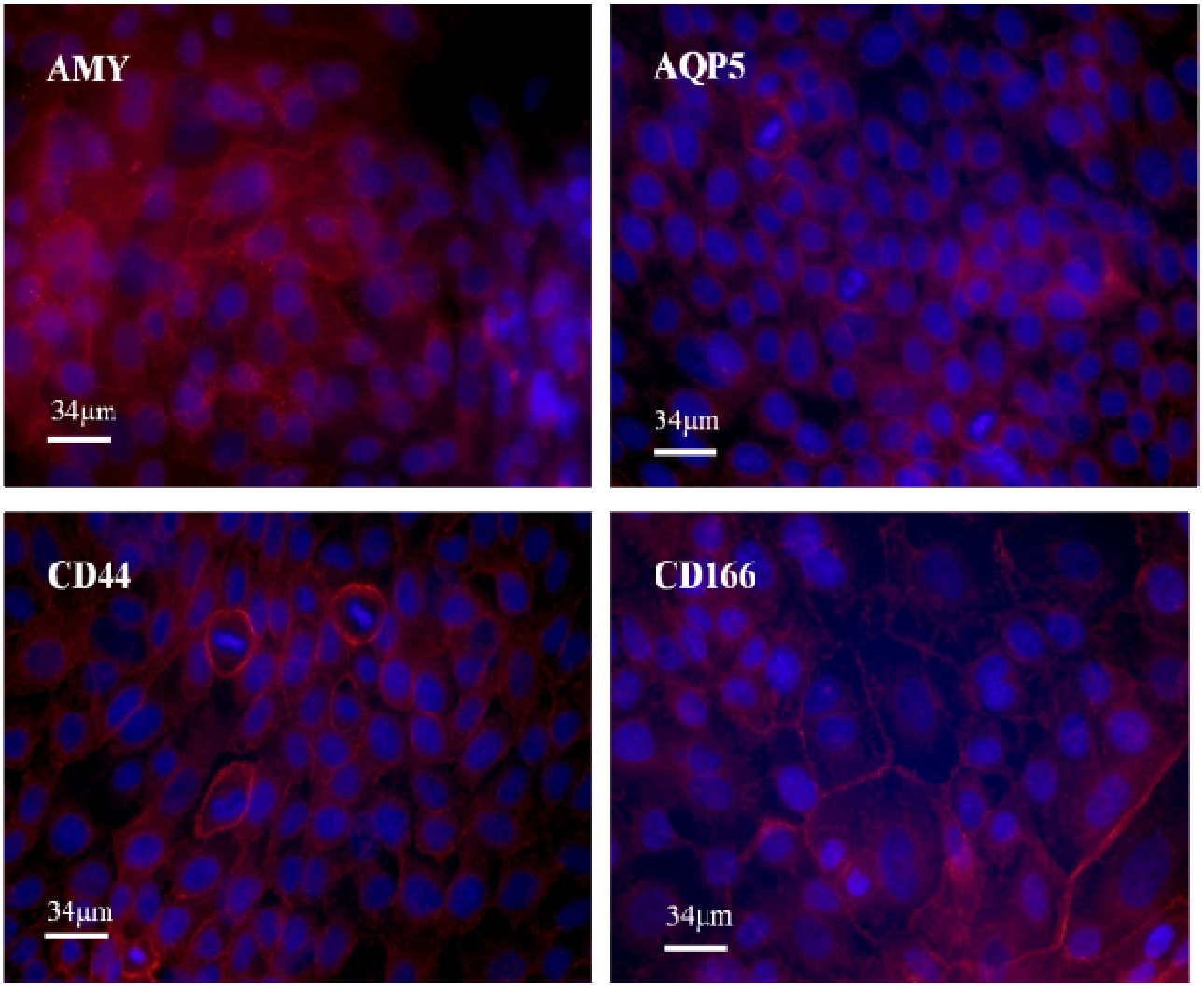

ZO-1
34µm

Figure 4.4: Immunofluorescence micrographs of huSG cultured on Matrigel. Expression of acinar secretory proteins: α–amylase (AMY) and AQP5, both expressed in 99% of huSG cells (shown in red). Adhesion-related proteins: CD44 and CD166 are expressed in 99% of huSG cells, (shown in red). [Scale bar= 34μm, X 400].


AMY
34μm
AQP5
34μm
CD44
34μm
CD166
34μm

Western Blot Analysis: We used 60µg total protein from each sample to evaluate the synthesis (in supernatants from cell lysates) and secretion (in media) of α–amylase. At day 1, 3, 5 and 7, both huSG culture types could synthesize and secrete α–amylase protein into media (supernatant samples showed similar results to those from media). However, Matrigel increased the synthesis and secretion of α–amylase protein to 2-5 folds relative to huSG cultured on plastic (Fig. 4.5).

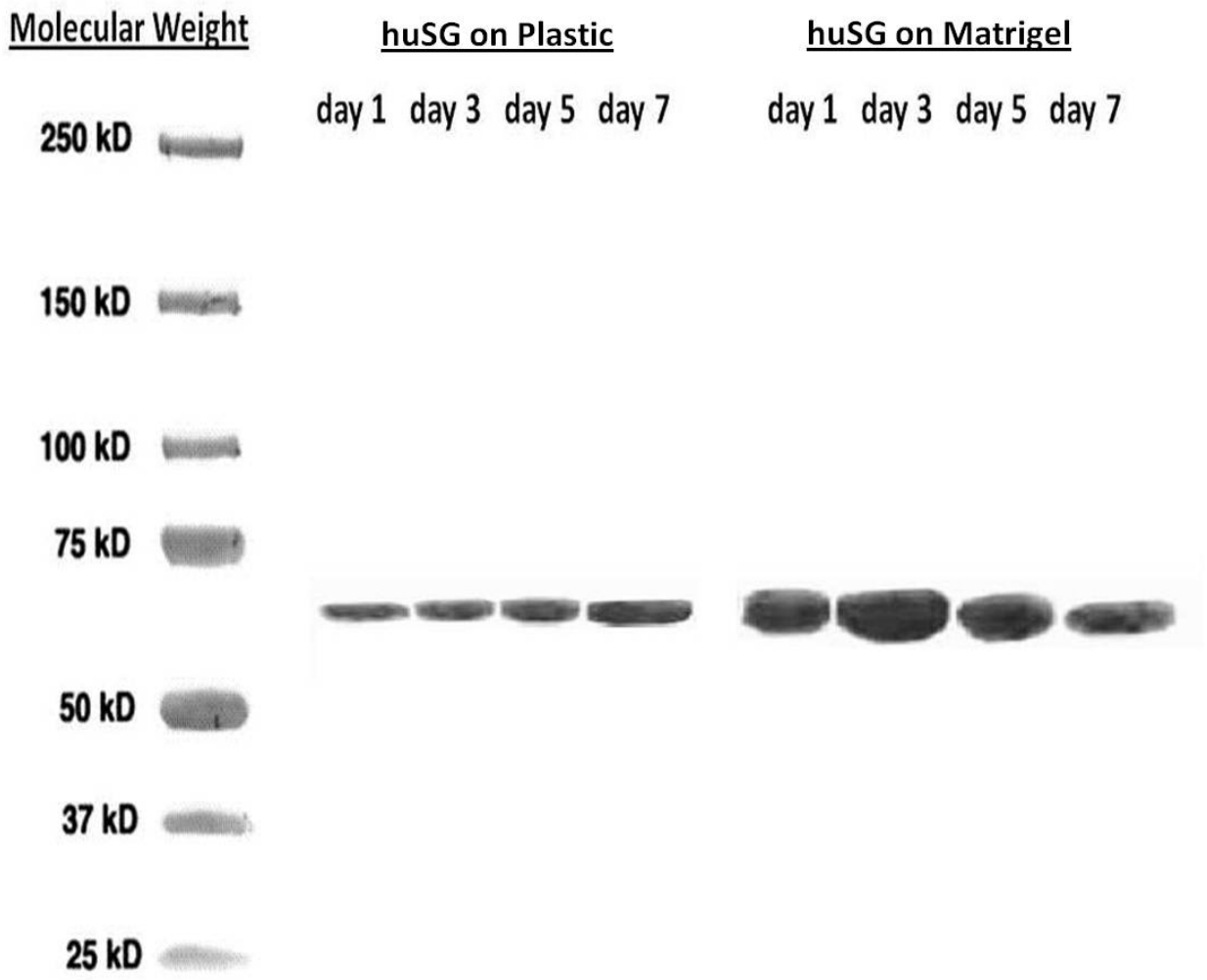
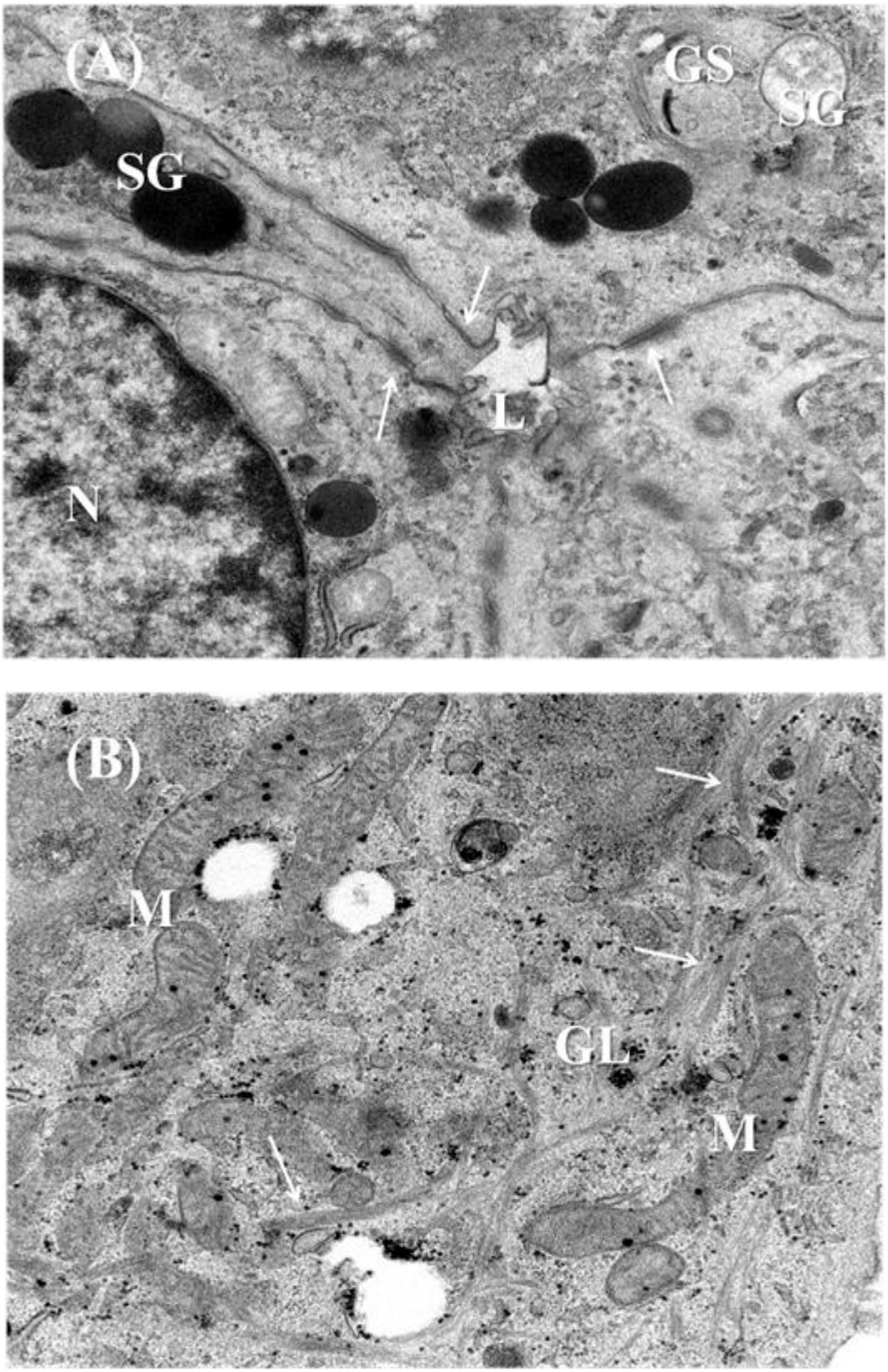
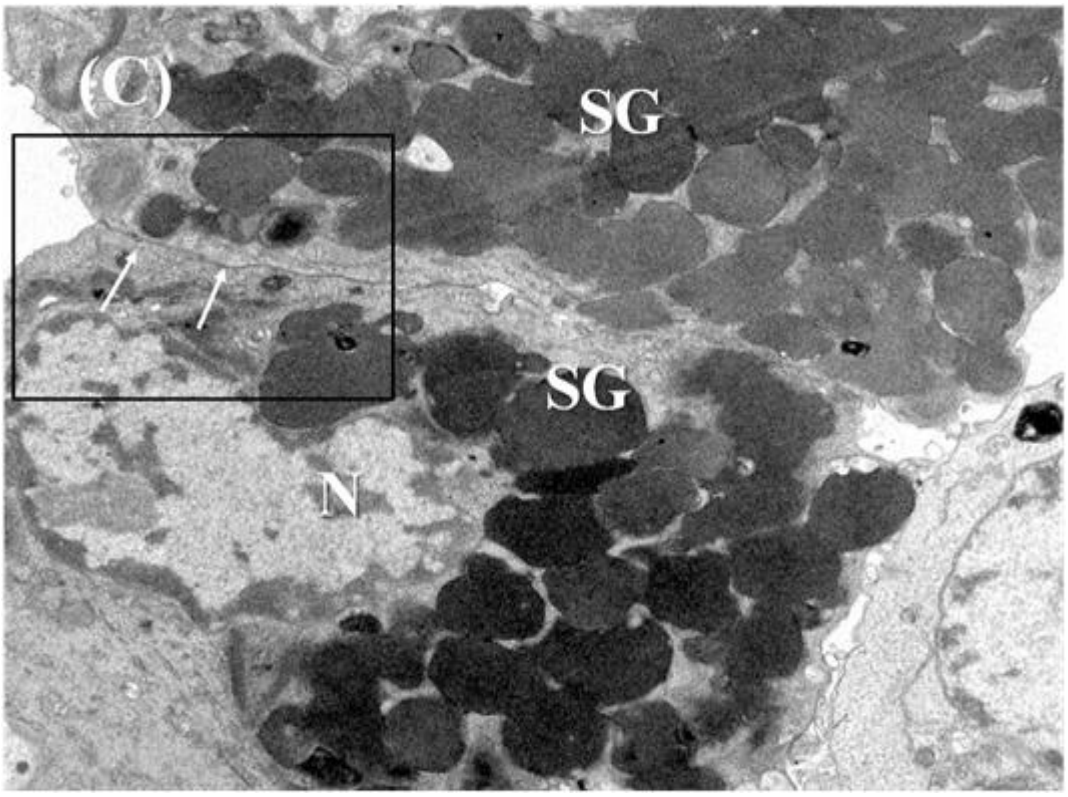


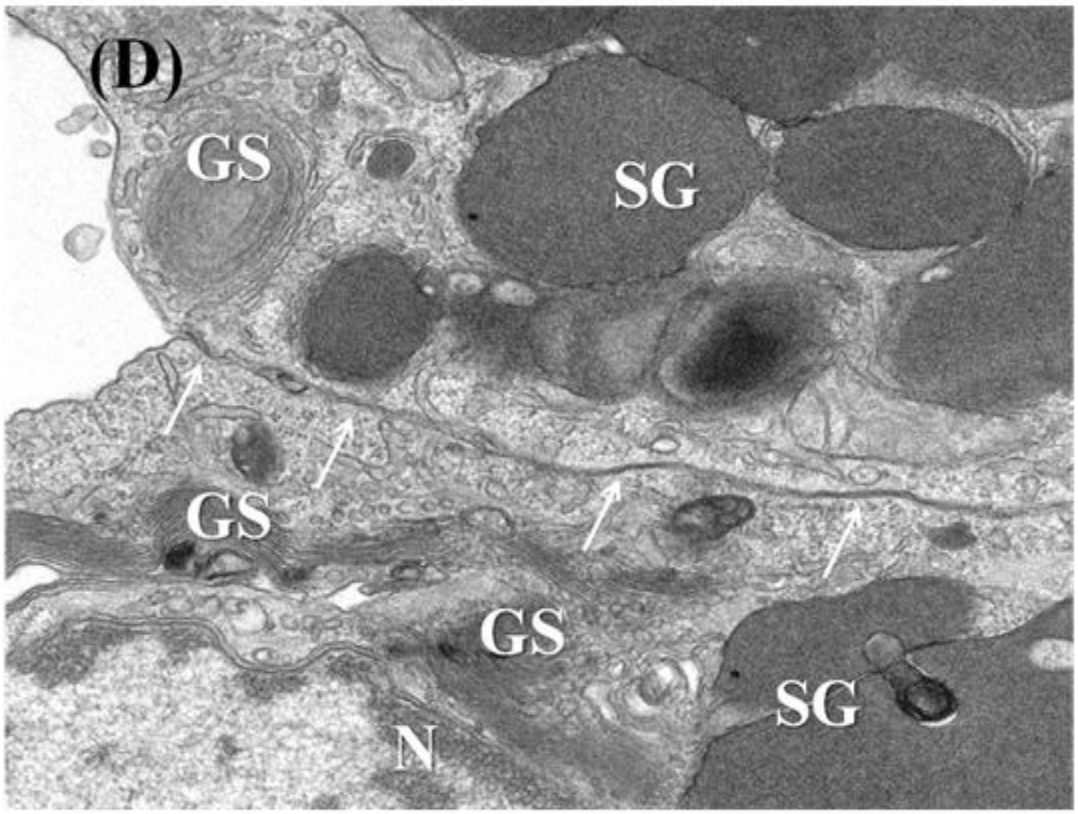
Figure 4.5: Western blot analysis of α–amylase protein (60 kDa) as evaluated in conditioned media from huSG cells (at day 1,3,5,7). When cultured on Matrigel, huSG cells could synthesize and secrete α–amylase protein in media with a concentration of 2-5 times that secreted when huSG cells were cultured on plastic.

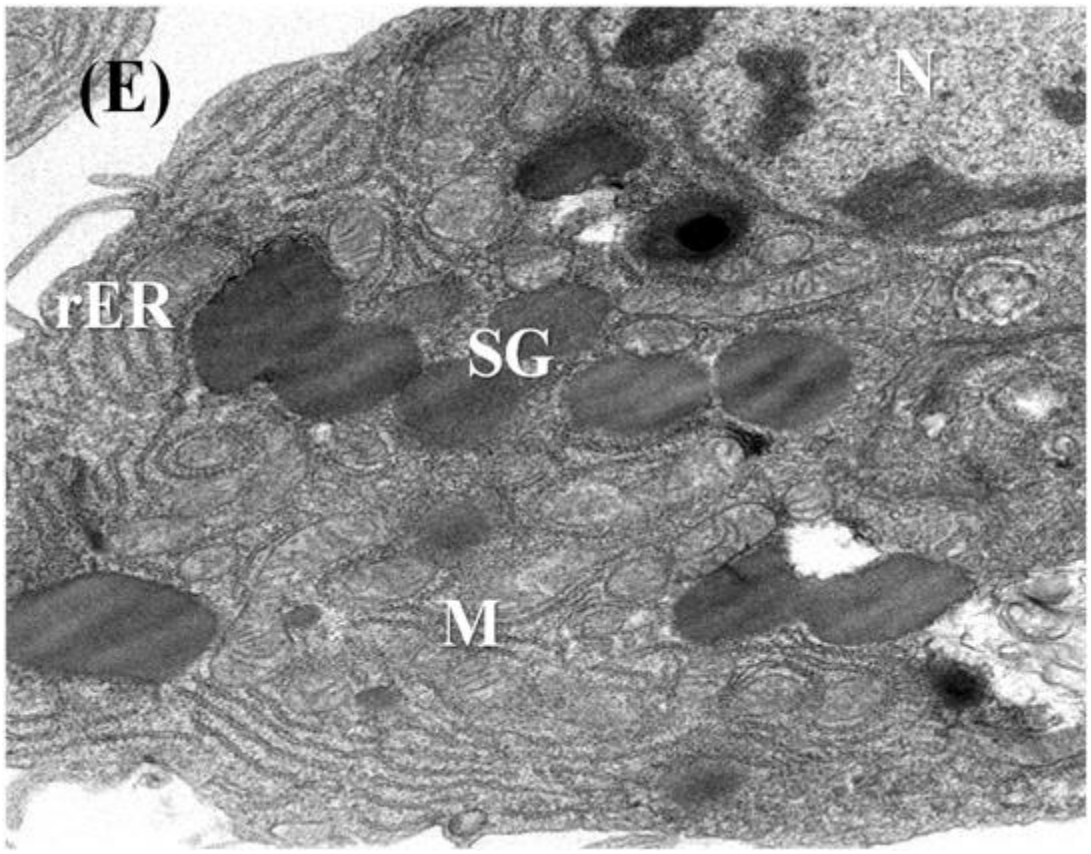
TEM: Ultrastructural analysis by TEM confirmed that huSG cells cultured on Matrigel were similar to normal human acinar cells shown in (Fig.4.6A). However, huSG cultured on plastic were similar to stiated duct cells (11): the cytoplasm contains multiple large mitochondria, tonofilaments, and small isolated island of glycogen, while no Golgi apparatus, rough endoplasmic reticulum, secretory granules or microvilli could be seen (Fig.4.6B). Both huSG culture types exhibited TJ structures at the apicolateral cell membranes confirming that huSG cells were polarized; however, no basal lamina could be observed (Fig.4.6C shows huSG cells on Matrigel). Only huSG cells cultured on Matrigel showed features of active protein synthesis (at day1-7); where many Golgi saccules (Fig.4.6D) and rough endoplasmic reticulum (Fig.4.6E) were obvious. Moreover, the apical cytoplasm was packed with electron-dense secretory granules resembling those of normal human salivary secretory granules[46] (Fig.4.6C, 4.6F). On Matrigel, approximately 90% of huSG cells (at day 5 and 7) produced secretory granules; both monolayers and 3-D units while earlier at day 3 only 60% of cells showed secretory granules. The 3-D units were well-organized around central lumens where cells at the interior had more secretory granules than those at the surface or in monolayer, suggesting that they adopted a typical acinar phenotype (Fig.4.6F). Cells in the middle of the 3-D unit showed signs of apoptosis including detachment of chromatin from the nuclear envelope and chromatin condensation at one side of the nucleus along with degradation of cytoplasmic organelles enclosed into multiple lysosomes (Fig. 4.6F).

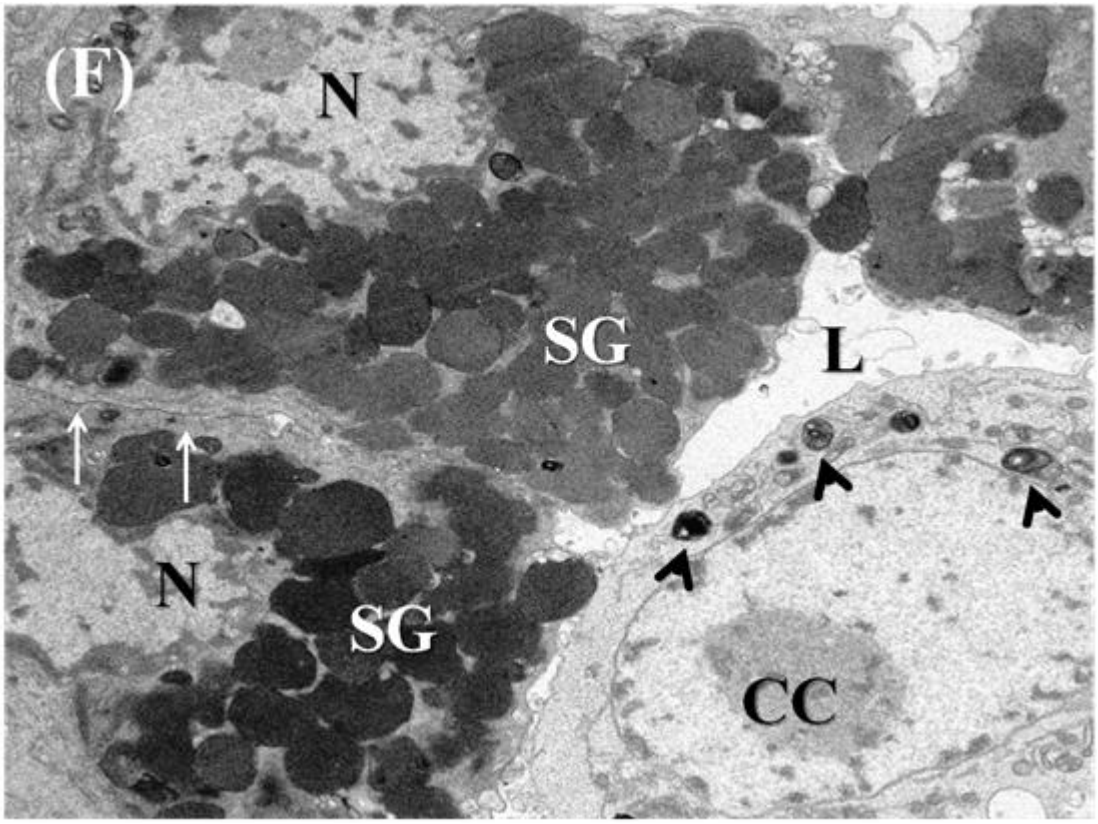
Figure 4.6: Transmission Electron Microscope (TEM) micrograph of huSG cells stained with lead citrate and uranyl acetate staining. Panel A: Normal human acinus structure (presented here as a positive control) showing features of secretory cells: active protein synthesis; Golgi saccules (GS), electron-dense and lucent secretory granules (SG), tight junction structures (arrows) surrounding a central lumen (L), and an euchromatic nucleus (N) [X 16,500]. Panels B shows huSG cultured on plastic (at day 5): exhibiting features of ductal cells; its cytoplasm contained multiple large elongated mitochondria (M), tonofilaments (arrows), and small isolated island of glycogen (GL) [X 16,500]. Panels C, D, E, and F show huSG cells grown on Matrigel. (C) Two huSG cells (at day 3) attached by tight junction structures (arrows) and packed with multiple electron-dense secretory granules (SG) around the nucleus (N) [X 8,200]. (D) Inset of the lateral area of the two huSG cells at a higher magnification showing features of active protein synthesis; Golgi saccules (GS) and electron-dense secretory granules (SG) all swimming close to the nucleus (N), tight junction structures (arrows) holding both cells together [X 26,500]. Panel E shows features of active protein synthesis in acinar-like huSG cell at day 1; electron-dense secretory granules (SG) swimming among mitochondria (M) and multiple rough endoplasmic reticulum (rER) cisternae, all close to the nucleus (N) [X 16,500]. Panel F shows 3-D acinar-like structure of huSG cells cultured on Matrigel (at day 3) exhibiting features of apoptosis in the middle of the 3-D acinar structure for the formation of a central lumen (L); membrane-bound apoptotic bodies (arrow heads), apoptotic nucleus showing chromatin detachment from the nuclear membrane and its condensation (CC) at one side of the nucleus. The surrounding differentiated active acinar cells show multiple secretory granules (SG), tight junction structures (arrows) and euchromatic nuclei (N). [X 6,000].


(A)
GS
SG
SG
L
N
(B)
M
GL
M


(C)
SG
SG
N


(D)
GS
SG
GS
GS
SG
N


(E)
N
rER
SG
M


(F)
N
SG
L
N
SG
CC

Quantitative Real-Time PCR Analysis: Gene expression levels were measured (in five patients) using quantitative RT-PCR. Results are expressed as fold-changes in gene expression relative to control sample (huSG cultured on plastic for 9 days) of at least 3 separate experiments. On Matrigel, the expression of tight junctions gene; CLDN-1, acinar-specific; AMY, AQP5, and MUC-7, and ductal-specific; CK-18, EGF and KLK-1 genes were decreased, (for CLDN-1, AQP5 and EGF genes down-regulation were statistically significant, $P < 0.05$ (Fig. 4.7). Similar trend was observed at day 3, 5 and 7 post-plating. These data suggest that the protein expression pattern associated with huSG cultured on Matrigel is achieved through regulation of translation, rather than transcription of relevant genes. Gene expression levels were different between all patients which might be related to patients' age or general health.

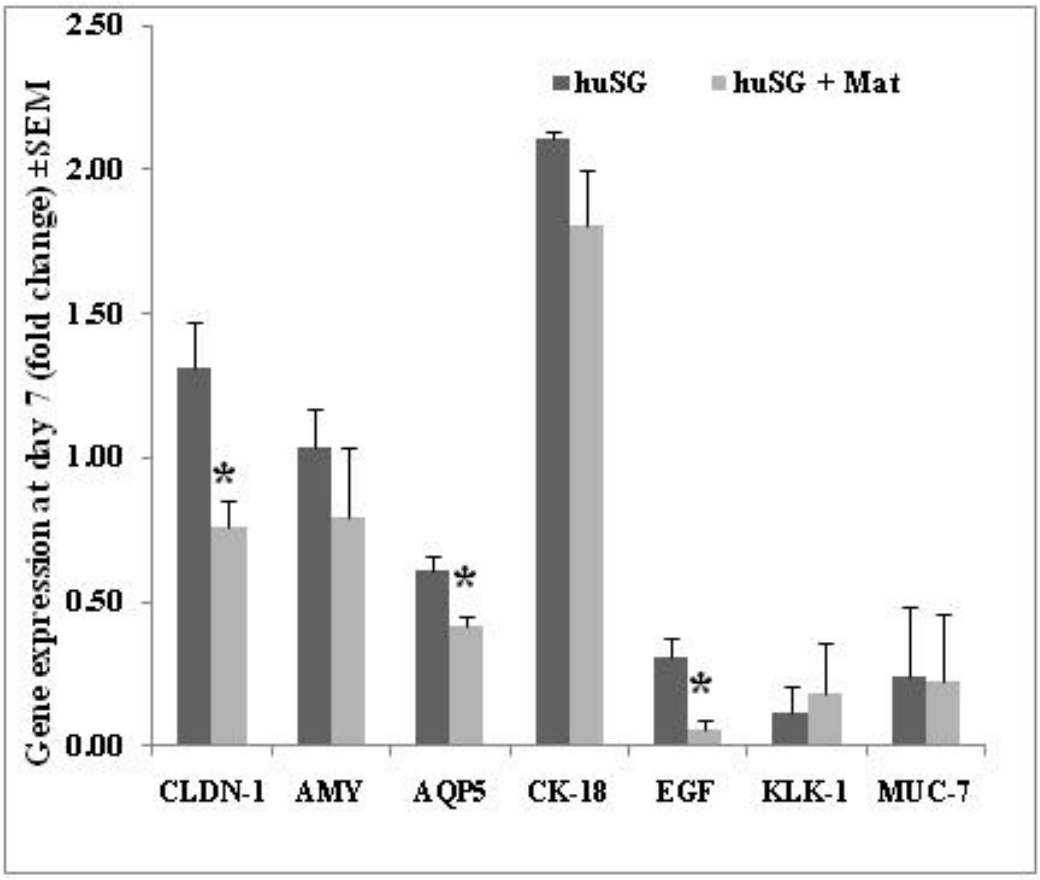


Figure 4.7: Gene expression levels (at day 7) indicated in fold change for huSG cultured on Matrigel and on plastic for; claudin-1 (CLDN-1), α-amylase (AMY), Aquaporin-5 (AQP-5), cytokeratin-18 (CK18), epidermal growth factor (EGF), kallikrein (KLK-1) and mucin-7 (MUC-7) genes, relative to samples from cultures grown on plastic for 9 days and normalized to GAPDH levels using quantitative real-time polymerase chain reaction (at least 3 separate experiments were performed/gene). Results are reported as means ± standard error of the mean of parallel cultures, (*$P<0.05$).

DISCUSSION

This study describes a convenient and reproducible method on Matrigel to grow and maintain polarized acinar units from primary human salivary cells that would help in regenerating salivary glands. Cells from passage 2 and 3 formed polarized acinar units when grown on Matrigel however on plastic they were polarized ductal cells. We tested several criteria: morphological (formation of 3-D acinar units revealed by phase contrast microscopy and TEM), immunological (positive for TJs; claudin-1, -2, -3, -4, occludin, JAM-A, ZO-1, acinar secretory proteins; α-amylase, AQP5, mucin-1, and adhesion-related mesenchymal stem cells; CD44, CD166), functional (exhibiting adequate TER and α-amylase secreted into media using western blot), and gene expression (quantitative RT-PCR).

Serous acini are the most sensitive salivary cell type to irradiation whereas ucous and ductal cells are more radio-resistant. Radio-sensitivity is caused mainly by lethal DNA damage upon exposure to radiation (282). The graft cell component in our envisioned artificial salivary gland device should have acinar phenotype (being polarized and secretory) to be properly functional for the treatment of xerostomic patients. Primary huSG cells were isolated and cultured according to our previous protocol (11) with little modification. Here, we wanted to test more heterogeneous population as our original protocol gave us cultures enriched for ductal phenotype when grown on plastic. Development of salivary glands is based mainly on epithelial-mesenchymal interactions, leading to morphogenesis and cytodifferentiation (283, 284) that are regulated by growth factors supplied by surrounding extracellular matrix, (285) accordingly we wanted to simulate the in vivo model. In this study, huSG cells were initially cultured using little medium volume to allow most cells from all types to attach on plastic. We used Hepato-STIM serum-free medium to prevent overgrowth of fibroblast. The use of serum-rich media obscures/restricts the study of the effects of growth factors, cytokines, and hormones on morphogenesis, growth, and

differentiation (286). Thereafter, huSG cells were passaged 2 times to be plated either on plastic or on Matrigel-coated plates. This method resulted in a mixed cell culture (mainly epithelial) capable of forming monolayer with tight junctions on filters.

Other investigators have reported successful cultures of human salivary epithelial cells (268, 287-289) However, cultures from these reports were not well-characterized and none of these reports has used functional assays of polarization (TER) to examine their cells. We began to culture huSG cells for the purpose of seeding a tissue-engineered artificial salivary device. Accordingly, it was critical for us to establish that huSG cells could form tight junctions and were appropriately polarized. Here we confirm successful cultures of well-characterised polarized acinar units of huSG cells grown on Matrigel.

Reduction in the mitotic rate of huSG cultures grown on Matrigel is consistent with the findings of studies on the HSG cell line (165, 166, 243) and on primary human salivary gland (272). During embryonic development, the acini are formed when the outer layer of cells becomes polarized on its basement membrane then cells growth arrests where cells at the interior undergo apoptosis to form the lumen (290,291). We found that huSG cells on plastic divide significantly ($p<0.01$) faster than cells grown on Matrigel as recorded at day 7. Moreover, the apoptotic activity was significantly ($p<0.01$) higher among 3-D cells cultured on Matrigel comparable to those grown on plastic.

Our immunofluorescence analysis of huSG cells cultured on Matrigel revealed the presence of mesenchymal, ductal (99% vimentin positive), epithelial (99% cytokeratin) and acinar cells (99% α-amylase/AQP5 positive), as well as adhesion-related mesenchymal stem cell-like (99% $CD44^{+}/CD166^{+}$) however, myoepithelial cells were absent. Vimentin protein, a component of intermediate filaments in mesenchymal cells (292) is known to be expressed in immature salivary acinar cells, their immediate precursors, all myoepithelial cells, and some ductal cells (293). The

3D acinar units were vimentin-negative, suggesting that they consisted mainly of mature acinar cells. TEM confirmed the establishment of TJ structures among huSG cells forming both monolayers and 3-D acinar units therefore both types of cells were polarized. TJs act as selective barriers for the passage of fluid, salts, and proteins through the paracellular milieu. Moreover, TJs are involved in the regulation of proliferation, (294) differentiation, (295) and cell signalling (296). Western blot analysis confirmed the synthesis and secretion of α-amylase by functional acinar cells with 2-5 fold increase on Matrigel. Although lumens of 3-D acinar units were formed on Matrigel, and higher α-amylase concentrations were secreted into the media, we do not know whether α-amylase secretion occurred through these lumens; especially that we could observe single cells bearing secretory granules.

Epithelial cells must be polarized to secrete fluid unidirectionally. The basement membrane guides epithelial cells to polarize and differentiate (297). Consequently, it maintains the establishment of a unidirectional secretion. On Matrigel, the expression of AQP5 at sites of cell-junction confirmed cell polarization along with secretory potential however on plastic all cells were AQP5 negative. For cells to differentiate and self-assemble, they must receive appropriate signals from the extracellular matrix. On the other hand, cultures on plastic appear to cause de-differentiation of acinar cells into ductal cells (11, 272, 298). The intercalated duct cells are believed to be the stem cells of ductal, acinar and myoepithelial cells (168). On Matrigel, the reservoir of stem/progenitor cells might decrease while more cells differentiate into acini thus the mitotic activity is being reduced. Here we report that adhesion-related mesenchymal stem cell markers CD44 and CD166 were expressed on plastic and on Matrigel in comparable percentages. In unpublished study on adult human salivary gland tissue, we have observed that CD44 and CD166 are expressed by serous and mucous acinar cells, respectively (240) Expansion and differentiation of multipotent salivary stem cells would improve the treatment of xerostomia.

Herein, the huSG culture appears to contain a mixed population of cells: salivary progenitors, differentiated epithelial cells (acinar, ductal), and mesenchymal cells.

The level of down-regulation in gene expression (quantitative RT-PCR results for AMY, AQP5, MUC-7, CLDN-1, KLK-1, EFG, CK-18) on Matrigel varied between cultures derived from different patients, similar results were reported by Szlavik et al 2008 (272). However, TJ proteins immunoreactivity was evident in comparable percentages in both culture types from the five patients we had. We think that down-regulation of such genes suggests that Matrigel plays a role in the regulation of relevant proteins translation and consequently the associated-expression pattern. It was reported that α-amylase gene induction is regulated by growth factors provided in Matrigel (161) and that α-amylase gene expression is independent on the acinar morphology (243, 299). The overall data presented here showed that Matrigel tends to direct the cells towards an acinar phenotype. However, longer-term (> 9 days) experiments would be useful to determine the effect of other factors such as patient's age, and associated-diseases on salivary cell regeneration capacity.

We conclude that these findings would have a strong impact on the development of a prototype artificial salivary device to be tested in vivo (animal models) afterward. As shown herein, primary huSG cultures provide an excellent model for such studies when cultured on Matrigel. Thereafter, a scaffold carrying primary huSG cells (as a graft cell type) seeded on Matrigel (as an extracellular matrix) would be implanted via a small surgically created pouch into the buccal submucosa of an animal model, and tested for secretion. Although promising, this model cannot be used currently for clinical application (human tissue-engineering) because Matrigel is derived from mouse sarcoma. In analysing safer substitutes of Matrigel, we are currently working on different gel types that would be acceptable for clinical applications. We believe that using 3-D cell-based models will help in a wide variety of applications in cancer biology, tissue-

engineering, as well as gene and drug therapy, all together would help in the treatment of xerostomic patients.

In summary, we report herein, the development of 3-D functional acinar units from huSG cells cultured on Matrigel using a serum-free medium. The huSG cells were capable of attaining a polarized orientation, expressing TJ structures and exhibiting adequate TER, all essential requirements for graft cells in our artificial salivary device. These 3-D units closely mimic the in vivo polarized functional salivary acinar cells. Moreover, these units expressed acinar-specific (α-amylase, AQP5, mucin-1), and adhesion-related (CD44, and CD166) proteins and were able to secrete α-amylase. Additionally, certain genes were down-regulated in association with translation of their corresponding proteins. This stable and differentiated multicellular huSG culture provides a unique approach for studying the expression of specific salivary proteins as well as the epithelial–mesenchymal interactions. Innovative cell culture techniques are important to generate sufficient amounts of cells with the capacity to differentiate for replacing damaged salivary tissues.

AKNOWLEDGEMENTS

The authors are grateful to Dr. Christopher Moskaluk for the histopathological analysis of salivary glands and CHTN for providing the salivary tissue samples. We are most appreciative of the technical help provided by Mrs. Jeannie Mui and FEMR at McGill University for TEM samples preparation and Mr. David Hori the pathologist assistant at RVH. The authors would like to thank the funding institutions; Canada Research Chair, CIHR, NSERC, RSBO and Egyptian Ministry of Higher Education and Research.

Author Disclosure Statement: No competing financial interests exist.

CHAPTER 5

CELLS FROM BONE MARROW THAT EVOLVE INTO ORAL TISSUES AND THEIR CLINICAL APPLICATIONS.

Published as: Maria OM, Khosravi R, Mezey E, Tran SD. Cells from bone marrow that evolve into oral tissues and their clinical applications. Oral Diseases, 2007;13(1):11-16.

The Ph.D. Candidate performed the following contributions in this manuscript

- Wrote the manuscript based on the available literature.

Cells from Bone Marrow that Evolve into Oral Tissues and their Clinical Applications

Ola M. Maria[1], Roozbeh Khosravi[1], Eva Mezey[2], Simon D. Tran[1*]

[1] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada.

[2] National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

*_Correspondence Author_:

Simon D Tran

Faculty of Dentistry, McGill University

3640 University Street, Room M-43

Montreal, Quebec, Canada, H3A 2B2

E-mail: simon.tran@mcgill.ca

ABSTRACT

There are two major well-characterized populations of post-natal (adult) stem cells in bone marrow: hematopoietic stem cells which give rise to blood cells of all lineages, and mesenchymal stem cells which give rise to osteoblasts, adipocytes, and fibroblasts. For the past 50 years, strict rules were taught governing developmental biology. However, recently, numerous studies have emerged from researchers in different fields suggesting the unthinkable - that stem cells isolated from a variety of organs are capable to ignore their cell lineage boundaries and exhibit more plasticity in their fates. Plasticity is defined as the ability of post-natal (tissue-specific adult) stem cells to differentiate into mature and functional cells of the same or of a different germ layer of origin. There are reports that bone marrow stem cells can evolve into cells of all dermal lineages, such as hepatocytes, skeletal myocytes, cardiomyocytes, neural, endothelial, epithelial, and even endocrine cells. These findings promise significant therapeutic implications for regenerative medicine. This paper will review recent reports of bone marrow cells that have the ability to evolve or differentiate into oral and craniofacial tissues, such as the periodontal ligament, alveolar bone, condyle, tooth, bone around dental and facial implants, and oral mucosa.

STEM CELLS IN BONE MARROW

Stem cells are defined as clonogenic, self-renewing, and capable of generating one or more specialized cell types (78). Developmentally, stem cells are categorized either as embryonic stem cells or as post-natal stem cells (they are also called organ-specific, tissue-specific, or adult stem cells) (79). Embryonic stem cells are derived from the inner cell mass of a developing blastocyst and are considered as pluripotent cells since they are able to form all the body's cell lineages (endoderm, mesoderm and ectoderm) (82). Post-natal stem cells (derived from specific tissues or organs) are considered multipotent as they can form multiple lineages that constitute an entire tissue or tissues (82).

According to our present knowledge there are two distinct populations of post-natal stem cells in the bone marrow: the hematopoietic stem cells (HSC) and the mesenchymal stem cells. HSC were recognized more than 40 years ago as they have the ability to reconstitute the hematopoietic system of a lethally irradiated host (79) since it gives rise to all blood cell lineages. Their unique ability to continuously self-renew permits HSC to sustain blood cell production throughout life. The frequency of HSC is one in 10,000-15,000 bone marrow cells (105). Under physiological conditions, quiescent HSC are interspersed with other cells within the bone marrow. However, under stressful conditions such as massive bleeding or acute bacterial infections, HSC rapidly proliferate, differentiate, and migrate from the bone marrow to circulate throughout the body (106,107). Mesenchymal stem cells originate from the mesodermal layer of the fetus and in the adult they reside in the bone marrow as well as in a variety of tissues. Mesenchymal stem cells constitute only a small portion (one in 10^4-10^6) of bone marrow (109, 110). The pivotal characteristic of mesenchymal stem cells is their ability to differentiate in vitro into several cell types based on culture conditions (110). It has been demonstrated that these cells possess a multilineage differentiation capability (bone, cartilage, adipose, tendon, and muscle tissues)

(89,111). Several studies have reported that mesenchymal stem cell clones comprise a heterogeneous cell population with respect to their self-renewal characteristic (304). However, this self-renewal potential is unclear mainly due to the different approaches used to derive populations of mesenchymal stem cells.

PLASTICITY OF POST-NATAL STEM CELLS

For the past 50 years, we were taught that post-natal stem cells have a limited developmental repertoire. Once a cell made a commitment to a dermal lineage during development, this was irrevocable (79, 320). A stem cell residing in a particular tissue (i.e. tissue-specific stem cell) could only differentiate into cells of that tissue. For example, a hematopoietic stem cell would give rise to new blood cells; a liver stem cell would make new liver cells, etc. However, for the past 7 years, a large number of studies emerged from researchers in different fields suggesting the unthinkable - that post-natal stem cells isolated from a variety of organs may be able to ignore its (dermal lineage) origin and exhibit more *plasticity* in their fate choices. Plasticity is defined as the ability of post-natal (tissue-specific adult) stem cells to differentiate into mature and functional cells of the same or of a different germ layer of origin (79). There are reports that bone marrow stem cells can differentiate into hepatocytes (112), skeletal myocytes (89), cardiomyocytes (318, 114), neural cells (87, 88), endothelial cells (114), epithelial cells (115), and pancreatic endocrine cells (116). These findings on the plasticity of post-natal stem cells carry great hope for regenerative medicine (117-119). As an example, because HSC can reconstitute the entire blood system, bone marrow transplantations have long been used in the clinic to treat hematopoietic diseases (108). Several companies are competing to market a variety of cell-based therapies based on post-natal bone marrow-derived stem cells for treating cancers, autoimmune, neurological, stroke and heart diseases (329).

Four explanations for the phenomenon of plasticity in post-natal stem cells have been proposed (135-139). First, there might be persistent stem cells from embryonic development with broad developmental potentials which are maintained within the adult bone marrow (140). When transplanted into other organs, these cells are instructed to differentiate into tissue-specific cells under inductive signals from that specific tissue. A second possibility is that true precursors of post-natal stem cells with embryonic stem cell-like properties persist in adult bone marrow, such as the multipotent adult progenitor cells (141). A third explanation may be that the nuclei of the transplanted stem cells undergo reprogramming of the existing genetic information, expressing new genes and proteins that are consistent with the novel lineage, and this might be a result of de-differentiation, and re-differentiation (142,143,139). A final explanation is when cell fusion occurs, which is a rare phenomenon reported *in vitro* and *in vivo* in tissues where polyploidy is common, such as hepatocytes, skeletal muscle, cardiac muscle and Purkinje cells of the cerebellum (144). As a result the genetic information of both fused donor and host cells is partially changed (139,145,326.

The objective of this review is to evaluate recent reports of cells from the bone marrow (hematopoietic stem cells and mesenchymal stem cells) that have the ability to evolve or differentiate into orofacial structures and their clinical applications for oral tissue regeneration (Table 5.1 and Figure 5.1). The readers are cautioned with the widely used term *mesenchymal stem cells* as the International Society for Cellular Therapy (ISCT) has stated that the current data are insufficient to characterize unfractionated plastic adherent marrow cells as stem cells (313). Therefore ISCT suggests the use of the term *multipotent mesenchymal stromal cell* to indicate these unique properties without ascribing homogeneity or stem cell activity; while the term *mesenchymal stem cells* is reserved for long-term self-renewing cells that are capable of differentiation into specific, multiple cell types in vivo (313). For both of these cell populations,

the acronym MSC may be used, as is the current practice. Therefore it is crucial that future publications clearly define the acronym that they are describing. The studies reported in this review are derived from experiments using multipotent mesenchymal stromal cell (MSC). It is not the goal of this review to report on the use of MSC from other oral tissues in tissue regeneration. Such MSC populations are from the human exfoliated deciduous teeth (321), dental pulp (311) and periodontal ligament (180,314). These post-natal stem cells have common characteristics with bone marrow MSC in addition to be readily accessible in the oral cavity.

Table 5.1: Reports describing bone marrow stem cells evolving into craniofacial tissues.

Origin	*Differentiated tissues*	*Reference*
MSC	Periodontium	Kawaguchi *et al* (2004, 2005)
MSC	Condyle	Abukawa *et al* (2003)
MSC	Dental implant	Yamada *et al*, 2004a,b
BM or MSC	Bone	Abukawa *et al* (2004), Warnke *et al* (2004), De Kok *et al* (2005)
BM	Tooth	Ohazama *et al* (2004)
BM or HSC	Buccal mucosa	Tran *et al* (2003), Metaxas *et al* (2005)

BM: bone marrow stem cells; HSC: hrmatopoeitic stem cells; MSC: multipotent mesenchymal stromal cells

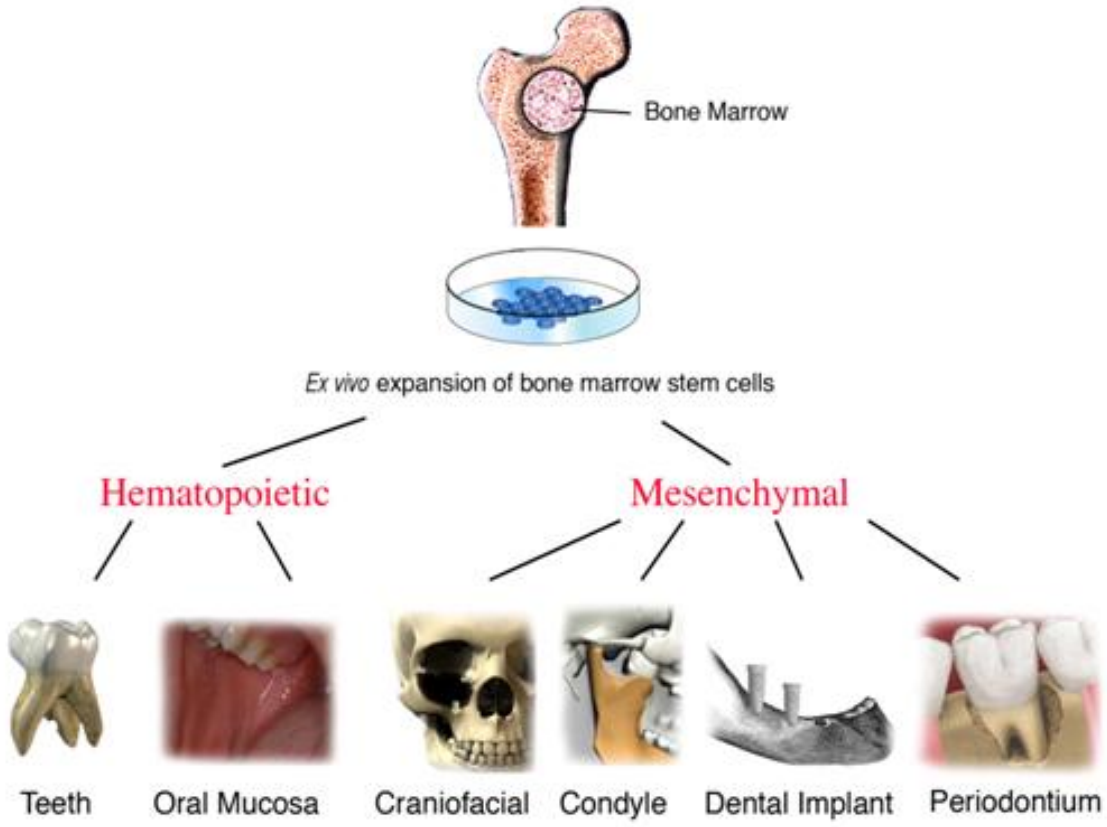


Figure 5.1. Oral tissues that evolved from bone marrow cells.

CELL-BASED THERAPIES FOR TISSUE REGENERATION

Cell encapsulation is an intervention in cell-based regenerative medicine. In brief, cells are delivered to a donor with the goal of improving the regeneration process. Initial reports in the 1970's by WT Green, a pediatric orthopedic surgeon, demonstrated that implanted spicules of bone and cartilage seeded with chondrocytes into animals could generate new cartilage (309). Today, the two common methods of cell delivery are intravenous injections (direct delivery of cells) and cell encapsulation systems (indirect delivery of cells using a carrier). The cell encapsulation approach uses a biodegradable material, which is a biocompatible product that is gradually resorbed once implanted in the body, due to enzymatic or hydrolytic degradation. This biodegradable construct is seeded with cells (ideally progenitor cells) and is implanted into defects in order to regenerate lost tissues (208). Bone marrow-derived MSC have a significant but highly variable self-renewal potential during in vitro experiments and this property has made them attractive as a source for cell-based therapies aiming at the regeneration of orofacial tissues, especially when the size of the lost tissue is large and that the body can no longer repair this defect (305, 306). Future advancements in stem cell research (either embryonic or post-natal) and in biomaterial science will allow cell encapsulation methods to be utilized in the clinic to regenerate both hard and soft tissues of the craniofacial complex.

PERIODONTIUM

Periodontal diseases are highly prevalent worldwide and the main signs are bone tissue destruction and subsequent tooth loss. Regenerating the periodontium has always been a high priority in craniofacial regenerative biology. Due to the complex structure of the periodontium (consisting of hard and soft tissues: cementum, bone, periodontal ligament, and gingiva), its complete regeneration would require a multipotent cell population (303, 310), Kawaguchi and

colleagues 2004 (316) demonstrated that transplantations of *ex vivo* expanded autologous MSC can regenerate new cementum, alveolar bone, and periodontal ligament in class III periodontal defects in dogs. Morphometric analysis revealed a 20% increase in new cementum length and bone area in animals treated with MSC. In a subsequent study the same group reported a similar approach in humans (317) when they transplanted $2x10^7$ cells/ml autologous expanded bone marrow–derived MSC mixed with Atelocollagen into periodontal osseous defects. All patients showed a significant improvement.

DENTAL IMPLANT

A sound and mature bone is an essential factor to achieve successful osseointegration of dental and facial implants. Very frequently, the quality and quantity of the remaining bone (that was destroyed due to trauma or diseases such as an enucleated tumor) is not suitable to allow a complete osseointegration of these implants. In a canine model, Yamada and colleagues (2004a, 330) extracted premolars and first molars. After one month of healing, they created four 10 mm-diameter defects on each side of the mandible. These surgically created defects were filled with 1) platelet rich plasma (PRP), 2) autologous MSC and PRP (MSC/PRP), 3) autologous particulate cancellous bone and marrow (PCBM), or 4) empty (control defect). After 8 weeks, dental implants were placed in the healed defects. The authors hypothesized that the presence of MSC in the surgical site would enhance wound healing and osseointegration. Higher marginal bone levels were recorded on dental implants placed in MSC/PRP- or PCBM-filled defects as compared to control defects. Bone-implant contact was significantly increased in MSC/PRP and PCBM groups. Histological results showed a well-formed lamellar and woven bone and new vascularization around dental implants of the MSC/PRP group. However, PCBM-filled defects exhibited bone resorption. In a similar study, Yamada et al., (2004b), (331) tested the application

of an autologous "scaffold" for delivering MSC to the surgical site. Using the same study design, they monitored the quality of regenerated bone in each defect. The MSC/PRP and PCBM groups showed a substantial increase in mature regenerated bone tissue. Their findings suggest that the insoluble gel generated from mixing platelet rich plasma and thrombin-calcium chloride can be a clinically feasible method to deliver MSC to the surgical sites. Other studies have combined progenitor cells with different growth factors such as bone morphogenetic proteins (although not in the orofacial area) (312, 315) or enamel matrix proteins (Murai et al., 2005). These growth factors promoted tissue regeneration but the exact role of the MSC alone remains unknown.

MANDIBLE

Autologous bone grafts have been a "gold standard" in craniofacial reconstruction. However, donor site morbidity and a limited quantity/supply are still substantial hurdles with this method. Bone tissue engineering can fully replace lost bone tissues through the use of three-dimensional biodegradable scaffold materials carrying osseous progenitor cells and bioactive agents (growth factors, hormones, etc). Abukawa et al., (2004) (300) used scaffolds to reconstruct bony defects in pig mandibles. They seeded MSC into a biodegradable polymer and incubated for 10 days. Complete bone growth was observed in the experimental group. De Kok et al., (2005), (307) studied safety and potential efficacy of utilizing MSC for alveolar bone repair in Beagle dogs. They showed that bone marrow MSC seeded on either hydroxyapetite/tricalcium phosphate biomaterials or not can increase bone formation in dental sockets. Improvements in cell encapsulation techniques along with new generations of smart biodegradable scaffolds (325) will lead to the reconstruction of new and well-differentiated bone.

Human mandibles with major discontinuity defects (more than 5 cm) caused by an ablative tumor surgery can be repaired with autologous vascularised fibula, scapula, iliac crest, or rib bone grafts. However this approach may create skeletal defects at the donor site which can be

associated with serious morbidity. Warnke et al., (2004) (328) reported the fabrication of a mandibular transplant for a patient who had a large resection of his mandible (from left paramedian region to the right retromolar region). The transplant was made of a titanium mesh cage filled with bone mineral blocks that were infiltrated with a combination of the patient's own iliac bone marrow and recombinant human bone morphogenetic protein-7. The transplant was implanted into the right latissimus dorsi muscle of the patient for seven weeks. The skeletal scintigraphy showed bone remodeling and mineralization inside the mandibular transplant both before and after transplantation. CT provided an evidence of new bone formation. Seven weeks post-transplantation, the transplant was excised with an adjoining part of the latissimus dorsi muscle containing the thoracodorsal artery and vein that had supplied blood for the entire transplant, and transplanted to repair the mandibular defect. The patient had an improved degree of mastication and was satisfied with the aesthetic outcome.

CONDYLE

The cartilaginous and osseous structures of the temporomandibluar joint (TMJ) can deteriorate due to injuries, rheumatoid arthritis, and osteoarthritis. Tissue engineering of the TMJ can overcome drawbacks of joint replacement such as immunological rejection, donor site morbidity, transmission of pathogens, or metal loosening. Abukawa et al., (2003) (301) fabricated a model of porcine mandibular condyle using porous biodegradable polymer scaffolds. The authors encapsulated differentiated osteoblasts (originating from cultured minipig bone marrow-derived MSC) into polymer scaffolds and incubated the construct in an oxygen-permeable bioreactor system for six weeks. Histological observations revealed uniform new bone formation and densely stained osteoid and osteocytes in lacunae surrounded by bone matrix in deeper layers. Radiographic assessment revealed higher radiodensity of the construct when compared to the control scaffold but lower density than the control minipig cadaver condyle. Alhadlaq et al.,

(2004) (302) designed a bi-layer model to engineer cartilage and bone of the mandibular condyle. They harvested rat bone marrow-derived MSC and differentiated them into chondrocytes and osteocytes *ex vivo*. Chondrocytes and osteocytes were then seeded in a two-layer biocompatiable poly (ethylene glycol)-based hydrogel. The construct was implanted in subcutaneous dorsal pockets of immuno-deficient mice. Histological observations of the harvested constructs showed stratified layers of chondrogenesis and osteogenesis.

TOOTH

Ohazama et al., (2004) (324) reported significant progress toward the creation of tissue-engineered embryonic tooth primordia (tooth buds) using cultured cells. In a mouse model, they tested different mixtures of non dental-derived mesenchymal cells (embryonic stem cells, neural stem cells, and adult bone marrow cells) with embryonic oral epithelium cells. These mesenchymal-epithelial mixtures were transplanted into the adult mouse renal capsules. All mixtures resulted in the development of a tooth structure and bone. They observed that the host tissues make no contribution to the donor tissue. Moreover, transfer of embryonic tooth primordia into the adult jaw resulted in the development of tooth structures, showing that an embryonic primordium can develop in its adult environment. They concluded that bone marrow-derived cells can form all mesenchymal-derived cells in a tooth structure. In vitro control of the shape of the tissue-engineered dental primordia will be a crucial step to bring this therapy to the clinic (322).

ORAL MUCOSA

Tran et al., (2003) (327) reported an example of transdifferentiation of human bone marrow-derived stem cells into buccal epithelial cells. Using fluorescence in-situ hybridization (FISH) and immunohistochemistry, they identified Y-chromosome positive buccal cells in 5 females who

had received either a bone marrow transplant or an allogeneic mobilized peripheral blood stem cell transplant from male donors. Y-chromosome positive cells in these female patients were morphologically distinguishable as buccal epithelial cells and they also expressed cytokeratin 13, a recognized epithelial marker located in the superficial layer of the cheek. These results were confirmed by Metaxas et al., (2005) (319) who reported the presence of 1.8% donor-derived buccal epithelial cells in cheek scrapings of 12 of the 13 female patients who received a male-to-female hematopoietic cell transplantation 56 to 1964 days ago. The cheek scrapings were done when no oral mucositis or oral graft-versus-host disease were present. The donor-derived buccal epithelial cells were identified by epithelial morphological characteristic, cytokeratins expression, positive Y-chromosome, and negative CD45 (blood lineage marker).

The plasticity of adult bone marrow-derived cells has been questioned by studies suggesting that fusion between donor and host cells gave the appearance of transdifferentiation (145,326). However, in vivo studies (319,327) did not observe cell fusion. Tran et al. (2003) (327) examined over 9,700 buccal cells and reported no evidence of fusion. These findings were also confirmed in the study by Metaxas and colleagues (2005) (319) who reported that none of the buccal cells examined had more than one X-chromosome, which excludes fusion as the answer to cell plasticity.

SUMMARY

In this review we have discussed studies reporting successful applications of bone marrow stem cells to reconstruct different craniofacial tissues such as the periodontal ligament, cementum, bone, condyle, tooth, and oral mucosa. Plasticity of adult stem cell is controversial and more research is needed before any safe implementation of these cell-based therapies can be utilized in the clinic.

ACKNOWLEDGEMENTS

Canada Research Chair and CIHR (for SDT), Egyptian Cultural and Educational Department (for OMM).

CHAPTER 6

OM Maria, SM Maria, Tran SD. Identification of Specific Population of Human Salivary Acini and their Progenitors Using Mesenchymal Stem Cell Markers. (S*ubmitted 2010*).

The Ph.D. Candidate performed the following contributions in this manuscript:

- All the experimental and technical work.
- Samples examination.
- Wrote the manuscript.

Identification of Specific Population of Human Salivary Acini and their Progenitors Using Mesenchymal Stem Cell Markers

O. M. MARIA, D.M.D.,[1,2] S. M. MARIA, D.V.M.,[1] S. D. TRAN, D.M.D., Ph.D.[1*]

[1] Faculty of Dentistry, McGill University, Montreal, Canada.

[2] Faculty of Dentistry, Mansoura University, Mansoura, Egypt.

O. M. MARIA, D.M.D.

Address: 3640 University Street, Room M33, Montreal, Quebec, H3A 2B2, Canada. E-mail: ola.maria@mail.mcgill.ca, Tel: +1 514 398 7203 ext. 00056, Fax: + 1 514 398 8900

S. M. MARIA, D.V.M.

Address: 740 Dr. Penfield Avenue, Room 2301, Montreal, Quebec, H3A 1A4, Canada. E-mail: salwa.maria@mail.mcgill.ca, Tel:+1 514 398 7203 ext. 094817, Fax: +1 514 398 8900

[*] *Correspondence Author*:

S. D. TRAN, D.M.D., Ph.D.

Address: 3640 University Street, Room M43, Montreal, Quebec, H3A 2B2, Canada. E-mail: simon.tran@mcgill.ca, Tel: +1 514 398 7203 ext. 09182, Fax: + 1 514 398 8900

ABSTRACT

Xerostomia results from irreversible destruction of salivary parenchyma in patients afflicted with Sjogren's syndrome or who received therapeutic irradiation for head and neck cancer. The development of an artificial salivary gland device or stem cell-based therapy would benefit these patients. Accordingly, we need to expand acinar cells and/or their progenitors. We aim to identify and characterize human salivary gland (huSG) stem/progenitor cells, and to track them in a duct-ligated parotid gland model in the rabbit. Human salivary tissue was tested for the expression of stem cell markers: Musashi-1, Stro-1, CD146, CD106, CD44, CD166, CD90, CD105 using immunofluorescence technique. Moreover, CD44 expression was tracked in rabbit parotids after duct-ligation. In huSG tissue, serous acini expressed the mesenchymal adhesion-related stem/progenitor cell marker CD44, while mucous acini expressed CD166. In rabbit controls, serous acini also expressed CD44. However upon ligation, CD44 expression increased in both acini and ducts at day 1, 7, 14 and only in duct-like structures at day 30 and 60 post-ligation. These CD44 stem/progenitor cells are more resistant to atrophy caused by duct-ligation than the differentiated cells. This duct-ligation model might be used to isolate a greater number of stem/progenitor cells while CD44/CD166 could be used, either to isolate stem cells or differentiated serous/mucous acini from human salivary glands. The ability to identify and isolate huSG stem/progenitor cells would provide a cell-based therapy for the treatment of xerostomia.

Keywords: Mesenchymal Stem Cells, Animal Models, Adult Stem Cells, Cell Adhesion.

INTRODUCTION

Irreversible salivary gland damage followed by hypofunction results from either an autoimmune disorder, Sjögren's syndrome, affecting 1 million patients in the United States or from therapeutic irradiation of patients with head and neck cancer (30,000 new cases each year) (194,201). Both categories of patients, suffer from altered salivary quantity and quality that leads to considerable morbidity, including dry mouth, dysphagia, dental caries, candidiasis, oropharyngeal infections, and mucositis (193). We have been working to develop a tissue-engineered artificial salivary gland device that would be implanted into a surgically created pouch in the patient's mouth (10,11). This device would contain autologous graft cells (acinar cells) secreting fluid towards the oral cavity. For these cells to function properly, they must be polarized and form an adequate epithelial barrier (10,11). We have succeeded in culturing and expanding primary human salivary cells that are polarized and provide an adequate functional epithelial barrier. However, most of these cells have a ductal phenotype and thus, are not fluid secretory cells (11). Salivary glands, like other parenchymal tissues contain progenitor cells (167-170), but their exact source is not yet understood. The patterns of repair and regeneration in adult salivary glands suggest that they contain a stem cell-like population within the intercalated ducts (168). In addition, almost all differentiated cell types of the adult glands have the ability to divide. It appears that division by both stem cells and differentiated cells contribute to the proliferation/ turnover of salivary cells, depending on the gender and cell/ gland type (168). The identification and in vitro expansion of acinar stem/ progenitor cells that would be used as autologous graft cells in our artificial salivary gland device would be an important achievement for the treatment of xerostomic patients.

The ligation of the main salivary excretory duct causes progressive atrophy in acinar and ductal cells and alters their physiology. This uniform atrophy is used as a model to study several salivary gland conditions such as: aging (332). radiotherapy, Sjogren's syndrome, sialadenitis

(122), atrophy and regeneration (333). Moreover, Hisatomi et al., (2004) (334) isolated salivary progenitor cells after submandibular duct-ligation in a mouse model, and induced these cells to differentiate into hepatic and pancreatic cells. Many investigators (167,168,335-337) have examined salivary glands of rodents for the presence of progenitor/ stem cells. However, a specific cell type capable to restore salivary parenchymal cells has not been identified. It has been suggested that salivary epithelial cells expressing the α6β1 integrin may have stem-like properties (169.170). The lack of defined markers for phenotypic characterization of salivary stem/ progenitor cells continues to be a major limitation. Our current knowledge in stem cells is significantly based on studies from the bone marrow (hematopoietic) and neural tissues. Our group has unpublished data that cells from the bone marrow can mobilize to the human salivary glands, and differentiate into acinar, ductal, and endothelial cells. Consequently, the objective of this study is to test if currently available stem cell markers from the bone marrow system can also be utilized to localize salivary stem/ progenitor cells, in situ, from human salivary gland biopsies. Once this stem cell marker is identified, the second objective of this study is to track (in time) this cell marker in an animal (rabbit) salivary gland duct ligation-induced tissue damage model that is known to increase the population of stem/ progenitor cells in the gland (i.e. we expect to see a higher numbers of salivary progenitor cells expressing this stem cell marker in rabbits which had their parotid ducts ligated). We report here the in situ identification of human acinar cells using mesenchymal stem cell (MSC) surface markers. CD44 specifically identified populations of serous acinar cells from human submandibular and parotid glands, while the marker CD166 can be used to identify mucous acinar cells. CD44 also identified serous acinar cells in the rabbit. The expression of CD44 is increased significantly upon the ligation of parotid ducts. We propose that these two cell surface markers (CD44 and CD166) could be used to isolate specific populations

of acinar cells from human salivary glands. Also CD44 can be used as a marker to identify progenitor cells of the acinar phenotype.

MATERIALS & METHODS

Source of Salivary Glands: Portions of human submandibular (n=9) and parotid (n=2) salivary glands, from both men (n=8) and women (n=3), were obtained from the Department of Pathology, University of Virginia, through the Cooperative Human Tissue Network (CHTN). For tissue use, we received an approval from the Institutional Review Boards at McGill University. This report describes results obtained with glands that were excised as part of the resection of head and neck cancers. Patients, from whom samples were obtained, had received no prior irradiation/ chemotherapy treatment. Pathologist at the University of Virginia (Charlottesville, VA) performed surgical pathologic examinations of all glands after their excision. We were provided with representative tissue slides after histopathological examination by CHTN. All glandular tissues received and reported here were judged as histopathologically normal. The salivary tissues were either frozen in liquid nitrogen or fixed in 10% buffered formalin by CHTN.

Animals & Surgery: We studied the expression of the mesenchymal stem cell marker CD44 in rabbits which had bilateral parotid duct-ligation for 1, 7, 14, 30, and 60 days (5 time points). Sixty male New Zealand rabbits (3-3.5 Kg) were divided into 5 groups, according to their follow-up time post-ligation. Each group had 10 duct-ligated (experimental) animals and 2 non-ligated (control) animals (n=12 per group). These rabbits were kept in the Faculty of Medicine, Mansoura University animal facility, and maintained on a standard diet. The rabbits were fasted overnight before surgery. The animals were anesthetized by sodium pentobarbitone intra-peritoneally, 40mg/kg body weight (Sominaletta 40mg/ampoule, Alex. Co., Egypt). We followed an extra-oral approach to free the parotid duct from the fascia and to exclude the facial nerve before ligation; the duct is superficial, just under the skin. Two separate sutures were tied on each

duct using 3-0 silk suture while the control animals were operated but no duct-ligation procedure was performed (sham-operated). The skin was replaced with three interrupted sutures. The rabbits were fasted overnight and re-anesthetized before being sacrificed. Tissue fixation by intra-cardiac perfusion technique was performed using 10 % buffered neutral formalin. The animal protocol for this study was approved by the Office of the Graduate Studies and Research Affairs at the Faculty of Dentistry, Mansoura University. In this study, the surgery and euthanasia of the animals were done under general anesthesia.

Immunofluorescence Staining: Frozen tissue (3µm-thickness) sections from human salivary glands (submandibular and parotid glands) were fixed in cold methanol (over dry ice) for 10 min, followed by three washing steps in PBS for 5 min each. Endogenous peroxidase and biotin activities were blocked with hydrogen peroxide 3% and the Universal Blocking (BioGenex, San Roman, CA), respectively. We used the following primary antibodies to test human submandibular and parotid gland sections: monocolonal goat anti-human Musashi-1, mouse anti-human multipotent mesenchymal/ stromal cell marker antibody panel kit (R&D Systems, Minneapolis, MN). The panel contains a group of antibodies for the positive identification of human MSCs; anti-Stro-1, anti-CD90 (Thy-1, a glycosylphosphatidylinositol-anchored glycoprotein) (338), anti-CD106 (vascular cell adhesion molecule) (339), anti-CD105 (endoglin, transforming growth factor-β receptor complex) 340, anti-CD146 (melanoma cell adhesion molecule, marker for endothelial cell lineage) (341), anti-CD166 (activated leukocyte cell adhesion molecule) [Swart, G.W. Activated leukocyte cell adhesion molecule (CD166/ALCAM): (339), and anti-CD44 (hyaluronic acid receptor) (342), plus leukocytes markers; anti-CD19 and anti-CD45 (leukocyte common antigen/ cell marker of hematopoietic origin) (341). In addition, we used goat (R&D Systems), rabbit and mouse (Zymed Labs, San Francisco, CA) isotype control antibodies, these antibodies are reactive against the respective proteins from human and

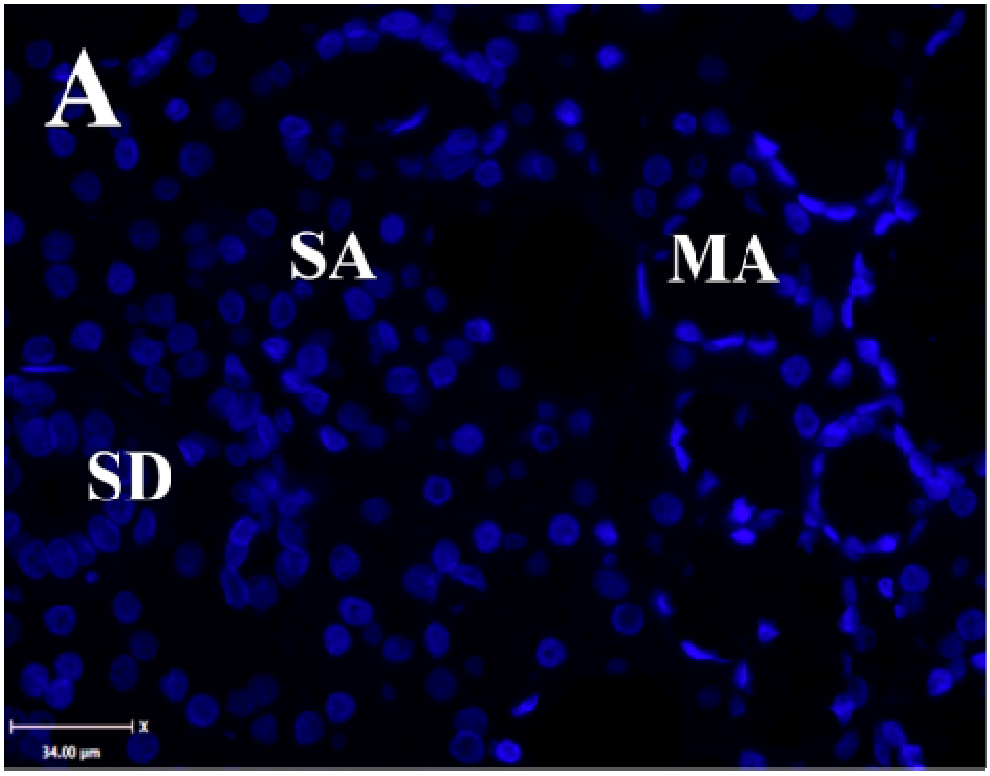
other species. Immunofluorescence stained sections do not provide clear morphology for identification of specific salivary cell sub-populations. For example in salivary tissue, acinar and ductal structures look similar without obvious cellular outline/ details. In order to overcome this problem, we co-localized the tested stem cell markers with either polyclonal goat anti-human Aquaporin-5 (AQP5) antibody (an acinar marker, Santa Cruz) or polycolonal rabbit anti-human α-amylase antibody (a serous acinar marker, Sigma-Aldrich). For duct-ligated parotid sections of the rabbit, we used monocolonal mouse anti-rabbit CD44/HCAM (3H1331) antibody (Santa Cruz). Donkey anti- mouse-FITC, rabbit-RRX and goat-RRX secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. All primary antibodies used were diluted (1:100) in PBS containing 5% donkey serum (Jackson ImmunoResearch Laboratories). Slides were incubated with primary antibodies overnight at 4^{o}C, and with secondary antibodies for 1 hour at room temperature in the dark. Then, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) was added for 3-5 minutes. For formalin fixed paraffin-embedded (FFPE) tissue slides, we used the same steps as described above, in addition to a boiling step (15 min) using an EDTA solution (pH 8.0, Zymed Labs) for heat-induced epitope retrieval. As a positive control, MSCs from human donors (25-35 years old) were purchased (Cambrex, Canada) and were stained in three forms: frozen, FFPE and fresh cell culture suspension. Rabbit slides from all groups were stained with Hematoxylin and Eosin (Sigma-Aldrich, Canada) for routine morphologic examination.

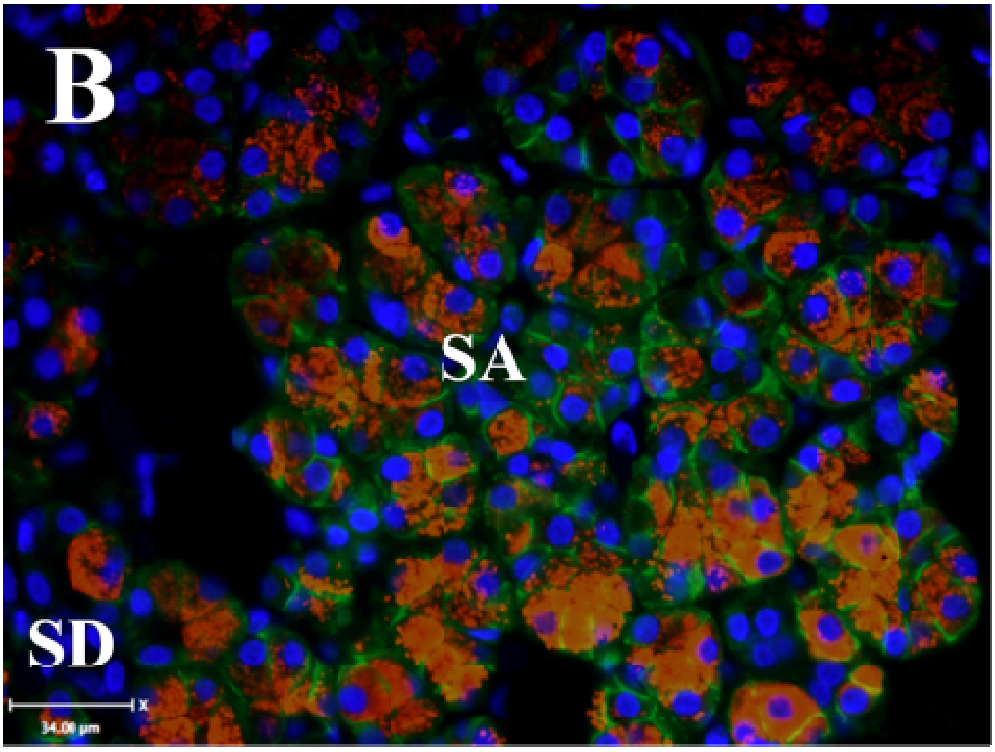
RESULTS

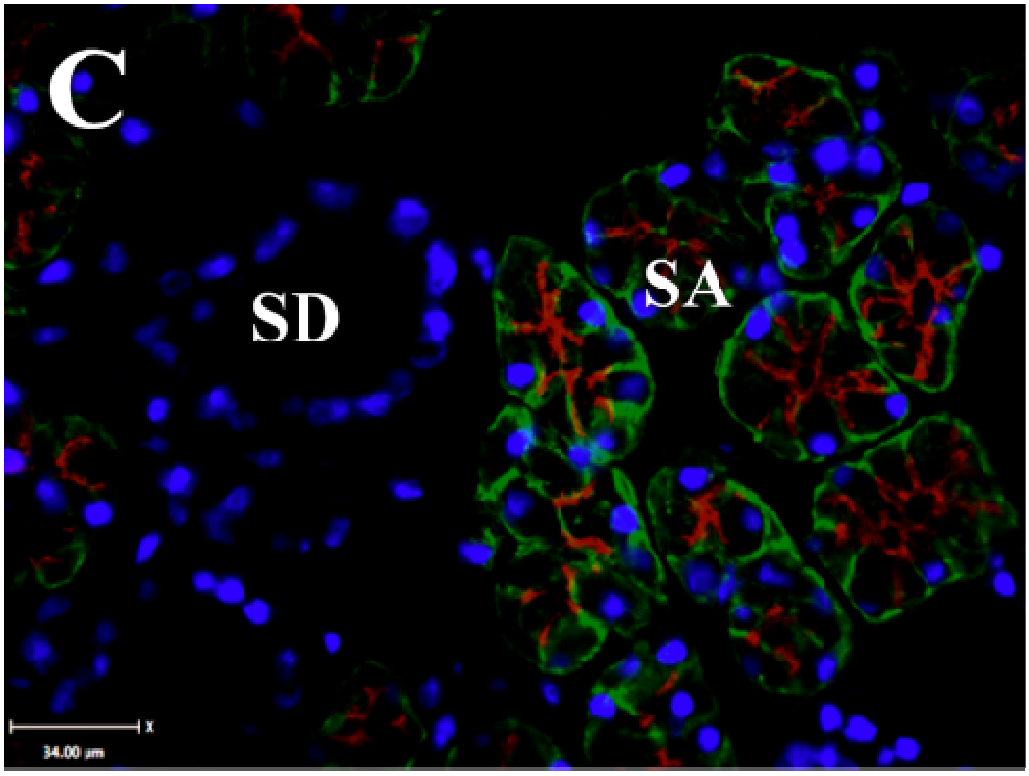
We examined slides of glands from both male (n=7) and female (n=4) patients, using both frozen and FFPE tissue sections (3 slides per gland). Patient age ranged between 38 and 73 years (average: 55.3 years). No differences in results were seen between the sexes or in regard to patient age. For duct-ligated parotid tissue from male rabbits we tested only FFPE tissue sections

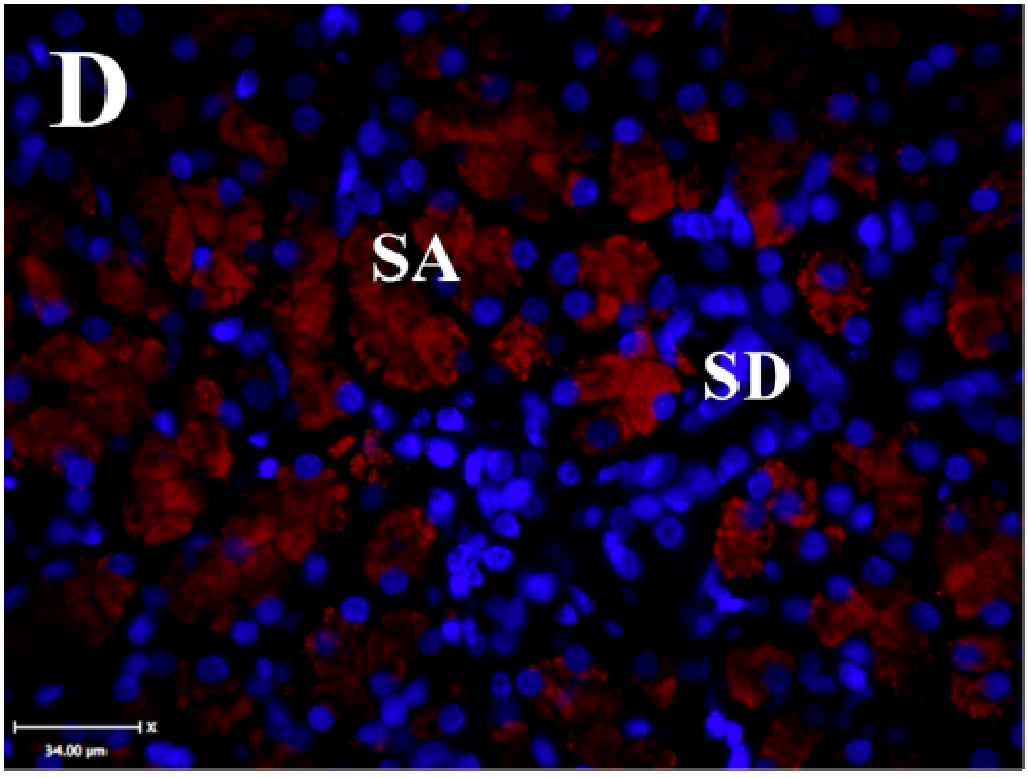
(3 slides per gland). In comparison to isotype control antibodies (Fig 6.1A), our results showed that 100% of human salivary serous acinar cells were CD44-positive ($CD44^+$; a summary of all cell markers tested is shown in Table 1). The CD44 signal is strongly localized along the basal and lateral cell membrane of acinar cells, while a weaker cytoplasmic signal could be detected in FFPE sections (Fig 6.1B, 6.1C). On the other hand, neither the ducts nor the mucous acini showed any reaction to CD44. Myoepithelial cells and blood vessels were negative to CD44. The CD166 marker was not expressed in any cell type in the parotid glands (Fig 6.1D). However CD166 was expressed in 100% of mucous acinar cells of the submandibular gland. CD166 localized along the apical and lateral cell membrane (Fig 6.1E). None of the serous, ductal, myoepithelial cells, or blood vessels expressed the CD166 marker. All other tested human stem cell or cell markers (Musashi-1, Stro-1, CD90, CD106, CD105, CD146, CD19, CD45) were not expressed by any cell in both submandibular and parotid glands (Table 6.1). We used AQP-5 water channels (expressed by acinar cells) and α-amylase (expressed specifically in serous acinar cells) antibodies to co-localize/ confirm the salivary cell type expressing the stem cell markers being tested. Results from two parotid glands (1 male and 1 female) were comparable to those from all submandibular gland samples (6 males and 3 females). Human MSCs used as positive control were (95±2%) positive for Stro-1, CD90, CD106, CD105, CD146, CD44, and CD166 markers however were negative for Musashi-1, CD19, and CD45 markers (Table 1). There was no difference in the positive results obtained between the three forms of human MSCs examined.

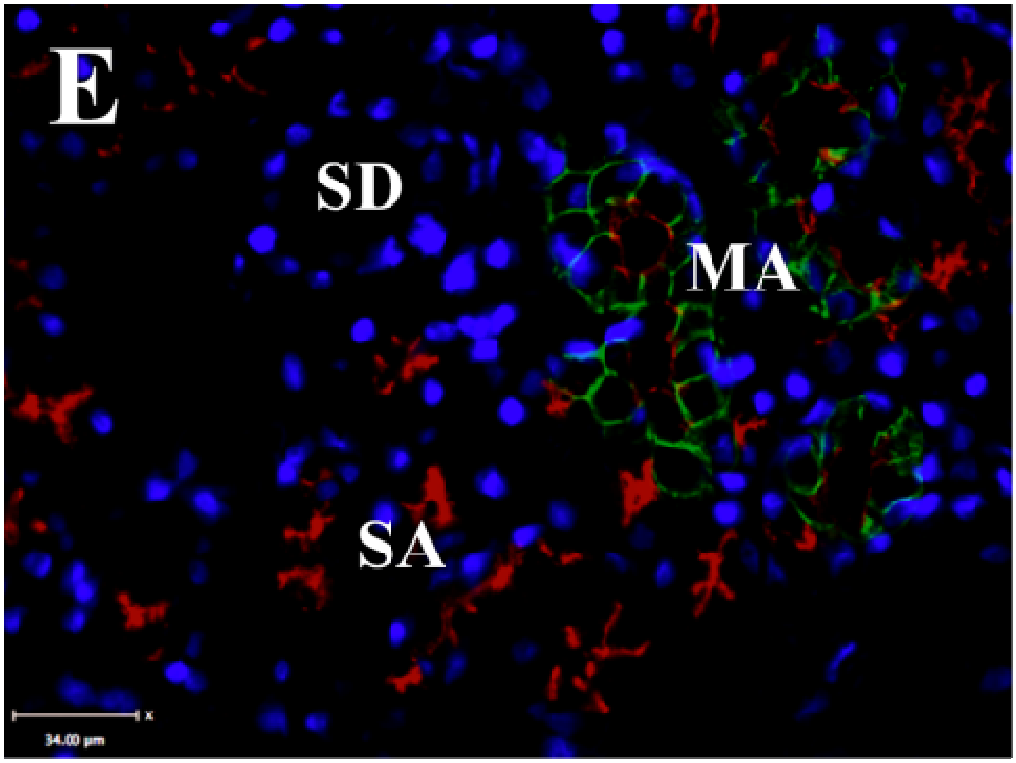
Figure 6.1: Immunofluorescence micrographs of human (A-E) salivary gland tissue sections showing expression of CD44 or CD166 in green; α-amylase or AQP-5 in red; DAPI nuclei in blue. (A) Isotype control antibody labeling: serous acinus (SA), mucous acinus (MA), and striated duct (SD). (B) CD44 (in green) and α-amylase (in red; a marker for serous acinar cells) expression in parotid gland: CD44 is positive in SA but negative in SD. (C) CD44 (in green) and AQP-5 (in red; a marker for acinar cells) expression in submandibular gland: CD44 is positive SA but negative SD. (D) CD166 (in green) and α-amylase (in red) expression in parotid gland: no expression of CD166 (no green signal) in α-amylase positive SA cells. (E) CD166 (in green) and AQP-5 (in red) expression in submandibular gland: CD166 is positive in MA cells but negative in SA and SD cells, AQP-5 is positive in MA and SA cells. [Scale bar= 34 μm, X 400, for all panels].


A
SA
MA
SD
34.00 µm


B
SA
SD
34.00 µm


C
SD
SA
34.00 µm


D
SA
SD
34.00 µm


E
SD
MA
SA
34.00 µm

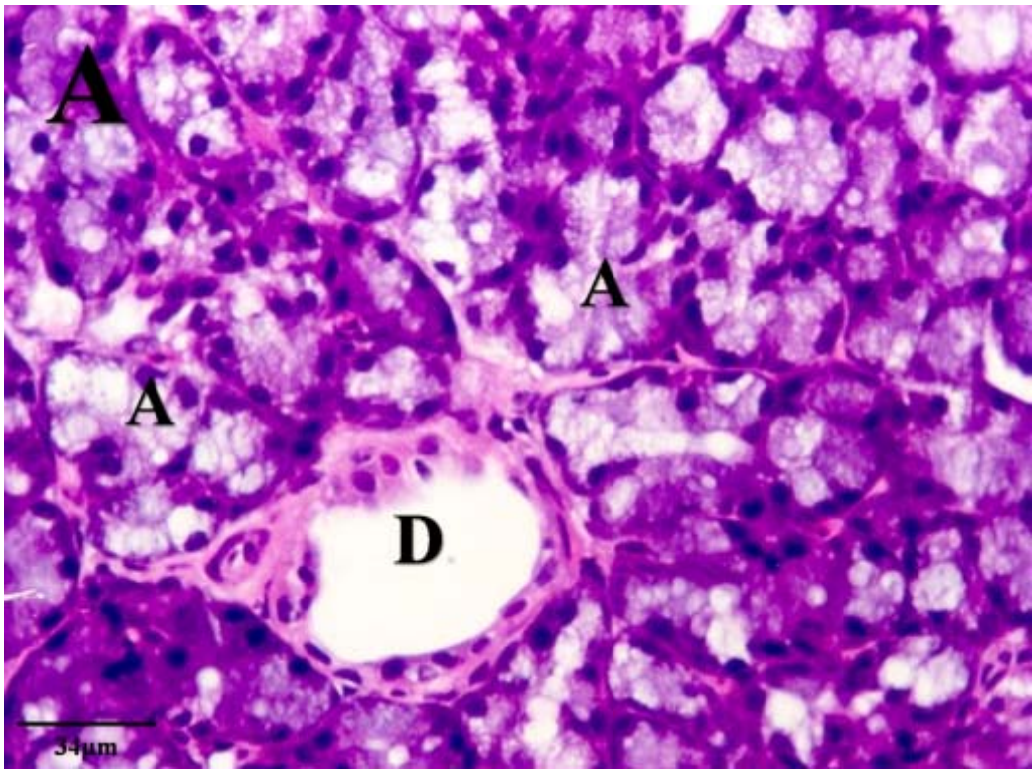
Stem Cell Marker	Acini		Ducts		Myoepithelial Cells	Blood Vessels
	Serous	Mucous	Intercalated	Striated		
Musashi-1	-	-	-	-	-	-
Stro-1	-	-	-	-	-	-
CD44	100%	-	-	-	-	-
CD166	-	100%	-	-	-	-
CD90	-	-	-	-	-	-
CD105	-	-	-	-	-	-
CD106	-	-	-	-	-	-
CD146	-	-	-	-	-	-
CD166	-	-	-	-	-	-
CD19	-	-	-	-	-	-
CD45	-	-	-	-	-	-

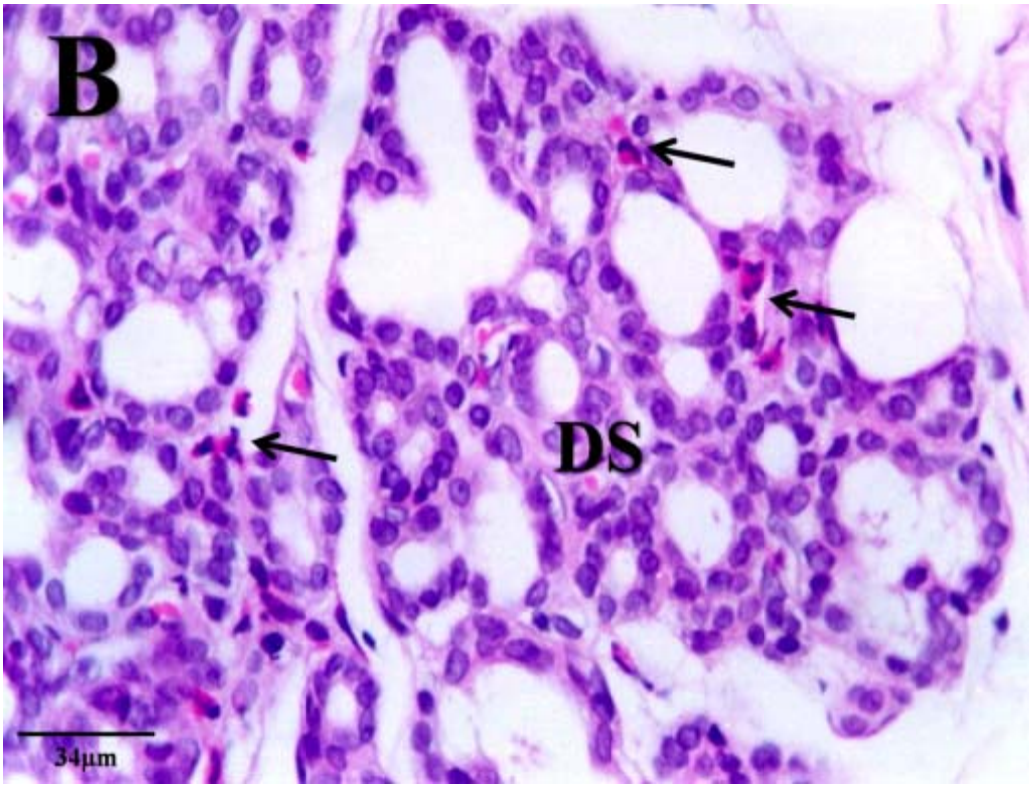
Table 6.1: Expression of stem cell markers in adult human submandibular and parotid glands: Results are based on the examination of 3 sections per salivary gland. At least 1,000 cells were examined per salivary cell type. * "100%" means that all cells observed expressed this stem cell marker; "-" means none (0%) of the observed cell types expressed this stem cell marker.

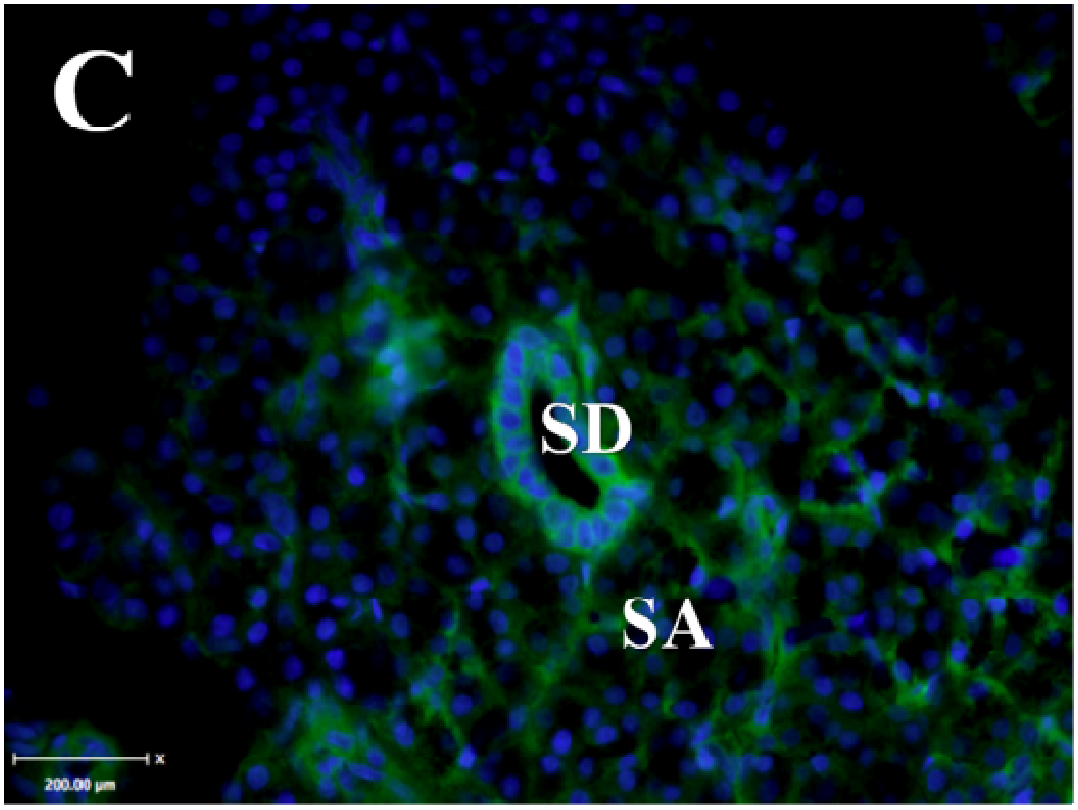
From our screening of stem cell markers in human salivary gland biopsies, we decided to investigate the CD44 marker in acinar cells - at different time intervals - using an animal model and method known to increase the population of stem/ progenitor cells in salivary glands. The method was to ligate salivary ducts to induce salivary glandular tissue (acinar cell) damage. This injury would induce local progenitor cells to proliferate and repair/ regenerate the gland. We chose the rabbit as the animal model to test because the histology of the rabbit parotid (a mixed gland consisting of sero-mucous secretory units) resembles that of human salivary glands (343). Also, the rabbit parotid gland undergoes atrophy faster than its submandibular and sublingual glands (i.e. faster for us to induce injury to the gland). The second objective of this study was to test if CD44 can be used as a biomarker to identify progenitors of acinar cells, in the rabbit model described above. During the period following duct ligation, we expect a decrease number of acinar cells and eventually, their complete loss in the gland. However, if we observe an increased CD44 expression in the surviving ductal cells, then this marker can be used to identify or isolate progenitor cells of the salivary glands. Rabbits were sacrificed at different time intervals post-ligation: 1, 7, 14, 30, and 60 days. Tissue morphology at day 1 post-ligation revealed distended acini and ducts; it was difficult to distinguish between serous and mucous acini (Fig 6.2A). At day 7 and 14 post-ligation, acini appeared completely separated with widened interstitial spaces. Duct-like structures increased at the expense of acini and it was difficult to identify a particular duct type. At day 30 and 60 post-ligation, acinar cells disappeared completely. Only duct-like structures with shrunken cytoplasm and pyknotic nuclei were observed (Fig 6.2B). Cellular debris, neutrophils and monocytes were observed among duct-like structures and intra-luminal as well. The shrinkage of acini and duct system could be considered as an adaptive response from cells to the persistent pressure caused by duct ligation. In rabbit parotid gland controls (no ligation, but sham-operated), only acini expressed the CD44 marker at the basal and lateral cell

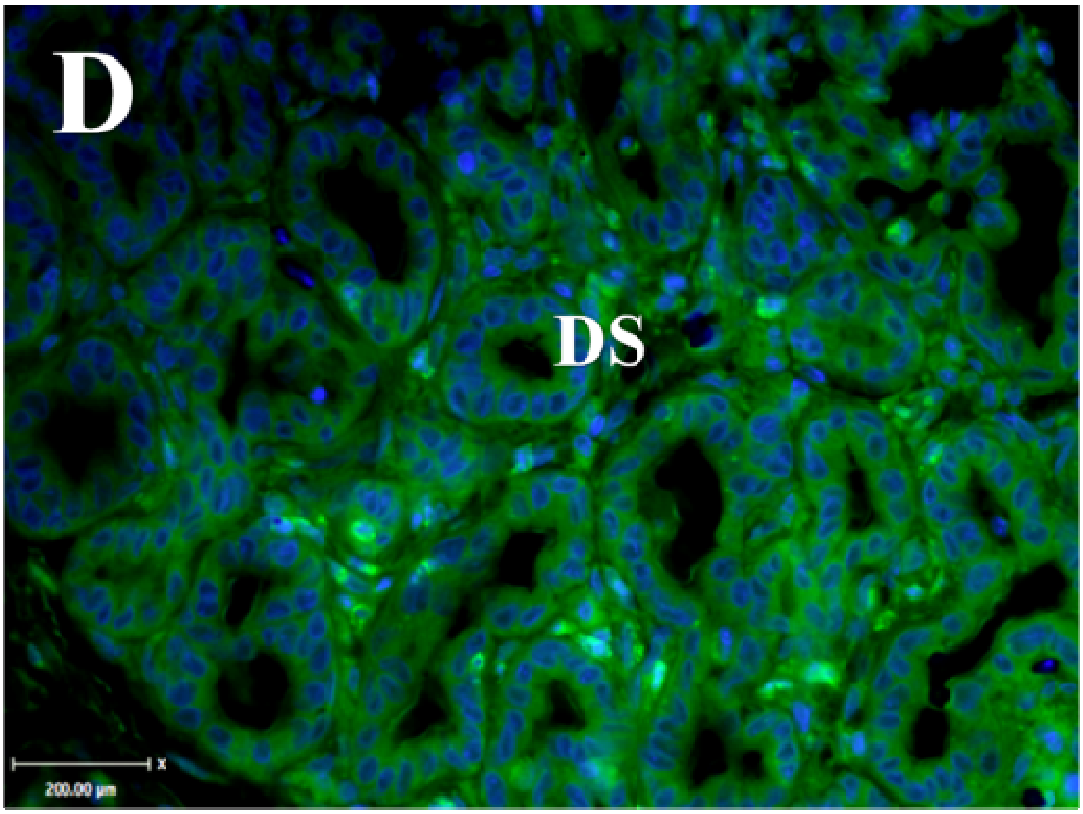
membrane (not shown). After duct-ligation, CD44 expression started to increase from day 1 (Fig 6.2C) to day 7 (not shown) and 14 (Fig 6.2D) in both acini and ducts. The CD44 signal was localized on cell membranes as well as in the cytoplasm. While at day 30 (Fig 6.2E) and 60 post-ligation, only duct-like structures remained in the gland, and showed high expression of CD44 at both the cell membrane and cytoplasm.

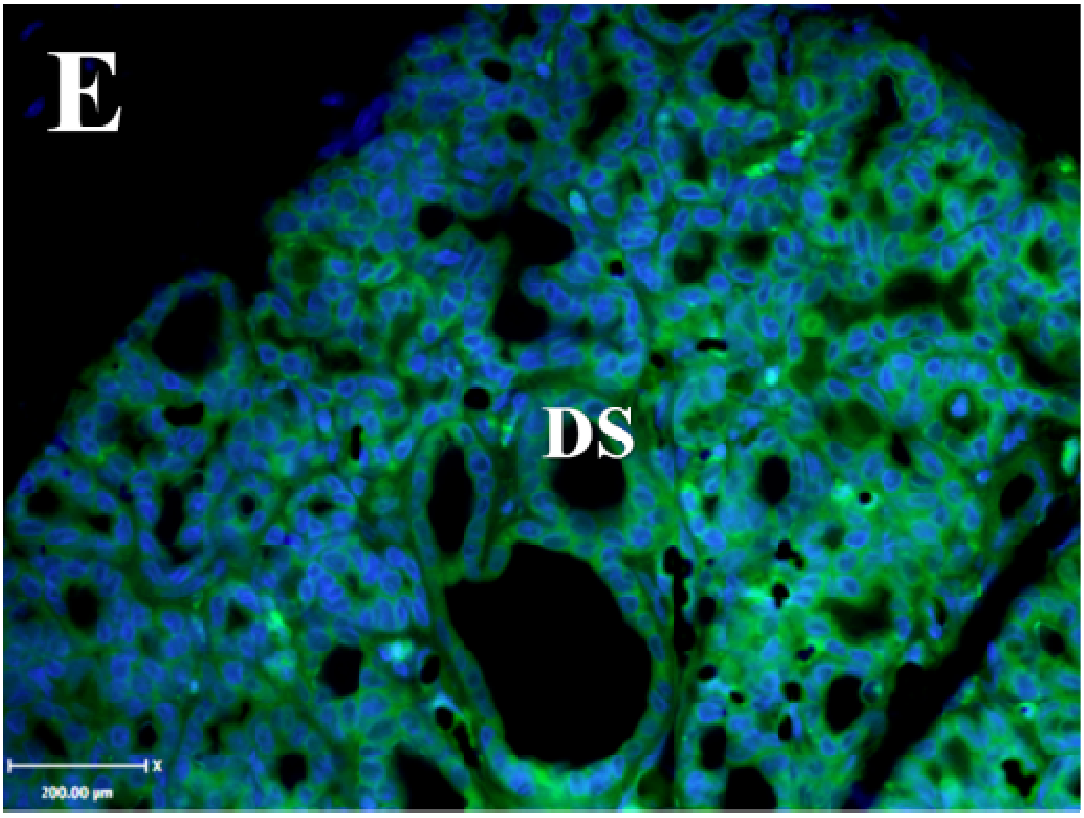
Figure 6.2: Light micrographs of rabbit duct-ligated parotid gland tissue sections. Panels A and B show tissue stained with hematoxylin and eosin. (A) At day 1 post-ligation: showing distended acini (A) and duct (D). (B) At day 30 post-ligation: showing duct-like structures (DS) and inflammatory cells (arrows). Panels C, D, and E show immunofluorescence micrographs of rabbit duct-ligated parotid gland tissue sections, CD44 expression shown in green, DAPI nuclei in blue. (C) At day 1 post-ligation: showing CD44 positive serous acinus (SA) and striated duct (SD). (D and E) At day 14 and 30 post-ligation: showing CD44 positive duct-like structures (DS). [Scale bar= 34 μm, X 400, for panels A and B; Scale bar=200 μm, X 200, for panels C to E].


A
A
A
D
34µm


B
DS
34µm


C
SD
SA
200.00 µm


D
DS
200.00 µm


E
DS
200.00 µm

DISCUSSION

We demonstrated the presence of a sub-population of cells within the human salivary glands (submandibular and parotid) that could be identified using markers for mesenchymal/stromal stem/progenitor cells. The salivary acinar cells expressed the adhesion-related proteins CD44 and CD166, and might play a role in the regeneration/repair of salivary glands. Also, both CD44 and CD166 markers can be used to isolate (FACS sorting) serous and mucous salivary cells, respectively. In addition, we were able to identify acinar progenitor cells in a rabbit duct-ligated parotid gland model using the CD44 marker.

During early fetal development, salivary gland morphogenesis involves interactions between the oral epithelium and the condensing neural crest-derived mesenchyme (168, 344).

Thus, the salivary tissue is formed from a heterogeneous mix of ectomesenchymal cells. This led us to test stem cell antibodies against: Musashi-1 (neural); Stro-1, CD146, CD106, CD44, CD166, CD90, CD105 (mesenchymal); and CD45 (hematopoietic/leukocyte) using immunofluorescence methods. Stem cells are thought to reside in a niche which regulates the balance between stem cell self-renewal and tissue regeneration (345). Genetic and histological analyses revealed the existence of stem/progenitor cell-related markers: either intracellular (Musashi-1) or cell surface proteins (cluster of differentiations; CD105, CD90, CD44, CD166, CD146, CD106). Some of these markers are tissue-specific while others are expressed in several tissues. However, because a single marker that defines a stem/progenitor cell has not been found yet, several markers need to be combined. Musashi 1, an RNA-binding protein that is expressed by epithelial progenitors in the intestine (346), gastric mucosa (347), mammary glands (348), hair follicles (349), endometrium (350), germ lines, CNS (351), plays an important role in controlling asymmetric cell division, differentiation, and tumorigenesis (352). Mesenchymal/stromal stem cells (MSCs) that express a large scale of adhesion molecules (have role in cell-cell as well as

cell-extracellular matrix interactions) have now been isolated from adult human bone marrow, retina, skeletal muscle and dermis, dental pulp and periodontal ligament (180,353-356).

Additionally, there are controversies regarding the presence of tissue-specific stem cells and cancer stem cells. Cancer stem cells are subpopulations selectively endowed with tumorigenic potential (357) that arise from normal tissue-specific stem cells that underwent mutation (358). The combined expression of CD44/ CD166 was suggested for the isolation of colorectal cancer-initiating stem cells (357, 359). Nevertheless, using these markers for the prognosis and diagnosis of cancer remain elusive until specific mechanisms governing tumor initiation and progression are elucidated. In addition, none of our 11 patients' glands examined were diagnosed with salivary gland tumor.

Several studies have reported the identification of salivary stem cells in mice (360), rats (361) and humans (184, 362,363). However, there are only two reports (362,364) in the literature indicating "direct identification" of human salivary stem cells into the glandular tissue. Intercalated ducts are thought to be the stem/progenitor cells for salivary tissue (127,128,365,366). However, Denny et al 1993, Schwartz-Arad et al 1988, Redman 1994, and Redman 1995 concluded that well-differentiated acini would self-renew to maintain their population in adult male mouse submandibular glands (334) and in developing rat parotids (335, 367,368). Moreover, Ihrler et al 2004, reported that acinar, intercalated duct and myoepithelial cells regenerate independently, with neither cell transition nor participation of other cell populations (369). These authors concluded that the columnar cells of striated and excretory ducts regenerate by proliferation and differentiation of basal cells (369). Lombaert et al 2008 reported that excretory ducts of mice salivary glands might contain the actual stem cells necessary for salivary tissue regeneration (360). Here we demonstrated that serous and mucous acini harbor a mesenchymal-like stem/progenitor cell population which might support previous

findings by Denny et al 1993 (337), Schwartz-Arad et al 1988 (369), Redman 1994 (368) and Redman 1995 (335).

The regeneration of adult rat submandibular glands following duct-ligation supports the existence of multipotent cells within ducts, the only surviving component in atrophied glands (370). Kishi et al 2006 have used clonal analysis to demonstrate that multipotent stem/progenitor cells isolated from rat submandibular gland would generate acini, ductal and myoepithelial cells - in vitro - suggesting that colony-forming cells are ductal-derived cells (361). However, other reports concluded that regeneration of acini would mainly occur by self-division of surviving acini when the ligature is released (121,122). We observed in the rabbit that progenitor/stem cell subpopulations increased upon ligation and are more resistant to atrophy. Although all CD44 positive cells were only acini (and none were ductal) before ligation, the ductal cells started to express CD44 from day 1 post-ligation and afterward, even after day 30 and 60 post-ligation when no acini could be detected in the gland. The expression of the CD44 adhesion protein in duct-like cells upon ligation might be a mechanism for the surviving cells: to strengthen their connection/communication to each other along with the extracellular matrix, or a response related to the immune system. CD44 is a trans-membrane glycoprotein with multi-functions including: cellular adhesion (aggregation and migration), hyaluronate degradation, lymphocyte activation, angiogenesis, cytokines release, and in metastatic spread of tumours (371). CD166, a trans-membrane glycoprotein (called activated leukocyte cell adhesion molecule) mediates heterophilic and hemophilic cell-cell interactions (339). It has been reported that upon activation by pro-inflammatory cytokines, the adhesion molecules are rapidly expressed on the surface of inflamed endothelium, initiating leukocyte rolling and tethering ()372.Stem cells that are believed to reside in cell niche is thought to be regulated by cell adhesion molecules as well as interaction between stem cells and extracellular matrix (373) Although the exact role of both $CD44^+$ and $CD166^+$

adhesion proteins in salivary glands still need to be elucidated, their distribution in serous and mucous acini respectively, suggests a potential role in the secretory functions of both acinar types.

Immuno-cytochemistry and flow cytometric analyses revealed that rat (169), mouse (334) and swine (242) salivary gland stem cells express CD44, CD90, and additional antigens which we did not test. Sato et al 2007 (364); Rotter et al 2008 (184); Gorjuupa et al 2009 (363) reported the isolation and characterization of adult human salivary progenitor cells (using FACS) that were CD90+ (364), CD105+/CD166+/CD44+/CD90+/Stro-1+ (184), and CD105+/CD90+/CD44+ (363), respectively. However we did not identify any positive cells for CD105, CD90, and Stro-1 markers in situ in our human salivary biopsies. Salivary tissue contains a population of mixed cell types and FACS sorting of dissociated fresh salivary tissue and of primary salivary cell culture might have been contaminated with cells from the capillary bed/ blood supply. Sato et al 2007 used immuno-fluorescence to localize CD90+ periductal cells. However, immuno-localization of antigens in FFPE tissue sections is complicated by paraffin auto-fluorescence and this might be a false-positive result (364). Lombaert et al 2008 identified Musashi-1 positive excretory and striated ductal cells in murine salivary glands (360), while we could not identify any positive Musashi-1 cells in human salivary tissue using immuno-fluorescence technique. There might be species variety in stem cell distribution and/ or markers expression. Another possibility is the different sensitivity in the detection techniques used (they used immuno-histochemistry while we used immuno-fluorescence). Additional studies with standardized methodologies would confirm/ exclude these inconsistent observations.

In conclusion, we report here, direct localization of mesenchymal-like stem/progenitor cells inside human salivary tissue that are positive for CD44/ CD166. We also conclude that duct-ligation would be a feasible way of isolating and analyzing $CD44^{+}$ salivary stem/progenitor cells

from rabbits. Whether detection of $CD44^+$ and $CD166^+$ salivary cells is sufficient for stating the presence of mesenchymal-like stem/progenitor cells remains to be elucidated. Because we observed a high prevalence of cells expressing CD44 and CD166 in human and rabbit salivary glands (well beyond the rare stem/progenitor cell population reported by other groups to date), we propose to call these as markers to "identify/ isolate serous and mucous acini". However, considering that acini were reported to be a self-expanding population capable of de-differentiation into duct-like structures upon salivary duct-ligation, there might be a sub-population of progenitor cells within this CD44/ CD166 positive population of acinar cells. This would be of therapeutic significance since the availability of sufficient multipotent cells - expanded in vitro to be implanted into an artificial salivary gland device - would provide "off the shelf" cell-based therapy for patients suffering from xerostomia.

ACKNOWLEDGEMENTS

The authors would like to thank the funding institutions; Canada Research Chair, CIHR, NSERC, RSBO and Egyptian Ministry of Higher Education and Research. The authors thank Dr. Christopher Moskaluk for the histopathological analysis of salivary glands and Cooperative Human Tissue Network for providing the salivary tissue samples.

Author Disclosure Statement: No competing financial interests exist.

CHAPTER 7

Maria OM, Tran SD. Mesenchymal Stem Cells from Human Bone Marrow Differentiate into Functional Salivary Gland Cells. (*Submitted 2010*).

The Ph.D. Candidate performed the following contributions in this manuscript:

- All the experimental and technical work.
- Examinations and analysis of data.
- Wrote the manuscript.

Human Mesenchymal Stem Cells Differentiate into Functional Salivary Acinar Cells

O.M. MARIA, D.M.D., S.D. TRAN, D.M.D., Ph.D.*

Faculty of Dentistry, McGill University, Montreal, Canada.

O. M. MARIA, D.M.D.

Address: 3640 University Street, Room M33, Montreal, Quebec, H3A 2B2, Canada. E-mail: ola.maria@mail.mcgill.ca, Tel: +1 514 398 7203 ext. 00056, Fax: + 1 514 398 8900

*_Correspondence Author_:

S. D. TRAN, D.M.D., Ph.D.

Address: 3640 University Street, Room M43, Montreal, Quebec, H3A 2B2, Canada. E-mail: simon.tran@mcgill.ca, Tel: +1 514 398 7203 ext. 09182, Fax: + 1 514 398 8900

ABSTRACT

Sjogren's syndrome and radiotherapy for head and neck cancer result in irreversible loss of functional salivary acini and xerostomia, for which no effective treatment is available. Implantation of autologous functional salivary acini would be a major achievement toward solving this problem. However, tissue-engineering of primary human salivary glands (huSG) would require the generation of a great number of such acini. When huSG cells were expanded, they exhibited a ductal phenotype and grew slowly. Here, we aim to explore the potential of the easily expandable human mesenchymal stem cells (MSCs, from bone marrow) to differentiate into functional salivary acini for further use as a graft in a tissue-engineered artificial salivary gland device. Adult human MSCs were cocultured with fresh huSG cells (biopsies) using a double chamber Transwell-clear system. As revealed by quantitative real time-PCR, MSCs in cocultures expressed α-amylase (acinar gene) and claudin-1 (tight junction [TJ] gene). Immunocytochemistry revealed that 20-40% of MSCs express TJ proteins as well as acinar-specific proteins; AQP-5 (water channel) and α-amylase. Moreover MSCs exhibited a noticeable level of transepithelial electrical resistance (TER), and were able to secrete α-amylase into the media when removed from the coculture system for up to 7 days, indicating successful differentiation into polarized functional salivary acini. Additionally, TEM examination confirmed the formation of secretory granules and TJ structures resembling those of huSG. Our culture model represents a proof-of-concept of differentiation of MSCs into huSG acini using serum-free culture medium. These data support the potential and feasibility of using MSCs as a novel source of graft for an implantable tissue-engineered artificial salivary gland device for the treatment of xerostomic patients.

INTRODUCTION

Xerostomia; salivary glands hypofunction, is a result of an irreversible salivary gland damage that is caused mainly by two conditions; the autoimmune exocrinopathy Sjogren's syndrome affecting 1-4 millions in US and radiotherapy for head and neck cancers (30,000 new cases each year). Moreover, some systemic diseases such as diabetes mellitus and pernicious anemia would cause xerostomia (1-5). In addition, in 20% of xerostomic patients the cause is idiopathic (6,7). The clinical negative sequelae of salivary gland hypofunction include; an increased incidence of dental caries, periodontitis, candidiasis, mucositis, gastric and esophageal ulcers (2). Currently, the available treatment for xerostomic patients includes saliva-stimulants and artificial saliva (8,9). This symptomatic treatment is temporary as permanent curatives for such condition are not available yet.

We have been working to develop a tissue-engineered artificial salivary gland device that would be implanted into a surgically created pouch in the patient's mouth (10,11). The standard design for such device includes; a blind-end tube fabricated from a slowly biodegradable scaffold coated with extracellular matrix on its inner (luminal) surface in order to promote attachment and polarization of epithelial cell monolayer; the graft cells that should be capable of unidirectional fluid secretion (147). The autologous graft cells (acinar cells) should be able to secret fluid towards the oral cavity. For these cells to function properly, they must be polarized and form an adequate epithelial barrier (10,11). To secrete fluid unidirectionally, cells must express a group of transport proteins (tight junctions; TJs) that generate an osmotic gradient and control the paracellular movement of water, proteins, and small solutes (148-151,153,154,158). Human salivary cells do express these TJ proteins at their apico-lateral membranes (155). The major hurdle has been to obtain a suitable graft cell type. We have succeeded in culturing and

expanding primary human salivary gland (huSG) cells that are polarized and can provide an adequate functional epithelial barrier. However, they grow very slow; consequently do not provide adequate cells for the envisioned device. Moreover, most of these cells have a ductal phenotype and thus, are not fluid secretory cells (11). When huSG cells were cultured on Matrigel (a basement membrane extract containing extracellular matrix components), they formed acinar-like units (unpublished data, 374). However, Matrigel is derived from mice sarcoma and would not be used in clinical applications (as a part of the artificial salivary gland device). While testing other extracellular matrix sources that would be safe and efficient for clinical applications, we thought about using stem/progenitor cells that have the potential to differentiate into polarized functional salivary acinar cells.

Salivary glands, like other parenchymal tissues contain stem/progenitor cells (167-170), but their exact source is not yet understood. The patterns of repair and regeneration in adult salivary glands suggest that they contain a stem cell-like population within the intercalated ducts (168). In addition, almost all differentiated cell types of adult glands have the ability to divide. It appears that division by both stem cells and differentiated cells contribute to the proliferation/ turnover of salivary cells (168). The identification and in vitro expansion of acinar stem/ progenitor cells that would be used as autologous graft cells in our artificial salivary gland device would be an important achievement for the treatment of xerostomic patients. In an attempt to identify and localize stem/progenitor cells human salivary gland tissue, CD44+/CD166+ cells were localized in serous and mucous acini, respectively (unpublished data, 240). Therefore, we would conclude that salivary gland tissue harbors mesenchymal stem/progenitor cell-like populations. However, the use of salivary gland stem cells might be difficult for clinical application if an insufficient number of stem cells are obtained from the patient's gland biopsies. Both patients with either

Sjogren's syndrome or head and neck cancers are old and the gland tissues tend to be atrophic in older patients.

Another source of stem cells that have been suggested is the bone marrow that shelters two types of stem cells; hematopoietic (117) and mesenchymal (171). The capacity to differentiate into mesodermal (110,173), ectodermal (173) and endodermal (174) cell lineages characterizes mesenchymal stem cells (MSCs) as pluripotent cells. MSCs were reported to differentiate in vitro into several cell types based on culture conditions (110). They have a tendency to acquire tissue-specific characteristics when cocultured with specialized cell types or exposed to tissue extracts in vitro (175-177). Many reports highlighted the heterogeneity of MSCs population (304), therefore, a panel of key markers is used to isolate MSCs (178) as no specific stem cell marker has been identified yet. Many studies have successfully isolated MSCs-like populations from different tissues; adipose tissue (179,180), bone marrow (171,172,181), synovial membrane (182), lungs (181,183), brain, spleen, liver, kidney, large blood vessels, muscle, thymus, pancreas (181), and salivary glands (184). Owing to the great expansion and differentiation potential of MSCs that are mainly present in the bone marrow (185), the aim of this study was to explore the potential of human MSCs to differentiate into huSG in Transwell-clear coculture system. Here, we report that 30-40% of human MSCs were successfully induced to differentiate into functional polarized salivary acini as confirmed by morphological, phenotypical and functional analyses. Therefore, these differentiated MSCs would be used as a graft in an envisioned artificial salivary gland device, ready for in vivo trials in animal models before clinical application.

MATERIALS & METHODS:

MSCs Culture: human MSCs from bone marrow were purchased from Cambrex (Charles City, IA, USA). As indicated by the company, MSCs received were from healthy adult donors (n=5; 3males and 2 females) with age ranging between 20-35 years. Cells were expanded in T-75 tissue

culture flasks containing 15 mL media: the MesenCult® Proliferation Kit that contains MesenCult® MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC, Canada). The flasks were incubated at 37°C in 5% CO_2 and 50% of medium was changed every 3-4 days until cells reached 80-90% confluence (generally within 7 days). MSCs were passaged using 0.05% trypsin-EDTA (Gibco, Invitrogen, Grand Island, NY). Cells used in the current experiments were from passages 5-11.

Source of huSG Tissue: Portions of human submandibular (n=7) and parotid (n=3) salivary glands, from both men (n=6) and women (n=4), were obtained from the Department of Pathology, University of Virginia, through the Cooperative Human Tissue Network (CHTN) and the Department of Pathology, Royal Victoria Hospital (RVH), McGill University. The patients' age ranged between 20 and 73 years. For tissue use, we received an exemption from the Institutional Review Boards at McGill University. This report describes results obtained with glands that were excised as part of the resection of head and neck cancers. Patients, from whom samples were obtained, had received no prior irradiotherapy or chemotherapy. Pathologists at the University of Virginia (Charlottesville, VA) and RVH (Montreal, Quebec) performed surgical pathologic examination of the glands at the time of excision. Further detailed histopathologic examination confirmed that all glandular tissues reported here were judged as normal tissues. The tissue was shipped on wet ice in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1% antibiotic-antimycotic solution.

Preparation of huSG for Culture: The received tissue pieces (0.5-1.5g) of huSG were washed twice in F-12 medium (Gibco, Invitrogen, Grand Island, NY) supplemented with antibiotic–antimycotic solution (200U/mL penicillin, 200µg/mL streptomycin, and 5µg/mL Fungizone; BioFluids, Rockville, MD). The pieces were mechanically minced with a pair of curved scissors until the tissue gave an appearance of a slurry thick paste. The minced tissue was transferred into

35mL of dissociation buffer and incubated for 4–5 h at 37°C incubator on a shaker, additionally, vigorous vortexing was applied every 30 min. The dissociation buffer contained Liberase Blendzyme-3 (0.2U/mL, Roche Diagnostic, Indianapolis, IN) in Dulbecco's modified Eagle's medium (DMEM; BioFluids, Rockville, MD). The cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and cold DMEM was used to wash the cell pellet (two times). After washing, the cell pellet was suspended in 5mL of culture medium: serum-free Hepato-STIM medium (BD Biosciences Discovery Labware, Bedford, MA), supplemented with antibiotic–antimycotic solution (500U/mL penicillin, 500µg/mL streptomycin, and 12.5µg/mL Fungizone) and 1% glutamine. This cell suspension was then filtered through a cell strainer (70µm pore size; BD Biosciences Discovery Labware), plated onto the lower compartment of Transwell-Clear system, and incubated at 37°C in 5% CO_2. The culture medium was changed twice a week.

MSCs Coculture with huSG: For induction of biological MSCs differentiation into huSG, cocultures of MSCs (passages 5-11) and freshly dissociated huSG cells were performed, using Transwell-Clear system plates with polyester membranes (Corning Life Sciences, Acton, MA) which allows direct visualization under light microscope. These culture plates are commonly used to produce a cell culture microenvironment that closely simulates the in vivo state. The huSG cells ($6x10^4$ cell/cm^2) were cultured in the lower compartment while MSCs ($2x\ 10^4$ cells/cm^2) were cultured on the membrane (in the upper compartment). Both types of cells were exposed to the same culture media conditions without direct cell-cell contact. The porous membrane of the insert is formed of clear polyester that allows clear examination and picturing of cells. Here, we used plates of membranes with a 0.4 µm pore size and 12mm and 24mm insert diameter. In these conditions, the small pore size, relative to any human cell size (10µm, 375) did not allow the migration of either huSG or MSCs to the other chamber compartment. We used

serum-free Hepato-STIM medium for the cocultures. For control, MSCs were cultured alone; on the membrane in the same cell density with serum-free Hepato-STIM medium and no huSG cells were added to the lower compartment. These cultures and cocultures were maintained at 37°C in 5% CO2 for up to 14 days. Both MSCs and huSG cells were analyzed at different time points (1, 2, 3, 5, 7, 9, 14 days).

Osteogenic Differentiation: MSCs from passage 5-10 were cultured in 6-well plastic plates ($1x10^4$cell/cm^2) with an osteogenic medium composed of; DMEM/F-12 supplemented with 10% FBS (Hyclone), 10nM dexamethasone, 5µg/ml sodium ascorbate-2phosphate, 10mM β-glycerol-phosphate and 100U penicillin/100 µg streptomycin/0.25 µg fungizone. Cultures were fed with fresh media every 3 days. After 21 days, media were removed and cells were washed with 0.09% NaCl and fixed in 70% ethanol. Afterward, cells were rinsed in distilled H_2O and stained with Von Kossa stain; incubated in a 1% (wt/vol) silver nitrate (Sigma Aldrich, Oakville, ON, Canada) solution in the dark for 60 min and washed several times with distilled H_2O then developed under direct light for 60 min.

Measurement of TER: huSG cells were seeded ($6x10^4$ cell/cm^2) on 24-mm Transwell-Clear polyester filters and grown as mentioned above. The upper chamber containing the cells received 1.5mL of medium, and the lower chamber (no cells) received 2.6mL of medium. For huSG and MSCs (either in cocultures or in control cultures), TER was measured at day 7 and 14 in 6 separate determinations, using a Millicell ERS epithelial volt-ohmmeter (Millipore Corp., Allen, TX) as described by the manufacturer. MDCK-II cells cultured on filters were used as positive control (MDCK-II cells have high TER and express TJs). MDCK-II cells obtained from BD Biosciences Clontech (Palo Alto, CA) were maintained in DMEM/F-12 supplemented with 10% FBS (HyClone), 100U/mL penicillin, 100µg/mL streptomycin, and 2.5µg/mL Fungizone and

incubated at 37°C in 5% CO2. TER readings from filters without cells (blank) were subtracted from readings obtained from filters seeded with cells.

Immunocytochemistry & Confocal Imaging: to analyze MSCs either in cultures or cocultures, at day 7, cells were fixed with 10% paraformaldehyde in PBS (pH 7.4) for 30 min. Cells were rinsed with PBS, and permeabilized with methanol at 20°C for 5 min followed by incubation with 0.2% Triton X-100 for 10 min at room temperature, followed by three washing steps in PBS for 5 min each. The following primary antibodies were used to characterize huSG huSG and MSCs: rabbit anti-claudin-1, claudin-2, claudin-3, occludin, JAM-A and mouse anti-ZO-1, claudin-4 from Zymed; Goat anti-aquaporin-5 from Santa Cruz; rabbit anti-α-amylase from Sigma Aldrich. Moreover, the following antibodies were used to characterize/confirm the mesenchymal stem cell phenotype of control MSCs; Mouse multipotent mesenchymal/stromal cell marker (MSCs) antibody panel kit from R&D Systems. The panel contains a group of antibodies for the positive identification of human MSCs; anti-Stro-1, anti-CD90, anti-CD106, anti-CD105, anti-CD146, anti-CD166, anti-CD44, plus leukocytes markers; anti-CD19 and anti-CD45. In addition, we used goat (R&D Systems), rabbit and mouse (Zymed Labs) isotype control antibodies. The antibodies used are reactive against the respective proteins from human and other species. The cells were incubated with blocking solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and 0.5% bovine serum albumin in PBS for 1h at room temperature. Cells were incubated with the primary antibodies (diluted 1:100 in blocking solution) overnight at 4^{o}C while incubated with either donkey anti-mouse/rabbit/goat-Rhodamine Red-X-conjugated (RRX), (Jackson ImmunoResearch Laboratories) secondary antibodies for 1h at room temperature (diluted 1:100 in blocking solution) in dark. Finally, 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) was added for 3-5 minutes. The membranes on which MSCs were cultured and processed for immunocytochemistry were cut-off the wall of

their inserts at their periphery using fine tweezers. The membranes were then mounted on glass slides with cover-slips. Fluorescence images were taken using Zeiss LSM 510 laser scanning confocal microscope (Jena, Germany). Images shown are representative of at least 3 separate experiments with multiple images taken per slide.

Western Blot Analysis: MSCs and huSG both in cultures (control) and in cocultures (at 1,3,5,7 days) were washed twice with cold PBS and lysed in 200 µl/well of cold RIPA buffer and kept on ice for 5 minutes. Lysates were collected into microcentrifuge tubes and centrifuged at 10,000 x g for 20 minutes to pellet the cell debris and keep the supernatants for further analysis. Protein samples, 30µg each, from supernatants and collected media were subjected to 10% SDS-PAGE on mini-gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% fat-free dry milk in Tris-buffered saline [0.137 M NaCl, 0.025 M Tris (hydroxymethyl)-aminomethane, pH 7.4] containing 0.1% Tween-20 (TBST) and immune-blotted overnight with rabbit anti-α-amylase antibody (1:2000 dilution; Sigma Aldrich) at 4°C in TBST containing 5% BSA and 0.02% sodium azide. After incubation with primary antibody, membranes were washed 3 times for 15 min each with TBST and incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The membranes were washed 3 times for 15 min each with TBST then treated with chemiluminescence detection reagent containing 20 mM Tris buffer (pH 8.5), 250 mM Luminol and 90 mM coumaric acid (Sigma Aldrich). Protein bands were visualized on X-ray film. All experiments were performed in duplicate and repeated at least 3 separate times. Conditioned media from different wells of MSCs control cultures and huSG cocultures/cultures were collected and analyzed with western blot. In addition, inserts of MSCs in cocultures were transferred (as they are after coculture for 1,3,5,7 days) into new plates with no cells in the lower compartment. Their media were discarded, then MSCs were washed 3 times with DMEM media

and new serum-free Hepato-STIM media were added to the cells. Afterward, MSCs were maintained at their new condition for additional 1,3,5,7 days then their media were collected and analyzed by western blot.

TEM: huSG cells and MSCs from cocultures and control cultures in 12-well plates were fixed (at 1, 3, 5, 7, 9,14) in 2% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in 0.1 M cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1 h. After rinsing in cacodylate buffer, samples were dehydrated through an ethanol series and infiltrated and embedded in Epon812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections (70 nm) were cut with a diamond knife and mounted onto copper grids. Grids were stained with 3% uranyl acetate for 30 min and 2% lead citrate for 5 min, then examined at various magnifications using a 100-CXII TEM (JEOL, Tokyo, Japan) at an accelerating voltage of 120 kV.

Quantitative Real-Time PCR Analysis: At day 1, 2, 3, 5, 7, 9, huSG cells and MSCs from cocultures and control cultures were washed in PBS, then total RNA was isolated using the RNeasy micro kit (Qiagen Ltd, Crawly, UK) with in-column DNase digestion. The concentration of RNA was determined using Qubit (Molecular Probes). Total RNA (2 μg per sample) was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in 20 μL volume. For polymerase chain reaction (PCR) amplification, 5% of the cDNA was used with real-time PCR primers and 6-carboxy-fluorescein (FAM)-labelled minor groove binder probes (MGB). The probes and primers for claudin-1(CLDN-1), α-amylase (AMY) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an endogenous reference) were selected from the Applied Biosystems. PCR reactions (20 μL) were performed in duplicates using TaqMan Universal Master Mix (Applied Biosystems) on a Prism Sequence Detection System 7500 (Applied Biosystems) with the default settings (50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles

[95°C for 15 s, 60°C for 1min]). Every culture experiment was repeated at least 5 times. Gene expression levels were calculated by normalizing the target RNA value to the value of GAPDH in the same sample. Results are expressed as fold-changes in gene expression relative to control huSG samples cultured on plastic for 9 days (expressing the lowest gene levels).

Statistical Analysis: Data are presented as means ± SEM of results from 3 or more separate experiments. Our data were analyzed by Student's *t*-test where P value < 0.05 represents the significant differences between groups at specified times. Cell percentages presented in this study have been determined upon examination of at least 500 cells per slide or grid.

RESULTS

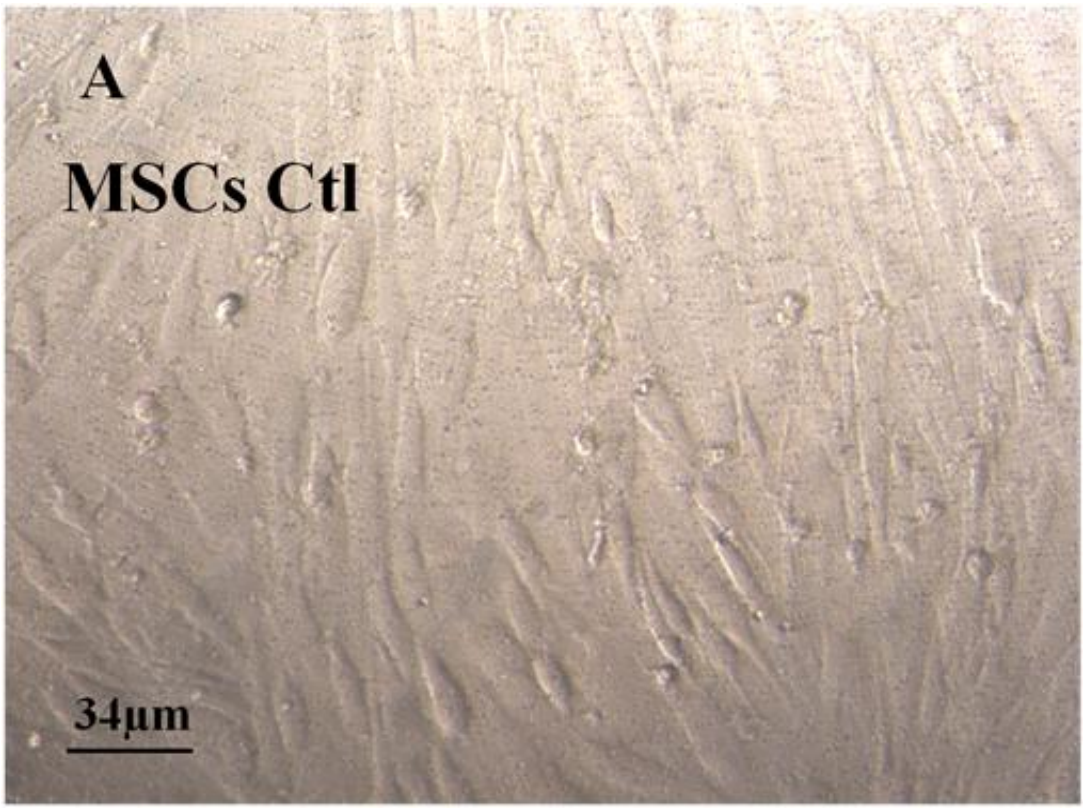
Morphology of MSCs in Culture: around 70% MSCs expanded in T-75 flasks attached within 24h, started spreading; most of the non-adherent cells were removed when culture media were changed after 24h of culture. Small colonies of 8-10 cells were formed at day 3 after incubation and extended very quickly thereafter. The cells morphology appeared to be compact, uniform, fibroblast-like colonies. At day 5-7, colonies began to fuse and form monolayer upon reaching 80-90% confluence. All cells showed typical MSCs morphology. The same findings were observed with control MSCs cultured alone in Transwell-clear system (Fig.7.1A).

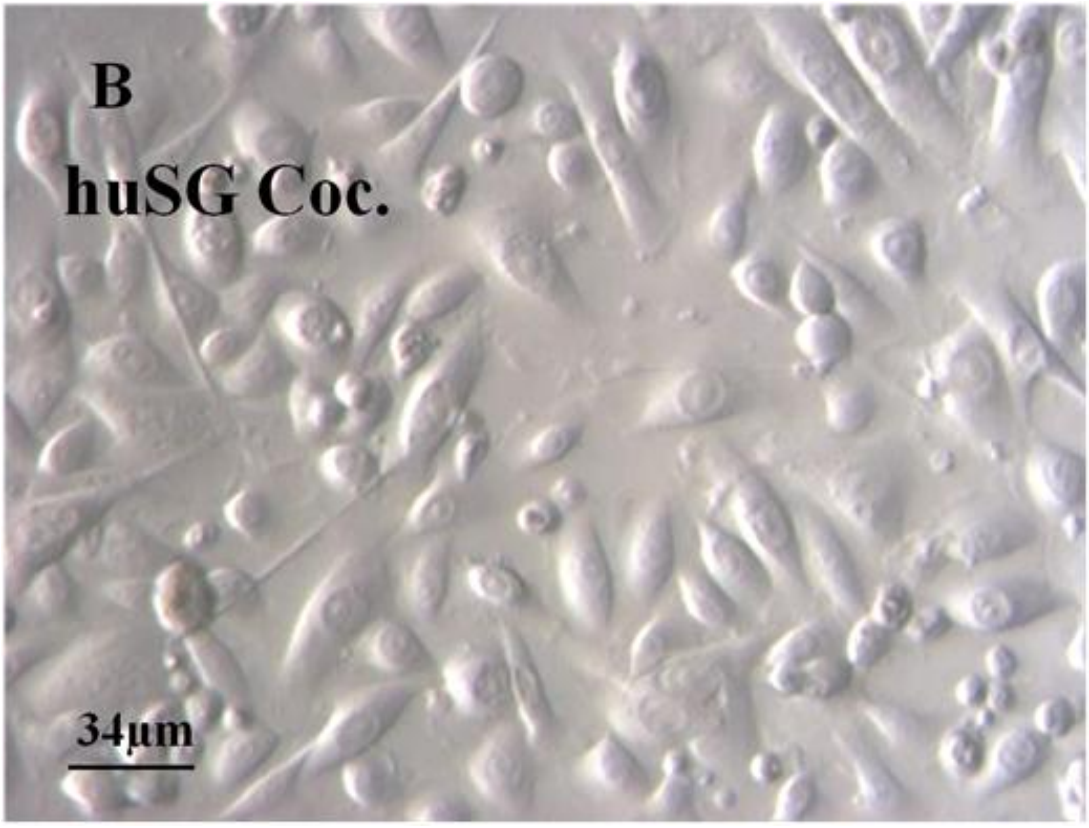
Morphology of huSG Cells in Coculture: The mechanically and enzymatically digested huSG tissue initially consisted of floating single and clumps of (10-20 cell/clump) cells. After 24h of initial plating on the plastic surface of Transwell lower compartment; 10-30% of cells attached to the dish, most of which were clumps. Small cobblestone islands (20-50 cells) of epithelial-like cells were observed at day 2-7. As cells proliferated, these islands enlarged until fused to form epithelial-like sheets of round, oval, and polygonal cells (Fig.7.1B). At 7-14 days, 30% of the superficial cells from those forming the huSG clumps started to degenerate, disunite and became

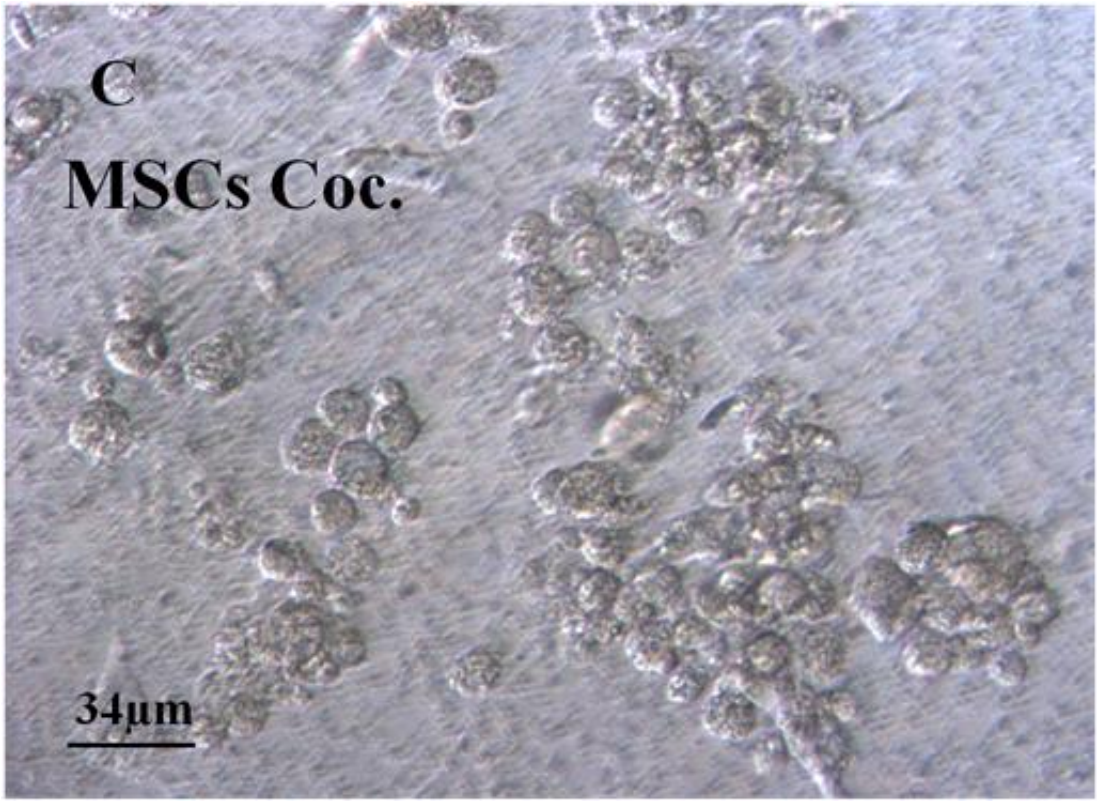
floating in the media while the lower cells stayed intact. The huSG cells took approximately 7-14 days to become 80-90% confluent. No differences in results were seen between the sexes or gland type. However, in regard to age; the huSG cells derived from old patients (60-73 years) appeared slower to attach and become confluent relative to those from younger patients (20-52 years).

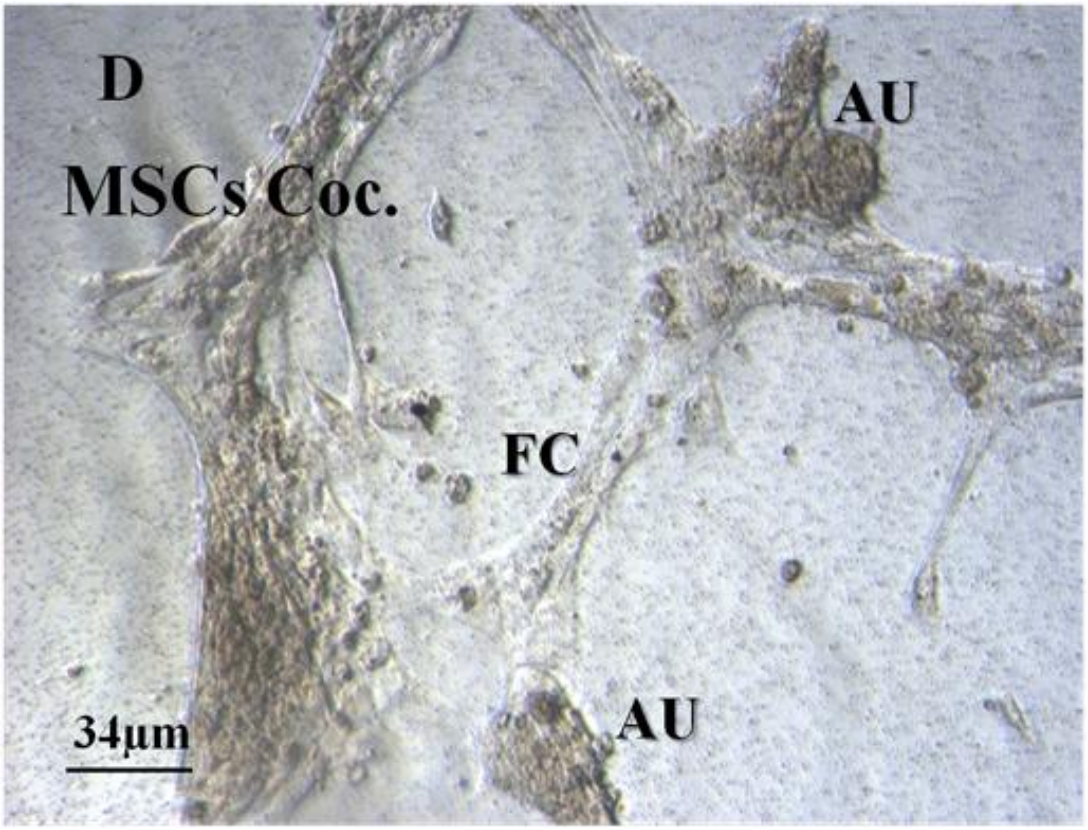
MSCs Differentiation into huSG: in vitro, to further test the hypothesis that MSCs are capable of differentiation into functional epithelial salivary cells, we co-cultured MSCs with freshly minced and enzymatically digested huSG cells (using Transwell-clear system) and analyzed both types of cells at day 1, 2, 3, 5, 7, 9, 14. Rapidly, 40% of MSCs lost their characteristic fibroblast-like morphology when initially cocultured with huSG cells and became round after 24h; where 6-10 cells clumped together (Fig.7.1C). At day 3, MSCs clumps formed salivary-like ductal/acinar units (8-12 cell/unit) that appeared connected to fibroblast-like MSC-cord-like structures (Fig.7.1D). At day 7-14, some of the superficial cells forming the salivary-like ductal/acinar units started to degenerate and disunite leaving only flattened polygonal epithelial-like MSCs (which initially attached to the filters and supported the whole salivary-like ductal/acinar units, Fig.7.1E). The degenerating cells were seen floating in media. No differences in results were seen between the sexes or gland type or in regard to the patient's age (20-35 years).

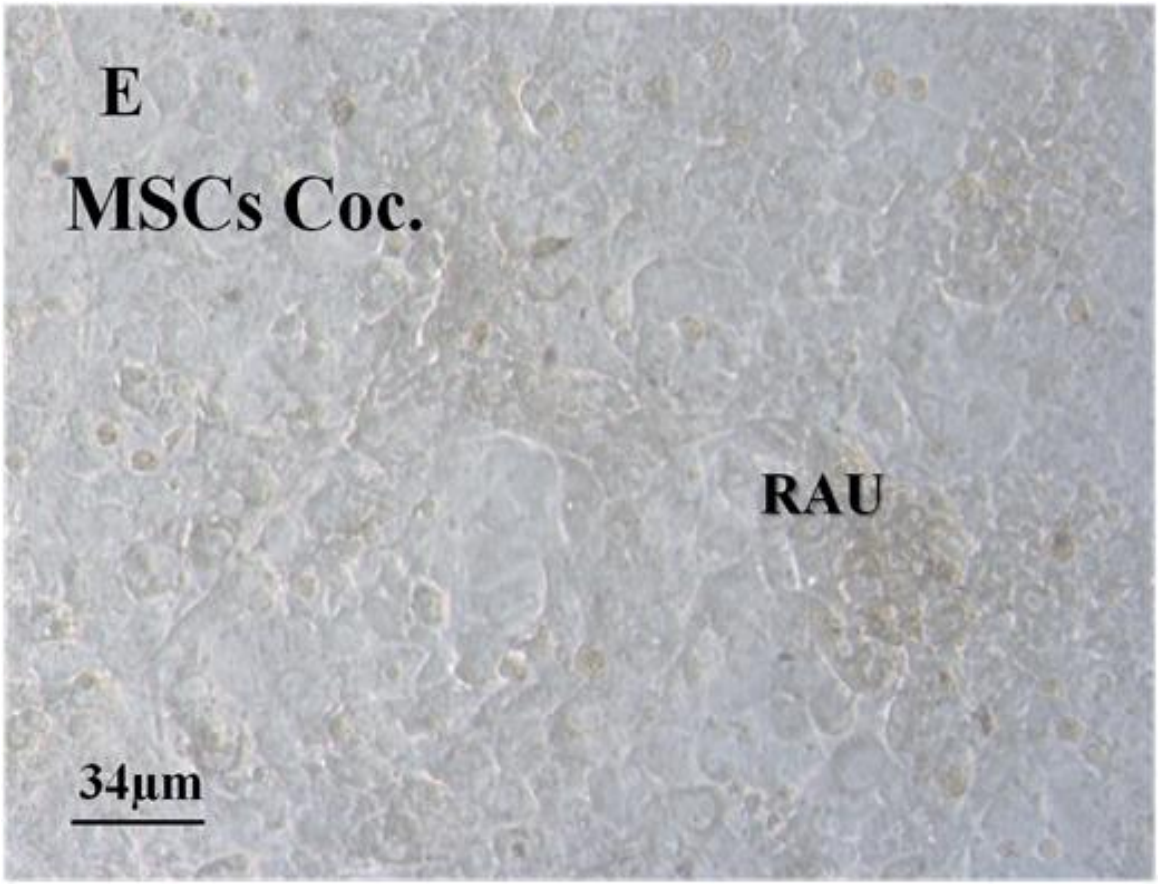
Figure 7.1: (A) MSCs control cultured alone on Transwell-clear filters for 5 days showing fibroblast-like morphology. (B): huSG cells cocultured in the lower compartment of Transwell-clear system for 7 days, showing small cobblestone epithelial-like cells. (C): MSCs cocultured with huSG on Transwell-clear filters for 1 day showing rounded-clumps morphology. (D): MSCs cocultured for 3 days forming acinar-like units (AU) connected together via fibroblast-like cords (FC). (E): MSCs cocultured for 7 days showing epithelial-like polygonal cells that established the attachment of the acinar-like units; some remnants of previous acinar-like units exist (RAU) [Scale bar= 34μm, X 200].


A
MSCs Ctl
34µm


B
huSG Coc.
34µm


C
MSCs Coc.
34µm


D
AU
MSCs Coc.
FC
34µm
AU


E
MSCs Coc.
RAU
34µm

Osteogenic Differentiation of MSCs: To confirm that MSCs used in these experiments were mesenchymal stem cells phenotypically, their osteogenic differentiation capability was examined.. MSCs changed their morphology in the osteogenic media from spindle-shaped to cubical accompanied by mineral deposition. Approximately 50-60% of MSCs were positive to Von Kossa stain; the calcium phosphate formed in MSCs precipitated along the cell membrane and showed up as black large aggregated particles in the extracellular matrix even after 10 passages in culture (Fig. 7.2A). No mineral deposition was detected by Von Kossa stain in MSCs cultured with ordinary expanding media (Fig. 7.2B).

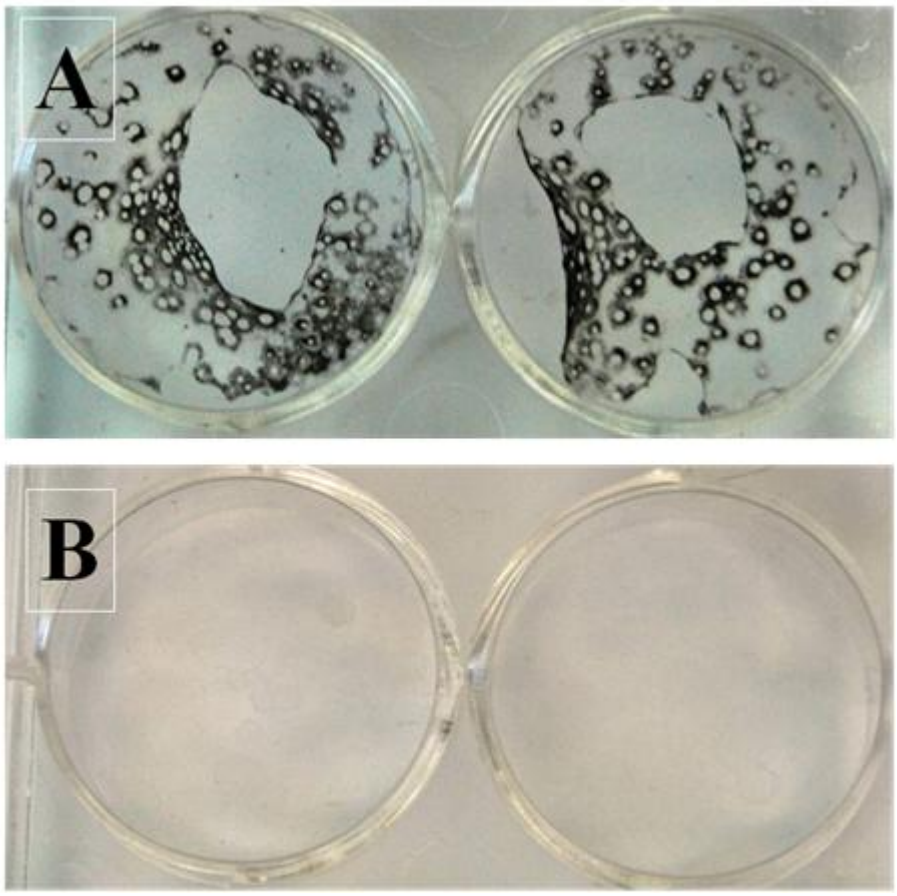


Figure 7.2: Van Kossa-stained 6-well plates to evaluate the osteogenic differentiation of MSCs after 21 days of culture. (A): MSCs cultured in osteogenic media showing 50-60% of MSCs became osteoblasts and deposited calcium phosphate that precipitated along the cell membrane and showed up as black large aggregated particles in the extracellular matrix. (B): MSCs cultured with ordinary expanding media negatively stained (no mineral deposition) by Von Kossa stain.

Measurement of TER: as a standard measure of TJs formation and functional development of acini, TER was assessed across MSCs (in cocultures and in control cultures) and huSG cells seeded on 24-mm Transwell-Clear polyester filters and grown as mentioned above. We compared TER of huSG and MSCs with those obtained by MDCK-II (positive control) monolayers at day 7 and 14 each from 6 separate determinations. Both MSCs in cocultures and huSG exhibited TERs comparable to MDCK-II (126 & 169 Ω.cm^2 for MSCs and 376 & 464 Ω.cm^2 for huSG). TER values of MSCs in cocultures were significantly different ($P<0.05$) from control MSCs cultured alone on filters (Fig.7. 3).

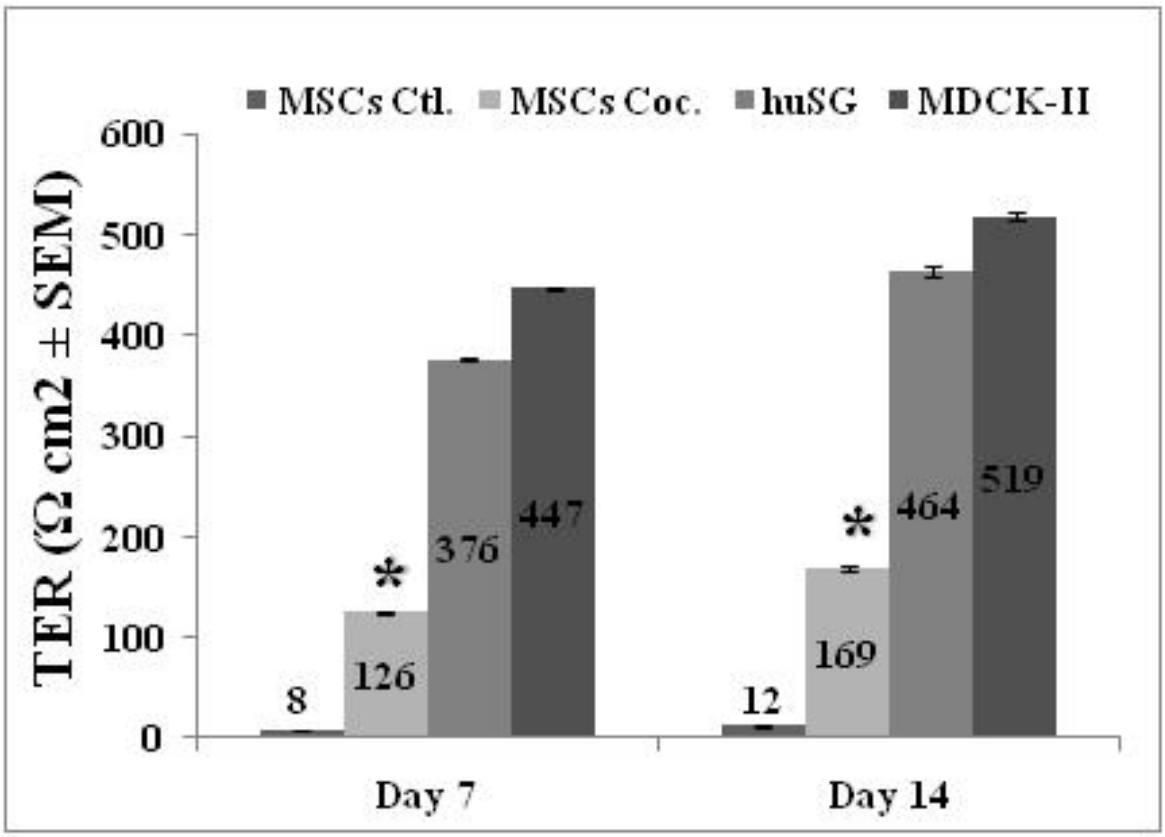
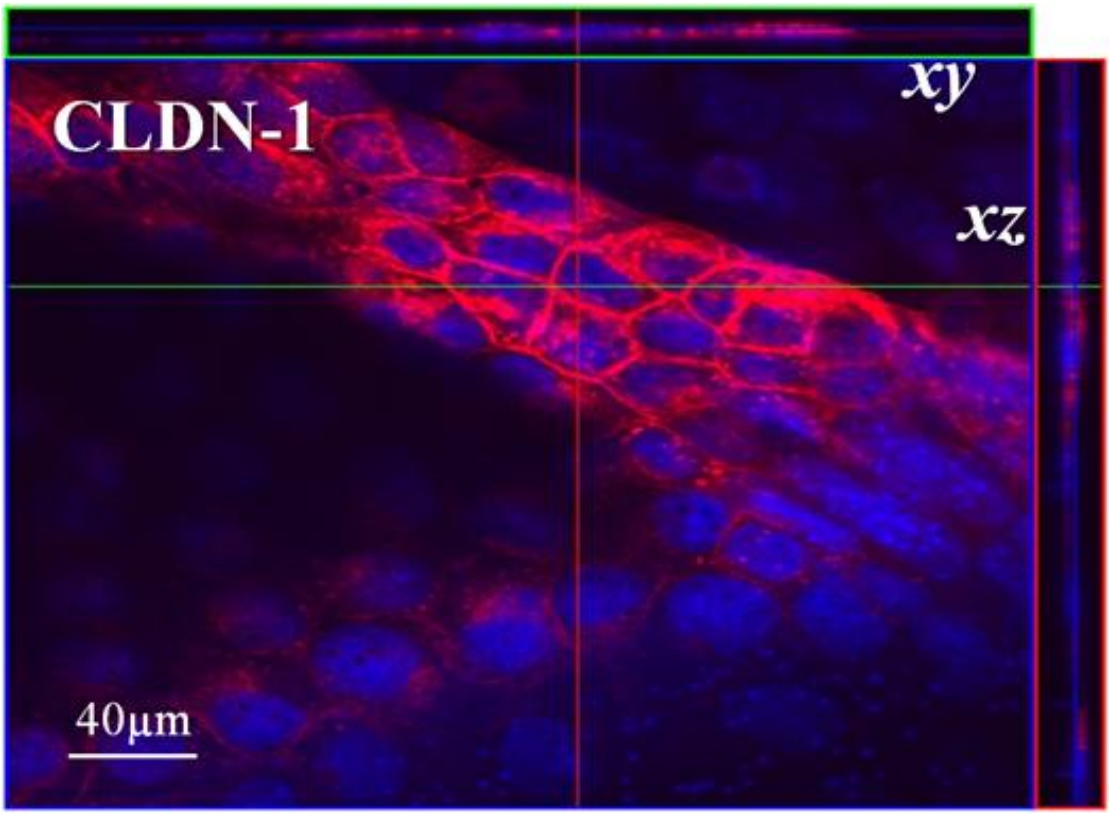
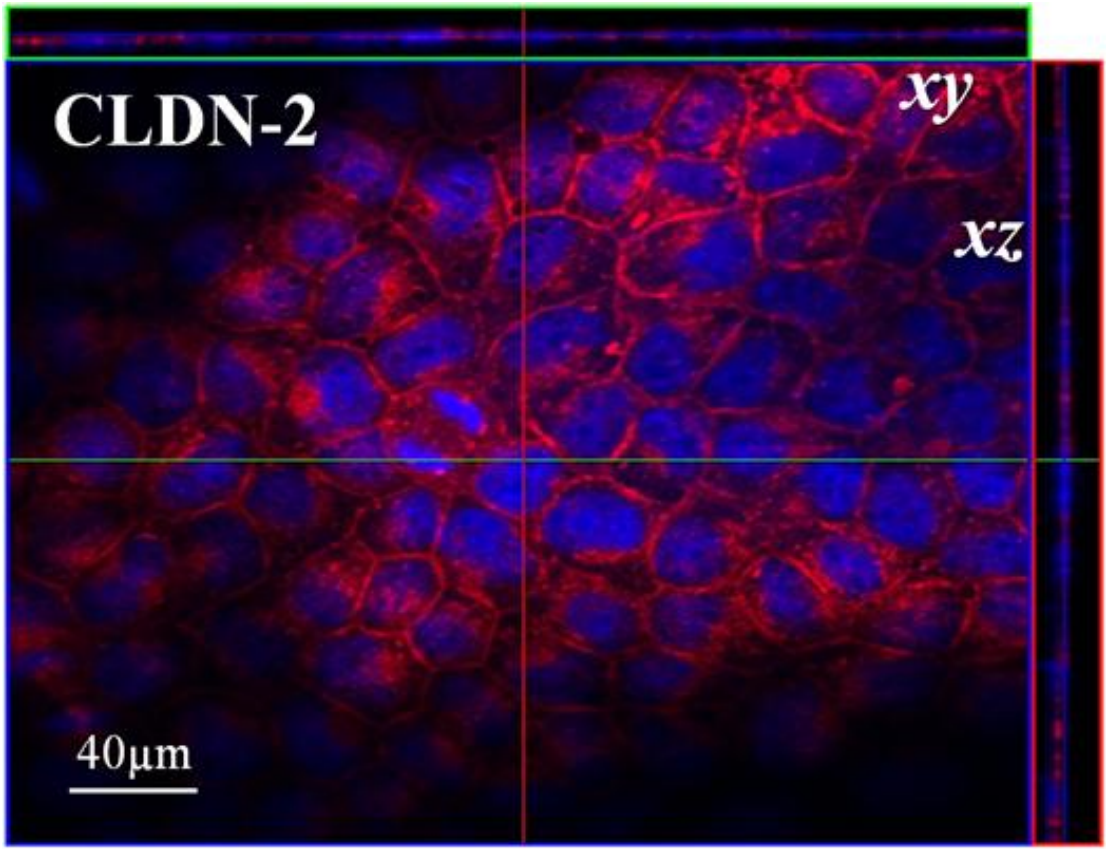


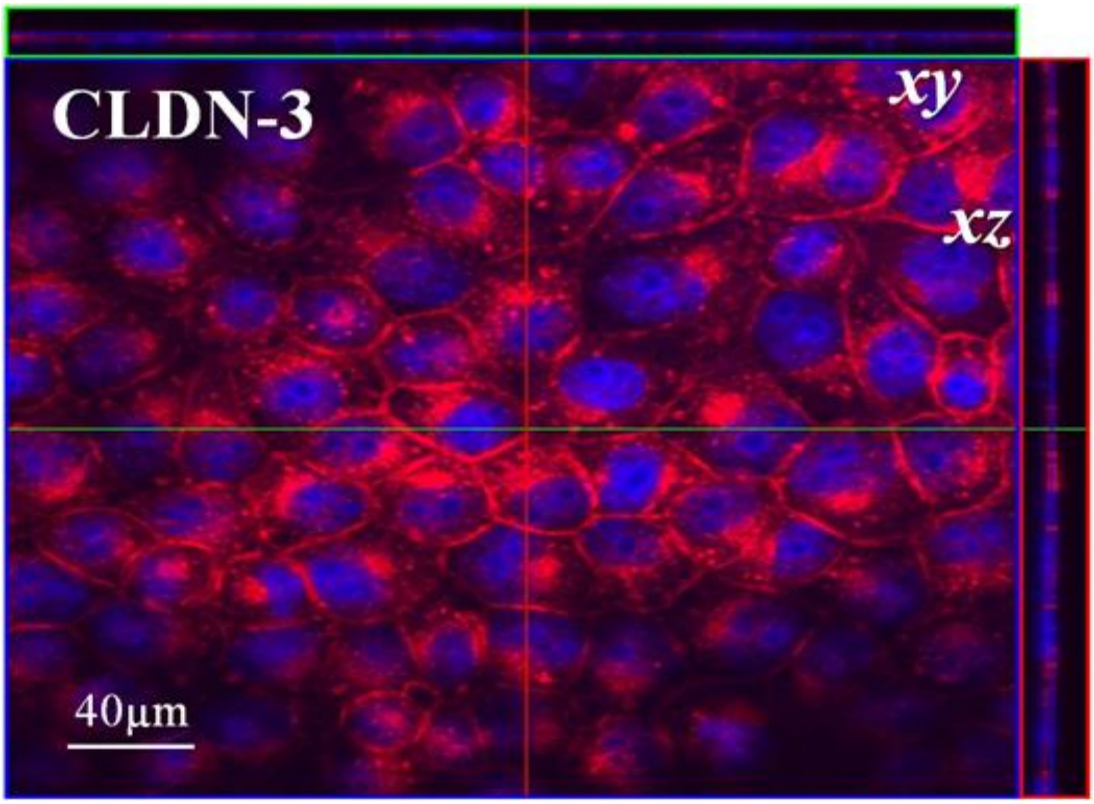
Figure 7.3: Transepithelial Electrical Resistance (TER) measurements at day 7 and 14. MSCs control (MSCs Ctl.) exhibited extremely low TERs while MSCs grown in cocultures (MSCs Coc.), exhibited a noticeable TERs levels comparable to control MSCs. Results represent the average ± standard error of means of 6 separate determinations for all cell types. TER values of MSCs in cocultures were significantly different from those of control MSCs. (*P<0.05).

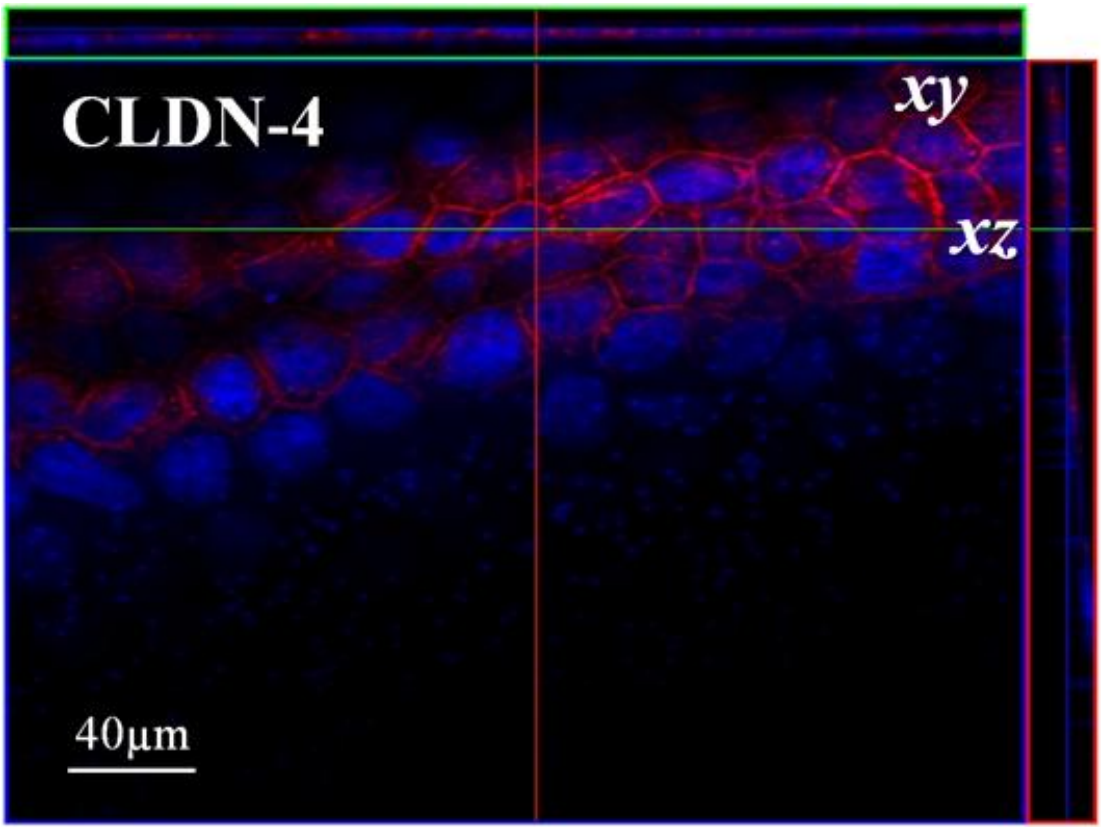
Immunocytochemistry and Confocal Imaging: The examined control MSCs cultured alone on Transwell filters were confirmed to be phenotypically mesenchymal stem cells: negative to CD45 and CD19 expression while positive for CD105, CD90, CD44, CD166, CD146, CD106 and Stro-1 (not shown). On the other hand, 20-40% MSCs in cocultures expressed all TJ proteins tested; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1, in addition to acinar specific proteins; α–amylase and AQP-5 (Fig. 7.4 and Table 7.1). The means difference was significant between each experimental trial and its control ($P<0.01$). Importantly, the Rhodamine Red-X-conjugated (RRX) labeled all TJ antibodies indicated that TJs are localized at the apico-lateral sides of the cells, therefore, these MSCs cells are correctly polarized on the coculture filters. The relative position of TJs to the medium-facing (analogous to "apical" or "lumenal," *in vivo*) and filter-facing ("basolateral") sides of the cells is shown in Fig. 7.4 (*xz* & *xy* planes). For huSG cells, they expressed all TJ proteins tested in 80-90% of cells as well as acinar-specific proteins; α–amylase (60%), AQP5 (50%) as shown in (Table. 7.1).

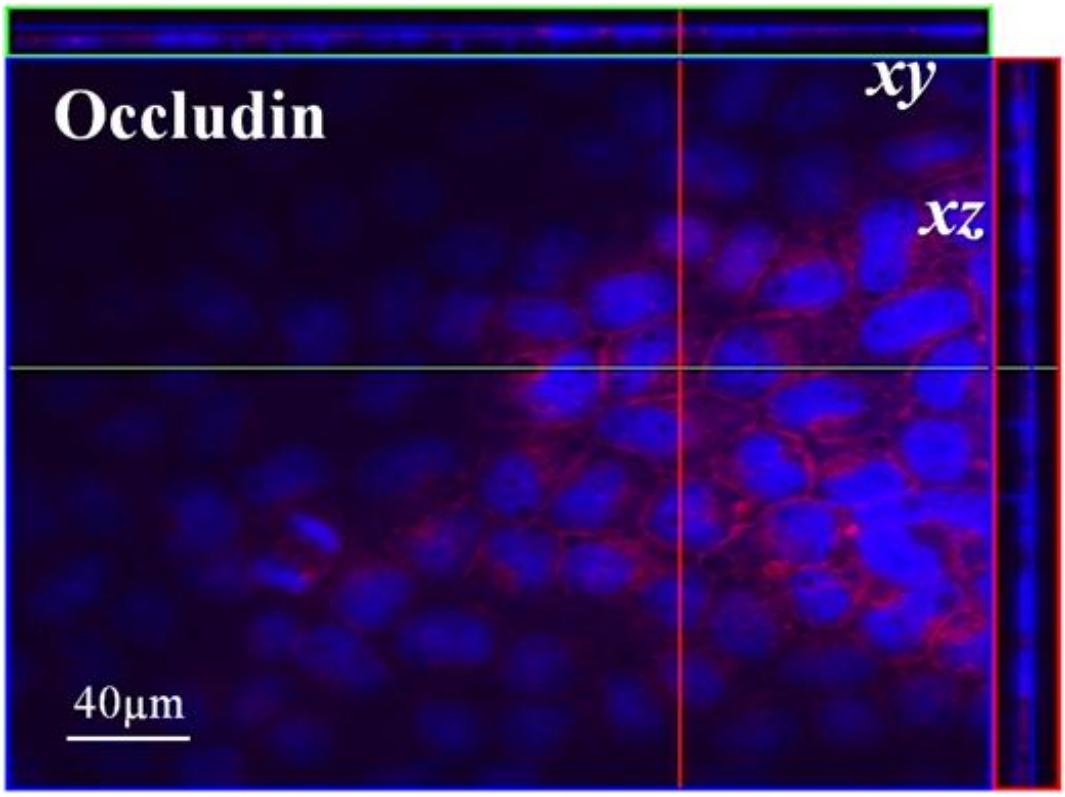
Figure 7.4: Confocal micrographs of MSCs cocultured with huSG for 7 days expressed all TJ proteins tested. The *xy* plane demonstrating the presence of TJ proteins; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1, respectively (shown in red). The nuclei are stained with DAPI (shown in blue). Both *xz* and *xy* planes show TJ proteins as correctly localized at the apicolateral cell membranes of MSCs cocultured on the Transwell polyester filters. In addition to the expression of acinar secretory α–amylase and AQP-5 water channels proteins (shown in red) in 40% and 20% of MSCs respectively. [Scale bar= 40μm, X 630].

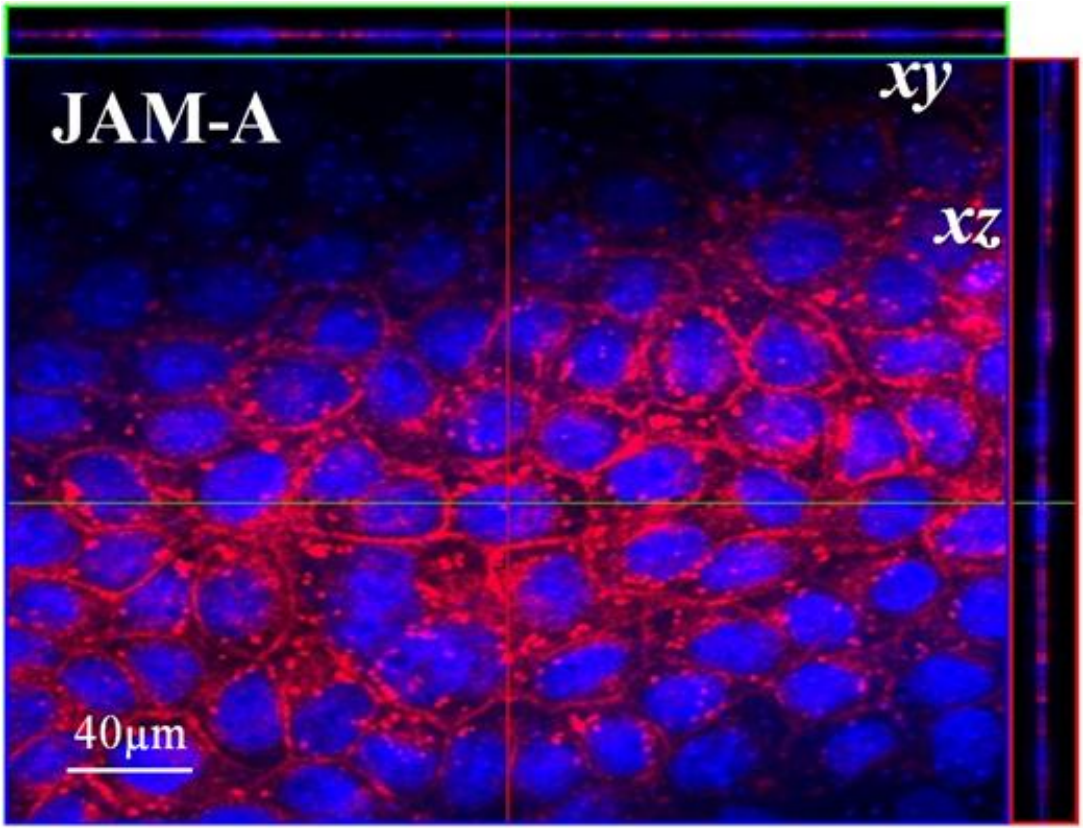

CLDN-1
xy
xz
40µm

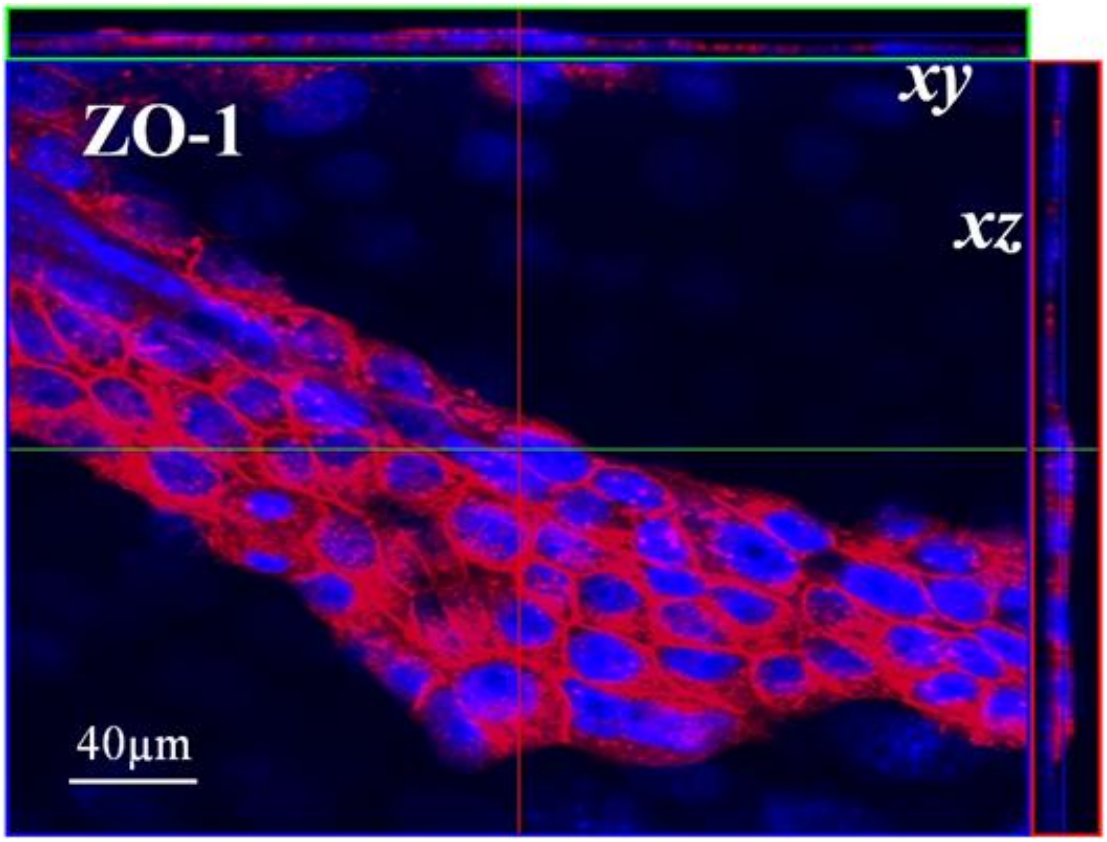

CLDN-2
xy
xz
40µm

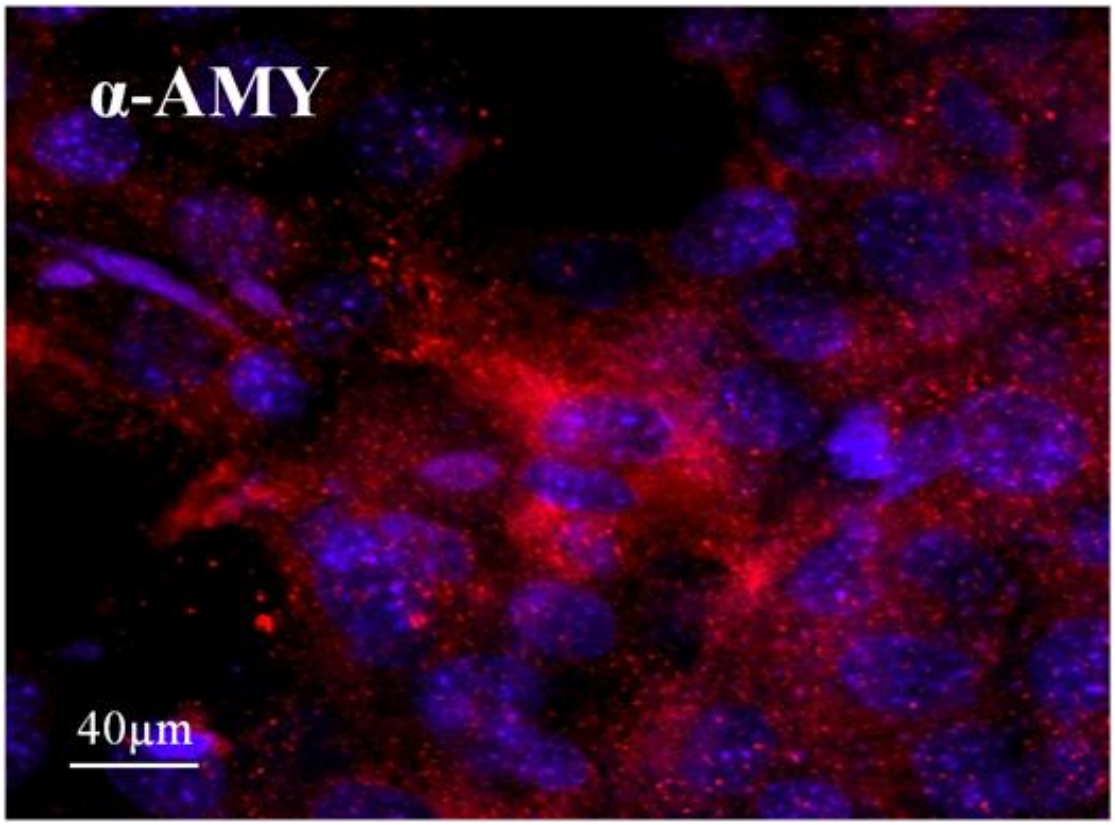

CLDN-3
xy
xz
40µm

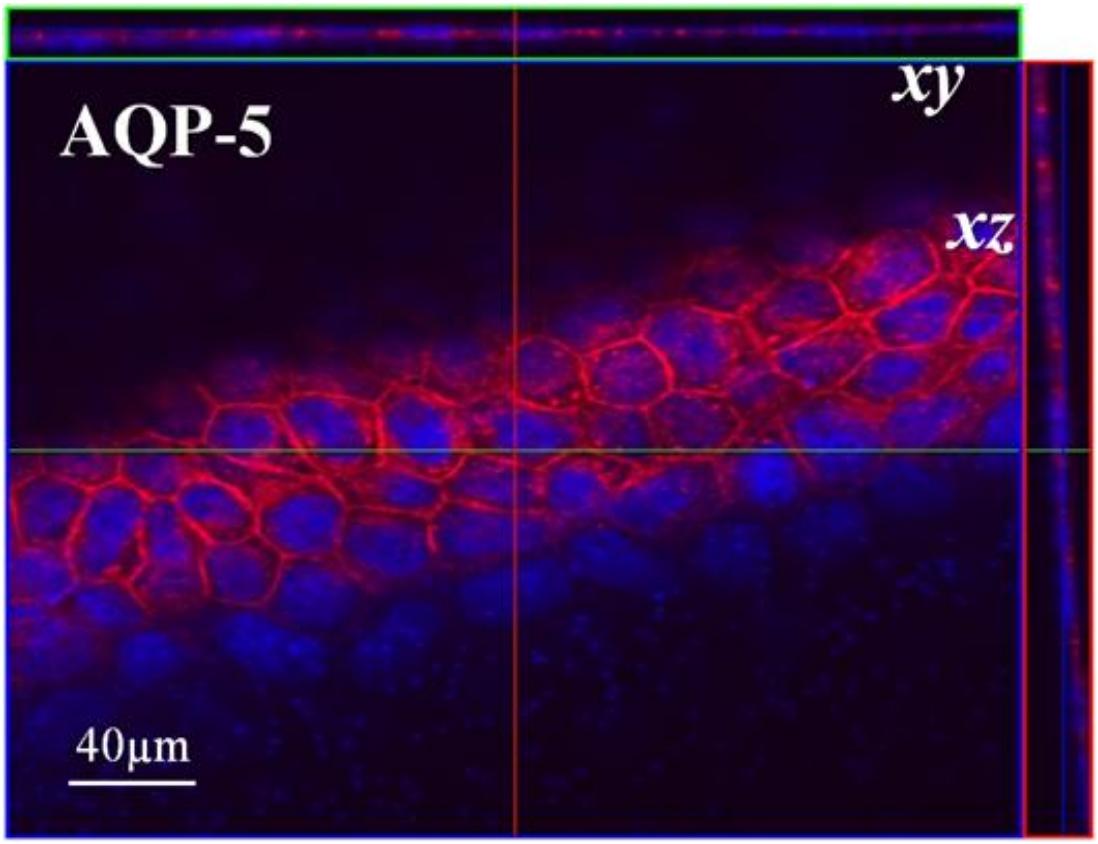

CLDN-4
xy
xz
40µm


Occludin
xy
xz
40µm


JAM-A
xy
xz
40µm


ZO-1
xy
xz
40μm


α-AMY
40μm


AQP-5
xy
xz
40µm

Marker	CLDN-1	CLDN-2	CLDN-3	CLDN-4	Occludin	JAM-A	ZO-1	α-AMY	AQP-5
MSCs Control	0%	0%	0%	0%	0%	0%	0%	0%	0%
MSCs Coculture	36%	40%	39%	30%	25%	35%	20%	40%	20%
huSG Coculture	89%	90%	90%	90%	80%	86%	80%	60%	50%

Table 7.1: Expression of different markers/proteins in MSCs and huSG cells grown in cocultures and control MSCs grown alone in cultures. Results are based on the examination of 5 filters mounted on slides per culture type. At least 500 cells were examined per slide. * The expression level of each marker/protein is indicated as percentage ±SEM. Tight junction proteins; claudin-1, -2, -3, -4 (CLDN), Occludin, junctional adhesion molecule-A (JAM-A), and zonula occludens-1 (ZO-1); in addition to α–amylase (α-AMY, salivary secretory serous) and aquaporin-5 (AQP5, acinar water channel) protein.

Western Blot Analysis: A 30µg total protein from each sample to evaluate the synthesis (in supernatants from cell lysates) and secretion (in media) of α–amylase protein. At day 1, 3, 5 and 7, both MSCs and huSG cells in cocultures could synthesize and secrete α–amylase protein (supernatant as well as media samples showed similar results). The synthesis and secretion of α–amylase protein detected in MSCs were comparable to those detected in huSG cells (Fig. 7.5). However, control MSCs cultures were not able to synthesize or secrete α–amylase protein. We noticed that when huSG were cocultured with MSCs their α-amylase synthesis and secretion has increased 2-3 folds than the secretion of huSG cultured alone. Western blot results obtained from conditioned media are presented in Fig.7.5

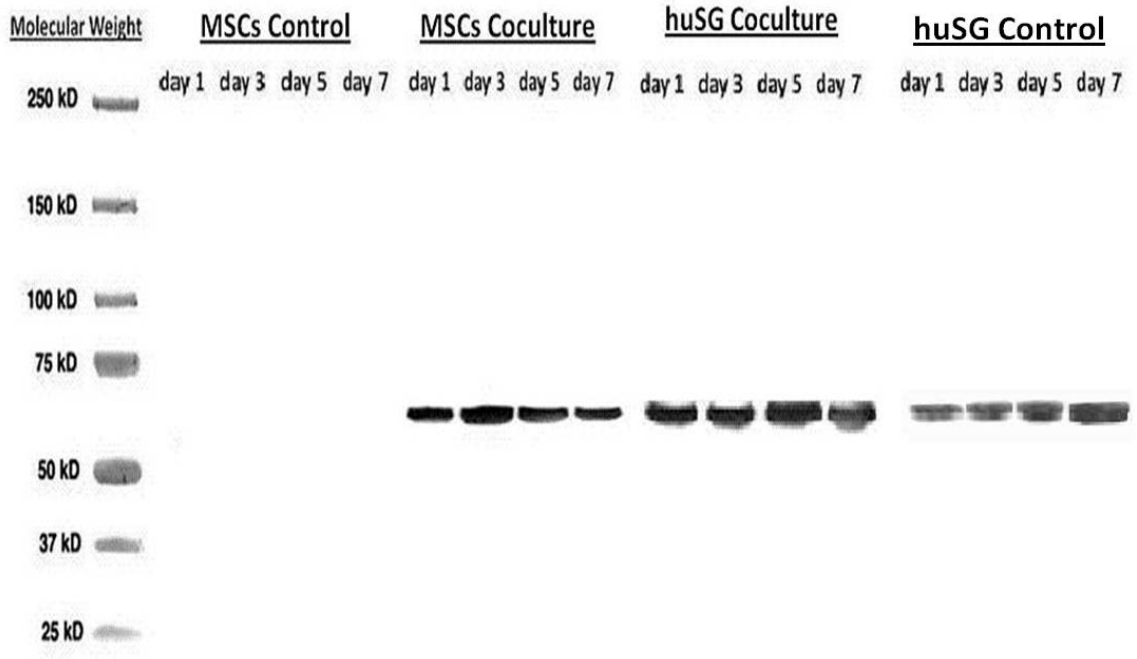
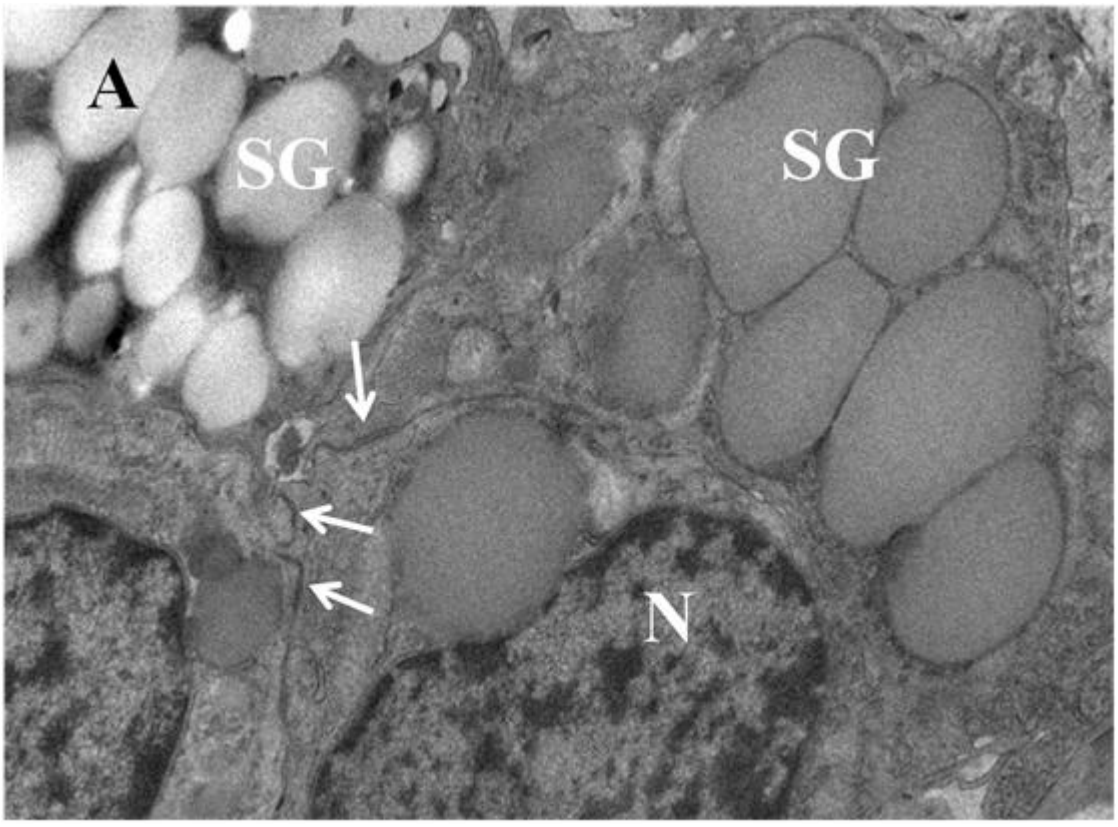
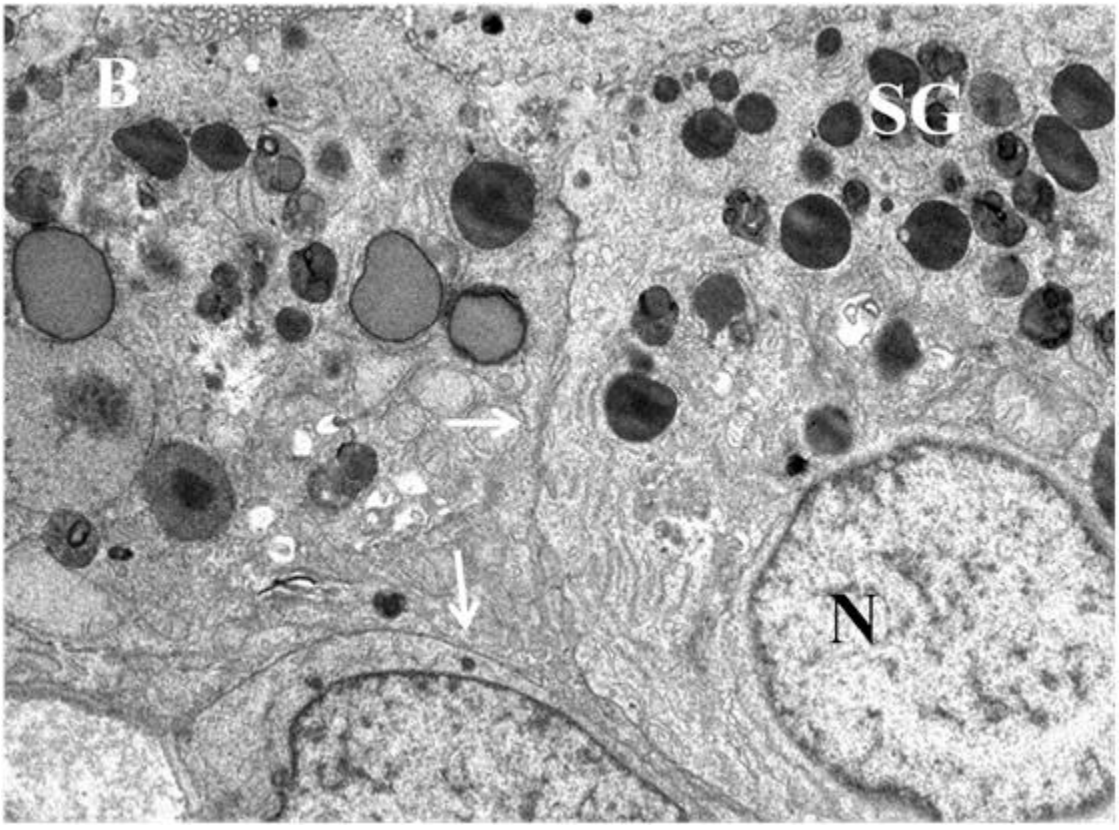


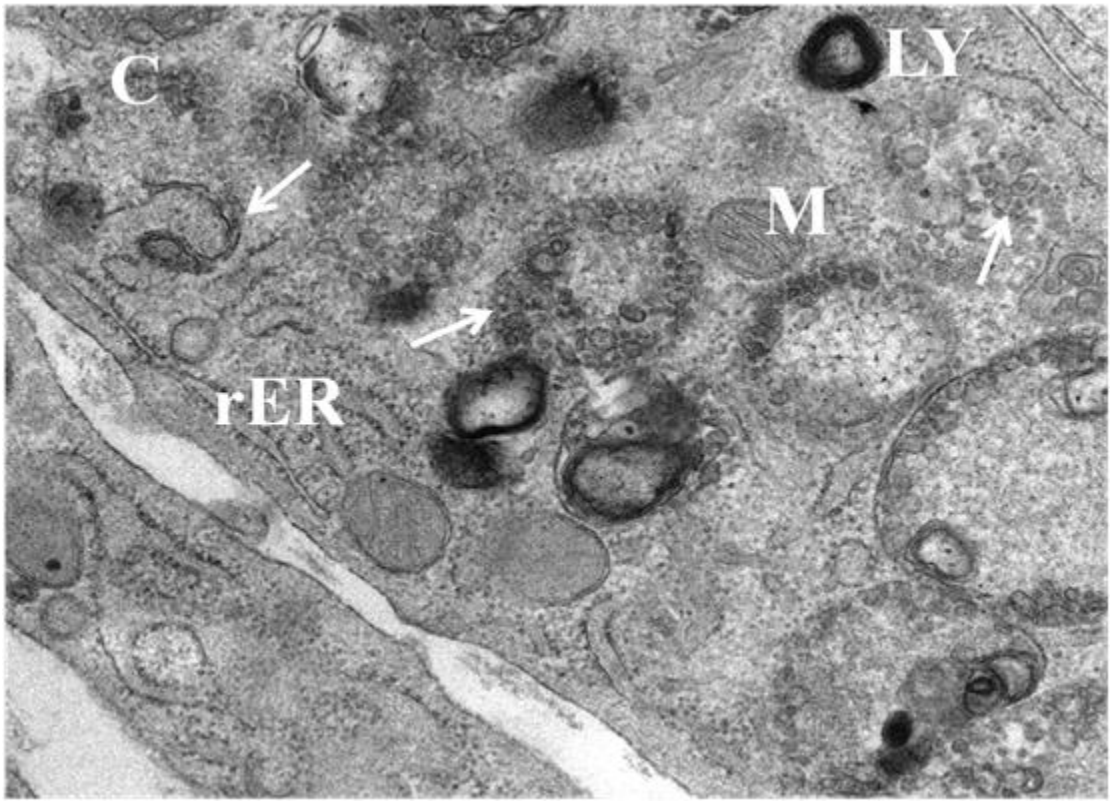
Figure 7.5: Western blot analysis of α–amylase protein (30 kDa) as evaluated in conditioned media from huSG cells in cocultures and huSG cultured alone (at day 1,3,5,7). The conditioned media taken from MSCs cocultures were new media added after the transfer of MSCs inserts into new separate plates and incubated alone for 1,3,5,7 days. MSCs were able to synthesize and secrete α–amylase protein into media in concentrations comparable to those of huSG cells. Of notice; the MSCs had increased α–amylase concentration secreted by huSG in the same coculture to 2-3 folds more that produced by huSG cultured alone (huSG Ctl.).

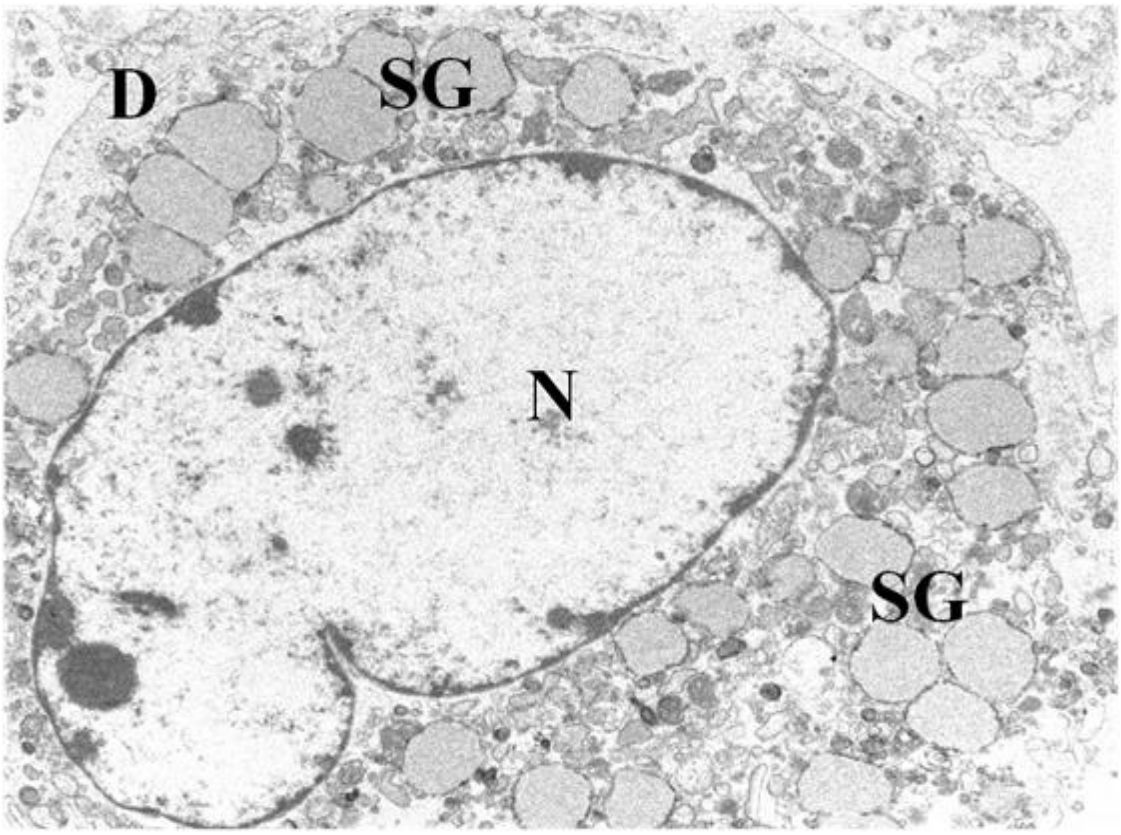
TEM: Ultrastructural analysis by TEM confirmed that huSG cells cocultured with MSCs were similar to normal human acinar cells at day 1; showing well-developed TJ structures and electron-lucent/-dense secretory granules (Fig.7.6A). However, most of the huSG cells started to lose their acinar phenotype gradually forming monolayers. Around 40% of huSG cells kept some secretory granules at day 14 (Fig. 7.6B) while the rest of huSG were similar to ductal cells. Although "control MSCs" were active in protein formation as revealed by well-developed rough endoplasmic reticulum, Golgi apparatus and multiple mitochondria, their cytoplasm did not show any salivary-like secretory granules or microvilli or TJ structures (Fig.7.6C). Interestingly, around 30-40% of MSCs in cocultures examined were able to synthesize salivary-like secretory granules similar in their electron-density to those of normal huSG cells and appeared budding beside Golgi saccules at day 1 through day 14 (Fig.7.6D and 7.6E respectively). In addition, they were able to form TJ structures (Fig. 7.6F) similar to those found in normal huSG confirming that they became polarized as revealed from immunocytochemical analysis.

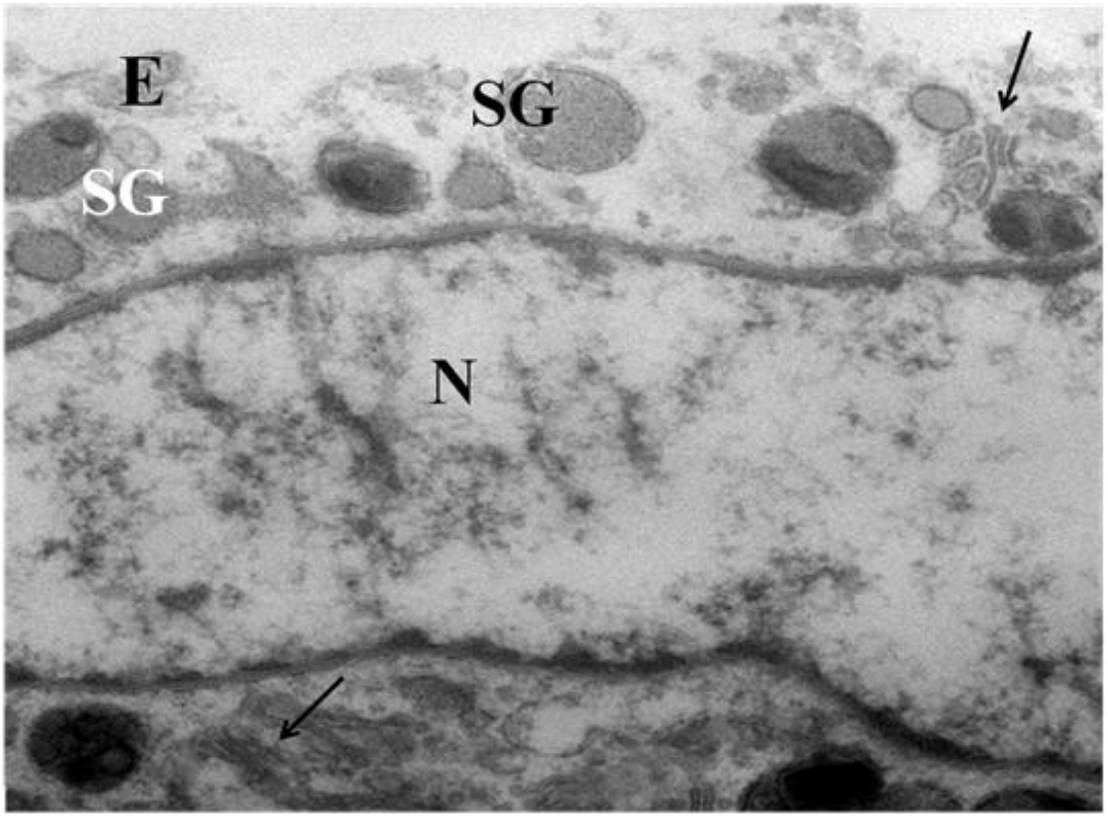
Figure 7.6: Transmission Electron Microscope (TEM) micrograph stained with lead citrate and uranyl acetate staining. Panel (A): huSG acinus structure (at day 1, from submandibular gland) in coculture showing features of secretory cells: electron-dense and -lucent secretory granules (SG), tight junction structures (arrows) surrounding a central lumen (*), and an euchromatic nucleus (N) [X 8,200]. Panel (B) shows huSG cocultured for 14 days): exhibiting features of acinar cells; showing electron-dense ecretory granules (SG), tight junction structures (arrows), and an euchromatic nucleus (N) [X 6,000]. Panels C shows MSCs cultured alone (control) for 14 days; active in protein synthesis; well-developed rough endoplasmic reticulum (rER), Golgi apparatus (arrows) and mitochondria (M); their cytoplasm showed some lysosomes (LY) while did not show any salivary-like secretory granules or microvilli or TJ structures [X 26,500]. Panels (D, E, and F) show MSCs in cocultures. (D): one MSC (at day 1) with a cytoplasm packed with multiple secretory granules (SG) around a central nucleus (N) [X 6,000]. (E): one MSC (at day 14) showing some electron-dense secretory granules (SG) around a central nucleus (N) and Golgi saccules (arrows) [X 26,500]. (F): shows four MSCs at day 7 attached to each others at some areas by tight junction structures (arrows) and some mitochondria (M) are in the field. [X 43,000].

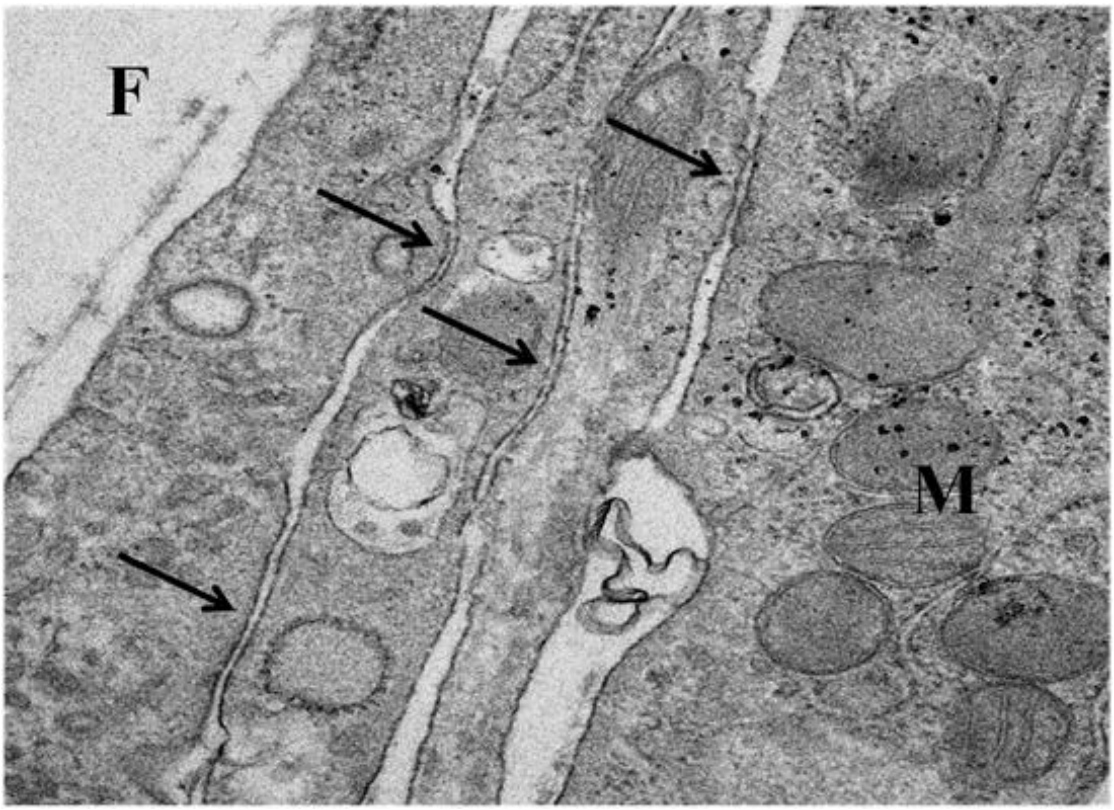

A
SG
SG
N


B
SG
N


C
LY
M
rER

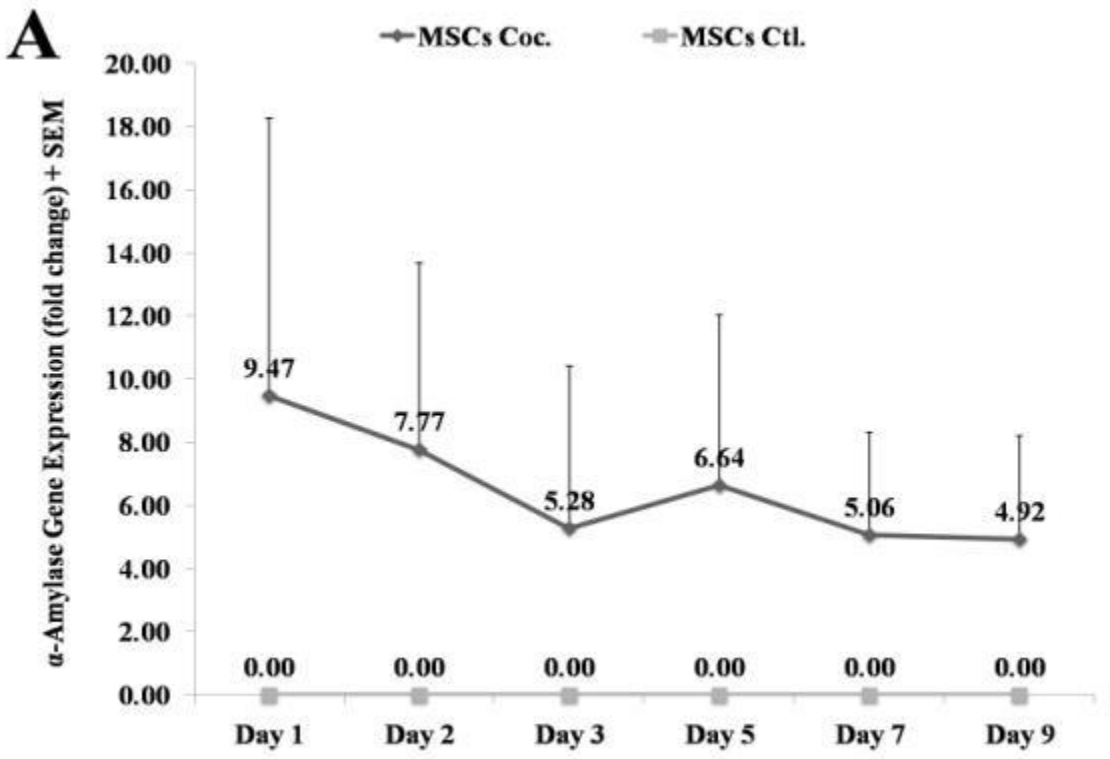

D
SG
N
SG

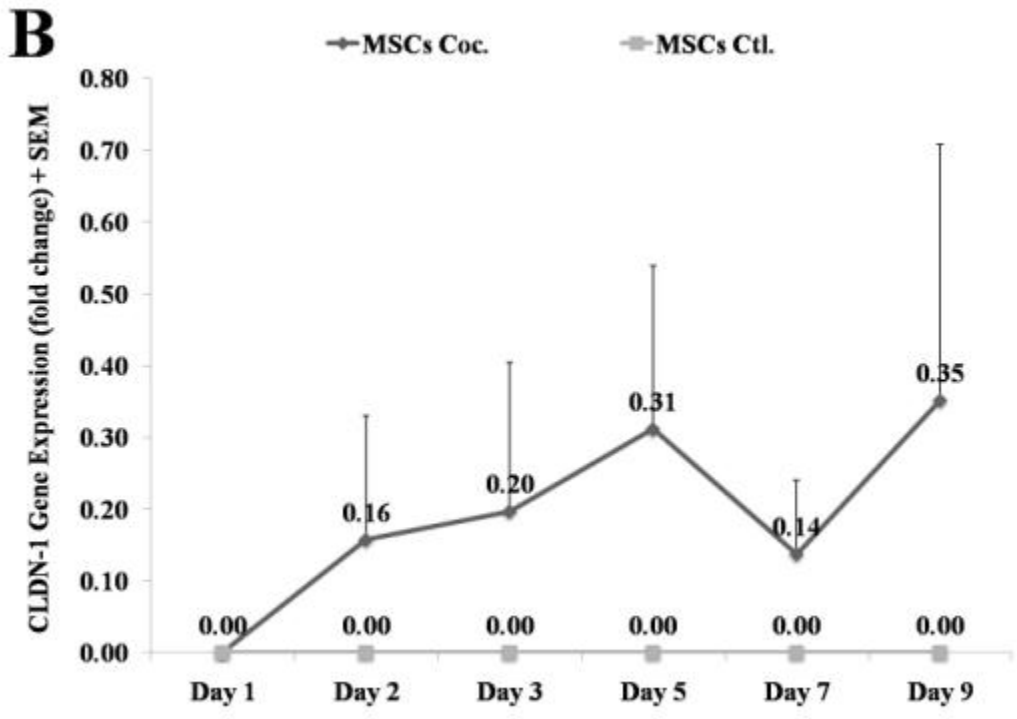

E
SG
SG
N


F
M

Quantitative Real-Time PCR Analysis: Gene expression levels in MSCs (5 patients) and huSGs (10 patients) were measured using quantitative RT-PCR. Results are expressed as fold-changes in gene expression relative to control sample (huSG cocultured for 9 days) of at least 5 separate experiments. MSCs in cocultures expressed the secretory acinar-specific α–amylase gene (AMY) starting at day 1 (Fig. 7.7A). In addition, the tight junction gene claudin-1 (CLDN-1) was expressed starting at day 2 (Fig. 7.7B). On the other hand, MSCs control cultures did not express any of the two genes tested. The huSG cells in cocultures expressed both genes in higher fold-changes (please refer to the supplemental data). Results from immunofluorescence technique and TEM confirm the proteins (claudin-1 and α–amylase) expression pattern and structures formation (TJ structures) respectively. All together, these data suggest that MSCs cocultured with huSG were able to achieved biological differentiation (into polarized functional salivary acini) through the induction of new gene expression including α–amylase and claudin-1 followed by the regulation of translation of relevant genes. Gene expression levels were different between all patients which might be related to patients' age or general health.

Figure 7.7: Gene expression levels (at day 1,2,3,5,7,9) indicated in fold-changes for MSCs in cocultures (MSCs Coc.) and MSCs control in cultures (MSCs Ctl.); Panel (A) shows α-amylase (AMY) gene expression and Panel (B) shows claudin-1 (CLDN-1) gene expression relative to samples from huSG in same cocultures grown for 9 days and normalized to GAPDH levels using quantitative real-time polymerase chain reaction (at least 3 separate experiments were performed/gene). Results are reported as means + standard error of the mean of parallel cultures.


A
MSCs Coc.
MSCs Ctl.
α-Amylase Gene Expression (fold change) + SEM
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
9.47
7.77
5.28
6.64
5.06
4.92
0.00
0.00
0.00
0.00
0.00
0.00
Day 1
Day 2
Day 3
Day 5
Day 7
Day 9


B
MSCs Coc.
MSCs Ctl.
CLDN-1 Gene Expression (fold change) + SEM
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00
0.16
0.20
0.31
0.14
0.35
0.00
0.00
0.00
0.00
0.00
Day 1
Day 2
Day 3
Day 5
Day 7
Day 9

DISCUSSION

In this study we have successfully induced MSCs to differentiate into polarized functional salivary acinar cells using coculture double chamber Transwell-clear system. As revealed from morphological and phenotypical analyses; 20-40% of MSCs were able to express TJ proteins; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1 and attain a noticeable level of TER. Moreover, they expressed salivary acinar-specific proteins; α–amylase and AQP-5 and were able to secrete α–amylase into media when removed from the coculture system for up to 7 days. The quantitative RT-PCR analysis revealed the expression of claudin-1 gene as well as α–amylase. In Addition, TEM examination confirmed the cytodifferentiation of 30 - 40% MSCs into polarized salivary acini; containing electron-dense secretory granules and exhibiting TJ structures. The stem cell fate is thought to depend on both internal and external signals. The external signals are sent from the surrounding microenvironment via either the direct cell-cell interactions or the soluble bioactive factors (185). Herein, huSG created an inductive extrinsic microenvironment that drove the MSCs into a salivary acinar differentiation pathway. As the Transwell culture system used does not allow direct cell-cell contact, the soluble bioactive molecules secreted by huSG cells into the coculture medium induced the MSCs differentiation. Similar results were reported by Spees et al 2003, who found that human MSCs would differentiate into epithelial cells after coculture with heat-shocked airway epithelial cells (376). In addition, Jang et al 2004 reported that 2.6% hematopoietic stem cells were converted to liver cells within 48h after coculture without fusion (377). Moreover, Zurita et al 2008 reported irreversible morphological and phenotypical differentiation of MSCs into Schwann cells upon coculture (378).

It has been reported that single cell-derived colonies of undifferentiated human MSCs express mRNA of multiple cell lineages (379) confirming the molecular heterogeneity and complexity of

MSCs (178). The MSCs used in this study expressed a panel of key surface markers; CD44, CD166, CD90, CD105, CD106, CD146, Stro-1 and were negative to the hematopoietic markers CD19 and CD45. In addition, MSCs used were able to differentiate into functional osteoblasts, thus confirming a mesenchymal stem cell phenotype (178).

Owing to their easy expansion without losing their multipotency (111), MSCs are attracting many researchers at the area of tissue-engineering and cell-based therapy. Bone marrow represents the main source of MSCs available for in vitro and in vivo studies; MSCs constitute 0.01- 0.001% of marrow resident nucleated cells (380) where they provide a biophysical microenvironment essential for hematopoiesis (381). The use of MSCs in experimental and clinical studies is preferred to embryonic stem cells for ethical, practical, technical and economical purposes. In an envisioned artificial salivary gland device, autologous MSCs obtained from healthy patient, would be used as graft cells, whereas, in old or sick patients (those with bone marrow or hematological disorders), allogeneic MSCs from healthy donors would be safely used owing to the low/non-immunogenicity of MSCs (382). MSCs from bone marrow are readily accessible and provide an easy and minimally-invasive procedure to harvest from patients with head and neck cancers, before their chemo-irradiation therapy. We have previously reported that bone marrow-derived cells (BMDCs) from healthy male donors can differentiate into buccal (oral) epithelial cells of female transplant recipients (327), therefore, we expect that MSCs would differentiate in vivo into huSG cells.

Culture-propagated MSCs were reported to secrete anti-apoptotic, immune-regulatory, angiogenic, anti-scaring, chemo-attractant molecules, (382,383) in addition to extracellular matrix components, therefore, through this paracrine effect, MSCs would support in vivo tissue regeneration (384). The mesenchymal-epithelial transition which occurs during development (130,131), although limited in adult tissues, it might explain how MSCs would share in tissue

repair (344). During early fetal development, salivary gland morphogenesis involves interactions between the oral epithelium and the condensing neural crest-derived mesenchyme (168,385). Thus, the salivary tissue is formed from a heterogeneous mix of ectomesenchymal cells. Moreover, CD44/CD166 positive cells were localized in serous and mucous acini in human salivary gland tissue, respectively (unpublished data, 240), concluding that huSG tissue harbors mesenchymal stem/progenitor-like cells. Herein, the coculture ratio used was 1:3 MSCs to huSG to provide 3-fold inductive signals exerted by huSG to guide MSCs differentiation. MSCs in cocultures differentiated into functional huSG acini, secreting α-amylase protein into media, and as noticed, huSGs cocultured with MSCs had their α-amylase synthesis/secretion increased 2-3 folds than huSGs cultured alone (Fig.7.5). It seems that MSCs have reciprocal inductive signals on huSGs in the coculture conditions. After 3 days of coculture, 40% of MSCs clumped into acinar-like units (Fig.7.1D) that were connected with MSCs cords of fibroblast-like cells. These structures resemble the branching morphogenesis and cytodifferentiation of normal salivary glands (386). Moreover, TEM examination revealed the presence of electron-dense secretory-like granules in MSCs (Fig. 7.6D, 7.6E) resembling those of normal huSG granules (387,388). In addition, 40% of MSCs were able to build TJ structures among them (Fig. 7.6F). Tight junction structures are a critical requirement for the graft cell component needed for the envisioned artificial salivary gland device. In order for these cells to secrete fluid unidirectionally, they must express these TJs that generate an osmotic gradient and control the paracellular movement of water, proteins, and small solutes (147-151,153,154,148). Human salivary cells do express TJ proteins at their apicolateral membranes (155), therefore, huSG cells form a polarized monolayer in cultures and maintain an adequate TER. In this study, MSCs in cocultures expressed a group of TJ proteins (Fig.7.4) and as revealed by the confocal microscope the TJs formed in 20-40% of MSCs were correctly positioned at the apicolateral membranes; resembling their distribution in

normal human salivary glands (155). Therefore, MSCs successfully exhibited noticeable TER values (at day 7 and 14) essential for saliva secretion in vivo (249). In addition, MSCs were able to express AQP-5 (in 20% of cells) water channel protein necessary for water secretion in human salivary glands (247,248) and synthesize/secrete α–amylase protein necessary for starch digestion (Fig.7.4, Fig.7.5). In unpublished data, we demonstrated that transplantation of BMDCs via intra-venous injections can regenerate radiation-damaged salivary glands and rescue its functions, where 9% of salivary tissue originated from these BMDCs (389).

Undoubtedly, to prove the differentiation of certain stem cell, its functionality should be examined. It is not sufficient to test the expression of specific proteins that might be reversible, but absolutely to test whether it carries out the function of a differentiated cell. Here we confirmed the polarization and the acinar secretory properties of differentiated MSCs in cocultures using TER and western blot (from media) analyses. We have shown that MSCs can secrete α–amylase into their media after being separated from the cocultures for up to 7 days. Differentiation must involve genetic reprogramming with turning off/on some genes. True differentiation can only be considered to have taken place when a new characteristic cellular phenotype is stably established (134). Herein, the differentiated MSCs expressed two salivary gland key genes; claudin-1 (TJ) gene and α–amylase (acinar) gene, thus confirming the polarization as well as the acinar phenotype MSCs achieved.

Many reports suggest that most adult tissues harbor stem-like progenitor cells that are recruited initially during the repair process. In addition, bone marrow progenitors participate in the repair of most if not all major organ systems (84,390) including salivary glands (unpublished data, 389) *via* the bloodstream.

However, other reports indicate that tissue repair by marrow progenitors occur via hidden mechanisms guided by their secreted cytokines and growth factors (18,391-394). In addition,

some reports indicate that tissue repair occurs by cell fusion (375,395, 396). Cell fusion is a rare phenomenon that was reported in vitro and in vivo in tissues where polyploidy is common, such as hepatocytes, skeletal muscle and cardiac muscle (139). Herein, we report differentiation of human MSCs into polarized salivary acini without cell fusion. It was reported that up to 12% of the epithelial cells harbored the Y chromosome in five female bone marrow recipients (327), thus representing in vivo differentiation of bone marrow cells into epithelial cells. These observations suggest the possibility of regenerating glandular structures destroyed by disease or radiation by using stem cells from the patient's own stem cells reservoir. MSCs provide a promising tool for medical applications involving osteogenesis imperfecta (397) and cardiotherapy (398). The rationale of MSCs use in clinical applications would not depend only on their differentiation capacity but also on their reparative microenvironment created through induction of angiogenesis, immune-inflammatory regulation, reduction of apoptosis supporting the survival of injured cells as well as recruiting the proliferation of tissue-specific progenitors (178,399). In unpublished study, the restoration of function and morphology of irradiated salivary glands arose from a combination of several factors such as vasculogenesis, paracrine effect, and cell differentiation by BMDCs (389). Although it has been demonstrated here that MSCs differentiated into huSG, this is a short-term study (14 days). MSCs are multipotent stem cells whereas huSG are terminally differentiated epithelial cells and do not belong to the same germ layer. The precise mechanism governing this differentiation process remains to be elucidated, further studies will be needed. MSCs are promising candidates for cell-based therapy and would be of great interest for the treatment of xerostomia: 1) MSCs are obtained easily from bone marrow and can be expanded tremendously in vitro; 2) MSCs are low/non-immunogenic cells allowing allogeneic transplantation into mismatched patients when autogenic MSCs are unavailable; 3) MSCs are favorable over embryonic stem cells regarding ethical issues.

Differentiated MSCs would provide "off-the-shelf" therapy for xerostomic patients. Depending on each patient's conditions MSCs would be injected directly into the gland, or be induced to differentiate into salivary gland cells in vitro, then injected into the damaged gland. Another option is to use the differentiated MSCs as graft cells in the artificial salivary gland device.

In summary, we report successful induction of MSCs to differentiate into polarized functional salivary acini upon coculture with huSG cells. Morphologically and phenotypically 20-40% of MSCs expressed TJ proteins; claudin-1,-2,-3,-4, occludin, JAM-A, ZO-1 and attained a noticeable level of TER. Moreover, they expressed salivary acinar-specific proteins; α–amylase and AQP-5 and were able to secrete α–amylase into media when removed from the coculture system for up to 7 days. Furthermore, they expressed claudin-1 and α–amylase salivary specific genes. In Addition, ultrastructural analysis confirmed the cytodifferentiation of 30 - 40% of MSCs into polarized salivary acini; containing secretory granules and exhibiting TJ structures. Importantly, this model represents a proof-of-concept of differentiation using serum-free culture medium.

AKNOWLEDGEMENTS

The authors are grateful to Dr. Christopher Moskaluk and Dr. Olga Gologan for the histopathological analysis of salivary glands, and CHTN and Dr. Anthony Zeitouni at RVH for providing the salivary tissue samples. We are most appreciative of the technical help provided by Mrs. Jeannie Mui and the facility of electron microscopy research (FEMR) at McGill University for TEM samples preparation. The authors would like to thank Dr. John Presley, Dr. Judith Lacoste and Cell Imaging and Analysis Network (CIAN) for Confocal Microscopy use. The authors would like to thank the funding institutions; Canada Research Chair, CIHR, NSERC, RSBO, L'Ordre des dentistes du Québec and Egyptian Ministry of Higher Education and Scientific Research.

CHAPTER- 8

GENERAL DISCUSSION AND CONCLUSIONS

IDENTIFICATION AND CHARACTERIZATION OF A CELL SOURCE TO REGENERATE SALIVARY GLANDS

Xerostomia; salivary glands hypofunction, is a result of an irreversible salivary gland damage that is caused mainly by two conditions; the autoimmune exocrinopathy Sjogren's syndrome affecting 1-4 millions in US and radiotherapy for head and neck cancers (30,000 new cases each year). Moreover, some systemic diseases such as diabetes mellitus and pernicious anaemia would cause xerostomia (1-5). In addition, in 20% of xerostomic patients the cause is idiopathic (6,7). The clinical negative sequelae of salivary gland hypofunction include; an increased incidence of dental caries, periodontitis, candidiasis, mucositis, gastric and esophageal ulcers (2). Currently, the available treatment for xerostomic patients includes saliva-stimulants and artificial saliva (8). This symptomatic treatment is not that effective as permanent curatives for such condition are not available yet. We have been working to develop a tissue-engineered artificial salivary gland device that would be implanted into a surgically created pouch in the patient's mouth (10,11). The standard design for such device includes; a blind-end tube fabricated from a slowly biodegradable scaffold coated with extracellular matrix on its inner surface in order to promote attachment and polarization of epithelial cell monolayer; the graft cells that should be capable of unidirectional fluid secretion (147). In order for certain cells to secrete fluid unidirectionally, they must express a group of transport proteins (tight junctions; TJs) that generate an osmotic gradient and control the paracellular movement of water, proteins, and small solutes (148,150,151,153,158). The major hurdle we faced was to find a suitable graft cell type that must establish tight junctions (TJs) and exhibit an adequate transepithelial electrical resistance (TER) in addition to being functional acinar secretory cells. Therefore, the aim of this thesis was to identify and characterize a suitable graft cell type to regenerate salivary glands.

We identified the key tight junction proteins distributed in adult human salivary gland tissue (Chapter 2) to serve as a reference for future studies to assess the presence of appropriate TJ proteins in the graft cells we are looking for. Next to this, we characterized two candidate graft cells; HSG cell line and huSG cells cultured individually on Matrigel (Chapter 3&4). We showed that Matrigel supported their morphogenesis and cytodifferentiation into 3-D polarized functional acinar units However, neither Matrigel nor HSG would be used in clinical applications. Nevertheless, they would be implanted in animal models to further examine our envisioned artificial salivary gland device. Mesenchymal stem cells (MSCs) can differentiate into cells from all dermal lineages; carrying new promises and significant therapeutic implications. A review paper on cells from bone marrow that can evolve into oral tissues and their clinical applications has been included (Chapter 5). Importantly, MSCs have been isolated from different adult tissues including salivary glands, however; no reports localized such cells in situ. Following this way, we localized CD44+ and CD166+ cells in human serous and mucous acini, respectively (Chapter 6). Therefore, huSG tissue harbors MSC-like population, thus, these markers would be used; either to isolate MSCs or differentiated serous/mucous acini from huSGs. Implantation of autologous functional acini would be a major achievement to treat xerostomic patients, however, this would require the generation of a great number of such acini. Autologous MSCs would be expanded in vitro and induced to differentiate into functional salivary acini. Accordingly, we showed differentiation of 40% of MSCs into polarized functional salivary acini when cocultured with huSG (Chapter 6). These results represent a proof-of-concept that supports the potential and feasibility of using MSCs as a novel alternative source of graft for an implantable artificial salivary gland device to treat xerostomic patients.

TIGHT JUNCTION PROTEINS IN ADULT HUMAN SALIVARY GLANDS

Tight junctions, together with adherens junctions and desmosomes, form the apical junctional complex in epithelial and endothelial cellular sheets. Adherens junctions and desmosomes are responsible for the mechanical adhesion between adjacent cells, whereas TJs are essential for the tight sealing of the cellular sheets, thus controlling paracellular ions flux and maintaining tissue homeostasis (154). TJs also play a crucial role in the maintenance of cell polarity by forming a fence that prevents lateral diffusion of membrane proteins and lipids, thereby maintaining the differential composition of the apical and basolateral domains. Finally, because of the ability of TJ proteins to recruit signalling proteins (205), TJs have been hypothesized to be involved in the regulation of proliferation, differentiation, and other cellular functions. Given these cellular biological roles, the presence and function of TJ proteins in the cellular component of an artificial salivary gland is critically important. We have evaluated the presence of multiple TJ proteins in human parotid and submandibular salivary glands in chapter 2. We found that claudin-2, -3, -4, JAM-A, Occludin, and ZO-1 are expressed in both acinar and ductal cells while claudin-1was expressed mainly in ducts and only 25% of serous acini. This report should be a useful guide for evaluating the presence of appropriate TJ proteins in the cellular component of our artificial salivary gland device. However, we did not examine all possible TJ proteins in these tissues for practical reasons, but rather selected the most common TJ proteins for which antibodies were readily available.

MATRIGEL EFFECT ON HSG CELL LINE

The extracellular matrix components present in Matrigel, play a major role in morphogenesis where they regulate different signal pathways across the cell membrane in addition to the physical support related to its rigidity (245). In chapter 3, we report that Matrigel induced obvious morphologic changes and cytodifferentiation in HSG cells including; the formation of 3-D acinar-like polarized units expressing TJs, showing reasonable TER and containing a well-

developed Golgi apparatus, rough endoplasmic reticulum and secretory-like granules. TJ proteins as well as salivary acinar markers; α-amylase, AQP5, mucin-1, CD44, CD166 were evident. The α-amylase protein secretion into conditioned media was confirmed. Moreover, quantitative RT-PCR analysis revealed down-regulation of TJ, acinar and ductal-specific genes accompanied by protein translation (claudin-1,-2, α-amylase, AQP5, and cytokeratin), therefore, confirming acinar differentiation of cells grown on Matrigel. Furthermore, HSG cells expressed claudin-1 and claudin-2 genes when cultured on plastic therefore, the lack of TJ structures formation might be a protein translation defect. In addition, we report that the mitotic activity was significantly reduced while apoptotic activity significantly increased ($P<0.01$), (Chapter 3).

HSG cells derived from intercalated ducts were initially reported to express markers of myoepithelial or acinar cell type after chemical induction (233). In our study the 3-D acinar-like structures formed were similar in size, shape and number of cells/3-D structure to the normal human salivary acini, although our 3-D structures were not connected via ductal structures. Similar results were reported by Hoffman et al 1996 (166). Expression of acinar-specific markers, such as α-amylase (162,165,246) and cystatin (162), indicated that HSG cells differentiate as well, however, there direct evaluation of secretion of these proteins into the medium is necessary to confirm functional differentiation of the HSG cells. We confirmed that HSG on Matrigel are functional secretory acini as they were able to secrete α-amylase protein into the conditioned media. In addition to α-amylase acinar protein, we report that the 3-D acinar-like as well as monolayer cells expressed AQP-5 (in 98% of cells) water channel protein necessary for water secretion in human salivary glands (247,248). Moreover, 3-D structures expressed new serous and mucous adhesion-related proteins CD44, CD166 respectively, unpublished data (240); these proteins are considered mesenchymal stem/progenitor cell markers as well.

On Matrigel, the establishment of reasonable TER essential for saliva secretion in vivo, (249) was confirmed by the distribution of TJ proteins to the apicolateral membrane of the HSG 3-D acinar-like structures and monolayer. TJs distribution in HSG cells resembled their distribution in normal human and rodent salivary glands (155,208). Aframian et al 2002 (160), reported that HSG cells did not express TJ structures and therefore have low TER even after transfection with claudin-1 and claudin-2 genes; the cells expressed both proteins at their membranes but did not acquire adequate TER. It seems that Matrigel drives a specific mechanism through which the translation of specific proteins as TJ proteins is triggered. Ultrastructural analysis confirmed the establishment of TJ structures at the apicolateral cell membrane in polarized HSG cells cultured on Matrigel. On plastic, no TJ structures could be observed. On Matrigel, many secretory granules were detected in the cytoplasm along with features of active protein synthesis. Approximately 60-90% of HSG cells (both monolayers and 3-D structures) were acinar (3-7 days), while on plastic HSG cells were all ductal. Membrane-bound apoptotic bodies were obvious in cells at the middle of 3-D acinar-like structures for the formation of central lumen. Apoptosis was observed in cells that lost contact with the Matrigel as they became internalized in the 3-D acinar structures leading to central lumen formation (166,260,261). During salivary gland development balanced mechanisms govern the interaction between cell proliferation, apoptosis and cytodifferentiation (262).

Neither Matrigel nor HSG cell line would be used in clinical applications as both are derived from mouse sarcoma and human adenocarcinoma, respectively. However, together they represent an excellent and easily reproducible model to further study salivary-specific gene expression, organization, morphogenesis, carcinogenesis and the mechanisms governing their biology. In addition, HSG cell line as a long-term dividing human salivary cell type would allow long-term studies in animal models. Our next step would be testing HSG (as a graft cell type) grown on

Matrigel (as an extracellular matrix) carried on a scaffold designed to be implanted in animal models (mouse, rat, rabbit, monkey, etc.) to further examine our envisioned artificial salivary gland device in vivo. Certainly, these studies would help to improve the treatment of salivary glands hypofunction.

MATRIGEL EFFECT ON PRIMARY HUMAN SALIVARY GLAND CELLS

Development of salivary glands is based mainly on epithelial-mesenchymal interactions, leading to morphogenesis and cytodifferentiation (283,284); both are regulated by growth factors supplied by surrounding extracellular matrix (285), accordingly we aimed to simulate the in vivo model. Our culture method resulted in a mixed cell culture (mainly epithelial) capable of forming monolayer with TJs on filters. In chapter 4 we described a convenient and reproducible method to grow and maintain polarized acinar units from primary human salivary cells that would help in regenerating salivary glands. Cells from passage 2 and 3 formed polarized acinar units when grown on Matrigel however on plastic they were polarized ductal cells. We tested several criteria: morphological (formation of 3-D acinar units), immunological (positive for TJs, acinar secretory proteins; α-amylase, AQP5, mucin-1, and adhesion-related MSCs; CD44, CD166), functional (exhibiting adequate TER and α-amylase secreted into media), and gene expression. Other investigators have reported successful cultures of human salivary epithelial cells (268, 287-289). However, cultures from these reports were not well-characterized and none of these reports has used functional assays of polarization (TER) to examine their cells. For the purpose of seeding a tissue-engineered artificial salivary gland device, it was critical for us to establish that huSG cells could form tight junctions and be appropriately polarized. Here we confirm successful cultures of well-characterised polarized acinar units of huSG cells grown on Matrigel. Reduction in the mitotic rate of huSG cultures grown on Matrigel is consistent with the findings of studies on the HSG cell line (165, 243, 400,401) and on primary human salivary gland (272). During

development, the acini are formed when the outer layer of cells becomes polarized on its basement membrane then cells growth arrests where cells at the interior undergo apoptosis to form the lumen (290,291).

Our immunofluorescence analysis of huSG cells cultured on Matrigel revealed the presence of mesenchymal, ductal (99% vimentin positive), epithelial (99% cytokeratin) and acinar cells (99% α-amylase/AQP5 positive), as well as adhesion-related mesenchymal stem cell-like (99% $CD44^{+}/CD166^{+}$) however, myoepithelial cells were absent. Vimentin protein, a component of intermediate filaments in mesenchymal cells (292) is known to be expressed in immature salivary acinar cells, their immediate precursors, all myoepithelial cells, and some ductal cells (293). The 3-D acinar units were vimentin-negative, suggesting that they consisted mainly of mature acinar cells. TEM confirmed the establishment of TJ structures among huSG cells forming both monolayers and 3-D acinar units therefore both types of cells were polarized. Western blot analysis confirmed the synthesis and secretion of α-amylase by functional acinar cells with 2-5 fold increase on Matrigel. We do not know whether α-amylase secretion occurred through these lumens; especially that we could observe single cells bearing secretory granules. The basement membrane guides epithelial cells to polarize and differentiate (297). Consequently, it maintains the establishment of a unidirectional secretion. On Matrigel, the expression of AQP5 at sites of cell-junction confirmed cell polarization along with secretory potential however on plastic all cells were AQP5 negative. The intercalated duct cells are believed to be the stem cells of ductal, acinar and myoepithelial cells (168). On Matrigel, the reservoir of stem/progenitor cells might decrease while more cells differentiate into acini thus the mitotic activity is being reduced. Here we report that adhesion-related MSCs markers CD44 and CD166 were expressed on plastic and on Matrigel in comparable percentages. Expansion and differentiation of multipotent salivary stem cells would improve the treatment of xerostomia.

We think that down-regulation of certain genes (TJ, ductal, acinar) suggests that Matrigel plays a role in the regulation of relevant proteins translation and consequently the associated-expression pattern. The overall data presented in chapter 4 showed that Matrigel tends to direct the cells towards an acinar phenotype. However, longer-term (> 9 days) experiments would be useful to determine the effect of other factors such as patient's age, and associated-diseases on salivary cell regeneration capacity. Our findings would have a strong impact on the development of a prototype artificial salivary device to be tested in vivo (animal models) afterward. Thereafter, a scaffold carrying primary huSG cells (seeded on Matrigel would be implanted via a small surgically created pouch into the buccal submucosa of an animal model, and tested for secretion. Although promising, this model cannot be used currently for clinical application (human tissue-engineering) because Matrigel is derived from mouse sarcoma. For analysing safer substitutes of Matrigel, we are currently working on different gel types that would be acceptable for clinical applications. We believe that using 3-D cell-based models will help in a wide variety of applications in cancer biology, tissue-engineering, as well as gene and drug therapy, all together would help in the treatment of xerostomic patients.

BONE MARROW-DERIVED CELLS THAT EVOLVE INTO ORAL TISSUES AND THEIR CLINICAL APPLICATIONS

Stem cells are defined as clonogenic, self-renewing, and capable of generating one or more specialized cell types (78). Developmentally, stem cells are categorized either as embryonic stem cells or as post-natal stem cells (tissue-specific, adult stem cells) (79). Embryonic stem cells are derived from the inner cell mass of a developing blastocyst and are considered as pluripotent cells since they are able to form all the dermal cell lineages (endoderm, mesoderm and ectoderm). Post-natal stem cells are considered multipotent as they form cells from multiple lineages that constitute an entire tissue or tissues (82).

Adult bone marrow harbors two stem cell types; the hematopoietic stem cells (HSC) and the mesenchymal stem cells. HSC were recognized by having the ability to reconstitute the hematopoietic system of a lethally irradiated host (79) since it gives rise to all blood cells. Their unique ability to continuously self-renew permits HSC to sustain blood cell production throughout life. Mesenchymal stem cells originate from the mesodermal layer of the fetus and in the adult they reside in the bone marrow as well as in a variety of tissues (109,110). MSCs were demonstrated to possess a multilineage differentiation capability (bone, cartilage, adipose, tendon, and muscle tissues (89, 111). Plasticity is defined as the ability of post-natal stem cells to differentiate into mature and functional cells of the same or of a different germ layer of origin (79). There are reports that bone marrow stem cells can differentiate into hepatocytes (112), skeletal myocytes (89), cardiomyocytes (113,114), neural cells (87,88), endothelial cells (114), epithelial cells (115), and pancreatic endocrine cells (116). These findings on the plasticity of post-natal stem cells carry great promises for regenerative medicine applications (117-119). As an example, because HSC can reconstitute the entire blood system, bone marrow transplantations have long been used in the clinic to treat hematopoietic diseases (119). Cell-based therapies based on post-natal bone marrow-derived stem cells would be used for treating cancers, autoimmune, neurological, stroke and heart diseases (108). Although cell fusion occurs when stem cell reside into a damaged tissue, it is considered a rare phenomenon that occurs commonly in polyploidy tissues; such as hepatocytes, skeletal muscle, cardiac muscle and Purkinje cells of the cerebellum (402).

Nowadays, the two common methods of cell delivery are intravenous injections (direct delivery of cells) and cell encapsulation systems (indirect delivery of cells using a carrier). The cell encapsulation approach uses a biodegradable material, which is a biocompatible product that is gradually resorbed once implanted in the body, due to enzymatic or hydrolytic degradation. This

biodegradable construct is seeded with cells (ideally progenitor cells) and is implanted into defects in order to regenerate lost tissues (308). Bone marrow-derived MSC have a significant but highly variable self-renewal potential during in vitro experiments and this property has made them attractive as a source for cell-based therapies aiming at the regeneration of orofacial tissues (305, 306). Future advancements in stem cell research and in biomaterial science will allow cell encapsulation methods to be utilized in the clinic to regenerate both hard and soft tissues of the craniofacial complex. In chapter 5, we review the plasticity and the clinical applications of bone marrow-derived stem cells that can evolve into oral tissues.

IDENTIFICATION OF HUMAN SALIVARY STEM/PROGENITOR CELLS USING MSCs MARKERS

In chapter 6, we demonstrated the presence of a sub-population of cells within the human salivary glands that are positive for mesenchymal/stromal stem/progenitor cell markers. The salivary acinar cells expressed the adhesion-related proteins CD44 and CD166; might play a role in the regeneration/repair of salivary glands. Also, both CD44 and CD166 markers can be used to isolate (FACS sorting) serous and mucous salivary cells, respectively. In addition, we were able to identify acinar progenitor cells in a rabbit duct-ligated parotid gland model using the CD44 marker.

During early fetal development, salivary gland morphogenesis involves interactions between the oral epithelium and the condensing neural crest-derived mesenchyme (168, 344). Thus, the salivary tissue is formed from a heterogeneous mix of ectomesenchymal cells. This led us to test stem cell antibodies against: Musashi-1 (neural); Stro-1, CD146, CD106, CD44, CD166, CD90, CD105 (mesenchymal); and CD45 (hematopoietic/leukocyte) using immunofluorescence methods. Stem cells are thought to reside in a niche which regulates the balance between stem cell self-renewal and tissue regeneration (345), however, the salivary stem cell niche has not been

localized yet. Genetic and histological analyses revealed the existence of stem/progenitor cell-related markers: either intracellular or cell surface proteins. Some of these markers are tissue-specific while others are expressed in several tissues. However, because a single marker that defines a stem/progenitor cell has not been found yet, several markers need to be combined. Intercalated ducts are thought to be the stem/progenitor cells for salivary tissue (127,128,365,366). However, Ihrler et al 2004, reported that acinar, intercalated duct and myoepithelial cells regenerate independently, with neither cell transition nor participation of other cell populations (369). Here we demonstrated that serous and mucous acini harbor a mesenchymal-like stem/progenitor cell population which might support previous findings reported (335,337, 368,369).

The regeneration of adult rat submandibular glands following duct-ligation supports the existence of multipotent cells within ducts, the only surviving component in atrophied glands (370). However, other reports concluded that regeneration of acini would mainly occur by self-division of surviving acini when the ligature is released (121,122). We observed in the rabbit that progenitor/stem cell subpopulations increased upon ligation thus surviving $CD44^+$ cells are more resistant to atrophy. Stem cells that are believed to reside in cell niche are thought to be regulated by cell adhesion molecules as well as interaction between stem cells and extracellular matrix (373). Although the exact role of both $CD44^+$ and $CD166^+$ adhesion proteins in salivary glands still need to be elucidated, their distribution in serous and mucous acini respectively, suggests a potential role in the secretory functions of both acinar types. Duct-ligation would be a feasible way for isolating and analyzing $CD44^+$ salivary stem/progenitor cells from rabbits. Whether detection of $CD44^+$ and $CD166^+$ salivary cells is sufficient for stating the presence of mesenchymal-like stem/progenitor cells remains to be elucidated. However, considering that acini were reported to be a self-expanding population capable of de-differentiation into duct-like

structures upon salivary duct-ligation, there might be a sub-population of progenitor cells within this CD44/ CD166 positive population of acinar cells. This would be of therapeutic significance since the availability of sufficient multipotent cells - expanded in vitro to be implanted into an artificial salivary gland device - would provide "off the shelf" cell-based therapy for patients suffering from xerostomia.

MESENCHYMAL STEM DIFFERENTIATE INTO FUNCTIONAL SALIVARY GLAND CELLS

Neither HSG cell line nor Matrigel would be used in clinical application to treat xerostomic patients. In addition, huSG cells exhibited ductal phenotype of slowly growing cells and would not form salivary acinar unit unless Matrigel is provided; we can conclude that huSG is not suitable for clinical applications in its current status either. Therefore, we assumed that if MSCs would be induced to differentiate into functional salivary acini, they would be the suitable novel graft for our envisioned artificial salivary gland device, especially that MSCs can be expanded easily without losing their multipotency. The stem cell fate is thought to depend on both internal and external signals. The external signals are sent from the surrounding microenvironment via either the direct cell-cell interactions or the soluble bioactive factors (185). In chapter 6, we confirm that huSG created an inductive extrinsic microenvironment that drove the MSCs into a salivary acinar differentiation pathway. As the Transwell culture system we used does not allow direct cell-cell contact, the soluble bioactive molecules secreted by huSG cells into the coculture medium induced the MSCs differentiation. MSCs were induced to differentiate into polarized functional salivary acinar cells using serum-free Hepato-STIM medium. As revealed from morphological and phenotypical analyses; 20-40% of MSCs were able to express TJ proteins and attain a noticeable level of TER. Moreover, they expressed salivary acinar-specific proteins; α–amylase and AQP-5 and were able to secrete α–amylase into media when removed from the

coculture system for up to 7 days. The expression of claudin-1 and α–amylase genes in MSCs confirms their differentiation into huSG acini. In Addition, TEM examination confirmed the cytodifferentiation of 30 - 40% MSCs into polarized salivary acini; containing electron-dense secretory granules and exhibiting TJ structures. Similar results were reported by Spees et al 2003, who found that human MSCs would differentiate into epithelial cells after coculture with heat-shocked airway epithelial cells (376). In addition, Jang et al 2004 reported that 2.6% hematopoietic stem cells were converted to liver cells within 48h after coculture without fusion (377). Moreover, Zurita et al 2008, reported irreversible morphological and phenotypical differentiation of MSCs into Schwann cells upon coculture (378). Owing to their easy expansion without losing their multipotency (111), MSCs are attracting many researchers at the area of tissue-engineering and cell-based therapy. The use of MSCs in experimental and clinical studies is preferred to embryonic stem cells for ethical, practical, technical and economical purposes. In our envisioned artificial salivary gland device, autologous MSCs obtained from healthy patient, would be used as graft cells, whereas, in old or sick patients (those with bone marrow or hematological disorders), allogeneic MSCs from healthy donors would be safely used owing to the low/non-immunogenicity of MSCs (382). From our previous study, we reported that bone marrow-derived cells (BMDCs) from healthy male donors can differentiate into buccal (oral) epithelial cells of female transplant recipients (327). The mesenchymal-epithelial transition which occurs during development (130), although limited in adult tissues, it might explain how MSCs would share in epithelial tissue repair (403). During early fetal development, salivary gland morphogenesis involves interactions between the oral epithelium and the condensing neural crest-derived mesenchyme (101,168). Thus, the salivary tissue is formed from a heterogeneous mix of ectomesenchymal cells. Moreover, we have localized CD44/CD166 positive cells in serous and mucous acini in human salivary gland tissue, respectively (Chapter 6), concluding that huSG

tissue harbors mesenchymal stem/progenitor-like cells. After 3 days of coculture, 40% of MSCs clumped into acinar-like units that were connected with MSCs cords of fibroblast-like cells. These structures resemble the branching morphogenesis and cytodifferentiation of normal salivary glands (386). Moreover, TEM examination revealed the presence of electron-dense secretory granules in MSCs resembling those of normal huSG granules (387,388). In addition, 40% of MSCs were able to build TJ structures among them. Tight junction structures are a critical requirement for the graft cell component that we need to employ in our envisioned artificial salivary gland device. MSCs in cocultures expressed a group of TJ proteins and as revealed by the confocal microscope; TJs were correctly positioned at the apicolateral membranes; resembling their distribution in normal human salivary glands (155). Thereafter, MSCs successfully exhibited noticeable TER values (at day 7 and 14) essential for saliva secretion in vivo (249). In addition, MSCs were able to express AQP-5 (in 20% of cells) water channel protein necessary for water secretion in human salivary glands (247,248) and synthesize/secrete α–amylase protein necessary for starch digestion. In unpublished data, we have demonstrated that transplantation of BMDCs via intra-venous injections can regenerate radiation-damaged salivary glands and rescue its functions, where 9% of salivary tissue originated from these BMDCs (389). Differentiation must involve genetic reprogramming with turning off/on some genes. True differentiation can only be considered to have taken place when a new characteristic cellular phenotype is stably established (134). Herein, the differentiated MSCs expressed two salivary gland key genes; claudin-1 (TJ) gene and α–amylase (acinar) gene, thus confirming the polarization as well as the acinar phenotype MSCs achieved. Many reports suggest that most adult tissues harbor stem-like progenitor cells that are recruited initially during the repair process. In addition, bone marrow progenitors participate in the repair of most if not all major organ systems (84,390) including salivary glands (389) via the bloodstream. However,

other reports indicate that tissue repair by marrow progenitors occur via hidden mechanisms guided by their secreted cytokines and growth factors (18,393). In addition, some reports indicate that tissue repair occurs by cell fusion (376,395,396). However, we reported differentiation of human MSCs into polarized salivary acini without cell fusion (chapter7). In one of our studies we found up to 12% of the epithelial cells harbored the Y chromosome in five female bone marrow recipients (98). These observations suggest the possibility of regenerating glandular structures destroyed by disease or radiation by using stem cells from the patient's own stem cells reservoir. The rationale of MSCs use in clinical applications would not depend only on their differentiation capacity but also on their reparative microenvironment (178,399). In unpublished data, we concluded that the restoration of function and morphology of irradiated salivary glands arose from a combination of several factors such as vasculogenesis, paracrine effect, and cell transdifferentiation by BMDCs (389).

CONCLUSIONS

Certain tight junction proteins (chapter 2) are essential key components for unidirectional fluid movement in human salivary glands as well as in the candidate suitable graft cells for our envisioned artificial salivary gland device. Matrigel induces the morphogenesis and cytodifferentiation of both HSG cell line and huSG cells into functional polarized salivary acinar units. Although neither Matrigel nor HSG would be applied clinically, they would be implanted (carried on scaffolds) into animal models to further test an artificial salivary gland device. Human salivary gland tissue harbors mesenchymal stem/progenitor-like cells positive for CD44 and CD166 markers that are localized in serous and mucous acini, respectively. MSCs are promising candidates for cell-based therapy and would be of great interest for the treatment of xerostomia: 1) MSCs are obtained easily from bone marrow and can be expanded tremendously in vitro; 2) MSCs are low/non-immunogenic cells allowing allogeneic transplantation into mismatched patients when autogenic MSCs are unavailable; 3) MSCs are favourable over embryonic stem cells regarding ethical issues. Differentiated MSCs would provide “off-the-shelf” therapy for xerostomic patients. Depending on each patient’s conditions MSCs would be injected directly into the gland, or be induces to differentiate into salivary gland cells in vitro, then injected into the damaged gland. Further option is to use the differentiated MSCs as graft cells in the artificial salivary gland device.

FUTURE PERSPECTIVES

Further studies are required to elucidate the mechanisms governing salivary cell differentiation when cultured on Matrigel. As culture components obtained from animal and/or cancerous origin are not relevant to be applied clinically, other comparable components to those of Matrigel need to be obtained. Currently, we are testing different gel types with the huSG cells that would be relevant for clinical applications. In the meantime, testing the functionality of our envisioned artificial salivary gland device in animal models using Matrigel and HSG/huSG cells would provide new useful insights. Both CD44 and CD166 would be used to isolate serous and mucous acinar cells, respectively, (and may be their progenitors) to further study the biology and physiology of salivary gland stem/progenitor cells and their similarities/differences to/with mesenchymal stem cells. Successful differentiation of MSCs into functional huSG acinar units provides a novel suitable graft cell type to be used in the regeneration of salivary glands. What mechanisms/pathways involved in stem cell differentiation need to be elucidated.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The data presented in this thesis have provided several original contributions to the existing body of scientific knowledge:

1. Identification of key tight junctions proteins and their appropriate distribution in human salivary gland tissue. A reference to assess candidate graft cells for artificial salivary gland device.
2. Successful induction of HSG cell line to differentiate into functional 3-D polarized salivary acinar units on Matrigel; HSG cells on Matrigel formed tight junction structures and exhibited adequate TER necessary for candidate graft cells for the artificial salivary gland device. In addition, HSG cells (on plastic) expressed TJ genes (claudin-1 and claudin-2) although they cannot translate the relevant TJ proteins unless Matrigel is provided; meaning that certain problem (s) might prevent the translation process. Furthermore, HSG cells cultured on Matrigel expressed CD44 and CD166 adhesion-related mesenchymal stem cell proteins.
3. Successful induction of previously reported huSG ductal cells into 3-D polarized functional salivary acinar units on Matrigel. In addition, both huSG cells cultured on plastic and or Matrigel express CD44 and CD166 adhesion-related mesenchymal stem cell proteins.
4. Adult human salivary gland tissue harbors mesenchymal stem/progenitor-like cells; expressing CD44 and CD166 markers in serous and mucous acini, respectively.
5. Successful induction of human MSCs from bone marrow into functional polarized salivary acinar units when cocultured with huSG cells in a serum-free medium using a Transwell-clear system; MSCs expressed TJ proteins as well as acinar-specific proteins

and secreted α-amylase into the media. In addition, exhibited a noticeable TER. MSCs provides a novel source of graft to regenerate salivary glands.

REFERENCES

1. Fox RI. (2005). Sjogren's syndrome. Lancet 366: 321–331.
2. Melvin JE. (1991). Saliva and dental diseases. Curr Opin Dent 1: 795–801.
3. Nederfors T, B Nauntofte and S Twetman. (2004). Effects of furosemide and bendroflumethiazide on saliva flow rate and composition. Arch Oral Biol.49: 507–513.
4. Ship JA and K Hu. (2004). Radiotherapy-induced salivary dysfunction. Semin Oncol 31: 29–36.
5. Jemal A, T Murray, A Samuels, A Ghafoor, E Ward and MJ Thun. (2003). Cancer statistics. CA Cancer J Clin. 53: 5-26.
6. Field EA, LP Longman, R Bucknall, SB Kaye, SM Higham and WM Edgar. (1997). The establishment of a xerostomia clinic: a prospective study. Br J Oral Maxillofac Surg 35: 96–103.
7. Longman LP, SM Higham, K Rai, WM Edgar and EA Field. (1995). Salivary gland hypofunction in elderly patients attending a xerostomia clinic. Gerontology 12: 67–72.
8. Atkinson JC and BJ Baum. (2001). Salivary enhancement: current status and future therapies. J Dent Educ 65: 1096–1101.
9. Fox RI, Y Konttinen and A Fisher (2001). Use of muscarinic agonists in the treatment of Sjogren's syndrome. Clin Immunol 101: 249–263.
10. Tran SD, T Sugito, G Dipasquale, AP Cotrim, BC Bandyopadhyay, K Riddle, D Mooney, MR Kok, JA Chiorini and BJ Baum. (2006). Re-engineering primary epithelial cells from rhesus monkey parotid glands for use in developing an artificial salivary gland. Tissue Eng. 12: 2939-2948.
11. Tran SD, J Wang, BC Bandyopadhyay, RS Redman, A Dutra, E Pak, WD Swaim, JA Gerstenhaber, JM Bryant, C Zheng, CM Goldsmith, MR Kok, RB Wellner and BJ Baum. (2005). Primary culture of polarized human salivary epithelial cells for use in developing an artificial salivary gland. Tissue Eng. 11: 172-181.
12. Jemal, A., Siegel, R., Ward, E. et al. (2006) Cancer statistics, 2006. Ca Cancer J. Clin. 56 [2], 106-130.
13. Parkin, D., Bray, F., Ferlay, J. et al. (2005). Global ancer statistics, 2002. Ca Cancer J. Clin. 55, 74-108.
14. Feber, T. (2000). Head and neck oncology nursing. Whurr Publishers Ltd., London.

15. Vissink, A., Jansma, J., Spijkervet, F.K. et al. (2003). Oral sequelae of head and neck radiotherapy. Crit Rev. Oral Biol. Med. 14 [3], 199-212.
16. Vissink, A., Burlage, F.R., Spijkervet, F.K. et al. (2003) Prevention and treatment of the consequences of head and neck radiotherapy. Crit Rev. Oral Biol. Med. 14 [3], 213-225.
17. Cooper, J.S., Fu, K., Marks, J. et al. (1995) Late effects of radiation therapy in the head and neck region. Int. J. Radiat. Oncol. Biol. Phys. 31 [5], 1141-1164.
18. Lombaert IM, Wierenga PK, Kok T, Kampinga HH, deHaan G, Coppes RP. (2006) Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands. Clin Cancer Res.; 12(6):1804-12.
19. Cherry CP, Gluckman A. (1959) Injury and repair following irradiation of salivary glands in male rats. Br J Radiol; 32:596–608.
20. Phillipe RM. (1970) X-ray-induced changes in function and structure of the rat parotid gland. J Oral Surg; 28:431–437.
21. Sholley MM, Sodicoff M, Pratt NE. (1974) Early radiation injury in the rat parotid gland. Reaction of acinar cells and vascular endothelium. Lab Invest; 31:340–354.
22. Fajardo, LF. (1982) Salivary glands and pancreas. In: Fajardo, LF., editor. Pathology of Radiation Injury. Masson Publishing USA; New York: p. 77-87.
23. Abok K, Brunk U, Jung B, Ericsson J. (1984) Morphologic and histochemical studies on the differing radiosensitivity of ductular and acinar cells of the rat submandibular gland. Virchows Arch Cell Pathol; 45:443–460.
24. Vissink, A., Kalicharan, D., Gravenmade, E.J. et al. (1991). Acute irradiation effects on morphology and function of rat submandibular glands. J. Oral Pathol. Med. 20: 449-456.
25. Nagler RM. (2002) The enigmatic mechanism of irradiation-induced damage to the major salivary glands. Oral Dis.;8:141–146
26. Coppes RP, Zeilstra LJW, Kampinga HH, Konings AWT. (2001)Early to late sparing of radiation damage tothe parotid gland by adrenergic and muscarinic receptor agonists. Br J Cancer; 85:1055–1063.
27. Dirix P, Nuyts S, Van den Bogaert W. (2006) Radiation-induced xerostomia in patients with head and neck cancer. A literature review Cancer; 107:2525–2534.

28. Chomette G, Auriol M, Vaillant JM, Bertrand JC, Chenal C. (1981) Effects of irradiation on the submandibular gland of the rat. Virchows Arch (Pathol Anat); 391:291–299.
29. Farbman, AI. (1972) The taste bud: A model system for developmental studies. In: Slavkin, HC.; Bavetta, LA.,editors. Developmental Aspects of Oral Biology. Academic Press; New York: p. 109-123
30. Franke RM, Herdly J, Phillipe E. (1965) Acquired dental defects and salivary gland lesions after irradiation for carcinoma. J Am Dent Assoc.;70:868–883
31. Bartel-Friedrich S, Friedrich RE, Lautenschlager C, Holzhausen HJ. (1993) Dose-response relationships and the effect of age and latency period on the expression of laminin in irradiated rat mandibular glands. Anticancer Res.; 20:221–5228.
32. Redman RS. (2008) On approaches to the functional restoration of salivary glands damaged by radiation therapy for head and neck cancer, with a review of related aspects of salivary gland morphology and development. Biotech Histochem; 83:103-30.
33. Zajicek, G., Yagil, C., and Michaeli, Y. (1985). The streaming submandibular gland. Anat. Rec. 213: 150-158.
34. Li Z, Zhao D, Gong B, Xu Y, Sun H, Yang B, et al. (2006) Decreased saliva secretion and down-regulation of AQP5 in submandibular gland in irradiated rats. Radiat Res; 165:678–87.
35. Baum BJ, Zheng C, Cotrim AP, Goldsmith CM, Atkinson JC, Brahim JS, et al. (2006) Transfer of the AQP1 cDNA for the correction of radiation-induced salivary hypofunction. Biochim Biophys Acta;1758:1071–7
36. Manoussakis MN, Georgopoulou C, Zintzaras E, et al. (2004) Sjögren's syndrome associated with systemic lupus erythematosus: clinical and laboratory profiles and comparison with primary Sjögren's syndrome. Arthritis Rheum; 50: 882–91.
37. Andoh Y, Shimura S, Sawai T, Sasaki H, Takishima T, Shirato K. (1993) Morphometric analysis of airways in Sjogren's syndrome. Am Rev Respir Dis.; 148:1358–62.
38. Bacman S, Sterin-Borda L, Camusso JJ, Arana R, Hubscher O, Borda E. (1996) Circulating antibodies against rat parotid gland M3 muscarinic receptors in primary Sjo¨gren's syndrome. Clin Exp Immunol; 104:454–9.

39. Steinfeld S, Cogan E, King LS, Agre P, Kiss R, Delporte C. (2001) Abnormal distribution of aquaporin-5 water channel protein in salivary glands from Sjogren's syndrome patients. Lab Invest;81:143–8
40. Tsubota K, Hirai S, King LS, Agre P, Ishida N. (2001) Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjogren's syndrome. Lancet; 357:688–9.
41. Aps JK, Martens LC. (2005) Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int.; 150(2-3):119-31.
42. Young, JA. van Lennep, EW. (1978) The Morphology of Salivary Glands. Academic Press; New York: p. 8-32.p. 69p. 80-93.p. 166-170.
43. Hand 1987, Robert M. Klein, Development, structure, and function of the salivary glands, (1994), Avery J.K., Essential of Histology and Embryology, 3rd edition, Copyright Mosby Year book, 292-329.
44. Quarnstrom EE, Hand AR. (1987) A granular cell at the acinar-intercalated duct junction of the rat submandibular gland. Eur J Morphol; 206:181–187.
45. Leblond CP, Puchtler H, Clermont Y. (1960) Structures corresponding to terminal bars and terminal web in many types of cells. Nature; 186:784–788
46. Chang WWL. (1974) Cell population changes during acinus formation in the postnatal rat submandibular gland. Anat Rec; 178:187–202.
47. Tamarin A, Sreebny LM. (1965) The rat submaxillary gland. A correlative study by light and electron microscopy. J Morphol;117:295–352,
48. Hand, AR. (1987) Functional ultrastructure of the salivary glands. In: Sreebny, LM., editor. The Salivary System. CRC Press; Boca Raton, FL: p. 43-67
49. Tandler B. (1993) Structure of the duct system in mammalian salivary glands. Microsc Res Tech; 26:57–74.
50. Tandler B, Pinkstaff CA, Phillips CT. (2006) Interlobular excretory ducts of mammalian salivary glands: structure and histochemistry review. Anat Rec ; 288A:498–526.
51. Garrett, JR. (1987) Innervation of salivary glands: neurohistochemical and functional aspects. In: Sreebny, LM., editor. The Salivary System. CRC Press; Boca Raton, FL: p. 69-93.
52. Hand AR. Salivary Glands. Antonio Nanci. (2003).Ten Cate's Oral Histology development, structure, and function. 6th Edition. Mosby. p. 299-328

53. Douglas, F. (2000) Histology & cell biology: examination and board review. Lange Medical Books, McGraw-Hill, New York.
54. Zussman, E., Yarin, A.L., and Nagler, R.M. (2007) Age- and flow-dependency of salivary viscoelasticity. J. DENT. RES. 86 [3], 281-285.
55. Inoue, H., Ono, K., Masuda, W. et al. (2006) Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. Arch. Oral Biol. 51 [12], 1055-1060.
56. Dowd, F.J. (1999) Saliva and dental caries. Dent. Clin. North Am. 43 [4], 579-597.
57. Veerasarn, V., Phromratanapongse, P., Suntornpong, N. et al. (2006) Effect of Amifostine to prevent radiotherapy-induced acute and late toxicity in head and neck cancer patients who had normal or mild impaired salivary gland function. J. Med. Assoc. Thai. 89 [12], 2056-2067.
58. Brizel D, Wasserman T, Henke M. (2000) Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. J Clin Oncol; 18:3339–3345.
59. Fox PC. (2004) Salivary enhancement therapies. Caries Res; 38:241–246.
60. Jham BC, Teixeira IV, Aboud CG, Carvalho AL, Coelho MD, Freiere AR. (2007) A randomized phase III prospective trial of bethanechol to prevent radiotherapy-induced salivary gland damage in patients with head and neck cancer. Oral Oncol; 43:137–142.
61. Johnson, J.T., Ferretti, G.A., Nethery, W.J. et al. (1993) Oral pilocarpine for post-irradiation xerostomia in patients with head and neck cancer. N. Engl. J. Med. 329 [6], 390-395.
62. Taylor, S.E. and Miller, E.G. (1999). Preemptive pharmacologic intervention in radiation-induced salivary dysfunction. Proc. Soc. Exp. Biol. Med. 221 [1], 14-26.
63. Braam, P.M., Terhaard, C.H., Roesink, J.M. et al. (2006) Intensity-modulated radiotherapy significantly reduces xerostomia compared with conventional radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 66 [4], 975-980.
64. MacLeod A, Kumar PAV, Hertess I, Newing R. (1990) Microvascular submandibular gland transfer; an alternative approach for total xerophthalmia. Br J Plast Surg; 43:437–439.
65. Regelink, G., Vissink, A., Reintsema, H. et al. (1998) Efficacy of a synthetic polymer saliva substitute in reducing oral complaints of patients suffering from irradiation-induced xerostomia. Quintessence. Int. 29 [6], 383-388.

66. Kagami H, Wang S , Hai B. (2007) Restoring the function of salivary glands. Oral diseases 14:15-24.
67. Vergara MN, Arsenijevic Y, Del Rio-Tsonis K (2005). CAS regeneration: a morphogen's tale. J Neurobiol 64: 491–507.
68. Burgo RD, Bedi KS, Nurcombe V (2002). Current concepts in central nervous system regeneration. J Clin Neurosci 9:613–617.
69. Fausto N (2000). Liver regeneration. J Hepatol 32:19–31.
70. Taub R (2004). Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5: 836–847.
71. Delporte C, O'Connell BC, He X, Lancaster H, O'Connell A, Agre P, et al. (1997) Increased fluid secretion following transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc Natl Acad Sci USA; 94:3268–73.
72. Adesanya et al. 1995, Baccaglini et al. 2001, Vitolo and Baum 2002. Baum BJ, Zheng C, Cotrim AP, Goldsmith CM, Atkinson JC, Brahim JS, et al. (2006) Transfer of the AQP1 cDNA for the correction of radiation-induced salivary hypofunction. Biochim Biophys Acta; 1758:1071–7.
73. O'Connell AC, Baccaglini L, Fox PC et al (1999). Safety and efficacy of adenovirus-mediated transfer of the human aquaporin-1 cDNA to irradiated parotid glands of nonhuman primates. Cancer Gene Ther 6: 505–513.
74. Kok MR, Yamano S, Lodde BM et al (2003). Local adenoassociated virus-mediated interleukin 10 gene transfer has disease-modifying effects in a murine model of Sjogren's syndrome. Hum Gene Ther 14: 1605–1618.
75. Lodde BM, Mineshiba F, Wang J et al (2006). Effect of human vasoactive intestinal peptide gene transfer in a murine model of Sjogren's syndrome. Ann Rheum Dis 65: 195–200.
76. Sugito T, Kagami H, Hata H et al (2004). Transplantation of cultured salivary gland cells into an atrophic salivary gland. Cell Transplant 13: 691–699.
77. Sharawy M, O'Dell NL. Regeneration of submandibular salivary gland autografted in the rat tongue. Anat Rec 1981;201:499–511.
78. Anderson DJ, Gage FH, Weissman IL (2001). Can stem cells cross lineage boundaries? Nat Med 7:393-395.

79. Leung AYH and Verfaillie CM (2005). Chapter 17. Stem cell model of hematopoiesis. In: Hoffman R et al, eds. Hematology: Basic Principles and Practice. 4th Editon. Philadelphia, Churchill Livingstone. pp. 200-214.
80. http://en.wikipedia.org/wiki/Stem_cell
81. Morrison, S.J. and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441 [7097], 1068-1074.
82. Smith A (2006). A glossary for stem-cell biology. Nature 441:1060.
83. Cohnheim, J. (1867) Pathol. Anat. Physiol. Klin. Med. 40, 1–79.
84. Prockop DJ, Carl A. Gregory, and Jeffery L. Spees. (2003) One strategy for cell and gene therapy: Harnessing the power of adult stem cells to repair tissues. PNAS, 100:11917–11923.
85. Körbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albitar M, Champlin RE, Estrov Z. (2002) Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med.; 346:738–746.
86. Tran SD, Pillemer SR, Dutra A, Barrett AJ, Brownstein MJ, Keys S, Pak E, Leakan RA, Kingman A, Yamada KM, Baum BJ, Mezey E. (2003) Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study. Lancet; 361:1084–1088.
87. Eglitis MA, Mezey E (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. Proc Natl Acad Sci U S A 94: 4080—4085.
88. Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B (2003). Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad Sci U S A 100: 1364–1369.
89. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528-1530
90. Brazelton, T.R., Rossi, F.M., Keshet, G.I. Et Al. (2000). From Marrow To Brain: Expression Of Neuronal Phenotypes In Adult Mice. Science 290 [5497], 1775-1779.
91. Priller, J. Robert Feulgen Prize Lecture. (2003). Grenzganger: Adult Bone Marrow Cells Populate The Brain. Histochem. Cell Biol. 120 [2], 85-91.

92. Lagasse, E., Connors, H., Al-Dhalimy, M. Et Al. (2000). Purified Hematopoietic Stem Cells Can Differentiate Into Hepatocytes In Vivo. Nat. Med. 6 [11], 1229-1234.

93. Theise, N.D., Badve, S., Saxena, R. Et Al. (2000). Derivation of Hepatocytes From Bone Marrow Cells In Mice After Radiation-Induced Myeloablation. Hepatology 31 [1], 235-240.

94. Theise, N.D., Nimmakayalu, M., Gardner, R. Et Al. (2000). Liver From Bone Marrow In Humans. Hepatology 32 [1], 11-16.

95. Yannaki, E., Athanasiou, E., Xagorari, A. Et Al. (2005). G-Csf-Primed Hematopoietic Stem Cells Or G-Csf Per Se Accelerate Recovery And Improve Survival After Liver Injury, Predominantly By Promoting Endogenous Repair Programs. Exp. Hematol. 33 [1], 108-119.

96. Ishizawa, K., Kubo, H., Yamada, M. Et Al. (2004). Bone Marrow-Derived Cells Contribute To Lung Regeneration After Elastase-Induced Pulmonary Emphysema. Febs Lett. 556 [1-3], 249-252.

97. Kotton, D.N., Ma, B.Y., Cardoso, W.V. Et Al. (2001). Bone Marrow-Derived Cells as Progenitors Of Lung Alveolar Epithelium. Development 128 [24], 5181-5188.

98. Yamada, M., Kubo, H., Kobayashi, S. Et Al. (2004). Bone Marrow-Derived Progenitor Cells Are Important For Lung Repair After Lipopolysaccharide-Induced Lung Injury. J. Immunol. 172 [2], 1266-1272.

99. Cho, S.W., Lim, S.H., Kim, I.K. Et Al. (2005). Small-Diameter Blood Vessels Engineered With Bone Marrow-Derived Cells. Ann. Surg. 241 [3], 506-515.

100. Herrera, M.B., Bussolati, B., Bruno, S. Et Al. (2004). Mesenchymal Stem Cells Contribute To The Renal Repair of Acute Tubular Epithelial Injury. Int. J. Mol. Med. 14 [6], 1035-1041

101. Nishida, M., Fujimoto, S., Toiyama, K. Et Al. (2004). Effect of Hematopoietic Cytokines on Renal Function In Cisplatin-Induced Arf In Mice. Biochem. Biophys. Res. Commun. 324 [1], 341-347.

102. Poulsom, R., Forbes, S.J., Hodivala-Dilke, K. Et Al. (2001) Bone Marrow Contributes To Renal Parenchymal Turnover And Regeneration. J. Pathol. 195 [2], 229-235.

103. Borue, X., Lee, S., Grove, J. Et Al. (2004). Bone Marrow-Derived Cells Contribute To Epithelial Engraftment During Wound Healing. Am. J. Pathol. 165 [5], 1767-1772.

104. Adachi, Y., Imagawa, J., Suzuki, Y. Et Al. (2004). G-Csf Treatment Increases Side Population Cell Infiltration After Myocardial Infarction In Mice. J. Mol. Cell Cardiol. 36 [5], 707-710.

105. Weissman IL (2000a). Stem cells: units of development, units of regeneration, and units in evolution. Cell 100:157-168.

106. Bordignon C (2006). Stem-cell therapies for blood diseases. Nature 441:1100-2.

107. Domen J, Weissman IL (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol Med Today 5:201-208.

108. Mayhall EA, Paffett-Lugassy N, Zon LI (2004). The clinical potential of stem cells. Curr Opin Cell Biol 16:713-720.

109. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2:83-92.

110. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR(1999). Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

111. Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D (2002). Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46:3349-3360.

112. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP (1999). Bone marrow as a potential source of hepatic oval cells. Science 284: 1168–1170.

113. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S (1999). Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697-705.

114. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ (1999). Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 100: II247–256.

115. Krause DS, Thiese ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105: 369–377.

116. Ianus A, Holz GG, Theise ND, Hussain MA (2003). In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 111: 843–850.

117. Weissman IL (2000b). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287:1442-1446.

118. Pittenger MF, Martin BJ (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95:9-20.

119. Kan I, Melamed E, Offen D (2005). Integral therapeutic potential of bone marrow mesenchymal stem cells. Curr Drug Targets 6:31-41.

120. Hanks, C.T. and Chaudhry, A.P. (1971). Regeneration of rat submandibular gland following partial extirpation. A light and electron microscopic study. Am. J. Anat. 130 [2], 195-207.

121. Burford-Mason, A.P., Cummins, M.M., Brown, D.H. et al. (1993) Immunohistochemical analysis of the proliferative capacity of duct and acinar cells during ligation-induced atrophy and subsequent regeneration of rat parotid gland. J. Oral Pathol. Med. 22 [10], 440-446.

122. Burgess, K.L. and Dardick, I. (1998) Cell population changes during atrophy and regeneration of rat parotid gland. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 85 [6], 699-706.

123. Scott, J., Liu, P., and Smith, P.M. (1999) Morphological and functional characteristics of acinar atrophy and recovery in the duct-ligated parotid gland of the rat. J. Dent. Res. 78 [11], 1711-1719.

124. Takahashi, S., Shinzato, K., Nakamura, S. et al. (2004) Cell death and cell proliferation in the regeneration of atrophied rat submandibular glands after duct ligation. J. Oral Pathol. Med. 33 [1], 23-29.

125. Takahashi, S., Nakamura, S., Domon, T. et al. (2005) Active participation of apoptosis and mitosis in sublingual gland regeneration of the rat following release from duct ligation. J. Mol. Histol. 36 [3], 199-205.

126. Kimoto, M., Yura, Y., Kishino, M. et al. (2007) Label-retaining Cells in the Rat Submandibular Gland. J. Histochem. Cytochem.

127. Denny, P.C. and Denny, P.A. (1999) Dynamics of parenchymal cell division, differentiation, and apoptosis in the young adult female mouse submandibular gland. Anat. Rec. 254 [3], 408-417.
128. Man, Y.G., Ball, W.D., Marchetti, L. et al. (2001) Contributions of intercalated duct cells to the normal parenchyma of submandibular glands of adult rats. Anat. Rec. 263 [2], 202-214.
129. Perez-Pomares JM, Munoz-Chapuli R. (2002) Epithelial-mesenchymal transitions: A mesodermal cell strategy for evolutive innovation in Metazoans. Anat Rec; 268:343–351.
130. Hay ED. (1990) Epithelial-mesenchymal transitions. Semin Dev Biol; 1: 347–356.
131. Hay ED. (1995) An overview of epithelio-mesenchymal transformation. Acta Anat; 154:8 –20.
132. Davies JA. (1996) Mesenchyme to epithelium transition during development of the mammalian kidney tubule. Acta Anat; 156:187–201.
133. Timmerman LA, Grego-Bessa J, Raya A et al. (2004) Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev; 18:99 –115.
134. Krabbe C, Zimmer J, Meyer M. (2005) Neural transdifferentiation of mesenchymal stem cells- a critical review. APMIS.; 113(11-12):831-44.
135. Grove JE, Bruscia E, Krause DS (2004). Plasticity of bone marrow-derived stem cells. Stem Cells; 22:487-500.
136. Kashofer K, Bonnet D (2005). Gene therapy progress and prospects: stem cell plasticity. Gene Ther 12:1229-1234.
137. Verfaillie CM (2002). Adult stem cells: assessing the case for pluripotency. Trends Cell Biol 12:502–508.
138. Martin-Rendon E, Watt SM (2003). Exploitation of stem cell plasticity. Transfus Med 13:325-349.
139. Lakshmipathy U, Verfaillie CM (2005). Stem cell plasticity. Blood Rev 19:29-38.
140. Dao M, Verfaillie CM (2005). Chapter 18. Bone marrow microenvironment. In: Hoffman R et al, eds. Hematology: Basic Principles and Practice. 4th Editon. Philadelphia, Churchill Livingstone. pp.215-232.

141. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41-49.

142. Brockes JP (1997). Amphibian limb regeneration: rebuilding a complex structure. Science 276:81–87.

143. Hochedlinger K, Jaenisch R (2006). Nuclear reprogramming and pluripotency. Nature 441:1061-1067

144. Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW (2001). Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. J Cell Biol 155:733–738.

145. Ying QL, Nichols J, Evans EP, Smith AG (2002). Changing potency by spontaneous fusion. Nature 416:545–548.

146. CORTI, S., Locatelli, F., Papadimitriou, D. et al. (2005) Nuclear reprogramming and adult stem cell potential. HISTOL. HISTOPATHOL. 20 [3], 977-986.

147. Baum BJ, S Wang, E Cukierman, C Delporte, H Kagami, Y Marmary, PC Fox, DJ Mooney and KM Yamada. (1999). Re-engineering the functions of a terminally-differentiated epithelial cell in vivo. Ann. N.Y. Acad. Sci. 875: 294- 300.

148. Baum BJ. Principles of saliva secretion. (1993). Ann. N.Y. Acad. Sci. 694: 17-23.

149. Mostov KE, M Verges and Y Altschuler. (2000). Membrane traffic in polarized epithelial cells. Curr. Opin. Cell Biol. 12:483-490.

150. Mitic LL, CM Van Itallie and JM Anderson. (2000). Molecular physiology and pathophysiology of tight junctions. I. Tight junction structure and function: Lessons from mutant animals and proteins. Am. J. Physiol. 279: G250-G254.

151. Cereijido M, L Shoshani and RG Contreras. (2000). Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. Am. J. Physiol. 279: G477-G482.

152. Anderson JM. (2001). Molecular structure of tight junctions and their role in epithelial transport. News Physiol. Sci. 16: 126-130.

153. Tsukita S, M Furuse and M Itoh. (1999). Structural and signalling molecules come together at tight junctions. Curr. Opin. Cell Biol. 11: 628- 633.

154. Tsukita S and M Furuse. (2000). Pores in the wall: Claudins constitute tight junction strands containing aqueous pores. J. Cell Biol. 149: 13-16.

155. Maria OM, JW Kim, JA Gerstenhaber, BJ Baum and SD Tran. (2008). Distribution of tight junction proteins in adult human salivary glands. J Histochem Cytochem. 56:1093-1098.

156. Baum BJ and Tran SD. (2000) Synergy between genetic and tissue engineering: creating an artificial salivary gland. Periodontology, Vol. 41, 2006, 218–223.

157. Shirasuna K, Sato M, Miyazaki T. (1981) A neoplastic epithelial duct cell line established from an irradiated human salivary gland. Cancer; 48: 745-752.

158. Aframian DJ, Cukierman E, Nikolovski J, Mooney DJ, Yamada KM, Baum BJ. (2000) The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. Tissue Eng.; 6: 209-216.

159. Aframian DJ, Zheng C, Goldsmith CM, Nikolovski J, Cukierman E, Yamada KM, Mooney DJ, Birkedal-Hansen H, Baum BJ. (2001) Using HSV thymidine-kinase for safety in an allogeneic salivary graft cell line. Tissue Eng.; 7: 405-413.

160. Aframian DJ, Tran SD, Cukierman E, Yamada KM, Baum BJ. (2002) Absence of tight junction formation in an allogeneic graft cell line used for developing an engineered artificial salivary gland. Tissue Eng.; 8: 871-878.

161. Vukicevic S, Kleinman HK, Luyten FP, Roberts AB, Roche NS, Reddi AH. (1992) Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp. Cell Res.;202: 1-8

162. Lam K, Zheng L, Bewick M, Lafrenie RM. (2005) HSG cells differentiated by culture on extracellular matrix involves induction of s-adenosylmethione decarboxylase and ornithine decarboxylase. Journal of Cellular Physiology;203: 353-361

163. Roberts AB, Sporn MB. (1990) The Transforming Growth Factor-bs. In Peptide Growth Factors and Their Receptors. Part 1, ch. 8, ed. M. B. Sporn and A. B. Roberts. Springer-Verlag, Berlin, p. 419-472.

164. Kleinman HK, Martin JR. (2005) Matrigel: Basement membrane matrix with biological activity. Seminars in Cancer Biology; 15: 378-386.

165. Royce LS, Kibbey MC, Mertz P, Kleinman HK, Baum BJ. (1993) Human neoplastic submandibular intercalated duct cells express an acinar phenotype when cultured on a basement membrane matrix. Differentiation; 52: 247-255.

166. Hoffman MP, Kibbey MC, Letterio JJ, Kleinman HK. (1996) Role of laminin-1 and TGF-b3 in acinar differentiation of a human submandibular gland cell line (HSG). J. Cell Sci.; 109: 2013-2021.

167. Zajicek G, D Schwartz-Arad, N Arber and Y Michaeli. (1989). The streaming of the submandibular gland II: parenchyma and stroma advance at the same velocity. Cell Tissue Kinet. 22: 343-348.

168. Denny PC, WD Ball and RS Redman. (1997). Salivary glands: a paradigm for diversity of gland development. Crit Rev Oral Biol Med. 8: 51-75.

169. Okumura K, K Nakamura, Y Hisatomi, K Nagano, Y Tanaka, K Terada, T Sugiyama, K Umeyama, K Matsumoto, T Yamamoto and F Endo. (2003). Salivary gland progenitor cells induced by duct ligation differentiate into hepatic and pancreatic lineages. Hepatology. 38: 104-113.

170. David R, E Shai, DJ Aframian and A Palmon. (2008). Isolation and cultivation of integrin α6β1-expressing salivary gland graft cells: a model for use with an artificial salivary gland. Tissue Eng. 14: 331-337.

171. Prockop DJ. (1997). Marrow stromal cells as stem cells for non-hematopoietic tissues. Science 276: 71-74.

172. Nardi NB and L da Silva Meirelles. (2006). Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb. Exp. Pharmacol. 174: 249-282.

173. Kopen GC, DJ Prockop and DG Phinney. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc. Natl. Acad. Sci. USA 96: 10711-10716.

174. Sato Y, H Araki, J Kato, K Nakamura, Y Kawano, M Kobune, T Sato, K Miyanishi, T Takayama, M Takahashi, R Takimoto, S Iyama, T Matsunaga, S Ohtani, A Matsuura, H Hamada and Y Niitsu. (2005). Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood 106: 756-763.

175. Houghton J, C Stoicov, S Nomura, AB Rogers, J Carlson, H Li, X Cai, JG Fox, JR Goldenring and TC Wang. (2004). Gastric cancer originating from bone marrow-derived cells. Science 306: 1568-1571.

176. Choi KS, JS Shin, JJ Lee, YS Kim, SB Kim and CW Kim. (2005). In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. Biochem. Biophys. Res. Commun. 330: 1299-1305.

177. Lange C, P Bassler, MV Lioznov, H Bruns, D Kluth, AR Zander and HC Fiegel. (2005). Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. World J. Gastroenterol. 11: 4497-4504.

178. Phinney DG and DJ Prockop. (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells. 25:2896-902.

179. Zuk PA, M Zhu, H Mizuno, J Huang, JW Futrell, AJ Katz, P Benhaim, HP Lorenz. And MH Hedrick. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 7: 211-228.

180. Seo BM, M Miura, S Gronthos, PM Bartold, S Batouli, J Brahim, M Young, PG Robey, CY Wang and S Shi. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364: 149-155.

181. da Silva Meirelles L, PC Chagastelles and NB Nardi. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 119: 2204-2213.

182. De Bari C, F Dell'Accio, P Tylzanowski and FP Luyten. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. 44:1928-1942

183. Sabatini F, L Petecchia, M Tavian, VJ de Villeroche, GA Rossi and D Brouty-Boye. (2005). Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab. Invest. 85: 962-971.

184. Rotter N, J Oder, P Schlenke, U Lindner, F Böhrnsen, J Kramer, J Rohwedel, R Huss, S Brandau, B Wollenberg and S Lang. (2008). Isolation and characterization of adult stem cells from human salivary glands. Stem Cells Dev. 17: 509-518.

185. Watt FM and BL Hogan. (2000). Out of Eden: stem cells and their niches. Science 287: 1427-1430.

186. Aframian DJ, Redman RS, Yamano S, Nikolovski J, Cukierman E, Yamada KM, Kriete MF, Swaim WD, Mooney DJ, Baum BJ (2002a) Tissue compatibility of two biodegradable tubular scaffolds implanted adjacent to skin or buccal mucosa in mice. Tissue Eng 8: 649-659.

187. Aframian DJ, Zheng C, Goldsmith CM, Nikolovski J, Cukierman E, Yamada KM, Mooney DJ, Birkedal-Hansen H, Baum BJ (2001) Using HSV-thymidine kinase for safety in an allogeneic salivary graft cell line. Tissue Eng 7:405-413

188. Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M (2002) Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci 115:4969-4976

189. Augustus J, Bijman J & Van Os CH (1978) Electrical resistance of rabbit submaxillary main duct: a tight epithelium with leaky cell membranes. Journal of Membrane Biology 43: 203-226

190. Baron S, Poast J, Cloyd MW (1999). Why is HIV rarely transmitted by oral secretions? Saliva can disrupt orally shed, infected leukocytes. Arch Intern Med 159:303-310

191. Bazzoni G, Martinez-Estrada OM, Mueller F, Nelboeck P, Schmid G, Bartfai T, Dejana E, Brockhaus M (2000) Homophilic interaction of junctional adhesion molecule. J Biol Chem 275:30970–30976

192. Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendle GG, Vestweber D (2000) Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. J Biol Chem 275:27979–27988

193. Fox PC (1998) Acquired salivary dysfunction. Drugs and radiation. Ann NY Acad Sci 842:132-137

194. Fox PC, Speight PM (1996) Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjogren's syndrome. Crit Rev Oral Biol Med 7:144-158

195. Furuse M, Furuse K, Sasaki H, Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 153:263-272

196. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156:1099-1111

197. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123:1777-1788

198. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE (2003). Tight junction proteins. Prog Biophys Mol Biol 81:1-44

199. Gonzalez-Mariscal L, Namorado MC, Martin D, Luna J, Alarcon L, Islas S, Valencia L, Muriel P, Ponce L, Reyes JL (2000) Tight junction proteins ZO-1, ZO-2, and occludin along isolated renal tubules. Kidney Int 57:2386-2402

200. Gresz V, Kwon TH, Gong H, Agre P, Steward MC, King LS, Nielsen S (2004) Immunolocalization of AQP-5 in rat parotid and submandibular salivary glands after stimulation or inhibition of secretion in vivo. Am J Physiol Gastrointest Liver Physiol 287:G151-G161

201. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ (2003) Cancer statistics. CA Cancer J Clin 53:5-26

202. Kriegs JO, Homann V, Kinne-Saffran E, Kinne RK(2007). Identification and subcellular localization of paracellin-1 (claudin-16) in human salivary glands. Histochem Cell Biol. 128:45-53.

203. Lourenço SV, Coutinho-Camillo CM, Buim ME, Uyekita SH, Soares FA (2007). Human salivary gland branching morphogenesis: morphological localization of claudins and its parallel relation with developmental stages revealed by expression of cytoskeleton and secretion markers. Histochem Cell Biol. 128 61-369.

204. Mandell KJ, Parkos CA (2005) The JAM family of proteins. Adv Drug Deliv Rev 57:857–67

205. Mitic LL, Anderson JM (1998) Molecular architecture of tight junctions. Annu Rev Physiol 60:121–42

206. Morita K, Furuse M, Fujimoto K, Tsukita S (1999b) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96:511–516

207. Morita K, Sasaki H, Furuse M, Tsukita S (1999a) Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 147:185-194
208. Peppi M, Ghabriel MN (2004) Tissue-specific expression of the tight junction proteins claudins and occludin in the rat salivary glands. J Anat 205:257-266
209. Schneeberger EE, Lynch RD. (2004) The tight junction: A multifunctional complex. Am J Physiol Cell Physiol 286: C1213–1228
210. Shin K, Fogg VC, Margolis B (2006) Tight junctions and cell polarity. Annu Rev Cell Dev Biol 22: 207-235
211. Shin K, Margolis B (2006) Zoning out tight junctions. Cell 126: 647-649
212. Smaje LH, Henderson JR (1984) Microcirculation of the exocrine glands. In The Physiology and Pharmacology of the Microcirculation, Vol. 2, ed. Martillaro NA, New York, Academic Press, 325–380
213. Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S (1999) Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J Cell Biol 147:195-204
214. Stevenson BR, Anderson JM, Goodenough DA, Mooseker MS (1988) Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. J Cell Biol 107:2401-2408
215. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103:755-766
216. Tsukita S, Furuse M, Itoh M (2001) Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2:285-293
217. Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126: 741-754
218. Van Itallie C, Rahner C, Anderson JM (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest 107:1319-1327

219. Cook DI, Van Lennep EW, Roberts ML, Young JA. (1994) Secretion by the major salivary glands. In: Johnson LR, editor. Physiology of the gastrointestinal tract. New York: Raven, pp. 1061-1117.
220. Fox PC, Van der Ven PF, Sonies BC, Weiffenbach JM, Baum BJ. (1985) Xerostomia: evaluation of a symptom with increasing significance. J Am Dent Assoc.; 110: 519-525.
221. Fox RI, Maruyama T. (1997) Pathogenesis and treatment of Sjögren's syndrome. Curr. Opin. Rheumatol ; 9: 393-399.
222. Fox RI, Michelson P, Casiano CA, Hayashi J, Stern M. (2000) Sjogren's syndrome. Clin. Dermatol; 18: 589-600.
223. Kashima HK, Kirkham JR, Andrews JR. (1965) Post-irradiation sialadenitis. A study of the clinical features, histopathologic changes and serum enzyme variations following irradiation of human salivary glands. Am. J. Roentgenol. Radium Ther. Nucl. Med.; 77-94
224. Baum BJ, O'Connell BC. (1995) The impact of gene therapy on dentistry. J Am Dent Assoc; 126: 179-189.
225. Fox PC, Brennan M, Pillemer S, Radfar L, Yamano S, Baum BJ. (1998) Sjogren's syndrome: a model for dental care in the 21st century. J Am Dent Assoc; 129:719-728.
226. Wang S, Cukierman E, Swaim WD, Yamada KM, Baum BJ. (1999) Extracellular matrix protein-induced changes in human salivary epithelial cell organization and proliferation on a model biological substratum. Biomaterials; 20: 1043-1049.
227. Redman RS, Quissell DO. (1993) Isolation and maintenance of submandibular gland cells. In Biology of the Salivary Glands. ed. K. Dobrosielski-Vergona. Boca Raton, FL. CRC Press, Inc., pp. 285-306.
228. Oliver C, Water JF, Tolbert CC, Kleinman HK. (1987) Growth of exocrine acinar cells on a reconstituted basement membrane gel. In Vitro Cell. Dev. Biol ;23: 465-473.
229. Durban EM. (1990) Mouse submandibular salivary epithelial cell growth and differentiation in long term culture: influence of the extracellular matrix. In Vitro Cell. Dev. Biol; 26: 33-43.
230. Batsakis JG. (1980) Salivary gland neoplasia: an outcome of modified morphogenesis and cytodifferentiation. Oral Surg Oral Med Oral Pathol; 49:229-232.
231. Pierce GB. (1974) Neoplasms, differentiations and mutations. Am J Pathol;77:103-118.

232. Eversole LR. (1971) Histogenic classification of salivary tumors. Arch Pathol. 92:433-443.

233. Sato M, Azuma M, Hayashi Y, Yoshida H, Yanagawa T, Yura Y. (1987) 5-Azacytidine induction of stable myoepithelial and acinar cells from a human salivary intercalated duct cell clone. Cancer Res; 47: 4453-4459.

234. Hayashi Y, Yanagawa T, Azuma M, Yura Y, Yoshida H, Sato M. (1985) Induction of cells with a myoepithelial cell phenotype by treatment with dibutyryl cyclic AMP in human salivary adenocarcinoma cells grown in 41. athymic nude mice. Virchows Arch. Zellpathol; 50: 1- 11.

235. Yoshida H, Azuma M, Yanagawa T, Yura Y, Hayashi Y, Sato M. (1986) Effect of dibutyryl cyclic AMP on morphologic features and biologic markers of a human salivary gland adenocarcinoma cell line in culture. Cancer; 57: 1011- 1018.

236. Azuma M, Hayashi Y, Yoshida H, Yanagawa T, Yura Y, Ueno A, Sato M. (1986) Emergence of differentiated subclones from a human salivary adenocarcinoma cell clone in culture after treatment with sodium butyrate. Cancer Res; 46: 770-777.

237. Motegi K, Azuma M, Tamatani T, Ashida Y, Sato M. (2005) Expression of aquaporin-5 in and fluid secretion from immortalized human salivary gland ductal cells by treatment with 5-Aza-2'-Deoxycytidine: a possibility for improvement of xerostomia in patients with Sjogren's syndrome. Laboratory Investigation; 85: 342-353.

238. Azuma M, Kawamata H, Kasai Y, Nagamine S, Yoshida H, Yanagawa T, Sato M. (1988) Effects of Retinoic Acid on Morphological Features and Biological Markers of a Neoplastic Human Salivary Intercalated Duct Cell Line in Culture. Cancer Research; 48: 7219-7225.

239. Maria OM, Maria O, Liu Y, Komarova SV, Tran SD. (2010) Matrigel improves functional properties of human salivary cells. Abstract ID: 129684, Session Sequence: 97, Presentation: 699, AADR 39th Annual Meeting, Washington DC, USA.

240. Maria OM, Maria SM, Tran SD. Identification of Specific Population of Human Salivary Acini and their Progenitors Using Mesenchymal Stem Cell Markers, Submitted.

241. Takano K, Bogert M, Malamud D, Lally E, Hand AR. (1991) Differential Distribution of Salivary Agglutinin and Amylase in the Golgi Apparatus and Secretory Granules of the Human Salivary Gland Acinar Cells. The Anatomical Record; 230: 307-318.

242. Matsumoto S, Okumura K, Ogata A, Hisatomi Y, Sato A, Hattori K, Matsumoto M, Kaji Y, Takahashi M, Yamamoto T, Nakamura K, Endo F. (2007) Isolation of tissue progenitor cells from duct-ligated salivary glands of swine. Cloning Stem Cells; 9: 176-190.

243. Vag J, Byrne EM, Hughes DH, Hoffman M, Ambudkar I, Maguire P, O'Connell BC. (2007) Morphological and functional differentiation of HSG cells: role of extracellular matrix and trpc 1. J Cell Physiol; 212: 416-423.

244. Barka T, Gresik ES, Miyazaki Y. (2005) Differentiation of a mouse submandibular gland-derived cell line (SCA) grown on matrigel. Experimental Cell Research;308: 394-406.

245. Dang H, Dehghan PL, Goodwiler K, Chen S, Zardeneta G, Zhang B, Yeh C. (2006) Inhibition of CD95-Mediated Apoptosis through β1-Integrin in the HSG Epithelial Cell Line. Cell Communication and Adhesion;13: 223-232.

246. Zheng C, Hoffman MP, Mcmillan T, Kleinman HK, O'connell BC. (1998) Growth factor regulation of the amylase promoter in a differentiating salivary acinar cell line. J. Cell. Physiol;177: 628-635.

247. Gresz V, Kwon TH, Hurley PT, Varga G, Zelles T, Nielsen S, Case RM, Steward MC. (2001) Identification and localization of aquaporin water channels in human salivary glands. Am J Physiol Gastrointest Liver Physiol; 281: G247-G254.

248. Beroukas D, Hiscock J, Jonsson R, Waterman SA, Gordon TP. (2001) Subcellular distribution of aquaporin 5 in salivary glands in primary Sjogren's syndrome. Lancet;358:1875-1876.

249. Turner RJ, Sugiya H. (2002) Understanding salivary fluid and protein secretion. Oral Dis.;8:3-11.

250. Joraku A, Sullivan CA, Yoo J, Atala A. (2007) In-vitro reconstitution of three dimensional human salivary gland tissue structures. Differentiation; 75: 318-324.

251. Wei C, Larsen M, Hoffman MP, Yamada KM. (2007) Self-organization and branching morphogenesis of primary salivary epithelial cells. Tissue Eng.; 13:721-735.

252. Szlávik V, Vág J, Markó K, Demeter K, Madarász E, Oláh I, Zelles T, O'Connell BC, Varga G. (2008) Matrigel-induced acinar differentiation is followed by apoptosis in HSG cells. J Cell Biochem.;103: 284-295.

253. Sato M, Hayashi Y, Yanagawa T, Yoshida H, Yura Y, Azuma M, Ueno A. (1985) Intermediate-sized Filaments and Specific Markers in a Human Salivary Gland Adenocarcinoma Cell Line and Its Nude Mouse Tumors. Cancer Research;45: 3878-3890.
254. Ogawa, Y. (2003) Immunocytochemistry of myoepithelial cells in the salivary glands. Prog Histochem Cytochem.; 38:343-426.
255. Cutler LS, Chaudhry AP. (1973) Intercellular contacts at the epithelial-mesenchymal interface during the prenatal development of the rat submandibular gland. Dev Biol.; 33: 229-240.
256. Pedersen AM, Bardow A, Jensen SB, Nauntofte B. (2002) Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis.; 8: 117- 129.
257. Fisher DA, Lakshmanan J. (1990) Metabolism and effects of epidermal growth factor and related growth factors in mammals. Endocr. Rev.; 11: 418-442.
258. Quissell DO, Watson E, Dowd FJ. (1992) Signal Transduction Mechanisms Involved in Salivary Gland Regulated Exocytosis. Critical Review in Oral Biology and Medicine; 3; 83-107.
259. Hoffman MP, Nomizu M, Roque E, Lee S, Jung DW, Yamada Y, Kleinman HK. (1998) Laminin-1 and laminin-2 Gdomain synthetic peptides bind syndecan-1 and are involved in acinar formation of a human submandibular gland cell line. J Biol Chem. 273: 28633-28641.
260. Boudreau N, Sympson CJ, Werb Z, Bissell MJ. (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science; 267: 891-893.
261. Dirami G, Ravindranath N, Kleinman HK, Dym M. (1995) Evidence that basement membrane prevents apoptosis of sertoli cells in vitro in the absence of known regulators of sertoli cell function. Endocrinology; 136: 4439-4447.
262. Jaskoll T, Melnick M. (1999) Submandibular gland morphogenesis: Stage-specific expression of TGF-alpha/EGF, IGF, TGF-beta, TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF-beta2, TGF-beta3, and EGF-r null mutations. Anat. Rec. 256:252-268.
263. Amerongen, A.V., and Veerman, E.C. (2002) Saliva–the defender of the oral cavity. Oral Dis. 8, 12.

264. Melvin, J.E., Yule, D., Shuttleworth, T., and Begenisich, T. (2005) Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Annu. Rev. Physiol. 67, 445.

265. Aframian, D.J., David, R., Ben-Bassat, H., Shai, E., Deutsch, D., Baum, B.J., and Palmon, A. (2004) Characterization of murine autologous salivary gland graft cells: a model for use with an artificial salivary gland. Tissue Eng. 10, 914.

266. Wellner, R.B., Hong, S., Cotrim, A.P., Swaim, W.D., and Baum, B.J. (2005) Modifying the NH2 and COOH termini of aquaporin-5: effects on localization in polarized epithelial cells. Tissue Eng. 11, 1449.

267. Chen, M.H., Chen, R.S., Hsu, Y.H., Chen, Y.J., and Young, T.H. (2005) Proliferation and phenotypic preservation of rat parotid acinar cells. Tissue Eng. 11, 526.

268. Quissell, D.O., Flaitz, C.M., Redman, R.S., and Barzen, K.A. (1994) Primary Culture of Human Labial Salivary Gland Acini. In Vitro Cell. Dev. Biol. 30A,736.

269. Bücheler, M., Wirz, C., Schütz, A., and Bootz, F. (2002) Tissue engineering of human salivary gland organoids. Acta Otolaryngol. 5, 541,2002.

270. Pradhan, S., Zhang, C., Jia, X., Carson, D.D., Witt, R., and Farach-Carson, M.C. (2009) Perlecan domain IV peptide stimulates salivary gland cell assembly in vitro. Tissue Eng. Part A 11, 3309.

271. Joraku, A., Sullivan, C.A., Yoo, J.J., and Atala, A. (2005) Tissue engineering of functional salivary gland tissue. Laryngoscope 115,244.

272. Szlavik, V., Szabo, B., Vicsek, T., Barabas, J., Bogdan, S., Gresz, V., Varga, G., O'Connell, B., and Vag, J. (2008) Differentiation of primary human submandibular gland cells cultured on basement membrane extract. Tissue Eng. Part A. 14,1915.

273. Benton, G., George, J., Kleinman, H.K., and Arnaoutova, I.P. (2009) Advancing Science and Technology Via 3D Culture on Basement Membrane Matrix. J. Cell. Physiol. 221, 18.

274. Hadley, M.A., Weeks, B.S., Kleinman, H.K., and Dym, M. (1990) Laminin promotes formation of cord-like structures by Sertoli cells in vitro. Dev. Biol. 140, 318, 1990.

275. Woodcock-Mitchell, J., Rannels, S.R., Mitchell, J., Rannels, D.E., and Low, R.B. (1989) Modulation of keratin expression in type II pneumocytes by the extracellular matrix. Am. Rev. Respir. Dis. 139, 343.

276. Opas, M., and Dziak, E. (1990) Effects of a tumour promoter, 12- 0-tetradecanoyl-phorbol-13-acetate (TPA), on expression of differentiated phenotype in the chick retinal

pigmented epithelial cells and on their interactions with the native basement membrane and with artificial substrata. Differentiation 43, 20.

277. Fong, C.J., Sherwood, E.R., Sutkowski, D.M., Abu-Jawdeh, G.M., Yokoo, H., Bauer, K.D., Kozlowski, J.M., and Lee, C. (1991) Reconstituted basement membrane promotes morphological and functional differentiation of primary human prostatic epithelial cells. Prostate 19, 221.

278. Schuetz, E.G., Li, D., Omiecinski, C.J., Muller-Eberhard, U., Kleinman, H.K., Elswick, B., and Guzelian, P.S. (1988) Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. J Cell Physiol. 134, 309.

279. Sieminski, A.L., Padera, R.F., Blunk, T., and Gooch, K.J. (2002) Systemic delivery of human growth hormone using genetically modified tissue-engineered microvascular networks: prolonged delivery and endothelial survival with inclusion of nonendothelial cells. Tissue Eng. 8, 1057.

280. Vugman, I. and Hand, A.R. (1995) Quantitative immunocytochemical study of secretory protein expression in parotid glands of rats chronically treated with isoproterenol. Microsc. Res. Tech. 31, 106.

281. O'Brien, L.E., Zegers, M.M., and Mostov, K.E. (2002) Opinion: building epithelial architecture: insights from three-dimensional culture models. Nat. Rev. Mol. Cell Biol. 3,531.

282. Sullivan, C.A., Haddad, R.I., Tishler, R.B., Mahadevan, A., and Krane, J.F. (2005) Chemoradiation-induced cell loss in human submandibular glands. Laryngoscope 115, 958.

283. Kashimata, M., and Gresik, E.W. (1996) Contemporary approaches to the study of salivary gland morphogenesis. Eur J Morphol 34,143.

284. Cutler, L.S. (1990) The role of extracellular matrix in the morphogenesis and differentiation of salivary glands. Adv. Dental Res. 4,27.

285. Wu, J.E., and Santoro, A.S. (1996) Differential expression of integrin and alpha subunits supports distinct roles during lung branching morphogenesis. Dev. Dyn. 206,169.

286. Vanaken, H., Vercaeren, I., Claessens, F., De Vos, R., Dewolf-Peeters, C., Vaerman, J.P., Heyns, W., Rombauts, W., and Peeters, B. (1998) Primary Rat Lacrimal Cells Undergo

Acinar-like Morphogenesis on Reconstituted Basement Membrane and Express Secretory Component under Androgen Stimulation. Experimental Cell Research 238, 377.

287. Hiraki, A., Shirasuna, K., Ikari, T., Shinohara, M., and Garrod, D.R. (2002) Calcium induces differentiation of primary human salivary acinar cells. J Cell Physiol. 1,55.

288. Dimitriou, I.D., Kapsogeorgou, E.K., Abu-Helu, R.F., Moutsopoulos, H.M., and Manoussakis, M.N. (2002). Establishment of a convenient system for the long-term culture and study of non-neoplastic human salivary gland epithelial cells. Eur. J. Oral Sci. 110, 21.

289. Okura, M., Shirasuna, K., Hiranuma, T., Yoshioka, H., Nakahara, H., Aikawa, T., and Matsuya, T. (1993) Characterization of growth and differentiation of normal human submandibular gland epithelial cells in a serum-free medium. Differentiation 54, 143.

290. Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J., and Brugge, J.S. (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol. 3,785.

291. Debnath, J., Walker, S.J., and Brugge, J.S. (2003) Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol. 163,315.

292. Kong, W., Li, S., Liu, C., Bari, A.S., Longaker, M.T., and Lorenz, H.P. (2006) Epithelial-mesenchymal transition occurs after epidermal development in mouse skin. Exp. Cell Res. 312, 3959.

293. Ogawa, Y. (2003) Immunohistochemistry of Myoepithelial Cells in the Salivary Glands. Progr. Histochem. Cytochem. 4, 343.

294. Bilder, D. (2004) Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes Dev. 18,1909.

295. Matter, K., Aijaz, S., Tsapara, A., and Balda, M.S. (2005) Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. Curr. Opin. Cell Biol. 17, 453.

296. Zahraoui, A., Louvard, D., and Galli, T. (2000) Tight junction, a platform for trafficking and signaling protein complexes. J Cell Biol. 151,F31.

297. Schittny, J.C., Timpl, R., and Engel, J. (1988) High resolution immunoelectron microscopic localization of functional domains of laminin, nidogen, and heparan sulfate

proteoglycan in epithelial basement membrane of mouse cornea reveals different topological orientations. J Cell Biol. 107, 1599.

298. Fujita-Yoshigaki, J., Matsuki-Fukushima, M., and Sugiya, H. (2008) Inhibition of Src and p38 MAP kinases suppresses the change of claudin expression induced on dedifferentiation of primary cultured parotid acinar cells. Am. J Physiol. Cell Physiol. 294,C774.

299. Hecht, D., Jung, D., Prabhu, V.V., Munson, P.J., Hoffman, M.P., and Kleinman, H.K. (2002) Metallothionein promotes laminin- 1-induced acinar differentiation in vitro and reduces tumor growth in vivo. Cancer Res. 62, 5370.

300. Abukawa H, Michael S, Williams WB, Vacanti JP, Kaban LB, Troulis MJ (2004). Reconstruction of Mandibular Defects with Autologous Tissue-Engineered Bone. *J Oral Maxillofac Surg* 62:601-606.

301. Abukawa H, Terai H, Hannouche D, Vacanti JP, Kaban LB, Troulis MJ (2003). Formation of a Mandibular Condyle in Vitro by Tissue Engineering, *J Oral Maxillofac Surg* 61:94-100.

302. Alhadlaq A, Elisseeff JH, Hong L, Williams CG, Caplan AI, Sharma B, Kopher RA, Tomkoria S, Lennon DP, Lopez A, Mao JJ (2004). Adult stem cell driven genesis of human-shaped articular condyle. *Ann Biomed Eng* 32:911-923.

303. Bartold PM, McCulloch CAG, Narayanan AS, Pitaru S (2000). Tissue-engineering: A new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol* 22:169-189.

304. Bianco P, Riminucci M, Gronthos S, Robey PG (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* 19: 180-192.

305. Caplan AI, Bruder SP (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* 7:259-264.

306. Colter DC, Class R, Di Girolamo CM, Prockop DJ (2000). Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci USA* 97: 3213-3218

307. De Kok IJ, Drapeau SJ, Young R, Cooper LF (2005). Evaluation of mesenchymal stem cells following implantation in alveolar sockets: A canine safety study. Int J *Oral Maxillofac Implants* 20:511-518.

308. Fuchs JR, Hannouche D, Terada S, Zand S, Vacanti JP, Fauza DO (2005). Cartilage engineering from ovine umbilical cord blood mesenchymal progenitor cells. *Stem Cells* 23:958-64.

309. Green WT (1977). Articular cartilage repair. Behavior of rabbit chondrocytes during tissue culture and subsequent allografting. *Clin Orthop Relat Res* 124:237-50.

310. Grezesik WJ, Narayanan AS (2002). Cementum and periodontal wound healing and regeneration. *Crit Rev Oral Biol Med* 13:474-484.

311. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 97:13625-13630.

312. Higuera CA, Inoue N, Lim JS, Zhang R, Dimaano N, Frassica FJ, Chao EY (2005). Tendon reattachment to a metallic implant using an allogenic bone plate augmented with rhOP-1 vs. autogenous cancellous bone and marrow in a canine model. *J Orthop Res* 23:1091-1099.

313. Horowitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. The International Society for Cellular Therapy (2005). Clarification of the nomenclature for MSc: The International Society for Cellular Therapy position statement. Cytotherapy 7: 393–395.

314. Ivanovski S, Gronthos S, Shi S, and Bartold P (2006). Stem cells in the periodontal ligament. *Oral Diseases* 12:358-363.

315. Kataoka H, Urist MR. (1993). Transplant of bone marrow and muscle-derived connective tissue cultures in diffusion chambers for bioassay of bone morphogenetic protein. *Clin Orthop Relat Res* 286:262-270.

316. Kawaguchi H, Hirachi A, Hasegawa N, Iwata T, Hamaguchi H, Shiba H, Takata T, Kato Y, Kurihara H (2004). Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol* 75:1281-1287.

317. Kawaguchi H, Hirachi A, Mizuno N, Fujita T, Hasegawa NH, Shiba, Nakamura S, Hino T, Yoshino H, Kurihara H (2005). Cell transplantation for periodontal diseases: A novel periodontal tissue regenerative therapy using bone marrow mesenchymal stem cells. *Clin Calcium* 15:1197-1202.

318. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S (1999). Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 103:697-705.

319. Metaxas Y, Zeiser R, Schmitt-Graeff A, Waterhouse M, Faber P, Follo M, Bertz H, Finke J, Spyridonidis A (2005). Human hematopoietic cell transplantation results in generation of donor-derived epithelial cells. *Leukemia 19*:1287-1289.

320. Mezey E (2004). Commentary: on bone marrow stem cells and openmindedness. *Stem Cells Dev* 13:147-152.

321. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003). SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 100:5807-8512.

322. Modino SAC, Sharpe PT (2005). Tissue engineering of teeth using adult stem cells. *Arch Oral Biol* 50:255-258.

323. Murai M, Sato S, Koshi R, Yokoyama K, Ikeda K, Narukawa M, Takayama T (2005). Effects of the enamel matrix derivative and beta-tricalcium phosphate on bone augmentation within a titanium cap in rabbit calvarium. *J Oral Sci* 47:209-217.

324. Ohazama A, Modino SAC, Miletich I, Sharpe PT (2004). Stem-cell-based Tissue Engineering of Murine Teeth. *J Dent Res* 83:518-522.

325. Simon CG Jr, Guthrie WF, Wang FW (2004). Cell seeding into calcium phosphate cement. *J Biomed Mater Res A* 68:628-639.

326. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416:542-545.

327. Tran SD, Pillemer SR, Dutra A, Barrett AJ, Brownstein MJ, Key S, Pak E, Leakan RA, Kingman A, Yamada KM, Baum BJ, Mezey E (2003). Differentiation of human bone marrow derived cells into buccal epithelial cells in vivo: a molecular analytical study. *Lancet* 361:1084–1088.

328. Warnke PH, Springer ING, J Wiltfang, Acil Y, Eufinger H, Wehmöller M, Russo PAJ, Bolte H, Sherry E, Behrens E, Terheyden H (2004). Growth and transplantation of a custom vascularised bone graft in a man. *Lancet* 364:766–770.

329. Wilan KH, Scott CT, Herrera S (2005). Chasing a cellular fountain of youth. *Nat Biotechnol* 23:807-815.

330. Yamada Y, Ueda M, Naiki T, and Nagasaka T (2004a). Tissue-engineered injectable bone regeneration for osseointegrated dental implants. *Clin Oral Implants Res* 15:589-597.

331. Yamada Y, Ueda M, Naiki T, Takahashi M, Hata K, Nagasaka T (2004b). Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: tissue-engineered bone regeneration. *Tissue Eng* 10:955-964.

332. Scott, J., Baum, B.J., Woods, K., and Berry, M.R. (1989) Chloride handling by submandibular cells from young and old rats with and without duct ligation. Mech Ageing Dev. 48, 231.

333. Martinez, J.R., Bylund, D.B., and Cassity, N. (1982) Progressive secretory dysfunction in the rat submandibular gland after excretory duct-ligation. Arch Oral Biol. 27, 443.

334. Hisatomi, Y., Okumura, K., Nakamura, K., Matsumoto, S., Satoh, A., Nagano, K., Yamamoto, T., and Endo, F. (2004) Flow cytometric isolation of endodermal progenitors from mouse salivary gland differentiate into hepatic and pancreatic lineages. Hepatology 39, 667.

335. Redman, R.S. (1995) Proliferative activity by cell type in the developing rat parotid gland. Anat. Rec. 241, 529.

336. Schwartz-Arad, D., Michaeli, Y., and Zajicek, G. (1991) Compensatory hyperplasia of the rat submandibular gland following unilateral extirpation. J Dent Res. 70, 1328.

337. Denny, P.C., Chai, Y., Klauser, D.K., and Denny, P.A. (1993) Parenchymal cell proliferation and mechanisms for maintenance of granular duct and acinar cell populations in adult male mouse submandibular gland. Anat Rec. 235, 475.

338. Summers, K.L., O'Donnell, J.L., Hoy, M.S., Peart, M., Dekker, J., Rothwell, A, and Hart, D.N. (1995) Monocyte–macrophage antigen expression on chondrocytes. J Rheumatol. 22, 1326.

339. Swart, G.W. (2002) Activated leukocyte cell adhesion molecule (CD166/ALCAM): Developmental and mechanistic aspects of cell clustering and cell migration. Eur J Cell Biol. 81, 313.

340. Jacob, S.S, Shastry, P., and Sudhakaran, P.R. (2002) Monocyte–macrophage differentiation in vitro: Modulation by extracellular matrix protein substratum. Mol Cell Biochem. 233, 9.

341. Karaöz, E., Doğan, B.N., Aksoy, A., Gacar, G., Akyüz, S., Ayhan, S., Genç, Z.S., Yürüker, S., Duruksu, G., Demircan, P.C., and Sariboyaci, A.E (2010). Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. Histochem Cell Biol, 133, 95.

342. Wang, L., Verbruggen, G., Almqvist, K., Elewaut, D., Broddelez, C., and Veys, M (2001). Flow cytometric analysis of the human articular chondrocyte phenotype in vitro. Osteoarthritis Cart 9, 73.

343. Menghi, G., Bondi, A.M,, Marchetti, L., and Fumagalli, L (1992). On the fine structure and complex carbohydrate cytochemistry of the rabbit parotid gland. Biol. Struct. Morphog. 4, 1.

344. Cutler, L.S., and Gremski, W. Epithelial-mesenchymal interactions in the development of salivary glands. Crit Rev Oral Biol. Med. 2, 1, 1991.

345. Moore, K.A., and Lemischka, I.R (2006). Stem cells and their niches. Science 311, 1880.

346. Potten, S.C., Booth, C., Tudor, G.L., Booth, D., Brady, G., Hurley, P., Ashton, G., Clarke, R., Sakakibara, S., and Okano, H. (2003). Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation 71, 28.

347. Nagata, H., Akiba, Y., Suzuki, H., Okano, H., and Hibi, T (2006). Expression of Musashi-1 in the rat stomach and changes during mucosal injury and restitution. FEBS Lett. 580, 27.

348. Clarke, R.B., Spence, K., Anderson, E., Howell, A., Okano, H., and Potten, C.S. (2005). A putative human breast stem cell population is enriched for steroid receptor-positive cells. Dev Biol. 277, 443.

349. Sugiyama-Nakagiri, Y., Akiyama, M., Shibata, S., Okano, H., and Shimizu, H. (2006). Expression of RNA-binding protein Musashi in hair follicle development and hair cycle progression. Am J Pathol. 168, 80.

350. Gotte, M., Wolf, M., Staebler, A., Buchweitz, O., Kelsch, R., Schuring, A.N., and Kiesel, L (2008). Increased expression of the adult stem cell marker Musashi-1 in endometriosis and endometrial carcinoma.J Pathol. 215, 317, 2008.

351. Okano, H., Kawahara, H., Toriya, M., Nakao, K., Shibata, S., and Imai, T. (2005). Function of RNA-binding protein Musashi-1 in stem cells, Exp. Cell Res. 306, 349.

352. Okano, H., Imai, T., and Okabe, M. (2002). Musashi: a translational regulator of cell fates, J. Cell Sci. 115, 1355.

353. Doherty, M.J., Ashton, B.A., Walsh, S., Beresford, J.N., Grant, M.E., and Canfield, A.E. (1998). Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res. 13, 828.

354. Young, H.E., Steele, T.A., Bray, R.A., Hudson, J., Floyd, J.A., Hawkins, K., Thomas, K., Austin, T., Edwards, C., Cuzzourt, J., Duenzl, M., Lucas, P.A., Black, A.C.Jr (2001). Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec. 264:51.

355. Gronthos, S., Zannettino, A.C., Hay, S.J., Shi, S., Graves, S.E., Kortesidis, A., and Simmons, P.J. (2003). Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow. J Cell Sci. 116, 1827.

356. Shi, S., and Gronthos, S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res. 18, 696.

357. Dalerba, P., Dylla, S.J., Park, I.K., Liu, R., Wang, X., Cho, R.W., Hoey, T., Gurney, A., Huang, E.H., Simeone, D.M., Shelton, A.A., Parmiani, G., Castelli, C. and Clarke, M.F (2007). Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 104, 10158.

358. Boman, B.M., Fields, J.Z., Cavanaugh, K.L., Guetter, A., and Runquist, O.A. (2008). How dysregulated colonic crypt dynamics cause stem cell overpopulation and initiate colon cancer, Cancer Res. 68, 3304.

359. Weichert, W., Knosel, T., Bellach, J., Dietel, M., and Kristiansen, G (2004). ALCAM/CD166 is over-expressed in colorectal carcinoma and correlates with shortened patient survival, J. Clin. Pathol. 57, 1160.

360. Lombaert, I.M.A., Brunsting, J.F., Wierenga, P.K., Faber, H., Stokman, M.A., Kok, T., Visser, W.H., Kampinga, H.H., de Haan, G., and Coppes, R.P (2008). Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One 3, e2063.

361. Kishi, T., Takao, T., Fujita, K., and Taniguchi, H (2006). Clonal proliferation of multipotent stem/ progenitor cells in the neonatal and adult salivary glands. Biochem Biophys Res Commun. 340, 544.

362. Feng, J., van der Zwaag, M., Stokman, M.A., van Os, R., and Coppes, R.P. (2009). Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. Radiotherapy and Oncology. 92, 466.

363. Gorjup, E., Danner, S., Rotter, N., Habermann,,J., Brassat, U., Brummendorf, T.H., Wien, S., Meyerhans, A., Wollenberg, B., Kruse, C., and Von-Briesen, H (2009). Glandular tissue from human pancreas and salivary gland yields similar stem cell populations. Eur. J. Cell Biol. 88, 409.

364. Sato, A., Okumura, K., Matsumoto, S., Hattori, K., Hattori, S., Shinohara, M., and Endo, F (2007). Isolation, tissue localization, and cellular characterization of progenitors derived from adult human salivary glands. Cloning and Stem Cells. 9, 191.

365. Yagil, C.H., Michaeli, Y., and Zajicek, G (1985). Compensatory Proliferative Response of the Rat Submandibular Salivary Gland to Unilateral Extirpation, Virchow's Arch Cell Pathol 49, 83.

366. Peter, B., Van Waarde, M.A., Vissink, A.,'s-Gravenmade, E.J., and Konings, A.W (1994). Radiation-induced cell proliferation in the parotid and submandibular glands of the rat. Radiat Res. 140, 257.

367. Redman, R.S (1994). Myoepithelium of salivary glands. Microsc Res. Tech. 27, 25.

368. Schwartz-Arad, D., Arber, L., Arber, N., Zajicek, G., and Michaeli, Y (1988). The rat parotid gland- a renewing cell population. J Anat. 161, 143.

369. Ihrler, S., Blasenbreu-Vogt, S., Sendelhofert, A., Rössle, M., Harrison, J.D., and Löhrs, U (2004). Regeneration in chronic sialadenitis: an analysis of proliferation and apoptosis based on double immunohistochemical labeling. Virchows Arch. 444, 356.

370. Takahashi, S., Schoch, E., and Walker, N.I. (1998) Origin of acinar cell regeneration after atrophy of the rat parotid induced by duct obstruction. Int. J Exp. Pathol. 79, 293.

371. Sneath, R.J. and Mangham, D.C. (1998). The normal structure and function of CD44 and its role in neoplasia. J Clin Pathol Mol Pathol. 51, 191.

372. Middleton, J., Patterson, A.M., Gardner, L., Schmutz, C., and Ashton, B.A. (2002). Leukocyte extravasation: chemokine transport and presentation by the endothelium. Blood 100, 3853.

373. Campbell, D.J., Kim, C.H., and Butcher, E.C. (2003). Chemokines in the systemic organization of immunity. Immunological reviews 195:58-71.

374. Maria OM, AG Zeitouni, O Gologan and SD Tran. Matrigel improves functional properties of primary human salivary cells. Submitted.

375. http://en.wikipedia.org/wiki/Cell_%28biology%29

376. Spees JL, SD Olson, J Ylostalo, PJ Lynch, J Smith, A Perry, A Peister, MY Wang and DJ Prockop. (2003). Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow. Proc Natl Acad Sci USA 100: 2397–2402.

377. Jang YY, MI Collector, SB Baylin, AM Diehl and Sharkis SJ. (2004). Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol. 6:532-539.

378. Zurita M, C Bonilla, L Otero, C Aguayo and J Vaquero. (2008).Neural transdifferentiation of bone marrow stromal cells obtained by chemical agents is a short-time reversible phenomenon. Neurosci Res. 60:275-280.

379. Tremain N, J Korkko, D Ibberson, GC Kopen, C DiGirolamo and DG Phinney. (2001). MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stem Cells. 19:408-18.

380. Uccelli A, L Morettaand V Pistoia. (2006). Immunoregulatory function of mesenchymal stem cells. Eur J Immunol. 36:2566-73.

381. Zhao Z, L Liao, Y Cao, X Jiang and RC Zhao. (2005). Establishment and properties of fetal dermis-derived mesenchymal stem cell lines: plasticity in vitro and hematopoietic protection in vivo. Bone Marrow Transplant. 36:355-365.

382. Meirelles Lda S and NB Nardi. (2009). Methodology, biology and clinical applications of mesenchymal stem cells. Front Biosci. 14:4281-4298.

383. Iso Y, JL Spees, C Serrano, B Bakondi, R Pochampally, YH Song, BE Sobel, P Delafontaine and DJ Prockop. (2007). Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun. 354:700-706.

384. Larouche D, A Lavoie, S Proulx, C Paquet, P Carrier, A Beauparlant, FA Auger and L Germain. (2009). Regenerative medicine: stem cells, cellular and matricial interactions in

the reconstruction of skin and cornea by tissue engineering. Pathol Biol (Paris). 57:299-308.

385. Cutler LS and W Gremski. (1991). Epithelial-mesenchymal interactions in the development of salivary glands. Crit Rev Oral Biol. Med. 2: 1-12.

386. Sakai T, M Larsen and KM Yamada. (2003). Fibronectin requirement in branching morphogenesis. Nature. 423:876-881.

387. Riva A, G Motta and F Riva-Testa. (1974). Ultrastructural diversity in secretory granules of human major salivary glands. Am J Anat. 139:293-8.

388. Tandler B and DK MacCallum. (1972). Ultrastructure and histochemistry of the submandibular gland of the European hedgehog, Erinaceous europaeus L. I. Acinar secretory cells. J Ultrastruct Res. 39:186-204.

389. Sumita Y, Y Liu, S Khalili, OM Maria, D Xia, S Key, AP Cotrim, E Mezey and SD Tran. Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. Submitted.

390. Herzog EL, L Chai, L and DS Krause. (2003). Plasticity of marrow-derived stem cells. Blood 102: 3483-3493.

391. Chen J, Y Li, L Wang, M Lu, X Zhang and M Chopp. (2001). Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. J. Neurol. Sci. 189: 49-57.

392. Chen X, Y Li, L Wang, M Katakowski, L Zhang, J Chen, Y Xu, SC Gautam and M Chopp. (2002). Ischemic rat brain extracts induce human marrow stromal cell growth factor production. Neuropathology 22: 275-279.

393. Chopp M, and Y Li. (2002). Treatment of neural injury with marrow stromal cells. Lancet. Neurol. 1: 92-100.

394. Mahmood A, D Lu and M Chopp. (2004). Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. Neurosurgery 55:1185–1193.

395. Weimann JM, CB Johansson, A Trejo and HM Blau. (2003). Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat.Cell Biol. 5, 959–966.

396. Alvarez-Dolado M, R Pardal, JM Garcia-Verdugo, JR Fike, HO Lee, K Pfeffer, C Lois, SJ Morrison and A Alvarez-Buylla. (2003). Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425: 968-973.

397. Horwitz EM, PL Gordon, WKK Koo, JC Marx, MD Neel, RY McNall, L Muul and T Hofmann (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci USA 99:8932-8937.

398. Fraser JK, RE Schreiber, PA Zuk and MH Hedrick. (2004). Adult stem cell therapy for the heart. Int J Biochem Cell Biol. 36:658-666.

399. Uccelli A, V Pistoia and L Morett. (2007). Mesenchymal stem cells: a new strategy for immunosuppression? Trends Immunol. 28:219-226.

400. Hoffman, M.P., Nomizu, M., Roque, E., Lee, S., Jung, D.W., Yamada, Y., and Kleinman, H.K (1998). Laminin-1 and laminin-2 G domain synthetic peptides bind syndecan-1 and are involved in acinar formation of a human submandibular gland cell line. J Biol. Chem. 273, 28633.

401. Maria, O.M., Maria, O., Liu, Y., Komarova, S.V., and Tran, S.D. Matrigel improves functional properties of human submandibular salivary gland cell line. (Submitted).

402. Denny, P.C., Chai, Y., Klauser, D.K., and Denny, P.A (1993). Parenchymal cell proliferation and mechanisms for maintenance of granular duct and acinar cell populations in adult male mouse submandibular gland. Anat Rec. 235: 475.

403. Zou Z, Y Zhang, L Hao, F Wang, D Liu, Y Su and H Sun. (2010). More insight into mesenchymal stem cells and their effects inside the body. Expert Opin Biol Ther. 10:215-30.

APPENDICES

- Reprints of Published Manuscripts

Distribution of Tight Junction Proteins in Adult Human Salivary Glands

By

Ola M Maria, Jung-Wan M Kim, Jonathan A Gerstenhaber, Bruce J Baum, Simon D Tran.

Journal of Histochemistry and Cytochemistry. 2008; 56(12):1093-1098.

Volume 56(12): 1093–1098, 2008
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

The Journal of Histochemistry & Cytochemistry

ARTICLE

Distribution of Tight Junction Proteins in Adult Human Salivary Glands

Ola M. Maria, Jung Wan Martin Kim, Jonathan A. Gerstenhaber, Bruce J. Baum, and Simon D. Tran

Faculty of Dentistry, McGill University, Montreal, Canada (OMM,J-WMK,SDT), and National Institute of Dental and Craniofacial Research, Bethesda, Maryland (JAG,BJB)

SUMMARY Tight junctions (TJs) are an essential structure of fluid-secreting cells, such as those in salivary glands. Three major families of integral membrane proteins have been identified as components of the TJ: claudins, occludin, and junctional adhesion molecules (JAMs), plus the cytosolic protein zonula occludens (ZO). We have been working to develop an orally implantable artificial salivary gland that would be suitable for treating patients lacking salivary parenchymal tissue. To date, little is known about the distribution of TJ proteins in adult human salivary cells and thus what key molecular components might be desirable for the cellular component of an artificial salivary gland device. Therefore, the aim of this study was to determine the distribution of TJ proteins in human salivary glands. Salivary gland samples were obtained from 10 patients. Frozen and formalin-fixed paraffin-embedded sections were stained using IHC methods. Claudin-1 was expressed in ductal, endothelial, and ~25% of serous cells. Claudins-2, -3, and -4 and JAM-A were expressed in both ductal and acinar cells, whereas claudin-5 was expressed only in endothelial cells. Occludin and ZO-1 were expressed in acinar, ductal, and endothelial cells. These results provide new information on TJ proteins in two major human salivary glands and should serve as a reference for future studies to assess the presence of appropriate TJ proteins in a tissue-engineered human salivary gland. (J Histochem Cytochem 56:1093–1098, 2008)

KEY WORDS
salivary gland
tight junction
claudins
occludin
junctional adhesion molecules
zonula occludens
epithelial barriers

IRREVERSIBLE SALIVARY GLAND parenchymal damage and hypofunction result from two major causes. The first is therapeutic irradiation of patients with head and neck cancer, affecting almost 30,000 new patients each year in the United States. The second is an autoimmune disorder, Sjögren's syndrome, affecting ~1 million patients in the United States (Fox and Speight 1996; Jemal et al. 2003). In these two types of patients, both the quantity and the quality of saliva are altered to the extent that considerable morbidity occurs, such as dry mouth, dysphagia, dental caries, oropharyngeal infections, and mucositis (Fox 1998).

We have been working to develop an orally implantable fluid secretory device (an artificial salivary gland) that would be suitable for treating patients lacking salivary parenchymal tissue (Baum et al. 1999; Aframian et al. 2000,2001,2002a,b). Our initial choice of an allogeneic human submandibular gland cell line (HSG) was unable to attain a polarized monolayer organization and did not express any tight junction (TJ) proteins or generate a transepithelial electrical resistance (TER) (Aframian et al. 2002b). We re-engineered HSG cells to express individual or a combination of key TJ proteins (claudin-1 or/and claudin-2) by establishing stable clones of transfected cells. Nonetheless, when we examined these transfectants for functional tight junctional activity (TER or fluid barrier), none was observed (Aframian et al. 2002b). The inability of the HSG cell line to form TJs led to the development of a method of culturing and expanding primary human salivary gland (huSG) cells. The cultured huSG cells have been shown to possess tight and intermediate junctions between cells, a transepithelial electrical resistance, and a low paracellular fluid movement, all of which are characteristics of polarized cells (Tran et al. 2005).

Correspondence to: Simon Tran, 3640 University Street, Room M43, Montreal, Quebec, Canada H3A 2B2. E-mail: simon.tran@mcgill.ca

Received for publication May 7, 2008; accepted August 22, 2008 [DOI: 10.1369/jhc.2008.951780].

0022-1554/08/$3.30

Epithelial cells with primary secretory functions, such as in salivary glands, must be polarized to achieve a unidirectional secretion. TJs are the primary structure that forms a barrier between the apical and basal region of the cell. In the apical face of the cell, the transport proteins produce an osmotic concentration gradient, in addition to providing a facilitated water permeability pathway. TJs also promote a "fence" function that maintains the differential composition of the basolateral and apical domains by preventing the free diffusion of lipids and proteins between these compartments (Baum 1993; Mostov et al. 2000). In salivary glands, this gradient separation becomes important for the effective movement of ions, such as sodium and chloride, required for the production of saliva. Water and solutes that will form the exocrine secretion must pass across three different barriers: the vascular endothelium, the glandular interstitium, and the secretory epithelium (Smaje and Henderson 1984). This movement in effect is regulated by tissue-specific TJs, with permissive and barrier mechanisms, to achieve the chemical specificity of saliva (Baron et al. 1999). TJs are built from almost 40 different proteins, including members from multigene families (Morita et al. 1999b). Among these proteins are three types of transmembrane proteins: claudins, occludin, and junctional adhesion molecules (JAMs), as well as cytoplasmic proteins fulfilling roles in scaffolding, cytoskeletal attachment, cell polarity, signaling, and vesicle trafficking. The most important cytoplasmic protein is the peripheral membrane protein, zonula occludens (ZO) (Tsukita et al. 2001). The composition of TJs is quite complex and diverse, apparently much more than the other epithelial junctions: gap, adherens junctions, and desmosomes. These proteins have been comprehensively reviewed by Gonzalez-Mariscal et al. (2003) and Schneeberger and Lynch (2004).

There are few reports on the distribution of TJ proteins in human salivary glands in the literature (Kriegs et al. 2007; Lourenço et al. 2007). Accordingly, in this study, we evaluated the presence and distribution of various TJ proteins in human submandibular and parotid glands to serve as a reference for future studies where it may be necessary to assess the presence of specific TJ proteins in a tissue-engineered human salivary gland.

Materials and Methods

Source of Human Salivary Glands

Portions of human submandibular (n=9) and parotid (n=1) salivary glands, from both men and women, were obtained from the Department of Pathology, University of Virginia, through the Cooperative Human Tissue Network (CHTN). For tissue use, we received an exemption from the Institutional Review Boards at McGill University and the National Institutes of Health. This report describes results obtained with glands that were excised as part of the resection of head and neck cancers. Patients, from whom samples were obtained, had received no prior irradiation treatment. A pathologist at the University of Virginia (Charlottesville, VA) performed a surgical pathologic examination of all glands after their excision. We were provided with representative tissue slides after histopathological examination by CHTN. All glandular tissues received and reported here were judged as histopathologically normal. The salivary tissues were either frozen in liquid nitrogen or fixed in 10% buffered formalin by CHTN.

IHC

Frozen tissue slides were fixed in methanol over dry ice for 10 min, followed by two washing steps in PBS for 5 min each. Endogenous peroxidase and biotin activities were blocked with hydrogen peroxide 3% and the Avidin D/Biotin kit (Cat. SP-2001; Vector Laboratories, Burlingame, CA), respectively. All primary antibodies were obtained from Zymed Labs (South San Francisco, CA). We used the following antibodies: rabbit anti-claudin-1 (Cat. 51-9000), claudin-2 (Cat. 51-6100), claudin-3 (Cat. 34-1700), occludin (Cat. 42-2400), JAM-A (Cat. 36-1700) and mouse anti-ZO-1 (Cat. 33-9100), claudin-4 (Cat. 32-9400), and claudin-5 (Cat. 35-2500). In addition, we used rabbit and mouse isotype control antibodies (Cat. 08-6599 and 08-6199). These antibodies are reactive against the respective proteins from human and other species. Secondary broad spectrum antibodies (Cat. 95-9743) were also obtained from Zymed Labs. All primary antibodies used were diluted 1:20 in PBS containing 5% goat serum (Cat. S-1000; Vector Laboratories). The slides were incubated with the primary antibodies for 60 min. A secondary broad spectrum antibody kit was used with adding either 3-amino-9-ethyl-carbazole (AEC; Cat. 00-1111, Zymed Labs) or diaminobenzidine (DAB; Cat. 00-2014, Zymed Labs) chromogen to observe the reaction color in either red or brown, respectively. The slides were counterstained with Mayer's hematoxylin (Cat. 245-653; Fisher Scientific, Kalamazoo, MI). For formalin-fixed paraffin-embedded (FFPE) tissue slides, we used the same steps as described above, in addition to a boiling step (15 min) using an EDTA solution (pH 8.0, Cat. 00-5500; Zymed Labs) for heat-induced epitope retrieval. Primary antibodies were incubated overnight with the FFPE slides at 4C.

Results

We examined slides of glands from both male (n=7) and female (n=3) patients, using both frozen and FFPE samples from each patient (~25 5-μm sections per salivary gland). Patient age ranged between 52 and

73 years. No differences in results were seen between the sexes or in regard to patient age. In comparison to isotype control antibodies (Figure 1A), our results (Table 1) showed that claudin-1 was present in ductal (intercalated and striated ducts) and endothelial cells and also in ~25% of serous acini (Figure 1B). Claudin-2 (Figure 1C), claudin-3 (data not shown), and claudin-4 (Figure 1D) were expressed in acinar (serous and mucous) and ductal (intercalated and striated) cells, whereas endothelial cells were negative. Claudin-5 was detected only in endothelial cells (Figure 1E). JAM-A protein was detected in acinar and ductal salivary cells (Figure 1F). Occludin (Figure 1G) and ZO-1 Figure 1H) were detected in both ductal and acinar salivary cells, in addition to endothelial cells. None of the TJ proteins were expressed in myoepithelial cells. Results from the single parotid gland available were comparable to those from all submandibular gland samples.

Discussion

In this study, we evaluated the presence of multiple TJ proteins (claudins-1, -2, -3, -4, and -5, JAM-A, occludin, and ZO-1) in human parotid and submandibular salivary glands. The summary of our results, shown in Table 1, provides the most detailed examination of TJ proteins in human major salivary glands reported to date (Kriegs et al. 2007; Lourenço et al. 2007) and should be a useful guide for evaluating TJ proteins present in the cellular component of any artificial salivary gland device. However, we did not examine all possible TJ proteins in these tissues for practical reasons, but rather, selected the most common TJ proteins for which antibodies were readily available.

TJs, together with adherens junctions and desmosomes, form the apical junctional complex in epithelial and endothelial cellular sheets. Adherens junctions and desmosomes are responsible for the mechanical adhesion between adjacent cells, whereas TJs are essential for the tight sealing of the cellular sheets, thus controlling paracellular ion flux and therefore maintaining tissue homeostasis (Tsukita et al. 2001). TJs also play a crucial role in the maintenance of cell polarity by forming a fence that prevents lateral diffusion of membrane proteins and lipids, thereby maintaining the differential composition of the apical and basolateral domains. Finally, because of the ability of TJ proteins to recruit signaling proteins (Mitic and Anderson 1998), TJs have also been hypothesized to be involved in the regulation of proliferation, differentiation, and other cellular functions. Given these cellular biological roles, the presence and function of TJ proteins in the cellular component of an artificial salivary gland is critically important.

Thus far, 24 members of the claudin family have been identified in mouse and human (Morita et al. 1999b; Tsukita et al. 1999). Recent findings indicate the importance of claudin-1 in essential physiological functions and in the formation of barriers. For example, claudin-1 knockout mice survive only for 1 day after birth and present with altered epidermal barrier properties (Furuse et al. 2002). We observed claudin-1 in salivary ductal, endothelial, and in ~25% of serous acinar cells. Why claudin-1 was apparently not detected in the remaining serous acinar or in mucous acinar cells is not clear. It may reflect differences in the functional state of the cells at the time of surgery or represent an example of different acinar cell subpopulations. Lourenço et al. (2007) reported that claudin-1 was expressed only in ductal cells of adult human minor salivary glands. The presence of claudin-1 protein may suggest that it functions as a barrier to prevent the loss of salivary tonicity through the walls of the salivary system.

We found a ubiquitous distribution of claudin-2 in salivary gland sections. The IHC staining was stronger in the acinar cells (both serous and mucous) than ductal cells. Claudin-2 has been reported to weaken the tightness of TJs compared with the proposed barrier function of the claudin-1 protein (Stevenson et al. 1988). For example, Madin-Darby Canine Kidney (MDCK) type I and II cells have similar numbers of TJs expressed; however, MDCK I cells have a tighter barrier, reflected by a higher TER, compared with MDCK II cells (Stevenson et al. 1988). MDCK I cells mainly express claudin-1, whereas MDCK II cells express claudin-2 in addition. Because epithelial cells of salivary glands are considered to be relatively leaky exocrine tissues (Augustus et al. 1978), our observation seems to be in agreement with this organ's function. Results from human minor salivary glands indicate the absence of claudin-2 in both ductal and acinar cells (Lourenço et al. 2007).

In our study, claudin-3 and claudin-4 were present in both acinar and ductal cells. Similar results were reported from studies in rats (Peppi and Ghabriel 2004) and in human minor salivary glands (Lourenço et al. 2007). Claudin-3 is not involved directly in barrier properties of epithelial cells (Furuse et al. 2001; Amasheh et al. 2002), although the presence of claudin-4 in TJs is known to decrease sodium permeability, causing a decreased paracellular conductance (Van Itallie et al. 2001). When the claudin-4 in MDCK I cells was removed, there was a decrease in the number of TJ strands along with an increase in cellular permeability (Sonoda et al. 1999). Claudin-5 seems to have an important role in the vascular permeability of endothelial cells (Morita et al. 1999a). In this study, the detection of claudin-5 only in endothelial cells of human salivary glands is consistent with the reported specificity of this molecule. However, Lourenço et al. (2007) reported the expression of claudin-5 in both ductal and acinar cells. This difference might be a reflection of technical differences from our study. Herein, we used a claudin-5 monoclonal

The Journal of Histochemistry & Cytochemistry

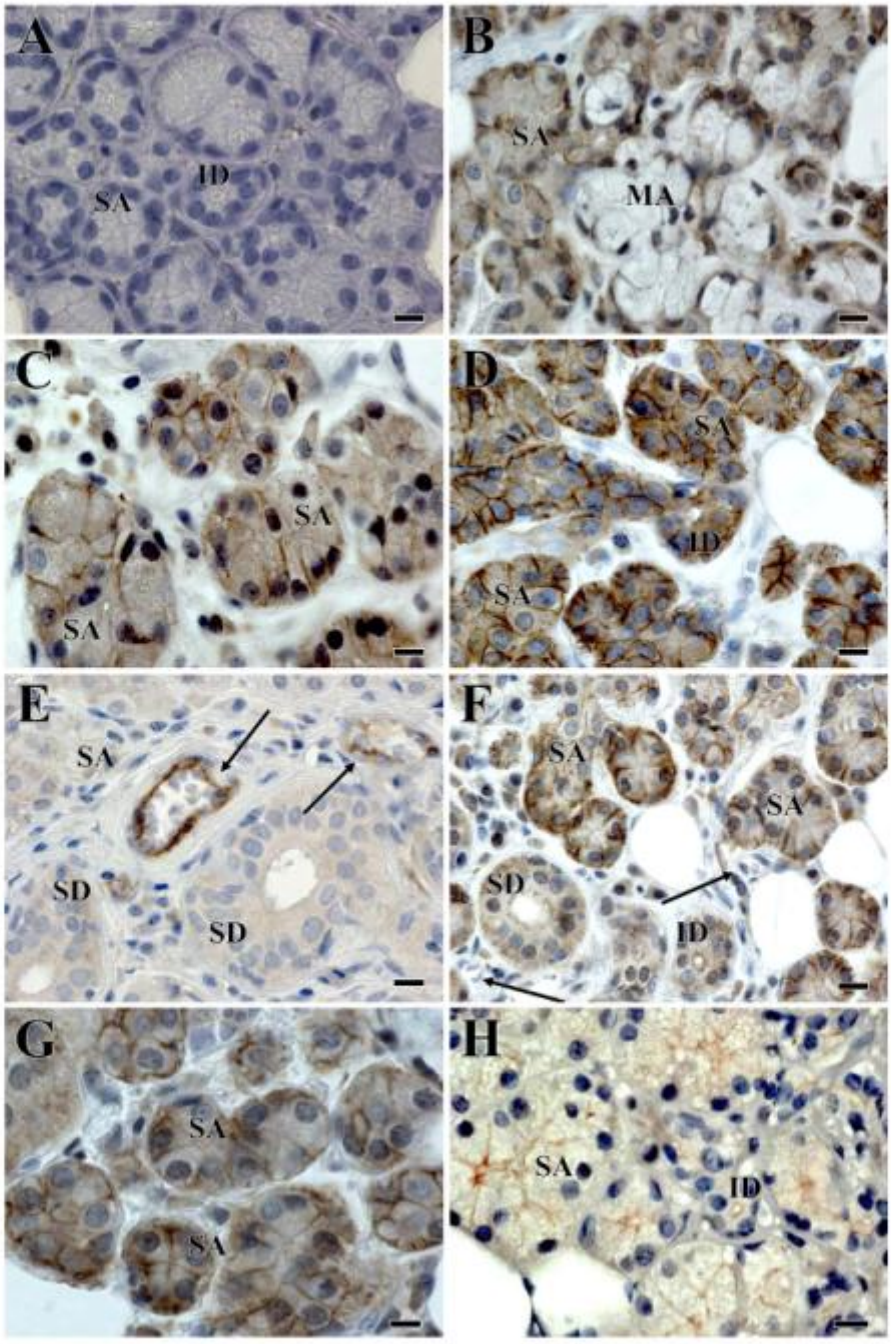


Table 1 Summary of tight junction proteins present in adult human submandibular and parotid salivary glands

	Ducts		Acini			
Antibody	Intercalated	Striated	Mucous	Serous	Myoepithelial cells	Blood vessels
Claudin-1	+	+	−	+25%	−	+
Claudin-2	+	+	+	+	−	−
Claudin-3	+	+	+	+	−	−
Claudin-4	+	+	+	+	−	−
Claudin-5	−	−	−	−	−	+
Junctional adhesion molecule A	+	+	+	+	−	−
Occludin	+	+	+	+	−	+
Zonula occludens 1	+	+	+	+	−	+

+, presence of protein; −, absence of protein.

antibody, whereas in the study of Lourenço et al. (2007), a claudin-5 polyclonal antibody was used.

The JAMs are four glycosylated transmembrane proteins (Shin et al. 2006), but in this study, we examined the expression of JAM-A (also known as JAM-1) protein only. JAMs were recently shown to bind directly to ZO-1 (Bazzoni et al. 2000; Ebnet et al. 2000), although the full role of JAM-related proteins still needs to be clarified. Herein, JAM-A protein was detected in ductal and acinar epithelial cells.

Occludin, a phosphoprotein, was the first transmembrane protein to be identified in TJs (Furuse et al. 1993). We localized occludin in ductal, acinar, and endothelial cells of human salivary glands. These results were consistent with the results reported by Kriegs et al. (2007) with regard to acinar and ductal cells but not for endothelial cells. The presence of occludin may provide the TJs with the ability to form seals between cells, preventing the leakage of saliva. Similarly, the expression of occludin has been reported in ductal and acinar cells of rat salivary glands (Peppi and Ghabriel 2004). Occludin interacts directly with ZO proteins (ZO-1, -2, and -3) (Gonzalez-Mariscal et al. 2000). Also, occludin interacts with the actin cytoskeleton and JAMs proteins indirectly through ZO proteins (Mandell and Parkos 2005). Tsukita et al. (2001) indicated that occludin might contribute to the electrical barrier function of TJs and possibly to the formation of aqueous pores within TJ strands; however, TJs can be present independent of occludin.

There are three isoforms of ZO proteins: ZO-1, -2, and -3. Because ZO proteins bind to actin, they act as scaffolds to link other TJ proteins and cytoskeleton (Shin and Margolis 2006). We only evaluated the distribution of ZO-1 in human salivary gland tissue. Unlike other TJ proteins, ZO-1 is not a transmembrane protein; rather, it is a large cytosolic phosphoprotein (Stevenson et al. 1986), which is critically important for claudin polymerization (Umeda et al. 2006). ZO-1 was found in endothelial as well as ductal and acinar cells of human salivary glands. Gresz et al. (2004) and Kriegs et al. (2007) reported that ZO-1 seemed to be restricted to the apical lateral region of acinar cells, presenting spider-like distribution, similar to that of aquaporin-5, in rat salivary glands, and our findings are generally comparable to their results.

In summary, we presented new data on the localization of several TJ proteins (claudin-1 to -5 and JAM-A) in normal human submandibular and parotid glands, as well as confirmed the presence of occludin and ZO-1 in these tissues (Kriegs et al. 2007). In showing the presence and localization of these proteins, we expect that our findings will allow a more critical assessment of TJ proteins in cultured primary salivary gland cells for use in an envisioned tissue-engineered salivary gland. Further studies are needed to find the exact roles and interactions of TJ proteins essential for physiological salivary gland functions.

Acknowledgments

This study was supported by Canada Research Chair and Canadian Institutes of Health Research, Egyptian Cultural and Educational Department (to OMM), and the Division of Intramural Research, National Institute of Dental and Craniofacial Research.

The authors thank Dr. Christopher Moskaluk for the histopathological analysis of salivary glands obtained from Cooperative Human Tissue Network and Dr. Younan Liu for technical assistance.

Figure 1 Light micrographs of IHC staining of human salivary gland sections. **(A)** Isotype control antibody labeling: serous acinus (SA), intercalated duct (ID), and blue nuclei stained with hematoxylin are indicated. **(B)** Distribution of claudin-1: positive SA but negative mucous acinus (MA). **(C)** Claudin-2: positive SA. **(D)** Claudin-4: strong positive ID and SA. **(E)** Claudin-5: positive blood vessels (arrows) but negative striated ducts (SD) and SA. **(F)** Junctional adhesion molecule (JAM)-A: positive SA, ID, and SD but negative blood vessel (arrow). **(G)** Occludin: positive SA. **(H)** Zonula occludens (ZO)-1: positive SA expressed in a spiderweb distribution and ID. **A**, **C**, and **D** are parotid gland sections, whereas **B**, **E**, **F**, **G**, and **H** are submandibular gland sections. The results observed are comparable in both submandibular and parotid glands. Bar = 100 μm.

Literature Cited

Aframian DJ, Cukierman E, Nikolovski J, Mooney DJ, Yamada KM, Baum BJ (2000) The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. Tissue Eng 6:209–216

Aframian DJ, Redman RS, Yamano S, Nikolovski J, Cukierman E, Yamada KM, Kriete MF, et al. (2002a) Tissue compatibility of two biodegradable tubular scaffolds implanted adjacent to skin or buccal mucosa in mice. Tissue Eng 8:649–659

Aframian DJ, Tran SD, Cukierman E, Yamada KM, Baum BJ (2002b) Absence of tight junction formation in an allogeneic graft cell line used for developing an engineered artificial salivary gland. Tissue Eng 8:871–878

Aframian DJ, Zheng C, Goldsmith CM, Nikolovski J, Cukierman E, Yamada KM, Mooney DJ, et al. (2001) Using HSV-thymidine kinase for safety in an allogeneic salivary graft cell line. Tissue Eng 7:405–413

Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M (2002) Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci 115: 4969–4976

Augustus J, Bijman J, Van Os CH (1978) Electrical resistance of rabbit submaxillary main duct: a tight epithelium with leaky cell membranes. J Membr Biol 43:203–226

Baron S, Poast J, Cloyd MW (1999) Why is HIV rarely transmitted by oral secretions? Saliva can disrupt orally shed, infected leukocytes. Arch Intern Med 159:303–310

Baum BJ (1993) Principles of saliva secretion. Ann NY Acad Sci 694:17–23

Baum BJ, Wang S, Cukierman E, Delporte C, Kagami H, Marmary Y, Fox PC, et al. (1999) Re-engineering the functions of a terminally differentiated epithelial cell in vivo. Ann NY Acad Sci 875:294–300

Bazzoni G, Martinez-Estrada OM, Mueller F, Nelboeck P, Schmid G, Bartfai T, Dejana E, et al. (2000) Homophilic interaction of junctional adhesion molecule. J Biol Chem 275:30970–30976

Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendle GG, Vestweber D (2000) Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. J Biol Chem 275:27979–27988

Fox PC (1998) Acquired salivary dysfunction. Drugs and radiation. Ann NY Acad Sci 842:132–137

Fox PC, Speight PM (1996) Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjogren's syndrome. Crit Rev Oral Biol Med 7:144–158

Furuse M, Furuse K, Sasaki H, Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 153:263–272

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, et al. (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156:1099–1111

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123:1777–1788

Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE (2003) Tight junction proteins. Prog Biophys Mol Biol 81:1–44

Gonzalez-Mariscal L, Namorado MC, Martin D, Luna J, Alarcon L, Islas S, Valencia L, et al. (2000) Tight junction proteins ZO-1, ZO-2, and occludin along isolated renal tubules. Kidney Int 57: 2386–2402

Gresz V, Kwon TH, Gong H, Agre P, Steward MC, King LS, Nielsen S (2004) Immunolocalization of AQP-5 in rat parotid and submandibular salivary glands after stimulation or inhibition of secretion in vivo. Am J Physiol Gastrointest Liver Physiol 287: G151–161

Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ (2003) Cancer statistics. CA Cancer J Clin 53:5–26

Kriegs JO, Homann V, Kinne-Saffran E, Kinne RK (2007) Identification and subcellular localization of paracellin-1 (claudin-16) in human salivary glands. Histochem Cell Biol 128:45–53

Lourenço SV, Coutinho-Camillo CM, Buim ME, Uyekita SH, Soares FA (2007) Human salivary gland branching morphogenesis: morphological localization of claudins and its parallel relation with developmental stages revealed by expression of cytoskeleton and secretion markers. Histochem Cell Biol 128:361–369

Mandell KJ, Parkos CA (2005) The JAM family of proteins. Adv Drug Deliv Rev 57:857–867

Mitic LL, Anderson JM (1998) Molecular architecture of tight junctions. Annu Rev Physiol 60:121–142

Morita K, Furuse M, Fujimoto K, Tsukita S (1999b) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96: 511–516

Morita K, Sasaki H, Furuse M, Tsukita S (1999a) Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 147:185–194

Mostov KE, Verges M, Altschuler Y (2000) Membrane traffic in polarized epithelial cells. Curr Opin Cell Biol 12:483–490

Peppi M, Ghabriel MN (2004) Tissue-specific expression of the tight junction proteins claudins and occludin in the rat salivary glands. J Anat 205:257–266

Schneeberger EE, Lynch RD (2004) The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286:C1213–1228

Shin K, Fogg VC, Margolis B (2006) Tight junctions and cell polarity. Annu Rev Cell Dev Biol 22:207–235

Shin K, Margolis B (2006) Zoning out tight junctions. Cell 126: 647–649

Smaje LH, Henderson JR (1984) Microcirculation of the exocrine glands. In Martillaro NA, ed. The Physiology and Pharmacology of the Microcirculation. Vol. 2. New York, Academic Press, 325–380

Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S (1999) Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. J Cell Biol 147:195–204

Stevenson BR, Anderson JM, Goodenough DA, Mooseker MS (1988) Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. J Cell Biol 107:2401–2408

Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103:755–766

Tran SD, Wang J, Bandyopadhyay BC, Redman RS, Dutra A, Pak E, Swaim WD, et al. (2005) Primary culture of polarized human salivary epithelial cells for use in developing an artificial salivary gland. Tissue Eng 11:172–181

Tsukita S, Furuse M, Itoh M (1999) Structural and signalling molecules come together at tight junctions. Curr Opin Cell Biol 11: 628–633

Tsukita S, Furuse M, Itoh M (2001) Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2:285–293

Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, et al. (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126:741–754

Van Itallie C, Rahner C, Anderson JM (2001) Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest 107:1319–1327

Cells From Bone Marrow That Evolve Into Oral Tissues And Their Clinical Applications.

By

Maria OM, Khosravi R, Mezey E, Tran SD.

Oral Diseases, 2007;13(1):11-16.

Oral Diseases (2007) 13, 11–16. doi:10.1111/j.1601-0825.2006.01324.x

http://www.blackwellmunksgaard.com

INVITED REVIEW

Cells from bone marrow that evolve into oral tissues and their clinical applications

OM Maria[1], R Khosravi[1], E Mezey[2], SD Tran[1]

[1]*Faculty of Dentistry, McGill University, Montreal, Quebec, Canada;* [2]*National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*

There are two major well-characterized populations of post-natal (adult) stem cells in bone marrow: hematopoietic stem cells which give rise to blood cells of all lineages, and mesenchymal stem cells which give rise to osteoblasts, adipocytes, and fibroblasts. For the past 50 years, strict rules were taught governing developmental biology. However, recently, numerous studies have emerged from researchers in different fields suggesting the unthinkable – that stem cells isolated from a variety of organs are capable of ignoring their cell lineage boundaries and exhibiting more plasticity in their fates. Plasticity is defined as the ability of post-natal (tissue-specific adult) stem cells to differentiate into mature and functional cells of the same or of a different germ layer of origin. There are reports that bone marrow stem cells can evolve into cells of all dermal lineages, such as hepatocytes, skeletal myocytes, cardiomyocytes, neural, endothelial, epithelial, and even endocrine cells. These findings promise significant therapeutic implications for regenerative medicine. This article will review recent reports of bone marrow cells that have the ability to evolve or differentiate into oral and craniofacial tissues, such as the periodontal ligament, alveolar bone, condyle, tooth, bone around dental and facial implants, and oral mucosa.

Oral Diseases (2007) **13**, 11–16

Keywords: bone marrow; cell plasticity; cell-based therapies; oral tissues; stem cells; tissue engineering

Stem cells in bone marrow

Stem cells are defined as clonogenic, self-renewing, and capable of generating one or more specialized cell types (Anderson *et al*, 2001). Developmentally, stem cells are categorized either as embryonic stem cells or as post-natal stem cells (they are also called organ-specific, tissue-specific, or adult stem cells) (Leung and Verfaillie, 2005). Embryonic stem cells are derived from the inner cell mass of a developing blastocyst and are considered as pluripotent cells as they are able to form all the body's cell lineages (endoderm, mesoderm, and ectoderm) (Smith, 2006). Post-natal stem cells (derived from specific tissues or organs) are considered multipotent as they can form multiple lineages that constitute an entire tissue or tissues (Smith, 2006).

According to our present knowledge there are two distinct populations of post-natal stem cells in the bone marrow the hematopoietic stem cells (HSC) and the mesenchymal stem cells. HSC were recognized more than 40 years ago as they have the ability to reconstitute the hematopoietic system of a lethally irradiated host (Leung and Verfaillie, 2005) as it gives rise to all blood cell lineages. Their unique ability to self-renew continuously permits HSC to sustain blood cell production throughout life. The frequency of HSC is 1 in 10 000 15 000 bone marrow cells (Weissman, 2000a). Under physiologic conditions, quiescent HSC are interspersed with other cells within the bone marrow. However, under stressful conditions such as massive bleeding or acute bacterial infections, HSC rapidly proliferate, differentiate, and migrate from the bone marrow to circulate throughout the body (Domen and Weissman, 1999; Bordignon, 2006). Mesenchymal stem cells originate from the mesodermal layer of the fetus and in the adult they reside in the bone marrow as well as in a variety of tissues. Mesenchymal stem cells constitute only a small portion (1 in 10^4 10^6) of the bone marrow (Friedenstein *et al*, 1974; Pittenger *et al*, 1999). The pivotal characteristic of mesenchymal stem cells is their ability to differentiate *in vitro* into several cell types based on culture conditions (Pittenger *et al*, 1999). It has been demonstrated that these cells possess a multilineage differentiation capability (bone, cartilage, adipose, tendon, and muscle tissues; Ferrari *et al*, 1998; Jones *et al*, 2002). Several studies have reported that mesenchymal stem cell clones comprise a heterogeneous cell population with respect to their self-renewal characteristic (Bianco *et al*, 2001). However, this self-renewal potential is unclear mainly due to the different approaches used to derive populations of mesenchymal stem cells.

Correspondence: SD Tran, Faculty of Dentistry, McGill University, 3640 University Street, Room M-43, Montreal, Quebec, Canada H3A 2B2. Tel: +1 514 398 7203 Ext. 09182, Fax: +1 514 398 8900, E-mail: simon.tran@mcgill.ca
Received 17 March 2006; revised 22 July 2006; accepted 7 August 2006

Plasticity of post-natal stem cells

For the past 50 years, we were taught that post-natal stem cells have a limited developmental repertoire. Once a cell made a commitment to a dermal lineage during development, this was irrevocable (Mezey, 2004; Leung and Verfaillie, 2005). A stem cell residing in a particular tissue (i.e., a tissue-specific stem cell) could only differentiate into cells of that tissue. For example, a hematopoietic stem cell would give rise to new blood cells; a liver stem cell would make new liver cells, etc. However, in the past 7 years, a large number of studies emerged from researchers in different fields suggesting the unthinkable that post-natal stem cells isolated from a variety of organs may be able to ignore its (dermal lineage) origin and exhibit more *plasticity* in their fate choices. Plasticity is defined as the ability of post-natal (tissue-specific adult) stem cells to differentiate into mature and functional cells of the same or of a different germ layer of origin (Leung and Verfaillie, 2005). There are reports that bone marrow stem cells can differentiate into hepatocytes (Petersen *et al*, 1999), skeletal myocytes (Ferrari *et al*, 1998), cardiomyocytes (Makino *et al*, 1999; Tomita *et al*, 1999), neural cells (Eglitis and Mezey, 1997; Mezey *et al*, 2003), endothelial cells (Tomita *et al*, 1999), epithelial cells (Krause *et al*, 2001), and pancreatic endocrine cells (Ianus *et al*, 2003). These findings on the plasticity of post-natal stem cells carry great hope for regenerative medicine (Weissman, 2000b; Pittenger and Martin, 2004; Kan *et al*, 2005). As an example, because HSC can reconstitute the entire blood system, bone marrow transplantations have long been used in the clinic to treat hematopoietic diseases (Mayhall *et al*, 2004). Several companies are competing to market a variety of cell-based therapies based on post-natal bone marrow-derived stem cells for treating cancers, autoimmune, neurologic, stroke, and heart diseases (Wilan *et al*, 2005).

Four explanations for the phenomenon of plasticity in post-natal stem cells have been proposed (Verfaillie, 2002; Martin-Rendon and Watt, 2003; Grove *et al*, 2004; Kashofer and Bonnet, 2005; Lakshmipathy and Verfaillie, 2005). First, there might be persistent stem cells from embryonic development with broad developmental potentials which are maintained within the adult bone marrow (Dao and Verfaillie, 2005). When transplanted into other organs, these cells are instructed to differentiate into tissue-specific cells under inductive signals from that specific tissue. A second possibility is that true precursors of post-natal stem cells with embryonic stem cell-like properties persist in adult bone marrow, such as the multipotent adult progenitor cells (Jiang *et al*, 2002). A third explanation may be that the nuclei of the transplanted stem cells undergo reprogramming of the existing genetic information, expressing new genes and proteins that are consistent with the novel lineage, and this might be a result of de-differentiation, and re-differentiation (Brockes, 1997; Lakshmipathy and Verfaillie, 2005; Hochedlinger and Jaenisch, 2006). A final explanation is when cell fusion occurs, which is a rare phenomenon reported *in vitro* and *in vivo* in tissues where polyploidy is common, such as hepatocytes, skeletal muscle, cardiac muscle and Purkinje cells of the cerebellum (Priller *et al*, 2001). As a result, the genetic information of both fused donor and host cells is partially changed (Terada *et al*, 2002; Ying *et al*, 2002; Lakshmipathy and Verfaillie, 2005).

The objective of this review was to evaluate recent reports of cells from the bone marrow (HSC and mesenchymal stem cells) that have the ability to evolve or differentiate into orofacial structures and their clinical applications for oral tissue regeneration (Table 1 and Figure 1). The readers are cautioned with the widely used term *mesenchymal stem cells* as the International Society for Cellular Therapy (ISCT) has stated that the current data are insufficient to characterize unfractionated plastic adherent marrow cells as stem cells (Horwitz *et al*, 2005). Therefore, the ISCT suggests the use of the term *multipotent mesenchymal stromal cell* to indicate these unique properties without ascribing homogeneity or stem cell activity; while the term *mesenchymal stem cells* is reserved for long-term self-renewing cells that are capable of differentiation into specific, multiple cell types *in vivo* (Horwitz *et al*, 2005). For both of these cell populations, the acronym MSC may be used, as is the current practice. Therefore, it is crucial that future publications clearly define the acronym that they are describing. The studies reported in this review are derived from experiments using multipotent mesenchymal stromal cells (MSC). It is not the goal of this review to report on the use of MSC from other oral tissues in tissue regeneration. Such MSC populations are from the human exfoliated deciduous teeth (Miura *et al*, 2003), dental pulp (Gronthos *et al*, 2000), and periodontal ligament (Seo *et al*, 2004; Ivanovski *et al*, 2006). These post-natal stem cells have common characteristics with bone marrow MSC in addition to be readily accessible in the oral cavity.

Cell-based therapies for tissue regeneration

Cell encapsulation is an intervention in cell-based regenerative medicine. In brief, cells are delivered to a donor with the goal of improving the regeneration process. Initial reports in the 1970s by WT Green, a pediatric orthopedic surgeon, demonstrated that implanted

Table 1 Reports describing bone marrow stem cells evolving into orofacial tissues

Origin	*Differentiated tissues*	*Reference*
MSC	Periodontium	Kawaguchi *et al* (2004, 2005)
MSC	Condyle	Abukawa *et al* (2003)
MSC	Dental implant	Yamada *et al*, 2004a,b
BM or MSC	Bone	Abukawa *et al* (2004), Warnke *et al* (2004), De Kok *et al* (2005)
BM	Tooth	Ohazama *et al* (2004)
BM or HSC	Buccal mucosa	Tran *et al* (2003), Metaxas *et al* (2005)

BM, bone marrow stem cells; HSC, hematopoietic stem cells; MSC, multipotent mesenchymal stromal cells.

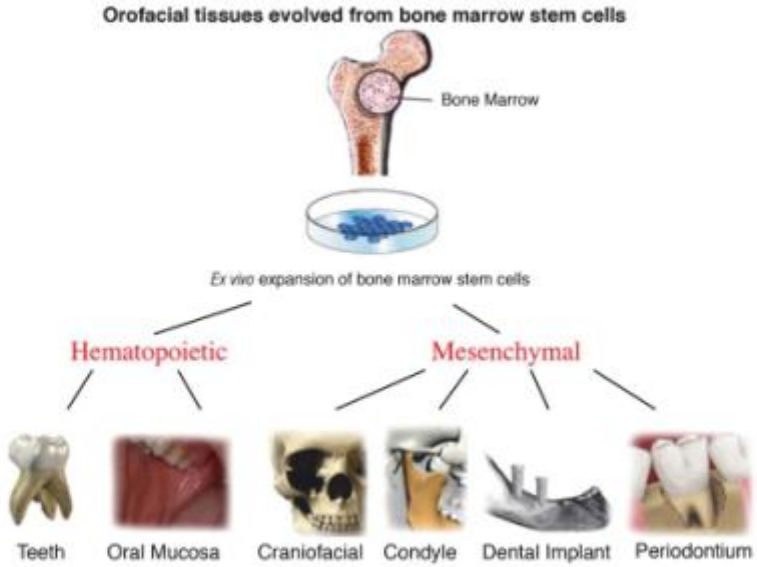


Figure 1 Oral tissues that evolved from bone marrow cells

spicules of bone and cartilage seeded with chondrocytes into animals could generate new cartilage (Green, 1977). Today, the two common methods of cell delivery are intravenous injections (direct delivery of cells) and cell encapsulation systems (indirect delivery of cells using a carrier). The cell encapsulation approach uses a biodegradable material, which is a biocompatible product that is gradually resorbed once implanted in the body, due to enzymatic or hydrolytic degradation. This biodegradable construct is seeded with cells (ideally progenitor cells) and is implanted into defects in order to regenerate lost tissues (Fuchs *et al*, 2005). Bone marrow-derived MSC have a significant but highly variable self-renewal potential during *in vitro* experiments and this property has made them attractive as a source for cell-based therapies aiming at the regeneration of orofacial tissues, especially when the size of the lost tissue is large and that the body can no longer repair this defect (Colter *et al*, 2000; Caplan and Bruder, 2001). Future advancements in stem cell research (either embryonic or post-natal) and in biomaterial science will allow cell encapsulation methods to be utilized in the clinic to regenerate both hard and soft tissues of the craniofacial complex.

Periodontium

Periodontal diseases are highly prevalent worldwide and the main signs are bone tissue destruction and subsequent tooth loss. Regenerating the periodontium has always been a high priority in craniofacial regenerative biology. Due to the complex structure of the periodontium (consisting of hard and soft tissues: cementum, bone, periodontal ligament, and gingiva), its complete regeneration would require a multipotent cell population (Bartold *et al*, 2000; Grezesik and Narayanan, 2002). Kawaguchi *et al* (2004) demonstrated that transplantations of *ex vivo* expanded autologous MSC can regenerate new cementum, alveolar bone, and periodontal ligament in class III periodontal defects in dogs. Morphometric analysis revealed a 20% increase in new cementum length and bone area in animals treated with MSC. In a subsequent study the same group reported a similar approach in humans (Kawaguchi *et al*, 2005) when they transplanted 2×10^7 cells ml^{-1} autologous expanded bone marrow-derived MSC mixed with Atelocollagen into periodontal osseous defects. All patients showed a significant improvement.

Dental implant

A sound and mature bone is an essential factor to achieve successful osseointegration of dental and facial implants. Very frequently, the quality and quantity of the remaining bone (that was destroyed because of trauma or diseases such as an enucleated tumor) are not suitable to allow a complete osseointegration of these implants. In a canine model, Yamada *et al* (2004a) extracted premolars and first molars. After 1 month of healing, they created four 10-mm diameter defects on each side of the mandible. These surgically created defects were filled with (1) platelet-rich plasma (PRP), (2) autologous MSC and PRP (MSC/PRP), (3) autologous particulate cancellous bone and marrow (PCBM), or (4) empty (control defect). After 8 weeks, dental implants were placed in the healed defects. The authors hypothesized that the presence of MSC in the surgical site would enhance wound healing and osseointegration. Higher marginal bone levels were recorded on dental

implants placed in MSC/PRP- or PCBM-filled defects when compared with control defects. Bone-implant contact was significantly increased in MSC/PRP and PCBM groups. Histologic results showed a well-formed lamellar and woven bone and new vascularization around dental implants of the MSC/PRP group. However, PCBM-filled defects exhibited bone resorption. In a similar study, Yamada *et al* (2004b) tested the application of an autologous 'scaffold' for delivering MSC to the surgical site. Using the same study design, they monitored the quality of regenerated bone in each defect. The MSC/PRP and PCBM groups showed a substantial increase in mature regenerated bone tissue. Their findings suggest that the insoluble gel generated from mixing PRP and thrombin calcium chloride can be a clinically feasible method to deliver MSC to the surgical sites. Other studies have combined progenitor cells with different growth factors such as bone morphogenetic proteins (although not in the orofacial area; Kataoka and Urist, 1993; Higuera *et al*, 2005) or enamel matrix proteins (Murai *et al*, 2005). These growth factors promoted tissue regeneration but the exact role of the MSC alone remains unknown.

Mandible

Autologous bone grafts have been a 'gold standard' in craniofacial reconstruction. However, donor site morbidity and a limited quantity/supply are still substantial hurdles with this method. Bone tissue engineering can fully replace lost bone tissues through the use of three-dimensional biodegradable scaffold materials carrying osseous progenitor cells and bioactive agents (growth factors, hormones, etc.). Abukawa *et al* (2004) used scaffolds to reconstruct bony defects in pig mandibles. They seeded MSC into a biodegradable polymer and incubated for 10 days. Complete bone growth was observed in the experimental group. De Kok *et al* (2005) studied the safety and potential efficacy of utilizing MSC for alveolar bone repair in beagle dogs. They showed that bone marrow MSC seeded on either hydroxyapetite/tricalcium phosphate biomaterials or not can increase bone formation in dental sockets. Improvements in cell encapsulation techniques along with new generations of smart biodegradable scaffolds (Simon *et al*, 2004) will lead to the reconstruction of new and well-differentiated bone.

Human mandibles with major discontinuity defects (more than 5 cm) caused by an ablative tumor surgery can be repaired with autologous vascularized fibula, scapula, iliac crest, or rib bone grafts. However, this approach may create skeletal defects at the donor site which can be associated with serious morbidity. Warnke *et al* (2004) reported the fabrication of a mandibular transplant for a patient who had a large resection of his mandible (from the left paramedian region to the right retromolar region). The transplant was made of a titanium mesh cage filled with bone mineral blocks that were infiltrated with a combination of the patient's own iliac bone marrow and recombinant human bone morphogenetic protein-7. The transplant was implanted into the right latissimus dorsi muscle of the patient for 7 weeks. The skeletal scintigraphy showed bone remodeling and mineralization inside the mandibular transplant both before and after transplantation. Computed tomography provided an evidence of new bone formation. Seven weeks post-transplantation, the transplant was excised with an adjoining part of the latissimus dorsi muscle containing the thoracodorsal artery and vein that had supplied blood for the entire transplant, and transplanted to repair the mandibular defect. The patient had an improved degree of mastication and was satisfied with the esthetic outcome.

Condyle

The cartilaginous and osseous structures of the temporomandibular joint (TMJ) can deteriorate because of injuries, rheumatoid arthritis, and osteoarthritis. Tissue engineering of the TMJ can overcome drawbacks of joint replacement such as immunologic rejection, donor site morbidity, transmission of pathogens, or metal loosening. Abukawa *et al* (2003) fabricated a model of porcine mandibular condyle using porous biodegradable polymer scaffolds. The authors encapsulated differentiated osteoblasts (originating from cultured minipig bone marrow-derived MSC) into polymer scaffolds and incubated the construct in an oxygen-permeable bioreactor system for 6 weeks. Histologic observations revealed uniform new bone formation and densely stained osteoid and osteocytes in lacunae surrounded by bone matrix in deeper layers. Radiographic assessment revealed higher radiodensity of the construct when compared with the control scaffold but lower density than the control minipig cadaver condyle. Alhadlaq *et al* (2004) designed a bi-layer model to engineer cartilage and bone of the mandibular condyle. They harvested rat bone marrow-derived MSC and differentiated them into chondrocytes and osteocytes *ex vivo*. Chondrocytes and osteocytes were then seeded in a two-layer biocompatible poly (ethylene glycol)-based hydrogel. The construct was implanted in the subcutaneous dorsal pockets of immunodeficient mice. Histologic observations of the harvested constructs showed stratified layers of chondrogenesis and osteogenesis.

Tooth

Ohazama *et al* (2004) reported significant progress toward the creation of tissue-engineered embryonic tooth primordia (tooth buds) using cultured cells. In a mouse model, they tested different mixtures of non-dental-derived mesenchymal cells (embryonic stem cells, neural stem cells, and adult bone marrow cells) with embryonic oral epithelium cells. These mesenchymal epithelial mixtures were transplanted into the renal capsules of adult mice. All mixtures resulted in the development of a tooth structure and bone. They observed that the host tissues make no contribution to the donor tissue. Moreover, transfer of embryonic tooth primordia into the adult jaw resulted in the development of tooth structures, showing that an embryonic

primordium can develop in its adult environment. They concluded that bone marrow-derived cells can form all mesenchymal-derived cells in a tooth structure. *In vitro* control of the shape of the tissue-engineered dental primordia will be a crucial step to bring this therapy to the clinic (Modino and Sharpe, 2005).

Oral mucosa

Tran *et al* (2003) reported an example of transdifferentiation of human bone marrow-derived stem cells into buccal epithelial cells. Using fluorescence *in situ* hybridization and immunohistochemistry, they identified Y-chromosome-positive buccal cells in five female patients who had received either a bone marrow transplant or an allogenic mobilized peripheral blood stem cell transplant from male donors. Y-chromosome-positive cells in these female patients were morphologically distinguishable as buccal epithelial cells and they also expressed cytokeratin 13, a recognized epithelial marker located in the superficial layer of the cheek. These results were confirmed by Metaxas *et al* (2005) who reported the presence of 1.8% donor-derived buccal epithelial cells in cheek scrapings of 12 of the 13 female patients who received a male-to-female hematopoietic cell transplantation 56 to 1964 days ago. The cheek scrapings were made when no oral mucositis or oral graft-*vs*-host disease was present. The donor-derived buccal epithelial cells were identified by epithelial morphologic characteristics, cytokeratin expression, positive Y-chromosome, and negative CD45 (blood lineage marker).

The plasticity of adult bone marrow-derived cells has been questioned by studies suggesting that fusion between donor and host cells gave the appearance of transdifferentiation (Terada *et al*, 2002; Ying *et al*, 2002). However, *in vivo* studies (Tran *et al*, 2003; Metaxas *et al*, 2005) did not observe cell fusion. Tran *et al* (2003) examined more than 9700 buccal cells and reported no evidence of fusion. These findings were also confirmed in the study by Metaxas *et al* (2005) who reported that none of the buccal cells examined had more than one X-chromosome, which excludes fusion as the answer to cell plasticity.

Summary

In this review we have discussed studies reporting successful applications of bone marrow stem cells to reconstruct different craniofacial tissues such as the periodontal ligament, cementum, bone, condyle, tooth, and oral mucosa. Plasticity of adult stem cell is controversial and more research is needed before any safe implementation of these cell-based therapies can be utilized in the clinic.

Acknowledgements

The authors acknowledge the Canada Research Chair and CIHR (for SDT), Egyptian Cultural and Educational Department (for OMM).

References

Abukawa H, Terai H, Hannouche D, Vacanti JP, Kaban LB, Troulis MJ (2003). Formation of a mandibular condyle in vitro by tissue engineering. *J Oral Maxillofac Surg* **61:** 94–100.

Abukawa H, Michael S, Williams WB, Vacanti JP, Kaban LB, Troulis MJ (2004). Reconstruction of mandibular defects with autologous tissue-engineered bone. *J Oral Maxillofac Surg* **62:** 601–606.

Alhadlaq A, Elisseeff JH, Hong L *et al* (2004). Adult stem cell-driven genesis of human-shaped articular condyle. *Ann Biomed Eng* **32:** 911–923.

Anderson DJ, Gage FH, Weissman IL (2001). Can stem cells cross lineage boundaries? *Nat Med* **7:** 393–395.

Bartold PM, McCulloch CAG, Narayanan AS, Pitaru S (2000). Tissue-engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol 2000* **22:** 169–189.

Bianco P, Riminucci M, Gronthos S, Robey PG (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* **19:** 180–192.

Bordignon C (2006). Stem-cell therapies for blood diseases. *Nature* **441:** 1100–1102.

Brockes JP (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science* **276:** 81–87.

Caplan AI, Bruder SP (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* **7:** 259–264.

Colter DC, Class R, Di Girolamo CM, Prockop DJ (2000). Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci U S A* **97:** 3213–3218.

Dao M, Verfaillie CM (2005). Chapter 18. Bone marrow microenvironment. In: Hoffman R, Benz Jr EJ, Shattil SJ, Furie B, Cohen HJ, Silbertstein LE, McGlave P, eds. *Hematology: basic principles and practice*, 4th edn. Churchill Livingstone: Philadelphia, PA, pp. 215–232.

De Kok IJ, Drapeau SJ, Young R, Cooper LF (2005). Evaluation of mesenchymal stem cells following implantation in alveolar sockets: a canine safety study. *Int J Oral Maxillofac Implants* **20:** 511–518.

Domen J, Weissman IL (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol Med Today* **5:** 201–208.

Eglitis MA, Mezey E (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A* **94:** 4080–4085.

Ferrari G, Cusella-De Angelis G, Coletta M *et al* (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279:** 1528–1530.

Friedenstein AJ, Deriglasova UF, Kulagina NN *et al* (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. *Exp Hematol* **2:** 83–92.

Fuchs JR, Hannouche D, Terada S, Zand S, Vacanti JP, Fauza DO (2005). Cartilage engineering from ovine umbilical cord blood mesenchymal progenitor cells. *Stem Cells* **23:** 958–964.

Green WT (1977). Articular cartilage repair. Behavior of rabbit chondrocytes during tissue culture and subsequent allografting. *Clin Orthop Relat Res* **124:** 237–250.

Grezesik WJ, Narayanan AS (2002). Cementum and periodontal wound healing and regeneration. *Crit Rev Oral Biol Med* **13:** 474–484.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* **97:** 13625–13630.

Grove JE, Bruscia E, Krause DS (2004). Plasticity of bone marrow-derived stem cells. *Stem Cells* **22:** 487–500.

Higuera CA, Inoue N, Lim JS *et al* (2005). Tendon reattachment to a metallic implant using an allogenic bone plate augmented with rhOP-1 vs. autogenous cancellous bone and marrow in a canine model. *J Orthop Res* **23:** 1091–1099.

Hochedlinger K, Jaenisch R (2006). Nuclear reprogramming and pluripotency. *Nature* **441:** 1061–1067.

Horowitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. The International Society for Cellular Therapy (2005). Clarification of the nomenclature for MSc: The International Society for Cellular Therapy position statement. *Cytotherapy* **7:** 393–395.

Ianus A, Holz GG, Theise ND, Hussain MA (2003). In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest* **111:** 843–850.

Ivanovski S, Gronthos S, Shi S, Bartold P (2006). Stem cells in the periodontal ligament. *Oral Dis* **12:** 358–363.

Jiang Y, Jahagirdar BN, Reinhardt RL *et al* (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418:** 41–49.

Jones EA, Kinsey SE, English A *et al* (2002). Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* **46:** 3349–3360.

Kan I, Melamed E, Offen D (2005). Integral therapeutic potential of bone marrow mesenchymal stem cells. *Curr Drug Targets* **6:** 31–41.

Kashofer K, Bonnet D (2005). Gene therapy progress and prospects: stem cell plasticity. *Gene Ther* **12:** 1229–1234.

Kataoka H, Urist MR (1993). Transplant of bone marrow and muscle-derived connective tissue cultures in diffusion chambers for bioassay of bone morphogenetic protein. *Clin Orthop Relat Res* **286:** 262–270.

Kawaguchi H, Hirachi A, Hasegawa N *et al* (2004). Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol* **75:** 1281–1287.

Kawaguchi H, Hirachi A, Mizuno N *et al* (2005). Cell transplantation for periodontal diseases: a novel periodontal tissue regenerative therapy using bone marrow mesenchymal stem cells. *Clin Calcium* **15:** 1197–1202.

Krause DS, Thiese ND, Collector MI *et al* (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105:** 369–377.

Lakshmipathy U, Verfaillie CM (2005). Stem cell plasticity. *Blood Rev* **19:** 29–38.

Leung AYH, Verfaillie CM (2005). Chapter 17. Stem cell model of hematopoiesis. In: Hoffman R, Benz Jr EJ, Shattil SJ, Furie B, Cohen HJ, Silbertstein LE, McGlave P, eds. *Hematology: basic principles and practice*, 4th edn. Churchill Livingstone: Philadelphia, PA, pp. 200–214.

Makino S, Fukuda K, Miyoshi S *et al* (1999). Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* **103:** 697–705.

Martin-Rendon E, Watt SM (2003). Exploitation of stem cell plasticity. *Transfus Med* **13:** 325–349.

Mayhall EA, Paffett-Lugassy N, Zon LI (2004). The clinical potential of stem cells. *Curr Opin Cell Biol* **16:** 713–720.

Metaxas Y, Zeiser R, Schmitt-Graeff A *et al* (2005). Human hematopoietic cell transplantation results in generation of donor-derived epithelial cells. *Leukemia* **19:** 1287–1289.

Mezey E (2004). Commentary: on bone marrow stem cells and open-mindedness. *Stem Cells Dev* **13:** 147–152.

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B (2003). Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* **100:** 1364–1369.

Miura M, Gronthos S, Zhao M *et al* (2003). SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* **100:** 5807–8512.

Modino SAC, Sharpe PT (2005). Tissue engineering of teeth using adult stem cells. *Arch Oral Biol* **50:** 255–258.

Murai M, Sato S, Koshi R *et al* (2005). Effects of the enamel matrix derivative and beta-tricalcium phosphate on bone augmentation within a titanium cap in rabbit calvarium. *J Oral Sci* **47:** 209–217.

Ohazama A, Modino SAC, Miletich I, Sharpe PT (2004). Stem-cell-based tissue engineering of murine teeth. *J Dent Res* **83:** 518–522.

Petersen BE, Bowen WC, Patrene KD *et al* (1999). Bone marrow as a potential source of hepatic oval cells. *Science* **284:** 1168–1170.

Pittenger MF, Martin BJ (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* **95:** 9–20.

Pittenger MF, Mackay AM, Beck SC *et al* (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284:** 143–147.

Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW (2001). Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. *J Cell Biol* **155:** 733–738.

Seo BM, Miura M, Gronthos S *et al* (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364:** 149–155.

Simon CG Jr, Guthrie WF, Wang FW (2004). Cell seeding into calcium phosphate cement. *J Biomed Mater Res A* **68:** 628–639.

Smith A (2006). A glossary for stem-cell biology. *Nature* **441:** 1060.

Terada N, Hamazaki T, Oka M *et al* (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416:** 542–545.

Tomita S, Li RK, Weisel RD *et al* (1999). Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* **100:** II247–II256.

Tran SD, Pillemer SR, Dutra A *et al* (2003). Differentiation of human bone marrow derived cells into buccal epithelial cells in vivo: a molecular analytical study. *Lancet* **361:** 1084–1088.

Verfaillie CM (2002). Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol* **12:** 502–508.

Warnke PH, Springer ING, Wiltfang J *et al* (2004). Growth and transplantation of a custom vascularised bone graft in a man. *Lancet* **364:** 766–770.

Weissman IL (2000a). Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100:** 157–168.

Weissman IL (2000b). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287:** 1442–1446.

Wilan KH, Scott CT, Herrera S (2005). Chasing a cellular fountain of youth. *Nat Biotechnol* **23:** 807–815.

Yamada Y, Ueda M, Naiki T, Nagasaka T (2004a). Tissue-engineered injectable bone regeneration for osseointegrated dental implants. *Clin Oral Implants Res* **15:** 589–597.

Yamada Y, Ueda M, Naiki T, Takahashi M, Hata K, Nagasaka T (2004b). Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: tissue-engineered bone regeneration. *Tissue Eng* **10:** 955–964.

Ying QL, Nichols J, Evans EP, Smith AG (2002). Changing potency by spontaneous fusion. *Nature* **416:** 545–548.

Bone Marrow-Derived Cells Rescue Salivary Gland Function In Mice With Head And Neck Irradiation.

By

Yoshinori Sumita, Younan Liu, Saeed Khalili, **Ola M. Maria**, Dengsheng Xia, Sharon Key, Ana P. Cotrim, Eva Mezey, Simon D. Tran.

(*Submitted* 2010)

The Ph.D. Candidate performed the following contributions in this manuscript:

- Immunocytochemistry techniques; PCNA, Apoptosis, blood vessels staining, PAS, Stem cell stains.
- Examination of slides and analysis of data related to the above mentioned stains.
- Shared writing and revising the manuscript.

Bone Marrow-Derived Cells Rescue Salivary Gland Function in Mice with Head and Neck Irradiation.

Yoshinori Sumita[1,2], Younan Liu [1], Saeed Khalili[1], Ola M. Maria[1], Dengsheng Xia[1], Sharon Key[3], Ana P. Cotrim[3], Eva Mezey[3], Simon D. Tran[1*]

[1] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada.

[2] Department of Regenerative Oral Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

[3] National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA.

*****_Corresponding author_:

Simon D. Tran, D.M.D., Ph.D.

Faculty of Dentistry - McGill University

3640 University Street, M-43, Montreal, Quebec, H3A 2B2 Canada, Tel: +1 514 398 7203, Fax: +1 514 398 2431, E-mail: simon.tran@mcgill.ca

ABSTRACT

Treatment for most patients with head and neck cancers includes ionizing radiation. A consequence of this treatment is irreversible damage to salivary glands (SGs), which is accompanied with a loss of fluid-secreting acinar-cells and a considerable decrease of saliva output. While there are currently no adequate conventional treatments for this condition, cell-based therapies are receiving increasing attention to regenerate SGs. In this study, we investigated whether bone marrow-derived cells (BMDCs) can differentiate into salivary epithelial cells and restore SG function in head and neck irradiated mice. BMDCs from male mice were transplanted through the tail-vein of female mice post gamma-ray irradiation (IR) of 15 or 18 Gy. After 8 and 16 weeks, salivary output was increased in mice treated by BMDCs transplantation. At 24 weeks after IR, harvested SGs (submandibular and parotid glands) of BMDC-treated mice had greater weights than those of non-treated mice. On histological analysis, SGs of treated mice possessed an increased level of tissue regenerative activity (blood vessel formation, cell proliferation, and epidermal growth factor activity), while apoptosis activity was increased in non-treated SGs. The expression of stem cell markers (Sca-1 or C-kit) was detected in BMDC-treated SGs. Finally, we detected an increased ratio of acinar-cell area and approximately 9% of Y-chromosome-positive salivary epithelial cells (derived from donor) in BMDC-treated mice. We propose here that cell therapy using BMDCs can rescue the functional damage of irradiated SGs by direct differentiation of donor BMDCs into salivary epithelial cells.

1. INTRODUCTION

Ionizing radiation is a key component of therapy for most patients with head and neck cancers. Salivary glands, particularly the acinar cells, in the ionizing radiation field suffer severe damage. These cells are the principal site of fluid secretion in salivary glands, and such patients cannot produce adequate levels of saliva, leading to severe salivary gland hypofunction and extreme discomfort. Severe hypofunction causes symptoms such as xerostomia (dry mouth), dysphagia, severe dental caries, oro-pharyngeal infections, and mucositis (Vissink et al., 2003a, 2003b). In many patients, all salivary parenchymal tissue is lost. These patients suffer considerable morbidity and a severe reduction in their quality of life. However, at present, there are no adequate treatments for patients with such irreversible gland damage. Current pharmacological approaches are to increase the secretory capacity of the surviving acinar cells. However, this approach is not feasible if little or no acinar cells remain in the glands. Therefore, developing an adequate treatment by using alternative strategies, such as gene therapy, tissue engineering, or cell-based therapy, is required (Baum et al., 2002; Baum and Tran, 2006; Kagami et al., 2008).

For functional restoration of damaged salivary glands due to irradiation, two main regenerative approaches have been tested to date. The first approach is to develop an artificial salivary gland using tissue engineering principles (Tran et al., 2005; Joraku et al., 2005; Tran et al., 2006; Aframian et al., 2007; Yang et al., 2008; Aframian et al., 2008; Pradhan et al., 2010). We recently reported that it was feasible to culture salivary epithelial cells for their eventual use in a prototype artificial salivary gland (Tran et al., 2006). However, this strategy can generate only one portion of the salivary parenchymal tissue (the ductal cells), and it has been difficult for our group to regenerate functional salivary tissue (both ductal and acinar cells). A second approach has been to apply stem cell-based therapy to damaged salivary gland tissue. Currently, stem cells from two different organs have been investigated: a) from the salivary gland, or 2) from the bone marrow

(Sugito et al., 2004; Lombaert et al., 2006; Tran et al., 2007; Lombaert et al., 2008a, 2008b; Tatuishi et al., 2009; Coppes et al., 2009; Feng et al., 2009). Using cells from salivary glands, Sugito and colleagues (2004) demonstrated that cultured rat salivary epithelial cells can be successfully transplanted to atrophic salivary glands. These cells mobilized to the damaged salivary tissue, and remained there. Moreover, Lombaert et al. (2008a) developed an *in vitro* culture system to enrich, characterize, and harvest primitive salivary gland stem cells. These cultured cells could rescue the gland functions after transplantation. However, this strategy may be difficult for clinical use if an insufficient number of stem cells are obtained from the patient's gland biopsies. Also, to establish an adequate culture condition for each patient may be challenging. Many patients with head and neck cancers are old. Gland tissues tend to be atrophic in older patients. Therefore, to expand these patients' salivary stem cells in vitro (after surgical removal) may be difficult as cell viability has already decreased. Another source of stem cells that have been suggested to potentially differentiate or repair non-hematopoietic organs are bone marrow-derived cells (BMDCs) (Lagasse et al., 2000; Orlic et al., 2001a; Nishida et al., 2004; Couzin., 2006). Specifically for the salivary glands, Lombaert and colleagues (2006) reported that granulocyte colony-stimulating factor (G-CSF) treatment induced mobilization of a large number of BMDCs to mouse salivary glands that had received irradiation to their neck area. Their results suggest that BMDCs could home to injured salivary glands and induce repair processes that improved gland's function and morphology. From our previous study, we reported that BMDCs from healthy male donors can differentiate into buccal (oral) epithelial cells of female transplant recipients (Tran et al., 2003). Our observations lead us to hypothesize that transplanted BMDCs may also differentiate into salivary epithelial cells and improve the gland functions post-irradiation. BMDCs are readily accessible and provide an easy and minimally-

invasive procedure to harvest from patients with head and neck cancers, before their chemo-irradiation therapy.

The aim of this study was to assess the regenerative capacity of BMDCs for salivary gland regeneration by their direct transplantations through intra-venous injections. This study is a pre-requisite step for future clinical trials aiming at developing cell-based therapy for salivary glands. We believe that using enriched BMDCs, without an in vitro culture system, is a simple and direct approach for transplantation; and that this strategy should be investigated as a priority to regenerate salivary glands.

2. MATERIALS AND METHODS

2.1. Animals

C3H female mice of 8 weeks old (Charles River, Montreal, QC, Canada) were used as recipient mice in a gender-mismatched bone marrow transplantation strategy. Donor mice were age-matched C3H male mice. All mice were kept under clean conventional conditions at the McGill University animal resource center. We received an approval for the animal use from the University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Irradiation

Mice were treated at 8 weeks of age and salivary gland damage was elicited by local head and neck radiation exposure (0, 15 and 18 Gy) using a gamma cell cesium-137 unit. For irradiation, the mice were anesthetized with 1 μl/kg body weight of a 60 mg/ml ketamine (Phoenix Scientific) and 8 mg/ml xylazine (Phoenix Scientific) solution given **intra-peritoneally (**i.p.), and placed in a 3-mm-think Perspex box, which was aligned in the radiation unit. The dose rate to the local head and neck was calculated monthly according to the decay formula provided by the

company. The rest of the body was shielded with 3 cm of lead to reduce the beam strength to 3 % in this area. Female mice were divided into 5 different groups (5 mice per group) and were followed for 24 weeks post irradiation with either: a) irradiation plus bone marrow cells treated groups (group 1: 15Gy + BMDCs; group 2: 18Gy + BMDCs), b) irradiation and no-cell transplant groups (group 3: 15Gy ; group 4: 18Gy), c) no-irradiation and no cell-transplant group (group 5: normal control group).

2.3. Bone Marrow Derived-Cells (BMDCs) Transplantation

8-week old male C3H mice were the donors. Bone marrow was harvested as follows: a) dissecting connective tissue to obtain clean femur and tibia bones, b) cutting off each end of the femur and tibia bones to expose their marrow, c) inserting a 25-gauge needle in the marrow opening, d) flushing out the marrow using a syringe filled with filtered Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 2% antibiotic-antimycotic reagent (Sigma), and e) resuspending the bone marrow in DMEM and filtering it through a 40-um filter to remove any particulates. The mixture was centrifuged at 1500 rpm for 10 minutes at 4°c. Cells were resuspended with medium kept on ice before transplantation. The injection of $1x10^7$ BMDCs was done via the tail vein immediately after irradiation, and the transplantations were repeated two times per week during 6 consecutive weeks.

2.4. Salivary Flow Rate

Secretory function of the salivary glands (salivary flow rate) was obtained by i.p. injection of 1 µl/kg body weight of a 60 mg/ml ketamine and 8 mg/ml xylazine mixture. Whole saliva was collected after stimulation of secretion using 0.5 mg Pilocarpine (Sigma) /kg body weight administered subcutaneously. Saliva was obtained from the oral cavity by micropipette, placed into pre-weighed 0.5 ml microcentrifuge tubes. Saliva was collected for a 10-minute period and

its volume determined gravimetrically. Saliva flow rate was determined at baseline (i.e. week 0; when mice were 7 week old, a week pre-irradiation) and at week 8, 16, and 24 post-irradiation.

2.5. Measurement of Gland Weight

At 24 weeks, the mice were sacrificed and their submandibular glands were harvested. These procedures were performed in accordance with the institutional guidelines. Before fixation (10% formalin), the weights of harvested gland tissues were measured.

2.6. Morphological and Histological Examination

2.6.1. Apoptosis

Mice glands (five mice per group) were fixed in 10% neutral buffered formalin and embedded in paraffin. ApopTag® *In Situ* Apoptosis Detection Kit (Chemicon, MA, USA) was used to evaluate apoptotic cells by detecting DNA cleavage and chromatin condensation associated with apoptosis using a mixed molecular biological-histo-chemical system. After deparaffinization and rehydration, slides were pre-treated with protein digestion enzyme (proteinase) for 15 minutes at room temperature. Endogenous peroxidase activity was blocked for 10 min by H_2O_2 in methanol. TdT enzyme was incubated with the slides for 1 hour, and then, Anti-Digoxigenin Conjugate for 30 min at room temperature. Peroxidase substrate was applied to develop the reaction color and the slides were counterstained in 0.5% (w/v) methyl green for 10 minutes. The nuclei of apoptotic cells were stained dark brown. Two examiners independently counted the absolute number of apoptotic cells each in five randomly chosen fields per section at the magnification of X 400 and the means of all groups were calculated.

2.6.2. **Blood vessel staining**

Five-micrometer thickness tissue sections were stained by immuno-histochemistry using the Blood Vessel Staining Kit (Chemicon, MA, USA). After deparaffinizing and rehydration, tissue

sections were treated three times with a citrate buffer solution (9 ml of 0.1 M citric acid and 41 ml of 0.1 M sodium citrate plus 450 ml distilled water) in a 600 W microwave and then allowed to cool down to room temperature for 30 min before blocking overnight at 4^{o}C. Rabbit anti-human vWF was used (1:100) for 2 hours at room temperature. After that, we followed the manufacturer's instructions. The percentage of surface area occupied by blood vessels was assessed by light microscopy under X400 magnification using 3 sections for each slide. At least 10 fields per section were accounted using NIH J Image software (NIH, Bethesda, USA).

2.6.3**. Cell proliferation staining**

After deparaffinization and rehydration, tissue sections were blocked for endogenous peroxidase activity with 3% H_2O_2 in methanol for 10 min. PCNA staining was performed with the Zymed PCNA staining kit (Invitrogen, Carlsbad, CA, USA). Before antibody labeling, the slides were treated three times with a citrate buffer solution (mentioned previously) in a 600 W microwave for 5 min. Thereafter, slides were processed with routine indirect immunoperoxidase techniques. Two examiners independently counted the absolute number of PCNA positive cells in a blinded manner in five randomly chosen sections (n=5 glands/group) at the magnification of X 400. For each group, the mean of all PCNA positive cells was then calculated.

2.6.4. **PAS staining**

Tissue sections were analyzed using Periodic Acid-Schiff (PAS) method (Sigma-Aldrich). The percentage of surface area occupied by acinar cells was assessed by light microscopy under X 400 magnification; for 3 tissue sections per slide. At least 10 random fields per section were analyzed by NIH J Image software (NIH).

2.7. Saliva Compositions

2.7.1. **Epidermal growth factor (EGF) concentration**

Concentration of EGF in saliva was measured by ELISA method (R&D System, Minneapolis, MN, USA) at baseline (week 0, pre-irradiation) and at the end of experiment (week 24 post-irradiation). This assay employed a quantitative sandwich enzyme immunoassay technique. The intensity of the color measured is in proportion to the amount of EGF. The sample values were compared to the EGF standard curve.

2.7.2. **Total protein concentration**

Total protein from saliva was assayed by bicinchoninic acid assay (BCA) kit (Thermo Scientific, Pierce, IL, USA) at week 24 post irradiation. Three ul from each saliva sample was used for the assay. OD values were measured at 562 nm and the results were expressed in ug/ml, assuming that 1 g weight difference of saliva is equal to 1 ml.

2.8. Immuno-histochemical Staining

Frozen salivary tissue sections (5μm thickness) from mice were fixed in cold methanol for 10 min, followed by three washing steps in PBS for 5 min each. Endogenous peroxidase and biotin activities were blocked with hydrogen peroxide 3% and the Universal Blocking solution (BioGenex, San Roman, CA, USA), respectively. We used the following primary antibodies to test the expression of stem/progenitor cells; Sca-1 and c-Kit (R&D Systems). We used isotype control antibodies (Invitrogen, Carlsbad, CA, USA) that are reactive against the respective proteins from mice and other species. Donkey anti-goat-FITC and anti-rat-FITC secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. All primary antibodies used were diluted 1:100 in PBS containing 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The slides were incubated with the primary antibodies overnight at 4^{o}C, and in the dark with the secondary antibodies for 1 hour at room temperature. Then 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, Carlsbad, CA, USA)

was added for 3-5 minutes. For formalin-fixed paraffin-embedded (FFPE) tissue slides, we used the same steps as described above, in addition to a boiling step (15 min) using an EDTA solution (pH 8.0, Zymed Labs) for heat-induced epitope retrieval.

2.9. Immunostaining and Fluorescence In Situ Hybridization (FISH)

Serial frozen tissue sections were fixed in a 2% paraformaldehyde fixative for 10 minutes. Immunostaining was performed by primary antibody that was detected by the Sternberger peroxidase antiperoxidase (PAP) method followed by a fluorescein isothiocyanate-conjugated (FITC)-tyramide signal amplification (TSA System, Invitrogen). The primary antibody used was against the $Na^+/K^+/2Cl^-$ co-transporter, (NKCC1, a salivary acinar cell marker; graciously donated by Dr. James Turner, NIH, USA) and Cytokeratins 8,-18 and 13 (salivary ductal markers; Biogenenx, USA) to detect salivary epithelial cells. A digoxigenin-labeled riboprobe was then added to recognize a repeated sequence (pY353B) on the mouse Y chromosome. The samples were blocked with endogenous peroxidase (DAKO, CA, USA). The riboprobe was then detected using an antibody to digoxigenin conjugated to peroxidase (Roche, Indianapolis, USA). This peroxidase was visualized by tyramide signal amplification with an Alexa Fluor 594 fluorochrome-tyramide reagent (TSA System, Invitrogen). All sections were stained with DAPI to label all nuclei and then mounted with Tris (hydroxymethyl)- aminomethane, [pH 7.4]) buffer. Finally the slides were visualized using a Leica DM6000 fluorescent microscope equipped with Volocity software.

2.10. Statistical Analysis

To determine the significance ($P< 0.05$), Linear Mixed Models and ANOVA analysis (Tukey test) were used. Subjects between and within the groups were compared in different time points by SPSS version 17 (IBM, USA).

3. RESULTS

3.1. Salivary Production and Gland Weight

Saliva output (salivary flow rate; SFR) was assessed to determine the effect of BMDCs transplantation for functional restoration of damaged glands. Overall SFR increased in BMDC-treated mice (15 Gy + BMDC or 18 Gy + BMDC groups) at week 8 and 16 post-irradiation, when compared to mice in non-transplanted groups (15 Gy or 18 Gy groups) (Fig.1 A). Remarkably, SFR of BMDC-treated mice at 8 weeks post-irradiation were increased to comparable SFR levels of those from normal non-irradiated control mice. However, SFR of BMDC-treated mice at 16 weeks after irradiation decreased to approximately 70% of SFR measured at 8 weeks. After the final SFR measurements (at week 24 post-irradiation), all animals were sacrificed and their salivary glands were weighed. Submandibular glands of BMDC-treated mice had greater weights than those of non-treated mice ($p<0.05$) (Fig.1 B).

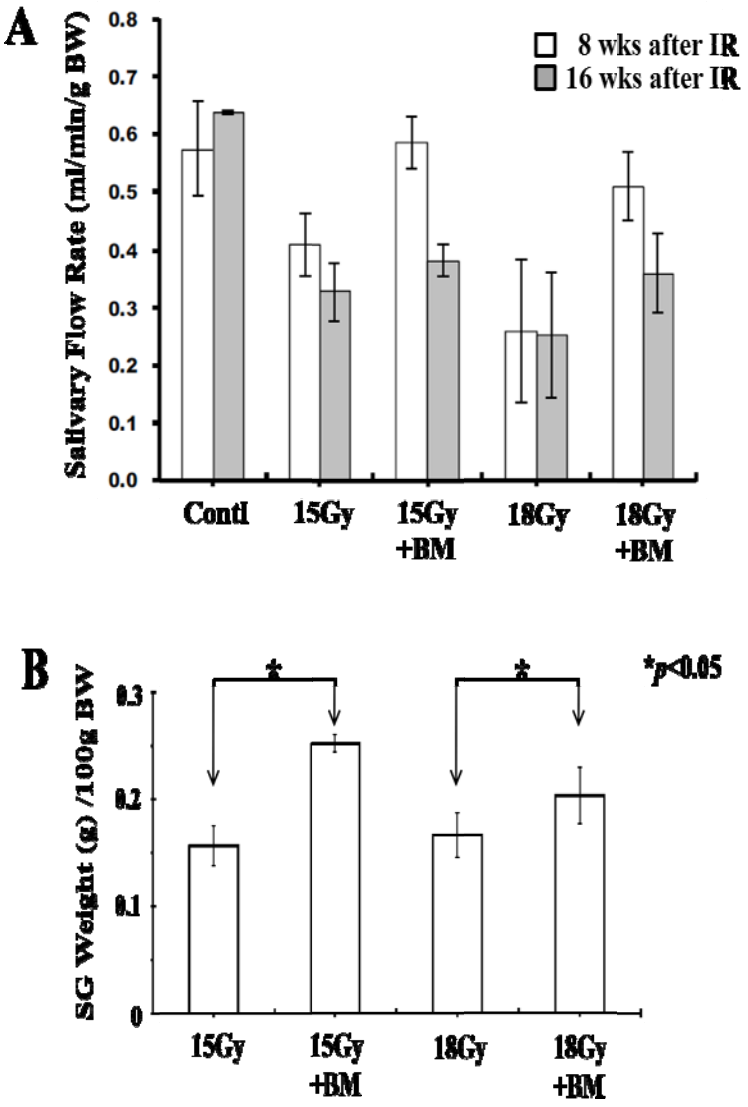


Figure 1.

Chart (A) shows the salivary flow rate (SFR) collected at 8 and 16 weeks after 15 and 18 Gy irradiation. SFR increased in BMDC-treated mice (15 Gy + BM or 18 Gy + BM groups) when compared to non-treated mice.

Chart (B) shows the weight of salivary glands harvested at 24 weeks after irradiation. The glands of BMDC-treated mice had greater weights than those of non-treated mice.

3.2.Tissue Restoration Activity in Glands at 24 Weeks Post-Irradiation

Firstly, apoptotic activity was assessed to determine the effect of BMDCs transplantation on the prevention of apoptosis in damaged salivary epithelial tissues. At 24 weeks post-irradiation, BMDC-treated mice (18 Gy + BMDCs group) showed a significantly lower apoptotic activity when compared to non-treated mice (18 Gy) ($p<0.01$) (Fig. 2 A).

Vascularization in damaged tissues was assessed by measuring the percentage of surface area occupied by blood vessels in each gland. The total surface area occupied by blood vessels in glands of BMDCs-treated mice was approximately 2.5-fold larger than that from non-treated mice (Fig. 2 B). This difference was statistically significant between BMDC- and non-treated mice which received 18Gy of irradiation ($p<0.01$).

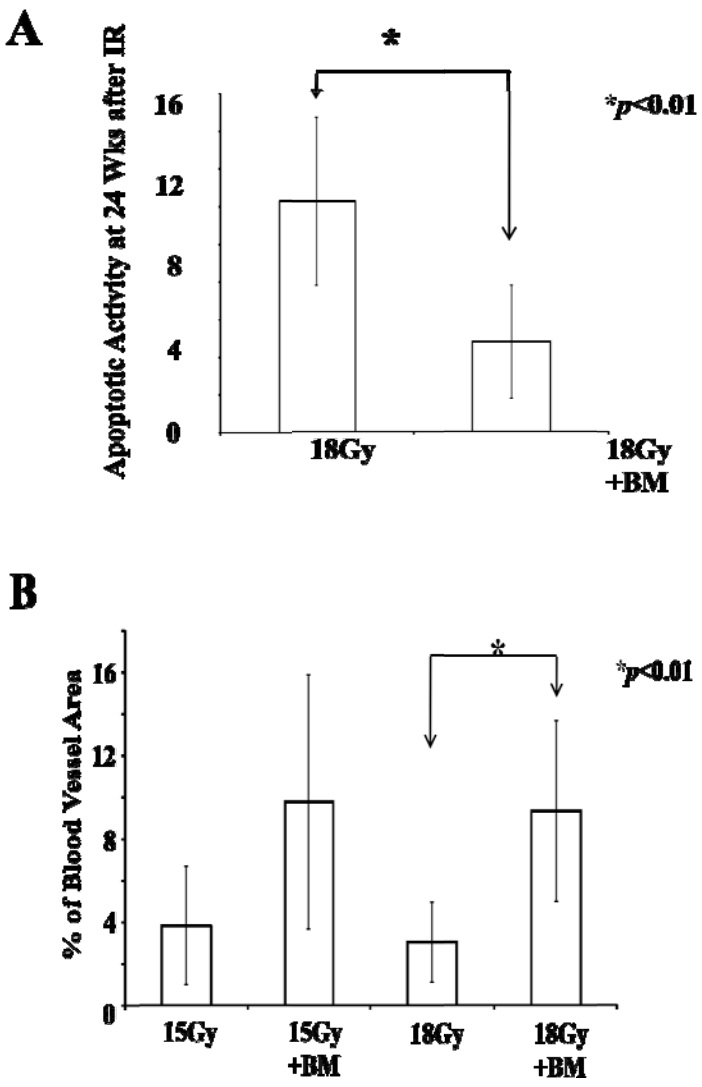


Figure 2.

Chart (A) shows the cell apoptotic activity evaluated at 24 weeks after 18 Gy of irradiation in treated- and non-treated mice. There is a decrease in the number of apoptotic cells in the BMDC-treated mice after irradiation. This decrease is statistically significant in comparison to the untreated mice.

Chart (B) shows the area (in percentage) of intact blood vessels in the salivary tissue, evaluated at 24 weeks after 15 Gy and 18 Gy irradiation in four groups of mice. There is an increase in the percentage of blood vessels area in mice treated with BMDCs. This difference was statistically significant in mice which received 18Gy, in comparison to the untreated mice.

PCNA positive cells and EGF concentration were measured to assess the regenerative activity of salivary epithelial cells in damaged tissues. At 24 weeks post-irradiation, the number of proliferating cells was significantly increased in both groups of BMDC-treated mice ($p<0.05$) (Fig. 3 A). The concentration of EGF in saliva secreted from BMDC-treated mice was markedly increased compared with that of non-treated or normal control mice ($p<0.01$) (Fig.3 B).

To detect the stem/progenitor cells which contribute to the regeneration of gland tissues, the expression of stem cell markers were analyzed by immunocytochemistry. The expression of c-Kit and Sca-1 was found mainly in the ductal portion of normal control and BMDC-treated mice (Fig. 4 A, B). In contrast, no expression of either c-Kit or Sca-1 were detected in glands of non-transplanted (18 Gy) mice.

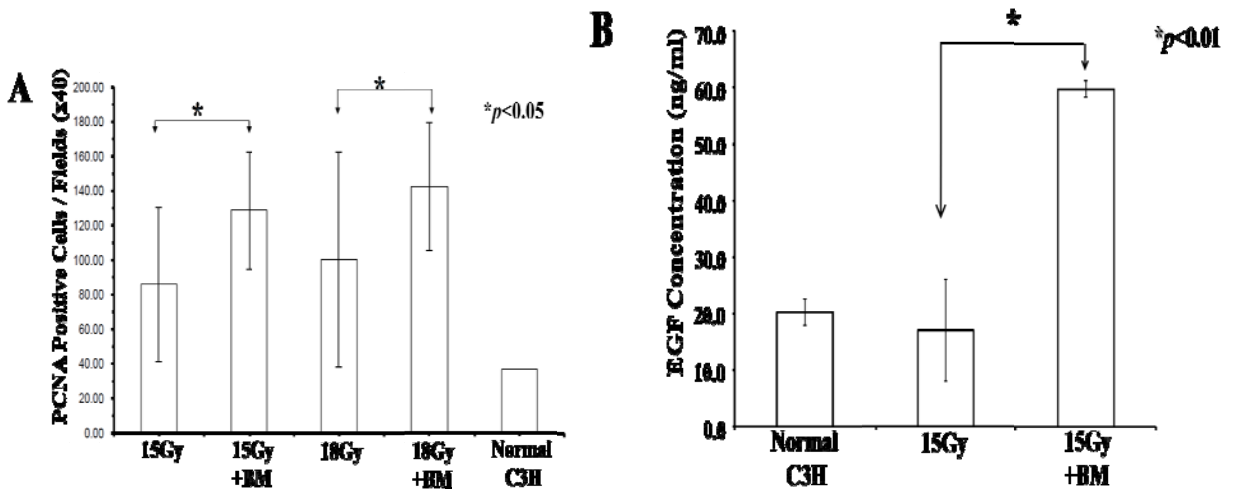


Figure 3.

Chart (A) shows the cell mitotic activity (PCNA stained) evaluated at 24weeks after either 15Gy or 18Gy irradiation in four groups of mice in comparison to non-irradiated normal C3H mice. There is an increase in the number of dividing cells in the mice treated with BMDCs after irradiation. This increase is statistically significant when compared to untreated mice.

Chart (B) shows the concentration of EGF in saliva. The saliva secreted from BMDC-treated mice was markedly increased when compared to untreated or normal C3H mice, and this difference is statistically significant.

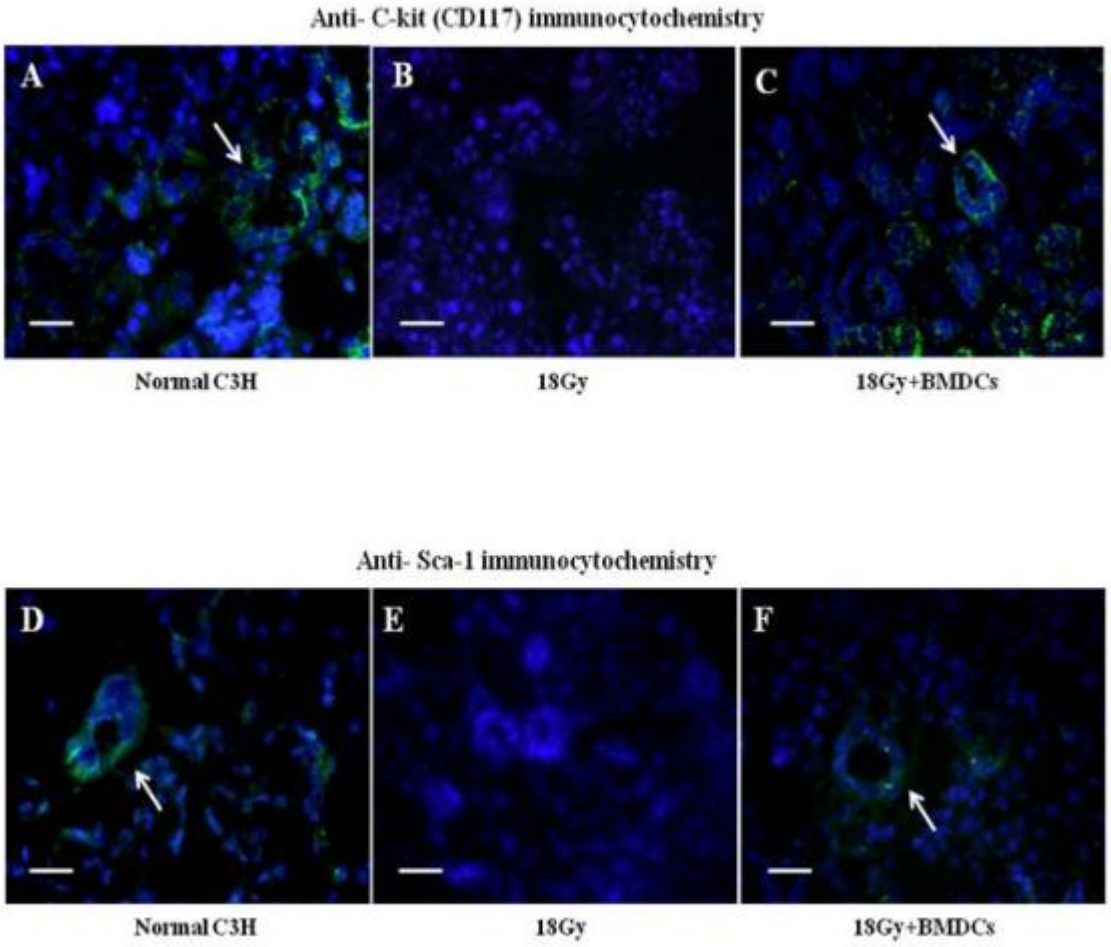


Figure 4.

Stem cell markers in mouse salivary tissue. (A) C-Kit (CD117; stained in green) is expressed in striated and excretory duct cells (shown by arrow) of non-irradiated submandibular glands. (B) C-Kit is absent in 18Gy-irradiated submandibular glands, (C) while highly expressed by all ductal cells in 18Gy-irradiated mouse treated with BMDCs injection. (D) Sca-1 (stained in green) is detected in some striated duct cells (arrow) of non-irradiated C3H mouse submandibular gland. (E) Sca-1 is absent in 18Gy-irradiated glands, (F) but is highly expressed by some ductal cells in 18Gy-irradiated mouse treated with BMDCs. The Sca-1 signal in all tissue sections is weaker than that of the C-Kit signal. These stem cell markers are labeled by FITC (green); nuclei are labeled with DAPI (blue). Scale bar = 34 μm.

3.3. Analysis of the Mechanism increasing saliva output by BMDCs transplant

Total protein concentration of non-treated groups was higher than that of BMDC-treated groups (Fig. 5 A). However, these differences were not statistically significant. On the other hand, the regeneration of acinar cells was prominent in BMDC-treated mice when compared to non-treated mice ($p<0.05$) (Fig. 5 B). Furthermore, we combined fluorescence in situ hybridization with immunohistochemical staining to allow for the simultaneous detection of both the Y-chromosome and specific markers for salivary epithelial cells. Approximately 9 % of Y-chromosome positive cells derived from donor BMDCs were detected in BMDC-treated mice, but none in non-transplanted or normal control mice. These positive cells were observed in salivary epithelial cells which expressed NKCC1 (an acinar cell marker; Fig 6 A), cytokeratin 8&18 (salivary ductal cell marker; Fig 6 B), cytokeratin13 (salivary epithelial cell marker; Fig 6 C).

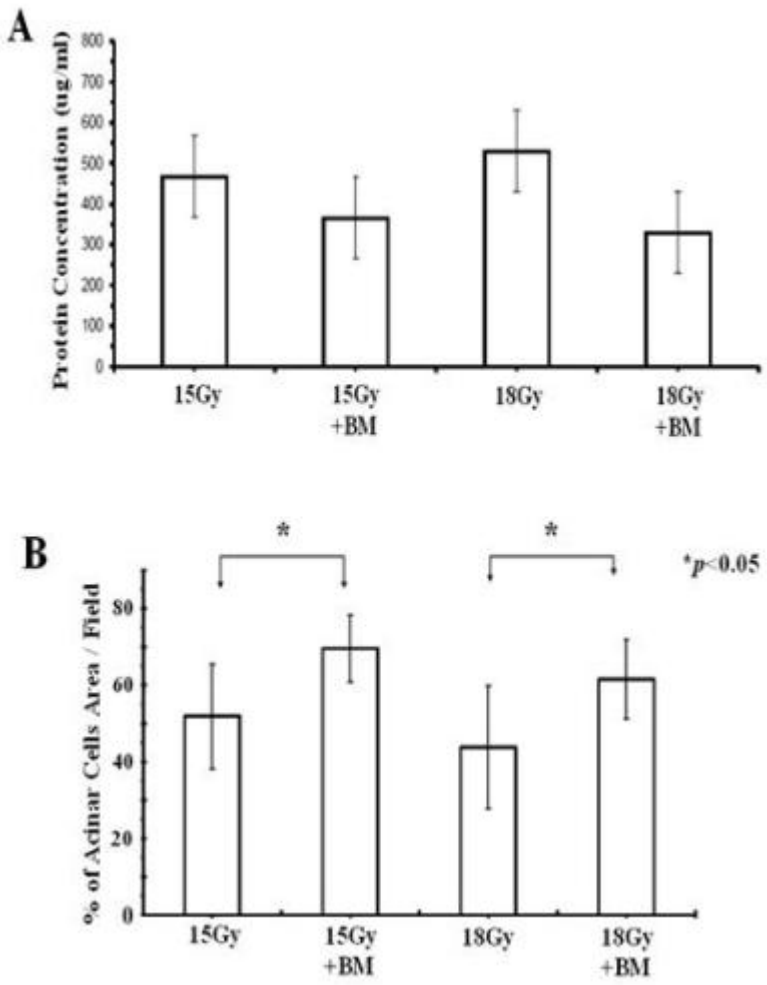
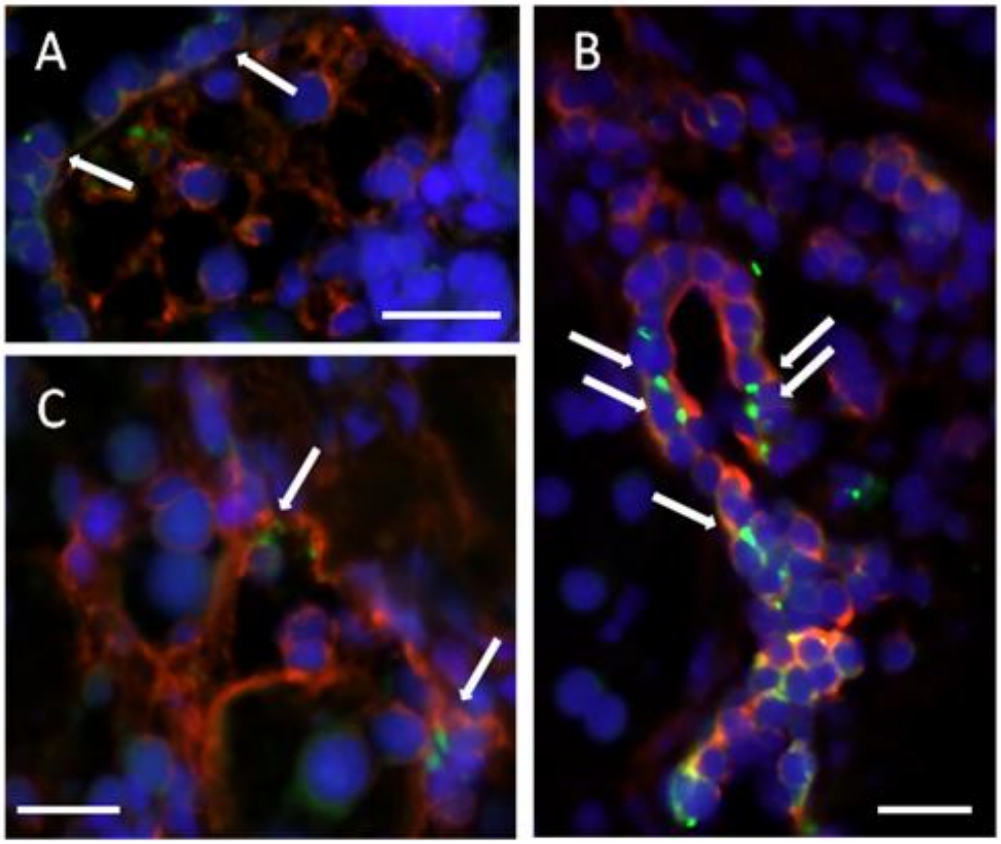


Figure 5.

Chart (A) shows the total protein concentration in saliva secreted at 24 weeks after irradiation. The concentrations of non-treated groups were slightly higher than that of BMDC-treated groups. Chart (B) shows the area (in percentage) of acinar cell occupying the salivary tissue at 24 weeks after 15Gy and 18Gy irradiation in four groups of mice. The areas are increased in BMDC-treated mice when compared with that of non-treated mice, and there are significant differences between BMDC- and non-treated groups.



D	NKCC1	CK8&18	CK13
Y-chromosome Positive Cells (%)	6.92	12.38	8.49

Figure 6.

Double immunostaining in irradiated salivary glands of female mice transplanted with BMDCs. (A) The Y chromosome signal is a green dot (shown by the arrow) and the co-transporter for Na-K-2Cl type 1 is a red signal that surrounds the cell membrane (NKCC1 is a marker of salivary acinar cells). Nuclei are stained in blue with DAPI. (B) We observed several Y chromosome cells (arrows) that are cytokeratins 8 and 18 positive, or (C) cytokeratin 13 positive. Cytokeratins 8, 18 and 13 are used here as markers of salivary epithelial cells. The scale bar represents 34 μm in all three panels. (D) Percentage of Y-chromosome positive cells in salivary glands of BMDC-treated mice. Scale bar = 34 μm.

4. DISCUSSION

Our results demonstrate that transplantation of bone marrow-derived cells (BMDCs) via intravenous injections can regenerate radiation-damaged tissue and rescue salivary gland functions. In previous studies, BMDCs were reported to serve as a valuable factor for the regeneration of damaged tissues (Kuehnle et al., 2002). Homing and engraftment of BMDCs in damaged non-hematopoietic organs, such as vascular tissue, myocardium, liver, kidney, lung, and skin, have been observed and suggested to contribute to tissue repair and function improvement (Lagasse et al., 2000; Krethe et al., 2004; Ohtuka et al.,2004; Orlic et al., 2001a, 2001b; Stamm et al., 2003; Minatoguchi et al., 2004; Nishida et al., 2004). These studies have provided evidence for the use of BMDCs in cell-based therapy. Furthermore, we initially observed that BMDCs from healthy male donors can differentiate into buccal (oral) epithelial cells of female transplant recipients (Tran et al., 2003). Therefore, our work has focused on the potential use of BMDCs to repair oral tissues (Tran et al., 2007; Crain et al., 2005; Maria et al., 2007).

Using a cell-therapy approach, we transplanted BMDCs of male C3H mice into female mice, after irradiation of the neck area (with 15 Gy and 18 Gy). Our successful treatment criteria were: 1) restoration of saliva production, 2) promotion of tissue regenerative activity, 3) direct differentiation of donor BMDCs into salivary epithelial cells. Our findings are comparable with those reported by Lombeart et al. (2006). They reported that the granulocyte colony-stimulating factor (G-CSF) treatment induced mobilization of a large number of BMDCs from mouse bone marrow to salivary glands that had received irradiation at their neck area. Although no transdifferentiation of BMDCs to salivary epithelial cells were observed in their study, they speculated that both hematopoietic and mesenchymal cells mobilized from bone marrow by G-CSF could stimulate the recovery of the salivary gland cells. To date, several mechanisms by which BMDCs improve organ functions have been reported. These are from cell fusion, cell

transdifferentiation, induction of vasculogenesis, or paracrine effect (Burt et al., 2008). The exact mechanisms remain unclear. However, Lombaert's and our findings supported the hypothesis that BMDCs could, at least, induce the vasculogenesis and paracrine effect in salivary gland. We found that the inhibition of apoptosis and the increase of tissue regenerative activity (blood vessel formation, cell proliferation, and EGF activity) occurred in salivary glands after BMDCs transplantation. Furthermore, the expression of salivary gland stem cell markers was observed in the damaged tissues of BMDCs-transplanted mice. More recently, Tatsuichi et al. demonstrated that the salivary gland stem/ progenitor cells could survive and remain dormant after irradiation, but their survival rate depended on the radiation dosage and cell age (Tatuishi et al., 2009). We used young (8-week old) C3H mice and a radiation dosage that caused approximately 50 % decline in saliva production. The presence of c-Kit and Sca-1 positive cells in treated-mice suggest that the transplanted BMDCs may provide a local paracrine effect for re-activation of dormant stem cells which survived the irradiation process.

A main difference from our results was the detection of 9 % Y-chromosome positive cells in salivary epithelial cells of BMDC-treated mice, as compared to none in the Lombaert's studies (Lombeart et al., 2006). Our results demonstrated that donor BMDCs could differentiate into the salivary gland epithelial cells. Moreover, this might suggest that transplanted exogenous-BMDCs can differentiation into salivary cells, when compared to endogenous-BMDCs mobilized from bone marrow by G-CSF. In general, somatic stem/ progenitor cells possess tissue-specific characteristics which inhibit their transdifferentiation in the body. However, once isolated into single cells, they tend to be more multipotent (Widera et al., 2009; Yalvac et al., 2010). In this study, we harvested the bone marrow from the male donor mice, and isolated BMDCs by 40-μm cell strainer. Then, these BMDCs were transplanted as a model of cell therapy.

BMDCs have the potential to be an effective cell source, not only for xerostomia, but also for other negative side effects of chemo-radiotherapy in the oral area, such as osteo-necrosis, skin and oral mucosa ulcers. For example, superselective intra-arterial infusion and chemo-radiotherapy have been introduced as a strategy for oral cancer (to avoid surgical resection) (Tohnai et al., 1998). Resection of oral cancer, especially in advanced lesions (T3-4), causes loss of functions including speech and swallowing, and is associated with disfigurement. In order to avoid these disadvantages, an effective, non-surgical strategy for oral cancer is required. Lately, our surgical unit reported that superselective intra-arterial infusion of anti-cancer agents with concurrent delivery of external beam radiotherapy is quite effective for oral cancer at both the primary site and metastatic lymph nodes, and has the potential to be curative in advanced cases which are inoperable (Uehara et al., 2010). However, for this treatment, the total radiation dose to the primary tumor and neck are 60 and 40 Gy, respectively. Therefore, these patients suffer from severe xerostomia immediately after the irradiation treatment. Furthermore, other sequelae such as osteonecrosis of the jaw bones occur after the irradiation treatment. To prevent or cure such severe sequelae due to irradiation treatment, BMDCs can be harvested before and after the chemo-radiotherapy (which will be restricted to the oral cavity). Therefore, for the amelioration of radiation-induced damages by cell therapy, the simple strategy of direct transplantation by using enriched BMDCs without in vitro culture system should be investigated further.

5. CONCLUSION

In conclusion, we demonstrated the capacity of BMDCs for salivary gland regeneration by their direct transplantation through the intra-vein injection. Although the mechanisms of transplanted BMDCs that led to the regeneration of damaged tissue remain unclear, we observed the phenomenon of differentiation of BMDCs to salivary epithelial cells. We believe that the

restoration of function and morphology arose from a combination of several factors such as vasculogenesis, paracrine effect, and cell transdifferentiation by hematopoietic and/or mesenchymal cells derived from bone marrow. For future clinical applications, additional investigations are needed to understand the mechanisms of transplanted bone marrow-derived cells that lead to radiation-damaged tissue regeneration.

REFERENCES

- Aframian DJ, Amit D, David R, Shai E, Deutsch D, Honigman A, Panet A, Palmon A. Reengineering salivary gland cells to enhance protein secretion for use in developing artificial salivary gland device. Tissue Eng. 2007;13:995-1001.
- Aframian DJ, Palmon A. Current status of the development of an artificial salivary gland. Tissue Eng Part B Rev. 2008;14:187-98.
- Baum BJ, Kok M, Tran SD, Yamano S. The impact of gene therapy on dentistry: a revisiting after six years. J Am Dent Assoc. 2002;133:35-44.
- Baum BJ, Tran SD. Synergy between genetic and tissue engineering – creating an artificial salivary gland. Periodontology2000. 2006;41:218-23.
- Burt RK, Loh Y, Pearce W, Beohar N, Barr WG, Craig R, Wen Y, Rapp JA, Kessler J. Clinical applications of blood-derived and marrow-derived stem cells for nonmalignant disease. JAMA. 2008;299:925-36.
- Coppes RP, van der Goot A, Lombaert IM. Stem cell therapy to reduce radiation-induced normal tissue damage. Semin Radiat Oncol. 2009;19:112-21.
- Couzin J. Clinical trials. A shot of bone marrow can help the heart. Science. 2006;313:1715-6.
- Crain BJ, Tran SD, Mezey E. Transplanted human bone marrow cells generate new brain cells. J Neurol Sci. 2005;233:121-3.
- 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. Radiother Oncol. 2009;92:466-71.
- Joraku A, Sullivan CA, Yoo JJ, Atala A. Tissue engineering of functional salivary gland tissue. Laryngoscope. 2005;115:244-48.

- Kagami H, Wang S, Hai B. Restoring the function of salivary glands. Oral Dis. 2008;14:15-24.
- Kuehnle I, Goodell MA. The therapeutic potential of stem cells from adults. BMJ. 2002;325:372-6.
- Kuethe F, Figulla HR, Voth M, Richartz BM, Opfermann T, Sayer HG, Krack A, Fritzenwanger M, Höffken K, Gottschild D, Werner GS. [Mobilization of stem cells by granulocyte colony-stimulating factor for the regeneration of myocardial tissue after myocardial infarction]. Dtsch Med Wochenschr. 2004;129:424-8.
- Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med. 2000;6:1229-34
- Lombaert IM, Wierenga PK, Kok T, Kampinga HH, deHaan G, Coppes RP. Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands. Clin Cancer Res. 2006;12:1804-12.
- Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, Visser WH, Kampinga HH, de Haan G, Coppes RP. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One. 2008a;3:e2063.
- 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. Clin Cancer Res. 2008b;14:7741-50.
- Maria OM, Khosravi R, Mezey E, Tran SD. Cells from bone marrow that evolve into oral tissues and their clinical applications. Oral Dis. 2007;13:11-6.
- Minatoguchi S, Takemura G, Chen XH, Wang N, Uno Y, Koda M, Arai M, Misao Y, Lu C, Suzuki K, Goto K, Komada A, Takahashi T, Kosai K, Fujiwara T, Fujiwara H. Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation. 2004;109:2572-80.
- Nishida M, Fujimoto S, Toiyama K, Sato H, Hamaoka K. Effect of hematopoietic cytokines on renal function in cisplatin-induced ARF in mice. Biochem Biophys Res Commun. 2004;324:341-7.

- Ohtsuka M, Takano H, Zou Y, Toko H, Akazawa H, Qin Y, Suzuki M, Hasegawa H, Nakaya H, Komuro I. Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization. FASEB J. 2004;18:851-3.
- Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature. 2001a;410:701-5.
- Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA. 2001b;98:10344-9.
- Pradhan S, Liu C, Zhang C, Jia X, Farach-Carson MC, Witt RL. Lumen formation in three-dimensional cultures of salivary acinar cells. Otolaryngol Head Neck Surg. 2010;142:191-5.
- Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schümichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 2003;361:45-6.
- Sugito T, Kagami H, Hata K, Nishiguchi H, Ueda M. Transplantation of cultured salivary gland cells into an atrophic salivary gland. Cell Transplant. 2004;13:691-9.
- Tatsuishi Y, Hirota M, Kishi T, Adachi M, Fukui T, Mitsudo K, Aoki S, Matsui Y, Omura S, Taniguchi H, Tohnai I. Human salivary gland stem/progenitor cells remain dormant even after irradiation. Int J Mol Med. 2009;24:361-6.
- Tohnai I, Fuwa N, Hayashi Y, Kaneko R, Tomaru Y, Hibino Y, Ueda M. New superselective intra-arterial infusion via superficial temporal artery for cancer of the tongue and tumour tissue platinum concentration after carboplatin (CBDCA) infusion. Oral Oncol. 1998;34:387-90.
- Tran SD, Pillemer SR, Dutra A, Barrett AJ, Brownstein MJ, Key S, Pak E, Leakan RA, Kingman A, Yamada KM, Baum BJ, Mezey E. Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study. Lancet. 2003;361:1084-8.
- Tran SD, Wang J, Bandyopadhyay BC, Redman RS, Dutra A, Pak E, Swaim WD, Gerstenhaber JA, Bryant JM, Zheng C, Goldsmith CM, Kok MR, Wellner RB, Baum BJ.

Primary culture of polarized human salivary epithelial cells for use in developing an artificial salivary gland. Tissue Eng. 2005;11:172-81.

- Tran SD, Sugito T, Dipasquale G, Cotrim AP, Bandyopadhyay BC, Riddle K, Mooney D, Kok MR, Chiorini JA, Baum BJ. Re-engineering primary epithelial cells from rhesus monkey parotid glands for use in developing an artificial salivary gland. Tissue Eng. 2006;12:2939-48.
- Tran SD, Kodama S, Lodde BM, Szalayova I, Key S, Khalili S, Faustman DL, Mezey E. Reversal of Sjogren's-like syndrome in non-obese diabetic mice. Ann Rheum Dis. 2007;66:812-4.
- Uehara M, Shiraishi T, Tobita T, Nonaka M, Asahina I. Antitumor effects on primary tumor and metastatic lymph nodes by superselective intra-arterial concurrent chemoradiotherapy for oral cancer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010;Apr 8 [Epub ahead of print].
- Vissink A, Burlage FR, Spijkervet FK, Jansma J, Coppes RP. Prevention and treatment of the consequences of head and neck radiotherapy. Crit Rev Oral Biol Med. 2003a;14:213-225.
- Vissink A, Jansma J, Spijkervet FK, Burlage FR, Coopes RP. Oral sequelae of head and neck radiotherapy. Crit Rev Oral Biol Med. 2003b;14:199-212.
- Widera D, Zander C, Heidbreder M, Kasperek Y, Noll T, Seitz O, Saldamli B, Sudhoff H, Sader R, Kaltschmidt C, Kaltschmidt B. Adult palatum as a novel source of neural crest-related stem cells. Stem Cells. 2009;27:1899-910.
- Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, Palotas A, Kose GT. Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo-, and neurogenesis. 2010;10:105-13.
- Yang TL, Young TH. The enhancement of submandibular gland branch formation on chitosan membranes. Biomaterials. 2008;29:2501-8.

Bone Marrow-Derived Cells: A Source Of Undifferentiated Cells To Prevent Sjögren's Syndrome And To Preserve Salivary Glands Function In The Non-Obese Diabetic Mice.

By

Saeed Khalili, Younan Liu, Yoshinori Sumita, **Ola M. Maria**, David Blank, Sharon Key, Eva Mezey, Simon D. Tran.

(*Submitted* 2010)

The Ph.D. Candidate performed the following contributions in this manuscript:

- Isolation and transplantation of bone marrow cells.
- Shared writing and revising the manuscript.

Bone Marrow-Derived Cells: A Source Of Undifferentiated Cells To Prevent Sjögren's Syndrome And To Preserve Salivary Glands Function In The Non-Obese Diabetic Mice.

Saeed Khalili[1], Younan Liu[1], Yoshinori Sumita[1,2], Ola M. Maria[1], David Blank[3], Sharon Key[4], Eva Mezey[4], Simon D. Tran[1*]

[1] McGill University, Faculty of Dentistry, Montreal, Canada

[2] Nagasaki University, Department of Regenerative Oral Surgery, Nagasaki, Japan.

[3] McGill University, Faculty Medicine, Montreal, Canada

[4] National Institute of Dental and Craniofacial Research, Bethesda, Maryland, USA

***** *Corresponding Author*:

Simon Tran. McGill University, Faculty of Dentistry, 3640 University Street, room M-43, Montreal, H3A 2B2, Canada,

Tel: 5143987203 ext. 09182; Fax: 514 398 8900

Email: simon.tran@mcgill.ca

ABSTRACT

Non-obese diabetic (NOD) mice develop Sjögren's-like syndrome (Ss) and a gradual loss of saliva secretory function. Our previous study showed that injections of matched normal spleen cells with Complete Freund's Adjuvant (CFA) reversed salivary gland dysfunction in 14-week-old NOD mice, which had established Ss. The spleen and bone marrow are closely related organs, and both are among the first sites of hematopoiesis during gestation. Noticing a rapidly increasing number of clinical trials using bone marrow-derived cell (BMDC) treatments for autoimmune diseases, we tested if BMDCs can prevent Ss and restore salivary glands' function. We tried treatments of CFA and MHC class I-matched normal BMDCs in 7-week-old NOD mice, which had not yet developed Ss, and observed its long-term effects. We found at week 52 post-treatment that all NOD mice receiving BMDCs and CFA had a recovery of salivary flow and were protected from Ss and diabetes. BMDC-treated mice had their salivary function restored quantitatively and qualitatively. Saliva flow was higher ($p<0.05$) in BMDC-transplanted mice when compared to control mice, which continued to deteriorate over time. Total proteins, epidermal growth factor, amylase, and electrolytes concentrations in saliva of BMDC-treated mice were not significantly changed at week 44 and 52 post-therapy when compared to pre-therapy (when the mice did not have Ss). Restoration of salivary flow could have resulted from a combination of rescue and paracrine effects from BMDCs. This study suggests that cellular therapy can permanently prevent Ss and restored salivary function in NOD mice.

Key words: Sjögren's syndrome, bone marrow-derived cells, cell therapy, xerostomia, salivary gland, saliva.

1. INTRODUCTION

Sjögren's syndrome, which affects people with a frequency of 1 in 100, is characterized by an autoimmune destruction of the salivary and lacrimal glands. As a result, patients with Sjögren's syndrome suffer from dry mouth and dry eyes (Delaleu et al., 2005, Lee et al., 2009). Salivary glands have various cell types: acinar cells which are responsible for water and proteins secretion, ductal cells that principally regulate the composition of saliva, and myoepithelial cells surrounding the acini and ducts (Lombaert et al., 2008). In Sjögren's syndrome the immune system attacks the salivary glands, particularly the acinar cells. This leads to a loss of saliva secretion and the patients' quality of life is severely compromised due to xerostomia (dry mouth), severe dental caries, and oral infections (Delaleu et al., 2005, Lee et al., 2009, Lombaert et al., 2008, Fox and Speight, 1996, Nikolov and Illei, 2009). Unfortunately, there is no suitable treatment for Sjögren's syndrome, because the current pharmacological therapy that depends on the stimulation of residual acinar cells frequently fails, since in many cases all the salivary secretory tissue has already been lost (Tran et al., 2006). Regeneration of destroyed salivary glands and restoration of their function would greatly improve the quality of life for these patients.

Non-obese diabetic (NOD) mice, a frequently used animal model of Sjögren's syndrome and type 1 diabetes mellitus (T1DM), both exhibit infiltrates of lymphocytes in the salivary glands (sialadenitis) with a gradual loss of salivary function and in the pancreas (insulitis) (Lee et al., 2009, Kodama et al., 2003, Tran et al., 2007, Soyfoo et al., 2007b, Jonsson et al., 2007). The reduced saliva output is similar to what is observed in patients (Tran et al., 2007). Previous studies have shown that a two-limb intervention can permanently restore lost function in Sjögren's syndrome and T1DM in NOD mice (Tran et al., 2007, Kodama et al., 2003). This two-

limb intervention consists of using complete Freund's adjuvant (CFA) as the first limb, and then combining this with matched Major Histocompatibility Complex (MHC) class I spleen cells, as the second limb. The rationale of this two-limb intervention is that cellular immunity (T lymphocytes) plays a major role in the pathophysiology of Sjögren's syndrome (Katsifis et al., 2007). NOD mice have a defect in the production of the low-molecular-weight protein 2, LMP2, leading to a problem in T cell selection. This results in the presence of autoreactive T cells and development of autoimmune diabetes and Sjögren's-like syndrome (Hayashi and Faustman, 1999). LMP2 is a catalytic subunit of the proteosomes, which are very large protein complexes inside all eukaryotes and their role is to degrade all proteins that the cell has no more use for. This proteasome defect also impairs the processing of nuclear factor κB (NF-κB), a transcription factor that stimulates the expression of genes involved in a wide variety of biological functions, including the protection from TNF-α induced apoptosis. The disruption of its function in antigen presenting cells results in the escape of autoreactive T cells from proper immune selection. NF-κB defect also increases the apoptosis of misselected T-cells by TNF-α-induced apoptosis (Ryu et al., 2001). Treatment of NOD mice with a TNF-α inducer such as CFA, promotes the apoptosis of autoreactive T-cells and eventually removes the autoimmunity (Tran et al., 2007, Kodama et al., 2003). Once the autoimmunity is removed, restoration of salivary glands' function can be achieved. Humans with Sjögren's syndrome also have a similar genetic problem resulting in improper T cell selection (Krause et al., 2006, Tran et al., 2007, Fu et al., 1993, Fu et al., 1998).

The spleen and bone marrow are closely related organs, and both are among the first sites of hematopoiesis during gestation. There are reports that bone marrow-derived cells (BMDCs) have been used to treat autoimmune diseases (Tran et al., 2003, Van Laar and Tyndall, 2006, Oyama et

al., 2005, Oyama et al., 2007, Burt et al., 2009, Saccardi et al., 2006, Bocelli-Tyndall et al., 2007, Miniati et al., 2009, Lowenthal et al., 1993). BMDCs have been suggested as a source of multipotent stem cells; particularly the marrow derived stromal cells, also known as Mesenchymal Stem Cells (MSCs) with their ability to repair non-hematopoietic organs, including the salivary gland and pancreas (Lombaert et al., 2006, Lombaert et al., 2008, Orlic et al., 2001, Couzin, 2006, Urban et al., 2008). MSCs that can be isolated from a variety of tissues were shown to interact with all cells of the innate and adaptive immune system to modulate their function. Following systemic administration they home to injured tissues and can suppress the pro-inflammatory cytokines to help survival. In addition to immuno-modulation they can also regenerate bone, fat, cartilage and cells of other lineages (Uccelli A and Pistoia V , 2008). The plasticity and immunosuppressive capability of MSCs have made them a novel therapeutic choice in autoimmune diseases (Zhao et al., 2009, Aguayo-Mazzucato and Bonner-Weir, 2010). MSCs can assist in the regeneration of the pancreas and salivary glands in mice (Lombaert et al., 2008, Urban et al., 2008). Transplantation of BMDCs boosted levels of serum insulin and decreased blood sugar levels in diabetic mice by mechanisms such as the re-construction of β-cell islets (the insulin-secreting cells), secretion of growth factors to endothelial progenitor cells, or by direct cell differentiation (Aguayo-Mazzucato and Bonner-Weir, 2010, Uccelli A and Pistoia V, 2008). In other words, a population of BMDCs is capable to differentiate into other cell types, as well as to provide a beneficial effect by secreting cytokines and /or growth factors (Burt et al., 2009, Coppes et al., 2009, Uccelli A and Pistoia V, 2008). The challenge of successful cellular therapy for autoimmune diseases is not only to regenerate the tissue but also to prevent it from the same autoimmune attack that is responsible for its destruction at the first place (Aguayo-Mazzucato and Bonner-Weir, 2010).

Noticing a rapidly increasing number of clinical trials using BMDC treatments for autoimmune diseases, we wish to assess whether the two-limb intervention described above can be used with MHC class I-matched normal BMDCs. The objective of this study was to test if a two-limb intervention, using CFA and BMDCs as source of undifferentiated cells, can prevent Sjögren's syndrome and restore salivary glands' function in NOD mice.

2. MATERIALS AND METHODS

2.1. ANIMALS

Seven-week old female NOD mice (which had not yet developed Sjögren's syndrome; Taconic Farms, Germantown, NY) and aged-matched CByF1B6F1/J (CByB6F1) mice (Jackson Laboratory) were maintained under pathogen-free conditions in the Animal facility at McGill University. Female NOD mice were divided into three different groups (10 mice per group) and were followed for 52 weeks after treatments with either: a) bone marrow cells transplant plus CFA (BMDC group), b) only CFA injected (CFA group), or c) no cell injection, no CFA, but daily injections of insulin to control blood sugar levels (Control group).

2.2. CELL TRANSPLANTATION

Bone marrow cells of male CByB6F1 mice ($1x10^7$ cells) were harvested and freshly injected into female NOD recipients (of the BMDC group) through the tail vein, twice a week for six consecutive weeks (Tran et al., 2007, Kodama et al., 2003). No bone marrow cells were injected into NOD mice in the CFA group or Control groups. Complete Freund's adjuvant (CFA, Difco, Detroit, MI) was freshly mixed with an equal volume of physiological saline and then injected (50 µl) into each hind footpad simultaneously with the first bone marrow cells injection. CFA was also injected once in NOD mice of the CFA group. No CFA was injected in mice of the Control group.

2.3. SALIVARY FLOW RATE

Secretory function of the salivary glands (salivary flow rate) was obtained by inducing mild gas anesthesia to NOD mice with 5% Isoflurane, 5% Halothane, and 0.5-1 L/min Oxygen (as per animal facility protocols at McGill University). Whole saliva was collected after stimulation of secretion using 0.5 mg Pilocarpine/kg body weight administered subcutaneously. Saliva was obtained from the oral cavity by micropipette, placed into pre-weighed 0.5-ml microcentrifuge tubes. Saliva was collected for a 10-minute period and its volume determined gravimetrically. Salivary flow rate was determined at baseline (week 0, when NOD mice were 7-week old; before transplantation started) and at weeks 2, 12, 34, 38, and 52 post-transplantation.

2.4. BLOOD SUGAR

Blood glucose levels from NOD mice were monitored once a week (Accu-Check, Roche Diagnostics, Laval, QC, Canada). The mice were diagnosed with diabetes after observing two consecutive daily blood glucose concentrations of >200 mg/dl. These diabetic mice were injected with insulin on a daily basis (Humulin N, Lilly, ON, Canada).

2.5. SALIVA COMPOSITIONS

2.5.1. TOTAL PROTEINS CONCENTRATION

The concentration of proteins in saliva was measured by Bicinchoninic Acid Assay (BCA) method following the manufacturer's instructions (Thermo Fisher Scientific, Ottawa, ON, Canada, Cat no. 23225). It was measured at the beginning of experiment in week 0 and at the end in week 52.

2.5.2. EPIDERMAL GROWTH FACTOR (EGF)

Concentration of EGF in saliva was measured by ELISA method at baseline (week 0) and at the end of the experiment (week 52 post-transplantation) (R&D System, Minneapolis, MN, USA, Cat no. MGE00).

2.5.3. AMYLASE ACTIVITY

Amylase activity in saliva was measured by colorimetric method at the beginning (week 0) and at the end of study (week 52) (Salimeterics, PA, USA, Cat no. 1-1902).

2.5.4. ELECTROLYTES [Na^+, K^+, Cl^-, and Ca^{2+}] CONCENTRATION

Salivary sodium, potassium, chloride, and calcium were analyzed on a Beckman Coulter DXc 800 automated chemistry analyzer using the urine chemistry mode. The sodium assay was an indirect potentiometric method based on a sodium sensitive electrode. Potassium was measured with a potassium sensitive electrode consisting of a valinomycin PVC membrane cast. An indirect potentiometry utilizing a solid-state chloride electrode was used to measure chloride. Calcium was also measured by an ion selective electrode consisting of a calcium ionophore membrane cast (Beckman Coulter Synchron systems, CA USA).

2.6. HISTOLOGY ANALYSIS

2.6.1. IMMUNOSTAINING COMBINED WITH FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Salivary glands were collected, immersed in Optimal Cutting Temperature (OCT) compound, and then frozen at –80°C. Serial frozen sections were cut in a cryostat at 4-8μm thickness, mounted on poly l lysine coated microscope slides and immersion fixed in 2% paraformaldehyde fixative for 10 minutes (Tran et al., 2007, Toth et al., 2008). Immunostaining was performed by primary antibody that was detected by the Sternberger peroxidase antiperoxidase (PAP) method followed by a Fluorescein Isothiocyanate-Conjugated (FITC)-Tyramide signal amplification (TSA System,

Invitrogen, Carlsbad, USA). The primary antibody used was against $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) (graciously donated by Dr. James Turner, NIH) to detect salivary acinar cells. A digoxigenin-labeled riboprobe was then added to recognize a repeat sequence (pY353B) on the mouse Y chromosome. Then the samples were blocked with endogenous peroxidase (DAKO, CA, USA#S2001). The riboprobe was then detected using an antibody to digoxigenin conjugated to peroxidase (Roche, Indianapolis, USA). This peroxidase was visualized by tyramide signal amplification with a Alexa Fluor 594 fluorochrome-tyramide reagent (TSA System, Invitrogen, Carlsbad, USA). All sections were stained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen#D3571), a chromosomal marker to label all nuclei and then mounted with buffer Tris. Finally the slides were visualized using a Leica DM6000 fluorescent microscope equipped with Volocity software (Tran et al., 2007)**.**

2.6.2. FOCUS SCORE

Focus score is defined as the number of foci, infiltrated lymphocytes (at least 50 inflammatory cells), per 4 mm^2 of tissue. After NOD mice were sacrificed, their submandibular glands were removed immediately. Half of a gland per mouse was fixed in 10% formalin and embedded in paraffin. Sections were cut at 5-8 μm thicknesses and subsequently stained with hematoxylin and eosin. This method is an accepted way to determine the severity of the sialadenitis (the inflammatory cells infiltrate found in the salivary glands).

2.7. STATISTICAL ANALYSIS

To determine statistical significant differences ($P< 0.05$), Linear Mixed Models and ANOVA analysis (Tukey's Post-Hoc test) were used. Subjects between and within the groups were compared at different time points using SPSS version 17 (IBM, Chicago, IL, USA).

3. RESULTS

3.1. SALIVARY FLOW RATE

Overall salivary flow rates (SFR) gradually increased in the BMDC-treated and CFA injected groups for up to one year post transplantation. SFR of non-treated NOD mice, on the other hand, continued to decrease until the end of experiment (Fig 1). Mice in BMDC-treated group had a decline in their SFR for 2 weeks post-treatment. After that, SFR started to recover and continued to improve until the end of the experiment, reaching comparable levels of the SFR in baseline. The SFR was significantly higher in BMDC-transplanted group than in the non-treated group ($P<0.05$).

Fig. 1.

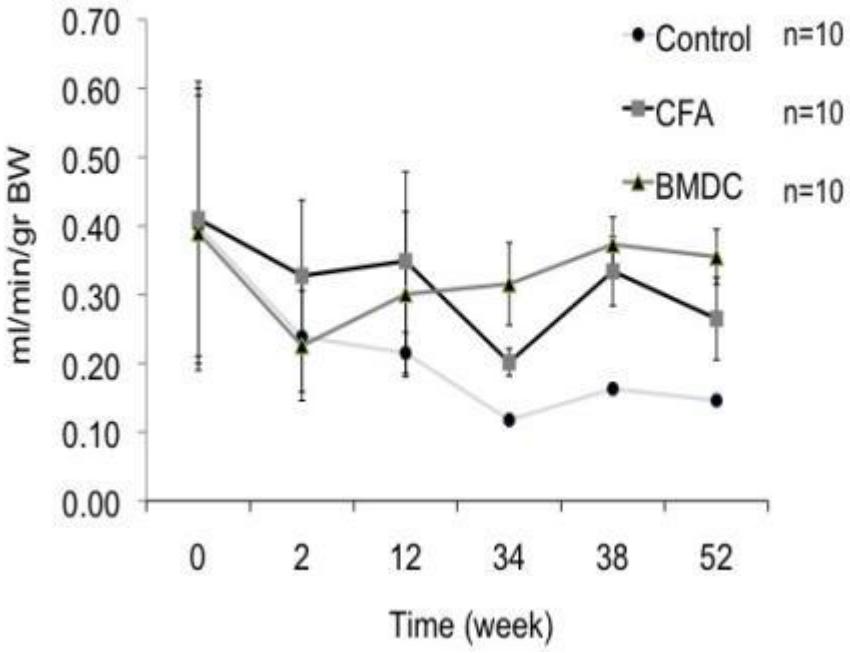


Fig. 1. Effects of treatment on the restoration of salivary flow rates (SFR). Stimulated salivary flow rate of BMDCs-treated (triangle), CFA-treated (square), and untreated NOD mice (circle) during the 52-week time course following therapy. SFR in BMDCs-treated mice has recovered. Mice in BMDC-treated group (triangle) had a decline in their SFR for 2 weeks post-treatment. After that period, SFR started to recover and continued to improve until the end of the experiment, reaching comparable levels to before Sjögren's-like syndrome. SFR was significantly different in BMDCs-transplanted group when compare to SFR in control-group ($P < 0.05$).

3.2. BLOOD GLUCOSE

All mice were normoglycaemic at the start of the experiment, at 7 weeks of age (week 0). Non-treated NOD mice (i.e. only receiving injections of insulin; no CFA or BMDCs) developed diabetes during the course of the experiment and 90% of them died by 30 weeks post-transplantation (Fig. 2). The first mouse in the control group was diagnosed with diabetes within 2 weeks of the follow-up period. Animals in the 2 treated groups (CFA+BMDCs; or CFA alone), however, showed a relatively stable blood sugar level and survived during the observation period of 52 weeks post-transplantation.

Fig. 2.

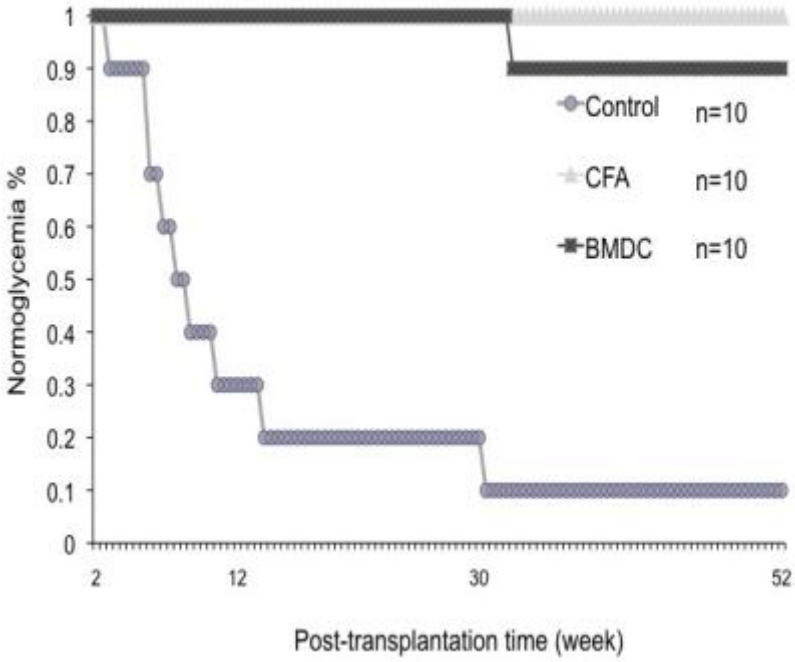


Fig. 2. Kaplan–Meier plot for normoglycaemia in BMDCs-treated (square), CFA-treated (triangle), and untreated (circle) NOD mice monitored for 66 weeks (for 52 weeks post-transplantation). All mice were normoglycaemic at the start of the experiment, at 7 weeks of age (week 0). The first diabetic mouse was diagnosed in the control group (circle) at 2 weeks post-therapy and 90% of mice in this group died within 30 weeks post-therapy. Mice in the treated groups (square and triangle) on the other hand, had a stable level of blood sugar during the course of the experiment. Only one mouse in the BMDC-treated group showed high level of blood sugar at 30 weeks post-transplantation.

3.3. TOTAL PROTEINS CONCENTRATION

Total proteins concentration in saliva has not changed significantly from week 52 (end of experiment) when compared to week 0 (when mice were healthy) among the treated groups ($p>0.05$) (Fig 3). Total proteins concentration in saliva of control mice could not be measure at week 52 as 90% of these mice had died due to their diabetes.

Fig. 3.

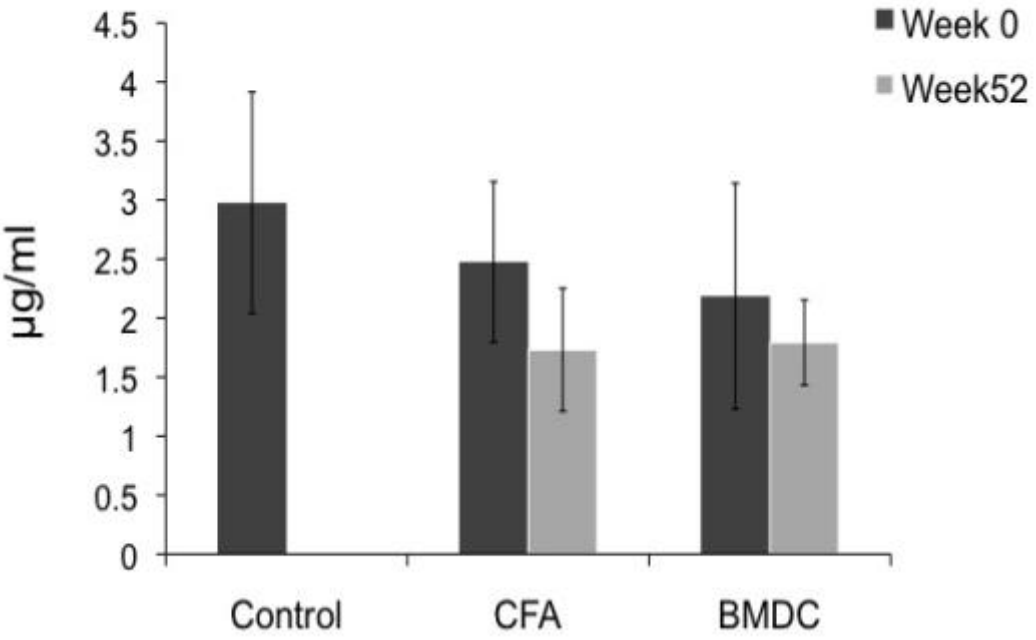


Fig. 3. Total proteins concentration in saliva. The concentration of total proteins did not significantly changed in NOD mice of treated groups (CFA and BMDC) between week 0 (when the mice were healthy) and week 52 following therapy ($P > 0.05$). Total proteins concentration in saliva of control mice could not be measured at week 52 as 90% of these mice had died due to their diabetes.

3.4. EPIDERMAL GROWTH FACTOR (EGF) AND AMYLASE ACTIVITY

No statistically significant differences were observed in the EGF concentration and amylase activity of saliva in treated groups during the course of experiment (Fig 4 and 5).

Fig. 4.

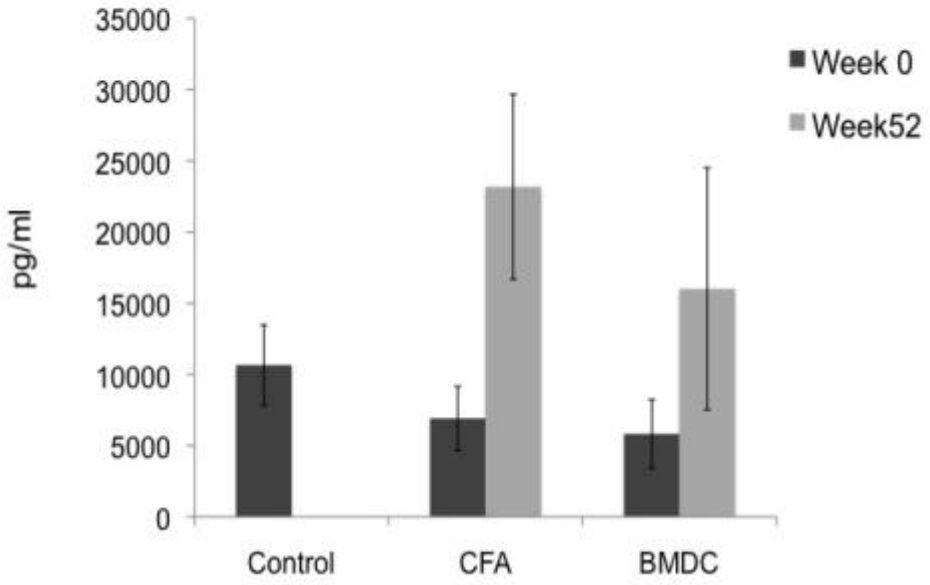


Fig. 4. Concentration of EGF in saliva. EGF concentration was not significantly different among the experimental groups in week 0 (baseline) and week 52, one year after treatment ($P > 0.05$).

Fig. 5.

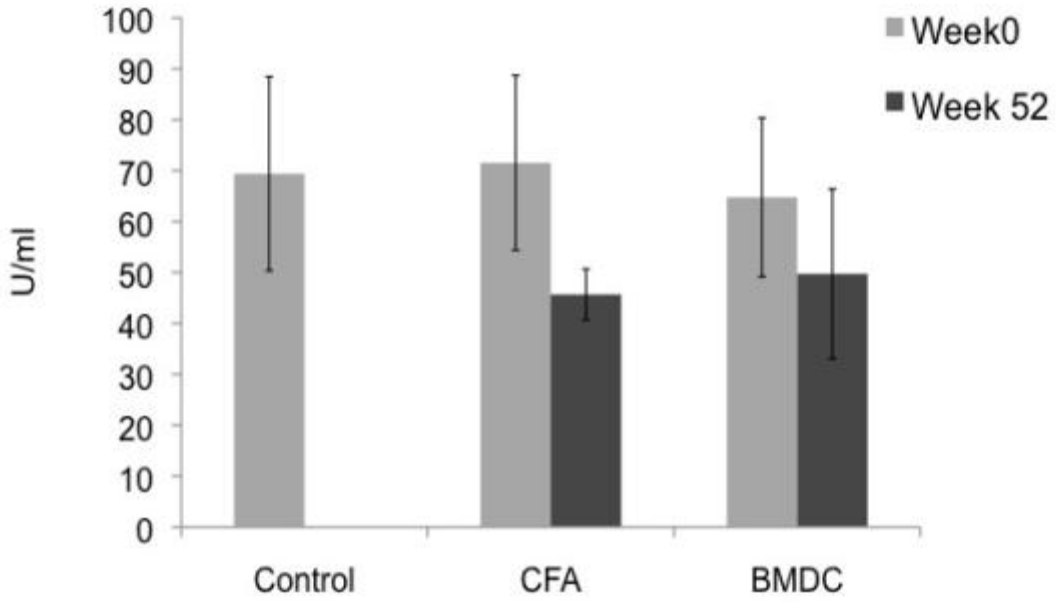


Fig. 5. Amylase activity in saliva. No statistical significant differences were observed in the amylase activity of saliva in treated groups (CFA and BMDC) at week 0 (baseline) when compared to week 52 post-therapy.

3.5. ELECTROLYTES IN SALIVA

In general, the electrolyte concentrations decreased in the BMDC-transplanted group. The levels of Na^+ and Cl^- decreased in week 44 compared to their levels in week 4 post-transplantation (Fig 6A). This may be due to the increase of SFR in the BMDC-treated mice during the 52-week follow-up period. The level of K^+, on the other hand, had a slight increase. The level of Na^+, Cl^- and $Ca2^+$ did not change in the CFA-treated group during the same interval of observation (Fig 6B). The concentration of salivary electrolytes (Na^+, K^+, Cl^- and $Ca2^+$) in the control-group mice increased during the course of the study (Fig 6C). This can be explained by the decrease of saliva secretion measured from these mice.

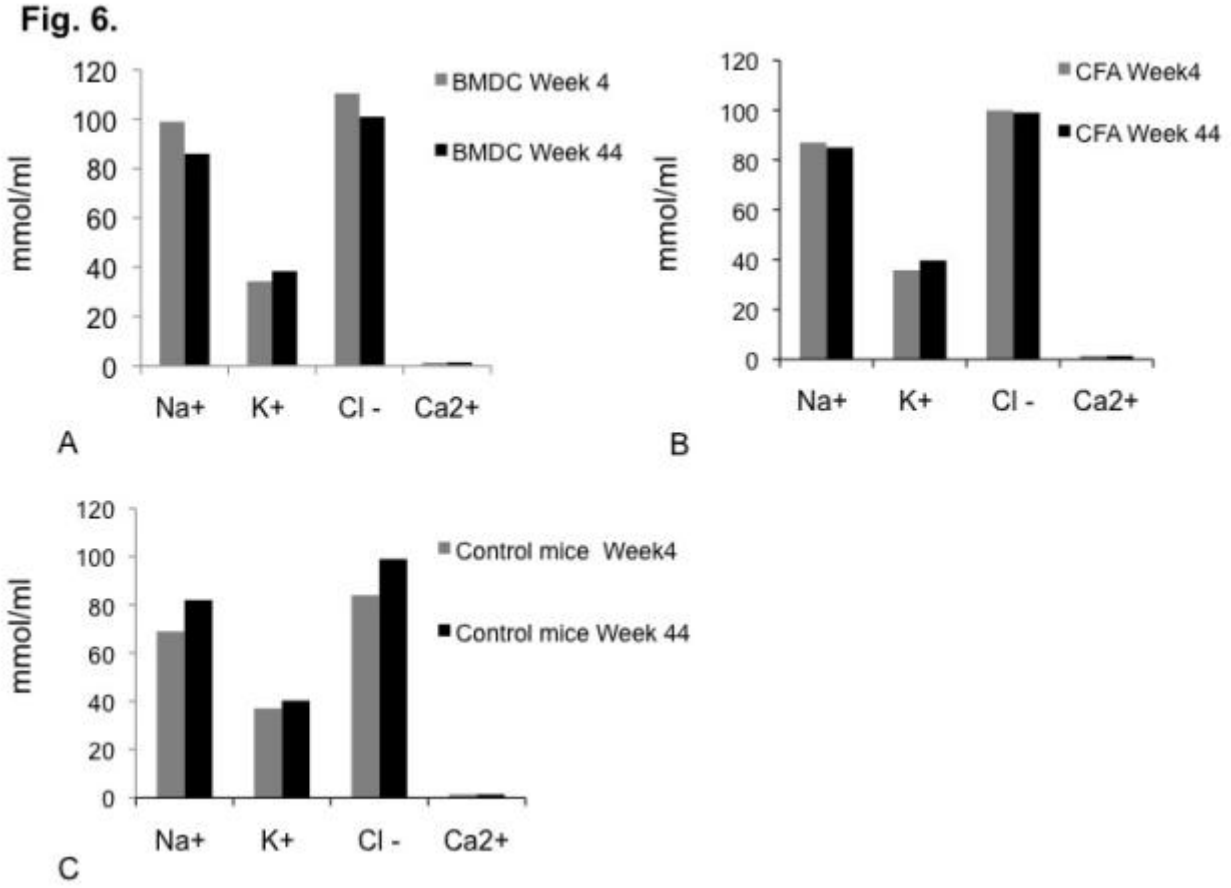


Fig.6. Effect of treatment on the saliva electrolytes. Overall, the level of electrolytes decreased in BMDC-treated group during the post-transplantation period when compared to their levels at the beginning of experiment. (A) Levels of Na^+ and Cl^- decreased at week 44 post-treatment in BMDC-transplanted group compared to week 4. The level of K^+, however, slightly increased. (B) Levels of Cl^- and Ca^{2+} did not change in CFA group. (C) Electrolytes concentration increased in control mice over the course of experiment. However, none of these changes from panels A, B, C were statistically significant.

3.6. IMMUNOSTAINING COMBINED WITH FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

To investigate the mechanism of the return of SFR in BMDC-treated NOD mice, we examined their salivary tissues for cell chimerism and inflammatory signs. Restoration of SFR could be the result of rescue of the salivary gland by removing autoimmunity, or by a combination of rescue and tissue regeneration that overcomes the destruction caused by autoimmunity. Regeneration could mainly be a de novo process, in which the glands are being regenerated by their endogenous stem cells (internal source). Another possibility is if the male donor BMDCs are able to differentiate into salivary epithelial cells. We observed a very small number of male donor cells which had differentiated into salivary epithelial cells (<0.1%), including acinar cells (detected by the NKCC1 antibody) (Fig 7).

Fig. 7.

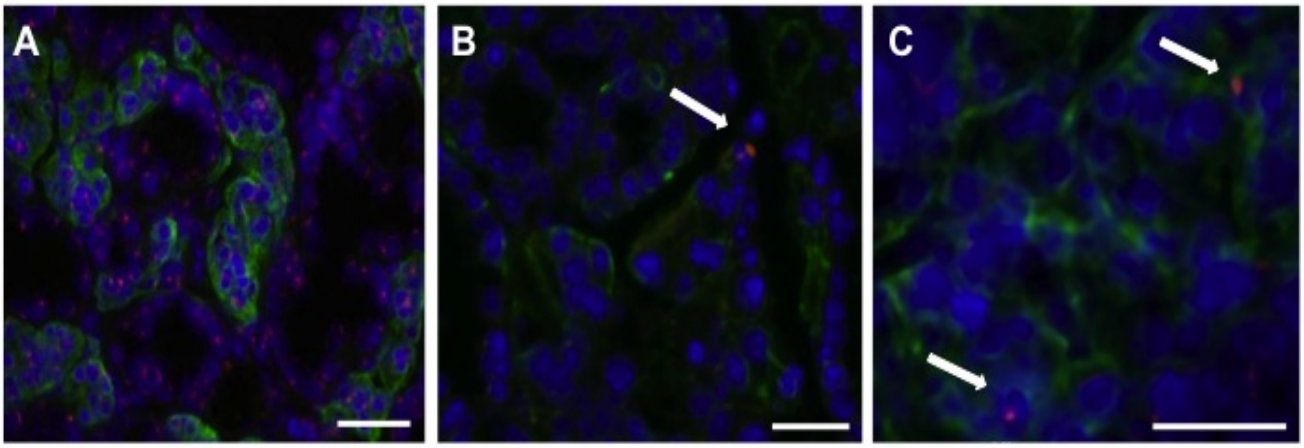


Fig. 7. Double immunostaining in mouse salivary glands. (A) Male mouse salivary gland tissue used here as a positive control for the Y chromosomal probe. The Y chromosome signal is a pink dot, and the co-transporter for Na-K-2Cl type 1 is a green signal that surrounds the cell membrane (NKCC1 is a marker of salivary acinar cells). Nuclei are stained in blue with DAPI. (B and C) We observed very few (<0.1%) Y chromosome positive cells in NKCC1-positive cells (shown by the arrows) in several BMDCs-treated female NOD mice. The scale bar represents 34 μm in all three panels.

3.7. FOCUS SCORE

Focus score in the salivary glands was not significantly different between non-treated and treated groups ($P>0.05$). The average focus scores were 3.13 in the control group, 2.4 in the CFA-treated group, and 2.3 in the BMDC-treated group. The size of the average lymphocytic infiltrate, however, was slightly larger in the non-treated mice when compared to the treated groups (Fig 8).

Fig. 8.

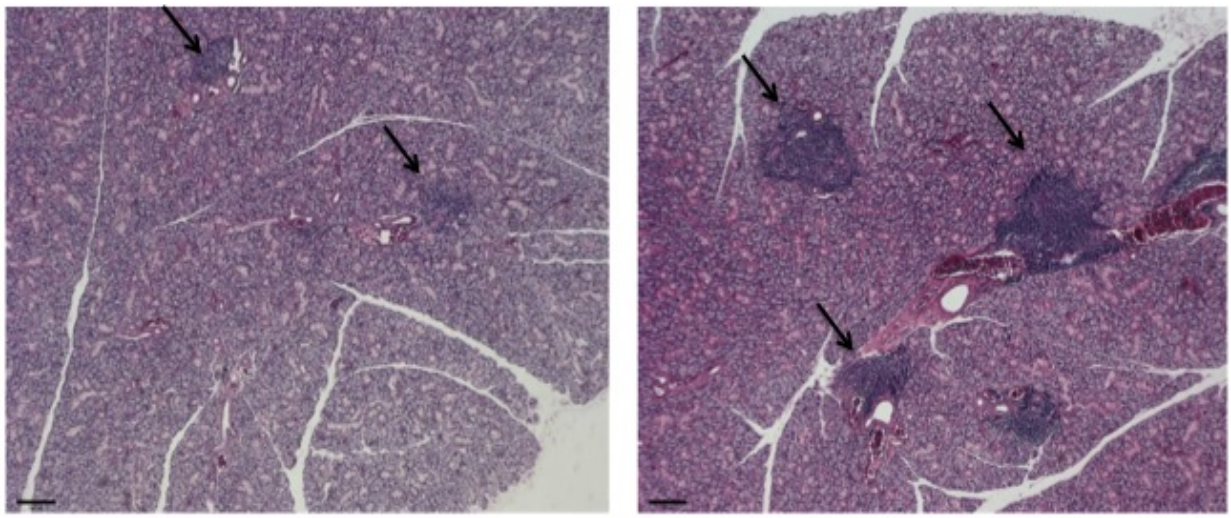


Fig. 8. Focus score between treated and non-treated mice. Light micrographs of mouse salivary glands stained with hematoxylin and eosin. Focus score was not significantly different between non-tread and treated groups. The size of the focus score, however, was slightly bigger in the non-treated mice (B) when compared to the BMDC-treated mice (A) ($P > 0.05$).

4. DISCUSSION

This study demonstrates that a two-limb therapy, simultaneous administration of CFA and BMDCs can prevent the development of Sjögren's syndrome and type I diabetes mellitus in female NOD mice. We transplanted 7-week-old female NOD mice (which had normal glucose and saliva production levels) with CFA and BMDCs. We measured effectiveness of this treatment using these criteria: (1) monitoring the progression to Sjögren's syndrome and diabetes, (2) measuring salivary flow rate (SFR), and (3) examining saliva composition. Our results showed that the treatment administered in young (7-week-old) NOD mice completely protected mice (100%) from progression to Sjögren's syndrome and diabetes. This percentage is higher than the 85% reported by Kodama (Kodama et al., 2003) for end-stage diabetic NOD mice treated with this two-limb therapy at 22 to 40 weeks of age using splenocytes. Our data points at the importance of an early intervention using this two-limb therapy. We have demonstrated SFR improvements from our previous study when treatment started for 14-week old NOD mice (with already established Sjögren's syndrome). However, our previous study did not evaluate saliva compositions. In the current study, we assessed the salivary glands' functions both quantitatively (SFR), and qualitatively (electrolytes, amylase activity, etc).

In general, 60-80% of female NOD mice develop Sjögren's syndrome and type 1 diabetes mellitus around 8 weeks of age, even though almost all mice exhibit sialadenitis and insulitis (Yamano et al., 1999, Lee et al., 2009, Jonsson et al., 2007). Symptoms from this disorder become more severe with time, and the majority of these animals die due to the complications caused by their diabetes and Sjögren's syndrome (Jonsson et al., 2007, Yamano et al., 1999, Hu et al., 1992). We, nevertheless, were able to keep the BMDC+CFA treated animals alive for more than one-year post-transplantation. Salivary gland dysfunction occurs in NOD mice with

increasing age and this trend was reported by Jonsson and Soyfoo (Jonsson et al., 2006, Soyfoo et al., 2007a). Our finding showed that salivary glands' function was quantitatively and qualitatively restored in the BMDC+CFA treated mice. Saliva compositions including total proteins, epidermal growth factor, amylase activity, and electrolyte concentrations in treated mice were restored to their initial levels as those at baseline (week 0) of the experiment, when the mice were still healthy. Small number of male donor BMDC cells (less than 0.1%) settled in the salivary gland and differentiated into salivary epithelial cells. The restoration mechanism of salivary function and its composition are probably driven by gland rescue and paracrine stimulation done by BMDCs (Tran SD, Submitted) (Coppes et al., 2009, Tran et al., 2007). Transplanted BMDCs, including mesenchymal and epithelial cells secrete cytokines and growth factors, which might stimulate the remaining progenitor cells in the glands to proliferate and generate new cells as well as inhibit the secretion of pro-inflammatory cytokines by the infiltrating lymphocytes. Therefore regeneration of the salivary is a result of de novo regeneration (Coppes et al., 2009). A paracrine mechanism has also been reported for pancreatic regeneration in a murine model by Aguayo-Mazzucato (Aguayo-Mazzucato and Bonner-Weir, 2010). Restoration of the glands' function could have resulted from mediators secreted by the donor cells when they were systemically transplanted, as explained by Horwitz and Aguayo-Mazzucato (Horwitz EM and Dominici M, 2008, Aguayo-Mazzucato and Bonner-Weir, 2010). BMDCs can also increase angiogenesis by regenerating endothelial cells, as reported by Lombaert (Lombaert et al., 2008). We, however, could not find any endothelial cell of donor origin in the NOD mice. In contrast to the pancreas, as reported by Kodama we did not find a significant change in focus score (lymphoid infiltrates within the glands) among the experimental groups (BMDC, CFA, or control group). This data is similar to our previous study (Tran et al., 2007). The size of the salivary lymphocytic infiltrates (focus score) in our study was relatively bigger in the control group as compare to the BMDC-

transplanted group; although this did not achieve a statistical significant difference. There seems to be no obvious association between focus score appearance and salivary glands dysfunction (Jonsson et al., 2006, HJ, 2002)

5. CONCLUSION

In conclusion, we demonstrated that a two-limb, CFA and BMDCs, intervention if applied at an early age can permanently prevent diabetes and Sjögren's-like syndrome, two forms of autoimmune diseases in the NOD mice. With the recent finding that human patients with Sjögren's syndrome have the same proteasome defects in their LMP2 subunit as the NOD mice, they might likely benefit from this treatment. However, further investigation is needed to find the mechanism of how and which population of cells from transplanted bone marrow leads to resolution of the autoimmune disease.

ACKNOWLEDGMENTS

We thank Drs. Mari Kaartinen, Dieter Reinhardt, Noreen Jane Bider, Jose Correa, Yonit Rebibo, and Neena Hanif. We also thank the staff at the McGill University animal facility. This study has been funded by the Canadian Institutes of Health Research (CIHR).

REFERENCES

- Aguayo-Mazzucato, C. & Bonner-Weir, S. (2010). Stem cell therapy for type 1 diabetes mellitus. *Nat Rev Endocrinol, 6***,** 139-148.
- Bocelli-Tyndall, C., Bracci, L., Spagnoli, G., Braccini, A., Bouchenaki, M., Ceredig, R., Pistoia, V., Martin, I. & Tyndall, A. (2007). Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. *Rheumatology (Oxford), 46***,** 403-408.
- Burt, R. K., Loh, Y., Cohen, B., Stefoski, D., Balabanov, R., Katsamakis, G., Oyama, Y., Russell, E. J., Stern, J., Muraro, P., Rose, J., Testori, A., Bucha, J., Jovanovic, B., Milanetti, F., Storek, J., Voltarelli, J. C. & Burns, W. H. (2009). Autologous non-myeloablative haemopoietic stem cell transplantation in relapsing-remitting multiple sclerosis: a phase I/II study. *Lancet Neurol, 8***,** 244-253.
- Coppes, R. P., van der Goot, A. & Lombaert, I. M. (2009). Stem cell therapy to reduce radiation-induced normal tissue damage. *Semin Radiat Oncol, 19***,** 112-121.
- Couzin, J. (2006). Clinical trials. A shot of bone marrow can help the heart. *Science, 313***,** 1715-1716.
- Delaleu, N., Jonsson, R. & Koller, M. M. (2005). Sjogren's syndrome. *Eur J Oral Sci, 113***,** 101-113.
- Fox, P. C. & Speight, P. M. (1996). Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjogren's syndrome. *Crit Rev Oral Biol Med, 7***,** 144-158.
- Fu, Y., Nathan, D. M., Li, F., Li, X. & Faustman, D. L. (1993). Defective major histocompatibility complex class I expression on lymphoid cells in autoimmunity. *J Clin Invest, 91***,** 2301-2307.
- Fu, Y., Yan, G., Shi, L. & Faustman, D. (1998). Antigen processing and autoimmunity. Evaluation of mRNA abundance and function of HLA-linked genes. *Ann N Y Acad Sci, 842***,** 138-155.
- Hayashi, T. & Faustman, D. (1999). NOD mice are defective in proteasome production and activation of NF-kappaB. *Mol Cell Biol, 19***,** 8646-8659.

- HJ, H. (2002). Clinical and immunological factors associated with low lacrimal and salivary flow rate in patients with primary Sjögren's syndrome. *J Rheumatol, 29*, 305-308.
- Horwitz EM, & Dominici M. (2008). How do mesenchymal stromal cells exert their therapeutic benefit? *Cytotherapy, 10*, 771-774.
- Hu, Y., Nakagawa, Y., Purushotham, K. R. & Humphreys-Beher, M. G. (1992). Functional changes in salivary glands of autoimmune disease-prone NOD mice. *Am J Physiol, 263*, E607-614.
- Jonsson, M. V., Delaleu, N., Brokstad, K. A., Berggreen, E. & Skarstein, K. (2006). Impaired salivary gland function in NOD mice: association with changes in cytokine profile but not with histopathologic changes in the salivary gland. *Arthritis Rheum, 54*, 2300-2305.
- Jonsson, M. V., Delaleu, N. & Jonsson, R. (2007). Animal models of Sjogren's syndrome. *Clin Rev Allergy Immunol, 32*, 215-224.
- Katsifis, G. E., Moutsopoulos, N. M. & Wahl, S. M. (2007). T lymphocytes in Sjogren's syndrome: contributors to and regulators of pathophysiology. *Clin Rev Allergy Immunol, 32*, 252-264.
- Kodama s, K. W., Fujimura S, Dale E.A, Faustman DL (2003). Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science, 302*, 1223-1227.
- Krause, S., Kuckelkorn, U., Dorner, T., Burmester, G. R., Feist, E. & Kloetzel, P. M. (2006). Immunoproteasome subunit LMP2 expression is deregulated in Sjogren's syndrome but not in other autoimmune disorders. *Ann Rheum Dis, 65*, 1021-1027.
- Lee, B. H., Tudares, M. A. & Nguyen, C. Q. (2009). Sjogren's syndrome: an old tale with a new twist. *Arch Immunol Ther Exp (Warsz), 57*, 57-66.
- Lombaert, I. M., Brunsting, J. F., Wierenga, P. K., Faber, H., Stokman, M. A., Kok, T., Visser, W. H., Kampinga, H. H., de Haan, G. & Coppes, R. P. (2008). Rescue of salivary gland function after stem cell transplantation in irradiated glands. *PLoS One, 3*, e2063.
- Lombaert, I. M., Wierenga, P. K., Kok, T., Kampinga, H. H., deHaan, G. & Coppes, R. P. (2006). Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands. *Clin Cancer Res, 12*, 1804-1812.

- Lowenthal, R. M., Cohen, M. L., Atkinson, K. & Biggs, J. C. (1993). Apparent cure of rheumatoid arthritis by bone marrow transplantation. *J Rheumatol, 20***,** 137-140.
- Miniati, I., Guiducci, S., Conforti, M. L., Rogai, V., Fiori, G., Cinelli, M., Saccardi, R., Guidi, S., Bosi, A., Tyndall, A. & Matucci-Cerinic, M. (2009). Autologous stem cell transplantation improves microcirculation in systemic sclerosis. *Ann Rheum Dis, 68***,** 94-98.
- Nikolov, N. P. & Illei, G. G. (2009). Pathogenesis of Sjogren's syndrome. *Curr Opin Rheumatol, 21***,** 465-470.
- Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A. & Anversa, P. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature, 410***,** 701-705.
- Oyama, Y., Barr, W. G., Statkute, L., Corbridge, T., Gonda, E. A., Jovanovic, B., Testori, A. & Burt, R. K. (2007). Autologous non-myeloablative hematopoietic stem cell transplantation in patients with systemic sclerosis. *Bone Marrow Transplant, 40***,** 549-555.
- Oyama, Y., Craig, R. M., Traynor, A. E., Quigley, K., Statkute, L., Halverson, A., Brush, M., Verda, L., Kowalska, B., Krosnjar, N., Kletzel, M., Whitington, P. F. & Burt, R. K. (2005). Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology, 128***,** 552-563.
- Ryu, S., Kodama, S., Ryu, K., Schoenfeld, D. & Faustman, D. (2001). Reversal of established autoimmune diabetes by restoration of endogenous beta cell function. *J Clin Invest, 108***,** 63-72.
- Saccardi, R., Kozak, T., Bocelli-Tyndall, C., Fassas, A., Kazis, A., Havrdova, E., Carreras, E., Saiz, A., Lowenberg, B., te Boekhorst, P. A., Gualandio, F., Openshaw, H., Longo, G., Pagliai, F., Massacesi, L., Deconink, E., Ouyang, J., Nagore, F. J., Besalduch, J., Lisukov, I. A., Bonini, A., Merelli, E., Slavino, S., Gratwohl, A., Passweg, J., Tyndall, A., Steck, A. J., Andolina, M., Capobianco, M., Martin, J. L., Lugaresi, A., Meucci, G., Saez, R. A., Clark, R. E., Fernandez, M. N., Fouillard, L., Herstenstein, B., Koza, V., Cocco, E., Baurmann, H., Mancardi, G. L. & Autoimmune Diseases Working Party of, E. (2006). Autologous stem cell transplantation for progressive multiple sclerosis: update of the European Group for Blood and Marrow Transplantation autoimmune diseases working party database. *Mult Scler, 12***,** 814-823.

- Soyfoo, M. S., De Vriese, C., Debaix, H., Martin-Martinez, M. D., Mathieu, C., Devuyst, O., Steinfeld, S. D. & Delporte, C. (2007a). Modified aquaporin 5 expression and distribution in submandibular glands from NOD mice displaying autoimmune exocrinopathy. *Arthritis Rheum, 56*, 2566-2574.
- Soyfoo, M. S., Steinfeld, S. & Delporte, C. (2007b). Usefulness of mouse models to study the pathogenesis of Sjogren's syndrome. *Oral Dis, 13*, 366-375.
- Toth, Z. E., Leker, R. R., Shahar, T., Pastorino, S., Szalayova, I., Asemenew, B., Key, S., Parmelee, A., Mayer, B., Nemeth, K., Bratincsak, A. & Mezey, E. (2008). The combination of granulocyte colony-stimulating factor and stem cell factor significantly increases the number of bone marrow-derived endothelial cells in brains of mice following cerebral ischemia. *Blood, 111*, 5544-5552.
- Tran, S. D., Kodama, S., Lodde, B. M., Szalayova, I., Key, S., Khalili, S., Faustman, D. L. & Mezey, E. (2007). Reversal of Sjogren's-like syndrome in non-obese diabetic mice. *Ann Rheum Dis, 66*, 812-814.
- Tran, S. D., Pillemer, S. R., Dutra, A., Barrett, A. J., Brownstein, M. J., Key, S., Pak, E., Leakan, R. A., Kingman, A., Yamada, K. M., Baum, B. J. & Mezey, E. (2003). Differentiation of human bone marrow-derived cells into buccal epithelial cells in vivo: a molecular analytical study. *Lancet, 361*, 1084-1088.
- Tran, S. D, S. T., Dipasquale G, Cotrim AP, Bandyopadhyay BC, Riddle K, Mooney D, Kok MR, Chiorini JA, Baum BJ (2006). Re-engineering primary epithelial cells from rhesus monkey parotid glands for use in developing an artificial salivary gland. *Tissue Eng, 12*, 2939-2948.
- Uccelli A, M. L., Pistoia V (2008). Mesenchymal stem cells in health and disease. *Nat Rev Immunol, 8*, 726-736.
- Urban, V. S., Kiss, J., Kovacs, J., Gocza, E., Vas, V., Monostori, E. & Uher, F. (2008). Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. *Stem cells, 26*, 244-253.
- Van Laar, J. M. & Tyndall, A. (2006). Adult stem cells in the treatment of autoimmune diseases. *Rheumatology (Oxford), 45*, 1187-1193.
- Yamano, S., Atkinson, J. C., Baum, B. J. & Fox, P. C. (1999). Salivary gland cytokine expression in NOD and normal BALB/c mice. *Clin Immunol, 92*, 265-275.

- Zhao, S., Wehner, R., Bornhauser, M., Wassmuth, R., Bachmann, M. & Schmitz, M. (2009). Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. *Stem Cells Dev, 2***,** 130-138.