SALIVARY FORMING UNIT

ISOLATION AND CULTURE

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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. However, to be able to achieve this future therapeutic option for xerostomia, we need to be able to provide an available pool of acinar cells for transplantation or engineering of an artificial gland. Therefore, the aim of this thesis was to develop a reproducible protocol to isolate, cultivate and expand human salivary gland acinar cells. Salivary acinar cells were purified by isopycnic centrifugation after collagenase/hyalyronidase digestion of human salivary glands. The resulting acinar and ductal fractions were put into separate low-attachment cultures for up to 30 days. Survival and proliferation rates were tested using MTT assay and cell counting techniques. Immunohistochemistry staining for acinar (AQP5, CD44, NKCC1) and ductal (CK5, CFTR) markers were used to characterize and monitor the phenotypal change of the cultured cells. The acinar fraction was highly enriched in aquaporine 5 (APQ5), CD44 and NKCC1; and contained very little cytokeratine 5 (CK5) and cystic fibrosis transmembrane conductance regulator (CFTR). On the other hand, the ductal fraction was enriched in CK5 and CFTR; but contained no trace of AQP5 and NKCC1. Under low-attachment condition, acinar cells developed into clusters of cells that we've defined as salivary forming units (SFUs). MTT assay showed that the cells were proliferating up to 15 days and could survive up to 21 days. SFUs were increasing in size and number for up to 15 days. In conclusion, we have developed a reproducible protocol to isolate and expand human salivary gland acinar cells. SFUs could provide a potential source of functional secretory units that could be used in future therapeutics for xerostomia.

RÉSUMÉ

Le syndrome de Sjögren et la radiothérapie de la tête et du cou peuvent engendrer une perte irréversible des acini salivaires. Ce phénomène, connue sous le nom de xérostomie, ne possède actuellement aucun traitement disponible. Notre groupe a testé différents biomatériaux, protéines de la matrice extracellulaire et cellules de greffon afin de développer un dispositif de glandes salivaires artificielles implantable dans la cavité buccale. Cependant, afin d'accomplir cette tâche, nous devons obtenir une sources de cellules acinaires disponible pour la transplantation ou le développement d'une glande artificielle. L'objective de cette thèse est de développer un protocole productible pour isoler, cultiver et multiplier les cellules acinaires des glandes salivaire humaines. Les cellules salivaires acinaires ont été purifié par centrifugation isopycnique après une digestion enzymatique des glandes salivaires humaines avec la collagénase/hyalunoridase. Les fractions acinaires et canalaires résultants ont été mis en culture séparément dans des milieux à faible attachement pendant 30 jours. Les taux de survie et de prolifération ont été testés par l'analyse du MTT et des techniques de comptage de cellules. La coloration immunohistochimique pour les marqueurs acinaires (AQP5, CD44, NKCC1) and canalaires (CK5, CFTR) ont été utlisé pour caractériser et suivre les changements phénotypiques des cellules. La fraction acinaire était fortement enrichie en aquaporine 5 (AQP5), CD44 et NKCC1 ; et contenait très peu de cytokératine 5 (CK5) et CFTR. D'autre part, la fraction canalaire était enrichi en CK5 et CFTR ; mais ne contenait aucune trace de AQP5 et NKCC1. Dans des condition de faible attachement, les cellules acinaires ont développés en des ams de cellules que nous avons défini comme unités formant salivaires (UFS). L'analyse MTT a démontré que les cellules proliféraient jusqu'à 15 jours et peuvent survivre jusqu'à 21 jours. Les UFS ont augmenté en taille et en nombre pour un maximum de 15 jours. En conclusion, nous avons développé un protocole reproductible pour isoler et multiplier les cellules acinaires des glandes

salivaires humaines. Les UFS pourraient fournir une source potentielle d'unités sécrétoires fonctionnelles qui pourraient être utilisés dans les futures thérapies pour la xérostomie.

To my grand mother, Vo Thi An (1922-2009)

ACKNOWLEDGEMENTS

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 and for helping me to pursue my dream. He was always full of support and encouragements. I would like also to express my sincere gratitude to my thesis committee members who helped me in the elaboration and guidance of my M.Sc. objectives: Dr. Peter Chauvin, and Dr. Falleh Tamimi. Many thanks to my collaborators: Dr. Anthony Zeitouni, Dr. Michel El-Hakim, Dr. Genevieve Bonin; and all my fellow laboratory members who were there to teach, guide, and support me: Dr. Younan Liu, Dr. Saeed Khalili, Dr. Ola Maria, André Charbonneau, Dr. Ghada Abughanam, Dr. Jin Choon Lee and Nicole Seo. I would like to thank the Co-operative Human Tissue Network, Royal Victoria Hospital and Montreal General Hospital staff members for providing human salivary gland samples. I am also grateful to the funding institutions; CIHR, NSERC, RSBO, FODQ. Finally, I would like to thank my Mother, Thi Tuyet Trinh Huynh; words cannot say enough to thank you for everything that you have done for me. I would like to thank my family members for their continuous support and prayers; Thi Tuyet Nga Huynh and Virginie Nguyen.

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
CFTR	cystic fibrosis tranmembrane conductance regulator
SFU	salivary forming unit

PREFACE

Following the work of previous members in my laboratory, the aim of this thesis was to develop a method to separate and culture human salivary gland acinar cells. By expanding this population of cells, we will be able to provide a suitable cell source to use as a graft in an orally implantable artificial device to regenerate salivary gland in xerostomic patients. I have elected to present my research in a thesis format in accordance with the guidelines for thesis preparation from the Faculty of Graduate Studies and Research at McGill University [Guidelines for Thesis Preparation].

CONTRIBUTION OF AUTHORS

Michel El-Hakim is a surgical oncologist at the Montreal General Hospital, Faculty of Dentistry, 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.

Younan Liu is a research assistant at Dr. Simon Tran's laboratory;

Ola Maria was a PhD candidate at Dr. Simon Tran's laboratory, she shared writing and reviewing this manuscript;

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

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.

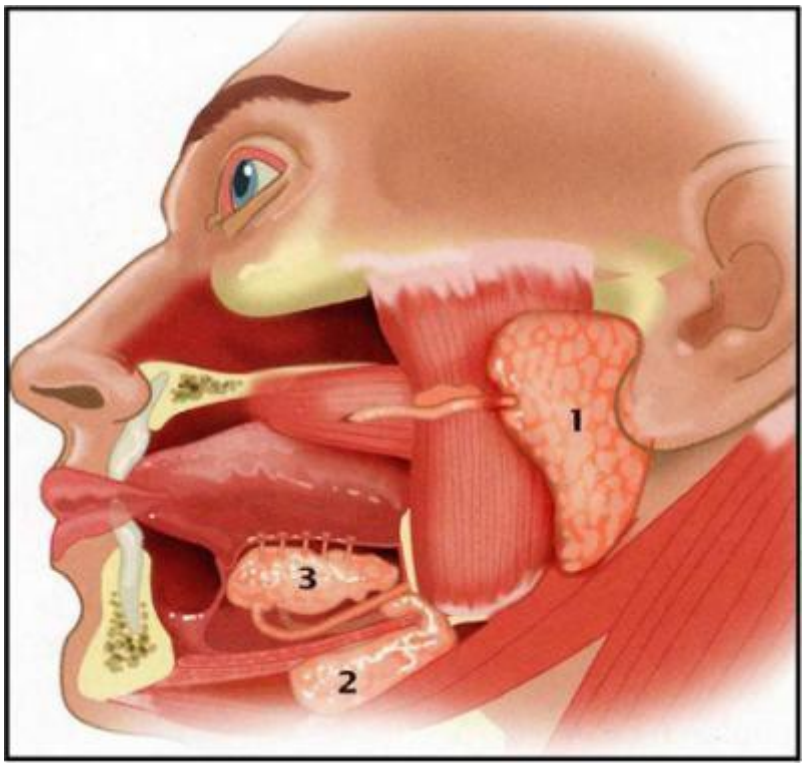
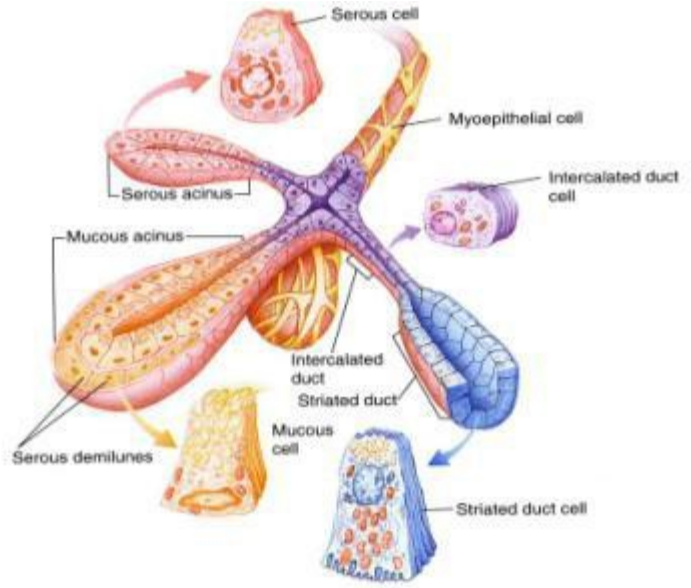


Figure.1.1: shows human major salivary glands anatomical positions; parotid (1), submandibular and sublingual (3) glands. This picture is copied from reference (41)

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



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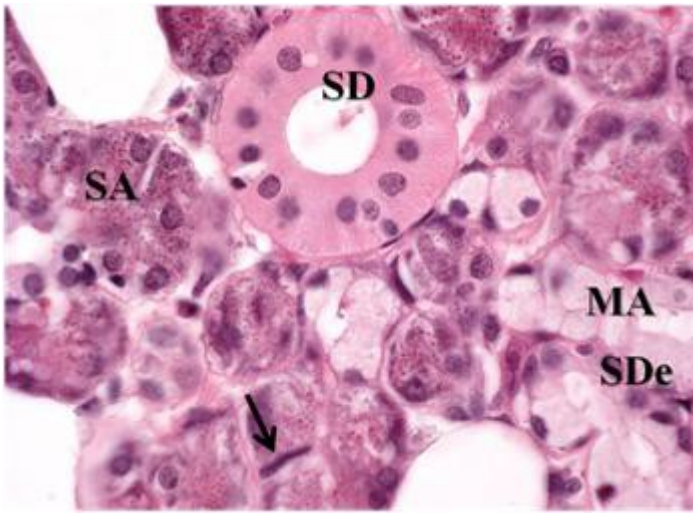


Figure.1.2: shows; (A) a diagram of salivary gland epithelial cell types, this picture is copied from reference (52). (B) a light micrograph showing the histology of human salivary gland tissue; striated duct (SD), serous acinus (SA), Mucous acinus (MA), serous demilunes (SDe) and myoepithelial cell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

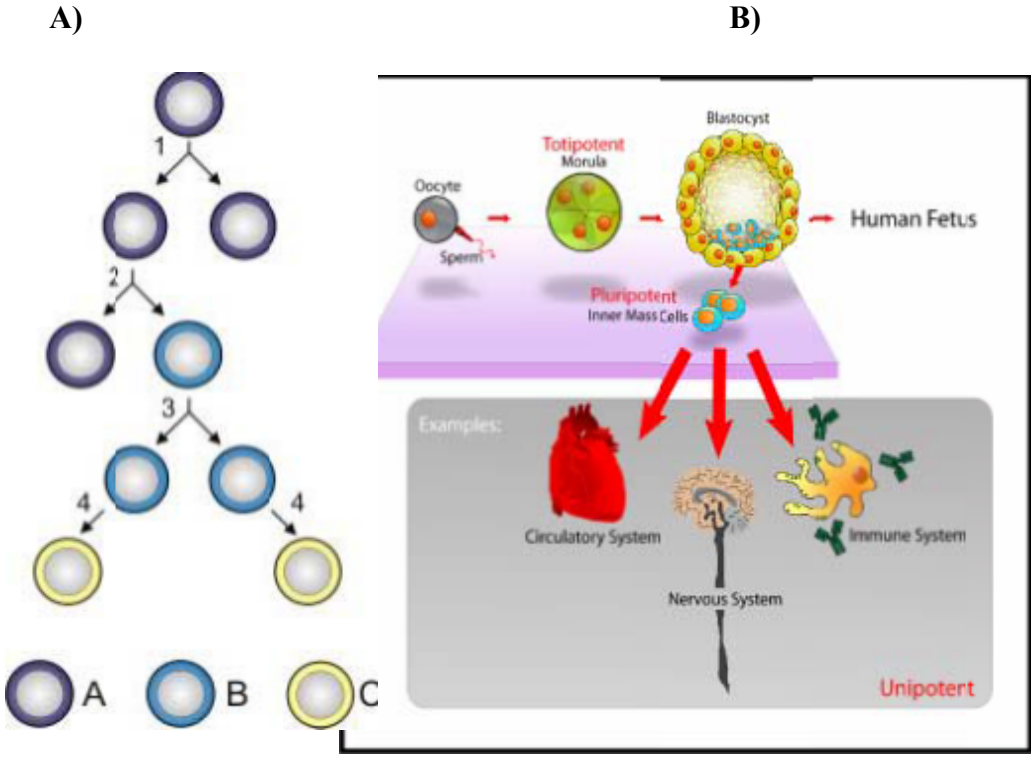


Figure.1.3: shows A); Stem cell division and differentiation diagram; 1 (symmetric stem cell division), 2 (asymmetric stem cell division); 3 (progenitor division), 4 (terminal differentiation) giving A (stem cells); B (progenitor cell), C (differentiated cell). B) Embryonic stem cells (ES, pluripotent)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

MECHANISMS 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).

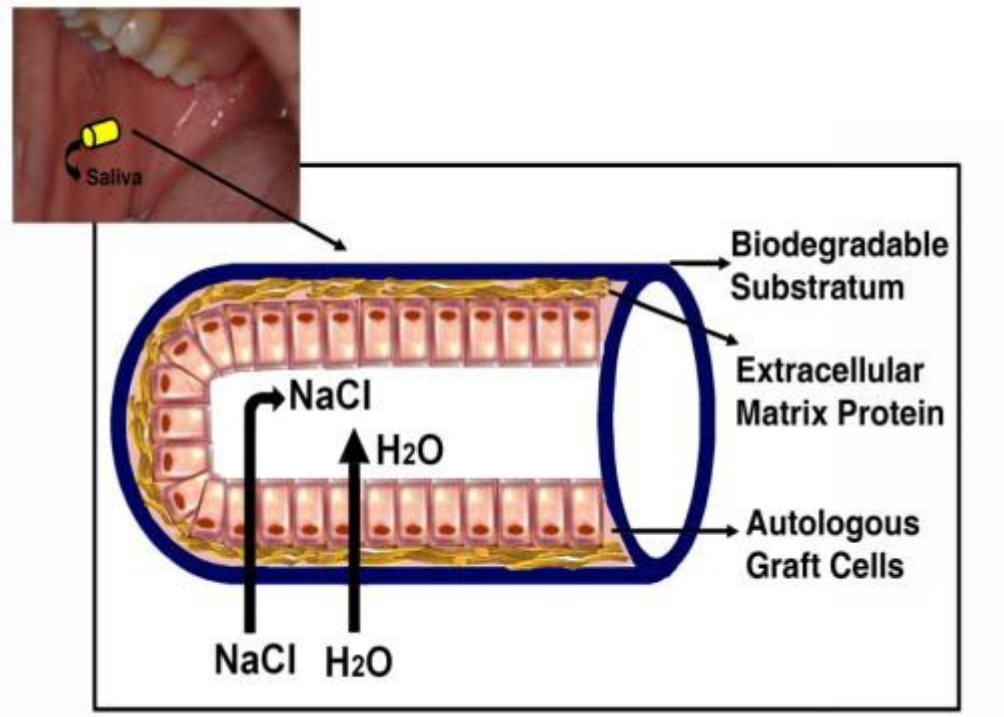


Figure.1.4: shows the design of the artificial salivary gland device as envisioned. The device is formed of a blind-end tube and composed of three essential elements: a biodegradable substratum; a coating of an extracellular matrix protein on the luminal surface of the substratum; and a polarized epithelial 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. 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. 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 previously able to culture primary huSG cells; however, these cells grow slowly and exhibit ductal phenotype. Nevertheless, it was thought by our group that if we could isolate acinar cells and cultivate them in a seperate environment, they will be able to maintain their acinar phenotype. The isolation and culture of acinar cells would serve as a model for future studies and provide a pool of salivary producing cells for transplantation or engineering of an artificial salivary gland.

In addition, characterization of the graft cell types from human salivary glands ; when cultured in a low-attachment culture environment would be beneficial ; to test salivary gland morphogenesis and cytodifferentiation and to further examine our envisioned articial salivary gland device.

HYPOTHESIS

Isolation and culture of acinar cell in a separate environment will help them maintain their phenotype and function. The culture and expansion of those cells will provide an available pool of salivary forming cells that could be used in future therapy against xerostomia.

AIM OF THE THESIS

The aim of this thesis is to develop a protocol to isolate, cultivate and expand human salivary gland acinar cells. This new knowledge will help in procuring an available pool of acinar cells for transplantation or engineering of an artificial salivary gland in future therapeutic options for xerostomia patients.

OBJECTIVES

1. Develop a method to digest and separate human salivary gland acinar cells.
2. Cultivate those cells in low attachement culture to produce salispheres.
3. Using neurotransmitters to stimulate salivary gland cells:
4. Characterize two human salivary gland cell types; acinar cells and ductal cells when cultured in a low-attachemnt environment.
5. Assess the growth and survival potential of enriched acinar cells cultured in a low-attachment environment.

CHAPTER 2

MATERIAL & METHODS

MATERIALS & METHODS

Source of human salivary gland tissue:

Human submandibular (n=9) salivary glands [men (n=5), women (n=4)], were obtained from the Department of Pathology, Royal Victoria Hospital (RVH); the Department of Oral and Maxillofacial surgery, Montreal General Hospital (MGH); and 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. 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), RVH and MGH (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.

Human salivary gland tissue digestion:

The received tissue pieces (0.5-3.5g) 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 (Figure

2.1A). The minced tissue was washed in normal saline solution (9.0g NaCl/L) and shaken thoroughly for 30 seconds. The test tube was put to rest for 60sec for the salivary gland tissue to separate from the connective tissue and fat (Figure 2.1B). The floating connective tissue and fat, along with the used saline solution, were discarded. This process was repeated 3 times. The washed salivary gland tissue was transferred to gentleMACS tube C (Miltenyi Biotec, Germany) in digestion buffer containing Liberase (0.1mg/mL) and Hyaluronidase (0.1mg/mL) in Dulbecco's modified Eagle's medium (DMEM; BioFluids, Rockville, MD). The tissue was further minced using the gentleMACS dissociator (Miltenyi Biotec, Germany) and incubated for 2 hours at 37°C incubator on a shaker. Additionally, vigorous vortexing was applied every 30 min. This cell suspension was then filtered through a cell strainer (70µm pore size; BD Biosciences Discovery Labware), then centrifuged at 800 rpm for 1 min, the supernatant was discarded, and 20mL of cold DMEM was used to re-suspend the cell pellet. This process was repeated 3 times. The resulting cell pellet was suspended in 8 mL 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.

Separation of ductal and acinar cells:

An isotonic 40% Percoll density centrifugation media (GE Healthcare Life Sciences, UK) solution in Ca^{2+}/Mg^{2+}-free PBS was prepared as described by Vincent et al. 1984 (187). Two 6-mL aliquots of this solution were kept at 4°C for 1h. 4 mL of cell suspension was carefully layered on top of the 40% Percoll density centrifugation media

(Figure 2.1C) and centrifuged for 10min at 12 000g. Two distinct bands, consisting predominantly of ducts (upper band) and acini (lower band) were observed in this final gradient (Figure 2.1D). These were collected and washed three times with 20ml of Ca^{2+}/Mg^{2+}-free PBS. The final pellets were suspended separately in serum-free Hepato-STIM medium.

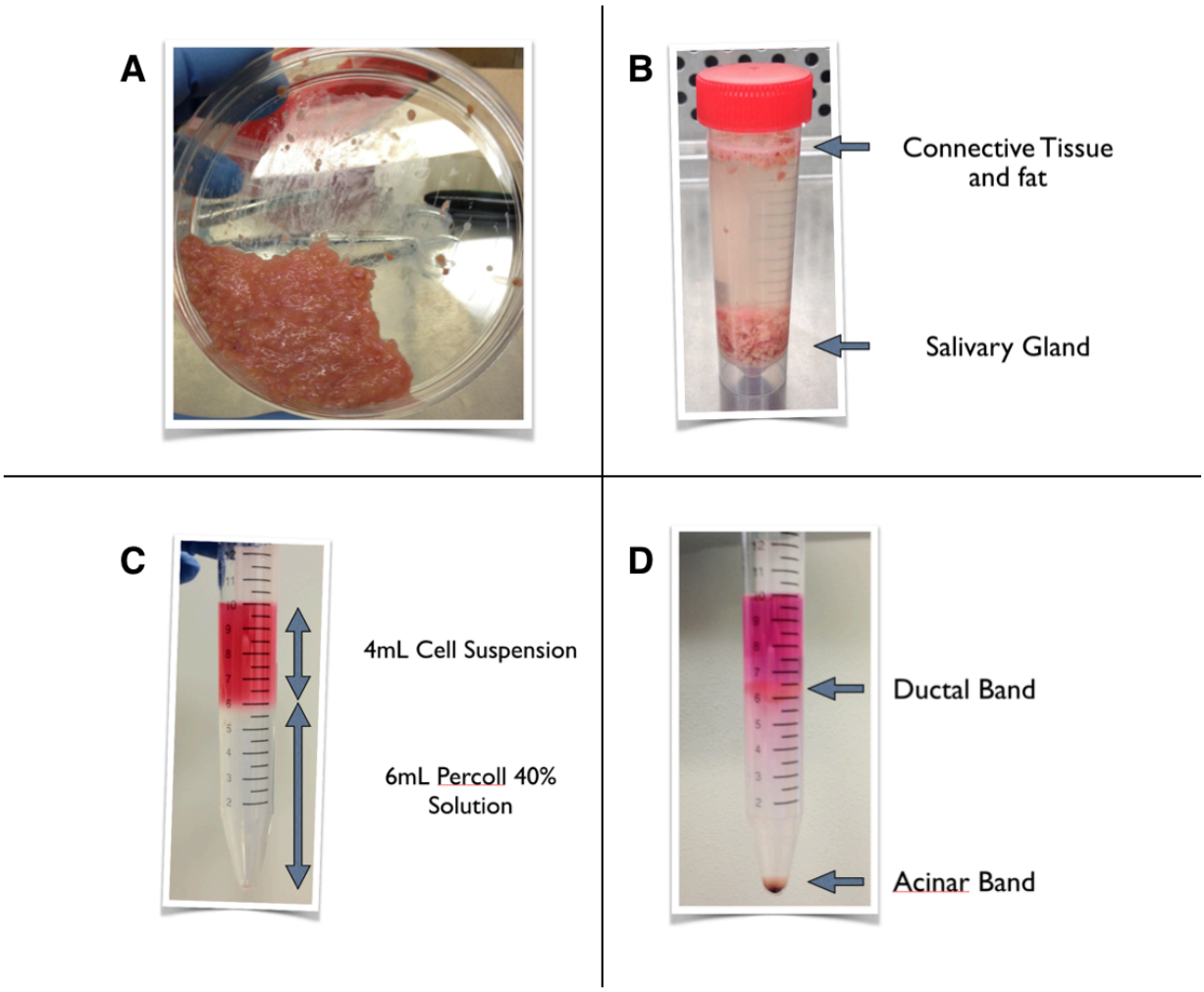


Figure 2.1: huSG tissue digestion and cell separation

A) The human salivary gland specimen was mechanically minced with a pair of curved scissors until the tissue gave an appearance of a slurry thick paste.

B) The minced tissue was washed in normal saline solution (9.0g NaCl/L) and shaken

thoroughly for 30 seconds. The test tube was put to rest for 60sec for the salivary gland tissue to separate from the connective tissue and fat.

C) 4 mL of cell suspension was carefully layered on top of 6mL of 40% Percoll density centrifugation media and centrifuged for 10min at 12 000g.

D) Two distinct bands, consisting predominantly of ducts (upper band) and acini (lower band) were observed in this final gradient

Tissue culture:

Acinar and ductal cell suspensions were plated onto a low-attachment 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. The passaged cells were cultured on low attachment, non-coated or Matrigel-coated (19.6 mg/ml, BD Biosciences, Bedford, MA) 12-well, 6-well or 96-well tissue culture dishes or 8-well slide chambers.

Matrigel preparation:

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.

Immunofluorescence Imaging:

huSG cells 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 cells; primary antibody used was against $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) (graciously donated by Dr. James Turner, NIH) to detect salivary acinar cells, Goat anti- aquaporin-5, from Santa Cruz; mouse anti-CD44 from R&D Systems, rabbit anti- CFTR from Cell Signaling Technology. 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^oC 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 Velocity software (PerkinElmer Inc.). Images shown are representative of at least 3 separate

experiments with multiple images taken per slide.

Table 2.1: Antibodies for acinar and ductal cell markers.

Acinar cell markers	Localization	Function
Aquaporin 5 (AQP5)	Cell surface	AQP5 is a water channel protein that plays a role in the generation of saliva, tears and pulmonary secretions.
CD44	Cell surface	CD44 is a cell-surface glycoprotein involved in cell-cell interactions.
NKCC1	Cell surface	NKCC1 is a membrane transport protein that transports Na, K, and Clions across cell membrane. NKCC1 is widely distributed throughout the body, especially in organs that secrete fluids.

Ductal cell markers	Localization	Function
Cytokeratin 5 (CK5)	Cytoplasmic	CK5 is an intermediate filament protein that is expressed during differentiation or simple or stratified epithelial tissues.
Cystic fibrosis transmembrane conductance regulator (CFTR)	Cell surface	CFTR is an ABC transporter-class ion channel that transports chloride and thiocyanate ions across epithelial cell membranes.

MTT colorimetric assay for cell proliferation:

MTT stock solution: 5mg/ml MTT (Promega) in RPMI-1640 without phenol red. This solution is filtered through a 0.2 mm filter and stored at 2-8°C. Wash cultured cells with warm RPMI-1640 without phenol red. Prepare MTT working solution (1:10 dilution of the 5mg/ml stock MTT in RPMI without phenol red). Add MTT working solution into wells being assayed, for example 1.0ml for each well of 12-well plate. Incubate at 37°C for 30min to 3 hrs. At the end of the incubation period, the medium can be moved if working with attached cells. The converted dye is solubilized with 1ml acidic isopropanol (0.04 M HCl in absolute isopropanol). Pipette up and down several times to make sure the converted dye dissolves completely. Transfer the dye solution with the cells into a 1.5 ml eppendorf tube and centrifuge at 13,000 rpm for 2 min. Transfer the supernatant into a new eppendorf tube. Absorbance of the converted dye is measured at a wavelength of 570nm with background subtraction at 650nm. For the measurement, use Beckman DU-600 Spectrophotometer and disposable plastic cuvettes.

Evaluation of proliferation:

The evaluation of the proliferation of salivary forming units (SFU) was done under inverted light microscopy (Leica DM16000B). Pictures were taken everyday, from day 0 to day 9, of acinar cells cultured in low-attachment 100mm tissue culture dish. Acinar cells in a low attachment environment tend to cluster together and form small acinar balls. Those balls were counted and categorized as small (<200um) and larger (>200um).

CHAPTER 3

RESULTS

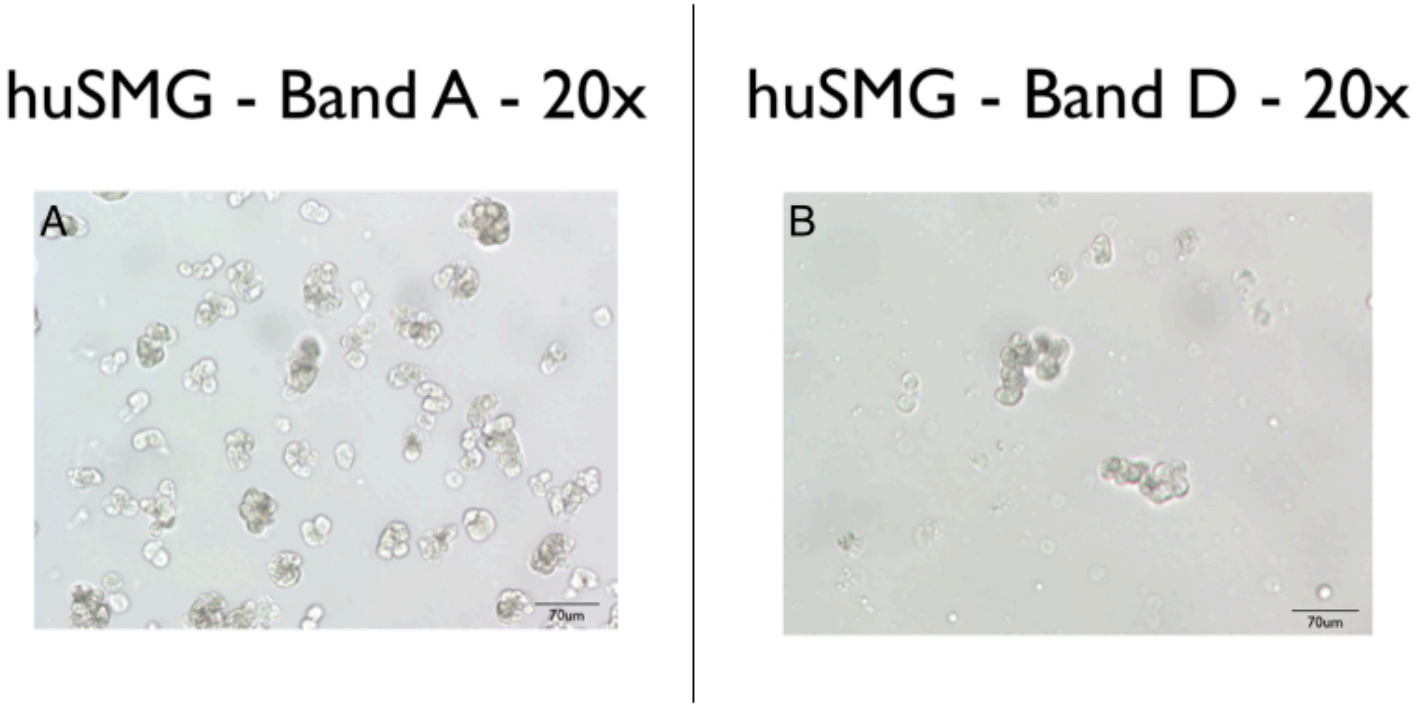
RESULTS

Acinar and ductal cell separation

Light microscopy

After tissue digestion of the huSG specimen and cell separation using 40% density centrifugation media, each band was collected, suspended in Hepato-STIM media and observed under inverted microscope (Leica DM16000B). Under light microscopy at 20x magnification, the lower band showed numerous circular clusters of 5-20 cells, smaller than 70um, consistent with the morphology of acini. The upper band exhibited fewer and more elongated aggregation of 10-22 cells, closed to 70um, reminding the morphology of ducts.

Figure 3.1 Acinar and ductal cell separation under light microscopy



A) At 20x magnification under light microscopy, the lower band showed numerous circular clusters of 5-20 cells consistent with the morphology of acini.

B) At 20x magnification under light microscopy, the upper band exhibited fewer and more elongated aggregation of 10-22 cells reminding the morphology of ducts.

Immunofluorescence imaging:

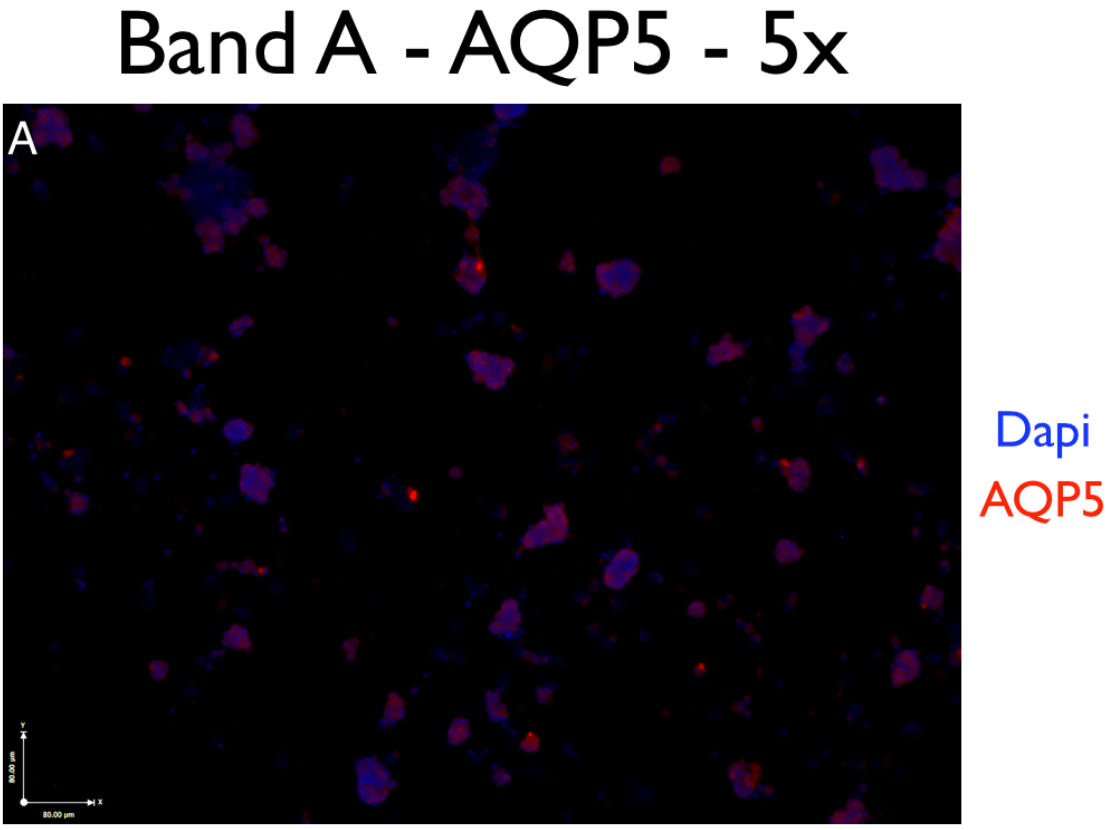
Acinar and ductal markers were used under immunofluorescence imaging in order to characterize the upper band and the lower band of Percoll density separation. Markers such as aquaporin 5 (AQP5), a water channel protein that plays an important role in the generation of saliva, tears and pulmonary secretions; NKCC1, a membrane transport protein that transports Na, K and Cl ions across cell membrane, that is widely distributed throughout the body in organs that secrete fluids; and CD44, a cell-surface glycoprotein involved in cell-cell interactions that was previously proven be closely related to acinar cells; were used to characterize acinar cells. Cytokeratin 5 (CK5), an intermediate filament protein that is expressed during differentiation or in simple or stratified epithelial tissues; and cystic fibrosis transmembrane conductance regulator (CFTR), an ABC transporter-class ion channel that transports chloride and thiocyanate ions across epithelial cell membranes; were used to characterize ductal cells.

As seen on figure 3.1, when staining for AQP5, the majority of the cells in the lower band were positive to the marker. On the other hand, the upper band did not show any reaction to AQP5. Similarly, the lower band presented cluster of cells positive to the NKCC1 marker. However, only around 25% of those cells showed reactivity when compared to AQP5. Once again, the upper band did not display any reaction to NKCC1. CD44 was positive on the majority of the cells in the lower band. However, it was also present on a few clusters of cells in the upper band. This finding may indicate two things: 1) the Percoll density separation technique may not be perfect and will only give enriched band

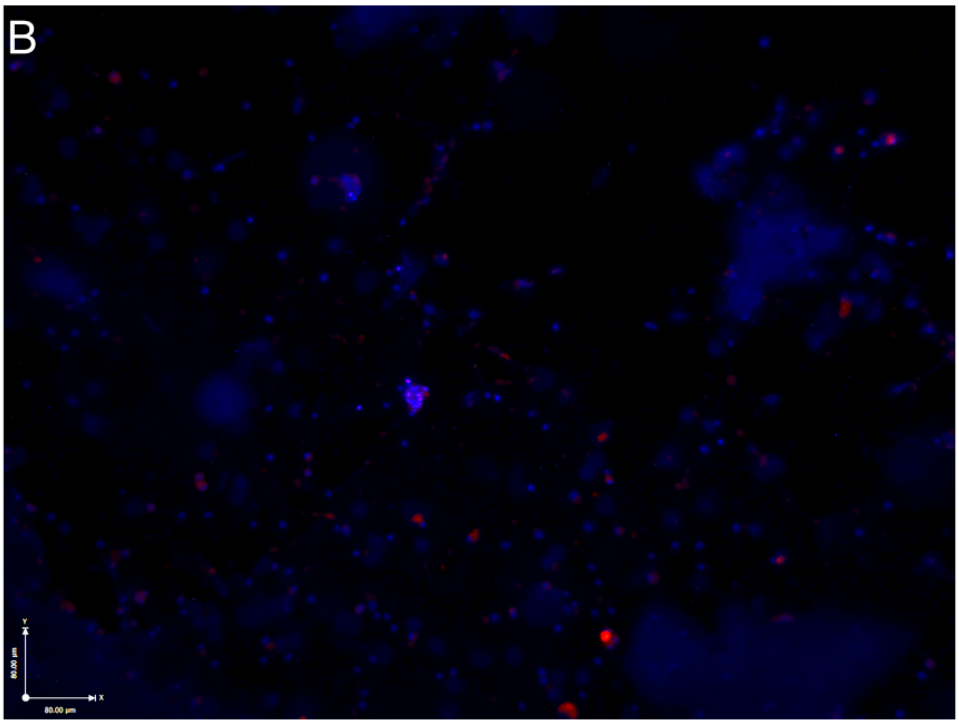
of acinar cells and ductal cells 2) CD44 may not be an accurate acinar cell marker. The upper and lower bands have also been stained with ductal cell markers.

As shown on figure 3.2, the upper and lower bands are both reacting equally to CK5. Other the other hand, the upper band is positive for CFTR and the lower band showed no reaction. It could be concluded that CFTR is an appropriate ductal cell marker. Furthermore, these results have showed that the upper band was in fact enriched with cells that are positives to ductal cell markers and the lower band was enriched with cells that are positives to acinar cell markers.

Figure 3.2: Acinar cell markers:

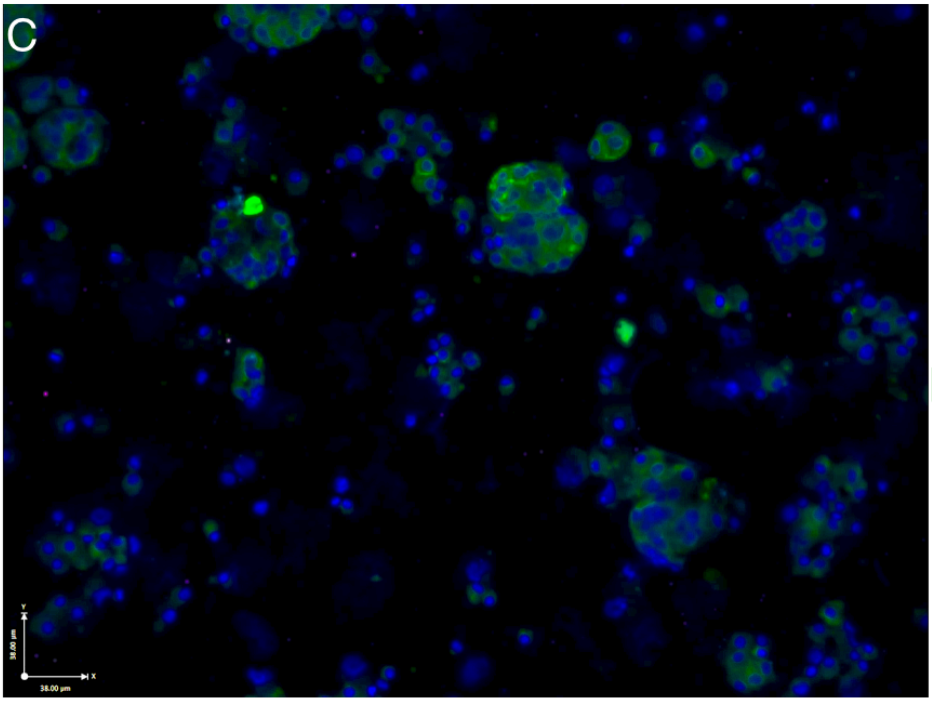


Band D - AQP5 - 5x



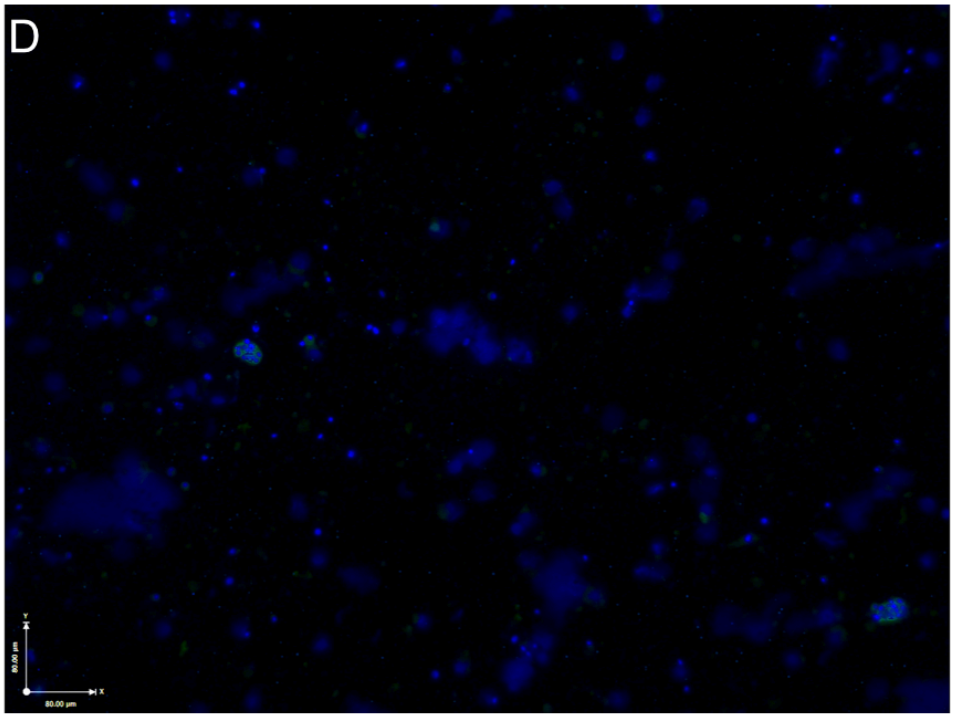
Dapi
AQP5

Band A - NKCC1-10x



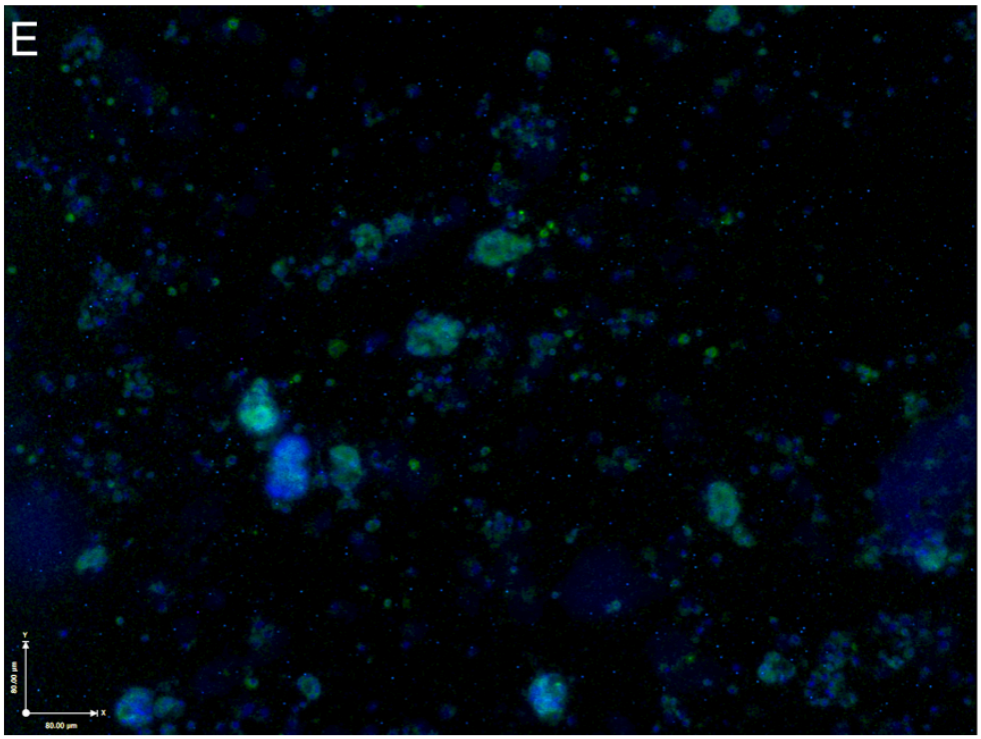
Dapi
NKCC1

Band D - NKCC1- 5x



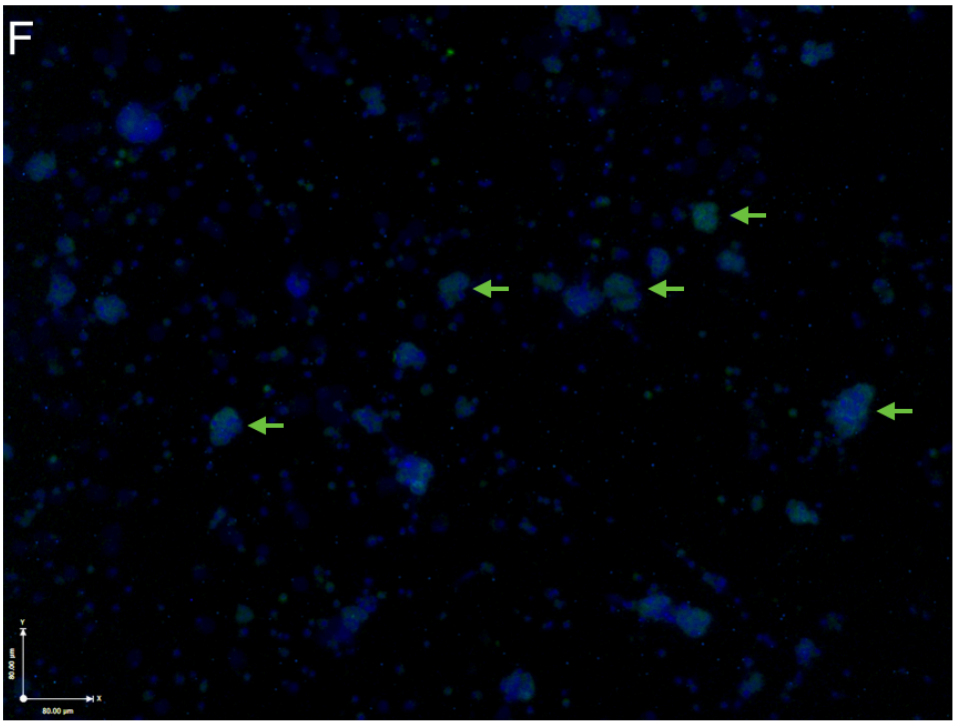
Dapi
NKCC1

Band A - CD44 - 5x



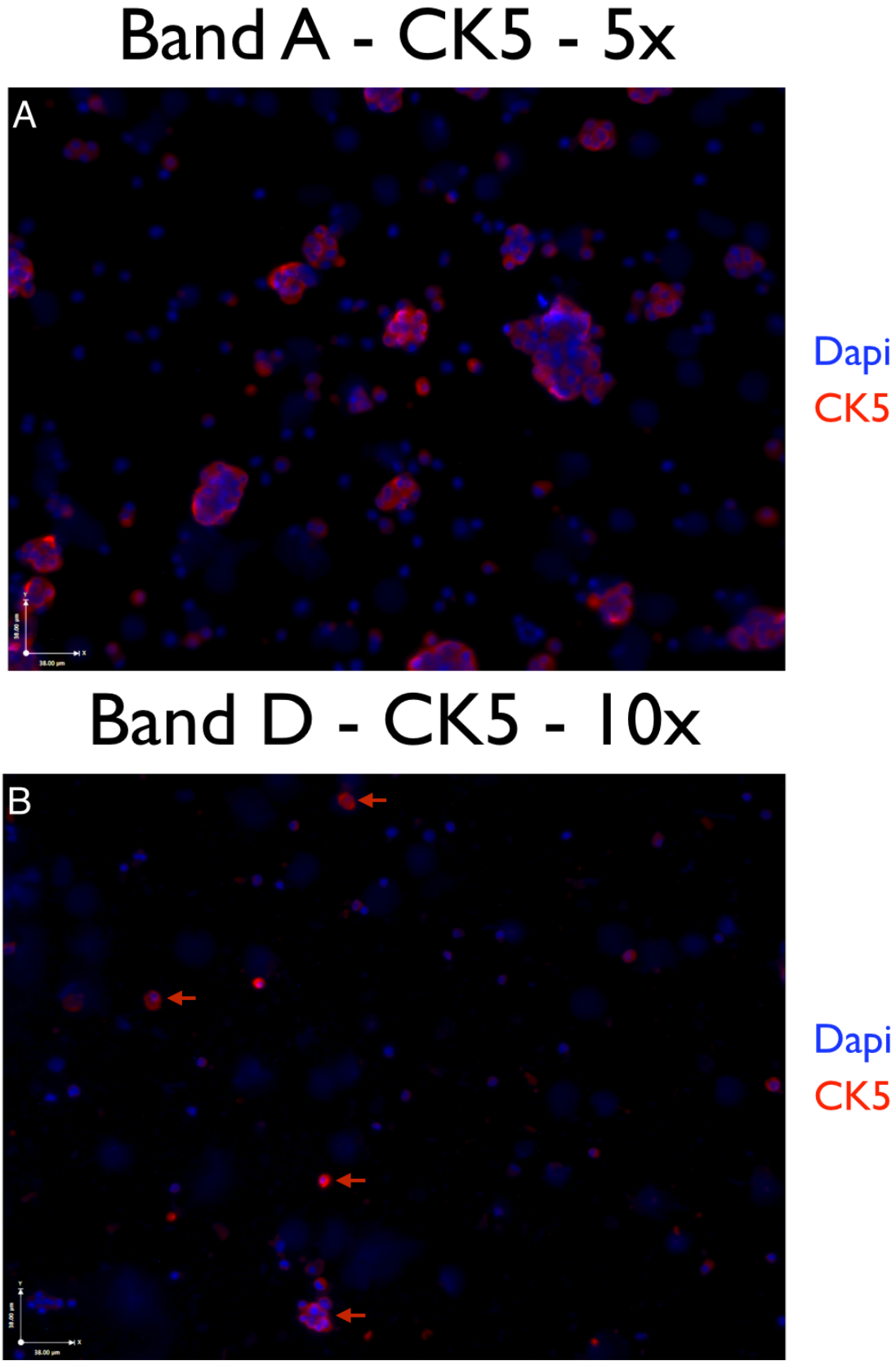
Dapi
CD44

Band D - CD44 - 5x

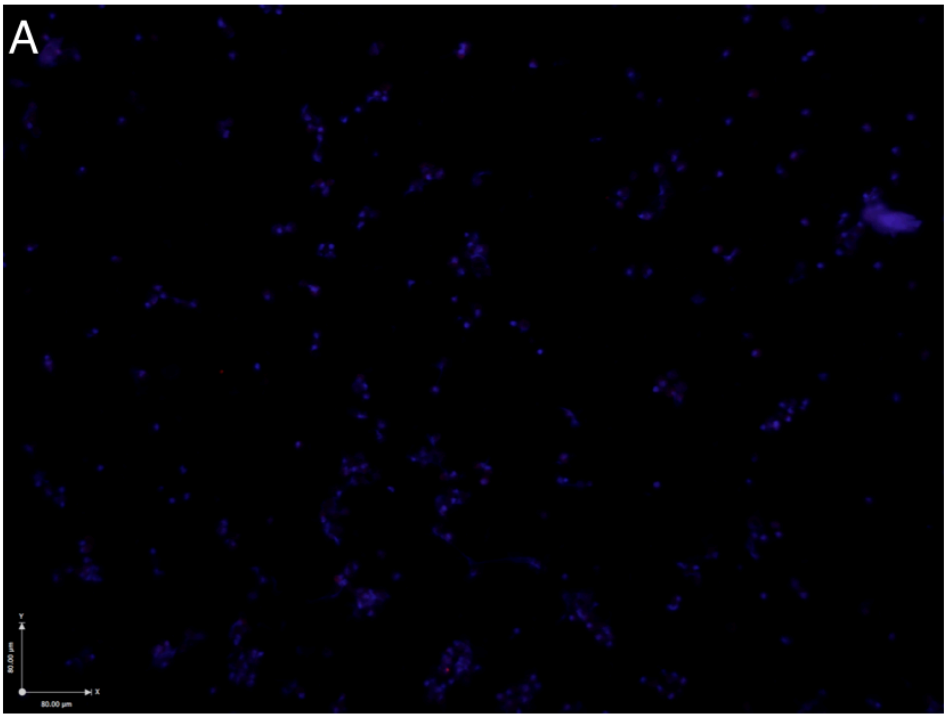


A) Immunofluorescence imaging of the lower band at 5x stained with AQP5, showing clusters of cells positive to aquaporin 5.
B) Immunofluorescence imaging of the upper band at 5x stained with AQP5, showing a negative reaction to aquaporin 5.
C) Immunofluorescence imaging of the lower band at 5x stained with NKCC1, showing clusters of cells positive to NKCC1.
D) Immunofluorescence imaging of the upper band at 5x stained with NKCC1, showing a negative reaction to NKCC1.
E) Immunofluorescence imaging of the lower band at 5x stained with CD44, showing clusters of cells positive to CD44.
F) Immunofluorescence imaging of the upper band at 5x stained with CD44, displaying a few clusters of cells positive to CD44.

Figure 3.3 Ductal cell markers:

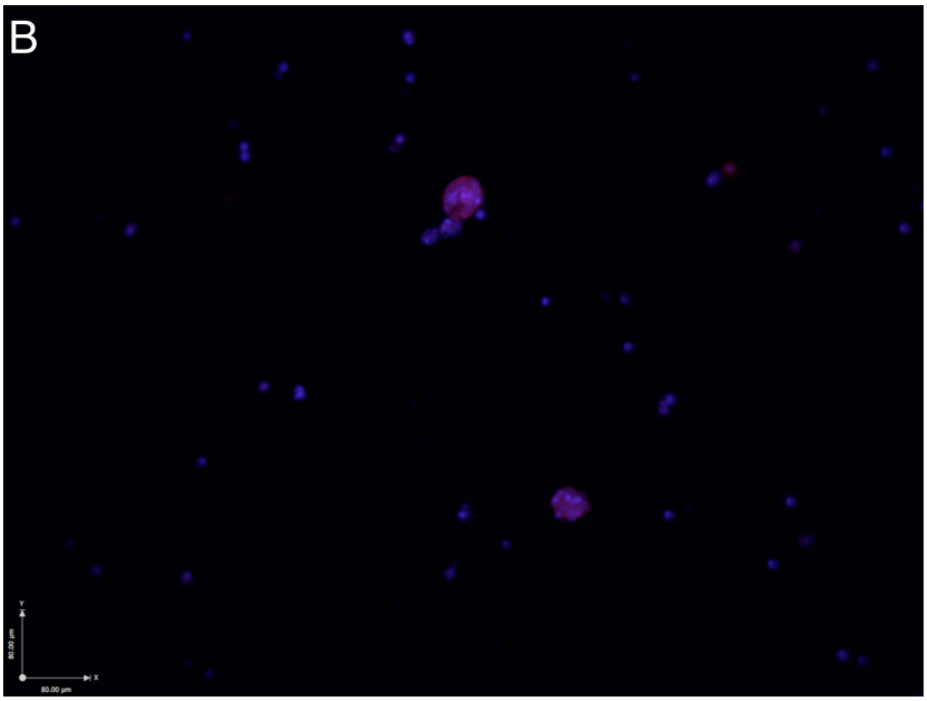


Band A - CFTR - 10x



Dapi
CFTR

Band D - CFTR - 10x



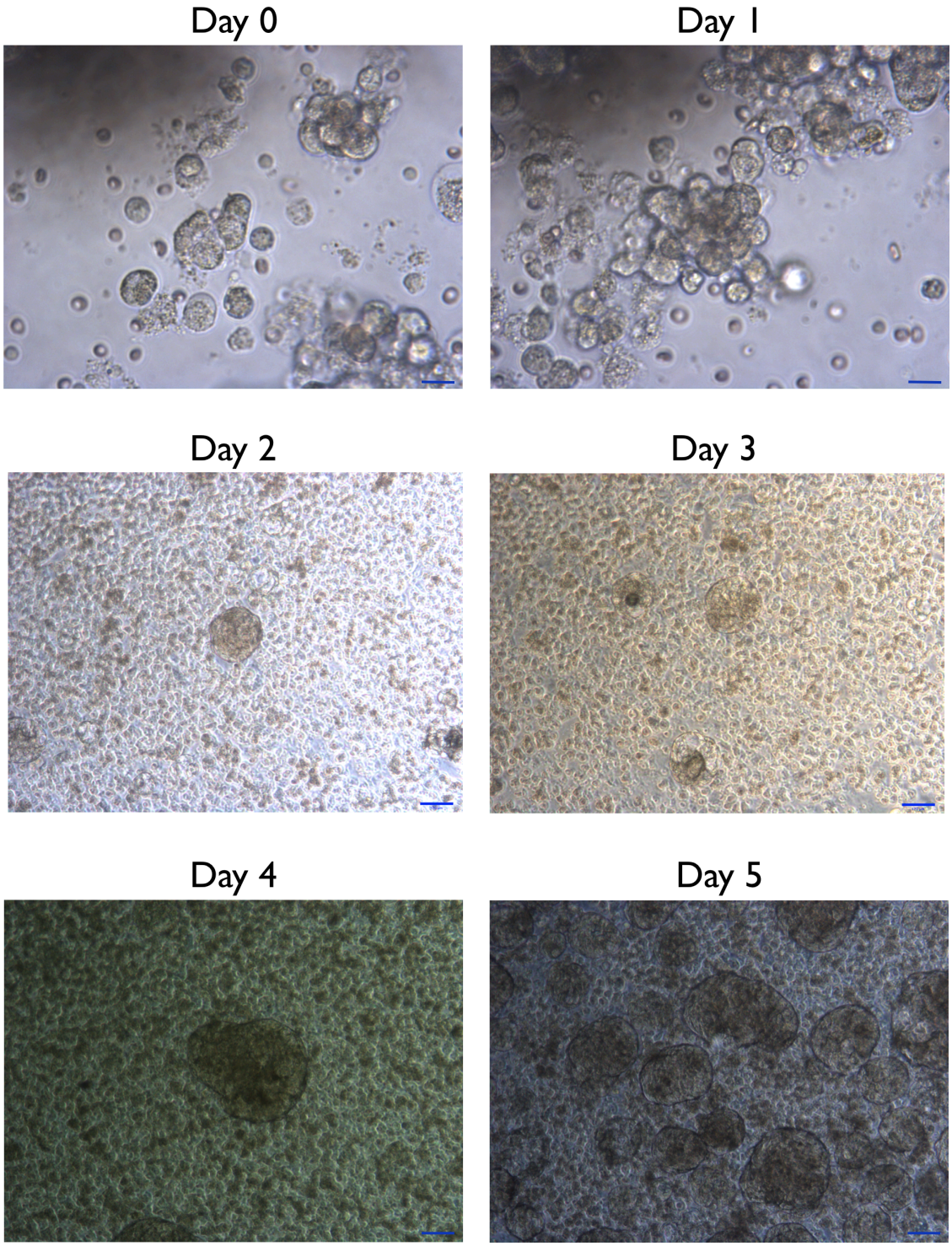
Dapi
CFTR

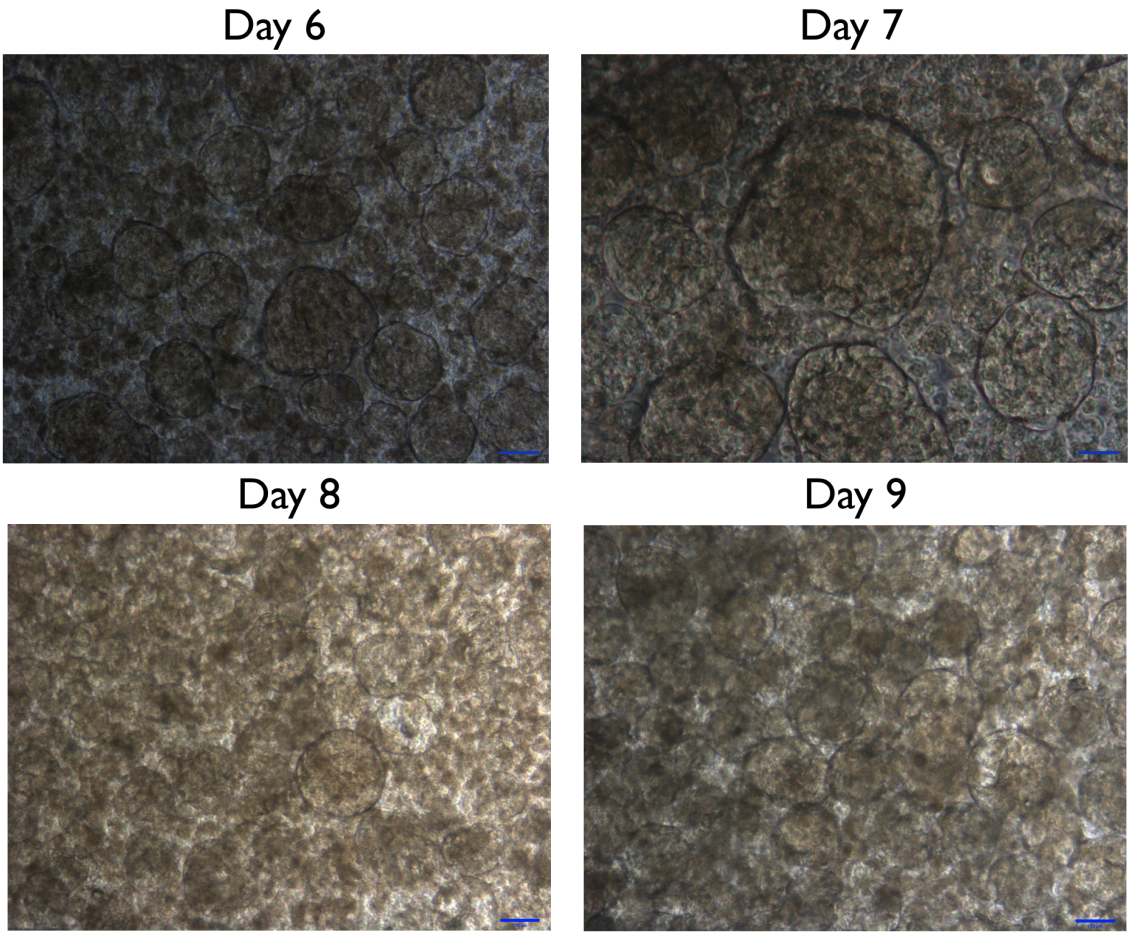
A) Immunofluorescence imaging of the lower band at 5x stained with CK5, showing clusters of cells positive to cytokeratin 5.
B) Immunofluorescence imaging of the lower band at 10x stained with CK5, showing clusters of cells positive to cytokeratin 5.
C) Immunofluorescence imaging of the lower band at 10x stained with CFTR, showing a negative reaction to the CFTR marker.
D) Immunofluorescence imaging of the lower band at 10x stained with AQP5, showing clusters of cells positive to CFTR.

Low attachment culture*:*

The acinar band was cultured in low-attachment 100mm tissue culture dish. Acinar cells in a low attachment environment tend to cluster together and form small acinar balls that we've called salivary forming units (SFU). An SFU is defined as a cluster of cells that can secrete fluid and proteins. The evaluation of the proliferation of SFUs was done under inverted light microscopy (Leica DM16000B). Pictures were taken everyday from day 0 through day 9, when the cells need to be passage. As we can see on figure 3.3, at day 0 single cells are floating separately in the low attachment culture dish. By day 1, cells are starting to cluster together. By day 2 and 3, we can see a denser population of cells and round cluster of cells starting to form measuring around 100um. From day 4 to day 5, more SFUs are forming, as they grow in size and density, now measuring around 200um. From day 6 to day 8, multiple SFUs are forming; some measuring up to 500um, and the cell population has reached a density level that is blocking the light of the inverted microscope. By day 9, most of the culture dish became dense SFUs and the cells are ready to be passaged. The proliferation rate of SFUs was quantified manually by counting the SFUs and categorizing them into small (<200um) and large (>200um) clusters. Figure 3.4 shows that small SFUs were increased by 5 folds from day 3 to day 6, and by 2 folds from day 6 to day 9. Similarly, large SFUs have started to appear by day 6 and have increased by 2 folds at day 9.

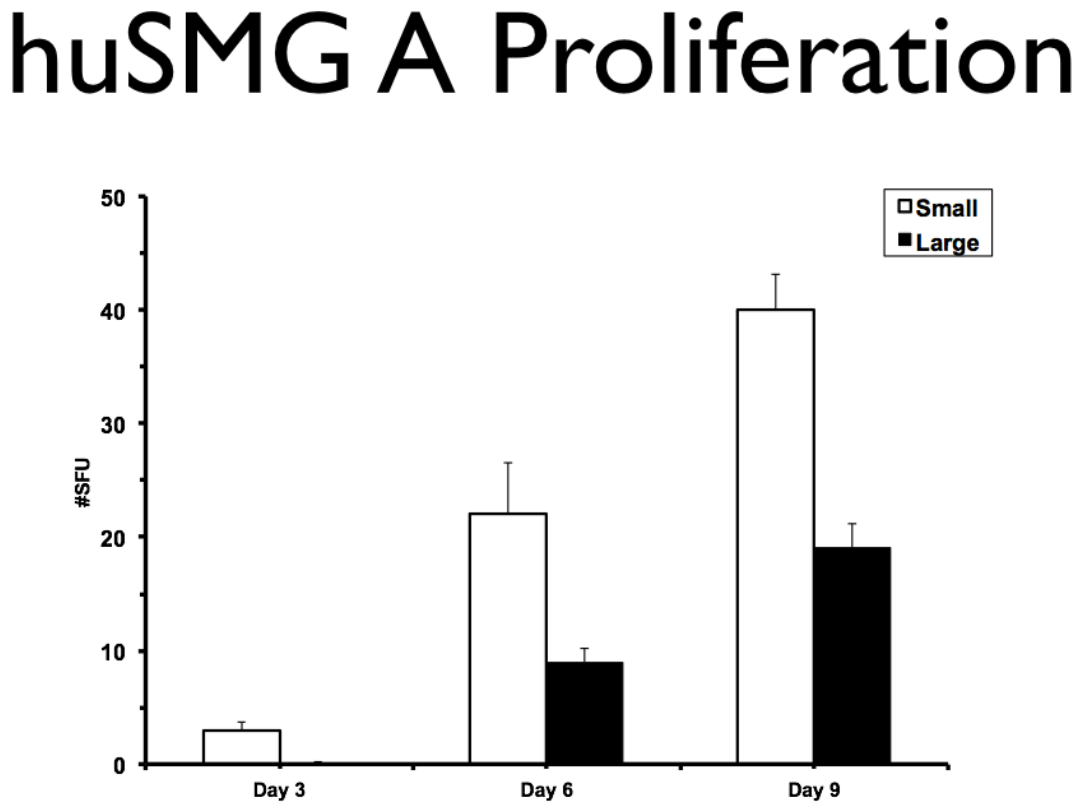
Figure 3.4 Acinar cells in low attachment culture from day 0 to day 9





Day 0: Single cells are floating separately in the low attachment culture dish.
Day 1: Cells are starting to cluster together.
Day 2-3: We can see a denser population of cells and round cluster of cells starting to form measuring around 100um.
Day 4-5: More SFUs are forming, as they grow in size and density, now measuring around 200um.
Day 6-8: Multiple SFUs are forming; some measuring up to 500um, and the cell population has reached a density level that is blocking the light of the inverted microscope.
Day 9: Most of the culture dish became dense SFUs and the cells are ready to be passaged.

Figure 3.5 SFUs proliferation



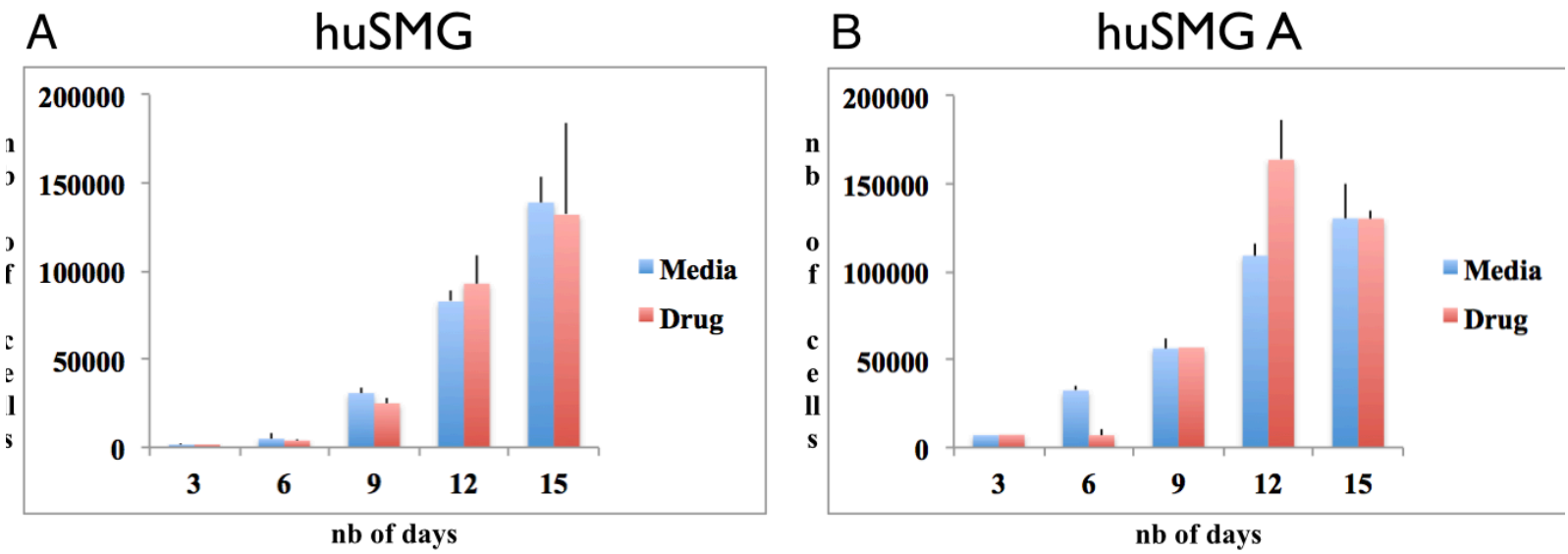
The proliferation rate of SFUs was quantified manually by counting the SFUs and categorizing them into small (<200um) and large (>200um) clusters. Figure 3.4 shows that small SFUs were increased by 5 folds from day 3 to day 6, and by 2 folds from day 6 to day 9. Similarly, large SFUs have started to appear by day 6 and have increased by 2 folds at day 9.

MTT colorimetric assay:

The MTT assay is a colorimetric assay for assessing cell viability. This test was performed at different time points (day 3, day 6, day 9, day 12 and day 15), on human submandibular salivary gland cells (huSMG) and human submandibular salivary gland acinar cells alone (huSMG A). The assay was also compared between cells treated with culture media only and cells stimulated with isoproterenol, a non-selective beta-

adrenergic agonist; pilocarpine, a non-selective muscarinic receptor agonist in the parasympathetic nervous system; and carbachol, a cholinergic agonist that binds and activates the acetylcholine receptors. As seen on figure 3.5, there was no significant difference, in the huSMG group, between stimulated and unstimulated cells. For both sub-groups, cells were increasing steadily from day 3 through day 15. In the huSMG A group, there was a significant difference between unstimulated cells and cells stimulated every day with drugs. The unstimulated subgroup was increasing, in a very similar fashion as the huSMG group, from day 3 to day 15. On the other hand, stimulated cells did not increase from day 3 to day 6, was 50% higher than unstimulated cells by day 12, and decreased to a level similar to unstimulated cells by day 15. This experiment has showed that acinar cells were in fact increasing in vitality and numbers up to 15 days. It was also concluded that there were no additional benefits in stimulating cells with isoproterenol, pilocarpine and carbachol.

Figure 3.6 MTT colorimetric assay



A) MTT assay was performed at different time points for the huSMG group. There were no significant differences between the stimulated and unstimulated cells.

B) MTT assay was performed at different time points for huSMG A group. There was a significant difference between stimulated and unstimulated cells at day 6 and day 12.

CHAPTER 4

DISCUSSION

DISCUSSION

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). Therefore, the aim of this thesis was to develop a reproducible protocol to isolate, cultivate and expand human salivary gland acinar cells.

We have received a total of nine human submandibular salivary glands [men (n=5), women (n=4)], however only five glands (n=5) were successfully cultivable. Our

results have showed that we were in fact able to dissociate fresh human submandibular salivary glands, harvested from head and neck dissections in cancer patients, into single and smaller clusters of cells. Furthermore, we were able to sort those cells using the Percoll density centrifugation technique into an acinar band and a ductal band of cells. As showed under light microscopy, the acinar band was formed of small round clusters of about 5-20 cells that resemble small acini; and the ductal band was formed of fewer and more elongated clusters of 10-22 cells reminding the morphology of ducts. These results were very similar to what was previously described by Dehaye & Turner in 1991 on acinar and ductal cells separation in mice using the Percoll density centrifugation technique. We have characterized those two bands with acinar and ductal cell markers under immunofluorescence imaging. We have showed that AQP5 and NKCC1 were effective acinar cells markers. However, contrary to previous studies, we have showed that CD44 was positive for the majority of the acinar band but also for a few clusters of cells in the ductal band. This finding leaves us to hypothesize that 1) the Percoll density separation technique is not perfect and will only result in an enriched band of acinar cells and ductal cells; 2) CD44 may not be an accurate acinar cell marker. If the later was true, more research will need to be done in order to understand what type of cells are positive to CD44. We have also showed that CFTR was an effective ductal cell marker and that CK5 was not specific to ductal cells. Knox et al. 2010 have reported that CK5 could be a progenitor cell marker, which could explain the reason why it showed positive reaction in both the acinar and ductal band. Further research will have to be done with the same markers (AQP5, NKCC1, CFTR) at different time point in order to monitor the differentiation of acinar and ductal cells.

We have also showed that we were able to effectively cultivate acinar cells in a low attachment culture. Under light microscopy, we have showed that acinar cells put into a low attachment culture could proliferate and form SFUs. From day 0 to day 9, we can see those SFUs grow in size, number and density. We have also characterized and quantify the amount of proliferation by counting the SFUs. Finally, with the MTT colorimetric assay, we have test the vitality and proliferation of those acinar cells at different time point. As described by Knox et al. 2013 epithelial cells secrete neurotransmitters to stimulate neuronal innervation, which is essential to maintain epithelial progenitor cells. Our group has tried to reproduce those neurotransmitter stimulations by using a combination of isoproterenol, pilocarpine and carbachol to arouse acinar cells in culture. However, our results have showed that acinar cell had a similar proliferation rate than unsorted salivary gland cells and that there was no significant benefit in using those drugs.

Our results with the SFUs are pretty similar to salispheres, which were first described by Lombaert et al. in 2008. Salipheres were obtained by growing rodent salivary progenitor cells in a gel-like three-dimensional culture system. Lombaert has reported that no salispheres were formed from single suspended cells and thought that cell-cell contact was necessary for salivary gland sphere formation. A similar observation has been made for intestinal stem cells (Marshman et al. 2002). This research group has also reported that they were unable to generate secondary spheres from the initial spheres, as has been shown for other tissues. This indicates that the self-renewal capacity of the salivary stem cells is restricted in the current culture system. Similar findings have also

been observed with our SFU culture. We were unable to obtain SFUs from single cell suspension and thought that a certain degree of cell-cell contact was necessary to induce cell agglomeration. Furthermore, as our SFUs were growing and size and number up to 9 days, we were never able to generate secondary spheres from our initial SFUs. Feng et al. 2009 have studied the presence and in vitro potential of human salivary gland stem cells positive to the c-kit marker. Feng has noted that although human and mouse salivary glands are not exactly the same, the tissue architecture after irradiation looks remarkably similar. In both species, the ductal compartment necessary for stem cell engraftment largely remains intact. Moreover, salisphere formation of human salivary gland cells was very similar to mice. They have also found that human salispheres could form duct and acinar-like cells and were able to self-renew for at least seven passages in culture. These results indicate that human salispheres do contain cells with stem cell-like properties. However, they have noted that these cells could be isolated from human salispheres in lower percentages than from rodent salispheres. This may be due to a lower stem cell number in older people such as the patients with head and neck cancer used in this study. Because of these restrictions when working with stem cells and progenitor cells, our group has opted to work on growing and expanding acinar cells, since those cells are more clinically available in patients with head and neck cancer. Lalitha et al. 2013 have demonstrated that c-kit$^+$ cells co-expressing CD24 and CD49f showed enhanced functional recovery compared to as few as 400 general c-Kit$^+$ cells. They have concluded that this subpopulation of c-Kit$^+$ cells is enriched for salivary gland stem/progenitor cells. c-Kit$^+$ cells survive in an irradiated environment and are able to create pools of differentiated acinar cells and

putative stem/progenitor cells. Additionally, they improve the morphology of irradiation-compromised niches by enhancing reciprocal communication within niches to establish repair and homeostasis of the tissue. These multiple similarities between SFUs and salispheres may indicate that a subpopulation of stem cell or progenitor cells, as described by Lombaert, Feng and Lalitha, may be present in our acinar cell separation culture. If this is the case, we may have successfully found a simple and cost effective alternative to isolate and grow progenitor cells. Further experimentation on SFUs will be necessary in order to confirm the presence c-Kit+ stem cells and the subpopulation of c-Kit+ cells co-expressing CD24 and CD49f.

CHAPTER 5

CONCLUSION

CONCLUSION

Through our quest of trying to expand and grow acinar cells, in order to create an available pool of secreting cells for future therapeutic options to xerostomia, we have developed a reproducible protocol to isolate human salivary acinar cells. Through a digestion process of fresh human submandibular gland and a cell density sorting technique, we've managed to isolate enriched clusters of acinar cells. We have proven that those cells were in fact acinar cells by using acinar cell markers such as AQP5 and NKCC1. We have also concluded that CD44 and CK5 may not be respectively specific to acinar and ductal cells. Further studies will be needed in order to understand the role of those markers. We have found that by cultivating acinar cells in a low attachment culture, those cells will cluster together and develop into salivary forming units. We have developed a method to culture SFUs and have showed with light microscopy, cell counting and MTT colorimetric assay that those SFUs are proliferating and multiplying in number and in size for up to 15 days. Further research will need to be done in order to characterize the SFUs and to prove that they are functional and secreting saliva and proteins. In order 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 (148,150,151,153,158). The major hurdle we will face is to demonstrate that SFUs establish tight junctions (TJs) and exhibit an adequate transepithelial electrical resistance (TER) in addition to being functional acinar secretory cells. Expanding SFUs from a patient's salivary gland before receiving radiation therapy may provide a suitable source of acinar cells for future therapy as injecting the cells into the damaged

gland or engineering an artificial salivary gland device. Furthermore, this new knowledge could provide an in-vitro human salivary acinar study model for future research.

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