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De-differentiation of parotid acinar cells in vitro.

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DE-DIFFERENTIATION OF PAROTID ACINAR CELLS *IN VITRO*

By

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B.S., University of Georgia, 2006
D.M.D., University of Louisville, 2010

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A Thesis Approved on

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by the following Thesis Committee:

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ABSTRACT

DE-DIFFERENTIATION OF PAROTID ACINAR CELLS *IN VITRO*

Andrew S. Currie

July 16, 2010

Background: When rat salivary gland cells are cultured in vitro, they de-differentiate within 24 h.

Hypothesis: Growth factors will prevent de-differentiation of primary cultures of parotid gland cells, and they will induce differentiation of ParC5 cells. DNA methylation drives de-differentiation and the loss of expression in cultured parotid cells.

Methods: Rat parotid glands were collected and cultured in media using different combinations of growth factors. DNA was evaluated for methylation at various time points.

Results: No media tested prevented the de-differentiation of parotid cells, and none induced differentiation of ParC5 cells. Limited variation in methylation of CpG sites was seen.

Conclusion: Growth factors do not prevent de-differentiation in primary cell culture, nor do they activate differentiation in ParC5 cells. Global methylation of Mist1 does not cause de-differentiation; evidence supports that methylation of PSP increases over time.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

LIST OF TABLES

TABLE **PAGE**

CHAPTER I

INTRODUCTION- PART I

Serum Growth Factors Do Not Prevent De-Differentiation of Salivary Acinar Cells

Human saliva is produced by three pairs of major salivary glands, and a variety of minor salivary glands. The major glands are the parotid glands, the submandibular glands, and the sublingual glands. Each set of major glands are located bilaterally: the largest set of glands, the parotids, are a somewhat inverted pyramid shape, located anterior and inferior to the ear, wrapping around the posterior border of the mandible; the submandibular glands are roughly egg-shaped, approximately half the size of the parotid glands, and located in the submandibular fossa on the medial surface of the body of the mandible, inferior to the mylohyoid line; and the smallest pair, the sublingual glands, are almond shaped, resting just beneath the mucous membrane in the floor of the mouth between the genioglossus muscle and the body of the mandible [1]. The minor salivary glands are found in many locations in the mouth, including in the tongue, lips, buccal mucosa, and throughout the palate.

The three pairs of major glands produce the vast majority of saliva, as was demonstrated by Hand in 1986, who found that 60-70% of saliva comes from the

submandibular glands, 25-35% from the parotid glands, and 5-8% from the sublingual glands [2]. All of these glands are exocrine in nature, secreting their respective serous fluid or mucous from clusters of cells known as acini into ducts. Each gland also has a major duct by way of which the saliva enters the mouth: the parotid empties into the oral cavity through Stensen's duct, which opens between the cheek and the maxillary second molar. The submandibular gland empties through Wharton's duct, which has a small opening near the lingual frenum at the sublingual caruncle. The sublingual gland has smaller ducts, known as the ducts of Rivinus, some of which join the submandibular duct, some of which open directly into the mouth. There is also a larger sublingual duct, known as the duct of Bartholin, which also joins Wharton's duct to exit at the sublingual caruncle.

The saliva secreted by each of these major glands differs in makeup. Over 1,100 proteins have been identified in salivary gland secretions, some of which are being tested for diagnostic value [3]. Parotid acinar cells secrete a wide variety of proteins with varied functions, including amylase, histatins, and parotid secretory protein (PSP) [3]. The parotid glands are almost completely serous (proteinaceous) in nature, whereas the other major glands, the submandibular and sublingual glands, are considered mixed glands, because they contain mucous acini capped with serous demilunes, allowing them to secrete both serous fluid as well as mucous. The submandibular gland produces significant amounts of both fluids, although the majority is serous, whereas the sublingual gland is predominantly a mucous secreting gland [4, 5].

The functions of saliva and its contents are extensive and clinically important. The most obvious role saliva plays is to moisten food and aid in swallowing, but it also functions in protection of the hard and soft tissues, digestion of carbohydrates, development and function of taste buds, and immunity against bacteria and fungus. As reported by Mandel in 1987, saliva lubricates the hard and soft tissue of the oral cavity, allowing food to pass into the esophagus smoothly. The frequent lavage of saliva also helps prevent food from being trapped between teeth. Enamel, when hydrated, is the strongest material in the body, but when desiccated (as happens when there is no saliva), it becomes brittle. Brittle teeth are much more prone to fractures and there is a greatly increased vulnerability to dental caries. Not only are dry teeth more susceptible to decay, but they allow a faster buildup of plaque and calculus, as a constant flow of saliva helps minimize accumulation of bacteria on teeth. This is due not only to the physical state of the teeth being more lubricated and more difficult for bacteria to adhere, but also because saliva itself has anti-bacterial and anti-fungal characteristics thereby reducing the number of colonies forming on a well lubricated tooth [6]. Another contributing factor of saliva's protection against bacteria is the pH buffering system present. As bacteria metabolize sugars, acids are produced that begin to destroy tooth structure. However, buffering components, like bicarbonate and phosphate, in saliva help raise the pH to minimize harm to the teeth by the acid [7]. Other minerals found in saliva help re-mineralize the teeth if any demineralization has taken place, or in post-eruption maturation of teeth [6]. The moisture provided by saliva also

significantly influences one's perception of taste. Taste buds have chemoreceptors that are stimulated by the chemical makeup of the food consumed. As the saliva present in one's mouth moistens food, chemicals in the food are solubilized, allowing the taste buds to detect the flavor [8].

Salivary glands are the focus of this research because there are numerous pathological conditions where functions of the salivary glands are lost. Sjögren's syndrome (OMIM 270150) is a condition that results in the partial to complete loss of salivary gland function [9-11] and it occurs in primary and secondary forms. The primary form of Sjögren's syndrome is an autoimmune reaction involving a lymphocytic infiltration of the major and minor salivary glands, which results in a decrease in salivary flow and often atrophy of the gland itself [12, 13]. Secondary Sjögren's syndrome is sequelae to another pre-existing condition, which is usually rheumatoid arthritis or another autoimmune connective tissue disease [12]. In about one-third of Sjögren's sufferers, there is an enlargement of the parotid or submandibular glands known as benign lymphoepithelial lesion, or Mukulicz's disease. Although the gland hypertrophies as opposed to atrophies, there is still an irreversible decline in function of the gland and resultant decrease in salivary production [12, 14].

Head and neck radiation used in the treatment of cancer patients is also a common cause of the destruction of the salivary glands [14]. According to Cooper, *et al.* [15], "irradiated mucocutaneous tissues demonstrate increased vascular permeability that leads to fibrin deposition, subsequent collagen formation, and eventual fibrosis. Irradiated salivary tissue degenerates after

relatively small doses, leading to markedly diminished salivary output." In addition, tumors of the parotid gland require partial to total excision of the gland due to the high probability of recurrence [16].

Parotid agenesis has also been seen alone and in addition to a variety of congenital conditions including: hemifacial microstomia, mandibulofacial dysostosis (Treacher-Collins syndrome), cleft palate, lacrimo-auriculo-dento-digital (LADD) syndrome, anophthalmia, ectodermal dysplasia, whereas hypoplasia of the parotid gland has been associated with Melkersson-Rosenthal syndrome [9].

Although there is a wide range in the etiologies behind the loss of salivary function, the oral complications are similar throughout, and they cause a variety of problems including: extreme xerostomia; extensive dental caries (especially in the cervical and cusp tip areas of teeth); rampant fungal infections; altered taste sensations; trouble speaking; sensitivity to acids; difficulty eating dry foods; erosion and ulceration of inflamed mucosa; sensitivity to spicy foods; fissured erythematous tongue; dsyphagia; cracked, peeling, or atrophic lips; corrugated and discolored buccal mucosa; loss of papillation on the tongue; difficulty wearing dentures and oral prostheses; and an overall vast decrease in the quality of life [9, 14, 17-21]. A common result of the dry oral cavity is the increase in number and proportion of *Streptococci mutans* and lactobacilli, the main bacteria responsible for dental decay [9, 21]. An increase in *Candida albicans* with resulting chronic erythematous oral candidiasis has been reported in about one-

third of Sjögren's patients [22]. This frequent candidiasis has been linked to a common complaint of angular cheilitis [20] and problems with dentures.

According to the Sjögren's syndrome Foundation, an estimated four million people in the United States suffer from Sjögren's alone, 85-90% of whom are females, with a mean age of 50 [20]. Along with radiation therapy being a common treatment for cancers found in the head and neck, and patients who have congenital problems or loss of the gland due to excision, the population affected by lack of salivary gland function is far from small and deserves the attention and efforts of the medical, dental, and research fields.

A focus of this laboratory is to effectively study these cells, so that progress can be made towards helping this patient pool. However, we must first be able to maintain parotid cells in culture. Unfortunately, we find that they lose cell-specific function, or de-differentiate, within twenty-four hours, rendering them useless [23]. As the body develops, cells that are less specialized become increasingly more specialized, or differentiate, to become cells with specific functions such as liver, brain, skin, glandular cells, etc. When a cell de-differentiates, as we see with parotid cells placed in culture, the cells no longer behave like parotid cells, although they do not die. This can be observed by measuring expression levels of mRNAs that serve as markers of differentiation, i.e., mRNAs that are only present in a specific cell type, such as a seromucous parotid acinar cell.

A stem cell is a cell that is able to differentiate into any type of cell, and much of today's research investigates the use of stem cells to replace missing or

damaged tissue. In a promising study in 2008, Lombaert showed that implantation of salivary stem cells into submandibular glands of mice that had been irradiated, allowed the stem cells to differentiate into acinar and ductal cells to restore the destroyed glands [24]. If it were possible to perform similar procedures in parotid glands in human patients, this large patient population could be treated successfully. As previously mentioned, parotid cells have proven extremely difficult to study for any prolonged amount of time, which limits the ability to develop procedures such as Lombaert used on submandibular cells.

Quissell reported in 1994, "Nontransformed adult rat salivary acinar cells cannot be sustained *in vitro* without an extracellular matrix substrate and they will not survive on plastic [25]." As mentioned previously, a way to verify that parotid acinar cells are not maintaining a differentiated state in culture is to measure expression of cell-specific mRNAs as markers of differentiation. For this purpose, I have used the mRNAs for three different genes; Mist1, PSP, and amylase. Mist1 is a transcription factor expressed only in serous and seromucous cells of exocrine glands, which is required for complete differentiation of those glandular cells. Pin reported in 2000, that not only do all serous-secreting exocrine cells typically express high levels of Mist1, but also all other cell types are Mist1-negative. Thus, Mist1 represents the first transcription factor that is unique to serous-secreting cells, suggesting that Mist1 may be involved in the differentiation of seromucous cells [26].

Parotid Secretory Protein (PSP) is another marker of differentiation. PSP is known to have antibacterial properties, and was shown by Shaw in 1986 to be

the most abundant gene product in the mouse parotid gland. After analyzing 11 different tissue types, Shaw concluded that the parotid secretory protein gene is expressed exclusively in the parotid gland [27].

The acinar cells of the pancreas and the salivary glands, especially the parotid gland, produce amylase. Salivary amylase is required for the initial step of chemical breakdown of carbohydrates for digestion, whereas pancreatic amylase continues the same process after food has passed through the upper portion of the alimentary canal. Meisler and Ting, in 1993, demonstrated that pancreatic and salivary amylase are almost identical in structure, except for a 1 kb fragment in the 5' promoter region of the gene, which is sufficient to function as an marker of cell-specific expression [28, 29]. Therefore, Mist1, amylase, and PSP are markers of terminal differentiation of parotid acinar cells. Importantly, the rapid loss of the mRNAs for these markers of differentiation provides a quantitative measure for changes in the differentiation status of the cells.

In an attempt to establish a medium that would maintain a differentiated state, we have cultured primary rat parotid cells in various media supplemented with species-specific growth factors, proteins, and steroids. These supplements included: rat serum (or calf serum, depending on the trial), epithelial growth factor (EGF), cystatin, retinoic acid (RA), triiodothyronine (T_3), hydrocortisone, trace element mixtures, fibroblast growth factor (FGF), insulin, transferrin, and selenium.

We also tested the different growth conditions with a rat parotid cell line, known as ParC5 cells. As Bockman noted in 2001, "one important obstacle to a

better understanding of...salivary gland secretion has been the lack of an immortalized cell line maintaining the phenotypical characteristics of an epithelial cell of acinar origin" [30]. However, in 1998, Quissell *et al.* and Turner *et al.*, reported the immortalization of clonal rat parotid gland acinar cell lines (ParC5 and ParC10) that manifest similar characteristics as those seen in native parotid acinar cells on morphological, biochemical, and functional levels [30, 31, 32]. These cells were transformed with simian virus 40, allowing them to persist *in vitro* indefinitely without much loss of differentiation over time or over the course of multiple passages [31]. The ParC5 line was used for this experiment, as Quissell considers it to be the most highly differentiated cell line. Since this is a well-characterized parotid acinar cell line, we used modern molecular tools to define whether these cells maintain a differentiated state.

The objective to establish a medium that will maintain primary rat parotid acinar cells in a differentiated state was focused to examine the efficacy of the supplements in preventing the loss of expression of the markers of differentiation, Mist1, amylase, and PSP, in primary adult rat parotid acinar cells. The study also looked at the efficacy of the supplements in inducing expression of these markers of differentiation in the immortalized parotid cell line, ParC5. We hypothesized that growth factors will prevent the de-differentiation of primary cultures of parotid salivary gland cells, and they will induce differentiation of ParC5 cells. This is a robust approach since two models systems (primary cells and ParC5 cells) were used to address the role of these supplements in differentiation.

CHAPTER II

MATERIALS AND METHODS- PART I

Harvest and purification of primary rat parotid glands

Adult rats were euthanized with CO_2 according to approved methods of UofL IACUC (IACUC Approval # 08050). Parotid glands were collected from Sprague-Dawley rats (Harlan, In.) and minced finely. The parotid tissue was suspended in Hank's Buffered Salt Solution (HBSS; Biowhittaker)+0.5% BSA with oxygen bubbled through the solution constantly. The cell solution was centrifuged at 700 rpm for 1 minute, and the pellet was collected. The pellet was re-suspended in 10 ml of HBSS+0.5% BSA. Cells were then digested with 2.5 units of Collagenase A and 2 mg Hyaluronidase (Worthington Biochemical Co., N.J.) for 2 h at 37°C under oxygen. The solution was filtered though a 70-micron cell filter (B.D. Falcon), and the filtrate was layered onto 5 ml of HBSS+4% BSA. The solution was then centrifuged again at 700 rpm for 2 min. The cell pellet was re-suspended in 10 ml of HBSS+0.5% BSA and centrifuged at 700 rpm for 1 min. The pellet was then collected and washed twice with Waymouth's medium (Cambrex, MD) without serum. After the second wash, the cell pellet was collected and re-suspended in Waymouth's medium with 10% rat serum (Equitech Bio. Inc, TX). The cells were then plated on plastic for 2 h to allow fibroblasts to attach to the plate. The non-adherent cells are primarily acinar

cells, and were collected by centrifugation, re-suspended in the various test media (the independent variables), and plated on collagen-I coated plates (BD Biosciences). The cells were cultured at 37 °C with 5% CO_2.

Culture media and conditions

After reviewing multiple journal articles involving prolonged cultures of primary rat parotid cells, various supplements that the authors considered essential to the success of their cultures were selected for this project's media [17, 21, 22]. The four different types of media used are as follows:

Standard Control Media (SCM): Waymouth's solution with L-Glutamine, Penicillin (100 U/ml), Streptomycin (0.1 mg/ml) (Invitrogen), ITS-X Supplement (100X) (Gibco), 10% Rat Serum, and Hydrocortisone (1 μM) (Sigma). A set of trials was done comparing bovine and rat serum, which used 10% fetal calf serum (Atlanta Biologicals) in this medium.

Rat Serum Medium (RSM): Waymouth's solution with L-Glutamine, Penicillin (100 U/ml), Streptomycin (0.1 mg/ml), ITS-X Supplement (100X), 10% Rat Serum, Hydrocortisone (1 μM), T_3 (10 nM), and EGF (10 ng/ml) (Invitrogen).

Cystatin/Rat Serum Medium (CRS): Waymouth's solution with L-Glutamine, Penicillin (100 U/ml), Streptomycin (0.1 mg/ml), ITS-X Supplement (100X), 10% Rat Serum, Hydrocortisone (1 μM), and cystatin (10 nM).

RA/FGF/Trace Element Medium (RFT): Waymouth's solution with L-Glutamine, Penicillin (100 U/ml), Streptomycin (0.1 mg/ml), ITS-X Supplement (100X), 10% Rat Serum, Hydrocortisone (1 μM), T_3 (10 nM), EGF (10 ng/ml), cystatin (10 nM),

retinoic acid (0.1 μM) (Sigma), Fibroblast Growth Factor (100 ng/ml), and Trace Element Mixture (ICN Biomedicals).

Plated primary parotid acinar cells were incubated at 37 °C with 5% CO_2 for twenty-four hours.

Collection of cells

Parotid acinar cells were collected from each culture and centrifuged (3 min at 3000 rpm) at 0 h and 24 h time points. The wells were washed with autoclaved PBS, and that solution was collected and centrifuged as well. Finally, the wells were washed with Trizol and mercaptoethanol. This solution was collected and added to the pellets from the previous steps. This approach was necessary because the cells are not adherent (at 0 h) or weakly adherent. Samples were stored at -80 °C when not being used.

RNA extraction and analysis of expression levels

The RNA was then extracted from the cells using the RNA Extraction with RNAprotect Cell Minikit from Promega. The RNA was quantified using a Nanodrop (Thermo Scientific), qualified using an Agilent bioanalyzer 2100 and the Agilent RNA 6000 Nano Marker, and used to prepare cDNA. Reverse transcription was performed for 60 min at 42 C using oligo dT(15) (Roche Diagnostics, IN). The reaction was stopped by heat inactivation for 5 min at 99 C. Using specific primers and ABI TaqMan probes for GAPDH, Mist1, amylase, TCF12, and PSP, the level of their mRNA in culture was quantified by real-time

TaqMan RT-PCR on an ABI 7900 System (Applied Biosystems, CA). The fold decrease in expression of the mRNAs was calculated after normalizing to the endogenous control (GAPDH).

ParC5 cell cultures

The same methods were used for trials with rat parotid ParC5 cells, except the cells were incubated in different media at 37 ^{o}C with 5% CO_2 for 1 week before RNA isolation, and the Standard Control Medium was a standard ParC5 medium.

Statistical analyses

Three independent trials were done for both primary rat parotid acinar cells, and ParC5 cells, with each trial plated in duplicate. Differences in the expression levels of the markers of differentiation were determined by ANOVA and paired t-tests, assuming a significant difference at $p < 0.05$.

CHAPTER III

RESULTS- PART I

Historically, primary parotid acinar cells have proven exceptionally difficult to culture for any prolonged period of time, due to the loss of cell specific function. This inability to maintain parotid cells that behave like parotid cells seriously limits the extent to which they can be studied, and it poses a significant problem to investigators of this gland. We have found that within the first twenty-four hours, the expression of mRNAs for proteins that function as markers of differentiation, Mist1, salivary amylase, and PSP quickly decrease to a level that is almost undetectable. This exponential decrease is demonstrated in **Figure 1**, and it shows that within one day of culture, the cells are no longer behaving like parotid acinar cells, rendering them useless for study.

Figure 1. Expression of Amylase, Mist1, and PSP mRNAs in primary parotid cell cultures

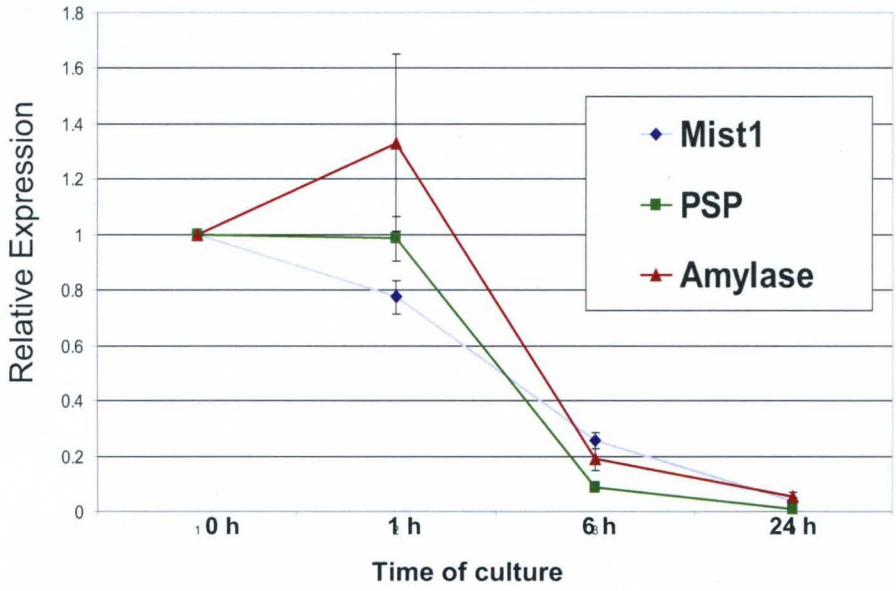

Figure 1. The expression of mRNAs for Mist1, PSP and Amylase are significantly and rapidly lowered during the first twenty-four hours of culture. The control mRNA for GAPDH and 18S RNA did not change in these experiments. This provides a quantitative measure of de-differentiation of acinar cells.

This de-differentiation within twenty-four hours creates the questions of, why does this happen? And, how can we prevent it? It was hypothesized that the de-differentiation is due to a lack of proper growth factors and hormones in the culture media, and that the addition of those supplements would prevent the de-differentiation of primary parotid acinar cells. This background allows a straightforward experimental design by comparing the expression of these markers at 0 h and after 24 h culture in various media.

For the primary parotid acinar cells, after twenty-four hours of culture in the different test media, expression levels of the mRNAs for the markers of terminal differentiation, Mist1, amylase, and PSP were measured and normalized against expression levels of the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control, as it is not a marker of differentiation. This is because GAPDH is present in every cell in the body, and its expression is not decreased as the parotid cells de-differentiate.

In three independent experiments, three different combinations of reagents were tested. When testing primary parotid acinar cells, two replicates were completed in each experiment. The reagents included rat serum (or calf serum, depending on the trial), epithelial growth factor (EGF), cystatin, retinoic acid (RA), triiodothyronine (T_3), hydrocortisone, trace element mixtures, fibroblast growth factor (FGF), insulin, transferrin, and selenium. The different combinations of the reagents are described in the Methods section. Regardless of the media tested, no significant differences were observed between the media

for any of the markers of differentiation. Every trial for each marker once again resulted in an exponential decrease in expression over the first 24 hours in culture (**Figure 2)**. As had been demonstrated previously (**Figure 1**), compared to the initial cells (0 h), there is a dramatic and significant ($p<0.05$) decrease in the total Amylase, Mist1, and PSP mRNA content of parotid cells cultured for 24 h.

Figure 2. Expression of Amylase, Mist1, and PSP mRNAs in primary rat parotid gland cells cultured in test media

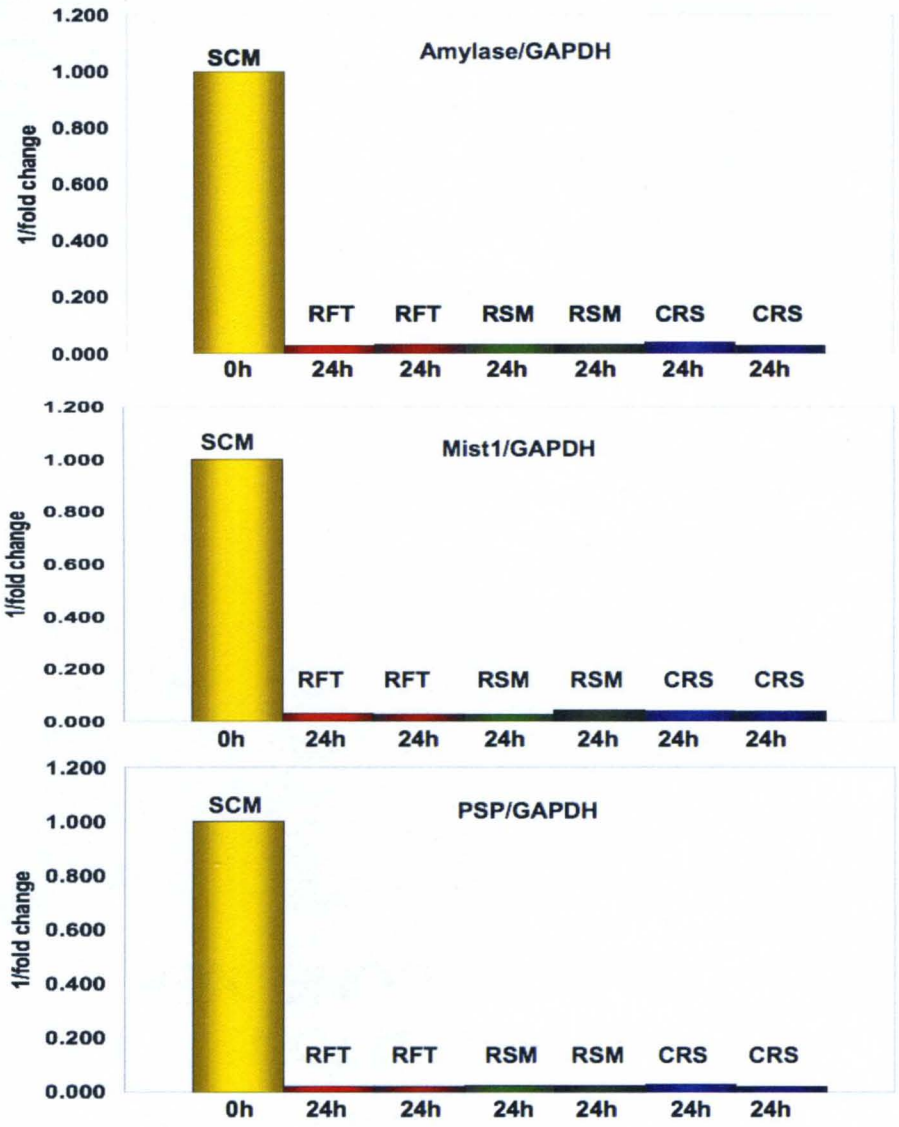

Figure 2. In this experiment, there is a dramatic and significant (*$p<0.05$) decrease in the total Amylase, Mist1, and PSP mRNA content of parotid cells in culture for 24 h. Each bar represents RT-PCR results from cells grown in a single well except for the SCM, which is an average of two control wells at 0 hr. Media are labeled as: Standard Control Media **(SCM),** Rat Serum Medium **(RSM),**

Cystatin/Rat Serum Medium **(CRS)**, and RA/FGF/Trace Element Medium **(RFT)**. All media consistently showed the exponential decrease, as seen in **Figure 1**.

Due to the continued inability to prevent de-differentiation in trials using primary rat parotid acinar cells, we decided to attempt inducing differentiation of ParC5 cells with a hope to increase their expression of markers of differentiation to a similar level of that seen in primary cells. It was hypothesized that the supplements added to the media would induce further differentiation in the less-specialized cells of the ParC5 parotid cell line. **Figure 3** depicts the extreme differences in levels of expression of the mRNAs for the markers of differentiation comparing parotid tissue and the ParC5 cell line. Since expression of these markers of differentiation is so low in ParC5 cells, this provides a very sensitive approach to test the effects of the supplements.

Figure 3. Expression of Amylase, Mist1, Tcf12, and PSP in ParC5 cells, normalized against GAPDH

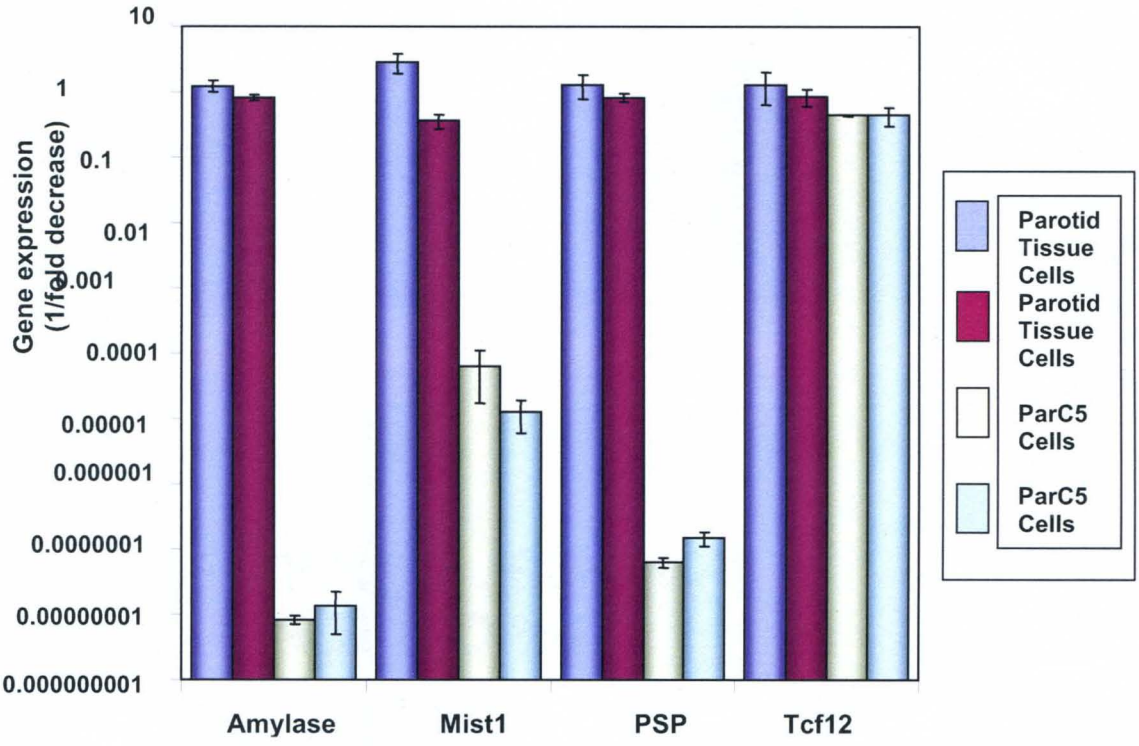

Figure 3. The expression of mRNAs for amylase, Mist1, PSP, and Tcf12 in rat parotid tissue was exponentially higher than seen in ParC5 cells. As a control, Tcf12, which is not a marker of differentiation, showed no change. These data show that the rat parotid cell line ParC5 have de-differentiated, but not completely. To reestablish levels of expression seen in parotid tissue, a remarkable increase of ParC5 expression would be required.

As mentioned in Chapter II, ParC5 cells were cultured in various media for 1 week instead of 24 hours, due to the stability of the cell line. The media used in the ParC5 trials followed the same recipes as used in the primary acinar cultures, which included: rat serum (or calf serum, depending on the trial), epithelial growth factor (EGF), cystatin, retinoic acid (RA), triiodothyronine (T_3), hydrocortisone, trace element mixtures, fibroblast growth factor (FGF), insulin, transferrin, and selenium. The Standard Control Medium is a standard ParC5 medium.

ParC5 cells were cultured in different media in three separate experiments, each of which was plated in duplicate for the control medium and the experimental media. In one additional trial using ParC5 cells, media containing rat serum was compared to media containing fetal calf serum. Total RNA was isolated for each well as described in Methods. mRNAs for marker genes were quantified using TaqMan assays, and normalized against GAPDH. Results for ParC5 cells in the various test media failed to demonstrate any major induction of differentiation, and there was no significant difference between media for all markers of differentiation, as seen in **Figure 4.**

Figure 4. Average Expression of Amylase, Mist1, and PSP mRNA in ParC5 cell cultures

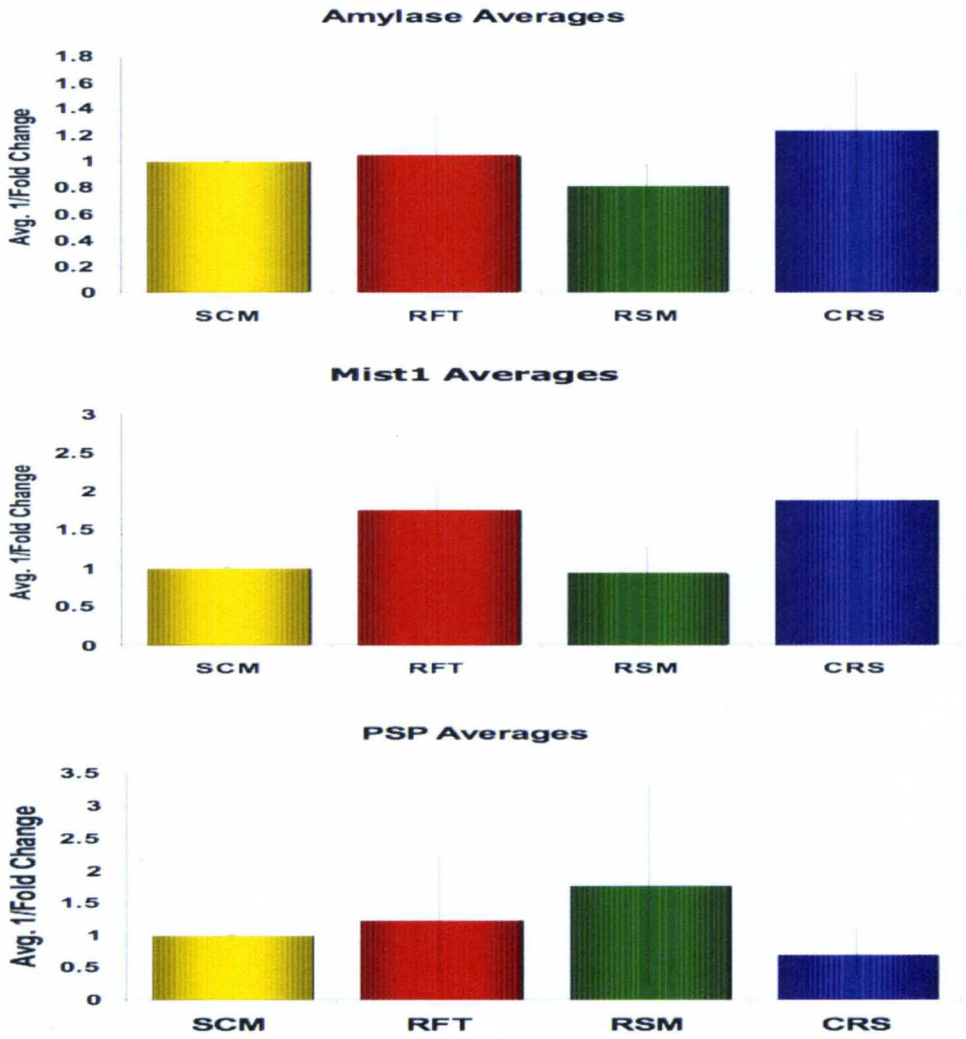

Figure 4. Each graph shows the level of expression of a marker gene in cells grown in 4 different media. Each bar represents the average of 3 experiments. The RT-PCR data are normalized to GAPDH and expressed relative to the control medium (SCM). No significant differences were found, $p > 0.05$ as compared to SCM.

For each media and each marker gene, expression levels were not induced exponentially as hypothesized. This means that there was no change in the level of differentiation, and there is no direct regulation of individual genes by the supplements in the media. **Figure 5** demonstrates that even when each individual trial is compared to the others, there is no significant induction of expression. When levels of expression in ParC5 cells for each individual experiment in each media are compared, the differences are minimal. Despite the appearance of some variability, no consistent changes in expression of the marker genes were found. Even the outliers are still 10,000 to 100 million fold shy of primary cells expression levels, which shows the supplements in the media do not directly regulate these genes.

Figure 5. Expression of Amylase, Mist1, and PSP mRNA in individual ParC5 cell cultures

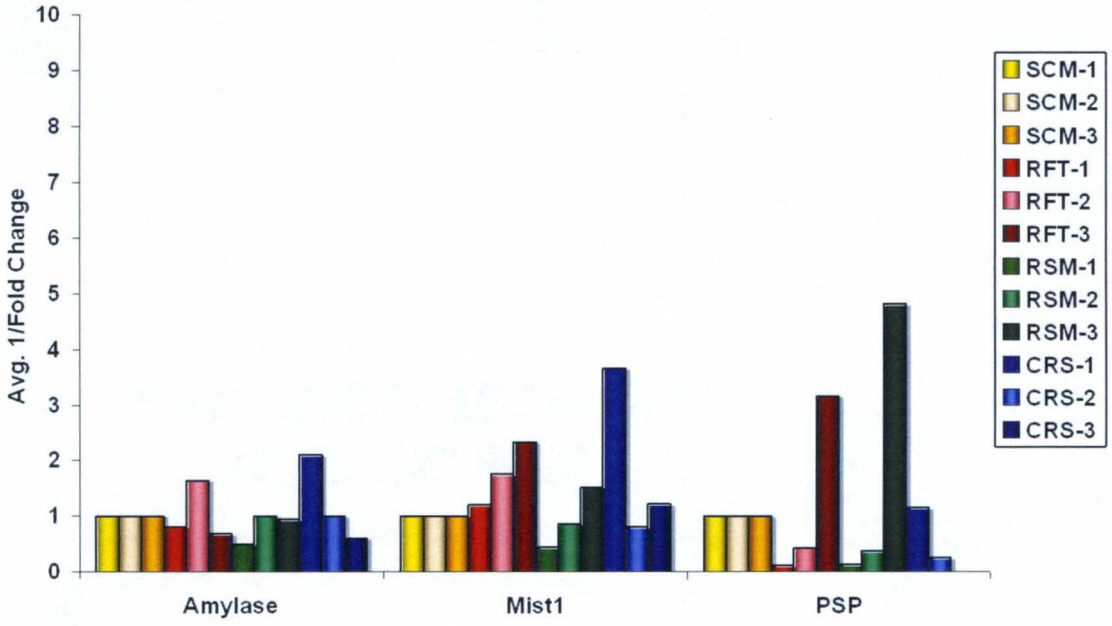

Figure 5. The bars are the averages of the expression levels for the control media (SCM) and each test media treatment (RFT, RSM, and CRS), in each of the three experiments, for the indicated genes. All trials were normalized against GAPDH. Normal differentiated expression levels would be 10^6-fold higher, as shown in Figure 3.

Depending on who conducts the research, there is variation in the components of the culture media. Some investigators, such as Humphries and Reyland *et al.*, have historically used fetal calf serum to culture parotid cells [33], whereas others, such as Yoshigaki *et al.*, have used rat serum [34]. Calf serum is sometimes used due to increased amount of growth factors found in the serum of young animals as opposed to adults, despite the fact that the cells being cultured are from rats. In 1998, Zhu *et al.* showed that use of rat serum in media causes an increased expression of amylase in parotid acinar cell lines [35], but expression levels were still significantly low compared to parotid tissue. Zhu's study did not investigate if rat serum would increase expression of amylase in cultured primary cells. To verify this, and to test the effects of the increased growth factors from the calf serum, another experiment with ParC5 cells was done comparing the test media to another set of identical test media, except that it contained 10% calf serum instead of the 10% rat serum in the original recipes. As done in the previous ParC5 trials, all samples and controls were plated in duplicate and allowed to grow for 1 week. Control media was the same standard ParC5 media used in previous trials. RNA was isolated and subsequently used to make cDNA for RT-PCR for Amylase and PSP mRNAs, normalized against GAPDH. Contrary to Zhu's findings, no significant differences in the levels of expression of markers of differentiation were noted between the samples cultured in calf serum media compared to those cultured in the rat serum media.

CHAPTER IV

DISCUSSION- PART I

Due to the large patient population that suffers from salivary gland problems such as Sjögren's syndrome, there is a definite need for parotid glands to be studied. However, when placed in culture, parotid acinar cells de-differentiate so that within twenty-four hours they lack the cell-specific characteristics that identify them as parotid cells. This de-differentiation makes studying these cells extremely difficult, and it creates a problem for investigators that needs to be solved. Using primary cells in culture is an excellent model, but only if the cells maintain their natural identity, which makes understanding the differentiation, and de-differentiation, process of these cells important.

Parotid acinar cells show a significant and dramatic decline in the expression of markers of differentiation such as Mist1, amylase, and PSP within 6 h of cell culture, and by the 24 hour time point, these markers are almost undetectable (**Figure 1**). Because of this loss of expression, there is no good model for the study of differentiated acinar cells. There are various hypotheses about why primary parotid acinar cells de-differentiate in culture, including the lack of growth factors *in vitro* that are normally present *in vivo*, and it was our goal to determine if these factors regulated the genes for markers of differentiation. This study strongly argues against this hypothesis, as all data

showed no decrease in severity, or rate, of de-differentiation of the parotid cells once placed in culture, despite different combinations of eleven growth factors and hormones. These reagents had been used in various publications that claimed to have differentiated cultures of acinar cells, however they did not directly compare cultured cells to parotid gland cells [35, 34, 33]. The reagents included: rat serum (or calf serum), epithelial growth factor (EGF), cystatin, retinoic acid (RA), triiodothyronine (T_3), hydrocortisone, trace element mixtures, fibroblast growth factor (FGF), insulin, transferrin, and selenium. No significant change in expression of markers of differentiation was noted throughout the study, and none of the factors prevented the de-differentiation of acinar cells in culture.

Since there was no prevention of de-differentiation of the primary rat cells, we made an attempt to induce differentiation in the stable parotid cell line, ParC5 cells. Although these cells maintain some characteristics of acinar cells, the levels of expression of the markers of differentiation are stable, but exponentially lower than those seen in parotid tissue. The goal was to increase the levels of expression towards that of the acinar cells by culturing the ParC5 cells in a medium that contained the growth factors present in the body. Depending on the marker of differentiation, the level of increase needed to mimic that of primary cells ranged from 10,000 fold to 100 million fold, as can be seen in **Figure 3**.

After culturing the cells for a week in the enriched media, compared to the control medium, the average increase in expression was 1.031 fold for amylase, 1.529 fold for Mist1, and 1.299 fold for PSP. Obviously this is not the exponential

rise in expression that was needed to be comparable to differentiated acinar cells, which argues that these growth factors and hormones can not induce differentiation in parotid gland cell lines such as ParC5 cells.

Also, we found that some investigators have chosen to use fetal calf serum [33] instead of rat serum [35] to culture parotid cell lines. *Zhu et al.* reported in 1998 that there was an induced increase in expression of amylase when using rat serum compared to that of fetal bovine serum. During this study of ParC5 cells comparing identical culture conditions, except for the variable of calf serum or rat serum, there was no significant difference in levels of expression for any markers, including amylase.

Within the limits of this experimental design, these findings indicate that a lack of growth factors is not the causative issue behind the loss of differentiation of primary parotid acinar cells. This study also shows that growth factors do not induce differentiation of parotid cell lines, such as ParC5. Therefore, alternative mechanisms to explain this loss of cell specific function must be explored with further investigation. The following chapters of this thesis address the possibility that epigenetic changes influence the differentiation of parotid gland acinar cells.

CHAPTER V

INTRODUCTION – PART II

DNA Methylation of Marker Genes During De-differentiation of Parotid Cells

Genetic information is contained in the linear strands of DNA in each cell, and encoded in this information are mechanisms that determine which genes will be expressed. However, there are factors other than just the DNA itself that regulate gene expression, such as epigenetic mechanisms [36]. Epigenetics, by definition, is "above or beyond the genome," which includes mechanisms that "establish heritable states of gene expression without altering the DNA sequence" [36, 37]. There are various epigenetic processes including post-translational modification of histones, generally including acetylation, phosphorylation, and/or ubiquitinylation. Histones are proteins found in eukaryotic cell nuclei that help package the DNA into units known as nucleosomes [38, 39]. DNA is wrapped around the histones, similar to thread on a spool, which allows the very long (1.8 meters) strand of DNA to be condensed down to about 90 millimeters on the histone [40]. This strand of DNA, wrapped around many histones, is often referred to as a string of pearls. This combination of nucleosomes and DNA, plus additional structural proteins, is known as chromatin. The chromatin then condenses further to form chromosomes, allowing for the 1.8 meters of DNA to be about 120 micrometers of chromosomes

[40]. When histones are modified by acetylation, phosphorylation, and/or ubiquitinylation, it can change the way the DNA is wrapped around them; thereby changing what DNA is accessible to complexes like DNA polymerases. DNA polymerases are enzymes that function to replicate and transcribe the contents of a DNA strand [41]. This explains why these epigenetic modifications of histones interact to alter chromatin structure and function, which subsequently alters expression [36], and why the modifications can be passed on through replication to subsequent generations without modifying the DNA sequence itself.

A second class of epigenetic regulation involves modification of the DNA itself. According to Bird in 2002, the most studied epigenetic modification in mammals is not histone modifications, but DNA methylation [42]. DNA methylation is thought to play many roles in cell physiology, "including genome stability, repression of endogenous retroviral and transposable elements, genomic imprinting, and developmental gene regulation" [42-47].

Interestingly, DNA methylation can only occur at one specific base pair, which is the 5' site of a cytosine that immediately precedes a guanine. This cytosine-guanine dinucleotide is referred to as a CpG site, and the enzyme DNA methyltransferase, using S-adenosyl methionine as the methyl group donor, is responsible for the conversion of cytosine to 5' methylcytosine [36]. Once the DNA is methylated, the methyl group protrudes into the major groove of the DNA subsequently displacing transcription factors that ordinarily bind to the DNA [36, 48]. Also, the sites that have been methylated can attract methyl-binding proteins, known as methyl-CpG-binding domain proteins (MBDs), which are

involved in 'reading' methylation marks [49]. These MBDs then recruit histone deacteylases that modify the tails of the histone proteins, resulting in chromatin condensation [36, 47]. Once chromatin has been compacted, there is often the result of gene silencing [34].

CpG sites occur throughout the genome, but there are areas that include much higher concentrations of these sites, known as "CpG islands." These are variably defined, but generally have a region of 200 base pairs with at least 50% G or C content. CpG islands often encompass promoter regions and transcriptional start sites of the associated gene, and they have been shown to be present in the promoter region of 50-70% of human genes [50, 51]. As the many possible sites in CpG islands undergo methylation, it becomes increasingly difficult for the transcription factors to bind, and more and more MBDs are attracted and recruit transcriptional repressors [52-54]. Due to these mechanisms, there is a well-established relationship between methylation of the promoter region and transcriptional repression [55, 52, 56, 57]. Also, according to Jones and Laird in 1999, not only may an increase in methylation in promoter regions lead to decreased expression, but a decrease in promoter region methylation may possess the potential for up-regulating gene expression [58]. This re-affirms that there is a clear link between epigenetic modifications and regulation of gene expression.

Not only can DNA methylation affect gene expression, but also there is evidence to support that it may play roles in the differentiation of cells [50]. In 1979, Taylor and Jones showed that by inhibiting DNA methylation in fibroblasts,

the cells can transform into muscle cells and other types of differentiated cells, which suggests that gene methylation regulates the process of differentiation [50, 59]. Considering this fact, and the difficulty in maintaining a differentiated state of primary rat parotid acinar cells when placed in culture (described in Part I, above), the second part of my study was directed at trying to determine if DNA methylation is causing a loss in differentiation of these cells. If promoter regions of the genes that serve as markers of terminal differentiation are becoming methylated during culture, it may cause a repression in gene expression and therefore a loss of differentiation.

The first step of my epigenetics research was designed to establish the presence of CpG sites and islands located in the promoter regions of the genes that encode terminal markers of differentiation such as Mist1 and PSP in adult rats. The next stage was to determine the level of methylation of these CpG sites. This study also examined the methylation status of the known CpG sites after the cells had been placed in culture for 0 h and 18 h time points so as to evaluate if there was a change in the level of methylation over time.

In order to identify whether or not a CpG site has been methylated, bisulfite conversion is a very useful procedure. When treated chemically with bisulfite, any cytosine that is <u>not</u> methylated will be converted to uracil [60, 61]. Unmethylated cytosines are chemically converted to uracils, which are amplified as thymidine by the PCR step. Thus, if DNA is treated with bisulfite, amplified with PCR, and analyzed by sequencing, any remaining cytosines indicate a CpG site that had been methylated. By first testing the level of methylation of adult rat

parotid cells immediately after harvest, a baseline will be established so that changes in methylation during culture can be recognized.

The main objective of this part of the study is to establish if DNA methylation prevents transcription of terminal markers of differentiation in primary rat parotid cells in culture. I hypothesized that global methylation of the genes such as Mist1 drives the loss of expression and loss of differentiation in cultured parotid cells.

CHAPTER VI

MATERIALS AND METHODS- PART II

Identification of CpG Islands

The first step of this part of the study required identifying the presence, number, and location of CpG islands in the promoter regions of the desired genes, Mist1 and parotid secretory protein (PSP). Using the National Center for Biotechnology Information's (NCBI) rat genome database (http://www.ncbi.nlm.nih.gov/guide/rat), the transcriptional start site in the DNA sequence for each desired gene was located. A 4,000-nucleotide section of DNA sequence was copied spanning from 2,000 nucleotides upstream of the transcriptional start site to 2,000 nucleotides downstream. This ensured that if a CpG island located in the promoter region continued somewhat past the start site, it would still be completely contained within the section of DNA sequence. Using the European Bioinformatics Institute's EMBOSS (http://www.ebi.ac.uk/Tools/emboss/cpgplot/), the 4,000-nucleotide sections of DNA were analyzed for the presence of CpG islands. Conveniently, PSP lacked any CpG islands in its promoter region, allowing it to act as a negative control.

Polymerase Chain Reaction (PCR) primer design

Once CpG islands were located, PCR primers were designed so that the individual islands could be amplified for analysis. Mist1 had a 387 base-pair CpG island in its promoter region that primers were designed to amplify. Since PSP lacked any CpG islands, PCR primers were designed for a 692 base-pair section of DNA that was centered around the transcription start site. The Mist1 gene CpG island was amplified using the following primers: a) the sense primer 5'-TGTTGGTGATGGTAATGTTGGTA and b) the anti-sense primer 5'-CCAATCAAACTCAAAAACATCAA. The primers for the PSP gene DNA were as follows: a) the sense primer 5'-TGAGTTTTTAAAAGATGATTGGGTTA and b) the anti-sense primer 5'-CCCACTATCTATCTCCACCAAAC.

Harvest and purification of rat parotid glands

Parotid glands were collected from adult rats and minced finely, as described in Chapter II. The parotid tissue was suspended in Hank's Buffered Salt Solution (HBSS) + 0.5% BSA with oxygen bubbled through the solution constantly.

At this point, there were two different paths taken, depending on the goal of the experiment. In order to evaluate the level of methylation present in parotid cells before any time in culture, 20 μL of proteinase K (600 mAU/ml) was added, vortexed, and then placed in a 56 °C water-bath for overnight lysis in preparation for DNA isolation.

The other route was to determine the amount of change in methylation during culture, comparing a 0 h and 18 h time point. For the cells used in this

type of experiment, cells were placed in Waymouth's medium, pelleted, digested with hyaluronidase and collagenase, and plated on plastic to allow for attachment of fibroblasts, as was described in detail in Chapter II, Materials and Methods. As soon as the cells were ready to be plated, the 0 h time point samples were harvested, and DNA isolation was initiated. The remaining samples were plated in Waymouth's medium + 10% rat serum for 18 h at 37 ^{o}C before being harvested and having DNA isolated.

The samples that were not prepared for culture, but were taken from the tissue and immediately prepared for DNA isolation will be referred to as "gland" or "gland samples." The samples that were prepared for culture and were taken at specific time points will be referred to at "0 h" and "18 h" samples.

DNA Isolation

Adult rat DNA was isolated from the parotid glands following the QIAGEN Mini Kit protocols exactly according to the manufacturer's instructions. Nuclease-free water was used to elute each sample. All DNA isolations were quantified using the Nanodrop.

Bisulfite Conversion

Using ZYMO Research EZ DNA Methylation-Direct Kit, isolated DNA was treated with bisulfite to convert all unmethylated cytosines to uracils. 500 ng of DNA was used for each round of bisulfite conversion. 20 μl of DNA & CT Conversion reagent was used for each column of bisulfite treatment, which was

eluted with 15 μl of PCR grade water instead of 10 μl of M Elution Buffer. Each sample of bisulfite converted DNA was quantified using the Nanodrop. Multiple (23) bisulfite conversions were completed for this experiment to provide independent samples for PCR.

Amplification of DNA using Polymerase Chain Reaction

Using between 50-100 ng of DNA, a PCR was run with 45 μl of PCR Supermix and 2.5 μl (1:20 ratio of primer to PCR grade water) of each primer. The PCR was run at 95 °C for 10 minutes, then 40 cycles involving 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C 1 minute 30 seconds, and finally 72 °C for 10 minutes before moving into a 4 °C infinite holding temperature. PCR products were verified to be single bands of the expected size by running samples through a 1% agarose gel.

Cloning of PCR products into *E. coli*

Using the fresh PCR products that were confirmed to be successful, PCR fragments were cloned into Topo® vector and One Shot® Top10 competent bacteria using the Invitrogen Topo TA Cloning® Kit for Sequencing. 12-15 μl of Top10 cells was used per clone, depending on how many PCR products were being cloned during the trial. 10-15 μl of the Top10 cell solution was used to run a negative control that received no DNA or Topo®. Protocols for "Chemically competent E. coli" were followed, which calls for 2 μl of PCR DNA. Cells were transformed using the heat-shock method protocol. Once ready for plating, two

sterile Luria Broth + Ampicillin agar plates were used for every clone: 50 μl of clone was spread on one plate, 100 μl of the clone was spread on the other. 100 μl of the Negative Control was spread on another plate. All plates were incubated overnight at 37 °C, and remaining bacteria solutions were stored at 4 °C.

The following day, colonies were counted, selected, and harvested using sterile pipette tips. Each harvested colony was placed in its own 50 ml centrifuge tube with 10 ml of the LB broth and 50 μl of Ampicillin. The tubes were then placed in a shaker at 200 rpm at 37 °C overnight to grow the bacteria.

A master plate was made with each colony selected using 1 μl of the bacteria filled media on a sterile Luria Broth + Ampicillin agar plate, and stored at 4 °C. A total of 112 clones were made from fresh PCR products during this experiment.

Plasmid Isolation from Clones

After allowing the culture tubes to incubate in the shaker overnight, plasmid DNA was isolated following the Promega Wizard Plus SV Mini Prep protocols. All isolated DNA samples were characterized and quantified using the Nanodrop. Isolated DNA was divided into 10 samples for each Mist1 and PSP for the gland samples. However, DNA was isolated and divided into four samples for each Mist1 and PSP at both the 0 h and 18 h time points.

DNA Sequencing

In order to identify which CpG sites had been methylated, the isolated DNA was sequenced and compared to the known rat genome sequence. To do this, 400 ng of isolated DNA from the Promega Mini Preps was added to a 0.2 ml PCR tube. The total volume was brought up to 10.4 μl using Nuclease Free Water. Samples were then taken to the Center for Genetics and Molecular Medicine at the University of Louisville for DNA sequencing. Once sequences were received, they were compared to each other and to the rat genome from NCBI for accuracy and to evaluate which CpG sites were methylated.

CHAPTER VII

RESULTS- PART II

Before being able to identify changes in methylation over time, we needed to define the methylation status of each CpG site in the CpG island in the Mist1 gene in adult rat. To do this, DNA from harvested parotid glands was immediately isolated ("gland samples"), as opposed to being plated in culture for any amount of time before DNA isolation (0 h and 18 h time points), as mentioned in the Chapter VI, Materials and Methods, Part II.

Bioinformatic analysis identified a CpG island in the promoter region of Mist1, spanning 387 base pairs, and terminating 863 base pairs upstream of the transcriptional start site. There are 23 CpG sites within this CpG island, as is illustrated in **Figure 6**.

Figure 6. Schematic representation of the CpG island located ~1250 bp upstream from Mist1's transcription start site

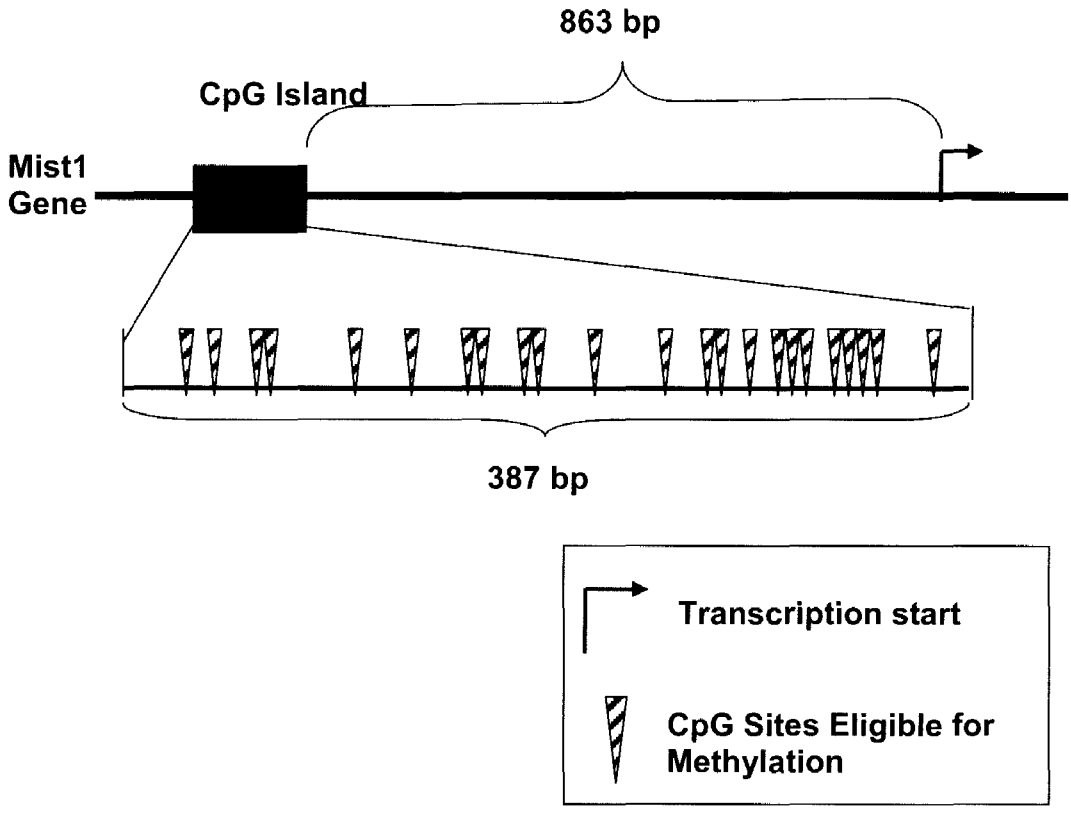

Figure 6. This figure shows the location of the CpG sites, which are eligible for methylation, in relation to each other and to the transcriptional start site. The methylation status of each site was determined for the gland samples so that it could be compared to methylation statuses seen at later time points (0 h and 18 h) when tissue had been placed in culture media.

As previously mentioned, PSP functioned as the negative control, because there was no identifiable CpG island present, so a 692 base pair section of DNA, centered around the transcription start site, was amplified with PCR. While designing PCR primers, a successful set of primers was found that encompassed the 692 bp, which was large enough to have a high probability of including the majority of CpG sites near the start site, while still being small enough that PCR would likely be successful. There are seven CpG sites in the 692 bp segment, as illustrated in **Figure 7**.

Figure 7. A schematic illustration of the location of the CpG sites located around the transcriptional start site of the Parotid Secretory Protein (PSP) gene

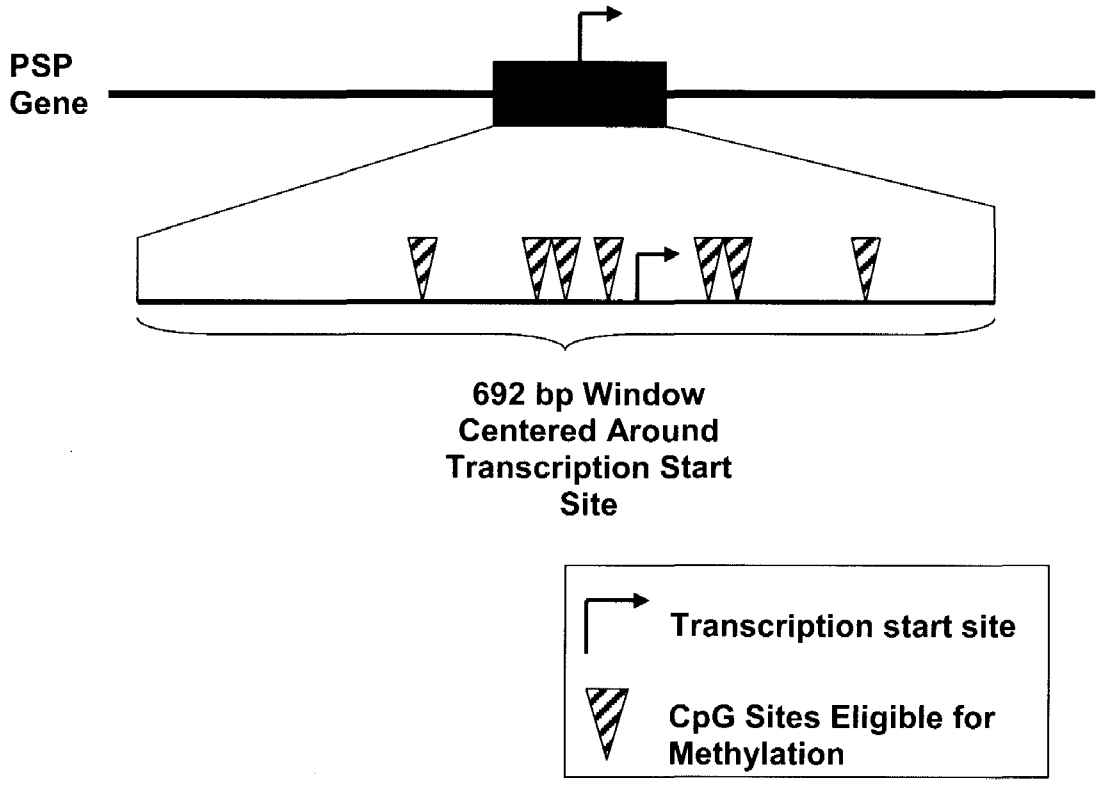

Figure 7. No CpG island was identified, so a 692 bp segment centered on the transcriptional start site was amplified so that the methylation status of individual sites near that start site could be evaluated.

To verify that the appropriate size section of DNA had been amplified with PCR, samples were electrophoresed on 1% agarose gels. **Figure 8** shows an example of the resulting gel.

Figure 8. An example of the verification of size of PCR products

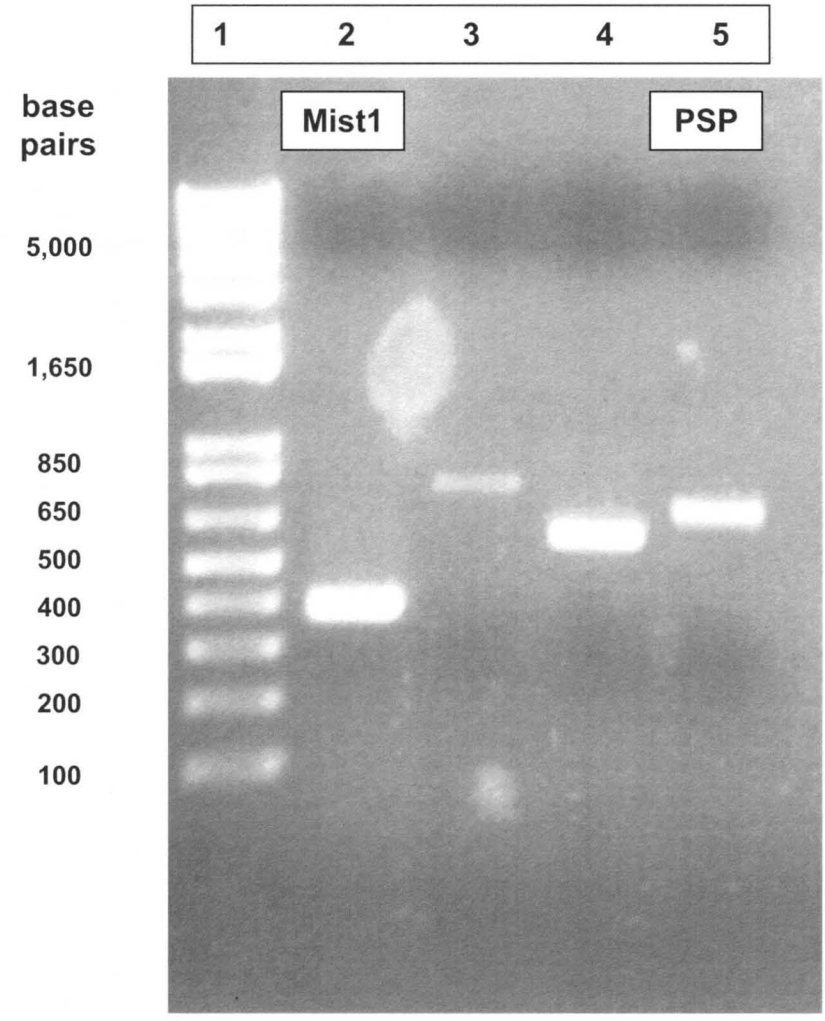

Figure 8. This example is a 1% agarose gel for PCR products Mist1 and PSP run for 40 cycles at 55 °C annealing. Lane 1 contains the DNA ladder for band size reference, Lane 2 contains the Mist1 387 bp amplicon, and Lane 5 contains the 692 bp PSP product. Samples in lanes 3 and 4 are the correct size amplicons for different genes, not described here.

Once the PCR reactions were validated, DNA samples were cloned into the Topo®TA plasmid, DNA was purified again, and it was then taken for sequencing at The Center for Genetics and Molecular Medicine at the University of Louisville. Upon receiving results, the sequences were evaluated to determine which CpG sites were methylated. This was done by comparing the returned sequences to the rat genome sequence as reported by the National Center for Biotechnology Information (NCBI). Any cytosine that remained visible in the sequences indicated a CpG site that had been methylated, as any unmethylated cytosine remains vulnerable to the bisulfite conversion and would have been converted to a uracil. These uracils are amplified as thymidines in the PCR step, so every unmethylated cytosine, regardless of being a CpG site or not, will appear as thymidine (T) in sequencing. All sequences were compared to the genome reported by NCBI to verify the accuracy as well as to ensure that any remaining cytosine did in fact correlate to a CpG site, which are the only sites eligible for methyl-protection from bisulfite conversion. Essentially all single cytosines in the genomic sequence appeared as T in these cloned sequences, demonstrating the high efficiency of the bisulfate reaction.

Table 1 and 2 illustrate the findings for the Mist1 and PSP genes in the gland samples. The methylated (M) or unmethylated (U) status of each CpG dinucleotide is shown in the table for each clone (labeled as the name of gene being investigated, followed by the sample number). Differences between clones are taken to represent the different methylation status of DNA from different cells in the sample. Therefore, for the Mist1 island in parotid tissue, while CpG #5 is

always unmethylated, CpG #19 is methylated in some cells but not in other cells. In general, these results show that this island is highly methylated in vivo in the parotid.

Adult Rat Gland - Mist1 CpG Island	Clone # / CpG #	Mist-1	Mist-2	Mist-3	Mist-4
	1	M	M	M	M
M = Methylated	2	M	M	M	M
U = Unmethylated	3	M	M	M	U
M* = C is methylated but G has a sequencing error	4	M	M	M	M
	5	U	U	U	U
	6	M	M	M	M
	7	M	M	M	M
	8	M	M	M	M
	9	M	M	M	M
	10	U	M	M	M
	11	M	M	M	M
	12	M	M	M	M
	13	M	M	M	M*
	14	M	U	M	M
	15	M	U	M	M
	16	M	U	M	M
	17	M	U	M	M
	18	M	U	M	M
	19	M	U	M	U
	20	M	U	M	M*
	21	M	U	M	M
	22	M	M	M	M
	23	M	M*	M	M*

Table 1. Methylation status of the 23 CpG sites in the CpG island located in the adult rat Mist1 gene's promoter region. Results show a predominantly methylated state throughout the CpG island for the gland samples.

Adult Rat Gland - PSP Control	Clone #	PSP - 1	PSP - 2	PSP - 3	PSP - 4
	CpG #				
M = Methylated	1	U	U	U	U
U = Unmethylated	2	U	M	U	U
N/A = Sequencing error covered area or sequencing stopped before this point	3	U	U	U	U
	4	U	M	U	U
	5	N/A	U	N/A	U
	6	N/A	M	N/A	N/A
	7	N/A	M	N/A	N/A

Table 2. Methylation status of the 7 CpG sites surrounding the transcriptional start site in the adult rat parotid secretory protein gene. Results show a predominantly unmethylated state in the gland, although sequencing terminated before completion in some of the samples.

As a next step, we compared the results shown in **Tables 1 & 2**, to the 0 h time point results to evaluate if much change occurred in the few hours of preparing the cells for culture. There is very little variation between the two sets of data for Mist1. However, for the sites sequenced for PSP, there is an over three-fold increase in methylation in the 0 h samples compared to the gland samples. The 0 h samples did continue closer to completion during sequencing, allowing for more chances at methylated sites. Interestingly, CpG site #5 of the Mist1 island remains unmethylated in all samples, despite the heavy concentration of methylated sites throughout the island. This consistency strengthens the veracity of the data as it reflects reproducibility in laboratory techniques. See **Tables 3 & 4**.

M = Methylated CpG site U = Unmethylated CpG site
M* = C is methylated but G has a sequencing error

	Gland				0 h Time Point		
Clone # / CpG #	Mist - 1	Mist - 2	Mist - 3	Mist - 4	Mist - 5	Mist - 6	Mist - 7
1	M	M	M	M	M	M	M
2	M	M	M	M	M	M	M
3	M	M	M	U	M	M	M
4	M	M	M	M	M	M	M
5	U	U	U	U	U	U	U
6	M	M	M	M	M	M	M
7	M	M	M	M	M	M	M
8	M	M	M	M	M	M	M
9	M	M	M	M	M	M	M
10	U	M	M	M	M	M	M
11	M	M	M	M	M	M	M
12	M	M	M	M	M	M	M
13	M	M	M	M*	M	M	M
14	M	U	M	M	M	M	M
15	M	U	M	M	M	M	M
16	M	U	M	M	M	M	M
17	M	U	M	M	M	M	M
18	M	U	M	M	M	M	M
19	M	U	M	U	U	M	M
20	M	U	M	M*	M	M	M
21	M	U	M	M	M	M	M
22	M	M	M	M	M	M	M
23	M	M*	M	M*	M	M	M

Table 3. Comparing methylation status of CpG sites for Mist1 in adult rat gland samples to methylation status at the 0 h time point.

M = Methylated CpG site U = Unmethylated CpG site
N/A = Sequencing error covered area or sequencing stopped before this point

	Gland				0 h Time Point			
Clone #	PSP-1	PSP-2	PSP-3	PSP-4	PSP-5	PSP-6	PSP-7	PSP-8
CpG #								
1	U	U	U	U	M	U	M	M
2	U	M	U	U	M	U	M	M
3	U	U	U	U	M	U	M	M
4	U	M	U	U	M	U	M	M
5	N/A	U	N/A	U	M	U	U	M
6	N/A	M	N/A	N/A	M	U	U	M
7	N/A	M	N/A	N/A	M	U	N/A	N/A

Table 4. Comparing methylation status of CpG sites for PSP in adult rat gland samples to status at 0 h time point.

Finally, 0 h and 18 h time points were compared to determine the amount of change in methylation over time in culture, which was hypothesized to increase in correlation with the decrease in expression of terminal markers of differentiation. As **Table 5** shows, there is very little variation between the two sets of data for Mist1, but overall a slight decrease in methylation is seen over time, contrary to my hypothesis. Once again, the Mist1 CpG site #5 consistently remained unmethylated throughout all samples, including the 18 h time point, which shows strong data. However in **Table 6**, which illustrates the PSP findings, there is continued increase in methylated CpG sites over the 18 hours, which corresponds with my hypothesis that a significant global increase of methylation correlates with the simultaneous decrease in expression of PSP mRNA. Results for Mist1 and PSP are shown in **Tables 5 & 6** respectively.

M = Methylated CpG site U = Unmethylated CpG site
M* = C is methylated but G has a sequencing error

	0 h Time Point			18 h Time Point			
Clone #	Mist-5	Mist-6	Mist-7	Mist-8	Mist-9	Mist-10	Mist-11
CpG #							
1	M	M	M	M	M	M	M
2	M	M	M	M	M	M	M
3	M	M	M	M	U	M	M
4	M	M	M	M	M	M	M
5	U	U	U	U	U	U	U
6	M	M	M	M	U	M	M
7	M	M	M	M	M	M	M
8	M	M	M	M	M	M	M
9	M	M	M	M	M	M	M
10	M	M	M	M	M	M	M
11	M	M	M	M	U	U	M
12	M	M	M	M*	M	M	M
13	M	M	M	M	M	M	M
14	M	M	M	M	U	M	M
15	M	M	M	M	M	M	M
16	M	M	M	M	U	M	M
17	M	M	M	M	M	M	M
18	M	M	M	M	M	M	M
19	U	M	M	M	M	U	M
20	M	M	M	M	M	M	M
21	M	M	M	M	M	M	M
22	M	M	M	M	M	M	M
23	M	M	M	M	M	M	M

Table 5. Comparing methylation status of CpG sites of the Mist1 gene CpG island at 0 h and 18 h time points.

M = Methylated CpG site U = Unmethylated CpG site
N/A = Sequencing error covered area or sequencing stopped before this point

	0 h Time Point				18 h Time Point	
Clone #	PSP - 5	PSP - 6	PSP - 7	PSP - 8	PSP - 9	PSP - 10
CpG #						
1	M	U	M	M	M	M
2	M	U	M	M	M	M
3	M	U	M	M	M	U
4	M	U	M	M	M	M
5	M	U	U	M	M	M
6	M	U	U	M	M	M
7	M	U	N/A	N/A	U	N/A

Table 6. Comparing methylation status of CpG sites for PSP in adult rat in culture at 0 h and 18 h time points.

CHAPTER VIII

DISCUSSION- PART II

As demonstrated in Part I, there is an almost complete loss of expression of terminal markers of differentiation for parotid acinar cells during the first twenty-four hours of being in culture (**Figure 1**). After confirming that a lack of proper growth factors and hormones in the culture media was most likely not the cause, a new direction of investigation was followed. Considering that epigenetics, specifically DNA methylation, has been linked to gene silencing [55, 52, 56, 57] and cell differentiation [50, 59] it was hypothesized that an increase in methylation in the promoter regions of the markers of differentiation would be seen over time in culture, which would correlate with the loss of expression and de-differentiation.

Upon evaluating the methylation status of the CpG sites found in the CpG island of Mist1, the overall trend was one of heavy methylation; very few of the total number of sites were in an unmethylated state, as can be seen in **Table 1**. When comparing methylation levels in gland samples to samples at the 0 h time point of culture, there was very little variation in the Mist1 gene (seen in **Table 3**). This was expected, as very little time had elapsed, which would theoretically not allow for much methylation to take place. These observations that this CpG island is heavily methylated in the Mist1 gene is very important. Heavy

methylation of the island under conditions when the Mist1 gene is strongly expressed demonstrates that this island is not a site where transcription factors must bind to activate the gene.

When 0 h and 18 h time points were compared for Mist1 in **Table 4**, very little change was evident, and what change was noticeable was actually a decrease in methylation. CpG site #5 remained unmethylated in every sample throughout this study, regardless of time elapsed. Besides site #5, only one Mist1 CpG site, #19, in only one sample, registered as being unmethylated at the 0 h time point. However, after 18 h had passed, CpG sites # 3, 6, 11, 14, 16, and 19 were also unmethylated in at least one sample. Site #11 was the only site to appear as unmethylated in more than one sample.

Although the number and location of unmethylated CpG sites increased over time, the expression level of Mist1 continued to decrease. This is contrary to the statement by Jones and Laird in 1999, that a decrease in methylation over time may posses the potential for up-regulating a gene [58]. These findings demonstrate that the loss of expression of Mist1, which does have a CpG island present in its promoter region, is not due to an increase in methylation of this island. This is contrary to my hypothesis, and there are at least 4 possible explanations for this: the Mist1 gene may have other CpG islands outside the 4,000 bp region I examined; the Mist1 gene may be regulated by individual critical CpG sites, as discussed below; a different transcription factor gene which regulates Mist1 expression may be regulated by methylation; or DNA methylation

may have no direct role in repression of Mist1 expression during culture of parotid acinar cells.

Currently, there are two viewpoints on DNA methylation: one thought is that a global increase of methylation at CpG sites is necessary to affect expression, which is what was tested in this study. The other opinion is that it is not dependent on a global increase, but that the epigenetic control comes from specific, individual independent CpG sites [42]. There are CpG sites in areas of the Mist1 promoter region that are not located in the CpG island itself, and one or more of those sites could be the sites that affect expression. CpG sites like site #5 in the Mist1 CpG island that remained unmethylated in each sample, regardless of time passed, indicate that the methylation status of the individual sites is not random. There could certainly be CpG sites elsewhere in the promoter region that show consistent unmethylated statuses at early time points, and those could lead to finding which of those sites consistently became methylated over time. Further studies of the Mist1 promoter region are necessary, but currently my data demonstrate that global methylation of the CpG island in the promoter region of Mist1 is not the cause of decreased expression and dedifferentiation.

The PSP gene lacks any clear CpG islands, but it does contain scattered individual CpG sites. Methylation of such individual CpG sites has recently been suggested to be able to regulate gene expression. In contrast to the Mist1 gene, PSP showed a predominantly unmethylated state for the seven CpG sites near the transcription start site.

PSP was initially developed as the negative control due to its lack of CpG islands and the subsequent rarity of CpG sites near the transcription site, but it did not turn out to be a control in the sense of having no change in methylation status. Of the sites sequenced for PSP, 20% are methylated in the gland samples compared to 65.4% in the 0 h samples, which is more than a three-fold increase. The fact that many of the sites were methylated at the 0 h time point, whereas very few were methylated in the gland samples taken 3 hours before that (2 hours for digestion with collagenase, and 1 hour for plating fibroblasts), could indicate that the methylation response is fairly rapid. Furthermore, comparing the 0 h and 18 h time points for PSP in **Table 5** shows a continued increase in methylation over time, from 65.4% at 0 h to 84.6% at 18 h, which corresponds to my hypothesis. If the 20% methylated state seen in the gland samples is also considered, there is a significant increase in methylation over time, which correlates with the dramatic decrease in expression of PSP that is seen in the first twenty-four hours of culture. Although PSP is lacking a CpG island, and the number of CpG sites near the transcriptional start site is considerably lower than what is seen in Mist1, there could certainly still be a link between methylation of the CpG sites that are present and the loss of expression.

The correlation seen in PSP between the passage of time, the increase in methylation, and the decrease in expression agrees with my hypothesis. However, as mentioned, PSP did not have a CpG island. This may be an example of a gene where methylation of individual CpG sites control the loss of

expression, as opposed to global methylation changes. Again, further investigation of the promoter region of the PSP gene should be pursued to evaluate more CpG sites, but the sites evaluated in this study should definitely be considered as possible controlling sites, as there was a positive correlation with their methylation status and the loss of expression.

In conclusion, significant differences were not seen between levels of methylation in gland cells compared to cultured parotid cells for Mist1, which indicates that methylation of the CpG island in the Mist1 promoter region evaluated in this study is not a controlling factor in expression. There may be, however, other CpG islands outside of the 4,000 bp area examined here that play a controlling role in the expression of Mist1. Significant differences were seen between levels of methylation gland cells compared to cultured parotid cells for Parotid Secretory Protein, which supports the theory that epigenetic control of PSP expression is present. Further testing of both Mist1 and PSP should be completed before a true correlation is drawn, but this study is evidence that may contribute to the theory that individual CpG sites in the promoter regions are where epigenetic control occurs in the PSP gene. Overall, this experiment suggests that global DNA methylation is not the cause of de-differentiation of parotid acinar cells in culture, which is contrary to my hypothesis, but it does indicate that increases in methylation at individual CpG sites seen over time may well correlate to decreases in expression for the PSP gene.

Future studies to further elucidate the role of DNA methylation in controlling expression of PSP will require different approaches than those used in

this study. The recognition that epigenetic silencing may play a major role in tumor biology has led to studies involving demethylating agents, such as DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine) [52]. Demethylating agents like decitabine may be employed in the continued study of PSP to evaluate if expression can be maintained when methylation is being prevented.

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LIST OF ABBREVIATIONS

EGF - Epithelial growth factor
RA - Retinoic acid
T_3 - Triiodothyronine
FGF - Fibroblast growth factor
ITS – X - Insulin, transferrin, and selenium mixture
ELISA - Enzyme-linked immunosorbent assay
PBS - Phosphate buffered saline
TNF - Tumor necrosis factor
HBSS - Hank's Buffered Salt Solution
BSA - Bovine Serum Albumin
PBS - Phosphate buffered saline
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
TCF12 - Transcription Factor 12
SCM - Standard Control Media
RSM - Rat Serum Medium
CRS - Cystatin/Rat Serum Medium
RFT - RA/FGF/Trace Element Medium
CpG - Cytosine-Guanine dinucleotide
MBD - Methyl-CpG-binding domain proteins
PCR - Polymerase Chain Reaction
h - Hours
bp - Base pairs
wk - Week

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