MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION *IN VITRO* BY $P2Y_2R$ ACTIVATION

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By

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TABLE OF CONTENTS

ACKNOWLEDGEMENT……………………………………………………………...…ii

LIST OF ILLUSTRATIONS…………………………………………………..…….v

LIST OF TABLES……………………………………………………………….……vi

LIST OF ABBREVIATIONS…………………………………………………………vii

ABSTRACT…………………………………………………………………….……...ix

CHAPTER

1. INTRODUCTION……………………………………………………………….1

2. MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION *IN VITRO* BY $P2Y_2R$ ACTIVATION

Introduction………………………………………………................…………9

Materials and Methods…………………………………………….………..…11

Results……………………………………………………….……………..….13

Discussion………………………………………………….………...…..21

Future aims……………………………………………………………………….24

References ………………………………………..……..……..………..……26

LIST OF ILLUSTRATIONS

Figures Page

Chapter 1

1. Mammalian salivary gland structure……………………………………………………..7

Chapter 2

1. Proliferation assay showing UTP-induced increase in HSG cell proliferation……...16

2. Effect of an inhibitor of Src on UTP-stimulated proliferation……………..……..…17

3. Effect of an inhibitor of ADAM 10 & 17 on UTP-stimulated proliferation…..….…18

4. Effect of an inhibitor of PKC on UTP-stimulated proliferation………………......…19

5. Effect of an inhibitor of ERK1/2 on UTP-stimulated proliferation………………….20

LIST OF TABLES

Table Page

CHAPTER 1

1. Mammalian P2Y receptor subtypes and relative agonist potencies…....………………….8

LIST OF ABBREVIATIONS

ADAM 10&17	a disintegrin and metalloproteinas 10&17
AQP-1	aquaporin-1
ATP	adenosine 5'-triphosphate
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
ERK	extracellular signal-regulated kinase
HB-EGF	heparin-binding epithelial growth factor
HCAEC	human coronary artery endothelial cells
HSG	human salivary gland
IP_3	inositol 1,4,5-triphosphate
MAPK	mitogen-activated protein kinase
MMP	metalloprotease
$P2Y_2R$	$P2Y_2$ receptor
PKC	protein kinase C
PLC	phospholipase C
SMG	submandibular gland
SS	Sjögren's syndrome
TAPI-2	tumor necrosis factor-α protease inhibitor

TGFα	transforming growth factor-α
UTP	uridine 5’-triphosphate
VCAM	vascular cell adhesion molecule
VEGFR	vascular epithelial growth factor receptor

MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION BY $P2Y_2R$ ACTIVATION

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Dr. Gary A. Weisman, Thesis Supervisor

ABSTRACT

Sjögren's syndrome (SS) is an autoimmune disease in which exocrine glands including the salivary gland are targeted and destroyed by the immune system (Voulgarelis and Tzioufas, 2010). SS and the side-effects of γ-radiation therapies for head and neck cancers cause salivary gland dysfunction, in particular hyposalivation (Atkinson *et al.*, 2005; Fox, 1998; O'Sullivan and Higginson, 2010). Among the diverse therapeutic strategies for replacement of non-functional tissue, salivary gland regeneration has been considered to be a very promising approach for restoring saliva secretion. To regenerate a functional salivary gland in a clinical setting, it is first necessary to gain a better understanding of the mechanisms involved in salivary gland regeneration. Salivary gland regeneration requires several functions to be sequentially activated in salivary epithelial cells, including increases in cell proliferation, migration and differentiation (Patel *et al.*, 2006). This thesis describes our attempts to understand mechanisms that increase

proliferation of salivary epithelial cells. These studies focus on the proliferative role of extracellular nucleotides via the activation of the $P2Y_2$ nucleotide receptor in salivary gland epithelial cells.

Activation of $P2Y_2$Rs has been shown to stimulate proliferation in many cell types, such as smooth muscle cells (Shen *et al.*, 2004) and lung epithelial tumor cells (Schafer *et al.*, 2003). Our previous studies have shown that the $P2Y_2$R interacts with many signaling molecules, such as growth factor receptors, integrins and metalloproteases to regulate multiple signaling pathways involved in cell proliferation (Liao *et al.*, 2007; Liu *et al.*, 2004; Ratchford *et al.*, 2010; Seye *et al.*, 2004). These studies support the overall hypothesis that expression and activation of $P2Y_2$Rs in damaged or diseased salivary gland cells promotes cell proliferation. In this project, we demonstrate that activation of the $P2Y_2$R increases the proliferation of human salivary gland (HSG) epithelial cells *in vitro*. Other data indicate that inhibition of Src prevents $P2Y_2$R-mediated proliferation of HSG cells, consistent with previous findings in our lab demonstrating that the $P2Y_2$R contains 2 Src homology-3 binding domains in the C terminus that enable nucleotides to induce Src-dependent transactivation of the epidermal growth factor receptor (EGFR) (Liu *et al.*, 2004; Seye *et al.*, 2004). The data also indicate that inhibition of ERK1/2 prevents $P2Y_2$R-mediated proliferation of HSG cells. Thus, the current results suggest that activation of $P2Y_2$Rs by extracellular nucleotides promotes proliferation of HSG cells by stimulating Src-dependent signaling pathway and activating ERK1/2.

Accordingly, the $P2Y_2R$ represents a promising target for salivary gland regeneration.

CHAPTER 1

INTRODUCTION

Salivary gland dysfunction and therapies

In mammals, the salivary gland is mainly comprised of the submandibular, sublingual and parotid glands (Fig. 1). Sjögren's syndrome (SS) is an autoimmune disease in which exocrine glands including salivary glands are targeted and destroyed by the immune system (Voulgarelis and Tzioufas, 2010). SS affects 0.1-0.4% of the population, with women being affected at a greater rate than men (Voulgarelis and Tzioufas, 2010). SS is characterized by infiltration of exocrine glands by lymphocytes and leads to xerophthalmia and xerostomia of the lacrimal and salivary glands, respectively (Fox and Kang, 1992; Ramos-Casals *et al.*, 2005; Schrader *et al.*, 2005). Side effects of γ-radiation therapy for head and neck cancers also cause xerostomia. The symptoms of xerostomia include a significant increase in dental caries, bacteria and fungal infections, difficulties with chewing and swallowing, loss of taste, and marked oral dryness (Chiorini *et al.*, 2009). These symptoms decrease the patients' life quality significantly. Many therapeutic strategies have been utilized to treat salivary gland dysfunction. Generally, the majority of treatments are medications. Medicines such as pilocarpine, cevimeline and interferon α, some of which are agonists of the muscarinic receptor, are used to increase saliva secretion (Fox, 2004). The limitation of these medicines is that they just relieve the symptoms temporarily instead of improving salivary gland function.

Besides medications, some new treatments for xerostomia have been considered, such as gene therapy. Gene therapy can increase the expression of specific proteins involved in the saliva secretion process. For example, the aquaporin-1 (AQP-1) protein, which is a transmembrane water channel, has been expressed in damaged salivary glands of rats (Delporte *et al.*, 1997) and rhesus monkeys (O'Connell *et al.*, 1999) by *in vivo* gene therapy. This technique that utilizes adenoviral vector-mediated transfer of AQP-1 cDNA to damaged salivary glands induces increased saliva flow (Baum *et al.*, 2006). Recent studies using AQP-1 gene therapy showed its success in two pre-clinical models (irradiated rats and miniature pigs) (Baum *et al.*, 2009). However, saliva is a complex mixture that contains water, various enzymes and antibacterial compounds. Therefore, it is difficult for gene therapy to increase expression of proteins that regulate the secretion of all of these components.

Regeneration is another new therapeutic approach that can potentially restore the function of damaged salivary glands (Carpenter and Cotroneo, 2010). Regeneration of a salivary gland requires optimization of multiple cellular responses, including proliferation, migration, differentiation, apoptosis and cytoskeletal reorganization (Hoffman *et al.*, 1996; Patel *et al.*, 2006). Approaches in regeneration include increasing the proliferation and differentiation of residual cells in damaged salivary glands (Okazaki *et al.*, 2000), or the transplantation of stem cells to salivary glands *in vivo* (Kagami *et al.*, 2008).

Although the propagation regeneration of residual salivary gland cells is more straightforward than stem cell transplantation, if the salivary gland is severely damaged, transplantation of stem cells is required for regeneration. Stem cell therapies have shown promise in the regeneration of severely damaged salivary glands. Studies have transplanted isolated stem cells to irradiated salivary glands in mice and successfully increased saliva production and partially recovered salivary gland function (Kojima *et a*l., 2011; Lin *et al*., 2011; Nanduri *et al*., 2011). However, current approaches using stem cell therapy are not yet practical for the clinical regeneration of salivary glands (Carpenter and Cotroneo, 2010). Generally, the mechanisms underlying salivary gland regeneration still need to be clarified.

P2 receptors in the salivary gland

P2 receptors are receptors for extracellular nucleotides (Erb *et al.*, 2006) and belong to two categories: P2X and P2Y receptors. P2X receptors are ion channels gated by ATP. They mediate potassium efflux and sodium and calcium influx, which cause depolarization of the plasma membrane (Erb *et al.*, 2006). P2Y receptors are G protein-coupled receptors. They can be activated by purine and/or pyrimidine nucleotides (Table 1). Now, 7 P2X ($P2X_{1\text{-}7}$) receptors and 8 P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors have been cloned and characterized (Erb *et al.*, 2006).

In mammalian salivary glands, five subtypes of P2 receptors have been identified (Fig. 2),

including the $P2X_4$ and $P2X_7$ receptors, which are ATP-gated ion channels, and the $P2Y_1$, $P2Y_2$ and $P2Y_{15}$ receptors (Inbe *et al*., 2004; Turner *et al*., 1999). The $P2Y_1$ and $P2Y_2$ receptors expressed in salivary gland cells are coupled to G proteins that activate phospholipase C (PLC) and generate inositol 1,4,5-trisphosphate that mobilizes intracellular calcium stores to stimulate saliva secretion (Turner *et al*., 1999). The $P2Y_{15}$ receptor is coupled to G_o and G_i proteins to induce Ca^{2+} mobilization (Inbe *et al*., 2004)

$P2Y_2R$ expression levels are relatively low in normal salivary glands, but the $P2Y_2R$ is upregulated in models of stress and disease of the salivary gland, which include *in vitro* primary cell culture, *in vivo* SMG duct ligation and the NOB.10 mouse model of SS (Ahn *et al*., 2000; Schrader *et al*., 2005; Turner *et al*., 1997). The $P2Y_2R$ has been shown to play an important role in cell proliferation of many cell types, *e.g.*, it stimulates proliferation of coronary artery smooth muscle cells (Shen *et al.*, 2004), human lung epithelial cells (Schafer *et al.*, 2003), C6 glioma cells (Tu *et al.*, 2000), and MCF-7 breast cancer cells (Bilbao *et al*., 2010). In contrast, $P2Y_2R$ activation leads to apoptosis of colon carcinoma cells (Hopfner *et al.*, 2001). Overall, the consequences of $P2Y_2R$ upregulation in salivary glands are still not well understood.

$P2Y_2R$-mediated signaling pathways

The $P2Y_2R$ is a $G\alpha_{q/11}$ protein-coupled receptor that can be activated by ATP or UTP (Erb *et al*., 2006). Upon ATP or UTP stimulation, the $P2Y_2R$ activates PLC which

produces diacylglycerol to activate PKC and IP_3 to increase intracellular calcium mobilization leading to many cellular responses (Turner *et al.*, 1999). Our recent studies have shown that PKC activation by the $P2Y_2R$ leads to ERK1/2 phosphorylation (Ratchford *et al.*, 2010).

Independent of $G\alpha_{q/11}$ protein, the $P2Y_2R$ also activates other signaling molecules, including growth factor receptors, integrins and metalloproteases and their associated signaling pathways. Our laboratory has demonstrated that $P2Y_2R$ activation in human salivary gland epithelial cells can transactivate the EGFR by activating the matrix metalloproteases (MMPs) ADAM10 and ADAM17 (Ratchford *et al.*, 2010). ADAM10 and ADAM17 belong to the adamalysin family of metalloproteases, which are membrane-anchored glycoproteins (Sahin *et al.*, 2004). They have multiple functions, including catalysis of the shedding of membrane-bound proteins from cells (Pierce *et al.*, 2001; Sahin *et al.*, 2004). Our laboratory has shown that ADAMs in human salivary gland cells catalyze the release of neuregulin-1, the only known ErbB3 ligand, which causes the formation of ErbB3 and EGFR heterodimers, leading to the activation of both receptors (Ratchford *et al.*, 2010). The activation of the EGFR results in stimulation of the ERK/MAPK signaling cascade (Jorissen *et al.*, 2003). The EGFR and ERK/MAPK mediated signaling pathways play essential roles in cell proliferation (Oda *et al.*, 2005). The ADAM10 and ADAM17 dependent transactivation of EGFR by $P2Y_2R$ activation is a potential signaling pathway that regulates salivary gland cell proliferation.

It has been previously demonstrated by our group that the $P2Y_2R$ contains two SH3 binding sites (PXXP motifs) in its intracellular C-terminal domain which mediate the binding of Src and the Src-dependent transactivation of growth factor receptors (Liu *et al.*, 2004). In human coronary artery endothelial cells (HCAECs), the SH3 binding sites of the $P2Y_2R$ also mediate the Src-dependent transactivation of vascular endothelial growth factor receptor-2 (VEGFR-2) (Seye *et al.*, 2004), and the VEGFR-2-dependent upregulation of the cell adhesion molecule VCAM-1 that promotes monocyte binding to endothelium (Baker *et al.*, 2008b). Similar studies have shown that $P2Y_2R$ activation in human SMG cells mediates the binding of lymphocytes by EGFR-dependent upregulation of VCAM-1 (Baker *et al.*, 2008a). As stated above, EGFR is important to cell proliferation. Although the role of Src in the transactivation of EGFR after $P2Y_2R$ activation in salivary gland cells is not clear, it is still a potential pathway to regulate cell proliferation.

In summary, the role of the $P2Y_2R$ in salivary gland cell proliferation will be investigated in this project. Based on our previous studies, we will evaluate $P2Y_2R$ mediated signaling pathways for their possible involvement in salivary gland cell proliferation. These studies will help us understand the $P2Y_2R$ mediated signaling pathways involved in salivary gland regeneration and benefit the improvement of salivary gland regeneration therapy.

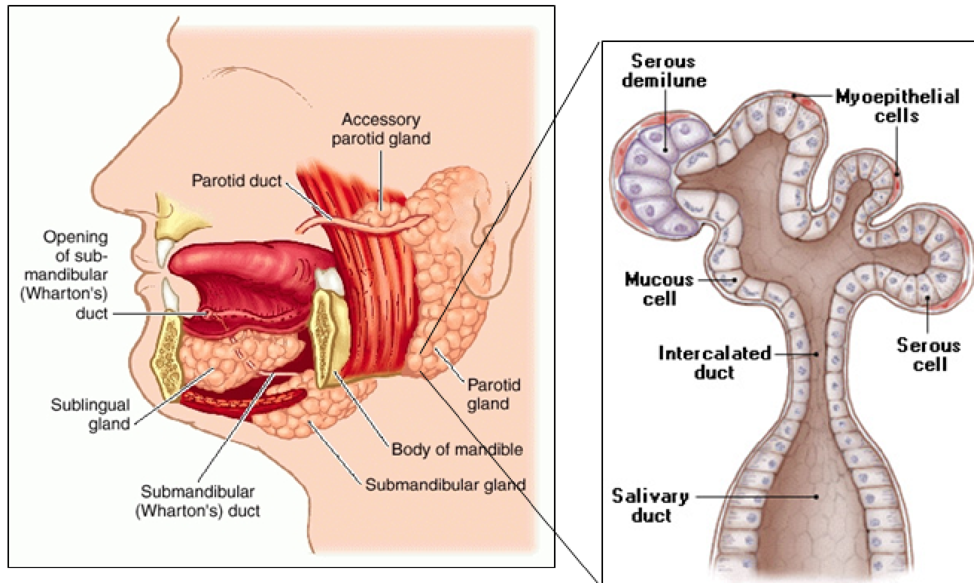

Figure 1. Mammalian salivary gland structure.

(Source: *http://medical-dictionary.thefreedictionary.com* *http://www.lib.mcg.edu*)

Mammalian salivary gland includes submandibular gland, sublingual gland and parotid gland. Each gland is comprised of acinar cells and duct cells.

P2Y Subtypes‡	Relative Agonist Potencies
$P2Y_1$ (P_{2Y}, P_{2T})	2MeSADP > ADP; UTP inactive
$P2Y_2$^{c} (P_{2U})	UTP = ATP >> 2MeSATP; ADP, UDP inactive
$P2Y_4$	UTP > ATP >> 2MeSATP
$P2Y_6$	UDP >> UTP > ADP = 2MeSATP > ATP
$P2Y_{11}$	ATP > 2MeSATP >> ADP; UTP, UDP inactive

‡ Harden *et al.*, 1997; Communi *et al.*, 1997; Weisman *et al.*, 1997.

Table 1. Mammalian P2Y receptor subtypes in salivary gland and relative agonist potencies (Turner *et al.*, 1999).

The P2Y receptors in mammalian salivary gland and their agonist potencies are shown in this table.

CHAPTER 2

MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION BY $P2Y_2R$ ACTIVATION

INTRODUCTION

We postulate that $P2Y_2Rs$ in dedifferentiated salivary gland cells are distributed throughout the plasma membrane where they interact with integrins, metalloproteases, growth factor receptors and cytoskeletal proteins to regulate multiple signaling pathways that potentially stimulate salivary gland regeneration (Giancotti and Ruoslahti, 1999; Ratchford *et al*., 2010; Weisman *et al*., 2005). Some studies have shown that these $P2Y_2R$-mediated signaling pathways regulate cell proliferation in many cell types, such as smooth muscle cells (Shen *et al*., 2004) and lung epithelial cells (Schafer *et al*., 2003). These results support the hypothesis that the expression and activation of $P2Y_2Rs$ in diseased or damaged salivary gland cells may promote regeneration by increasing cell proliferation. Accordingly, we quantified *in vitro* cell proliferation in the presence and absence of UTP, a $P2Y_2R$ ligand, to demonstrate that activation of the $P2Y_2R$ promotes salivary gland cell proliferation. In addition, we used chemical inhibitors of proteins downstream of the $P2Y_2R$ to determine the signaling pathways involved. These studies provide basic information that will be applied to optimizing regeneration of damaged

salivary glands.

Materials and Methods

Material--Dulbecco's modified Eagle's medium (DMEM), High Glucose and the PrestoBlue cell viability kit were purchased from Invitrogen. EGF, tumor necrosis factor-α protease inhibitor (TAPI-2) and PP2 were purchased from Calbiochem. UTP, GF109203X were purchased from Sigma-Aldrich. U0126 was purchased from Cell Signaling Technology.

Cell Culture--HSG cells were cultured in DMEM with 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell Viability Assay—HSG cells were seeded at 1×10^4 per well in Falcon 24-well plates and serum depleted for 24 h. Cells were then pretreated with inhibitors for 1 h and stimulated by different agonists for 48 h. After that, cell culture medium was removed, 500μl of cell viability test reagent was added (DMEM:PrestoBlue 10X reagent; 9:1), cells were incubated at 37°C for 1 h, and fluorescence was monitored at 560 nm (excitation) and 590 nm (emission) using EnSpire 2300 multilabel reader.

Data Analysis—Proliferation data are based on the fluorescence values obtained. The basal values were set at 100% and the effects of agents were expressed relative to basal values. For experiments with inhibitors, the inhibitor effects on $P2Y_2R$ activation were

compared to corresponding control groups. Statistical analysis was performed using GraphPad Prism 5.0. The means of two separate groups were compared by unpaired, two-tailed t test, where $P < 0.05$ was considered to be a significant difference.

RESULTS

Salivary gland regeneration requires proliferation of epithelial cells. We postulate that in undifferentiated salivary gland epithelial cells, upregulation and activation of $P2Y_2$Rs can stimulate signaling pathways that enhance cell proliferation. Human salivary gland (HSG) cells are a good cell line to study salivary gland proliferation, because the $P2Y_2$R is expressed in HSG cells (Yu and Turner, 1991) and the $P2Y_4$R and the $P2Y_6$R are either not expressed or have no physiological functions in HSG cells (Baker *et al.*, 2008b). Therefore, the possibility that the $P2Y_4$R or $P2Y_6$R are being activated can be excluded and observed results can be attributed to the $P2Y_2$R. The current study indicates that $P2Y_2$R activation by UTP stimulates HSG cell proliferation over 48 h, similar to the effect of EGF (Fig. 1). In response to UTP stimulation, HSG cell proliferation increased by about 25% compared to the basal control (Fig. 1).

Based on this result, we determined the $P2Y_2$R-mediated signaling pathway(s) responsible for the observed UTP-stimulated HSG cell proliferation. We focused on three $P2Y_2$R-related signaling pathways:

1. Our previous studies have shown that the $P2Y_2$R contains two Src homology 3 binding sites (PXXP motifs) in its intracellular C-terminal domain that mediate the binding of Src and the Src-dependent activation of growth factor receptors (Liu *et al.*, 2004; Seye *et al.*, 2003). In human coronary artery endothelial cells (HCAECs), the SH3 binding sites of

the $P2Y_2R$ mediate the Src-dependent transactivation of vascular endothelial growth factor receptor-2 (VEGFR-2) (Seye *et al.*, 2004).

2. The $P2Y_2R$ in HSG cells can activate ERK1/2 by a metalloprotease-dependent mechanism that promotes the release of the ErbB3 ligand, neuregulin-1, which leads to the formation of EGFR/ErbB3 heterodimers and the activation of both EGFR and ErbB3 (Ratchford *et al.*, 2010).

3. The $P2Y_2R$ in HSG cells can activate ERK1/2 by a fast PKC-dependent mechanism (Ratchford *et al.*, 2010). Activation of the $P2Y_2R$ by its agonist UTP induces the stimulation of PLC, thereby generating the second messengers inositol 1,4,5-trisphosphate and diacylglycerol that serve to increase calcium release from intracellular stores and activate PKC, respectively (Turner *et al.*, 1999).

To study whether these signaling pathways were involved in the observed UTP-stimulated HSG cell proliferation, selective inhibitors were used to inhibit these pathways and then test whether UTP-stimulated cell proliferation was changed.

HSG cells were pretreated with the Src inhibitor, PP2, and then UTP-induced cell proliferation was measured. PP2 decreased UTP-stimulated cell proliferation from about 135% to 110% (Fig. 2). The unpaired t-test shows that this reduction is statistically significant and suggests that Src plays an important role in UTP-stimulated cell proliferation.

Selective inhibitors of ADAM 10 & 17 (TAPI-2) and PKC (GF109203X) were used to study the involvement of these signaling pathways (Figs. 3 & 4). Figure 3 shows that the ADAM 10 & 17 inhibitor TAPI2 did not cause a significant decrease in UTP-stimulated HSG cell proliferation. GF109203X also did not cause a significant decrease in UTP-stimulated HSG cell proliferation. These results indicate that Src-mediated activation of growth factor receptors or other pathways in response to $P2Y_2R$ activation is more important for HSG proliferation than activation of metalloproteases or PKC.

The inhibitor U0126 was used to test the role of ERK1/2 in the observed proliferative response. UTP-stimulated HSG cell proliferation was significantly decreased by U0126, as compared to the UTP-treated control (Fig. 5). This result suggests that ERK1/2 plays an important role in UTP-stimulated HSG cell proliferation.

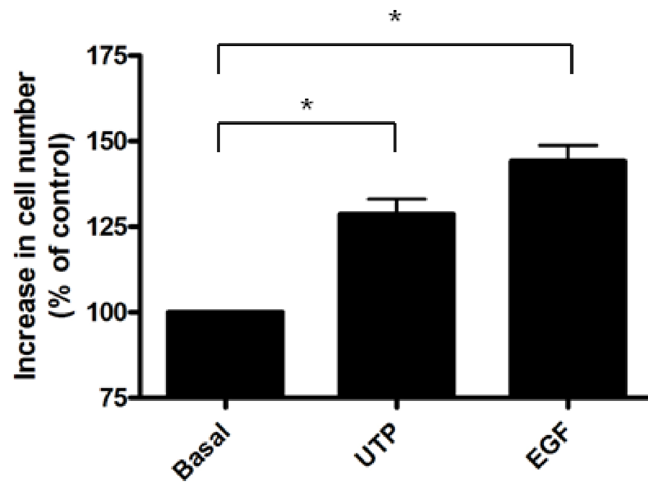

Figure 1. Proliferation assay showing UTP-induced increase in HSG cell proliferation. HSG cells were treated with or without UTP (100 μM) or EGF (100 ng/ml) for 48 h. Cell proliferation was measured by the PrestoBlue proliferation assay. Results are the means ± SEM of data from at least ten experiments. *, $p<0.05$ relatively.

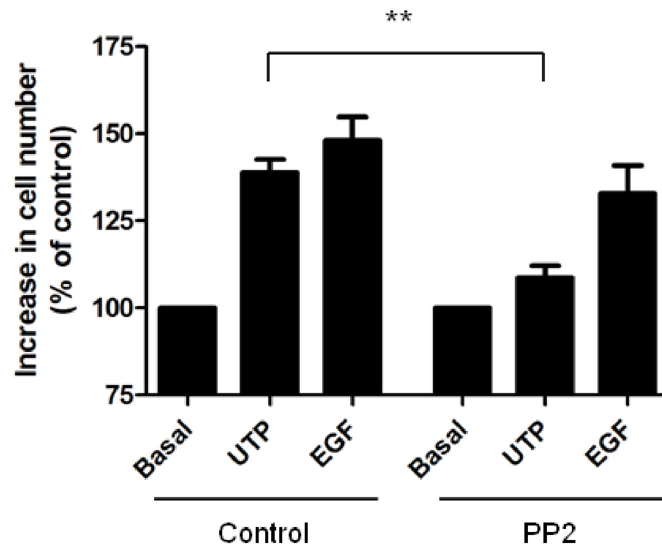

Figure 2. Effect of an inhibitor of Src on UTP-stimulated proliferation. HSG cells were pretreated with PP2 (2 μM) for 1 h, and then were treated with or without UTP (100 μM) or EGF (100 ng/ml) for 48 h. Cell proliferation was measured by the PrestoBlue cell viability assay. Results are the mean ± SEM of data from at least four experiments. **p<0.01.

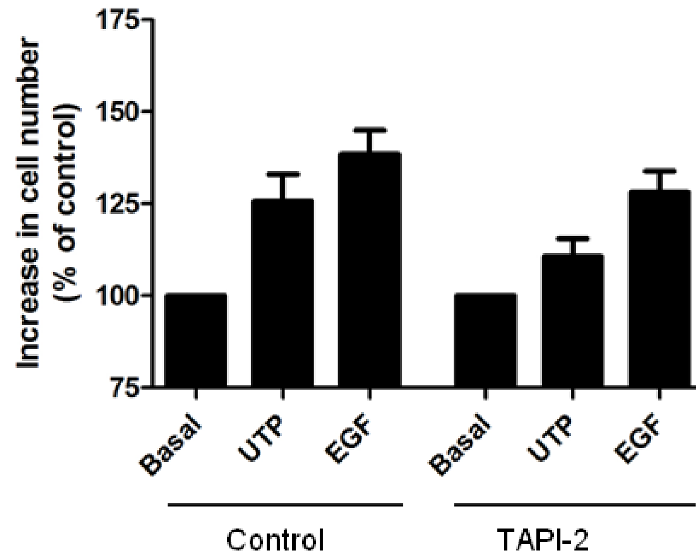

Figure 3. Effect of an inhibitor of ADAM 10 & 17 on UTP-stimulated proliferation. HSG cells were pretreated with TAPI-2 (10 μM) for 1 h, and then were treated with or without UTP (100 μM) or EGF (100 ng/ml) for 48 h. Cell proliferation was measured by the PrestoBlue cell viability assay. Results are the mean ± SEM of data from at least five experiments.

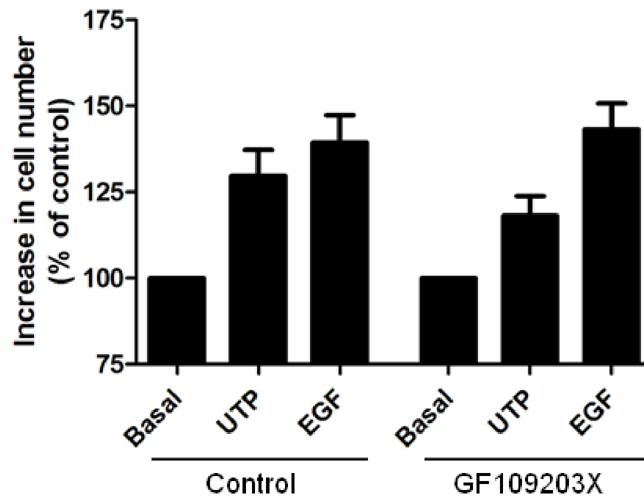

Figure 4. Effect of an inhibitor of PKC on UTP-stimulated proliferation. HSG cells were pretreated with GF109203X (10 μM) for 1 h, and then were treated with or without UTP (100 μM) or EGF (100 ng/ml) for 48 h. Cell proliferation was measured by the PrestoBlue cell viability assay. Results are the mean ± SEM of data from at least four experiments.

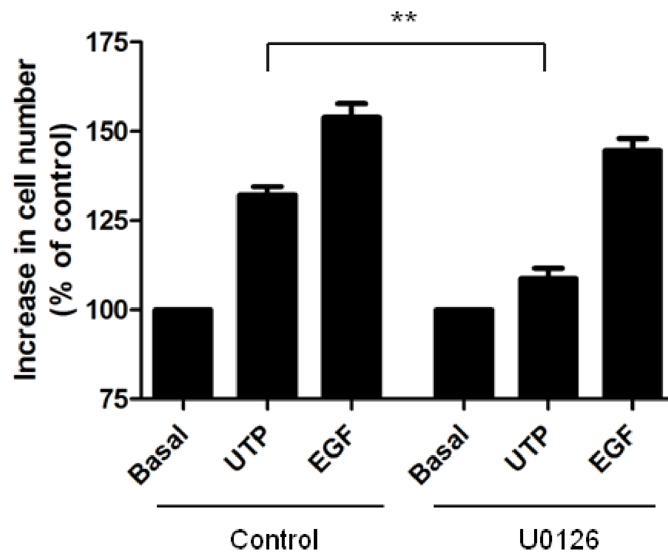

Figure 5. Effect of an inhibitor of ERK1/2 on UTP-stimulated proliferation. HSG cells were pretreated with U0126 (5 μM) for 1 h, and then were treated with or without UTP (100 μM) or EGF (100 ng/ml) for 48 h. Cell proliferation was measured by the PrestoBlue cell viability assay. Results are the mean ± SEM of data from at least three experiments. **p<0.01 relatively.

DISCUSSION

Cell proliferation is an important step during salivary gland regeneration. The $P2Y_2R$ has been shown to play important roles in the proliferation of many cell types, such as coronary artery smooth muscle cells (Shen *et al.*, 2004), human lung epithelial cells (Schafer *et al.*, 2003), C6 glioma cells (Tu *et al.*, 2000) and MCF-7 breast cancer cells (Wagstaff *et al.*, 2000). The current study focuses on the role of the $P2Y_2R$ in salivary gland cell proliferation.

First, we demonstrated that the $P2Y_2R$ agonist UTP can stimulate HSG cell proliferation (Fig. 1). Then, we demonstrated that inhibition of Src using PP2 significantly decreased UTP-stimulated HSG cell proliferation. This suggests that the interaction of the $P2Y_2R$ with Src stimulates a signaling pathway that promotes HSG cell proliferation. This result should be further confirmed using Src siRNA. Src has been shown to play an important role in the transactivation of VEGFR-2 in endothelial cells (Seye *et al.*, 2004), although the role of Src in transactivation of EGFR family members in epithelial cells has not been established (Ratchford *et al.*, 2010). The evaluation of mechanisms involving $P2Y_2R$-Src interactions in the proliferation of salivary epithelial cells requires further investigation.

Inhibition of metalloproteases with TAPI-2 and PKC with GF109203X, did not significantly decrease UTP-stimulated HSG cell proliferation. Previous studies have

shown that metalloprotease activity leads to stimulation of ErbB3 in HSG cells (Ratchford *et al*., 2010) and PKC is known to activate MAPKs (Ali *et al*., 2009). However, the reason of these non-significant cell proliferation decreases could be an incomplete inhibition of these pathways. Further studies are needed to determine whether the inhibitors of PKC and TAPI-2 completely inhibit their targets well before we can draw a firm conclusion.

Inhibition of ERK1/2 with U0126 significantly decreased UTP-stimulated HSG cell proliferation. This suggests that activation of ERK1/2 is responsible for UTP-stimulated HSG cell proliferation. The G-protein-coupled receptor (GPCR) has been shown to induce ERK activation through Src (Gavi *et al*., 2006; Keely *et al*., 2000; Luttrell *et al*., 1997). As the data also show that the inhibition of Src decreased UTP-stimulated HSG cell proliferation. It is possible that the $P2Y_2R$ promotes HSG cell proliferation via activation of ERK and this activation of ERK is mediated by Src. There was no significant effect of ERK inhibition on EGF proliferation, which suggests that multiple EGFR related signaling pathways are responsible for the EGF-stimulated cell proliferation. It has been shown that the activated epidermal growth factor receptor stimulates multiple signaling pathways in addition to ERK1/2 including JNK signaling pathways (Buzzi *et al*., 2009), phosphotidylinositol-3-kinase (PI3K) related pathways and phospholipase-Cγ1 (PLCγ1) related pathways (Kashimata *et al*., 2000). The JNK pathways has also been shown to stimulate cell proliferation (Carpenter and Cotroneo,

2010; Jaeschke *et al*., 2006) and the PI3K/Akt pathway has been shown to stimulated cell proliferation in smooth muscle cells (Wilden *et al*., 1998) and human glioma cells (Jacques-Silva *et al*., 2004) These are all possible signaling pathways that may have been responsible for the lack of an effect of ERK1/2 inhibition on proliferation in EGF stimulated cells.

In summary, the current data indicate that the $P2Y_2R$ agonist UTP stimulates HSG cell proliferation, which is decreased by the Src inhibitor PP2 and ERK1/2 inhibitor U0126. This suggests that interactions between the $P2Y_2R$ and Src, and the activation of ERK1/2 are required for UTP-stimulated HSG cell proliferation.

FUTURE AIMS

The future aims include the following studies:

Src siRNA will be used to inhibit Src and confirm the Src inhibitor studies showed above. Whether TAPI-2 and GF109203X inhibit their targets completely will be determined. Series of concentrations of TAPI-2 and GF109203X will be tried and the IC_{50} of these two inhibitors will be determined. These studies will help us draw firmer conclusions about the results showed in this thesis. The inhibitor of EGFR (AG1478) will also be used to determine whether UTP-stimulated HSG cell proliferation by Src is EGFR dependent.

An *In vivo* ligation model will be used to study the role of the $P2Y_2R$ during regeneration. Duct ligation causes salivary gland atrophy associated with the rapid loss of differentiated cell types, but retains progenitor cells (Carpenter and Cotroneo, 2010). Then, removal of the ligature induces spontaneous regeneration of salivary gland tissue. $P2Y_2$ receptor upregulation occurs in rat salivary glands *in vivo* 3 days after submandibular gland (SMG) duct ligation (Turner *et al*., 1997), and then is reduced to basal levels upon removal of the ligature (Ahn *et al*., 2000). Both wild type (WT) and $P2Y_2R$ knockout ($P2Y_2R$ KO) mice will be ligated and deligated to study whether the $P2Y_2R$ promotes regeneration. Effects on regeneration will be determined by weighing the glands, performing

immunohistochemistry experiments with regeneration markers, and collecting the saliva. Comparison of the differences between WT and $P2Y_2R$ KO mice in this *in vivo* study will help determine the role of the $P2Y_2R$ in salivary gland regeneration.

REFERENCES

Ahn, J.S., Camden, J.M., Schrader, A.M., Redman, R.S., and Turner, J.T. (2000). Reversible regulation of $P2Y_2$ nucleotide receptor expression in the duct-ligated rat submandibular gland. Am J Physiol Cell Physiol *279*, C286-294.

Ali, A.S., Ali, S., El-Rayes, B.F., Philip, P.A., and Sarkar, F.H. (2009). Exploitation of protein kinase C: a useful target for cancer therapy. Cancer Treat Rev *35*, 1-8.

Atkinson, J.C., Grisius, M., and Massey, W. (2005). Salivary hypofunction and xerostomia: diagnosis and treatment. Dent Clin North Am *49*, 309-326.

Baker, O.J., Camden, J.M., Redman, R.S., Jones, J.E., Seye, C.I., Erb, L., and Weisman, G.A. (2008a). Proinflammatory cytokines tumor necrosis factor-α and interferon-γ alter tight junction structure and function in the rat parotid gland Par-C10 cell line. Am J Physiol Cell Physiol *295*, C1191-1201.

Baker, O.J., Camden, J.M., Rome, D.E., Seye, C.I., and Weisman, G.A. (2008b). $P2Y_2$ nucleotide receptor activation up-regulates vascular cell adhesion molecule-1 expression and enhances lymphocyte adherence to a human submandibular gland cell line. Mol Immunol *45*, 65-75.

Baum, B.J., Zheng, C., Cotrim, A.P., Goldsmith, C.M., Atkinson, J.C., Brahim, J.S., Chiorini, J.A., Voutetakis, A., Leakan, R.A., Van Waes, C., *et al.* (2006). Transfer of the AQP1 cDNA for the correction of radiation-induced salivary hypofunction. Biochim Biophys Acta *1758*, 1071-1077.

Baum, B.J., Zheng, C., Cotrim, A.P., McCullagh, L., Goldsmith, C.M., Brahim, J.S., Atkinson, J.C., Turner, R.J., Liu, S., Nikolov, N., *et al.* (2009). Aquaporin-1 gene transfer to correct radiation-induced salivary hypofunction. Handb Exp Pharmacol, 403-418.

Bilbao, P.S., Santillan, G., and Boland, R. (2010). ATP stimulates the proliferation of MCF-7 cells through the PI3K/Akt signaling pathway. Arch Biochem Biophys *499*, 40-48.

Buzzi, N., Bilbao, P.S., Boland, R., and de Boland, A.R. (2009). Extracellular ATP activates MAP kinase cascades through a P2Y purinergic receptor in the human intestinal Caco-2 cell line. Biochim Biophys Acta *1790*, 1651-1659.

Carpenter, G.H., and Cotroneo, E. (2010). Salivary gland regeneration. Front Oral Biol *14*, 107-128.

Chiorini, J.A., Cihakova, D., Ouellette, C.E., and Caturegli, P. (2009). Sjögren syndrome: advances in the pathogenesis from animal models. J Autoimmun *33*, 190-196.

Delporte, C., O'Connell, B.C., He, X., Lancaster, H.E., O'Connell, A.C., Agre, P., and Baum, B.J. (1997). Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc Natl Acad Sci U S A *94*, 3268-3273.

Erb, L., Liao, Z., Seye, C.I., and Weisman, G.A. (2006). P2 receptors: intracellular signaling. Pflugers Arch *452*, 552-562.

Fox, P.C. (1998). Acquired salivary dysfunction. Drugs and radiation. Ann N Y Acad Sci *842*, 132-137.

Fox, P.C. (2004). Salivary enhancement therapies. Caries Res *38*, 241-246.

Fox, R.I., and Kang, H.I. (1992). Pathogenesis of Sjögren's syndrome. Rheum Dis Clin North Am *18*, 517-538.

Gavi, S., Shumay, E., Wang, H.Y., and Malbon, C.C. (2006). G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. Trends Endocrinol Metab *17*, 48-54.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028-1032.

Hoffman, M.P., Kibbey, M.C., Letterio, J.J., and Kleinman, H.K. (1996). Role of laminin-1 and TGF-β3 in acinar differentiation of a human submandibular gland cell line (HSG). J Cell Sci *109 (Pt 8)*, 2013-2021.

Hopfner, M., Maaser, K., Barthel, B., von Lampe, B., Hanski, C., Riecken, E.O., Zeitz, M., and Scherubl, H. (2001). Growth inhibition and apoptosis induced by $P2Y_2$ receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. Int J Colorectal Dis *16*, 154-166.

Inbe, H., Watanabe, S., Miyawaki, M., Tanabe, E., and Encinas, J.A. (2004). Identification and characterization of a cell-surface receptor, $P2Y_{15}$, for AMP and adenosine. J Biol Chem *279*, 19790-19799.

Jacques-Silva, M.C., Bernardi, A., Rodnight, R., and Lenz, G. (2004). ERK, PKC and PI3K/Akt pathways mediate extracellular ATP and adenosine-induced proliferation of U138-MG human glioma cell line. Oncology *67*, 450-459.

Jaeschke, A., Karasarides, M., Ventura, J.J., Ehrhardt, A., Zhang, C., Flavell, R.A., Shokat, K.M., and Davis, R.J. (2006). JNK2 is a positive regulator of the cJun transcription factor. Mol Cell *23*, 899-911.

Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W., and Burgess, A.W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res *284*, 31-53.

Kagami, H., Wang, S., and Hai, B. (2008). Restoring the function of salivary glands. Oral Dis *14*, 15-24.

Kashimata, M.W., Sakagami, H.W., and Gresik, E.W. (2000). Intracellular signalling cascades activated by the EGF receptor and/or by integrins, with potential relevance for branching morphogenesis of the fetal mouse submandibular gland. Eur J Morphol *38*, 269-275.

Keely, S.J., Calandrella, S.O., and Barrett, K.E. (2000). Carbachol-stimulated transactivation of epidermal growth factor receptor and mitogen-activated protein kinase in T_{84} cells is mediated by intracellular Ca^{2+}, PYK-2, and $p60^{src}$. J Biol Chem *275*, 12619-12625.

Kojima, T., Kanemaru, S., Hirano, S., Tateya, I., Ohno, S., Nakamura, T., and Ito, J. (2011). Regeneration of radiation damaged salivary glands with adipose-derived stromal cells. Laryngoscope *121*, 1864-1869.

Liao, Z., Seye, C.I., Weisman, G.A., and Erb, L. (2007). The $P2Y_2$ nucleotide receptor requires interaction with αv integrins to access and activate G_{12}. J Cell Sci *120*, 1654-1662.

Lin, C.Y., Chang, F.H., Chen, C.Y., Huang, C.Y., Hu, F.C., Huang, W.K., Ju, S.S., and Chen, M.H. (2011). Cell therapy for salivary gland regeneration. J Dent Res *90*, 341-346.

Liu, J., Liao, Z., Camden, J., Griffin, K.D., Garrad, R.C., Santiago-Perez, L.I., Gonzalez, F.A., Seye, C.I., Weisman, G.A., and Erb, L. (2004). Src homology 3 binding sites in the $P2Y_2$ nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. J Biol Chem *279*, 8212-8218.

Luttrell, L.M., Della Rocca, G.J., van Biesen, T., Luttrell, D.K., and Lefkowitz, R.J. (1997). Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J Biol Chem *272*, 4637-4644.

Nanduri, L.S., Maimets, M., Pringle, S.A., van der Zwaag, M., van Os, R.P., and Coppes, R.P. (2011). Regeneration of irradiated salivary glands with stem cell marker expressing cells. Radiother Oncol *99*, 367-372.

O'Connell, A.C., Baccaglini, L., Fox, P.C., O'Connell, B.C., Kenshalo, D., Oweisy, H., Hoque, A.T., Sun, D., Herscher, L.L., Braddon, V.R*., et al.* (1999). Safety and efficacy of adenovirus-mediated transfer of the human aquaporin-1 cDNA to irradiated parotid glands of non-human primates. Cancer Gene Ther *6*, 505-513.

O'Sullivan, E.M., and Higginson, I.J. (2010). Clinical effectiveness and safety of acupuncture in the treatment of irradiation-induced xerostomia in patients with head and neck cancer: a systematic review. Acupunct Med *28*, 191-199.

Oda, K., Matsuoka, Y., Funahashi, A., and Kitano, H. (2005). A comprehensive pathway map of epidermal

growth factor receptor signaling. Mol Syst Biol *1*, 2005 0010.

Okazaki, Y., Kagami, H., Hattori, T., Hishida, S., Shigetomi, T., and Ueda, M. (2000). Acceleration of rat salivary gland tissue repair by basic fibroblast growth factor. Arch Oral Biol *45*, 911-919.

Patel, V.N., Rebustini, I.T., and Hoffman, M.P. (2006). Salivary gland branching morphogenesis. Differentiation *74*, 349-364.

Pierce, K.L., Luttrell, L.M., and Lefkowitz, R.J. (2001). New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene *20*, 1532-1539.

Ramos-Casals, M., Loustaud-Ratti, V., De Vita, S., Zeher, M., Bosch, J.A., Toussirot, E., Medina, F., Rosas, J., Anaya, J.M., and Font, J. (2005). Sjögren syndrome associated with hepatitis C virus: a multicenter analysis of 137 cases. Medicine (Baltimore) *84*, 81-89.

Ratchford, A.M., Baker, O.J., Camden, J.M., Rikka, S., Petris, M.J., Seye, C.I., Erb, L., and Weisman, G.A. (2010). $P2Y_2$ nucleotide receptors mediate metalloprotease-dependent phosphorylation of epidermal growth factor receptor and ErbB3 in human salivary gland cells. J Biol Chem *285*, 7545-7555.

Sahin, U., Weskamp, G., Kelly, K., Zhou, H.M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P., and Blobel, C.P. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol *164*, 769-779.

Schafer, R., Sedehizade, F., Welte, T., and Reiser, G. (2003). ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. Am J Physiol Lung Cell Mol Physiol *285*, L376-385.

Schrader, A.M., Camden, J.M., and Weisman, G.A. (2005). $P2Y_2$ nucleotide receptor up-regulation in submandibular gland cells from the NOD.B10 mouse model of Sjögren's syndrome. Arch Oral Biol *50*, 533-540.

Seye, C.I., Yu, N., Gonzalez, F.A., Erb, L., and Weisman, G.A. (2004). The $P2Y_2$ nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). J Biol Chem *279*, 35679-35686.

Seye, C.I., Yu, N., Jain, R., Kong, Q., Minor, T., Newton, J., Erb, L., Gonzalez, F.A., and Weisman, G.A. (2003). The $P2Y_2$ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. J Biol Chem *278*, 24960-24965.

Shen, J., Seye, C.I., Wang, M., Weisman, G.A., Wilden, P.A., and Sturek, M. (2004). Cloning, up-regulation, and mitogenic role of porcine $P2Y_2$ receptor in coronary artery smooth muscle cells. Mol

Pharmacol *66*, 1265-1274.

Tu, M.T., Luo, S.F., Wang, C.C., Chien, C.S., Chiu, C.T., Lin, C.C., and Yang, C.M. (2000). $P2Y_2$ receptor-mediated proliferation of C_6 glioma cells via activation of Ras/Raf/MEK/MAPK pathway. Br J Pharmacol *129*, 1481-1489.

Turner, J.T., Landon, L.A., Gibbons, S.J., and Talamo, B.R. (1999). Salivary gland P2 nucleotide receptors. Crit Rev Oral Biol Med *10*, 210-224.

Turner, J.T., Weisman, G.A., and Camden, J.M. (1997). Upregulation of $P2Y_2$ nucleotide receptors in rat salivary gland cells during short-term culture. Am J Physiol *273*, C1100-1107.

Voulgarelis, M., and Tzioufas, A.G. (2010). Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome. Nat Rev Rheumatol *6*, 529-537.

Wagstaff, S.C., Bowler, W.B., Gallagher, J.A., and Hipskind, R.A. (2000). Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. Carcinogenesis *21*, 2175-2181.

Weisman, G.A., Wang, M., Kong, Q., Chorna, N.E., Neary, J.T., Sun, G.Y., Gonzalez, F.A., Seye, C.I., and Erb, L. (2005). Molecular determinants of $P2Y_2$ nucleotide receptor function: implications for proliferative and inflammatory pathways in astrocytes. Mol Neurobiol *31*, 169-183.

Wilden, P.A., Agazie, Y.M., Kaufman, R., and Halenda, S.P. (1998). ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. Am J Physiol *275*, H1209-1215.

Yu, H.X., and Turner, J.T. (1991). Functional studies in the human submandibular duct cell line, HSG-PA, suggest a second salivary gland receptor subtype for nucleotides. J Pharmacol Exp Ther *259*, 1344-1350.