MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION IN VITRO BY P2Y₂R ACTIVATION

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By
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MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION IN VITRO BY P2Y₂R ACTIVATION

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LIST OF ABBREVIATIONS

ADAM 10&17  a disintegrin and metalloproteinases 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
IP₃  inositol 1,4,5-triphosphate
MAPK  mitogen-activated protein kinase
MMP  metalloprotease
P2Y₂R  P2Y₂ receptor
PKC  protein kinase C
PLC  phospholipase C
SMG  submandibular gland
SS  Sjögren’s syndrome
TAPI-2  tumor necrosis factor-α protease inhibitor
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<th>Abbreviation</th>
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<tr>
<td>TGFα</td>
<td>transforming growth factor-α</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5’-triphosphate</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<tr>
<td>VEGFR</td>
<td>vascular epithelial growth factor receptor</td>
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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$_2$R 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 al., 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 (P2X1-7) receptors and 8 P2Y (P2Y1,2,4,6,11,12,13,14) 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 P2X₄ and P2X₇ receptors, which are ATP-gated ion channels, and the P2Y₁, P2Y₂ and P2Y₁₅ receptors (Inbe et al., 2004; Turner et al., 1999). The P2Y₁ and P2Y₂ 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 P2Y₁₅ receptor is coupled to Gₒ and Gᵢ proteins to induce Ca²⁺ mobilization (Inbe et al., 2004).

P2Y₂R expression levels are relatively low in normal salivary glands, but the P2Y₂R 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 P2Y₂R 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, P2Y₂R activation leads to apoptosis of colon carcinoma cells (Hopfner et al., 2001). Overall, the consequences of P2Y₂R upregulation in salivary glands are still not well understood.

**P2Y₂R-mediated signaling pathways**

The P2Y₂R is a Gₒ₁₉/₁₁ protein-coupled receptor that can be activated by ATP or UTP (Erb et al., 2006). Upon ATP or UTP stimulation, the P2Y₂R 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$_2$R leads to ERK1/2 phosphorylation (Ratchford et al., 2010).

Independent of Ga$_{q/11}$ protein, the P2Y$_2$R also activates other signaling molecules, including growth factor receptors, integrins and metalloproteases and their associated signaling pathways. Our laboratory has demonstrated that P2Y$_2$R 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$_2$R activation is a potential signaling pathway that regulates salivary gland cell proliferation.
It has been previously demonstrated by our group that the P2Y$_2$R 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$_2$R 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$_2$R 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$_2$R 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$_2$R in salivary gland cell proliferation will be investigated in this project. Based on our previous studies, we will evaluate P2Y$_2$R mediated signaling pathways for their possible involvement in salivary gland cell proliferation. These studies will help us understand the P2Y$_2$R mediated signaling pathways involved in salivary gland regeneration and benefit the improvement of salivary gland regeneration therapy.
Figure 1. Mammalian salivary gland structure.
Mammalian salivary gland includes submandibular gland, sublingual gland and parotid gland. Each gland is comprised of acinar cells and duct cells.
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.

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<thead>
<tr>
<th>P2Y Subtypes†</th>
<th>Relative Agonist Potencies</th>
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<tr>
<td>P2Y₁ (P₂y₁, P₂y₁)</td>
<td>2MeSADP &gt; ADP; UTP inactive</td>
</tr>
<tr>
<td>P2Y₂ (P₂y₂)</td>
<td>UTP = ATP &gt;&gt; 2MeSATP; ADP, UDP inactive</td>
</tr>
<tr>
<td>P2Y₄</td>
<td>UTP &gt; ATP &gt;&gt; 2MeSATP</td>
</tr>
<tr>
<td>P2Y₆</td>
<td>UDP &gt;&gt; UTP &gt; ADP = 2MeSATP &gt; ATP</td>
</tr>
<tr>
<td>P2Y₁₁</td>
<td>ATP &gt; 2MeSATP &gt;&gt; ADP; UTP, UDP inactive</td>
</tr>
</tbody>
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† Harden et al., 1997; Communi et al., 1997; Weisman et al., 1997.
CHAPTER 2
MECHANISMS OF SALIVARY GLAND CELL PROLIFERATION BY P2Y$_2$R ACTIVATION

INTRODUCTION

We postulate that P2Y$_2$Rs 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$_2$R-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$_2$Rs 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$_2$R ligand, to demonstrate that activation of the P2Y$_2$R promotes salivary gland cell proliferation. In addition, we used chemical inhibitors of proteins downstream of the P2Y$_2$R 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% CO₂.

Cell Viability Assay—HSG cells were seeded at 1×10⁴ 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 P2Y₂R 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 P2Y2Rs 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 P2Y2R is expressed in HSG cells (Yu and Turner, 1991) and the P2Y4R and the P2Y6R are either not expressed or have no physiological functions in HSG cells (Baker et al., 2008b).

Therefore, the possibility that the P2Y4R or P2Y6R are being activated can be excluded and observed results can be attributed to the P2Y2R. The current study indicates that P2Y2R 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 P2Y2R-mediated signaling pathway(s) responsible for the observed UTP-stimulated HSG cell proliferation. We focused on three P2Y2R-related signaling pathways:

1. Our previous studies have shown that the P2Y2R 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 P2Y2R mediate the Src-dependent transactivation of vascular endothelial growth factor receptor-2 (VEGFR-2) (Seye et al., 2004).

2. The P2Y2R 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 P2Y2R in HSG cells can activate ERK1/2 by a fast PKC-dependent mechanism (Ratchford et al., 2010). Activation of the P2Y2R 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$_2$R 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$_2$R in salivary gland cell proliferation.

First, we demonstrated that the P2Y$_2$R 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$_2$R 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$_2$R-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 P2Y2R 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,
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$_2$R 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$_2$R 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$_2$R 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$_2$R knockout (P2Y$_2$R KO) mice will be ligated and deligated to study whether the P2Y$_2$R 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$_2$R KO mice in this {	extit{in vivo}} study will help determine the role of the P2Y$_2$R in salivary gland regeneration.
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