The Role of the P2Y₂ Nucleotide Receptor in Salivary Gland Regeneration

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By: Farid G. El-Sayed
Dissertation Advisor: Dr. Gary A. Weisman
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The undersigned, appointed by the Dean of the Graduate Faculty, have examined the dissertation entitled:

**“The Role of the P2Y₂ Nucleotide Receptor in Salivary Gland
Regeneration”**

presented by Farid G. El-Sayed, a candidate for the degree of Doctor of Philosophy, and hereby certify that in their opinion it is worthy of acceptance.

_____ Dr. William Durante

_____ Dr. Michael Petris

_____ Dr. Laurie Erb

_____ Dr. Gary A. Weisman

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"The dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, sometimes poking you with a sharp stick called 'truth'."

- Dan Rather

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular free calcium concentration
ADAM	a disintegrin and metalloproteinases
Akt	protein kinase B
ANOVA	analysis of variance
AQP5	aquaporin 5
ATP	adenosine 5'-triphosphate
Bcl-2	B-cell lymphoma 2
BMDC	bone-marrow-derived cell
BSA	bovine serum albumin
Cdc42	cell division control protein 42 homolog
cDNA	complementary DNA
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GAP	GTPase activating protein
GDP	guanosine 5'-diphosphate
GEF	guanine nucleotide exchange factors
GFP	green fluorescent protein
GFR	growth factor receptor
GFR Matrigel	growth factor reduced Matrigel
GPCR	G protein-coupled receptor
GTP	guanosine 5'-triphosphate
HCAEC	human coronary artery endothelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSG	human salivary gland
IACUC	Institutional Animal Care and Use Committee
IP ₃	inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase

MMP	matrix metalloproteinase
MP	metalloproteinase
mRNA	messenger RNA
NOD	non-obese diabetic
P2Y ₂ R	P2Y ₂ nucleotide receptor
P2Y ₄ R	P2Y ₄ nucleotide receptor
P2Y ₆ R	P2Y ₆ nucleotide receptor
PAK1	p21-activated kinase-1
PBD	p21-binding domain
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PTB	phosphotyrosine-binding domain
Rac	Ras-related C3 botulinum toxin substrate
RGD	arginine-glycine-aspartic acid
Rho	Ras homology gene family

RT-PCR	real-time-polymerase chain reaction
RTK	receptor tyrosine kinase
S.E.M.	standard error of the mean
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGP	salivary gland-derived progenitor
SH2	Src homology 2
SH3	Src homology 3
SMG	submandibular gland
SS	Sjögren's syndrome
TBST	tris-buffered saline Tween-20
UDP	uridine 5'-triphosphate
UTP	uridine 5'-triphosphate
VEGFR-2	vascular endothelial growth factor receptor 2

The role of the P2Y₂ nucleotide receptor in salivary gland regeneration

Farid G. El-Sayed

Dr. Gary A. Weisman, Dissertation Supervisor

ABSTRACT

Salivary gland dysfunction affects millions of Americans whose quality of life is severely impacted by dry mouth, oral bacterial infections, poor nutrition, and other disorders that are associated with decreased saliva production. Over the past decade, progress has been made in cell-based reconstitution strategies for salivary glands. Understanding the mechanisms and signaling pathways that regulate the salivary gland reconstitution process is a necessity to enhance the ongoing efforts to develop better regenerative therapies for damaged salivary glands. The P2Y₂ nucleotide receptor (P2Y₂R), a G protein-coupled receptor equipotently activated by ATP and UTP, is upregulated in a variety of tissues, including salivary gland epithelium, in response to injury or stress and is proposed to play important roles in the regeneration of a variety of tissues. The results presented in this dissertation indicate that P2Y₂R activation with UTP enhances the migration, aggregation and self-organization of dispersed salivary epithelial cells forming spheres that display characteristics similar to differentiated acini in salivary glands. Moreover, our data suggest that the aforementioned P2Y₂R-mediated responses depend on the transactivation of the epidermal growth factor receptor (EGFR) via a disintegrin and metalloproteinases (ADAM10/ADAM17) and the $\alpha_5\beta_1$ integrin/Cdc42 Rho GTPase signaling pathway. This study sheds light on the P2Y₂R as a target in

salivary gland reconstitution strategies and introduces $\alpha_5\beta_1$ integrin and Cdc42 as novel downstream components in the P2Y₂R-mediated signaling network. Future studies will optimize the activation of P2Y₂R-mediated signaling pathways to promote the self-organization of salivary epithelial cells into acinar-like spheres that secrete saliva components and have utility for replacement of salivary gland tissue damaged by autoimmune disease or radiation therapy used to treat head and neck cancers.

CHAPTER I

Introduction

A. Salivary gland structure, function and dysfunction

Salivary glands are exocrine glands composed of multiple secretory endpieces called acini, each of which is formed of a cluster of cells (1). Acini can be formed of serous cells, which secrete a watery fluid, essentially devoid of mucus, or mucous cells, which produce a mucus-rich secretion (1). These acini are linked to the oral cavity via a system of ducts including intercalated ducts, striated ducts and a main excretory duct (1).

Almost 90% of the total amount of saliva is secreted from three major salivary glands: the parotid, submandibular and sublingual glands (2). The rest of the saliva (approximately 10%) is secreted from minor salivary glands (2). Saliva is composed mainly of water, electrolytes and numerous secretory proteins (1). Activation of M3 muscarinic receptors at the basolateral membrane of the acini stimulates apical Ca^{2+} -dependent Cl^- channels as well as basolateral K^+ channels, which leads to the efflux of Cl^- and K^+ (3). Cl^- and K^+ efflux generates a transepithelial potential difference (PD) that drives Na^+ transport and water diffusion across the tight junctions between epithelial cells, producing isotonic saliva in the lumen (3). As the saliva moves through the ducts towards the oral cavity, it becomes more hypotonic due to the reabsorption of Na^+ and the secretion of large amounts of K^+ and HCO_3^- (3). In addition to water and electrolytes, saliva is composed of secretory proteins (1, 4). The secretory proteins are synthesized in the rough endoplasmic reticulum and then transported to the Golgi apparatus, where the

secretory granules are formed. From the mature *trans*-Golgi network, secretory granules are transported to the acini. In response to the activation of β -adrenergic receptors, secretory granules discharge their protein content into saliva (5).

Hence, saliva provides the oral cavity with water, electrolytes and a variety of salivary proteins, including lubricants, antibacterials, antifungals, antivirals, anticarcinogens, remineralization agents, digestive enzymes, proteases, antiproteases and growth factors (4, 6). Saliva performs many protective and physiological functions in large part due to its aforementioned constituent proteins (4, 6). Saliva cleans and lubricates oral mucosal surfaces, buffers acids, provides antimicrobial protection, protects the enamel surface, initiates the digestion of carbohydrates through the action of amylase, and solubilizes food for the proper functioning of taste buds (4, 6). Consequently, the loss of normal salivary gland function, primarily resulting from irradiation therapy for head and neck cancers or Sjögren's syndrome (SS), an autoimmune exocrinopathy of unknown etiology characterized by lymphocytic infiltration of the salivary gland and ultimately tissue degeneration, can result in widespread deterioration of oral health that seriously affects the quality of life of patients and may lead to progression of lymphomas and other autoimmune diseases (7-9).

B. Current therapies for hyposalivation

Current treatments for hyposalivation are limited to saliva substitutes in the form of gels or sprays and medications that increase the overall fluid output, such as the parasympathomimetic secretagogues pilocarpine and cevimeline, which induce saliva secretion from residual salivary gland cells due to muscarinic receptor activation. These

treatments are largely ineffective due to their transient nature or systemic side effects that are unendurable for many patients (10, 11). In addition, these treatments do not address the underlying inflammatory processes associated with SS or the loss of functional acinar cells that follows radiation therapy, and consequently have very limited success in patients with advanced salivary dysfunction (10, 11). In contrast, regenerative therapy can provide an effective radical cure that overcomes the limitations and inefficiency of current treatments.

C. Regenerative therapy to regain salivary gland function

It has been shown that salivary glands lose acini after ligation of the main excretory duct in rodent models, but the glands have a very high potential to regenerate after deligation (12-14). Ligation of the main excretory duct leads to atrophy of the salivary gland due to apoptosis of the differentiated acinar cells. This likely occurs via the Fas ligand signaling pathway and activation of caspases 3 and 8 (15). The undifferentiated ductal cells, particularly intercalated duct cells, resist apoptosis by overexpressing the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) (16). Upon deligation, new acini differentiate from the remaining ductal cells. Subsequently, the number of acinar cells increases and the parasympathetic nerves are re-attached, resulting in almost complete restoration of salivary function and control levels of saliva secretion with normal ion and protein content (16).

Different experimental approaches have been pursued to restore salivary gland function following injury. Gene therapy has been considered to augment the expression of proteins involved in saliva secretion, such as the water channel aquaporin 5 (AQP5)

(17-19). Stem cell therapy seems to be a promising approach where the transplantation of bone-marrow-derived cells (BMDCs) has been shown to effectively maintain salivary gland function after γ -irradiation in mice, as indicated by salivary flow rate, epidermal growth factor (EGF) levels in saliva, limited apoptotic activity and normal acinar cell area in the irradiated glands (20). An alternative approach to regain the function of salivary glands is to induce the proliferation, migration and differentiation of residual cells in the damaged salivary glands to promote tissue regeneration (21). This approach can be further applied to bioengineer artificial salivary glands that closely resemble the native organ in both structure and function (21).

D. Cell-based salivary gland reconstitution

To provide insights into the regeneration process, tissue reconstitution studies have been performed to determine the ability of dispersed cells or tissue fragments to reassemble and self-organize into structures that resemble the native organ with regards to differentiation markers and/or functional responses. Over the past six decades, tissue reconstitution studies have been done using a variety of experimental models, including chick embryo cells (22), sea urchin cells (23, 24) and mammary epithelial cells (25, 26). The ability of dispersed salivary epithelial cells to reassemble and self-organize into acinar-like spheres has been tested using salivary gland tissue isolated from embryonic mice (27) and humans (28) or human salivary gland progenitor (SGP) cells (29). Salivary tissue reconstitution studies demonstrated the ability of dissociated cells to migrate towards each other and self-organize into acinar-like aggregates with structural features and differentiation markers that resemble the native gland. However, the mechanisms and

signaling pathways underlying salivary tissue reconstitution and the formation of acinar-like aggregates are not well understood. Cellular mechanisms and components that enhance the formation of these acinar-like aggregates would likely be important factors in salivary gland reconstitution and regeneration.

E. P2Y₂ nucleotide receptor

In this study, we investigated the role of the G_q protein-coupled P2Y₂ nucleotide receptor (P2Y₂R) in salivary gland reconstitution/regeneration. The P2Y₂R is a member of the P2 nucleotide receptor family that is activated by extracellular nucleotides, such as adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) (30, 31). P2 receptors are classified in two categories: P2X receptors that are ATP-gated ion channels and G protein-coupled P2Y receptors (30, 31). Currently, seven P2X receptors (P2X₁₋₇) and eight P2Y receptors (P2Y_{1, 2, 4, 6, 11-14}) have been cloned (30). Of these, only P2Y_{2, 4} and 6 receptors can be activated by uridine nucleotides (30).

F. P2Y₂ nucleotide receptor and tissue repair

In response to injury, stress or pro-inflammatory cytokines, the P2Y₂R is upregulated in a variety of tissues (32-35), including salivary gland epithelium (36-38). Previous findings have suggested roles for the P2Y₂R in corneal epithelia wound healing by triggering cell migration (39), liver regeneration by promoting hepatocyte proliferation (40), inflammatory bowel disease by enhancing epithelial repair (41), intestinal re-epithelialization following experimental colitis (42) and reduction of infarct size

following myocardial infarction (43). In addition, the P2Y₂R is upregulated upon disruption of salivary gland tissue homeostasis (37) and in salivary glands of the NOD.B10 mouse model of SS-like autoimmune exocrinopathy (38). In a classic model of salivary gland regeneration, rat submandibular gland (SMG) duct ligation for three days results in the atrophy of the majority of acinar cells, which is associated with increases in the expression and activity of the P2Y₂R. Fourteen days after duct deligation, P2Y₂R expression and activity return to basal levels as the tissue recovers to normal morphology and function (36). Collectively, these findings suggest that P2Y₂R upregulation plays a role in salivary gland regeneration.

G. P2Y₂ nucleotide receptor-mediated signaling pathways

The P2Y₂R interacts with diverse signaling networks via several structural motifs (30, 31) (Figure I-1). The P2Y₂R regulates G_q protein-dependent activation of phospholipase C (PLC) via its intracellular loops. PLC activation generates inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which act as secondary messengers to trigger intracellular calcium mobilization and protein kinase C (PKC) activation, respectively (30, 31). Moreover, the P2Y₂R has two Src homology 3 (SH3) binding domains in its C-terminus that promote Src activation that can initiate the transactivation of receptor tyrosine kinases (RTKs), including growth factor receptors (GFRs) such as EGF receptor family members (EGFR) (30, 44). In addition to Src-dependent mechanisms, the P2Y₂R has been shown to transactivate the EGFR through a disintegrin and metalloproteinases ADAM10/ADAM17 (45).

- **Growth factor receptors (ErbB family)**

Growth factor receptors are a family of receptor tyrosine kinases comprised of four homologous members: ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (46). In this class of receptors, signal transduction is initiated through the binding of growth factors to the extracellular domain of the receptor, which activates the intracellular kinase domain leading to autophosphorylation of tyrosine residues (46). Proteins containing Src homology 2 (SH2) binding and phosphotyrosine binding (PTB) domains dock at the phosphorylated sites leading to the propagation of growth signaling through a variety of pathways, including the mitogen-activated protein kinases (MAPK) (46). Growth factor receptor stimulation regulates numerous physiological processes, including cell proliferation, migration and differentiation (46).

The P2Y₂R has been shown to transactivate various GFRs, including the EGFR (44, 45, 47, 48) and the platelet-derived growth factor receptor (PDGFR) (44), through Src- or metalloprotease-dependent mechanisms, to regulate physiological responses, including cell migration and tissue repair. The P2Y₂R also has been shown to induce the phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR-2) in human coronary artery endothelial cells (HCAECs) to generate inflammatory responses (49). In human salivary gland (HSG) cells, the P2Y₂R has been shown to transactivate the EGFR via the activation of the metalloproteinases ADAM10/ADAM17 (45).

- **A disintegrin and metalloproteinases (ADAMs)**

ADAMs represent a member of the metzincin family of metalloproteinases that includes matrix metalloproteinases (MMPs) and astacins (50). ADAMs

consist of an extracellular domain with an N-terminal prodomain that is removed during maturation, a metalloproteinase (MP) domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic domain that comprises phosphorylation sites or proline-rich regions with SH3 domains (50, 51). ADAMs' characteristic domain structure enables them to perform adhesive functions as well as proteolytic functions (52) to mediate the shedding of transmembrane proteins, such as GFR ligands, leading to the release of soluble extracellular domains (50, 53, 54). The released extracellular domain may bind to certain cell surface receptors, such as GFRs, either in the same cell or other cells leading to the initiation of autocrine or paracrine extracellular signaling cascades, respectively (50, 53, 54). The membrane bound fragment can be further processed through γ -secretase activity leading to the intracellular release of soluble cleavage products, which can modulate intracellular signaling (50, 55-57). ADAM-mediated shedding of transmembrane proteins can also provide a mechanism for downregulating cell surface proteins (50).

In addition to the SH3-binding domains that mediate the transactivation of GFRs, the P2Y₂R has an Arg-Gly-Asp (RGD) sequence in its first extracellular loop (58) (Figure I-1) through which it can interact with a class of integrins called RGD-binding integrins, which leads to activation of the Rho and Rac GTPases and cytoskeletal rearrangements (30, 59, 60).

- **Integrins**

Integrins are cell adhesion receptors composed of heterodimers of α and β subunits (61-63). Different combinations of 18 α subunits and 8 β subunits form 24 different integrins (61-63). Upon ligand binding, integrins undergo a conformational change from the inactive form to a more extended active form (62-64). Integrins have large extracellular domains and generally short intracellular domains (62). On the extracellular face, integrins bind to the extracellular matrix (ECM) and other cell surface receptors. On the cytoplasmic side, integrins bind to cytoskeletal polymers and many signaling complexes that allow integrins to be involved in intracellular signaling. Integrins signal bidirectionally controlling diverse cell processes and functions (61, 62). Integrins are able to bind to a wide variety of ligands, including collagen, laminin and RGD-containing molecules (61). Upon ligand binding, integrins act as classical receptors, transmitting signals from outside to inside the cell (62, 65-67). Outside-in integrin signaling regulates cell polarity, survival, proliferation and the expression of a variety of genes (62, 65-67). On the other hand, integrins can transmit signals from inside to outside the cell upon talin binding (62, 65, 68). Inside-out signaling regulates extracellular matrix assembly, cell adhesion and cell migration (62, 65, 68).

Through an RGD-sequence in its first extracellular loop, the P2Y₂R is able to interact with RGD-binding integrins. Although interactions between the P2Y₂R and the $\alpha_v\beta_{3/5}$ RGD-binding integrins have been reported to regulate cell migration (60), interactions between the P2Y₂R and other RGD-binding integrins have not been investigated to date. Among the RGD-binding integrins that can be

potential targets of interest in salivary gland regeneration is the $\alpha_5\beta_1$ integrin, the major receptor for the extracellular matrix protein fibronectin that has been shown to be crucial for salivary gland morphogenesis (69).

- **Rho GTPases**

Rho GTPases are small monomeric GTPases that constitute one of the five families of the Ras superfamily (70, 71). Rho GTPases act as molecular switches by cycling between an inactive guanosine 5'-diphosphate- (GDP-)bound form and an active guanosine 5'-triphosphate- (GTP-)bound form to control a variety of signal transduction pathways (70, 72). Guanine nucleotide exchange factors (GEFs) catalyze GDP hydrolysis, which allows GTP that has a higher intracellular concentration than GDP to bind. In their active GTP-bound state, Rho GTPases bind to target (effector) proteins to regulate various cellular responses, including cell adhesion and migration, primarily through regulating the assembly and disassembly of actin cytoskeleton (72, 73). Eventually, GTPase activating proteins (GAPs) enhance the intrinsic GTP hydrolyzing activity of the Rho proteins, which completes the cycle and switches Rho GTPases back to their inactive GDP-bound form (72, 74, 75).

Rho, Rac and Cdc42 are three members of the Rho GTPases family, each controlling a distinct signaling pathway that provides a link between membrane receptors, including integrins, and actin cytoskeleton to regulate cell migration (72). Rho regulates the formation of stress fibers and focal adhesions, Rac induces the formation of lamellipodia and membrane ruffles, whereas Cdc42 triggers the formation of filopodia at the cell periphery (72, 76). There is a reciprocal balance

between Rac/Cdc42 and Rho activities, where Rac/Cdc42 activation has been shown to downregulate Rho activity in many cell types (72, 76, 77).

Our group has previously shown that P2Y₂R/ α_v integrin interactions regulate astrocytoma cell migration through the downstream activation of Rac and Rho via the GEF Vav2 (60). However, Cdc42 has not been previously reported to be a downstream component of the P2Y₂R signaling network.

Transactivation of the GFRs and the integrin signaling pathways by the P2Y₂R also leads to the activation of MAPKs, such as c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (78, 79). Upon activation, JNK and ERK1/2 shuttle to the nucleus and regulate the expression of genes involved in cell proliferation, migration, differentiation (80) and tissue regeneration including in the salivary gland (81-95).

- **Mitogen activated protein kinases (MAPKs)**

MAPKs comprise a family of cytoplasmic Ser/Thr kinases that participate in transmitting extracellular signals from membrane receptors through a sequence of phosphorylation events to elicit a wide range of cellular responses (96-99).

Conventional MAPKs include ERK (1/2), JNK (1/2/3) and p38 ($\alpha/\beta/\gamma/\delta$), crucial players in regulating cell proliferation, migration and differentiation during embryogenesis as well as tissue repair following injury in adults (96-99). The role of ERK1/2 and JNK in regeneration has been elucidated in a wide variety of tissues, including salivary glands (94), kidney (81), liver (91, 92) and dopaminergic neurons (93).

In HSG cells, our group has previously shown that the P2Y₂R activates ERK1/2 through two independent pathways. The first pathway involves Src and EGFR activation and the second pathway involves PKC activation (45). In addition to ERK1/2, P2Y₂R has been reported to induce JNK activation in HCAECs (100) and primary rat hepatocytes (101). However, the P2Y₂R-mediated activation of JNK has not been previously considered in salivary epithelial cells.

Overall, the P2Y₂R plays a central role in intracellular signaling to regulate a wide variety of cellular responses including cell proliferation, migration and differentiation, crucial steps in tissue regeneration (102).

H. Research goals and experimental approaches

Based on the role of the P2Y₂R in the regulation of intracellular signaling pathways crucial to tissue repair, we determined whether the P2Y₂R has a similar role in the salivary gland using *in vitro* and *ex vivo* approaches. Our goals were to test whether P2Y₂R activation enhances the migration, aggregation and self-organization of dispersed salivary epithelial cells into acinar-like aggregates and to determine the underlying mechanisms. We hypothesized that P2Y₂R-mediated formation of acinar-like spheres involves both the transactivation of the EGFR and $\alpha_5\beta_1$ integrin/Cdc42 Rho GTPase signaling pathways by extracellular nucleotides, leading to the downstream activation of MAPKs.

In the following studies, we used the rat parotid acinar (Par-C10) cell line, an established *in vitro* model of salivary gland differentiation and other functions (3, 103).

Par-C10 cells express endogenous $P2Y_2$ Rs, the primary receptor that responds to UTP in these cells (103). Unlike the majority of salivary cell lines, including HSG cells, Par-C10 cells are able to differentiate into three-dimensional (3D) acinar-like spheres on Matrigel that display characteristics similar to differentiated acini in salivary glands, including cell polarization and tight junction formation, which are required to maintain the transepithelial potential difference and responsiveness to muscarinic receptor agonists (3). In addition, we used primary SMG cells isolated from wild-type and the $P2Y_2R^{-/-}$ mice to corroborate the results obtained with Par-C10 cells.

Figure I-1. P2Y₂ nucleotide receptor structure (31). The P2Y₂ receptor (P2Y₂R) has structural motifs that enable interactions with diverse signaling pathways. The P2Y₂R regulates G_q protein-dependent activation of phospholipase C (PLC) via its intracellular loops. PLC activation generates inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which act as secondary messengers to trigger intracellular calcium mobilization and protein kinase C activation, respectively. The P2Y₂R has two Src homology 3 (SH3) binding domains on its C-terminus that enables the P2Y₂R to activate Src signaling pathways, such as Src-dependent activation of growth factor receptors (GFRs) (44). In its first extracellular loop, the P2Y₂R has an Arg-Gly-Asp (RGD) domain, which enables interactions with a class of integrins called RGD-binding integrins, such as $\alpha_v\beta_{3/5}$ integrins, that lead to downstream activation by ATP or UTP of the Rho and Rac GTPases and cytoskeletal rearrangements.

CHAPTER II

P2Y₂ nucleotide receptor activation enhances the aggregation and self-organization of dispersed salivary epithelial cells forming acinar-like spheres

Abstract

Hyposalivation resulting from salivary gland dysfunction leads to poor oral health and greatly reduces the quality of life of patients. Current treatments for hyposalivation are limited. However, regenerative medicine to replace dysfunctional salivary glands represents a revolutionary approach. The ability of dispersed salivary epithelial cells or salivary gland-derived progenitor cells to self-organize into acinar-like spheres or branching structures that mimic the native tissue holds promise for cell-based reconstitution of a functional salivary gland. However, the mechanisms involved in salivary epithelial cell aggregation and tissue reconstitution are not fully understood. This study investigated the role of the P2Y₂ nucleotide receptor (P2Y₂R), a GPCR that is upregulated following salivary gland damage and disease, in salivary gland reconstitution. *In vitro* results with the rat parotid acinar Par-C10 cell line indicate that P2Y₂R activation with the selective agonist UTP enhances the self-organization of dispersed salivary epithelial cells into acinar-like spheres. Other results indicate that the P2Y₂R-mediated response is dependent on EGFR activation via the metalloproteases ADAM10/ADAM17 or the $\alpha_5\beta_1$ integrin/Cdc42 signaling pathway, which leads to activation of the MAPKs JNK and ERK1/2. *Ex vivo* data using primary submandibular

gland (SMG) cells from wild-type and *P2Y₂R*^{-/-} mice confirmed that UTP-induced migratory responses required for acinar cell self-organization are mediated by the P2Y₂R. Overall, this study suggests that the P2Y₂R is a promising target for salivary gland reconstitution and identifies the involvement of two novel components of the P2Y₂R signaling cascade in salivary epithelial cells, the $\alpha_5\beta_1$ integrin and the Rho GTPase Cdc42.

Introduction

Salivary glands are exocrine glands composed of multiple secretory endpieces called acini, which secrete saliva into the oral cavity via a system of branched ductal cells, including intercalated ducts, striated ducts and a main excretory duct (1). Saliva performs many protective and physiological functions by providing the oral cavity with water and electrolytes along with essential proteins, including lubricants, antibacterial, antifungal, antiviral and remineralization agents, digestive enzymes and growth factors (1, 4, 6). Accordingly, hyposalivation due to salivary gland dysfunction resulting from the autoimmune disease SS or irradiation therapy for head and neck cancers leads to a significant deterioration of oral health and seriously decreases the quality of life of these patients (7, 8). Current treatments for hyposalivation are limited to saliva substitutes in the form of gels or sprays and medications, such as the muscarinic receptor agonists pilocarpine and cevimeline, which induce saliva secretion from residual salivary gland cells. However, these treatments are largely ineffective due to their transient nature or systemic side effects that are poorly tolerated by many patients (10, 11). Therefore, the development of new therapeutic approaches to treat salivary hypofunction is a necessity.

Experimental approaches for restoring salivary gland function have been considered, including gene therapy to augment the expression of proteins involved in saliva secretion (17-19). An alternative approach to regain the function of salivary glands is to induce the proliferation, migration and differentiation of residual cells in the damaged salivary glands to promote tissue regeneration (21). This approach can be further applied to bioengineer artificial salivary glands that closely resemble the native organ in both structure and function (21).

Reconstitution studies, using salivary gland tissue isolated from embryonic mice (27) and humans (28) or human SGP cells (29), have demonstrated the ability of dissociated cells to migrate towards each other and self-organize into acinar-like aggregates with structural features and differentiation markers that resemble the native gland. Cellular mechanisms and components that enhance the formation of these acinar-like aggregates would likely be important factors in salivary gland reconstitution and regeneration. In this study, we investigated the role in salivary gland reconstitution/regeneration of the P2Y₂R for extracellular ATP and UTP, since previous findings have suggested roles for the P2Y₂R in corneal epithelia wound healing by inducing cell migration (39), liver regeneration by promoting hepatocyte proliferation (40), inflammatory bowel disease by enhancing epithelial repair (41), intestinal re-epithelialization following experimental colitis (42) and reduction of infarct size following myocardial infarction (43). In addition, the P2Y₂R is upregulated upon disruption of salivary gland tissue homeostasis (37) and in salivary glands of the NOD.B10 mouse model of SS-like autoimmune exocrinopathy (38). In a classic model of salivary gland regeneration, P2Y₂R expression and activity increase due to tissue atrophy caused by a three-day ductal ligation in rat SMG, whereas

P2Y₂R expression and activity levels and glandular morphology resemble unligated controls fourteen days after deligation (36). Collectively, these findings suggest that P2Y₂R upregulation plays a role in salivary gland regeneration.

The P2Y₂R has structural motifs that enable interactions with diverse signaling pathways, such as SH3 binding domains that mediate transactivation of GFRs (44) and an RGD domain that binds directly to $\alpha_v\beta_{3/5}$ integrins to activate the Rho and Rac GTPases and cytoskeletal rearrangements (30, 60, 104). The P2Y₂R also has been shown to activate metalloproteases that induce EGFR (ErbB1) and ErbB3 phosphorylation in HSG cells (45). P2Y₂R signaling pathways can induce downstream activation of MAPKs, including JNK (100, 101) and ERK1/2 (45, 100, 105) in a variety of cell types. P2Y₂R interactions and signaling pathways enable extracellular ATP and UTP to regulate numerous physiological processes, such as cell proliferation, migration and differentiation (30, 31, 39, 59, 60, 106-108).

Based on the role of the P2Y₂R in the regulation of intracellular signaling pathways that are crucial to tissue repair, we investigated whether the P2Y₂R plays a similar role in the salivary gland using *in vitro* and *ex vivo* approaches. Our goals were to test whether P2Y₂R activation enhances the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like aggregates and to determine the underlying mechanisms. We found that P2Y₂R-mediated formation of acinar-like spheres involves the transactivation of the EGFR through activation of the metalloproteases ADAM10/ADAM17 and the $\alpha_5\beta_1$ integrin/Cdc42 Rho GTPase signaling pathway, leading to downstream activation of MAPKs.

In the following studies, we used the rat parotid acinar (Par-C10) cell line, an established *in vitro* model of salivary gland differentiation and function (3, 103). Par-C10 cells express endogenous P2Y₂Rs, but not P2Y₄R (unpublished results) or P2Y₆R (112). Therefore, P2Y₂R is the only P2Y or P2X receptor subtype that responds to UTP in these cells (103). Unlike the majority of salivary cell lines, including HSG cells, Par-C10 cells are able to differentiate on Matrigel into three-dimensional (3D) acinar-like spheres that display characteristics similar to differentiated acini in salivary glands, including cell polarization and tight junction formation, which are required to maintain the transepithelial potential difference and responsiveness to muscarinic receptor agonists (3). In addition, we used primary SMG cells isolated from wild-type and *P2Y₂R*^{-/-} mice to corroborate the results obtained with Par-C10 cells.

Results

P2Y₂R activation enhances Par-C10 cell aggregation and the formation of acinar-like spheres.

When plated on extracellular matrices, such as Matrigel, dispersed salivary epithelial cells isolated from embryonic mice (27) or adult humans (28) as well as cultured Par-C10 (3) and HSG (109) cells migrate towards each other and self-organize into aggregates that display structural and/or functional features similar to the native salivary gland. Since activation of the P2Y₂R has been shown to enhance the migration of a variety of cell types (59, 60, 108), including epithelial cells (102, 105), we investigated whether P2Y₂R activation enhances the migration, aggregation and self-organization of salivary epithelial cells. Par-C10 single-cell suspensions seeded on growth factor reduced (GFR) Matrigel-

coated 24-well plates (2×10^5 cells/well) were treated with or without UTP (100 μ M) and cells were monitored for 36 h by time-lapse live cell imaging (Figure II-1A), as described in Materials and Methods. During the first 2 h of the time course, UTP-treated single Par-C10 cells showed enhanced migratory responses, as indicated by the distance that single cells traveled from the origin (Figure II-1B), the total distance that cells migrated (Figure II-1C) and the increase in the cell velocity (Figure II-1D). After 2 h, single Par-C10 cells began to form aggregates that were quantified, as described in Materials and Methods, where one aggregation event represents the coalescence/fusion of two or more cells at the same time point. UTP-treated Par-C10 cells exhibited enhanced aggregation (Figures II-1A and 1E) with 100% forming acinar-like spheres that display lumen formation and an organized distribution of the tight junction protein ZO-1 (Figure II-1F) at the end of 36 h. Although untreated (basal) Par-C10 cells can aggregate and express ZO-1, they did not form differentiated acinar-like spheres until ~72 h in culture (data not shown), as previously described (3). Notably, the majority of aggregation events took place in the first 12 h after addition of UTP (Figure II-1G). Therefore, subsequent experiments investigating aggregation events were performed for 12 h.

Inhibition of EGFR decreases UTP-induced Par-C10 cell aggregation.

EGFR regulates a wide variety of cellular responses, including cell migration and differentiation (110, 111). In HSG cells, the P2Y₂R has been shown to activate EGFR (45). To determine if UTP-induced enhancement of Par-C10 cell aggregation is dependent on EGFR activation, cells were pretreated with AG1478 (1 μ M), a potent EGFR inhibitor, 2 h prior to UTP stimulation. The results show that EGFR inhibition

decreased UTP-induced Par-C10 cell aggregation by 69% (Figure II-2A) and, as expected, completely inhibited EGF-induced enhancement of Par-C10 cell aggregation (Figure II-2A). EGFR inhibition also prevented UTP- and EGF-induced phosphorylation of the EGFR (Figure II-2B).

Inhibition of ADAM10/ADAM17 metalloproteases decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation.

In HSG cells, the P2Y₂R has been shown to activate EGFR via ADAM10 and ADAM17 metalloproteases (45) that promote shedding of EGF-like ligands, such as neuregulin (NRG), which bind to and activate members of the EGFR family. To determine whether ADAM10/ADAM17 are involved in the UTP-induced enhancement of Par-C10 cell aggregation, cells were pretreated with the selective ADAM10/ADAM17 inhibitor TAPI-2 (10 μ M), which partially (52%) decreased UTP-induced Par-C10 cell aggregation (Figure II-3A), whereas ADAM10/ADAM17 inhibition did not affect the EGF-induced increase in aggregation (Figure II-3A). Consistent with the role of metalloproteases in the P2Y₂R-mediated generation of EGFR agonists (45), ADAM10/ADAM17 inhibition decreased UTP-induced, but not EGF-induced, phosphorylation of the EGFR (Figure II-3B). The partial loss of UTP-induced Par-C10 cell aggregation by ADAM10/ADAM17 inhibition suggests that other P2Y₂R-mediated signaling pathways contribute to the cell aggregation response.

Inhibition of the $\alpha_5\beta_1$ integrin/Cdc42 signaling pathway decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation.

The P2Y₂R contains an extracellular-oriented RGD sequence that interacts with RGD-binding integrins (58) to activate Rho GTPases that regulate cell migration (59, 60). The RGD-binding $\alpha_5\beta_1$ integrin has been shown to regulate SMG branching morphogenesis (69) and stimulate migration of a variety of cell types (112-117). To test whether UTP-induced Par-C10 cell migration and aggregation require activation of $\alpha_5\beta_1$ integrin, cells were pretreated with $\alpha_5\beta_1$ integrin function-blocking antibody (100 mg/ml) prior to addition of 100 μ M UTP. Results indicate that inhibition of $\alpha_5\beta_1$ integrin function decreased UTP-induced aggregation by 49%, but had no effect on the EGF-induced response (Figure II-4A). Integrin-dependent activation of the Rho GTPase Cdc42, Rac1 and RhoA, have been shown to modulate cytoskeletal reorganization and cell migration, where there is a reciprocal relationship between Cdc42/Rac1 and RhoA activities (76, 77, 118). Our results show that stimulation of Par-C10 cells with 100 μ M UTP activates Cdc42 in a time-dependent manner (Figure II-4B, top). Inhibition of Cdc42 with the selective antagonist ML141 (10 μ M) inhibited UTP-induced aggregation by 90% (Figure II-4B, bottom) and inhibited the UTP-induced phosphorylation of EGFR to a similar extent (Figure II-4D), but had no effect on EGF-induced responses, which are independent of metalloproteases (Figure II-3) and $\alpha_5\beta_1$ integrin/Cdc42 (Figures II-4A-B and II-4D). However, inhibition of RhoA with SR3677 (10 μ M), significantly increased basal cell aggregation by almost 2-fold and had no additional effect on the UTP- or the EGF-induced enhancement of Par-C10 cell aggregation (Figure II-4C). Inhibition of Rac1 using NSC23766 (100 μ M) did not affect UTP-induced or basal Par-C10 cell aggregation (data not shown). These data suggest that the P2Y₂R-mediated activation of the $\alpha_5\beta_1$ integrin/Cdc42 signaling pathway enhances the self-organization of Par-C10

cells into acinar-like spheres through the activation of the EGFR pathway. Moreover, these data show that RhoA plays an inhibitory role in the basal aggregation of dispersed Par-C10 cells.

UTP-induced Par-C10 cell aggregation depends on the activation of JNK and ERK1/2.

The activation of the EGFR leads to downstream activation of the MAPKs ERK1/2 and JNK, a response shown to modulate cell migration (80). Initially, we determined that P2Y₂R activation with 100 μ M UTP stimulates the time-dependent phosphorylation of JNK and ERK1/2 in Par-C10 cells (Figure II-5A). Inhibition of JNK with 10 μ M SP600125 or ERK1/2 with 10 μ M U0126, a MEK inhibitor, decreased UTP-induced Par-C10 cell aggregation (Figure II-5B), suggesting that JNK and ERK1/2 are regulators of Par-C10 acinar-like sphere formation. The EGFR pathway is apparently involved in the regulation of JNK- and ERK1/2-dependent Par-C10 cell aggregation induced by 100 μ M UTP, since inhibition of the EGFR with 1 μ M AG1478 (Figure II-5C) significantly reduced UTP-induced JNK and ERK1/2 phosphorylation.

UTP stimulates the migration of primary murine SMG cells from wild-type but not P2Y₂R^{-/-} mice.

To test whether the UTP-induced salivary epithelial cell migration and aggregation are mediated by the P2Y₂R, SMGs from wild-type and P2Y₂R^{-/-} mice were isolated, enzymatically dispersed and cultured for 3 days to allow for upregulation of the P2Y₂R, as previously described (37). Similar to primary rat SMG cell aggregates (37), P2Y₂R

mRNA expression is upregulated with time in cells cultured from wild-type mice (Figure II-6A), consistent with an increase in the $[Ca^{2+}]_i$ induced by UTP in these cells (Figure II-6B), responses not seen in SMG cell aggregates from *P2Y₂R*^{-/-} mice (data not shown). UTP (100 μ M) stimulated the migration of SMG cell aggregates from wild-type but not *P2Y₂R*^{-/-} mice (Figure II-6C). UTP-induced migration of wild-type primary SMG cells occurred only during the first 4 h, as indicated by the distance that cell aggregates traveled from the origin (Figure II-6D), the total distance that cell aggregates migrated (Figure II-6E) and an increase in the velocity of the cell aggregates (Figure II-6F). A lack of these responses in SMG from *P2Y₂R*^{-/-} mice (Figures II-6C-F) confirms that UTP-induced SMG cell migration is mediated by P2Y₂R activation. Furthermore, inhibition of EGFR with 1 μ M AG1478 impaired the UTP-induced migratory responses in wild-type primary SMG cells (Figure II-7) corroborating our findings using Par-C10 cells that the P2Y₂R-induced responses are dependent on EGFR activation.

Discussion

Recent progress has been made in the development of strategies for regeneration and engineering of a variety of tissues, including skin (119, 120), corneal epithelium (121), cartilage (122), bone (123, 124), bladder (125), and lacrimal (126) and salivary glands (127). Determining the capacity of dispersed cells or tissue fragments to reassemble into native structures and the underlying mechanisms involved should provide novel insights to improve tissue regeneration approaches. The capacity of dispersed salivary epithelial cells or SGP cells to reassemble into acinar-like spheres or branching structures has been previously assessed (27-29), but little is known about the signaling events involved in

these differentiation processes. In the present study, we determined that the P2Y₂R, known to be upregulated during salivary gland damage and disease (36-38), plays a role in the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres. Our *in vitro* data using the rat parotid acinar (Par-C10) cell line show that P2Y₂R activation by UTP significantly enhances the migration, aggregation and self-organization of dispersed Par-C10 cells into acinar-like spheres (Figures II-1A-E) that display structural features and differentiation markers similar to those of acini in the native gland (Figure II-1F), as previously described (3). In addition, our *ex vivo* data show that P2Y₂R deletion prevents the UTP-induced migration of primary murine SMG cell aggregates (Figure II-6), demonstrating that UTP-induced migratory responses of salivary epithelial cells are primarily mediated by P2Y₂R activation.

In this paper, we demonstrate that UTP-induced enhancement of dispersed salivary epithelial cell aggregation occurs by two distinct signaling pathways coupled to activation of the P2Y₂R: (1) the activation of metalloproteases (*i.e.*, ADAM10/ADAM17) and (2) the activation of the $\alpha_5\beta_1$ integrin/Cdc42 Rho GTPase pathway. Both of these signaling pathways activate EGFR, which leads to the downstream activation of JNK and ERK1/2 that we demonstrate increases UTP-induced aggregation of Par-C10 cells. A schematic outlining these P2Y₂R-mediated signaling pathways involved in salivary epithelial cell migration and aggregation is shown in Figure II-8.

It is well-established that the EGFR and its signaling pathways are critical for stimulating cell migration and the regeneration of a variety of tissues (111, 128-132). In salivary tissue reconstitution studies, exogenous EGF has been shown to be crucial for the self-organization of dispersed salivary gland-derived progenitor cells into branching

structures (29). Several studies have shown that P2Y₂R activation enhances epithelial cell migration, thereby accelerating wound healing and tissue regeneration (42, 48, 102, 105-107, 133) in part due to transactivation of the EGFR (48, 102, 106, 107). Our group has previously shown that the P2Y₂R mediates transactivation of the EGFR in HSG cells through metalloprotease-dependent NRG release (45). In the present study, we demonstrate that the ADAM10/ADAM17/EGFR signaling pathway is required for P2Y₂R-mediated aggregation of salivary epithelial cells (Figures II-2, II-3 and II-8), suggesting P2Y₂R as a potential therapeutic target for promoting salivary gland regeneration or the *ex vivo* bioengineering of salivary glands, which represent promising alternative approaches to replace the current ineffective therapies for hyposalivation resulting from SS or irradiation therapy for head and neck cancers.

In addition to metalloprotease-dependent activation of the EGFR, the P2Y₂R can activate EGFR through the $\alpha_5\beta_1$ integrin/Cdc42 signaling pathway (Figure II-4). Our group has previously shown that the P2Y₂R contains an RGD motif in its first extracellular loop that enables receptor interaction with RGD-binding $\alpha_v\beta_{3/5}$ integrins to stimulate cell migration (42, 59, 60, 134). However, P2Y₂R interactions with other RGD-binding integrins have not been previously reported. In this study, we show for the first time that the $\alpha_5\beta_1$ integrin, a known mediator of SMG branching morphogenesis (69), cell migration and tissue regeneration (112-117, 135-141), also plays a role in P2Y₂R-mediated salivary epithelial cell aggregation (Figure II-4A). We also have shown that the P2Y₂R/ α_v integrin interaction leads to the activation of Rac (60), a Rho GTPase critical for regulating cell migration (70, 75, 142-145), epithelial morphogenesis (70, 73) and salivary acinar formation (146). In this study, we found no evidence that Rac1 is required

for P2Y₂R-mediated salivary epithelial cell aggregation (data not shown), but rather Cdc42, another Rho GTPase known to regulate cell migration (70, 75, 142-145, 147) and tissue regeneration (148, 149), regulates the aggregatory response to P2Y₂R activation (Figure II-4B). Previous reports from our lab have linked P2Y₂R-mediated cell migration to the activation of the Rho GTPase RhoA, as well as Rac1 (49, 60, 104, 134).

Interestingly, RhoA inhibition in Par-C10 salivary epithelial cells increased basal cell aggregation by almost 2-fold and had no additional effect on the UTP- or EGF-induced enhancement of cell aggregation (Figure II-4C), suggesting that RhoA GTPase is a negative regulator of migratory responses in these cells. A reciprocal relationship between Cdc42 and RhoA has recently been described for mammary epithelial acinar morphogenesis (118). In contrast, another study has shown that inhibition of RhoA does not affect acinus formation by HSG cells (146).

It is well-established that MAPKs, including JNK and ERK1/2, regulate cell proliferation, migration and differentiation (80, 96-99), processes important for salivary gland morphogenesis (150, 151) and regeneration of a wide variety of tissues (81-95). The P2Y₂R-mediated activation of ERK1/2 has been reported in HSG cells (45), corneal epithelial cells (105) and HCAEC (100). However, the ability of the P2Y₂R to activate JNK has only been reported for HCAEC (100) and primary rat hepatocytes (101). Our data indicate that the P2Y₂R agonist UTP activates JNK and ERK1/2 (Figure II-5A) through the canonical EGFR pathway (Figure II-5C) to enhance salivary epithelial cell aggregation and self-organization (Figure II-5B).

Growth factor receptors and integrins represent major signaling pathways that interact at different levels to regulate various physiological processes (152, 153). The present

study indicates that the P2Y₂R transactivates the EGFR through the $\alpha_5\beta_1$ integrin/Cdc42 signaling pathway as well as the activation of the metalloproteases ADAM10/ADAM17 enabling extracellular nucleotides to enhance the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres on GFR Matrigel by increasing the activities of the MAPKs JNK and ERK1/2. Future work will investigate whether other P2Y₂R signaling pathways are involved in acinar-like sphere formation, such as the activation of the MAPK p38 that has been reported to promote the regeneration of salivary glands (94), skeletal muscle (154) and sciatic nerve (155) and to regulate corneal epithelial wound healing (83). Other potential P2Y₂R signaling pathways involved in salivary epithelial cell migration include the activation of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) (59) and the G_o signaling pathway (60). Understanding the signaling events responsible for the aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres should provide insights into novel approaches for the bioengineering of salivary glands (127) and should lead to better regenerative/replacement strategies for salivary glands damaged in human autoimmune diseases or as an unintended side effect of radiation treatments for head and neck cancers.

Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Par-C10 cell culture

Par-C10 cells transfected with cDNA encoding the green fluorescent protein (GFP)-tagged human P2Y₂R (GFP-hP2Y₂R) (3) were cultured in a 1:1 mixture of DMEM/Ham's F-12 medium (Life Technologies, Grand Island, NY) supplemented with 2.5% (v/v) fetal bovine serum (FBS) (Life Technologies), insulin (5 µg/ml), transferrin (5 µg/ml), selenite (5 ng/ml), retinoic acid (0.1 µM), EGF (80 ng/ml) (Calbiochem, Billerica, MA), triiodothyronine (2 nM), hydrocortisone (1.1 µM), glutamine (5 mM), gentamicin (50 µg/ml), cholera toxin (8.4 ng/ml) and G418 (0.5 mg/ml) (Mediatech, Inc., Manassas, VA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Mice

C57BL/6 (wild-type) and *P2Y₂R*^{-/-} mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the Christopher S. Bond Life Sciences Center Animal Facility of the University of Missouri, Columbia, MO. Animals were housed in vented cages with 12 h light/dark cycles and received food and water *ad libitum*. All animals were handled using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri.

Preparation of dispersed cell aggregates from mouse submandibular gland (SMG)

Dispersed cell aggregates from SMGs of wild-type (wt) C57BL/6 and *P2Y₂R*^{-/-} mice were prepared, as previously described (45). Briefly, the mice were anesthetized with

isofluorane and SMGs removed. The glands were finely minced and incubated in dispersion medium consisting of DMEM/Ham's F-12 medium (1:1), 0.2 mM CaCl₂, 1% (w/v) bovine serum albumin (BSA), 50 units/ml collagenase (Worthington Biochemical, Freehold, NJ), and 400 units/ml hyaluronidase at 37°C for 40 min with aeration (95% air and 5% CO₂). Cell aggregates in dispersion medium were suspended by pipetting at 20, 30 and 40 min of the incubation period. The dispersed cell aggregates were washed with enzyme-free assay buffer (120 mM NaCl, 4 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, 15 mM HEPES, pH 7.4) containing 1% (v/v) FBS, filtered through nylon mesh, and cultured in DMEM/Ham's F-12 medium (1:1) containing 2.5% (v/v) FBS and the following supplements: retinoic acid (0.1 µM), EGF (80 ng/ml), triiodothyronine (2 nM), hydrocortisone (1.1 µM), glutamine (5 mM), insulin (5 µg/ml), transferrin (5 µg/ml), selenite (5 ng/ml), gentamicin (50 µg/ml) and cholera toxin (8.4 ng/ml). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 0, 24, 48 or 72 h.

Migration assay

Par-C10 single-cell suspensions in DMEM/Ham's F-12 medium (1:1) containing 0.1% (v/v) FBS were seeded (2×10^5 cells/well) on a 24-well plate coated with GFR Matrigel (BD Biosciences, San Jose, CA) and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 4 h. Then, the cell culture plate was mounted on a Nikon Eclipse Ti-E microscope equipped with a digital camera, a motorized x-y stage, an automatic shutter and an *in vivo* incubation chamber (37°C, 5% CO₂ and 95% air). Within each well, a field of cells was located with a 10x objective and marked for

monitoring over the duration of the experiment using Nikon NIS-Elements imaging software. The exposure time was kept constant for all positions and all time points. Cells were treated with or without UTP (100 μ M) or EGF (100 ng/ml). In inhibitor studies, cells were pretreated with EGFR inhibitor AG1478 (1 μ M) (Cell Signaling Technology, Beverly, MA), ADAM10/ADAM17 inhibitor TAPI-2 (10 μ M) (Peptides International, Louisville, KY), $\alpha_5\beta_1$ integrin blocking antibody (100 μ g/ml) (Biolegend, San Diego, CA), Cdc42 inhibitor ML141 (10 μ M) (Tocris Bioscience, Minneapolis, MN), RhoA inhibitor SR3677 (10 μ M) (Tocris Bioscience), MEK/ERK pathway inhibitor U0126 (10 μ M) (Cell Signaling Technology) or JNK inhibitor SP600125 (10 μ M) (Tocris Bioscience) for 2 h before UTP, EGF or vehicle (basal) treatment. Unless otherwise noted, inhibitors at the concentrations employed had no effect on the responses measured under basal conditions. Transmitted light images of cells were obtained every 10 min for the time indicated. Cellular aggregation was monitored by manually counting the number of aggregation events, where one aggregation event was defined as the coalescence/fusion of two or more cells at the same time point. ZO-1 tight junction protein was detected in Par-C10 cell aggregates formed after 36 h using immunofluorescence as previously described (3).

For primary SMG cells isolated from wild-type and *P2Y₂R*^{-/-} mice, SMGs were enzymatically dispersed and incubated for 3 days (37°C, 5% CO₂ and 95% air) to allow the P2Y₂R to upregulate. After 3 days, cells were serum-starved overnight and on day 4, cells were seeded on GFR Matrigel for 8 h, treated with or without UTP and monitored by time-lapse live cell imaging, as described above. In inhibitor studies, cells were pretreated with EGFR inhibitor AG1478 (1 μ M) (Cell Signaling Technology) for 2 h

before UTP or vehicle (basal) treatment. Primary cell migration was assessed by measuring the distance traveled from the origin, total distance traveled and average velocity using the tracking software provided with the NIS-Elements imaging software.

SDS-PAGE and Western blot analysis

Par-C10 cells (2×10^5 cells/well) were seeded on 24-well culture dishes, grown to 70% confluence and then incubated overnight in DMEM/Ham's F-12 medium (1:1) without serum. When indicated, the cells were pretreated with or without inhibitors for 2 h at 37°C before stimulation with agonists for the indicated times. Then, the medium was removed and 100 µl of 2X Laemmli lysis buffer (20 mM NaH₂PO₄, pH 7.0, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 100 mM dithiothreitol) were added. The samples were sonicated for 5 sec with a Branson Sonifier 250 (microtip; output level, 5; duty cycle, 50%), heated at 95°C for 5 min and subjected to SDS-PAGE on 7.5% (w/v) polyacrylamide gels. The proteins resolved on the gel were transferred to nitrocellulose membranes and blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST). The blots were incubated overnight at 4°C in blocking solution or TBST with the following rabbit polyclonal antibodies used at 1:1,000 dilutions: anti-phospho-EGFR (Tyr1068) (Cell Signaling Technology), anti-phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology) or anti-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control. The membranes were washed three times with TBST and incubated with horseradish peroxidase-linked goat anti-rabbit IgG antibody (1:2,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h.

The membranes were washed three times with TBST, incubated with enhanced chemiluminescence reagent and the protein bands detected on x-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad, Hercules, CA). The intensities of phosphorylated protein bands in cells treated with agonists or other agents were normalized to total ERK1/2 and expressed as a percentage of normalized data from untreated controls.

Cdc42 activation assay

A Cdc42 activation assay kit (Cell Biolabs, San Diego, CA) was used to assess Cdc42 activity according to the manufacturer's instructions. Briefly, Par-C10 cells were cultured in 100 mm culture dishes and grown to 70% confluence. Then, cells were starved overnight in serum-free DMEM/Ham's F-12 medium (1:1) before being stimulated with UTP (100 μ M) for the indicated times. Cell lysates were collected using 1X assay/lysis buffer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM MgCl₂, 5 mM EDTA, 10% glycerol) and incubated for 1 h at 4°C with p21-activated kinase-1 p21-binding domain (PAK1 PBD) agarose beads, which bind the GTP-bound form of Cdc42. GTP-bound Cdc42 was analyzed by Western analysis using mouse monoclonal anti-rat Cdc42 antibody (1:1,000 dilution; Cell Biolabs). The membranes were washed three times with TBST and incubated with horseradish peroxidase-linked goat anti-mouse IgG antibody (1:2,000 dilution) at room temperature for 1 h. The membranes were washed three times with TBST, incubated with enhanced chemiluminescence reagent and the protein bands were detected on x-ray film.

Reverse Transcription and Real-Time PCR analysis of P2Y₂R mRNA expression

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) from SMG aggregates cultured for 0, 24, 48 or 72 h at 37°C in 5% CO₂ and 95% air. cDNA was synthesized from 1 µg of purified RNA using the Advantage RT for PCR kit (Clontech Laboratories, Mountain View, CA). Ten percent of the synthesized cDNA was used as a template in 25 µl Real-Time PCR (RT-PCR) reactions and samples were run in duplicate for the *P2Y₂R* target and the endogenous *18S* RNA control. The relative levels of *P2Y₂R* and *18S* RNA in each sample were determined and expressed as a ratio of *P2Y₂R* to *18S* RNA (normalized to 1) using Applied Biosystems software.

Intracellular free Ca²⁺ concentration measurements

Changes in the intracellular free Ca²⁺ concentration ([Ca²⁺]_i) in SMG cell aggregates were quantified as previously described (38). Briefly, dispersed SMG aggregates from wild-type or *P2Y₂R*^{-/-} mice were cultured for 72 h and loaded with 2 µM fura 2-AM (Calbiochem) for 30 min at 37°C in assay buffer (120 mM NaCl, 4 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, 15 mM HEPES, pH 7.4) containing 0.1% (w/v) BSA. Then, the SMG aggregates were washed and adhered to chambered coverslips coated with Cell-Tak (BD Biosciences) for an additional 30 min in the absence of fura 2-AM. SMG aggregates were stimulated with UTP (100 µM) and changes in the 340/380 nm excitation ratio (505 nm emission) were monitored using an InCyt dual-wavelength fluorescence imaging system (Intracellular Imaging, Cincinnati,

OH). Fluorescence ratios were converted to $[\text{Ca}^{2+}]_i$ (nM) using a standard curve created with known concentrations of Ca^{2+} .

Statistical analysis

The quantitative results are presented as the means $\pm$ S.E.M. of data from three or more experiments. Two-tailed t test or ANOVA followed by Bonferroni or Dunnett's test was performed, as indicated, where $P < 0.05$ represents a significant difference.

Figure II-1. UTP enhances Par-C10 cell aggregation and the formation of acinar-like spheres on GFR Matrigel. (A) Par-C10 single-cell suspensions were cultured on GFR Matrigel for 4 h, then treated with or without UTP (100 μM) and monitored by

time-lapse live cell imaging for 36 h, as described in Materials and Methods. The migration of single Par-C10 cells was monitored for 2 h and (B) the distance migrated from the origin, (C) the total distance traveled and (D) the average velocity of single cells were quantified with the tracking software provided with the NIS-Elements imaging software. The data represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) indicates a significant increase over basal levels using the two-tailed t test. (E) Quantification of the number of aggregation events in response to UTP (100 μ M) after 36 h. (F) After 36 h, UTP-treated Par-C10 cell aggregates formed acinar-like spheres that display lumen formation and an organized distribution of the tight junction protein ZO-1 (red) detected by immunofluorescence using rabbit anti-ZO-1 antibody, as previously described (3), features not observed in the Par-C10 cell aggregates formed under basal conditions. (G) Quantification of the aggregation events from 0–12 h, 12–24 h and 24–36 h indicates that the majority of aggregation events take place in the first 12 h with or without UTP treatment. The data shown represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) indicates a significant increase over basal levels using the two-tailed t test.

E

F

G

Figure II-2. Inhibition of EGFR activation decreases UTP-induced enhancement of Par-C10 cell aggregation. (A) Par-C10 single-cell suspensions plated on GFR Matrigel for 4 h were pretreated with or without the EGFR inhibitor AG1478 (1 μ M) for 2 h, then incubated with or without UTP (100 μ M) or EGF (100 ng/ml). Par-C10 cell aggregates were monitored by time-lapse live cell imaging for 12 h. The data are expressed as percentages of the maximal number of aggregation events induced by UTP or EGF in the absence of AG1478 and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.01$ (**) or $P < 0.001$ (***) indicates a significant difference from the UTP- or EGF-induced response using the two-tailed t test. (B) Par-C10 cells were serum-starved overnight, pretreated with AG1478 (1 μ M) for 2 h, and then treated with or without UTP (100 μ M) or EGF (100 ng/ml). Five min after UTP or EGF addition, protein extracts were prepared from Par-C10 cell aggregates and EGFR phosphorylation (Y1068) was determined by Western analysis. Representative blots are shown (top). Quantification of protein levels in blots (bottom) was performed using Quantity One software, as described in Materials and Methods. The data are expressed as the percentage increase in EGFR phosphorylation induced by UTP or EGF, as compared to untreated controls, and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) or $P < 0.01$ (***) indicates a significant difference from the UTP- or EGF-induced response using the two-tailed t test.

A

B

Figure II-3. Inhibition of metalloproteases ADAM10/ADAM17 decreases UTP-

induced enhancement of Par-C10 cell aggregation and EGFR phosphorylation. (A)

Par-C10 single-cell suspensions plated on GFR Matrigel for 4 h were pretreated with or without the ADAM10/ADAM17 inhibitor TAPI-2 (10 μ M) for 2 h, then incubated with or without UTP (100 μ M) or EGF (100 ng/ml). Par-C10 cell aggregates were monitored by time-lapse live cell imaging for 12 h. The data are expressed as percentages of the maximal number of aggregation events induced by UTP or EGF in the absence of TAPI-2 and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.001$ (***) indicates a significant difference from the UTP- or EGF-induced response using the two-tailed t test. (B) Par-C10 cells were serum-starved overnight, pretreated with TAPI-2 (10 μ M) for 2 h, and then treated with or without UTP (100 μ M) or EGF (100 ng/ml). Five min after UTP or EGF addition, protein extracts were prepared from Par-C10 cell aggregates and EGFR phosphorylation (Y1068) was determined by Western analysis. Representative blots are shown (top), where a black line represents noncontiguous lanes from the same gel. Quantification of protein levels in blots (bottom) was performed using Quantity One software, as described in Materials and Methods. The data are expressed as the percentage increase in EGFR phosphorylation induced by UTP or EGF, as compared to untreated controls, and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.01$ (**) indicates a significant difference from the UTP- or EGF-induced response using the two-tailed t test.

A

B

Figure II-4. Inhibition of the $\alpha_5\beta_1$ /Cdc42 signaling pathway decreases UTP-induced Par-C10 cell aggregation and EGFR phosphorylation, whereas RhoA inhibition increases basal cell aggregation. Par-C10 single-cell suspensions were plated on GFR Matrigel for 4 h, pretreated for 2 h with or without (A) $\alpha_5\beta_1$ integrin function-blocking antibody (100 $\mu\text{g/ml}$), (B, bottom) the Cdc42 inhibitor ML141 (10 μM) or (C) the RhoA inhibitor SR3677 (10 μM). Then, cells were treated with or without UTP (100 μM) or EGF (100 ng/ml), as indicated, and cell aggregation was monitored by time-lapse live cell imaging for an additional 12 h. The data are expressed as percentages of (A, B) the maximal number of aggregation events induced by UTP or EGF or (C) the total number of aggregation events. The data represent the means $\pm$ S.E.M. of results from at least 3 experiments. (A, B) $P < 0.05$ (*) or $P < 0.01$ (**) indicates a significant difference from the UTP- or EGF-induced response using the two-tailed t test. (C) Two-way ANOVA was performed followed by the Bonferroni test, where $P < 0.05$ (*) or $P < 0.01$ (**) indicates a significant difference in the number of aggregation events between SR3677-treated and untreated cells under basal conditions or SR3677-treated cells stimulated with or without UTP or EGF, as indicated. (B, top) GTP binding by Cdc42 was determined in serum-starved Par-C10 cells treated with or without UTP (100 μM) for 1, 5 or 10 min, as described in Materials and Methods. (D) Par-C10 cells were serum-starved overnight, pretreated with the Cdc42 inhibitor ML141 (10 μM) for 2 h, and then treated with or without UTP (100 μM) or EGF (100 ng/ml) for 5 min. EGFR phosphorylation (Y1068) was determined by Western analysis. A representative blot is shown (top), where a black line represents noncontiguous lanes from the same gel. Quantification of protein levels in blots (bottom) was performed using Quantity One software, as described in Materials and

Methods. The data are expressed as the percentage increase in EGFR phosphorylation induced by UTP, as compared to untreated controls, and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) indicates a significant difference from the UTP-induced response using the two-tailed t test.

A

B

C

D

Figure II-5. UTP-induced enhancement of Par-C10 cell aggregation is dependent on the activation of JNK and ERK1/2 by the EGFR. (A) Par-C10 cells were serum-starved overnight and treated with or without 100 μ M UTP for 1, 5, 10 or 15 min. Protein extracts were subjected to SDS-PAGE and p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 (loading control) were detected by Western analysis. (B) Par-C10 single-cell suspensions plated on GFR Matrigel for 4 h, were pretreated with or without the JNK inhibitor SP600125 (10 μ M) or the MEK/ERK inhibitor U0126 (10 μ M) for 2 h. Then, cells were treated with or without UTP (100 μ M) and cell aggregation monitored by time-lapse live cell imaging for 12 h. The data are expressed as percentages of the maximal number of aggregation events induced by UTP and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.01$ (**) or $P < 0.001$ (***) indicates a significant difference from the UTP-induced response using the two-tailed t test. (C) Par-C10 cells were pretreated for 2 h with or without the EGFR inhibitor AG1478 (1 μ M) then treated with or without UTP (100 μ M) for 5 min. Protein extracts were subjected to SDS-PAGE and p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204) and ERK1/2 (loading control) were detected by Western analysis (representative blots are shown on the left, where a black line represents noncontiguous lanes from the same gel). Quantification of protein levels was performed (right), as described in Materials and Methods. The data are expressed as the percentage increase in JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) phosphorylation induced by UTP, as compared to untreated control, and represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) or $P < 0.01$ (**) indicates a significant difference from the UTP-induced response using the two-tailed t test.

Figure II-6. The P2Y₂R mediates UTP-induced migration of primary SMG cell aggregates. (A) P2Y₂R mRNA was isolated from SMG cell aggregates from wild-type and *P2Y₂R*^{-/-} mice after 0, 24, 48 or 72 h in culture, as described in Materials and Methods. The data are expressed as fold increase in P2Y₂R mRNA levels over the 0 time point and represent the means of $\pm$ S.E.M. of results from 4 experiments, where $P < 0.05$ (*) or $P < 0.001$ (***) indicates a significant increase in P2Y₂R mRNA expression, as compared to the 0 time point using one-way ANOVA followed by the Dunnett's test. (B) Changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) induced by 100 μ M UTP in SMG cell aggregates at 0 and 72 h in culture were determined, as described in Materials and Methods. The data represent the means $\pm$ S.E.M. of results from 7 experiments, where $P < 0.001$ (***) indicates a significant difference from the 0 time point using the two-tailed *t* test. (C) Primary SMGs isolated from wild-type and *P2Y₂R*^{-/-} mice were enzymatically dispersed, incubated for 3 days (37°C, 5% CO₂ and 95% air) to enable upregulation of the P2Y₂R. After 3 days, cells were serum-starved overnight, seeded on GFR-Matrigel for 8 h and treated with or without 100 μ M UTP. Cell aggregates of similar size were monitored by time-lapse live cell imaging for 24 h and the point of origin (arrow) and the migration path (red line) are indicated. Quantification of (D) the distance migrated from the origin, (E) the total distance traveled and (F) the average velocity of aggregates throughout the first 4 h of the time course was performed with the tracking software provided with the NIS-Elements imaging software. The data represent the means $\pm$ S.E.M. of results from at least 3 experiments, where $P < 0.05$ (*) indicates a significant increase over basal levels using the two-tailed *t* test.

Figure II-7. The P2Y₂R-induced migration of primary SMG cell aggregates is dependent on EGFR. Primary SMGs isolated from wild-type and *P2Y₂R*^{-/-} mice were enzymatically dispersed, incubated for 3 days (37°C, 5% CO₂ and 95% air) to enable upregulation of the P2Y₂R. After 3 days, cells were serum-starved overnight, seeded on GFR-Matrigel for 8 h, and pretreated for 2 h with or without the EGFR inhibitor AG1478 (1 μM). Then, cells were treated with or without UTP (100 μM). Cell aggregates of similar size were monitored by time-lapse live cell imaging. Quantification of (A) the distance migrated from the origin, (B) the total distance traveled and, (C) the average velocity of aggregates during the first 4 h of the time course was performed with the tracking software provided with the NIS-Elements imaging software. The data represent the means ± S.E.M. of results from at least 3 experiments, where *P* < 0.05 (*) indicates a significant increase over basal levels using the two-tailed *t* test.

A **B** **C**

Figure II-8. Proposed mechanisms for P2Y₂R-mediated enhancement of salivary epithelial cell aggregation and formation of acinar-like spheres. The P2Y₂R enhances the aggregation of dispersed salivary epithelial cells into acinar-like spheres through the activation of the EGFR and subsequent downstream activation of JNK and ERK1/2. The P2Y₂R mediates EGFR activation through two distinct pathways, the first pathway involves the P2Y₂R-mediated activation of matrix metalloproteases (*i.e.*, ADAM10/ADAM17) , which cleave membrane-bound EGFR ligands (45) leading to the activation of the EGFR, and the second pathway involves the P2Y₂R-mediated activation of the RGD binding $\alpha_5\beta_1$ integrin, which leads to activation of the Rho GTPase Cdc42 that also activates the EGFR. RhoA activation has an inhibitory effect on the basal aggregation of dispersed Par-C10 salivary epithelial cells. *P2Y₂R*: P2Y₂ receptor; *ADAM*: a disintegrin and metalloproteinase; *GFR*: growth factor receptor; *NRG*: neuregulin; *EGFR*: epidermal growth factor receptor; *RhoA*: Ras homolog gene family member A; *Cdc42*: cell division control protein 42 homolog.

CHAPTER III

Summary and Future Directions

A. Summary

Hyposalivation due to salivary gland dysfunction primarily resulting from radiation therapy for head and neck cancers or SS causes serious oral health issues, including bacterial infection, dental carries, mastication/swallowing dysfunction and overall reduced quality of life. Current therapies for hyposalivation are mainly symptomatic. In contrast, regenerative medicine has revolutionized disease management by offering radical cures to a number of human diseases. Progress has been made in strategies for the regeneration of a variety of tissues including skin (119, 120), corneal epithelium (121), cartilage (122), bone (123, 124), bladder (125), and lacrimal (126) and salivary glands (127). In this study, we aimed to extend this progress to promote cell-based reconstitution of salivary glands *in vitro*. Understanding the signaling events responsible for the migration, aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres should provide insights into novel approaches for the bioengineering of salivary glands (127) and should lead to better regenerative/replacement strategies for damaged salivary glands.

Recently, evidence from different model systems revealed that GFRs and integrins cooperate at different levels to regulate cell migration during development as well as tissue repair following injury (152, 153). The coordination of signals from growth factors and the extracellular matrix is required to achieve full biological responses. The present study suggests that the P2Y₂R links GFR and integrin signaling by transactivating the

EGFR through $\alpha_5\beta_1$ integrin/Cdc42, enabling extracellular nucleotides to enhance the migration, aggregation and self-organization of dispersed salivary epithelial cells into acinar-like spheres on GFR Matrigel by increasing the activities of the MAPKs JNK and ERK1/2. These results introduce the P2Y₂R as an important target in the reconstitution of salivary glands and possibly other tissues. In addition, the fact that the EGFR and $\alpha_5\beta_1$ integrin are crucial players in metastasis and cancer progression in various tissues sheds light on the P2Y₂R as a potential therapeutic target in cancer biology and goes along with many reports demonstrating the involvement of the P2Y₂R in different types of cancer (156-162).

In summary, the results presented in this dissertation suggest the following novel findings:

1. These studies have demonstrated the ability of extracellular nucleotides, specifically UTP, to enhance salivary tissue reconstitution through the activation of the P2Y₂R, in contrast to previous salivary tissue reconstitution studies that have focused only on growth factors to enhance this process.
2. Novel findings indicate that the P2Y₂R signals through the fibronectin receptor $\alpha_5\beta_1$ integrin to induce salivary epithelial cell migration. These results demonstrate an interesting link between extracellular nucleotides, which are released from cells in response to disease or stress, and extracellular matrix interactions that are required for the healing process.
3. These studies have identified the Rho GTPase Cdc42 as a novel downstream component of the P2Y₂R signaling network and a regulator of P2Y₂R-mediated transactivation of the EGFR.

4. Novel findings demonstrate that the P2Y₂R mediates JNK activation in salivary epithelial cells through EGFR transactivation, which corroborates previous studies on P2Y₂R-induced activation of JNK in HCAEC and primary rat hepatocytes.

B. Future directions

The research project presented in this dissertation can be further extended by pursuing the following tracks:

1. Investigating the role of the P2Y₂R in salivary gland regeneration in the murine SMG duct ligation *in vivo* model

To extend our *in vitro* and *ex vivo* data and to confirm the role of the P2Y₂R in salivary gland regeneration *in vivo*, ongoing studies are being performed using the classical SMG duct ligation/deligation model with both wild-type and *P2Y₂R*^{-/-} mice. *In vivo* studies will determine the expression and/or activation levels of EGFR, $\alpha_5\beta_1$ integrin, Cdc42, JNK and ERK1/2 in SMGs isolated from wild-type and *P2Y₂R*^{-/-} mice at different time points following duct ligation (which is associated with overall atrophy of acinar cells) and after ligature removal (which is associated with gland regeneration), using Western analysis. Moreover, we will investigate the subcellular localization of JNK and ERK1/2 activated by the P2Y₂R signaling pathway to determine their nuclear levels using immunohistochemistry. Other *in vivo* studies will compare gland weights, general morphology and the amount of saliva secreted in both wild-type and *P2Y₂R*^{-/-} mice at different time points following SMG duct ligation and ligature removal.

2. Investigating the role of the P2Y₂R in enhancing the self-organization of dispersed SGP cells into branched organ-like structures

It has been recently shown that dispersed SGP cells cultured on Matrigel in the presence of exogenous EGF were able to self-organize into branching structures characterized by short thick stalks, round buds and cleft-like portions, features of the pseudoglandular stage during salivary gland development (29). These results hold promise for the cell-based reconstitution of salivary glands *in vitro*. Future work will determine whether P2Y₂R activation with UTP enhances the self-organization of dispersed SGP cells into branched organ-like structures and/or induces further terminal differentiation of these branching structures.

3. Delineating other potential P2Y₂R-mediated mechanisms to enhance salivary epithelial cell migration and the formation of acinar-like spheres

Future work will investigate whether other P2Y₂R signaling pathways are involved in acinar-like sphere formation by inhibiting target proteins of the hypothesized pathways using pharmacological inhibitors or RNA interfering strategies and monitoring the effect of inhibition on the P2Y₂R-induced responses.

EGFR activation leads to the phosphorylation of β -catenin at the Y654 residue, the dissociation of β -catenin from the E-cadherin catenin complex at the cell membrane and consequent nuclear accumulation of β -catenin, which activates Wnt signaling pathway target genes to enhance cell migration (163, 164). In addition, Wnt signaling has been reported to play a role in salivary gland regeneration in the *in vivo* SMG duct ligation mouse model (165). The P2Y₂R has been shown to transactivate the EGFR in a variety of tissues (44, 48, 107), including salivary gland (45), but

possible interactions between the P2Y₂R and Wnt/ β -catenin signaling have not been addressed to date. Thus, it would be significant to investigate possible cross-talk between the P2Y₂R and the Wnt/ β -catenin signaling pathways to further enhance salivary epithelial cell migration and aggregation into acinar-like spheres.

The MAPK p38 is another downstream target that might be involved in the P2Y₂R-mediated enhancement of salivary tissue reconstitution since p38 has been previously reported to be activated by the P2Y₂R in astrocytic cells (166) and human neutrophils (167), in addition to its suggested roles in promoting the regeneration of various tissues, including salivary glands (94), skeletal muscle (154) and sciatic nerve (155).

Other potential P2Y₂R signaling pathways involved in salivary epithelial cell migration include the activation of PI3K/Akt (59) and the G_o signaling pathways (60), which have been previously shown to mediate P2Y₂R-induced migration of astrocytic cells.

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VITA

Farid Galal El-Sayed was born May 8th, 1985, in Cairo, Egypt. After graduating from high school in his hometown, Farid joined School of Pharmacy, Cairo University, Egypt and received a B.S. degree in Pharmaceutical Sciences in 2007. In the fall of 2009, Farid started his Ph.D. program in the Department of Biochemistry at the University of Missouri-Columbia, USA, under the supervision of Dr. Gary A. Weisman.