Characterization of the ATCC α-TC1-6 Pancreatic Alpha Cell Line to Study Glucagon Secretion

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Characterization of the ATCC α-TC1-6 Pancreatic Alpha Cell Line to Study Glucagon Secretion

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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Advisor: Robert M. Dores
ABSTRACT

The endocrine pancreas is responsible for maintaining glycemic equilibrium in the body. Given the importance of this blood-glucose homeostasis and the implication an unbalance has on Diabetes mellitus, the study of the glucose-sensing alpha and beta cells in the pancreas is a popular field for scientific researchers. In this study, we use immunofluorescence, qPCR analysis, intracellular calcium experiments, and biochemical glucagon secretion assays to determine if the commercially available tumor cell line clone, α-TC1-6 obtained from American Type Culture Collection, is an appropriate model system for glucagon secretion in pancreatic alpha cells. We confirm the production of the hormone peptide glucagon as well as increased intracellular calcium activity associated with a decrease in extracellular glucose. However, the resultant stimulated secretion of glucagon at low glucose levels is much less than expected from an endocrine cell and indicates deficiency of the pathway. The American Type Culture Collection α-TC1-6 cell line also retains the activity of the $K_{\text{ATP}}$ channel and an agatoxin-sensitive P/Q-type calcium channel, but does not exhibit activity of a TTX-sensitive sodium channel and lacks the expression of the receptor of a key paracrine regulator, epinephrine. Paracrine control via an increase in cytosolic cAMP is observed but not coupled with glucagon secretion. Due to these shortcomings, the ATCC α-TC1-6 cell line is not a good model system for the study of regulated glucagon secretion in pancreatic alpha cells.
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I dedicate this thesis to my beloved pet and best friend, Bailey. He always offered unconditional love and provided me with emotional relief after difficult days. I love you and miss you every day.

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INTRODUCTION

Approximately 10% of the American population is living with diabetes. With 1.5 million new cases diagnosed each year, diabetes has become an epidemic in the United States as well as the rest of the world (Sheik Abdulazeez 2015). Due to this statistic, many researchers are working to control the physiological effects of the disease through an increased understanding of the secretion mechanisms behind the specialized cells of the pancreas responsible for the maintenance of glucose homeostasis. Type 1 and type 2 diabetes have been characterized by the body’s inability to respond to high blood-glucose levels with the secretion of appropriate amounts of insulin bringing the blood glucose levels to a normal range. However, recent research has indicated that unregulated glucagon secretion by the alpha cells plays a major role in the progression of diabetes (Lee et al. 2011). In type 1 diabetes, this is due to an autoimmune destruction of the beta cells, while type 2 diabetes is characterized by the impairment of the beta cells. Both cases lead to inefficient secretion of insulin and uncontrolled secretion of glucagon (Frandsen et al. 2016).

Regulation of Blood-Glucose by the Pancreas

A change in glycemic equilibrium is detected in the pancreas by a group of cells called the islets of Langerhans. The islets of Langerhans make up 1-2% of the pancreas and are comprised of four main cell types, each responsible for secreting a specific endocrine hormone (Rorsman and Braun 2013). Beta cells secrete insulin; alpha cells
secrete glucagon; delta cells secrete somatostatin; PP cells secrete pancreatic polypeptides; and epsilon cells secrete ghrelin. In rodents, the β-cells are in highest abundance at 60-80%, followed by the α-cells at 15-20%, with delta and PP cells in low abundance at <10% and <1%, respectively. The architecture of the islets varies greatly between species. In laboratory mice and rats, the β-cells are found in a cluster at the center of the islet, while the other cell types are dispersed throughout the mantle of the islet. Conversely, in human islets, there is no β-cell core as all of the cells are distributed evenly throughout the islet (Steiner et al. 2010). The islet is also highly vascularized, receiving approximately 10% of the total pancreatic blood supply (Rorsman and Braun 2013). These five cell types work in coordination to sustain biological blood-glucose equilibrium.

An increase in blood-glucose levels in the body, or hyperglycemia, stimulates the β-cells to secrete insulin, which in turn stimulates glucose absorption by the tissues for storage and lipid synthesis, thereby resulting in a drop in the amount of glucose circulating in the blood. Conversely, when there is a drop in normal blood-glucose levels, or hypoglycemia, it is detected in the pancreas by the alpha cells, which then secrete glucagon. Glucagon stimulates the release of glucose from glycogen stores in adipose cells, muscle cells, and the liver, resulting in an increase in blood glucose. Working in coordination, these two hormones are largely responsible for sustaining biological glycemic equilibrium.

Type 1 and type 2 diabetes are characterized by the body’s inability to respond to high blood-glucose levels by secreting the appropriate amounts of insulin necessary for bringing the blood-glucose levels into the normal range. In type 1 diabetes, this is due to
an autoimmune destruction of the beta cells (Sheik Abdulazeez 2015). In type 2 diabetes, an impairment of the beta cells causes ineffective insulin synthesis, and the body is unable to respond to insulin appropriately (Sheik Abdulazeez 2015). Following the rediscovery of the role of glucagon in diabetes, there is renewed interest in researching glucagon secretion by alpha cells (Moon and Won 2015). A recent study revealed that glucagon-receptor-knockout mice, whose beta cells were destroyed with streptozotocin, were able to maintain normal glycemic levels in the blood (Lee et al. 2011). In other words, animals that were incapable of producing insulin due to the loss of β-cells and that could not respond to the release of glucagon from α-cells because of the absence of the glucagon receptor had circulating blood-glucose levels that remained stable. The implication of these observations is that the regulation of glucagon secretion by the alpha cell is as important for the management of diabetes as the regulation of insulin secretion by the beta cells.

**Physiology of the Pancreatic Beta Cell**

*Glucose-regulated Insulin Secretion*

The mechanism of glucose-induced insulin secretion in the beta cell has been well studied and is largely understood. At high glucose concentrations, glucose enters through the GLUT2 transporter in the plasma membrane and is metabolized, resulting in an increase in the adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio in the cell which triggers closure of the ATP-sensitive potassium (K\textsubscript{ATP}) channel. This closure of the K\textsubscript{ATP} channel leads to depolarization of the membrane and the opening of voltage-gated L-type Ca\textsuperscript{2+} channels, resulting in an influx of calcium and the release of docked secretory granules containing insulin. Once secreted, insulin initiates cellular uptake of
glucose at fat, liver, and skeletal muscle cells and the incorporation of glucose moieties into glycogen in the cytoplasm for storage (Rorsman and Braun 2013).

**Physiology of the Pancreatic Alpha Cell**

In contrast to the beta cell, how the alpha cell regulates glucagon secretion is highly debated and not well understood. The regulation of secretion appears to be complex, and current research indicates that there are multiple possible mechanisms. The theories are approached from two perspectives: the intrinsic response to circulating levels of glucose and the influence of paracrine and endocrine factors (Rorsman et al. 2014b).

**Glucose-regulated Glucagon Secretion**

In low glucose conditions, glucagon secretion is increased due to low activity of K\textsubscript{ATP} channels, which results in the firing of large-amplitude action potentials. Brief or partial opening of K\textsubscript{ATP} channels hyperpolarizes the membrane to a negative membrane potential and removes the nearby Tetrodotoxin-sensitive Na\textsuperscript{+} channel inactivation. Opening of voltage-gated T-type Ca\textsuperscript{2+} channels depolarizes the membrane. When the membrane potential reaches action potential threshold, a large population of P/Q-type channels is able to enter the conductive state, triggering calcium influx and glucagon secretion (Göpel et al. 2000). Alternatively, in high glucose conditions an increased cytosolic ATP/ADP ratio causes the closure of K\textsubscript{ATP} channels, keeping the membrane potential depolarized below action potential threshold. At this depolarized potential, a large portion of T-type Ca\textsuperscript{2+} channels and Tetrodotoxin-sensitive Na\textsuperscript{+} channels are inactive and enter a nonconductive state. Consequently, low-amplitude action potential spikes are generated and the reduced Ca\textsuperscript{2+} influx is insufficient to trigger glucagon secretion (Figure 1) (Gromada et al. 2004).
Figure 1: Glucose-regulated glucagon secretion (Ashcroft and Rorsman 2013).

Paracrine, Autocrine, and Endocrine Regulation of the Alpha Cell

The spatial distribution of alpha cells in the islet, the high level of vascularization, and innervation allow for alpha cells to be regulated by nutrients, hormones, and neurotransmitters (Brereton et al. 2015). Insulin and zinc from the β-cell have been shown to be important paracrine regulators of alpha cells. Glucagon secretion is inhibited via the PIK3 pathway as well as the alteration in the sensitivity of the K<sub>ATP</sub> channel (Lee et al. 2011). Somatostatin, which is produced in islet δ-cells as well as other tissues, has been shown to inhibit glucagon secretion and insulin secretion. Glucagon itself can also exert control via an autocrine positive-feedback mechanism that stimulates secretion by increasing exocytosis-associated cAMP. GLP-1 from beta cells and L-cells in the stomach also act via GPCR, elevating cAMP levels and resulting in the suppression of glucagon. Alternatively, epinephrine, again acting through the cAMP pathway, increases glucagon secretion. The neurotransmitter γ-aminobutyric acid (GABA) is released by the
beta cell and activates GABA$_A$ receptors on the alpha cell. Activation of these receptors is coupled to inward chloride currents that hyperpolarize the alpha cell membrane and therefore decrease glucagon secretion. In addition, the neurotransmitter L-glutamate is co-secreted with glucagon, triggering GABA release from β-cells and resulting in a decrease in glucagon secretion (Figure 2).

**Figure 2: Autocrine, paracrine, and neuroendocrine regulation of the alpha cell** (Quesada et al. 2008).

**cAMP Pathways in Endocrine Cells**

Many of the paracrine signals that regulate glucagon secretion do so via the second messenger, cAMP (Figure 2). This occurs through G-protein coupled receptors.
(GPCR) that initiate the signaling cascade that generates cAMP. Following the activation of the GPCR by the ligand, the $G_s$-alpha subunit dissociates from the $\beta$ and $\gamma$ subunits and exchanges GDP for GTP. The now activated $G_s$-alpha subunit binds to adenylyl cyclase (AC), causing the catalytic conversion of ATP to cyclic adenosine monophosphate (cAMP). cAMP, now a second messenger, is able to activate the PKA and Epac pathways.

Protein kinase A (PKA) is a tetramer consisting of two catalytic subunits and two cAMP binding sites. Four molecules of cAMP are required to induce the conformational change that results in the regulatory subunits dissociating from the activated catalytic subunits. Once activated, these catalytic subunits are capable of phosphorylating target proteins, including ion channels, enzymes, and transcription factors (Wu et al. 2015).

Epac (exchange protein activated by cAMP) is a cAMP-regulated guanine nucleotide exchange factor (GEF) (Holz, 2004). Two isoforms, Epac1 and Epac2, have been discovered and exhibit different affinities for cAMP binding. Both isoforms of Epac are multi-domain proteins containing an N-terminal regulatory domain and a C-terminal catalytic domain, which is the site for cAMP binding. The Epac isoforms act as GEFs for the GTPases Rap1 and Rap2. The nucleotide exchange is initiated by the binding of cAMP to the catalytic domain of the protein (Bos, 2006).

**Ca$^{2+}$-induced Glucagon Secretion in Pancreatic Alpha Cells**

Due to the important role endocrine peptides and hormones play in maintaining homeostasis, their secretion occurs through the regulated secretory pathway. The message for propeptides and prohormones is first translated in the rough endoplasmic reticulum. The peptide is then transferred to the Golgi apparatus for further processing and finally
packaged into dense-core vesicles (DCV) (Lin and Salton 2013). The exocytosis of DCV and secretion of hormone is tightly controlled by both Ca\(^{2+}\) and cAMP, signaling pathways within the endocrine cells. In alpha cells, synaptotagmin 7 (SYT7) functions as the primary Ca\(^{2+}\)-sensor for exocytosis and acts via Ca\(^{2+}\)-dependent interactions with both the fusing of phospholipid membranes and the membrane fusion machinery (Gustavsson et al. 2009a).

In order to study the many facets of pancreatic alpha cell secretion, we used the Alpha TC1.6 immortal cell line clone purchased from American Type Culture Collection (ATCC).

**Usage of Immortal Cell Lines for Biological Study**

The usage of immortal cell lines for biological study is appealing to scientists because they are easy to obtain, offer an unlimited supply of cells, are cost effective, and avoid the ethical concerns of using animal and human tissue. American Type Culture Collection (ATCC) has stock of over 3,600 cell lines from over 150 different species (Kaur and Dufour 2012). Despite the many advantages of using cell lines for study, there are also drawbacks. In order for a cell line to be considered an appropriate model system, the cell line must retain many of the same functional characteristics of the primary cell and be properly maintained to preserve these characteristics (Geraghty et al. 2014).

**Study of Pancreatic Alpha Cell Line Clone Alpha TC1.6**

Alpha TC1.6 is one of the pancreatic alpha clones derived from an adenoma created in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat preproglucagon promoter. Previous literature indicates the presence of high levels of glucagon and a proglucagon fragment, glucagon-like peptide I (GLP-1), the
presence of processing enzyme PC2 ((Powers et al. 1990) and tissue specific homeotic genes in alpha TC1.6 cells (Mizusawa et al. 2004), as well as the evidence of insulin inhibition of glucagon secretion (Shen et al. 2012).

Previously in our lab, experiments were performed using the alpha TC1.6 clone obtained from Dr. Don Steiner (University of Chicago). This clone was used to study intracellular calcium activity and cAMP activity associated with the melanocortin 4 receptor (Liang 2009), as well as cAMP production and the presence of phosphodiesterases (Crane 2011). However, this clone of alpha TC1.6 cells is no longer available.

The objective of my research was to test the hypothesis that the current alpha TC1.6 clone available through the American Type Culture Collection (ATCC) has the same properties as the alpha TC1.6 clone provided to our lab by Dr. Steiner several years ago. To this end, we will analyze the relative expression of genes crucial for glucagon secretion, the intracellular calcium activity and biochemical glucagon secretion at different extracellular glucose concentrations, and the intracellular calcium activity and secretion in the presence of pharmacological drugs that modulate cAMP levels and ion channel activity.

The subsequent experiments outlined in this thesis help characterize glucagon secretion in a commercially available alpha TC1.6 clone from American Type Culture Collection (ATCC).


**METHODS**

**Cell Culture**

Mouse pancreatic α cell line αTC1-6 cells (American Type Culture Collection, ATCC, purchased in 2014, Manassas, VA) were cultured in DMEM containing 15 mM glucose, 10% heat-inactivated fetal bovine serum, 1.0 mM sodium pyruvate, 100 unit/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml normacin, and MEM non-essential amino acids and maintained in a humidified incubator with 95% air and 5% CO₂ at 37 °C.

**Islet Dissociation and Culture**

Mouse pancreas was perfused with 4 mL (1,000 U ml⁻¹) collagenase in HBSS via the joint site of the hepatic duct and cystic duct. (All procedures were approved by University of Denver, IACUC.) The pancreas was removed and digested in 1 mL of collagenase for 15 minutes at 37 °C. Adding ice-cold 1mM CaCl₂ in 1x HBSS terminated collagenase digestion. Islets were handpicked and plated on tissue culture dishes. Islets were cultured until use at 37 °C in RPMI medium containing 10.4 mg/L-glutamate, 10% FBS, 10 mM HEPES, 1 mM Na pyruvic acid, 7 µl/L β-mercaptoethanol, 1% pen/strep, and 3.7 g/L sodium bicarbonate.

**Immunofluorescence**

For single cell immunofluorescence experiments, the ATCC αTC1-6 cells were plated in 8-well chamber slides. After 24 hours, the cells were fixed with a 4% PFA solution for 20 minutes, and then all wells were washed with a 1xPBS solution. The cells
were then permeablized with 0.4% Triton X-100/1X PBS for 15 minutes, followed by a blocking step (20% donkey serum, 1% BSA, in 1X PBS) for 30 minutes at room temperature. Next, the primary antibody was added in 1X PBS with 10% donkey serum and 5% BSA, and the cells were incubated in a humidified chamber at 40 °C for 90 minutes. After three washes with 1X PBS, cells were incubated at RT for 45 minutes with the secondary antibody in 1X PBS with 20% donkey serum and 1% BSA. After three washes with 1X PBS, the slides were mounted with Vecta-Shield + DAPI (Vector Laboratories Inc., Burlingame, CA) and coverslips were applied to the slides and sealed with clear nail polish. The slides were imaged using a laser-scanning Olympus FluoView FV1000 confocal microscope using the 60x oil objective (N.A. 1.42).

Isolated islets were immunostained in solution. First they were fixed in 4% PFA for 15 minutes at RT, followed by a wash with 1X PBS. The cells were then permeablized with 0.4% Triton X-100/1X PBS for 15 minutes. Next, the primary antibody was added in 1X PBS with 10% donkey serum and 10% BSA, and the islets were incubated overnight at 4 °C. After three washes with 1X PBS, islets were incubated at RT for 60 minutes with the secondary antibody in 1X PBS with 20% donkey serum and 10% BSA. After three washes with 1X PBS, islets were affixed to ECL-coated slides, then mounted with Vecta-Shield + DAPI (Vector Laboratories Inc., Burlingame, CA) and coverslips were applied to the slides and sealed with clear nail polish. The slides were imaged using a laser-scanning Olympus FluoView FV1000 confocal microscope using the 60x oil objective (N.A. 1.42).
mRNA Isolation and cDNA Synthesis

Prior to extracting messenger RNA (mRNA), the adherent cells were detached using 0.25% trypsin (Thermo Fisher Scientific, Waltham, MA), pelleted low speed and washed with 1X PBS. The mRNA was isolated using the RNAqueous-4PCR kit (Invitrogen, Carlsbad, CA). The mRNA was then extracted and converted into complimentary DNA (cDNA) using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

Quantitative Polymerase Chain Reaction (qPCR)

Thermocycling and fluorescence detection were done using a Bio-Rad iQ5 Real-Time PCR Detection System linked to a PC running iQ5 Optical System Software version 1.1. Each reaction was performed in quadruplicate. qPCR Taqman primers were obtained from Applied Biosystems (Foster City, CA). Inventoried primers were ordered for all genes of interest. Each qPCR reaction consisted of 10 µL Bio-Rad iQ5 2X Supermix (Hercules, CA), 1 µL 20X primer and probe, 2 µL cDNA template, and 7 µL DEPC treated water. Final reaction conditions were as follows: 100 ng template DNA, 50 mM KCl, 20 mM Tris-HCL, 0.8 mM deoxynucleotide triphosphates, 0.5 U iTaq DNA polymerase, 3 mM MgCl₂, and 400 nM primer and 250 nM probe. The QPCR program consisted of 2 steps: initial denaturation for 3 minutes at 95 °C, followed by 40 cycles of a denaturing step at 95 °C for 10 seconds and an annealing/extension step at 60 °C for 30 seconds. Fluorescence was measured at the end of every annealing/extension step. The housekeeping gene, GAPDH, was run in quadruplicate along with all the samples.
**Relative Expression and Statistics**

Relative expression was calculated using the $2^{\Delta Ct}$ method, described in the iQ5 software manual. The calculations are:

$$\text{Relative expression} = 2^{\Delta Ct} \times 10000; \quad \Delta Ct = \text{Ct}_{\text{control}} - \text{Ct}_{\text{sample}}$$

The control was the average Ct (threshold cycle) of the housekeeping gene and the sample. The housekeeping gene used for all genes tested was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Significance between samples was determined using an unpaired student-T test. Results with $p<0.05$ were deemed significant. Statistical tests were performed using Prism software (GraphPad Software Inc., La Jolla, CA).

**Glucagon Secretion Biochemical Assay**

An ANF-GFP plasmid used to monitor secretion was obtained as a gift from Dr. Cedric Asencio (University of Denver, Denver, CO). Alpha TC1.6 cells were plated 72 hours prior to performing the secretion assay on ECL-coated (37 °C for 1 hour followed by a serum-free media rinse) 24-well plates. A confluent flask of the adherent cells was detached using 0.25% trypsin (Thermo Fisher Scientific, Waltham, MA), pelleted at low speed and supernatant was removed. Cells were resuspended in 24 mL of fresh culture media. 1 mL of the cell suspension was pipetted into each well. Plated cells were incubated in a humidified incubator with 95% air and 5% CO$_2$ at 37 °C. 24 hours following plating, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfection conditions per reaction were as follows: 250 ng plasmid DNA, 100 µl optimum, 2 µl Lipofectamine 2000. Solution was incubated at room temperature for 15 minutes and added to 1 well containing 500 µl of fresh culture media. Transfected cells were incubated in a humidified incubator with 95% air and 5% CO$_2$ at 37 °C for 48 hours.
Following incubation period, the culture media was removed and cells were incubated at room temperature for 30 minutes in standard resting solution. Buffer was then removed and cells were incubated with 250 µl testing solution for 10 minutes. After incubation, solution was immediately removed and placed into 1.5 mL microcentrifuge tubes which were kept at 4 °C. 250 µl of lysis buffer (1% triton-x, 20 mM Tris pH 7.5, 50 mM NaCl, protease inhibitor tablet). A 24-well plate containing cells and lysis buffer was incubated, rotating, at 4 °C for 30 minutes. Cell lysate was removed and placed into 1.5 mL microcentrifuge tubes. All 1.5 mL microcentrifuge tubes from both stimulation and lysis were pelleted at 14,000 RPM for 10 minutes at 4 °C. 150 µl of each solution was pipetted into a 96-well plate (Corning, Corning, NY) and read at 480/530 nm using a TECAN M1000 plate reader. Results were plotted as a % of total using Prism software. Significance between samples was determined using an unpaired student-T test. Results with p<0.05 were deemed significant.

**Intracellular Calcium Imaging**

Alpha TC1.6 cells were plated 48 hours prior to imaging on ECL-coated (37 °C for 1 hour followed by a serum-free media rinse) in 12 mm glass bottom imaging dishes. Prior to imaging, culture media was removed and replaced with 5 µM Fura-2 AM (Invitrogen, Carlsbad, CA) in standard resting solution for 30 minutes at room temperature. Following a 30-minute incubation period, the glass dishes were thoroughly washed with standard resting solution (1.0 mM MgCl₂, 10.0 mM HEPES, 5.0 mM CaCl₂, 140 mM NaCl₂, 2.0 mM KCl, 8.0 mM D-glucose, at a pH of 7.2). As controls for cellular health, a plate was imaged for total experimental time in resting glucose (8 mM) prior to beginning experiments, and a high potassium (60 mM) solution was applied to cells via
perfusion following experimental imaging. All experiments were conducted at ambient temperature (~22 °C). Images were acquired by 40X oil immersion objective (Zeiss) with an inverted fluorescence Zeiss Axiovert microscope equipped with Cooke SensiCam CCD camera. 340 nm and 380 nm excitation filters controlled by a Sutter lambda 10-2 filter wheel were used for Fura-2 AM dual excitation ratio imaging. Image pairs were obtained every 3 seconds for 100 ms and binned with a setting of 4x4. Images were acquired and analyzed with Slidebook software (Intelligent Imaging Innovations, Denver, CO). Perfusions of the indicated test external solutions were performed using a gravity flow system. All test solutions were applied after establishing baseline calcium activity. Images from Fura-2 AM microscopy experiments were analyzed in Slidebook. Values were exported as Microsoft Excel files and imported in Igor software (Wavemetric, Portland, OR). All traces were blindly scored as responders and non-responders.

**Pharmacology of Channel Complexes and Modulation of cAMP in Alpha Cells**

Endocrine cells undergo regulated secretion in response to physiological signals and must utilize ion channels on the plasma membrane as well as activate chemical pathways. Following is a brief description of the pharmacological tests utilized in this study. A summary of experimental conditions can be found in Appendix A.

**cAMP**

Forskolin is a biochemical activator of the enzyme adenylyl cyclase (AC), which catalyzes the conversion of adenosine triphosphate (ATP) to the second messenger, cyclic adenosine monophosphate (cAMP).

3-isobutyl-1-methylxanthine (IBMX) is a competitive, non-selective inhibitor of cAMP phosphodiesterases and therefore increases cytosolic levels of cAMP.
8-pCPT-2'-O-Me-cAMP is a cAMP analog and Epac-selective agonist, resulting in activation of the Epac pathway. Conversely, 6-Bnz-cAMP, also a cAMP analog, is a PKA-selective agonist.

**Voltage-gated Calcium Channel Blockers**

The toxins listed below block the calcium channel activity and therefore alter the influx of calcium ions into the cell. The voltage sensor in the α1 subunit, which forms the Ca$^{2+}$-selective pore, classifies voltage-gated calcium channels.

Conotoxins are a group of neurotoxins isolated from marine snails. Two isoforms, ω-conotoxin CVIB and ω-conotoxin CVIC, are moderate inhibitors of P/Q-type calcium channels as well as a less effective N-type calcium channel blocker.

ω-Agatoxin is a neurotoxin isolated from spider venom. It selectively blocks P/Q-type calcium channels.

Nifedipine is a dihydropyridine and is a high affinity L-type calcium channel blocker.

**Sodium Channel Blocker**

Tetrodotoxin (TTX) is a neurotoxin that blocks the activity of voltage-gated sodium channels, inhibiting the flow of sodium ions into the cell, and therefore prevents of action potentials.

**Potassium-sensitive Channel Modulators**

The pharmaceutical drugs tolbutamide and diazoxide act via the $K_{ATP}$ channel by binding to the sulfonylurea receptor SUR1 to close or open the channel, respectively. As a result, this alters the flow of potassium ions out of the cell, affecting membrane potential.
RESULTS

Immunofluorescence of Glucagon in ATCC αTC1-6 Cells

Immunofluorescence was the first step in determining the efficacy of the cell line we received from ATCC and whether or not it could be used as a model for secretion. This was performed using an antibody against the hormone glucagon. As a control for the primary antibody specificity to the antigen of interest, using the anti-glucagon primary antibody at the same concentration and similar conditions as the experimental, whole islets dissected from a wild-type FVB mouse were analyzed for the presence of glucagon (Figure 3A). As expected, the data indicate the presence of glucagon in the anticipated localization on the periphery of the islet from the rodent pancreas (Steiner et al. 2010). As a control for the specificity of the secondary antibody to the primary antibody, performed simultaneously with the experiment and under the same conditions, ATCC αTC1-6 cells were not treated with the primary antibody against the hormone of interest (Figure 3B). No reaction was observed for the secondary antibody, indicating that the anti-mouse secondary antibody used is specific for the mouse glucagon primary antibody. Using a primary antibody against the hormone glucagon on ATCC αTC1-6 cells, a strong signal that is characteristic of endoplasmic reticulum, Golgi apparatus, and dense-core vesicle staining was observed (Figure 3C).
Relative Expression of Significant Genes in ATCC αTC1-6 Cells

For a cell line to be considered a good model for secretion in pancreatic alpha cells, it needs to express the genes of established key players of the primary cells. In order to determine which representative genes are expressed in the ATCC αTC1-6 cells, qPCR analysis was performed using cDNA generated from mRNA of cell lysates. As expected, the gene encoding the intrinsic hormone glucagon was detected in high abundance relative to the other genes tested (Figure 4).

Next, the presence of the gene encoding the ATP-sensitive potassium channel, which is known to help maintain the resting potential and depolarization of the cellular membrane under glucose-mediated glucagon secretion, was investigated. It is comprised of 2 subunits: an inward rectifying (Kir6.2) subunit and a sulfonylurea-1 (SUR1) subunit. qPCR analysis indicated the presence of the genes encoding both subunits (Figure 4).

Many hormones, including glucagon, are secreted in dense-core vesicles in a calcium-dependent manner from alpha cells (Gustavsson et al. 2009a). SYT7 mediates this secretion by inserting into the membrane and inducing a positive curvature of the membrane, initiating the fusion of the vesicle membrane and the plasma membrane. Studies have shown that SYT7 is responsible for the calcium-dependent secretion of glucagon in alpha cells (Gustavsson et al. 2009b). qPCR analysis indicated the presence of SYT7 in ATCC αTC1-6 cells (Figure 4).

The neurotransmitter γ-aminobutyric acid (GABA) has been shown to be a paracrine-signaling molecule in pancreatic islets. The GABA_A receptor is present on the islet alpha cell membrane, but is not present on islet beta cells. Therefore, when released from beta cells via Ca^{2+}-dependent exocytosis, inhibition of glucagon secretion is
observed (Wendt et al. 2004). qPCR analysis of the gene GABRA1 is found to be below detectable limits in ATCC αTC1-6 cells (Figure 4).

Electrical activity and ion channels largely regulate the secretion of glucagon. At low glucose concentrations, the action potentials resulting from the opening of voltage-activated L- and P/Q-type Ca\(^{2+}\) channels, tetrodotoxin (TTX)-sensitive Na\(^+\) channels, and K\(_{ATP}\) channels lead to increased glucagon secretion. One of these channels, the P/Q-type Ca\(^{2+}\) channel has been found to be sensitive to the peptide toxin ω-agatoxin IVA (Ramracheya et al. 2010). Therefore, we wanted to determine if this ω-agatoxin-sensitive channel was expressed in ATCC αTC1-6 cells. The data indicate that the gene CACNA1A was not expressed at detectable limits (Figure 4).

A previous study demonstrating the stimulatory effect of adrenaline on glucagon secretion in primary rodent alpha cells revealed that the beta-1 adrenergic receptor and the beta-2 adrenergic receptor were expressed in the alpha cell at a 25- and 40-fold increase relative to beta cells (Rorsman et al. 2014c). The expression for the genes ADRB1 and ADRB2 were below detectable limits in ATCC αTC1-6 cells (Figure 4).
**Figure 3: Immunofluorescence analysis of glucagon in ATCC αTC1-6 cells.** In all experiments, nuclei were stained with DAPI and from a mouse Alexa 488 secondary antibody (1:800) was used for visualization. (A) Intact islet of WT mouse immunostained with a mouse anti-glucagon primary antibody (1:100). (B) ATCC αTC1-6 cells not exposed to anti-glucagon primary antibody. (C) ATCC αTC1-6 cells immunostained with a mouse anti-glucagon primary antibody (1:100).
**Figure 4: qPCR analysis of ATCC αTC1-6 cell cDNA.** Genes are listed by common names followed by scientific names in parentheses. The genes encoding glucagon, Kir6.2, SUR1, and SYT7 were detected. Genes encoding the GABA_A receptor, an agatoxin-sensitive voltage-gated calcium channel, β-1 adrenergic receptor, and β-2 adrenergic receptor were below detection limits. All expression is normalized to GAPDH. Relative expression was determined using the $2^{ΔΔCt} \times 10000$. 
Effect of Extracellular Glucose Concentrations on Glucagon Secretion in ATCC αTC1-6 Cells

Previous studies of isolated intact islets have demonstrated that maximal glucagon secretion occurs at low glucose concentrations within the range of 0 mM-5 mM and that maximal inhibition of glucagon secretion occurs between 6 mM and 8 mM glucose. As the extracellular glucose concentration increases from 8 mM to 20 mM, a reduction in this inhibition is observed (Salehi, Vieira, and Gylfe 2006). Glucagon secretion at varying glucose concentrations was tested using the dense-core vesicle-targeted ANF-GFP plasmid. It is assumed that the GFP fluorescence detected is directly correlated with the secretion of glucagon. ATCC αTC1-6 cells were stimulated for 10 minutes at room temperature with test solution containing 2 mM, 5 mM, 8 mM, and 20 mM glucose. The analysis of the fluorescence of the secreted fraction divided by the fluorescence of the lysis fraction is depicted as a percentage in Figure 5. Minimal glucagon secretion is observed at 8 mM glucose. An elevated level of glucagon secretion is observed at low (2 mM glucose) as well as high (20 mM) glucose conditions. There is a significant change in secretion between 2 mM and 8 mM glucose (p-value<0.05).

Effect of Extracellular Glucose Concentrations on [Ca^{2+}]_i in ATCC αTC1-6 Cells

The next set of experiments analyzed the effect that different extracellular glucose concentrations have on the intracellular Ca^{2+} activity in the ATCC αTC1-6 cell. Intracellular Ca^{2+} activity ([Ca^{2+}]_i) was investigated using the ratiometric calcium sensor Fura-2 AM. Alpha cells are electrically excitable cells as they contain many different ion channels and are known to have spontaneous action potentials and sporadic Ca^{2+} influx under resting conditions (Ramracheya et al. 2010). These experiments characterized the
effect that glucose concentration has on the electrical activity, specifically the influx of calcium. In Figure 6, the cells were initially in a bath of 8 mM glucose establishing a baseline calcium activity. Next, via bath perfusion, low (2 mM) or high (15 mM) glucose was applied to the cells. Intracellular calcium activity was monitored for 16.6 minutes. Prior to the end of data acquisition in all intracellular calcium experiments, a bath solution containing 60 mM K+ was perfused over the cells to provide a maximal calcium influx, thus indicating the cells remained healthy throughout the experiment (data not shown). Following the addition of 2 mM glucose, an increase in calcium oscillations was observed in 27% of cells analyzed (n=60) and 19% (n=62) after the addition of 15 mM glucose (Figure 6).
Figure 5: Glucagon secretion in ATCC αTC1-6 cells at different extracellular glucose concentrations. 72 hours after cells were transfected with an ANF-GFP plasmid, the secretion experiment was performed. Data is represented as % of total GFP fluorescence. Maximal inhibition occurs at 8 mM glucose. Maximal secretion is observed at 2 mM glucose and 20 mM glucose. Significant change is detected between 2 mM glucose and 8 mM glucose (p-value<0.05).
Figure 6: Effect of glucose on $[\text{Ca}^{2+}]_i$ in ATCC αTC1-6 cells. Traces are representative $[\text{Ca}^{2+}]_i$ responses to 8 mM, 2 mM, and 15 mM extracellular glucose. Calcium activity is visualized using Fura-2 AM. Cells were imaged in 8 mM glucose for 10 minutes, followed by perfusion of 2 mM (A) or 15 mM (B) glucose and imaged an additional 16.6 minutes. α-cell $[\text{Ca}^{2+}]_i$ is expressed as a ratio of 340/380 nm. An increase in $[\text{Ca}^{2+}]_i$ is observed in 27% of cells analyzed (n=60) at 2 mM glucose (A) and 19% (n=62) at 15 mM glucose (B).
Effect of $K_{ATP}$ Channel on $[Ca^{2+}]_i$ in ATCC $\alpha$TC1-6 Cells

The $K_{ATP}$ channel is thought to be responsible for the regulation of membrane potential in the pancreatic alpha cell, leading to or preventing action potentials responsible for glucagon secretion (Rorsman et al. 2014c). The next set of experiments was directed at analyzing the effect the channel activity has on intracellular calcium levels. ATCC $\alpha$TC1-6 cells were loaded with the ratiometric calcium-indicator dye FURA2-AM. $[Ca^{2+}]_i$ signal was recorded every 3 seconds for 10 minutes at 8 mM glucose. This was followed by a bath perfusion of the $K_{ATP}$ channel modulators and recorded for an additional 16.6 minutes. Figure 7A illustrates the effect of $K_{ATP}$ channel blocker tolbutamide on ATCC $\alpha$TC1-6 cells. An increase in calcium-spiking activity was observed in 53% of the cells analyzed (n=74). Figure 7B depicts the effect of the $K_{ATP}$ channel activator diazoxide on ATCC $\alpha$TC1-6 cells. A decrease in calcium-spiking activity was observed in 4% of the cells analyzed (n=90).

Effect of a Na$^+$ Channel Blocker on $[Ca^{2+}]_i$ in ATCC $\alpha$TC1-6 Cells

The sodium channels in alpha cells play a major role in reaching action potential, resulting in the secretion of glucagon (De et al. 2010). The next experiment investigated the effect the Na$^+$ channel blocker tetrodotoxin (TTX) has on the spontaneous activity of ATCC $\alpha$TC1-6 cells in resting solution. ATCC $\alpha$TC1-6 cells were loaded with the ratiometric calcium-indicator dye FURA2-AM. $[Ca^{2+}]_i$ signal was recorded every 3 seconds for 10 minutes at 8 mM glucose. Next, Tetrodotoxin was applied to the cells via bath perfusion, and the resultant calcium activity was monitored every 3 seconds for 16.6 minutes. No decrease in intracellular calcium activity was detected (n=97) (Figure 8).
Figure 7: Effect of K\textsubscript{ATP} channel modulators on [Ca\textsuperscript{2+}]\textsubscript{i} in ATCC αTC1-6 cells. Traces are representative of [Ca\textsuperscript{2+}]\textsubscript{i} responses to tolbutamide and diazoxide at 8 mM glucose. Calcium activity is visualized using Fura-2 AM. Cells were imaged in 8 mM glucose for 10 minutes, followed by perfusion of 8 mM glucose + tolbutamide (100 µM) (A) or 8 mM glucose + diazoxide (1 µM) (B) and imaged an additional 16.6 minutes. α-cell [Ca\textsuperscript{2+}]\textsubscript{i} is expressed as a ratio of 340/380 nm. An increase in [Ca\textsuperscript{2+}]\textsubscript{i} is observed in 53% of cells analyzed (n=74) in 8 mM glucose + tolbutamide (A). A decrease in [Ca\textsuperscript{2+}]\textsubscript{i} is observed in 4% of cells analyzed (n=90) in 8 mM glucose + diazoxide (B).
Figure 8: Effect of a Na\(^+\) channel blocker on \([Ca^{2+}]_i\) in ATCC αTC1-6 cells. Traces are representative of \([Ca^{2+}]_i\) responses to tetrodotoxin at 8 mM glucose. Calcium activity is visualized using Fura-2 AM. Cells were imaged in 8 mM glucose for 10 minutes, followed by perfusion of 8 mM glucose + tetrodotoxin (100 nM) and imaged an additional 16.6 minutes. α-cell \([Ca^{2+}]_i\) is expressed as a ratio of 340/380 nm. No change in \([Ca^{2+}]_i\) is observed in any cells analyzed (n=97).
Effect of Voltage-gated Ca^{2+} Channel Blockers on [Ca^{2+}]_i in ATCC αTC1-6 Cells

Voltage-gated calcium channels are important for the calcium influx associated with alpha cell excitability and glucagon secretion (Ramracheya et al. 2010). The next experiments determined the presence or absence of N-type, P/Q-type, and L-type channels in ATCC αTC1-6 cells. The cells were loaded with the ratiometric calcium-indicator dye FURA2-AM. [Ca^{2+}]_i signal was recorded for 10 minutes at 8 mM glucose. Next, a 2 mM glucose solution was perfused across the cells, followed by an additional 10 minutes of [Ca^{2+}]_i signal recording. The calcium channel antagonists were then added to the perfusion solution. Nifedipine, ω-agatoxin, and ω-conotoxin (CVIC and CVIB) were used to selectively block L-type, P/Q-type calcium channels, and N-type calcium channels, respectively. In Figure 9, a representative FURA2-AM experiment shows that neither ω-conotoxin CVIB (100 nM, n=53) nor ω-conotoxin CVIC (50 nM, n=65) has any apparent effect on ATCC αTC1-6 cells [Ca^{2+}]_i at low glucose levels. Figure 10A indicates that in 70% of cells analyzed, there was a decrease in calcium oscillations in the presence of nifedipine (10 µM, n=20) at low glucose concentrations. Figure 10B shows that 15% of cells exposed to ω-agatoxin (100 µM, n=19) under low glucose concentrations resulted in a decrease in calcium-spiking activity.
**Figure 9: Effect of N-type Ca\(^{2+}\) channel blockers on [Ca\(^{2+}\)]\(_i\) in ATCC \(\alpha\)TC1-6 cells.**

Traces are representative [Ca\(^{2+}\)]\(_i\) responses to \(\omega\)-conotoxin CVIB (A) and \(\omega\)-conotoxin CVIC (B) at 2 mM glucose. Calcium activity is visualized using Fura-2 AM. \(\alpha\)-cell [Ca\(^{2+}\)]\(_i\) is expressed as a ratio of 340/380 nm. Cells were imaged in 8 mM glucose for 10 minutes, followed first by perfusion of 2 mM for 10 minutes, followed second by perfusion of 2 mM glucose + \(\omega\)-conotoxin CVIB (100 \(\mu\)M) and 2 mM glucose + \(\omega\)-conotoxin CVIC (50 \(\mu\)M), and then imaged an additional 10 minutes. No significant decrease in calcium oscillations is observed.
Figure 10: Effect of P/Q- and L-type Ca\(^{2+}\) channel blockers on [Ca\(^{2+}\)]\(_i\) in ATCC αTC1-6 cells. Traces are representative [Ca\(^{2+}\)]\(_i\) responses to nifedipine (A) and ω-agatoxin (B) at 2 mM glucose. Calcium activity is visualized using Fura-2 AM. α-cell [Ca\(^{2+}\)]\(_i\) is expressed as a ratio of 340/380 nm. Cells were imaged in 8 mM glucose for 10 minutes, followed first by perfusion of 2 mM for 10 minutes, followed second by perfusion of 2 mM glucose + nifedipine (10 µM) and 2 mM glucose + ω-agatoxin (100 µM), and imaged an additional 10 minutes. A decrease in [Ca\(^{2+}\)]\(_i\) is observed in 70% of cells analyzed (n=20) in the presence of 2 mM glucose + nifedipine (A) and in 15% of cells analyzed (n=19) in the presence of 2 mM glucose + ω-agatoxin (B).
Effect of cAMP on Intracellular Ca\textsuperscript{2+} Activity in ATCC αTC1-6 cells

cAMP is known to potentiate glucagon secretion in rodent alpha cells through a PKA-dependent pathway as well as through Epac2, a PKA-independent pathway (De et al. 2010). Experiments were performed to test how each pathway affects the intracellular calcium activity of the ATCC αTC1-6 cells. ATCC αTC1-6 cells were loaded with the ratiometric calcium-indicator dye FURA2-AM. [Ca\textsuperscript{2+}]\textsubscript{i} signal was recorded for 10 minutes at 8 mM glucose, followed by a perfusion of the cAMP modulators and recorded for an additional 16.6 minutes. First, forskolin, which increases cAMP by activating adenylyl cyclase in conjunction with the non-selective phosphodiesterase inhibitor IBMX, was applied to the cells. 35% of cells analyzed (n=55) showed an increase in intracellular calcium activity following the addition of forskolin/IBMX (Figure 11A). Next, 6-Bnz-cAMP, a cell permeable cAMP analog that selectively activates the cAMP-dependent PKA pathway, was applied. 2% of cells analyzed (n=72) showed an increase in intracellular calcium activity following the addition of 6-Bnz-cAMP (Figure 11B). Finally, 8-pCPT-2'O-Me-cAMP, a cell permeable cAMP analog that selectively activates the EPAC signaling pathway, was tested. 13% of cells analyzed (n=55) showed in increase in intracellular calcium activity following the addition of 8-pCPT-2'O-Me-cAMP (Figure 11C).

Effect of cAMP Agonists on Glucagon Secretion in ATCC αTC1-6 Cells

The previous experiments indicated that, to varying degrees, an increase in cAMP agonists led to an increase in intracellular calcium. To determine if the increases in cAMP and [Ca\textsuperscript{2+}]\textsubscript{i} are linked to an increase in glucagon secretion, the following experiment was performed. Using the ANF-GFP system for glucagon secretion analysis,
three conditions were tested: 8 mM glucose, the cAMP agonist forskolin/IBMX in 8 mM glucose, and the cAMP inhibitor Rp-8-Br-cAMPS in 8 mM glucose. No significant difference was observed among any of the conditions (Figure 12).
Figure 11: Effect of cAMP modulators on $[\text{Ca}^{2+}]_i$ in ATCC αTC1-6 cells. Traces are representative of $[\text{Ca}^{2+}]_i$ responses to forskolin/IBMX (A), 6-Bnz-cAMP (B), and 8-pCPT-2'-O-Me-cAMP (C) at 8 mM glucose. Calcium activity is visualized using Fura-2 AM. Cells were imaged in 8 mM glucose for 10 minutes, followed by perfusion of 8 mM glucose + forskolin/IBMX (50 µM), 8 mM glucose + 6-Bnz-cAMP (20 µM), and 8-pCPT-2'-O-Me-cAMP (10 µM), then imaged an additional 16.6 minutes. α-cell $[\text{Ca}^{2+}]_i$ is expressed as a ratio of 340/380 nm. An increase in $[\text{Ca}^{2+}]_i$ is observed in 35% of cells analyzed ($n=55$) in the presence of forskolin/IBMX, 2% ($n=72$) in the presence of 6-Bnz-cAMP, and 13% ($n=55$) in the presence of 8-pCPT-2'-O-Me-cAMP.
Figure 12: Glucagon secretion in ATCC αTC1-6 cells stimulated by cAMP modulators. The cAMP agonist forskolin/IBMX and cAMP antagonist RP-8-Br-cAMPS were analyzed and compared to 8 mM glucose. 72 hours after cells were transfected with an ANF-GFP plasmid, the secretion experiment was performed. Data is represented as % of total GFP fluorescence. No significant difference was observed among any of the conditions.
DISCUSSION

Tumor cell lines are appealing for use in scientific research because they are cost effective, readily available, and not encumbered by the ethical concerns of working with primary tissue. However, in order for a tumor cell line to be considered an appropriate model system for endocrine research, it must retain the functionality of the primary endocrine cell and meet the following criteria: (1) Be able to detect characteristic physiological change through receptors that respond to this change and regulate secretion in the endocrine cell. (2) Possess channel complexes that respond to the extracellular signal and (3) Have a functional signal transduction pathway (i.e., appropriate second messenger system). The objective of this study was to analyze how the tumor cell line clone αTC1-6 obtained from ATCC responds to a change in circulating glucose levels and the resulting effect on the release of glucagon, the presence and activity of important ion channels in glucose-sensing and glucagon secretion, and the function of the cAMP signaling pathway leading to glucagon secretion.

Confirmed Expression of Glucagon in ATCC αTC1-6 Cells

The pancreatic alpha cell is responsible for the regulated secretion of the peptide hormone glucagon in response to hypoglycemia. This secretion of glucagon initiates the synthesis and mobilization of glucose, returning the blood-glucose concentrations to levels within the normal range. Our results indicate that the gene for glucagon is
expressed in high abundance in the clonal cell line ATCC αTC1-6 (Figure 4) and is transcribed into protein (Figure 3).

**Expression Detected for Synaptotagmin 7, but No Expression Detected for the Adrenergic Receptor in ATCC αTC1-6**

The pancreatic alpha cell is responsible for secreting glucagon when there is a decrease in circulating glucose levels in the blood. The hormone is packaged into dense-core vesicles and secreted in a Ca\(^{2+}\)-dependent manner following the binding of calcium to the Ca\(^{2+}\)-sensor synaptotagmin 7. Our results indicate that the gene encoding synaptotagmin 7 is present in ATCC αTC1-6 cells (Figure 4).

An important paracrine regulator of the alpha cell is the neurotransmitter epinephrine. Research has shown that epinephrine stimulates glucagon secretion through the activation of the β-1 and β-2 adrenergic receptor (De et al. 2010). Our results indicate the genes encoding the β-1 and β-2 adrenergic receptors are not detectable in the ATCC αTC1-6 cells (Figure 4).

**Increase in Intracellular Calcium Oscillations Coupled with Glucagon Secretion at Low Levels of Glucose Concentration**

The alpha and beta cells in the pancreas work in coordination to maintain normal blood-glucose levels. The ability of the alpha cell to detect a drop in circulating glucose is vital in regulating glycemic levels in the blood. At resting glucose concentrations (8 mM), minimal calcium oscillations were observed along with correlating minimal glucagon secretion. At low glucose concentrations (2 mM), an increase in calcium activity was observed and was associated with an increase in glucagon secretion (Figure 5). These results demonstrate that ATCC αTC1-6 exhibits a glucose-mediated response to
glucagon secretion. However, the magnitude of glucagon secretion at low glucose levels was much smaller than anticipated from an endocrine cell, and we are most likely observing basal secretion in all conditions tested (Rorsman et al. 2014a). This indicates that the regulated secretory pathway in the ATCC cells is present, but impaired.

**Voltage-gated K$_{ATP}$ and P/Q-type Calcium Channel Activity Present, but TTX-sensitive Na$^+$ Channel Absent in ATCC αTC1-6 Cells**

Ion channels play an important role in alpha cell activity during the response to a change in extracellular glucose. In low glucose conditions, glucagon secretion is increased due to low activity of K$_{ATP}$ channels, resulting in the firing of large-amplitude action potentials that inactivate nearby Na$^+$ channels. The results of this study confirm the presence of the genes encoding the subunits of the K$_{ATP}$ channel, SUR1 and KCNJ11, in ATCC αTC1-6 cells (Figure 4). Intracellular calcium experiments also indicated the channel was present and active. Tolbutamide, a K$_{ATP}$ channel inhibitor, increased intracellular calcium activity (Figure 7A). Alternatively, the K$_{ATP}$ channel activator diazoxide decreased the calcium oscillation activity (Figure 7B).

Brief or partial opening of K$_{ATP}$ channels hyperpolarizes the membrane to a negative membrane potential and removes the nearby Tetrodotoxin-sensitive Na$^+$ channel inactivation. Intracellular calcium experiments indicated there was no effect on the calcium spiking using the drug Tetrodotoxin in ATCC αTC1-6 cells (Figure 8). If the channel were present, a decrease in the spontaneous calcium activity in the cell would be expected. This result could indicate that the Tetrodotoxin-sensitive channel is not present in this cell line or it is not responsible (or activity is not detectable) for the spontaneous spiking at 8 mM glucose.
Following the removal of the inactivation of the Tetrodotoxin-sensitive Na\(^+\) channel, voltage-gated T-type Ca\(^{2+}\) channels open and the resultant calcium influx depolarizes the membrane. When the membrane potential reaches action potential threshold, a large population of P/Q-type channels is able to trigger calcium influx and glucagon secretion. The gene for the ω-agatoxin IVA-sensitive P/Q-type Ca\(^{2+}\) channel, CACNA1A, was not detected in ATCC αTC1-6 cells (Figure 4). This implies that either there is no message for the gene (CACNA1A) or that it is below the detection limit using qPCR as a method of analysis. However, the decrease in calcium oscillations observed at 2 mM glucose suggests that there is protein expression and functional channel activity (Figure 10).

**cAMP-Induced Intracellular Calcium Not Coupled to Glucagon Secretion in ATCC αTC1-6 Cells**

In endocrine cells, cAMP is an intracellular second messenger activated by the binding of hormones to G protein-coupled receptors (GPCRs). cAMP exerts many of its physiological effects by activating cAMP-dependent protein kinase (PKA) or exchange protein activated by cAMP (Epac), which regulates the functions of downstream protein targets.

The experiments in this study revealed that all three pharmacological drugs that increase the production of cAMP and activate the PKA-dependent and Epac-dependent pathways leading to glucagon secretion increased calcium oscillations in ATCC αTC1-6 cells at 8 mM glucose (Figure 11). However, the secretion assay indicated that this increase in cAMP activity did not result in an increased secretion of glucagon (Figure...
These results suggest that all required exocytosis machinery for cAMP secretion is not present in the ATCC αTC1-6 cells.

**Limitations of Immortal Cell Lines Used for Biological Study**

A common problem when using an immortal cell line such as the one used in these experiments is the evolution of the functional characteristics of the cells. The most prominent example of this type of evolution is the HeLa cell line. The first immortal cell line, HeLa, was derived from a cervical tumor and has been used in scientific research for over 70 years. Over time, however, researchers have discovered variation in new and old stocks of HeLa cells, thus limiting the cell line for experimental usage (Sato et al. 2016). Unending proliferation can be beneficial for analysis, but with the increased cellular division, there is a higher probability of phenotypic drift (Geraghty et al. 2014). As a result, clonal cell lines must be continually evaluated for changes in the expected functional properties of the primary cells when used for scientific research.

**Major Conclusions**

The focus of this study was to evaluate the clonal pancreatic alpha cell line ATCC αTC1-6 for use as a model cell line to study glucagon secretion. Our research shows that the ATCC αTC1-6 cell line has not retained essential functional characteristics of the original alpha cells. While several important alpha cell components are present (i.e., production of glucagon, K$_{ATP}$ channel activity, and voltage-gated calcium channels), it does not appear to have completely functional glucagon secretion via glucose sensing, a cAMP-mediated glucagon secretion pathway, a Tetrodotoxin-sensitive sodium channel, or the expected adrenergic receptor.
Due to these shortcomings, it has been determined that the ATCC $\alpha$-TC1-6 cell line is not a good model system for the study of regulated glucagon secretion in pancreatic alpha cells.
REFERENCES


Crane, Colette. 2011. “cAMP AND CALCIUM INTERACTIONS IN ENDOCRINE CELLS.”


Putative Cancer Stem Cells and Their Heterogeneous Progeny.” *Scientific Reports* 6 (March). Nature Publishing Group: 23328. doi:10.1038/srep23328.


### APPENDIX A: PHARMACOLOGICAL DRUGS USED IN EXPERIMENTS

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<td>100 µM</td>
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APPENDIX B: LIST OF ABBREVIATIONS

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<tr>
<td>[Ca(^{2+})](_i)</td>
<td>Intracellular calcium activity</td>
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<tr>
<td>AC</td>
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</tr>
<tr>
<td>ANF-GFP</td>
<td>Atrial natriuretic peptide-green fluorescent protein</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>Adenosine triphosphate/adenosine diphosphate</td>
</tr>
<tr>
<td>BSA</td>
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</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
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<td>Deoxyribonucleic acid</td>
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<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>K(_{\text{ATP}}) Channel</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIK3</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SYT7</td>
<td>Synaptotagmin 7</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
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