Improving the Differentiation of Human Pluripotent Stem Cells to Beta-Cells

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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April 2012

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Acknowledgements

Many friends made this work possible. Primarily, my advisor, Meri Firpo, who always found time to help with all issues related to science and school. Also, Zhoahui Geng, who years ago excellently performed two of the experiments presented, then taught me how to perform them myself, Kristin Voltzke, who taught me how not to kill cultured cells, and do just about everything else lab-related, and Ben Becker, who was a resource for PCR. Our collaborator, Bob Sorenson, who kindly spent hours discussing how to present and analyze data, along with opening up his lab to me, and, finally, Nick Baghroo, who taught me everything I know about human islet culture. These people, along with every member of the Firpo lab, made going through this work the enjoyable experience that it was.

Abstract

Human pluripotent stem cells (hPSCs) have the ability to differentiate into any cell type within the body, thus holding the potential for treating the beta-cell loss in type 1 diabetes through a cell replacement therapy. Though effective protocols have been produced for creating beta-cells from hPSCs, the efficiency of this process can be improved to yield more beta-cells. Here, we hypothesized that the addition of the hormones prolactin and human growth hormone to our established differentiation protocol will result in more beta-cells or beta-cell progenitors. This is based on work with isolated pancreatic islets, which showed that these hormones are able to stimulate proliferation of mature beta-cells and increase islet volume. And as much of the work with these hormones has been in rodent islets, we also present data showing that prolactin is able to stimulate cell division and islet growth in human islets. Any influences of the hormones were observed through gene expression analysis and a cell-death assay. This work will inform future work on creating beta-cells from hPSCs, and hopefully move the potential therapy closer to the clinic.

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Introduction

In the United States alone, 25.8 million people, or 8.3% of the total population, are afflicted with either type one or type two diabetes. Further, diabetes is the leading cause of kidney failure and new cases of blindness in the US, and the seventh leading cause of death (Center of Disease Control and Prevention, 2011).

Type 1 diabetes is caused by an autoimmune destruction of beta-cells, the cells within the pancreas that regulate blood glucose through the secretion of the hormone insulin. Normally, when the amount of glucose in the blood rises, insulin is released to signal tissues, such as fat, muscle, and liver, to take-up and store glucose. This is a very efficient system, keeping the concentration of glucose in the blood tightly regulated between 80 and 110 mg/dL during fasting conditions, and below 140 mg/dL even after a meal (Gunst & Van de Burghe, 2010). Without beta-cells, this tight regulation is not possible, and concentrations can reach toxically high levels, and lethally low levels.

With such a life-threatening disease affecting so many people, much work has been done on developing treatments. The first treatment introduced was the administration of exogenous insulin, first done in 1922 (Rosenfeld, 2002). And while the insulin used is no longer derived from animals, as it was initially, this remains the most effective treatment for diabetics. Now recombinant insulin that is more stable is used, administered through either multiple daily injections or a sensor-augmented insulin pump. Although the use of insulin therapy is effective and life-saving, the patients are still at risk for hypoglycemia and secondary complications, including retinal, renal, and

vascular damages (de Kort et al., 2011), not to mention potential social and lifestyle hurdles. This leaves other, more complete treatments desired.

Another treatment option is the introduction of new, exogenous islets, which has had the most success and is essentially a temporary cure for the patients able to receive them. The first transplant therapy done to treat diabetes was in 1967 (Kelly et al, 1967), transplanting an entire pancreas into two patients, with one becoming insulin-independent for six months. This procedure has been improved so that now the graft survival rates are 70-80% after three years, and patients' survival rates are over 90% for this same time (Robertson, 2010). However, whole pancreas transplants are normally only done in patients already receiving a kidney transplant due to the morbidity and costs associated with going through whole organ transplants.

In an attempt to create a less invasive treatment, isolated islets began being transplanted in 1980 (Najarian et al), although this was an autoislet transplant. Alloislet transplants had little success until 2000, when 9 out of 12 patients who received an allograft were insulin independent after a median time of 10 months (Ryan et al, 2001). This procedure has now been used for just over a decade, and outcomes are improving (de Kort et al, 2011). A report in 2005, looking at a cohort of 44 patients, saw that the average length of their exogenous insulin-independence was 15 months, but after five years only 7.5% remained off insulin (Ryan et al.), however 83% of the patients still had detectable C-peptide levels after five years, indicating that the grafts were still functioning, although insufficiently. Also, these patients usually became more stable

despite the regression back to insulin injections. A more recent report of 14 patients receiving a transplant showed that, after all becoming insulin-independent after 14 days, after two years post-transplant nine remained insulin-independent, and after three years eight (57%) remained off injections (Vantyghem et al, 2009). This is an example of the improvement in techniques and treatment protocol that are making this an even more promising therapy. But, there are limiting factors for islet transplants. The lack of available islets for transplant, combined with the need for typically more than one donor per recipient, leaves this as still a last-resort option for only the few patients who are still very unstable even with insulin injections.

Xenotransplants are also an option for an islet-replacement therapy. Islets derived from pigs hold a lot of promise for a therapy, as they have already been shown to be effective in diabetic-induced primate studies (reviewed by Dufrane & Gianello, 2009). Pig islets are attractive for multiple reasons: the porcine insulin protein differs from humans by only a single amino acid, the system would allow genetic modification to allow better engraftment, and porcine islets are more resistant to immunologic attacks (Koulmanda et al, 2003). However, there remain doubts about using xenotransplants, in particular the introduction of pathogens. To help both human islets and xenotransplants to be better accepted by the donor, work has been done creating devices to house the grafts. These devices protect the graft from the patient's immune system while still allowing the regulation of glucose in the blood (Zhi et al, 2012; reviewed by Souza et al, 2011).

As transplanted human islets have shown such success, but are limited in availability, pluripotent stem cells are an option for creating new human islets. Human pluripotent stem cells (hPSCs), by definition, are able to divide indefinitely and turn into any cell type within the body. The first type of hPSCs derived were embryonic stem cells (ESCs). These cells are extracted from the developing embryo in the blastocyst stage, specifically from the inner cell mass. At this stage of development, the cells have yet to become specified toward any cell or tissue lineage. ESCs were first cultured in 1981, being derived from mice (Evans and Kaufman, 1981; Martin, 1981). Since then, many species have had embryonic stem cells created from them, including primates (Thomson et al, 1995) and, of course, humans (Thomson et al, 1998).

After years of research examining how to create tissue- and cell-replacement therapies from these cells, and their safety if transplanted, clinical trials are currently underway in the United States. One of these trials is transplanting retinal pigment epithelium into patients with macular dystrophy or dry macular degeneration (Schwartz et al, 2012), while another, sponsored by the company Geron, is using oligodendrocyte progenitor cells to treat severe spinal cord injury.

However, using ESCs as an acceptable clinical therapy has two hurdles to overcome: the immune system and ethical issues. As the cells transplanted are still likely to be derived from a non-HLA-matching donor, any cells created from the ESCs and subsequently transplanted would be recognized as foreign and attacked by the patient's immune system, requiring indefinite use of immunosuppressants. And,

regarding the second obstacle, many people and groups have ethical or religious issues with creating a cell line from a fertilized embryo, believing that life has already begun at this stage of development.

In an attempt to circumvent these concerns, a similar cell type to ESCs was created in 2006 (Takahashi & Yamanaka), first from mice, then from human cells the following year (Takahashi et al, 2007; Yu et al, 2007). By inserting four genes into cultured skin cells using viruses, the cells were essentially reprogrammed to a state nearly identical to ESCs, and have been labeled induced pluripotent stem cells (iPSCs). Much research has been done comparing how similar these iPSCs are to ESCs (Kim et al, 2010; Phanstiel et al, 2011; Christodoulou et al, 2011; Narsinh et al, 2011), and it is generally accepted that although there are some differences, iPSCs still are just as therapeutically relevant as ESCs. And as iPSCs avoid both issues raised with ESCs, this cell type is an exciting candidate for treating diabetes.

As iPSCs were not created until 2006, and human ESCs were cultured in 1998, the first attempts at creating beta-cells from stem cells were done using ESCs. Initially, using mouse ESCs, a gene-trapping system was used. After being transfected with a DNA construct containing an insulin promoter-controlled drug-resistance gene, ESCs were allowed to differentiate spontaneously for 8-10 days before selecting for insulin-expressing cells (Soria et al, 2000). While 12 out of 18 mice implanted with these cells were able to normalize their blood glucose levels, the process of cells selection is not

amenable to a clinical setting, as it requires exogenous DNA insertion. Also, the insulin content of these cells was much less than that found in typical beta-cells.

After a few years of trying, unsuccessfully, to develop a reproducible protocol for creating beta-cells from ESCs (Hansson et al, 2004), one emerged in 2006 (D'Amour et al). After work by Kubo and colleagues (2004) showed that efficient endoderm differentiation was possible, establishing a more developmental paradigm for differentiating the cells, D'Amour and colleagues (2006) demonstrated an efficient method of producing pancreatic endocrine cells from ESCs, and later iPSCs, by moving the cells through sequential developmental stages.

To briefly discuss the developing pancreas, specification begins at gastrulation, when the three germ layers are established: ectoderm, mesoderm, and definitive endoderm. The formation of the definitive endoderm can be monitored by the expression of *Sox17* (Hudson et al, 1997), which is expressed following gastrulation. Soon after, the gut-tube is formed as the endoderm folds around itself. Anterior-posterior and dorsal-ventral patterning of the gut occurs here, specifying areas for organ development. Many of the signals responsible for this are released from surrounding mesoderm and overlying notochord (Grapin-Botton & Melton, 2000). From the foregut, all the digestive and respiratory organs develop: esophagus, trachea, stomach, lungs, liver, and pancreas. The midgut and hindgut form the small and large intestines, respectively (Zorn & Wells, 2009).

Specifically, the pancreas arises from the dorsal-most region of the foregut, originating as separate dorsal and ventral buds. This area of the gut tube first expresses the homeobox gene *Hlxb9*, which activates the transcription of pancreatic and duodenal homeobox protein 1 (*Pdx1*), another transcription factor. *Pdx1*-expressing cells will eventually give rise the entire pancreas, including exocrine, endocrine, and duct tissue (Gu et al, 2002). Each bud then develops somewhat uniquely. For example, the gene *Hlxb9* is required only for the dorsal bud to develop, while *hnf1β* and *ptf1a* are required by only the ventral bud (Zaret, 2008). Once established, the buds eventually rotate toward each other, fusing into one organ. Finally, the pancreas matures and forms its branched structure, while forming specified exocrine and endocrine cells. The expression of the transcription factor *Neurogenin3 (Ngn3)* first marks the endocrine-cell progenitors (Gu et al, 2002), followed by each endocrine cell's respective hormone, e.g. *insulin* is expressed by beta-cells, *glucagon* by alpha-cells. However, the cues responsible for specifying the various endocrine cells are poorly understood.

D'Amour, followed by others (Shim et al, 2007; Jiang et al, 2007; Phillips et al, 2007; Kroon et al, 2008) applied what is known about pancreas development, and using the signals responsible for each of these developmental stages in vitro created a protocol that produced endocrine cells. Each of these protocols, and the one we used in the following studies, directs the cells from pluripotent stem cells to definitive endoderm, then to primitive gut tube, the pancreas, and finally beta-cells, with each stage monitored my gene expression.

This strategy creates the possibility of replacing the need for cadaver-derived islets with those produced from iPS cells, which is the ultimate goal of this research. However, the process is not yet perfected. Many obstacles need to be overcome before this process is clinically applicable. First, the population of cells created from the differentiation is not homogenous, and some of these cells might still be in a stem-cell state and maintain the ability to divide continually, possibly creating tumors. Also, there is room for improvement in consistently and efficiently producing beta-cells from the differentiation. For the process of growing these cells to be economically feasible, the protocol must be as efficient as possible, yielding as many usable beta- or beta-like cells as possible.

We suspected that culture conditions could be a reason for fewer beta-cells being created, thinking that they were dying as they were being created due to poor culture conditions. This stems from a few intrinsic properties of beta-cells. First, beta-cells are not adept to handling oxidative stress (Lenzen, 2008). So as reactive oxygen species are created through normal metabolism, beta-cells cannot clear these as efficiently as most other cells in the body. If the reactive oxygen species' levels get too high, they begin interfering with protein function within the cell, and, if too high, cause that cell to undergo apoptosis. Thus, if beta-cells are being created by the differentiation, but our culture conditions are causing too much oxidative stress on the cells, perhaps they are dying as fast as they are being created. An alternative hypothesis

is that the cells are not maturing enough to become mature beta-cells, stopping somewhere along the developmental track.

To address the first question, we examined the resulting embryoid bodies (EBs; the ball of cells formed while culturing the stem cells in suspension) from the differentiation with a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay to assess cell death, combined with immunoflorescence. These showed whether beta-cells, or beta-cell precursors, were selectively dying within the EB. Further, gene-expression analysis was done on many of the cell-cycle inhibitor genes to see how their expression changed throughout the differentiation, addressing, in part, how senescent the cells had become. For example, more mature beta-cells express higher levels of the *p16/ink4a* gene (Krishnamurthy et al, 2006).

To address the second question, on whether or not the cells are not becoming mature beta-cells, we hypothesized that adding the hormones prolactin and human growth hormone to the late stages of the differentiation would improve maturation while also reducing cell death. Much of the research into these hormones on islets have stemmed from gestational diabetes research, with both of these hormones proving to be effective in causing proliferation and growth of rodent and human islets in vitro (Brelje et al, 1991; Brelje et al, 1994; Terra et al, 2010; Yamamoto et al, 2010), reflecting their roles that they have on islets during pregnancy, when insulin demands are increased.

And as most of the research on these hormones has been done on rodent islets, we did experiments on cadaveric human islets to assess any effects on the volume rate of replication occurring within islets. To examine the integrity of the isolated islets through the culture period, immunoflorescence was also done to examine continued expression of endocrine markers.

If successful, this work would allow, or at least inform, more efficient production of beta-cells from pluripotent stem cells, and thereby progress the use of pluripotent stem cells as a therapy for type 1 diabetes.

Methods

Cell culture

Undifferentiated ESCs and iPSCs were cultured and expanded upon mouse embryonic fibroblasts (MEFs) from E12.5 mice. The MEFs were derived in-lab from CF-1 mice (Charles River Laboratory International, Inc., Wilmington, MA). After expanding the MEFs for four passages, they were irradiated and frozen until use. Before plating ESCs or iPSCs, MEFs were thawed and plated onto a gelatin-coated plate, and allowed to attach. During this attachment period, typically at least 8 hours, the MEFs were cultured in medium comprised of DMEM-High Glucose (Gibco, Life Technologies Grand Island, NY), 10% (vol/vol) fetal bovine serum (HyClone, Rockford, IL), 5mM L-Glutamine (Gibco). The stem cells were then plated onto these, this “feeder medium” was removed, and the stem cell culture medium was added, which differed for ESCs and iPSCs. For ESCs, the medium consisted of KnockOut-DMEM, 20% (vol/vol) KO-Serum Replacer, 5mM L-Glutamine, 0.1mM Non-essential amino acids (all from Gibco), 0.1mM beta-mercaptoethanol (Sigma-Aldrich Co., St. Louis, MO), and 4ng/ml FGF2 (R&D Systems Inc., Minneapolis, MN). For iPSCs, the KO-DMEM was replaced by DMEM/F12 (Gibco). The day after either thawing or passaging the cells, their medium was changed, and thereafter whenever their medium was observed to be too acidic (by the medium’s color change). To passage the cells, a collagenase solution was used, composed of the normal culture medium, minus the FGF, plus 1mg/mL of lyophilized Type IV collagenase (Gibco). The cells had their culture medium removed, followed by a rinse in PBS

(Ca^{2+}/Mg^{2+} -free), then incubated with the collagenase solution for 15-20 minutes. Following incubation, the cells were scraped from the plate with a cell-lifter (Fisher Scientific Inc., Pittsburg, PA), centrifuged, then plated onto previously-plated MEFs with their appropriate culture medium. Incubation conditions were 5% CO_2 for iPSCs and 10% CO_2 for ESCs, with both at 37°C and ambient oxygen.

Differentiation

The differentiation was carried out in suspension cultures in non-adherent six-well plates, with each well assigned to a particular sample day, e.g. one well provided the day 3 sample, another the day 6, etc. The cells received no special treatments before beginning the differentiation. Each differentiation factor was added to a basal differentiation medium of DMEM-High Glucose (Gibco), 2% (vol/vol) FBS, 5mM L-glutamine (Gibco), and 0.1mM Non-essential amino acids (Gibco) and 2-mercaptoethanol (Sigma). The first day of the differentiation, the medium is supplemented with 100ng/ml Activin A, 50 ng/ml Wnt3a, and 2.5µg/ml anti-Shh (N-terminus specificity) (all from R&D Systems), while days 3-9 include only Activin A and anti-Shh. Activin A is responsible for inducing pluripotent cells to become endoderm (Kubo et al, 2003), while Wnt3a is normally present during the patterning of the blastula and has been shown to improve the differentiation in our lab. Inhibition of Sonic Hedgehog (Shh) signaling by the anti-Shh antibody is able to increase the differentiation toward the gut tube and improve the efficiency of the differentiation (Kroon et al,

2008). In the next stage, from day 9 through day 15, 50ng/ml EGF (R&D Systems) and 50ng/ml heparin sulfate (Sigma) are added to the medium to expand the pancreatic progenitors (Zhang et al, 2009), with heparin sulfate added to increase the binding efficiency of EGF to its receptor (Bernfield et al, 1999). The final stage of the protocol, from day 15 through day 21 is an attempt to mature the pancreatic progenitor cells into endocrine cells, hopefully with mostly beta-cells. This involves 10nM Exendin4-4 (Sigma), 50ng/ml GDF-11 (R&D Systems), and 50ng/ml Betacellullin (R&D Systems). Figure 1 shows the standard differentiation protocol.

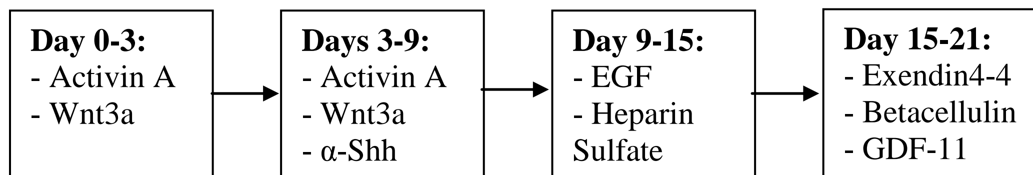


Figure 0. Our standard differentiation protocol used to differentiate pluripotent stem cells to beta-cells.

Medium was changed in the differentiation every three days by tilting the culture plates, allowing the EBs to settle to a corner, then carefully removing nearly the entire medium with a pipette. Fresh medium was added back as quickly as possible. The differentiation was incubated at 37°C and 5% CO_2, with ambient oxygen concentrations.

Quantitative PCR

Samples were collected on each day that medium was changed, or every three days of the differentiation, along with a day 0, undifferentiated sample. The sample was taken from the differentiation, rinsed twice with PBS (with Ca^{2+} and Mg^{2+}), then lysed with RLT Buffer with 2-mercaptoethanol. This sample was then stored at -80°C until processed

further. The RNeasy mini kit (Qiagen, Valencia, CA) was used to isolate the RNA, following the provided instructions. The RNA was then either stored at -80°C or immediately used to synthesize cDNA. The cDNA was made using and following the Superscript III First Strand Synthesis System (Invitrogen, Life Technologies). The final cDNA was stored at -20°C. Primers used were designed using the Primer-BLAST tool on the NCBI website, ncbi.nlm.nih.gov. They were subsequently synthesized at the University of Minnesota Biomedical Genomics Center. Upon receipt of the primers, they were suspended to their stock concentrations using nuclease free water, then stored at -80°C. To make the working primer solutions, 6.25µl of the forward and reverse primers were added to 237.5µl of nuclease-free water, then stored at -20°C. The PCR reactions were carried out in duplicates, using 1µl of cDNA (1/20th of total synthesized), 4.75µl of nuclease-free water, 6.25µl Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies), and 0.5µl of primer solution per sample, in 96 well PCR plates. Once made, each plate was centrifuged for four minutes at 1000 rpm. The reaction was carried out in a MasterCycler EP RealPlex (Eppendorf, Hamburg, Germany), using the following program: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 1 minute at 59°C, . Subsequent data analysis was done in Microsoft Excel, using the gene *gapdh* as a reference. Quantifications are all relative to the value at day zero of the differentiation. This value was calculated as the difference in cycle number (C_t) between the gene assayed and *gapdh* (ΔC_t), with that difference representing the difference in cycles where the sample was doubled. Thus, this is quantified as $2^{\Delta Ct}$. Each

subsequent sample's relative change was then quantified by first calculating the ΔC_t value for each well, then finding the difference between this sample's ΔC_t value and that for day zero. This difference was then used as the exponent over two, i.e. $2^{(DayX\ \Delta C_t - d0\ C_t)}$. The average of the two wells done per sample and standard deviation were then calculated. Positive and negative controls on each plate were human fetal pancreas and nuclease-free water, respectively.

Primers:

Gene	Forward Primer	Reverse Primer
Retinoblastoma (Rb)	CCCTTGCATGGCTCTCAGATTCACC	TGCTTGTGTCTCTGCATTTGCAGT
p16/Ink4A	TCGGAGGCCGATCCAGGTCA	TGCAGCACCACCAGCGTGTC
p27	TCTACTACAGACCCCCGCGGC	GAATCGTCGGTTGCAGGTCGCTTCC
p21	AGGCACTCAGAGGAGGCGCCATG	CCTGGATGCAGCCCGCCATTAG
p53	CTGGCTGCCAATCCAGGGAAGC	CCATGTGCTCAAGACTGGCGCT
Pdx-1	CATTGCAAGGCTCCCTAACACA	GGCATCAATTTCACGGGATC
Insulin	CTACCTAGTGTGCGGGGAAC	GCTGGTAGAGGTAGCAGATG
FoxA2	ATTGCTGGTCGTTTGTTGTG	TACGTGTTCATGCCGTTCAT
Sox17	CGCACGGAATTTGAACAGTA	GGATCGGGGACCTGTCACAC

Sample Embedding, Sectioning, and Mounting

Samples to be sectioned were washed twice in PBS (with Ca^{2+} and Mg^{2+}), then fixed in 4% paraformaldehyde for 20 minutes. The samples were then stored at 4°C until all the samples had been collected and were ready to be embedded. To embed the samples in wax, they were first dehydrated by progressing through the following solutions for the indicated amount of time: 50% ethanol (EtOH) for 5 minutes, 70% EtOH for 5 minutes, 85% EtOH for 5 minutes, twice in 95% EtOH for 5 minutes, twice in 100% EtOH for 5 minutes, then two separate xylene baths for 10 minutes each. Samples were then incubated at 60-65°C in a mixture of half xylene and half paraffin wax for 45-60 minutes,

then in only paraffin for another 30 minutes. Each sample was then placed into the middle of a wax mold, covered in wax, and allowed to set overnight on the cooling block.

Sectioning was done on a microtome set to a 5° cut angle and for 7µm sections. Before sectioning, the slides to be used were heated on a heating block to 37°C and covered with deionized water. As sections were made, between three and five sections were placed atop the water on each slide. They were then left until the water was evaporated, adhering the sections to the slides. The slides were then stored at room temperature until use.

TUNEL Assay

The TUNEL assay was performed using the In Situ Cell Death Labeling Kit (Roche Applied Science, Indianapolis, IN) following the supplied instructions and suggestions. However, some modifications were made. First, the slides were deparaffinized by heating the slides to 65-70°C for 30 min. The slides were then rehydrated moving through a series of baths: In a fume hood, two times in a Xylene bath for 1 minute, then 1 minute in 100% ethanol. Then they were placed in a second 100% ethanol bath, and brought out to the bench to finish the rehydration. The final baths were each for one minute in 90% ethanol, 85% ethanol, 70% ethanol, and finally a few rinses of distilled water followed by a 1 minute bath. Samples were then permeabilized in a solution of 0.1% Sodium

Citrate, 0.1% Triton X-100 solution, which was added to the slides for 8 minutes at room temperature. The slides were then rinsed twice with PBST (PBS + 0.5% Tween-20). After making a positive control with a short DNase 1 treatment, as directed, the TUNEL reaction mixtures were added to the slides. The slides were incubated at 37°C for one hour. Slides were rinsed twice, treated with the primary and secondary antibodies as described in the Immunohistochemistry procedures. The images of the TUNEL slides were quantified using the open-sourced CellProfiler program, which is further described in the Results section.

Immunohistochemistry

The slides to be used were deparaffinized by heating the slides to 65-70°C for 30 minutes. The slides were then rehydrated as they described above. Antigen retrieval was then done using Antigen Retrieval Reagent, Basic (R&D Systems) at 95°C for 5 minutes. Slides were then rinsed once in PBST for 10 minutes. Blocking followed for about one hour, in a solution of PBS (with Ca^{2+} and Mg^{2+}), 10% (vol/vol) normal donkey serum, 0.1% Triton X-100, and 1% (mass/vol) bovine serum albumin (Sigma). After pouring off the blocking buffer, the diluted primary antibodies were added to the slides then incubated overnight at 4°C. The following day, three washes were done with PBST, each lasting at least 5 minutes. Secondary antibodies were diluted in PBST, and incubated with the slides at room temperature, in the dark, for four to seven hours. One more wash was done with PBST for five minutes, followed by the addition of

Hoechst dye for 30 min. After washing, Prolong Gold Mounting Medium (Invitrogen) was added with a coverslip. Antibodies used:

Antigen	Host Species	Dilution	Cat. Number	Company
Insulin	Guinea Pig (GP)	1:1000	*Made in lab*	Sorenson Lab
Glucagon	Rabbit	1:800	G2654-.2mL	Sigma
PRL-R	Rabbit	1:800	*Made in lab*	Sorenson Lab
Pdx1	Mouse	1:200	562160	BD Pharmingen
Anti-Rabbit-A488	Donkey	1:500	A21206	Invitrogen
Anti-GP-Cy3	Donkey	1:600	41618	Jackson Immunolab
Anti-Mouse-Cy3	Donkey	1:600	19132	Jackson Immunolab

Islet Culture

(Add isolation procedures) After receiving the islets from the isolation group, they were immediately cultured for four days in a medium of RPMI-1640 with 0mM Glucose (Sigma), 3% GlutaMax (Gibco), 1% HEPES, 1% PSF, 5.0mM Pyruvate, 16.7mM $ZnSO_4$, 5.6mM Glucose, and 10% fetal bovine serum. After four days, the cells left in suspension were used for experiments, while those that attached were discarded. All subsequent culture conditions were in the above medium, except with the use of 10% horse serum rather than fetal bovine serum (FBS).

Islet Growth Experiment

Single islets were moved into one well each of a 96-well plate, filled with 250µl of the above medium with 10% FBS. For each group tested, 10 wells of one row of the 96-well plate was used, so 10 individual islets per group. The groups treated with oleate had both the oleate and fatty-acid-free bovine serum albumin (BSA) added to the medium

before it was added to the wells. To make the islet culture medium with oleate, the oleate stock solution was added to a 10% BSA stock solution, in appropriate quantities, then warmed to 50°C for one hour. The mixture could then be added to the normal culture medium. This brought the final concentration to 0.60mM oleate. In the groups treated with prolactin, a stock solution was added to each well after the new medium was added, the final concentration being 500ng/ml. Every 2-3 days half of the medium was removed, only doing half to minimize the potential of accidentally removing the islet.

Pictures were analyzed manually on ImagePro 5.1. Based on a calibration, the program produced islet area (A_{islet}) and X and Y axes measurements, which were then extrapolated to produce the islet volumes by the following formula: $V_{islet} = A_{islet} (4/3)r$, where r=1/2 the shortest axis of the islet. Data collection and statistical analysis were done within Microsoft Excel.

Short Prolactin Exposure Experiments

These experiments were done by first selecting about 100 islets that had good morphology. These were transferred to a small, 35mm culture dish, with either regular medium, medium supplemented with 500ng/ml of prolactin, or 500ng/ml of human growth hormone (HGH). The medium was allowed to come to 37°C before beginning the experiment, by placing it in the incubator for about 10 minutes. After the 30 minute

exposure, the islets were either fixed and stained as a whole islet, or sectioned by the Histology Core Facility.

Results

Prolactin and human growth hormone in human islet culture

There has been well documented work using rodent islets (Brelje et al, 1991; Brelje et al, 1994) that prolactin (PRL) and human growth hormone (HGH) are able to stimulate beta-cell proliferation and increase islet volume. However, there is still work to be done demonstrating the same effects are had on human islets. This information will inform the application of these hormones to the stem cell differentiation.

One of the problems preventing more research in this area is that human islets are notoriously difficult to culture, not lasting longer than a month in a dish. This makes optimizing the culture conditions difficult, forcing more of a trial-and-error paradigm for approaching the problem. Their short life also creates other problems that are avoidable with typical laboratory animal models or established cell lines, the foremost being the innate variance between each organ donor, but also the conditions surrounding a donor's death, which can create hypoxic, or other conditions that can damage the organ. With rodent models, the procedures are highly standardized to avoid any unnecessary variance between experiments. Nonetheless, the culture is still possible and can provide valuable information for the stem cell culture.

Human islets were received from the University of Minnesota Pancreatic Islet Core Facility, and immediately placed into RGX56 culture medium for four days. After four days, the islets are more uniform and pure, and respond better to experimental conditions (lab observation).

A growth experiment was done to show how the volume of the islets changed after exposed to the hormone PRL. Islets were individually imaged every two or three days, with the image analyzed by software to calculate the islet's area and dimensions, which was then used to calculate the volume of the islet. Over the course of the

experiment, the islets treated with PRL showed significantly more growth than the islets culturing on only the regular culture medium (Figure 1).

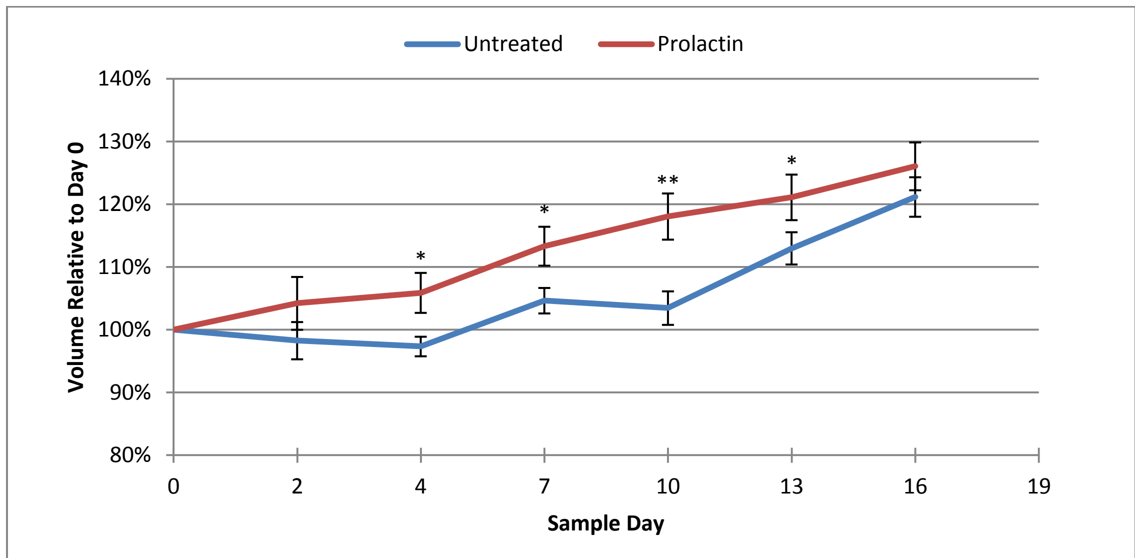


Figure 1. Increase of human islet volume, in normal medium vs. medium supplemented with 500ng/ml of recombinant human PRL. * P<0.05, ** P<.005.

The islets (n=10) showed consistent growth throughout the experiment, averaging a 26.1% increase in volume by day 16 when treated with PRL, and a 21.2% increase for the untreated group. After day 16 there was too much contamination in the wells to continue the experiment. Combined with this experiment, which does not necessarily indicate cell division within the islet, a bromodeoxyuridine (BrdU)-labeling experiment was done to compare the amount of replication in the islets (Figure 2). This data was consistent with the growth experiment, showing an increase the BrdU-labeled nuclei by about 50% in the PRL-treated group. Previous work (Brelje et al, 1994) has shown that the dividing cells in these islets are exclusively beta-cells, so any division is assumed to only beta-cell division. These results are also similar to what has been seen in past experiments from the lab.

Immunoflorescence (IF) was also on sections of islets from this donor at two different time points: after four days in the "pre-culture", then after another ten days in standard conditions (Figure 3). This was done to gauge the health of the islets, as well as to characterize them as much as possible. The islets were collected, fixed, embedded, and sectioned as described in the methods section. The slides were stained for the antigens Pdx1, insulin, glucagon, PRL-Receptor (PRLr).

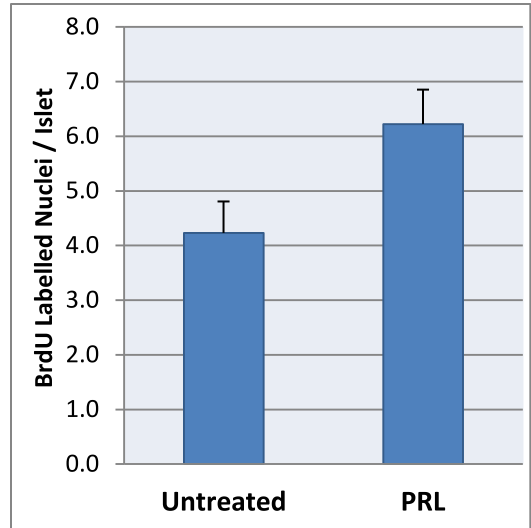
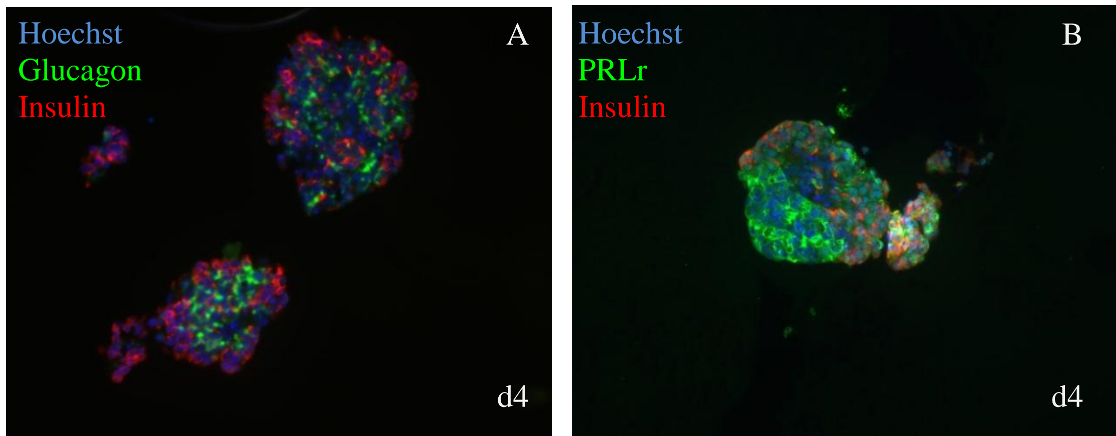


Figure 2. The amount of cell division occurring per islet with or without 500ng/ml of PRL, after four days of treatment. BrdU was added for the last 16 hours.

The islets showed expression of both insulin and glucagon through the 14 days of culture, though it had become fainter, suggesting a decline in the health of the islets. In each islet, about half of the cells were insulin-positive, while the other half was showed glucagon, with no co-expression. This amount of glucagon-positive cells was more than expected, as islets are normally mostly beta-cells.



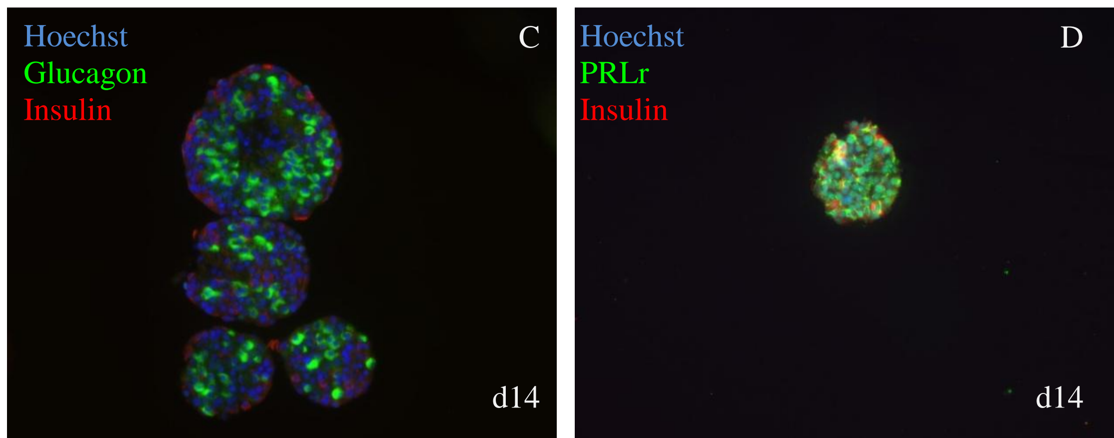


Figure 3. Immunoflorescence of human islets after 4 or 14 days in culture. A) and C) insulin (red) and glucagon (green). B) and D) show insulin (Red) and PRLr (Green). Both images were taken at 20X magnification.

The expression of PRLr was surprising, appearing in the nuclei of insulin-negative cells, then in the cytoplasm and cell-border in insulin-positive cells. This could be a unique feature of islets that has not yet been observed, as no previous literature could be found describing this in beta-cells. There has been work done on the role of PRLr in cancer, identifying a function of the receptor in the nucleus (Fiorillo et al, 2011), but nothing specific to an islet. In addition, Pdx1 was found in about half the cells in the islets from both time points (not shown), consistent with the insulin staining.

While these experiments have not yet been done in the differentiating stem cell cultures, they suggest that PRL could still be beneficial for increasing the final yield of beta-cells. The results coincide well with the TUNEL assay, suggesting that the addition of PRL both prevents the death of cells while inducing cell division, as has been shown (Terra et al, 2011), and is consistent with the role of PRL in pregnant rodent (Brelje et al, 2008).

Cell-death is occurring in differentiating stem cell cultures

In our differentiation, we hypothesized that there was cell death occurring in the EBs, and any cell death occurring could be reduced by adding PRL. Previous differentiations done in the lab, by Zhoahui Geng, following the normal protocol, had

fixed and sectioned EBs, so these were analyzed using terminal deoxynucleotide transferase dUTP nick end-labeling (TUNEL) assay, combined with an anti-Pdx1 antibody to label any beta-cells or beta-cell precursors that were dying (Figure 4). The differentiations had all been done following the same protocol, but there were differences in cell type used and the culture volumes, as noted in the graphs below.

Initially the TUNEL assay had to be optimized for use with a primary antibody. Changing the order of application for the steps involved in the immunohistochemistry and TUNEL procedures resulted in the methods used for all the following TUNEL assays, as described in the methods section.

After the assay was finished, the slides were photographed and then analyzed using CellProfiler, an open-sourced cell counting software. To be brief, the software works by scanning the image for brightness or color differences, identifying the bright or different colored areas as an object. The objects are then filtered by their size and brightness, to parameters entered and adjusted using the positive and negative controls created during the TUNEL experiment. Finally, the objects are all counted. For a quantification that allowed comparisons, the number of TUNEL positive cells are shown as a percentage of total cells in the picture, counted using a Hoescht dye. Shown below is an example of what the program produces and counts during the process, compared to the original picture (Figure 5). The data produced is then showed in the following graphs, grouped by their original experiment (Figure 6).

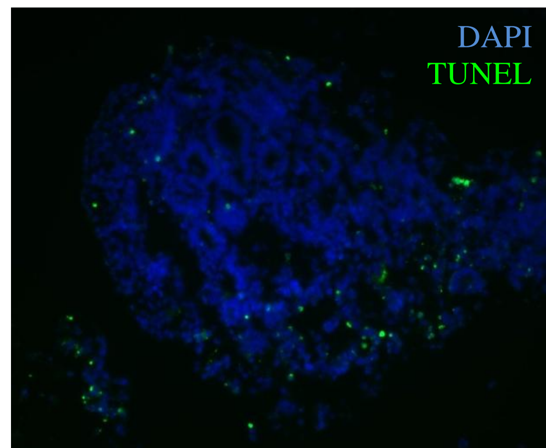


Figure 4. Example picture from slide that was subjected to TUNEL assay.

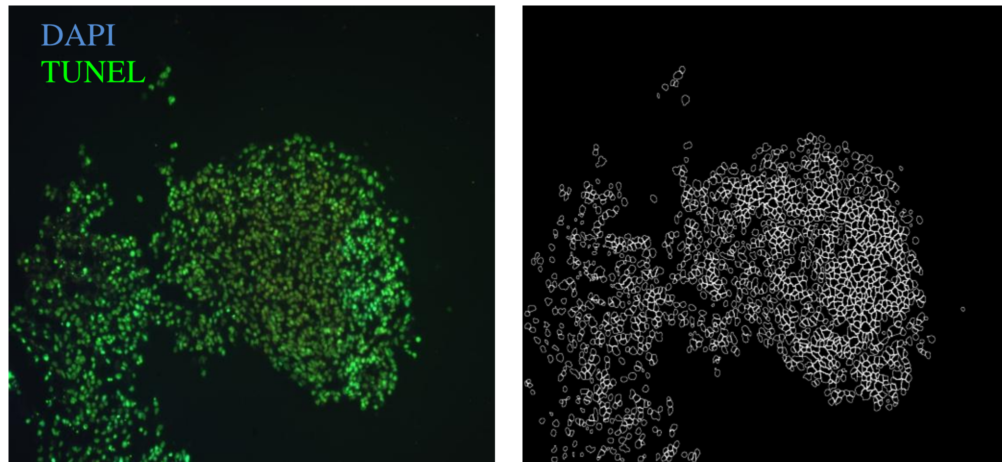


Figure 5. Example of the output from the cell-counting software. The image on the left is the raw image taken from the positive control slide, treated with DNase I prior to TUNEL. The image on the right shows what the program recognized as individual cells (each circle) before counting.

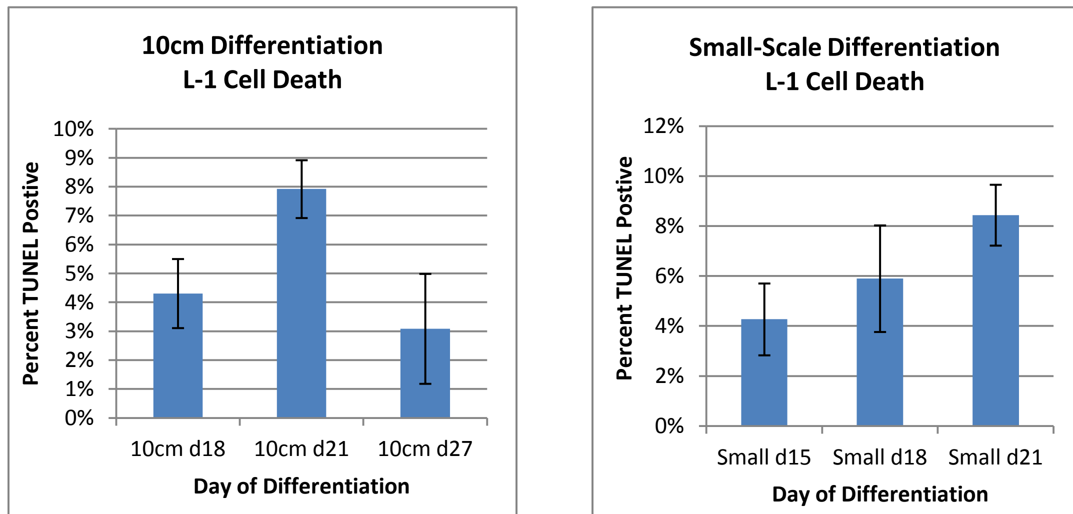


Figure 6. Cell death is occurring in the differentiating cell cultures, and it tends to increase over time. Error bars represent SEM. "L-1" is an iPS cell line. The 10cm Differentiation was done in a 10cm culture dish, while the Small-Scale Differentiation was done in multiple wells of 6-well plates. The data represents an average count from at least four images of separate sections.

While the results are not statistically significant from one another, the results are nonetheless consistent between the cultures, with days 18 and 21 both having similar percentages of cell death. And although the images are not shown, the EBs appear to be healthy and robust. The cells that did end up marked as TUNEL-positive were

dispersed throughout the EB, and generally not located at the core of the EB, which is thought to occur when the EBs get too large. Also, although this was not a question asked by this study, the differences in culture conditions (10cm plate vs. 6-well plate) do not make a difference in the cell viability.

Importantly, there were no Pdx1-positive cells in any of the L-1 sections. As we hypothesized that the *Pdx1*-expressing cells were selectively dying in the EB, this is clearly not the case. There was not a single cell observed in any of the slides that showed positivity for both Pdx1 and TUNEL.

These analyses conclude that the cells undergoing apoptosis within the differentiating embryoid bodies are probably not pancreatic precursors, as suspected. They also showed that the L-1 differentiation did not produce any Pdx1-positive cells at day 21, and although this could be a result of cells expressing P*dx1* earlier in the differentiation, then turning it off, it would be unusual for the entire EB to do this in synchrony.

The data above showed that there is indeed cell death occurring in the differentiation. While no tests or comparisons were done to understand the amount of death occurring in normal cultures, and although this is not as high of a number as we expected, this was an area that could be improved during the differentiation.

Decreasing the Expression of Cell-Cycle Inhibitors during the Differentiation

Past differentiations were done by another lab member, Zhoahui Geng, examining the influence of the hormones prolactin (PRL) and growth hormone (HGH), as well as zinc (Zn^{+}) on the differentiation. The two hormones have been shown to prevent apoptosis and expand islets in vitro, and also play a role in islet growth during pregnancy in multiple species. They are also thought to help islet maturation. With all of these attributes in mind, the two hormones were added to the differentiation with the hypothesis that they would aid islet maturation, observed through the expression of

Pdx1 and *insulin,* while also stimulating proliferation, observed by down-regulation of cell-cycle inhibitors *Retinoblastoma protein (Rb), p16*, and *p53*.

Likewise, Zn^+ can prevent apoptosis through inhibition of caspase activity, while also being necessary to bind hormones to their export proteins. Without sufficient Zn^+ present, the differentiating beta-cells might not be able to fully mature. This question is also tested here, hypothesizing that Zn^+ is a limiting factor in the differentiation, and the added supplement will allow more mature cells to form. Because the other cell-cycle inhibitors were also of interest, their expression was also tested to see if Zn^+ also prevented senescence.

The third aspect of these experiments is the change in serum concentration. Previous to these studies, the proportion of serum in the media was 20%. However, this high concentration was also hypothesized to be inhibiting the maturation of the differentiation beta-cells, so a lower concentration of 2% was tested.

Finally, these experiments also compared the differentiations of different cell lines. These included two ESC lines and two iPSC lines: HSF6, H9, IMR-90-DL-1, and L-1, respectively. Some variation is expected between the potential for these lines to differentiate, and hopefully will be observed through the PCR analysis.

Expression of Cell Cycle Inhibitors during the Standard Differentation Protocol

The first question addressed was how the genes p16, p53, and Rb were all expressed during the typical differentiation protocol. Each of the three cell lines was tested: H9, HSF6, and IMR-90.

Experiment	**D0**	**D3-D9**	**D9-D15**	**D15-D21**
Control *(No factors)*				
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin44 GDF-11 Betacellulin

Figure 7. A summary of both the experimental groups in this differentiation. The control group contained cells cultured in the basal medium without any of the differentiation

factors added, while the Standard Protocol group received the typical factors, as explained in the Methods section.

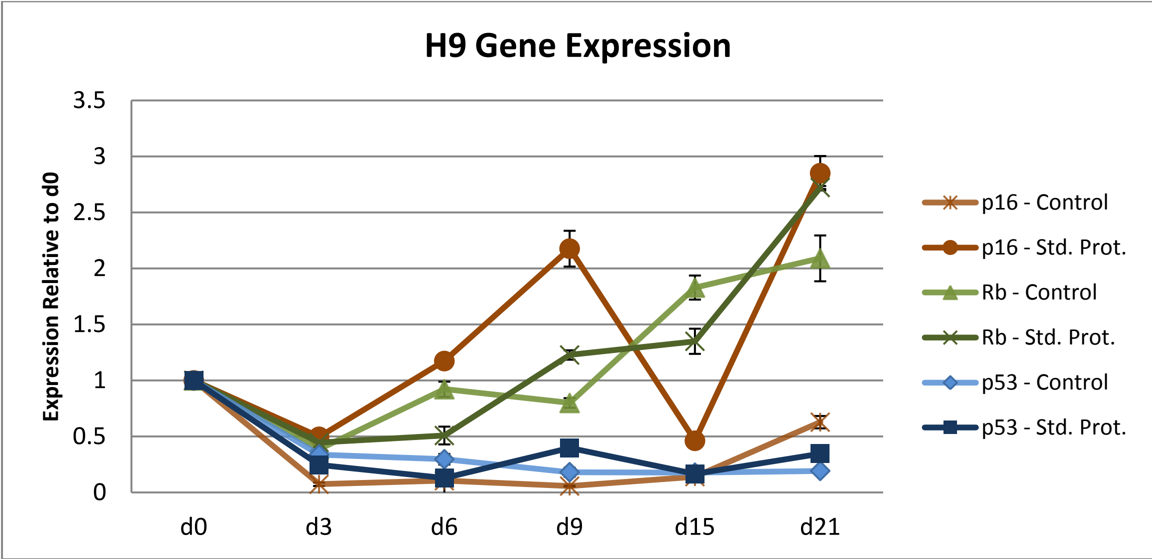


Figure 8. The expression of *p16, Rb,* and *p53* throughout the differentiation period, for the H9, ES cell line.

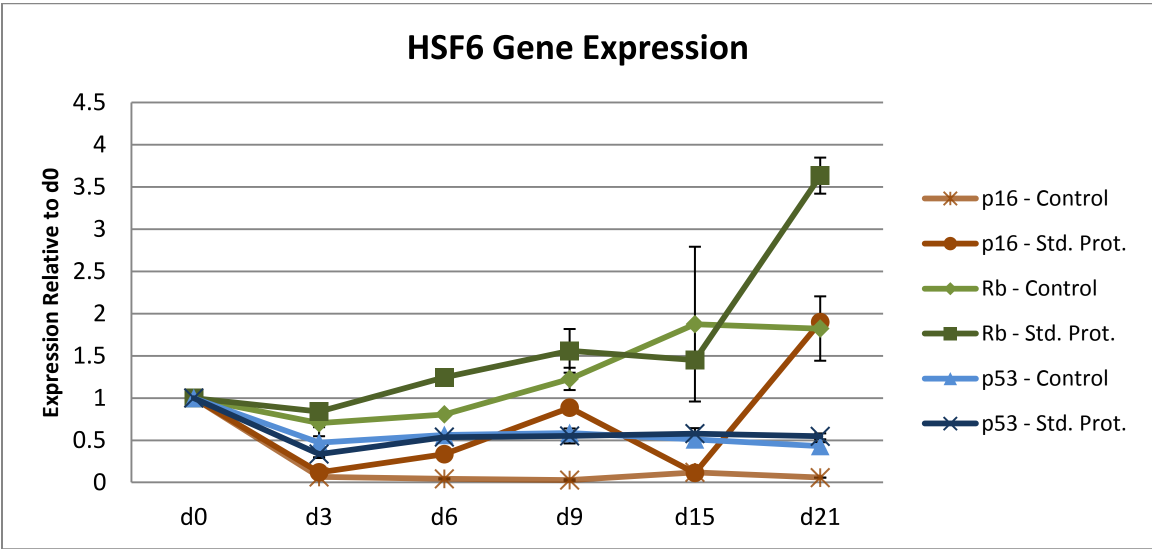


Figure 9. The expression of *p16, Rb,* and *p53* throughout the differentiation period, for the HSF6, ES cell line.

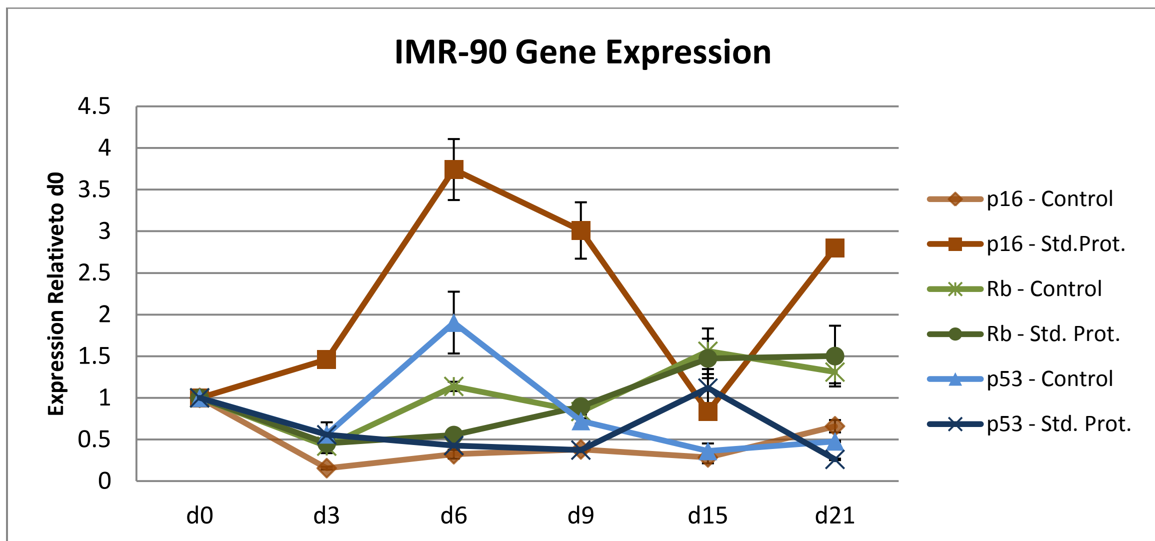


Figure 10. The expression of *p16, Rb,* and *p53* throughout the differentiation period, for the IMR-90, iPS cell line.

From these results, p53 is likely not going to be susceptible to extrinsic control, as the standard protocol does not increase the expression above the levels present at day zero in any of the cell lines. This could have been expected, as p53 is activated and primarily regulated post-translationally, not at the level of transcription. Thus, further attempts to reduce the expression of cell-cycle inhibitors were not focused on p53.

The other two genes analyzed here, p16 and Rb, both seem to have high enough expression to be worth the attempts to reduce them, especially p16, which showed a large difference between the control and standard protocol groups in every cell line.

<u>Influence of Increased Serum Concentration on Cell Cycle Inhibitor Expression</u>

As stated above, at the time of this experiment, the standard differentiation conditions used 20% FBS in the basal culture medium. There was the hypothesis that this high concentration was inhibiting the differentiation of the cells, which was analyzed by the expression of two markers of mature beta-cells, *Pdx1* and *insulin*. Testing the high concentration of 20% serum against a low concentration of 2% serum was done on all three cell lines (H9, HSF6, and IMR-90) again, also done by Zhaohui

Geng. The *Pdx1* and *insulin* PCR results from the IMR-90 differentiation are shown here (Figure 11) to demonstrate the effectiveness of the protocol, and the success of the experiment.

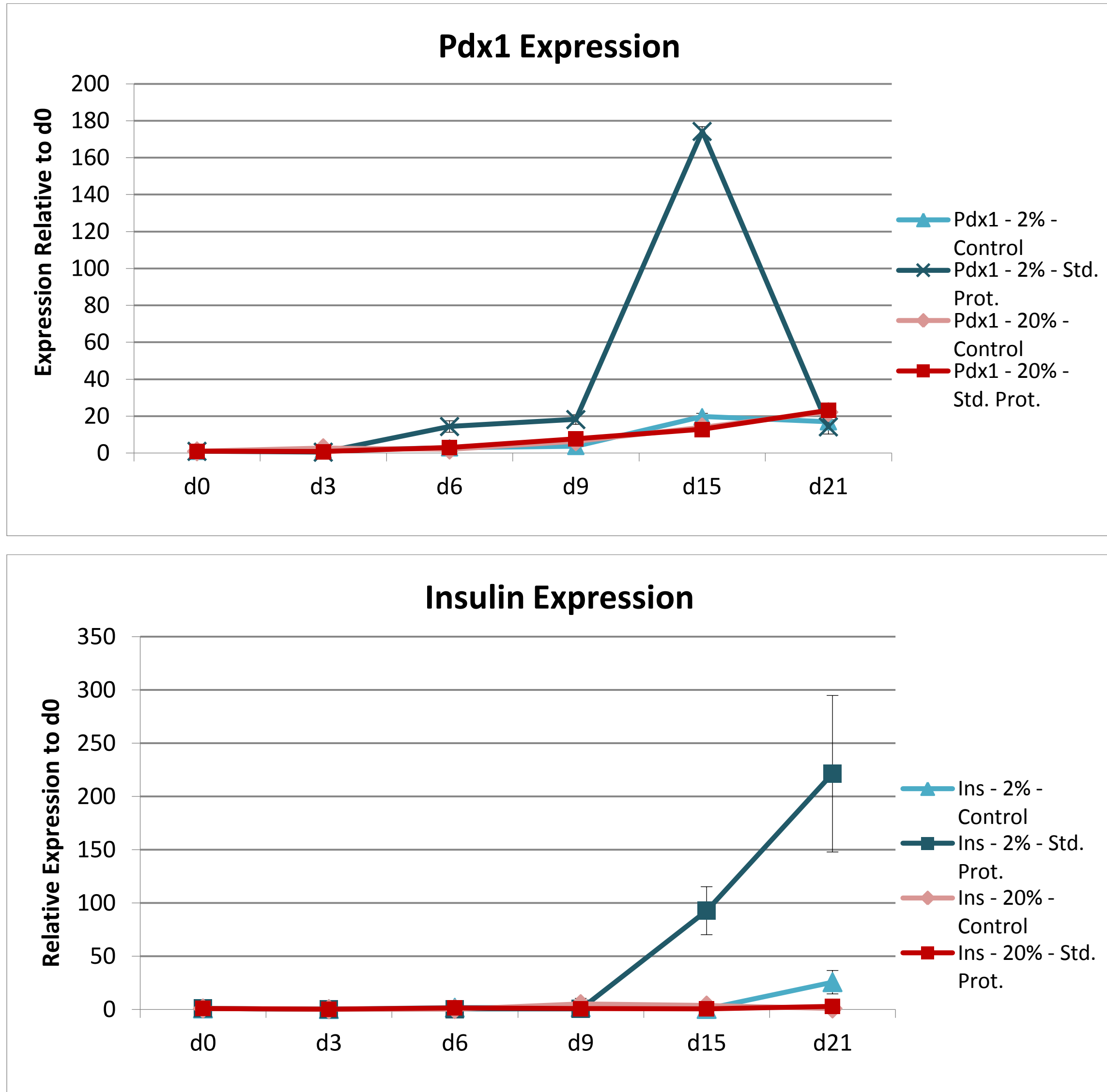


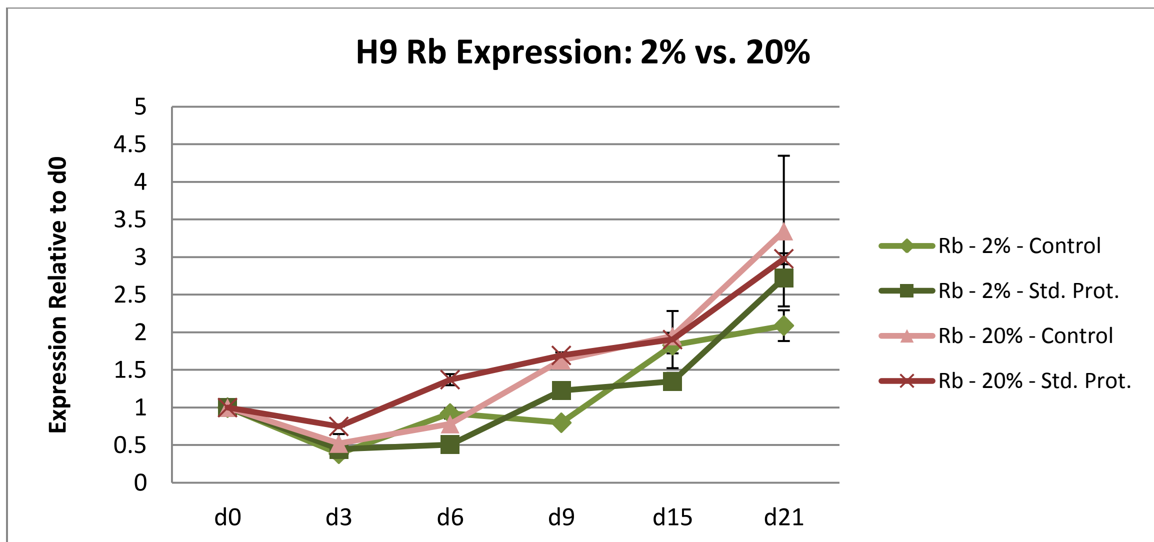
Figure 11. Lower serum concentration (2%) improves the differentiation, as shown by increased *Pdx1* and *insulin* expression over the high concentration (20%). This was from just one cell line used in this experiment, the IMR-90 cell line.

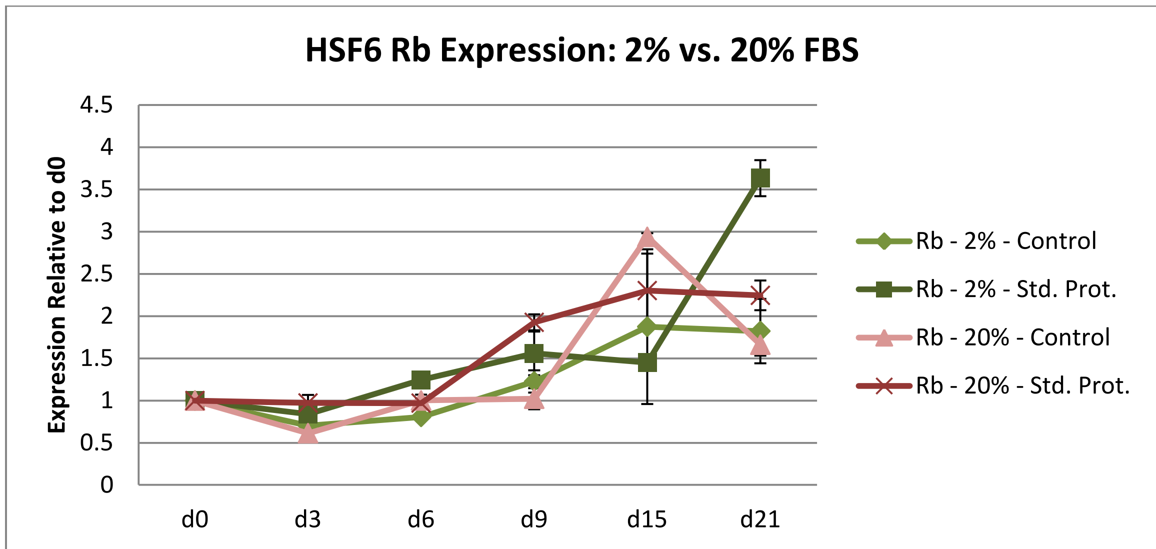
With the expression of both *Pdx1* and *insulin* showed to be obviously higher in the group receiving only 2% FBS, the 20% concentration was reduced to only 2% as the

standard condition. However, further analysis was done on how the change in serum concentration influenced the expression of the cell-cycle inhibitor genes *p16* and *Rb*. Figure 12 shows a table summarizing the experimental groups.

Experiment	D0	D3-D9	D9-D15	D15-D21
Control – ***2% FBS*** *(No factors)*				
Standard Protocol – ***2% FBS***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Control – ***20% FBS*** *(No factors)*				
Standard Protocol – ***20% FBS***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin

Figure 12. A summary of the four experimental groups in testing the influence of high and low serum concentrations. The control groups contained cells cultured in the basal medium without any of the differentiation factors added, though differed by the serum concentration in their medium. The Standard Protocol groups received the typical factors, as explained in the Methods section, but also had different serum concentrations. Three cell lines were used again: H9, HSF6, and IMR-90 lines.





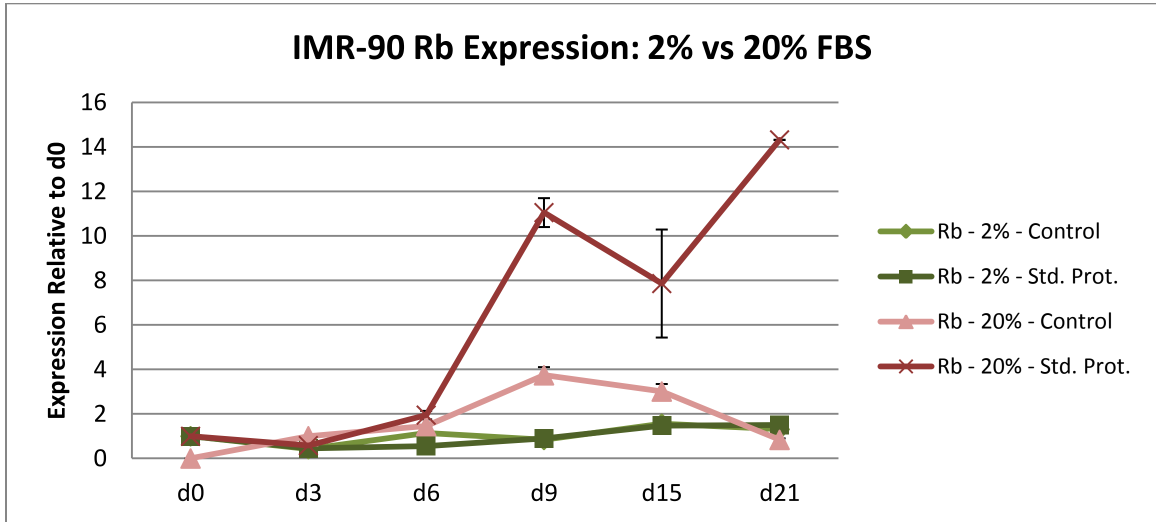
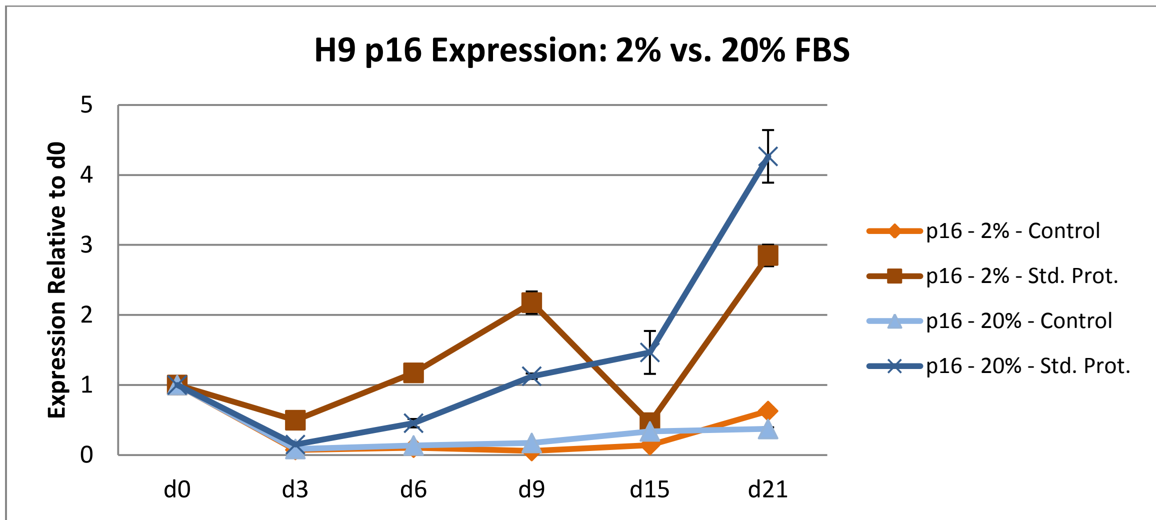
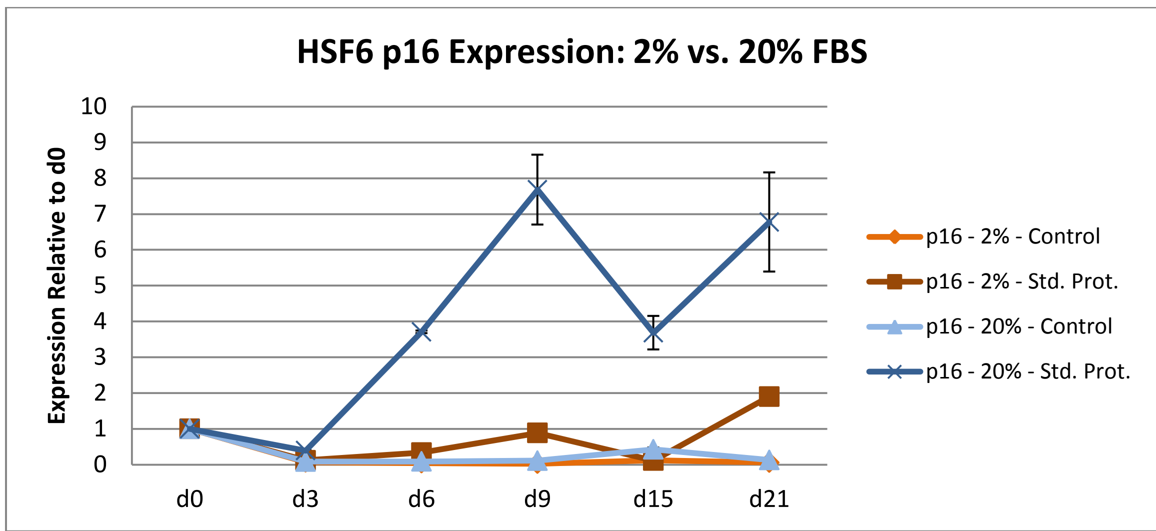


Figure 13. The influence of low and high serum concentrations on the expression of *Rb* in three different pluripotent cell lines. Each of the cell lines' groups received identical treatment, and the experiments were all done in parallel.


H9 p16 Expression: 2% vs. 20% FBS
Expression Relative to d0
5
4
3
2
1
0
d0
d3
d6
d9
d15
d21
p16 - 2% - Control
p16 - 2% - Std. Prot.
p16 - 20% - Control
p16 - 20% - Std. Prot.


HSF6 p16 Expression: 2% vs. 20% FBS
Expression Relative to d0
10
9
8
7
6
5
4
3
2
1
0
d0
d3
d6
d9
d15
d21
p16 - 2% - Control
p16 - 2% - Std. Prot.
p16 - 20% - Control
p16 - 20% - Std. Prot.

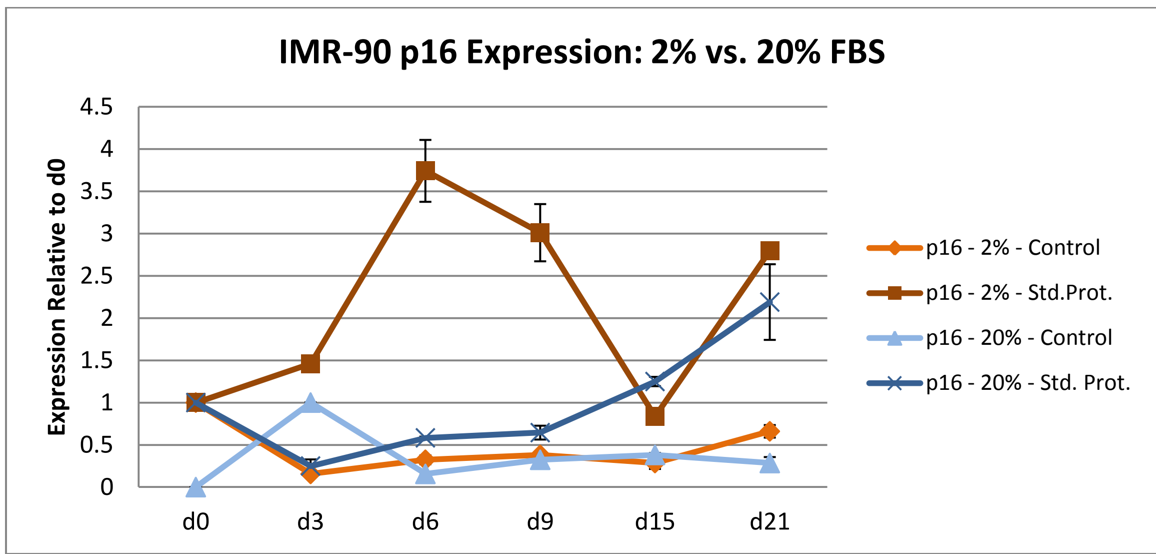

IMR-90 p16 Expression: 2% vs. 20% FBS
Expression Relative to d0
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
d0
d3
d6
d9
d15
d21
p16 - 2% - Control
p16 - 2% - Std.Prot.
p16 - 20% - Control
p16 - 20% - Std. Prot.

Figure 14. The influence of low and high serum concentrations on the expression of *p16* in three different pluripotent cell lines. Each of the cell lines' groups received identical treatment, and the experiments were all done in parallel.

There was some variability, as expected, between each cell type, and between the 2% and 20% FBS conditions, though there was one consistency: a drastic and consistent dip in the expression of p16 at day 15. This comes directly after the six day exposure to epidermal growth factor (EGF). The EGF is then removed for the final six days of culture, during which time the levels of p16 began to rise again, usually to their highest point. This makes a strong suggestion that the addition of EGF to the differentiation inhibited the expression of p16, which could indicate that it allowed more proliferation to occur. Also, and perhaps more importantly, at these times of low levels of p16 expression, the expression of *Pdx1* and insulin is usually at its peak, as was seen in these differentiations by previous PCR analysis. This correlation makes decreasing the levels of p16 an attractive goal.

Of note is that the dip in *p16* expression was only observed in the group cultured with 20% FBS in one cell line, the HSF6 line, suggesting that the lower serum concentration is allowing the cells to be more responsive to the factors added to the differentiation.

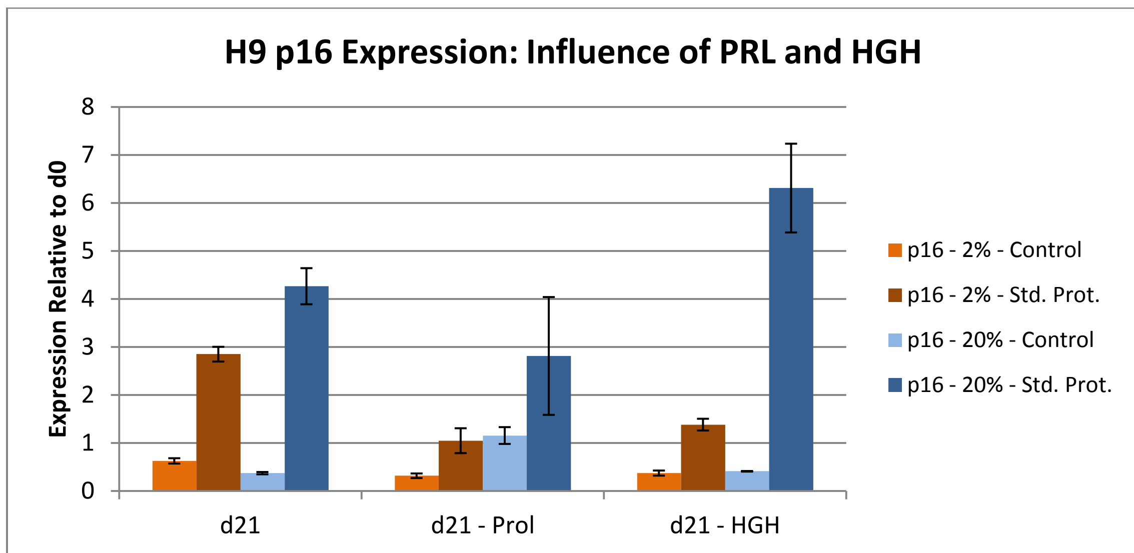
<u>The influence of PRL or HGH on the expression of p16 and Rb</u>

To test the influence of either PRL or HGH on the differentiating cell, they were added individually to differentiating cells at day 15. This time was chosen because we expected either of the hormones to only act on more mature cells, which become present at later stages of the differentiation. This experiment was done in conjunction with the above experiments, and, again, by Zhoahui Geng, so the influence of PRL and HGH was tested in both low 2% and 20% serum concentrations. The groups from the previous experiment were used, with an additional two groups being created for each

condition, as one received PRL and the other HGH. All three cell lines were used again as well. We hypothesized that the addition of each of these factors would reduce the expression of each of the cell-cycle inhibitors.

Experiment	D0	D3-D9	D9-D15	D15-D21
Control – ***2% FBS*** *(No factors)*				
Control – ***2% FBS – Only*** ***PRL or HGH***				*PRL* / *HGH*
Standard Protocol – ***2% FBS***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Standard Protocol – ***2% FBS –*** ***w/PRL or HGH***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin *PRL or* / *HGH*
Control – ***20% FBS*** *(No factors)*				
Control – ***20% FBS - Only*** ***PRL or HGH***				*PRL* / *HGH*
Standard Protocol – ***20% FBS***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Standard Protocol – ***20% FBS –*** ***w/PRL or HGH***	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin *PRL or* / *HGH*

Figure 15. A summary of the twelve groups in testing the influence of PRL and HGH in both low and high serum concentrations. Testing the influence of PRL and HGH on the experiment, each of the four groups present in the previous experiment were split into three groups at day 15, with one group receiving PRL, another HGH, and the last continuing the Standard Protocol. The same treatment was done to the control groups. This decreased the variability between the experiments, as the population of cells going into each group at day 15 was very similar. Three cell lines were used: H9, HSF6, and IMR-90 lines.



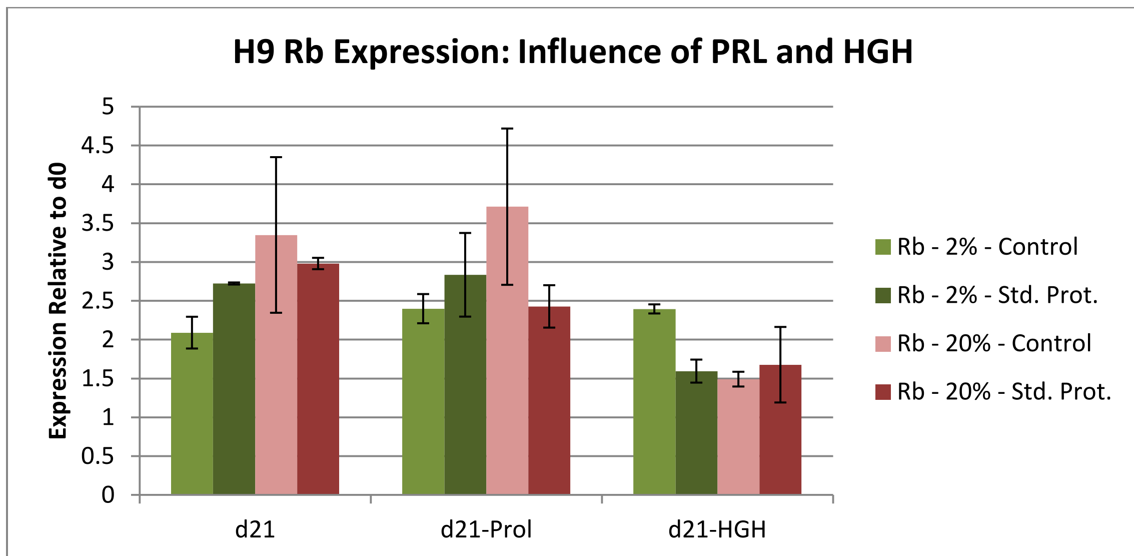
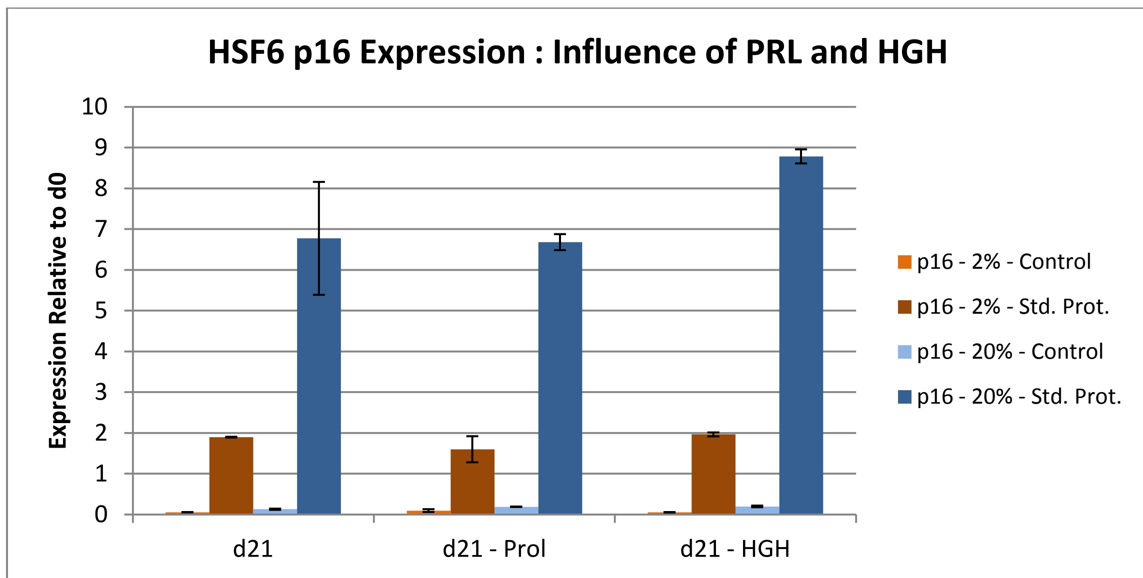


Figure 16. The influence of PRL and HGH, in both low and high serum concentrations, on the expression of *p16* and *Rb* in the differentiating H9 cell line. In the PRL and HGH groups, the hormone was added at day 15, allowing for six days of treatment before analysis.



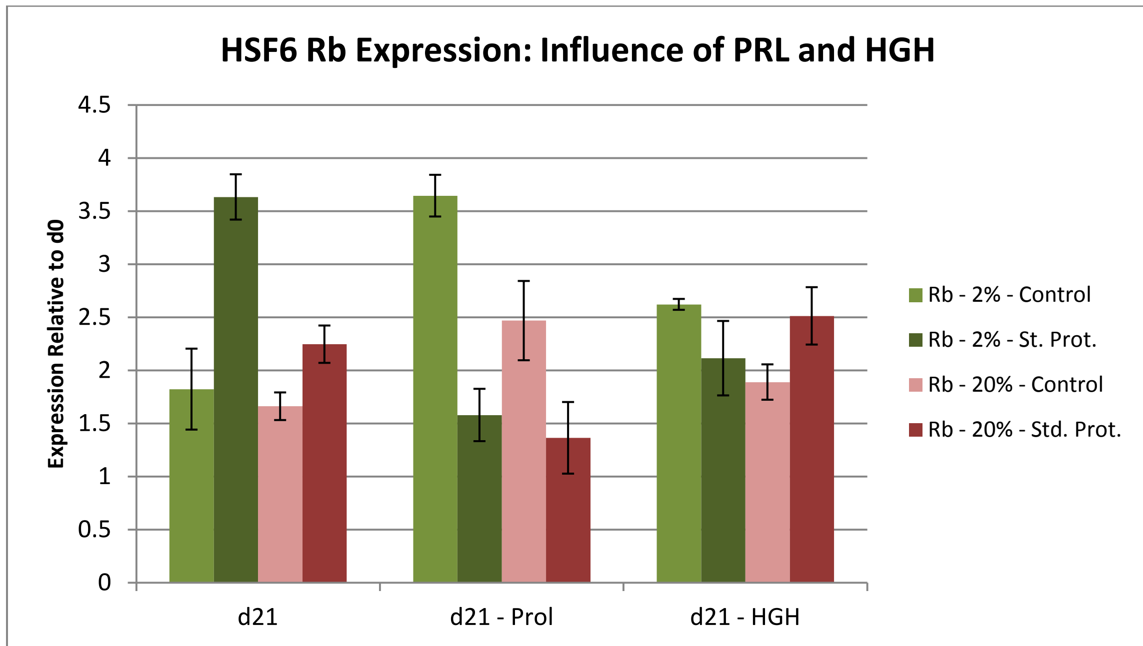
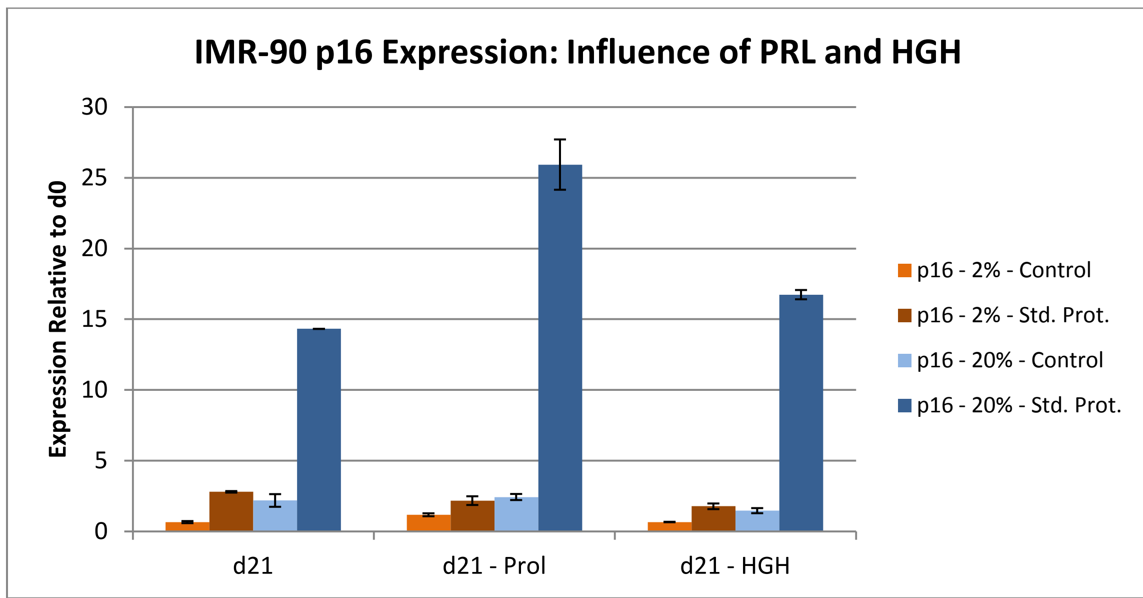


Figure 17. The influence of PRL and HGH, in both low and high serum concentrations, on the expression of *p16* and *Rb* in the differentiating H9 cell line. In the PRL and HGH groups, the hormone was added at day 15, allowing for six days of treatment before analysis.



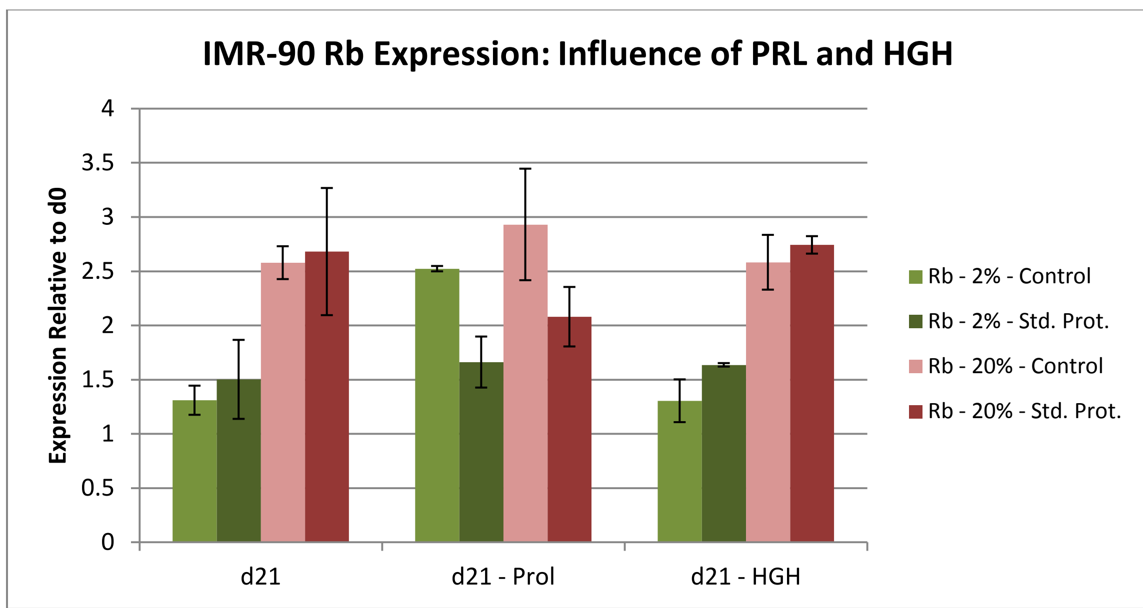


Figure 18. The influence of PRL and HGH, in both low and high serum concentrations, on the expression of *p16* and *Rb* in the differentiating H9 cell line. In the PRL and HGH groups, the hormone was added at day 15, allowing for six days of treatment before analysis.

Aside from a few experiments, the addition of PRL or HGH did not influence to expression of either p16 or Rb. However, there was a significant increase in p16 expression between the group cultured in 2% serum and the group in 20% serum, as

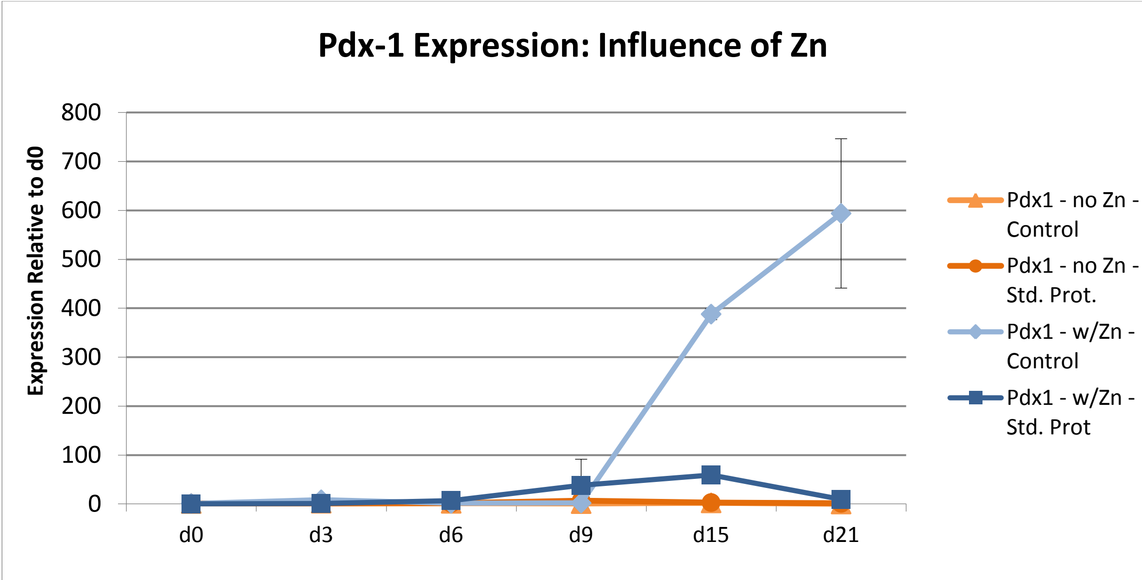
seen in every cell line. This increase was not reduced in the groups treated with PRL or HGH, actually increasing the expression of p16 in the IMR-90 cells treated with PRL. Also, HSF6 cells were the one group that showed a decreased Rb expression in response to PRL treatment, although in only the cells cultured with 2% serum.

Influence of PRL, and HGH on the Expression of *p16, p21,* and *p27* with Zn^+ present

This initial experiment was also done by Zhoahui Geng, but the samples here are re-analyzed. The experiment compared the effect of many factors: prolactin (PRL), human growth hormone (HGH), zinc (Zn), both PRL and HGH together, and finally all three, using the L-1, iPS cell line. The reasons for testing PRL and HGH were the same as for the previous experiments, and the addition of Zn was tested due to its requirement in binding hormones (e.g. insulin) to their cellular export machinery. The initial focus of this experiment was whether the addition of these three factors improved the maturation of the differentiating cells, as shown by *Pdx1* expression. The expression of *Pdx1* is shown here, along with the expression data for *p16, p21*, and *p27*, to see if Zn also influenced whether the cells began to enter a state of senescence.

Experiment	**D0**	**D3-D9**	**D9-D15**	**D15-D21/24**
Control – (No factors)				
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Standard Protocol + PRL/HGH	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin PRL and/or HGH
Control – w/ Zn				
Standard Protocol – w/ Zn	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Standard Protocol – w/ Zn + PRL/HGH	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin PRL and/or HGH

Figure 19. A summary of the groups used in testing both the influence of Zn on the differentiation, as well as the groups with PRL, HGH, and PRL and HGH together. The control groups received no added differentiation factors, though the one control had Zn added throughout the differentiation. Similarly, the standard protocol groups had the typical differentiation factors added, though one also received Zn throughout. In the groups receiving PRL and HGH, the hormone(s) were added at day 15. The L-1 cell line was used.



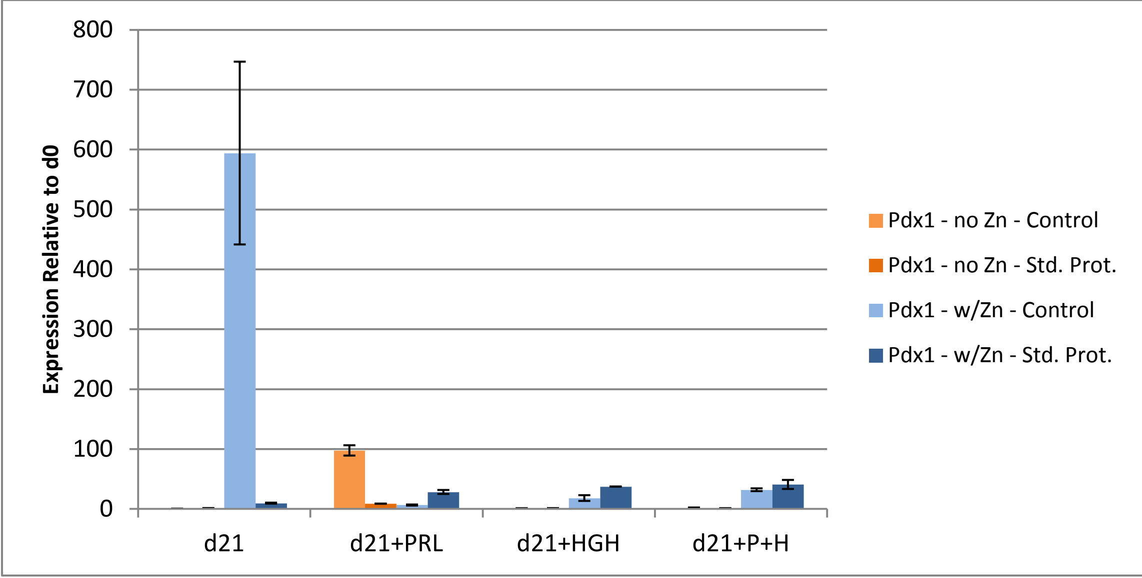
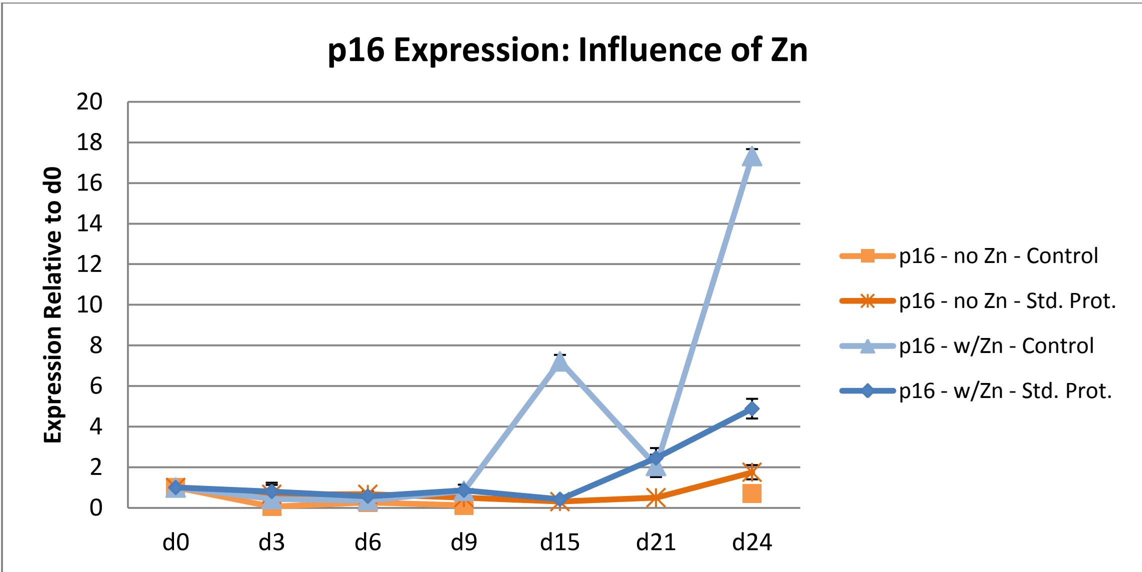


Figure 20. The influence of Zn, added throughout the differentiation, on the expression of *Pdx1*. While there was an unusually high peak in the Control group, there is still a

typical peak around d15 in the Std. Prot. group, with Zn present. The influence of PRL and HGH on the expression of *Pdx1* is shown below.

Figure 20 shows the expression of Pdx-1 throughout the experiment, which showed an insignificant rise in Pdx-1 in the Standard Protocol group without Zn added. In the group that was treated with Zn, a 59-fold increase in Pdx-1 expression was observed at day 15. This level then dropped down to a 10-fold increase by day 21, though when PRL of HGH was added, this drop was attenuated, staying between a 28-fold and a 40-fold increase.



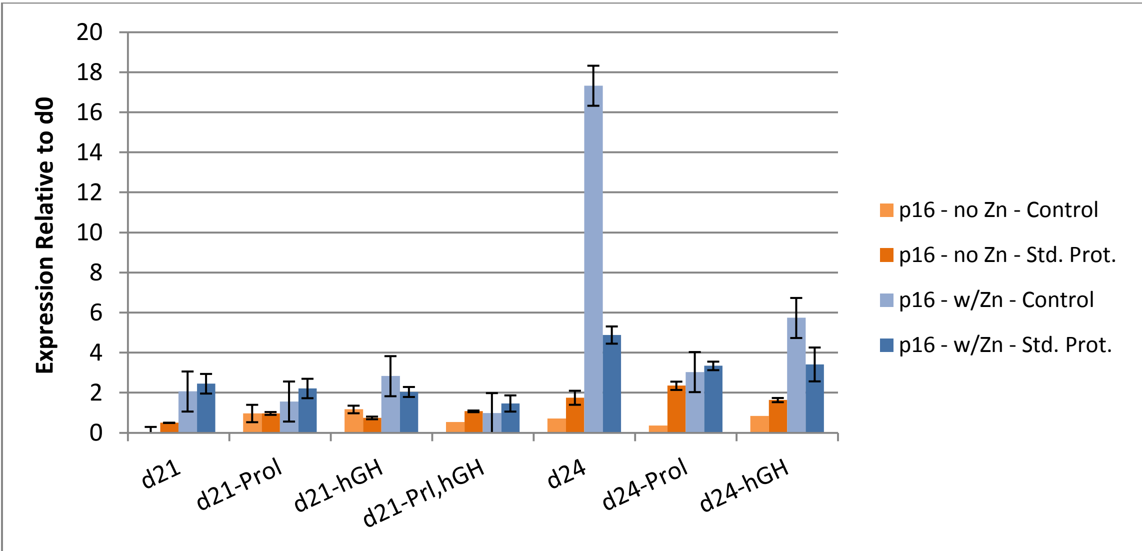
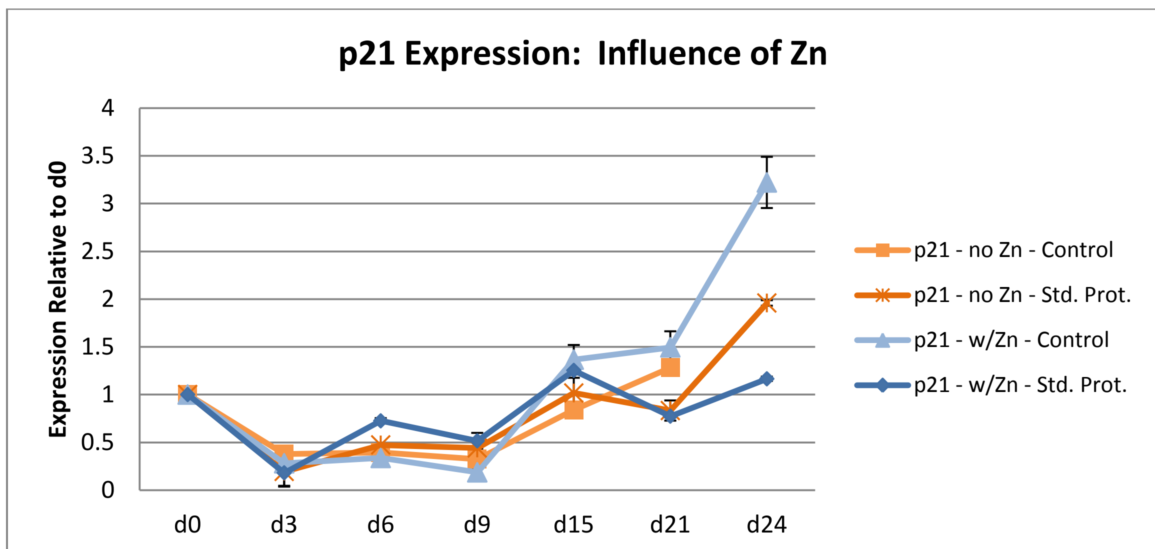


Figure 21. The influence of Zn, added throughout the differentiation, on the expression of *p16*. The influence of PRL and HGH, added at day 15, on the expression of *p16* is shown below.

The first gene analyzed (Figure 21), which stayed relatively constant through each of the differentiations was p16 (INK4A). This protein is a cell-cycle inhibitor that arrests the cell cycle in the G_1 phase. In this differentiation, the highest levels were observed in the Control group, peaking at day 15, then at day 24 at a 17-fold increase over the day 0 value. In the Standard Protocol groups, nothing changed significantly during the first 21 days. But, by day 24, the regular differentiation group had about a 5-fold increase, while the PRL, HGH, and PRL+HGH conditions were all at about a 3-fold increase with very small error bars. Very similar observations were had for the other cell-cycle inhibitor p21 (Figure 22).

While p21 expression dropped to almost zero at d3, it then remained between its original level and a two-fold increase for the rest of the experiment. In both of these genes, there were no significant differences apparent between the groups with and without Zn.



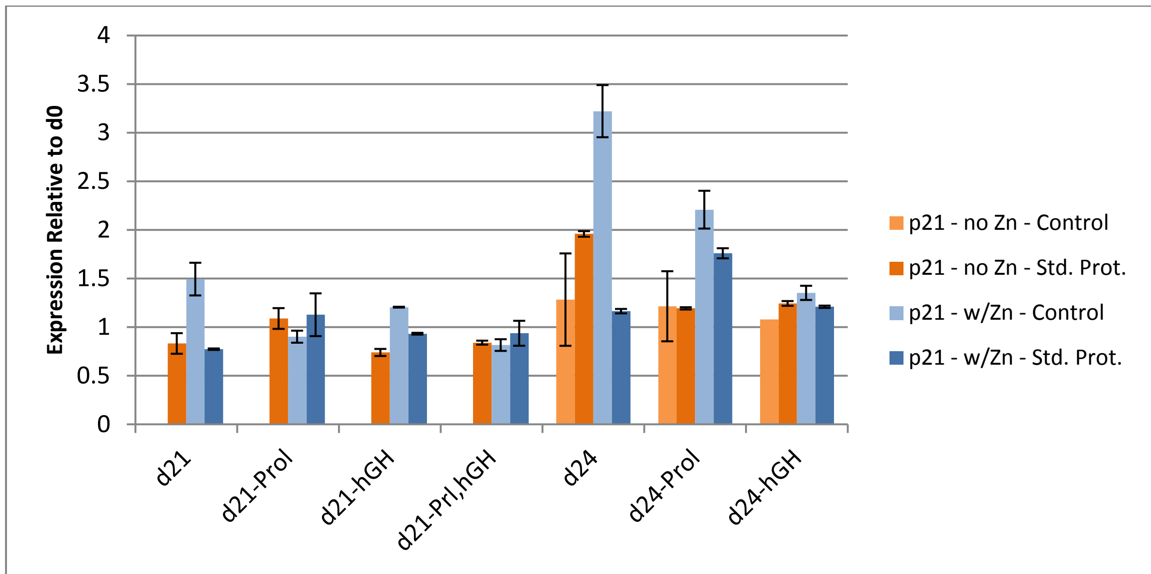
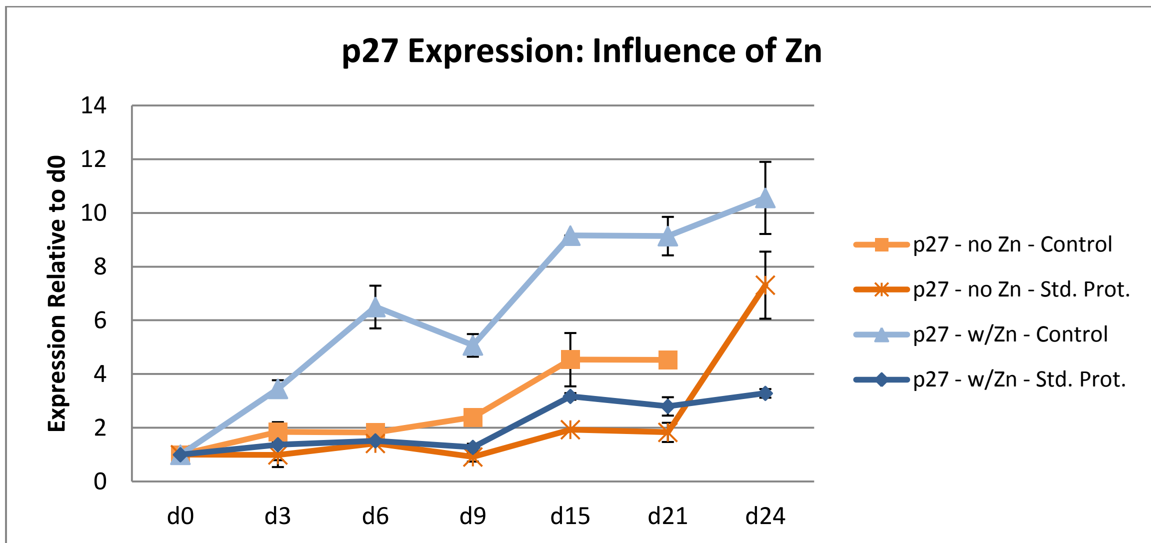


Figure 22. The influence of Zn, added throughout the differentiation, on the expression of *p21*. The influence of PRL and HGH, added at day 15, on the expression of *p21* is shown below.



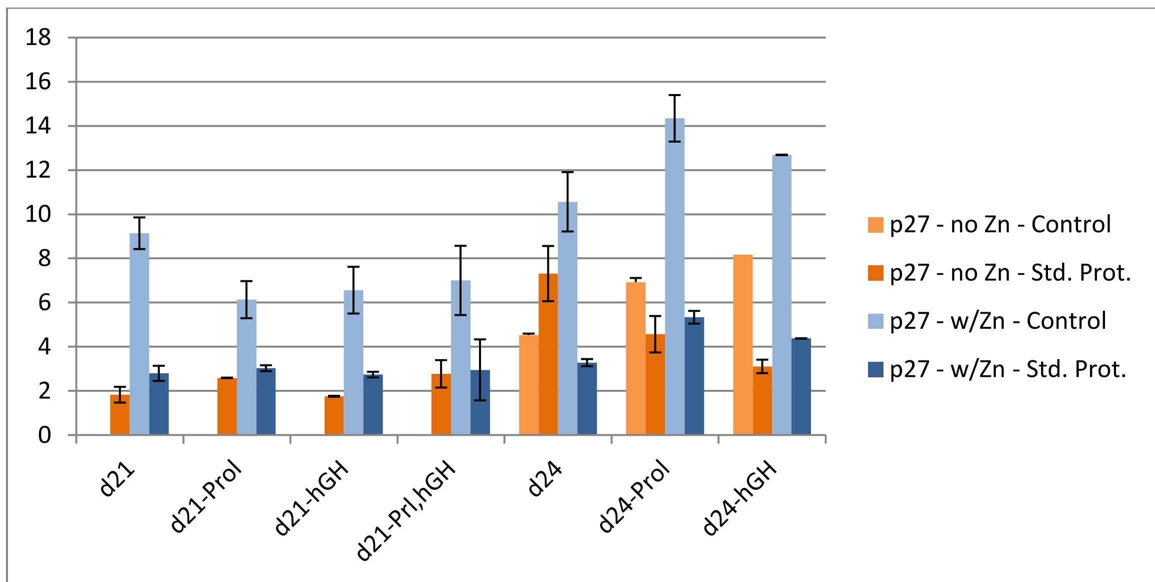


Figure 23. The influence of Zn, added throughout the differentiation, on the expression of *p27*. The influence of PRL and HGH, added at day 15, on the expression of *p27* is shown below.

In another cell-cycle inhibitor gene, p27, a gradual increase in expression was observed (Figure 23). While Zn did not make a difference, the expression rose gradually to a 2-fold increase, then jumped to about a 7-fold increase at d24 without Zn present, and only about 3 with Zn. Like earlier, the hormones and glucose only had an effect at day 24, with these groups having about half the p27 expression as the normal stimulation group.

In addition to these genes being analyzed, both insulin and *Pdx1* were also assessed back when the differentiation was originally done. Unfortunately, there was no observed expression of insulin, and very low levels of *Pdx1* (below a 10-fold increase at the maximum). The expression of *FoxA2* was also analyzed, showing typical expression of very high early in the differentiation, as expected, then tapering off.

In summary, the addition of Zn did not make a difference in the differentiation for the cell cycle inhibitors. The expression of these cell-cycle regulators did not show a significant difference when any of the hormones were added. This is unfortunate, as we had hoped to see a decrease in these genes' expression. From these analyses alone, we

concluded that these hormones do not influence the cells through regulation of these cell cycle inhibitors, though from this data, we were able to plan new experiments that could retest some of the same conditions, and attempt to tailor the time the factors are added.

Differentiation experiments with added Prolactin, Human Growth Hormone, and prolonged EGF

Based on the reported and observed effect on islet cultures, the addition of prolactin (PRL) and human growth hormone (HGH) were retested in the differentiation. Also, because of the possible influence of EGF on both *p16* and *Pdx1* expression, experiments were done with longer periods of EGF exposure to try to increase the *Pdx1* expression. All further work is of new experiments, as opposed to previous data based on re-analysis of past experiments.

Prolonged EGF Treatment

This experiment tested whether increasing the duration of EGF exposure by three days, both before and after its original time frame, would allow for the increased *Pdx1* expression. This is based on the inverse correlation between the *p16* expression and *Pdx1* expression observed in the past experiments. Figure 24 shows the two conditions tested using the L-1, iPS cell line.

Experiment	D0	D3	D6	D9	D12	D15	D18
Control *No Factors*							
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin	Exendin4 GDF-11 Betacellulin
Factors added to Std. Prot.							
EGF d6-d15			EGF + Heparin Sulfate				
EGF d9-d18						EGF + Heparin Sulfate	

Figure 24. A summary of the groups used in testing the influence of extending the exposure of the differentiating L-1 cells to EGF. The control group received no added differentiation factors, and the standard protocol groups had the typical differentiation factors added. The "EGF d6-d15" received all the same factors as the standard protocol, though, in addition, EGF and Heparin Sulfate, from day six through day nine. The "EGF d9-d18" group was also identical to the standard protocol group, but added EGF and Heparin Sulfate from day 15 through day 18.

EGF was added from either day 6 through day 15, day 9 through day 15 (Normal), or day 9 through day 18. Results from the experiment were analyzed by qPCR. Figure 25 displays the expression of *Pdx1* in each of the groups.

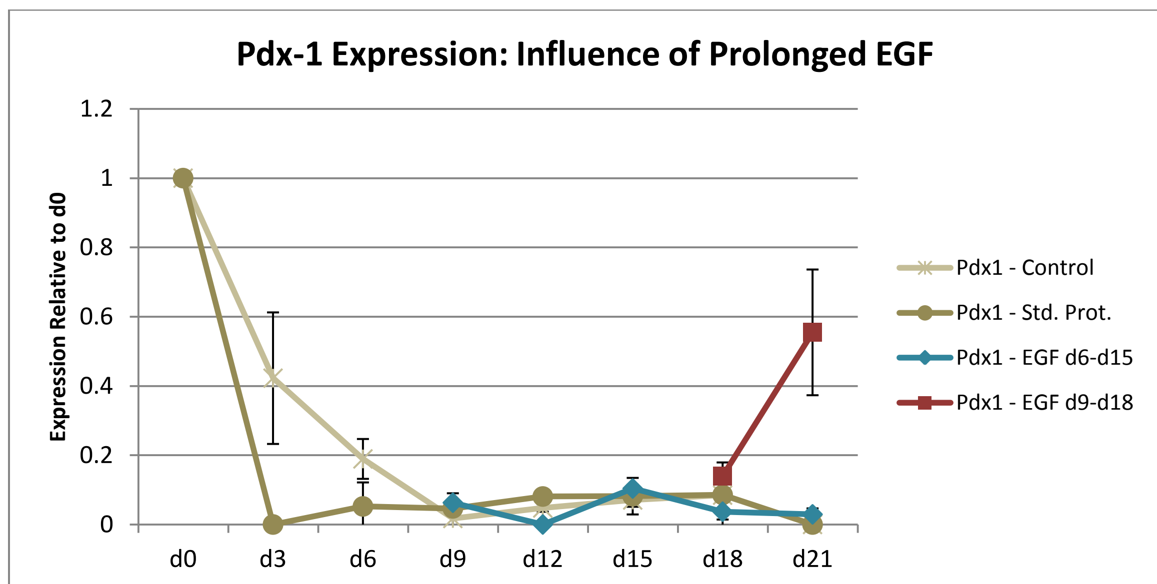


Figure 25. Expression of *Pdx1* throughout the experiment under normal conditions and early and prolonged EGF exposure. The normal time of EGF exposure is d9-d15. Because the prolonged EGF groups were treated the same as the Std. Prot. group until either day nine or day 18, only the data from after they differed is showed.

The expression profile of *Pdx1* suggested that there was some background differentiation, as the cells were either unable to differentiate or differentiated but were unable to re-achieve the amount of expression present at the first day of the experiment. Though even with the lack of an increase late in the differentiation, the extended treatment with EGF, from day 9 through day 18, caused a much higher amount of *Pdx1* expression.

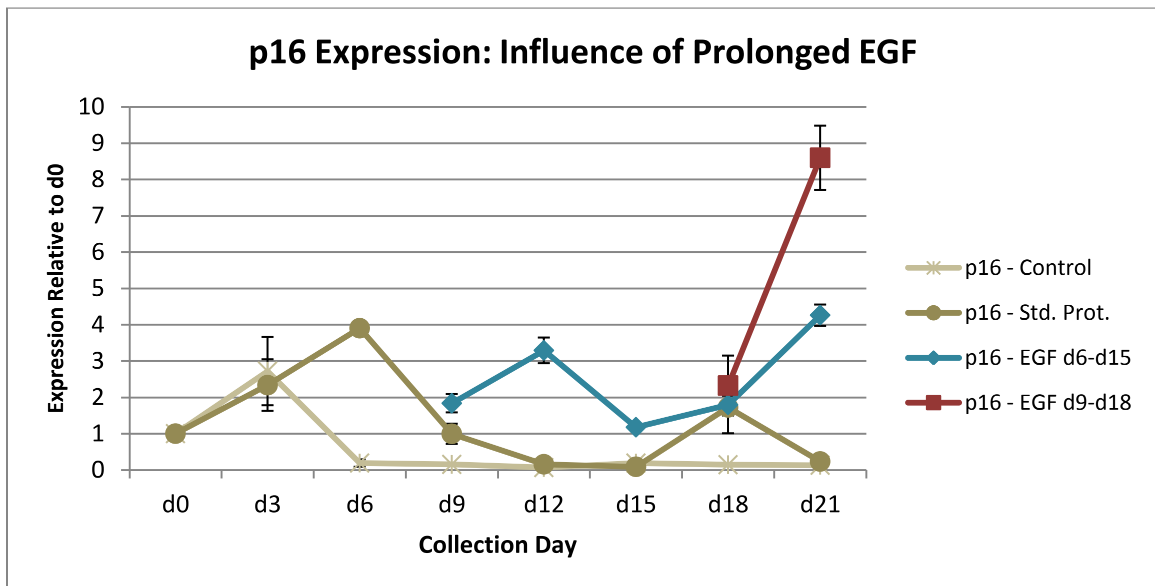


Figure 26. Expression of *p16* throughout the experiment under normal conditions and early and prolonged EGF exposure. The normal time of EGF exposure is d9-d15. Again, because the prolonged EGF groups were treated the same as the Std. Prot. group until either day nine or day 18, only the data from after they differed is showed.

The expression of *p16* (Figure 26) did not correlate as expected with the *Pdx1* data. The group with an extended EGF exposure, the only that showed an increase in *Pdx1* expression, showed an increase in *p16* expression at day 21. Previous data suggested that the increase in *p16* would not be concurrent with an increase in *Pdx1*, however this was not the case. Because of the low *Pdx1* expression in the experiment,

no correlations could be had between the two genes for the other groups. The amount of *insulin* expressed was also tested, but it was too low to be useful.

This experiment's results cannot be taken as definitive, as the low amount of *Pdx1* expressed shows that proper differentiation did not occur. Therefore, the desired effect of EGF on increasing the amount of maturation was not able to be tested. To answer the question, the experiment will need to be repeated.

Influence of PRL and HGH on maturation of differentiating beta-cells

Multiple experiments were done to compare the effects of PRL and HGH on iPS cell differentiation. We hypothesize that either or both of these added factors will improve the outcome of the differentiation, as assessed by PCR gene-expression analysis. Figure 27 shows a chart of the normal protocol, and the experimental conditions added.

Experiment	**D0**	**D3 – D9**	**D9 – D15**	**D15 – D21**
Control *No Factors*				
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Factors added to Std. Prot.				
Prolactin			PRL	PRL
HGH			HGH	HGH

Figure 27. Summary of experimental conditions testing the effect of PRL and HGH on the differentiation of the L-1 iPS cell line. There was a negative control group, which received none of the differentiation factors, labeled "Control" in the graphs. The "Standard Protocol" group received the usual differentiation factors. The Prolactin and HGH groups each received the factors present in the Standard Protocol, but then either PRL or HGH was also added from day nine until the end of the experiment.

Opposed to the past experiments, PRL and HGH were added at day 12. Because the expression levels of *Pdx-1* typically peak around day 15, if these hormones were added then, they could be missing the period where they have the greatest effect, that

being before day 15. Also, samples were taken every day that the medium was changed, as opposed to every six days after day 9.

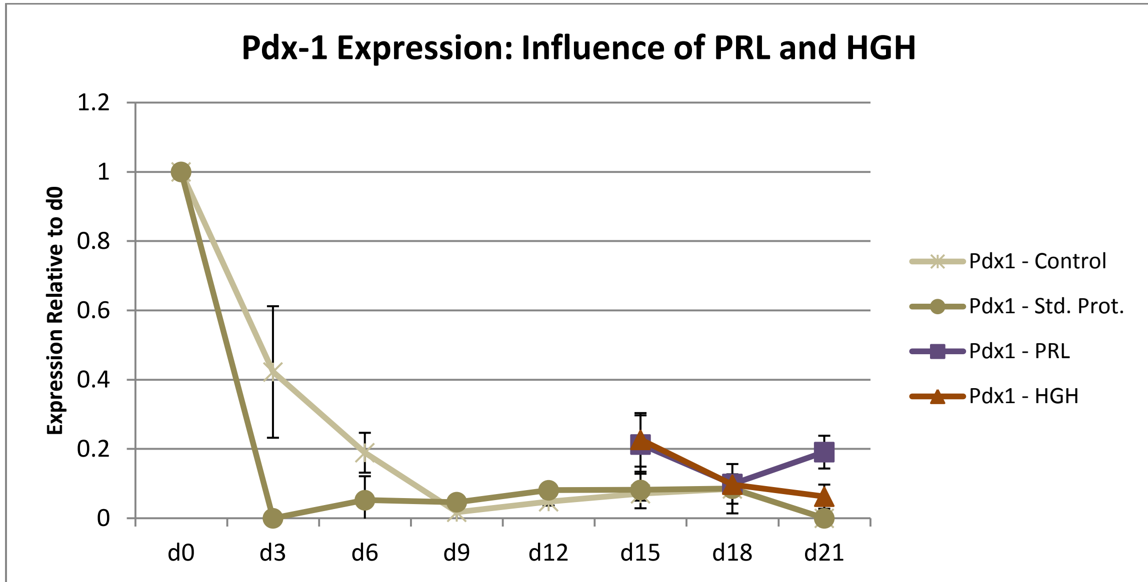


Figure 28. Expression of *Pdx1* throughout the experiment under normal conditions and with either PRL or HGH added from day 12 through day 21. Because the PRL and HGH groups were treated the same as the Std. Prot. group until day 12, only the data from after day 12 is shown.

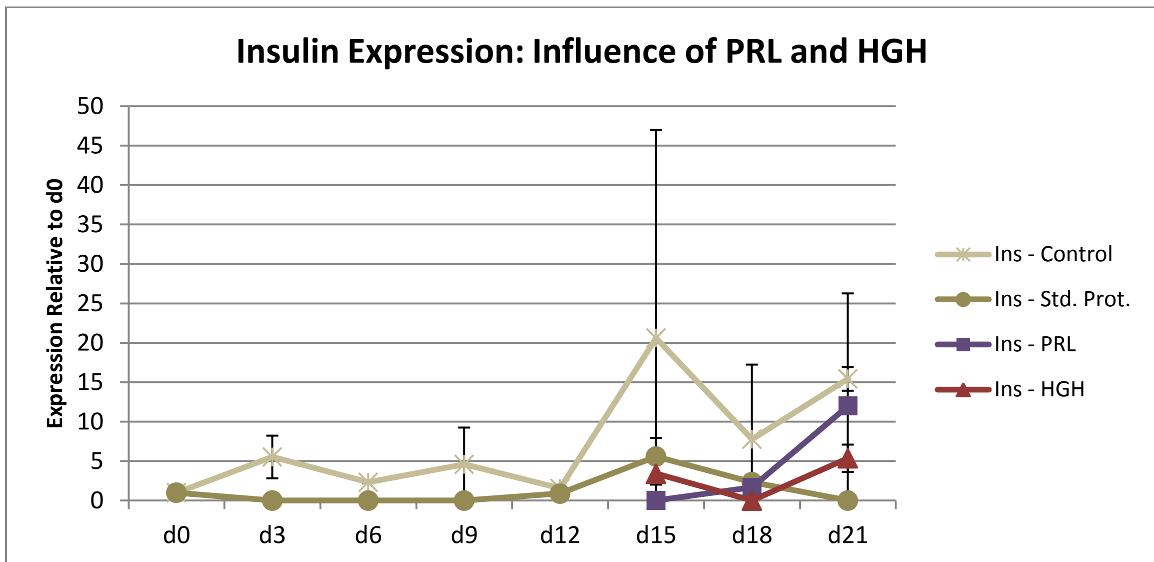


Figure 29. Expression of *insulin* throughout the experiment under normal conditions and with either PRL or HGH added from day 12 through day 21. Because the PRL and HGH

groups were treated the same as the Std. Prot. group until day 12, only the data from after day 12 is shown.

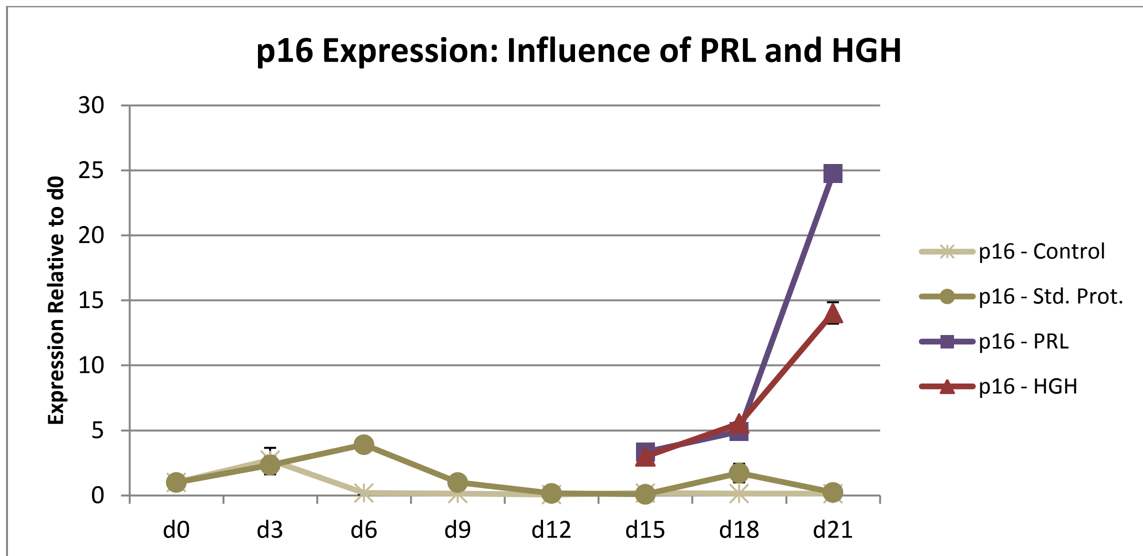


Figure 30. Expression of *p16* throughout the experiment under normal conditions and with either PRL or HGH added from day 12 through day 21. Because the PRL and HGH groups were treated the same as the Std. Prot. group until day 12, only the data from after day 12 is shown.

From the qPCR analysis, it is obvious that the first stages of the differentiation went well, with *Foxa2* and *Sox17* (not pictured) both being expressed at high levels for the first 6 days of differentiation. In development, both of these genes are expressed in the definitive endoderm, which develops further into the gut tube, segregated into the foregut, midgut, and hindgut.

From the foregut endoderm, the pancreatic buds are induced, expressing the genes *Pdx1* and *Ptf1a* initially. Unfortunately, *Pdx1*, which is more of a definitive marker of the pancreas, did not increase during the differentiation (Figure 28). Rather unusually, it dropped down to almost no detectable expression. I suspect this is due to a poor starting population of cells, which might have already been expressing *Pdx1* at day zero. As the differentiation progressed, there was less *Pdx1* expressed relative to

the first day. Another explanation is that the cells were simply not induced to become pancreas. All the groups were similar, in that they all showed poor *Pdx1* expression.

The other marker for early pancreas specification is *Ptf1a* (also known as *p48*). This marker did not rise until after day 18 (not shown), and only to about a level 6 times that of day 0. Taken together, the *Pdx1* and *Ptf1a* data suggest that there was not sufficient induction of the pancreas lineage during this differentiation. This is echoed by the poor *insulin* expression (Figure 29).

Finally, the expression of *p16* expression was also not what was expected (Figure 30). This gene's expression did not change much during the first 15 days of the differentiation in any of the groups. But, in each of the PRL and HGH treated groups, a small increase occurred at day 18, followed by an even larger increase by day 21. This was most pronounced in the PRL group, but a significant jump was made in the HGH group too. As these hormones were added at day 12, this suggests that either they require at least 6 days for the cells to respond, or the cells are not susceptible to the hormones until day 18. But with the almost undetectable levels of *Pdx1* and *insulin*, drawing strong conclusions about what these hormones do is not possible. That said, recent reports have shown p16 to be expressed higher in the aged pancreas and other tissues (Krishnamurthy et al, 2008), so this could be taken as a more mature cell type is being produced.

Overall, this was an unsuccessful differentiation, shown by both *Pdx1* and *insulin* gene expression. However, they did have an effect on the level of p16 expression, which, in the future, should be monitored for a correlation with pancreas-specific genes.

The above experiment was then repeated, with the same conditions as the previous experiment, except with the H9 ESC line (Figure 31).

Experiment	**D0**	**D3 – D9**	**D9 – D15**	**D15 – D21**
Control *No Factors*				
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Factors added to Std. Prot.				
Prolactin			PRL	PRL
HGH			HGH	HGH

Figure 31. Summary of experimental conditions testing the effect of PRL and HGH on the differentiation, though in the H9, ES cell line. There was a negative control group, which received none of the differentiation factors, labeled "Control" in the graphs. The "Standard Protocol" group received the usual differentiation factors. The Prolactin and HGH groups each received the factors present in the Standard Protocol, but then either PRL or HGH was also added from day nine until the end of the experiment.

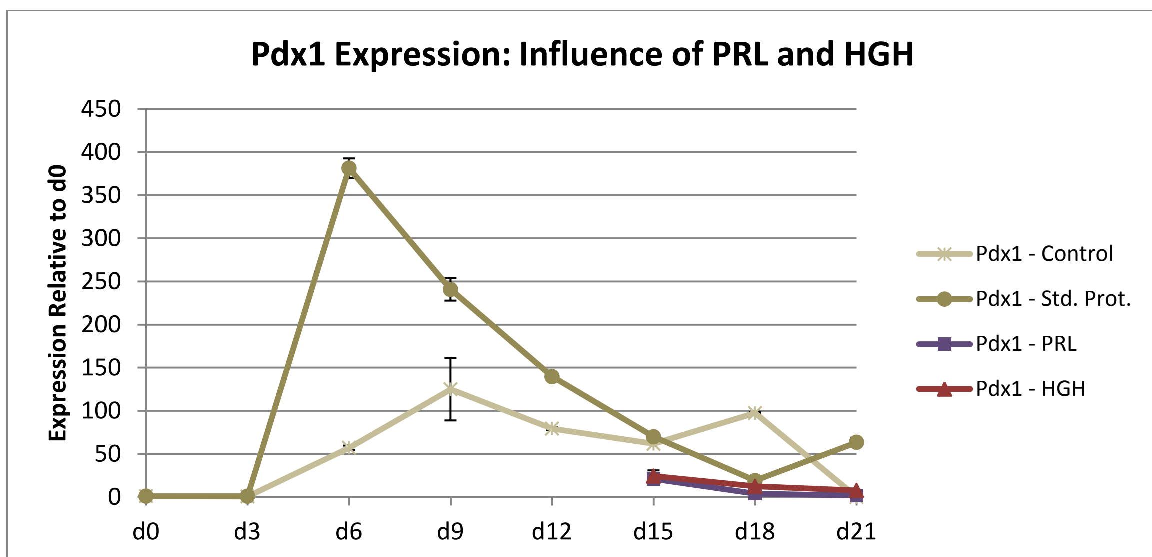


Figure 32. Expression of *Pdx1* throughout the experiment, in H9 cells, under normal conditions and with either PRL or HGH added from day 12 through day 21. Because the PRL and HGH groups were treated the same as the Std. Prot. group until day 12, only the data from after day 12 is shown.

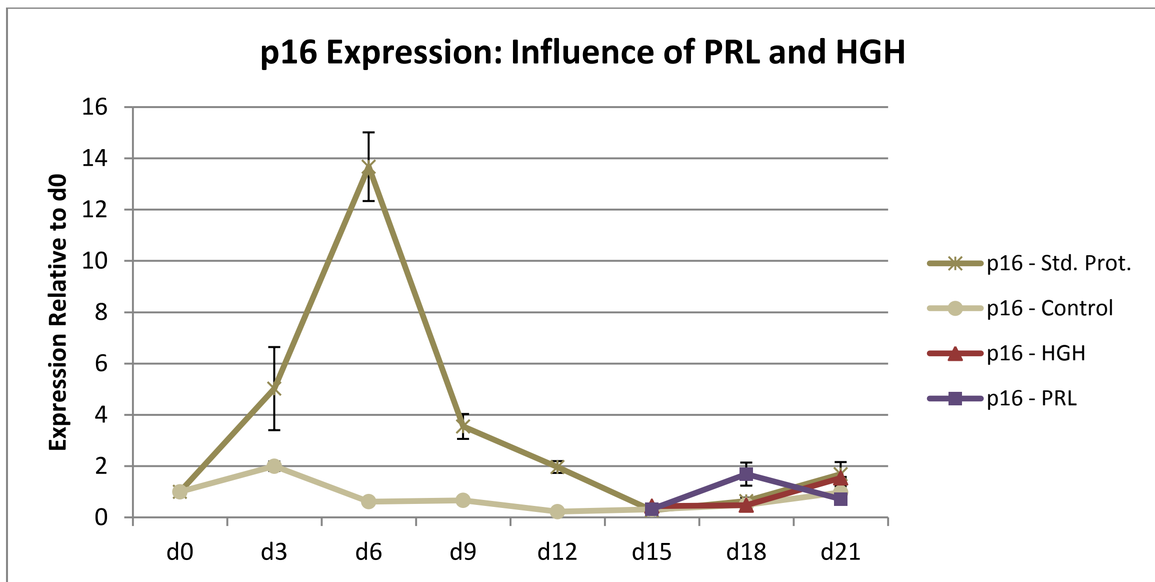


Figure 33. Expression of *p16* throughout the experiment, in H9 cells, under normal conditions and with either PRL or HGH added from day 12 through day 21.

Again, the results of this experiment were surprising. Early developmental genes were not assessed, as there has been consistent good expression of *Foxa2* and *Sox17*. For *Pdx1* the expression showed a sharp rise at day 3, to a level near 375 times that at day zero by day 6 (Figure 32). After this, the level dropped steadily until day 18, where it was back to nearly the same level as day 0. Another increase then occurred by day 21 to a level of 50. This is what occurred in the Standard Protocol group, receiving the normal differentiation factors. In both the PRL- and HGH-treated groups, poor expression of *Pdx1* was shown throughout the later days, staying at less than 25.

Surprisingly, when the levels of the cell-cycle inhibitor p16 were analyzed (Figure 33), they showed a pattern reflecting the expression of Pdx1, rather than the inverse correlation that was observed in the past experiments. The expression peaked at day 6, at about 14, then dropped steadily to day 18 before rising again a bit. Again, this was in the Standard Protocol group, with the hormone-treated groups showing a steady expression of p16, hovering near a level similar to day 0.

However, despite *Pdx1* expression, no insulin expression was observed. This showed that no beta-cells were created, but more likely a cell type from earlier in

pancreatic development. Thus, if allowed to differentiate longer, and with the correct signals, perhaps the cells could form endocrine cell types.

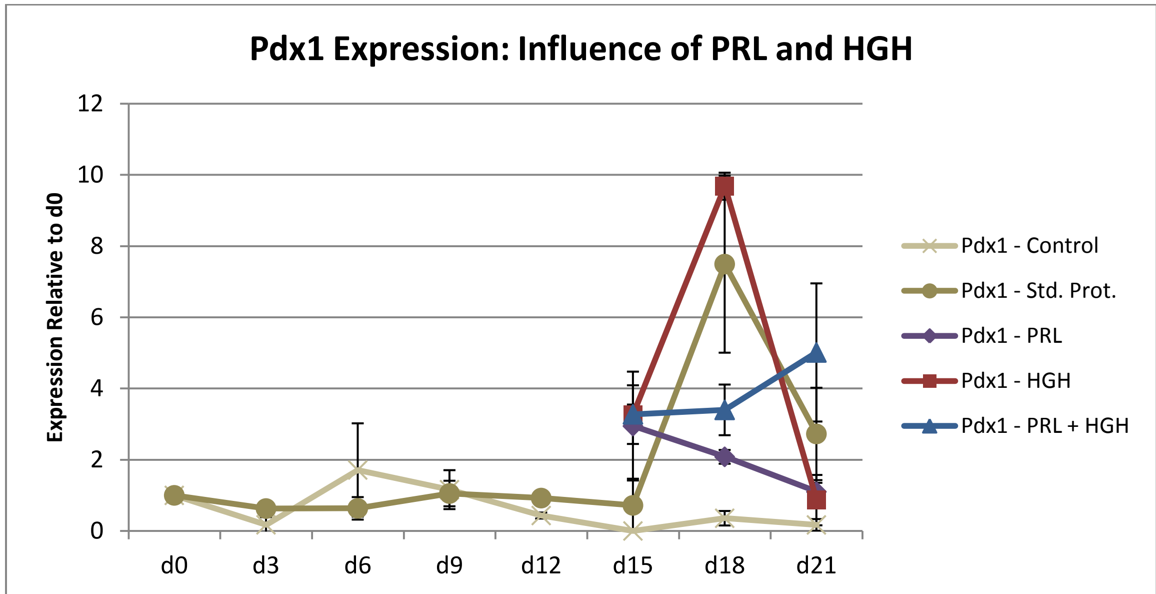
Other cell-cycle inhibitors were also examined: *Rb, p21,* and *p27* (data not shown). Of these, the only gene showing significantly less expression in the presence of the hormones is *p27*, and only with the PRL-treated group. This could indicate that PRL is signaling is suppressing *p27* transcription.

As a whole, this experiment did not support the hypothesis that PRL and HGH improve the outcome of the differentiation. The addition of either of these hormones did not result in a higher level of *Pdx1* or *insulin* expression. But because this is only one experiment, it was repeated to draw some conclusions.

The following repeats were done under the same conditions as the previous experiment, testing both PRL and HGH, except that an experimental group containing both PRL and HGH together was added. This was to hopefully increase the effect of one or the other, so any effect would be more easily observed. Also, these experiments used the L-1 iPS cell line, rather than the H9 ES cell line used in the previous experiment. Below are the *Pdx1* and *insulin* expression from each of the four experiments.

Experiment	**D0**	**D3 – D9**	**D9 – D15**	**D15 – D21**
Control *No Factors*				
Standard Protocol	Activin-A Wnt3a	Activin-A Wnt3a α-Shh	EGF Heparin Sulfate	Exendin4 GDF-11 Betacellulin
Factors added to Std. Prot.				
Prolactin			PRL	PRL
HGH			HGH	HGH
Prolactin + HGH			PRL + HGH	PRL + HGH

Figure 34. Summary of experimental conditions testing the effect of PRL and HGH on the differentiation, though in the H9, ES cell line. There was a negative control group, which received none of the differentiation factors, labeled "Control" in the graphs. The "Standard Protocol" group received the usual differentiation factors. The Prolactin and HGH groups each received the factors present in the Standard Protocol, but then either PRL and/or HGH was also added from day nine until the end of the experiment.



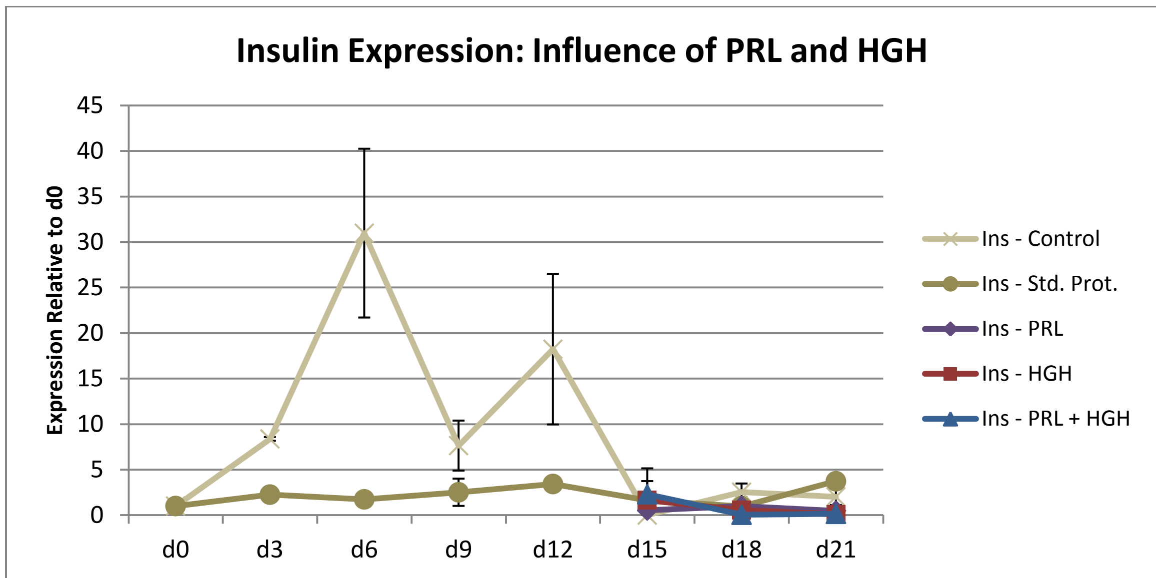
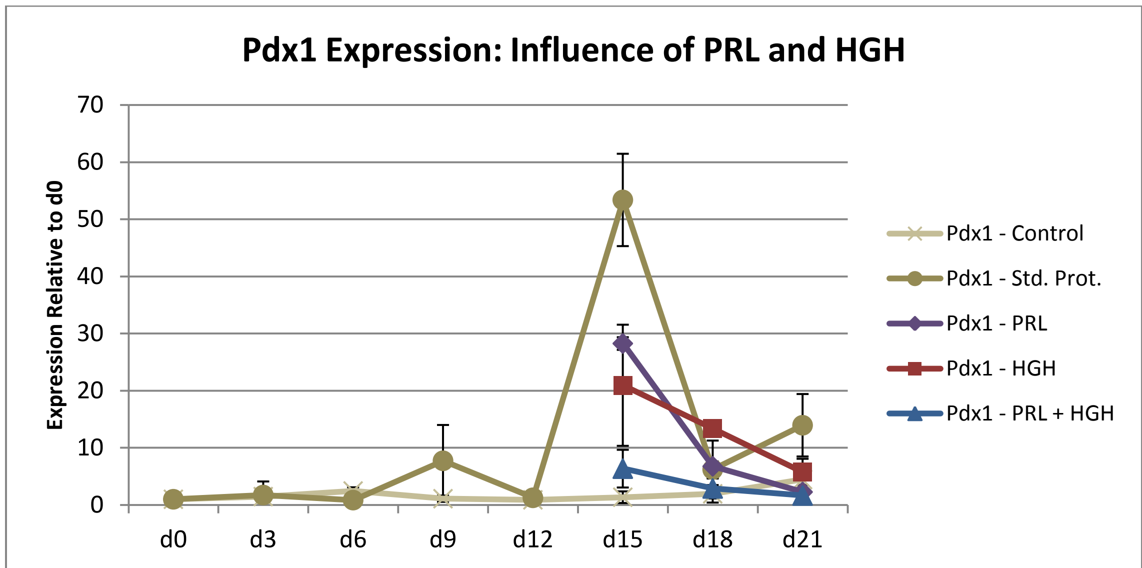


Figure 35. Expression of *Pdx1* and *Insulin* throughout the first repeat experiment.



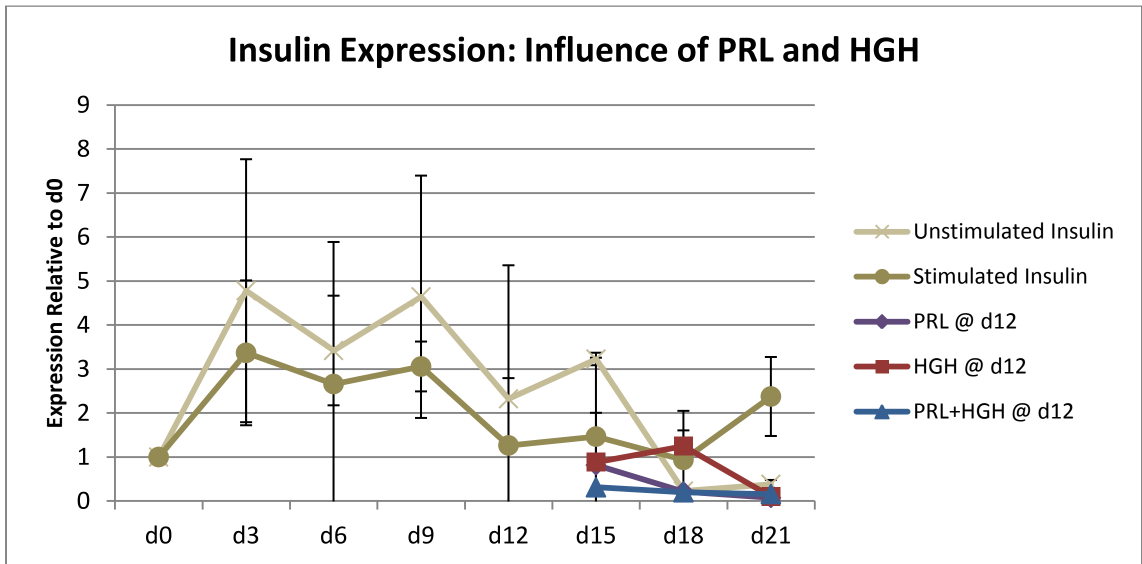
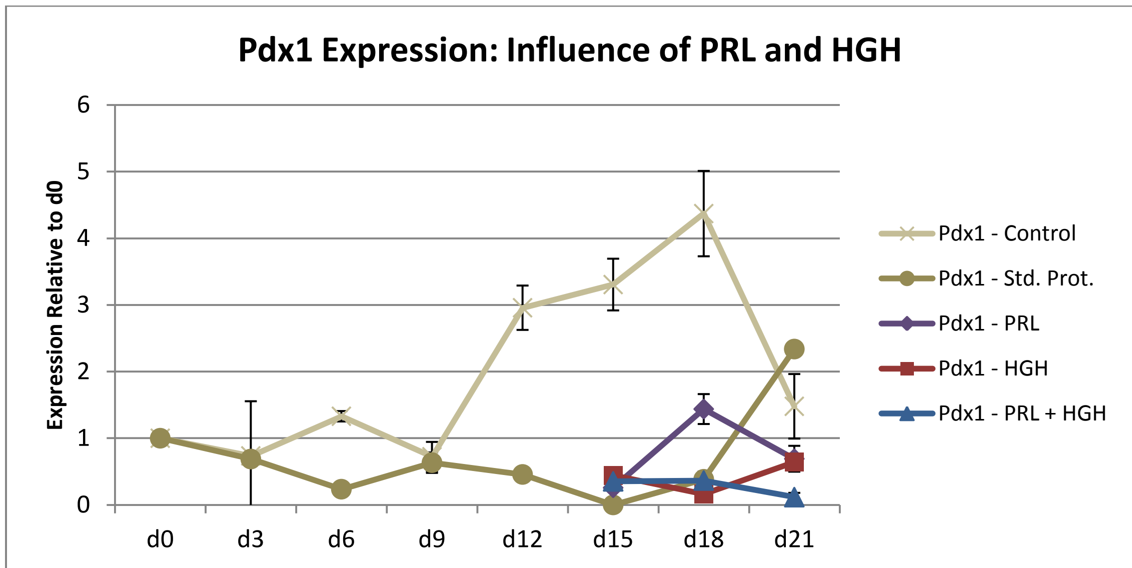


Figure 36. Expression of *Pdx1* and *Insulin* throughout the second repeat experiment.



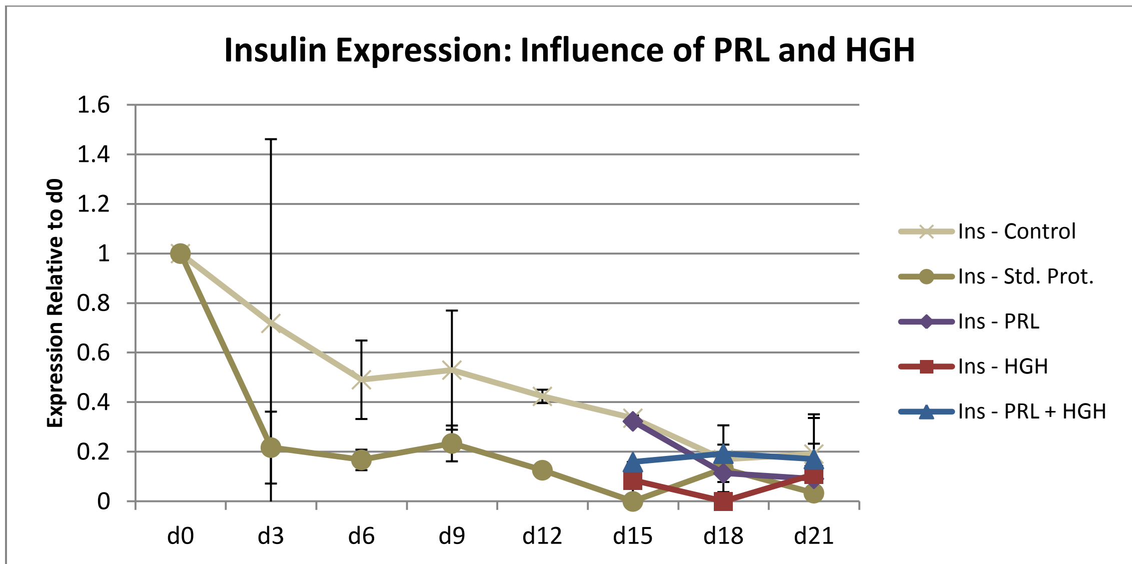
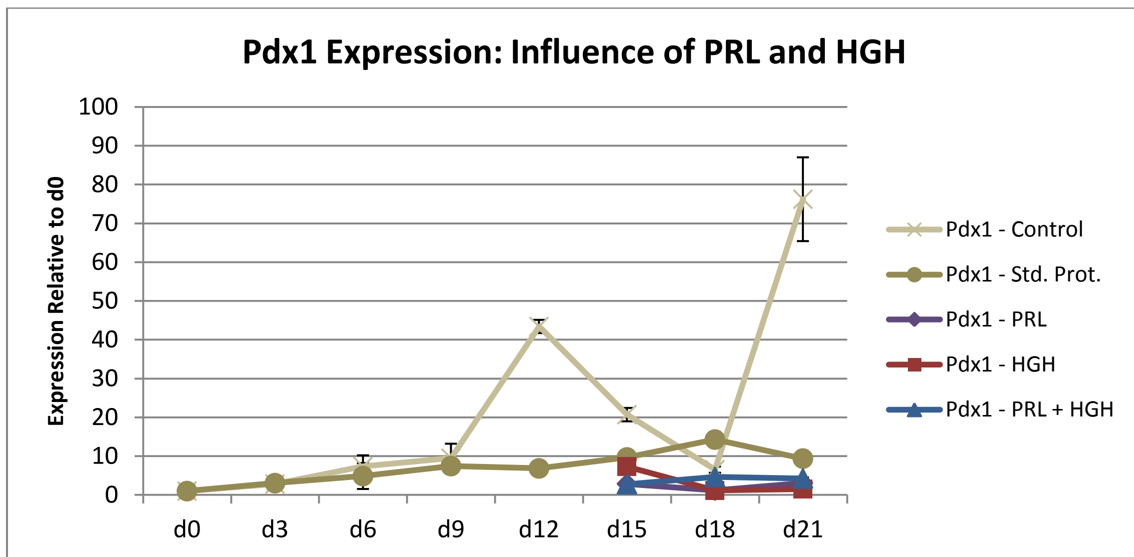


Figure 37. Expression of *Pdx1* and *Insulin* throughout the third repeat experiment.



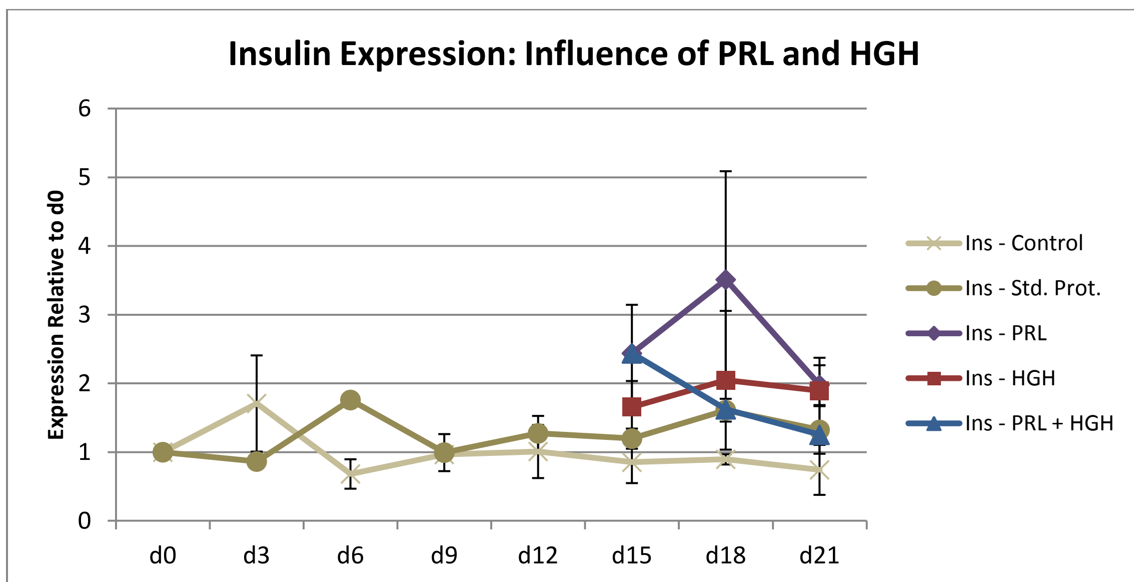
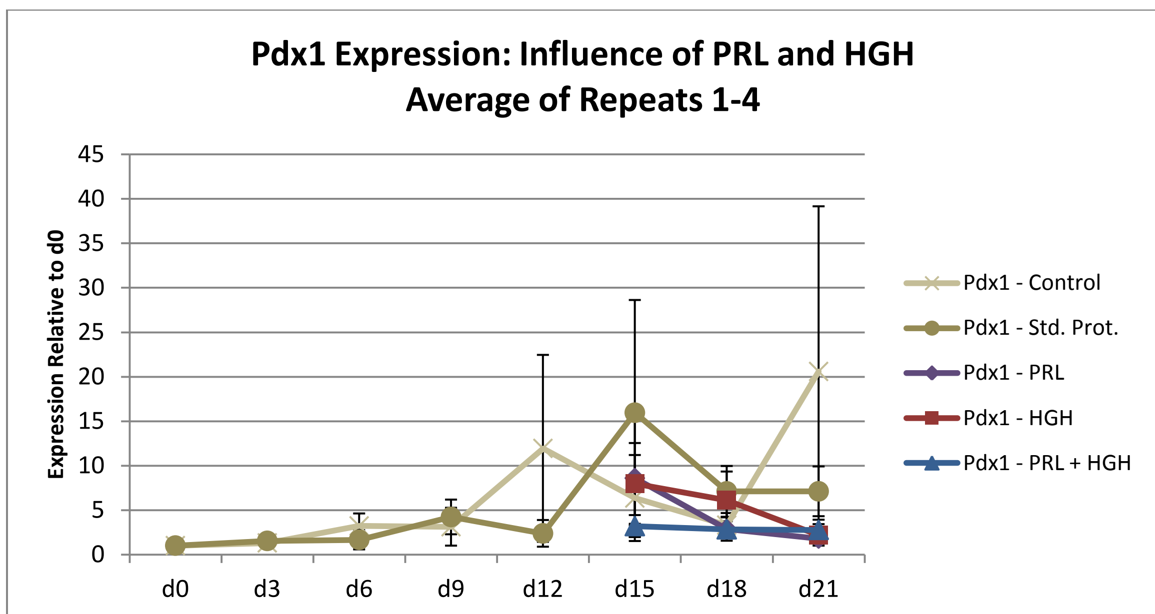


Figure 38. Expression of *Pdx1* and *Insulin* throughout the fourth repeat experiment.

From these four experiments, unfortunately, it is clear that the addition of either of these hormones does not increase the expression of *Pdx1* or *insulin.* For *insulin*, the levels never got high enough to get even close to those found in a pancreas sample. Often the levels of the Standard Protocol group were even less than that of Control group.

The expression levels of *Pdx1* were better, but only in the first and second repeats (Figures 35 and 36). In these experiments there was a spike in *Pdx1* levels at either day 15 or day 18, which is more consistent with what has been found in past experiments. However, in third and fourth repeats (Figures 37 and 38) the levels remained consistently low. The addition of the hormones also did not help in the third repeat, but in fourth repeat there was a unique increase in *Pdx1* expression for the group treated with HGH at day 15 to a level about seven times that at day 0. This is not the largest increase that has been observed, but it was a large difference between what the Standard Protocol group showed.

As each of these experiments were all repeats, testing the same hypothesis, their data can be presented together. Consolidating the data into one graph (Figure 39), with an *n* of four for each data point, allows any trends underlying the repeats to be observed. However, unfortunately, there were no new conclusions to be had from the data.



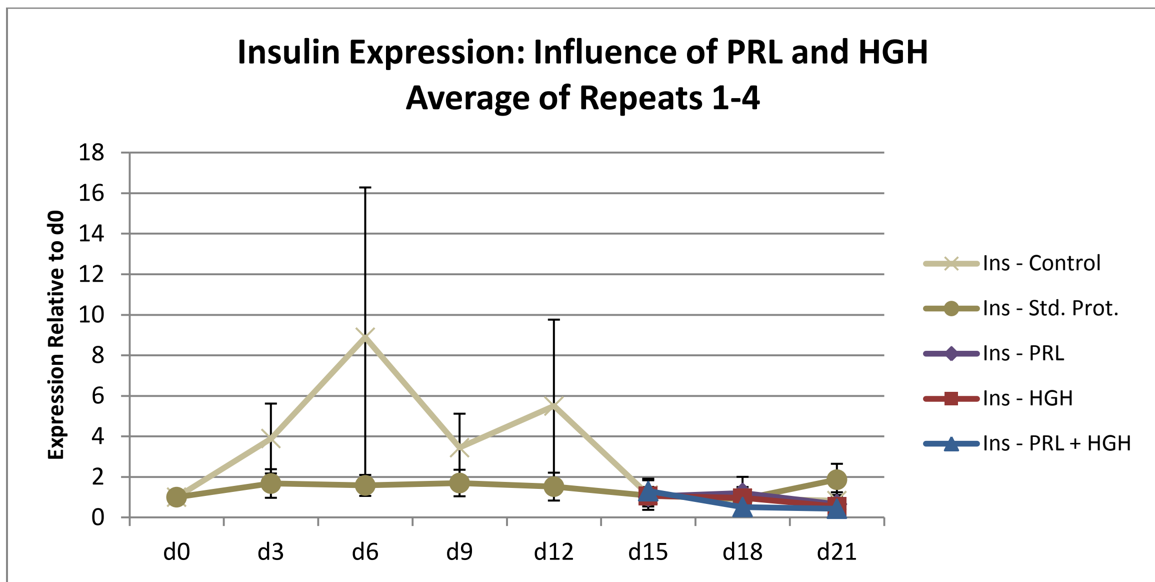


Figure 39. Consolidation of the data from repeats 1-4, testing the influence of either PRL or HGH on the differentiation. Each data point therefore represents an average the four experiments at that time-point, with the error bars the SEM.

One caveat to presenting data as values relative to the first day of the differentiation is that if there is a higher-than-normal level at that first day, an increase later in the differentiation will not be obvious. When the raw numbers for qPCR analysis for *Pdx1* are compared between these differentiations (Figure 40), one can see that, excluding Differentiation #1, the "Peak ΔC_t" values show that more similar levels of *Pdx1* transcript are present than the "Fold over d0" value suggests, which is the value shown in each of the charts (lower ΔC_t number indicates higher expression).

Exp. #	d0 Δ C_t	Peak Δ C_t	Day of Peak	Fold over d0
1	14.62	14.62	d0	1
2	19.78	10.5	d6	596
3	17.53	14.665	d18	7.5
4	19.655	13.925	d15	53.4
5	15.645	14.42	d21	2.3
6	19.925	16.095	d18	14.3

Figure 40. The levels of *Pdx1* expression are more similar than the previous charts suggest. The data in the far right column are the values presented in each respective

graph, while the "Peak ΔC_t" value represents the point of the differentiation when the highest level of *Pdx1* was detected by qPCR.

The groups treated with PRL and PRL+HGH also showed a higher level of *Pdx1* expression in this experiment, with the PRL+HGH group maintaining a higher level, around four times that of the Standard Protocol group. Although this increase is obvious, the level of *Pdx1* expression is still weak compared to past experiments done in the lab, which showed levels well over a 100 times that at day 0.

Aside from this last experiment, the levels of gene expression in the hormone-treated groups were all similar to, or even less than, the Standard Protocol group. From this data, I would deem these hormones as ineffective at improving the differentiations.

In addition to the PCR analysis, however, a TUNEL was done on sections of the EBs that resulted from Differentiation #4. While not enough were done to make any statistical conclusions, the data is still informative (Figure 41). Thus, even though the addition of the hormones does not improve the gene expression profile of the differentiating EBs, they could improve the viability of the cells. However, with only 4.22% of the cells showing positive for the TUNEL assay in the Standard Protocol group, the increase in the resulting cells might not be large enough to warrant the cost of the added hormone. Nonetheless, repeating the TUNEL assay on the other experiments might show more convincing evidence for the hormones as having a protective effect.

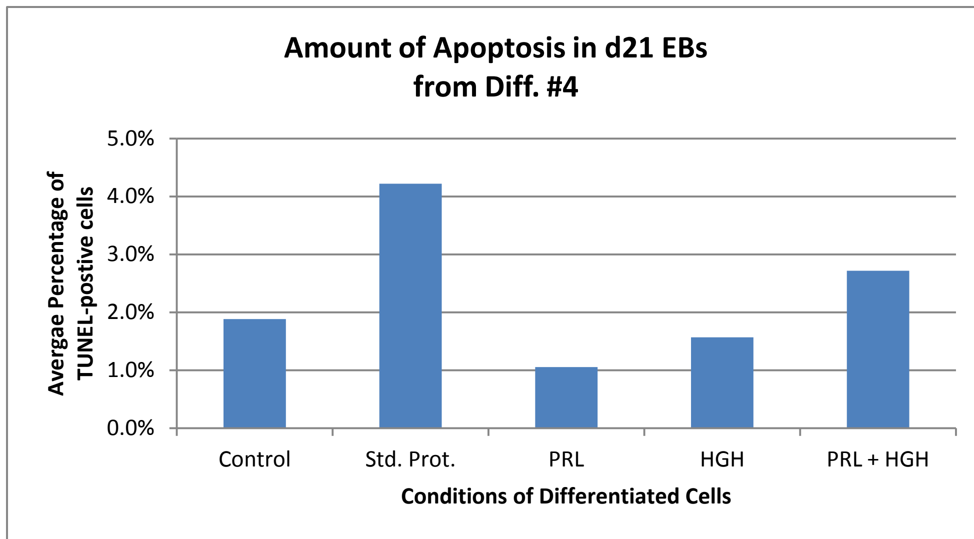


Figure 41. Results of TUNEL analysis done on EBs from d21 of Differentiation #4. The number of images analyzed was not high enough for statistical comparisons.

Discussion

The first question asked here was how much cell death was occurring during the differentiation, and if those cells dying are indeed beta-cells or beta-cell progenitors. The TUNEL assay showed that the cells undergoing apoptosis are not *Pdx-1* positive, providing strong evidence that the cells dying are simply not, and never were, a beta-cell or beta-cell progenitor. But, with no Pdx1 positivity, this could also mean other, less optimistic conclusions. One, that the differentiation conditions were not effective in this differentiation, or, two, that the cells which became Pdx1-positive had already died. It is unknown how quickly Pdx1 is down-regulated once a cell is committed to undergoing apoptosis. And with the TUNEL assay assessing only the later stages of the apoptosis, it is possible that the few cells which showed positivity had previously expressed *Pdx1*. Future work into this topic should first establish the behavior of a dying beta-cell, which could be used to assess the behavior of the differentiating cells.

From the past differentiation samples analyzed by PCR, we primarily found that the period of EGF treatment causes a decrease in expression of the cell-cycle inhibitor p16. Around the time of this down-regulation at day 15, the expression of *Pdx1* typically peaks, suggesting a relationship between low p16 levels and high Pdx1. This could be an interesting avenue to pursue in the future. While we began to investigate changing the exposure period of EGF, we stopped because of the increased number of cells required, and to avoid swaying too far from the original research question.

In the following six differentiation experiments, unfortunately, drawing conclusions is difficult. This is because the hormones were added to influence the maturation and viability of beta-cell progenitors specifically, but the differentiations arguably did not produce the typical amount of *Pdx1*-expressing beta-cell progenitors, so how these cells reacted to the hormones could not be tested. However, in Repeats #1 and #2, there was an observed peak in *Pdx1* expression, and in these experiments the addition of prolactin and growth hormone did not improve the subsequent expression of neither *Pdx1* nor *insulin*, suggesting that these hormones do not have a

significant influence on the differentiation. But another aspect of the differentiation questioned was final cell viability. From the TUNEL assay performed on the experiment Repeat #2, there was an obvious decrease in apoptosis in the groups treated with the hormones compared to the normal protocol. But, more testing will need to be done to allow statistical comparisons to be made.

An obvious question to ask, since there was such little observed response of the cells to either PRL or HGH, is whether the cells are expressing the correct receptors that make them susceptible to such signals. In a preliminary analysis of this, doing immunoflorescence on a few sections from Repeat Experiment #2 for the PRL receptor (PRLr), there was no observed staining that occurred. This was just a single experiment, though if future work continues with the hormones, examining when the cells tend to begin to be susceptible to the signals would be helpful.

Finally, the whole islet research is still an ongoing project. Determining what effect the hormones have on islet and beta-cell growth and replication is being done in combination with other factors that stimulate growth, particularly fatty acids. The combination of prolactin and the fatty acid oleate has a synergistic effect on beta-cell replication and, subsequently, an increase in islet volume which is well documented in rodent islets (Brelje et al, 2008), but not in human islets. Currently data is being collected from more human islet cultures to see if the influence of the same affect is seen. However, the data so far suggests that the response to these signals is not as robust as what was observed in neonate rodents. This decreased response could be due to interspecies differences, or it could be because of age. The human islets come from adult, cadaveric donors while the rodent islets are typically from neonates. Future experiments examining adult rodent islets, and continued research on human islets, will give more insight into this question.

Should the production of patient-specific beta-cells be moved to the clinic, this type of technical research will aid in making it more accessible to patients by reducing production costs. For instance, adding a factor like prolactin to the differentiation might

increase the final yield of cells enough to offset the cost of adding it. Similarly, anything that improves the efficiency of the differentiation by producing more beta-cell progenitors or mature beta-cells will also reduce the cost of treatment. These are not obstacles that necessarily have to be overcome, but there is room for improvement in the procedures. The work presented here attempted to make an improvement, but at most can be taken as inconclusive, and more attempts should be made to test the hypothesis.

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