UNIVERSITY OF CALIFORNIA, SAN DIEGO

Novel improvements to the differentiation of human iPS and ES cells for the derivation of pancreatic beta cells

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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Professor Elvira Tour

2011

The Thesis of Megan Elizabeth Simpkinson is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

DEDICATION

I would like to dedicate this thesis to my parents, my sister, and my Sam. My parents fostered my education from the start and have lovingly supported me throughout my life. My sister has been my confidant and friend, always believing in my craziest ideas and making me feel like I can do anything. And my Sam has been there for me with all the love, inspiration, words of encouragement, and popcorn a girl could hope for in a partner. This thesis would have never come to be without all of you. All my love and thanks.

I would also like to dedicate this thesis to my eight-year-old self. It may not be a cure, but we have made our mark!

EPIGRAPH

"It had long since come to my attention that people of accomplishment rarely sat back and let things happen to them. They went out and happened to things."

Leonardo da Vinci

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ACKNOWLEDGMENTS

I would first like to express my gratitude to Dr. Hayek for giving me the opportunity to work in his lab. I am lucky to have found his lab and thankful for the invaluable gift of the experiences I have had as part of the team. Dr. Hayek has encouraged me throughout my studies and always treated me like a scientist, not just a student.

I would next like to thank Iva Afrikanova for being my teacher. She has taught me throughout my time in the lab and the work described in this thesis would never have come to fruition without her. Working with stem cells is an art form, and Iva is an artist. I am grateful for the knowledge she has passed along to me.

I would also like to thank all of the members of the Hayek lab. They have all been my teachers at some point during my time here and have made me feel welcome. I especially appreciated all of their advice and lunch time conversation.

I am grateful also to Dr. Montgomery for giving me the opportunity to do work for what was to become my first published paper. I learned a lot from the process of publishing and am thrilled that I could put my name on a paper so soon in my scientific career.

Next, I would like to thank Dr. Murre and Dr. Tour for serving on my thesis committee. I appreciate their time and guidance throughout the process of preparing this thesis.

Finally, I am ever grateful for the patience and encouragement of my family and friends during the duration of my studies. Their support was a constant source of strength.

Figures 4 and 5 contain a reprint of material that appears in the article “Inhibitors of Src and Focal Adhesion Kinase Promote Endocrine Specification: Impact on the Derivation of Beta-Cells from Human Pluripotent Stem Cells,” Afrikanova, Iva; Yebra, Mayra; Simpkinson, Megan; Xu, Yang; Hayek, Alberto; and Montgomery, Anthony; Journal of Biological Chemistry, August 18, 2011. The Thesis writer was an author on this paper.

ABSTRACT OF THE THESIS

Novel improvements to the differentiation of human iPS and ES cells for the derivation of pancreatic beta cells

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Professor Alberto Hayek, Chair

Type 1 diabetes is caused by pancreatic β cell destruction due to autoimmunity. The prospect of utilizing hESC or hiPSC derived β cells as cell replacement therapy is a promising potential cure, but proof of principle in animal models has yet to be achieved. In this study, we sought to improve the D'Amour et al., 2006 protocol for deriving β cells from hESCs and hiPSCs by targeting the duration of the developmental stages in hiPSCs, testing small molecules for their effect on the generation of pancreatic hormone progenitors in both hESCs and hiPSCs, and transplanting differentiated hiPSCs into immune-suppressed mice to evaluate their ability to

secrete insulin *in vivo*. We found that by optimizing the duration of the developmental stages of the protocol for the hiPSCs, we could generate higher levels of hormone-producing cells by the end of the protocol. We also found that the addition of the Src family kinase inhibitor, PP2, improved the generation of NGN3+ hormone progenitors and the eventual yield of hormone-producing cells. Though we were able to improve the *in vitro* yields of endocrine progenitors and the eventual yield of hormone-producing cells, after transplantation, the cells produced teratomas in about 50% of the mice that received them and no C-peptide release was found, up to four months after transplantation. While our studies have found novel ways to improve the differentiation protocol, further studies are needed to test methods for reducing teratoma formation and improve endocrine progenitor survival after transplantation.

I.

INTRODUCTION

In patients with type 1 diabetes mellitus (T1DM), autoimmunity destroys the insulin-producing β cells of the pancreas, leaving diabetic individuals dependent on injections of insulin to maintain normal blood glucose levels. Although the technologies and treatments for diabetes have improved greatly over time, the disease takes constant vigilance and even with careful monitoring diabetics may still encounter complications later in life. While patients can live with the disease, it often causes debilitating complications later in life, such as kidney failure, cardiovascular disease, retinopathy, and neuropathy, well characterized in many previous studies (Abbott et al., 2011; Kubin et al., 2011; and Brownlee et al., 1988). In addition, acute complications such as hypoglycemic episodes may endanger life to the patient himself and those around, such as in vehicular accidents. With so much at stake for people living with diabetes, it is a pressing necessity that the biomedical community finds a cure for this frustrating and destructive disease.

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) may hold the promise of a cure for the millions living every day with T1DM. The ability to culture and differentiate hESCs is still a fairly recent development; their isolation from the intercellular mass of the blastocyst of the early embryo was achieved first in 1998 by Jamie Thomson and colleages (Thomson et al., 1998). Prior studies of stem cells were limited to animal species, such as mouse ESCs, isolated for the first time in 1981 (Evans and Kauffman, 1981 and Martin, 1981). While hESCs hold the promise of pluripotency, the more recently developed hiPSC has the same potential as hESCs, as well as the added benefit of patient-specificity. The hiPSC, first derived from human somatic cells in 2007 by Shinya Yamanaka and in the same year by Jamie Thomson, is generated by the reprogramming of somatic cells with the addition of a handful of pluripotency transcription factors (Yamanaka et al., 2007). With the development of this technology, patient-specific hiPSCs can be generated and have the potential to become a source for cell-replacement therapy for the treatment of certain diseases,

like diabetes. T1DM is an excellent example of a disease that may be cured by stem cell derived replacement cell therapy because it is the loss of one cell type, the β cell, which causes disease onset. Theoretically, the disease could be cured by replacing the endogenous β cells with those generated by hiPSCs or hESCs. This is supported by the success of replacement islet cell transplant studies, which provide a replacement of β cells, though this type of replacement therapy is not a long-term solution due to immune rejection and a lack of sustainability since they are from cadavers (Shapiro et al., 2000 and Langer, 2010).

Therefore, much of the search for a cure for diabetes has been focused on the development of replacement insulin-producing beta cells from hESCs, for transplant into diabetics. Stem cell biologists have been working to derive glucose-responsive insulin-producing β cells from hESCs and have successfully derived insulin-producing cells (D'Amour et al., 2006; Zhang et al., 2009). There has also been some ability to generate glucose-responsiveness in the cells once they are transplanted into immune-suppressed mice that have been induced to a diabetic state (Kroon et al., 2008).

However, current protocols for deriving β cells from hESCs and hiPSCs are not yet perfected. Two issues of the current protocols are a lack of ability to generate high yields of pancreatic hormone producing cell progenitors and a lack of knowledge about hESC vs. hiPSC differences during differentiation. In the following studies, we have attempted to address both of these issues with the over-arching goal of improving the yield of pancreatic endocrine precursors and in turn, β cells generated during differentiation.

Generating insulin-producing β cells at a high efficiency from hiPSCs was one goal of our research. Currently, there is some published data on hiPSCs being used to generate insulin-producing cells, but the efficiency of the derivations is not as high as in hESC derivations (Zhang et al., 2009). Also, successful transplantation of hiPSC-derived β cells into mice with

the effect of regulating blood glucose levels upon glucose challenge has not been achieved yet. Because of the similarities of hESCs and hiPSCs, it is postulated that hiPSCs can be differentiated into insulin-producing cells at an efficiency equal to that of hESCs. Our research focused partially on optimizing the protocols designed to generate insulin-producing cells from hiPSCs.

This work is grounded in the protocols that my lab uses to differentiate hESCs into insulin-producing β cells, which is a modified form of the protocol first published by D'Amour and colleagues (D'Amour et al., 2006). This protocol induces the cell to differentiate by mimicking what is known about the natural development of pancreatic β cells in the embryo. It utilizes several growth factors and chemicals to guide the cell first into becoming definitive endoderm, primitive gut tube, then into posterior foregut tissue, next into pancreatic endoderm and endocrine precursors, and finally into pancreatic hormone-producing cells, as seen in figure 1 (D'Amour et al., 2006). While there are a few protocols for generating insulin-producing cells, this protocol has been the most effective in our results, with modifications to a couple of the stages.

The effectiveness of the protocols is evaluated by looking at the transcription factors and proteins expressed at each of the stages of the development of the cells by using QPCR and at the end of the protocol by fluorescense-activated cell sorting (FACS). Immunofluorescent staining of the cells at different developmental stages is also used to visualize the expression in the cells of the hormones or transcription factors of interest. Important transcription factors that are seen in pancreatic and β cell development are well mapped already, so looking for them was simply a matter of selecting those of interest (Habener et al., 2005).

The two most important transcription factors that were sought in my analyses are *Ngn3*, which is expressed in cells that are going to become hormone-producing pancreatic tissue, and

Pdx1, which is expressed in β cells, as well as at an earlier stage in development that indicates that the cells are pancreatic progenitors (Habener et al., 2005).

Optimizing the D'Amour protocol for use in differentiating the hiPSCs has been the initial step towards the goal of having a high enough amount of insulin-producing cells at the end of the protocol to be transplanted into mice. To optimize the protocol for hiPSCs, experiments with the timing of the different developmental stages, by keeping the cells in certain stages for different amounts of time, as well as looking for new small molecules to add to the protocol to increase the rate of efficiency, were performed. The addition of small molecules could improve the efficiency of differentiation because pancreatic development is not yet fully understood and further manipulation of signaling pathways may hold the key to increased yields of β cells.

While the established protocol attempts to mimic embryonic development, it has several bottlenecks, which detract from its efficiency. One such bottleneck that I worked on improving was that of the production of pancreatic hormone-producing cell progenitors. It has been shown that cells expressing the transcription factor NGN3 are progenitors of the hormone producing cells of the pancreas (Jensen et al., 2000; Schwitzgebel et al., 2000; Gu et al. 2002). Improving the yield of *Ngn3*-expressing cells directly improves the yield of β cells, so finding a chemical that could improve the derivation of these *Ngn3*-expressing progenitors is of great interest.

Some small compound inhibitors have been tested and seen to improve the differentiation of β cells, such as inhibitors of phosphatidylinositol 3-kinase (PI3K) (Zhang et al., 2009; McLean et al., 2007, Ptasznik et al., 1997). However, many other signaling pathway contributions to pancreatic endocrine development have yet to be explored. One such pathway is that of Src family kinases (SFKs). Since it has been implicated in the development of other types of cells, such as cardiomyocites and chondrocytes, we chose to investigate the use of the

SFK inhibitor, PP2, by introducing it to the differentiation protocol (Pala et al., 2008 and Hakuno et al., 2005).

By working to optimize the ability to generate insulin-producing cells, this study seeks to find whether or not hESCs and hiPSCs can fulfill their promising outlook, and ultimately, whether or not they have the potential to be the cure diabetics so desperately need.

II.

RESULTS

Altering the duration of the developmental stages improves hiPSC differentiation

Since little is known about how hiPSCs differentiate compared to hESCs, we began investigating the differentiation of hiPSCs by adjusting the lengths of time that the cells were kept in each stage of the D'Amour modified protocol. The duration of the primitive gut tube (stage 2) and posterior foregut (stage 3) generating stages were altered systematically. It was found that modifying stage 2 so that it was only two days instead of 3 and expanding stage 3 by one day improved the levels of expression of *Ngn3* and *Pdx1* over the course of the later stages of the protocol (figure 3a and b), and the expression of *Ins*(figure 3c), *Gcg* (figure 3b), and *Sst* (figure 3c) at the end of the protocol. This was confirmed in repeated experiments on multiple hiPSC lines (not shown).

Staining for PDX1 and insulin, which are both present in the mature β cell, confirms the expression of these proteins at the end of the protocol in both 38HI and 2I21 hiPSC lines (figure 2a and b). The staining shows that insulin is expressed in PDX1 expressing cells, but that there are some PDX1 expressing cells that do not express insulin. These may be immature β cells, as mature β cells are known to express both PDX1 and Insulin.

Addition of the small molecule inhibitor PP2 improves hESC and hiPSC differentiation

The addition of the Src kinase inhibitor, PP2, improves the differentiation of both hESCs and hiPSCs. PP2 added during the first 48 hours of the posterior foregut stage (stage 3) at an optimized concentration improves *Ngn3* mRNA levels in three hESC cell lines and all hiPSC lines tested (figure 4a-d). It also improves *Ins* expression and induction as a direct result of the higher *Ngn3* expression (figure 5a). In addition, the induction of Gcg and Sst is higher, as expected (figure 5b).

Immunofluorescent staining of the cells at the end of the protocol confirms that PP2 improves the expressed protein levels of Insulin and glucagon in hESCs (figure 5d) and higher expression of PDX1 and Insulin in hiPSCs (figure 5e). Staining at the end of Stage 3, during which levels of NGN3 are expressed as the pancreatic endocrine progenitors are formed, shows higher expression of NGN3 in PP2 treated cells (figure 4e).

FACS analyses also show levels of NGN3 positive cells increased by 6% at the end of Stage 3 (figure 4f), while levels of C-peptide positive cells increased by 16% at the end of the protocol with the addition of PP2 (figure 5c).

Transplantation of hiPSCs after differentiation to posterior foregut stage is ineffective

An hiPSC line derived from 38H1 and known as Chip2, was tested for its ability to differentiate successfully and was then used for transplantation experiments in immune-suppressed mice.

The cells were found to have the ability to successfully differentiate under the hiPSC version of the modified D'Amour protocol, as confirmed by their mRNA levels of *Pdx1* and *Ngn3* after stage 3, and the levels of *Ins*, *Gcg*, and *Sst* mRNA expression after the end of the protocol (not shown). The expression of these proteins was confirmed by immunofluorescent staining that was performed on the cells at the end of stages 3 and 5 (figure 6a and b).

After confirming their ability to differentiate, more cells were put through the protocol until the end of stage 3, when they were transplanted into mice to evaluate whether or not they were able to finish developing into hormone producing cells in vivo, as their hESC counterparts have been previously shown to do (Kroon et al., 2008). Samples of cells from the batch that were being transplanted were evaluated for their levels of mRNA expression for both *Ngn3* and

Pdx1 (figure 7a and b). Their levels were confirmed to be the same level as previous differentiations.

Levels of the pluripotency transcription factors Oct4 and Nanog were also tested to see that their levels were lower than in Day 0 samples (figure 7c and d). These results showed that the majority of the cells were no longer expressing these transcription factors at significant levels, indicating that they may have lost their pluripotency.

With these results, it was established that the cells were differentiated to the posterior foregut stage, were expressing pancreatic endocrine progenitor transcription factors, and they could be transplanted. The mice were monitored after transplant, and up to four months after when they were sacrificed, they appeared healthy. They were tested for circulating levels of human C-peptide after glucose stimulation every six weeks after receiving the cells, but significant circulating C-peptide levels were not found in any of the mice that had received the iPS-generated cells (data not shown). Upon sacrifice and dissection at four months post transplant, large, hard teratomas (figure 8a) were found in the vicinity of the transplant site in 50% of the mice. The teratomas were found to have a multitude of tissues upon histological analysis (figure 8b-d). One was found to be a cancerous fibrosarcoma (figure 8a). Even those mice without teratomas did not have C-peptide release.

Figures 4 and 5 contain a reprint of material that appears in the article "Inhibitors of Src and Focal Adhesion Kinase Promote Endocrine Specification: Impact on the Derivation of Beta-Cells from Human Pluripotent Stem Cells," Afrikanova, Iva; Yebra, Mayra; Simpkinson, Megan; Xu, Yang; Hayek, Alberto; and Montgomery, Anthony; Journal of Biological Chemistry, August 18, 2011. The Thesis writer was an author on this paper.

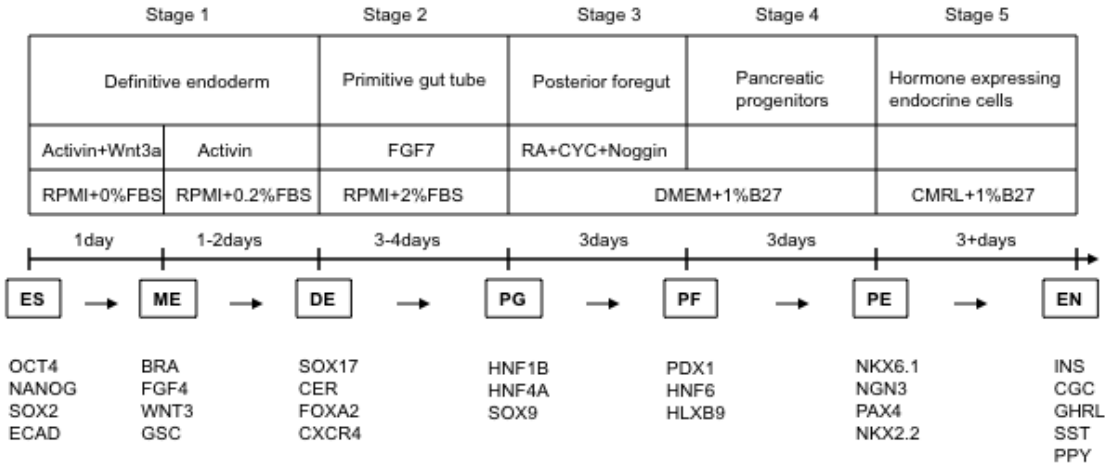

Figure 1. The modified D'Amour protocol for hESC differentiation.

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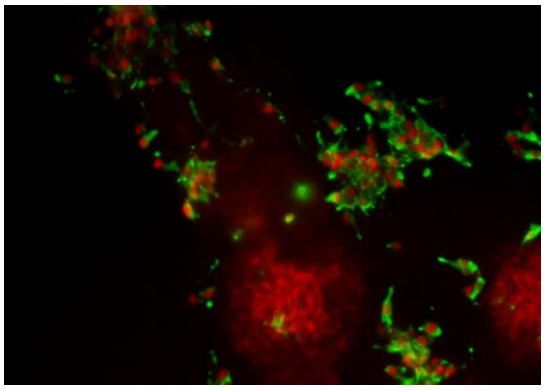

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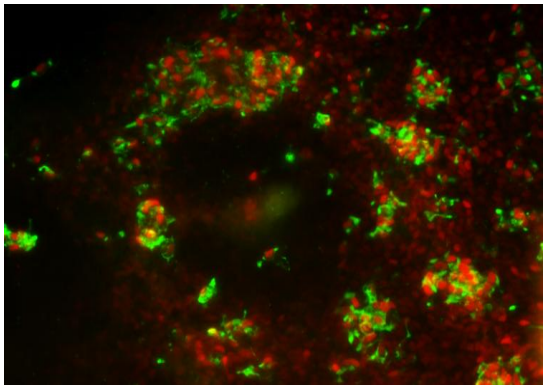
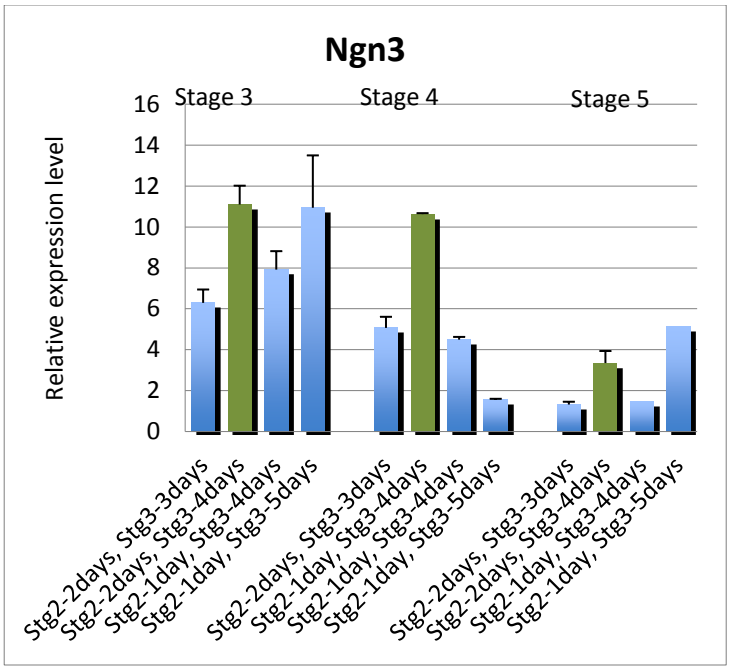


Figure 2. Immunofluorescent staining for PDX1 and C-peptide

Immunofluorescent staining for PDX1 (red) and C-peptide (green) at 20x magnification in **a.)** 38HI- and **b.)** 2I21-derived cells at the end of the modified D'Amour protocol.

a.



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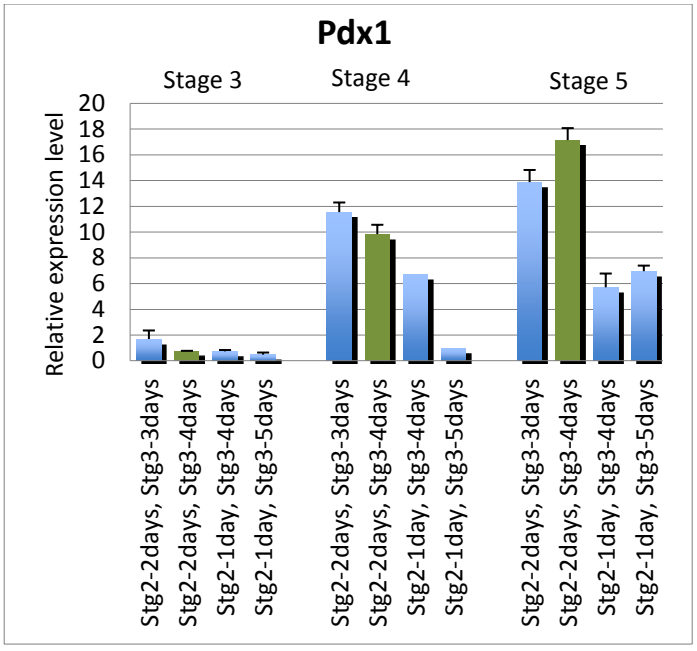
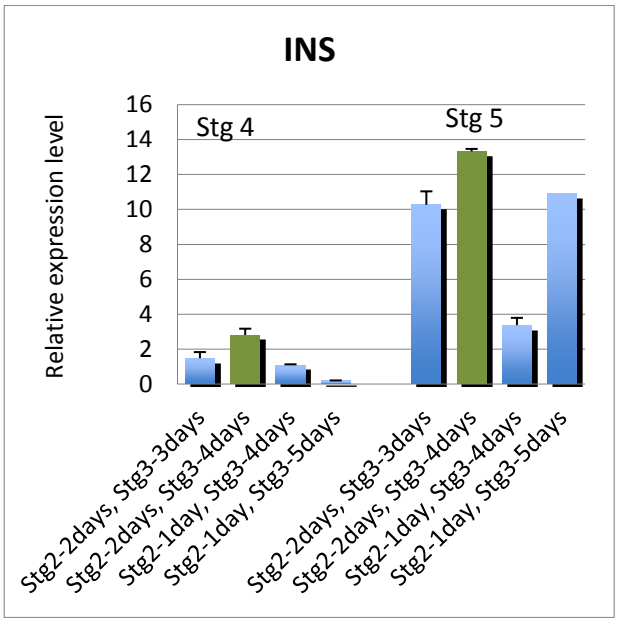


Figure 3. mRNA expression of various developmental transcription factors. Error bars represent standard deviation between averages of the quantity of mRNA expressed between duplicate wells. The relative levels of **a.)** *Ngn3* and **b.)** *Pdx1* are compared over the course of the differentiation by measuring mRNA expression in samples taken at the end of stages 3, 4, and 5 of the modified D'Amour protocol. The levels of **c.)** *Ins*, **d.)** *Gcg*, and **e.)** *Sst* are compared between stages 4 and 5. The amount of time that the cells were kept in stages 2 and 3 is indicated on the x-axis of each figure.

c.



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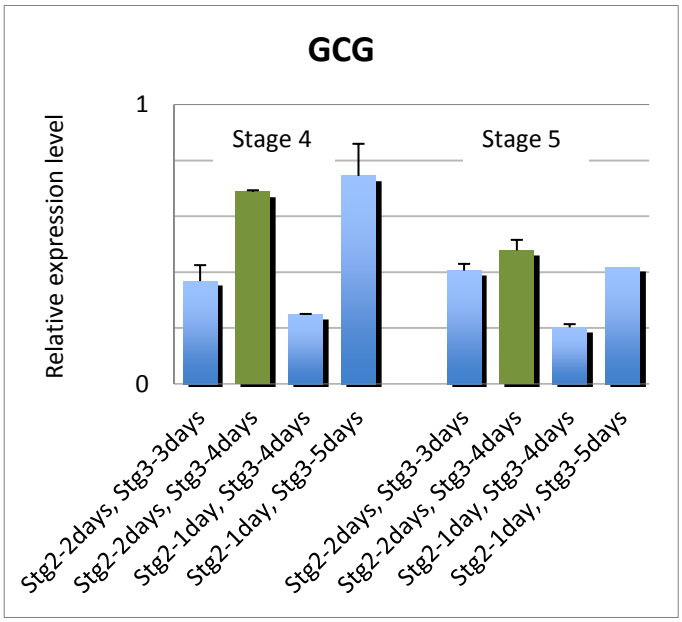

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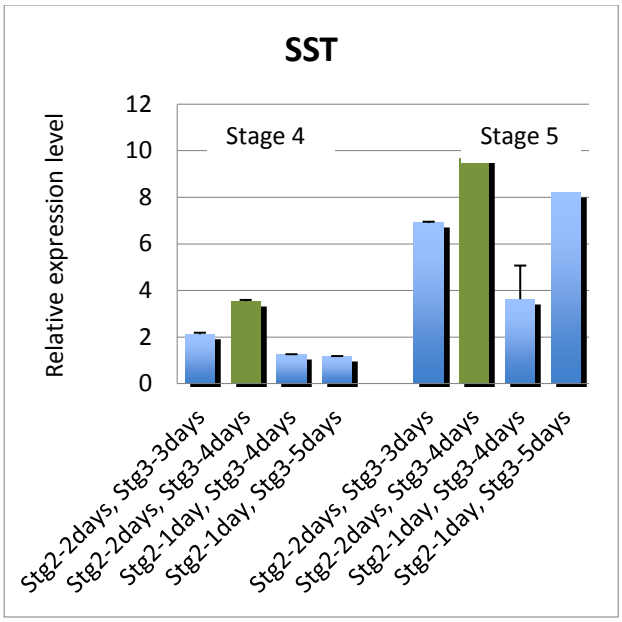

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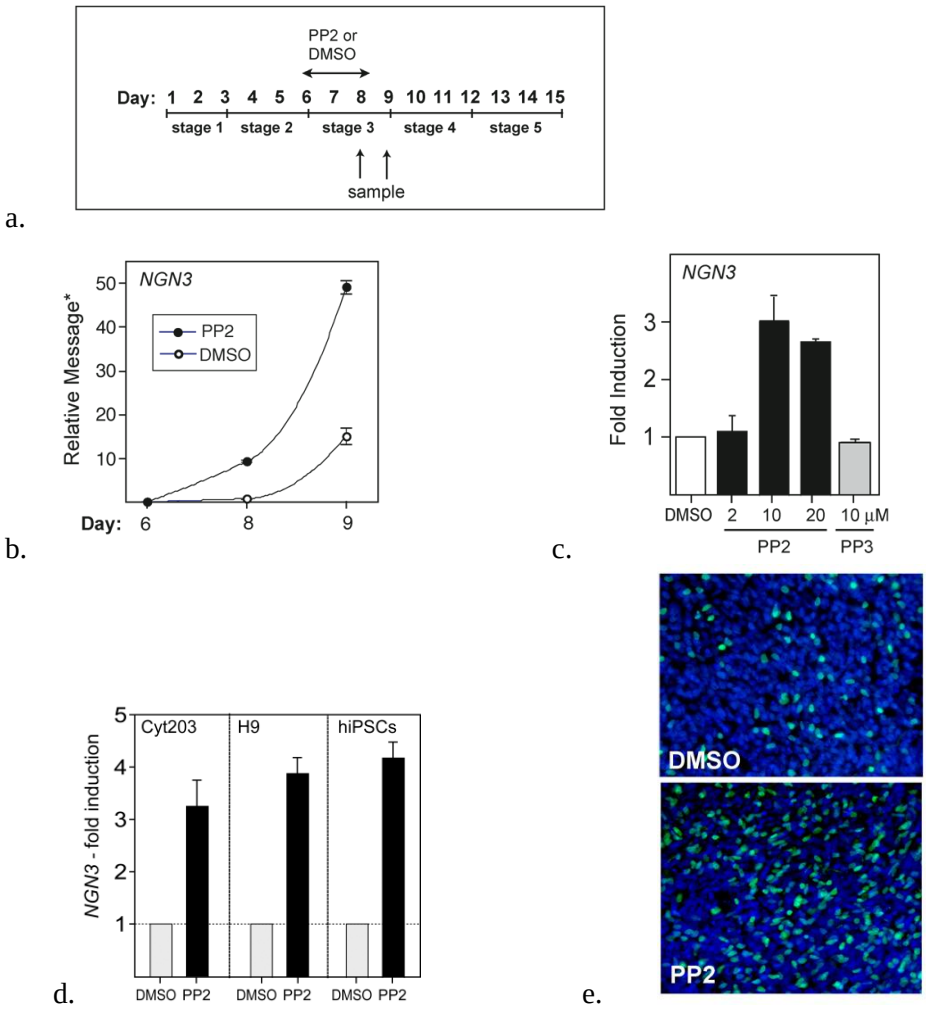
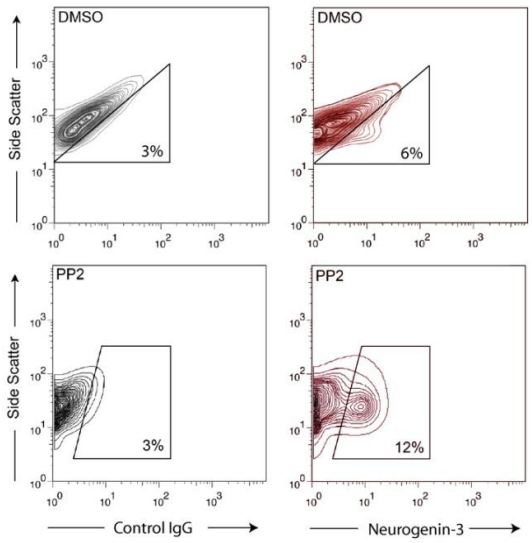


Figure 4. PP2 induces *Ngn3* expression and NGN3+ cells. a.) PP2 is added during the first two days of stage 3. Samples are taken on days 6, 8, and 9 of the protocol to see **b.)** the relative mRNA message of *Ngn3* expression. **c.)** The concentration of PP2 was optimized and found to be 10 μM. Also, PP2's structural analog, but inactive form, PP3, was found to have no effect on induction of *Ngn3*. **d.)** PP2 also increased the induction of *Ngn3* in Cyt203 and H9 hESC lines, as well as the hiPSC lines tested. **e.)** Immunofluorescent staining shows an increase in NGN3 expressing cells in green, with Dapi staining in blue. **f.)** FACS shows a 6% increase in the number of NGN3 positive cells between control DMSO and PP2 treated cells.



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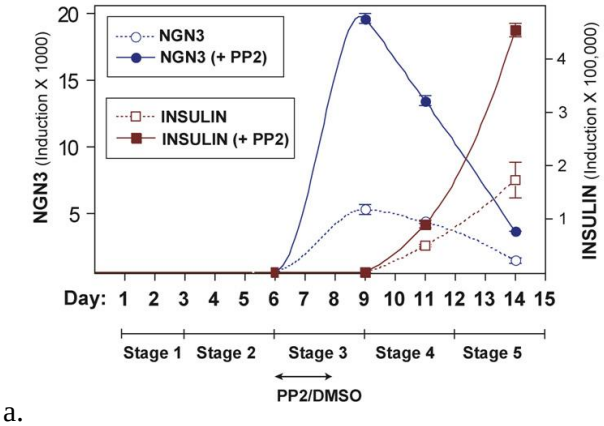

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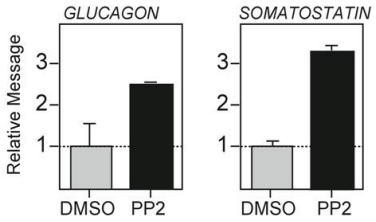

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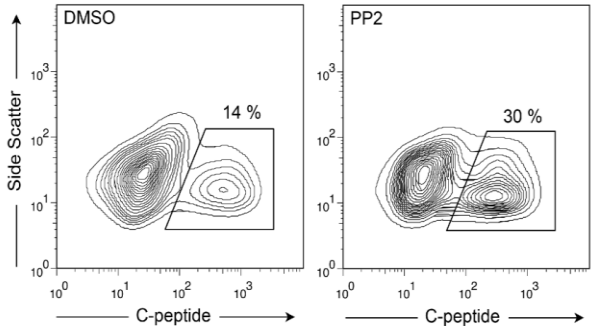

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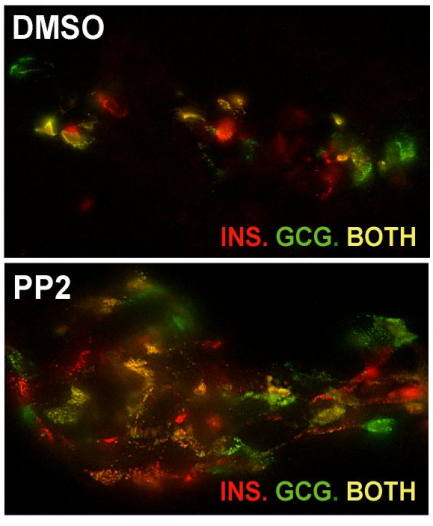

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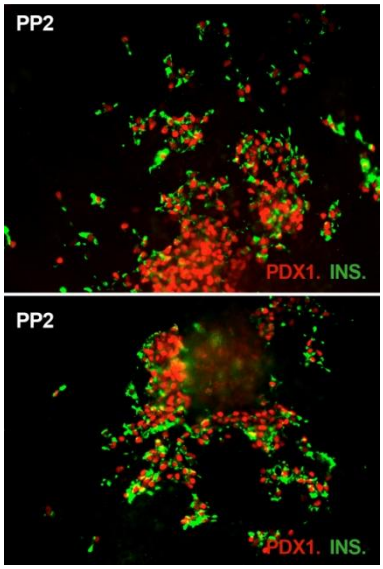
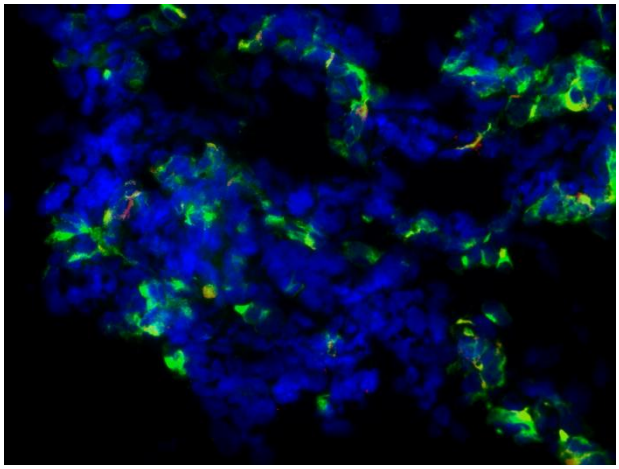


e.

Figure 5. PP2 increases the expression of insulin, glucagon, and somatostatin. a.) A time course of insulin and *Ngn3* induction with PP2 or control DMSO treatment. **b.)** Glucagon and somatostatin expression is also increased in PP2 treated cultures. **c.)** FACS shows an increase in C-peptide positive cells of 16% by the end of the protocol with PP2 treatment. **d.)** Immunofluorescent staining for insulin (red) and glucagon (green) shows increased expression of both at the protein level with PP2 treatment compared with DMSO control. **e.)** Staining of 38H1 derived cells at the end of the protocol for PDX1 (red) and C-peptide (green).

a.



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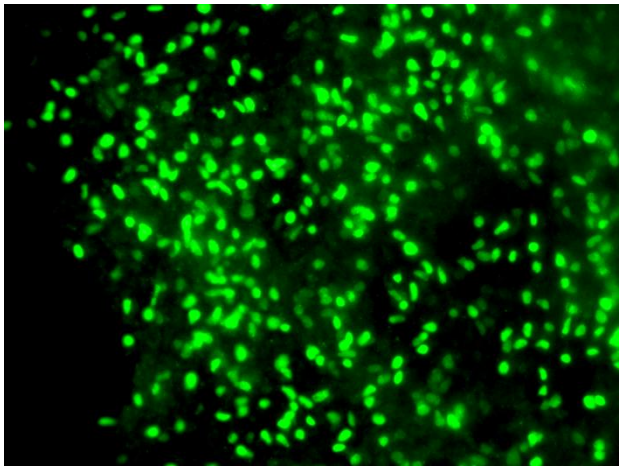


Figure 6. Immunofluorescent staining of Insulin, Somatostatin, and NGN3 in differentiated Chip2 cells. a.) Insulin (green), somatostain (red), and Dapi (blue) in Chip 2 cells after stage 5 of the modified hiPSC version of the D'Amour protocol and **b.)** NGN3 staining in green after Stage 3 of the modified D'Amour protocol in the same line.

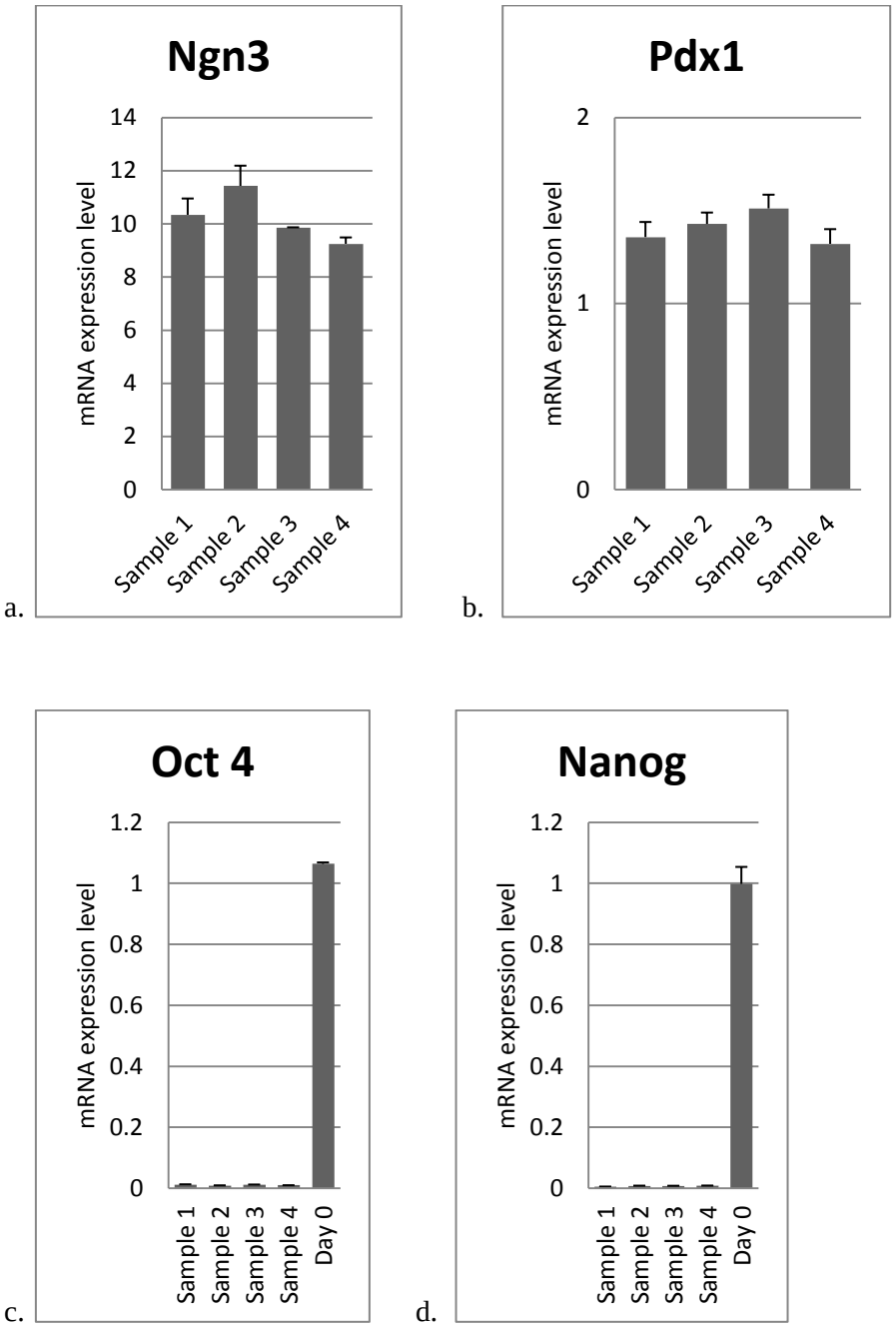
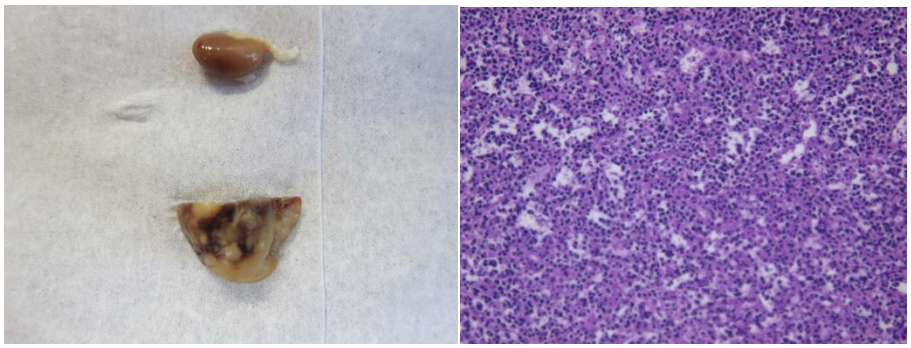


Figure 7. mRNA expression levels of *Ngn3*, *Pdx1*, *Oct4*, and *Nanog* prior to transplantation. a.) *Ngn3* and **b.)** *Pdx1* in stage 3 Chip2 cultures prior to transplant and comparison of **c.)** Oct4 and **d.)** Nanog mRNA expression in samples taken from Chip2 cells to be transplanted vs. day 0 control.

a.



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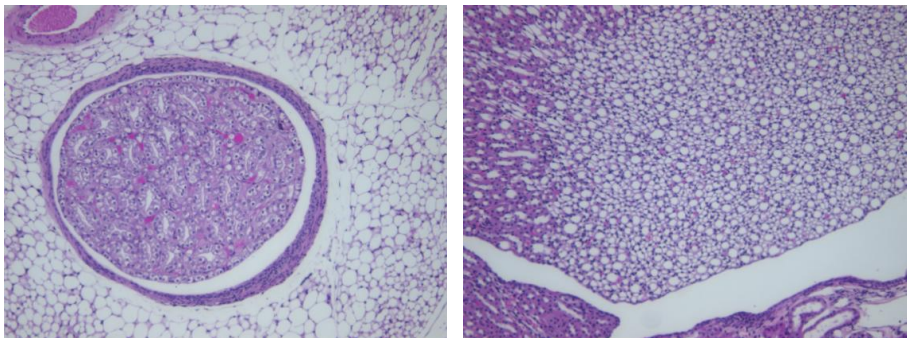
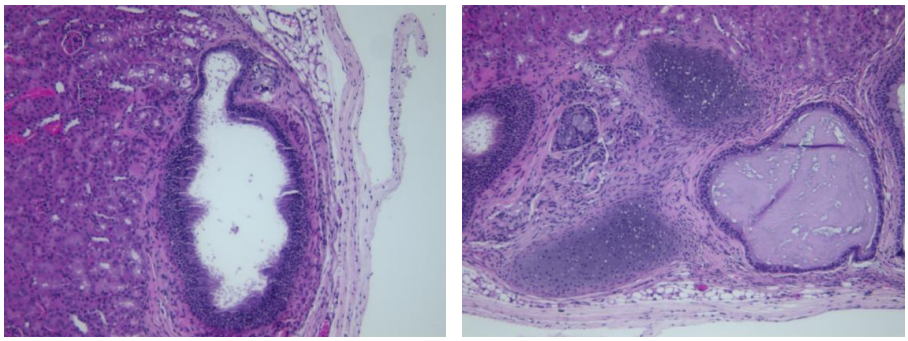


c.



d.

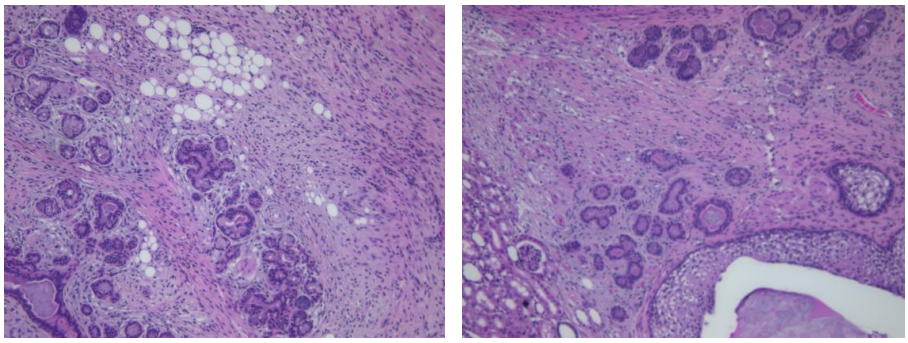


Figure 8. Teratomas from hiPS transplants. In **a.)** a teratoma can be seen positioned below the kidney of another mouse that received hiPS cells but did not grow a teratoma, and the histology of the teratoma, which was found to be mostly fibrosarcoma cells is also pictured. In addition, **b-d** show histology of three other teratomas. The teratomas were found to have tissues of **b.)** brain, intestine, cartilage, and respiratory track; **c.)** intestine, respiratory track, and cartilage; and **d.)** gastric, respiratory track, pancreas, ducts, skin, hair follicle, and esophagus.

IV.

DISCUSSION

In these studies, we found that altering the differentiation protocol for forming hormone producing pancreatic cells from human pluripotent stem cells by changing the timing of their developmental stages or adding in new small molecule compounds has significant effects on the efficiency of the protocol. This indicates a couple of things about the D'Amour protocol. First, it indicates that the protocol can be further improved by continuing to explore the signaling pathways that influence pancreatic development. Second, our studies of altering the protocol for hiPSCs show that there are differences between the hESCs and hiPSCs that need to be explored to continue optimizing the protocol.

As can be seen by the results of the hiPSC timing experiments (figures 2 and 3), altering the amount of time that the cells are exposed to the various growth factors made a significant difference in the amount of endocrine progenitors and hormone producing cells developed. Development *in utero* is a dynamic process that is difficult to recapitulate *in vitro*, especially when the origin of the pluripotent cells is as different as it is between hESCs and hiPSCs. Since little is known about the differentiation of hiPSCs in terms of β cell development, we began optimizing the protocol with these developmental kinetics experiments. We have not looked at optimizing the concentrations of the growth factors and small molecule inhibitors from the modified D'Amour protocol, and those tests might further yield improved differentiation efficiency, considering the impact that altering the timing of the developmental stages made.

In addition to addressing the differences in developmental rates for the hESCs and hiPSCs, our studies sought to improve the points in the D'Amour differentiation protocol that were not yet as efficient as they might become by the addition of small molecule inhibitors. It was found that the SFK inhibitor PP2 was able to significantly induce higher expression of endocrine precursors when it was added to the differentiation protocol during the stage in which

hormone progenitors are first formed (figure 4), and was therefore able to generate more hormone producing cells by the end of the protocol (figure 5). These results indicate that there are gaps in the knowledge of signaling during development, and that the exploration of these developmental pathways is necessary to recapitulate *in utero* pancreatic development. Here we uncovered just one way to modify the protocol with an inhibitor and were able to improve the amount of C-peptide positive cells at the end of the protocol by 16% in CyT49 cells (figure 5C). However, the final yield of C-peptide positive cells was only 30%, indicating that there are still further ways that the D'Amour protocol could be improved by the addition of other small molecule compounds. The mechanism by which PP2 acts on the cells to encourage endocrine precursor development is elucidated by other studies we have performed published in Afrikanova et al., 2011.

The eventual goal of this research is to be able to transplant the hESC or hiPSC derived endocrine progenitor cells into type 1 diabetics to act as β cell replacements. To accomplish this goal, successful transplants into animal models must first be achieved. While other studies have shown the ability to transplant hESC derived progenitor cells into mice, and have demonstrated those cells' ability to generate a somewhat controlled response to glucose, as β cells would, this has not yet been accomplished with cells derived from hiPSCs (Kroon et al., 2008). Also, we have not yet been able to replicate the results of the Kroon et al. paper, as the mice that receive the cells we derive from CyT49 hESCs grow large teratomas at the transplant site.

The attempts to transplant hiPSC derived cells have thus far resulted in no detection of C-peptide release up to four months later and about half of the mice that received the cells develop large teratomas (figure 8). The generation of teratomas that contain many different types of tissues indicates that at least a small percentage of the cells are still pluripotent upon transplantation, while the lack of C-peptide release could point to a few different issues with the

current methods. It is possible that the endocrine progenitors do not continue to develop into hormone producing cells once they have been transplanted into the mice, although this is what they have been shown to do in previous studies, such as the Kroon et al., 2008 study. Also, it is possible that the endocrine progenitor cells are damaged somehow during the process of dissociating them from the extra-cellular matrix and aggregating them prior to transplant. We have observed that the hiPSCs behave differently than the hESCs during dissociation and aggregation, and it may be that new techniques to better perform these processes need to be developed to prevent damage to the endocrine progenitor cells.

To address the problem of teratoma formation, a few different methods are being explored and may hold the solution. Two of these are the development of methods to remove cells with pluripotency cell surface markers, and the use of zinc finger nuclease technology to select specific cells prior to transplant (Kelly et al., 2011 and Moehle et al., 2007). Both of these methods would allow for more specificity when transplanting pluripotent cell derived cells, and may allow for the elimination of pluripotent cells from the cells that are selected for transplant, reducing the rate of teratoma formation.

While further studies are necessary to successfully accomplish transplantation of hESC and hiPSC derived pancreatic endocrine precursor cells for cell replacement therapy, our studies show that we are making strides *in vitro* to improving the efficiency of β cell derivation. There are problems to be overcome, but hESCs and hiPSCs hold the promise of potential cell replacement therapy for type 1 diabetics. With every incremental improvement to the differentiation of β cells, we are one step closer to better understanding β cell development, and diabetics are one step closer to a cure.

V.

MATERIALS AND METHODS

hiPSC and hESC culture and differentiation

Human hiPSC lines, 38HI, 2I21 and Chip2 were maintained in DMEM/F12 media supplemented with FGF2 (20ng/ml; R&D Systems, Minneapolis, MN), Activin A (10-20ng/ml; R&D Systems), KnockOut serum replacement (20% vol/vol; Invitrogen, La Jolla, CA), non-essential amino acids (1mM), 2-mercaptoethanol (0.55mM), Glutamax, and penicillin/streptomycin. Growth media was replaced daily and the hiPSC lines were grown in the presence of a monolayer of mitomycin-C-treated mouse embryonic fibroblasts (~$2x10_5$ cells/60mm plate). Cultures were passaged using TrypLE (Gibco) and were routinely split at a 1:6 -1:9 ratio every 5-7 days. They were cultured in the presence of ROC inhibitor (10 μM) for the first day after splitting.

Human ESC line CyT49 was maintained in the same media, but with 4ng/ml FGF2 and half the amount of mitomycin-C-treated mouse embryonic fibroblasts (~$1x10^5$ cells/60mm plate) in the monolayer. Cultures were mechanically passaged using a StemPro EZPassage passaging tool (Invitrogen) and were routinely split at a 1:6 -1:9 ratio every 5-7 days.

Differentiation was carried out essentially as described (D'Amour et al., 2006 and Kroon et al., 2008) with minor modifications. Media conditions used, and the duration of individual differentiation steps, are shown in Fig. 1. Basal media used included RPMI (Mediatech, Manassas, VA), DMEM high glucose (Invitrogen) and CMRL 1066 (Invitrogen). These media were supplemented with Glutamax and with either FBS (0.2-2%; HyClone, Lakewood, NJ) or B27 supplement (1% vol/vol; Invitrogen). Human recombinant activin A (100ng/ml), mouse recombinant Wnt3a (25ng/ml), mouse recombinant Noggin (50ng/ml) and human recombinant KGF (50ng/ml) were all purchased from R&D Systems. KAAD-cyclopamine (0.25μM) was purchased from EMD Biosciences (Gibbstown, NJ) and *all-trans* retinoic acid (2μM) from Sigma (St. Louis, MO). Cultures were variously treated with PP2 (2-20μM; EMD Biosciences). Please note that ESC cultures were grown to a high cell density prior

to initiating differentiation and cultures were washed with PBS (Invitrogen) at the beginning of stages 1 and 2.

Real-time qPCR

Q-PCR analysis was performed on differentiated hiPSC and hESC cultures. Total RNA was extracted using an RNeasy Plus Mini Kit 50 (Qiagen, Valencia, CA) and cDNA was synthesized from 100-300ng of RNA using an iScript cDNA synthesis kit (Bio-Rad). cDNA reactions (20µl) were further diluted 1:5. PCR reactions (20µl total volume) were then run in duplicate using 3µl of cDNA, combined with TaqMan® Universal PCR Master Mix (#4324018, Applied Biosystems) and unlabeled PCR primers and TaqMan FAM™ dye-labeled probes, listed in the supplemental material of Afrikanova et al., 2011. RT-qPCR was performed using an Applied Biosystems StepOnePlus Real-Time PCR System machine. Results were analyzed by the standard curve method. Standard curves were generated using cDNA prepared from pooled 11-17 week human fetal ICCs (provided by University of Washington, Seattle, WA). Quantified values for each gene of interest were normalized to Cyclophilin A. Q-PCR results shown in this study are the average value of 2-5 repeat experiments (mean ±1SD).

Immunofluorescence

Differentiated hiPSC and hESC cultures, were fixed with 4% PFA and then treated for 45 minutes with PBS containing 0.2% TritonX-100 (Sigma) and 5% normal donkey serum (Jackson ImmunoResearch). The cells were subsequently washed and incubated with primary monoclonal antibodies (mAbs) or polyclonal antibodies (pAbs) to insulin (15µg/ml sheep pAb #PC059, Binding Site, UK), glucagon (15µg/ml mouse mAb, Clone K7961310, Sigma), NGN3 (5µg/ml sheep pAb #AF3444, R&D Systems), or to PDX1 (1:5000 goat pAb) (kindly provided by Dr. Christopher Wright, Vanderbilt University Medical Center, Nashville, TN). The sheep pAb to insulin was used in combination with the mouse mAb to glucagon. Cells were incubated with primary antibodies overnight at 4°C in PBS containing 0.1% Triton X-100 and 0.5%

normal donkey serum. Bound antibodies were subsequently detected using donkey anti-sheep Alexa Fluor 488 (1:500 #A11015, Molecular Probes-Invitrogen), donkey anti-goat Rhodamine RedTM-X (1:500; Jackson Immnoresearch), donkey anti-rabbit Alexa Fluor 488 or 568 (1:500 #A21206/10042, Molecular Probes-Invitrogen) or donkey anti-mouse Alexa Fluor 488 or 568 (1:500 # A21202/A10037, Molecular Probes-Invitrogen). Secondary immunoconjugates were used alone or in combination as required and staining was performed for 90 minutes at room temperature.

Flow cytometry

Differentiated hESC cultures were harvested as single cells using 0.25% Trypsin/EDTA (Invitrogen) and were permeablized and fixed using IntraPrep permeabilization reagent kit (#PN IM2388, Beckman Coulter) according to the manufacturer's instructions. After fixation and permeabilization, the cells were blocked with 5% normal donkey serum in the presence of the permabilization reagent, for 20 min at room temperature. Cells were subsequently stained with antibodies to NGN3 (5µg/ml sheep pAb; #AF3444, R&D Systems), human C-peptide (10µg/ml mouse mAb Clone C-PEP-01, AbB Serotec), insulin (15µg/ml sheep pAb, #PC059, Binding Site), or glucagon (12µg/ml mouse mAb, Clone K7961310, Sigma). Incubations were performed for 1-2 hours at room temperature. For detection the cells were incubated with donkey anti-sheep Alexa Fluor 488 (1:500 #A11015, Molecular Probes-Invitrogen) or donkey anti-mouse Alexa Fluor 488 (1:500 #A21202, Molecular Probes-Invitrogen) for 1 hour. Washing and incubation steps were performed using PBS containing 0.5% BSA. Cells were analyzed on a Becton Dickinson FACScan using CellQuest v. (3.3) software.

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