Regulation of Sonic hedgehog expression and activity during differentiation of human pluripotent stem cells

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Introduction

Diabetes mellitus has become a strikingly prevalent and deadly endocrine disease. Characterized by the inability of the pancreas to produce insulin and thus maintain blood glucose levels, diabetes has spurred current efforts in science to strive for better understanding and treatment of this ailment. There has been moderate success in the treatment of diabetes through the implantation of pancreatic islet cells of the Langerhans. However, the grave lack of islet donors does not make this a reliable or sustainable treatment option for those whose lives depend on the transplantation of islets. Thus, current efforts have begun to seek new sources for insulin-producing β-cells. Embryonic stem cells and induced pluripotent stem cells have been considered as potential sources for these transplantable cells.

Stem cells are known for their pluripotency, that is, their ability to develop, or differentiate, into more than one cell type while in an undifferentiated state. Stem cells can be derived from a variety of sources. Embryonic stem cells, for example, are primarily obtained from the inner cell mass of the early blastocyst. Recent developments have shown that normal cells, such as skin cells, can be stimulated to become pluripotent through the forced expression of certain genes. Such cells, induced to become pluripotent, are rightly called induced pluripotent stem cells. Pluripotent cells, with the potential to differentiate into the components of all germ layers—ectoderm, endoderm, and mesoderm—have been highly considered as a source for insulin-producing, transplantable β-cells. The application of pluripotent stem cells in this manner relies on the reproducibility and efficiency of differentiation. Many different procedures have been employed to stimulate stem cell differentiation directly into β-cells and as such, there is a lack of consistency and straightforwardness. Furthermore, the number of β-cells generated through these faulty procedures has been insubstantial and variable.

A new approach to β-cell generation calls for stem cell differentiation into definitive endoderm before differentiation into insulin-producing cells. This method resembles the developmental pathway of a normal fetus; more significant achievements have been made in the derivation of definitive endoderm from stem cells. The endoderm, a germ layer responsible for developing into gut organs such as the stomach, is also responsible for formation of the pancreas during early vertebrate development. Recently, a study with human embryonic stem cells established that the differentiation process into definitive endoderm cells can be induced up to 80% purity and that these cells can be coaxed toward development into pancreatic insulin-producing cells via *in vitro* by the use of certain pancreatic precursor factors (D'Amour et al., 2006). As this method is explored, it is important to consider the traits of a developing pancreas.

Realizing the importance of Sonic hedgehog (Shh) in endoderm development, as established by previous studies (Hebrok et al., 2000), was the prime motivation for further analysis. This project was an effort to better understand the role and regulatory ability of Shh, a protein responsible for vertebrate organ development, in the context of human

pluripotent stem cell differentiation into pancreatic precursor and insulin-producing β (beta) cells *in vitro*. The hypothesis for this study was as follows: Altering conditions during stem cell differentiation will induce sonic hedgehog expression in gut endoderm and increase expression of Shh target genes.

Materials and Methods

Overview

In this experiment, the degree of expression for the genes Shh, Gli-1, Patched (Ptc-1), PDX1, and insulin was measured for each of three different types of pluripotent stem cells. Each type, of the three used, was further divided into four groups, with each group of cells grown in a different set of conditions. The three types of cells used were: HSF6 human embryonic stem cells, H9 human embryonic stem cells, and induced pluripotent stem cells (iPS).

Growing Conditions

For all of the cell types used, the cells were organized into groups numbered 1 through 4. The cells in group 1 were grown in 20% fetal bovine serum (FBS) with no additional factors; any differentiation that occurred was unstimulated. The cells in group 2 were grown in 20% FBS and sequentially differentiated with the addition of Activin and BMP4 at day 0, the addition of Activin, BMP4, and anti-Sonic hedgehog (α-Shh) at day 3, the addition of EGF and heparan sulfate (HS) at day 9, and the addition of GDF-11, Betacellulin, and Exendin-4 at d15. The cells in group 3 were grown in 2% FBS with no additional factors to aid differentiation. The cells in group 4 were grown and sequentially differentiated in conditions identical to those in group 2 with the replacement of 20% FBS with 2% FBS and the replacement of BMP4 with Wnt3a. The three cell types, HSF6, H9, and iPS were divided identically and grown under the same conditions. Data was collected at d0, d3, d9, d15, and d21 for all cells. Thus, the cells were organized and grown according to the following diagram, for all cell types:

Group	% FBS	Differentiation
1	20%	unstimulated
2	20%	sequential
3	2%	unstimulated
4	2%	sequential

cDNA Synthesis

After it was isolated from the cells, RNA was reverse-transcribed in order to synthesize complimentary DNA (cDNA). This was necessary because the qPCR machine is only able to quantify gene expression from strands of DNA, not RNA. In order to synthesize cDNA, a series of directions must be followed to ensure that it is done accurately and effectively. First, a "master" mix composed of treated water, dNTP mix, and random hexamers was made. After each sample of RNA was added to its respective 0.2 ml PCR tube containing the master mix, the entire sample was incubated for 5 minutes at 65° C and then placed on ice for one minute. Next, a cDNA synthesis mix was made by the mixing of RT buffer, $MgCl_2$, DTT, RNaseOUT, and SuperScript III RT. 10µl of this mix was added to each RNA mixture, followed by a brief

centrifugation and incubation. The final step, in which 1µl of RNase H is added to each tube and then incubated, completes the cDNA synthesis process. The resulting cDNA could be stored at -20° C or immediately used for quantitative PCR.

Quantitative Polymerase Chain Reaction

To analyze levels of gene expression for each cell, its respective cDNA was used in the qPCR machine. However, a series of procedures must be followed before proper analysis can begin. First, a template for each qPCR plate must be setup. Refer to the following blank template:

GAPDH		Primer		GAPDH		Primer		GAPDH		Primer	
<	#1		>	<	#2		>	<	#3		>
<	Pos.		>	<	Neg.		>				

Master mix

6.25µl SYBR green
4.75µl RNase-free water x # of samples for each primer = total amount in each mix
0.5µl primer

Each box represents a well of the PCR plate; there are 96 in all. To set up the template, each sample of cDNA was designated four boxes. For example, the d0 cDNA sample for HSF6 cells would be placed in the range of four boxes labeled with "#1." The next sample, in this case, HSF6 d3, would occupy the next four wells, and so on until all the samples had four wells dedicated to it. Of the four wells for each sample, two would be analyzed for the gene, Gapdh while the other two would be analyzed for target gene. For this experiment, the target genes were Shh, Gli-1, Patched, PDX1, and insulin, replacing the columns labeled "Primer."

Each well, as outlined by the template, was filled with SYBR green, a dye that binds to DNA and allows it absorb blue light, RNase-free water, and a primer (either Gapdh, Shh, Gli-1, Patched, PDX1, insulin, or HFP). Added to this was 1µl of cDNA, also in accordance with the template.

After a 5-minute centrifugation at 1000rpm, the PCR plate could be either stored in a deli fridge or immediately used in the qPCR machine.

Positive and negative controls play a significant role in qPCR. The primer Gapdh serves as a control itself. Expressed by all cells in the body, regardless of size, type, or function, Gapdh serves as a positive control; it is virtually guaranteed to be expressed. Another positive control used in the qPCR process is the primer for human fetal pancreas, HFP. Added to the first four wells of the last row instead of Gapdh and the primer for the target gene, HFP serves as a positive control. RNase-free water serves as a negative control and is used in the second set of four wells in the last column. Since water is not expected to have any gene expression whatsoever, its use as a negative control is justified.

qPCR Data Analysis

The qPCR machine indirectly measures the cycle threshold, the number of cycles it takes to obtain relatively substantial expression, for each well in the PCR plate. The more cycles it takes, the less the gene is expressed. This data, collected in the form of a spreadsheet from the machine, can be further processed to

determine specific expression values as they relate to the harvest day.

In order, the following values are calculated: Gapdh Ct, Delta Ct, fold, average fold, and the standard deviation of the fold. The Gapdh Ct and Delta Ct values are irrelevant but are important in calculating the fold, average fold, and standard deviation. These values can be plotted to visually represent gene expression relative to d0.

Results

The data were obtained from the qPCR machine and processed further to render the following graphs. Error bars were determined from the standard deviation of fold value pairs.

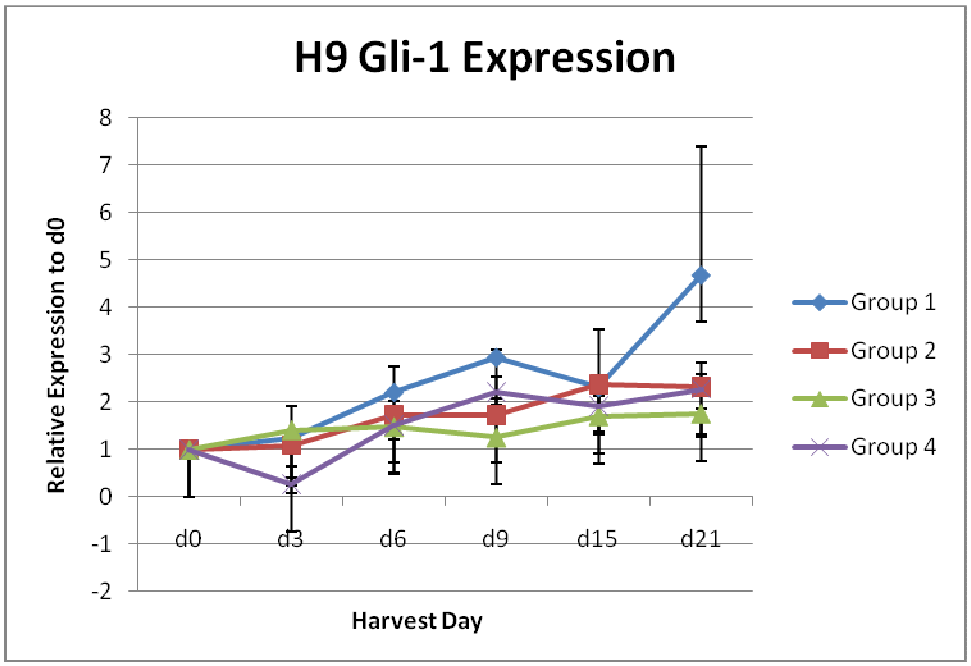


Figure 1

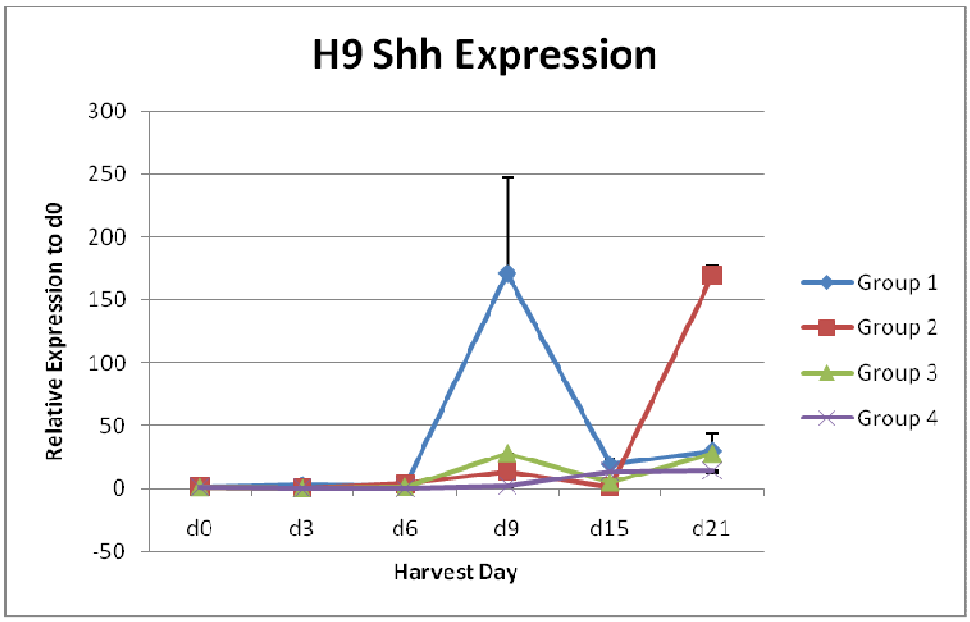
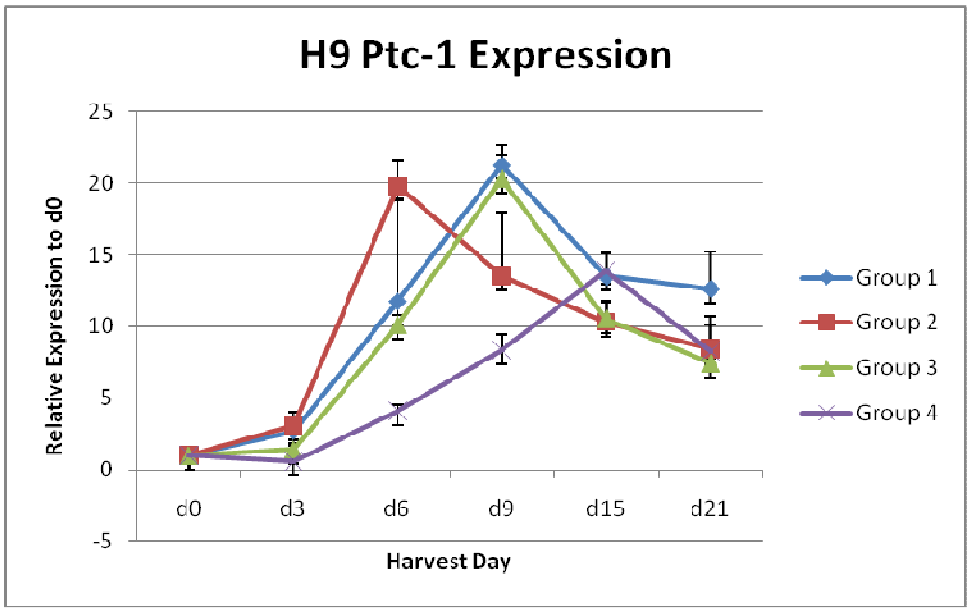


Figure 2



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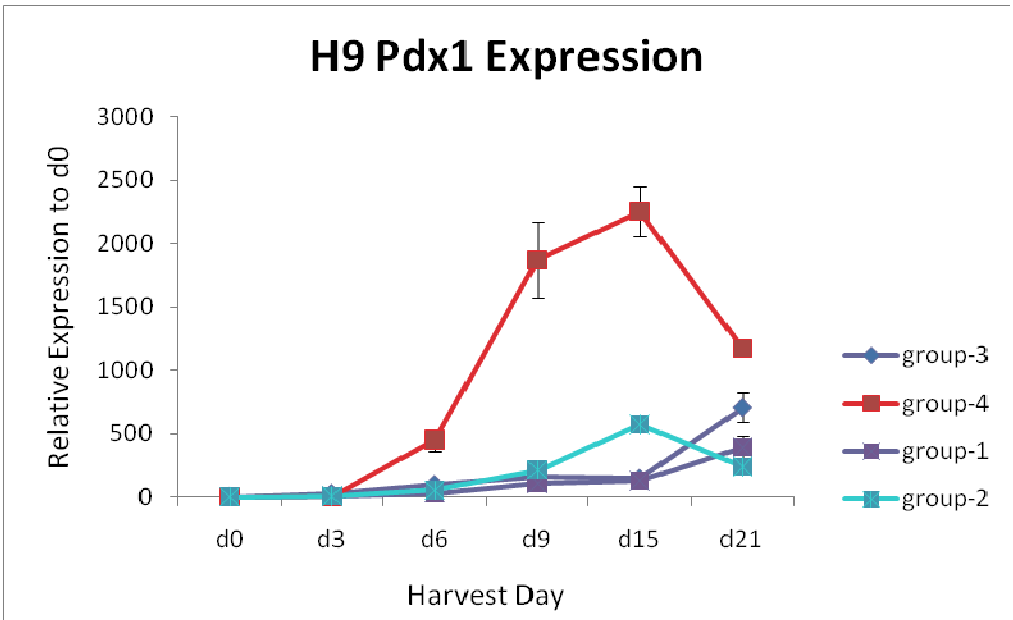
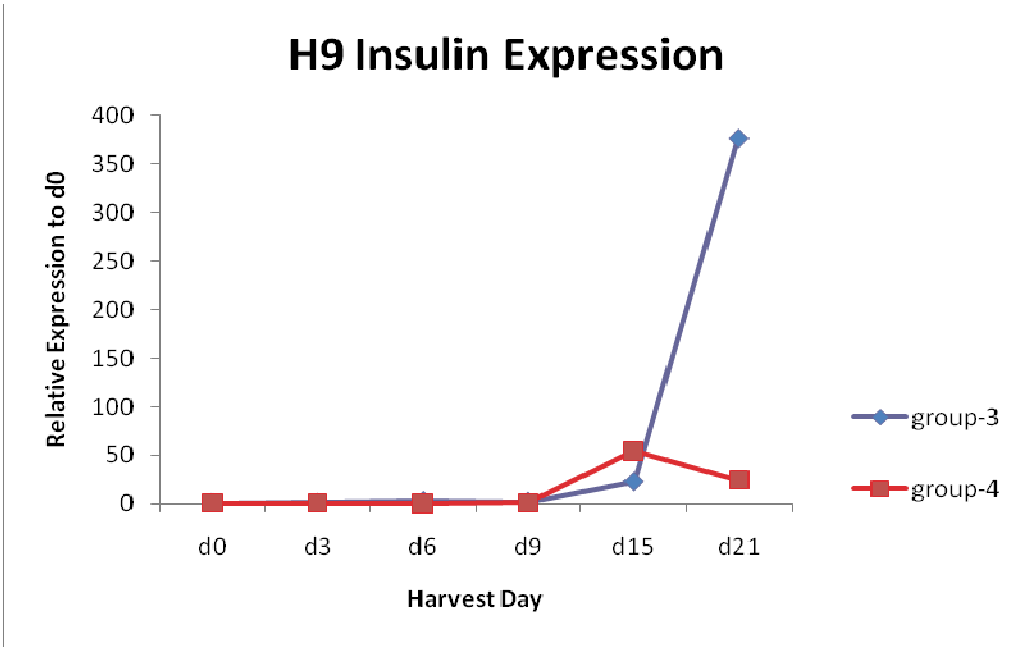


Figure 4



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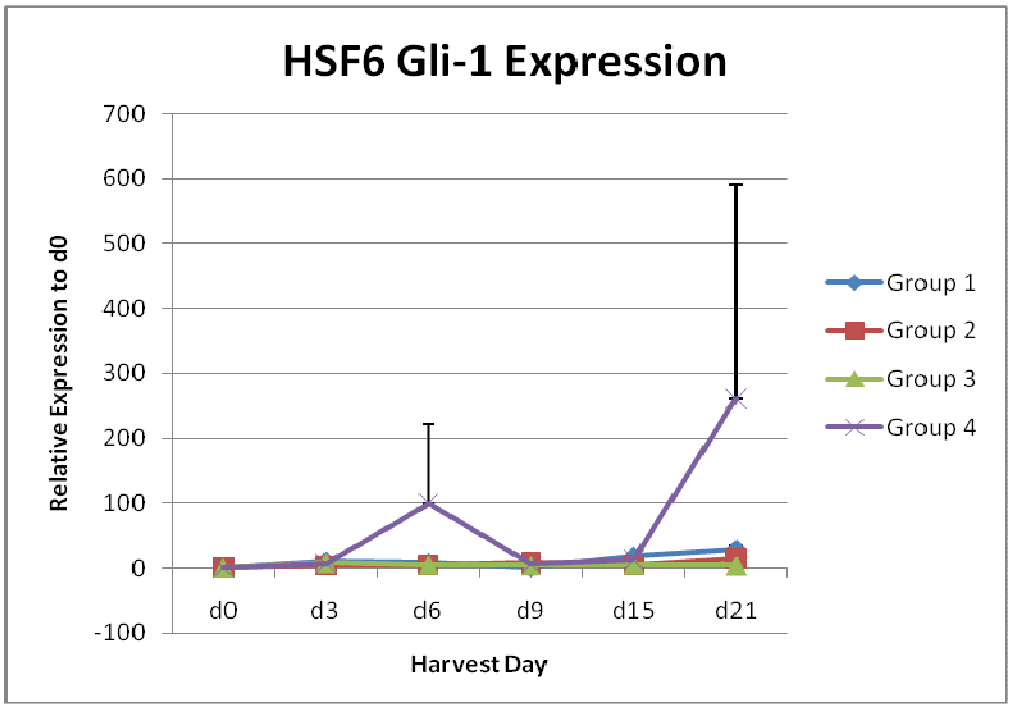

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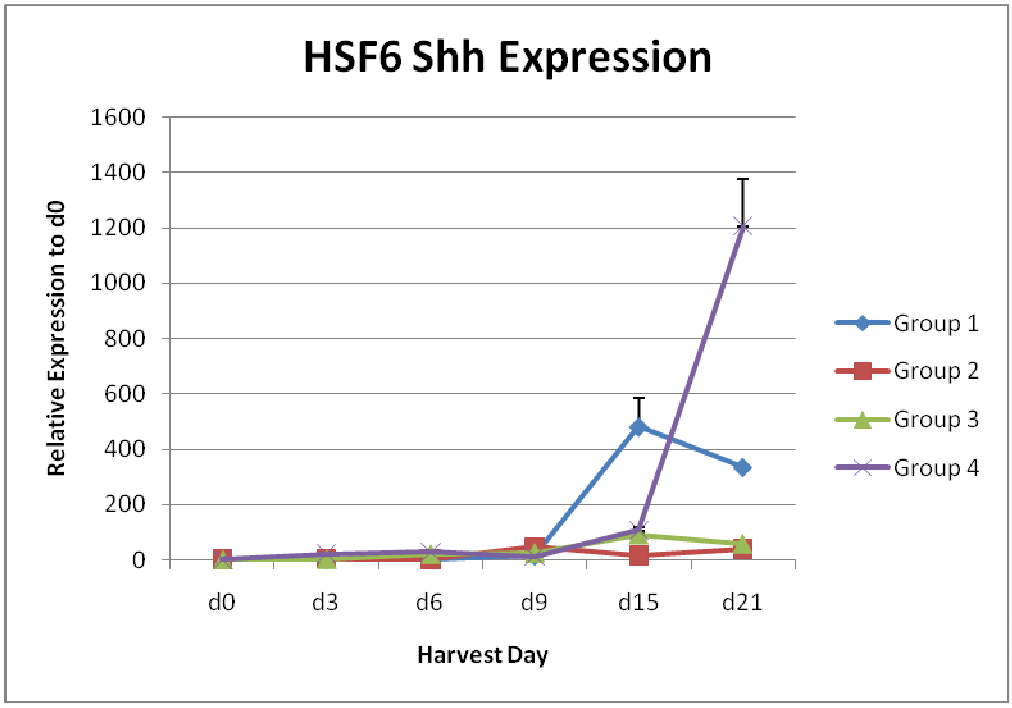

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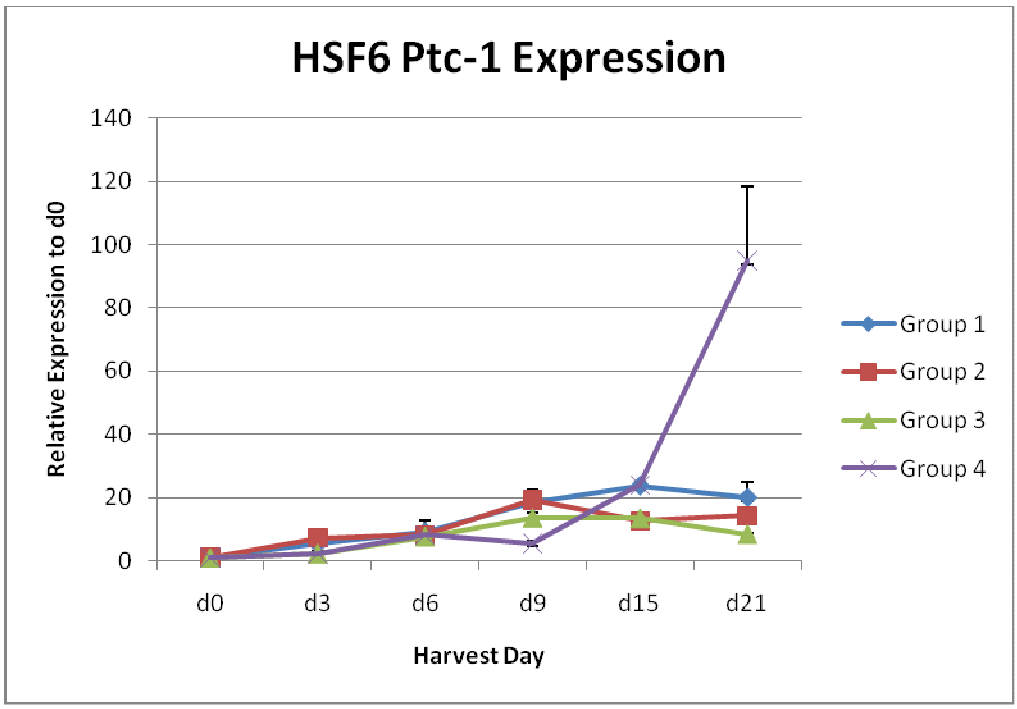

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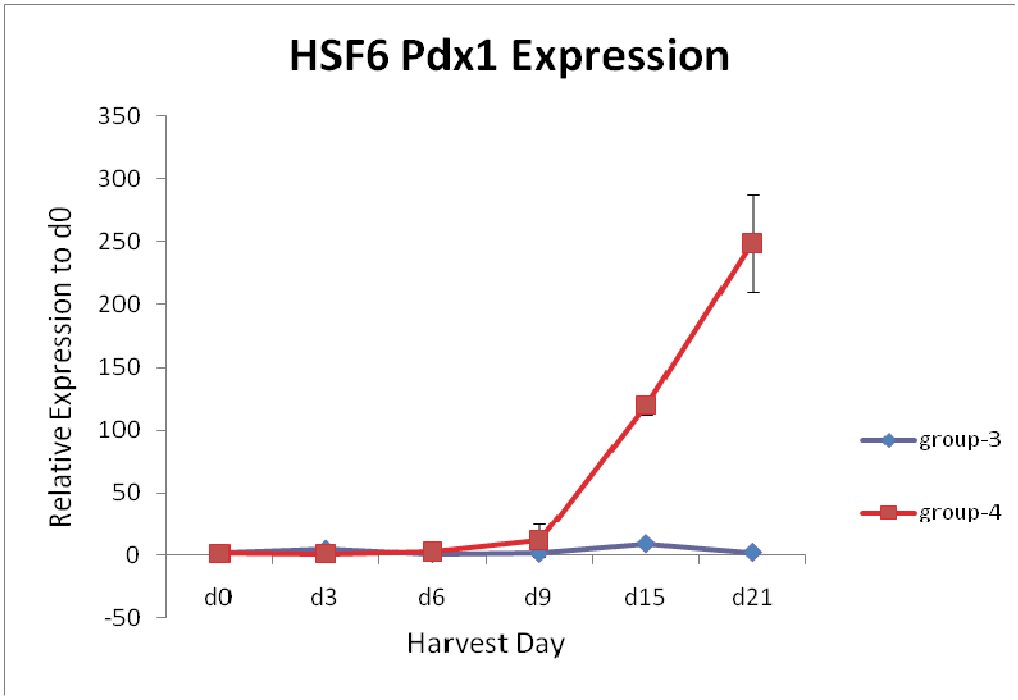


Figure 9

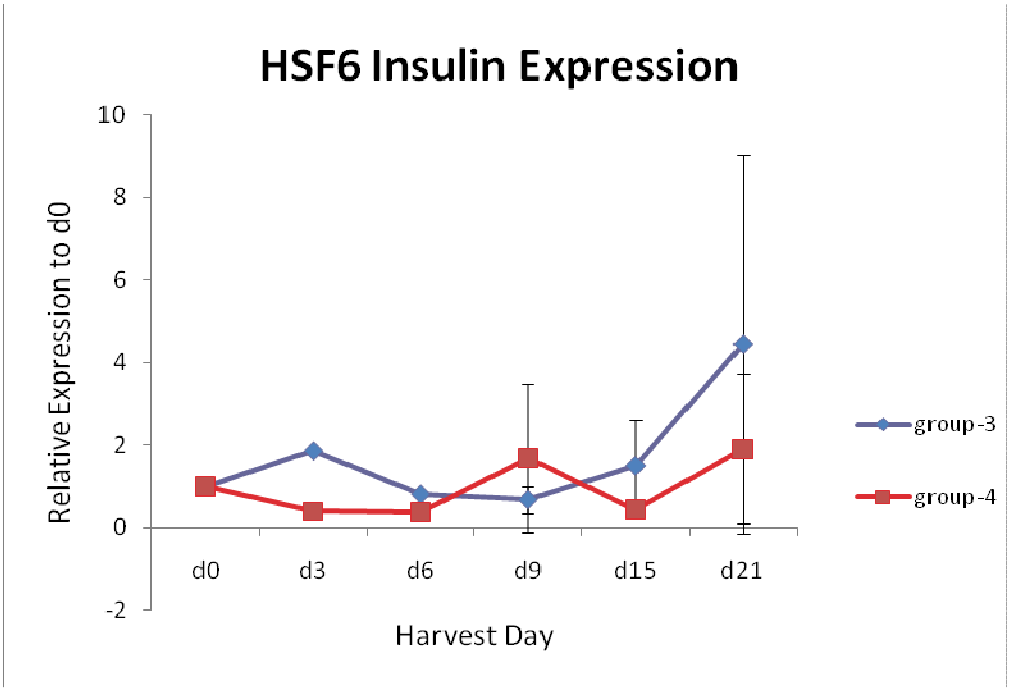


Figure 10

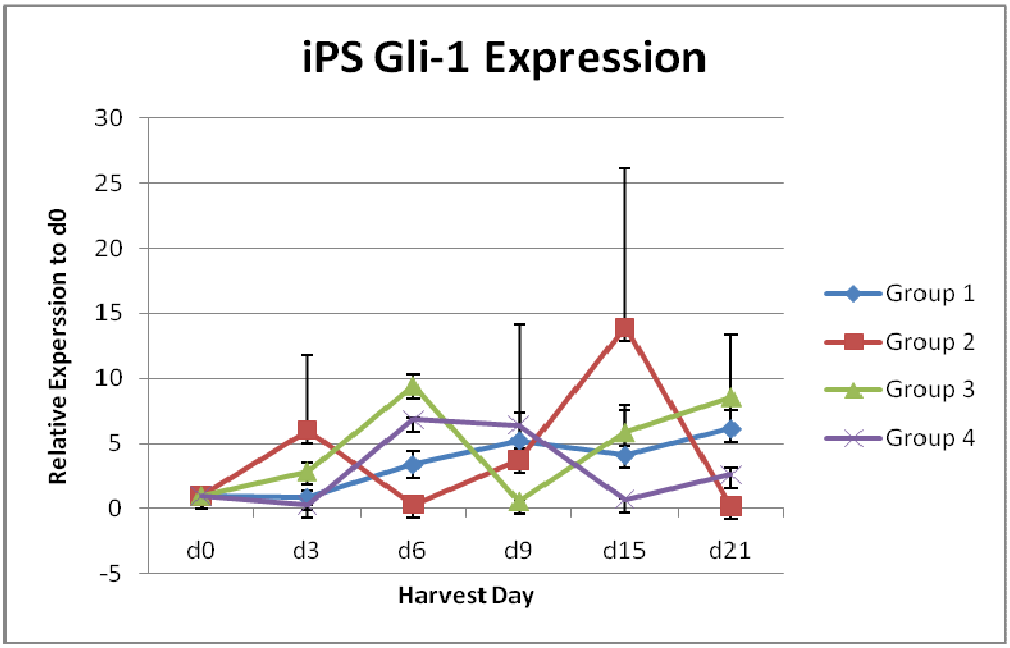
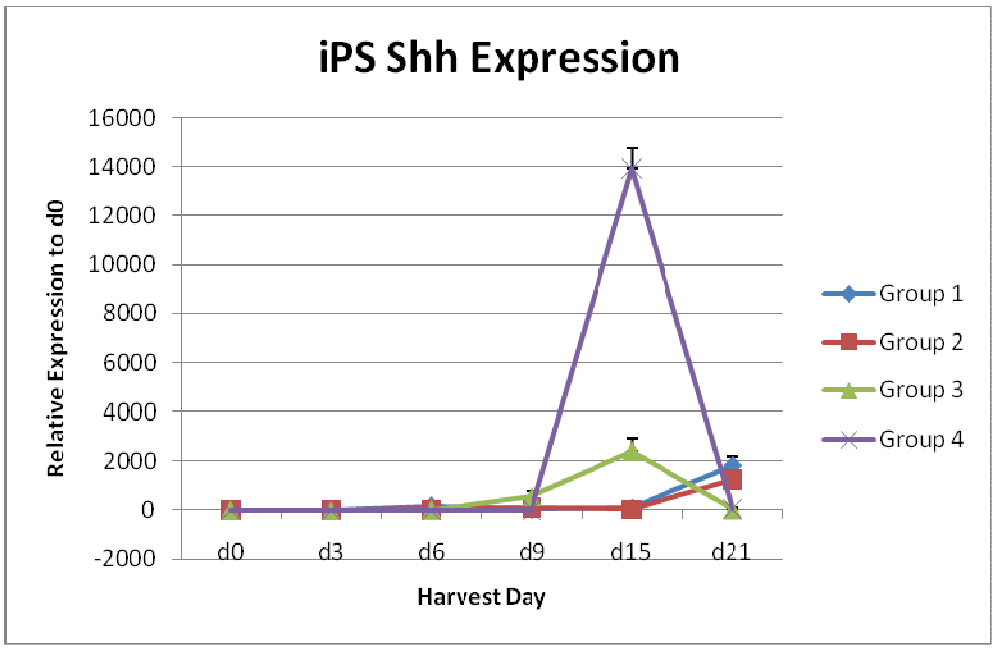


Figure 11



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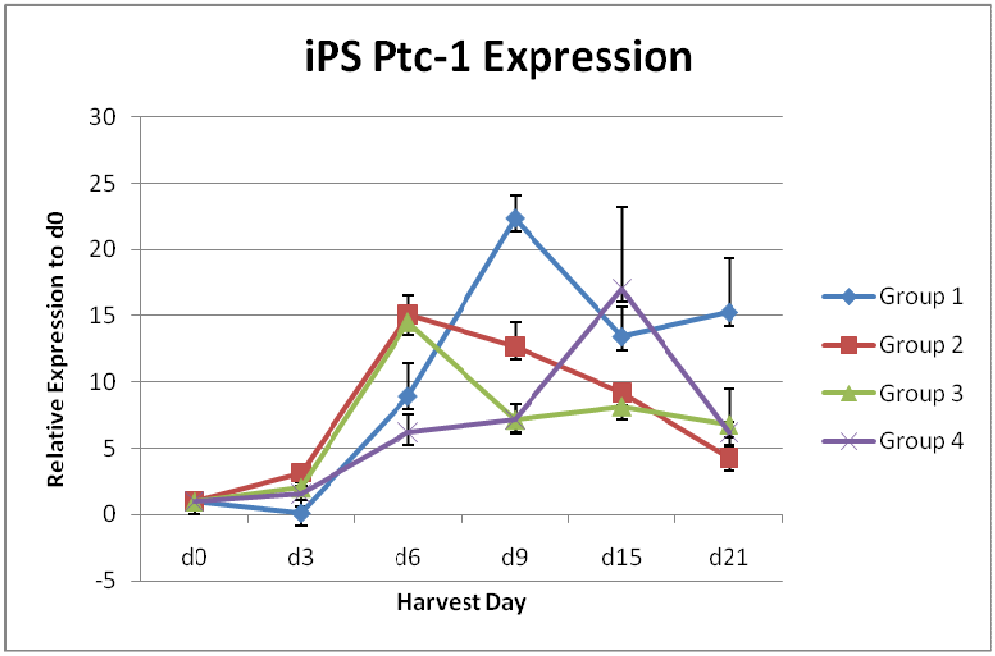

Figure 13

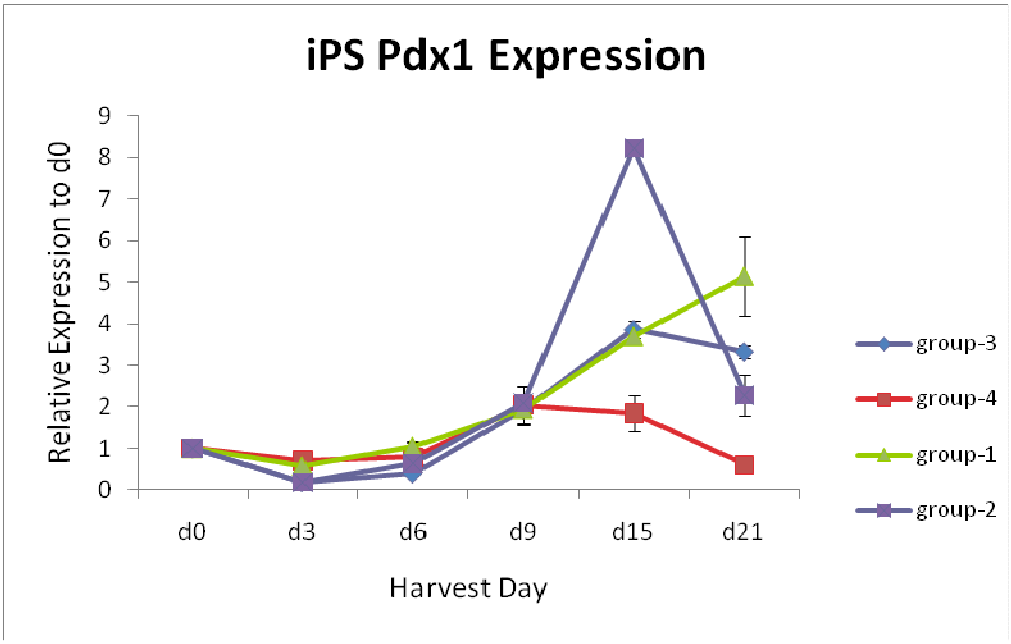

Figure 14

[Note: no significant insulin expression data was detected by PCR for the iPS cells]

The following is an example of how values were calculated from the spreadsheet provided by the qPCR machine. Normally, the machine presents the "Ct SYBR" values for all samples on the plate. However, only the data for Shh expression in H9 cells for d3 are shown below.

Pos	Name	Ct SYBR	Amount SYBR	Target SYBR	GAPDH Ct	Delta Ct	Fold	Avg Fold	Stan Dev
A9	d6	16.42	-	GAPDH					
A10		16.7	-	GAPDH	16.56				
A11		26.64	-	Shh		10.08	1.952064		
A12		26.19	-	Shh		9.63	2.666597	2.30933	0.505252

Figure 15

The values present in the third column are collected directly from the machine. "Pos" refers to the sample's position on the qPCR plate. A5 refers to row A, column 5. The "Name" column specifies the condition and harvest day of the sample. "Target SYBR" refers to the primer used in the corresponding cell. "GAPDH Ct" is the average value of the pair of "Ct SYBR" values for Gapdh. "Delta Ct" is the difference between the "Ct SYBR" value for the target gene, in this case Shh, and the "GAPDH Ct" for that sample, in this case d3. "Fold" assigns a relative expression value for each gene and is calculated by raising 2 to the power of the difference between the d0 average Delta Ct and the Delta Ct of the target

gene. Thus, 2^(d0 average Delta Ct – Delta Ct of target gene). The “Average Fold” determines the average value between the pair of “Fold” values. A standard deviation is calculated for the two “Fold” values; the standard deviation values are used to construct error bars.

Discussion and Conclusion

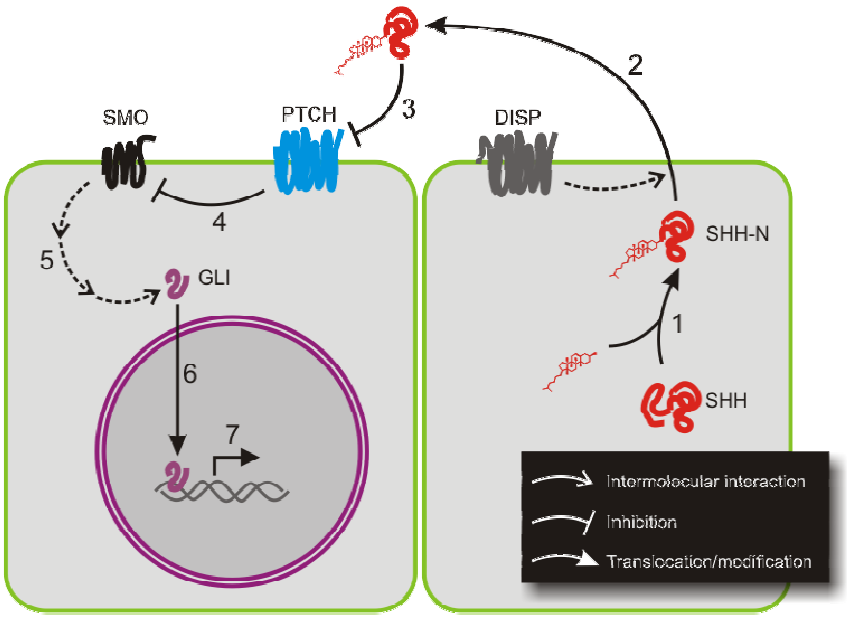

Figure 16 http://commons.wikimedia.org/wiki/File:Sonic_hedgehog_pathway.png

Many conclusions can be drawn from the trends present in the data shown above. It must first be recognized that embryonic stem cells provide a reasonable model of human development. Following formation of the three germ layers and patterning of the endoderm, pluripotent cells are responsible for organogenesis, including formation of the embryonic gut and glands, such as the liver and pancreas.

It is known from previous studies that Shh plays an inhibitory role in pancreatic development from the endoderm. However, this is somewhat surprising. As stated in *Hedgehog signals in pancreatic differentiation from embryonic stem cells: revisiting the neglected* by Mfopou et. al, “The absence of Hedgehog expression from the pancreatic anlagen is striking, as components of this pathway are widely expressed by nearly all epithelial cells of the oral ectoderm and entire gut endoderm, except at the level of Rathke’s pouch and pancreas (Apelqvist et al., 1997; Hebrok et al., 1998, 2000; Treier et al., 2001).” Additionally, this article states: “Indeed, as little as 50 ng/ml of recombinant Shh peptide diverts isolated ventral endoderm from pancreatic fate, whereas inhibition of the pathway in the foregut endoderm by Cyclopamine treatment can extend the pancreatic anlagen up to the stomach and duodenum (Kim and Melton, 1998; Deutsch et al., 2001).” Thus, it is understood that Shh signals inhibit pancreas development from gut endoderm.

It is also important to understand the Shh hedgehog signaling pathway when considering its effects on gene expression of surrounding cells. Consider figure 16.

With this information in mind, and considering the data obtained in this study, it is possible to verify certain aspects of Shh's effects on surrounding cells and how these patterns compare to normal vertebrate development. By looking at the data above, it can be verified that during endoderm differentiation into pancreas, Shh is absent and PDX1 is highly expressed. This is seen to a greater extent in conjunction with the Shh signaling pathway. Furthermore, it can be verified that Activin reduces Shh expression but does not increase PDX1 expression. This indicates that PDX1 is highly indicative of endoderm differentiation into pancreas. Further, it can be understood from the data that as Shh expression decreases, or as its prevalence in the cell decreases, the activity of Patched increases. This can be justified by again, looking at the signaling pathway of Shh; Patched is a "downstream" protein of Shh. Similarly, Gli-1 expression corresponds to Shh expression in a similar manner, most apparently in Group 4 of the H9 cells. By observing trends in relative expression, the effect of Shh on surrounding cells can be concluded via the correlation of expression values. Thus the hypothesis: Altering conditions during stem cell differentiation will induce sonic hedgehog expression in gut endoderm and increase expression of Shh target genes, was proven to be correct in a broad sense.

Future directions for this research include identifying the pathway of Shh signaling when treated with an antibody or series of antibodies, optimizing embryonic stem cell differentiation as a model of pancreatic differentiation, combining factors to induce insulin expression, and combining factors to induce insulin expression *during* differentiation.

In sum, it can be concluded that Shh plays an important role in pluripotent stem cell differentiation into pancreatic cells. It is highly expressed in the endoderm during *in vitro* differentiation but strikingly absent during development of the pancreas from endoderm. Additionally, blocking Gli signaling prevents Shh signaling and lastly, blocking Shh binding sites increases Shh expression and secretion—as in the case of Patched.

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