Generating Mature Beta Cells from Patient-Specific Pluripotent Stem Cells

Stephanie Micucci

Nyack High School

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ABSTRACT

Type 1 diabetes is an autoimmune disease characterized by the autoimmune destruction of one's insulin-producing, glucose-responsive cells, or beta cells. By generating beta cells from a diabetic's own somatic cells, these can be used in cell replacement therapy to cure an individual of diabetes. In this study, pluripotent cells were generated from cells derived from the process of human somatic cell nuclear transfer and differentiated to beta cells and transplanted into immunodeficient mice. The efficiency of differentiation was shown to be moderately successful; however, mice with the transplanted cells developed teratomas at the transplantation site. Teratomas from a separate but related experiment were sectioned on microscopy slides with paraffin and analyzed via immunohistochemistry. They were found to express some pancreatic hormones and transcription factors such as insulin and pdx1. Differentiation protocols must be refined to generate more successful beta cells and to prevent the formation of teratomas after cell transplantation.

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INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by the destruction of the insulin producing cells in the pancreas. As many as three million people in the United States alone have the disease, and more than 15,000 adults and 15,000 children are diagnosed each year [Statistics: JDRF and Diabetes, 2014]. Type 1 diabetes was previously called juvenile diabetes, as many people are diagnosed in adolescence. Unlike type 2 diabetes, which is caused by strain on the pancreas from obesity or lack of exercise, type 1 diabetes cannot be prevented.

With type 1 diabetes, one's beta cells, the insulin producing cells of the pancreas, become unable to produce any or enough insulin, the hormone that controls blood glucose levels. Without an adequate amount of insulin in the body, glucose levels rise to potentially dangerous levels, which could be fatal if left untreated. Type 1 diabetes can be managed with the administration of insulin via injections and monitoring of blood glucose levels, but there is still not a cure. As beta cells are better understood, it will lead to more successful protocols to cure the disease.

Within diabetes research, cell replacement therapy is a viable prospect for the cure. To replace the dysfunctional beta cells of a diabetic, insulin-producing, glucose-responsive cells must first be created. Human embryonic stem cells (hESCs) have the potential to replicate into a countless number of cell types [Stanekzai, 2012]. However, hESCs, which are from a human blastocyst, are genetically different from a type 1 diabetic patient.

To generate genetically identical stem cells, nuclear transfer embryonic stem cells have been created [Tachibana, 2013]. Through the process of somatic cell nuclear transfer, a human egg turns a skin cell into a stem cell. Once these stem cells have been created, they can then be differentiated to beta cells. The differentiated cells are often polyhormonal [Basford, 2012 and

Micallef, 2011] expressing other hormones such as glucagon and somatostatin, and therefore resemble immature beta cells [Hrvatin, 2013]. Differentiation protocols must therefore be adapted in order to create mature, functional beta cells.

An obstacle in using embryonic stem cells is the formation of teratomas following transplantation in mice [Stanekzai, 2012]. Protocols have been developed to attempt to avoid such formations [Rong, 2012] but they are not entirely successful. Analysis of such teratomas would allow for the understanding of how to change differentiation protocol to avoid the formations.

The aim of this study was to test the efficiency of the differentiation protocol on human nuclear transfer somatic cells, to observe the differentiated cells after transplantation into mice, and to analyze the content of transplanted-mouse teratomas to further understand how they form and how they can be prevented.

PROCEDURES

PRIOR TO EXPERIMENTATION

Prior to experimentation, hESCs were generated via nuclear transfer.

CELL PASSAGE

hES media was warmed at 37°C and the existing media was aspirated from the cell culture plate. 2 ml of TrypLE were added for 4-5 minutes to detach and dissociate hES. Once the TrypLE was removed, the plate was washed with DPBS (w/o Ca-Chloride or Mg Chloride). hES with ROCK inhibitor (Y27632) was added and the cells were detached by pipetting up/down. The cells were

transferred to a new plate and the volume was adjusted. Cells grew in huES medium for 1 or 2 days and then differentiation was started (the culture should be confluent).

DIFFERENTIATION

After cell passaging, 4 6-well plates and 4 4-well plates were differentiated (Figure 1B) following the procedure shown in Figure 1A. At day 9, differentiation was stopped for 1 4-well plate for immunostaining and at day 13, it was also stopped for 2 4-well plates (Figure 1C).

IMMUNOFLUORESCENCE ANALYSIS

Plates were washed once in 1X PBS. They were then fixed in approximately 2 ml of 4% PFA for 20 minutes at room temperature. Plates were washed twice in 1X PBS. The plates were sealed with parafilm and stored at 4°C. They were then blocked with 3% normal serum (Donkey Serum) with 0.1% Triton-X in 1X DPBS (without Ca/Mg) at room temperature. Blocking solution was removed after 30 minutes and primary antibodies in blocking solution were added. The plates were incubated at 4°C overnight. In the morning, the plates were washed 3 times for 15 minutes, each wash in PBST (PBS with 0.1% Tween-20). Secondary antibodies diluted in blocking solution were then added. The plates were incubated for 1 hour at 4°C. The plates were then washed 3 times in PBST for 15 minutes each wash. During the second wash, DAPI solution was added.

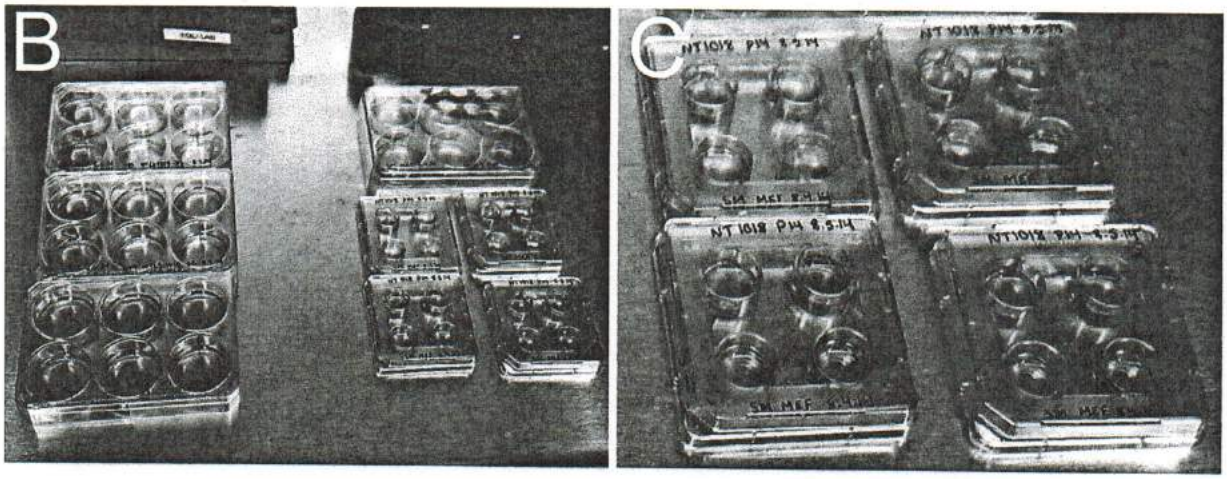
TRANSPLANTATION

Due to laboratory policy, Dr. Lina Sui transplanted the differentiated cells into subcutaneous space of immunodeficient mice.

FIGURE 1: CELL DIFFERENTIATION

A

Stage 1		Stage 2	Stage 3	Stage 4	Stage 5
Endoderm Diff Kit		RPMI-1640 +PS +Glutamax	DMEM-HG +PS	DMEM-HG +PS	CMRL +PS +Glutamax
		2% FBS	B27	B27	B27
A	B	KGF (50 ng/ml)	KAAD-cyclopamine (0.25 uM)	exendin-4 (50 ng/ml)	
B			RA (2uM)	ALK5 inh (1uM)	
			LDN (250 nM)		
Day 1	Day 2-3	Day 4-5	Day 6-8	Day 9-12	Day 13-15



(A) Process of differentiation followed: 5 stages and 15 days
(B) Total cell plates differentiated
(C) Cell plates used for immunofluorescence analysis

IMMUNOHISTOCHEMISTRY (PARAFFIN SLIDES)

Prior to experimentation:

Teratomas were obtained from mice possessing transplanted beta cells and sectioned on microscopy slides with paraffin and stored at room temperature (Figure 2).

Day 1

Slide de-paraffinization and rehydration:

A slide holder with select paraffin slides was placed into Xylene for 5 minutes. The slides were moved to a different jar of Xylene for another 5 minutes. They were then placed in 100% EtOH for 3 minutes and moved to another jar of 100% EtOH for 3 minutes. Afterwards, the slides were placed in 95%, 75%, and 50% EtOH for 3 minutes each. They were then washed with water for 3 minutes.

Antigen Revival:

Slides were placed into mailers and 10 mM sodium acetate pH 6 was added until the mailers were almost full. The mailers were submerged in water in a 500 mL beaker and microwaved for 1 minute and 30 seconds 3 times with a 1 minute cool off period in between microwaving.

Blocking Solution:

The slides were individually dried off slightly with a Kim wipe as they were removed from the mailers. Perimeters were drawn around the tissue on the slides with a hydrophobic pen. 200 µl of blocking medium was added to each slide. Once the blocking medium was added, the slides were placed in a closed slide box at room temperature with wet paper towels inside underneath the slides, which created a humidified chamber. After 30 minutes, the blocking solution was gently knocked off from each slide.

TABLE 1: Blocking Solution			
2T - 1	**2T - 4**	**2T - 8**	**Total**
200 µl PBST	200 µl PBST	200 µl PBST	600 µl PBST
10 µl Donkey Serum (5%)	10 µl Donkey Serum (5%)	10 µl Donkey Serum (5%)	30 µl Donkey Serum (5%)

Primary Antibodies:

200 µl of primary antibody solution was then added to each slide. The slide box was closed and stored overnight at 4°C.

TABLE 2: Primary Antibody Solution			
2T - 1	**2T - 4**	**2T - 8**	**Total**
200 µl PBST	200 µl PBST	200 µl PBST	600 µl PBST
4 µl Donkey Serum (5%)	4 µl Donkey Serum (5%)	4 µl Donkey Serum (5%)	12 µl Donkey Serum (5%)
1 µl rabbit x insulin	1 µl rabbit x insulin	1 µl rabbit x insulin	3 µl rabbit x insulin
1 µl mouse x glucagon	1 µl sheep x ngn3	1 µl goat x pdx1	

Day 2

Secondary Antibodies:

The following day, the primary antibody solution was gently knocked off and the slides were washed in PBST 3 times for 5 minutes each wash. The border around the tissue on each slide was reapplied with a hydrophobic pen and 200 µl of secondary antibody solution was added to each slide. The slides were placed back into the slide box and stored at room temperature for 2 hours.

TABLE 3: Secondary Antibody Solution			
2T - 1	**2T - 4**	**2T - 8**	**Total**
200 µl PBST	200 µl PBST	200 µl PBST	600 µl PBST
4 µl Donkey Serum (4%)	4 µl Donkey Serum (4%)	4 µl Donkey Serum (4%)	12 µl Donkey Serum (4%)
0.2 µl 488 x mouse	0.2 µl 488 x rabbit	0.2 µl 488 x goat	
0.2 µl 555 x rabbit	0.2 µl 555 x sheep	0.2 µl 555 x rabbit	

DAPI Solution:

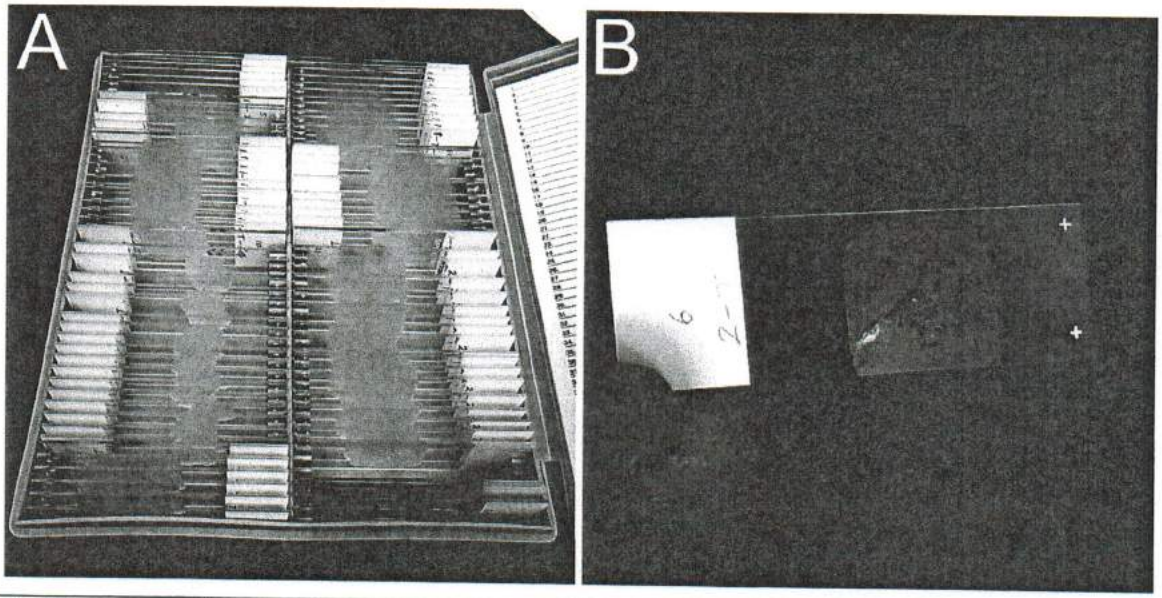
Afterwards the secondary solution was gently knocked off and 200 µl DAPI solution was added to each slide. They were then returned to the closed box.

TABLE 4: DAPI Solution			
2T - 1	**2T - 4**	**2T - 8**	**Total**
200 µl PBS	200 µl PBS	200 µl PBS	600 µl PBS
0.2 µl DAPI	0.2 µl DAPI	0.2 µl DAPI	0.6 µl DAPI

Final:

After 10 minutes the slides were washed in PBS 3 times for 5 minutes each wash. The slides were individually taken out and dried gently before 1 drop of mounting media was added to the middle of the slide. A coverslip was then carefully placed over the mounting media. The slides were placed in the slide box without wet paper towels and were stored at 4°C.

FIGURE 2: TERATOMA SECTION SLIDES



(A) Slide box with teratoma section slides
(B) Individual unstained teratoma slide (2T – 6 not used in experiment)

RESULTS

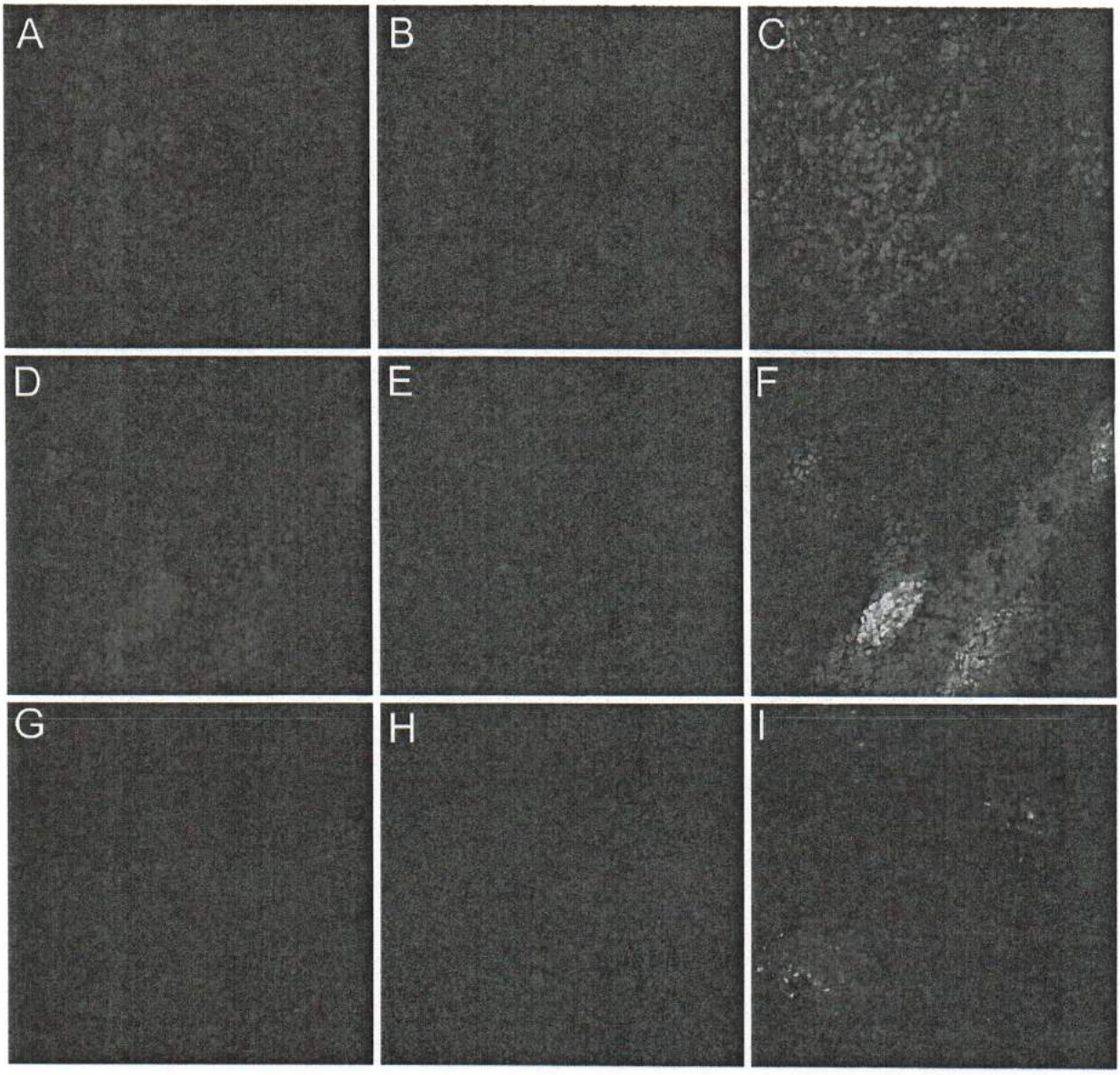
IMMUNOFLUORESCENCE ANALYSIS

To assess the efficiency of differentiation, immunofluorescence analysis was performed during different stages throughout differentiation (Figure 1C). 1 4-well plate of day 9 differentiated cells were stained for DAPI, pdx1, and nkx6.1. DAPI staining allows for the visibility of the nuclei as the fluorescent stain attaches to DNA. Pdx1 and nkx6.1 were stained for at day 9 of differentiation as the expression of these transcription factors is found in beta cells of equivalent development. The cells showed high expression of pdx1, but very low expression of nkx6.1, although still visible (Figure 3A, 3B, 3C). 1 4-well plate of day 13 differentiated cells was also stained for DAPI, pdx1, and nkx6.1 while another day 13 plate was stained for DAPI, glucagon, and C-peptide. The day 13 differentiated cells showed high expression of both pdx1 and nkx6.1in concentrated areas (Figure 3D, 3E, 3F), but showed moderate, isolated expression of both glucagon and C-peptide (Figure 3G, 3H, 3I).

TRANSPLANTATION

Stem cell-derived beta-like cells were transplanted into subcutaneous space of immunodeficient mice to evaluate the functionality of the cells. However, the grafted cells developed teratomas two months after transplantation. These teratomas formed in the transplantation site. Therefore, the in vivo functional tests of grafted stem cell-derived pancreatic beta-like cells could not be performed.

FIGURE 3: IMMUNOFLUORESCENCE ANALYSIS OF CELL DIFFERENTIATION



Differentiated cells at day 9 stained with (A) pdx1 (B) nkx6.1 and (C) pdx1, nkx6.1, and DAPI
Differentiated cells at day 13 stained with (D) pdx1 (E) nkx6.1 and (F) pdx1, nkx6.1, and DAPI
Differentiated cells at day 13 stained with (G) C-peptide (H) glucagon and (I) C-peptide, glucagon, and DAPI

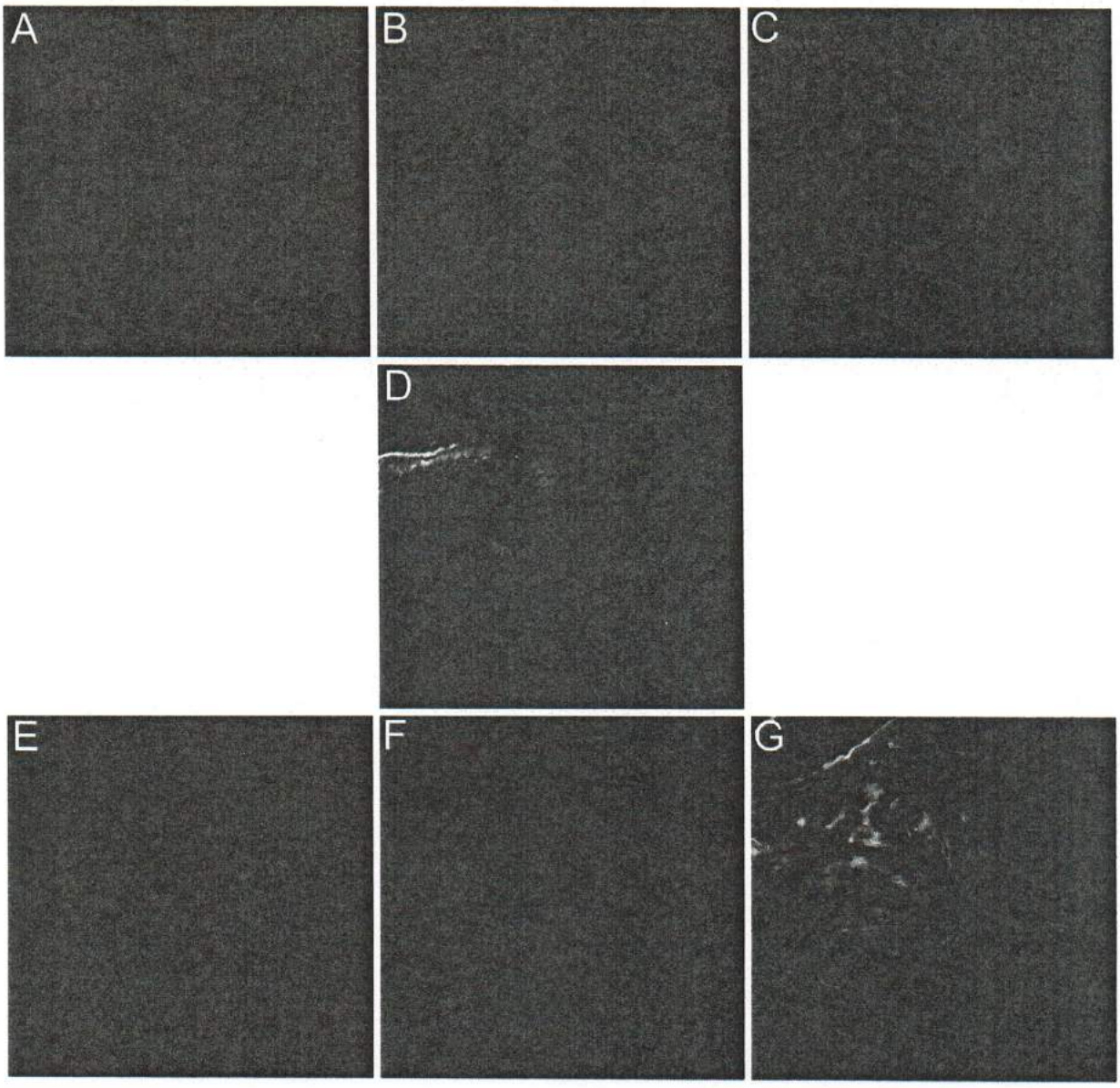
TERATOMA ANALYSIS

Teratoma sections in individual slides were analyzed from 1 mouse (mouse ID #2) via immunohistochemistry. One slide, 2T – 1, was stained for glucagon and insulin, another, 2T – 4, was stained for ngn3 and insulin, and a third, 2T – 8, was stained for pdx1 and insulin. Slide 2T – 1 showed very little expression of glucagon, and showed moderate to high levels of insulin in a concentrated area (Figure 4A and 4B). Using the DAPI staining as a guide for the present cells, glucagon was not significantly expressed, while insulin was in an isolated area of the teratoma (Figure 4C). Slide 2T – 4 showed concentrated and isolated expression of both ngn3 and insulin (Figure 4D). Using the DAPI staining as a guide, it is shown that expression of ngn3 and insulin is limited to a very select number of cells. Slide 2T – 8 showed moderate, uniform expression of pdx1, and moderate, isolated expression of insulin (Figure 4E and 4F). There is a small amount of insulin expressed uniformly throughout the section, however, certain areas show higher expression which overlaps with the expression of pdx1 (Figure 4F and 4G).

DISCUSSION AND CONCLUSIONS

The efficiency of differentiation on human nuclear transfer somatic cells was performed and observed. Pdx1 and nkx6.1are first expressed at day 9 of differentiation in beta cells. At day 9 of differentiation, cells expressed desired levels of pdx1, although they did not express nkx6.1. At day 13 of differentiation, cells expressed less pdx1 than at day 9 and expressed isolated, concentrated amounts of nkx6.1, glucagon, and C-peptide. Expression of pdx1 in successful differentiation should decrease at day 12, which began to occur in the differentiated cells of the study (Figure 3D). At day 13, however, cells expressed both C-peptide and insulin. The expression of both pancreatic hormones represents the immature state of the cells. The

FIGURE 4: TERATOMA IMMUNOHISTOCHEMISTRY



Teratoma section 1 from mouse 2 stained with (A) glucagon (B) insulin and (C) glucagon, insulin, and DAPI
Teratoma section 4 from mouse 2 stained with (D) ngn3, insulin, and DAPI
Teratoma section 8 from mouse 2 stained with (E) pdx1 (F) insulin and (G) pdx1, insulin, and DAPI

efficiency of in vitro differentiation was therefore moderately successful.

Cells were transplanted into mice at day 14 of differentiation to complete maturation. However, 2 months after transplantation the mice developed teratomas at the transplantation site. In vivo functional tests of the cells could therefore not be completed. Although differentiation was moderately successful, the protocol of differentiation and transplantation must be investigated and observed to avoid formation of teratomas after transplantation.

Immunohistochemistry shows that some pancreatic transcription factors and hormones are present in the teratoma sections removed from mice who received beta cell transplants. Each section expressed at least moderate levels of insulin. While glucagon was very minimally expressed, pdx1 was expressed uniformly and moderately throughout the stained section. Ngn3, a transcription factor important to the differentiation of beta cells, was moderately expressed along with insulin in a concentrated area of the teratoma section. Although the content and formation of teratomas formed after beta-like cell transplantation are still largely unknown, some pancreatic transcription factors and hormones are expressed within them.

For the future, the differentiation protocol must be adjusted to ensure that pancreatic transcription factors and hormones are expressed at the correct amount at the corresponding stage of differentiation, and to possibly prevent the formation of teratomas after transplantation. The content of the teratomas formed must also be investigated further in order to understand why and how they form.

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