Case Report
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Transplantation of human pluripotent stem cells overexpressing ERRγ can efficiently improve the symptoms of type 2 diabetes patient

Abstract

Since the discovery that insulin can reduce blood glucose levels in 1922, exogenous insulin administration becomes the standard method to treat human diabetes patients. Yet, this method cannot efficiently prevent the development of diabetes complications. Here, we report a combinatorial strategy that combines human pluripotent stem cells and overexpression of estrogen-related receptor γ (ERRγ) together, which, in theory, not only can repair the damaged tissues and restore the function of human pancreatic β cells, but also can synthesize and secrete human insulin, so that to reduce the blood glucose levels of the diabetic patients after transplantation. Our preliminary investigation demonstrated that our method can efficiently secrete human insulin, increase the secretion of human C-peptide, and improve the patient's health conditions physically and mentally, thus, with the potential to prevent and even to reverse human diabetic complications with more transplantations. Our reports laid an important foundation for treating human diabetes and eventually cure this disease.

Keywords: directly generated human pluripotent stem cells, ERRγ, insulin secretion, C-peptide increase, human type 2 diabetes

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Abbreviations: hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; ERRγ, estrogen-related receptor γ; hADSCs, human adipose-derived stem cells; dgHPSCs: directly generated human pluripotent stem cells

Introduction

In 1922, Banting et al.,[1] first discovered that daily insulin injection could decrease the blood glucose levels and other associated symptoms of type I diabetes, and since then, insulin is generally used for treatment of diabetes with absolute insulin deficiency.[1,2] However, with the progressing of the symptoms, exogenous insulin administration need to be intensified with more and more dosages, and even worse, the only administration of insulin cannot maintain blood glucose levels within the narrow physiological range that protects from development of various diabetic complications.[2] In addition, currently available therapies for diabetes have limited effects in preventing the progression of diabetes complications and repairing existing tissue damages.[3] Therefore, more efficient strategies for diabetes treatment are urgently needed to develop.

To generate insulin-producing pancreatic β cells from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) *in vitro* provides an unlimited cell source for transplantation therapy in diabetes.[4,5] So far, a number of groups have generated immature or mature human insulin-producing pancreatic β cells from hESCs/hiPSCs.[4–16] After transplantation of these β cells, they can restore the glucose homeostasis in diabetic mice shortly after or after a period of *in vivo* maturation.[4,5,16] Although the protocols to generate pancreatic β cell-like cells are complicated and challenging, these achievements are encouraging and promising with the potential for better treatment for human type I and type 2 diabetes. Yet, very few reports for successful treatment of human diabetes patients are known with this strategy.

Estrogen-related receptor γ (ERRγ) is a master regulator of β cell maturation *in vivo*. Forced expression of ERRγ in hiPSC-derived β-like cells enables glucose-responsive secretion of human insulin *in vitro*, and can further restore the glucose homeostasis in type 1 diabetes mouse models after transplantation, without the need for kidney capsule maturation, to achieve functionality immediately.[16] Human adipose-derived stem cells (hADSCs) were first confirmed by Zuk et al.,[17] to have the potentials to differentiate toward the osteogenic, adipogenic, myogenic, chondrogenic, and putative neurogenic cells. Sun et al.[18] reported the successful induction of hiPSCs from hADSCs with lentivirus transduction containing human Oct4, Sox2, Klf4, and c-MYC.[18] Yet the use of oncogene c-MYC as one of the inducing factors still remained to be a potential concern for the clinical application of these hADSC-derived iPSCs.

During the past years, we invented a novel protocol, which can directly generate human pluripotent stem cells from hADSCs without

any genetic modification. We termed these cells as directly-generated human pluripotent stem cells (dgHPSCs). Then, we transduced human ERRγ gene into dgHPSCs (termed as dgHPSCs-ERRγ) via a lentivirus vector, and the forced expression of ERRγ conferred the dgHPSCs secrete human insulin immediately after transduction. When clinically delivered these dgHPSCs-ERRγ cells into type 2 diabetes patients, they can not only decrease the blood glucose levels, but also increase the secretion of C-peptide, which indicates the improvement of the functions of the pancreatic β-cells of the patient. Our data provided an efficient strategy for treatment of diabetes with the potential to repair the malfunctioned pancreatic β cells by human stem cell therapy.

Materials and methods

Statement of ethical approval

The treatments for the patients and the use of human stem cells were approved by the Ethics Committee of Interventional Hospital of Shandong Red Cross Society (Shengjieyi 2003, No. 26) in compliance with Helsinki Declaration. The Ethics Committee of Interventional Hospital of Shandong Red Cross Society approved this clinical study and treatments. The participants provided their written confirmed consent to participate the clinical study and treatments. The Ethics Committee of Interventional Hospital of Shandong Red Cross Society approved this consent procedure. All the treatments for the patients and use of human stem cells were performed in accordance with the guidelines established in Interventional Hospital of Shandong Red Cross Society approved by the Ethics Committee. After traditional daily insulin injection for about four years, the patient agreed to try the stem cell therapy with overexpression of ERRγ in our hospital to control his blood glucose levels and wanted to restore his physical conditions and potential complications of type 2 diabetes, including his pancreatic β cell functions. The stem cells used in these clinical treatments are dgHPSCs Line #1 stored at our Stem Cell Bank. All these stem cells were isolated and proliferated with the written confirmed consent of the participants and their parents.

Patient case

The patient (designed as patient #1, initials T. S. D., male, born at 1958) was first diagnosed as type 2 diabetes with high blood glucose values in 2005, and the value was about 7.0mmol/L. Subsequently, his blood glucose levels increased with the time going on, and the testing results were approximately above 7.0mmol/L in 2006, above 9.0mmol/L in 2007, about 12.0mmol/L in 2012, and above 13.0mmol/L in 2013, respectively.

In September of 2014, he was diagnosed with coronary heart disease, and three cardiac stents were implanted at 11th of September. He began to inject insulin (Humalog Mix25, Eli Lilly Italia S.P.A., Italy) in October of 2014 twice a day, 10IU in the morning and 8IU at night, respectively, together with taking Repaglinide (NovoNorm, Novo Nordisk A/S, Denmark) orally to control his blood glucose levels for about a month. After stop taking Repaglinide, the insulin injected was increased to 12IU in the morning and 10IU at night daily. Beginning at 2016, the insulin injected was intensified as 12IU in the morning and 12IU at night daily. From January 19th of 2018 on, he gave up insulin injection and accepted stem cell therapy to treat his type 2 diabetes.

Cell preparation

We isolated processed lipoaspirate cells from one of the volunteered author (G.Z.) and cultured the ADSCs according to the method described by Zuk et al.[17] Then, we directly generated dgHPSCs using our invented novel protocol without any genetic modifications. These cells are termed as dgHPSCs Line #1 and show TRA-1-60 positive, which is a pluripotent marker of human stem cells (Data not shown).[19] A detailed report of the method of the generation and identification of dgHPSCs will be published at an early date after finishing the patenting procedure.

Lentivirus vector construction, production and infection

A clinical level third generation of lentivirus vector pWPI/ERRγ was constructed according to the combinatorial strategy previously described[20–23] from original vector pWPI/hPLKWT/Neo (Addgene plasmid #35385).[24] The pWPI/ERRγ lentivirus vector was produced, and infected into dgHPSCs Line #1 cells according to a previous report.[24] Briefly, one 15-cm dish of dgHPSCs was infected with 25ml lentiviruses, and two 15-cm dishes of dgHPSCs were infected with 50ml lentiviruses.

dgHPSCs transplantation

After clinical treatment of dgHPSCs-ERRγ cells, the cells were transplanted into the patient intravenously. Each time, approximately 8x107 to 1.5x108 cells were transplanted into the patient, and the intervals between transplantations were around three to five days, respectively. Totally, five times of transplantation were performed (Table 1).

Table 1 Time table of human stem cell transplantations

Transplantation date	Cell type	Cell number
20-01-2018	dgHPSCs+pWPI/ERRγ	$8.1x10^7$
24-01-2018	dgHPSCs+pWPI/ERRγ	$1.4x10^8$
29-01-2018	dgHPSCs+pWPI/ERRγ	$9.36x10^7$
02-02-2018	dgHPSCs+pWPI/ERRγ	$1.53x10^8$
05-02-2018	dgHPSCs+pWPI/ERRγ	$1.12x10^8$

Insulin secretion test

The insulin secreted into cell culture supernatant was tested via electrochemiluminescence method performed by Kingmed Diagnostics (Jinan, Shandong Province, China).

Clinical responses and treatment efficacy assessment

The venous blood C-peptide and insulin were tested by Jinan ADICON Clinical Center (Jinan, Shandong, China). The fasting venous blood glucose and glycosylated haemoglobin were tested by the Central Hospital of Zhangdian District (Zibo, Shandong, China) (Table 2). The fasting fingertip capillary blood glucose was monitored daily (Table 3). The subjective symptoms were reported by the patient during the following-up visit.

Table 2 Test for fasting venous blood glucose, insulin, C-peptide and glycosylated haemoglobin

Test	Result (19-01-2018)	Result (07-03-2018)
Fasting glucose	9.46mmol/L	11.23mmol/L
Glycosylated hemoglobin	6.00%	8.30%
Fasting insulin (pmol/L)	59.88	53.06
0.5hr insulin	141.78	99.2
1 hr insulin	298.42	179.69
2 hr insulin	359.1	240.35
3 hr insulin	219.58	203.74
Fasting C-peptide (nmol/L)	0.584	0.89
0.5 hr C-peptide	1.03	1.2
1 hr C-peptide	2.01	1.71
2 hr C-peptide	3.09	2.46
3 hr C-peptide	2.54	2.47

Table 3 Daily monitoring of fasting fingertip capillary blood glucose (FFT-CBG) levels

Date	FFT-CBG	Date	FFT-CBG	Date	FFT-CBG
20-01-2018	9.0	21-01-2018	8.8	22-01-2018	9.7
23-01-2018	8.8	24-01-2018	9.2	25-01-2018	9.7
26-01-2018	10.5	27-01-2018	10.4	28-01-2018	10.7
29-01-2018	10.6	30-01-2018	9.9	31-01-2018	10.3
01-02-2018	10.7	02-02-2018	11.2	03-02-2018	10.7
04-02-2018	11.1	05-02-2018	9.9	06-02-2018	9.0
07-02-2018	10.1	08-02-2018	10.0	09-02-2018	9.2
10-02-2018	9.5	11-02-2018	8.4	12-02-2018	10.1
13-02-2018	9.0	14-02-2018	9.7	15-02-2018	9.2
17-02-2018	11.1	18-02-2018	10.5	23-02-2018	10.4
24-02-2018	8.8	25-02-2018	9.4	26-02-2018	8.4
27-02-2018	8.6	28-02-2018	9.6	01-03-2018	9.1
02-03-2018	7.2	03-03-2018	10.1	04-03-2018	10.5
05-03-2018	9.4	06-03-2018	9.3	07-03-2018	10.7
08-03-2018	10.4	10-03-2018	10.6	11-03-2018	10.4
13-03-2018	10.9	15-03-2018	10.8	16-03-2018	10.2
18-03-2018	10.6	19-03-2018	10.2		

Results

dgHPSCs-ERRγ cells can secrete insulin immediately after transduction

Yoshihara et al.,[16] reported that forced expression of ERRγ gene in human iPSC-derived β-like cells enabled glucose-responsive secretion of human insulin *in vitro*, and demonstrated that ERRγ is a master regulator of β cell maturation *in vivo*. At the same time, this function of ERRγ could be recapitulated *in vitro*.[16] Inspired by this finding, we reasoned that the overexpression of ERRγ might drive the synthesis and secretion of human insulin from human pluripotent stem cells without differentiating into β-like cells *in vitro*. To confirm this hypothesis, we used 50ml clinical level third generation of lentiviruses pWPI/ERRγ to infect approximately 1x108 dgHPSCs, 2 days post infection, the insulin in the cell culture supernatants were tested. To our surprise, we found that the concentration of insulin in the supernatant was 30.84μIU/ml, whereas, the concentration of secreted insulin in the supernatant was 11.61μIU/ml when dgHPSCs were infected with pWPI/Insulin lentivirus vectors (designated as dgHPSCs-Insulin) using the same method. In addition, no human C-peptides were detected in the supernatant of dgHPSCs-ERRγ and dgHPSCs-Insulin, respectively. To our knowledge, this is the first time to discover that human pluripotent stem cells overexpression ERRγ can efficiently synthesize and secrete human insulin at the pluripotent stem cell state, and do not need to differentiate into β-like cells.

Transplantation of dgHPSCs-ERRγ can replace exogenous insulin administration, efficiently reduce the blood glucose levels and increase C-peptide secretion

The tested results were showed in Table 3, and the patient did twice tests at January 19th of 2018 before receiving stem cell transplantation and at March 7th of 2018 after five times transplantations, respectively. The patient stopped daily insulin injection at January 19th of 2018, and went to the Central Hospital of Zhangdian District to test his fasting blood glucose, glycosylated haemoglobin, fasting insulin, and fasting C-peptide. The results showed that, without the exogenous insulin administration, the patient's fasting blood glucose gradually increased from around 7mmol/L (with daily insulin injection) up to 9.46mmol/L in the morning of January 19th of 2018 (at January 18th of 2018, the patient injected insulin twice, morning and night, 12U, respectively). With daily injection of insulin, his glycosylated hemoglubin value was 6.0%, and the fasting insulin and C-peptide were 59.88pmol/L and 0.584nmol/L, respectively (Table 2). After receiving dgHPSCs-ERRγ cell transplantations, the patient could totally give up exogenous insulin administration, and maintained his fasting glucose level around 11.23mmol/L (compared with his fasting glucose levels above 13mmol/L in 2013 before treatment with insulin administration), and his glycosylated hemoglubin value about 8.3%, respectively. Surprisingly, his fasting C-peptide levels increased from 0.584nmol/L up to 0.890nmol/L, which is the hallmark for the repairing of the functions of pancreatic β cells. In addition, the daily monitoring of fingertip capillary blood glucose revealed that the effect of decreasing blood glucose by dgHPSCs-ERRγ cell transplantation could maintained at least for one and half months (Table 3). The exact duration of the effects of stem cell transplantations still needs to be determined further because the patient will take the second course of treatment using stem cell transplantation afterwards. The glucose

challenge tests before and after stem cell transplantations did not show significant improvements both for insulin and C-peptide secretions (Table 2). These data demonstrated that transplantations of dgHPSCs-ERRγ cells could potentially replace exogenous insulin administration and restore the functions of the patient's pancreatic β cells.

The follow-up visit of the patient

The patient reported that he had a transient fever after the transplantations of about 1x108 cells, and the body temperature reached up to 37°C. The fever faded away next day. At March 26 of 2018, about 50 days after the last transplantation, the patient described that he felt stronger physically, and his knees felt much better, he could exercise much longer than before and did not feel tired. His gross amount of food per meal did not change, but his body weight decreased slightly. In a word, his overall physical conditions were improved obviously.

Discussion

Type 2 diabetes is an increasing threat to human health span, and diabetes incidence and prevalence increase with the age. Approximately 25.9% of Americans of 65 years or older have diabetes, whereas, 9.3% of those in the general population.[3] In addition, diabetes is a major risk factor for premature onset of multiple age-related conditions, including renal dysfunction, cardiovascular disease, stroke, impaired wound healing, infection, depression, and cognitive decline.[25–27] Although, almost one hundred years ago, Banting et al.,[1] confirmed that administration of exogenous insulin can decrease blood glucose levels and improve the symptoms of diabetes, the administration of exogenous insulin cannot maintain blood glucose levels within the narrow physiological range and further protect from development of various diabetic complications, because normal pancreatic β cells adjust insulin secretion continually in response to varying blood glucose levels.[2] Therefore, to develop novel strategies for diabetes treatment to restore the normal functions of pancreatic β cells is critical to finally cure diabetes and protect from diabetic complications development. To date, different protocols are reported for *in vitro* differentiation and production of pancreatic β cells from hESCs and hiPSCs. Transplantations of these β cells produced can decrease the blood glucose levels of the animal models, and elongate their life spans. Although these results are encouraging and promising, these protocols are complicated and time consuming.[4–16] Worth of particular note, to our knowledge, there are no successful reports for clinically treatment of human diabetes patients using *in vitro* differentiated pancreatic β cells thus far.

ERRγ is a master regulator of β cell maturation *in vivo*, and *in vitro* overexpression of ERRγ in human iPSC-derived β-like cells can give rise to functional and transplantable glucose-responsive cells capable of restoring glucose hemeostasis in type 1 diabetic mouse models. More importantly, these transplanted β-like cells can control blood glucose levels in recipient mice chronically up to 56 days.[16] Surprisingly, we found that our transduced dgHPSCs-ERRγ cells could secrete insulin into cell culture supernatant, and the concentration of secreted insulin in the supernatant was 30.84μIU/ml. Since our transduction format was performed in 50ml volume, in theory, our transduced cells could continually secrete about 1,542μIU insulin into the recipient blood after intravenous transplantation. Furthermore, we found that the patient's fasting blood C-peptide is 0.890nmol/L after stem cell transplantations, much higher than 0.584nmol/L before those transplantations. Consequently, the fasting blood insulin concentration is 53.06pmol/L after transplantations, which is slightly lower than 59.88pmol/L before transplantations, with the exogenous insulin injection of 24IU daily before the test. These data demonstrated that the transplantation of dgHPSCs-ERRγ cells not only can decrease the blood glucose levels, but also increase the secretion of C-peptide by the pancreatic β cells, which is the hallmark for the improvement of the β cells functions. Therefore, we believe that the transplanted dgHPSCs-ERRγ cells can potentially differentiate into functional pancreatic β cells and further restore the injured patient's pancreatic β cell's normal functions. Although the patient's blood glucose, C-peptide and glycosylated haemoglobin levels are still higher than normal physiological range, with continued transplantation of these stem cells, it is very promising to totally restore the normal functions of pancreatic β cells, and effectively cure type 2 diabetes and possibly prevent and reverse diabetic complications.

There are two milestone works in differentiating hESCs/hiPSCs into functional pancreatic β cells *in vitro* by using 11 different factors or overexpressing ERRγ, respectively.[4,16] The produced functional adult pancreatic β-like cells share the common characteristics with bona fide pancreatic β cells, which include: 1) expressing markers found in mature β cells; 2) fluxing Ca^{2+} in response to glucose; 3) packaging insulin into secretory granules; 4) secreting quantities of insulin comparable to adult β cells in response to multiple sequential glucose challenges *in vitro*; 5) secreting human insulin into the serum of mice shortly after transplantation in a glucose-regulated manner; 6) transplantation of these cells ameliorates the hyperglucemia in diabetic mice.[4,16] Compared with these two important works, our strategy reported here have the following advantages. Firstly, our method can directly produce and secrete human insulin into the patient blood without the use of the complicated and time-consuming *in vitro* differentiation procedure. Secondly, our method can reduce the patient's blood glucose levels and stop exogenous insulin administration immediately after transplantation. In addition, our method can improve the patient's symptoms physically and mentally, not only by decreasing the glucose levels and improving glucose metabolism, but, more importantly, also by repairing the damaged tissues and organs, and further preventing the development of diabetic complications and even reversing the existed complications potentially. Furthermore, in our strategy, we use directly generated human pluripotent stem cells derived hADSCs without any genetic modifications, therefore, our stem cells are much safer than other reports.[4–16] Finally, we directly use our strategy to treat volunteered type 2 diabetes patient, and to our knowledge, this is the first report to directly use human pluripotent stem cells overexpressing ERRγ to treat human diabetes.

As for the physical and mental improvements of the patient after stem cell transplantations, it is beyond the words to describe the overall beneficial effects, including the strengthened muscles and the knees, and the vitality of the patient. All these changes indicate the positive effects of stem cell therapy. Therefore, our preliminary investigations laid an important foundation for stem cell therapy for human diabetes diseases.

Conclusion

Our work reported here demonstrated the following conclusions:

A. Human pluripotent stem cells can be generated directly from human adipose-derived stem cells without the need of any genetic modifications.

B. Human pluripotent stem cells overexpressing ERRγ can produce and secrete human insulin into cell culture medium *in vitro* immediately after transduction and do not need to differentiate into functional pancreatic β-like cells.

C. Transplantation of these stem cells can reduce patient's blood glucose levels, increase C-peptide levels, and replace exogenous insulin administrations.

D. Transplantation of these cells can improve the patient symptoms greatly physically and mentally.

E. Our strategy reported here potentially can prevent the development of diabetic complications and even reverse the existed complications with more transplantations

Declarations

Ethical approval and consent to participate

Described in Statement of ethical approval section.

Consent for publication

All the participated patients were consent for the publication of this work.

Availability of supporting data

The datasets generated and/or analysed during the current study are not publicly available due to the protection of the confidential information of the participated patients but are available from the corresponding author on reasonable request.

Author's contribution

T W and G Z instructed and supervised the whole experimental and clinical work. X W and X C performed the vector construction. B Z and L Z charged the lentiviral transduction. M F, X C, Z Y and X Y did the cell culture. R L, Q F, X D, L Z, G Y and Y M worked on the clinical treatments of the cells. All the authors discussed, wrote, read and approved the final manuscripts.

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Conflict of interest

The author declares they have no competing interests.

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