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Differentiation of embryonic stem cells towards pancreatic progenitor cells and their transplantation into streptozotocin-induced diabetic mice

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Abstract

Type I diabetes is characterized by the deficiency of endocrine β cells in the pancreatic islets of Langerhans and transplantation of islet cells can be an effective therapeutic approach. Embryonic stem cells can be differentiated into any cell type, and therefore represent an unlimited source of islet cells for the transplantation and treatment for type I diabetes. We have adopted an easy and reproducible in vitro differentiation system with a reduced serum concentration plus nicotinamide to generate early pancreatic progenitor cells from embryonic stem cells. Gene expression analysis indicated that the differentiated cells expressed not only endoderm markers such as GATA-4, HNF-3β, but also early markers of pancreatic development including key transcription factors PDX-1 and IAPP. Some pancreatic specific markers, such as insulin I, insulin II, Glu-2 and glucagon, were also expressed to some extent at the mRNA level. Differentiated ES cells showed low level immunoreactivity for insulin. However, transplantation of these early pancreatic progenitor clusters into STZ-induced diabetic mice failed to reverse the hyperglycemic state of the disease as reported previously. The results suggest that culture manipulation can direct ES cells to differentiate into early pancreatic progenitor cells committing to pancreatic islet cell fate, but these cells cannot function normally to reduce blood glucose of diabetic mice at this stage.

Keywords: Embryonic stem cell; Differentiation; Pancreatic progenitor; Type I diabetes; Streptozotocin; Transplantation

1. Introduction

Diabetes mellitus is a heterogeneous metabolic disorder affecting 5% of the adult population. Diabetes mellitus can be classified broadly into two groups: the insulin-dependent type (IDDM or type I) and the non-insulin-dependent type (NIDDM or type II). Type I diabetes is usually results from autoimmune-mediated destruction of insulin-secreting β cells in the islets of Langerhans of the pancreas, whereas Type II diabetes may be caused by systemic insulin resistance and reduced insulin secretion by pancreatic β cells.

Despite intensive insulin therapy, most individuals with type I diabetes are unable to maintain a blood glucose level in the normal range at all times. Transplantation of pancreatic islet cells can be a promising therapeutic option for the treatment of insulin-dependent diabetes. However, the lack of suitable donor tissues remains a major obstacle. Although transplantation of the whole pancreas or isolated islets of Langerhans is an effective strategy, it remains limited due to the low availability of human donor pancreas (Shapiro et al., 2000).

A promising alternative for the generation of pancreatic islets is the utilization of embryonic stem (ES) cells. ES cells are derived from the inner mass of the mammalian blastocyst, and are characterized by their self-renewal capacity and the ability to retain their developmental capacity *in vivo* (Bradley et al., 1984) and *in vitro* (Keller, 1995). To date, it has been reported that ES cells can be differentiated into insulin-producing cells

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by manipulating culture conditions *in vitro*. Several approaches have been used to obtain insulin-secreting cells: the selection for nestin-expressing ES cells (Lumelsky et al., 2001; Soria et al., 2000; Hori et al., 2002); overexpression of key transcription factors Pax4 (paired box gene 4) or PDX-1 (pancreatic and duodenal homeobox factor-1) (Blyszczuk et al., 2003; Miyazaki et al., 2004); and cell trapping with antibiotic resistance driven by the Nkx6.1 or insulin promoter to select cells (Soria et al., 2000; Leon-Quinto et al., 2004).

Although these studies suggested that mouse ES cells could be manipulated to express and secrete insulin *in vitro*, insulin-producing cell clusters derived from ES cells in these initial reports were obtained in a small quantity and these cells had lower insulin level when compared to pancreatic islets.

In the study, the differentiated protocol with reduced serum plus nicotinamide was used to promote the differentiation of ES cells towards progenitor cells of early pancreatic development. Then direct implantation of the differentiated clusters into STZ-induced diabetic mice was carried out in a hope that transplantation of early progenitor cells fully expressing β-cell markers can ameliorate hyperglycemia in diabetes.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice, 7–8 weeks of age, were purchased from Shanghai SLAC Laboratory (Shanghai, China). Mice were kept under controlled temperature (22–23 °C) and on a 12 h light/dark cycle with free access to water and pelleted food. Care and maintenance of all animals were in accordance with the Institutional Animal Care and Use Committee (IACUS) guidelines set by the Shanghai Institutes for Biological Sciences.

2.2. Culture of mouse embryonic stem cells

The D3 cell line was kindly supplied by Dr Y Jin and maintained on a mitomycin-C (Sigma, St. Louis, MO, USA)-treated mouse embryonic fibroblast (MEF) feeder layer. ES cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) containing 15% fetal bovine serum (FBS) (Hyclone), 2 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% non-essential amino acid, 1 mmol/L sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, USA), 1000 U/ml mouse recombinant leukemia inhibitory factor (LIF, Chemicon, Tenecula, CA, USA). Cells were grown in 5% CO_2 in air at 95% humidity. The culture medium was refreshed daily and cells were passaged every 2 days.

2.3. Differentiation of ES cells in vitro

To induce differentiation, ES cells were transferred and grown on gelatinized dishes for 2 passages to deplete feeder cells. For the generation of embryoid bodies (EBs), ES cells were trypsinized into a single-cell suspension, washed twice, and plated at 1×10^5 cells in 35 mm bacteriological Petri dishes with differentiation medium containing Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA), 2 mmol/L L-glutamine, 1% non-essential amino acid, and 3% FCS. On day 6 of EB formation, the resulting embryoid bodies were plated onto gelatin-coated 6-well plates and allowed outgrowth and differentiation for 10 days in the medium with 10% FBS, plus 10 mmol/L nicotinamide (Sigma, USA). The cultured medium was changed every day.

2.4. Real-time PCR

Total RNA was isolated from cell pellets using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was removed from total RNA using the RNase-free DNase set (Qiagen). The first strand cDNA was synthesized by using Sensiscript RT Kit (Qiagen). Random hexamers were used to prime cDNA synthesis. The gene expression levels were performed by real-time PCR using SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). Thermocycler conditions comprised an initial holding at 50 °C for 2 min, and 95 °C for 10 min followed by a two-step PCR program consisting of 95 °C for 15 s, and 60 °C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The β-actin gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All quantities were expressed as number of folds relative to the expression of β-actin. The sequences of primers were showed in the Table 1. Primers for real-time PCR were synthesized in Invitrogen Biotechnology.

Table 1
The primer sequence used for real-time PCR

Gene name	Acc Num	Forward primer	Reverse primer
β-actin	X 03672	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA
Insulin I	NM_008386	ACAACTGGAGCTGGGAGGAA	AAATGCTGGTGCAGCACTGA
Insulin II	NM_008387	ACCCAGGCTTTTGTCAAGCA	CGGGACATGGGTGTGTAGAAG
Glut2	NM_031197	AGCAATGTTGGCTGCAAACA	CACTTCGTCCAGCAATGATGA
PDX-1	NM_008814	TTCCAAAACCGTCGCATGA	CAATCTTGCTCCGGCTCTTC
Glucagon	NM_008100	CCACTCACAGGGCACATTCA	TCCGGTTCCTCTTGGTGTTC
IAPP	NM_010491	ACAACGCCTGGCAAACTTTT	CCGCATTCCTCTTGCCATAT
GATA-4	NM_008092	AAGGCTATGCATCTCCTGTCACT	CGGTGATTATGTCCCCATGACT
HNF-3β	NM_010446	CAGAGCCCCAACAAGATGCT	GAGAGTGGCGGATGGAGTTC
OCT-4	NM_013633	CTCACCCTGGGCGTTCTCT	GGCCGCAGCTTACACATGTT