

Transdifferentiation of mouse pancreatic ductal epithelial cells into islet-like cells[☆]

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Abstract

BACKGROUND: Islet transplantation is an effective method for the treatment of type 1 diabetes mellitus and parts of type 2 diabetes mellitus. However, its application is hindered by insufficient sources and immunologic rejection. Though transdifferentiation of pancreatic stem cells is at the starting step, it is thought to be the hopeful source for islet cell transplantation. **OBJECTIVE:** To look for a suitable cells-transplantation source for the treatment of diabetes mellitus. **METHODS:** The pancreatic ductal epithelial cells were separated from Kunming mice and cultured in DMEM/F12 medium supplemented with keratinocyte growth factor, hepatocyte growth factor and nicotinamide, etc. Samples were taken at different time points for light microscopy and electron microscope. The changes of CK-19 and PDX-1 were detected by immunocytochemistry at 1 and 16 days. The expressions of insulin and glucagon gene were detected by RT-PCR at 1 and 16 days. The physiologic function of these islet-like clusters was determined by dithizone staining and glucose stimulation at 21 days. **RESULTS AND CONCLUSION:** A large number of epithelial cells were CK-19 immunoreactive positive and few of them were PDX-1 positive at 1 day after isolation, then CK-19 positive cells proliferated quickly and formed substantial plaques of epithelial cells in cobblestone pattern. At 16 days later, these cells begin to form islet-like clusters gradually, while most of them were PDX-1 immunoreactive positive. The analysis of mRNA by RT-PCR showed very low levels of insulin and glucagon mRNA in the starting materials but increase was found as the process of transdifferentiation. At 21 day differentiated islet-like clusters were stained red by dithizone. In those samples exposed to a stimulatory 15 mmol/L glucose, there was a 1.6-fold increase in insulin compared with to 5.6 mmol/L glucose ($P < 0.05$). Pancreatic ductal cells of adult Kunming mice could proliferate quickly and have the potency of transdifferentiation into islet-like clusters when cultured *in vitro* under appropriate conditions.

INTRODUCTION

Treating diabetes mellitus with islet cells transplantation has made breakthrough progress recently. However, such an approach is limited by the scarcity of the transplantation material^[1]. In order to look for suitable cells-transplantation source and to improve the technique of stem cells culture, pancreatic ductal epithelial cells from Kunming mice were separated, cultured and transdifferentiated into insulin-producing cells in this experiment.

MATERIALS AND METHODS

Materials

Kunming mice were provided by the Center of Experimental Animals of Henan. Main reagents and instruments used are as follows:

Reagent and instrument	Source
DMEM/F12 culture medium (1:1)	Hyclone, USA
Bovine serum albumin (BSA), insulin, glucagon primer	Sangon Biotech (Shanghai) Co., Ltd., China
Fetal bovine serum (FBS)	T.B.D.
Type V collagenase, nicotinamide, dithizone, polylysine	Sigma, USA
Keratinocyte growth factor (KGF), hepatocyte growth factor (HGF)	PeptoTech EC, USA
Cytokeratin (CK-19), pancreatic duodenal homeobox-1 (PDX-1)	SantaCruz, USA
RNA extraction kit	QIAGEN, Germany
Insulin radio-immunity assay kit	Beijing Furui Bioengineering Company, China
EDTA	Life Technologies, USA

Methods

Pancreatic ductal epithelial cells separation and cultivation

Pancreases of Kunming mice were digested with type V collagenase, followed by filtrating to separate pancreatic ductal epithelial cells from islets and acinar tissue. Ductal epithelial cells were cultured in DMEM/F12 medium plus 10% FBS at 37 °C 5% CO₂. At 1–3 days, the nonadherent tissue (both viable and dead) was removed with a media change, the media was changed to DMEM/F12 medium with the additional of 10% FBS, 2.0 g/L BSA 10 µg/L KGF, cortril 4 mg/L (BBI), penicillium 0.1 U/L, streptomycin 100 mg/L. The adherent, or residual cells were expanded up to 7–10 days. A total of 10 µg/L KGF, 10 µg/L HGF and 10 mmol/L nicotinamide were added at 7–10 days when epithelial cells covered more than 50% of the flask bottom. The medium was replaced each other day. Samples were taken at different time points over the course of 3–4 weeks.

Morphological observation

Samples were taken at different time points for light microscopic examination in double days.

Immunocytochemical staining

Cultured cells were taken by trypsin at 1 day and 16 days. Harvested cells were washed by Hank's fluid, fixed by ice acetone, and stored at -80 °C for immunocytochemistry. The cells were incubated with antibodies (1:100) against protein of transdifferentiation gene PDX-1 and protein CK-19, PBS instead of antibody served as control. Then added with streptavidin-peroxidase and stained with 3'3-diaminobenzidine.

RT-PCR inspecting the expression of insulin and glucagons

Total RNA from samples at 1 and 16 days was extracted following manufacturer-suggested protocols using QIAGEN Co. cDNA synthesis was performed. PCR was carried out in 50 μ L reactions using 3 μ L of the diluted cDNA reaction product (corresponding to 20 ng RNA equivalent) as template mixed with 47 μ L of PCR mix [1 \times Taq buffer, 1.5 mmol/L $MgCl_2$, 10 pm of each insulin primers (forwards and backwards), 4 μ L of 4:6 ratio of 18 S primers/competimers, 80 μ mol/L cold dNTPs, 5 units AmpliTaq Gold DNA polymerase. Reverse transcription-polymerase chain reaction (RT-PCR) for insulin with 18 S ribosomal subunit as internal control was run on the samples. The thermal cycling protocol began with a denaturing step of 94 $^{\circ}$ C for 5 minutes, then 35 cycles of 94 $^{\circ}$ C 45 seconds, 55 $^{\circ}$ C 45 seconds, 72 $^{\circ}$ C 50 seconds, and finished with 72 $^{\circ}$ C for 5 minutes. Primers were as follows: mouse insulin: sense primer 5'-TCA GAG ACC ATC AGC AAG CAG-3', anti-sense primer 5'-GTC TGAAGG TCC CCG GGG CT-3', (which yield a 265 bp PCR product); Glucagon: 5'-ACT CAC AGG GCA CAT TCA CC-3', 5'-ACC AGC CAC GCAATG AAT TCC TT-3', (which yield a 214 bp PCR product); β -actin: 5'-TCT ACAATG AGC TGC GTG TG-3', 5'-AGC TGT AGC CAC GCT CGG TC-3', (which yield a 330 bp PCR product). DNA amplification products by 15 g/L agarose gel electrophoresis and the gels were taken photos in a gel imager for analysis. The relative amounts of the target gene were calculated as a percentage of target gene and internal control bands.

Dithizone staining

Remaining monolayer cells were collected by a solution of 0.05% trypsin and 0.02% EDTA. Dithizone staining was used to determine the purity of fresh tissue and to assess quickly the insulin-containing cells in differentiation experiments.

Glucose stimulation of insulin release

Cells cultured in 6-well plates were washed by D-Hanks three times at 21 days, then were incubated in RPMI (5.6 mmol/L glucose + 50 g/L FBS + 10 mmol/L HEPES) for 4 hours at 37 $^{\circ}$ C, the media were removed for measurement of preincubation insulin levels, and fresh media (15 mmol/L glucose + 50 g/L FBS + 10 mmol/L HEPES) were added for a 4-hour incubation. After this 4-hour period, media were again removed and measured for basal insulin secretion. At the end of this second 4-hour incubation, the final media were removed for measurement with a human insulin RIA kit.

Statistical analysis

All data were presented as Mean \pm SD. Comparisons between two means were made by Student's *t* test for group observations. Significance was evaluated using SPSS 10.0. A value of *P* < 0.05 was considered significant.

RESULTS

Primary culture of pancreatic ductal epithelial cells from Kunming mice

At the beginning of separation, typical epithelioid cells existed with single globular shape. These cells proliferated quickly when the medium had been changed to full-medium 48 hours

later. These cells then were grown for about 1–2 weeks until reaching near confluence or forming substantial plaques of epithelial cells in cobblestone pattern (Figure 1). 2–3 weeks later, epithelioid cells gathered gradually and formed islet-like clusters.

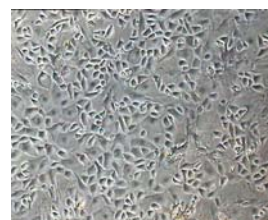


Figure 1 Pancreatic ductal cells form in cobblestone pattern ($\times 40$)

Immunocytochemistry

At the beginning of isolation, a large number of epithelioid cells were CK-19 immunoreactive positive and few of them were PDX-1 positive, while the number of CK-19 positive cells were increased significantly and most cells were PDX-1 positive at 16 days (Figure 2).

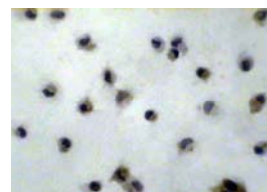


Figure 2 Positive expression of PDX-1 at 16 d after culture ($\times 400$)

RT-PCR inspecting the expression of insulin and glucagons

The analysis of mRNA by RT-PCR showed very low levels of insulin and glucagon mRNA in the starting material but increase were found as islet-like clusters developed (Figure 3, Table 1).



Line 1: marker; line 2 and line 4: the PCR production of insulin at 1 and 16 d; line 3 and line 5: the PCR production of glucagon at 1 and 16 d

Figure 3 Expression of insulin and glucagon mRNA at different time points

Table 1 Integrated absorbance value of insulin and glucagon prior to and after induction (x±s)

Group	Prior to induction	After induction	t	P
Insulin	0.363±0.038	1.960±0.117	-28.998	< 0.01
Glucagon	0.273±0.042	1.644±0.090	-30.990	< 0.01

Dithizone staining

At 1 day, few cells were positive to dithizone staining, while at 21 days, they developed islet-like clusters and were stained red. It illustrated islet-like cells have the ability to produce insulin (Figure 4).

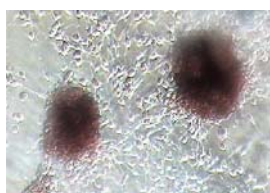


Figure 4 Islet-like cells was stained red by dithizone at 21 d after culture (x64)

Glucose stimulation of insulin release

In those samples exposed to a stimulatory 16 mmol/L glucose during the 4-hour period, there was a 1.6-fold increase in insulin (47.6±12.3 mU/L vs. 29.5±11.4 mU/L, $t=2.653$, $P<0.05$), displaying the glucose responsiveness of the islet-like clusters (Figure 5).

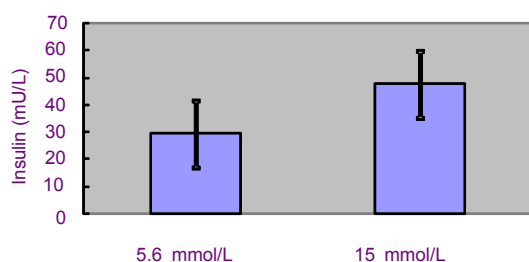


Figure 5 Glucose stimulation of insulin release

DISCUSSION

It was unclear that the mechanism for pancreatic ductal epithelial cells differentiated into islet-like cells^[2]. We have been able to expand mouse duct tissue and then to direct its differentiation to islet-like cells in vitro. These experiments were designed to start with the nonislet ductal tissue. Firstly, ductal epithelial cells have been demonstrated to give rise to islet tissue in various experimental models of pancreas regeneration^[3-4]. Secondly, β cells have been shown to have extremely low replication rate, and it is unlikely that adherent islets had lost all of their insulin or became dedifferentiated. In fact, because islets rarely adhere to the nonsticky flasks, the conditions did not favor their inclusion. KGF is known to be a

potent mitogen for a variety of epithelial cell types and to support the growth of embryonic pancreatic epithelium while repressing endocrine development^[5].

In our study the adherent cells during the early culture period seem to be ductal epithelial cells. There was little dithizone staining of the adherent cells. The large cytokeratin-19 positive cells that form in cobblestone pattern are characteristic of pancreatic ductal epithelium.

It is well known that PDX-1 is one of the important markers of pancreas stem cells^[6]. Ductal cells start to reexpress Pdx-1 when they proliferate, which has been taken as an indication of their precursor cell capacity^[7-9]. As the result presented here, most cells were PDX-1⁺ at 16 days, which proved that pancreatic ductal epithelial cells could proliferate quickly by this way and have the potency of transdifferentiation into pancreatic stem cells. There is a parallel increase of insulin and glucagons mRNA levels during the culture as seen by the RT-PCR, which demonstrated pancreatic ductal cells differentiating into stem cells or insulin-expressing cells. At 21 days, these cells developed islet-like clusters and were stained red. It illustrated islet-like cells have the ability to produce insulin.

The glucose-induced insulin response is immature as one would expect from newly formed islets^[10]. In the studies reported here, with modest expansion of tissue, insulin content was increased 1.6-fold, displaying the glucose responsiveness of the islet-like clusters. These data provide evidence of the potential to expand and differentiate mouse duct cells to islet-like cells. However, the amount of insulin released by cultures is limited and would be expected to have little clinical impact, how to control growth and differentiation of pancreas ductal cells so as to obtain enough islet cells is still needed further study. It will have important implications for making β cell transplantation therapy available to a larger number of people with type 1 and 2 diabetes mellitus.

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胰腺导管上皮细胞转分化为胰岛样细胞^{☆☆}

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摘要

背景: 胰岛移植是治疗 1 型糖尿病包括部分 2 型糖尿病的有效方法。然而, 供体来源的匮乏及移植免疫排斥反应极大地阻碍了该方法的推广和应用。最有希望的来源是从干细胞诱导分化出大量可供移植的胰岛细胞, 但目前胰腺干细胞转分化方面的研究尚处于起步阶段。

目的: 寻找胰岛移植治疗糖尿病合适的细胞来源。

方法: 分离培养昆明小鼠胰腺导管上皮细胞, 以添加有角化细胞生长因子、肝细胞生长因子和烟酰胺的 DMEM/F12 培养基培养, 不

同时间取样本于光镜和电镜下观察, 检测第 1, 16 天时 CK-19、PDX-1 免疫化学染色变化并以半定量 RT-PCR 检测第 1, 16 天时胰岛素和胰高血糖素基因表达情况, 21 d 时行双硫脲染色实验及葡萄糖刺激的胰岛素释放实验以检验胰岛样细胞的生理功能。

结果与结论: 分离第 1 天, 大部分细胞 CK-19 染色阳性, 偶可见 PDX-1 阳性细胞, 16 d 后, CK-19 阳性细胞快速增殖形成细胞团, 大部分细胞 PDX-1 染色阳性; RT-PCR 显示培养细胞胰岛素和胰高血糖素表达明显增强, 分别增加了 5.4 倍和 6.1 倍($P < 0.01$); 21 d 时胰岛样细胞团更加成熟, 双硫脲着色阳性, 且对高糖(15 mmol/L)刺激的胰岛素释放较低糖(5.6 mmol/L)时增加了 1.6 倍($P < 0.05$)。提示小鼠胰腺导管上皮细胞在体外培养条件下可增殖, 并具有干细胞潜能, 可转分化胰岛素分泌细胞。

关键词: 胰腺; 干细胞; 胰岛; 转分化; 小

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外国专家修饰的医学英语句型: 本刊英文部

原文	修饰前	修饰后
血脑屏障通透性为什么会增高? 何种物质能引起血脑屏障通透性增高? 其血脑屏障通透性增高与高原脑水肿是何关系?	It is not clear why BBB permeability increases. What is the reason for the increase in BBB permeability, and how about the relationship between the increase in BBB permeability and HACE remain poorly understood.	It remains unclear why BBB permeability increases with exposure to high altitude. The mechanisms for increased BBB permeability, and the relationship between increased BBB permeability and HACE, remain poorly understood.
不能完全解释	conventional idea cannot exactly explain	conventional theories fail to explain
与...一致	, which was in accordance with the increase in brain edema.	, which was consistent with previous reports of increased brain edema.
过表达	over-expressed VEGF	VEGF over-expression
经过均匀加工处理	Approximately 500 mg of brain sections were weighed, homogenated.	Approximately 500 mg of brain sections were weighed, homogenized.
发病率	attack rate	onset rate
同意	Stroke neurologists agreed topographic diagnosis	Stroke neurologists were in agreement with topographic diagnosis
诊断(可数)	every child with possible diagnosis of stroke	every child with a possible stroke diagnosis