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Rat Bone Marrow Mesenchymal Stem Cells Transdifferentiate into Islet-secreting Cells in Vitro

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Abstract: Objective To explore the protocol that induced marrow mesenchymal stem cells (MSCs) differentiating into islet-secreting cells in vitro and to provide new clues for the sources of islet transplantation. **Methods** Using a defined culture medium and technique for transdifferentiation, MSCs from adult SD rats were guided into specific insulin-secreting cells. The expressions of nestin and islet-specific hormones and proteins, such as insulin, glucagon, somatostatin and pancreatic duodenal homeobox 1 (Pdx-1) were analyzed by indirect immunofluorescence cytochemistry staining before and after induction. The expressions of pancreatic islet cell differentiation-related transcripts, such as nestin, insulin 1, glucose transporter 2 (GLUT 2), Isl-1, Pdx-1, Pax-4 and Pax-6 were detected by reverse transcription-PCR (RT-PCR). In addition, the quantity of insulin secretion was examined using radioimmunoassay. **Results** Five hours after induction, (44.6 ± 7.3)% of differentiated MSCs expressed nestin and it increased to (61.8 ± 8.4)% 24 hs after induction, but the expression of nestin almost disappeared at day 14. In the meantime, islet-like cellular clusters appeared after day 14 and became more apparent by day 28. Differentiated cells were found to be immunoreactive to insulin, glucagon, somatostatin and Pdx-1, and expressed insulin 1, GLUT 2, GK, Isl-1, PDX-1, Pax-4, Pax-6 mRNA. In addition, the results of cumulative quantities of insulin of 24 hs and the stimulation index showed that differentiated cells were able to produce insulin at higher levels, and displayed glucose-dependent insulin release in vitro. **Conclusions** Adult rat MSCs can be differentiated into insulin-secreting cells in vitro. This approach might lead to widespread cell replacement therapy for Type 1 diabetes. **[Chin J Contemp Pediatr, 2003, 5(5): 393-397]**

Key words: Marrow mesenchymal stem cells; Islet of Langerhans; Diabetes mellitus; Transdifferentiation

体外诱导大鼠骨髓间质干细胞分化为胰岛素分泌细胞

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[摘 要] 目的 探索大鼠骨髓间质干细胞体外诱导分化为胰岛素分泌细胞的方法。为解决胰岛移植物来源匮乏这一问题提供新的思路。**方法** 采用横向分化技术，将成年大鼠骨髓间质干细胞诱导成为胰岛素分泌细胞。间接免疫荧光法鉴定诱导前后细胞 nestin、胰岛素、胰高血糖素、生长抑素及 Pdx-1 的表达，RT-PCR 法检测诱导前后细胞 nestin，胰岛素-1，葡萄糖转运子-2，葡萄糖激酶及其转录因子 Isl-1，Pdx-1，Pax-4 和 Pax-6 mRNA 的表达；测定 24 h 胰岛素分泌量和胰岛素刺激实验评价诱导前后细胞的功能。**结果** 诱导 5 h，nestin 阳性细胞为(44.6±7.3)%；诱导 24 h，nestin 阳性细胞增至(61.8±8.4)%；此后，nestin 阳性细胞数目开始下降，诱导第 14 天后，nestin 表达基本消失。诱导后的胰岛素分泌细胞可以表达胰岛素、胰高血糖素、生长抑素和 Pdx-1 等蛋白；表达胰岛素-1、葡萄糖转运子-2、葡萄糖激酶及其多种转录因子 mRNA；胰岛素分泌量增加；胰岛素刺激实验反应敏感等。而诱导前 MSCs 不具备上述特点。**结论** 大鼠骨髓间质干细胞体外可以诱导成为胰岛素分泌细胞，为胰岛移植开辟新的研究思路。 **[中国当代儿科杂志，2003，5(5)：393-397]**

[关 键 词] 骨髓间质干细胞；胰岛；糖尿病；横向分化

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Islet transplantation is an efficient therapy for Type 1 diabetes[1]. Fetal pancreatic tissue and adult pancreas have all been used as potential sources for islet transplantation. It is restricted by the quantity and quality of transplants, immunologic rejection and ethical problems. The stem cell technique, especially the technique for transdifferentiation of the adult stem cells, provides a new way to solve theses problems. Mesenchymal stem cells (MSCs) are typical adult stem cells, possessing polytransdifferentiate potential, which can be induced to differentiate into a variety of cells, including osteoblasts, chondrocytes, adipocytes, myocardial cell, muscle tendon, hepatocyte, nerve cell, glial cell and so on[2]. Using the technique of transdifferentiation, MSCs from adult SD rats were guided into specific insulin-secreting cells, which is the basis for the research of the transplantation of islet stem cell.

Materials and methods

Reagents and animals

The DMEM medium, B27 and Trizol were from Gibco BRL. The fetal bovine serum (FBS) was from Hyclone. MCDB-201, ITS and LY294002 were supplied by Sigma. The basic fibroblast growth factor (bFGF) and HGF were from Pepro Tech EC. Matrigel was from BD Biosciences, USA. The insulin radiation immune kit was from the Atomic Energy Institution of China. The RT-PCR kit was from Promega. The other reagents were all imported analytical pure.

The mouse anti-rat nestin antibody was from R&D. The mouse anti-rat insulin, glucagons, somatostatin antibody were from Neomarks. The goat anti-Pdx-1 antibody was from Santa Cruz. The second fluorescence-labelled antibody was from Sigma.

Inbreeding and blocking adult SD rats (Clearing grade, weighing between 50 – 200 g and with no special limitation of gender) were afforded by the Department of Animal Experiment, Central South University.

Isolation, cultivation and proliferation of MSCs

The rat tibiae were obtained under sterile condition and cleaned by D-Hank's solution. The medullar cavities were flushed carefully by the 5 ml complete medium (60% DMEM, 40% MCDB-201, 2% FBS, 2 mmol/L glutamine, 0.1 mmol/L 2-mercaptoethanol, 0.1 mmol/L non-essential amino acid, 1 ng/mL bFGF and antibiotic).

The flushed cells were diluted and then inoculated into the plastic flask and cultivated in the container which contained 5% CO_2 and a temperature of 37℃. The medium after 24 hours in order to wipe off the non-adhered cells and thereafter every 72 hours. Once the adhered cells coalesced completely, 5 passages were transferred and the culture medium was changed every 72 hours. After the 6th passage, MSCs were cultivated using the low density proliferation[6]. The density of inoculation was 2 – 8 cells/cm^2 and the medium was changed every 48 – 72 hours. The induction experiments were done after the 10th passage.

Insulin-secreting cells induction in vitro

The induction was divided into 3 phases. The first phase: MSCs were induced to differentiate into nestin positive cells (0 – 5 hours), then they were washed with D-Hank's solution 3 times and cultivated for 5 hours in the inducing medium of the first phase (DMEM medium, 5 mmol/L β-mercaptoethanol, or 0.2 – 0.4 mmol/L baicalin[3]). The second phase: after the nestin positive cells proliferated and induced to precursor (the 6th hour to the 7th day), they were inoculated into 6-hole culture dishes which were besmeared with Matrigel and cultivated for 7 days using the culture medium of the second phase (DMEM medium, B_{27}, 10 ng/ml bFGF, 0.1 mmol/L β-mercaptoethanol, ITS and so on). The third phase (insulin-secreting cells maturation phase, the 8th day to the 28th day): Removing bFGF from the culture medium, the cells were cultivated in the medium of the third phase (DMEM medium, B_{27}, 10 ng/ml HGF, 20 mmol/L niacinamide, 0.1 mmol/L β-mercaptoethanol, LY294002 and so on) for 7 – 21 days.

Identification of the morphology and function of the induced cells

The cells before and after induction were fixed with 4% formaldehydum polymerisatum and processed with 0.5% H_2O_2-methanol for 30 minutes and

with 1% BSA-0.3% Triton X-100 37℃ for 30 minutes. Then the first antibody (1∶50 – 200) was added and reacted at 37℃ for 30 minutes and stored at 4℃ refrigerator overnight. After that, the fluorescence labelled second antibody (1:64) was added and were reacted at 37℃ for 30 minutes. Then the cells were flushed completely with 0.01 mol/L PBS. The cells were observed under fluorescence microscope. The first antibody was replaced by PBS in the negative control, the other steps were the same as previously. Two hundred cells on each section were counted randomly, then the percentage of the nestin positive cells was obtained.

The general cell RNA was extracted with Trizol reagent and detected by the UV spectrophotometer. The reaction system was 50 μl. The reverse transcription and PCR were completed in one step. Ten μl PCR products was electrophorised in 1.7% agarose gel. The results of the electrophoresis were observed under the UV transmission. The primers were synthesized by the Biological Engineering Company of Shanghai (Table 1).

After 24 hours the medium was collected and the quantities of insulin were detected using radioimmuoassay.

The insulin stimulating index was counted by the method previously described[4].

Statistical analysis

Analysis of variance and χ^2 test were used.

Results

Induction and differentiation

After the medium had been changed several times, the cells grew confluently and became adhered cells. Through 6 – 8 passages and low-density proliferation, these cells turned into the typical MSCs with the shape of a shuttle (See Figure 1).

After the 5th hour of the first inducing phase, the body of some cells contracted like an awl, possessing long and thin prominence which seemed like a three-dimensional structure. Some cells were surrounded by aula and seemed like nerve cells. Some cells fell and died. The cells quantity increased in the second phase and a few of them differentiated into nerve-like cells. After bFGF was removed in the 3rd phase, many cells fell. But there were some adhered cells which assembled and formed a mass of islet-like cells (the 14th day). When the time of the culture was prolonged, a large number of cells proliferated from the verge of the mass and formed one or more layers of cells on the surface of other cells. The previous mass structure remained the same or disappeared (the 28th day, See Figure 2 – 4).

Identification of nestin positive cell

The expression of nestin in MSCs before induction was weak, less than 5%. At the 5th hour of the first phase, (44.6 ± 7.3)% of cells expressed nestin. At the 24th hour of the 2nd phase, the quantity of nestin positive cells increased to (61.8 ± 8.4)%. But after the 48th hour it decreased to (38.1 ± 7.7)%. On the 7th day after induction, the quantity of nestin positive cells was only (15.8 ± 3.9)%. After the 14th day of the 3rd phase, there was no nestin expression. The result of RT-PCR suggested that there was no nestin expression before induction. The nestin positive cells appeared at the 5th hour after induction, decreased on the 7th day and disappeared on the 14 – 28th day (See Figure 5).

Identification of the cells after induction

The islet-specific hormones and protein such as insulin, glucagon, somatostain, pancreatic duodent homeobox 1 (Pdx-1) did not express in the MSCs before induction. There were low expressions of insulin, glucagon and somatostain on the 7th day of induction, and they increased with the time going. After the 14th day of induction there were a large number of insulin positive cells in the center of the mass structure. At the same time, the glucagon positive cells and somatostatin positive cells increased (See Figure 6 – 7). These cells were round and assembled to form a mass, with cytoplasms Pdx-1 positive staining and a few nucleuses Pdx-1 positive staining.

The mRNA of insulin-1 expressed on the 7th day after induction, and it increased with time. The expression of insulin-1 mRNA on the 28th day was higher than those on the 7th and 14th day. Glucose transporter 2 (GLUT-2) and glucose kinase (GK) participated in regulating the secretion of insulin and they expressed on the 7th day after induction and

continued to the 28th day. There were no expressions of the transcription factors such as Isl-1, Pdx-1, Pax-4 and Pax-6 before induction. The expressions of Pdx-1, Pax-4 and Pax-6 appeared on the 7th day after induction, Isl-1 appeared on the 14th day. The expressions of Pdx-1 and Pax-6 on the 28th day were higher than those on the 7th and 14th day (See Figure 8).

The secretion of insulin was very low before the 7th day of induction, but it increased sharply after that and mounted up with time. The difference between various time points was significant ($P < 0.01$) (See Figure 9).

The induced cells responded to the stimulation of glucose on the 7th day. The secretion of insulin before and after stimulation all increased markedly. The cells became more sensitive to the stimulation of glucose. The insulin stimulating indexes of the 14th and 28th days were much higher than that of the 7th day ($P < 0.01$), but there was no difference between the 14th day and the 28th day (See Figure 10).

Table 1 The primers of RT-PCR

Primers	Positive	Negative	Products
Nestin	5'-GCG GGG CGG TGC GTG ACT AC-3'	5'-AGG CAA GGG GGA AGA GAA GGA TGT-3'	329 bp
Insulin-1	5'-GCT ACA ATC ATA GAC CAT C-3'	5'-GGC GGG GAG TGG TGG ACT C-3'	350 bp
GLUT-2	5'-TTA GCA AGT GGG TCT GCA AT-3'	5'-GGT GTA GTC CTA CAC TCA TG-3'	343 bp
GK	5'-GTG GTG CTT TTG AGA CCC GTT-3'	5'-TTC GAT GAA GGT GAT TTC GCA-3'	340 bp
Isl-1	5'-GCG GAG GAT GGG CTT TTC TG-3'	5'-TTC TGC TTT TCG TTG AGC ACA G-3'	235 bp
Pdx-1	5'-GGT GCC AGA GTT CAG TGC TA-3'	5'-TTA TTC TCC TCC GGT TCT GC-3'	329 bp
Pax-4	5'-TGG CTT TCT GTC CTT CTG TGA GG-3'	5'-TCC AAG ACT CCT GTG CGG TAG TAG-3'	214 bp
Pax-6	5'-AAG AGT GGC GAC TCC AGA AGT TG-3'	5'-ACC ACA CCT GTA TCC TTG CTT CAG G-3'	545 bp
β-actin	5'-ACA CTG TGC CCA TCT AGG AGG-3'	5'-AGG GGC CGG ACA CGT CAT ACT-3'	621 bp

Discussion

Both embryonic stem cells and adult stem cells can be induced to differentiate into insulin-secreting cells[5,6]. However, immunologic rejection has to be faced if islet differentiated from embryonic stem cell as implantation is used. On the contrary, this problem may be avoided if islet from adult stem cell is used. Adult stem cells have widespread sources. They may come from either nestin-positive multipotential stem cells and duct epithelial cells in the pancreas[6,7] or liver mesenchymal stem cells (oogonium)[8]. Islet-secreting cells from bone marrow mesenchymal stem cells were used because MSCs were easy to obtain, easy to amplify in vitro, easy to induce and easy to modify with genes.

The strategy of induction is important. From Lumelsky's experiment it can be seen that the key point was to induce embryonic stem cell differentiate into nestin-positive cells[5]. There are nestin-positive stem cells in normal adult rat and human pancreas which can be induced to differentiate into islet and exocrine pancreas. Nestin is characteristic biological sign of neural stem cells, which indicates that the gene adjusting pathways in the earlier differentiation process of neuron and islet are similar[6]. In addition, during the process of MSCs differentiating into neuron, Woodbury reported that nestin-positive cells should be induced before differentiating into neuron[9]. Therefore in this experiment, MSCs were induced to differentiate into nestin-positive cell and then were induced to differentiate into insulin-secreting cells.

The process of induction was divided into 3 phases and different induced environments were set up in different phases. In the first phase, a medium free of serum was used to which β-mercaptoethanol, dimethyl-sulfoxide and baicalin were added in order to induce MSCs to differentiate into nestin-positive cell. Five to six hours after induction the expression of nestin was observed. The second phase included the amplification of nestin-positive cell and the formation of precursor of islet. Since there were continuous and overlapping expression and similar inducing microen-

vironment between nestin-positive cell and precursor of islet, no particular distinction was made. After being induced for 6 - 24 hours, the amplification peak of nestin-positive cells occurred, but it began to decrease after 48 hours. Simultaneously, precursor of islet formed and began to differentiate. In this phase, bFGF was added to facilitate the formation of islet and increase the induction efficiency. In addition, extra cellular matrix Matrigel was used to facilitate pancreas duct to differentiate into islet and establish reasonable stereo structure of islet[9]. Through these strategies, it was found that a small quantity of cells expressing insulin, glucagon and somatostatin. The secretion of insulin was detected 7 days after induction. In the third phase, bFGF was withdrawn. After this, the secretion of insulin increased on a large scale indicating that the cells in this phase differentiated and matured rapidly. Concomitant with this, many cells degenerated and fell away from the wall of the flask after which apoptosis took place. Because of this extra cellular matrix Matrigel was used to increase the number of adhesive cells and to elongate the time of induction. LY294002 and HGF can also facilitate the maturation of insulin-secreting cells and increase the quantity of insulin secretion.

Islet is a complex, special formation composed not only of α, β and δ cells but also of special vascular system and so it is difficult to induce MSCs to differentiate into a formation consistent with islet in constitution of cell and space formation.

There were some differences between the insulin-secreting cells induced in the experiment and genuine islet. They were just insulin, glucagon, somatostatin-positive cells and even included neurons and neuroglial cells. But they expressed some genes mediating insulin secretion such as GLUT-2 and GK. Because they could release some insulin and respond to glucose stimulation, they had the potential to replace islet transplantation.

In addition, there are many transcription factors involved in the regulation of the development of islet such as Isl-1, Pdx-1, Pax-4 and Pax-6[12]. The results indicated that with the maturation of induced cell, Isl-1, Psx-1, Pax-4 and Pax-6 expressed successively, which suggested that the inducing process of MSCs might follow the developing rule of islet.

Of course there were some shortcomings, such as the lower quantity of insulin secretion and the lack of animal experiment, and so further study is required. (All figures were on the back cover).

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