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World J Gastroenterol 2005;11(22):3426-3430
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Adult islets cultured in collagen gel transdifferentiate into duct-like cells

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Received: 2004-07-02 Accepted: 2004-09-09

Abstract

AIM: To establish a model of islet-ductal cell transdifferentiation to identify the transdifferentiated cells.

METHODS: Collagen was extracted from rat tail at first. Purified rat islets were divided into three groups, embedded in collagen gel and incubated respectively in DMEM/F12 alone (control group), DMEM/F12 plus epidermal growth factor (EGF), DMEM/F12 plus EGF and cholera toxin (CT). Transdifferentiation was proved by microscopy, RT-PCR, immunohistochemistry and RIA.

RESULTS: Islets embedded in collagen gel plus EGF and CT were cystically transformed and could express new gene cytokeratin 19 while still maintaining the expression of insulin and Pdx-1 genes. Immunohistochemistry demonstrated that the protein of cytokeratin 19 was only expressed in the third group. The insulin content secreted by islets in the third group decreased significantly during the transdifferentiation.

CONCLUSION: CT is a crucial factor for the islet-ductal cell transdifferentiation.

Key words: Islets of Langerhans; Ductal cell; Transdifferentiation

Lu J, Gu YP, Xu X, Liu ML, Xie P, Song HP. Adult islets cultured in collagen gel transdifferentiate into duct-like cells. *World J Gastroenterol* 2005; 11(22): 3426-3430
http://www.wjgnet.com/1007-9327/11/3426.asp

INTRODUCTION

Transdifferentiation is a process in which differentiated cells alter their identity to become other distinct cell types. This process has been proven by researches[1,2]. During pancreas development, both the exocrine and endocrine systems seem to originate from the ductal cells which are considered as a kind of pancreatic stem cells. This hypothesis is supported by the phenomenon that ductal cells have the ability to transdifferentiate into acinar cells and islets[3-5]. But this process is bilateral. On the contrary, acinar cells and islets can transdifferentiate into duct-like cells[6,7]. It has also been reported that islets in a long-term culture can transdifferentiate into exocrine and undifferentiated cells, which may be considered as pancreatic precursor cells[8]. The process of mature endocrine cells transdifferentiating into exocrine cells was also confirmed in our study. Our data show that, rat islets cultured in rat tail collagen gel plus EGF and CT can transdifferentiate into duct-like cells.

MATERIALS AND METHODS

Isolation and purification of islets

Male Sprague-Dawley (SD) rats, weighing 230-250 g, were obtained from Central South University Laboratory Animal Department. For each isolation, one rat was anesthetized with pentobarbital sodium (30 mg/kg) intraperitoneally. Isolation of pancreatic islets was performed as previously described[9]. Briefly, after the output site of the duct from pancreas to duodenum was clamped, the pancreas was distended maximally with 20 mL collagenase (type IV, 1.5 mg/mL, Invitrogen) using a pulsed infusion technique via the portal duct of pancreas. The whole pancreas was removed from male SD rats and placed in a stationary water bath at 37 ℃ for 25 min to allow it to digest enzymatically. Digestate was agitated gently for 1 min every 5 min during incubation. The digestion process was stopped by adding 30 mL of cold Hank's balanced salt solution (HBSS) supplemented with 5% fetal bovine serum (FBS). After having vortexed for 1 min to dissociate the islets from adherent acinar elements, the mixture was passed through a steel mesh filter with an aperture of 0.5 mm. The filtrate was subjected to the conventional method of histopaque density gradient centrifugation to separate islets from the digestion mixture.

DTZ staining and hand-picking

DTZ stock solution preparation Ten milligrams of DTZ were dissolved entirely in 5 mL of dimethyl sulfoxide (DMSO), then filtered through a 0.2-μm nylon filter and stored briefly at -20 ℃.

***In vitro* DTZ staining** Ten microliters of the stock solution was added to 1 mL of culture medium. The culture dishes were incubated at 37 ℃ for 15 min in the DTZ solution.

After the dishes were rinsed thrice with HBSS, clusters stained crimson red were identified as islets. These islets were hand-picked under a stereomicroscope. After hand-picking, the dishes were refilled with DMEM containing 10% FBS. The stain completely disappeared from the cells after 2 h.

Three-dimensional (3D) collagen gel matrix preparation and islet culture

Collagen preparation Collagen was extracted from rat tail tendons as previously described[10]. Tendons were excised from rat tails and sheared. Some connective tissues were removed carefully, washed twice with PBS and then soaked in 4 mmol/L acetic acid. After having stirred for two d at 4 ℃, collagen was extracted. The extracts were centrifuged for 30 min at 10 000 *g* and supernatant was collected into another distilled vessel for use. Protein concentration in the collagen was 1.0-1.5 mg/mL measured with an ultraviolet spectrophotometer.

Preparation of collagen gels Following sterile components (0.7 mL 10×DMEM/F12, 0.1 mL heat-inactivated low-endotoxin FCS, 0.1 mL 0.1 mol/L NaOH, 0.1 mL islet suspension) were carefully mixed to avoid bubbles. All components required to make collagen gels were placed on ice except for the islet suspension. Thus, the mixture resulted in a physiologic ionic strength and 1×DMEM/F12 in the final gel. The mixture was incubated at 37 ℃ in 50 mL/L CO_2 and then gelation was completed after 60 min.

Islet culture Islets embedded in collagen gel were divided into three groups and incubated under the following conditions.

3D culture alone (control group) DMEM/F12 (penicillin 100 U/mL, streptomycin 100 μg/mL, 10 mmol/L HEPES) +10% FCS+nicotinamide (10 mmol/L).

3D culture+EGF: DMEM/F12 (penicillin 100 U/ml, streptomycin 100 μg/mL, 10 mmol/L HEPES)+10% FCS +EGF (100 ng/mL)+nicotinamide (10 mmol/L).

3D culture + EGF + CT: DMEM/F12 (penicillin 100 U/mL, streptomycin 100 μg/mL, 10 mmol/L HEPES) +10% FCS+EGF (100 ng/mL)+CT (100 ng/mL) +nicotinamide (10 mmol/L).

Insulin content assay

Samples were taken at the 1st, 3rd, 5th, 16th, 28th, 40th, 52nd, 76th, 88th ,120th, 168th h. After old culture medium was piped out thoroughly, islets were rinsed twice and fresh medium was added. Culture media were sampled after 1 h and frozen at -20 ℃. Insulin content in samples was detected by radioimmunoassay (RIA).

Immunohistochemistry

Islets were hand-picked from slides pretreated with poly-L-lysine and fixed in 4% paraformaldehyde (PFA). Then the fixed islets were processed for routine histology and immunostained for insulin (mouse anti-insulin, Boster, Wuhan) and cytokeratin 19 (mouse anti-cytokeratin 19, Boster, Wuhan) using the streptavidin-biotin complex (SABC) method following the instructions of kit (Boster, Wuhan). For cytokeratin 19, islet cells were pretreated with 0.1% trypsin. The sections were incubated overnight at 4 ℃ with appropriate primary antibodies. Negative controls involved omission of the primary antibodies.

RNA extraction and RT-PCR analysis

The collagen matrix was dissolved in 0.25 mg/mL collagenase and islets were washed thrice. Total RNA was extracted from cultured rat islets using TRIzol (GIBCO BRL) and reverse-transcribed into cDNA with TaKaRa RNA PCR kit (AMV) Ver. 2.1 (TaKaRa, Japan) according to the standard procedure. cDNA samples were subjected to PCR amplification with specific primers. The cycling parameters were pre-denaturation at 94 ℃ for 2 min, denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and elongation at 72 ℃ for 1 min (34 cycles). Table 1 summarizes the sequences of the PCR primers used in this study.

Table 1 Primers used in study

Targeted mRNA	Primer	Length
Rat Pdx-1	F: gaggacccgtacagcctaca R: cgttgtcccgctactacgtt	201 bp
Rat Insulin	F: ccgtcgtgaagtggagga R: cagttggtagagggagcagat	154 bp
Rat Cytokeratin 19	F: atccccaaagacacgagatg R: gtgagctacaaccgcagctt	200 bp
Rat β-actin	F: taaagagaagctgtgctatgttgc R: atgatcttgatcttcatggtgcta	354 bp

Statistical analysis

All data were expressed as mean±SD with $n = 3$ at each time point. The difference between time points with respect to insulin content was evaluated by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Microscopy

We used DTZ to identify islets which were stained crimson while acinar and ductal tissue failed to incorporate the stain. Islets were stained with DTZ and hand-picked after density gradient centrifugation. The stain completely disappeared from the cells after 2 h (Figure 1).

Islets embedded in collagen gel could retain their natural shape, while those in monolayer cultures could not (Figure 2).

The islets in groups 1 and 2 were not different in shape on d 1, 3 and 5. But some islets in group 3 continually enlarged in these days (Figure 3). The percentage of islets undergoing cystic transformation was increased over the time-course of the culture period in group 3.

Secreted insulin content

During the culture time, the abilities of islets to secrete insulin in three groups were all decreased. Five hours later, islets lost their secreting ability faster in group 3 than in groups 1 and 2 ($P<0.05$ or 0.01) (Figure 4).

Immunohistochemistry

Islets cultured for 1, 3, 5 d were insulin positive and cytokeratin 19 negative in groups 1 and 2.

In group 3, islets were insulin positive and cytokeratin 19 negative on d 1. There was a decrease in expression of insulin mainly in the center of islets and some islet cells around the cystic spaces began to express protein cytokeratin 19

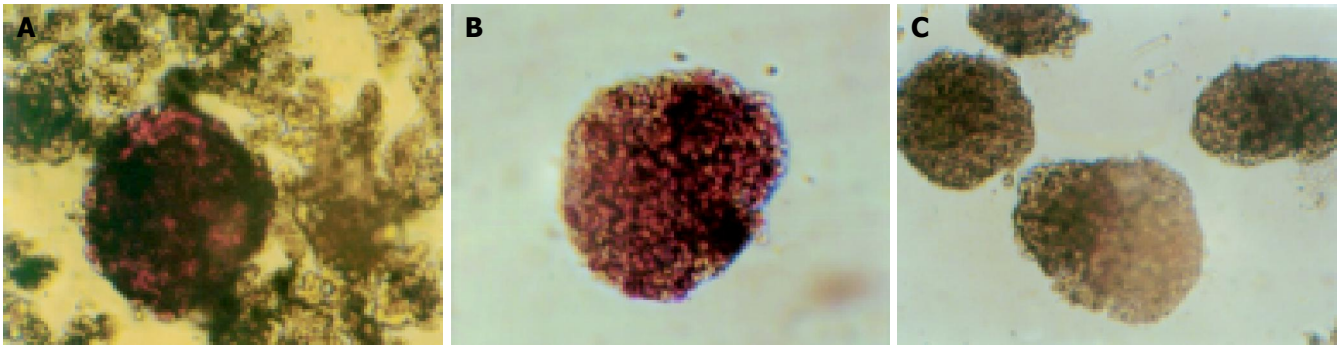

Figure 1 Islets stained crimson with DTZ (**A**), purified by density gradient separation and hand-picking (**B**), and loss of color 2 h after islet staining (**C**) (×100).

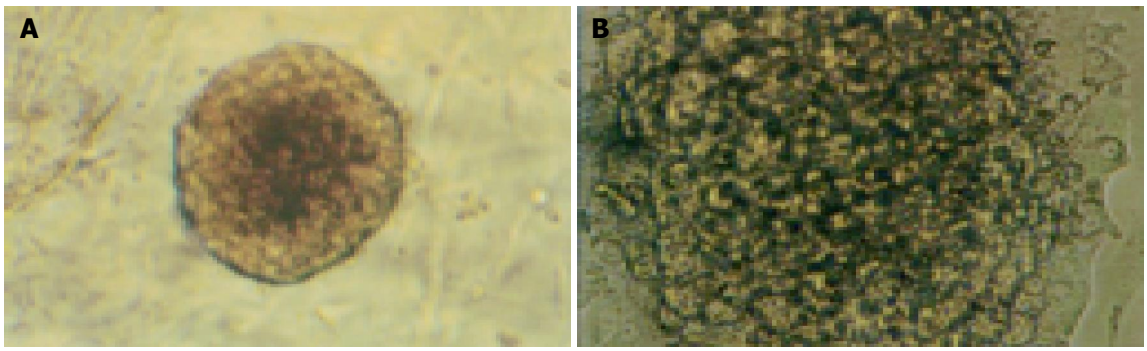

Figure 2 Shape of islets culture in collagen gel (**A** ×100) and in monolayer (**B** ×200).

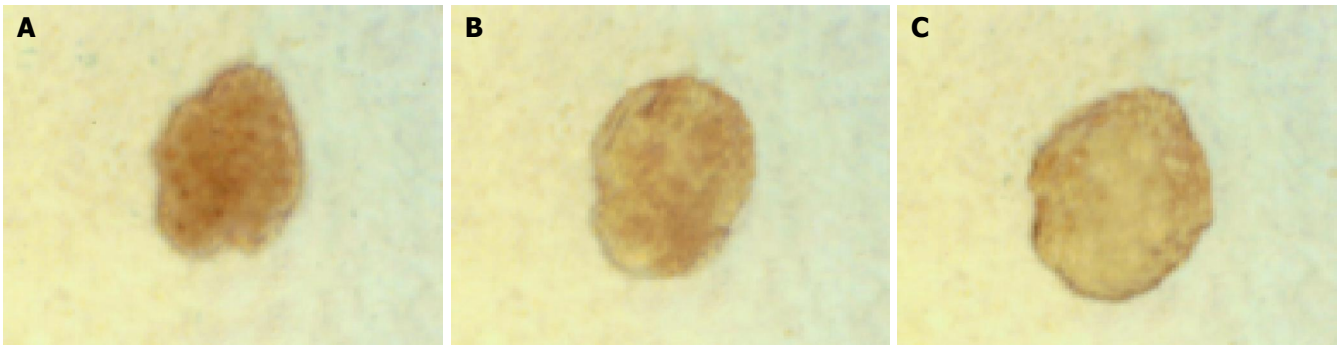

Figure 3 Islets of group 3 cystic transformed gradually in the process of culture. **A:** d 1 in culture; **B:** d 3 in culture; **C:** d 5 in culture (inverted microscope ×100).

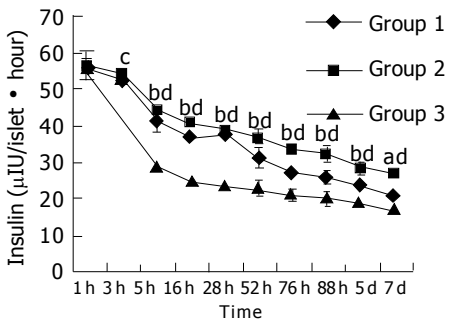

Figure 4 Insulin contents in culture media of three groups at different time points were detected using RIA. ($^{a}P<0.05$: group 1 *vs* group 3; $^{b}P<0.01$: group 1 *vs* group 3; $^{c}P<0.05$: group 2 *vs* group 3; $^{d}P<0.01$: group 2 *vs* group 3).

on d 3. Only a few cells in the islets expressed insulin continually on d 5, but most cells expressed cytokeratin 19 (Figures 5 and 6).

Reverse transcription and polymerase chain reaction

Freshly isolated islets expressed islet characteristic genes (pdx-1 and insulin) but did not express duct characteristic gene (cytokeratin 19) (Figure 7). After incubation for 7 d, islets in the three groups could express genes (insulin and pdx-1), but cytokeratin 19 was only expressed in group 3 (Figure 8).

DISCUSSION

Cells of both the exocrine (acinar cells) and endocrine systems (islet cells) seem to originate from the ductal cells. From

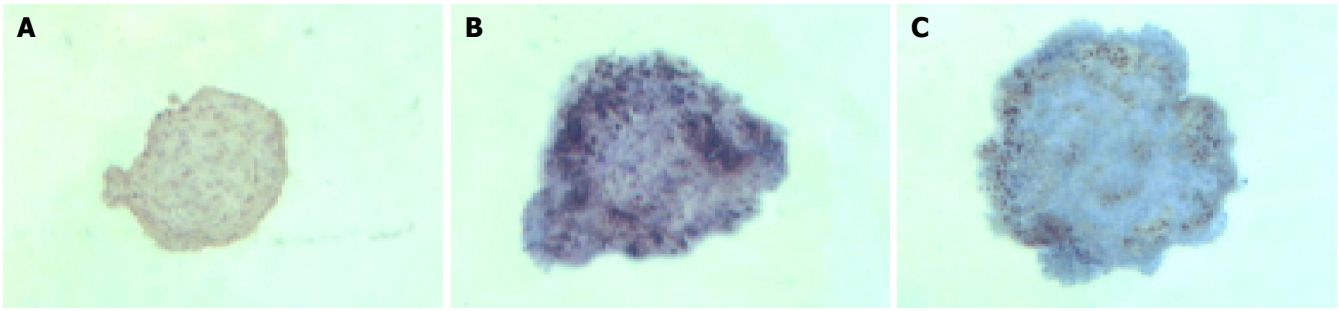

Figure 5 Islets of group 3 stained for insulin by immunocytochemistry. **A:** d 1 (strongly stain); **B:** d 3 (decreased stain); **C:** d 5 (lightly stain). (DAB stain and counterstained with hematine crystal ×100).

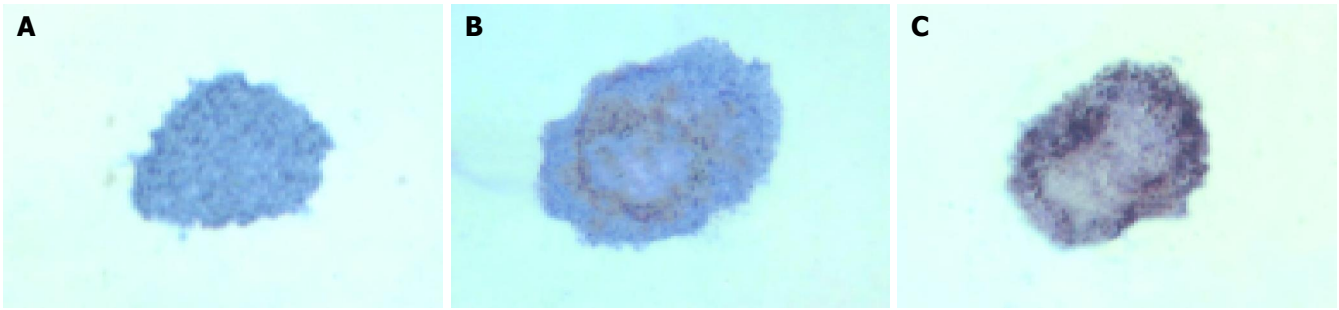

Figure 6 Islets of group 3 stain for cytokeratin 19. **A:** d 1 (did not stain); **B:** d 3 (lightly stain); **C**: d 5 (strongly stain).

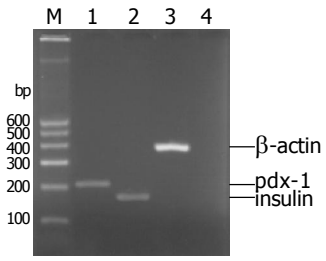

Figure 7 Pdx-1, insulin genes were expressed in freshly isolated islets, but cytokeratin 19 was not expressed (lane 1: Pdx-1; lane 2: Insulin; lane 3: β-actin; lane 4: cytokeratin 19).

experiments *in vitro* it is evident that during development, endocrine cells emerge from the pancreatic ducts and form aggregates that eventually form islets of Langerhans. Teitelman *et al*[11], removed pancreatic rudiments from E11 mouse embryos and maintained in culture, and found that pancreas (including exocrine and endocrine tissues) regenerates *in vitro* from E11 pancreatic ducts. Rosenberg *et al*[12], reported that partial obstruction of the pancreatic duct in adult hamsters leads to islet cell differentiation from cells in the interlobular ducts, followed by formation of new islets. More recently, Susan Bonner-Weir *et al*[5], cultivated human adult pancreatic duct cells *in vitro* and differentiated the duct cells into "cultivated human islet buds" (CHIBs) which have the ability to secrete glucose-response insulin.

On the other hand, islets can transdifferentiate into duct-like cells. Human and dog islets embedded in collagen gel and cultured in media plus EGF and CT for several days can transform and express duct cell marker CK-19[7,13]. In this study, we isolated rat islets and incubated them in the same conditions as above, and proved that rat islets could transdifferentiate into duct-like cells.

Extracellular matrix (ECM) is one of the most important components in creating cellular microenvironment. Among the ECM components, collagen can provide cells with a bio-

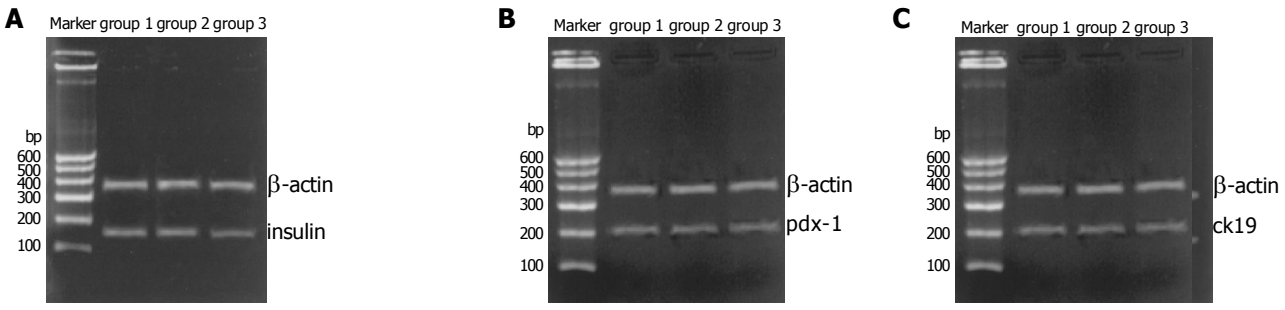

Figure 8 Gene's expression of islets of three groups cultured for 7 d; **A:** all of three groups expressed insulin; **B:** all of three groups expressed pdx-1; **C:** only group 3 expressed CK19 which group 1 and group 2 did not express.

mimic environment favorable for their reorganization, or maintenance of the three-dimensional structure. The use of collagen gel as an extracellular matrix material is an important part of the culture system in our study. Collagen gel matrix can help promote or maintain the differentiated state of cells in culture, such as liver cells[14] and mammary epithelial cells[15]. ECM may also promote the process of cell transdifferentiation[6,16]. Collagen plays an important role in the process. Islets cannot transdifferentiate in agarose gel[13]. ECM is the necessary condition for transdifferentiation. In our study, islets embedded in collagen gel without EGF and CT did not express CK-19, consistent with the report by Wang *et al*[13].

EGF can activate protein tyrosine kinase (PTK), protein kinase C and increase intracellular Ca^{2+} concentration after binding to EGF receptor[17]. CT can activate adenylate cyclase, and result in the increase of intracellular cAMP concentration[18]. It was reported that the process of cystic transformation requires both an elevation of intracellular cAMP and the presence of ECM proteins[13]. Both cAMP and Ca^{2+} are classic second messengers. Perhaps EGF and CT affect the signal transduction of islet cells in two ways by increasing the concentration of these messengers. In our study, we found that EGF alone had no ability to initiate islet cell transdifferentiation, but it could preserve islet's function. It is supported by the fact that both EGF and betacellulin are members of EGF-family of cytokines, and betacellulin is a kind of beta-cell growth factor, suggesting that EGF also can stimulate beta-cell growth.

In conclusion, the process of transdifferentiation is promoted by cooperation of collagen matrix, EGF and CT. But the exact mechanisms of interaction of these factors remain to be fully elucidated.

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Science Editor Wang XL and Guo SY **Language Editor** Elsevier HK