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MIN6 Is Not a Pure Beta Cell Line but a Mixed Cell Line with Other Pancreatic Endocrine Hormones

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Abstract. MIN6 cells retains glucose-stimulated insulin secretion (GSIS) as isolated islets. We comprehensively evaluated the gene expression and production of other islet hormones in MIN6 cells. Islet hormones were demonstrated by immunohistochemical staining and measured by ELISA. The gene expression profiles of MIN6 cells were compared with those in the mouse islets obtained by the laser capture micro-dissection (LCM). MIN6 cells excreted insulin, glucagon, somatostatin and ghrelin. They expressed mRNAs of insulin I and II, proglucagon, somatostatin, pancreatic polypeptide (PP) and ghrelin which were shown in the mouse pancreatic islet core and periphery obtained by LCM. A variety of genes closely related to the islet hormone producing cells were expressed in MIN6. Confocal laser scanning microscopy revealed that MIN6 cells included not only insulin positive cells but also insulin and glucagon or somatostin double positive cells. Glucagon, somatostatin and ghrelin were detectable in the culture medium. The present study clearly demonstrated that MIN6 produce pancreatic endocrine cells. It would be possible to use this cell line as a model to research the development, cell differentiation and function of pancreatic islets.

Key words: MIN6, Islet, Insulin, Glucagon, Somatostatin

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PANCREATIC endocrine cells include five subtypes: glucagon secreting α-cells, insulin producing β-cells, somatostatin yielding δ-cells, pancreatic polypeptide (PP) synthesizing γ-cells (PP cell) and ghrelin excreting ε-cells [1–5]. These cells form a cluster known as the pancreatic islet. The coexistence of different types of cells is thought to be required not only to maintain glucose-insulin homeostasis, a major role of the endocrine pancreas but also to develop the endocrine pancreas. In embryonic development of the pancreas, glucagon promotes the insulin producing cell development [6]. In the adult islets, these cells reveal a cooperative response to stimulation with nutrients, cytokines, chemical secretagogues, and neural input to secrete hormones [7].

To be freed from the bottleneck of using primary cultured β-cells in biochemical and molecular research, several insulin producing cell lines have been established, including HIT [8], MIN6 [9], βTC [10], RIN, and INS-1 [11]. Among these cell lines, MIN6 reveals the ability of glucose-stimulated insulin secretion (GSIS) similar to the isolated pancreatic islet, and has been widely used as the β-cell specific cell line [9, 12].

It is likely that insulin secreting cell lines form the islet-like structure including non-β cells to demonstrate their function and to possess an ability of highly active self-renewal. HIT-T15 and βTC-6 were reported to contain small amounts of glucagon and somatostatin [8, 10, 11]. MIN6 had been reported to have the character to form cell clusters [13]. It is well known that a monolayer culture showed less GSIS activity, and that the cell to cell contact was important to perform highly responsible GSIS [13–15].

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We comprehensively evaluated the gene expression, immunochemical staining of insulin, glucagon and somatostatin and hormone secretion of MIN6. To confirm the accuracy of findings in the MIN6 study, the gene expression profiles in MIN6 cells were compared with those in the mouse islets obtained by laser capture micro-dissection (LCM).

Materials and Methods

MIN6 cell culture

The MIN6 cell line was a kind gift from Dr. Junichi Miyazaki (Osaka University, Osaka, Japan) [9]. The cells, passage 22–42, were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml L-glutamine, and 5 μl/l β-mercaptoethanol in humidified 5% CO_2, 95% air at 37°C. Medium was changed every 48 hour, and cells were passaged once weekly following detachment using trypsin-EDTA.

The detached MIN6 cells were washed twice with PBS and pellets were frozen and stored at –80°C till the next use. MIN6 cells were also cultured in Lab-Tek Chemical Slide System (Nalge Nunc International, Naperville, IL) for the immunohistochemical staining of insulin, glucagon and somatostatin.

Mouse islet and laser capture micro-dissection (LCM)

The lean wild-type mice (BKS.Cg-m+/m+/Jcl) at 8 weeks of age were allowed free access to water and an ordinary laboratory diet. The mice were anesthetized by intraperitoneal injections of 0.05 mg/g pentobarbital, and the pancreas was removed and embedded in Tissue-Tek (Sakura Fine Technical Co. Ltd., Torrance, CA, USA) and frozen in liquid nitrogen within 30 sec. These studies were approved by the Animal Use Committee of the Kawasaki Medical School (no. 05-086) and were conducted in compliance with the Animal Use Guidelines of the Kawasaki Medical School.

The frozen pancreas was sectioned at 8 μm in a cryostat, mounted on uncoated glass slides, and the glass slides were placed on the dry ice, and then stored at –80°C. Slides containing the frozen section were immediately fixed in 70% ethanol for 30 sec stained with hematoxilin for 2 sec, followed by dehydration every 30 sec with 70, 95 and 100% ethanol and final 5 min dehydration with xylene. Once air-dried, the sections were laser micro-dissected with a PixCell system from Arctus Engineering (Mountain View, CA, USA) using 2 sec duration, 7.5 μm laser beam at 95 mV power. The islets, larger than 50 μm in diameter, were readily identified and captured the peripheral region of about 8 μm thickness, and then dissected the remained central core of the islet. By using this method, contamination of non-β cells and exocrine cells into the central core sample was absolutely avoided.

RNA isolation and real time RT-PCR

RNA was extracted from the mouse pancreatic islet dissected by LCM and MIN6 cells, and was purified using PicoPure RNA isolation Kit (Arcturus PN12206-01). The RNA which attached on the filter was exposed with RNase free DNase 1 (Qiagen, Kachidoki, Tokyo, Japan) according to the manufacture's instructions. For the real time RT-PCR, primers were designed according to the Primer Express software and the amplicon length was less than 151 bp (Table 1). The specificity of the amplicon sequence was confirmed by the nucleotide databank, NCBI-BLAST. The primers were put into a 96 well plate, and the plate was stored at –30°C until PCR analysis.

To use the amplicon, the length of which was shorter than 151 bp, led to the benefit of elevating the sensitivity using 45 cycles, resulting in the effective amplification of PCR. Instead of the previous method by Laybutt *et al.* [16], the use of the present method allows researchers to carry out gene expression analysis using small amounts of sample dissected by LCM. In addition, an application of the LCM method carries the more realistic *in vivo* information because of immediate freezing of the organ, and reduces the risk of artificial change of gene expression, especially apoptosis-related genes by collagenase digestion to isolate islets [17].

Reverse transcription was performed with random hexamers using TaqMan Reverse Transcription Reagents (AB N808-0234). PCR was performed with monitoring the increase in fluorescence of the SYBR Green dye (ABI, using the ABI 7700 sequence detection system, Applied Biosystems). The reverse-transcribed sample was applied on a 96 well plate to con-

Table 1. Primer sequences

target	GBC	Foreward and reverese primer sequence	Size
18SrRNA	X56974	GCGCTTCCTTACCTGGTTGAT GCCATTCGCAGTTTCACTGTAC	108
Ins1	NM_008386	CCCTTAGTGACCAGCTATAATCAGAGA ACCACAAAGATGCTGTTTGACAA	151
Ins2	NM_008387	CTGCTGGCCCTGCTCTTC AACCACAAAGGTGCTGCTTGA	70
proglucagon	NM_008100	GGCACATTCACCAGCGACTA TCATCAACCACTGCACAAAATCT	73
somatostatin	NM_009215	CTCTCCCCCAAACCCCATAT TTTCTAATGCAGGGTCAAGTTGAG	71
PP	NM_008918	GAAACTCAGCTCCGCAGATACA TGTTCTCCTCCTCGGCTCTCT	73
ghrelin	NM_021488	CCAAGAAGCCACCAGCTAAAC TGATCTCCAGCTCCTCCTCTGT	102
Iapp	NM_010491	GCCACGTGTGCCACACA GAGGACTGGACCAAGGTTGTTG	69
Glut2	NM_031197	TTGACTGGAGCCCTCTTGATG CACTTCGTCCAGCAATGATGA	73
Gck	NM_010292	CCCTGAGTGGCTTACAGTTC ACGGTAGTGAGTGTTGAAGC	144
Oct4	NM_013633	CCCGGAAGAGAAAGCGAACT TCGGGCACTTCAGAAACATG	75
Nanog	NM_028016	TGTGCACTCAAGGACAGGTTTC CAGGTTCAGAATGGAGGAGAGTTC	81
Sox2	NM_011443	CTGTTTTTTCATCCCAATTGCA CGGAGATCTGGCGGAGAATA	75
Kitl	NM_013598	CGTGTGACAGCATTTCTCATATTAGAC ACAGCACATCCTCTAAATTCTGGTT	92
Kit	NM_021099	ACACGTGCAGCAACAGCAAT TCGCTGTCTTCTTTGCCAAAC	109
nestin	NM_016701	AATGGGAGGATGGAGAATGGA GATCCTCGTCCCCATGCA	67
Lif	NM_008501	TCTGAAAACGGCCTGCATCTA CTGCGGCCAAGACCTTCAT	76
Lifr	NM_013584	TCCAGAGACTCCCGAGATCCT CCCTCTGTCGTTCCACTTCAG	79
Afp	NM_007423	TCTGCTGGCACGCAAGAAG TCGGCAGGTTCTGGAAACTG	66
Pdx1	NM_008814	CGGCTGAGCAAGCTAAGGTT TGGAAGAAGCGCTCTCTTTGA	71
Ngn3	NM_009719	GCGCAAGAAGGCCAATGA CAGCGCCGAGTTGAGGTT	58
NeuroD	NM_010894	AGGAACACGAGGCAGACAAGA CTCCCCCGTTTCTCAGAGAGT	78
Nkx6.1	NM_144955	CAAACCTCTGGACCCGAACTC GCTGCCACCGCTCGATT	76
Nkx2.2	NM_010919	CACAGGTCAAGATCTGGTTCCA GCGTCACCTCCATACCTTTCTC	78
Pax4	NM_011038	GGCTTCCTCCTCATATGTGGAA TATGGCCAGTTTGAGCAATGG	114
Pax6	NM_013627	TGGAAACAAACGCCCTAGCT GGACAGGGAACACACCAACTTT	71
Bran4	X66603	CCTGCTCCAAACTCGTCCAT GCCTGGCTGCGAGTACACAT	63
Cckbr	NM_007627	TGGTCCGTGCTTCTGCTAATACT CCATAGGCCACCGCCATA	65

GBC: GENBANK accession number, Size: Amplicon size (bp)

tain 0.5 μl of the sample in one well. Final primer concentrations were 50 nM and total reaction mixture was 10 μl. The reaction was carried out at 95°C for 15 sec, and annealing/extension at 60°C for 1 min in a PCR of 45 cycles. As an internal gene control, 18S rRNA was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The quantity of gene expression was calculated as relative mRNA levels normalized to the internal control 18S rRNA. The specificity of each primer pair was confirmed by a melting curve analysis and an agarose gel electrophoresis of PCR products. Agarose-gel electrophoresis was performed using the standard method of 3.0% gel concentration.

Assays of pancreatic hormone contents and amylase activity

MIN6 cells were cultured in the recommended condition and 80% confluent cells were used after washing. Pellets of MIN6 cells were dissolved by gentle pipetting with ice cold T-PER (Tissue Protein Extraction reagent; Pierce, Rockford, IL) containing Halt Protease Inhibitor Cocktail Kit and EDTA for the assay of insulin and somatostatin. Aprotinin, a final concentration 500 KIU/ml, was added for the assay of glucagon and ghrelin. After centrifugation at 15,000 rpm for 5 min, the supernatant was used to assay the peptide hormones. Insulin was measured by ELISA kit (Morinaga, Yokohama, Japan), glucagon by YK090 Glucagon EIA Kit (Yanaihara, Shizuoka, Japan), somatostatin by Enzyme Immunoassay Kit (Phoenix Pharmaceuticals, Burlingame, CA) and ghrelin by Active Ghrelin ELISA Kit (Mitsubishi Chemical and Yatron, Tokyo, Japan). Amylase activity was measured using the clinical laboratory kit, AMYY Gal-CNP (Mizuho Medy, Tosu, Saga, Japan). The concentrations of the samples were normalized by protein content of the lysate determined by a Bio-Rad Protein Assay kit.

Immunohistochemical staining

After being cultured in Lab-Tek Slide system for 3–4 days. MIN6 cells on the slide were rinsed with phosphate buffered saline (PBS) and fixed with 4.0% formaldehyde solution in PBS for 30 min. Then the cells were washed with 10 mM glycine in PBS and were incubated with 2% Donkey serum in PBS for 15 min to block nonspecific binding, followed by two sequential 50 min incubations at 37°C with primary and secondary antibodies following 5 times wash by 0.5% BSA in PBS, respectively. The primary antibodies were used at the indicated dilutions: rabbit anti-insulin (1 : 500), goat anti-glucagon (1 : 500) or goat anti-somatostatin (1 : 500) (Santa Cruz Biotech, Santa Cruz, CA). Corresponding secondary antibodies were Donkey anti-rabbit IgG Alexa Fluoro 488 and Donkey anti-goat IgG Alexa Fluoro 594 (Invitrogen, Eugene, OR) and each used at a 1 : 500 dilution. The cells were immersed with Vectashield with DAPI (Vector Labs, Burlingame, CA). The coverslips were mounted and sealed with manicure on object slides, and images were taken with a Leica TCS MP2 confocal laser scanning microscope (Leica Microsystems Inc., Tokyo). DAPI image for DNA was taken sequentially using two photon microscopy.

Statistical analysis

Each value represents the mean ± SEM. The experimental number is described in each figure legend.

Results

Immunohistochemical staining and hormone related genes in MIN6

Insulin-positive cells were predominantly seen, and insulin and glucagon/somatostatin double positive cells were also observed (Fig. 1A, 1B).

Gene expression profiles related to pancreatic hormones in MIN6 and mouse pancreatic islets. MIN6 expressed proglucagon, somatostatin, PP and ghrelin as well as insulin I and II (Fig. 2). Amylase mRNA was also detectable in MIN6 cells, and was confirmed to be identical in PCR product size with that of the periphery sample incidentally contaminated with acinar cells.

Using the LCM method, the core and peripheral regions of the islet were separately dissected. The peripheral cells expressed genes of both β-cell and non-β-cell such as insulin I, insulin II, proglucagon, somatostatin, PP, and islet amyloid polypeptide (IAPP) (Fig. 2 and Table 2). Gene expressions of insulin I and II, IAPP and ghrelin were shown in the core area, while proglucagon, somatostatin and PP

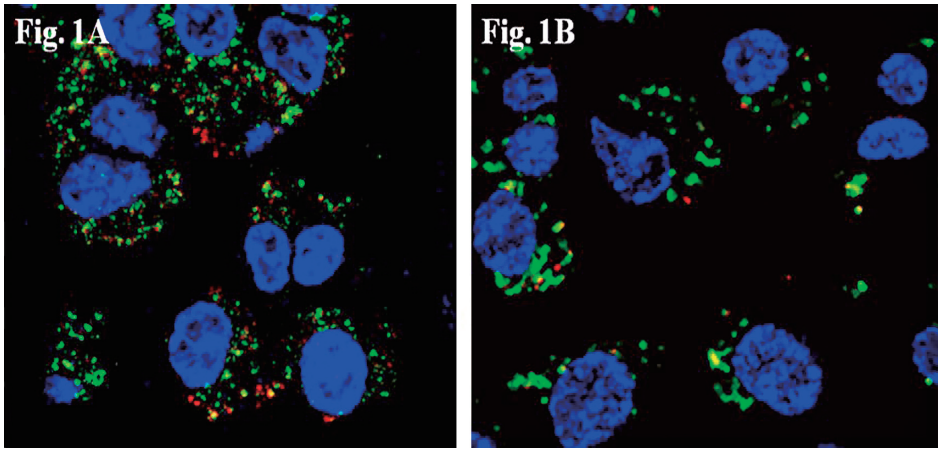


Fig. 1. MIN6 cells include insulin positive cells and insulin and glucagon/somatostatin double positive cells. (A) Confocal laser scanning and two photon microscopy of MIN6 cells (×1000). Green: insulin, Red: glucagon, Blue: nucleus. (B) Confocal laser scanning and two photon microscopy of MIN6 cells (×1000). Green: insulin, Red: somatostatin, Blue: nucleus.

were not in the core at all. Agarose gel electrophoresis of PCR products demonstrated that amplicon sizes of glucagon, somatostatin and PP from the islet periphery were identical to those from MIN6 cells (Fig. 2).

Gene expression profiles related to cell development, proliferation and differentiation in MIN6 and mouse pancreatic islets

MIN6 expressed Oct4, Nanog, Sox2, Kitl, Kit, Nestin, Lif, Lifr, and α-fetoprotein, which were reported to be observed in the embryonic development of pancreas [18–24] and a pancreatic undifferentiated-cell marker [19]. Furthermore, Pdx1, Ngn3, NeuroD, Pax4, Nkx6.1, Nkx2.2, Brain 4 and CckBr genes were expressed in MIN6 as shown in Table 2.

The Pdx1, NeuroD, Nkx6.1, Nkx2.2 and Pax6 genes were also expressed in the pancreatic islet, while Ngn3, Brain4 and cholecystokinin B receptor were not expressed in the islet (Table 2). A Kit gene expression was detected in the islet core area but not at the periphery, and glucokinase (Gck) and Glut2 genes specific to the β cell function were also expressed more significantly in the core than at the periphery (Table 2). These observations were supported by the previous result demonstrated that the β cell function is more active in the core than at the periphery [25, 26].

Interestingly, exocrine pancreas-related *Hes1*, *Ptf1a*, *Mist1*, Cpa, *Cx32* and *Amyl2* gene expressions were also detected in MIN6 cells (data not shown). The *Hes1*, *Ptf1a* and *Mist1* are known to promote a differentiation of the progenitor cell into the exocrine cell [22, 27, 28], and *Cx32* is necessary to maintain the structure and function of the pancreatic exocrine gland [29].

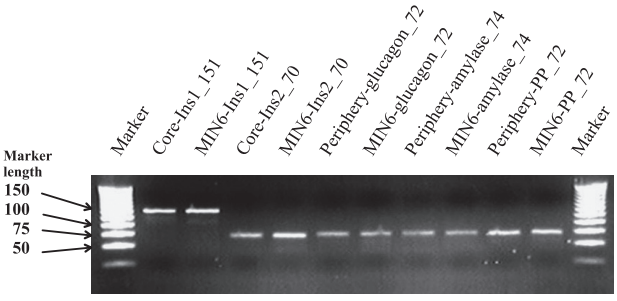


Fig. 2. Agarose gel electrophoresis of PCR products from MIN6 cells and the islet core and periphery tissues. The amplicon length of each gene is shown as follows. M (markers); 25, 50, 75, 100, 150 bp, 1. Ins1 (islet core); 151 bp, 2. Ins1 (MIN6); 151 bp, 3. Ins2 (islet core); 70 bp, 4. Ins2 (MIN6); 70 bp, 5. proglucagon (islet periphery); 73 bp, 6. proglucagon (MIN6); 73 bp, 7. Amylase (islet periphery) 74 bp 8. Amylase (MIN6) 74 bp, 9. PP (islet periphery); 73 bp, 10. PP (MIN6); 73 bp.

Pancreatic hormone production in MIN6

The hormone contents in the MIN6 cell lysate and the culture media were shown in Table 3. A significant amount of insulin, glucagon, somatostatin, and ghrelin were detected in the MIN6 cell lysate and culture media at 80% confluence.

Table 2. Relative mRNA levels of PCR products from MIN6 and the islet core and periphery were normalized to the internal control 18Sr RNA. Each result indicates the mean and SEM of 5 separate experiments

	islet core		islet periphery		NIN6	
	mean	SEM	mean	SEM	mean	SEM
Ins1	1.12836	0.61642	0.34564	0.18586	0.41716	0.20858
Ins2	7.52169	3.98105	3.16452	0.91440	6.21592	3.10796
proglucagon	0	0	0.06209	0.02665	0.00690	0.00345
somatostatin	0	0	0.00471	0.00263	0.01282	0.00459
PP	0	0	0.00254	0.00136	0.00882	0.00441
ghrelin	0.00238	0.00161	0	0	0.00045	0.00033
Iapp	0.26745	0.20043	0.15678	0.03253	0.16714	0.08357
Glut2	0.09311	0.07603	0.00002	0.00002	0.00885	0.00132
Gck	0.00011	0.00007	0.00005	0.00005	0.00020	0.00011
Oct4	0	0	0	0	0.00020	0.00008
Nanog	0	0	0	0	0.00574	0.00288
Sox2	0	0	0	0	0.00012	0.00008
Kitl	0	0	0	0	0.00471	0.00263
Kit	0.01585	0.00904	0	0	0.00629	0.00352
nestin	0.00008	0.00007	0.00742	0.00606	0.00988	0.00111
Lif	0	0	0	0	0.00050	0.00034
Lifr	0	0	0	0	0.01117	0.00698
Afp	0	0	0	0	0.00360	0.00190
Pdx1	0.00152	0.00064	0.00305	0.00111	0.01061	0.00211
Ngn3	0	0	0	0	0.00980	0.00218
NeuroD	0.00462	0.00427	0.00487	0.00072	0.08426	0.03722
Nkx6.1	0.00385	*	0.00037	*	0.02401	0.01575
Nkx2.2	0.00237	0.00151	0.00372	0.00205	0.01173	0.00475
Pax4	0	0	0	0	0.00820	0.00336
Pax6	0.00070	0.00053	0.00321	0.00317	0.01783	0.00641
Bran4	0	0	0	0	0.03250	0.00530
CckBr	0	0	0	0	0.00590	0.00130

* detected in only one sample

Table 3. The contents of insulin, glucagon, somatostatin and ghrelin, and amylase activity in the MIN6 cell lysate and the culture media of 80% confluent. Each result reveals the mean and SEM of 5 separate experiments

	Lysate			Medium		
	mean	SEM	units	mean	SEM	units
insulin	1578.9	552.7	μg/g protein	7179	872.3	μg/ml
glucagon	11.8	1.21	ng/g protein	3.08	3.29	ng/ml
somatostatin	46.4	9.4	pg/g protein	46.5	9.6	pg/ml
ghrelin	820.1	320.2	pg/g protein	22.1	6.6	pg/ml
amylase	134	2.1	IU/g protein	0		IU/ml

Deacyl ghrelin was not detected in the MIN6 lysate and culture medium, indicating that ghrelin was not degraded at all. The amylase activity was detectable in the lysate, but not in the culture medium (Table 3).

Discussion

The present study clearly demonstrated that MIN6 cells produced islet hormones such as insuin, glucagon, somatostatin, and ghrelin. The immunohistochemical study revealed that MIN6 cells included

predominantly insulin positive cells, and also insulin and glucagon/somatostatin double positive cells. These observations may suggest that MIN6 is primarily insulin producing cell line and may possess a suitable environment to secrete insulin by concomitant secretion of other islet hormones.

It remains to be elucidated how MIN6 cells acquire the ability to produce non-insulin hormones. One possible explanation would be that MIN6 is tumor derived cell line and possesses a phenotype of tumor atypism. In comparison with islet cells, MIN6 cells expressed the genes related to immature primitive cells and required to islet cell maturation.

In this study, MIN6 cells expressed Brain 4 and cholecystokinin B receptor. The Brain 4 is known to be the α cell differentiation factor, and cholecystokinin B receptor is specific to the δ cells, respectively [30, 31]. These results also support a hypothesis that MIN6 cells would produce a variety of pancreatic hormones. The existence of glucagon in the medium suggests that glucagon promotes a differentiation of insulin producing cells as well as shown in the embryonic development of pancreas [6].

Pdx1, NeuroD, Nkx6.1, Nkx2.2, Pax6, Ngn3, Pax4, Bran4 and CckBr are known to constitute a differentiation cascade in islet cells [22], whereas Oct4, Nanog, Sox2 and Lif/Lifr are thought to suppress a differentiation and to maintain a self-renewal activity. On the other hand, Ngn3, a multipotent islet stem cell marker, was not detected in the pancreatic islet, suggesting that the cell renewal mechanism in MIN6 is different from that in the adult pancreatic islet. Rachdi *et al.* demonstrated that Kit was expressed both in the mature β cells and in the insulin producing cell line, INS1[18]. In the adult islets, the Kit might act mainly in the signal transduction pathway as a membrane bound receptor tyrosine kinase [18]. Several reports demonstrated the presence of Nestin and Kit in islet cell development as pluripotential stem cells [19–21]. Thus it is possible that the MIN6 cell line may have pluripotential stem cells although we cannot make a conclusion in this study.

Ghrelin was originally identified in the rat stomach as the endogenous ligand for the growth hormone serectagogue-receptor, and was proposed as the fifth hormone of the pancreatic islet [1–5]. MIN6 cells produce ghrelin. The physiological function of ghrelin in the pancreatic islet has not been fully understood, but it might be possible that ghrelin displays a paracrine action on the adjunct β cells in regulating insulin secretion [5]. Granata *et al.* reported that HIT-T15 cells expressed ghrelin mRNA and peptide and suggested that acylated and unacylated ghrelin promote survival of β cells and islets [32]. We showed MIN6 cells excreted only acylated form. MIN6 thus might be suitable material for ghrelin research.

We observed Ptf1a and Cpa gene expressions, markers of exocrine pancreas, in MIN6 cells (data not shown). In addition to amylase gene expression, the amylase activity was observed in the cell lysate but not in the culture media (Table 3). A significant amount of insulin in the culture media may possibly activate the insulin responsive element (IRE) located in the pancreatic enhancer of amylase gene [33].

In conclusion, the present study clearly demonstrated that MIN6 produce pancreatic endocrine cells. It would be possible to use this cell line as a model to research the development, cell differentiation and function of pancreatic islets. We can analyze the culture media of MIN6 as a model of interstitial fluid in the islet for the autocrine/paracrine research.

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