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Transcriptome Analysis of Mesenchymal Stem Cells Differentiated into Insulin-Producing Cells Reveals Dissimilarities with Pancreatic Beta Cells in Response to Glucose

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Abstract

The efficient differentiation of mesenchymal stem cells (MSCs) into functional insulin-producing cells (IPCs) provides an attractive approach to strategies of cell transplantation for curing diabetes. However, it is noticed that differentiated MSCs to IPCs do not behave like native beta cells in view of insulin secretion quantity and response to glucose. Here, gene expression profiling by DNA microarray technology was recruited to compare 10K genes between rat pancreatic beta cells with IPCs differentiated from MSCs. Moreover, gene expression profiles of both were compared after glucose stimulation. Data were confirmed by RT-PCR and insulin secretion assay. The results revealed great gene profile differences between beta cells and differentiated IPCs under basal and stimulatory glucose conditions. Although IPCs were responsive to glucose stimulation, the insulin output and stimulatory index were lower than that in beta cells. These results suggest that the applied HGF-EGF differentiation protocol is insufficient for inducing beta-cell-similar IPCs from MSCs.

Keywords: Mesenchymal stem cells, gene expression profiling, DNA microarray, stem cell differentiation, insulin producing cells, pancreatic islets.

Introduction

Transplantation of insulin-producing cells (IPCs) derived from mesenchymal stem cells (MSCs) may represent an alternative to cure diabetes mellitus. Several studies have described the successful differentiation of bone marrow MSCs cells into IPCs [1-8]. MSCs are mostly differentiated into IPCs by one of 2 methods. The first depends on the chemical manipulation of the microenvironment around the cells by some modifications of the culture medium. In this method, which is mostly applied, the addition of some growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) and nicotinamide was shown to be sufficient for the conversion of MSCs to IPCs [8-14]. In the other method, differentiation can be achieved by genetic manipulations. As a result of both differentiation protocols, IPCs were able to secrete insulin, but so far these cells were not as efficient as native beta cells. Differences in cell shape, phenotypic characters and in insulin response to glucose have been observed.

Here, gene expression profiling may help to investigate the similarity of the transcriptome between beta cells and differentiated stem cells. The insulin-secreting beta cells express specific genes that are essential for the development and/or function of these cells. In addition to insulin gene, the signal for insulin release from the β-cells is mediated by uptake of glucose via the low affinity glucose transporter type 2, which is encoded by the gene *Glut2*. For *Insulin* expression to be achieved, a number of transcription factors have been shown to play important roles during differentiation of pancreatic endocrine cells and in insulin secretion process. *Nkx6.1* and *Nkx2.2* act to assure β-cell function. In mice mutant for these genes the specification of β-cells are specified but fail to terminally differentiate [15, 16]. *Pax4* is another transcription factor needed for the specification of both β-and δ-cells [17, 18]. *Pax6, Isl1* and *NeuroD* are expressed in differentiated pancreatic endocrine cells. Deletions of any of these genes resulted in mice with perturbed pancreatic endocrine cell differentiation [19-21]. Also, lacking *Ngn3* expression or function fail to generate any pancreatic endocrine cells and die postnatally from diabetes [22, 23]. *Pdx1* has a dual role being required both for early pancreas development and for the proper function of insulin secreting β-cells [24, 25]. All of these beta cell marker genes must be upregulated during the differentiation of insulin-secreting cells. On the other side, other genes such as stem cell – specific and cell cycle activation genes should be downregulated during the differentiation process. DNA microarray technology has served in several studies to follow the gene expression profiling during the embryonic stem cell differentiation [26-28], but little is known about the gene profile of MSCs differentiation to IPCs.

In the present study, the transcriptome of differentiated MSCs to IPCs has been compared with that of beta cells and undifferentiated MSCs. It is shown that validating the differentiation status of MSCs to IPCs by microarray analysis and real-time RT-PCR using beta cell markers revealed that a dissimilarity exists between the transcriptome of beta cells and the MSCs-derived insulin-secreting cells in response to glucose stimulation.

Material and Methods

Mesenchymal Stem Cell Isolation, Culture and Differentiation into Insulin-Producing Cells

Rat bone marrow was isolated by flushing femurs and tibias by DMEM as described by Zhang and Chan [29]. All bone marrow cells were cultured for 4 days and the plastic adhered cells were washed several times and cultured until reached confluency. MSCs in passage 3 were used for differentiation. The differentiation into insulin-producing cells (IPCs) followed the method applied by most laboratories [eg. 8-11] including ours [12-14]. The islet beta cell-conditioning medium was DMEM with 5.5 mmol/L glucose, and contained HGF (hepatocyte growth factor, Sigma-Aldrich, cat. H9661, 20 ng/ml), EGF (epidermal growth factor, Sigma-Aldrich, cat. E9644, 20 ng/ml) and nicotinamide (Sigma-Aldrich, 10 mmol/L). MSCs were cultured in this differentiation medium for 3 weeks, and then tested for insulin and other islet genes expression and insulin secretion. For investigating the effect of stimulation with glucose, cells (MSCs or IPCs) were cultured in RPMI media containing either 2.8 (basal) or 22.4 mmol/L (stimulatory) glucose for 24 hr.

Isolation and Culture of Rat Islets

Adult rat islets were isolated and cultured as described in details in our previous work [30]. For investigating the effect of stimulation with glucose, islets were cultured in RPMI media containing either 2.8 (basal) or 22.4 mmol/L (stimulatory) glucose for 24 hr.

Microarray Analysis

Three tissues under 2 conditions were used for gene profiling. Tissues were rat MSCs, rat differentiated IPCs and rat islets, while conditions were culturing in either low (2.8

mmol/L) or high (22.4 mmol/L) glucose for 24 hr. Total RNA was extracted from tested tissues using a RNeasy Mini Kit (Qiagen). DNase I treatment of isolated RNA was used after extraction to exclude any DNA interference in the labeling reaction and during hybridization. First strand cDNA and dsDNA were synthesized and labelled using Express Art mRNA Amplification Kit (Micro Version, Amp Tec, Germany). During reverse transcription, fluorescent-labeled nucleotides were incorporated into the produced first strand cDNA. The first strand cDNA was then separated from the RNAse-degraded template RNA, primers, unincorporated nucleotides, and RNA debris. A microarray chip (*Rat 10K OciChip,* Ocimum BioSolutions), which can be loaded with 2 different samples have been applied. The two sets of differently labeled cDNAs were combined and co-hybridized to the same *OciChip*. After hybridization, unbound and non-specific fixed cDNA was removed by thoroughly washing the array. After scanning of the array using a Gene Array scanner into a microarray image, the fluorescence intensity of each spot, and the ratio of the expression levels between the two cell populations were analyzed by ImaGene software (Biodiscovery).

Real Time PCR

To confirm microarray data, the expression of some specific beta cell genes has been analyzed by RT-PCR as described elsewhere [31]. The following primers have been used: rat *Ins1* 5'-AGGCTCTGTACCTGGTGTGT-3' (forward) and 5'-AGTTGGTAGAGGGAGCAGATG-3' (reverse), *Glucagon* 5'-CTTCCCAGACAGAACCACTTG-3' (forward) and 5'-CTGGCCCTCCAAGTAAGAACT-3' (reverse), *Glut2* 5'-AGCACATACGACACCAGACG-3' (forward) and 5'-TCAAGAGGGCTCCAGTCAAC-3 (reverse), *β-actin* 5'-ACCGTGAAAAGATGACCCAGATC-3' (forward) and 5'-GACCAGAGGCATACAGGGACAAC-3' (reverse).

Insulin Measurement

After the 24 hr incubation with either basal or stimulatory glucose, rat insulin was determined in the supernatant using ELISA kit from DRG diagnostics, Germany (EIA-2048).

Statistical Analysis

Data are presented as mean±SEM. Analysis of variance (ANOVA) was applied for the statistical analysis followed by t-test as a post-hoc test. A $p<0.05$ was considered as significant in all cases.

Results and Discussion

The aim of the present study was to investigate the change in the gene expression profile of bone marrow – derived mesenchymal stem cells that are differentiated to insulin-secreting cells and compare this profile with that of the native insulin-secreting pancreatic beta cells. Thus, the transcriptome of three tissues (bone marrow mesenchymal stem cells MSCs, differentiated insulin-producing cells IPCs, and pancreatic islets) have been examined after culture with low (basal) or high (stimulatory) glucose concentrations. This transcriptome has been analyzed by the DNA microarray technology, which can describe, in a semi-quantitative way, 10,000 of actually active genes in a target cell.

Before the application of microarray analysis, IPCs were proven to be functional, regarding both glucose-stimulated insulin secretion and insulin gene expression. Although IPCs were able to secrete insulin in response to glucose, the present results showed that none of the beta cell marker genes have been found in the uppermost upregulated genes (Table 1) during the differentiation of MSCs to IPCs. Instead, the uppermost upregulated genes were related to stress tolerance, indicating that the differentiation of MSCs to IPCs is a stressful process. For example, ubiquitin C, *Ubc*, which expression increased about 20x in IPCs more than MSCs, is known to be induced during stress. It provides ubiquitin protein necessary to remove damaged or unfolded proteins, kinase activity, DNA repair and many other related biological processes [32-34]. Similarly, peroxiredoxin 6 and glutathione S-transferase participate in the protection against oxidative injury [35], and thioredoxins reduce oxidative stress through their response to reactive oxygen species [36]. *Max* protein represses *MYC* transcriptional activity from E-box elements and negative regulation of G0 to G1 transition [37], which is a sign of differentiation. Most other activated genes were related to metabolism, oxidative phosphorylation and mitochondrial function.

Table 1. Upregulated and downregulated genes involved in the differentiation of mesenchymal stem cells into insulin-producing cells

	UPREGULATED Gene ID	Symbol	Signal ratio	DOWNREGULATED Gene ID	Symbol	Signal ratio
1	sodium channel, voltage-gated, type 6, alpha polypeptide	*Scn6a*	57.11	electron-transfer-flavoprotein, beta polypeptide	*Etfb*	-45.08
2	ubiquitin C	*Ubc*	19.81	protein tyrosine phosphatase, non-receptor type 11	*Ptpn11*	-28.54
3	S100 calcium binding protein A10 (calpactin)	*S100a10*	8.84	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	*Lrat*	-27.11
4	nuclear factor of kappa light chain enhancer in B-cell inhibitor, alpha	*Nfkbia*	7.84	glutathione S-transferase M4	*Gstm4*	-27.03
5	peroxiredoxin 6	*Prdx6*	7.18	gap junction membrane channel protein beta 3	*Gjb3*	-19.40
6	thioredoxin 1	*Txn1*	5.47	lysyl oxidase	*Lox*	-17.90
7	proteasome (prosome, macropain) subunit, beta type 4	*Psmb4*	5.34	chemokine (C-C motif) ligand 7	*Ccl7*	-17.75
8	heat shock protein 4	*Hspa4*	5.19	Protein kinase, interferon-inducible double stranded RNA dependent	*Prkr*	-15.61
9	keratin complex 1, acidic, gene 18	*Krt1-18*	5.14	Janus kinase 1	*Jak1*	-14.88
10	Max protein	*Max*	5.12	tumor necrosis factor receptor superfamily, 4	*Tnfrsf4*	-14.26
11	alpha-spectrin 2	*Spna2*	5.03	collagen, type 1, alpha 1	*Col1a1*	-13.51
12	annexin A2	*Anxa2*	4.91	clusterin	*Clu*	-13.38
13	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	*Ndufb9*	4.77	bone mar row stromal cell antigen 1	*Bst1*	-13.27
14	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5	*Ndufa5*	4.57	chemokine (C-C motif) ligand 20	*Ccl20*	-12.98
15	cytochrome c oxidase, subunit VIc	*Cox6c*	4.51	lipocalin 2	*Lcn2*	-11.68
16	platelet-activating factor acetylhydrolase, isoform Ib, alpha	*Pafah1b1*	4.50	matrix metallopeptidase 12	*Mmp12*	-10.39
17	myotubularin related protein 2 (predicted)	*Mtmr2*	4.50	acyl-CoA synthetase long-chain family member 4	*Acsl4*	-10.31
18	inositol hexaphosphate kinase 1	*Ihpk1*	4.35	regulating synaptic membrane exocytosis 1	*Rims1*	-10.14
19	tubulin, alpha 1	*Tuba1*	4.31	androgen regulated 20 kDa protein	*Andpro*	-9.40
20	ubiquinol-cytochrome c reductase binding protein	*Uqcrb*	4.20	biglycan	*Bgn*	-9.26
21	cytochrome c oxidase, subunit XVII assembly protein homolog	*Cox17*	4.17	transgelin	*Tagln*	-9.19
22	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	*Sdhb*	4.15	interleukin 6	*Il6*	-8.53
23	glutathione S-transferase, pi 2	*Gstp2*	4.14	coagulation factor III	*F3*	-7.54
24	desmin	*Des*	4.14	fibrinogen, B beta polypeptide	*Fgb*	-7.48
25	pancreatic lipase	*Pnlip*	4.10	striatin, calmodulin binding protein	*Strn*	-7.43

Signal ratio is the ratio of fluorescence signal of IPCs/MSCs. Only the first 25 genes of a total of 10K genes are mentioned in the table. Many data of ribosomal and tRNA related genes have been removed from the upregulated genes list.

Upregulated genes of pancreatic islet development and function were found in the middle of the list of upregulated genes. Table 2 summarizes the upregulation level of some of these genes. *Insulin 2* was the most upregulated gene and its expression increased by 3.17x in IPCs. The values of upregulation of insulin and other genes as glucagon, the glucose transporter *Glut2* and also that of other transcription factors seem to be lower than expected. To confirm this, data of stimulation by glucose for the same cells in comparison with islet cell data were also included in Table 2. Glucose-stimulated insulin and glucagon gene expressions in islets 28.68 x and 21 x times that in IPCs, respectively. This great difference was not repeated in most of the transcription factor genes. However, the expression of the essential genes *pdx1* and *Glut2* was also 2.55 x and 3.36 x times higher in islets than in IPCs. Taken together, these data reveal the unequal response of islet-specific genes in islets and IPCs differentiated from MSCs. Also, it is logically expected that due to this comparatively weak response in these gene expressions, the insulin – and also glucagon – secretions would be lower from IPCs than from islets.

Table 2 Genes involved in pancreatic islet differentiation and function

Gene Symbol	IPCs vs MSCs	islet vs IPCs, high glucose
Insulin 2	3.17	28.68
Glucagon	2.33	20.99
somatostatin	-0.12	-2.73
Pdx1	2.41	2.55
Glut2	2.55	3.36
Neurog3	1.63	0.34
c-Maf	1.32	-0.04
Isl2	2.13	0.09
Pax6	2.82	2.61
Nkx6-1	2.09	0.52
Neurod3	1.51	-0.21
Isl2	2.13	0.09
Cdx2	2.31	-0.08

Values are the signal ratio of IPCs : MSCs or islet : IPCs for each gene. The minus values mean MSCs was superior to IPCs, or IPCs was superior to islets.

The effect of stimulation with glucose on insulin gene and other islet-specific gene expressions in IPCs, islets, and MSCs has been compared (Table 3). Samples of the 3 tissues have been cultured for 24 hr in either basal or stimulatory glucose concentrations. Labeled samples of the same tissue but from both culture conditions were arrayed on the same microarray chip. The results (Table 3) revealed that glucose-induced a moderate increase in insulin gene (4 times more than the basal value) and hardly any change in glucagon gene expression. In islets, glucose-stimulated 21 times increases in insulin gene expression than the basal value. It inhibited glucagon gene 15x and also induced a 12x increase in *Glut2* expression. The effect on glucagon gene may be indirect. It was reported that glucose itself induces glucagon secretion from isolated alpha cells, but the inhibition in islets is derived by the inhibitory effect of the secreted insulin and its paracrine action [38]. Thus, although islet gene expressions in IPCs were highly responsive to glucose, their response was weak, as compared to that of islets.

Table 3. Gene profile of beta cell – specific genes in islets and IPCs after stimulation with glucose

	Glucose stimulation		
Gene Symbol	IPCs	islet	MSCs
Insulin 2	4.06	21.19	0.95
Glucagon	-0.27	-15.28	-0.86
somatostatin	2.31	2.78	1.07
Pdx1	2.61	2.47	0.43
Glut2	3.23	12.00	1.66
Neurog3	3.08	-0.63	0.34
c-Maf	2.15	1.99	-1.94
Isl2	1.99	2.38	0.67
Pax6	2.00	2.97	5.14
Nkx6-1	2.60	1.84	-1.87
Neurod3	2.06	1.50	1.29
Isl2	1.99	2.38	0.67
Cdx2	1.96	2.10	0.58

Values are the signal ratio of glucose-stimulated / basal conditions in IPCs, islet and MSCs for each gene. The minus values mean downregulation of gene expression by glucose.

The results showed in Table 4 that glucose worked variably in both systems (IPCs and islets). The uppermost genes upregulated by glucose in islets were *Insulin* and *Glut2*, whereas most upregulated enzymes in IPCs were metabolic enzymes, including some involved in glucose metabolism, but not insulin secretion. These results revealed dissimilarities in the glucose-stimulated change in the gene profile of both islets and IPCs.

The obtained results have been confirmed by PCR amplification of some islet-specific genes, and also by the determination of glucose-stimulated insulin secretion (Figure. 1). The PCR (Figure. 1A) showed the lower scale of expressions of *Ins1, Ins2, Glut2* and glucagon genes in IPCs than in islets cultured in basal glucose conditions.

Differentiated insulin-producing cells and pancreatic islets were cultured for 24 hr in either basal or stimulatory glucose media. Insulin secretion was measured in the supernatant and data were normalized by referring to the tissue protein-content (Figure. 1B and 1C). The results showed a significant difference between different groups (ANOVA, p=0.000001). Glucose could stimulate insulin secretion significantly in both islets and IPCs. However, the secreted insulin quantity was far less in IPCs than that in islets. As well, the stimulatory index, calculated as stimulated secretion / basal secretion, was significantly higher in islets than in IPCs.

Table 4. The uppermost 30 upregulated genes in response to glucose in IPCs (left) and islets (right)

	Gene ID (IPCs)	Symbol	SR	Gene ID (islet)	Symbol	SR
1	sodium channel, voltage-gated, type 6, alpha polypeptide	*Scn6a*	46.78	insulin 2	*Ins2*	21.19
2	Janus kinase 2	*Jak2*	18.51	Glucose transporter, member 2	*glut2*	12.00
3	connective tissue growth factor	*Ctgf*	16.34	glutamate receptor, ionotropic, kainate 3	*Gnik3*	9.36
4	protease, serine, 11 (Igf binding)	*Prss11*	12.77	cytochrome P450 4F5	*Cyp4f5*	8.95
5	hydroxysteroid 11-beta dehydrogenase 1	*Hsd11b1*	12.02	HGF-regulated tyrosine kinase substrate	*Hgs*	7.79
6	matrix metallopeptidase 3	*Mmp3*	11.78	inositol 1,4,5-trisphosphate 3-kinase A	*Itpka*	7.52
7	thioredoxin 1	*Txn1*	10.29	UDP-glucuronosyltransferase,	*Udpgtr2*	5.16
8	glyceraldehyde-3-phosphate dehydrogenase	*Gapd*	8.96	alkylglycerone phosphate synthase	*Agps*	5.05
9	chemokine (C-C motif) ligand 2	*Ccl2*	8.89	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	*Slc24a2*	4.98
10	tissue inhibitor of metalloproteinase 1	*Timp1*	8.87	lectin, galactose binding, soluble 8	*Lgals8*	4.87
11	heat shock 27kDa protein 1	*Hspb1*	8.71	ornithine decarboxylase 1	*Odc1*	4.85
12	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	*Atp5a1*	8.04	lactate dehydrogenase 3, C chain	*Ldhc*	4.82
13	proteasome (prosome, macropain) subunit, beta type 4	*Psmb4*	7.92	calcium/calmodulin-dependent protein kinase 1, alpha	*Camkk1*	4.81
14	heat shock protein 8	*Hspa8*	7.89	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	*Cyp2c*	4.65
15	glyceraldehyde-3-phosphate dehydrogenase	*Gapd*	7.76	Tumor necrosis factor receptor superfamily, member 6	*Tnfrsf6*	4.37
16	NCK-associated protein 1	*Nckap1*	7.70	cytochrome b-5	*Cyb5*	4.33
17	lysosomal membrane glycoprotein 1	*Lamp1*	7.65	mitochondrial acyl-CoA thioesterase 1	*Mte1*	4.14
18	annexin A1	*Anxa1*	7.63	Matrix metalloproteinase 23	*Mmp23*	3.98
19	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	*Nfkbia*	7.40	cytosolic acetyl-CoA hydrolase	*Cach*	3.93
20	ornithine decarboxylase antizyme 1	*Oaz1*	7.30	solute carrier family 6 (GABA), member 11	*Slc6a11*	3.92
21	aldolase A	*Aldoa*	7.26	phospholipase D1	*Pld1*	3.90
22	fibronectin 1	*Fn1*	7.17	proline-rich protein	*PRP-2*	3.85
23	UDP-glucose dehydrogenase	*Ugdh*	7.11	calcium-sensing receptor	*Casr*	3.82
24	proteasome (prosome, macropain) subunit, alpha type 2	*Psma2*	6.98	thioesterase domain containing 1	*Thedc1*	3.81
25	aldose reductase family 1, member B4	*Akr1b4*	6.82	neuropilin 2	*Nrp2*	3.79
26	ATP synthase, H+ transporting, mitochondrial F0 complex,	*Atp5h*	6.58	adipose differentiation-related protein	*ADRP*	3.78
27	NADH dehydrogenase (ubiquinone) flavoprotein 2	*Ndufv2*	6.48	steroidogenic acute regulatory protein	*Star*	3.78
28	lactate dehydrogenase A	*Ldha*	6.38	cytochrome P450, family 27, subfamily b, polypeptide 1	*Cyp27b1*	3.77
29	dual specificity phosphatase 1	*Dusp1*	6.37	cysteine conjugate-beta lyase	*Ccbl1*	3.77
30	glyceraldehyde-3-phosphate dehydrogenase	*Gapd*	6.35	Inositol (myo)-1(or 4)-monophosphatase 1	*Impa1*	3.76

Values are the signal ratio (SR) of glucose-stimulated / basal conditions in IPCs and islets

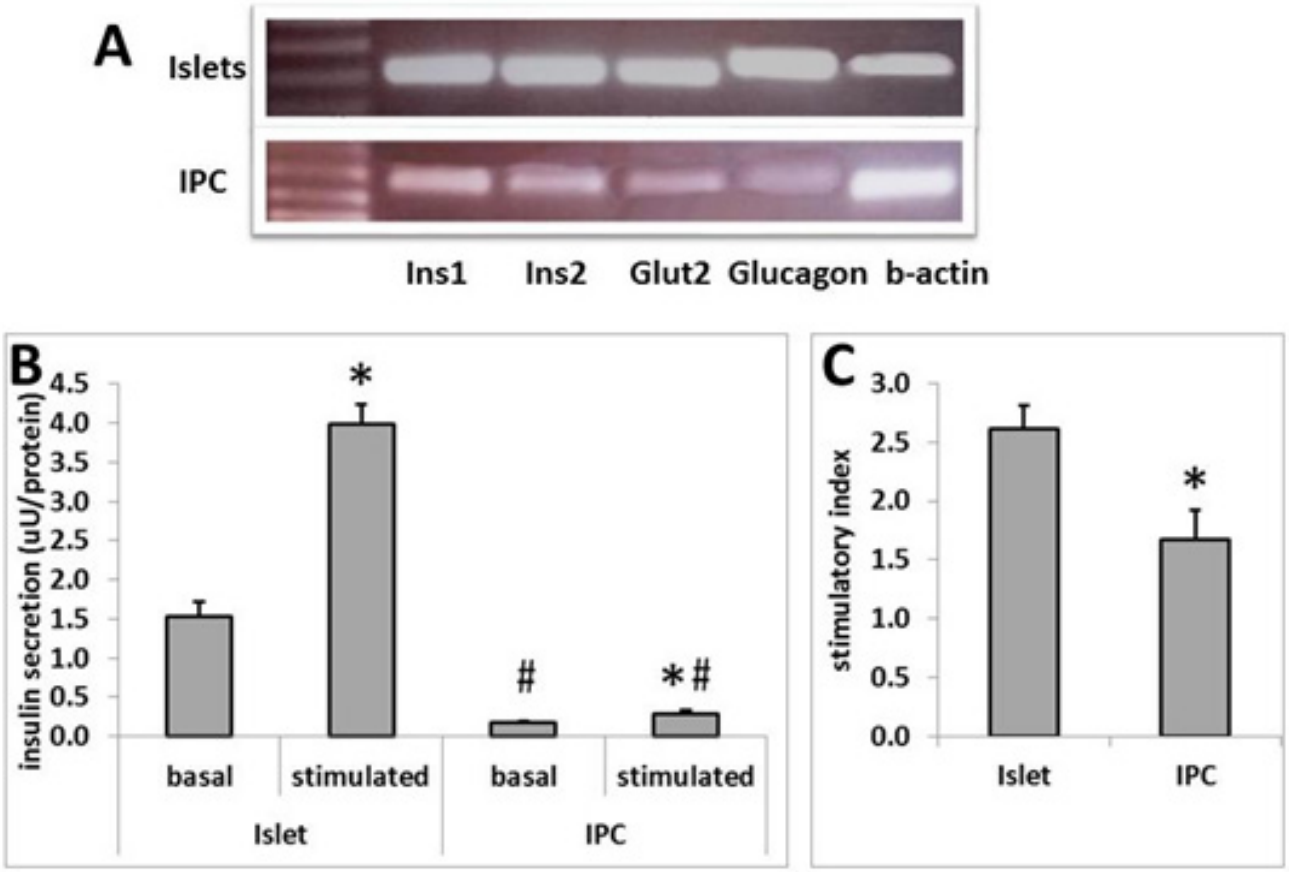

Figure 1. Islet-specific gene expression and glucose-stimulated insulin secretion in IPCs derived from bone marrow MSCs in comparison with that of pancreatic islets. Data are presented as mean±SEM. Statistical analysis: ANOVA $p<0.00001$, * denotes significantly different from basal value in B and from islet value in C, # denotes significantly lower than the corresponding islet value (t-test).

Conclusion

The present data demonstrate a different pattern of gene expression and different transcriptome in pancreatic islets and insulin-producing cells differentiated from bone marrow mesenchymal stem cells. More efforts are still required to develop differentiation protocols that make the transcriptome and the consequent glucose-stimulated insulin secretion in differentiated stem cells closer to that of pancreatic islets.

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