

Improved insulin-secreting properties of pancreatic islet mesenchymal stem cells by constitutive expression of Pax4 and MafA

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Received: 31.07.2017 • Accepted/Published Online: 21.10.2017 • Final Version: 18.12.2017

Abstract: For long-term treatment of diabetes type 1, transplantation of insulin-producing beta cells may be a promising method, but the limited number of islets for transplantation requires the development of different approaches. In this study, we aimed to generate beta-like insulin-producing cells. For this purpose, MafA, Pax4, and Ngn3 genes were transferred into pancreatic islet-derived mesenchymal stem cells, and the effect of their ectopic expressions on differentiation efficiency was examined. Stemness properties of pancreatic islet stem cells were characterized. The 3 genes were transfected by electroporation and expressed constitutively. The transfected cells were further stimulated to differentiate by using chemical induction. Pax4 expression had significant effects on differentiation into insulin-producing cells. Although it caused morphological alterations in cells, similar to epithelial cells, the insulin secretion levels remained lower than those of the cell line cotransfected with MafA and Pax4. Cotransfection of the 3 transcription factors did not further improve the beta-like cell generation. MafA and Pax4 ectopic expression resulted in improved differentiation efficiency into insulin-secreting cells. However, support of this differentiation process using additional chemical induction may suffice to overcome control by endogenous regulatory pathways.

Key words: Mesenchymal stem cells, beta cells, insulin-producing cells, mesenchymal epithelial transition, transcription factors

1. Introduction

Diabetes is a public health problem affecting all levels of society, and is generally characterized by a lack of balance in the blood sugar level. External supplementation of insulin is frequently used to treat patients with diabetes. Despite promising replacement therapy of beta cells, alternative methods are required to address the high number of patients and the limited number of pancreatic islet donors. Mesenchymal stem cells (MSCs) might be used as an alternative cell source for this approach. MSCs are multipotent cells that function in the maintenance and repair of tissue. Along with their regeneration activity, they also inhibit degenerative inflammatory reactions that limit both somatic and stem cell functions in the tissue. Their property of differentiating into various cell types in the presence of external stimulation increases the potential of MSCs in cell-based applications. This also provides the rationale for their use in the long-term treatment of type 1 diabetes (Gabr, 2013; Karaoz, 2013). There are an increasing number of studies demonstrating the use of these cells for the treatment of similar diseases (Trounson, 2011; Uccelli, 2011; Keating, 2012).

Tissue-specific MSCs may differentiate more effectively into the tissue cell types, which they are isolated from, compared to other stem cells. Pancreatic islet-derived stem cells were shown to have a protective effect on pancreatic islet cells by decreasing apoptosis and immune regulation (Karaoz, 2010a; Sariboyaci, 2014). However, their ability to differentiate into insulin-producing cells (IPCs) has not been demonstrated yet. Stem cells derived from rat pancreatic islets have previously been shown to share common features with rat bone marrow-derived MSCs with respect to their immune phenotype, differentiation potential, growth kinetics, and gene expression profiles (Karaoz, 2010a). Co-culture of streptozotocin-treated pancreatic islets with bone marrow MSCs had significant cytoprotective effects through paracrine actions (Karaoz, 2010b).

It is very important to understand the role of transcription factors in the development of the endocrine pancreas and highly specialized beta cells. Pdx1 determines the region of the primitive gut that will form the pancreas; Ngn3 provides endocrine lineage progression; and expression of Pax4, NeuroD1/beta2, Nkx2.2, and MafA leads to differentiation into mature beta cells (Bernardo,

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2008). Various types of studies have aimed to reprogram different cell types into a beta-like state (Tang, 2006; Nishimura, 2009; Akinci, 2012).

In the present study, rat pancreatic islet-derived MSCs (rPI-MSC) were differentiated into insulin-producing beta-like cells by transfection of cell-lineage genes with or without further chemical induction. MafA, Pax4, and Ngn3 transcription factors were overexpressed, and their effects on the cell transformations were compared by gene expressions.

2. Materials and methods

2.1. Genes

The genes were supplied by GeneScript (Piscataway, NJ, USA). For the ectopic expression, musculoaponeurotic fibrosarcoma oncogene homolog A (MafA; GeneBankAcc. No. XM_345846.2; 1086 bp), paired box gene 4 (Pax4; GeneBank Acc. No. NM_031799.1; 1050 bp), and neurogenin3 (Ngn3; GeneBank Acc. No. NM_021700.1; 645 bp) of rat origin were used. The genes were maintained on the pUC57 vector, and the plasmids were propagated in *E. coli* DH5 α (New England Biolabs, Hitchin, Herts, UK), according to the supplier's instruction.

2.2. Stem cell isolation and culture

All procedures performed in this study involving animals were in accordance with the ethical standards of the Experimental Animal Center of Kocaeli University (KOU-HADYEK1/4-2012). The MSC-isolation procedure for rPI-MSC was previously described in Karaoz et al. (2010a). Briefly, the pancreatic islets were isolated with collagenase P (Roche Applied Science, Mannheim, Germany) and suspended in RPMI 1640 basal medium (Gibco, Invitrogen, Paisley, UK). MSCs from pancreatic islets were obtained by explant culturing of pancreatic islets in plastic culture flasks. Characterization of the cells was performed according to the criteria described by Dominici et al. (2006). Cells were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Pen/Strep; Gibco). The medium was replaced with fresh medium every 2 days. After cells reached 70%–80% confluence, cells were detached by 0.025% trypsin–EDTA (Gibco). The cells were washed with Ca²⁺–Mg²⁺ free phosphate-buffered saline (PBS; Gibco) and re-plated at 1:5 ratios for subculturing.

2.3. Characterization of cells

rPI-MSC phenotypes were confirmed by flow cytometer analysis (FACS Calibur, BD Biosciences, San Jose, CA, USA), and their differentiation capacity was analyzed. After passage 3 (P3), MSCs were harvested and resuspended in PBS at a concentration of 1×10^6 cells/mL. Debris and dead cells were gated out during analysis of the results with CellQuest software (BD Biosciences). Immunophenotyping of rPI-MSCs was performed with antibodies against the following rat antigens: CD29,

CD90, CD54, CD106, CD45, and MHC class I. All of the antibodies were supplied by BD Biosciences. More than 10% staining was considered positive.

To induce adipogenic differentiation, cells were seeded onto 6-well plates (3000 cells/cm²) and cultured in adipogenic differentiation medium (Mesencult MSC Basal Medium [StemCell Technologies Inc., Vancouver, BC, Canada] supplemented with 10% adipogenic supplement and 1% Pen/Strep) for 3 weeks. The medium was refreshed every 3–4 days. The formation of intracellular lipid droplets was confirmed by staining with 0.5% Oil-Red-O (Sigma-Aldrich). For Oil-Red-O staining, cells were fixed for 15 min in 4% paraformaldehyde and 5 min in 60% isopropanol at room temperature.

For osteogenic differentiation, cells (3000 cells/cm²) were seeded in 6-well plates. The osteogenic differentiation medium (RPMI 1640 supplemented with 0.1 μ M dexamethasone [Sigma-Aldrich], 0.05 μ M ascorbate-2-phosphate [Wako Chemicals, Richmond, VA, USA], 10 mM beta-glycerophosphate [Sigma-Aldrich], 1% Pen/Strep, and 10% FBS) was replaced twice a week. After 4 weeks, osteogenic differentiation was estimated by Alizarin Red staining. For Alizarin Red-S staining, cells were fixed for 5 min in ice-cold 70% ethanol. The cells were stained with Alizarin Red-S solution (2%, pH 4.2). Stained cells were dehydrated in pure acetone, fixed in acetone–xylene (1:1) solution, and cleared with xylene.

2.4. Immune staining

Samples were fixed in ice-cold methanol for 10 min and permeabilized with 0.025% Triton X-100 (Merck, Darmstadt, Germany). Cells were incubated with 1.5% blocking serum (Santa Cruz Biotechnology, Heidelberg, Germany) in PBS for 30 min, and incubated overnight with the primary antibody (1:50 dilution) at 4 °C. After repeating the PBS washes, cells were incubated with secondary antibodies for 60 min, and were mounted with mounting medium containing DAPI (Santa Cruz Biotechnology). The antibodies for cell-specific markers are given in Table 1.

2.5. Preparation of expression vectors and transfection

All genes (MafA, Pax4, and Ngn3) were excised from the cloning vectors by using restriction enzymes HindIII, BamHI, or EcoRV (Thermo Scientific, Fermentas, Vilnius, Lithuania), and ligated into pIRES–DsRed (ClonTech, Mountain View, CA, USA) downstream of the CMV promoter. The plasmids were amplified in *E. coli* DH5 α and purified using an endotoxin-free plasmid isolation kit (Qiagen, Hilden, Germany). MSCs were transfected with the Neon Transfection System (Invitrogen/Life Sciences) according to the method described by the manufacturer, with the pre-optimized parameters of 990 V and 40 ms. Stable cell lines were maintained by continuous culture of transfected cells in the selective culture media with G418 (Gibco/Life Sciences; 200 μ g/mL).

Table 1. Primary antibodies used in the immune staining.

	Supplier	Cat. no.
ASMA	Thermo Fisher	(MS-113-P)
c-Fos	Santa Cruz Biotechnology	Sc-52
Desmin	Santa Cruz Biotechnology	Sc-23879
Fibronectin (FN)	Santa Cruz Biotechnology	Sc-8422
Vimentin	Santa Cruz Biotechnology	Sc-7557
VEGF	Santa Cruz Biotechnology	Sc-507
Pax4	Santa Cruz Biotechnology	Sc-98942
C-peptide	Cell Signaling Technology	4593
Insulin (Ins)	Santa Cruz Biotechnology	Sc-9168

Cells were transferred with either single genes ([MafA]; [Pax4]; [Ngn3]), or in different combination of genes ([MafA+Pax4]; [MafA+Ngn3]; [Pax4+Ngn3]; [MafA+Pax4+Ngn3]). In total, 7 different transfected cell lines were compared.

2.6. Gene expression analysis

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany). Adherent cells were detached by trypsin and washed twice with PBS, as described previously for the subculture. Cells were digested by the lysis buffer provided in the kit. The isolation procedure

was followed by column purification according to the manufacturer's instructions. After synthesis of single strand cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science), the gene expression levels were detected using the LightCycler 480 DNA SYBR Green I Master (Roche Applied Science) with gene-specific primers on a LightCycler 480 real-time PCR instrument (Roche Diagnostics), according to the manufacturer's protocol. Melting curve analysis for determining the dissociation of PCR products was performed from 60 °C to 95 °C to confirm the presence of a single peak. The primers are listed in Table 2.

Table 2. Primers used in RT-PCR.

Gene	GenBank acc. no.	Sequences (5'-3')
MafA	XM_345846.2	GCAAGCTTATGGCTTCAGAACTGGC
		GGATCCTCACATGAAAAATTCGGGAGAG
Pax4	NM_031799.1	GAAGCTTATGCAGCAGGACGGTCTC
		CGGATCCTTATGGCCAGTGTAAGTAATAG
Ngn3	NM_021700.1	ACAAGCTTATGGCGCCTCATCCCTTG
		CGGATCCTCACAAGAAGTCTGAGAACACC
Ins1	NM_019129.3	GACCTTGGCACTGGAGGTT
		CCAGTTGGTAGAGGGAGCAG
Ins2	NM_019130.2	CGAAGTGGAGGACCCACA
		TGCTGGTGCAGCACTGAT
Glut2	NM_012879.2	TAGAGCAGCTCTTATTCCAGATTT
		TTACTCTCCATTTCAAGTCCTTTGT
Pdx1	NM_022852.3	CGTAGTAGCGGGACAACGA
		CTCCTCGCCCGAGGTTAC
Gcg	NM_012707.2	GGTGAAAGGCCGAGGAAG
		GAGAAGGATCCATCAGCATGT
Ppy	NM_012626.2	ACTCGCTCAGGACACAGGAT
		CGGGTACATTGGCTCCAG
Sst	NM_012659.2	CTGGAGCCTGAGGATTTGC
		CTGCAGCTCCAGCCTCAT
G3PDH/ GAPDH	NM_017008.4	AGAGAGAGGCCCTCAGTTGCT
		TGGAATTGTGAGGGAGATGCT

2.7. Differentiation into endocrine cell lineages by chemical induction

For induction of endocrine differentiation by the chemical method, cells were cultured in the differentiation medium (RPMI 1640 medium supplemented with 10% FBS, 1% Pen/Strep, 25 ng/mL EGF, 10 mM nicotinamide, 2 nM activin-A, 100 ng/mL activin-B, 10 nM exendin-4, 100 pM HGF, 10 nM pentagastrin) for 14 days. After incubation, the cells were analyzed for the expression of beta cell markers by immune staining and gene expression.

2.8. Statistical analysis

All experiments were repeated a minimum of 3 times. All statistical analyses were performed using SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). The data were analyzed using one-way ANOVA and unpaired t-test. Differences between the experimental and control groups were regarded as statistically significant when $P < 0.05$.

3. Results

3.1. Characterization of rat PI-MSC

Cells migrating out of the pancreatic islets were spindle-shaped and had fibroblast-like morphology. Flow cytometer analysis indicated that they expressed CD29, CD90, CD54, and MHC Class-I, but not CD45 and CD106, and that they maintained their phenotype in the following passages. The cells differentiated successfully into adipocytes after 21 days of culture. In the osteogenic differentiation medium, the node formation was detected after 28 days of culture. The mineral deposits were detected by Alizarin-Red-S staining. The expression of the typical MSC-markers (ASMA, c-Fos, desmin, fibronectin, and vimentin) and differentiation into different cell types indicated that the isolated cells possessed the MSC character (Figure 1).

3.2. Cell morphology after ectopic expression of genes

At the initial passages, the morphology of the cells transfected with the different genes was not significantly different, and cells still preserved the previous MSCs' characters. After the 3–4 passages, an epithelial-like alteration in cell morphology was observed in the culture of the cell lines transfected with MafA and MafA+Pax4 genes. The colonies were composed of densely grown cells with spherical nuclei in the centers. This type of cell became dominant in later passages, while the other cell lines maintained their fibroblast-like MSC morphology in the culture (Figure 2). Flow cytometer analysis of all recombinant cell lines (which were transferred with the appropriate gene) was performed, and the results indicated that they expressed the same surface markers (CD29 and CD90) observed in rPI-MSCs (unmodified).

3.3. Differentiation of cells by gene transfer without chemical induction

After the transfection, the expression pattern of the beta cell and other islet cell markers was analyzed with real-

time PCR (Figure 3; Supplementary Figure). The results are summarized in Table 3. In the cells transfected only with Pax4 [Pax4], the expression of both MafA and Pax4 was increased. Ins1, Ins2, and Glut2 levels did not change. To improve these expressions, Pax4 and MafA genes were cotransfected. This cell line ([MafA+Pax4]) showed increased expression of Ngn3 and the alpha cell marker, glucagon, but the level of MafA was reduced to half compared to the [Pax4]-cell line. The expression of the Pax4 gene also declined in [MafA+Pax4] to the level of rPI-MSC before the gene transfection.

In cells transfected with Ngn3 alone, MafA and Pax4 expressions increased by 2- and 3.5-fold, respectively. Although the DsRed existence confirmed the ectopic expression of Ngn3 in the cell, total levels of both endogenous and ectopic Ngn3 were not significantly changed, and were equal to those of the untransformed cells. The level of Glut2 was decreased almost 3-fold compared to the untransfected rPI-MSCs. The ectopic expression of Ngn3 and Pax4 together in the cell line [Pax4+Ngn3] did not change the expression of MafA, Ins1, and Ins2 significantly compared to rPI-MSC, but the expression of Pdx1 and Pax4 declined by 4- and 3-fold, respectively. Only the glucagon level was slightly increased, but the beta cell markers were not improved in the [Pax4+Ngn3] cell line compared to [Ngn3] and untransfected rPI-MSCs.

The level of MafA expression improved more than 3-fold in the [MafA] cell line, but the Pax4 expression decreased almost 2-fold. Epithelial cell-like morphology was observed in these cells, but the decrease in Pax4 expression and the slight increase in the Ins1 and Ins2 expression did not support differentiation into the beta-like cells. The [MafA+Pax4] cells, which also showed epithelial cell-like morphology, had increased expression of MafA, Ngn3, and glucagon by 3-, 2.5-, and 2.5-fold, respectively. The increased level of glucagon might also indicate the differentiation into alpha cells, as previously described. The ectopic expression of both Ngn3 and MafA increased MafA, Pax4, and Glut2 expression by 8-, 5-, and 2.5-fold, which resembles cells differentiated into beta-like cells. However, the insulin gene expressions were not significantly increased in [MafA+Ngn3].

The cell line transfected with 3 genes showed the increased gene levels of MafA, Pax4, and Glut2 by 6-, 2.5-, and 4-fold, respectively, compared to untransfected rPI-MSCs. Compared to [MafA+Ngn3] cells, the ectopic Pax4 expression in [MafA+Pax4+Ngn3] decreased the expression level of the genes important to insulin production. Exceptionally, the ectopic Pax4 expression improved the level of Glut2, which is important for glucose response in beta cells. The expression level of glucagon was estimated to be unchanged in the 3-gene construct compared to the untransfected cells.

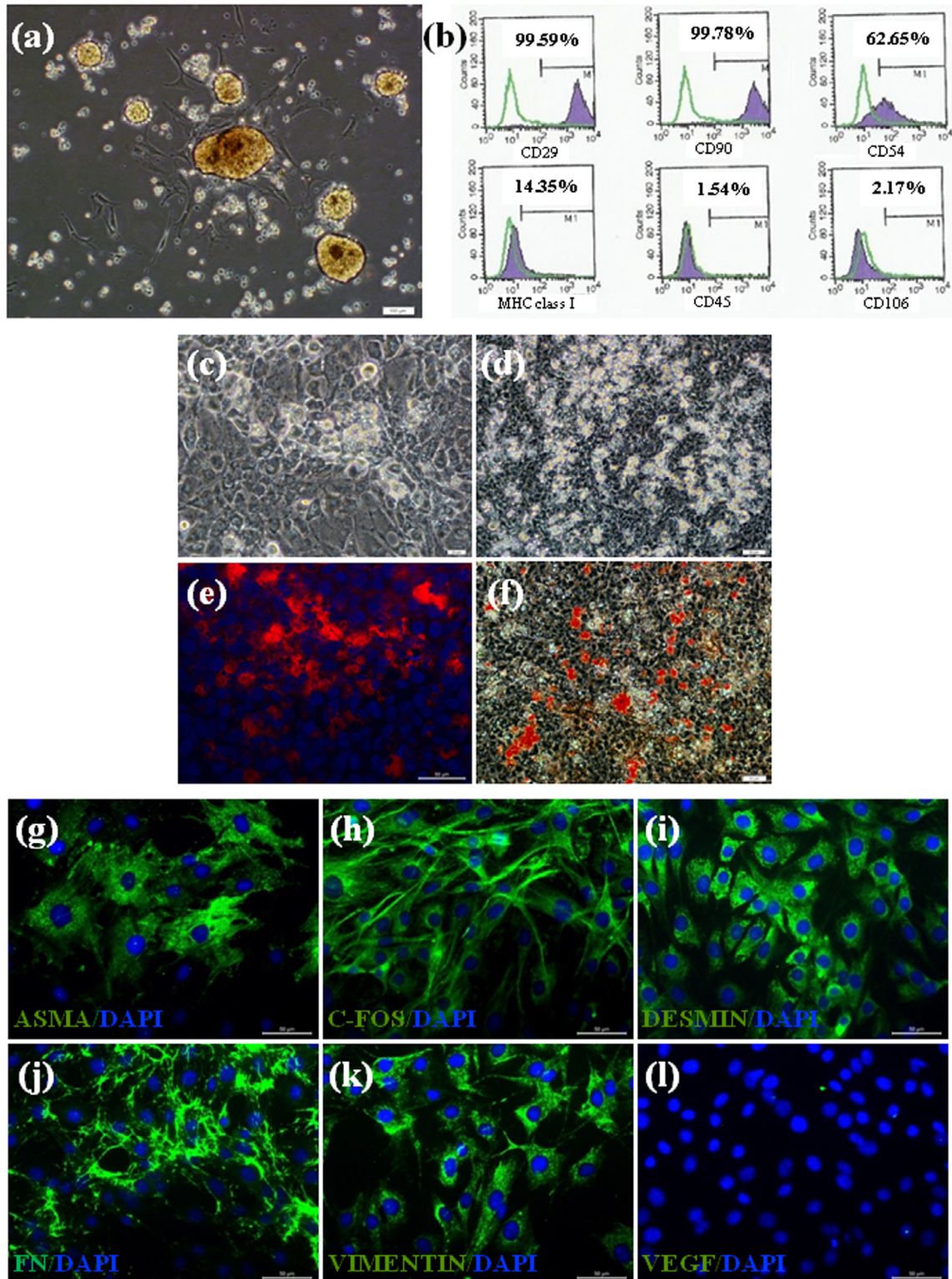


Figure 1. Characterization of rat pancreatic islet derived mesenchymal stem cells (rPI-MSC). The cells were isolated from pancreatic islets by the explant method (a). The isolated cells were analyzed by flow cytometer for mesenchymal stem cell specific surface markers after 3 passages of culture (b). The differentiation capacity of the cells into adipogenic (c, e) and osteogenic (d, f) cell lineages was proven after staining with Oil-Red-O and Alizarin-Red-S, respectively. The rPI-MSCs were further analyzed for the production of mesenchymal cell markers (green, g–k). The cells were also analyzed by immune staining for VEGF expression, but VEGF (green) was not positive (l). The nuclei were stained with DAPI (blue, g–l).

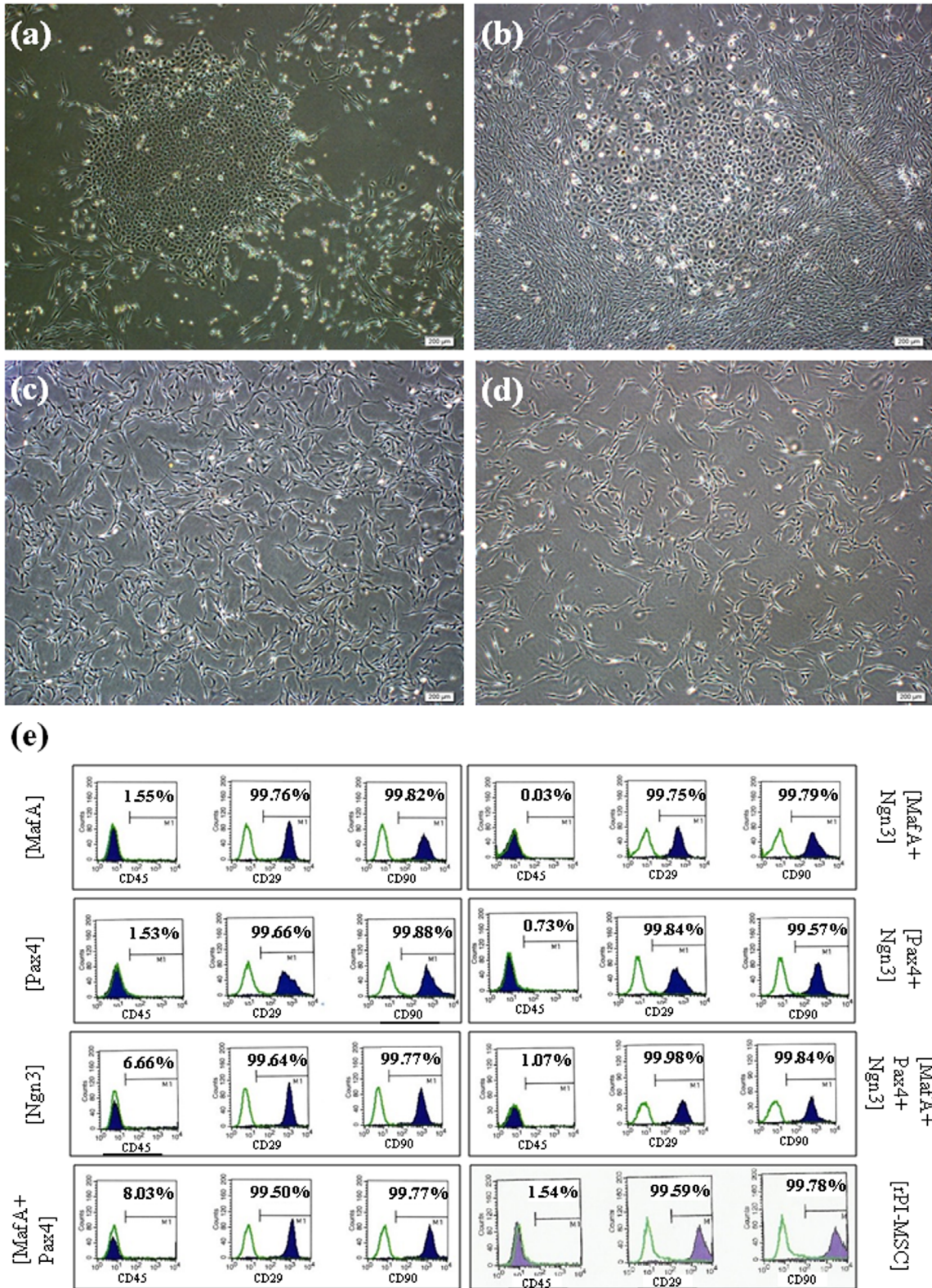


Figure 2. Cell morphology of the rPI-MSCs in the culture and characterization by flow cytometer. Three genes were transfected in 7 different combinations; some of the cells demonstrated distinct cell appearance following antibiotic selection with G418. Cell morphologies in the culture are shown after the gene transfer with MafA (a), MafA+Pax4 (b), MafA+Ngn3 (c), MafA+Pax4+Ngn3 (d). The cells were analyzed by flow cytometer with respect to the mesenchymal stem cell markers (e). Scale bar = 200 µm.

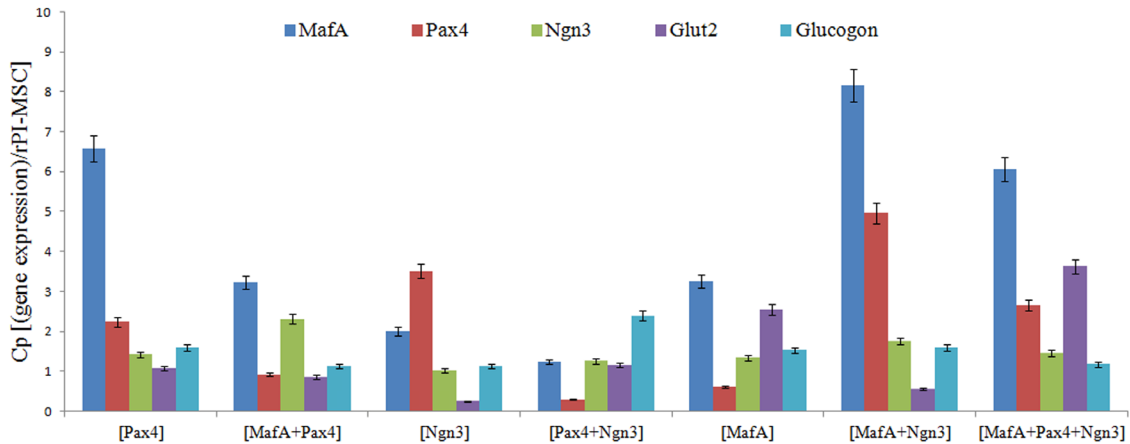


Figure 3. Gene expression analysis of transfected cells. The endogenous expression of Pax4, MafA, and Ngn3 genes after the transfection is shown. The results are represented as $\Delta\Delta C_p$ in folds with respect to GAPDH reference gene compared with the expression values of the untransfected rPI-MSCs.

Table 3. Summary of results.

Ectopic gene expression	Expression before endocrine differentiation	Expression after endocrine differentiation
Pax4		MafA, Pax4 ↑
MafA+Pax4	MafA, Ngn3, Gcg ↑ Pax4 ↓	MafA, Glut2 ↑ Sst ↓
Ngn3	MafA, Pax4 ↑ Glut2 ↓	MafA, Pax4, Ngn3 ↑↑ Glut2 ↑ Sst ↓
Pax4+Ngn3	Gcg ↑ Pdx1, Pax4 ↓	MafA ↑ Gcg ↓
MafA	MafA ↑ Pax4 ↓	MafA, Pax4, Ngn3 ↑↑
MafA+Ngn3	MafA, Pax4, Glut2 ↑	Gcg, Sst ↓
MafA+Pax4+Ngn3	MafA, Pax4, Glut2 ↑	MafA, Pax4 ↑ Ngn3, Sst ↓

Pax4 and C-peptide proteins were significantly expressed in [MafA+Pax4+Ngn3] cells (Figure 4). Insulin expression was not significantly altered and was limited to the clustered cells.

3.4. Differentiation of cells by gene transfer with chemical induction

Chemical induction was performed following the selection of stable cell lines. The expression of marker genes of the pancreatic islets cells was assessed by real-time PCR and compared with the cells that were not chemically induced (Figure 5).

Chemical induction promoted the MafA expression in the [Pax4]-cells, but the most significant effect was

observed in the Glut2 expression, which increased almost 2.5-fold compared to cells that were not chemically induced. Somatostatin expression decreased to 2-fold that of noninduced [Pax4]-cells. [MafA+Pax4]-cells also showed decreased somatostatin and increased Glut2 expressions. In addition, glucagon expression was slightly decreased. A significant effect was observed in the expression of MafA, Pax4, and Ngn3, which increased 2100-, 55-, and 1610-fold, respectively.

Chemical induction of [Ngn3]-cells increased the expression level of MafA substantially while decreasing the glucagon level by 2-fold. Other beta cell markers in these cells did not improve significantly. [Pax4+Ngn3]-

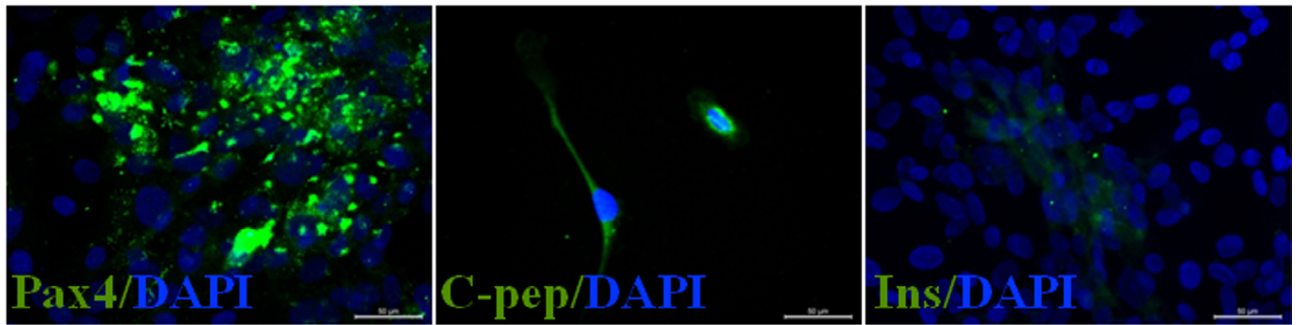


Figure 4. The immunofluorescence staining of [MafA+Pax4+Ngn3] cells with antibodies against the Pax4, C-pep, and Ins. The cells stained positively with appropriate antibodies are shown in green. The nuclei were stained with DAPI and are shown in blue. Scale bar = 50 μ m.

cells demonstrated increased expression of Ins1, Ins2, and Glut2 genes. The expression of MafA, Pax4, and Ngn3 genes was considerably increased: by 2400-, 155-, and 2610-fold, respectively. Pancreatic polypeptide (PPy) expression was also stimulated chemically in the [Pax4+Ngn3]-cells.

The effect of chemical induction on cells transfected with MafA alone was limited, but glucagon and somatostatin expression was decreased 2-fold. Unlike the [MafA+Pax4]-cells, the [MafA+Ngn3]-cells responded to chemical induction by increasing the expression of MafA and Pax4 further to 13-fold and 7-fold, compared to the untransfected cells without chemical induction. Chemical induction decreased the expression of the Ngn3 gene to normal levels in the untransfected cells, while the somatostatin expression was reduced 4-fold.

Cells transfected with 3-genes showed a significant increase in MafA and Ngn3 expression. Pax4 was also increased (4-fold), but less than MafA or Ngn3 (20.11- and 22.79-fold, respectively). After gene delivery, Glut2 expression increased about 4-fold in the cells, but after chemical induction, this expression decreased to 2-fold that of the untransfected cells without the chemical induction.

3.5. Cell morphology after chemical induction

Overexpression of MafA and Pax4 genes resulted in the appearance of epithelial-like cell morphology of rPI-MSCs. Chemical induction further improves this phenotype (Figure 6a–d). The formation of cell clusters became evident, especially in [MafA+Pax4] cells (Figure 6d). The cell clusters in the culture preserved their structure in the following days and did not transform into spheres. Cytoplasmic staining of the cell cultures for insulin showed positive results (Figure 6e–6h). The formation of insulin-secreting granulelike structures was only observed in a limited number of [MafA+Pax4+ Ngn3] cells (Figure 6h).

4. Discussion

A permanent solution to relieve patients from continuous insulin injection could be the generation and transplantation of insulin-producing cells that respond to blood glucose levels, similar to the beta cells in pancreatic islets (Wong, 2011; Akinci, 2012; Ricordi, 2012). The transformation of MSCs into IPCs can be used as an alternative long-term solution for the beta cell transplantation in therapy for type-1 diabetes. However, MSCs display inefficient differentiation into endodermal cell lineages (Czubak, 2014). Culturing under the specific defined conditions may result in commitment to differentiating into cells with the phenotype of beta cells or IPCs (Xie, 2009). Many protocols have been developed for the differentiation of MSCs in vitro. Along with single- or multistep chemical induction of differentiation into beta-like IPCs, differentiation via gene transfer has been used for MSCs. Although the cells gain a beta-cell-like character through these techniques, the differentiation efficiency of MSCs thus far has been relatively inefficient. Furthermore, although differentiation efficiency has been evaluated by insulin expression, insulin secretion in response to the varying glucose levels failed in most cases (Hashemian, 2015). Effective and functional differentiation could be achieved by the forced expression of transcription factors, which are important during the stage of embryonic pancreatic islet development (Johansson, 2007). The transcription factors Ngn3, Pdx1, Pax4, MafA, and NeuroD1 are mostly used for cloning studies due to their role in insulin synthesis, secretion, and pancreatic development. Some of these genes have been reported to stimulate the expression of other transcription factors in the signaling pathways of the insulin secretion mechanism.

The overexpression of transcription factors specific to beta cells in MSCs has been shown to be effective in the generation of beta-like cells (Ogihara, 2008; Guo, 2012; Zhou, 2013). Betacellulin, a ligand of epidermal growth factor receptor, is expressed abundantly in the pancreas

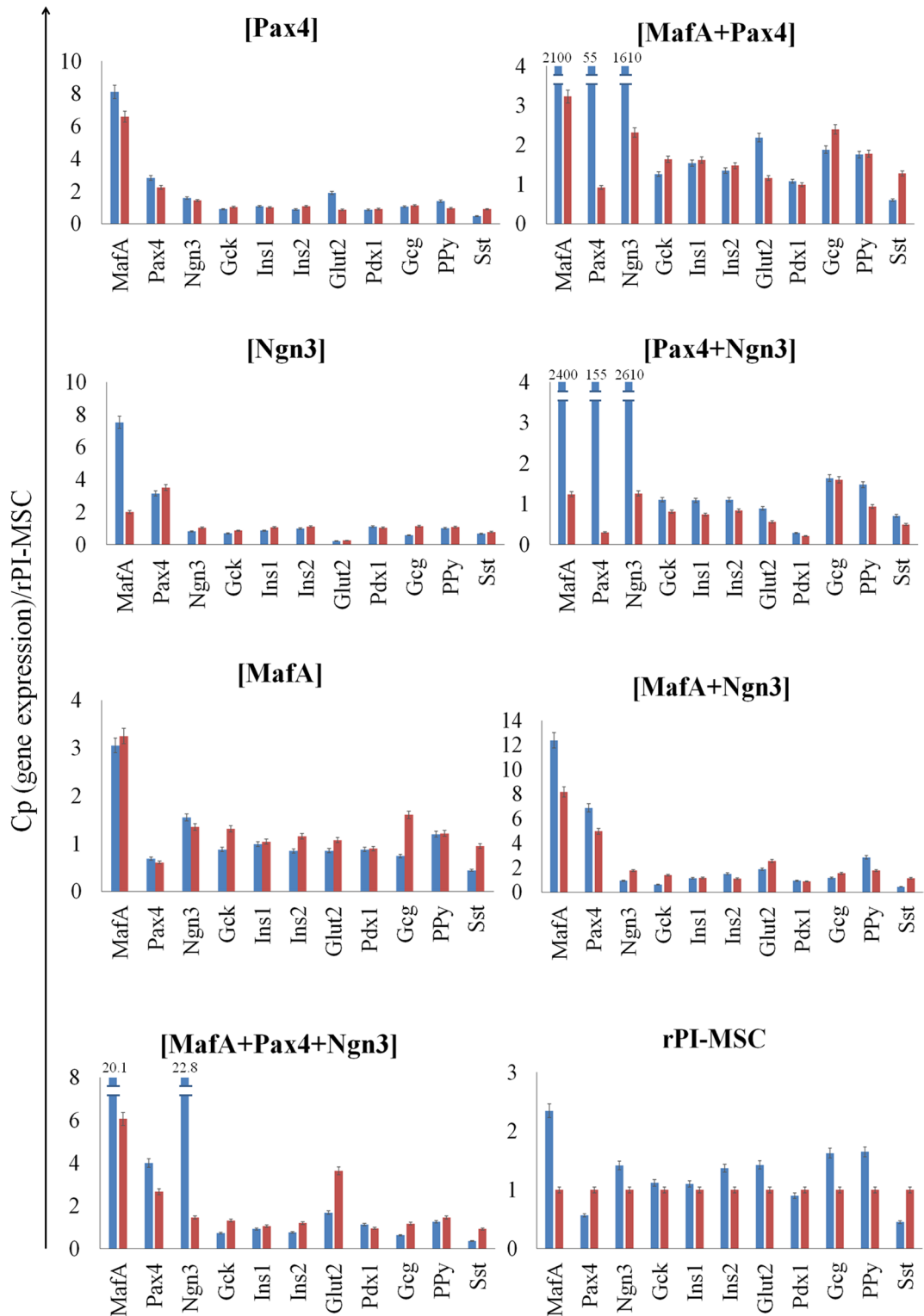


Figure 5. The evaluation of gene expressions of transfected cells before and after the chemical induction. The gene expression after the chemical induction (blue bars) was compared with the gene expression of cells before chemical induction (red bars). Besides the expression of the transfected genes (Pax4, MafA, Ngn3), the expression levels of beta cell markers (Ins1, Ins2, Glut2, Pdx1, and glucokinase [Gck]) and other islet cell markers (alpha cell [glucagon, Gcg], pancreatic polypeptide cells [PPy], and delta cells [somatostatin, Sst]) were analyzed. The results are represented as $\Delta\Delta C_p$ in folds with respect to GAPDH reference gene compared with the expression values of the untransfected and uninduced rPI-MSCs.

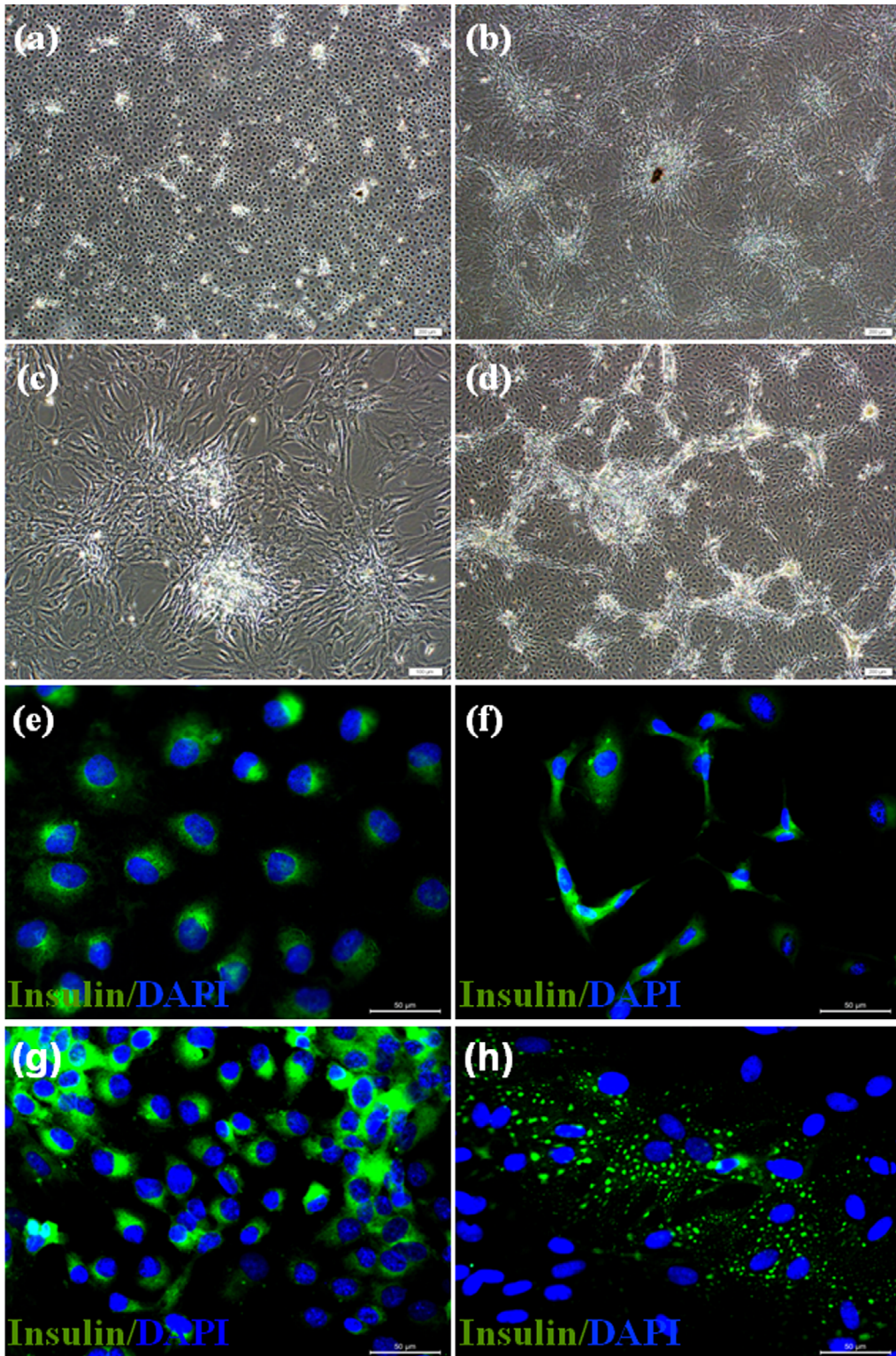


Figure 6. Morphologies of chemically induced transfected cells in the culture and immune staining. After chemical induction, [MafA] and [MafA+Pax4] cells (a, d) showed epithelial-like morphology, while [Pax4], [Ngn3], and [MafA+Pax4] had the tendency to form clusters (b–d). To compare the MafA transfected cells according to insulin production, the cells were stained against insulin (green) antibody (e, [MafA]; f, [MafA+Pax4]; g, [MafA+Ngn3]; h, [MafA+Pax4+Ngn3]). The nuclei were stained with DAPI (blue). Scale bar = 50 μ m (for e–h), 100 μ m (for c), and 200 μ m (for a, b, d).

and intestine. Paz et al. (2011) overexpressed betacellulin in rat bone-marrow-derived MSCs and reported the formation of IPCs. Stable expression of Pdx-1 in adipose tissue-derived MSC did not induce a pancreatic phenotype in vitro, but these cells were able to differentiate into IPCs and reduce hyperglycemia in streptozotocin-treated diabetic mice (Kajiyama, 2010).

In the present study, 3 transcription factors were used to induce differentiation of MSCs into insulin-secreting cells. MafA, Pax4, and Ngn3 were transfected with a single gene or in different combinations, and overexpressed under the control of CMV promoters. Following transfection, the cells responded variably to the altered gene expression pattern. Despite gene transfer, Pax4 was not highly expressed in [Pax4+Ngn3] and [MafA+Pax4], and this might indicate the feedback control mechanism that repressed endogenous Pax4 expression. Compared to its expression level in [Ngn3], Pax4 expression was decreased in [Pax4+Ngn3]. However, the Pax4 level was not decreased in [Pax4]. The highest expression of Pax4 was observed in [MafA+Ngn3], in which the Pax4 was not expressed ectopically. Similar feedback control mechanisms were also observed in other cellular processes, as in the regulation of the expression of the insulin receptor gene (Puig and Tjian, 2005). It was found that insulin receptor gene expression represses its own synthesis by the transcription factor, FOXO1, which regulates metabolism and insulin signaling in mammalian cells. Such a control mechanism has not yet been defined for Pax4 expression. This study has provided some preliminary information for such a mechanism for the transcription factor Pax4 in the rPI-MSC. This may indicate that there is a close control mechanism between MafA, Ngn3, and Pax4 transcription factors in the MSCs. During the beta cell reprogramming and differentiation, it was reported that the transcription factors Ngn3, Pax4, and Pdx1 were closely regulated (Li, 2015). Pdx1 induced Ngn3 expression, and Ngn3 regulated Pax4 expression (Courtney, 2011).

The exogenous overexpression of these transfection factor genes resulted in unexpected gene expression patterns. For example, in [Pax4], the expression of MafA was substantially increased. However, the simultaneous expression of Pax4 and MafA in [MafA+Pax4] repressed the total MafA expression. Similarly, in the [Pax4+Ngn3] line, total Pax4 gene expression was decreased compared to the [Ngn3] line. The cause of this may be the suppression of endogenous expression. The expression of Pax4 in [Pax4] was shown to induce expression of MafA and Ngn3. MafA expression was stimulated in [Pax4], but not in [MafA+Pax4]. It is possible that a control mechanism is activated when MafA exceeds a specific level. Although MafA has a self-regulating mechanism, its expression may be upregulated by Ngn3 and Pax4. Ngn3 expression

increased MafA and inactivated feedback repression. The level of MafA was limited by Pax4 expression at a specific limit, rather downregulating it.

MSCs preserved their stemness character even after the ectopic coexpression of the strong transcription factors MafA, Pax4, and Ngn3. This resistance to differentiating into terminal cells might be explained by the expression of Pdx1 in the cells, in which the expression of the notch signaling pathway prevents further differentiation and arrest in an undifferentiated multipotent form (Murtaugh, 2003). In other studies, Pdx1 was reported to induce differentiation into beta-like cells (He, 2011).

The significant decrease in Pdx1 level was observed in [Pax4+Ngn3], in which the glucagon level increased substantially, while the Ins1 and Ins2 levels decreased. A similar observation was also reported in the transformation of mesenchymal cells. Glucagon-secreting alpha-like cells were generated by transfecting monolayer mesenchymal cells derived from pancreatic tissue exocrine with adenoviruses containing Pdx1, Ngn3, MafA, and Pax4 genes (Lima, 2013). This study also suggested that the formation of epithelial-like clusters of exocrine-derived mesenchymal stromal cells promotes the generation of IPCs. In our study, epithelial-like cell clusters were observed in [MafA] and [MafA+Pax4] cell lines, but [MafA+Pax4+Ngn3] showed fibroblast-like cell morphology in the standard culture medium. [MafA+Pax4] showed epithelial-like clusters in the early passages after the transfection, but this formation was changed later and turned into cell morphology with typical mesenchymal character. At the same time, a higher expression of Ngn3 was observed in rPI-MSCs, resulting in the expression of glucagon, an alpha cell marker. Single Ngn3 expression was reported to be required for beta cell neogenesis, but recent reports have indicated that this procedure is highly inefficient for the transformation of progenitor cells into beta cells (Baeyens, 2006; Johansson, 2007). Ngn3 gene expression in rPI-MSC induces the endogenous expression of glucagon and promotes differentiation into alpha cells. Without chemical induction, cells with 3 co-expressed genes transformed more efficiently into IPCs and showed higher Glut2 expression.

Chemical induction positively affected endocrine differentiation, but the expression of the insulin gene remained limited. [MafA+Pax4], [Pax4+Ngn3], and [MafA+Pax4+Ngn3] cells showed increased gene expression. In particular, the MafA, Pax4, and Ngn3 genes were highly expressed in these lines. These cells share a common feature in that they were all transfected with Pax4. The transfection of a single Pax4 gene may be sufficient for the generation of IPCs, but [Pax4] cells displayed reduced gene expression of MafA and Pax4. [MafA+Pax4+Ngn3] cells after the chemical induction

showed a weak profile compared to [MafA+Pax4] and [Pax4+Ngn3] cell lines, and conserved their mesenchymal cell character. The best combination of transfected genes was found to be [MafA+Pax4]. In the chemical induction medium for endocrine differentiation, co-expression of [MafA+Pax4] substantially improved insulin secretion and Glut2 gene expression. However, compared to beta cells in the pancreatic islets, this insulin level was far lower than the required amount. The formation of epithelial-like cells during the chemical induction resembled the mesenchymal-epithelial transition, which was demonstrated to be critical in IPC production (Johansson, 2007).

In conclusion, the 3 genes were transformed either as single or as a set into the cells, and caused different responses. The transformation of MafA (single), Pax4 (single), or MafA+Pax4 (set) caused high expression of the MafA gene in the cells. Therefore, the process involved the gene transfer of MafA+Pax4, which induced the MafA expression, and this endogenous MafA expression played an important role in stem cell differentiation into insulin-

producing beta-like cells. The co-expression of MafA and Pax4 genes in rPI-MSCs is sufficient to generate IPCs, but it should be supported with chemical induction. The maturation of the [MafA+Pax4] cells is incomplete with respect to the beta cells. Currently, the transfection of 2 or 3 genes is still inadequate, and different chemical induction cocktails or methods are required. A multistage differentiation protocol may be required to control the signaling pathways for better IPC generation and to overcome endogenous regulation of gene expression in the MSCs.

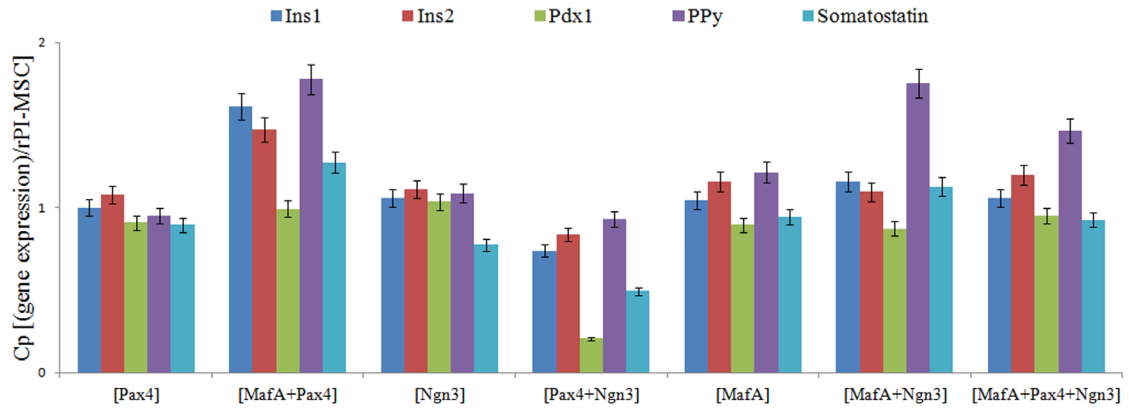
Acknowledgments

This work was supported by the Scientific and Technological Research Council of Turkey under Grant No. TÜBİTAK 112S125. We also thank Cansu Subaşı, Gülay Erman, Ayça Aksoy, and Alparslan Okçu for their precious technical assistance in immune staining, flow cytometer, and stem cell isolation procedure in this study. We would also like to express our thanks to Yusufhan Yazır for his support.

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Supplementary Figure. The expression levels of genes related to the endocrine cell lineage cells are shown. Beta cell markers (insulin 1 [Ins1], insulin 2 [Ins2], pancreatic and duodenal homeobox 1 [Pdx1], alpha cell (glucagon [Gcg]), delta cell ([somatostatin, Sst]), and pancreatic polypeptide cells (PPy) markers were analyzed. The results are represented as $\Delta\Delta C_p$ in folds with respect to GAPDH reference gene compared with the expression values of the untransfected and uninduced rPI-MSCs.