

ORIGINAL ARTICLE

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Differentiation of human bone marrow-derived mesenchymal stem cells into functional pancreatic beta cells **Ayla Eker Sariboyaci**,  **Onur Uysal**,  **Sibel Gunes***Eskisehir Osmangazi University, Cellular Therapy and Stem Cell Production Application and Research Center, ESTEM, Department of Stem Cell, Eskisehir, Turkey*

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**Abstract**

Two challenges must be overcome to achieve successful therapeutic strategies for type 1 diabetes mellitus (T1DM), as stated by the International Juvenile Diabetes Research Foundation. The first one is the regeneration of the destructed beta-cells and the presence of cell resources that will provide it, the second one is the usage of immunosuppressive drugs throughout life after the transplantation. To date, three different strategies have been developed, aiming at beta cell replacement; pancreatic transplantation, islet transplantation, and cellular therapy (islet-neogenesis). Stem cell-based therapy can be achieved by transplanting cells into a target organ to restore the functions of that organ. Experimental and clinical studies have provided promising results of the application of mesenchymal stem cells (MSCs) in diabetes therapy. MSCs have the potential to be applied in many clinical fields such as autoimmune and hereditary diseases treatment, transplantation, and regenerative medicine because of their immunoregulatory properties. Although there are many studies focused on β -cell replacement, it has been observed that beta cells have not been obtained yet at the desired morphological and functional level. This study aimed to differentiate hBM-MSCs in-vitro, into both morphologically and functionally pancreatic progenitor cells. For this purpose, hBM-MSCs were differentiated into beta-cells by a differentiation method in which culture conditions were modified. The differentiation efficiency of the obtained cells was analyzed. In our results, it was determined that the cells obtained when hBM-MSCs were differentiated with inducers added to the medium, were expressing beta-cell-specific markers and had morphological and functional features of the beta cells. Effective differentiation methods, including using different types of MSCs obtained from different tissues should be developed as the source of cell-based therapies for the treatment of type 1 diabetes.

Keywords: Bone marrow, mesenchymal stem cell, differentiation, pancreatic beta cells, diabetes**Introduction**

Diabetes mellitus (DM) is a chronic metabolic disease, associated with high blood sugar (hyperglycemia) which occurs due to the insufficient production of insulin by the pancreas, or the inadequate usage of the insulin by the body [1]. There are two types, insulin-dependent (type 1) and insulin-independent (type 2). In type 1 diabetes, pancreatic beta cells are destroyed as a result of immunodeficiency. In type 2 diabetes, insulin secretion deficiency from pancreatic beta cells is observed [2]. The incidence of DM has been raised in the world and in our country. While the number of adults aged 20-79 with diabetes in the world was 285 million in 2010, it is estimated that this number will increase to 700 million in 2045. An expectation of increasing the incidence number of adults with diabetes in the period from 2010 to 2030 by 69% in the developing countries and 20% in the developed countries [3].

Normally, there are approximately one million islets of Langerhans in the pancreas with endocrine cells. These islets are composed of different types of cells; the beta cells that produce insulin, alpha cells that produce glucagon, and cells that release somatostatin and pancreatic polypeptide [4,5]. Beta cells in the islets of Langerhans are a very important cell group for body homeostasis and digestive processes. The damage or the dysfunction of beta cells leads to many diseases, mainly diabetes [4,6]. Three different strategies have been developed to date, namely pancreatic transplantation, islet transplantation, and cell-based therapy aimed at the replacement of beta cells. The lack of sufficient donors for pancreas and islet transplantations has led researchers working in this field to focus on the production of islet cells from different sources [4,7]. The transplantation of the pancreas or the beta cells could lead to successful therapy for type 1 diabetes. But unfortunately, it could come up with a big challenge which is the rejection of it by the immune system. In such cases, the usage of patient's stem cells and achieve its differentiation into beta cells in vitro, after that the injection of it into the damaged site is seen as a promising issue for today's researchers.

Cells that can self-renew and at the same time differentiate into different cell types are defined as stem cells [8]. Stem cells are the

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cells responsible for maintaining homeostasis, repairing damage in the body, and differentiating into new cells when necessary. Multipotent mesenchymal stem cells (MSCs) constitute the stem cell group with the most common clinical use. MSCs can be isolated from most adult tissues. The criteria for defining MSCs were settled by the International Society for Cellular Therapy. The main MSCs characteristics are they can adhere to the plastic of culture surfaces, differentiate into many specific cells (pluripotency), and self-renewal. Also, MSCs express the cell surface markers CD105, CD73, and CD90, but lack expression of CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR surface molecules and can differentiate into different mesodermal cell lines such as adipocytes, chondroblasts and osteoblasts [9,10]. Important advantages of MSCs include their potential to differentiate into cell types of endodermal and ectodermal lineages, including connective tissue cells, renal tubular cells, skin cells, neural cells, hepatocytes, and insulin-producing cells. Thanks to the factors they secrete, they provide support to other cells by establishing adhesion connections between the extracellular matrix and cells. At the same time, they are of interest for clinical use due to their immunomodulatory (mostly immunosuppressive) effects, angiogenic, anti-apoptotic properties, easy reproducibility in vitro, and mobilization to damaged areas [11-15].

Two challenges must be overcome to achieve full therapeutic strategies for type 1 diabetes mellitus (T1DM), as stated by the International Juvenile Diabetes Research Foundation. The first is the replacement of damaged beta cells and the finding of cell sources to provide this. The second is to provide treatment without the use of immunosuppressive drugs throughout life after this transplantation procedure. In other words, permanent replacement is required. Three different strategies have been developed to date: pancreas transplantation, islet transplantation, and cellular therapy (cell replacement and islet neogenesis) aimed at beta cell replacement. The fact that there are not enough donors for pancreas and islet transplantations has caused researchers working in this field to focus on the production of islet cells from different sources. The aim of cell-based therapy, which has become an attractive therapeutic strategy for many diseases in humans, is to replace and repair the biological function of damaged tissue or organ. This goal can be succeeded by transplanting cells that have been isolated and whose characteristics have been determined in sufficient numbers and quality to restore the functions of that organ to a target organ. The main effective source used in cellular therapy is stem cells. Although there are many studies focused on the treatment of type 1 diabetes, it has been observed that beta cells at the desired morphological and functional level have not yet been obtained. Experimental and clinical studies have provided promising results of the application of mesenchymal stem cells (MSCs) in diabetes therapy. Due to their immunosuppressive properties, these cells have the potential to be applied in many clinical areas, including the treatment of autoimmune and hereditary diseases, transplantation, and regenerative medicine.

BM-MSCs are the first source used for obtaining mesenchymal stem cells, recently it can also be obtained from several tissues like; adipose tissue, cord blood, placenta, umbilical cord, amniotic fluid, peripheral blood, dermis connective tissue, and skeletal muscle [16,17]. Bone marrow-derived MSCs BM-MSCs are the most applied MSCs in tissue regeneration studies due to their

multipotential properties. However, until today, as a result of differentiation studies from MSCs to beta cells, obtaining beta cells with morphological and functional properties at the desired level for treatment protocols using these cells is not at the desired level. Therefore, this study, it was aimed to differentiate hBM-MSCs into pancreatic progenitor cells both morphologically and functionally under in vitro culture conditions as a source of cellular therapy to overcome pancreatic origin diseases, which is one of the biggest health problems of the 21st century, with cellular replacement therapy.

Materials and Methods

In this study, human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were used, they were isolated and characterized by the permission of the Eskişehir Osmangazi University Clinical Research Ethics Committee (12/06.09.2017), and cryopreserved to be used in future experiments.

Thawing and culture of hBM-MSCs

The cryovials were kept in a 37°C water bath for a few seconds and were resuspended with fresh medium. The cells in the cryovial were taken into a conical-bottom centrifuge tube were centrifuged at 200xg for 10 min and then washed more 3 times resuspended in 1 ml complete DMEM medium containing 2% FBS, 0.2% primocin (Invivogen), 1% (ml/ml) Glutamax (Sigma). They were plated in 75 cm² flasks at the density of 0.5×10⁶ cell/flask and cultured at 37°C and in an atmosphere containing 5%O₂ and 5%CO₂ and in an incubator with humidity. Microscopic controls were made every day and the medium was changed every 3 days after the cells adhered. When cells were 70-80% confluent they were subcultured, the cells were detached from the culture dish by trypsin-EDTA, and they were seeded into a new culture dish by dividing 1/3. Cells cultured to passage 4 (P4) by subculturing were made in the characterization analysis.

Characterization of hBM-MSCs

Cell phenotypic characteristics analyses was done to confirm that hBM-MSCs maintains them after cryopreservation, MSCs were analysed by flow cytometry, and immunocytochemical and differentiation (adipogenic and osteogenic) analyses were done.

Phenotypical characterization of hBM-MSCs

Flow cytometry analysis

Characterization analyses of MSCs cultured up to P4 were performed using specific surface markers. It was determined whether it expressed CD29, CD54, CD90, HLA-A, B, C (MHC class I), CD45, and DQ (MHC class II) markers.

Briefly, after the cells that had reached the 4th passage was removed by trypsinization, they were transferred to tubes containing complete medium DMEM and centrifuged. After centrifugation, the pellet was resuspended in complete DMEM at a rate of 1x10⁶/ml after counting. Cells are then treated with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE) and PerCP-conjugated monoclonal antibodies specific to the identified cell surface markers; It was incubated for 45 minutes at room temperature (in the dark) with antibodies to CD29, CD54, CD90,

HLA-A, B, C (MHC class I), CD45 and DQ (MHC class II). After adding washing solution and centrifuging at 500xg for 5 minutes, the supernatant was discarded. After the pellet was resuspended in 400 µl of washing solution, it was analyzed in the FACSCalibur (BD Biosciences) instrument. Analyses were done using the Cell Quest program (BD Biosciences).

Immunocytochemical (IHC) analysis

IHC staining was performed to identify cellular markers. First, the cells were seeded on the cover slips and waited for sufficient confluence. Afterward, cells were fixed with 0.3% hydrogen peroxide and methanol at +4°C, thus reducing non-specific background stains as a result of the use of endogenous peroxidase (Carlo Erba Reactifs, Val-De-Reuil Cedex, France). After the fixed cells were washed with PBS, they were incubated with the blocking solution for 30 minutes at room temperature. After incubation, it was incubated overnight at +4°C with the primary antibodies in Table 1. Cells were then treated with biotinylated secondary antibodies. After incubation, streptavidin peroxidase (Thermo Scientific, Welwyn Garden City, UK) treatment was performed and the AEC kit (Zymed Laboratories, San Francisco, CA, USA) was used. Cells were nuclear stained with haematoxylin (Sigma) and examined under a microscope (Leica, DM2500, Wetzlar, Germany).

Differentiation analysis of hBM-MSCs for determination of potency

As a further characterization analysis, cells were cultured into a differentiation culture medium. They were cultured for appropriate times to differentiate into bone and adipose cell lines. After differentiation, adipogenic cells were stained with Oil Red O dye and osteogenic cells with Alizarin Red S dye, and it was determined whether the cells obtained differentiated or not.

Adipogenic Differentiation

MSCs were plated into a culture dish with 3000 cells/cm² culture surfaces. Then, they were cultured for two weeks in DMEM culture medium containing 10% FBS, 0.5 mM isobutyl-methylxanthine, 10-6M dexamethasone, 10 µg/ml insulin, 200 µM indomethacin and 1% penicillin-streptomycin. At the end of the culture intracytoplasmic lipid droplets were stained histochemical with Oil Red O and their positivity was demonstrated by microscopic examinations.

Osteogenic Differentiation

MSCs were plated with 3000 cells/cm² culture surfaces. They were cultured for 4 weeks in DMEM culture medium containing 100 nM dexamethasone, 0.05 µM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 1% penicillin-streptomycin and 10% FBS. In addition, cells were histochemical stained with Alizarin Red S for osteogenic differentiation and their positivity was demonstrated by microscopic examinations.

Differentiation from hBM-MSC to progenitor pancreatic beta cells

Differentiation of hBM-MSCs into pancreatic progenitor cells was performed in 3 steps. First, hBM-MSCs were incubated for

24 hours in high glucose/H-DMEM containing 10% FBS, 10-6 mol/L retinoic acid (RA; Sigma-Aldrich, Darmstadt, Germany) and 1% penicillin/streptomycin. Then cells were incubated in DMEM containing 10% FBS for 2 days. Second, cells were treated with trypsin-EDTA. Culture dish coated with 0.1% gelatine (Sigma-Aldrich, Darmstadt, Germany) and the cells seeded the culture dish. The cells were incubated for one week in DMEM containing 10% FBS, 10 mmol/L nicotinic acid (Sigma-Aldrich, Darmstadt, Germany) and 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, Darmstadt, Germany). Third, cells were incubated for one week in DMEM containing 10% FBS, exendin-4 (Sigma-Aldrich, Darmstadt, Germany) and glucagon-like peptide-1 (GLP-1; Sigma-Aldrich, Darmstadt, Germany). For further differentiation, cells were cultured with DMEM/F12 containing 15% FBS and L-glucose. Cells cultured for the same period of time using mesenchymal stem cell medium were used as the control group. During the differentiation process, changes in the morphology of the cells were monitored and photographed daily under the microscope. At the end of the differentiation protocol, the differentiated cells were analysed as morphological and functional.

Morphological analysis of differentiated from hBM-MSC to progenitor pancreatic beta cells

For the immunohistochemical (IHC) analysis, differentiated hBM-MSC cells were labelled with INSULIN (Santa Cruz Biotechnology, Texas, USA), C-PEPTIDE (Cell Signalling, Leiden, The Netherlands), PDX1 (Santa Cruz Biotechnology, Texas, USA) and GLUT-2 (Santa Cruz Biotechnology, USA). Texas, USA) antibodies. IHC studies were performed using Ultravision Detection System Large Volume Anti-Polyvalent, HRP (RTU) and ABC Staining System immunohistochemistry kits (Thermo Scientific™, Massachusetts, USA; Immunocruz Santa Cruz, Texas, USA). The cells were rinsed with PBS then they were fixed with methanol for 20 minutes. After washing with PBS, 1.5% normal block (normal sera) was incubated for 30 min in PBS containing serum. Primary antibodies prepared in appropriate dilution ratios were added and incubated for 2 hours at room temperature. After washing 3 times with PBS, the procedure of the immunohistochemical kit was followed and in the last step, the enzyme complex was made visible with AEC chromogen (AEC (RED) substrate kit, Thermo Scientific™, Massachusetts, USA), nuclei were counterstained with Gill's Haematoxylin II (Gill II). The dried preparations were covered with Crystal Mounting Medium and analyzed under a light microscope (Leica DM2500 Microsystems Wetzlar, Germany).

In vitro functional analysis of differentiated from hBM-MSC to progenitor pancreatic beta cells

The functionality of differentiated hBM-MSCs in terms of glucose-induced insulin release was analyzed. Before starting the insulin secretion analysis, the differentiated cell population was cultured in insulin-free medium for 2 hours and washed periodically until insulin was completely removed from the medium. The trypsinized cells were then washed with PBS and a density of 3000 cells/cm² plated in 24 wells. Serum-free L-DMEM (low-DMEM; low glucose content DMEM; 5.5 mmol/L) containing 0.5% BSA was added to the wells and the cells were incubated at 37°C for 1 hour. The supernatant was collected for basal insulin secretion analysis

and frozen at -20°C . The cells were then cultured in the H-DMEM (high DMEM; high glucose content DMEM 25 mmol/L) medium for 1 hour. At the end of the period, supernatants were collected and frozen to determine the release of glucose-stimulated insulin. Insulin ELISA kit (Millipore) was used to determine the amount of immunoreactive insulin stimulated by glucose released from differentiated beta cells (experimental group) and non-differentiated hBM-MSCs (control). Absorbance was measured at 450 nm in Microplate Reader with monochromator system (BIOTEK ELx808IU, Vermont, USA). The values were normalized according to the total protein content of the cells for each experiment on the day of collection of media/supernatant samples. The stimulation index (SI) for both the control (non-differentiated MSCs) and the experiment (differentiated beta cells) group was calculated by dividing the normalized insulin values measured from high glucose samples into the normalized insulin values measured from low glucose samples. At the end of the period, supernatants were collected and frozen to determine the release of insulin stimulated by glucose.

Statistical analysis

Analyses were repeated at least 3 times. Data are reported as means $\pm$ SD. All statistical analyses were performed using SPSS

20.0 (or the later version, SPSS Inc., Chicago, IL, USA). Since we have one nominal and measurement variable, and want to compare the mean values of the measurement variable, Student's t-test was used. Differences between the groups were regarded as statistically significant when $p < 0.05$.

Results

After the freezing and thawing process of hBM-MSCs, daily inverted phase-contrast light microscopic examination and photographing were performed in the culture. In this microscopic examination of the isolated cells, it was observed that the cells were spindle-shaped, large or more fibroblast-like morphology. (Figure 1-I). When the immunophenotypic features of hBM-MSCs were analyzed by flow cytometry and immunocytochemical methods, it was determined that while they were positive for CD29, CD54, CD90, CD146, and MHC-I markers, negative for CD71, C-PEPTIDE, PDX1, CD146, Ki67, PCNA, CD45, and MHC-II markers. (Table 1, Figures 1-II and 1-III). Adipogenic and osteogenic differentiation of the cells isolated from human bone marrow was determined by staining with histochemical dyes Oil Red O and Alizarin Red S specific to intracellular lipid droplets and calcified bone nodules (Figure 2).

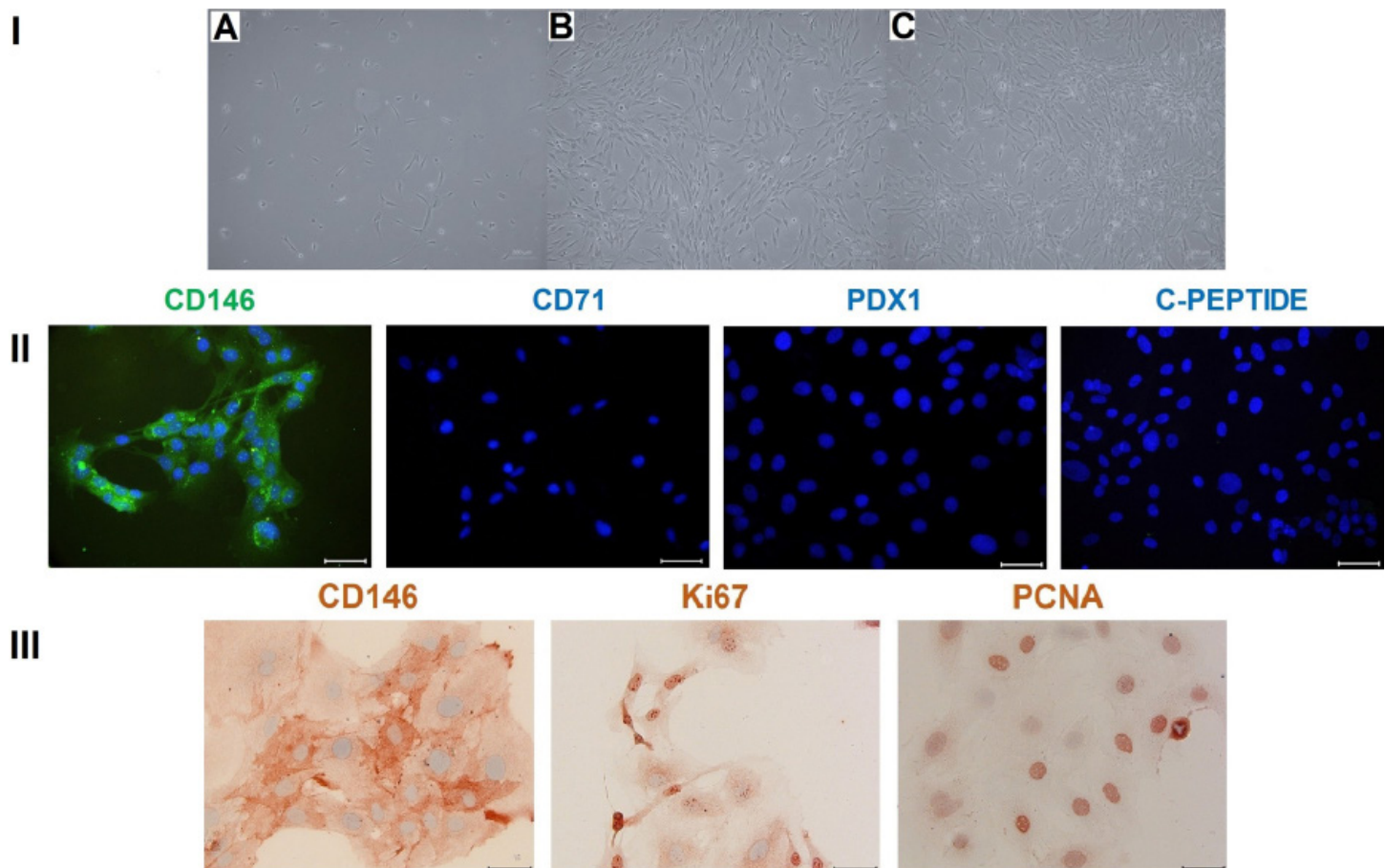


Figure 1. Morphological and immunophenotypic analyses of hBM-MSCs cells 1-I) Phase-contrast microscopic views of hBM-MSCs cells in the early (A: P0, 3rd day attached cells) and later stages (B: P1, 3rd days; 50-60% confluence, C: P3, 4th day; 70% confluence) of culture. The fibroblast-like, spindle-shaped and elongated structures of the hBM-MSCs are clearly visible. (Scale bars: 200 μm). 1-II) When human bone marrow-derived mesenchymal stem cells were labelled with immunofluorescence method, it was determined that while they expressed CD146 marker, which is positive specific to MSCs, it did not express CD71, which is also expressed in MSCs, and C-PEPTIDE and PDX1 markers, which are specific to beta cells of the pancreas. (Scale bars: 50 μm). 1-III) It was determined that when human bone marrow-derived mesenchymal stem cells were labelled by immunohistochemical method, they expressed CD146 marker, which is specific to MSCs, and Ki67 and PCNA markers, which are viability and proliferation markers (Scale bars: 50 μm)

Table 1. Flow cytometry and immunocytochemical analysis of immunophenotypical features of hBM-MSCs (P3)

Antibody/marker name	Phenotype in hBM-MSCs (% or +/- expression)
CD29	99.72%
CD54	98.38%
CD90	95.16%
MHC-I	74.38%
CD45	0.11%
MHC-II	0.67%
CD146	+
CD71	-
PDX1	-
C-PEPTIDE	-
CD146	+
Ki67	+
PCNA	+

+= positive antibody expression; -= negative antibody expression

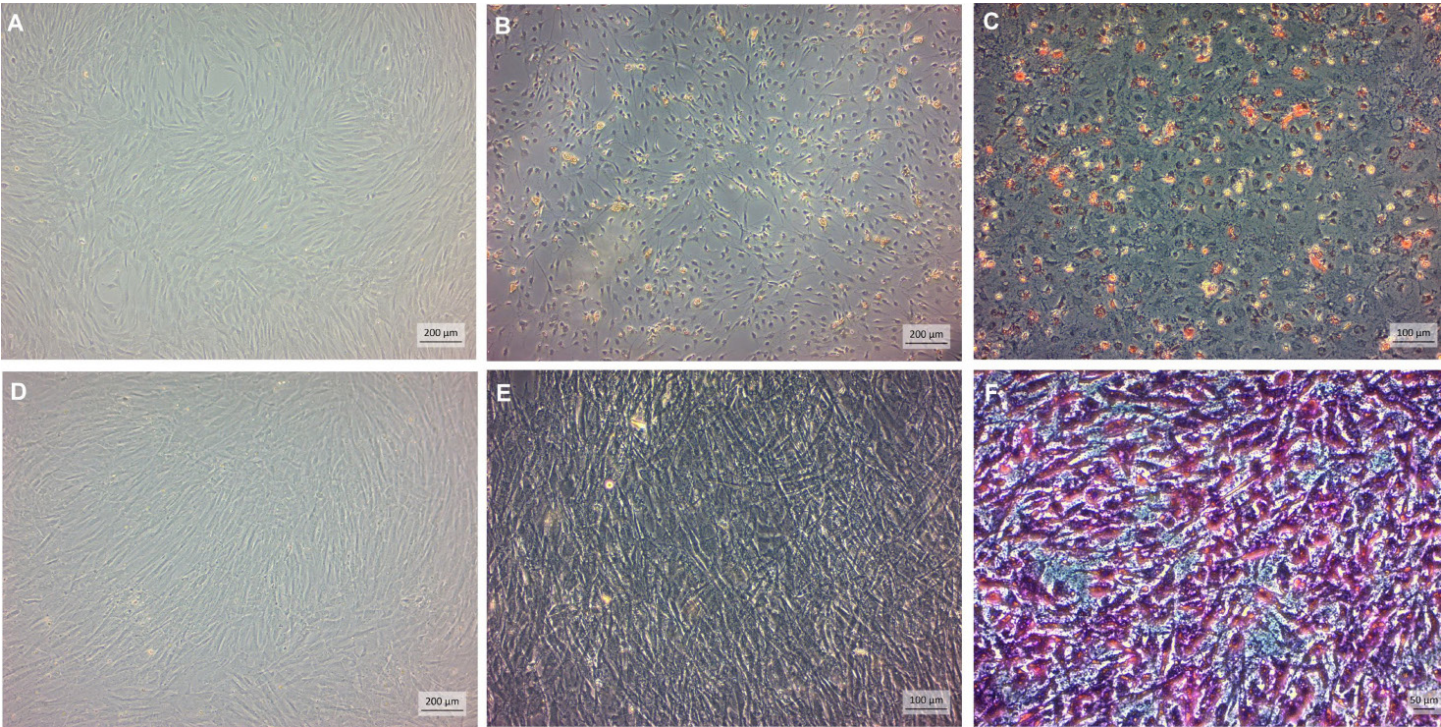


Figure 2. Microscopic views of adipogenic and osteogenic differentiation analyses of hBM-MSCs. (A) Phase-contrast microscopic view of adipogenic undifferentiated hBM-MSCs (control group). (B) Phase-contrast microscopic view of adipogenic differentiated hBM-MSCs after 20 days of incubation. (C) Phase-contrast microscopic view of adipogenic differentiation determined by accumulation of neutral lipid vacuoles after 25 days of culture (Oil Red O; ORO staining). (D) Phase-contrast microscopic view of osteogenic undifferentiated hBM-MSCs (control group). (E) Phase-contrast microscopic view of osteogenic differentiated hBM-MSCs after 34 days of incubation. (F) Osteogenic differentiation of hBM-MSCs after 34 days of osteogenic induction. Positive staining of mineral nodules with Alizarin Red-S staining. (Scale bars: A, B and D 200 µm; C and E 100 µm; F 50µm)

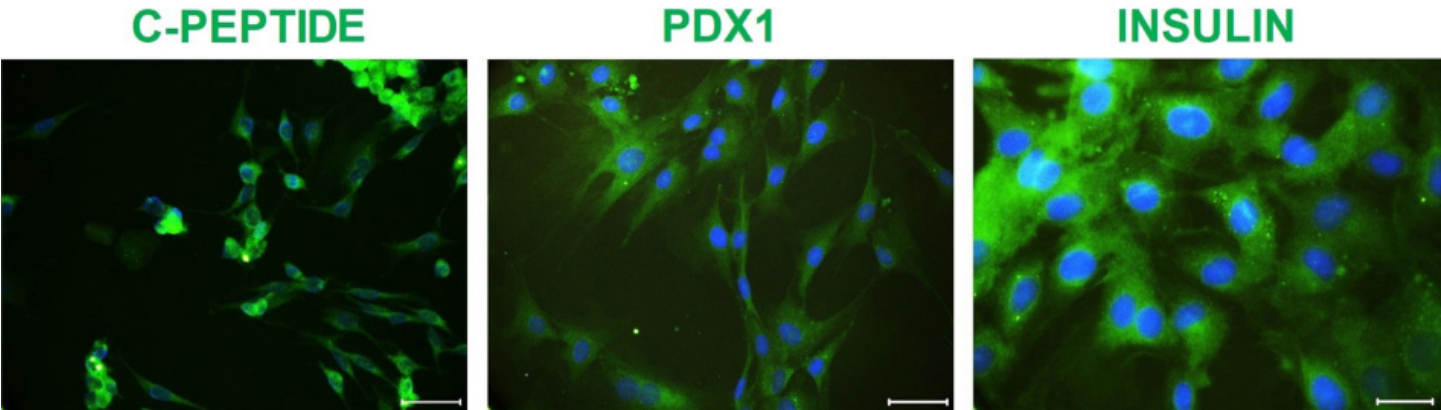


Figure 3. When cells differentiated from hBM-MSCs into pancreatic progenitor/beta cells were immunofluorescence labelled, it was determined that they expressed C-PEPTIDE, PDX1 and INSULIN markers specific to beta cells of the pancreas. Nuclei were labelled with DAPI (Scale bars: 50, 50 and 20 µm)

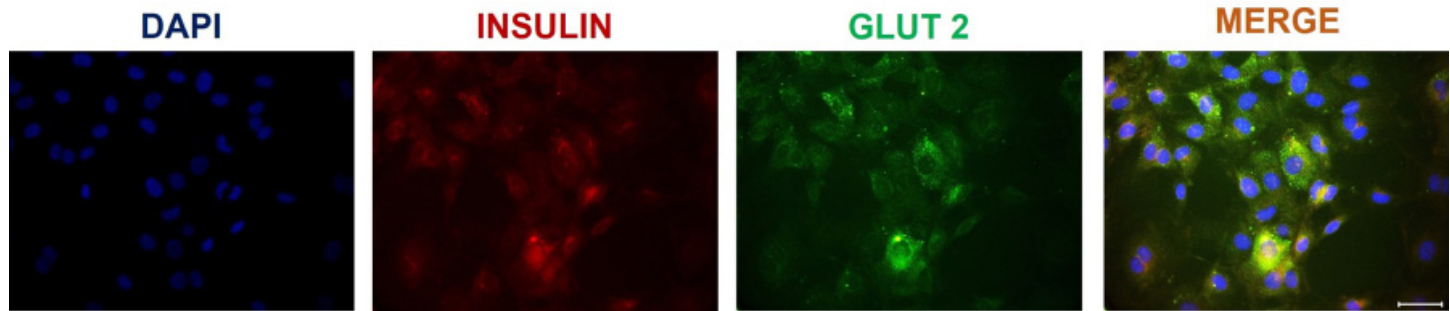


Figure 4. When cells differentiated from hBM-MSCs into pancreatic progenitor/beta cells were immunofluorescence labelled, it was determined that they expressed INSULIN and GLUT-2 markers specific to beta cells of the pancreas. Nuclei were labelled with DAPI (Scale bar: 50 μ m)

It was immunocytochemically determined that pancreatic progenitor/beta cells differentiated from hBM-MSCs expressed the markers INSULIN, C-PEPTIDE, PDX1 and GLUT-2, which are specific to beta cells (Figures 3 and 4). After differentiated hBM-MSC cells were determined morphologically differentiate into beta cells, their functionality was analyzed in terms of glucose-stimulated insulin release. When low (LDMEM) and high (HDMEM) concentrations of glucose were added to the medium of the cells in the control and experimental groups (MSCs and MSCs differentiated into beta cells, respectively), which were kept in insulin-free medium after removal of insulin, differentiated beta cells responded to changing glucose ratios by increasing insulin

secretion. When the amount of glucose was increased, an increase in the amount of insulin was observed in response ($p < 0.01$). The glucose stimulation index (SI) of beta cells differentiated from hBM-MSCs was higher than the control group ($p < 0.05$) (Figure 5).

Discussion

Diabetes mellitus (DM) is a chronic metabolic disease, which occurs due to the autoimmune destruction or dysfunction of the insulin-producing pancreatic beta cells. Diabetes is known as the "silent killer" because it progresses slowly and with no alarms in the body. Frequent urination (polyuria) and excessive thirst

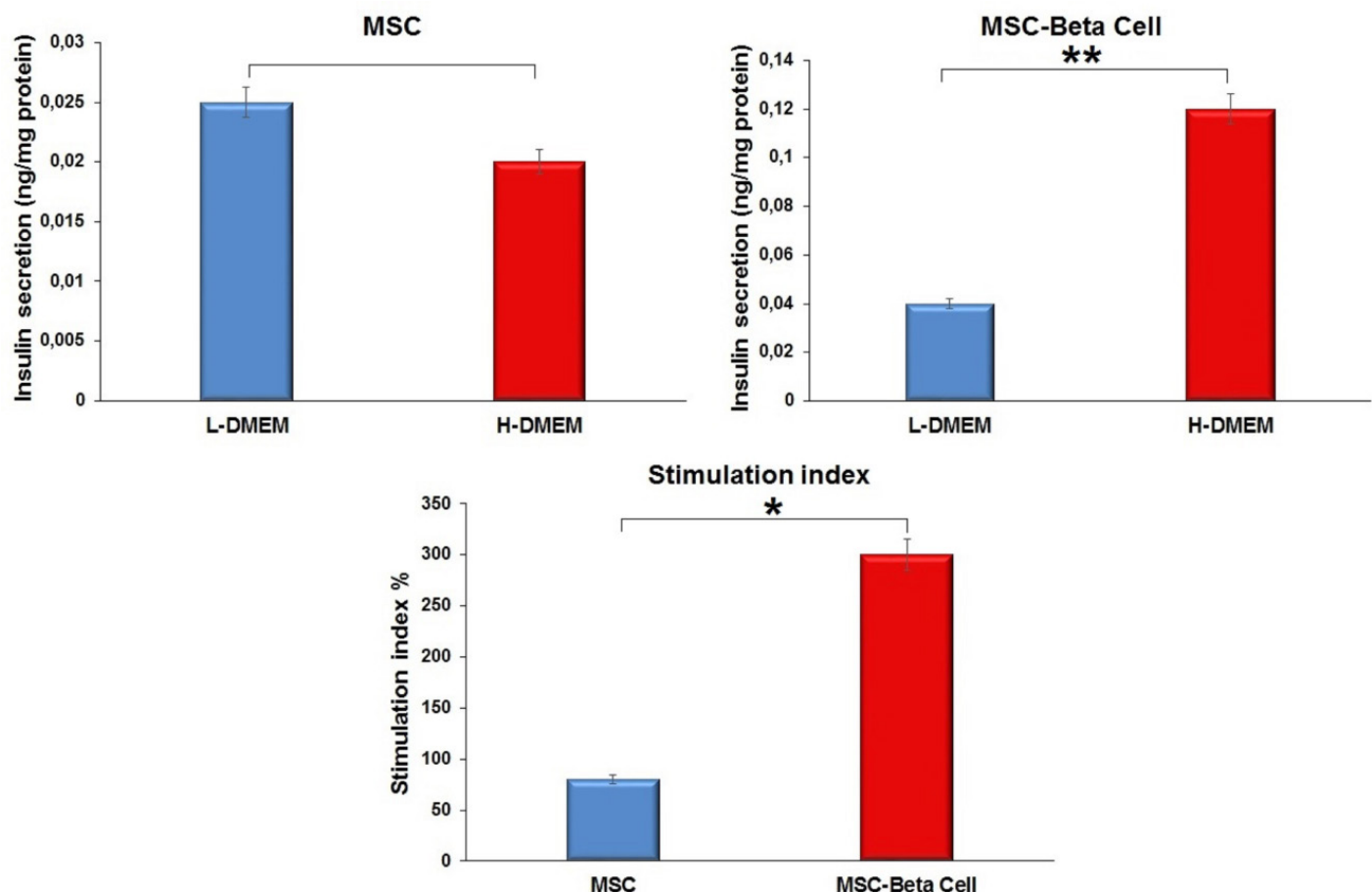


Figure 5. Glucose-stimulated insulin release and insulin content analysis (insulin producing glucose tolerance test, IPGTT) by ELISA on media after differentiation into beta cells. The response of both differentiated beta cells and control groups (MSCs) by secreting insulin to various concentrations of glucose added to the medium was compared. When glucose concentration was increased in the medium of beta cells differentiated from control group (MSCs) and MSCs (when switching from 3.3 mM glucose to 20 mM glucose), it was determined that the insulin secretion of differentiated beta cells increased ($*p < 0.01$) compared to the control group. At the same time, the stimulation index of differentiated beta cells was higher than the control group ($*p < 0.05$). (n=3, mean $\pm$ SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

(polydipsia) in newly diagnosed diabetic patients are not severe. And some peoples show no symptoms. But as time passes, the effects of both types of diabetes (type 1 and 2) become more and more serious and it could cause death, these symptoms include heart disease, eye problems, kidney failure, nerve damage, and erectile dysfunction. Hypoglycaemia (acute low blood sugar level) and hyperglycaemia (high blood sugar level) contribute significantly to the effects of diabetes [18].

Many ways can be applied to repair the physiological control of blood glucose levels: external insulin administration, insulin-stimulating drugs, drugs that reduce insulin resistance and/or replace beta cell mass (sensitizers) [19]. Antidiabetic drugs are used to provide glucose control. However, it is known that its effects on stopping the progression of the disease are limited. Alternatively, the generation of beta cells by transplantation of islets of Langerhans is a promising treatment option. However, the shortage of donors prevents the widespread application of this treatment (or along with this, to achieve continuous metabolic control for 1 year, at least 2 million beta cells per kg of body weight must be transplanted which results in a restricted number of healthy islets available for this application) [20]. The human pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells are considered as attractive alternative source for beta cells transplantation [19].

Recently, researches on stem cells have added an important part of the scientific understanding of type 1 diabetes. In type 1 diabetes, beta cells are being attacked by the patient's immune system, to prevent this, the patient has to take immunosuppressant drugs. It may be possible to grow islet cells from the patient's existing islet cells in the future, but a patient with type 1 diabetes will still need immunosuppressive drugs to prevent the cells from being damaged [21]. The stem cell replicates itself and can then divide asymmetrically to form another cell type (differentiation). Although initially thought to be derived only from embryos, non-embryonic stem cells can now be obtained without much difficulty from neonatal tissue, umbilical cord, as well as various adult tissues such as bone marrow, skin, and fat. Recent studies have shown that stem cells are prime candidates in the fight against diabetes, which affects millions of people worldwide, thanks to their ability to transform or differentiate into different cell types in the body and to their immunosuppression properties [22]. Regarding the treatment of diabetes with stem cells, there are studies on obtaining beta cells from stem cells of pancreatic or non-pancreatic (liver, bone marrow) origin [23,24,25,26,27,28,29]. However, as cells similar to beta cells produced in these studies were eliminated by autoimmunity, the expected results have not been obtained yet.

The general strategy for identifying bone marrow mesenchymal stem cells cultured in-vitro is to analyze the expressions of the cell surface markers such as CD29, CD44, and CD90 [30,31,32]. In our study, the hBM-MSCs were determined immunophenotypically as positive for CD29, CD54, CD90, and MHC-I markers, while it was negative for CD45 and MHC-II markers. When hBM-MSCs were labelled by the immunofluorescence method, it was determined that while they expressed CD146 marker, which is MSCs specific positive expressed marker, they did not express CD71 which is also expressed in MSCs, and also, they did not express the pancreatic beta cells specific markers; C-PEPTIDE and PDX1. On the other

hand, it was determined that the immunofluorescence labeled differentiated BM-MSCs into pancreatic progenitor/beta cells, expressed C-PEPTIDE, PDX1, and INSULIN markers, which are the pancreatic beta cells specific. In addition, it was found that it expresses the MSCs specific marker CD146, and the viability and proliferation markers; Ki67 and PCNA. The expression of cell surface antigens and markers results was similar to previous studies [33,34].

The main function of beta cells is releasing sufficient insulin to maintain the physiological ratio of glucose in plasma in response to nutrients, hormones, and neural stimuli for optimal functioning of all tissues in the body. However, insulin secretion is not specific to beta cells, it is known that insulin is also secreted from other cells [35,36]. Therefore, in our study, when the differentiated beta cells were induced in-vitro by adding glucose to their medium, we analyzed if they could stimulate a cellular response to lower glucose via secreting insulin. ELISA, and glucose-induced insulin secretion and insulin content analyses (IPGTT) were performed for the different concentrations of glucose added to the medium of both differentiated beta cells and the control group (MSCs), and the results were compared. Accordingly, it was determined that when glucose concentration was increased in the medium of beta cells differentiated the insulin secretion was increased ($p < 0.05$). Likewise, the stimulation index (SI) of differentiated beta cells was higher than the control group (MSCs) ($p < 0.01$). Our results showed that progenitor pancreatic beta cells differentiated from BM-MSCs secreted insulin at a significant level compared to the control group. So, generating surrogate beta cells suitable to replace the damaged beta cell function in diabetes is possible by this approach.

PDX1 (pancreatic and duodenal homeobox 1) is expressed in the pancreatic bud during embryogenesis and regulates the development of the pancreas [37]. In the postnatal period, in addition to its effect on insulin gene expression, it also determines beta cell survival and beta cell resistance to endoplasmic reticulum stress. It is known that the PDX1 protein, which is very important in the regulation of insulin secretion, also plays a critical role in the development of the endocrine and exocrine components of the pancreas. The PDX1 gene is also closely associated with the differentiation of insulin-producing cells from progenitor cells, and with the island neogenesis [38]. PDX1 has been recognized as a key transcription factor in pancreatic embryogenesis and it has an important role as a glucose-sensitive regulator of insulin expression in mature beta cells. Expressions of PDX1, GLUT-2, INSULIN, and C-PEPTIDE are expected to be detected in the late stages of pancreatic islet maturation. In our study, cells differentiated from human bone marrow to pancreatic progenitor cells were positive for C-PEPTIDE, PDX1, and INSULIN markers when immunofluorescence stained. Protein products required for insulin production and secretion in pancreatic beta cells such as PDX1, GLUT-2, and C-PEPTIDE were expressed by beta cells.

In our study, we aimed to differentiate human bone marrow derived MSCs into pancreatic progenitor cells both morphologically and functionally in vitro. In our results, it was determined that the cells obtained when human BM-MSCs were differentiated with inductors added to the medium, were expressing beta-cell-specific markers and had morphological and functional features of the beta

cells.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

This study was supported by Eskişehir Osmangazi University, Scientific Research Projects (ESOGU-BAP, ESOGU/201846D27).

Ethical approval

Ethical approval by the local ethical authority was indicated in the 'Material and methods' section for human and experimental studies. The human bone marrow-derived mesenchymal stem cells (hBM-MSCs) used in this study are cells that were isolated and characterized by the permission of the Eskişehir Osmangazi University Clinical Research Ethics Committee (12/06.09.2017), and cryopreserved to be used in future experiments.

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