Elucidation of adipogenic differentiation regulatory mechanism in human maxillary/mandibular bone marrow-derived stem cells

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Abstract

Mesenchymal stem cells (MSCs) are pluripotent stem cells present in various tissues. Previous studies have shown that maxillary/mandibular bone marrow-derived MSCs (MBMSCs) scarcely differentiate into adipocytes. However, the regulatory molecular mechanisms underlying adipogenic differentiation of MBMSCs remain unknown. We investigated the underlying molecular mechanisms that regulate adipogenic differentiation of MBMSCs. We observed no significant differences in cell surface antigen profiles and stem cell marker gene expression in MBMSCs and iliac bone marrow-derived MSCs (IBMSCs). MBMSCs and IBMSCs displayed similar osteogenic and chondrogenic differentiation potentials, whereas MBMSCs showed significantly lower lipid accumulation, adipocyte marker gene expression, and intracellular glucose uptake than that showed by IBMSCs. Expression of CCAAT/enhancer binding protein β (C/EBPβ), C/EBPδ, early B-cell factor 1 (Ebf-1), and Krüppel-like factor 5 (KLF5), which are transcription factors expressed early in adipogenic differentiation, was suppressed in MBMSCs compared to that in IBMSCs. Peroxisome proliferator-activated receptor-γ (PPARγ) and C/EBPα, which play important roles in the terminal differentiation of preadipocytes into mature adipocytes, were increased during adipogenic differentiation in MBMSCs and IBMSCs; however, the expression level of these genes in MBMSCs was lower than that in IBMSCs. Furthermore, the level of zinc finger protein 423 (Zfp423), which is involved in the commitment of undifferentiated MSCs to the adipocyte lineage, was significantly lower in undifferentiated MBMSCs than that in IBMSCs. These data indicate that MBMSCs are negatively regulated in the commitment of undifferentiated MSCs to the adipocyte lineage (preadipocytes) and in terminal differentiation into mature adipocytes. These results may elucidate the site-specific characteristics of MBMSCs.

1. Introduction

Mesenchymal stem cells (MSCs) can differentiate into various cells, such as osteoblasts, chondrocytes, and adipocytes [2, 3]. Therefore, MSCs are considered a promising cell source for the repair of damaged tissues. MSCs express several cell surface antigens, such as CD44, CD73, and CD90, and are negative for CD11, CD34, and CD45 [1]. MSCs are present in various tissues throughout the body, including the bone marrow, adipose tissue, muscle, and synovium [3]. MSCs also reportedly reside in various dental tissues, such as dental pulp, periodontal ligament, gingival, and exfoliated deciduous teeth [4]. Previously, we showed that MSCs are present in the maxillary/mandibular bone marrow [5]. Most of the bone tissue is derived from the mesoderm, whereas the craniofacial bone, including the maxillary/mandibular bone, is derived from the neural crest [6]. MSCs isolated from the orofacial region and iliac crest have been reported to exhibit site-specific characteristics owing to the differences in embryological origins [7–10]. Our previous study revealed that the osteogenic potential of MBMSCs was similar to that of iliac bone marrow-derived MSCs (IBMSCs); however, MBMSCs scarcely differentiated into adipocytes [5]. Consistent with our study, other groups have also reported that MBMSCs exhibit lower adipogenic differentiation potential than that exhibited by IBMSCs [7, 10]. However, the mechanisms through which the differentiation of MBMSCs is regulated remain unclear.

Adipogenesis is regulated by multi-step processes in which undifferentiated MSCs are committed to the adipocyte lineage (preadipocytes), followed by preadipocyte differentiation into mature adipocytes [11]. Each stage of adipogenic differentiation of MSCs is tightly regulated by various transcription factors [3]. CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ are immediately upregulated after adipogenic differentiation, and they function as the transcriptional activator of peroxisome proliferator-activated receptor-γ (PPARγ) and C/EBPα [12, 13]. Furthermore, early B-cell factor 1 (Ebf-1) and Krüppel-like factor 5 (KLF5), which are induced by C/EBPβ and C/EBPδ, promote PPARγ and C/EBPα expression, thereby inducing adipogenic differentiation [14, 15]. Expression of these early adipogenic transcription factors is essential for differentiation of preadipocytes into immature adipocytes. PPARγ and C/EBPα regulate the expression of adipocyte metabolic factors, including adipocyte protein 2 (aP2), lipoprotein lipase (LPL), adiponectin, and glucose transporter 4 (GLUT4), resulting in adipocyte maturation [11]. While the mechanisms that regulate terminal differentiation of preadipocytes into mature adipocytes are well elucidated, adipose lineage-determining factors in undifferentiated MSCs are not well understood. Recently, several studies have demonstrated that zinc finger protein 423 (Zfp423) is important for the adipocyte lineage commitment of MSCs [16, 17].

MBMSCs may be negatively regulated in the process of commitment of undifferentiated MBMSCs to the adipocyte lineage or after differentiation of preadipocytes into mature adipocytes; however, this is yet to be clarified. Therefore, we compared the adipogenic potential of MBMSCs and IBMSCs and elucidated the underlying molecular mechanisms that regulate adipogenic differentiation of MBMSCs. We believe our study findings will form an important basis for clarifying the site-specific characteristics of MBMSCs and elucidating the in vivo functions and the developmental of MBMSCs.

2. Materials And Methods

2.1. Isolation of MBMSCs from maxillary/mandibular bone marrow

Bone marrow was collected from three patients (aged 39–65 years) from a hole made in the maxillary or mandibular bone using a biopsy needle (GC, Tokyo, Japan) during dental implant surgery, according to the protocol approved by the ethical committee at Kagoshima University, Kagoshima, Japan (No. 170263 EKI-KAI3) [18]. The number of white blood cells in part of the bone marrow was counted. The bone marrow cells were then seeded at a density of $5 \cdot 10^4$ white blood cells/cm^2 in a cell-culture dish. The cells that adhered to the culture dish and proliferated were used as MBMSCs.

2.2. Cell culture

In the present study, we used three IBMSCs (donors aged 25–41 years; Lonza, Walkersville, MD, USA). MBMSCs and IBMSCs were cultured in α-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA) and 1% antibiotics (Life Technologies, Waltham, MA, USA) at 37°C with 5% CO_2. Cells between the 5th and 7th passages were used for further experimentation.

2.3. Flow cytometric analysis

Cell surface marker expression was measured using the Guava easyCyte™ 5 (Merck, Darmstadt, Germany). MBMSCs and IBMSCs were harvested from the cell culture dishes using Trypsin-EDTA (Thermo Fisher Scientific, Rockford, IL, USA), and the cells ($5 \cdot 10^4$ cells) were resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (Thermo Fisher Scientific), 2 mM EDTA, and FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Next, the cells were incubated with the following antibodies: fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated antibodies against human CD11, CD13, CD29, CD34, CD44, CD45, CD73, CD 90, CD166, HLA-DR, HLA-ABC, mouse IgG1 or IgG2a as a negative control (BioLegend, San Diego, CA, USA), and human CD105 (Miltenyi Biotec).

2.4. Osteogenic differentiation

MBMSCs and IBMSCs (1×10^4 cells) were seeded in 24-well cell culture plates. Once confluent, the cells were cultured in osteogenic induction medium (α-MEM supplemented with 10% FBS, 1% antibiotics, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL L-ascorbic acid-2-phosphate). After 14 days of culturing, the cells were stained with 1% alizarin red S solution (Sigma-Aldrich, St Louis, MO, USA) and then photographed using a phase-contrast microscope (Olympus, Tokyo, Japan).

2.5. Chondrogenic differentiation

Chondrogenic differentiation was induced using the pellet culture method. The cells (2×10^5) were centrifuged at 1500 rpm for 5 min, and the cell pellets were cultured in chondrogenic differentiation medium (α-MEM supplemented with 1% antibiotics, 1% GlutaMAX, 10 ng/mL of transforming growth factor β3, 100 nM of dexamethasone, 50 µg/mL of L-ascorbic acid 2-phosphate, 3.5 g/L of *D*-(+)-glucose, 100 µg/mL of pyruvate, and 1% ITS + Premix). After chondrogenic induction for 28 days, the pellets were fixed with 4% paraformaldehyde for 15 min and embedded in paraffin. The sections were stained with toluidine blue (Sigma-Aldrich) and photographed using a phase-contrast microscope (Olympus).

2.6. Adipogenic differentiation

Cells were cultured in adipogenic differentiation medium (DMEM-high glucose supplemented with 10% FBS, 1% antibiotics, 1 µM of dexamethasone, 0.5 mM of 3-isobutyl-1-methyl-xanthine, 0.2 mM of indomethacin, and 10 µg/mL of insulin) for 2 days, followed by culturing in adipogenic maintenance medium (DMEM-high glucose supplemented with 10% FBS, 1% antibiotics, and 10 µg/mL of insulin) for another 2 days. After adipogenic induction for 14 days, the cells were stained with 60% Oil Red-O solution (Sigma-Aldrich) and photographed using a phase-contrast microscope (Olympus). Lipid droplet area was quantified in five randomly chosen fields in each well using Image J software (National Institute of Health, Bethesda MD, USA.

2.7. Quantitative real-time polymerase chain reaction (PCR) analysis

Cells were seeded in 35 mm culture dishes and maintained in growth medium until confluent. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). In some experiments, cells were induced to undergo adipogenic differentiation for the indicated times, followed by RNA isolation. The resulting total RNA was reverse-transcribed into cDNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using the THUNDERBIRD SYBR Green PCR kit (TOYOBO) and CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). In the present study, *GAPDH* mRNA was used as an internal control to correct for relative gene expression. Table 1 shows the list of primer sequences used in this study.

2.8. Immunoblotting analysis

MBMSCs and IBMSCs were cultured in adipogenic induction medium for the indicated times and lysed in RIPA lysis buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The extracted total protein was separated by polyacrylamide gel electrophoresis on 10% gels, and proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with 5% nonfat milk (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) solution for 1 h. The membrane was then incubated with anti-C/EBPβ (Abcam, Cambridge, UK), anti-C/EBPδ, anti-Ebf-1, anti-KLF5 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-PPARγ, anti-C/EBPα, anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), and anti-Zfp423 (Novous Biologicals, Centennial, CO, USA) antibodies overnight at 4°C. After washing with PBS containing Tween-20 (PBS-T), the membrane was incubated with HRP-conjugated anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h. Enhanced chemiluminescence (ECL) western blot detection reagent (GE Healthcare, Buckinghamshire, UK) was used to visualize the target protein bands. Protein bands were visualized using the ChemiDoc Imaging System (Bio-Rad). β-Actin protein signals were used as an internal control to normalize protein expression.

2.9. Measurement of intracellular glucose

To measure intracellular glucose, we used the Glucose Assay Kit-WST (Dojindo, Kumamoto, Japan). MBMSCs and IBMSCs were induced to undergo adipogenic differentiation for the indicated days, and cell lysates were prepared using PBS containing 0.1% Triton-X-100. Equal volumes of cell lysates and assay working solution were mixed and incubated at 37°C for 30 min. Absorbance was measured at 450 nm using a microplate reader (Multiscan FC; Thermo Fisher Scientific).

2.10. Statistical analysis

All data analyses were performed using SPSS software (version 25.0, SPSS Inc., Chicago, IL, USA). The normality test for each data set was performed using the Shapiro–Wilk test. Comparisons between two groups of normally distributed data were performed using the Student's *t*-test. Multiple comparisons of normally distributed data were performed using two-way analysis of variance with Tukey's post hoc test. For non-normally distributed data, we used the nonparametric Kruskal–Wallis test followed by Tukey's test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Cell surface antigen and stem cell marker expression of *MBMSCs and IBMSCs*

First, we compared cell surface antigen expression between MBMSCs and IBMSCs. The expression levels of 12 cell-surface antigens in MBMSCs and IBMSCs were examined using flow cytometry. The cell surface antigen expression profiles were similar to those previously reported for MSCs (positive for CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC, and negative for CD11, CD34, CD45, and HLA-DR) in both MBMSCs and IBMSCs (Table 2). However, the expression patterns of CD105 and HLA-DR differed slightly between the MBMSCs and IBMSCs (Table 2). It has been reported that *OCT4*, *Nanog*, and *SOX2* gene expression are important for the maintenance of MSC pluripotency [19]. We evaluated whether the expression of these stem cell marker genes was suppressed in MBMSCs. There was no significant difference in *OCT4*, *SOX2*, or *Nanog* gene expression between undifferentiated MBMSCs and IBMSCs (Fig. 1).

3.2. Comparison of differentiation potential of MBMSCs and IBMSCs

MBMSCs and IBMSCs were cultured in osteogenic induction medium for 14 days, and the calcification level was estimated using alizarin red staining. The cell lines showed obvious calcium deposition, and there was no difference in osteogenic potential between MBMSCs and IBMSCs (Fig. 2A). The chondrogenic potential of MBMSCs and IBMSCs was examined in pellet culture for 28 days. Toluidine blue-stained cartilage-like tissue was confirmed in all MBMSCs and IBMSCs, and there was no difference in the chondrogenic differentiation potential between MBMSCs and IBMSCs (Fig. 2B). Next, we compared the adipogenic differentiation potential of MBMSCs and IBMSCs. IBMSCs displayed accumulation of many lipid droplets after adipogenic induction, but MBMSCs displayed only a few lipid droplets (Fig. 2C). In addition, lipid droplet area in MBMSCs were significantly smaller than that in IBMSCs (Fig. 2C).

3.3. Evaluation of early adipogenic differentiation factor expression in MBMSCs and IBMSCs

Adipogenic differentiation of MSCs is regulated by complex steps involving various transcription factors [2]. C/EBPβ and C/EBPδ are transcription factors that are induced in the early stages of adipogenic differentiation and play important roles in the differentiation of preadipocytes into adipocytes [12, 13]. We investigated C/EBPβ and C/EBPδ expression in MBMSCs and IBMSCs 72 h after adipogenic differentiation. In undifferentiated cells (0 h), the mRNA expression levels of *C/EBPβ* and *C/EBPδ* in MBMSCs were lower than that observed in IBMSCs; however, the difference was not significant (Fig. 3A). *C/EBPβ* and *C/EBPδ* mRNA expression was induced during adipogenic differentiation in both MBMSCs and IBMSCs, however, the expression level of each the mRNA in MBMSCs was lower than that in IBMSCs at all time points (Fig. 3A). C/EBPβ and C/EBPδ proteins were also increased in MBMSCs and IBMSCs

during adipogenic differentiation, but their expression levels in MBMSCs were lower than those observed in IBMSCs (Fig. 3B). Ebf-1 and KLF5, early adipogenic transcription factors, are regulated by C/EBPβ and C/EBPδ [14, 15]. Therefore, we investigated whether Ebf-1 and KLF5 are involved in the regulation of adipogenic differentiation in MBMSCs. *Ebf-1* mRNA expression was significantly lower in undifferentiated MBMSCs than that in IBMSCs. Although there was no significant difference, *KLF5* mRNA expression in undifferentiated MBMSCs was lower than that in undifferentiated IBMSCs (Fig. 3C). *Ebf-1* and *KLF5* mRNA expression in MBMSCs was lower than that in IBMSCs throughout the adipogenic differentiation period (Fig. 3C). The Ebf-1 and KLF5 expression in MBMSCs was also lower than that in IBMSCs (Fig. 3D).

3.4. Evaluation of late adipogenic differentiation factor expression in MBMSCs and IBMSCs

PPARγ and C/EBPα are essential transcription factors that promote the expression of various adipocyte metabolic factors and induce terminal differentiation of preadipocytes into mature adipocytes [3, 11]. Therefore, we evaluated their molecular expression during terminal differentiation of MBMSCs and IBMSCs into adipocytes. The induction of adipogenic differentiation increased *PPARγ* and *C/EBPα* mRNA and protein expression in both MBMSCs and IBMSCs, but their expression levels in MBMSCs were lower than those in IBMSCs at all time points (Fig. 4A, B). Next, we investigated the expression of adipogenic regulatory genes in MBMSCs and IBMSCs. In IBMSCs, *aP2*, *LPL*, *adiponectin*, and *GLUT4* expression levels were significantly increased after the 7th day of adipogenic differentiation, however, the expression of these adipogenic regulator genes was only slightly altered in MBMSCs (Fig. 4C). Figure 4D shows the intracellular glucose uptake by MBMSCs and IBMSCs on days 7, 10, and 14 after adipogenic differentiation. The intracellular glucose concentration by MBMSCs was significantly lower than that by IBMSCs on days 10 and 14 after adipogenic differentiation (Fig. 4D).

3.5. Evaluation of adipogenic lineage commitment factor expression in MBMSCs and IBMSCs

Next, we investigated the expression of Zfp423, which regulates adipocyte lineage determination, in undifferentiated MSCs. *Zfp423* mRNA levels were significantly lower in undifferentiated MBMSCs than that in IBMSCs (Fig. 5A). Figure 5B shows that Zfp423 protein expression was lower in all the three MBMSCs compared to the IBMSCs.

4. Discussion

Osteogenic and chondrogenic differentiation potential of MBMSCs have been reported to be equivalent to that of iliac and long bone marrow-derived MSCs [7, 8]. In contrast, MBMSCs show lower adipogenic differentiation potential than that showed by MSCs derived from the iliac or long bone marrow [5, 7, 10]. However, the molecular mechanisms that regulate adipogenic differentiation of MBMSCs are unclear. This is the first detailed study to evaluate the differences in adipogenic potential between MBMSCs and

IBMSCs. Furthermore, we elucidated the molecular mechanisms responsible for the lower adipogenic potential of MBMSCs compared to IBMSCs.

The International Society for Cellular Therapy defines human MSCs as positive for CD73, CD90, and CD105, and negative for CD11b, CD34, CD45, and HLA-DR [20]. We evaluated whether there was a difference in cell-surface antigen expression between the MBMSCs and IBMSCs. In this study, we compared the expression of 12 typical cell-surface antigens in MBMSCs and IBMSCs. The cell-surface antigen expression profiles of MBMSCs were similar to those of IBMSCs. However, individual differences were observed in the expression of CD105 and HLA-DR. CD105 expression in MSCs is affected by the reaction time of trypsin and seeding cell density [21, 22]. Human MSCs were defined as HLA-DR-negative. Previous reports have shown that osteoblasts express HLA-DR [23]. In the present study, 4.15% of HLA-DR-positive cells were detected in one batch of IBMSCs (IBMSC-2). It is possible that the contamination of osteoblast-like cells or differentiation of some cells into osteoblasts occurred during cell culture. Osteoblast contamination may account for the low adipogenic differentiation potential of MBMSCs. However, HLA-DR expression in all the MBMSCs was low (0–1.6%), suggesting that the possibility of contamination of MBMSCs with osteoblasts was low. Previous reports have demonstrated that MSCs derived from CD56 (neural cell adhesion molecule 1) knockout (KO) mice have significantly reduced adipogenic differentiation potential due to impaired insulin signaling [24]. Moreover, our previous study showed that CD56 is expressed in both MBMSCs and IBMSCs, with no difference in their expression levels [5]. Therefore, CD56 expression alone was not involved in the regulation of adipogenesis in MBMSCs. Another study reported that CD71-positive adipose-derived stromal cells (ASCs) showed lower adipogenic differentiation potential than that showed by CD71-negative ASCs [25]. Furthermore, previously, we showed that CD71 expression in MBMSCs is higher than that in IBMSCs [5]. Based on these results, CD71 expression may influence the regulation of adipogenic differentiation of MBMSCs, and future studies are required for further clarification.

OCT4, SOX2, and Nanog are important for maintaining pluripotency of MSCs [26, 27]. Overexpression of *SOX2* and *Nanog* genes can enhance the differentiation potential of MSCs [28]. In addition, knockdown of *OCT4* in human MSCs suppresses *SOX2* and *Nanog* expression, resulting in decreased differentiation potential [26]. We evaluated whether the expression of these pluripotent marker genes is involved in the regulation of adipogenic potential of MBMSCs; however, no significant differences in *OCT4*, *SOX2*, and *Nanog* gene expression was observed between undifferentiated MBMSCs and IBMSCs. A previous study reported that human amniotic membrane-derived MSCs (hAMSCs) and chorionic-derived MSCs (hCMSCs) have a lower adipogenic potential than that of IBMSCs. However, the expression levels of OCT4, Nanog, and SOX2 in hAMSCs and hCMSCs were confirmed to be comparable to that in IBMSCs [27]. Consistent with our study findings, it is possible that the expression of these pluripotent markers in MBMSCs does not directly affect the regulation of adipogenic differentiation.

Various transcription factors are involved in the differentiation of undifferentiated MSCs into pre-adipocytes. C/EBPβ and C/EBPδ act as initiators of adipogenic differentiation [29]. A previous study showed that C/EBPβ and C/EBPδ are upregulated within 48 h of adipogenic differentiation [29]. C/EBPβ

and C/EBPδ regulate PPARγ and C/EBPα expression and promote adipogenic differentiation [12, 13]. Knockdown of *C/EBPβ* and *C/EBPδ* reportedly suppresses adipogenic differentiation of 3T3-L1 cells [12, 30, 31]. We showed that C/EBPβ expression in MBMSCs was significantly lower than that in IBMSCs at undifferentiated stages, and that the expression of C/EBPβ and C/EBPδ in MBMSCs was significantly suppressed, even during adipogenic differentiation. A recent study showed that the transcription factor Ebf-1, expressed in the early stages of adipose differentiation, induces adipogenic differentiation by directly binding to the promoter regions of *PPARγ* and *C/EBPα* [14]. It has been reported that overexpression of Ebf-1 promotes adipogenesis in 3T3-L1 fibroblasts [14, 32]. Furthermore, the expression of the Ebf-1 dominant negative form suppresses adipocyte maturation [32]. A previous study demonstrated that *KLF5* promotes adipogenic differentiation by activating the *PPARγ* promoter in the early stage of adipogenic differentiation, and *KLF5* knockout mice have significantly less white adipose tissue formation [33]. Herein, *Ebf-1* and *KLF5* expression in MBMSCs was significantly suppressed compared to that in IBMSCs during adipogenic differentiation. The suppression of these transcription factors may also contribute to the low adipogenic differentiation potential of MBMSCs. Previous studies have demonstrated that Ebf-1 and KLF5 are regulated by C/EBPβ and C/EBPδ during adipogenic differentiation [14, 33]. Therefore, the low expression of C/EBPβ and C/EBPδ in MBMSCs may be due to the suppression of Ebf-1 and KLF5. These results indicate that MBMSCs may be negatively regulated during the early stages of differentiation of preadipocytes into immature adipocytes.

In MBMSCs, a few lipid droplets were formed during adipogenic differentiation; however, the lipid droplets were significantly smaller than those in IBMSCs. These results indicate that the terminal differentiation of immature adipocytes into mature adipocytes may also be suppressed in MBMSCs. PPARγ and C/EBPα are essential transcription factors involved in the process of terminal differentiation of preadipocytes into mature adipocytes [3, 11]. Previous studies have demonstrated that *PPARγ* knockout is lethal to mouse embryos, and adipose tissue-specific PPARγ-lacking transgenic mice displayed significantly reduced adipose tissue [34–36]. In addition, induction of *PPARγ* gene expression in non-adipogenic fibroblasts stimulates ectopic adipogenic differentiation [37]. C/EBPα plays an important role in regulating adipogenesis by interacting with PPARγ and upregulating the transcription of various adipogenic genes [38]. A previous study reported that *C/EBPα* knockout mice died shortly after birth [39], and induction of ectopic *C/EBPα* gene expression in various mouse fibroblasts enhanced the terminal differentiation of adipocytes [40]. Therefore, we investigated whether PPARγ and C/EBPα are involved in the mechanism responsible for the low adipogenic potential of MBMSCs. In MBMSCs and IBMSCs, there were no significant differences in PPARγ and C/EBPα expression in the undifferentiated stage. As expected, the expression levels of PPARγ and C/EBPα during adipogenic differentiation in MBMSCs were significantly lower than those in IBMSCs. Since C/EBPβ and C/EBPδ transactivate the expression of PPARγ and C/EBPα [12, 13], it is possible that the suppression of PPARγ and C/EBPα expression in MBMSCs is dependent on C/EBPβ and C/EBPδ. Activation of PPARγ and CEBPα induces the expression of various adipogenesis-related factors [11]. It has been reported that aP2 and LPL are important for fatty acid accumulation in the late stage of adipogenic differentiation [41, 42], and that adiponectin promotes GLUT4 expression and increases glucose uptake by adipocytes [43]. The expression levels of *aP2*, *LPL*,

and *adiponectin* in MBMSCs were significantly lower than those in IBMSCs, which may be due to suppressed *PPARγ* and *C/EBPα* expression in MBMSCs. Glucose is involved in adipocyte maturation [44], and our results showed that GLUT4 expression and glucose uptake were lower in MBMSCs than that in IBMSCs. It is possible that suppression of adiponectin in MBMSCs led to decreased GLUT4 expression and intracellular glucose uptake, which may have been partially involved in the suppression of differentiation into mature adipocytes. These results indicated that MBMSCs may also be negatively regulated during the late stages of adipogenic differentiation.

Although the expression of the master transcription factor that regulates the differentiation of preadipocytes into mature adipocytes is well understood [11, 38], the molecular mechanisms regulating lineage allocation in undifferentiated MSCs are unclear. Recently, it has been reported that Zfp423 expression is higher in preadipocytes than in non-adipogenic fibroblasts (NIH3T3 fibroblasts), and adipogenic differentiation is induced by transfecting the *Zfp423* gene into NIH3T3 fibroblasts [16]. Furthermore, the treatment of 3T3-L1 preadipocytes with *Zfp432* shRNA reduced adipogenic differentiation [16]. Moreover, there was no change in *Zfp423* expression during adipogenic differentiation in several preadipocyte cell lines [16]. These results indicate that Zfp423 is an essential determinant of preadipocyte commitment. Until now, the Zfp423 expression pattern in MBMSCs was unknown; however, this study revealed that undifferentiated MBMSCs display lower Zfp423 expression than that displayed by undifferentiated IBMSCs. These results indicate that adipogenic differentiation of MBMSCs may be suppressed due to the inability of undifferentiated cells to commit to the adipocyte lineage. In this study, we showed that attenuation of the expression of various transcription factors that function in the early and late stages of adipogenic differentiation may be a mechanism for the suppression of MBMSC adipogenesis. However, our study did not reveal the direct involvement of these transcription factors in MBMSC adipogenesis. Therefore, it is necessary to evaluate whether adipogenic potential is restored by overexpressing these transcription factors in MBMSCs in future studies.

In this study, the reason for the difference in expression of adipogenic differentiation regulators between MBMSCs and IBMSCs was not clarified. Previous studies have demonstrated that MBMSCs and IBMSCs have different gene expression profiles and MSCs may retain the site-specific memory of the original tissue [10]. Another report also showed that stem cells are able to store information about the physical environment they have encountered in the past and mechanical memory affects cell fate [45]. In addition, a recent report demonstrated that the ionic concentrations (pH) of the femur defect site and the tooth extraction socket differed, and the pH of the tooth extraction socket was higher than that of the femur defect site [46]. This result indicates that pH of the environments in the jaw and iliac bone marrow may differ. The stemness and differentiation potential of MSCs are affected by changes in the pH of the environment [46]. Therefore, the characteristic differentiation potential of MBMSCs may be regulated by the specific microenvironment of the jawbone. In the future, it will be necessary to elucidate the regulatory mechanisms involved in the suppression of adipogenic differentiation transcription factors in MBMSCs.

Taken together, our study revealed that the adipogenic differentiation of MBMSCs is regulated by characteristic molecular mechanisms. These results provide important information for understanding the

mechanisms underlying MBMSC differentiation.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. The study design was conceptualized by Masakazu Ishii. Funding was acquired by Masakazu Ishii and Masahiro Nishimura. Material preparation was performed by Fumio Suehiro, Naohiro Komabashiri, and Tomoaki Sakurai. Data collection and analysis were performed by Haruka Miyata and Masakazu Ishii. The first draft of the manuscript was written by Haruka Miyata, Masakazu Ishii, and Masahiro Nishimura. The manuscript was reviewed and edited by Masahiro Nishimura, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Kagoshima University Hospital Clinical Research Committee, Kagoshima, Japan (No. 170263 EKI-KAI3).

Consent to participate

Written informed consent was obtained from all patients.

Consent to publish

The patients involved in this study provided informed consent for publication of their data.

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

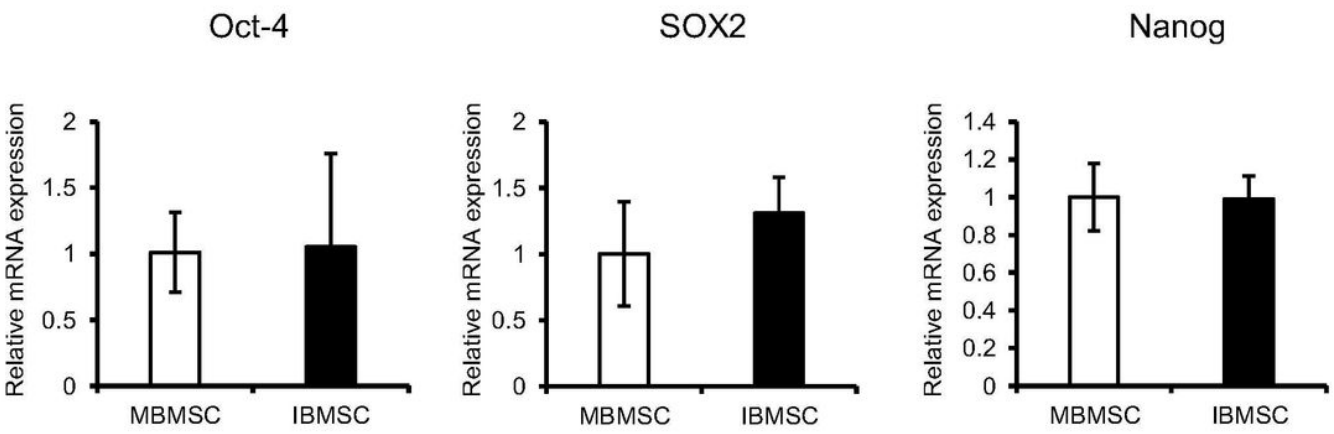

Figure 1

Pluripotency marker gene expression in maxillary/mandibular bone marrow-derived mesenchymal stem cells (MBMSCs) and iliac bone marrow-derived MSCs (IBMSCs). *Oct-4*, *SOX2*, and *Nanog* gene expression levels were measured in undifferentiated MBMSCs (n = 3) and IBMSCs (n = 3). Each gene expression was normalized against *GAPDH* expression levels, and all data are presented as fold change compared to MBMSCs. Results are presented as the mean ± standard deviation

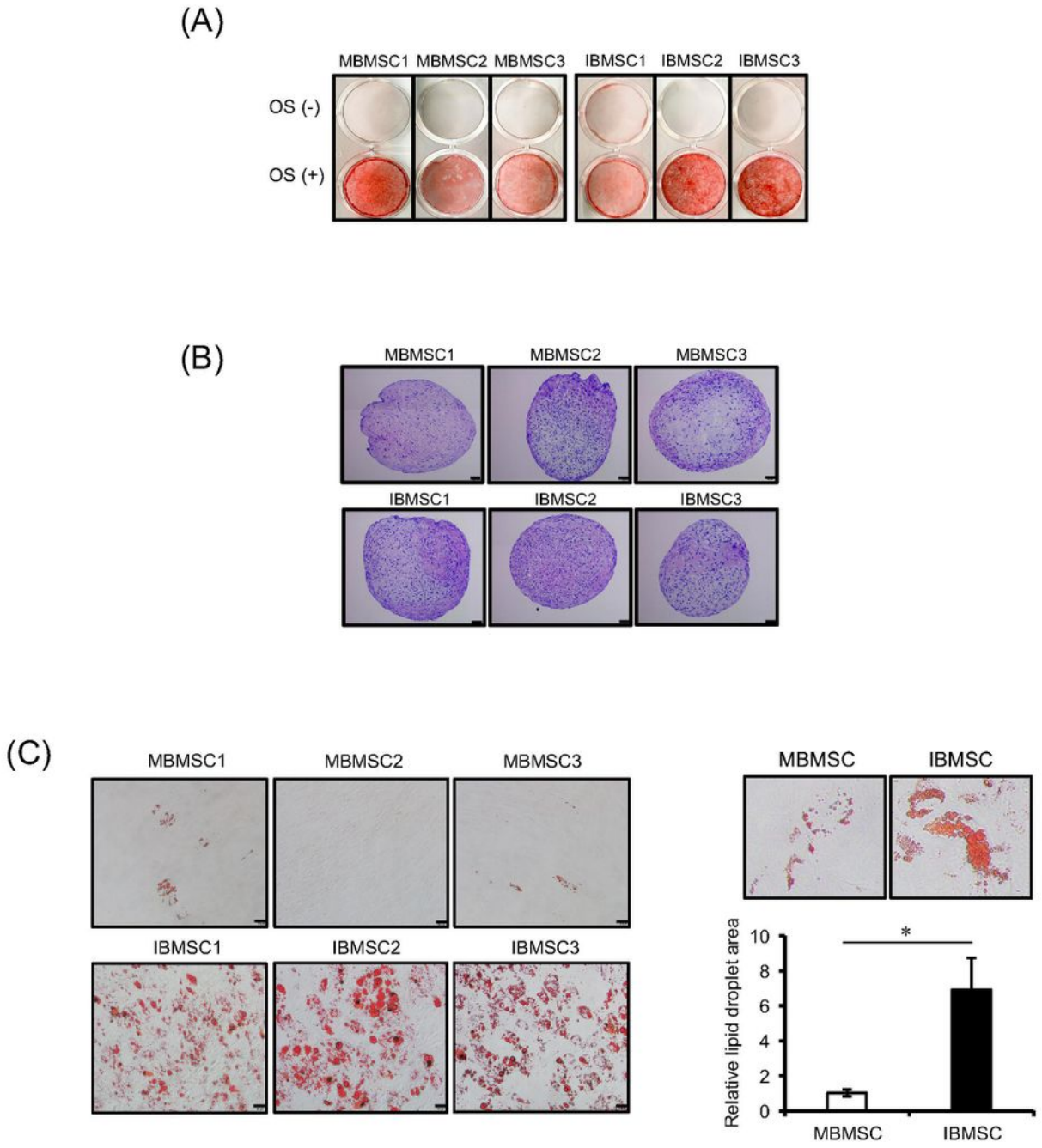

Figure 2

Comparison of differentiation potential between maxillary/mandibular bone marrow-derived mesenchymal stem cells (MBMSCs) and iliac bone marrow-derived MSCs (IBMSCs). (A) MBMSCs and IBMSCs were cultured using osteogenic induction medium. After 14 days of differentiation, calcified cells were evaluated using alizarin red staining. OS (-): osteogenic induction (-), OS (+): osteogenic induction (+). (B) Chondrogenic differentiation of MBMSCs and IBMSCs was performed using the pellet culture

method, and evaluated using toluidine blue staining 28 days after differentiation. Scale bar, 20 μm. (C) MBMSCs and IBMSCs were cultured using adipogenic induction medium. After 14 days of differentiation, lipid droplets were stained using Oil Red-O staining. Lipid droplet area in MBMSCs (n = 3) and IBMSCs (n = 3) was quantified, and the data are presented as fold change compared to the MBMSCs. Results are presented as the mean ± standard deviation. * $p < 0.05$. Scale bar, 20 μm.

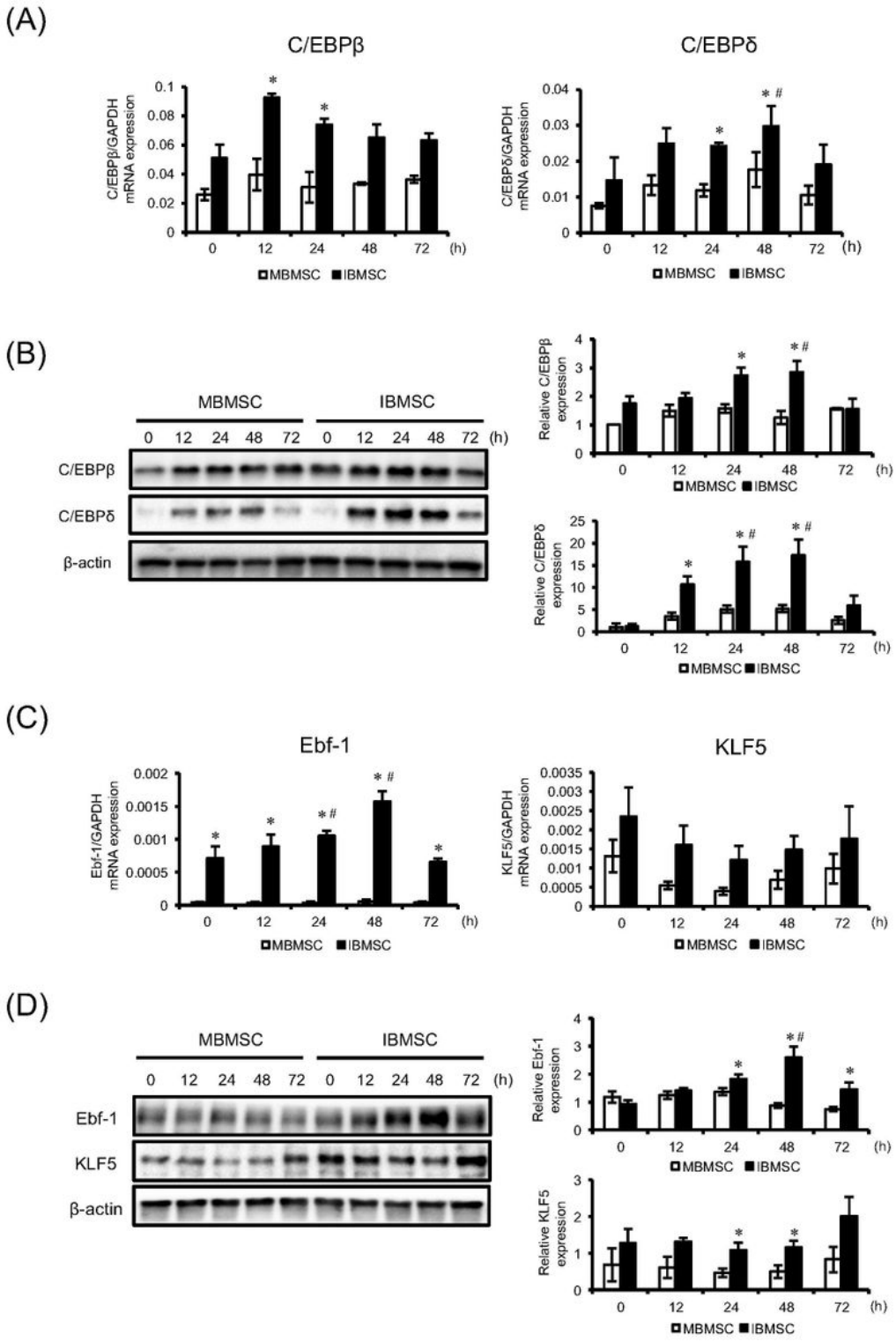

Figure 3

Evaluation of early adipogenic differentiation factor expression in maxillary/mandibular bone marrow-derived mesenchymal stem cells (MBMSCs) and iliac bone marrow-derived MSCs (IBMSCs). (A) mRNA and (B) protein expression of CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ, and (C) mRNA and (D) protein expression of early B-cell factor 1 and Krüppel-like factor 5 in the MBMSCs (n = 3) and IBMSCs (n = 3) cultured in adipogenic differentiation medium for indicated times. Each gene expression was normalized against *GAPDH* expression levels. Each protein expression was analyzed using western blotting. Representative immunoblots are shown. Relative protein levels were normalized to the β-actin protein signals, and all data are presented as fold change compared to MBMSCs (0 h). Results are presented as the mean ± standard deviation. * $p < 0.05$ vs MBMSCs in each time point. # $p < 0.05$ vs IBMSCs (0 h).

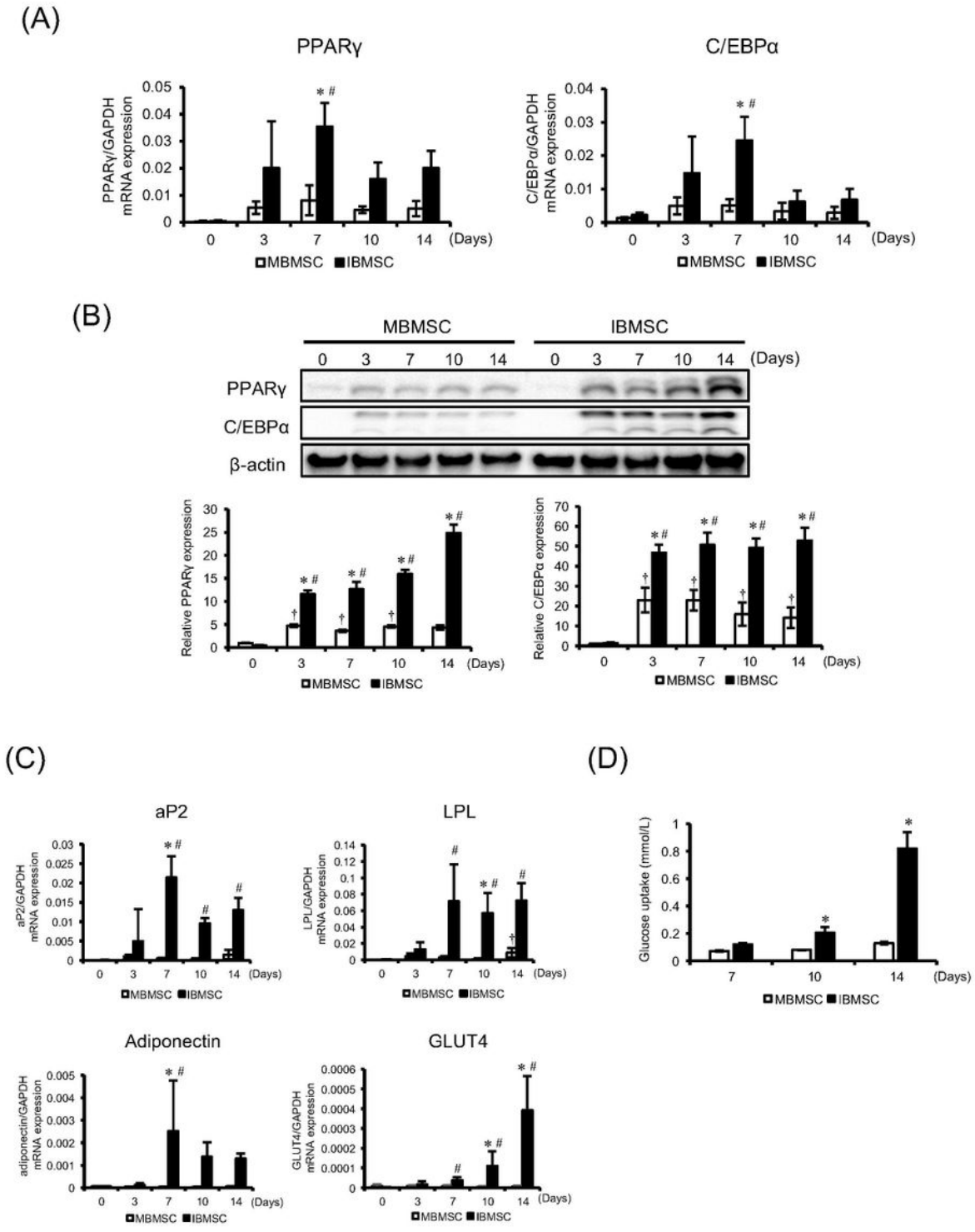

Figure 4

Evaluation of molecular expression involved in the late stages of adipogenic differentiation in maxillary/mandibular bone marrow-derived mesenchymal stem cells (MBMSCs) and iliac bone marrow-derived MSCs (IBMSCs). (A) mRNA and (B) protein expression levels of peroxisome proliferator-activated receptor-γ and CCAAT/enhancer binding protein α, that induced adipogenic differentiation for the indicated days, were analyzed in MBMSCs (n = 3) and IBMSCs (n = 3. In real-time PCR analysis, each

mRNA expression was normalized against *GAPDH* expression. Each protein expression was analyzed using western blotting. Representative immunoblots are shown. Relative protein levels were normalized to the β-actin protein signals, and all data are presented as fold change compared to MBMSCs (day 0). Results are presented as means ± standard deviation. * $p < 0.05$ vs MBMSCs in each time point. # $p < 0.05$ vs IBMSCs (day 0). † $p < 0.05$ vs MBMSCs (day 0). (C) *aP2*, *LPL*, *adiponectin*, and *GLUT4* mRNA expression levels in MBMSCs (n = 3) and IBMSCs (n = 3) that induced adipogenic differentiation for indicated days were analyzed. Each mRNA expression was normalized against *GAPDH* expression. Results are presented as means ± standard deviation. * $p < 0.05$ vs MBMSCs in each time point. # $p < 0.05$ vs IBMSCs (Day 0). † $p < 0.05$ vs MBMSCs (Day 0). (D) Measurement of intracellular glucose uptake by MBMSCs (n = 3) and IBMSCs (n = 3) that induced adipogenic differentiation for indicated days. Results are presented as means ± standard deviation. * $p < 0.05$ vs MBMSCs in each time point

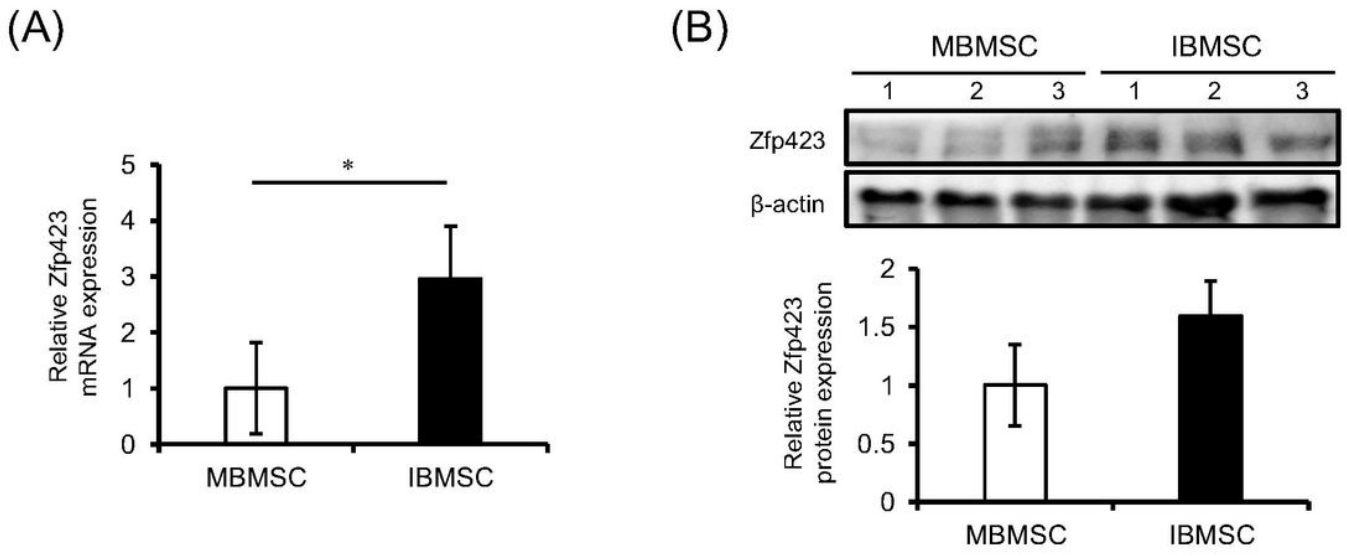

Figure 5

Evaluation of adipogenic lineage commitment factor expression in maxillary/mandibular bone marrow-derived mesenchymal stem cells (MBMSCs) and iliac bone marrow-derived MSCs (IBMSCs). (A) *Zfp423* gene expression was analyzed in undifferentiated MBMSCs (n = 3) and IBMSCs (n = 3). Each mRNA expression was normalized against *GAPDH* expression. All data are expressed as a relative value to MBMSCs. Results are presented as means ± standard deviation. * $p < 0.05$. (B) Zinc finger protein 423 protein expression was analyzed in undifferentiated MBMSCs (n = 3) and IBMSCs (n = 3). Relative protein levels were normalized to the β-actin protein signals. All data are expressed as a relative value to MBMSCs. Results are presented as means ± standard deviation.

Supplementary Files

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- Tables.docx