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Trophic factors from adipose tissue-derived multi-lineage progenitor cells promote cytodifferentiation of periodontal ligament cells

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ABSTRACT

Stem and progenitor cells are currently being investigated for their applicability in cell-based therapy for periodontal tissue regeneration. We recently demonstrated that the transplantation of adipose tissue-derived multi-lineage progenitor cells (ADMPCs) enhances periodontal tissue regeneration in beagle dogs. However, the molecular mechanisms by which transplanted ADMPCs induce periodontal tissue regeneration remain to be elucidated. In this study, trophic factors released by ADMPCs were examined for their paracrine effects on human periodontal ligament cell (HPDL) function. ADMPC conditioned medium (ADMPC-CM) up-regulated osteoblastic gene expression, alkaline phosphatase activity and calcified nodule formation in HPDLs, but did not significantly affect their proliferative response. ADMPCs secreted a number of growth factors, including insulin-like growth factor binding protein 6 (IGFBP6), hepatocyte growth factor and vascular endothelial growth factor. Among these, IGFBP6 was most highly expressed. Interestingly, the positive effects of ADMPC-CM on HPDL differentiation were significantly suppressed by transfecting ADMPCs with IGFBP6 siRNA. Our results suggest that ADMPCs transplanted into a defect in periodontal tissue release trophic factors that can stimulate the differentiation of HPDLs to mineralized tissue-forming cells, such as osteoblasts and cementoblasts. IGFBP6 may play crucial roles in ADMPC-induced periodontal regeneration.

1. Introduction

Periodontitis is a chronic inflammatory disease caused by a bacterial biofilm on the root surface. Progression of this disease leads to the destruction of periodontal tissues which support the teeth. Mechanical removal of the dental plaque allows for the control of inflammatory responses; however, this alone is not enough to induce regeneration of the damaged periodontal tissue.

Autogenous bone grafting, guided tissue regeneration (GTR) and local administration of an enamel matrix derivative (EMD), are some of the moderately effective approaches that have been used in general dental practice for the purpose of regeneration of the periodontal tissue. Furthermore, some recent studies have clinically assessed the effect of locally administered human recombinant growth factors, such as platelet-derived growth factor (PDGF) [1] and basic fibroblast growth factor (FGF-2) [2] on periodontal tissue regeneration.

Treatment strategies, such as GTR, EMD, or the local administration of growth factors, regenerate the periodontal tissue by activating the self-healing abilities of the endogenous stem cells within the periodontal tissues. The number of stem cells in the body is reported to decrease with age, likewise, the endogenous stem cell population in the periodontal tissues is believed to have decreased proliferative and differentiation ability [3]. Moreover, tissue destruction caused by severe periodontal disease results in the reduced number of endogenous stem cells. Therefore, there may be a need for “cell therapy”, wherein stem cells collected from other tissues are transplanted to the defective periodontal tissue.

Periodontal tissue regeneration therapies using bone marrow-derived mesenchymal stem cells (BMSCs) [4], periodontal

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ligament cells (PDLs) [5] and alveolar bone periosteal cells [6], have been discovered to have a regenerative effect. However, these therapies have certain limitations; PDLs and alveolar bone periosteal cells are limited in terms of the harvestable amount of tissue, while BMSCs involve an invasive harvesting procedure, and are known to show low amplification efficiency [7–9]. In contrast, mesenchymal stem cells (MSCs) from adipose tissue can be harvested less invasively and with greater ease compared to other tissues listed above. In addition, these cells have a higher self-renewal capacity and proliferative ability [8–10], and produce an abundance of humoral factors [11,12]. By transplanting adipose tissue-derived multi-lineage progenitor cells (ADMPCs) into canine experimental periodontal disease models, we have demonstrated that ADMPC induces significant periodontal tissue regeneration [13].

The cell-autonomous mechanism has been supported by many reports which indicate that MSCs directly differentiate into various cell types constituting the target tissues. In addition, recent studies have indicated that the transplanted MSCs have an important trophic effect, in which various growth factors and cytokines activate tissue regeneration at the transplant site [14,15]. These findings propose the importance of a non-cell-autonomous mechanism in addition to the cell-autonomous effects.

In this study, we investigated the effects of endogenous factors secreted by ADMPCs on the functionality of human PDLs (HPDLs), which play a central role in the induction of periodontal tissue regeneration, in order to clarify the mechanism of ADMPC-induced periodontal tissue regeneration.

2. Materials and methods

2.1. Cell culture

All human subjects provided informed consent according to a protocol that was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. ADMPCs were isolated from the subcutaneous adipose tissues collected from healthy volunteers. ADMPCs were prepared as described previously [16,17]. ADMPCs were seeded onto a dish coated with human fibronectin (BD Biosciences, Franklin Lakes, NJ, USA) in expansion medium (Exp-Med), which was composed of 60% Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco Life Technologies, Carlsbad, CA, USA), 40% MCDB-201 medium (Sigma–Aldrich, St. Louis, MO, USA), 1 nM dexamethasone (Sigma–Aldrich), 100 μM L-ascorbic acid (Sigma–Aldrich), 10 mg/L insulin-transferrin-selenium solution (Gibco), 10 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 60 μg/mL kanamycin (Wako Pure Chemical Industries) and 5% fetal bovine serum (FBS; Sigma–Aldrich). HPDLs were commercially purchased (Lonza Biosciences, Basel, Switzerland) and were cultured in α-Modified Eagle Minimum Essential Medium (α-MEM, Sigma–Aldrich) supplemented with 60 μg/mL kanamycin and 10% FBS. ADMPCs and HPDLs were analyzed *in vitro* at passages 4–8.

2.2. Preparation of ADMPC conditioned medium (ADMPC-CM)

A million of ADMPCs were seeded on to a 100 mm human fibronectin-coated dish, and grown to sub-confluence in Exp-Med. The culture medium was subsequently changed to fresh DMEM-high glucose (DMEM-HG) supplemented with 60 μg/mL kanamycin and 10% FBS. After 3-day culture, the supernatant was collected as ADMPC-conditioned medium (ADMPC-CM). In experiments requiring the addition of ADMPC-CM, an equal amount of 10% FBS and 60 μg/mL kanamycin-containing DMEM-HG was added and used as the control.

2.3. Osteogenic differentiation

Osteogenic differentiation was induced by culturing HPDLs in 12-well plates to confluence and subsequently exposing these cells to mineralization medium, consisting of α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 5 μM ascorbic acid, and 60 μg/mL kanamycin with ADMPC-CM or DMEM-HG which was replaced every 3 days.

2.4. Cell count assay

HPDL proliferation was determined by calculating the total number of cells in a hemocytometer (Minato-Medical, Tokyo, Japan). HPDLs were seeded into a 6-well plate containing α-MEM supplemented with 60 μg/mL kanamycin and 10% FBS at a density of 2.5×10^4 cells/well. The culture medium was then changed to α−MEM mixed at a 1:1 ratio with ADMPC-CM or DMEM-HG 6 h after seeding. Cells were counted every day up to day 4 after seeding.

2.5. Quantitative polymerase chain reaction (PCR)

Total RNA was isolated from cells using RNA-Bee™ (TEL-TEST, Friendwood, TX, USA), according to the manufacturer protocols. Total RNA was subjected to reverse transcription, using Random Hexamer Primers (Amersham Biosciences, Milwaukee, WI, USA) and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

The synthesized cDNA was used as the template for real-time PCR; the reaction mixture was composed of the template cDNA, the gene-specific primers and the components of the Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). *Alkaline phosphatase* (*ALPase*) (sense 5′-GGACCATTCCCACGTCTTCAC-3′; antisense 5′-CCTTGTAGCCAGGCCCATTG-3′), *Bone sialo protein* (*BSP*) (sense 5′-CTGGCACAGGGTATACAGGGTTAG-3′; antisense 5′-GCCTCTGTGCTGTTGGTACTGGT-3′), *Collagen type I alpha 1* (*Col1a1*) (sense 5′-CCCGGGTTTCAGAGACAACTTC-3′; antisense 5′-TCCACATGCTTTATTCCAGCAATC-3′), *IGFBP6* (sense 5′-CAGAGGAGAATCCTAAGGAGA-3′; antisense 5′-TGAGTCCAGATGTCTACGGCATGGGCC -3′), *Runt-related transcription factor 2* (*RUNX2*) (sense 5′-CACTGGCGCTGCAACAAGA-3′; antisense 5′-CATTCCGGAGCTCAGCAGAATAA -3′) and *hypoxanthine phosphoribosyltransferase* (*HPRT*) (sense 5′-GGCAGTATAATCCAAAGATGGTCAA-3′; antisense 5′-GTCAAGGGCATATCCTACAACAAAC-3′) gene specific primers were purchased from Takara-bio (Shiga, JPN).

Amplification was performed in the Step One Plus Real-time PCR System (Applied Biosystems) according to the manufacturer protocol. The relative expression was determined after normalization against *HPRT* expression.

2.6. Determination of ALPase activity

ALPase activity was measured as described previously [18]. ALPase from bovine intestinal mucosa was used as the standard; one unit (U) is defined as the amount of the enzyme that catalyzes the hydrolysis of 1 mM p-nitrophenyl phosphate per min at pH 9.8 and 37 °C. DNA concentration was determined using the modified Labarca and Paigen method [19], described in a previous study [20]; the results of this analysis were expressed as U/μg DNA.

2.7. Mineralization assay

Calcified nodules were stained with alizarin red using a method described by Dahl [21]. The density of the calcified nodules in each well was calculated using the WinRoof software program (Mitani Corporation, Fukui, Japan).

2.8. Determination of growth factor concentration in ADMPC-CM

Growth factor content in the ADMPC-CM was screened using the Human Growth Factor Array® (RayBiotech, Norcross, GA, USA) in accordance with the manufacturer protocol. Signals were detected using an Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, UK) and the density of each membrane dot was measured using image analysis software Image Quant TL (GE Healthcare), which allowed for screening of growth factors in ADMPC-CM, 10%FBS and 60 μg/mL kanamycin-containing DMEM-HG was used as the control. Each dot on a membrane was normalized with positive control (POS) and results were calculated as ratio to control.

2.9. RNA interference

Expression of human IGFBP6 was knocked-down by small interfering RNA (siRNA). The human IGFBP6 siRNA (5′-GCCCAAUUGUGACCAUCGATT-3′) and a negative-control siRNA (silencer select negative control siRNA) were purchased by Applied Biosystems. The negative-control siRNA was designed to express no significant sequence similarity to mouse, rat, or human transcript sequences. ADMPCs were incubated in a 6-well culture dish for 24 h. On reaching 40–50% confluence, ADMPCs were then transfected with 200 pM human IGFBP6 siRNA or negative-control siRNA using Lipofectamine® (Invitrogen), according to the manufacturer protocol.

2.10. ELISA assay

IGFBP6 concentration was measured using Human IGFBP6 ELISA kit (Raybiotech, Norcross, GA, USA) according to the manufacturer's instructions.

2.11. Statistical analysis

Results are presented as means ± standard deviation (SD) of triplicate assays. Statistical analyses were carried out using the Mann–Whitney test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. ADMPC-derived humoral factors stimulated cytodifferentiation of HPDLs

The effects of ADMPC-derived humoral factors on HPDL differentiation into mineralized tissue-forming cells was analyzed by culturing in ADMPC-CM-supplemented mineralization medium. Real-time PCR analysis revealed that the ADMPC-CM-

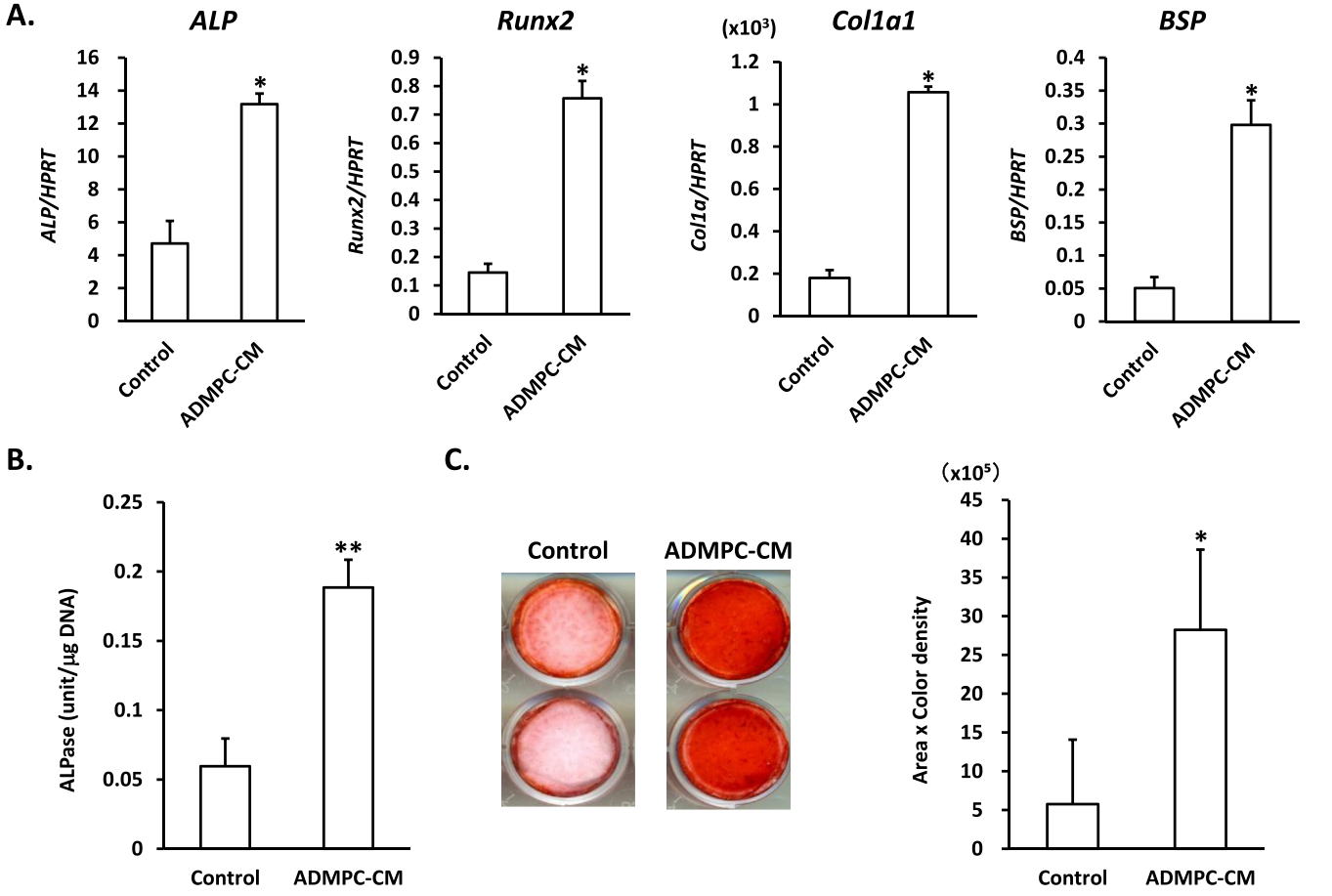

Fig. 1. Influence of ADMPC-derived humoral factors on cytodifferentiation of HPDLs. (A) Increased mRNA expression of *ALP*, *Runx2*, *Col1a1*, and *BSP* were observed in HPDLs cultured for 6 days in mineralization medium containing ADMPC-CM compared to the control. Values are indicated as ratios relative to *HPRT*. (B) Increased ALPase activity were detected in HPDLs cultured for 12 days in mineralization medium containing ADMPC-CM compared to control. (C) Enhanced calcified nodule formation in HPDLs cultured for 18 days in mineralization medium containing ADMPC-CM compared to control. Representative image of alizarin red (left) and quantified density of staining (right) is shown. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control. These results are representative of 3 independent experiments.

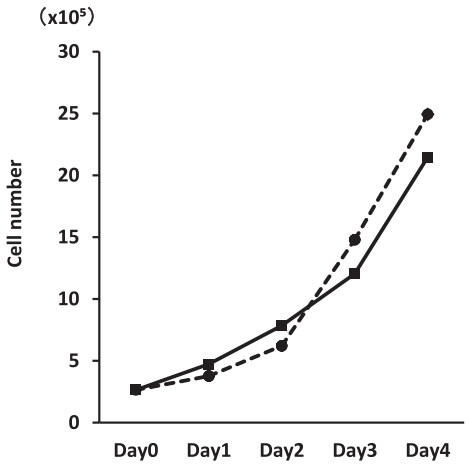

Fig. 2. Effect of ADMPC-derived humoral factors on HPDL proliferation. Cell numbers of HPDLs cultured in ADMPC-CM or DMEM-HG (control) medium at 1:1 ratio with 10% FBS-containing α-MEM. The solid line represents the ADMPC-CM-supplemented group and the dotted line represents the control group.

supplemented group showed a significantly higher *ALP*, *Runx2*, *Col1a1*, and *BSP* mRNA expression compared to the control group (Fig. 1A). Measurement of ALP activity demonstrated a significantly higher ALP activity in the ADMPC-CM-supplemented group compared to the control group (Fig. 1B). The mineralization assay revealed that the ADMPC-CM-supplemented group showed a significantly enhanced calcified nodule formation compared to the control group (Fig. 1C).

3.2. ADMPC-derived humoral factors did not impact HPDL proliferation

The effect of ADMPC-CM on HPDL proliferation was then investigated by adding ADMPC-CM to the HPDL culture medium (1:1 ratio); cell number was subsequently measured on days 0, 1, 2, 3, and 4 post-seeding. The results revealed that the addition of ADMPC-CM did not have a significant impact on cell proliferation compared with the control group (Fig. 2). These results indicate that ADMPC-derived humoral factors promote the differentiation of HPDLs into mineralized tissue-forming cells without affecting cell proliferation.

3.3. ADMPC-CM contained several growth factors

The growth factors expressed by the ADMPC-CM were screened using the Human Growth Factor Array®, in order to elucidate their action in promoting the differentiation of HPDLs into mineralized tissue-forming cells. As shown in Fig. 3A, the analysis revealed the presence of several growth factors, such as IGFBP6, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in ADMPC-CM, which are involved in wound healing and regeneration of damaged tissues. IGFBP6 was the most abundant factor expressed by ADMPCs (Fig. 3B).

3.4. IGFBP6 was involved in the promoting effects of ADMPC-CM on HPDL cytodifferentiation

We introduced IGFBP6 siRNA to the ADMPCs and the more than 95%knock-down efficiency was confirmed at mRNA and protein levels up to 72 h after siRNA-introduction (Fig. 4A). The supernatant was collected from the IGFBP6 and control siRNA-introduced

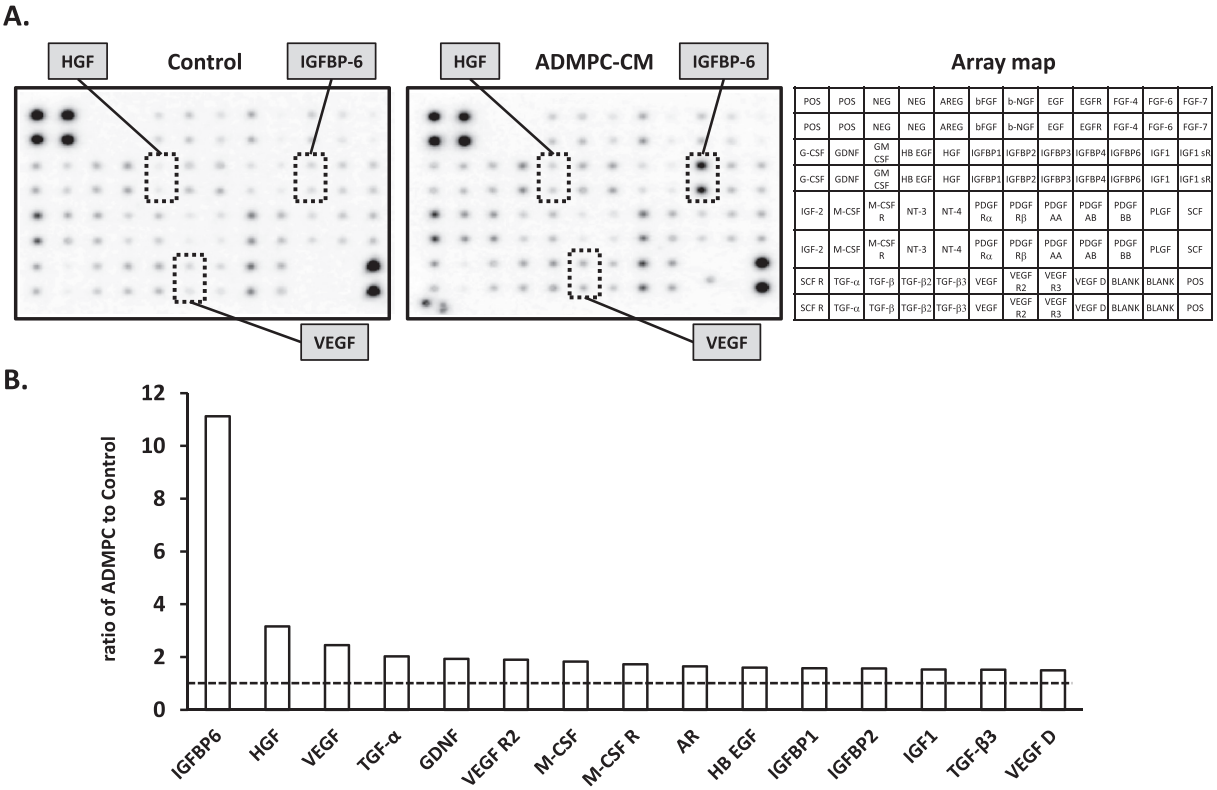

Fig. 3. Identification of growth factors secreted by ADMPCs. ADMPC-CM contained several kinds of growth factors. IGFBP6 was most highly presented in ADMPC-CM. A shows representative images of dot blots. B shows the semi-quantitative analysis of dot blot images.

ADMPC culture medium; these were termed BP6 si-CM and Con si-CM, respectively. BP6 si-CM or Con si-CM was added at a 1:1 ratio to the mineralization medium and the effects of ADMPC-derived IGFBP6 on HPDL differentiation was examined. The BP6 si-CM-supplemented HPDL showed significantly lower *Runx2*, *ALP* and *Col1a1* mRNA expression compared to the Con si-CM-supplemented HPDL (Fig. 4B). A functional measurement for ALPactivity revealed a significantly lower ALP activity in the BP6 si-CM-supplemented group compared to the Con si-CM-supplemented group (Fig. 4C). The mineralization assay showed a significantly inhibited calcified nodule formation in the BP6 si-CM-supplemented group compared to the Con si-CM-supplemented group (Fig. 4D). These results indicate that IGFBP6 plays a role in the ADMPC-CM-induced promotion of HPDL differentiation into mineralized tissue-forming cells.

4. Discussion

We have previously clarified the positive effects of ADMPC transplantation on periodontal tissue regeneration in preclinical studies [13]. To enhance the periodontal tissue regeneration by ADMPCs, a better understanding of the molecular mechanisms is required. As stated previously, stem cell transplantation therapy works on two principles. One is the cell-autonomous and the other is non-cell-autonomous. In previous *in vitro* studies, we have demonstrated the pluripotency of ADMPCs [17], as well as their potential to differentiate into mineralized tissue-forming cells and PDLs [20]. This suggests that ADMPC transplantation to periodontal defects stimulates tissue regeneration, at least in past, through the cell-autonomous effect.

However, the importance of the trophic effects of adipose tissue-derived MSCs has been reported in previous preclinical studies utilizing a hind limb ischemia model [22] and skin wound healing model [23]. In this study, we demonstrated that ADMPCs secreted various cytokines (Fig. 3). Among those, VEGF is known to play critical roles in blood vessel formation. In addition, the expression of VEGF and its receptors has been confirmed in PDLs and gingival fibroblasts in periodontal tissues [24]. VEGF is also known to promote proliferation and migration of HPDLs and their differentiation into osteoblasts [24,25]. HGF is involved in the proliferation and migration of vascular endothelial cells [26]. HGF secreted by human adipose tissue-derived MSCs transplanted into a mouse hind limb ischemia model is suggested to induce an improvement in blood flow at the site, by activating cells surrounding the transplant [27]. These findings suggest that VEGF and HGF secreted by ADMPCs can contribute to periodontal tissue regeneration in a non-cell-autonomous manner.

In this study, we have demonstrated for the first time that IGFBP6, an ADMPC-derived humoral factor, stimulates the differentiation of HPDLs. IGFBP is a protein that selectively binds to IGF and six subtypes have been reported (IGFBP1 to IGFBP6) [28]. Among those, IGFBP6 (molecular weight: 34 kDa) is reported to bind to IGF-2 via an IGFBP6-specific C-terminal region [29]. Interestingly, our preliminary experiments showed that HPDL-derived

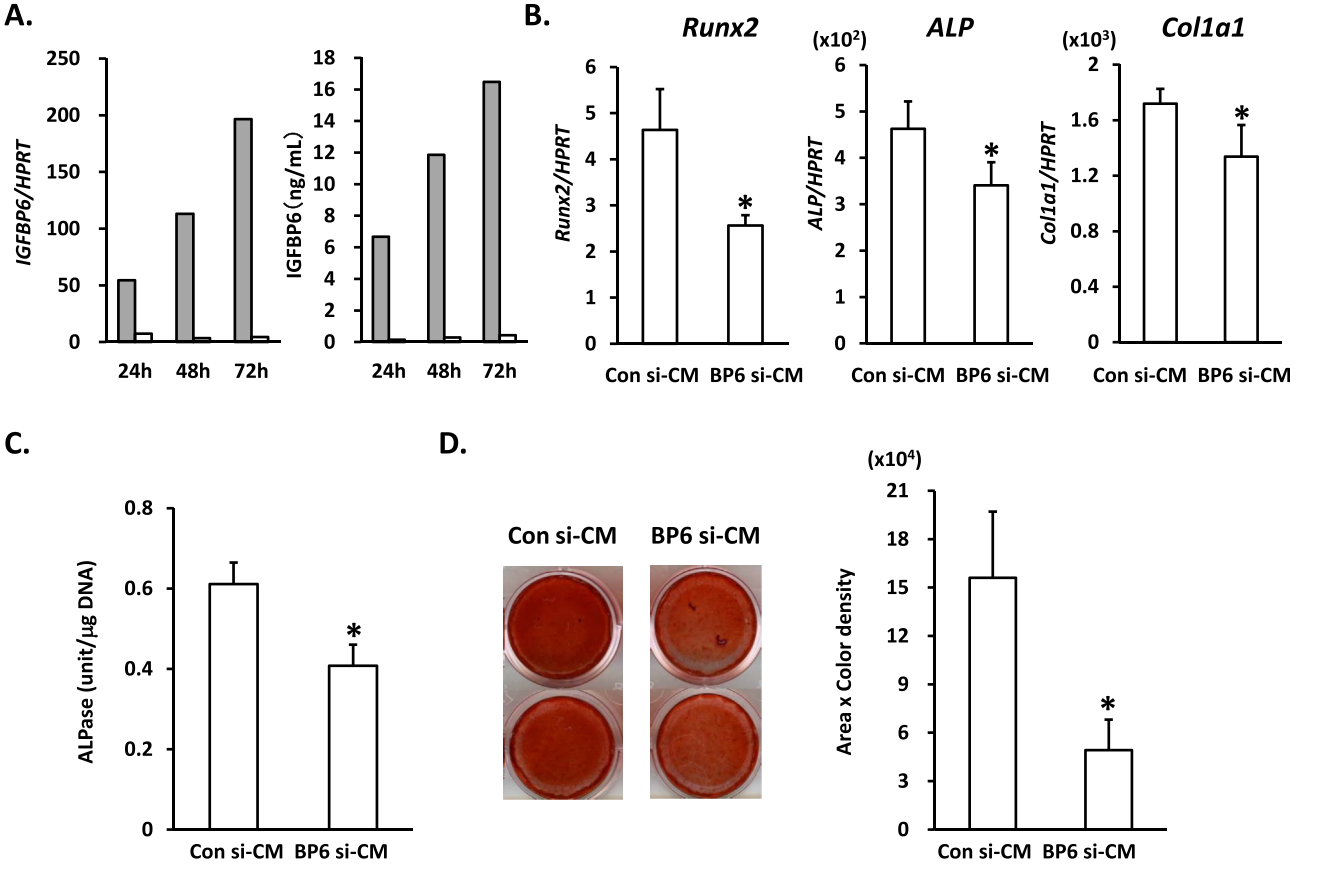

Fig. 4. Impact of ADMPC-derived IGFBP6 on differentiation of HPDLs into mineralized tissue-forming cells. (A) Suppressed expression of IGFBP6 in ADMPC was demonstrated in both mRNA and protein level by transfecting siIGFBP6 (white bar) compared to control siRNA (black bar). (B) Decreased mRNA expression of *Runx2*, *ALP* and *Col1a1* in HPDLs cultured for 12 days in mineralization medium containing BP6 si-CM compared to Con si-CM. (C) Decreased ALPase activity in HPDLs cultured for 12 days in mineralization medium containing BP6 si-CM compared to Con si-CM. (D) Suppressed calcified nodule formation on HPDLs cultured for 18 days in mineralization medium containing BP6 si-CM compared to Con si-CM. Representative image of alizarin red (left) and quantified density of staining (right) is shown.*$p < 0.05$ compared to Con si-CM.

IGF-2 levels are elevated when HPDLs are induced to differentiate into mineralized tissue-forming cells (data not shown). Together with positive effects of IGF-2 in osteoblast differentiation [30], it is reasonable to speculate that IGF-2 may be involved in IGFBP6-induced HPDL differentiation. However, simultaneous addition of IGF-2 and IGFBP6 did not demonstrate a coordinated action to promote HPDL differentiation (data not shown). This implies that IGFBP6 in ADMPC-CM may control HPDL differentiation via an IGF-2-independent mechanism. Interestingly, Cui et al. reported that IGFBP6 binds to vitamin D receptors and inhibits vitamin D signaling [31]. Although vitamin D is known to exert a stimulatory regulation on the differentiation of osteoblasts [32], it is also reported to inhibit the differentiation of PDLs into mineralized tissue-forming cells [33]. These findings suggest that vitamin D signaling may be involved in the mechanism of IGFBP6 action. Further studies should clarify the molecular mechanism of IGFBP6-dependent HPDL differentiation. In addition, the synergistic/additive effects of ADMPC-derived cytokines need to be clarified.

The concept of tissue engineering, which effectively combines the use of stem cells, scaffolds and signaling factors, is essential to develop ideal regenerative therapies that address severe periodontal tissue defects. The results of this study suggest that ADMPCs play a critical role, not only as the “stem cells” which can differentiate into the tissue-forming cells, but also as the source of humoral factors to exert stimulatory control on the periodontal tissue regeneration. We believe that the molecular basis of the interactions between ADMPCs and the component cells of periodontal tissue must be further analyzed in future studies, which will lead to better selection of a suitable scaffold and signaling factor(s) to allow maximization of the outcome of periodontal tissue regeneration.

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