免疫抑制剤の投与を必要とすることを報告している[22]。すなわち，MSC を用いても移植免疫を単純に回避できないことを示している。実際，筆者らの同種 MSC を用いての骨再生においては骨髄移植を初回に行い，それに伴い免疫抑制剤を患者に投与している[14]。最近のさらなる同種 MSC を用いての臨床経験により，同種 MSC の生着ならびに骨分化が向上する症例を経験しているが，確証するにはさらに臨床経験を積み重ねる必要がある。

5 将来展望

筆者らは骨再生の臨床において，骨髄から増殖された MSC を患者に移植している。他の組織からの MSC を用いる可能性もあるが，脂肪由来の MSC の比較研究より，現段階では我々は骨髄由来 MSC が骨再生に非常に有用であると考えている。また，ほとんどの症例は患者自身の骨髄細胞から MSC を培養増殖して用いている。しかし，骨再生を多くの患者に用いるにはストック保存された細胞，特に患者自身でない同種の保存細胞を用いることが期待される。この点において，筆者らは MSC が長期にわたり，増殖ならびに骨分化能を維持したまま冷凍保存（－80 ℃）できうることを報告している[23]。ただ，単純な移植による同種の MSC が患者体内で生着するのは期待できないが，患者に対しての種々の処置を施すことにより，同種の MSC の臨床応用の範囲も広がることが期待できる。

MSC の問題点の１つとして，移植に必要な細胞数を得るためには継代という培養ステップを必要とするが，その継代数が増えることにより増殖・分化能が低下する問題がある。これの解決法として，種々の方法が考えられる。例えば，我々が報告しているように Nanog といった単一の遺伝子を導入することも考えられるが[19]，さらなる増殖・分化能の高い細胞を創製するには iPS 細胞を視野に入れる必要がある。すなわち，iPS 細胞は多分化能を維持したまま，長期にわたり継代可能な高い増殖能を有する幹細胞である。また，iPS 細胞は MSC から作製可能であることを筆者らは報告しているが[20][21]，その逆のプロセス，すなわち iPS 細胞から間葉系幹細胞も誘導可能であり，iPS 細胞由来の MSC を用いての骨再生治療も将来実現可能と思われる。また，移植免疫の回避できうる同種の MSC が iPS 細胞から得られるなら，同じ細胞（MSC）を用いて多数の種々の骨疾患の再生に用いることが可能となり，その応用範囲は飛躍的に広がる。この点において，遺伝子マッチングされた iPS 細胞がバンキングされる構想も出ている。

このように，患者に対して移植免疫を回避できうる同種の iPS 細胞由来 MSC を用いての再生治療も将来可能になるかと思われる。この将来の iPS 細胞利用の骨再生医療を考える場合，現在の MSC を用いての臨床応用の研究成果により，MSC 移植の安全性と有用性を確認することが非常に重要である。なお，iPS 細胞そのものは造腫瘍性（テラトーマ発生）を有するので，この iPS 細胞由来の細胞（例えば MSC）を利用する再生医療においては，残余する未分化の iPS 細胞を除外する必要がある。現在，iPS 細胞ならびにその周辺技術が進歩しつつあり，この造腫瘍性の問題も解決できうると思われる。近い将来，iPS 細胞由来の MSC が多くの疾患患者に応用できうる時代が到達することを期待して本項を終える。

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文　献

1）A. Friedenstein et al.: *Exper Hematol,* **6**, 440–444（1978）.
2）H. Ohgushi et al.: *J Orthop Res,* **7**（4）, 568–578（1989）.
3）H. Ohgushi et al.: *Biomaterials,* **26**, 4654–4661（2005）.
4）M. Okumura et al.: *Biomaterials,* **12**（4）, 411–416（1991）.
5）H. Ohgushi and A. I. Caplan: *J Biomed Mater Res,* **48**（6）, 913–927（1999）.
6）H. Ohgushi et al.: *J Biomed Mater Res,* **32**（3）, 333–340（1996）.
7）A. Matsushima: *Artif Organs,* **33**（6）, 474–481（2009）.
8）T. Morishita et al.: *Artif Organs,* **30**, 115–118（2006）.
9）K. Kawate et al.: *Artif Organs,* **30**（12）, 960–962（2006）.
10）Y. Tohma et al.: *J Orthop Res,* **24**（4）, 595–603（2006）.
11）M. Ogawa et al.: *Int J Mol Sci,* **13**（5）, 5528–5541（2012）.
12）N. Mizuta et al.: *J Tissue Eng Regen Med,* **7**（1）, 51–60（2013）.
13）T. Matsumoto et al.: *Tissue Eng Part A,* **17**（1–2）, 171–180（2011）.
14）M. Tadokoro et al.: *J Pediatr,* **154**, 924–930（2009）.
15）T. Kihara et al.: *Biochem Biophys Res Commun,* **316**（3）, 943–948（2004）.
16）M. Akahane et al.: *J Tissue Eng Regen Med,* **2**（4）, 196–201（2008）.
17）A. Nakamura et al.: *Bone,* **46**（2）, 418–424（2010）.
18）O. Hayashi et al.: *Calcif Tissue Int,* **82**（3）, 238–247（2008）.
19）M. J. Go et al.: *Exp Cell Res,* **314**（5）, 1147–1154（2008）.
20）Y. Oda et al.: *J Biol Chem,* **285**, 29270–29278（2010）.
21）H. Ohnishi et al.: *J Tissue Eng Regen Med,* **6**（4）, 261–271（2012）.
22）N. Kotobuki et al.: *Cell Transplant,* **17**, 705–712（2008）.
23）N. Kotobuki et al.: *Tissue Eng,* **11**（5–6）, 663–673（2005）.

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RESEARCH ARTICLE

Osteogenic potential of rat stromal cells derived from periodontal ligament

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Abstract

Various mesenchymal stromal cells (MSCs) have been applied to regenerative medicine. MSCs derived from periodontal tissue could also be a useful cell source for alveolar bone regeneration. However, only a few attempts of direct comparisons have been made between MSCs from periodontal tissues and those from other somatic tissues. The purpose of this study was to clarify the osteogenic characteristics of mesenchymal stromal cells derived from bone marrow (BMSCs), adipose tissue (ASCs) and periodontal ligament (PDLSCs). BMSCs, ASCs and PDLSCs were isolated from Fisher 344 rats. After 1 week of primary culture, stromal cells were subjected to cell surface analysis and osteogenic differentiation. The cells were subcultured for 2 weeks with and without osteogenic supplements (OS), followed by biochemical and histological analyses. With regard to cell surface antigens, all MSCs were positive for CD29 and CD90 and negative for CD45. With regard to osteogenic differentiation, BMSCs with OS had the highest ALP activity, calcium uptake and osteocalcin content. Without OS, PDLSCs had the highest levels of these bone differentiation markers. RT–PCR analysis and histological analysis showed similar trends. These results indicate that PDLSCs are an ideal candidate for alveolar bone regeneration.

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Keywords **mesenchymal stromal cells; periodontal ligament; bone marrow; adipose tissue; osteogenesis; tissue engineering**

1. Introduction

Mesenchymal stromal cells (MSCs) are fibroblast-like cells that can be isolated from a variety of tissues, such as bone marrow (Pittenger *et al.*, 1999), periosteum (De Bari *et al.*, 2001a), synovium (De Bari *et al.*, 2001b) and adipose tissue (Zuk *et al.*, 2002). MSCs include multipotent stem cells; therefore, various MSCs have been applied to regenerative medicine. Bone marrow-derived MSCs (BMSCs) can differentiate into multiple lineages, such as osteoblasts, adipocytes, chondrocytes and hepatocytes (Pittenger *et al.*, 1999; Kotobuki *et al.*, 2004). In clinical practice, tissue-engineering methods using BMSCs have been applied to the regenerative cell therapy of bone, articular cartilage, liver, neurons and cardiovascular diseases (Ohgushi *et al.*, 1999). Adipose-derived MSCs (ASCs) have also been used to treat various diseases, including the treatment of widespread traumatic calvarial defects (Lendeckel *et al.*, 2004), cosmetic breast augmentation (Yoshimura *et al.*, 2008) and rectovaginal fistula in perianal Crohn's disease (García-Olmo *et al.*, 2010). Moreover, MSCs from synovial tissue and umbilical cord blood have been shown to be promising alternative sources of MSCs (Rosenbaum *et al.*, 2008).

In the periodontal field, the periodontal ligament, which is a soft connective tissue embedded between the cementum and the inner wall of the alveolar bone socket, contains heterogeneous cell populations that can differentiate multiple lineages (Seo *et al.*, 2004; Iwata *et al.*, 2009). Therefore, periodontal ligament-derived MSCs (PDLSCs) are also expected to be a useful cell

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source for alveolar bone regeneration (Seo *et al.*, 2004). At present, BMSCs, ASCs and PDLSCs are used for alveolar bone regeneration (Yamada *et al.*, 2006; Tobita *et al.*, 2008; Iwata *et al.*, 2009); however, only a few attempts at direct comparison (Lindroos *et al.*, 2008) have been made between PDLSCs and those from other somatic tissues under the same culture conditions, including the same donor tissue, passage number and culture environment. The purpose of this study was to clarify the osteogenic characteristics of BMSCs, ASCs and PDLSCs.

2. Materials and methods

2.1. Preparation and culture of rat BMSCs, ASCs and PDLSCs

We harvested BMSCs, ASCs and PDLSCs from 12 week-old Fischer 344 male rats purchased from Japan SLC (Shizuoka, Japan). All procedures used in the animal experiments conform with the Guidelines for the Care and Use of Laboratory Animals of the National Institute of Advanced Industrial Science and Technology of Japan. Three rats were sacrificed using 2% halothane (Takeda Pharmaceuticals, Osaka, Japan) in a glass jar.

BMSCs were isolated as described previously (Hayashi *et al.*, 2008). Briefly, both ends of the femora were cut away from the epiphysis and marrow was flushed out using 10 ml culture medium expelled from a syringe through a 20 gauge needle. The released bone marrow cells were collected in a T-75 flask for primary culture. ASC isolation was performed as described previously (Hayashi *et al.*, 2008). Briefly, rat inguinal adipose tissue was treated with 10 ml phosphate-buffered saline (PBS) containing 3 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 1 h, followed by centrifugation at 400 × *g* for 5 min after filtration through 40 μm cell strainers. The sediment was resuspended in culture medium and isolated adipose cells were collected in a T-75 flask for primary culture. PDLSC isolation was performed as described by Techawattanawisal *et al.* (2007), with some modifications. Briefly, rat maxillary and mandibular molars (M1, M2 and M3) were washed with PBS containing 5% antibiotics. Under microscopic inspection, the gingiva was carefully exfoliated and maxillary and mandibular molars were removed from the sockets using extraction forceps. The extracted molars were washed twice with PBS containing 5% antibiotics. Subsequently, these molars were treated with 10 ml PBS containing 3 mg/ml collagenase for 1 h. The digest was then centrifuged at 400 × *g* for 5 min following filtration with 40 μm cell strainers. The sediment was resuspended in culture medium. From 10 molars, 4×10^5 periodontal cells were isolated and collected in a T-75 flask for primary culture.

Primary cultures of BMSCs, ASCs and PDLSCs were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. In primary cultures, the medium was renewed three times/week. After 1 week of primary culture, cells were released from the substratum using trypsin–EDTA (0.05% trypsin, 0.53 mM EDTA-4Na; Invitrogen, Carlsbad, CA, USA) and were subjected to analysis of cell surface, proliferation rates and osteogenic differentiation.

2.2. Cell surface analysis

Harvested cells were suspended at 1×10^6 cells/ml in PBS. Cell suspensions (100 μl) were placed into 1.5 ml centrifuge tubes and incubated with anti-CD antibodies on ice for 20 min. PBS containing 10% Block Ace (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan) was then added to the tubes, followed by analysis using a FACSCalibur flow cytometer (BD). The antibodies used were CD29-FITC (fluorescein isothiocyanate), CD90-FITC and CD45-FITC (BioCarta Inc., San Diego, CA, USA). Mouse IgG-FITC (Beckman Coulter) was used as a negative control.

2.3. Cell proliferation rates

Cell proliferation rates of BMSCs, ASCs and PDLSCs were compared on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation, using an Amersham Cell Proliferation Biotrak ELISA System (GE Healthcare, Buckinghamshire, UK). After primary cultures, BMSCs, ASCs and PDLSCs were plated at 10^4 cells/well in 96-well plates and cultured for 24 h. BrdU labelling solution was added to the culture wells and incubated for 4 h. After fixing and blocking for 30 min, peroxidase-labelled anti-BrdU was added and incubated for 1 h, thenBrdU incorporation was measured using a microplate reader (Wallac 1420 ARVOsx; Perkin-Elmer, Boston, MA, USA).

2.4. *In vitro* osteogenic differentiation

After primary culture, BMSCs, ASCs and PDLSCs were subcultured for 2 weeks in 12-well tissue culture plates at a cell density of $2.0 \times 10^4/\text{cm}^2$ for histochemical staining and RT–PCR analysis, and in 24-well tissue culture plates at a cell density of $1.0 \times 10^4/\text{cm}^2$ for biochemical analyses. Under non-differentiation conditions, medium supplemented with 10 mM β-glycerophosphate (Calbiochem, San Diego, CA, USA) and 0.28 mM ascorbic acid-2-phosphate (Wako Pure Chemical Industries, Osaka, Japan) was renewed three times/week. Under osteogenic differentiation conditions, the medium was further supplemented with 10 nM dexamethasone (Nacalai Tesque, Kyoto, Japan).

2.5. Histochemical staining

After washing twice with PBS, the cultured cells were fixed in 10% paraformaldehyde and then were stained with alizarin red S (Sigma-Aldrich, St Louis, MO, USA) for 2 min, followed by ALP staining for 5 min. ALP staining

was performed as described previously (Ohgushi *et al.*, 1996).

2.6. Mineralization analysis by calcein uptake

To visualize the mineralized extracellular matrix of subcultured cells, the calcein uptake assay according to our previous report was performed (Uchimura *et al.*, 2003). For the calcein assay, 1 μg/ml calcein was added to each well whenever the culture medium was renewed. Prior to the assays, subcultured cell layers were washed twice with PBS. After 2 weeks of culture, calcein incorporated into the mineralization of the matrix was observed using a fluorescence microscope (Model IX70, Olympus, Tokyo, Japan) and was visualized and quantified using a Typhoon 8600 image analyser (Molecular Dynamics, Sunnyvale, CA, USA).

2.7. Measurements of ALP activities

ALP activity and DNA content were measured as described previously (Ohgushi *et al.*, 1996). The layers of subcultured cells were washed twice with PBS and were collected by scraping into sample tubes containing 500 μl 10 mM Tris buffer (pH 7.4, 1 mM EDTA and 100 mM NaCl). The samples were sonicated and used for DNA quantification. Quantification of DNA contents was performed using Hoechst 33 258 (Invitrogen). The sonicated suspension (20 μl) was mixed with 200 μl 0.25 μg/ml Hoechst 33 258 and incubated for 5 min at room temperature. Fluorescence was measured on the microplate reader. Standard DNA was prepared with salmon sperm DNA (Invitrogen). The remnant suspension was used for measurement of ALP activity. After centrifuging at 12 000 × *g* for 3 min at 4 °C, 20 μl supernatant was mixed with 100 μl *p*-nitrophenylphosphate substrate, followed by incubation for 30 min at 37 °C. The ALP activity represented by the amount of *p*-nitrophenol release was normalized against the DNA content. The remaining supernatant, including the sonicated cell suspension, was used for the osteocalcin assay.

2.8. Quantification of osteocalcin

After ALP measurements, 300 μl 20% formic acid was added to the tube in order to extract inorganic ions and organic matrix proteins, and the samples were stored at 4 °C for 48 h. The samples were then centrifuged at 15 000 × *g* for 10 min at 4 °C. Gel filtration was applied to the supernatant in order to eliminate inorganic ions, after which gel-filtered samples were evaporated. The resulting concentrated samples were added to an enzyme immunoassay plate immobilized with anti-rat osteocalcin antibodies in order to measure the concentrations of osteocalcin with an intact rat osteocalcin enzyme immunoassay kit (Biomedical Technologies, Stoughton, MA, USA).

2.9. Semi-quantification of mRNAs by RT–PCR analysis

After 2 weeks of subculture, total RNA was isolated from the cell layers using an RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan) in a 20 μl reaction volume. Gene-specific amplicons were then amplified by polymerase chain reaction (PCR). Semi-quantitative RT–PCR was performed for *osteocalcin*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control housekeeping gene (Gartland *et al.*, 2005). The PCR primers were as follows (forward and reverse, 5′ to 3′): *osteocalcin*, CATGAGGACCCTCTCTCTCTGC and CCTAAACGGTGGTGCCATAG; *GAPDH*, AACTCCCTCAAGATTGTTCAGCA and TCCACCACCCTGTTGGCTGTA. PCR conditions were as follows: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s for 30 cycles.

2.10. Statistical analysis

Statistical analyses were performed using the Excel Statistical Program File Ystat 2008 (developed by Yamazaki S, Igakutosyo Syuppan Co. Ltd, Tokyo, Japan). Calcein, ALP activity and osteocalcin contents were analysed by one-way repeated analysis of variance (ANOVA). In all analyses, the significance level was set at $p < 0.01$.

3. Results

3.1. Cell morphology, surface analysis and proliferation rates

BMSCs, ASCs and PDLSCs became largely confluent in T-75 flasks after 1 week of primary culture. These cells exhibited fibroblastic morphology, which is characteristic of MSCs (Figure 1A). Cell surface markers for rat MSCs are not as well understood as human MSCs. Therefore, cell surface markers were selected with reference to the literature (Figure 1B). All three cell types were positive for markers present in mesenchymal cells (CD29 and CD90) and were negative for haematopoietic markers (CD31, CD44, CD45 and CD172; data for CD31, CD44 and CD172 are not shown in the Figure). Therefore, all three cell types showed similar cell surface antigens and were mesenchymal cells (Nagaya *et al.*, 2004). The proliferation rates of three MSC types were compared on the BrdU (Figure 1C). As shown the figure, proliferation rates were different for each of MSCs. Although the proliferation rate of the PDLSCs was low compared with other cell types, the PDLSCs together with other cells become almost confluent after 1week. Therefore, a

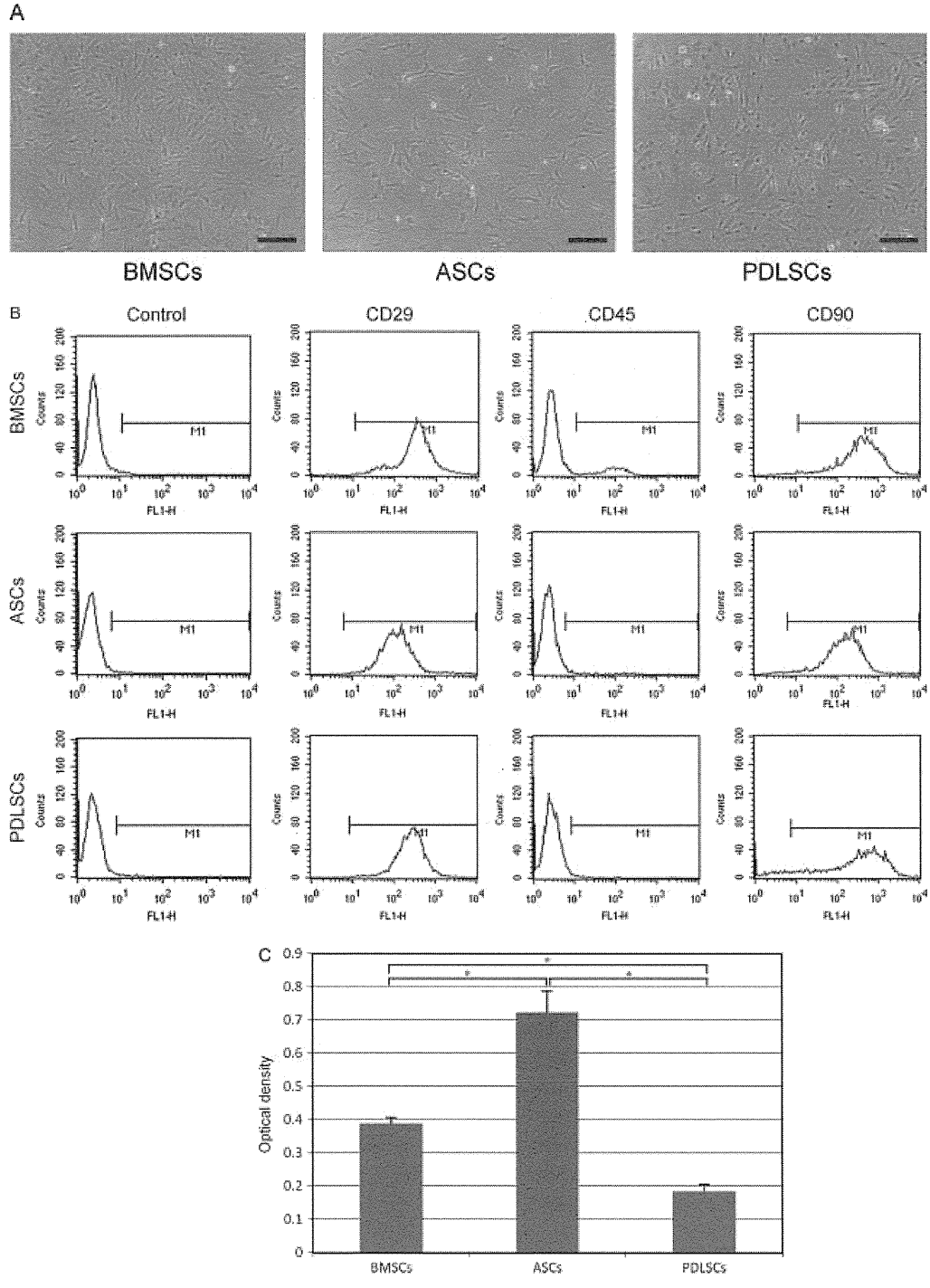

Figure 1. Characteristics of primary cultured MSCs. (A) Phase-contrast micrographs of BMSCs, ASCs and PDLSCs after 1 week of primary culture (scale bar = 200 μm). (B) Cell surface analysis of BMSCs, ASCs and PDLSCs after 1 week of primary culture. Brackets indicate positive cell population. BMSCs, ASCs and PDLSCs showed similar profiles; positive for CD29 and CD90 and negative for CD45. (C) Cell proliferation of BMSCs, ASCs and PDLSCs measured by BrdU incorporation. Values are means ± SD ($n = 8$). *$p < 0.01$ (one-way ANOVA)

sufficient number of cells was obtained using the culture method as described above.

3.2. Histochemical staining

Histochemical staining for BMSCs, ASCs and PDLSCs was performed after 2 weeks of subculture under osteogenic differentiation and non-differentiation conditions (Figure 2A). Under non-differentiation conditions, ALP staining was noted on BMSCs and PDLSCs, while alizarin red S-positive nodules were only seen in PDLSCs. Under osteogenic differentiation conditions, ALP staining was noted on BMSCs and PDLSCs, while alizarin red S-positive nodules were seen in BMSCs and PDLSCs. As shown in Figure 3, ALP and alizarin red S staining was

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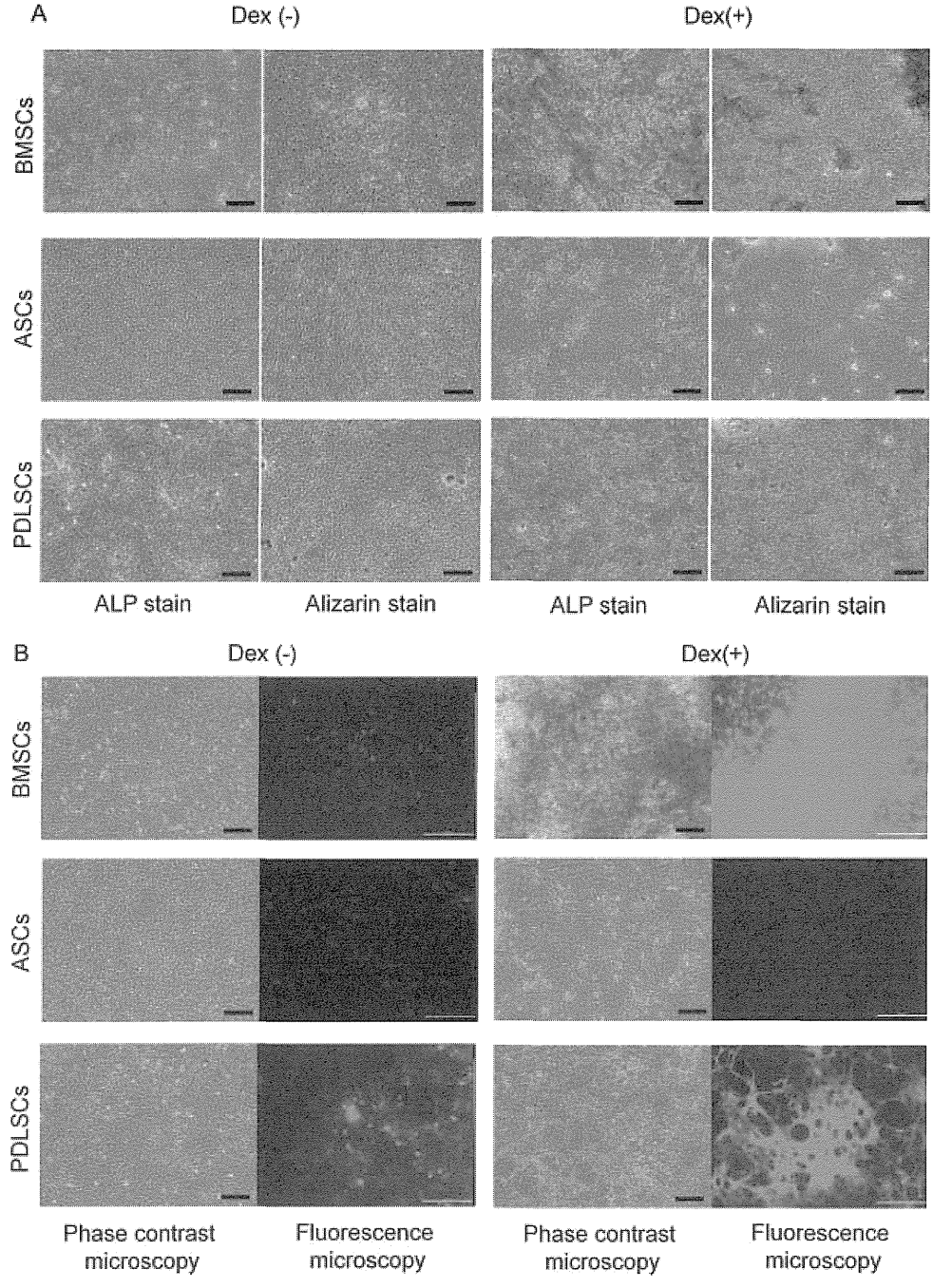

Figure 2. Histochemical staining. (A) ALP and alizarin red S staining under non-differentiation and osteogenic differentiation conditions (scale bar = 200 μm). (B) Phase-contrast micrographs and fluorescence micrographs of BMSCs, ASCs and PDLSCs under non-differentiation and osteogenic differentiation conditions (scale bar = 200 μm). Phase-contrast micrographs show extracellular bone mineral regions (brown areas). Fluorescence micrographs show calcein uptake (green areas)

more intense for BMSCs than PDLSCs. However, ALP staining and alizarin red S-positive nodules were not seen in ASCs under either non-differentiation or osteogenic differentiation conditions.

3.3. Calcein staining, quantification of mineralization and calcein uptake

In order to quantitatively analyse the mineralization of BMSCs, ASCs and PDLSCs, calcein, which is a calcium-binding fluorescent dye, was added to the culture medium. The calcein fluorescence intensities in the mineralized matrix of BMSCs, ASCs and PDLSCs were assessed 2 weeks after subculture. Under non-differentiation conditions, numerous fluorescent spots were noted on the PDLSCs and faint fluorescent spots could be detected in the BMSCs (Figure 2B). Under osteogenic differentiation conditions, fluorescence could be detected in PDLSCs and BMSCs. The fluorescence was more intense and extensive in BMSCs than in PDLSCs. The quantitative data for the fluorescence intensities

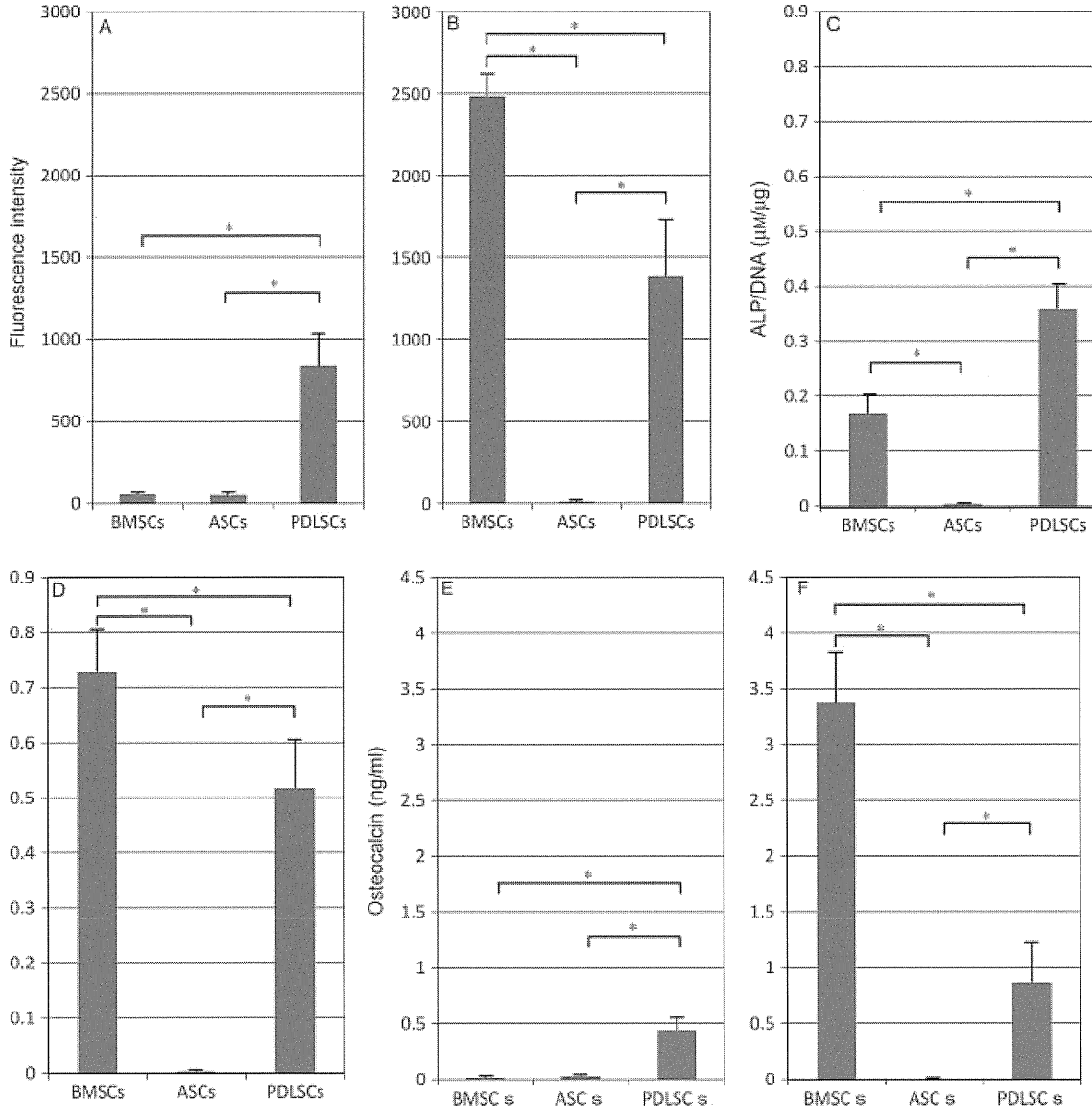

Figure 3. Quantification of osteoblastic markers. (A) Calcein uptake (fluorescence intensity) of BMSCs, ASCs and PDLSCs under non-differentiation and (B) osteogenic differentiation conditions. Values are means ± SD ($n = 6$). *$p < 0.01$ (one-way ANOVA). (C) ALP activity of BMSCs, ASCs and PDLSCs under non-differentiation and (D) osteogenic differentiation conditions. Values are means ± SD ($n = 6$). *$p < 0.01$ (one-way ANOVA). (E) Osteocalcin contents in BMSCs, ASCs and PDLSCs under non-differentiation and (F) osteogenic differentiation conditions. Values are means ± SD ($n = 6$). *$p < 0.01$ (one-way ANOVA)

are shown in Figure 3A, B. Under non-differentiation conditions, the fluorescence intensity of PDLSCs was highest among the three cell types (Figure 3A). Significant differences were observed between PDLSCs and other MSCs. Under osteogenic differentiation conditions, the fluorescence intensity of BMSCs was highest among the three cell types (Figure 3B). In contrast to BMSCs and PDLSCs, ASCs demonstrated remarkably low fluorescence intensity under either osteogenic differentiation or non-differentiation conditions.

3.4. Quantification of ALP activity

ALP activity is recognized as an early osteoblastic marker. The ALP activity of each sample was normalized against the DNA content of the cells in the sample. Under non-differentiation conditions, ALP activity of PDLSCs was highest among the three cell types (Figure 3C). Under osteogenic differentiation conditions, the ALP activity of BMSCs was highest among the three cell types (Figure 3D). Significant differences were observed between all pairs of three cell types under either osteogenic differentiation or non-differentiation conditions.

3.5. Quantification of osteocalcin and RT–PCR analysis

Osteocalcin is known to be a bone-specific protein and is used as a late marker of osteogenic differentiation. We analysed the osteocalcin contents of cultured cell layers

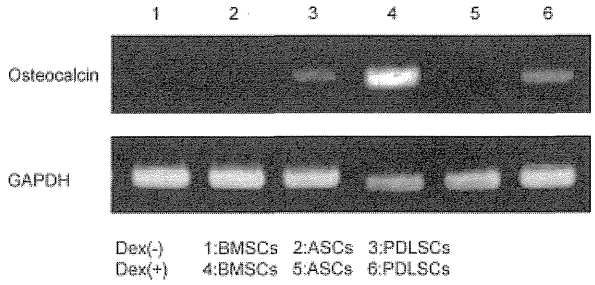

Figure 4. Osteocalcin and *GAPDH* gene expression by RT–PCR analysis

and mRNA expression levels were assessed after 2 weeks of subculture under non-differentiation and osteogenic differentiation conditions. The results for osteocalcin contents showed the same trend as calcein uptake and ALP activity. Under non-differentiation conditions, osteocalcin contents in PDLSCs were the highest among the three cell types (Figure 3E). Significant differences were observed between PDLSCs and other MSCs. Under osteogenic differentiation conditions, the osteocalcin contents of BMSCs were highest among the three cell types (Figure 3F). Significant differences were observed between all pairs of three cell types. RT–PCR analysis confirmed osteocalcin expression in PDLSCs under non-differentiation conditions and in BMSCs and PDLSCs under osteogenic differentiation conditions (Figure 4). Higher levels of osteocalcin expression of were confirmed in BMSCs when compared with PDLSCs under osteogenic differentiation conditions.

4. Discussion

In this study, we performed a direct comparison of three MSC types. On cell surface analysis, the three types of cell had similar characteristics at the end of primary culture, i.e. the three cell types had the same cell surface antigens and were of the mesenchymal type. After subculture, osteogenic supplements were shown to stimulate osteogenic differentiation of both BMSCs and PDLSCs. In addition, BMSCs had a higher osteogenic potential than PDLSCs. Under non-differentiation conditions, only PDLSCs showed osteogenic potential. However, ASCs demonstrated remarkably low osteogenic potential under either osteogenic differentiation or non-differentiation conditions. Our observations agreed with the results of other studies (Yoshimura *et al.*, 2007; Hayashi *et al.*, 2008). Therefore, BMSCs and PDLSCs are apparently ideal candidates for alveolar bone regeneration.

In 1982, guided tissue regeneration (GTR) was first applied to the regeneration of alveolar bone (Nyman *et al.*, 1982) and several surgical treatments have since been developed for the regeneration of periodontal tissue (Sculean *et al.*, 2008). GTR is a surgical procedure that utilizes a barrier membrane to direct the growth of periodontal ligament- and bone-derived cells, including bone. The principle is that the barrier membrane excludes unwanted cells from the healing site in order to allow growth of the desired tissue. According to the accumulated clinical data (Yamada *et al.*, 2006; Okuda *et al.*, 2009), research began to focus on the regeneration of new bone in an area where teeth are being extracted or have already been removed. Therefore, osteogenic cells from extracted teeth could be a useful tool for alveolar bone regeneration.

In this study we investigated the osteogenic characteristics of BMSCs, ASCs and PDLSCs and we demonstrated the osteogenic potential of BMSCs and PDLSCs. BMSCs are well-known multipotent cells that differentiate into adipocytes, chondrocytes and osteoblasts (Pittenger *et al.*, 1999). For bone regeneration using BMSCs, surgical stress (discomfort caused by bone marrow extraction) is induced when harvesting bone marrow, and osteogenic supplements may be required for osteoblastic differentiation. In contrast, PDLSCs can be expanded from extracted teeth, which are typically discarded, and have osteogenic potential without dexamethasone. Therefore, both PDLSCs and BMSCs are candidates for utilization in alveolar bone regeneration.

Although several studies have focused on the osteogenic potential of ASCs, there remains some controversy. Im *et al.* (2005) showed that human ASCs have inferior osteogenic capability when compared to BMSCs (Im *et al.*, 2005). Hayashi *et al.* (2008) reported that rat BMSCs showed distinct osteogenic differentiation capacity when compared with rat ASCs *in vivo* and *in vitro*. In contrast, De Ugarte *et al.* (2003) found no significant differences in osteogenic capacity between human BMSCs and ASCs. However, Im Gl *et al.* and De Ugarte *et al.* used human MSCs that had been passaged more than three times and ALP activity levels of human MSCs are very low when compared with the present study. Moreover, ALP activity analysis of human MSCs has low statistical power (Hayashi *et al.*, 2008). In rat ASCs, some studies show osteogenic capacity (Yoshimura *et al.*, 2007); however, these authors also used MSCs that had been passaged more than twice. Tobita *et al.* (2008) revealed that ASCs mixed with platelet-rich plasma can regenerate periodontal tissue, including alveolar bone. Therefore, for the osteogenic differentiation of ASCs, more osteogenic supplements in addition to dexamethasone may be required.

Dexamethasone is a steroid drug and acts as an anti-inflammatory and immunosuppressant. In bone tissue-engineering fields, dexamethasone has been used to promote undifferentiated stem cells to differentiate in the osteogenic lineage. However, the mechanism of osteogenic differentiation is not clear. In our study, BMSCs showed distinct osteogenic differentiation capability compared with PDLSCs under osteogenic differentiation conditions with dexamethasone. In contrast to BMSCs, PDLSCs showed osteogenic potential even under non-differentiation conditions. Based on these results, it is speculated that PDLSCs as a heterogeneous cell population contain many osteogenic cells and BMSCs contain many undifferentiated stem cells. The osteogenic property of the PDLSCs might show benefits for clinical

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situations because of pre-existing osteogenic cells in their population. In this regard, Feng *et al.* (2010) utilized cultured PLDSCs under non-differentiation conditions for three patients with periodontitis. They demonstrated the clinical, experimental evidences to supporting an efficacy and safety of PLDSCs transplantation.

In conclusion, we have demonstrated the utility of PDLSCs for periodontal bone regeneration. Seo *et al.* (2004) confirmed the multipotency of PDLSCs, which differentiate into cementoblast-like cells, adipocytes and collagen-forming cells). Therefore, PDLSCs are potentially useful for periodontal tissue regeneration. Due to their durability after freezing, cryopreserved PDLSCs may also become an ideal candidate for utilization in periodontal tissue regeneration (Oh *et al.*, 2005; Seo *et al.*, 2005).

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References

De Bari C, Dell'Accio F, Luyten FP. 2001; Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum* **44**: 85–95.

De Bari C, Dell'Accio F, Tylzanowski P, *et al.* 2001; Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**: 1928–1942.

De Ugarte DA, Morizono K, Elbarbary A, *et al.* 2003; Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* **174**: 101–109.

Feng F, Akiyama K, Liu Y, *et al.* 2010; Utility of PDL progenitors for *in vivo* tissue regeneration: a report of three cases. *Oral Dis* **16**: 20–28.

García-Olmo D, Herreros D, De-La-Quintana P, *et al.* 2010; Adipose-derived stem cells in Crohn's rectovaginal fistula. *Case Report Med* **2010**: 961758.

Gartland A, Mechler J, Mason-Savas A, *et al.* 2005; *In vitro* chondrocyte differentiation using costochondral chondrocytes as a source of primary rat chondrocyte cultures: an improved isolation and cryopreservation method. *Bone* **37**: 530–544.

Hayashi O, Katsube Y, Hirose M, *et al.* 2008; Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. *Calcif Tissue Int* **82**: 238–247.

Im Gl, Shin YW, Lee KB. 2005; Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthr Cartilage* **13**: 845–853.

Iwata T, Yamato M, Tsuchioka H, *et al.* 2009; Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. *Biomaterials* **30**: 2716–2723.

Kotobuki N, Hirose M, Takakura Y, *et al.* 2004; Cultured autologous human cells for hard tissue regeneration: preparation and characterization of mesenchymal stem cells from bone marrow. *Artif Organs* **28**: 33–39.

Lendeckel S, Jödicke A, Christophis P, *et al.* 2004; Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. *J Craniomaxillofac Surg* **32**: 370–373.

Lindroos B, Mäenpää K, Ylikomi T, *et al* 2008; Characterisation of human dental stem cells and buccal mucosa fibroblasts. *Biochem Biophys Res Commun* **368**: 329–335.

Nagaya N, Fujii T, Iwase T, *et al.* 2004; Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol Heart Circ Physiol* **287**: 2670–2676.

Nyman S, Lindhe J, Karring T, *et al.* 1982; New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* **9**: 290–296.

Oh H-Y, Che M-Z, Hong C-J, *et al.* 2005; Cryopreservation of human teeth for future organization of a tooth bank: a preliminary study. *Cryobiology* **51**: 322–329.

Ohgushi H, Caplan AI. 1999; Stem cell technology and bioceramics: from cell to gene engineering. *J Biomed Mater Res* **48**: 913–927.

Ohgushi H, Dohi Y, Katuda T, *et al.* 1996; *In vitro* bone formation by rat marrow cell culture. *J Biomed Mater Res* **32**: 333–340.

Pittenger MF, Mackay AM, Beck SC, *et al.* 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.

Rosenbaum AJ, Grande DA, Dines JS. 2008; The use of mesenchymal stem cells in tissue engineering: a global assessment. *Organogenesis* **4**: 23–27.

Sculean A, Nikolidakis D, Schwarz F. 2008; Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials – biological foundation and preclinical evidence: a systematic review. *J Clin Periodontol* **35**: 106–116.

Seo BM, Miura M, Gronthos S, *et al.* 2004; Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**: 149–155.

Seo BM, Miura M, Sonoyama W, *et al.* 2005; Recovery of stem cells from cryopreserved periodontal ligament. *J Dent Res* **84**: 907–912.

Techawattanawisal W, Nakahama K, Komaki M, *et al.* 2007; Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. *Biochem Biophys Res Commun* **357**: 917–923.

Tobita M, Uysal AC, Ogawa R, *et al.* 2008; Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng A* **14**: 945–953.

Uchimura E, Machida H, Kotobuki N, *et al.* 2003; *In situ* visualization and quantification of mineralization of cultured osteogenetic cells. *Calcif Tissue Int* **73**: 575–583.

Yamada Y, Ueda M, Hibi H, *et al.* 2006; A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: a clinical case report. *Int J Periodont Restor Dent* **26**: 363–369.

Yoshimura H, Muneta T, Nimura A, *et al.* 2007; Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* **327**: 449–462.

Yoshimura K, Sato K, Aoi N, *et al.* 2008; Cell-assisted lipotransfer for cosmetic breast augmentation: supportive use of adipose-derived stem/stromal cells. *Aesthet Plast Surg* **32**: 48–57.

Zuk PA, Zhu M, Ashjian P, *et al.* 2002; Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**: 4279–4295.

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Osteogenic Potential of Mesenchymal Stem Cells on Expanded Polytetrafluoroethylene Coated with Both a Poly-Amino-Acid Urethane Copolymer and Collagen

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We have developed an expanded polytetrafluoroethylene polymer (e-P) coated with both poly-amino-acid urethane copolymer and collagen (e-PPC) to be used for cell culture substrata. Rat mesenchymal stem cells (MSC) were cultured on the e-P and e-PPC polymers in the presence of dexamethasone. After 24 h, the MSC contacted well with the e-PPC surface and after 14 days, the MSC showed high levels of alkaline phosphatase activity, and calcium and bone-specific osteocalcin protein deposition. The MSC cultured on these polymers for 1 week were then implanted at rat subcutaneous sites and harvested after 4 weeks. Microcomputed tomography as well as histological analyses showed that any hard tissue could not be seen in implants of the MSC/e-P composites, whereas new bone formation could be detected in the MSC/e-PPC composites. These *in vitro* as well as *in vivo* results confirmed the importance of polymer surface to support the osteogenic differentiation, which resulted in new bone formation. The surface modification using poly-amino-acid urethane copolymer and collagen together with tissue engineering technology might facilitate bone anchoring to the polymers for dental and orthopedic applications.

Introduction

EXPANDED POLYTETRAFLUOROETHYLENE(e-P) is a porous form of a synthetic fluoropolymer with a microstructure that is characterized by nodes interconnected by fibrils. The e-P is biologically and chemically bioinert[1] and has sufficient strength/flexibility; therefore, it has been used as vascular graft prosthesis[2] and dental/orthopedic biomaterials.

In the dental field, e-P has been utilized clinically as a membrane for guided tissue regeneration (GTR) and guided bone regeneration (GBR).[3,4] The GTR has been carried out for periodontal tissue regeneration and the GBR for alveolar bone tissue regeneration. However, e-P membranes are used provisionally and need to be removed from the body at the end of the treatment. The e-P for the purpose of GTR and GBR tends to be exposed to oral cavity and due to hydrophobic nature; there is a fair of infection and insufficient bone tissue regeneration.[5,6]

In the orthopedic field, surgeons initially regarded the e-P as a very promising material and applied it for replacement of ligamentous tissues such as anterior cruciate ligament (ACL) prostheses in knee joint patients who sustain an injury to their ACL.[7] In the ACL reconstruction procedure, the e-P ligaments were placed through bone tunnels in the tibia or femur and fixed with screws to provide initial fixation. The short-term results of these ACL reconstruction were excellent and showed a low rate of failure.[7] However, with extended follow-up, the rate of complications increased remarkably.[8,9] Paulos *et al.*[8] reported that attenuation or rupture of the e-P grafts (12%), loosening (34%), chronic effusion (34%), and infection (2.7%) occurred during long-term follow-up. Fukubayashi *et al.*[9] noted that bone tunnel enlargement (tunnel osteolysis) was observed in most cases in their series. The loosening and bone tunnel enlargement might be due to inadequate affinity of the e-P ligaments for osteogenic cells, because the ligaments do not support osteoblast growth.[10]

These results of the e-P in dental/orthopedic applications indicate the necessity of surface chemical modification of e-P, which permits sufficient cell attachment followed by osteogenic differentiation. We have developed an e-P coated with both a poly-amino-acid urethane copolymer (PAU) and collagen (abbreviated as e-PPC). The aim of this study was to investigate the bone formation by osteogenic cells on e-PPC. To achieve this, we isolated rat bone marrow-derived

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mesenchymal stem cells (MSCs) and assessed their osteogenic differentiation on e-PPC *in vitro* and *in vivo*.

Materials and Methods

Preparation of the biomaterial

e-P was a porous form of a synthetic fluoropolymer with a microstructure characterized by nodes interconnected by fibrils. The fibrils was diameter of 10 mm and thickness of 50 μm (Sumitomo Denko, Tokyo, Japan). To fabricate e-PP, e-P fibrils were washed with ethanol, and immersed in a coating solution of 1% PAU in dichloroacetic. To fabricate e-PPC, the e-P fibrils were immersed in a solution of 1% PAU and 0.1% ocean collagen in dichloroacetic acid. The fabricated e-PP and e-PPC samples were washed in water to remove the excess coating solution, dried as much as possible, and sterilized with ethylene oxide. The samples were analyzed by scanning electron microscopy (SEM; SM-300; Topcon, Tokyo, Japan) . Figure 1 shows the SEM picture of the e-PPC.

Isolation and culture of MSC

The experimental protocol described in this study was approved by the Animal Experimentation Committee of National Institute of Advanced Industrial Science and Technology. Rat bone marrow cells were prepared according to previously reported methods.[11,12] Briefly, bone marrow cells were obtained from the femoral bone shafts of 7-week-old Fischer 344 male rats. Both ends of each femur were cut away from the epiphysis, and the bone marrow was flushed out using 10 mL of standard medium expelled from a syringe through a 21-gauge needle. The released cells were collected in a T-75 flask (BD Biosciences, Bedford, MA) containing 15 mL of standard medium. To remove nonadherent cells, the medium was renewed three times per week. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The standard medium consisted of Eagle's minimum essential medium (Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (JRH Bioscience, Lenexa, KS) and antibiotics (100 U/mL penicillin and 0.25 μg/mL amphotericin B; Sigma Chemical, St. Louis, MO). The adherent cells were regarded as MSC and initially cultured to 80% confluence in T-75 flasks. The cells were then detached from the flasks using 0.05% trypsin, harvested, and resuspended in the culture medium at 2×10^4 cells/mL. Next, 500-μL aliquots of the cell suspension were applied to the e-PPC scaffolds, which were then placed in a 24-well polystyrene plate. As controls, the cell suspension was also applied to e-P and e-P coated with 1% PAU (abbreviated as e-PP) scaffolds, which were also placed in the 24-well polystyrene plate. The cells on the scaffolds were cultured in the standard medium containing 10 nM dexamethasone (DEX, Sigma-Aldrich Corporation, St. Louis, MO), 10 mM β-glycerophosphate (Merck, Darmstadt, Germany), and 0.28 mM ascorbic acid 2-phosphate magnesium salt n-hydrate (Sigma-Aldrich Corporation) (osteogenic supplements). The culture medium was changed three times per week during the culture period.

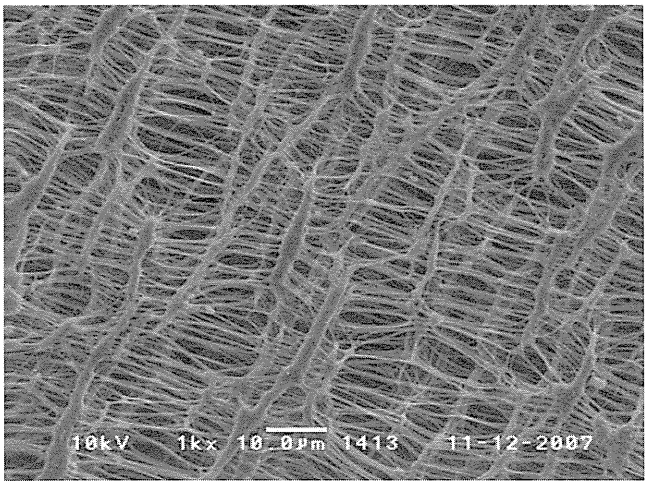

FIG. 1. Scanning electron micrograph of an expanded polytetrafluoroethylene polymer scaffold coated with both poly-amino-acid urethane copolymer and collagen.

Cell morphology

The morphologies of MSC after 24 h of culture on e-P, e-PP, and e-PPC were observed by SEM. Briefly, after 24 h of culture, each sample was rinsed twice with phosphate-buffered saline (PBS) and fixed with 10% neutral-buffered formaldehyde solution for 1 h at 4°C. The samples were then dehydrated through a series of ethanol solutions with increasing concentrations for 15 min and air-dried. The fixed samples were sputter-coated with platinum and observed by SEM.

Alkaline phosphatase staining

After 7, 14, and 21 days of culture, the cells on e-P, e-PP, and e-PPC were rinsed with PBS and fixed with 10% neutral-buffered formaldehyde solution for 10 min at 4°C. The fixed cells were soaked in 0.1% naphthol AS-MX phosphate and 0.1% fast red violet LB salt in 56 mM 2-amino-2-methyl-1, 3-propanediol (pH 9.9) for 10 min at room temperature, washed with PBS, and observed using an objective microscope (MZ FLIII; Leica, Tokyo, Japan).

Calcein stain (quantitative analyses of mineralization)

To quantitatively assess the mineralized extracellular matrix of the cultured cells, we performed calcein uptake assays according to our previous report.[13] For the calcein assays, 1 μg/mL of calcein was added every time the culture medium was renewed. Before the assays, the samples were washed twice with PBS. Calcein incorporated into the mineralized matrix was observed using a fluorescence microscope (Model IX70; Olympus, Tokyo, Japan) and then observed and scanned using an image analyzer (Typhoon 8600; Molecular Dynamics, Sunnyvale, CA). The scanned fluorescence images of each sample were quantified using Image Quant software (Molecular Dynamics) and presented as the fluorescence intensity per area.

Biochemical analyses

For quantitative analyses of osteogenic parameters, the samples were washed twice in PBS, scraped into 0.2% Triton-

X-100 solution, sonicated for 5 min, and then allowed to stand at room temperature for 5 min. The pulverized cell suspension (100 μL) was used for quantification of the DNA content. The DNA contents were measured fluorometrically with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Picogreen dsDNA Quantitation Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

After DNA measurements, an aliquot of each sample (100 μL) was mixed with p-nitrophenyl phosphate substrate (900 μL) and incubated for 10 min at 37°C to assay the alkaline phosphatase (ALP) activity. The ALP activity was represented by the amount of p-nitrophenol released during the 10-min incubation.[14]

After the quantification of the DNA content and ALP activity, 300 mL of 20% formic acid was added to the sonicated solution and incubated for 1 week at 4°C. Aliquots of the acid-hydrolyzed samples were diluted with ion-exchanged water. The calcium contents of the diluted samples were measured using an inductively coupled plasma atomic emission spectrometer 33 (SPS7800 Spectrometer; Seiko Instruments, Chiba, Japan).

The osteocalcin content was also measured in an aliquot of each sample extracted in 20% formic acid for 1 week at 4°C. The samples in formic acid were applied to a column of NAP 5 (GE Healthcare UK, Buckinghamshire, United Kingdom) and the protein fraction was eluted with 1 mL of 10% formic acid. The eluted fraction was lyophilized and prepared for analysis of intact osteocalcin using a Rat Osteocalcin EIA Kit (Biomedical Technologies, Stoughton, MA) as previously described.[14]

TABLE 1. SUBCUTANEOUS IMPLANTATION OF E-P, E-PP, AND E-PPC

Sample group	*Detection of bone formation*	*The average bone volume (mm^3)*
Control (without cell)		
e-P/control ($n=6$)	0/6	—
e-PP/control ($n=6$)	0/6	—
e-PPC/control ($n=6$)	0/6	—
Culture without DEX (DEX−)		
MSC/e-P composites ($n=8$)	0/8	—
MSC/e-PP composites ($n=8$)	0/8	—
MSC/e-PPC composites ($n=8$)	1/8	0.03
Culture with DEX (DEX+)		
MSC/e-P composites ($n=8$)	0/8	—
MSC/e-PP composites ($n=8$)	4/8	0.15 ± 0.25
MSC/e-PPC composites ($n=8$)	5/8	0.61 ± 0.79

e-P, expanded polytetrafluoroethylene polymer; e-PP, e-P coated with poly-amino-acid urethane copolymer; e-PPC, e-P coated with both poly-amino-acid urethane copolymer and collagen; DEX, dexamethasone; MSC, mesenchymal stem cell.

Subcutaneous implantation

After primary culture of MSC using the above-described method, the cells were subsequently cultured on e-P, e-PP, and e-PPC with or without DEX (DEX+, DEX−) for 1 week. Syngenic recipient rats were anesthetized by intraperitoneal injection of pentobarbital (Nembutal; Dainippon Pharmaceutical, Tokyo, Japan) at a final concentration of 35 mg/kg body weight. The cultured scaffolds (MSC/e-P, MSC/e-PP,

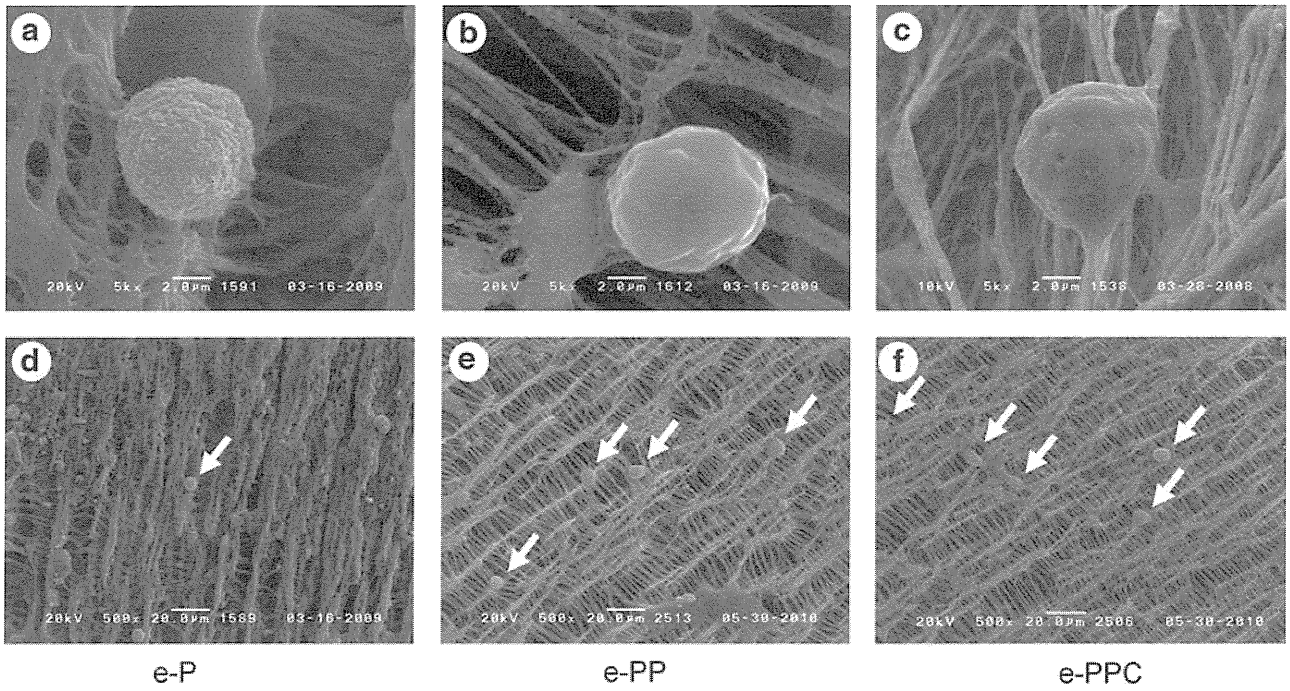

FIG. 2. Morphological appearances of MSC. Images of MSC on e-P **(a, d)**, e-PP **(b, e)**, and e-PPC **(c, f)** after 24 h of culture are shown. Upper row includes high magnification micrographs (5000×). Lower row includes low magnification micrographs (500×), and arrows indicate MSC. MSC, mesenchymal stem cell; e-P, expanded polytetrafluoroethylene polymer; e-PP, e-P coated with poly-amino-acid urethane copolymer; e-PPC, e-P coated with both poly-amino-acid urethane copolymer and collagen.

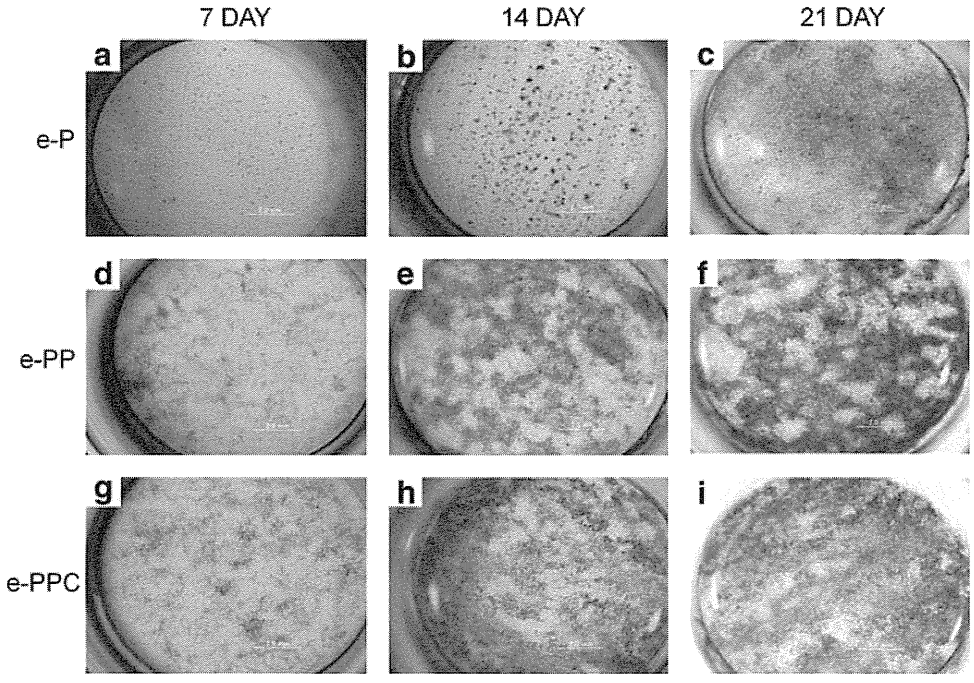

FIG. 3. ALP staining. Images of ALP staining of MSC on e-P after 7 **(a)**, 14 **(b)**, and 21 **(c)** days of culture; on e-PP after 7 **(d)**, 14 **(e)**, and 21 **(f)** days of culture; and on e-PPC after 7 **(g)**, 14 **(h)**, and 21 **(i)** days of culture are shown. Scale bars: 2 mm. ALP, alkaline phosphatase.

and MCS/e-PPC composites) were implanted subcutaneously into the back of the recipient rats (total 11 recipients) at different six sites, and then the implants (total 66) were harvested after 4 weeks. As controls, the scaffolds without cells (e-P/control, e-PP/control, and e-PPC/control) were also implanted subcutaneously into the back of the rat. Table 1 summarizes the number of implants. All implants were harvested after 4 weeks' implantation.

Microcomputed tomographic analysis

The capability of the scaffolds for newly bone formation was evaluated by microcomputed tomography (micro-CT, MCT-CB 100MF[Z]; Hitachi Medical, Tokyo, Japan). These samples were scanned at widths of every 10 μm at 55 kV and 150 mA. The analytical conditions were precision mode, ×3 magnification with an image intensifier field of 1.8 inches, and total 256 sagittal sections scanned. The intensity of a newly formed bone was defined as the same level intensity of the osseous tissue of syngenic rat (2400-4096 LUT).[15] In case of the detection of a newly formed bone into the scaffold, total volumes of the bone were measured using the software package TRI3D-BON (Ratoc System Engineering, Tokyo, Japan) according to the methods we previously reported.[15]

Histological analysis

After the micro-CT analysis, the samples were fixed with 10% neutral-buffered formaldehyde solution, decalcified with K-CX solution (Falma, Tokyo, Japan), and embedded in

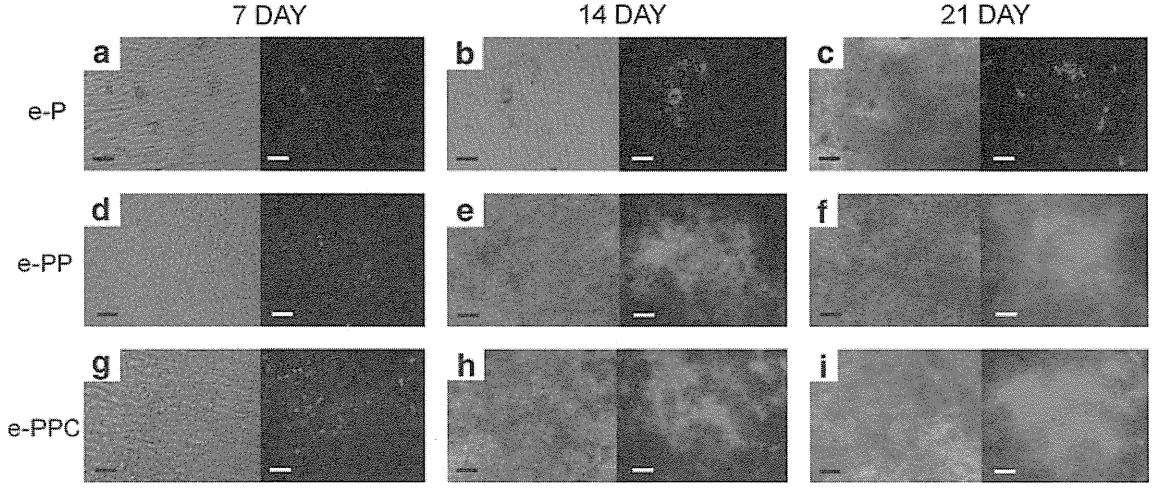

FIG. 4. Calcein staining of MSC. Images of calcein staining of MSC on e-P after 7 **(a)**, 14 **(b)**, and 21 **(c)** days of culture; on e-PP after 7 **(d)**, 14 **(e)**, and 21 **(f)** days of culture; and on e-PPC after 7 **(g)**, 14 **(h)**, and 21 **(i)** days of culture are shown. The images are phase-contrast of micrographs (left) and fluorescence micrographs (right). Scale bar: 50 μm.

paraffin. The paraffin blocks were cut into 4-μm-thick sections and stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan). Then, the section slides were examined by light microscopy.

Statistical analysis

For multiple comparisons, the groups were analyzed using one-way analysis of variance. When significant variance was detected, differences between individual groups were determined using the Student–Newman–Keuls test. The significance level was set at $p < 0.05$.

Results

Morphological findings of MSC

Figure 2 shows scanning electron micrographs of MSC on e-P, e-PP, and e-PPC after 24 h of culture. Compared with e-PP and e-PPC, small number of MSC appeared on e-P and the MSC did not show tight contact with e-P fibrils. In contrast, many MSC attached to the fibrils of e-PP and e-PPC. Further, the MSC on e-PPC contacted with e-PPC fibrils *via* extended dendritic-like process from the MSC.

ALP staining

ALP staining of MSC on e-P, e-PP, and e-PPC was performed after 7, 14, and 21 days of culture (Fig. 3). After 7 days of culture, the ALP stain was hardly detected on e-P, but was observed on both e-PP and e-PPC. After 14 and 21 days of culture, the ALP stain was observed on all scaffolds; however, the stains on e-PP and e-PPC were more intense than that on e-P.

Calcein staining (quantification of mineralization)

To perform quantitative analysis of extracellular mineralized matrix formation by culturing MSC on e-P, e-PP, and e-PPC over time during culture, calcein, which is a calcium-binding fluorescent dye, was added to the culture medium. The calcein fluorescence intensities in the mineralized matrix on e-P, e-PP, and e-PPC were assessed after 7, 14, and 21 days of culture (Figs. 4, 5). After 7 days of culture, the calcein fluorescence was faint on e-P, e-PP, and e-PPC. After 14 days of culture, the fluorescence remained faint on e-P, but increased on e-PP and e-PPC. After 21 days of culture, weak fluorescence was seen in only restricted areas on e-P; however, extensive fluorescence was seen homogeneously on both e-PP and e-PPC. The quantitative data for the fluorescence intensities are shown in Figure 5. After 7 days of culture, no significant differences were found among the three groups. After 14 and 21 days of culture, the intensities on both e-PP and e-PPC were significantly higher than that on e-P.

Biochemical analyses (quantification of osteogenic parameters)

The ALP activities were assessed after 7, 14, and 21 days of culture (Fig. 6). The ALP activity of each sample was normalized by the DNA content of the cells in the sample. The ALP activities of MSC on e-P, e-PP, and e-PPC increased rapidly, attained their highest levels after 14 days of culture, and decreased by 21 days of culture. After 7 and 14 days of culture, significant differences in the ALP activities were found among the three groups, and the highest activity was seen on e-PPC. After 21 days, the activities on both e-PP and e-PPC were significantly higher than that on e-P.

The calcium contents were measured after 7, 14, and 21 days of culture (Fig. 7). The calcium content of each sample was normalized by the DNA content of the cells in the sample. The calcium contents of the MSC on e-P, e-PP, and e-PPC increased during the time course of the culture. After 14 days of culture, significant differences in the calcium contents were found among the three groups, and the highest content was seen on e-PPC. After 21 days of culture, the contents on both e-PP and e-PPC were significantly higher than that on e-P.

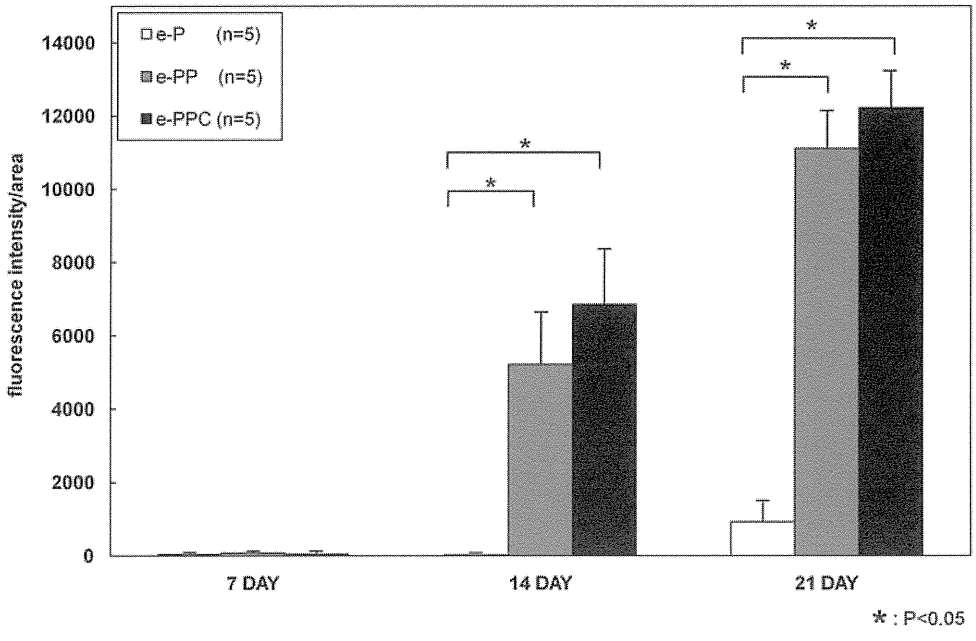

FIG. 5. Fluorescence intensities evaluated by quantitative calcein uptake of MSC cultured on e-P, e-PP, and e-PPC. Error bars represent the standard deviation for each group ($n = 5$). *$p < 0.05$.

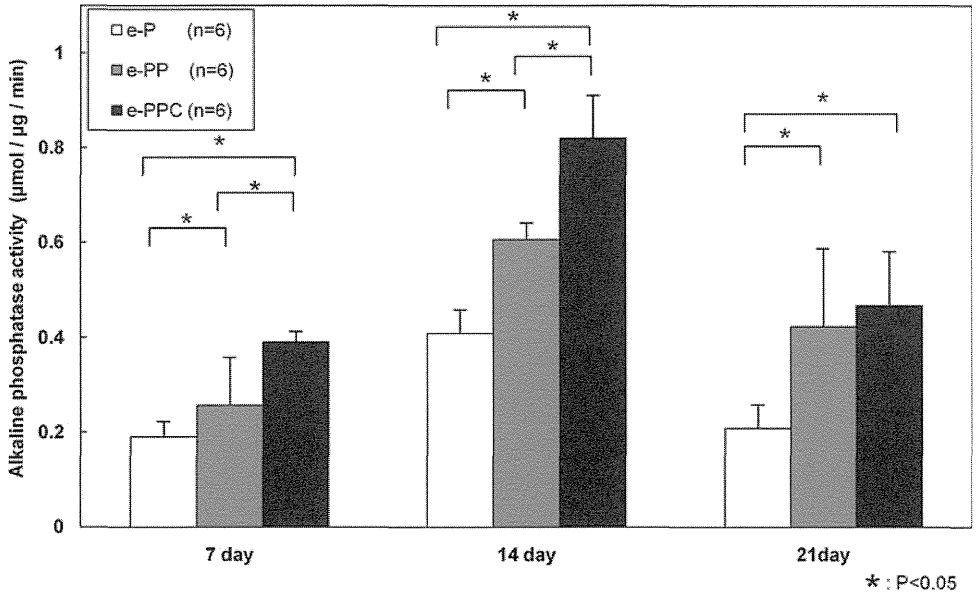

FIG. 6. ALP activities of MSC cultured on e-P, e-PP, and e-PPC. Error bars represent the standard deviation of each group ($n = 6$). *$p < 0.05$.

The osteocalcin contents were measured after 7, 14, and 21 days of culture (Fig. 8). The osteocalcin content of each sample was normalized by the DNA content of the cells in the sample. The data of the osteocalcin contents were quite similar to those of the calcium contents as described above. The contents of the MSC on all scaffolds increased during the time course of the culture and the highest content on e-PPC was detected after 14 days of culture. The contents on both e-PP and e-PPC were higher than that on e-P after 21 days.

Micro-CT and histological findings of in vivo *implants*

To evaluate *in vivo* new bone-forming capability of MSC on each scaffold, the MSC were cultured on the scaffolds with DEX (DEX+) or without DEX (DEX−) for 1 week, and then the cultured MSC/scaffolds composites were implanted subcutaneously into the back of the recipient rats. Scaffolds without MSC culture were also implanted as control. At 4 weeks after implantation, all implants were harvested and evaluated by micro-CT (Fig. 9). High-density areas could not be detected in any scaffolds without MSC and the area could be detected in only one out of eight implants of MSC/e-PPC composites cultured without DEX (DEX−). In contrast, when cultured with DEX (DEX+), the high-density areas were clearly seen in four and five out of eight implants of MSC/e-PP and MSC/e-PPC composites, respectively (Table 1). Average volume of the high-density areas was 0.03, 0.15 ± 0.25, and $0.61 \pm 0.79\,mm^3$ for MSC/e-PPC/(DEX−), MSC/e-PP/(DEX+), and MSC/e-PPC/(DEX+), respectively (Table 1).

To identify the high-density areas with bone tissue, harvested implants after micro-CT analyses were subjected to histological analyses. As seen in Figure 10, only implants having high-density areas showed new bone tissue formation. Extensive bone areas could be detected in MSC/e-PP/(DEX+) and MSC/e-PPC/(DEX+). These findings confirmed that the high-density areas seen in the CT analyses were newly formed bone tissue in the implants.

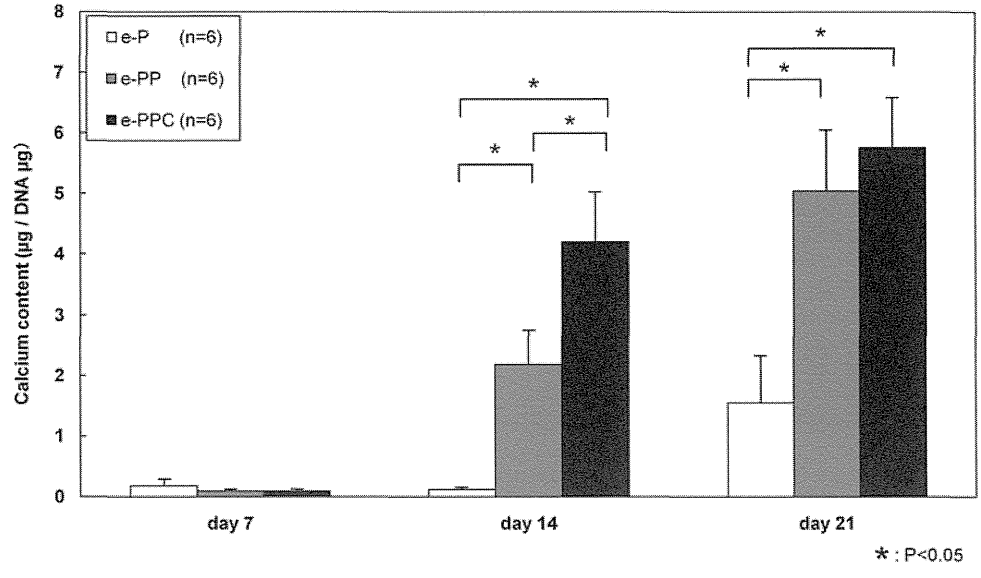

FIG. 7. Calcium contents of MSC cultured on e-P, e-PP, and e-PPC. Error bars represent the standard deviation of each group ($n = 6$). *$p < 0.05$.

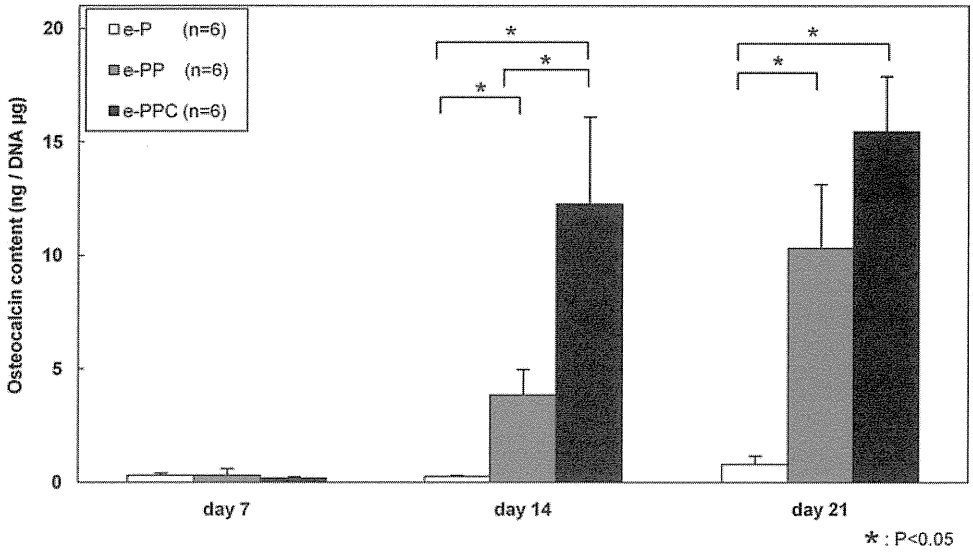

FIG. 8. Osteocalcin contents of MSC cultured on e-P, e-PP, and e-PPC. Error bars represent the standard deviation of each group ($n = 6$). $^*p < 0.05$.

Discussion

For the development of new biomaterials in the field of tissue engineering, it is important to assess cell–biomaterial surface interactions. The interactions of e-P with endothelial cells have been studied. Camilleri *et al.*[16] investigated the e-P grafts as an arterial bypass for up to 60 months and revealed the absence of neovascularization and limited neointimal proliferation on the graft surface. This report showed the poor cell attachment on the surface of e-P grafts and also implies limitations of the use of e-P for the purpose of vascular prostheses. To solve the problem, Lindblad *et al.*[17] reported that e-P pretreated with a blood clot enhanced cell attachment and Feugier *et al.*[18] reported that e-P precoated with various proteins showed superior endothelial cell adherence and spreading on the grafts. Wang *et al.*[19] reported that PAU-coated e-P vascular grafts enhanced endothelialization. Therefore, protein or PAU coating on the e-P graft showed advantage to improve the cell interactions. However, due to hydrophobic surface of the e-P, the blood/protein coating might be unstable and there was no comparative study between protein and PAU coatings. In our present paper, we seeded the MSC on e-PP or PAU/collagen (e-PPC) and compared the bone-forming capabilities of these coated materials. To fabricate e-PPC, e-P was immersed in a coating solution composed of collagen and PAU, which changes high hydrophobic surface (e-P) to hydrophilic surface. Therefore, using the technology, PAU could easily modified e-P with collagen; otherwise, e-P membranes cannot be efficiently coated with collagen. The other methods of collagen coating utilized cross-linking reagents such as glutaraldehyde and formaldehyde.[20,21] However, these reagents are known to induce cytotoxic reactions.[22] We also seeded the MSC on noncoated e-P as control, because Friedmann *et al.*[23] reported the good osteogenic response of noncoated e-P using osteoblastic cells. However, Walsh *et al.*[10] concluded that the surfaces of e-P did not support osteoblast growth.

Present study using *in vitro* culture experiments demonstrated that MSC well attached on the surfaces of both e-PP and e-PPC, showed osteogenic differentiation, and finally demonstrated bone matrix formation. The osteogenic differentiation was confirmed by high osteogenic parameters of ALP activities, and calcium and osteocalcin contents. In contrast, MSC did not show tight contact with the surface of e-P and showed low levels of these osteogenic parameters. Thus, compared with e-PP and e-PPC, the surfaces of e-P were hard to show the osteogenic response of MSC. With regard to the differences between e-PP and e-PPC, after 14 days of culture, the MSC on e-PPC showed high ALP activity, and calcium and osteocalcin contents compared

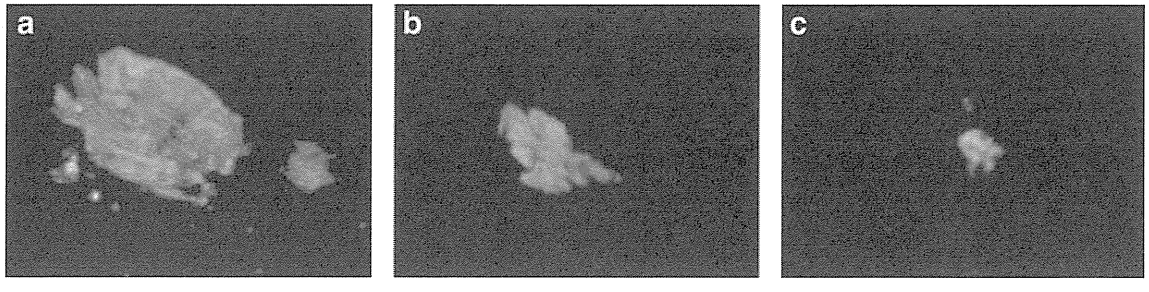

FIG. 9. Three-dimensional computed tomography images of MSC/scaffolds composites after 4 weeks' implantation. The composites were cultured with DEX (DEX+) or without DEX (DEX−) for 1 week, and then implanted at subcutaneous sites. MSC/e-PPC composite **(a:** DEX+**)**, MSC/e-PP composite **(b:** DEX+**)**, and MSC/e-PPC composites **(c:** DEX−**)**. DEX, dexamethasone.

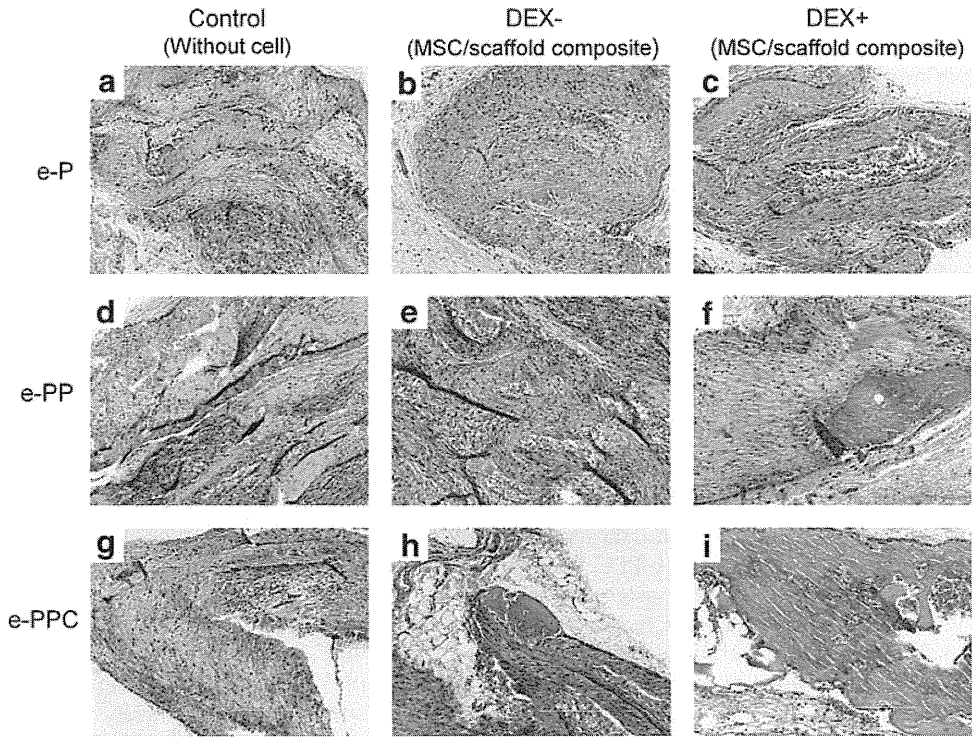

FIG. 10. Histological images of implants of scaffolds without MSC and scaffolds with MSC (MSC/scaffolds composites). Scaffolds used were e-P, e-PP, and e-PPC. The scaffolds without MSC **(a, d, g)** and with MSC **(b, c, e, f, h, i)** were implanted at subcutaneous sites for 4 weeks. Scaffolds with MSC (MSC/e-P, MSC/e-PP, and MSC/e-PPC composites were cultured with (DEX+) or without DEX (DEX−) for 1 week. Scale bar: 200 μm.

with the MSC on e-PP. After 21 days, though significant differences were not detected, all parameters on e-PPC were higher than those on e-PP. Therefore, collagen well improved the osteogenic differentiation of rat MSC. In this regard, we reported that the differentiation of rat MSC under the almost same condition of the present study was enhanced by adding collagen into the culture medium.[24] Therefore, collagen molecule used for e-PPC scaffold may directly influence the MSC to induce osteogenic differentiation.[24] *In vivo* implantation results confirmed these *in vitro* experiments. MSC/e-P composites did not show any bone formation regardless of DEX treatments during the culture before implantation. MSC/e-PPC composites showed higher frequency of bone formation than MSC/e-PP. Therefore, surface of e-PPC well supported the *in vivo* bone formation process of the MSC. These results proved that e-PPC accelerated the osteogenic differentiation cascade compared with e-PP and in considering the clinical application; the results may lead the surgeons to establish early rehabilitation programs for the e-PPC use in osseous reconstruction surgery.

It is important to know the fate of copolymer and collagen coating upon e-P for clinical applications. Christenson *et al.*[25] revealed that polyurethane film showed large shallow pit and surface cracking 3 months after implantation and extensive surface microcracking covering a majority of surface area after 1 year. Therefore, several months after implantation, PAU might show some degradation, Concerning the PAU coating (e-PP), Wang *et al.*,[19] using vascular graft model, reported that the effect of PAU coating continued 8 weeks after implantation. We fabricated e-PPC by immersing the e-P in a solution of PAU and collagen. The degradation of collagen might be depending on the degradation of PAU, because, as described, the PAU coating is relatively stable and also change the high hydrophobic surface to hydrophilic surface, which promotes collagen modification. Although, details of the collagen degradation need further studies.

Bone is formed by cells called osteoblasts, which arise from progenitor cells through the cascade of osteogenic differentiation. The osteoblast progenitors are derived from MSCs, which originally reside in the bone marrow. Therefore, the MSC have the principal role for the bone formation and thus were used in the present experiments.[26] The osteogenic differentiation cascade can be reproduced by *in vitro* culture of MSC in the presence of DEX, resulting in *in vitro* bone formation (the appearance of osteoblasts together with bone matrix formation). The osteoblasts/bone matrix further demonstrates *in vivo* new bone-forming capability. As seen in our *in vivo* subcutaneous implantation experiments, when MSC were cultured on e-PP or e-PPC with DEX (DEX+), extensive *in vivo* bone formation was detected (more bone was detected in MSC/e-PPC than in MSC/e-PP composites); however, when cultured without DEX (DEX−), only one out of eight MSC/e-PPC composites showed the bone formation. Further, none of MSC/e-P composites showed bone formation. These results indicate the importance of MSC culture in the presence of DEX, but the culture needs appropriated scaffolds to hold MSC and support their osteogenic differentiation. Here, we found that MSC well attached on the surface of e-PPC scaffold, which supported the osteogenic differentiation.

We also reported the importance of MSC culture using porous ceramics as scaffolds.[26,27] When, MSC/ceramic composites were cultured in the presence of DEX, early and extensive *in vivo* bone formation was detected after subcutaneous implantation of the composites.[26,27] We obtained the initial results using rat's MSC/ceramic composites[11,12,26]; then, we did serial *in vitro/in vivo* experiments using large animal to human MSC and based on these experiences; we finally succeeded in the use of patient's MSC/ceramic

composites for the bone tissue repair in clinical cases.[28,29] These experiences indicate the importance of rat MSC experiments at the level of *in vitro* culture and *in vivo* subcutaneous implantation for the future clinical applications. Especially, rat MSC in various scaffolds can show consistent *in vitro* as well as *in vivo* bone formation if the scaffolds are biocompatible and can hold the MSC to support the osteogenic differentiation.[26,30–32] This indicates the usefulness of rat cells to identify the material performance. Our present study using rat MSC clearly showed that e-PPC is a feasible scaffold for bone tissue regeneration and might imply future clinical applications of human MSC/e-PPC composites for bone tissue reconstruction in dental and orthopedic fields; however, further extensive experiments are needed to confirm the clinical relevance.

Conclusions

This study reports the first data regarding the osteogenic potential of MSC on e-P and e-PPC. Compared with e-P, the e-PPC well supported *in vitro* osteogenic differentiation of rat MSCs and further enhanced *in vivo* bone formation. These results indicate the usefulness of MSC/e-PPC composites for the purpose in bone tissue regeneration and possible applications as GBR in the dental and artificial ligaments in the orthopedic field.

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Disclosure Statement

No competing financial interests exist.

References

1. Simmermacher, R.K., van der Lei, B., Schakenraad, J.M., and Bleichrodt, R.P. Improved tissue ingrowth and anchorage of expanded polytetrafluoroethylene by perforation: an experimental study in the rat. Biomaterials **12,** 22, 1991.
2. Herring, M., Baughman, S., Glover, J., Kesler, K., Jesseph, J, Campbell, J, Dilley, R., Evan, A., and Gardner, A. Endothelial seeding of Dacron and polytetrafluoroethylene grafts: the cellular events of healing. Surgery **96,** 745, 1984.
3. Gottlow, J., Nyman, S., Karring, T., and Lindhe, J. New attachment formation as the result of controlled tissue regeneration. J Clin Periodontol **11,** 494, 1984.
4. Melcher AH. On the repair potential of periodontal tissues. J Periodontol **47,** 256, 1976.
5. Machtei EE. The effect of membrane exposure on the outcome of regenerative procedures in humans: a meta-analysis. J Periodontol **72,** 512, 2001.
6. Fritz, M.E., Jeffcoat, M.K., Reddy, M., Koth, D., Braswell, LD., Malmquist, J., and Lemons, J. Guided bone regeneration of large mandibular defects in a primate model. J Periodontol **71,** 1484, 2000.
7. Bolton, CW., and Bruchman, W.C. The GORE-TEX expanded polytetrafluoroethylene prosthetic ligament. An *in vitro* and *in vivo* evaluation. Clin Orthop Relat Res **196,** 202, 1985.
8. Paulos, L.E., Rosenberg, T.D., Grewe, S.R., Tearse, D.S., and Beck C.L. The GORE-TEX anterior cruciate ligament prosthesis. A long-term followup. Am J Sports Med **20,** 246, 1992.
9. Fukubayashi, T., and Ikeda K. Follow-up study of Gore-Tex artificial ligament—special emphasis on tunnel osteolysis. J Long Term Eff Med Implants **10,** 267, 2000.
10. Walsh, W.R., Olmedo, M., Kim, H.D., Zou, L., and Weiss, A.P. Human osteoblast response to PTFE surfaces. Clin Mater **16,** 201, 1994.
11. Ohgushi, H., Dohi, Y., Katuda, T., Tamai, S., Tabata, S., and Suwa, Y. *In vitro* bone formation by rat marrow cell culture. J Biomed Mater Res **32,** 333, 1996.
12. Ohgushi, H., Dohi, Y., Yoshikawa, T., Tamai, S., Tabata, S., Okumaga, K., and Shibuya, T. Osteogenic differentiation of cultured marrow stromal stem cells on the surface of bioactive glass ceramics. J Biomed Mater Res **32,** 341, 1996.
13. Maeda, M., Hirose, M., Ohgushi, H., and Kirita, T. *In vitro* mineralization by mesenchymal stem cells cultured on titanium scaffolds. J Biochem **141,** 729, 2007.
14. Tanaka, T., Hirose, M., Kotobuki, N., Ohgushi, H., Furuzono, T., and Sato, J. Nano-scaled hydroxyapatite/silk fibroin sheets support osteogenic differentiation of rat bone marrow mesenchymal cells. Mater Sci Eng C **27,** 817, 2007.
15. Nishikawa, M., Myoui, A., Ohgushi, H., Ikeuchi, M., Tamai, N., and Yoshikawa, H. Bone tissue engineering using novel interconnected porous hydroxyapatite ceramics combined with marrow mesenchymal cells: quantitative and three-dimensional image analysis. Cell Transplant **13,** 367, 2004.
16. Camilleri, J,P., Phat, V.N., Bruneval, P., Tricottet, V., Balaton, A., Fiessinger, J.N., and Cormier, J.M. Surface healing and histologic maturation of patent polytetrafluoroethylene grafts implanted in patients for up to 60 months. Arch Pathol Lab Med **109,** 833, 1985.
17. Lindblad, B., Wright, S.W., Sell, R.L., Burkel, W.E., Graham, L.M., and Stanley, J.C. Alternative techniques of seeding cultured endothelial cells to ePTFE grafts of different diameters, porosities, and surfaces. J Biomed Mater Res **21,** 1013, 1987.
18. Feugier, P., Black, R.A., Hunt, J.A., and How. T.V. Attachment, morphology and adherence of human endothelial cells to vascular prosthesis materials under the action of shear stress. Biomaterials **26,**1457, 2005.
19. Wang, C., Zhang, Q., Uchida, S., and Kodama, M. A new vascular prosthesis coated with poly -amino-acid urethane copolymer (PAU) to enhance endothelialization. J Biomed Mater Res **62,** 315, 2002.
20. Wissink, M.J.B. Endothelialization of collagen matrices: effects of crosslinking, heparin immobilization and bFGF loading. Ph.D. thesis. University of Twente. Enschede, Netherlands, 1999.
21. van Wachem, P.B., Plantinga, J.A., Wissink, M.J., Beernink, R., Poot, A.A., Engbers, G.H., Beugeling, T., van Aken, W.G., Feijen, J., and van Luyn, M.J. *In vivo* biocompatibility of carbodiimide-crosslinked collagen matrices: effects of crosslink density, heparin immobilization, and bFGF loading J Biomed Mater Res **55,** 368, 2001.
22. Huang-Lee, L.L., Cheung D.T., and Nimni, M.E. Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. J Biomed Mater Res **24,** 1185, 1990.
23. Friedmann, A., Dehnhardt, J., Klenber, B., Bernimoulin, J. and Bernimoulin, J. Cytobiocompatibility of collagen and

ePTFE membranes on osteoblast-like cells *in vitro*. J Biomed Mater Res A **86,** 935, 2008.
24. Kihara, T., Hirose, M., Oshima, A., and Ohgushi, H. Exogenous type I collagen facilitates osteogenic differentiation and acts as a substrate for mineralization of rat marrow mesenchymal stem cells *in vitro*. Biochem Biophys Res Commun **341,** 1029, 2006.
25. Christenson, E.M., Anderson, J.M., and Hiltner, A. Antioxidant inhibition of poly (carbonate urethane) *in vivo* biodegradation. J Biomed Mater Res A **76,** 480, 2006.
26. Ohgushi, H., and Caplan, A.I. Stem cell technology and bioceramics: from cell to gene engineering. J Biomed Mater Res **48,** 913, 1999.
27. Matsushima, A., Kotobuki, N., Tadokoro M., Kawate, K., Yajima, H., Takakura, Y and Ohgushi, H. *In vivo* osteogenic capability of human mesenchymal cells cultured on hydroxyapatite and on beta-tricalcium phosphate. Artif Organs **33,** 474, 2009.
28. Ohgushi, H., Kotobuki, N., Funaoka, H., Machida, H., Hirose, M., Tanaka, Y., and Takakura, Y. Tissue engineered ceramic artificial joint—ex vivo osteogenic differentiation of patient mesenchymal cells on total ankle joints for treatment of osteoarthritis. Biomaterials **26,** 4654, 2005.
29. Morishita, T., Honoki, K., Ohgushi, H., Kotobuki, N., Matsushima, A., and Takakura, Y. Tissue engineering approach to the treatment of bone tumors: three cases of cultured bone grafts derived from patients' mesenchymal stem cells. Artif Organs **30,** 115, 2006.
30. Hayashi, O., Katsube, Y., Hirose, M., Ohgushi, H., and Ito, H.Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. Calcif Tissue Int **82,** 238, 2008.
31. Oliveira, J.M., Silva, S.S., Malafaya, P.B., Rodrigues, M.T., Kotobuki, N., Hirose, M., Gomes, M.E., Mano, J.F., Ohgushi, H., and Reis, R.L. Macroporous hydroxyapatite scaffolds for bone tissue engineering applications: physicochemical characterization and assessment of rat bone marrow stromal cell viability. J Biomed Mater Res A **91,** 175, 2009.
32. Tanaka, T., Hirose, M., Kotobuki,N., Tadokoro, M., Ohgushi, H., Fukuchi, T., Sato, J. and Seto, K. Bone augmentation by bone marrow mesenchymal stem cells cultured in three-dimensional biodegradable polymer scaffolds. J Biomed Mater Res A **91,** 428, 2009.

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