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Bone Regeneration at Dental Implant Sites with Suspended Stem Cells

ABSTRACT

During the maintenance of bone marrow-derived mesenchymal stem cells (BMMSCs), suspended cells are discarded normally. We noted the osteogenic potential of these cells to be like that of anchorage-dependent BMMSCs. Therefore, we characterized suspended BMMSCs from rabbit bone marrow by bioengineering and applied the suspended BMMSCs to double-canaled dental implants inserted into rabbits. After primary isolation of BMMSCs, we collected the suspended cells during primary culture on the third day. The cells were transferred and maintained on an extracellular-matrix-coated culture plate. The cells were characterized and compared with BMMSCs by colony-forming-unit fibroblast (CFU-f) and cell proliferation assay, fluorescence-activated cell sorter (FACS), *in vitro* multipotency, and reverse transcription polymerase chain reaction (RT-PCR). We also analyzed the osteogenic potential of cells mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) and transplanted into immunocompromised mice. We compared the viability and proliferation of the suspended BMMSCs and BMMSCs on the titanium implant surface and observed cell morphology. Then, the cells mixed with HA/TCP were applied to the double-canaled implants during installation into rabbit tibia. Four weeks later, we analyzed bone formation inside the canal by histomorphometry. The suspended cells showed higher CFU-f on the extracellular matrix (ECM)-coated culture plate and similar results of proliferation capacity compared with BMMSCs. The cells also showed osteogenic, adipogenic, and chondrogenic ability. The suspended cells showed levels of attachment survival and proliferation on the surfaces of titanium implant discs to be higher than or similar to those of BMMSCs. The suspended cells as well as BMMSCs showed stronger bone formation ability in both upper and lower canals of the implants compared with controls on double-canaled implants inserted into rabbit tibia. In this study, we showed that suspended cells after primary BMMSC isolation have bone regeneration capacity like that of BMMSCs, not only *in vitro* but also *in vivo*. ECM was valuable for propagation of MSCs for cell-based bone regeneration. Therefore, the suspended cells could also be useful tools for bone regeneration after implant surgery.

INTRODUCTION

In modern dentistry, tooth loss caused by trauma and periodontal disease is considered a major health problem. For such patients, the use of dental implants for oral rehabilitation has become a standard treatment. Bone augmentation is required in implant placement sites with insufficient bone volume. Among the various techniques for the reconstruction of alveolar bone deficiency, autogenous bone grafting has become a reliable,

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well-documented surgical approach and is accepted as the gold standard treatment (Buser *et al.*, 1999); however, it is also associated with substantial morbidity, including infection, malformation, pain, and loss of function. Recent advancements in bone-engineering techniques have made it possible to provide alternatives for new regenerative treatment, including cell-based bone regeneration by autologous cell transplantation (Noshi *et al.*, 2001; Abukawa *et al.*, 2009; Park *et al.*, 2011), because this technique requires only a small amount of tissue from the patients and can be used to repair bone defects without leading to donor site morbidity.

Bone-marrow-derived mesenchymal stem cells (BMMSCs) have received attention because of their potential utility in bone regeneration (Ciapetti *et al.*, 2006; Brooke *et al.*, 2007). The cells have self-renewal as well as pluripotent capacities, which can differentiate into distinct cell types, including osteocytes, adipocytes, and chondrocytes (Prockop, 1997; Ferrari *et al.*, 1998; Dennis *et al.*, 1999). BMMSCs are involved in bone regeneration throughout life and could be useful for the treatment of bone-defect-related diseases (Petite *et al.*, 2000; Kassem, 2006; Banerjee and Bhonde, 2007). However, a major obstructive factor in the clinical application of BMMSCs has been their limited number, because they are rarely found in bone marrow (BM) tissue (~0.01-0.001%) (Wexler *et al.*, 2003). Obtaining a large number of functional osteogenic cells is essential for successful bone regeneration. Although it has been recognized that BMMSCs reside in adherent cell populations, this was overshadowed by the finding that the suspended cells were also rich in MSCs and could differentiate into osteogenic cells *in vitro* (Long *et al.*, 1990) and *in vivo* (Włodarski *et al.*, 2004; Zhang *et al.*, 2008).

Recently, ECM containing several components was found to favor cell proliferation, self-renewal, and multipotential differentiation, suggesting that ECM secreted by MSCs modulates cell behavior and facilitates the retention of stem cell properties (Prewitz *et al.*, 2013). It was reported that ECM prepared from mouse BMMSCs was capable of generating a new subset BMMSCs (Akiyama *et al.*, 2012), but it is necessary for cell utilization to be studied *in vitro* and *in vivo* in secondary animals, like the rabbit, before clinical application in humans.

During osseointegration, bone regeneration is a complicated process and involves different steps in the cortical and marrow areas of a surgical site. The easiest method for the study of bone regeneration has been to prepare a defect in the bone and retrieve the regenerated area of bone tissue. This method was rejected because of the difficulty in locating the defect as an implant insertion site. In our previous study, we reported that the double-canaled implant model can be considered to be a useful tool for quantifying bone regeneration, since it was not affected by the environment of the overlying tissue and mechanical stimulation (Lee *et al.*, 2011). In this study, we endeavored to prove the effectiveness of natural extracellular matrix (ECM) to generate a colony of suspended cells, designed the double-canaled implant for bioengineering application of the suspended cells, and identified the feasibility of the suspended cells for bone regeneration in the rabbit after implant surgery.

MATERIALS & METHODS

Characterization of Suspended BMMSCs

BMMSC Isolation, Reconstitution of ECM-coated Culture Plate, and Transferring the Suspended Cells onto the Plate

New Zealand white rabbits were used in this study and treated according to the Guidelines and Regulations for the Use and Care of Animals at the School of Dentistry of Seoul National University (Approval number: SNU-120312-5). BMMSCs were isolated from rabbit tibia. For reconstitution of the ECM-coated plate, after 7 days of BMMSC culture, the cells were decellularized with 0.005% Triton X-100 for 5 min at RT, and the remaining ECM-coated plate was dried. Then, after 3 days of primary mononuclear cell seeding, the suspended cells were collected and re-plated on the ECM-coated plate and cultured for 14 days (Fig. 1A). The colony-forming re-attached cells were subcultured on a plastic culture plate for the following experiments. The detailed methods are described in the Appendix.

In vitro and *in vivo* Assay

The colony-forming ability, cell proliferation, cell-surface molecules, and the differentiation potential of the suspended cells were investigated. Reverse transcription polymerase chain reaction (RT-PCR) analysis was conducted. For further characterization of the *in vivo* survivability and osteogenic potential of the suspended cells, the cells were mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) and subcutaneously transplanted into immunocompromised mice (BALB/c). The detailed *in vitro* and *in vivo* assays are described in the Appendix.

Biomechanical Analysis of the Suspended BMMSCs Cultured on Titanium Implant Surface Discs

To investigate the early osseointegration and biocompatibility of the cells, machined and anodized titanium surface discs were prepared for optical adherence of the MSCs (see Appendix). To determine the biomechanical properties of the suspended cells on the discs, we examined their proliferation, viability, and morphology in comparison with those of the BMMSCs.

Capacity of the Suspended BMMSCs to Regenerate Bone in Double-canaled Implants

In total, 40 screw-shaped, double-canaled implants made of grade 4 commercially pure Ti were prepared (Fig. 4A). The total length of each implant was 7 mm, with 5 mm threaded and 2 mm unthreaded at the coronal portion. The implants had an outer diameter of 3.75 mm, a thread pitch height of 0.6 mm, 2 canals of 1.5 mm in diameter passing through the threaded portion, and 1.0 mm apart from each canal, which was designed according to the position of bone quality (upper canal to cortical bone and lower canal to cancellous bone). The internal and external surfaces of the canal were machined. Defects and canals were filled with HA/TCP (control groups), and experimental groups filled with BMMSCs and the suspended BMMSC-loaded HA/TCP were prepared for examination of the capacity of cells to regenerate bone (Fig. 4B). The details are described in the Appendix.

Figure 1. Obtaining and characterization of suspended BMMSCs. **(A)** The image shows the scheme for obtaining the suspended BMMSCs on an ECM-coated plate. **(B)** The appearance of CFU-f. (a) Freshly isolated primary bone marrow mononuclear cells cultured on a plastic culture plate. (b) After 3 days of primary mononuclear cell culture, the suspended cells were collected and cultured on an ECM-coated culture plate. (c) After 3 days of primary mononuclear cell culture, the suspended cells were collected and cultured on a plastic culture plate. Inserted image shows expanded view of CFU-f, magnification 40x. (d) Increased CFU-f number of suspended cells on an ECM-coated culture plate (primary bone marrow cells on a plastic culture plate vs. suspended cells on an ECM-coated culture plate, $*p < .05$; suspended cells on an ECM-coated plate vs. suspended cells on a plastic culture plate, $*p < .05$; The graph bar represents mean $\pm$ SD). (e) The suspended cells were bound to the ECM-coated culture plate for colony generation; magnification, 200x. **(C)** The proliferation of BMMSCs (a) and the suspended BMMSCs (b) were cultured on a plastic culture plate after first subculture. (c) There was no significantly different percentage of BrdU-positive cell nuclei relative to the total nucleated cells; $p > .05$. The graph bar shows mean $\pm$ SD. **(D)** (a) Flow cytometry analysis of BMMSCs and suspended BMMSCs. Both groups of cells were positive for the MSC marker CD90, but the cells were negative for the hematopoietic stromal cell marker CD14. (b) Flow cytometry analysis revealed that the suspended BMMSCs showed higher expression of the MSC surface marker than did BMMSCs; $*p < .05$. However, there was no statistically significant difference in the expression of HSC surface markers between BMMSCs and suspended BMMSCs.

Implant Installation and Histomorphometric Analysis

All surgical procedures were performed according to the guidelines, and the details are described in the Appendix. After 4 wk of implant placement, 10 rabbits were sacrificed, and the 40 implants were prepared for histomorphometric analysis.

Statistical Analysis

Student's *t* test, two-way analysis of variance, and a multivariate analysis of variance were utilized, and the details are described in the Appendix.

RESULTS

Characterization of the Suspended BMMSCs by *in vitro* Assay

The suspended cells were bound to the ECM-coated plate (Fig. 1B-e) and formed colonies with fibroblasts, appeared larger, and tended to be more densely packed (Fig. 1B-b). Colony-forming-unit fibroblast (CFU-f) levels were 3- to 4-fold greater when the suspended cells were cultured on ECM-coated plates (Fig. 1B-b) compared with fresh BM mononuclear cells cultured on a plastic plate (Fig. 1B-a) (144 ± 41 vs. 44 ± 5 ; $p < .049$) and suspended cells re-plated on a plastic plate (Fig. 1B-c) (144 ± 41 vs. 34 ± 5 ; $p < .041$). The ECM-coated plate could bind greater numbers of suspended cells and generate a larger colony than the suspended cells on a plastic plate, as suggested by the higher number of CFU-f (Fig. 1B-d). There was no statistically significant difference in increased bromodeoxyuridine (BrdU) uptake rates between BMMSC (Fig. 1C-a) ($62.62\% \pm 6.99\%$) and suspended BMMSC (Fig. 1C-b) ($69.41\% \pm 5.17\%$) subculture on the plastic culture plate (Fig. 1C-c) ($p > .05$). In addition, the cells were positive for the MSC marker CD90 ($48.45\% \pm 0.54\%$, BMMSCs vs. $62.00\% \pm 0.67\%$, suspended BMMSCs), but failed to express the hematopoietic stem cell marker CD14 ($1.05\% \pm 0.08\%$, BMMSCs vs. $1.64\% \pm 0.04\%$, suspended BMMSCs) (Fig. 1D-a). The suspended BMMSCs showed enhanced expression of the MSC surface marker compared with BMMSCs (Fig. 1D-b) ($p < .05$). For differentiation potential, the suspended BMMSCs (Fig. 2A-b) ($53.32\% \pm 3.27\%$) were able to form mineralized nodules as were BMMSCs (Fig. 2A-a) ($61.42\% \pm 12.26\%$) and expressed the osteoblastic marker Runx2 (Fig. 2A-c). The osteogenic differentiation capacity of the suspended BMMSCs was similar to that of BMMSCs (Fig. 2A-d) ($p > .05$). The BMMSCs (Fig. 2B-a) and suspended BMMSCs (Fig. 2B-b) had the capacity to differentiate into Oil-red O-positive lipid droplets and expressed the adipogenic marker LPL (Fig. 2B-c). The adipogenic differentiation capacity of the suspended BMMSCs ($36.77\% \pm 4.89\%$) was similar to that of the BMMSCs ($42.55\% \pm 5.21\%$) (Fig. 2B-d) ($p > .05$). Alcian blue and Safranin-O staining showed that suspended BMMSCs (Figs. 2C-b, 2C-d) were capable of forming cartilage matrix in chondrogenic inductive cultures, as seen in BMMSCs (Figs. 2C-a, 2C-c). The suspended BMMSCs expressed the chondrogenic marker collagen II, as seen in BMMSCs (Fig. 2C-e).

Characterization of the Suspended BMMSCs by *in vivo* Assay

After 8 wk of transplantation, blood vessels were formed and grew into cells in the HA/TCP group. Newly formed bone tissues were seen in the experimental groups. The amounts of bone formation between BMMSCs (Fig. 2D-a) ($31.33\% \pm 3.06\%$) and the suspended BMMSCs (Fig. 2D-b) ($31.66\% \pm 4.16\%$) were not significantly different compared with the total area of the bone ($p > .05$) (Fig. 2D-c).

Biomechanical Analysis of the Suspended BMMSCs: Cell Morphology, Cell Viability, and Cell Proliferation on Titanium Discs

The characteristics of Ti implant surface discs are shown in Figs. 3A and 3B. A smooth configuration with machined marks was observed on untreated machined Ti discs (Figs. 3A-a, 3A-b). Scratch lines over the original surfaces were observed on treatment of anodized Ti discs (Figs. 3A-c, 3A-d). Morphology of the BMMSCs (Figs. 3C-a, 3C-b) and the suspended BMMSCs (Figs. 3C-c, 3C-d) showed more adherence on anodized Ti discs than on the machined Ti discs. The seeded BMMSCs (Figs. 3D-a, 3D-b) and the suspended BMMSCs (Figs. 3D-c, 3D-d) appeared to be healthy, showed spindle-like fibroblastic morphology, and increased in number over time. The percentages of formed formosans were calculated at 1, 4, and 7 days after cell seeding; there was no statistically significant difference in cell proliferation between BMMSCs on the machined and anodized Ti discs and the suspended BMMSCs on the machined Ti discs by time (Fig. 3E) ($p > .05$). There was greater proliferation of the suspended BMMSCs on the anodized Ti discs than the other groups at 7 days ($p < .05$). The total proteins produced by the cells on the discs were measured. As shown in Fig. 3F, there were no statistically significant results in all groups at 4 days ($p > .05$). The ALP activity of the BMMSCs and the suspended BMMSCs on the anodized Ti discs was higher than that of the cells on the machined Ti discs at 7 and 14 days ($p < .05$); however, there were no statistically significant results between BMMSCs and the suspended BMMSC groups ($p > .05$).

Capacity of the Suspended BMMSCs to Regenerate Bone in Double-canaled Implants: Histologic Findings and Histomorphometric Results and Analysis

The upper canals of the implants were situated in the cortical bone area in all sections, and the lower canals were placed in the marrow regions. Both the upper and lower canals demonstrated generalized bone growth, with more bone in the upper canals in all groups (Fig. 4C). The upper canals of defect-only groups (Figs. 4D-a, 4E-a) demonstrated newly formed bone growing into the canals from the outside. At higher magnification (Fig. 4F-a), the bone was located mainly in the lateral sides of the upper canals. In the lower canals of the defect-only groups (Figs. 4D-e, 4E-e), mostly fatty marrow spaces were observed. At higher magnification (Fig. 4F-e), only a few instances of newly formed bone could be detected. In the upper canals of the HA/TCP groups (Figs.

Figure 2. Characterization of the suspended BMMSCs. **(A)** (a) BMMSCs and (b) suspended BMMSCs formed nodule-like structures stained by Alizarin red S and showed up-regulation of (c) Runx2. The inserted image presents an expanded view of mineralized nodules. (d) BMMSCs and suspended BMMSCs possessed similar potential to differentiate into osteocytes after induction ($p > .05$). **(B)** In adipogenic culture, (a) BMMSCs and (b) suspended BMMSCs formed small lipid droplets and turned red upon Oil red O staining, and (c) lipoprotein lipase (LPL) genes were found to be expressed. (d) Oil red O staining presented similar adipogenic differentiation capacity in BMMSCs and suspended BMMSCs ($p > .05$). **(C)** (a, c) BMMSCs and (b, d) suspended BMMSCs were able to differentiate into chondrocytes as assessed by Alcian blue and Safranin-O staining for acidic sulfate mucosubstances. RT-PCR analysis indicated that cartilage-specific genes, including (e) collagen II, were largely expressed in chondrogenic cultures. **(D)** After 8 wk of subcutaneous transplantation of BMMSCs mixed with HA/TCP into immunocompromised mice, newly formed bone tissue was found in both BMMSCs (a) mixed with HA/TCP and suspended BMMSCs (b) mixed with HA/TCP. The graph bar represents mean $\pm$ SD. B, BMMSC; S, suspended BMMSC; U, undifferentiation.

Figure 3. Biomechanical properties analysis of the suspended BMMSCs. **(A)** SEM micrographs of the titanium discs. (a, b) Machined titanium implant surface discs. (c, d) Anodized titanium implant surface discs. Magnifications 500x (left column) and 1,000x (right column). **(B)** The surface characteristics of the examined area. (a) Machined control (no treatment). (b) Anodized treatment. **(C)** SEM micrographs of cells cultured on the titanium implant surface discs. BMMSCs seeded on machined titanium implant surface discs (a, b). Suspended BMMSCs seeded on machined titanium implant surface discs (c, d). **(D)** BMMSCs cultured on anodized titanium implant surface discs (a, b). Suspended BMMSCs cultured on anodized titanium implant surface discs (c, d). Magnifications 500x (left column) and 1,000x (right column). **(E)** MTT assays measuring cell proliferation on titanium implant surface discs. The proliferation of the suspended BMMSCs on the anodized Ti discs significantly enhanced at 7 days, $*p < .05$. **(F)** Alkaline phosphatase (ALP) activity of cultured BMMSCs and the suspended BMMSCs on titanium implant surface discs. Both groups of cells cultured on the anodized Ti discs showed increased ALP activity compared with the cells cultured on machined Ti discs at 7 and 14 days, $*p < .05$.

4D-b, 4E-b), the HA/TCP particles were surrounded by newly formed bone, and at higher magnification (Fig. 4F-b), the HA/TCP granules were seen to be sharp-edged and relatively dark-colored. Mature bone and connective tissue encapsulation appeared around the HA/TCP, and gaps were present at the bone particle interfaces. A few instances of mature bone could be observed in the canals from outside the canals. However, fibrovascular tissue invaded the center of the canal. In the lower canals

of the HA/TCP groups (Figs. 4D-f, 4E-f), a small amount of newly formed bone was observed, and at higher magnification (Fig. 4F-f), fibrous tissue appeared, with less bone to place around the HA/TCP particle surfaces and less invasion of fibrovascular tissue. In the upper canals of the BMMSCs (Figs. 4D-c, 4E-c) and the suspended BMMSC groups (Figs. 4D-d, 4E-d), mature bone could be detected in the central parts of the canals, with a little fibrovascular tissue, and at higher magnification (Figs. 4F-c, 4F-

d), individual HA/TCP particles were still clearly identifiable. The mineralized matrix of the regenerated bone consisted of lamellar bone together with numerous rounded osteocytes, which were very well-organized. HA/TCP particles were fully embedded and came into direct contact with the newly formed bone. There was no fibrous encapsulation around the HA/TCP. In the lower canals of the BMMSC (Figs. 4D-g, 4E-f) and the suspended BMMSC groups (Figs. 4D-h, 4E-h), there were also few quantities of mature bone; moreover, immature bone formation with fibrovascular tissues could be seen at higher magnification (Figs. 4F-g, 4F-h). The newly formed bone in the upper and lower canals of the implants was calculated (Fig. 4G). In all groups, new bone formation of the BMMSC and the suspended BMMSC groups exhibited higher percentages than in the HA/TCP and defect-only groups ($p < .05$). However, there were no significant differences between the BMMSC group ($28.34\% \pm 2.14\%$) and the suspended BMMSC group ($27.72\% \pm 4.62\%$) in the upper canal ($p > .05$), followed by the HA/TCP group ($17.11\% \pm 3.12\%$) and the defect-only group ($12.24\% \pm 2.04\%$). The new bone inside the lower canals showed significantly more new bone formation in the BMMSC group ($9.93\% \pm 1.42\%$) and the suspended BMMSC group ($8.97\% \pm 1.72\%$) than in the HA/TCP group ($5.04\% \pm 1.53\%$) and the defect-only group ($5.21\% \pm 1.45\%$) at 4 wk ($p < .05$); however, there was also no difference between the BMMSC and the suspended BMMSC groups in the lower canal (Fig. 4G). The detailed percentages of NB (new bone), GM (graft material), and MS (marrow space) are described in Appendix Table 2.

DISCUSSION

It has been well-recognized, from a cell-level study, that the new subset of mouse suspension BMMSCs has characteristics analogous to those of BMMSCs *in vitro* (Akiyama *et al.*, 2012). For

Figure 4. Bone regeneration of suspended BMMSCs in a double-canaled implant. **(A)** The image of a threaded titanium double-canaled implant. **(B)** The upper and lower implant canals were filled with: (a) defect only, (b) HA/TCP, (c) BMMSCs mixed with HA/TCP, or (d) suspended BMMSCs mixed with HA/TCP. **(C)** Histologic views of the threaded titanium double-canaled implant in the rabbit tibia for 4 wk (toluidine blue; magnification 12.5x). (a) Defect only. (b) HA/TCP. (c) BMMSCs mixed with HA/TCP. (d) Suspended BMMSCs mixed with HA/TCP. **(D)** Histological views of canals after 4 wk. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixed with HA/TCP, (d, h) suspended BMMSCs mixed with HA/TCP. Rows, top to bottom: upper canal, 40x; lower canal, 40x. **(E)** Histological views of canals after 4 wk. Columns, left to right: (a, e) defect only, (b, f) HA/TCP, (c, g) BMMSCs mixed with HA/TCP, (d, h) suspended BMMSCs mixed with HA/TCP. Rows, top to bottom: upper canal, 100x; lower canal 100x (toluidine blue staining). **(F)** High-power histological views inside the upper canal after 4 wk, magnification 200x. (a, e) Defect only, (b, f) HA/TCP, (c, g) BMMSCs mixed with HA/TCP, (d, h) suspended BMMSCs mixed with HA/TCP. New bone (NB), graft material (GM), and marrow space (MS) were observed, magnification 200x. **(G)** New bone formation inside the upper and lower implant canals implanted into the rabbit for 4 wk (the graph bar represents means $\pm$ SD).

clinical application of this new subset, it is essential to identify the effectiveness of the suspended BMMSCs in the secondary animal for additional utilization in human studies. In this study, we obtained new BMMSCs from rabbit tibia to compare the characteristics with those of BMMSCs *in vitro* and designed the novel double-canaled dental implant for useful application of the MSCs and quantification of bone regeneration in the cortical and marrow areas of the rabbit.

In this study, we cultured the suspended cells on BMMSC-derived natural ECM-coated plates. Our results showed that the suspended BMMSCs appeared to adhere and generated CFU-f and subsequently proliferated on plastic plates. To date, the CFU-f assay has been considered to be an effective way to investigate the characterization of BMMSCs. It is well-recognized that MSCs lose their ability to self-renew as well as their multipotentiality upon long-time culture on plastic plates (Izadpanah *et al.*, 2008). We showed that the suspended cells cultured on ECM-coated plates exhibited much higher CFU-f numbers compared with those of BM primary mononuclear cells or suspended cells cultured on plastic plates. The BMMSC-derived ECM-coated plate was effective in obtaining suspended cells. Similar results were also observed by other authors, who showed that BM-cell-derived ECM facilitated the expansion of BMMSCs (Chen *et al.*, 2007). Previous studies have reported that the ECM was a significant component of the cellular niche to sustain cellular function and was produced by BM stromal cells consisting of collagen types I and III, fibronectin, small leucine-rich proteoglycans, and major components of basement membrane (Yamaguchi *et al.*, 1990; Chen *et al.*, 2004; Ahmed *et al.*, 2005; Philp *et al.*, 2005). Therefore, we could speculate that the growth factors or cytokines that were produced by the suspended BMMSCs may bind to the specific adherence cytokines produced by ECM. In this study, the suspended BMMSCs were similar to the BMMSCs with respect to differentiation and their expression of CD90 and lack of expression of CD14, implying that the suspended BMMSCs might also be derived from a population of MSCs. In addition, the suspended BMMSCs promoted bone formation *in vivo*. After 8 wk of transplantation, new bone and many blood vessels were seen in groups of cells loaded with HA/TCP, indicating that the cells might produce vascular factors and improve vascularization of the bone formation, and that the suspended BMMSCs have osteogenic potential similar to that of BMMSCs *in vivo*. The results may imply that the loaded BMMSCs and the suspended BMMSCs might have a participatory role in the regeneration of new bone.

In general, osteoblast cells adhered closely to the biointeractivity of replacement bone on the implant material surface (Park *et al.*, 2011). In this study, to confirm the biomechanics of cells loaded onto implant surfaces, we cultured the suspended BMMSCs on machined and anodized discs and evaluated cell morphology and differentiation abilities. The suspended BMMSCs spread and firmly attached to the machined and anodized discs. The MTT assay and ALP activity assay demonstrated that both the BMMSCs and the suspended BMMSCs seeded on machined and anodized discs possessed higher cell proliferation numbers and differentiation potential. The results proposed that the suspended BMMSCs cultured on machined and anodized implant

surfaces were capable of differentiating into osteoblastic lineage, which promoted the healing process of osseointegration.

In this study, this double-canaled implant seemed appropriate for the evaluation of bone regeneration in the cortical and cancellous areas in the rabbit. A previous study reported that bone formation in the rabbit was noticeably higher in cortical bone than in marrow sites. In the same designed double-canaled implant model, only 17.11% and 5.03% bone areas were reported in the upper and lower canals, respectively (Lee *et al.*, 2011). In the current study, the regenerated bone areas in the HA/TCP group were about 17% in the upper canal and 5% in the lower canal. Our results exhibited that the transplantation of BMMSCs and the suspended BMMSCs loaded with HA/TCP into double-canaled implants was more effective for bone regeneration compared with pure HA/TCP and defect-only in the cortical and marrow spaces of the tibia. This observation implied that the osteogenic potential of BMMSCs and suspended BMMSCs remained at the graft area and participated in bone regeneration. These findings suggest that suspended BMMSC-based bone regeneration was effective. Additional studies should be carried out for further evaluation of the mechanisms of carrier-loaded living cells.

Taken together, the present findings suggest that suspended BMMSCs, which are usually discarded, have excellent support in terms of potential for bone regeneration in the rabbit. In the future, the bone newly formed from the suspended BMMSCs may offer an excellent environment for osseointegration of dental implants and bone regeneration.

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REFERENCES

- Abukawa H, Zhang W, Young CS, Asrican R, Vacanti JP, Kaban LB, *et al.* (2009). Reconstructing mandibular defects using autologous tissue-engineered tooth and bone constructs. *J Oral Maxillofac Surg* 67:335-347.
- Ahmed N, Riley C, Rice G, Quinn M (2005). Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment. *Clin Exp Metastasis* 22:391-402.
- Akiyama K, You YO, Yamaza T, Chen C, Tang L, Jin Y, *et al.* (2012). Characterization of bone marrow derived mesenchymal stem cells in suspension. *Stem Cell Res Ther* 3:40.
- Banerjee M, Bhande RR (2007). Autologous bone marrow transplantation/mobilization: a potential regenerative medicine for systemic degenerative disorders and healthy living. *Med Hypotheses* 68:1247-1251.
- Brooke G, Cook M, Blair C, Han R, Heazlewood C, Jones B, *et al.* (2007). Therapeutic applications of mesenchymal stromal cells. *Semin Cell Dev Biol* 18:846-858.
- Buser D, Dula K, Hess D, Hirt HP, Belser UC (1999). Localized ridge augmentation with autografts and barrier membranes. *Periodontol* 2000 19:151-163.
- Chen XD, Fisher LW, Robey PG, Young MF (2004). The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *FASEB J* 18:948-958.

- Chen XD, Dusevich V, Feng JQ, Manolagas SC, Jilka RL (2007). Extracellular matrix made by bone marrow cells facilitates expansion of marrow-derived mesenchymal progenitor cells and prevents their differentiation into osteoblasts. *J Bone Miner Res* 22:1943-1956.
- Ciapetti G, Ambrosio L, Marletta G, Baldini N, Giunti A (2006). Human bone marrow stromal cells: in vitro expansion and differentiation for bone engineering. *Biomaterials* 27:6150-6160.
- Dennis JE, Merriam A, Awadallah A, Yoo JU, Johnstone B, Caplan AI (1999). A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. *J Bone Miner Res* 14:700-709.
- Ferrari G, Angelis D, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, *et al.* (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528-1530.
- Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J, *et al.* (2008). Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 68:4229-4238.
- Kassem M (2006). Stem cells: potential therapy for age-related diseases. *Ann NY Acad Sci* 1067:436-442.
- Lee JE, Heo SJ, Koak JY, Kim SK, Han CH (2011). Bone regeneration with rabbit bone marrow-derived mesenchymal stem cells and bone graft materials. *Int J Oral Maxillofac Implants* 27:1389-1399.
- Long MW, Williams JL, Mann KG (1990). Expression of human bone-related proteins in the hematopoietic microenvironment. *J Clin Invest* 86:1387.
- Noshi T, Yoshikawa T, Dohi Y, Ikeuchi M, Horiuchi K, Ichijima K, *et al.* (2001). Recombinant human bone morphogenetic protein-2 potentiates the in vivo osteogenic ability of marrow/hydroxyapatite composites. *Artif Organs* 25:201-208.
- Park KH, Koak JY, Kim SK, Heo SJ (2011). Wettability and cellular response of UV light irradiated anodized titanium surface. *J Adv Prosthodont* 3:63-68.
- Petite H, Viateau V, Bensaid W, Meunier A, De Pollak C, Bourguignon M, *et al.* (2000). Tissue-engineered bone regeneration. *Nat Biotechnol* 18:959-963.
- Philp D, Chen SS, Fitzgerald W, Orenstein J, Margolis L, Kleinman HK (2005). Complex extracellular matrices promote tissue-specific stem cell differentiation. *Stem Cells* 23:288-296.
- Prewitz MC, Seib FP, von Bonin M, Friedrichs J, Stißel A, Niehage C, *et al.* (2013). Tightly anchored tissue-mimetic matrices as instructive stem cell microenvironments. *Nat Methods* 10:788-794.
- Prockop DJ (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276:71-74.
- Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM (2003). Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* 121:368-374.
- Włodarski K, Galus R, Włodarski P (2004). Non-adherent bone marrow cells are a rich source of cells forming bone in vivo. *Folia Biol (Praha)* 50:167-173.
- Yamaguchi Y, Mann DM, Ruoslahti E (1990). Negative regulation of transforming growth factor- β by the proteoglycan decorin. *Nature* 346:281-284.
- Zhang Z, Tong J, Lu R, Scutt A, Goltzman D, Miao D (2008). Therapeutic potential of non-adherent BM-derived mesenchymal stem cells in tissue regeneration. *Bone Marrow Transplant* 43:69-81.