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Injectable Bone Tissue Engineering Using Expanded Mesenchymal Stem Cells

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Abstract

Patients suffering from bone defects are often treated with autologous bone transplants, but this therapy can cause many complications. New approaches are therefore needed to improve treatment for bone defects, and stem cell therapy presents an exciting alternative approach. Although extensive evidence from basic studies using stem cells has been reported, very few clinical applications using stem cells for bone tissue engineering have been developed. We investigated whether injectable tissue-engineered bone composed of mesenchymal stem cells (MSCs) and platelet rich plasma was able to regenerate functional bone in alveolar deficiencies. We performed these studies in animals and subsequently carried out pilot trial cases in patients with long-term follow up; these showed good bone formation using minimally invasive MSC transplantation. All patients exhibited significantly improved bone volume with no side effects. Newly formed bone areas at 3 months was significantly higher than the pre-operation baseline (P <0.001) and reached levels equivalent to that of native bone. No significant bone resorption occurred during long term follow-up. Injectable tissue-engineered bone restored masticatory function in patients. This novel clinical approach represents an effective therapeutic utilization of bone tissue engineering.

Keywords

tissue engineering; regenerative medicine; bone; cell transplantation; clinical application

INTRODUCTION

Mastication and ingestion are critical for health and survival. Following tooth loss, alveolar bone resorption occurs in approximately 40-60% of cases within 3 years, and mastication in people wearing complete dentures is reduced to less than 20% compared to those with a natural dentition [1, 2]. In addition, occlusal or masticatory power decreases in patients with bone defects resulting from trauma, tumor, infections, periodontitis, or abnormal skeletal development. Consequently, bone tissue regeneration represents an important challenge for oral-maxillofacial surgeons, dentists, and orthopaedic and plastic surgeons. Autologous bone grafting has been frequently used for bone reconstruction because a patient's own bone lacks immunogenicity and provides bone-forming cells to the implant site directly. Although there are many advantages to the use of autologous bone, there are also major drawbacks to harvesting from a healthy bone, including post-operative pain, infection, hypersensitivity, paresthesia, and time constraints [3-8]. Additionally, autologous bone is occasionally not suitable for reconstruction because of poor quality or difficulty in shaping the graft bone, and complications affect 10–30% of patients undergoing autologous bone transplants [5].

In order to overcome these problems, alternative approaches have been attempted for bone regeneration. One of these is grafting of allogeneic bone from human cadavers, which can be obtained from tissue banks. The immunogenic potential of these allografts and risks of virus transmission to the recipient are serious disadvantages [6]. Processes such as

irradiation and freeze drying are used to decrease risks, but these procedures also eliminate the cellular component, resulting in reduced osteoinductivity [8]. Moreover, allogeneic bone has decreased revascularization and a higher resorption rate [2], resulting in a lower rate of new bone tissue formation as compared to autologous bone [8, 9]. Another alternative method is synthetic prostheses such as hydroxyapatite, beta-Tricalcium phosphate, and calcium phosphate cements. However, these approaches suffer from increased susceptibility to infection because of extrusion and an uncertain long-term interaction with the host's physiology, and the degree of osteogenic and osteoinductive properties are less than osteoprogenitor cells [8].

To overcome the drawbacks of bone graft materials, tissue engineering using stem cells has been suggested as a promising technique for reconstructing bone defects. To date, translational research using stem cells has reached only a few areas in which there has been long-standing insight into stem cell biology [10]. Furthermore, the maintenance of stem cell properties and the fate of biomaterials after transplantation has been and still is the subject of intensive research in the field. We developed an approach to bone regeneration using injectable tissue-engineered bone precursors (TEB) that was composed of cultured bone marrow-derived mesenchymal stem cells (BMMSCs) and platelet rich plasma (PRP) with good plasticity. TEB was used in patients in 104 clinical cases. Here, we demonstrate that BMMSCs can engraft in humans and generate donor-derived osteoblasts to contribute to bone regeneration, which resulted in the improvement of masticatory function.

MATERIALS AND METHODS

Animal Studies

The protocols and guidelines for this study were approved by the Institutional Animal Care Committee and the University Committee of Nagoya University. In canine guided bone regeneration (GBR) models, surgical procedure, preparation of grafting materials (BMMSCs, autogenous bone and PRP), and the retroviral vector with GFP that was used to label BMMSCs were performed according to previously described methods [11, 12]. In periodontitis models, experimental periodontitis was induced and TEB was injected into the bone defect. Details are presented in the Supplementary Appendix.

Clinical Studies

Participants—104 cases aged between 19 and 78 years (mean age: 57.7 years) were enrolled in this study. This study was approved by the Ethics Committee of Nagoya University (permission number 172). Verbal and written informed consent was obtained from the patients. After routine oral and physical examinations, patients who were healthy were selected. Patients with conventional problems of masticatory function because of severe alveolar ridge atrophy were eligible for inclusion (the treatment schema is shown in Figure 2A).

TEB preparation and surgical procedures—BMMSCs were isolated from the patient's iliac crest bone marrow aspirate and TEB was prepared using previously described techniques [13]. The mixture of BMMSCs and PRP solution was combined with human

thrombin (5000 units) that was dissolved in 10% calcium chloride. After the contents appeared gel-like, they were injected to the following applied operation sites. (1) GBR cases; TEB was transplanted to the bone resorption area (Supporting Information video 1). The grafted area was covered with a non-resorbable expanded polytetrafluoroethylene (ePTFE) membrane (Gore-Tex membrane, W.L. Gore and Associates, Newark, DE) in order to protect against mucosal flap compression. (2) Sinus floor elevation (SFE) cases; following traditional SFE procedure, TEB was injected into the sinus cavity (Supporting Information video 2). (3) Socket preservation cases; tooth extraction was performed using a careful traumatic technique. The socket was curetted to remove residual pathology and granulation tissue and filled with TEB. Following TEB transplantation, the membrane (Gore-Tex membrane) was used to cover the grafted area. (4) Periodontitis cases; periodontal surgery consisted of a traditional open-flap procedure and TEB transplantation. Buccal and lingual full-thickness flaps were elevated to expose the underlying bone and the roots of the involved teeth. TEB was injected into the bone defect adjacent to the root surface after meticulous debridement to remove bacterial deposits and inflamed tissues, and the flaps were replaced. The patients received antibiotics along with analgesics as needed.

Statistical Analysis

All statistical analyses were done with SPSS. We compared newly formed bone areas, cell viability, CT value with the one-way ANOVA and post-hoc least significant difference tests, and periodontal index with paired t-test. A p value of less than 0.05 was taken to be significant.

RESULTS

Transplantation of BMMSCs using a Canine in vivo Model

After transplantation into experimental bone defects in the canine model, good bone formation was found in TEB transplanted groups, and newly formed bone areas of the TEB and autogenous bone graft (ABG) groups was higher at all time points compared with control or PRP (Fig. 1A). Fluorescence microscopy showed that GFP-expressing cells were present within the transplanted area at 2, 4 and 8 weeks after transplantation, indicating that transplanted BMMSCs differentiated into osteoblasts and osteocytes and participated in bone regeneration (Fig. 1B-E). Analysis of fractured surfaces by SEM showed mineralized lamellar bone structures in the TEB and ABG groups at 2 weeks after transplantation (Fig. 1J, L). The amount of mineralized nodules appeared to increase in the TEB group over time. On the other hand, dense mineralized extracellular matrix and bone formation were rarely observed in the control and PRP groups (Fig. 1F-I). An energy dispersive X-ray spectrometer (EDS) was used to evaluate the elemental composition in regenerated tissues. In EDS mapping, calcium (Ca) and phosphate (P) (pixels highlighted in blue) were detected in mineralized areas (Supporting Information Fig.1) and Ca coverage and the Ca/P ratio were greater for TEB specimens than for control, PRP, or ABG specimens.

In the periodontitis model, the TEB technique was compared with Guided Tissue Regeneration (GTR), which is one of the most popular surgical procedures for periodontal regeneration. Histological observations showed not only formation of new alveolar bone and

inhibition of epithelial down growth, but also formation of a new cellular cementum attached to the underlying dentin and periodontal ligament with collagen fibers inserting into the cementum. The cementum induced by GTR was thin and composed of only acellular layers (Fig. 1O), whereas that by TEB was thick and composed of cellular and acellular layers (Fig. 1P), which correspond to the natural structure (Fig. 1N). Visualization of GFP-expressing cells indicated that these were present in areas with periodontal regeneration, and the cells participated in cementum regeneration (Fig. 1Q, R).

Characterization of TEB in a Clinical Application

In order to characterize BMMSCs used for TEB, we performed flow cytometry analysis using mesenchymal lineage markers (CD13, CD29, CD44, CD73, and CD105), a monocytic marker (CD14), an endothelial cell marker (CD31), and a hematopoietic lineage marker (CD45). The BMMSCs used for TEB were positive for mesenchymal stem cell markers and negative for hematopoietic lineage and monocytic markers (Supporting Information Fig. 2A). The expression levels of osteogenic markers (ALP and Runx2) in BMMSCs were up-regulated by osteoinduction (Supporting Information Fig.2B, C). *In vitro* differentiation and mineralization potential of BMMSCs was tested by alizarin red staining and von Kossa staining (Supporting Information Fig. 2E, F). No mycoplasma infection or karyotypic abnormalities were detected in cultured BMMSCs, and no tumorigenesis was found in the TEB groups (Supporting Information Fig. 3 and 4).

The cell viability of BMMSCs encapsulated in PRP gel was examined at 1, 3 and 7 days. There were no statistically significant differences in the percentage of surviving cells at these time points (Fig.2B-F). Next, SEM images were used to examine the microstructure of TEB. In the PRP gel, spherical BMMSCs had a breadcrumb-like appearance, comprising randomly arranged fibrillar elements (fibrin) and platelet cells [14] (Fig. 2G-J'). At day 3 and day 7, the spherical cells appeared to have "foot-like" cell projections extending along the PRP fiber surface. These results indicated that BMMSCs survived in and adapted to the PRP gel.

Analysis of Clinical Biopsy Specimens

Before implant placement or re-entry procedures, biopsies using a trephine burr were performed from part of the augmented area. The regenerated tissue showed hardness similar to native bone (Supporting Information video 3). The histological examination of TEB clinical biopsy samples showed new bone formation with a lamellar pattern, well-differentiated marrow cavity, and abundant vascularization (Fig. 2M). This structure resembled that of NB compared with ABG. Immunohistochemical staining revealed positive staining of osteocalcin within newly formed mineralized tissue in TEB and NB sections (Fig. 2N-S). Histomorphometric examination indicated that the newly formed bone areas of TEB was similar compared with NB control (Fig. 2T). There were no significant differences among the TEB and NB. On the other hand, ABG was significantly less dense than NB ($P=0.0021$).

Clinical Results of TEB Transplantation

TEB transplantation was applied in 104 cases that required bone regeneration (Table 1 and 2) comprising: guided bone regeneration (GBR); 36 cases (Fig. 3A-H), sinus floor elevation (SFE); 39 cases (Fig. 3I-P), socket preservation; 12 cases (Fig. 3Q-X), and periodontal regeneration; 17 cases (Fig. 3Y-HH). The GBR, SFE, socket preservation technique using TEB was used for the regeneration of osseous defects. Radiographs clearly showed that the bone defect was filled with newly generated bone after TEB injection, and little resorption occurred during the follow-up period (Fig. 3E-G, M-O, V-X). Histological observations of biopsy specimens indicated that newly formed tissue underwent good bone formation (Fig. 3H, P). The mean densitometric results (computed tomography; CT value) of regenerated bone by TEB (GBR; 309.1, 381.0, SFE; 354.3, 455.4 at 3, 6 months, socket preservation; 388.0 Hounsfield unit (HU) at 3 months, respectively) was higher than the pre-operation baseline ($p < 0.001$) (Table 1). A statistically significant difference in bone density was found between the baseline and all time points after operation ($p < 0.001$). The evaluation was equivalent to that of native bone as a control at 6 months in GBR and SFE, and at 3 months in socket preservation. No significant decrease was found up to 48 months in GBR, 60 months in SFE during the long follow-up period. Moreover, all dental implants placed in the regenerated region were functional and the success rate was 100%.

Periodontal treatment was associated with improvement in clinical variables by TEB application (Fig. 3Y-HH). To determine the degree of periodontal disease, probing depth, clinical attachment level, and bone gain were measured. The measurement of periodontal probing depth and clinical attachment level has played an integral part in the periodontal examination and the detection of periodontal diseases.Its use not only enables treatment to be planned appropriately, but also facilitates longitudinal monitoring, so that the response to treatment may be assessed and sites of possible disease progression dentified [15]. The average reduction in probing depth, gain in the clinical attachment level, and bone gain was 5.12, 4.29, and 3.12 mm, respectively (Table 2). The periodontal probing depth, clinical attachment level, and linear bone were significant improved compared with baseline levels ($p < 0.001$). Bone formation was confirmed by radiographic observation, which clearly showed that the bone around the tooth had regenerated and little resorption was observed during the follow-up period (Fig. 3DD-HH).

DISCUSSION

Alveolar ridge deficiency influences not only quality of life but also general health. Masticatory ability may affect dietary choices and nutritional intake and have consequences for overall health [16]. Therefore, the development of procedures to regenerate oral bone is desirable. Although autologous bone grafts are the method of choice for bone repair and regeneration [4], there are weaknesses to the harvesting procedure [17]. In addition, to overcome the faults of current bone graft materials such as allografts and synthetic prostheses, bone regeneration with cell therapy using tissue engineering provides a promising technique with minimal invasiveness.

In previous reports with small sample sizes [11, 13, 18], we demonstrated the potential ability of bone regeneration, and here, we provide a preclinical animal (Fig. 1) and *in vitro*

study together with the first large-scale and long-term clinical study. The *in vivo* differentiation capacity of BMMSCs has been assessed primarily by transplantation of cultured BMMSCs subcutaneously into the dorsal surface of immunodeficient mice in combination with osteoconductive composites of hydroxyapatite/tricalcium phosphate carrier particles [19]. Subsequent studies have demonstrated the bone-regenerating capacity of *in vitro* expanded and *in situ* implanted BMMSCs in several animal models of critical segmental bone defects [20]. These results have led to the approval of clinical trials for the implantation of human BMMSC-matrix composites for the treatment of large bone defects in humans [21]. However, little is known about the function of the BMMSCs in clinical use. In this study, one principal finding was that the transplanted cells contributed to the bone formation process (the lining osteoblasts, osteocytes, and mature bone formation) *in vivo* using GFP-expressing BMMSC transplantation. The results also indicated the direct participation of BMMSCs in osteogenesis, and the cells underwent gradual differentiation toward an osteoblastic lineage and contributed to the improvement of biomechanical properties *in vivo* (Fig. 1A-M and Supporting information Fig.1). These results are consistent with previous studies that indicated that BMMSCs can be directed towards osteogenic differentiation [20, 22, 23]. On the other hand, bone formation with PRP matrix alone without cells provided some improvement, but less than the matrix containing BMMSCs. Therefore, we concluded that the therapeutic BMMSCs indeed functioned as osteoblastogenic stem cells.

Since PRP contains cytokines and proteins carried within platelets [24], it likely provides an osteoconductive milieu for the cells to undergo accelerated differentiation and matrix production and enhanced bone formation. In addition, the other advantage of using PRP gel is that it is easy to manipulate in a coagulated form that can be applied for complicated bone defects (Supporting Information video. 1, 2). However, it must be applied soon after preparation to maintain growth factor activity. The life span of platelets and the direct influence period of growth factors were less than 5 days [25]. Cell survival is the most important requirement for achieving clinical success in cell-based bone regeneration (Fig. 2B-J'). TEB provided a favorable biological environment for the implanted cells, leading to good bone formation. These results were consistent with current applications for BMMSCs in tissue engineering of musculoskeletal tissue, which requires the use of scaffolding material for cell attachment and matrix deposition [26].

Until recently, clinical bone tissue engineering has not been a success [6, 8, 27]. The major concern in bone regeneration is resorption of the graft, because it can lead to insufficient bone volume and quality, which imply failure of the operation. Previous studies of autogenous grafts reported a high level of reduction in grafted bone after bone reconstruction, corresponding to 36–44% after 1–5 years [28], and the bone volume reduction of the transplants, which were evaluated using CT scans, was 47.5% within 6 months [29]. Our results also showed that grafted autogenous bone had less newly formed bone areas than native bone (NB) (Fig. 2T). However, TEB was comparable to NB in newly formed bone areas. These results indicated that bone quality of TEB was better than autogenous bone graft, and TEB transplantation resulted in bone regeneration that was equal to NB. Currently, the progress of bone formation is assessed mainly on the basis of

radiographic changes and CT scans, which offer the best radiological method for morphological and qualitative analysis of bone in the grafted region [30, 31] and facilitate the evaluation of bone density in HU [30]. A report for tissue engineered bone using periosteum cells seeded on polyglycolid–polylactid (PLGA) scaffolds showed that resorption rate was 90%, and sufficient mineralization was found in only one case (152 HU) out of 14 cases at 3 months [32]. Another study showed that bone density also significantly decreased in the first 3 months after grafting [30]. On the other hand, CT scans in this study showed that the mean bone density at 3 months after TEB transplantation was significantly higher than the pre-operation baseline ($p < 0.001$) (Table 1), and the bone density was equivalent to that of native bone. This result was supported by clinical and histological observations (Fig. 2M, P, S, T, and Fig. 3). We did not identify any decrease in bone density, and the density of the grafts remained at nearly the same level for 4 years and 5 years after GBR and SFE, respectively (Table 1). No failures of dental implants were found during the follow-up period. The survival rate of dental implants placed in autogenous bone grafted maxillary sinus was 88.9% [33], which was lower than our survival rate (100%). These results may involve bone deposition by both endogenous and donor cells or the paracrine actions of donor cells.

Periodontitis affects more than 20% of adults, is a major cause of tooth loss, and is associated with systemic disorders such as diabetes mellitus and cardiovascular disease [34, 35]. Previous studies of periodontal surgery have shown that gains in clinical attachment level of 0.2 to 1.5 mm, reductions in probing depth of 1.5 to 2.7 mm, and linear bone gains 0.3 to 1.1 mm can be expected 1 year after traditional periodontal surgery [36]. In our study, patients who received TEB treatment had mean gains in clinical attachment level of 4.29 mm, mean reductions in probing depth of 5.12 mm and bone gain of 3.12 mm at 1 year after treatment. These parameters were significant improvements compared with baseline levels ($p < 0.001$) (Table 2). Periodontal treatment by TEB led to significantly better clinical outcomes after surgery (Fig. 3Y-HH) and may have a positive effect on regenerating true periodontal tissue regeneration (Fig. 1P) with long-lasting effects.

A previous study that applied MSCs to distraction osteogenesis of the long bones also reported that the rate of complications was significantly lower in the MSC transplantation group as compared to the control group (without cell therapy) [37]. In addition, case reports using MSCs for the treatment of patients with conditions such as bone tumors, osteoarthritis, and spinal cord injury stated that no adverse reactions were apparent during the postoperative period [38-40]. These studies were consistent with our clinical results showing that none of the patients had secondary clinical side-effects. Taken together, regenerative bone therapies using MSC transplantation are highly effective and reduce associated complications by accelerating new bone formation and maintaining good functional quality.

CONCLUSION

We conclude that engrafted BMMSCs can be safely and effectively used as therapeutic agents after cell transplantation for long-lasting improvement. These improvements in bone structure and function likely reflect the activity of stem cells, and the regenerated bone mimics natural bone and maintains function. The therapeutic activity in engraftment of

MSCs in patients with bone defects indicates that TEB transplantation may also be feasible in other disorders, such as spinal fusion, augmentation of fracture healing, and the reconstruction of various bone defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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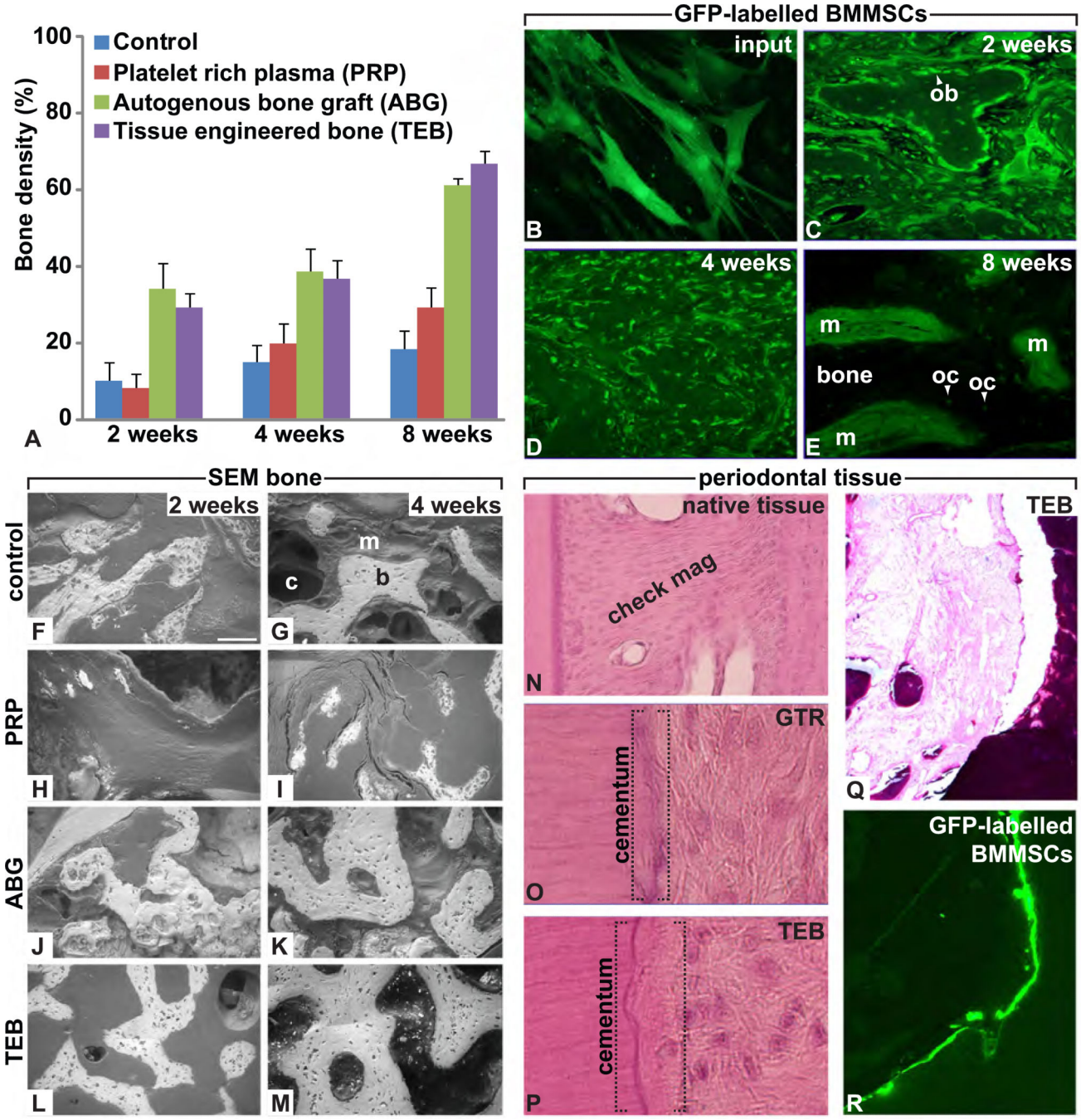

Figure 1. Bone regeneration using TEB in canine bone defect models

(A) The mean newly formed bone areas of TEB, ABG, and PRP implanted groups and no implant controls at 2, 4 and 8 weeks post-transplantation. Data shown in the bar graph are the means ± s.d. (B) GFP-expressing BMMSCs were created using a retroviral construct in order to trace the distribution of transplanted TEB. (C-E) GFP-expressing BMMSCs (green) were identified in the grafted area at 2, 4 and 8 weeks after transplantation and osteoblasts (ob) lined up beside the regenerated bone, osteocytes (oc) within it and marrow (m) were positive for GFP (magnification x 200). (F-M) SEM evaluations of control, PRP, ABG and

TEB implants were recorded at 2 and 4 weeks after transplantation. Increased bone formation (b) was observed in the ABG and TEB groups compared to control and PRP groups. (N-R) The effect of TEB transplantation in periodontitis models and representative histological images of the native tissue (N), GTR group (O), TEB transplantation group (P). (Q, R) GFP-expressing BMMSCs were detected within the regenerated tissue.

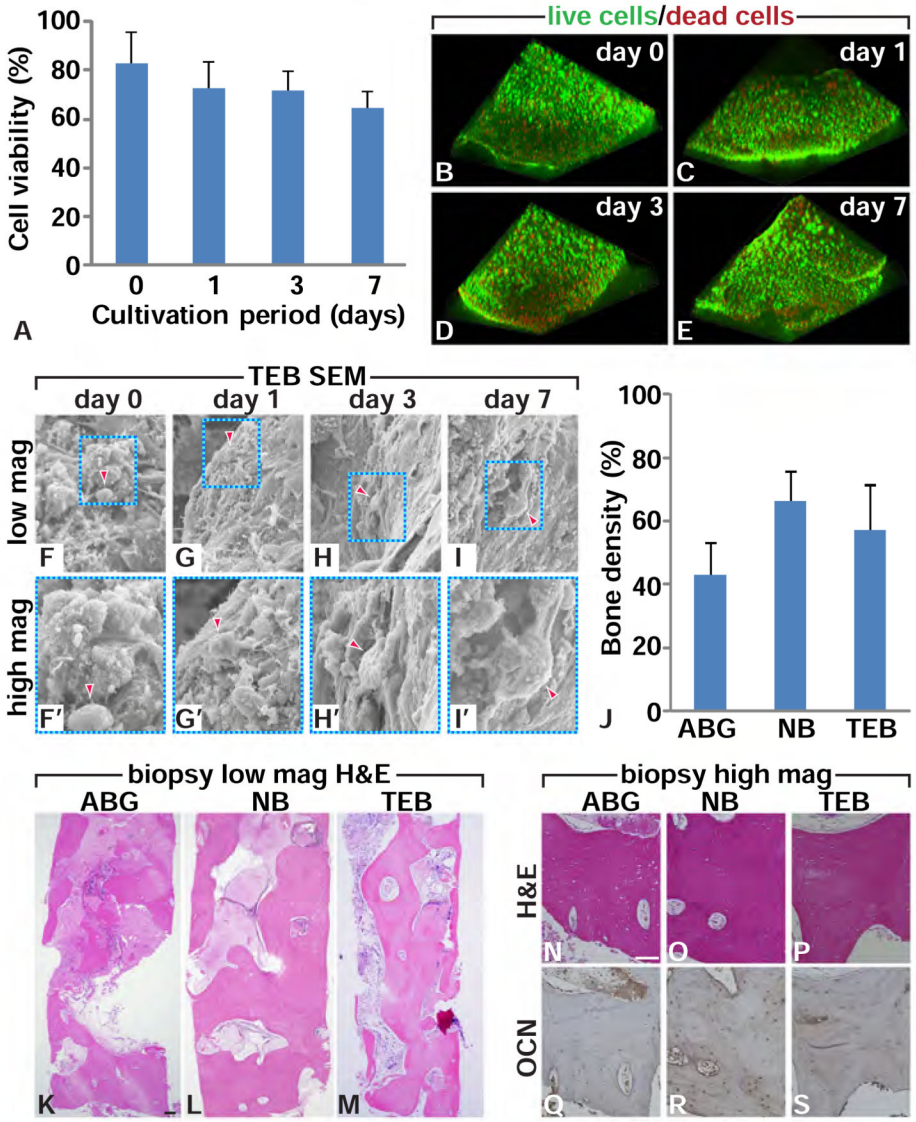

Figure 2. Transplantation of BMMSCs viability and clinical histological observation in human patients.

(A) Treatment protocol schema using injectable TEB. (B) Time course of hBMMSCs viability in TEB. Data are shown as mean ± s.d. (C-F) Representative image of live and dead staining of TEB. (G-J) The assessment of the microstructure of TEB using SEM. (G'-J') Higher magnification images of G-J. hBMMSCs are indicated by red arrowhead. Scale bars, 20 μm (G-J); 7.5 μm (G'-J'). (K-M) Representative histological images of human biopsy samples in ABG, NB, and TEB. Scale bar, 500 μm. Biopsy samples were analyzed by HE staining (N-P) and osteocalcin (OCN) immunostaining (Q-S). Scale bar, 100 μm. (T) The newly formed bone areas of a biopsy sample that was obtained at implant placement surgery. Data are shown as mean ± s.d.

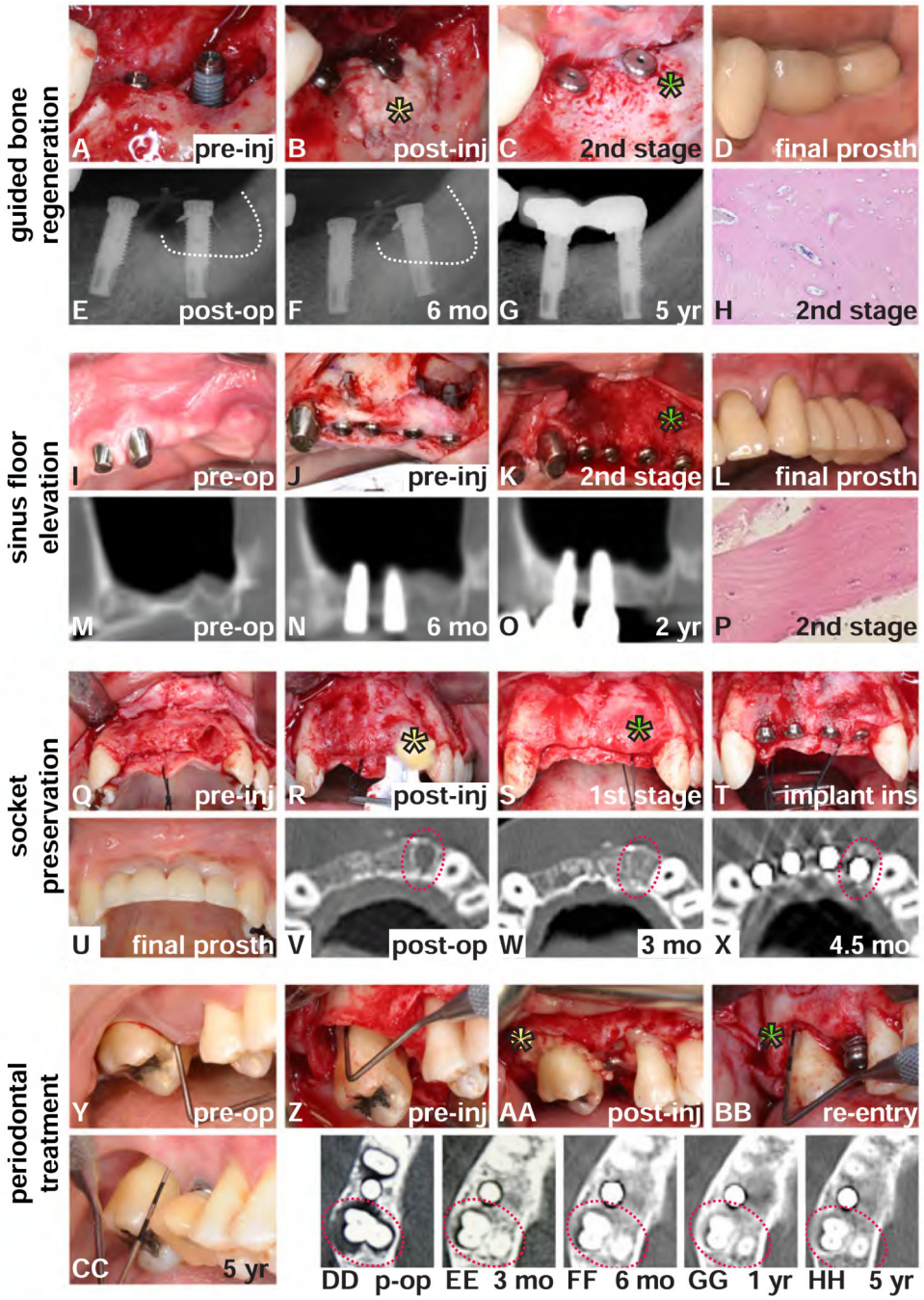

Figure 3. Clinical outcomes of TEB transplantation in human patients

(A-H) Representative images of GBR. Most of the implant threads were exposed (A). TEB (*) was transplanted into the bone cavity (B). In second-stage surgery, all space was completely filled with hard, bone-like tissue (*) (C). (D) The final prostheses. (E-G) X-ray images taken at post-operation immediately, 6 months, and 5 years. (H) HE staining of a biopsy sample at second-stage surgery. (i-p) Representative images of SFE. The maxillary bone was insufficient to place dental implants (I). After maxillary sinus floor augmentation and implants placement (J), TEB was transplanted into the sinus cavity where the implant

fixture was exposed. At second-stage surgery, adequate bone regeneration was observed, and it was filled with newly formed bone (*) (K). (L) The final prostheses. (M-P) CT image taken before surgery, at post-operation 6 months, 2 years. HE staining of a biopsy sample at second-stage surgery. (Q-X) Representative images of socket preservation. After tooth extraction (Q), TEB (*) was transplanted into the socket (R). At the time of the re-entry and dental implant placement procedures, the bone defect was fully filled with hard bone tissue (*) (S, T). (U) The final prostheses. (V-X) CT image taken at post-operation immediately, 3 months and the first-stage surgery for dental implantation. (Y-HH) Representative images of periodontal regeneration. Before surgery, deep periodontal probing depth (14 mm) and intraosseous defect (8 mm) with severe tooth mobility (Degree 3) was observed (Y, Z). TEB (*) was transplanted into the defect (AA). At 6 months after TEB surgery, the bone defect was filled with bone (*) and no tooth mobility was found (BB). cc shows magnified view at 5 years. (DD-HH) CT image taken at post-operation immediately, 3, 6 months, 1 year, and 5 years.