

## Effect of *E Coli* Expressed Recombinant Human Bone Morphogenetic Protein-2(rhBMP-2) on Stem Cells for Periodontal Tissue Regeneration: A Preliminary Study

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**Abstract :** Periodontal disease results in destruction of periodontal tissues(cementum, alveolar bone and periodontal ligament) which may ultimately lead to tooth loss. To date, there is no ideal treatment approach in achieving optimal periodontal tissue regeneration. Recruitment of progenitor cells to the site which could differentiate into cementoblasts, osteoblasts and periodontal ligament-forming cells are needed and recently, using pluripotent stem cells isolated from various tissues in conjugation with growth and differentiation factors have been presented to lead periodontal tissue regeneration. This study evaluated the effect of *E coli* expressed recombinant human bone morphogenetic protein-2(ErhBMP-2) to periodontal ligament stem cells(PDLSCs) and bone marrow stem cells(BMSCs) on periodontal tissue regeneration. rhBMP-2 those expressed from *E coli* was treated on *ex-vivo* expanded human BMSCs and PDLSCs to evaluate its regenerative effect *in vivo* and *in vitro*. Cementum, PDL-like tissues and bone were regenerated in transplanting PDLSCs and BMSCs, respectively. There was enhanced tissue regeneration when ErhBMP-2 was treated to PDLSCs, but not with BMSCs. Similar results were observed with *in vitro* experiments. Though further studies are needed, the results suggest a possible treatment approach in combining ErhBMP-2 and PDLSCs for periodontal tissues regeneration.

**Key words:** *Stem cells, bone morphogenetic protein, periodontal regeneration*

### 1. Introduction

Periodontal tissues are complex, composed of cementum, alveolar bone and periodontal ligament. Once periodontitis is established, destruction of these tissues occurs and regeneration of these tissues becomes one of the major goal in periodontal treatment. Efforts in regenerating damaged periodontal tissues has been carried out, such as root surface conditioning<sup>1</sup>, implantation of bone graft material<sup>2,3</sup>and guided tissue regeneration(GTR).<sup>4,5</sup> However, treatment outcome of such approaches were often unpredictable, showing repair healing pattern and mostly dependent on regenerative potential of existing periodontal defect morphology.<sup>6,7</sup> Recently, applying biological growth and differentiation factors with or without a biocompatible scaffold to defects has been a new approach in regenerating periodontal tissues.<sup>8-10</sup>

Bone morphogenetic proteins(BMPs) are members of the transforming growth factor- $\beta$  super-family which has the ability to induce bone.<sup>11</sup> When BMP is implanted on bone defects, new bone is effectively formed and when implanted on ectopic sites, ectopic bone formation is induced.<sup>12</sup> In 1988 Wozney<sup>11</sup> developed in recombining human BMP(rhBMP), and from then many clinical studies were carried out, introducing numerous products until today.<sup>13-18</sup> Most of the rhBMPs used today have been obtained from Chinese hamster ovary(CHO) cells.<sup>19</sup> However, unfortunately rhBMPs obtained from mammalian CHO cells are cost effective and therefore may not be easily applied clinically. In order to be a viable commercial product, BMPs recombined from low level animals such as *E coli* could be used. And several trials have presented its production and evaluation as an alternative to rhBMPs produced by mammalian cells.<sup>20-23</sup>

Periodontal ligament tissues are reported to have several cell types, including mesenchymal stem cells which serve as a source of renewing progenitor cells of cementoblasts,

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osteoblast and fibroblast.<sup>24,25</sup> Several studies presented periodontal tissue regeneration in transplanting periodontal ligament derived stem cells (PDLSCs) in periodontal defects of animals<sup>26-29</sup> and Seo *et al*<sup>28</sup> successfully isolated stem cells from human periodontal ligament, introducing a new approach to periodontal regeneration via tissue engineering. The stem cells isolated from periodontal ligament showed common characteristics to other stem cells. It formed mineralization deposits of alizarin red positive nodules *in vitro* and cementum/periodontal ligament structure formation following implantation *in vivo*, suggesting a potential use of stem cell-based therapies to damaged periodontal tissues.

In the present study, *E coli* expressed rhBMP-2(ErhBMP-2) was treated to periodontal ligament stem cells(PDLSCs) and bone marrow stem cells(BMSCs) to evaluate its effect on periodontal tissue regeneration.

## 2. Material and Methods

### 2.1 *In Vivo* Transplantation

In order to evaluate the effect of ErhBMP-2, *in vivo* transplantation model using nude mouse was carried out with human PDLSC for cementum, periodontal ligament, and BMSCs for bone regeneration.

#### 2.1.1 Stem Cell Isolation and Culture

Bone marrow stem cells(BMSCs) were obtained from a commercially available resource(AllCells LLC, Berkeley, CA, USA). Periodontal stem cell isolation was followed as the previously published paper.<sup>28</sup> Briefly, teeth extracted for orthodontic treatment were used and an informed consent was received by all patients undergoing teeth extraction. Periodontal ligament tissues were separated from the root surface by use of a scapel and were minced into smallest size as possible. Periodontal ligament membrane was digested three times for 1h in a solution of 3 mg/mL collagenase type I(WAKO, Tokyo, Japan) and 4 mg/mL dipase(GIBCO BRL, Grand Island, NY, USA) at 37°C. Single cell suspensions were obtained by passing through 70 µm pore size strainer(Falcon, BD Labware, Franklin Lakes, NJ, USA) and the cells( $1 \times 10^4$ ) were seeded on to 10 cm culture dishes containing alpha-modification of Eagle's medium( $\alpha$ -MEM, GIBCO BRL) supplemented with 15% fetal bovine serum(GIBCO BRL), 100 µmol/L ascorbic acid 2-phosphate(Sigma-Aldrich, St Louis, MO, USA), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin(GIBCO BRL) and incubated at 37°C in

5% carbon dioxide. Single cell colony was observed after 7 days and P0 passage of periodontal ligament stem cells was cultured. The P3 passage cells were used for the study and the cells were treated with 100 ng/mL of ErhBMP-2 for 3 days after reaching sub-confluence.

#### 2.1.2 Transplantation

A total of fifteen nude mice with 3 in each group was used. Animal selection, management, and surgical procedures followed a protocol approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

Particulated macroporous biphasic calcium phosphate(MBCP™, Biomatlante Sarl, France) bone graft material was used as a carrier. 80 mg of MBCP was mixed with  $4 \times 10^6$  human PDLSCs(P3) or MBSCs(P3) and was transplanted on to subcutaneous pocket of nude mouse. The animals received one of the four experimental conditions: MBCPonly(control), human periodontal ligament stem cells only(PDLSC/MBCP) or bone marrow stem cells only(BMSC/MBCP) and ErhBMP-2 treated human periodontal ligament stem cells(ErhBMP-2/PDLSC/MBCP) or bone marrow stem cells(ErhBMP-2/BMSC/MBCP).

The surgical procedure was performed under general anesthesia by an intramuscular injection of Ketamine hydrochloride(Ketalar, Yuhan Co., Seoul, Korea). The surgical site was disinfected with iodine and a vertical incision was made on the skin of the mouse back. After flap reflection, a subcutaneous pocket was prepared by a blunt dissection and respective treated materials according to the experimental design were implanted. The animals were healed for 8 weeks and were sacrificed to obtain block sections for histological analysis.

#### 2.1.3 Histological Analysis

Block sections were fixed in 10% formalin. The specimens were demineralized in 5% nitric acid, dehydrated in ethanol and embedded in paraffin. The central sections were reduced to a thickness of 7 µm and were stained in hematoxylin and eosin. The histological analysis were performed using a light microscope(Olympus BX50, Olympus Optical Co, Tokyo, Japan).

## 2.2 *In Vitro* Differentiation

### 2.2.1 Osteogenic and Adipogenic Differentiation.

The osteogenic and adipogenic differentiation of human PDLSCs and BMSCs with ErhBMP-2 treatment were

evaluated. PDLSCs were isolated following the procedure previously described. After obtaining single-cell suspensions, the cells were seeded into 24 well culture dish with  $1 \times 10^4$  cells on each well, and stem cells of P0 passage was able to be cultured. The cells were cultured until it reached an over confluent stage(P3) and 100 ng/mL of ErhBMP-2 were treated for 3 days. The culture medium was changed within 2~3 days of interval containing equal concentration of ErhBMP-2. The culture medium used for osteogenic induction was composed of  $\alpha$ -MEM with 15% FBS (GIBCO BRL), 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 1.8 mM  $\text{KH}_2\text{PO}_4$ , 10 nM dexamethasone(Sigma-Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin(GIBCO BRC). For adipogenic induction, the culture medium was composed of  $\alpha$ -MEM with 15% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 0.5 mM isobutyl-methylxanthine, 60 mM indomethacin, 0.5 mM hydrocortisone, 10mM insulin, 100 U/mL penicillin and 100 mg/mL streptomycin. After 2(adipogenic differentiation) and 4 weeks(osteogenic differentiation) of induction the cells were stained with oil red and alizarin red stain for evaluation.

### 2.2.2 Western Blotting

Runx-2, Osteocalcin, beta-Actin antibodies(Santa Cruz Biotechnology, SantaCruz, CA, USA) at dilutions ranging from

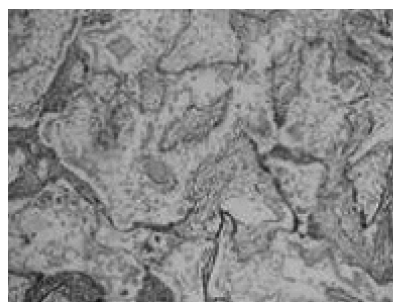


Figure 1. Histological section of control group(MBCP only). The MBCP was surrounded with loose connective tissue(H-E stain, original magnification  $\times 100$ ).

1:200 to 1:1000 were used for western blot. Western blot analyses were carried out as previously reported.<sup>30</sup>

## 3. Results

### 3.1 *In Vivo* Transplantation

In MBCP only implanted control group, the MBCP material particles were surrounded with loose connective tissues associated with multi-cellular giant cells. There were no findings of periodontal tissue regeneration(Fig 1). In hPDLSC only implanted group, a thin layer of cementum like tissues lined on the surface of MBCP particles(Fig 2). And along this

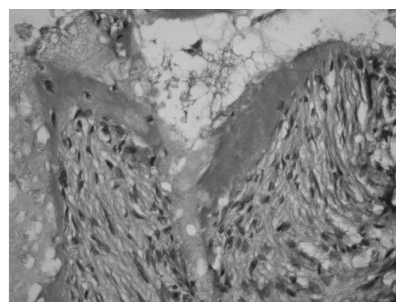
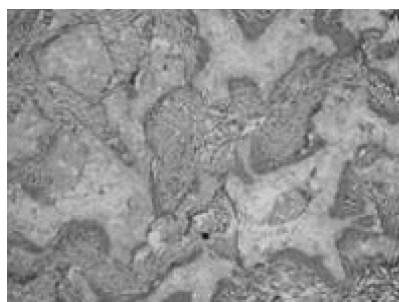


Figure 2. Histological section of PDLSCs implanted group. Regeneration of cementum and periodontal ligament like tissues were observed around MBCP(left). Higher magnification shows collagen fiber embedment(right)(H-E stain, original magnification; left  $\times 100$ , right  $\times 400$ ).

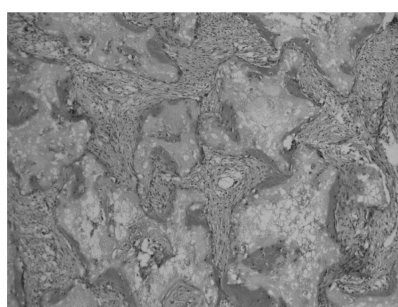
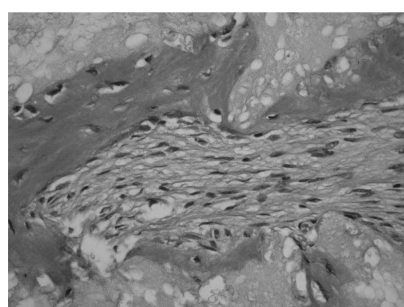
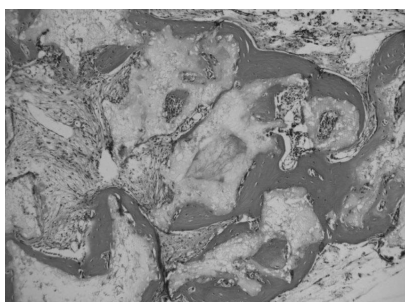
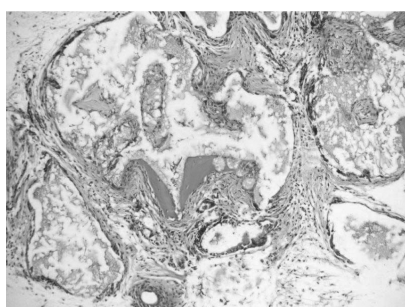


Figure 3. Histological section of ErhBMP-2 treated PDLSCs implanted group. Note thicker cementum formation and dense periodontal ligament like tissues around MBCP(H&E stain, original magnification; left  $\times 100$ , right  $\times 400$ ).



**Figure 4.** Histological section of BMSCs implanted group. Regeneration of bone structures were observed around MBCP(H&E stain, original magnification  $\times 100$ ).



**Figure 5.** Histological section of ErhBMP-2 treated BMSCs implanted group. The amount of bone regeneration was not significantly increased after ErhBMP-2 treatment(H&E stain, original magnification  $\times 100$ ).

cementum like tissues, dense collagen fibers with sparse cells were observed which resembled periodontal ligament tissues. These fibers resembled Sharpey's fiber, integrating and embedding with newly formed cementum like tissues(Fig 3). The alignment of periodontal ligament like fibers did not show any difference between groups presenting the fibers(Figs 2 and 3). In ErhBMP-2 treated PDLSCs implanted group newly formed cementum like tissues and periodontal ligament like tissues were presented along the surface of MBCP particles with thickest layer of cementum like tissue and more cementoblastic cells in ErhBMP-2 treated PDLSCs group than

the untreated(Figs 2 and 3).

In BMSCs only implanted group, a layer of bone was observed around the MBCP particles. The newly formed bone had osteocytes and was in lamellar form(Fig 4). Finding of bone marrow was also observed representing typical form of the bone structure. In ErhBMP-2 treated group, newly formed bone was also present. However, the amount of bone tissue formation seemed to be less than the untreated group(Fig 5).

### 3.2 *In Vitro* Differentiation

Alizarin red-positive nodules were observed on the culture dishes. The area of nodules formed per surface area was significantly greater when treated with ErhBMP-2 than untreated BMSCs(Fig 6).

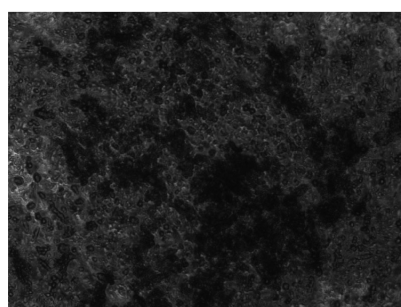
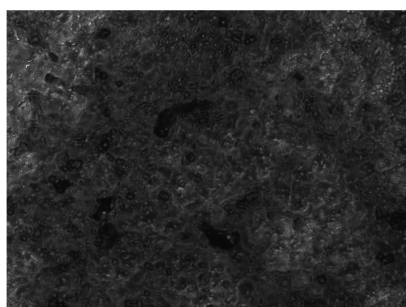
In adipogenic differentiation test, oil red O-positive adipocytes were formed and were significant with ErhBMP-2 treated BMSCs(Fig 7). However, there was no finding of oil red O-positive adipocytes formed with PDLSCs whether treated with ErhBMP-2 or not(Fig 8).

Western blot analysis showed osteoblastic markers(Runx-2, Osteocalcin, Beta-Actin) expression in both ErhBMP-2 treated and untreated BMSCs with less significant expression with treated BMSCs(Fig 9).

## 4. Discussion

Regeneration of periodontal tissues destroyed by periodontitis is the main goal in periodontal treatment. Regenerating periodontal tissues is challenging and many mechanical, chemical and surgery approaches have been performed to regenerate the tissues(PDL, cementum and bone).<sup>1-5</sup> However, the results of the conventional periodontal treatment were greatly dependent on defect morphology and residual vital periodontal cells, and sites with periodontitis are mostly deprived of vital periodontal cells.<sup>6</sup>

Cells in periodontal ligaments have multiple cell types which



**Figure 6.** Results of alizarin red stain of rhBMP-2 untreated(left) and treated(right) BMSCs.

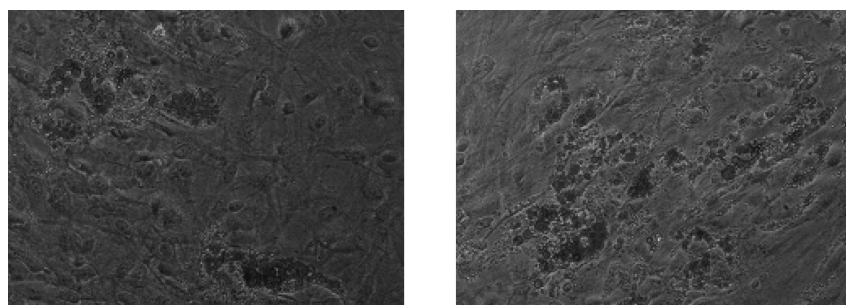


Figure 7. Oil red stain of ErhBMP-2 untreated(left) and treated(right) BMSCs.

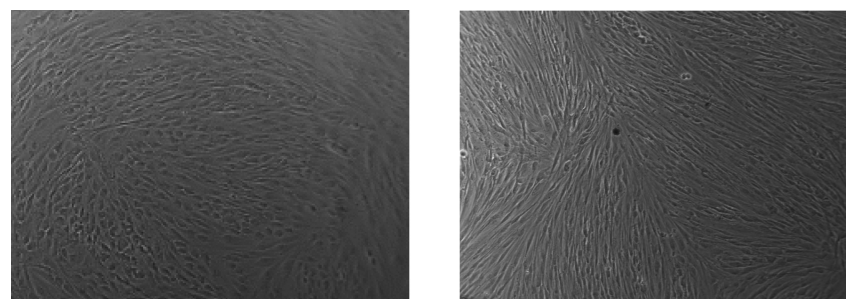


Figure 8. Oil red stain of ErhBMP-2 untreated(left) and treated(right) PDLSCs.

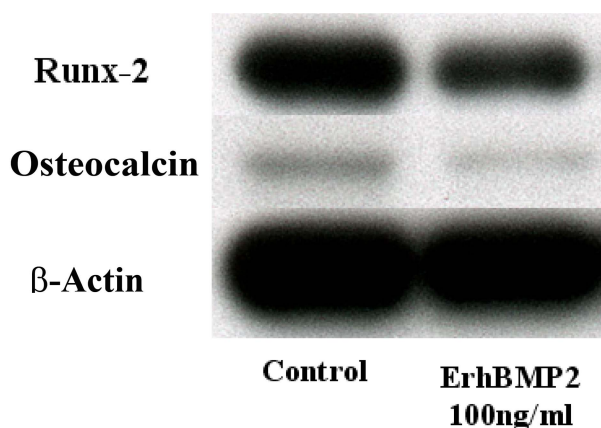


Figure 9. Western blot analysis of BMSCs.

contain progenitor cells in regenerating periodontal tissues, and studies have presented PDL cells to have osteoblastic and cementoblastic properties.<sup>24,25,28</sup> Seo *et al*,<sup>28</sup> presented that human periodontal ligament cells contain multipotent stem cells, regenerating cementum and periodontal ligament like tissues when transplanted into immunocompromised mice. However, unlike with other stem cells, periodontal ligament stem cells seem to present minimum bone forming property.<sup>27,28</sup>

BMPs induce bone formation, and many *in vivo* and *in vitro* studies with rhBMPs have shown ectopic bone and cartilage formation and osteoblastic makers expression<sup>11-15,17,18</sup> rhBMPs are usually recombined by using mammalian CHO cells.<sup>19</sup>

However, its high cost makes it difficult in clinical application and therefore, BMPs recombined by using *E coli* has been evaluated as an alternative.<sup>20,22,23</sup>

The objective of the present study was to evaluate the effect of *E coli* expressed rhBMP-2(ErhBMP-2) in tissue regeneration of BMSCs and PDLSCs. ErhBMP-2 was treated on *ex-vivo* expanded human BMSCs and PDLSCs to evaluate its regenerative effect *in vivo* and *in vitro*.

BMSCs and PDLSCs were mixed with MBCP bone material and were transplanted on subcutaneous skin of nude mouse. Histological findings after 8 weeks showed newly formed bone on the surface of MBCP particles with BMSCs and cementum and periodontal ligament like tissue formation with PDLSCs. In newly formed periodontal ligament like tissues, sparse fibers were observed, embedding to the newly formed cementum like tissue which resembled sharpey's fiber attachment of normal periodontal ligament(Fig 2). These results were consistent with other studies of PDLSCs and BMSCs.<sup>27,28,31,32</sup> Seo *et al*,<sup>28</sup> evaluated *ex-vivo* expanded PDLSCs, BMSCs and dental pulp stem cells(DPSCs) transplanted on to immunocompromised mouse. The histological findings presented cementum and PDL like tissues with PDLSCs, bone and marrow structures with BMSCs and dentin and pulp like tissue formation with DPSCs.

When ErhBMP-2 was treated to the stem cells, there was an enhancement in cementum and denser PDL like tissue

formation with PDLSCs. The layer of the newly formed cementum was thick with more cells. However, with BMSCs, the newly formed bone seemed to be less than untreated ErhBMP-2 cells instead of enhancement. These findings were also confirmed with the results of *in vitro* experiments. Alizarin red positive nodules and oil red O-positive lipid cells were more significant with untreated BMSCs cells in both osteogenic and adipogenic differentiation analysis. Results of the western blot also showed less significant expression of osteoblastic markers with ErhBMP-2 treated groups than untreated controls. These results show contrary with other studies of rhBMP and stem cells. Kim *et al*<sup>33</sup> presented synergistic bone formation with rhBMP-2 and human mesenchymal cells(MSC) in histological and immunochemical analysis. Friedman *et al*<sup>34</sup> also reported an upregulation of osteoblast-related gene expression when rhBMP-6 was treated to human MSCs.

The present study hypothesized the stem cells to show enhanced tissue regeneration when treated with ErhBMP-2. However, the enhanced effect was only observed with PDLSCs and not with BMSCs. The reason for such results could be assumed to be due to the use of *E coli* expressed rhBMP-2. rhBMP-2 expressed by prokaryotic cells such as *E coli* is presented to produce less bone induction than eukaryotic CHO cell expressed rhBMP-2.<sup>35</sup> However, contrary results are also reported, concluding that bone inductive effect of *E coli* derived rhBMP-2 was similar to that of CHO cell derived rhBMP-2.<sup>20,36,37</sup> Since controversy still exist on the bone inductive effect of *E coli* expressed rhBMP-2, further evaluation is needed. Another reason in explaining reduced bone inductive effect with ErhBMP-2 treatment could be due to treatment time and dose of ErhBMP-2. In the present study, *ex-vivo* expanded cells were treated with 100 ng/mL of ErhBMP-2 for 3 days before transplantation. Short treatment time and low dose of ErhBMP-2 may have not allowed the cells to induce bone actively. Though dose dependency is affected by several factors such as carrier system, species, experimental site and observation period<sup>38</sup>, controversial results are only currently present therefore, effect of dose of rhBMP-2 can also be a factor in producing such results.

In conclusion, within the limits of the present study, when *E coli* expressed rhBMP-2 was treated on to human PDLSCs and BMSCs, there was enhanced cementum and PDL-like tissue formation in PDLSCs but no significance in BMSCs. Though further studies are needed in relation to ErhBMP-2 and stem cells, the results suggest a possibility in combine use of ErhBMP-2 and PDLSCs for periodontal tissues regeneration.

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