SPANDIDOS PUBLICATIONS EXPERIMENTAL AND THERAPEUTIC MEDICINE 18: 358-365, 2019

Platelet-rich fibrin increases the osteoprotegerin/receptor activator of nuclear factor-κB ligand ratio in osteoblasts

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Received January 25, 2019; Accepted April 25, 2019

DOI: 10.3892/etm.2019.7560

Abstract. Platelet-rich fibrin (PRF) is a platelet concentrate derived from complete autologous blood rich in growth factors in the fibrin matrix. Although PRF has been used during oral surgery to optimize wound healing in soft and hard tissue, the precise role of PRF in bone healing remains unclear. The present study assessed the role of PRF in bone remodeling. PRF was prepared from whole blood by low speed centrifugation without any anti-coagulants. Culture of MC3T3-E1 cells with PRF induced the expression of osteoprotegerin (OPG), but had no effect on the expression of receptor activator of nuclear factor-κB ligand (RANKL), increasing the OPG/RANKL ratio. Expression of other osteoblastic differentiation makers, including BMP-2 and -4 and RUNX2, was not affected. PRF filling of a hole defect in the mental foramen bone of rats increased OPG positivity and decreased tartrate-resistant acid phosphatase positivity compared with unfilled control. In conclusion, PRF increased the OPG/RANKL ratio by inducing OPG expression, suggesting that PRF enhances early stage osteogenesis by optimizing osteoblastic differentiation. The present study provides a scientific basis for clinical findings showing that PRF can enhance bone regeneration such as sinus lift.

Introduction

Tissue engineering techniques are important for maxillofacial surgery. Current techniques involve combinations of cellular components, carrier/scaffold, and bioactive components. Platelets contain high intracellular concentrations of cytokines and growth factors, including platelet-derived growth factors (PDGF-AA, PDGF-BB and PDGF-AB), transforming growth factor-β (TGF-β1 and TGF-β2) and vascular endothelial growth factors (VEGFs), all of which can stimulate cell proliferation, matrix remodeling and angiogenesis (1). Following platelet aggregation, these molecules are released from intracellular pools and act to repair injured tissue. Platelets not only secrete cytokines from their cytoplasm but continue to synthesize cytokines using their mRNA reserves for at least another 7 days (2). Therefore, platelet concentrates, which are regarded as autologous alternatives to fibrin glue without anti-coagulants, contain fibrin glue rich in cytokines and are widely utilized for tissue regeneration following surgical treatment.

Platelet concentrates were originally utilized to treat haemorrhage-based severe thrombocytopenia, which is caused by medullar aplasia or acute leukaemia. Platelet concentrates, called platelet-rich plasma (PRP), have been used successfully for bone grafting in patients undergoing maxillofacial surgery (3) and for regeneration of periodontal tissue (4-6). PRP preparations are needed by subjects administered thrombin as an anti-coagulant. Some alternative preparations without anti-coagulant have been described, including platelet rich fibrin (PRF) (7) and concentrated growth factors (CGF) using a centrifuge (Medifuge) designated only for these preparations (8,9). Regardless of their methods of preparation, the resulting platelet concentrates are rich in the above-mentioned growth factors (10). Therefore, platelet concentrates, which act as a source of growth factors as well as containing a cellular scaffold, are thought to promote tissue regeneration.

Bone remodeling is maintained through a balance between bone formation and resorption (11). TGF-β and bone morphogenetic protein-2 (BMP-2) promote bone forming activity, by activating Smad1/2 and by increasing the production of type I collagen, alkaline phosphatase (ALP), and osteocalcin. The Wnt protein family also contributes to bone formation, either alone or combined with TGF-β/BMP-2 receptor signalling (12-15). Wnt signaling is initiated by its binding to its receptor, Frizzled. To date, two types of Wnt signaling pathways have been identified, the β-catenin-dependent 'canonical' pathway, involving, for example, Wnt3a; and the β-catenin-independent, 'non-canonical' pathway, involving, for example, Wnt5a (16-18). Phosphorylation of β-catenin by glycogen synthase kinase (GSK)-3β causes the former to become unstable, leading to its degradation under non-stimulating conditions. Inactivation of GSK-3β by Wnt signaling, however, stabilizes β-catenin and induces its binding to

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Key words: platelet-rich fibrin, osteoprotegerin, osteoblastic differentiation, MC3T3-E1, bone defect-healing

the transcription factor Lef1/Tcf, leading to osteoblastic differentiation (19).

In contrast, the differentiation and activation of osteoclasts are physiologically stimulated by osteoblasts through receptor activator of nuclear factor-κB (RANK) and its ligand RANKL (20). Because RANKL, a type II homotrimeric transmembrane protein, is expressed on the outer membrane of osteoblasts, osteoblasts play a central role in both the osteogenic and osteolytic activities of bone. Bone resorption is an important process, not only for bone remodeling but for calcium release from bone, which acts as a calcium store to maintain blood calcium concentrations. Calcium release is controlled by the RANK-RANKL system, which can be activated by an external signaling molecule such as parathyroid hormone (PTH) or $1\alpha,25(OH)_2D_3$, a stimulation that down-regulates the expression of osteoprotegerin (OPG). OPG is a decoy receptor for RANKL, inhibiting osteoclast differentiation and preventing excess bone resorption. Both Wnt/β-catenin and BMP-2 signaling activate Lef1/Tcf for transcription of *Tnfrsf11b*, the gene that encodes OPG (21). Treatment of human osteogenic sarcoma U2OS cells with PRF induced the production of OPG protein within one day, with production maintained for five 5 days, but rapidly decreasing at day 7 (22). The OPG/RANKL ratio has been reported to reflect osteoblastic differentiation status, with a high OPG/RANKL ratio resulting in a switch to osteoblast maturation (23). To date, however, the effect of PRF on OPG/RANKL ratio has not been widely accepted as a standard effect of PRF. The present study was designed to confirm that a PRF-induced increase in OPG/RANKL ratio resulted in osteoblastic differentiation of the mouse osteoblast MC3T3-E1 cell line and that PRF was involved in the healing process in a rat bone defect model. These findings confirm that PRF may play a role in bone defect/fracture healing.

Materials and methods

Cells and cell culture. The MC3T3-E1 cell line, a clonal pre-osteoblastic cell line derived from newborn mouse calvaria, was grown in α-minimum essential medium (α-MEM; ICN Pharmaceuticals, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 50 μg/ml ascorbate 2-phosphate, 10 mM β-glycerophosphate, and 40 mM HEPES (pH 7.4), as described (24). Mouse NIH3T3 fibroblasts were purchased from Riken (Tsukuba) and maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with FBS. All cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air.

Animals. Male Wistar rats, aged 8-10 weeks and weighing 400-450 g, were obtained from Clea Japan Inc., Tokyo, Japan). The rats were housed individually in a barrier facility for laboratory animals with a 12 h light-dark cycle and allowed food and water *ad libitum*. All surgical procedures were performed under general anesthesia with sevoflurane (Mylan; 4% for the induction and 3% for the maintenance), with local anaesthesia provided by 2% lidocaine (250 μg/kg) if necessary. Rats were sacrificed by intraperitoneal injection of over dose (120 mg/kg) of sodium pentobarbital (Kyoritsu) under general anaesthesia with sevoflurane.

All animal experiments were approved by the animal ethics committee of Ohu University (Koriyama, Japan) and done in the Animal Facility where animals were cared by the Animal Care Staff according to compliance by the ARRIVE guidelines (no. 2017-14). Number of rats used for preparation of PRF were as follows: One donor rat for a set of *in vitro* experiment and one donor rat for two recipient rats to treat defect. One *in vivo* experiment used eight rats as the recipient, which were divided into two groups: One group was used as PRF-grafted group and the other one was as the control. Thus, we minimized number of rats and used total 23 rats including repetition.

Preparation of PRF. PRF was prepared as described (7,25,26), with slight modifications. Briefly, rats were anaesthetized with sevoflurane. Whole blood (6 ml) was collected from the apex of the heart using a 23-gauge needle and BD Vacutainer® Blood Collection Tubes (Becton-Dickinson) without anti-coagulants. Immediately after collection, the blood was centrifuged at 890 x *g* at room temperature for 13 min. The intermediate layer was defined as PRF to be subjected to *in vitro* and *in vivo* experiments (27).

Osteoblastic differentiation and PRF treatment. We used preosteoblastic cell line MC3T3-E1 cells, which was well established model for osteoblastic differentiation, to analyze the effects of PRF on osteoblastic differentiation according to Ogino *et al* (27) with slight modifications. MC3T3-E1 cells that reached 70% confluence in 6-well culture plates were induced to undergo osteoblastic differentiation by the addition of 50 μg/ml ascorbate 2-phosphate and 10 mM β-glycerophosphate, as described (24), with the culture medium renewed every 2-3 days. PRF (21 $\mu g/cm^2$) was placed at the center of a cell monolayer sheet and the treatment was started at the same time.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from samples with acid guanidinium thiocyanate-phenol-chloroform (AGPC) and reverse-transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The resulting cDNA was PCR amplified using a GoTaq® Real-Time qPCR Kit (Bio-Rad) with specific primers (Table I) in a Thermal Cycler Dice Real Time System (TP-870; Takara). Results were normalized relative to *Actb* (β-actin) mRNA expression levels in the same samples.

TCF-4 binding activity. The TCF-4 binding motif was cloned by PCR and inserted into the pGL3-OT vector (Addgene #16558). Following transfection of this vector into MC3T3-E1 cells using Xfect Transfection Reagent (Takara), the cells were stimulated with PRF (0.2 g/well; 21 $\mu g/cm^2$) in 6-well plates for 24 h. TCF-4 binding activity was evaluated using Dual-Luciferase Reporter Assay System (Promega). Cells were co-transfected with the pGL4.75 [hRluc (*Renilla reniformis*)/CMV] vector to control for transfection efficiency.

Alizarin red S (AR-S). Mineralized matrix in culture plates was stained with AR-S as described (24). Briefly, cells were fixed in 70% ethanol for 1 h at room temperature and stained

Table I. Primer sets.

Genes (products)	Primer sequences (5'-3')
Alpl (ALP)	
Forward	GCAGTATGAATTGAATCGGAACAAC
Reverse	ATGGCCTGGTCCATCTCCAC
Runx2 (Cbfa1/Runx2)	
Forward	ACTCCAGGCATACTGTACAACT
Reverse	AGGCTGTTTGACGCCATAGT
Bmp2 (Bmp-2)	
Forward	TGACTGGATCGTGGCACCTC
Reverse	CAGAGTCTGCACTATGGCATGGTTA
Bmp4 (Bmp-4)	
Forward	AGCCGAGCCAACACTGTGAG
Reverse	TCACTGGTCCCTGGGATGTTC
Csf1 (M-CSF)	
Forward	AGTGCTCTAGCCGAGATGTG
Reverse	CTGCTAGGGGTGGCTTTAGG
Tnfsf11 (RANKL)	
Forward	AGCGCAGATGGATCCTAACA
Reverse	CCAGAGTCGAGTCCTGCAAAT
Tnfrsf11b (OPG)	
Forward	AGTGTGAGGAAGGGCGTTAC
Reverse	AATGTGCTGCAGTTCGTGTG
Wnt3a (Wnt3a)	
Forward	CTACCCGATCTGGTGGTCCT
Reverse	ACAGAGAATGGGCTGAGTGC
Wnt5a (Wnt5a)	
Forward	AAAGGGAACGAATCCACGCT
Reverse	CAGCACGTCTTGAGGCTACA
Actb (β-actin)	
Forward	CATCCGTAAAGACCTCTATGCCAAC
Reverse	ATGGAGCCACCGATCCACA

ALP, alkaline phosphatase; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; BMP, bone morphogenetic protein.

with 40 mM AR-S at pH 4.2 for 10 min at room temperature. After washing with deionized water and Ca^{2+}- and Mg^{2+}-free phosphate buffered saline [PBS(-)], AR-S-positivity was quantified using Molecular Imager (Bio-Rad).

Alkaline phosphatase (ALP) activity. Cells were washed twice with PBS(-) and sonicated in ice-cold 0.1 M Tris-HCl (pH 7.2) containing 0.1% Triton X-100. ALP activity was determined using a Lab Assay ALP kit (Wako), according to the Bessey-Lowry method.

Western blotting. Proteins in cell lysates (20 μg), prepared in RIPA buffer, and those in conditioned medium (CM, 10 μg), which were concentrated by acetone precipitation, were separated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were sequentially incubated with primary antibody, biotinylated anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch Laboratories) as secondary antibody, and horseradish peroxidase (HRP)-conjugated streptavidin (Bio-Rad). Signals were detected using Luminate™ Forte Western HRP Substrate (Merck Millipore). The primary antibodies used in this study included anti-RANKL antibody (BioLegend), anti-M-CSF antibody (Abcam), anti-OPG antibodies, and anti-β-actin antibodies (both from GeneTex).

Bone defect-healing model. A bone defect-healing model in calvaria was adapted to the mental foramen bone to assess the effects of PRF on bone regeneration (1,28-31). Briefly, following anesthetisation with 4% sevoflurane, both sides of the mental foramen bone were treated with 100 μg of lidocaine and each side was surgically drilled to make holes 2.0 mm in diameter while avoiding injury to the roots of the teeth. The bone defect was filled with PRF (doses equivalent to 0.3 g/defect area) on one side (PRF-grafted group, n=4) and without PRF on the other side (non-grafted or control group, n=4). Four days after surgery, rats were sacrificed by over dose of pentobarbital subject to histological analysis.

Histological analysis. Samples of mandibular tissue were obtained from rats under sodium pentobarbital anaesthesia and fixed in phosphate-buffered 4% paraformaldehyde (pH 7.2) at 4˚C for 24 h. The samples were decalcified with 10% EDTA (pH 7.0), which was changed every 2-3 days, at 4˚C for 4 weeks. The tissue samples were embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin (H&E) and then tartrate-resistant acid phosphatase (TRAP) using a commercial TRAP staining kit (Wako), according to the manufacturer's protocol.

OPG was detected by immunohistochemistry, as previously described (32-34). Specimens were blocked with 5% skim milk and incubated sequentially with anti-OPG antibodies (GeneTex), biotinylated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and fluorescein isothiocyanate (FITC)-conjugated streptavidin. Signals were observed with a fluorescence microscope (Axio Observer, Carl Zeiss Microscopy GmbH).

Protein assay. Protein concentration was determined by the Bradford method, using a Bio-Rad protein assay kit, with bovine serum albumin (BSA) as the standard.

Statistical analysis. Representative results from three independent experiments (unless otherwise noted) were shown and statistical significance between two groups was evaluated by Student's t-tests. Simple regression analysis was used to compare two slopes obtained by the least squares method. A P-value <0.05 was considered statistically significant.

Results

PRF induced expression of OPG-encoding mRNA in osteoblastic cells. MC3T3-E1 cells were treated with PRF to evaluate OPG production during osteoblastic differentiation. PRF treatment gradually but significantly increased expression of *Tnfrsf11b* mRNA, encoding OPG, for 12 days (Fig. 1A-C). In contrast, *Tnfsf11* mRNA, encoding RANKL, was constitutively expressed but not affected by treatment of cells with PRF.

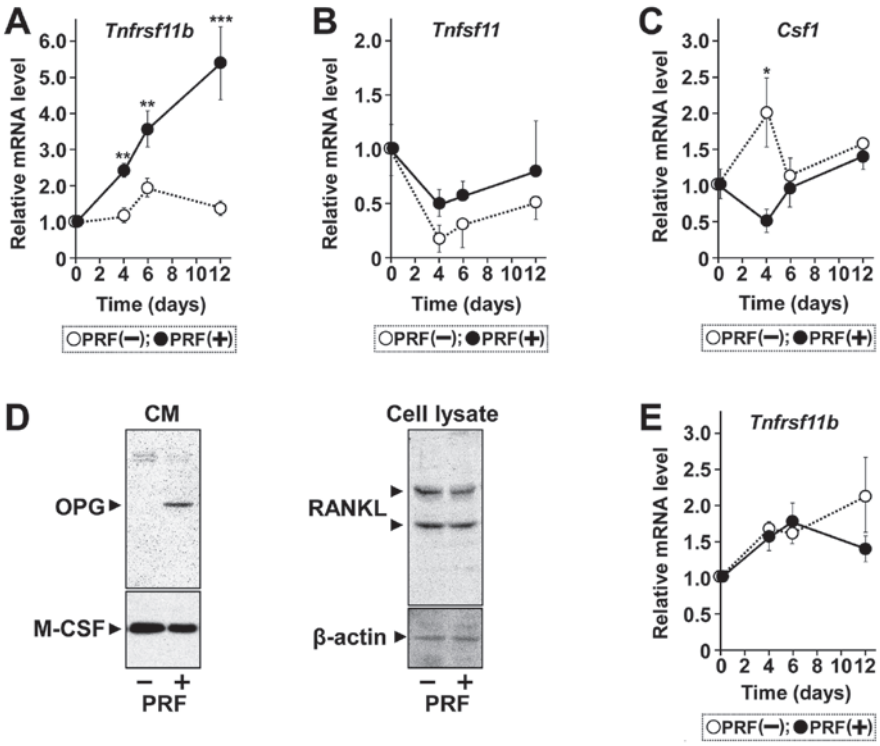

Figure 1. PRF suppresses osteoclast-differentiation factor expression in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the absence or presence of PRF for 6 days. Total RNA was extracted and the expression of (A) *Tnfrsf11b*, (B) *Tnfsf11* and (C) *Csf1* mRNAs was determined by reverse transcription-quantitative PCR. (D) The amount of RANKL in whole-cell lysates and of MCSF and OPG in concentrated CM was assayed by western blotting. (E) PRF did not affect *Tnfrsf11b* mRNA expression by NIH3T3 cells. Representative results were from three independent experiments. Data are presented as the mean ± standard error (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. samples incubated in the absence of PRF at each time point. PRF, platelet rich fibrin; CM, conditioned medium; OPG, osteoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand.

Expression of *Csf1* mRNA, encoding M-CSF was transiently lowered in PRF treated cells on day 4, with no significant differences at later times. Western blot analysis showed that PRF strongly upregulated the expression of OPG, but not of RANKL or M-CSF, by these cells (Fig. 1D). PRF, however, did not induce expression of *Tnfrsf11b* mRNA in non-osteoblastic NIH3T3 fibroblasts (Fig. 1E), although non-osteoblastic cells, such as gingival fibroblasts (35), adipocytes (36), and endothelial cells (37), can produce OPG. PRF also increased the ratio of OPG-encoding mRNA to RANKL-encoding mRNA in these cells (Fig. 2).

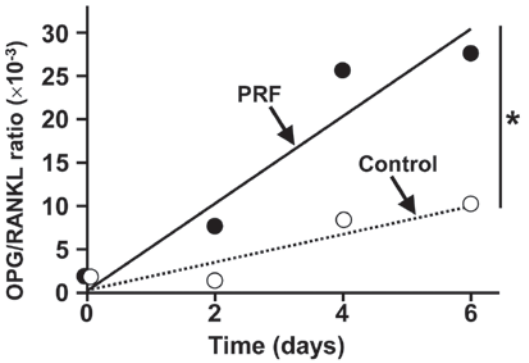

Figure 2. Effect of PRF on OPG/RANKL ratio. MC3T3-E1 cells were cultured in the absence or presence of PRF for 6 days. Total RNA was extracted and the expression of *Tnfrsf11b* and *Tnfsf11* mRNAs was determined by reverse transcription-quantitative PCR. Cq values of *Tnfrsf11b* and *Tnfsf11* mRNAs were normalized relative to that of *Actb* mRNA in the same samples to calculate OPG/RANKL ratios. Representative results were from three independent experiments. *P<0.05. PRF, platelet rich fibrin; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand.

PRF did not affect the expression of other osteoblastic marker encoding genes and mineralization in vitro. Treatment of MC3T3-E1 cells with PRF did not induce formation of nodules resulting from mineral deposition (Fig. 3A). PRF reduced *ALP* mRNA level on day 4, with low *ALP* mRNA level maintained through day 8, although *ALP* mRNA level was higher on day 8 than on day 4 (Fig. 3B). ALP activity was also lower in the presence than in the absence of PRF on day 8 (Fig. 3C), ALP activity in PRF-treated cells was higher on day 8 than on day 4, whereas ALP activity in control cells was higher on day 4 than on day 8, suggesting that PRF regulates bone regeneration by delaying the peak of osteoblast differentiation. The expression of other osteoblastic marker genes, encoding Runx2, BMP2, and BMP4, were not affected by PRF treatment (Fig. 3D-F).

In assessing the effect of PRF on Wnt signaling which plays a role in OPG expression, we found that PRF had no effect on the expression of Wnt3a and Wnt5a (Fig. 4A and B). In addition, PRF did not significantly activate the transcription of β-catenin from a TCF-4 binding motif containing a reporter vector (Fig. 4C). These findings suggested that the Wnt pathway is not involved in the induction of *Tnfrsf11b* mRNA in MC3T3-E1 cells.

PRF increases the number of OPG producing cells but reduces the number of active osteoclasts. To determine

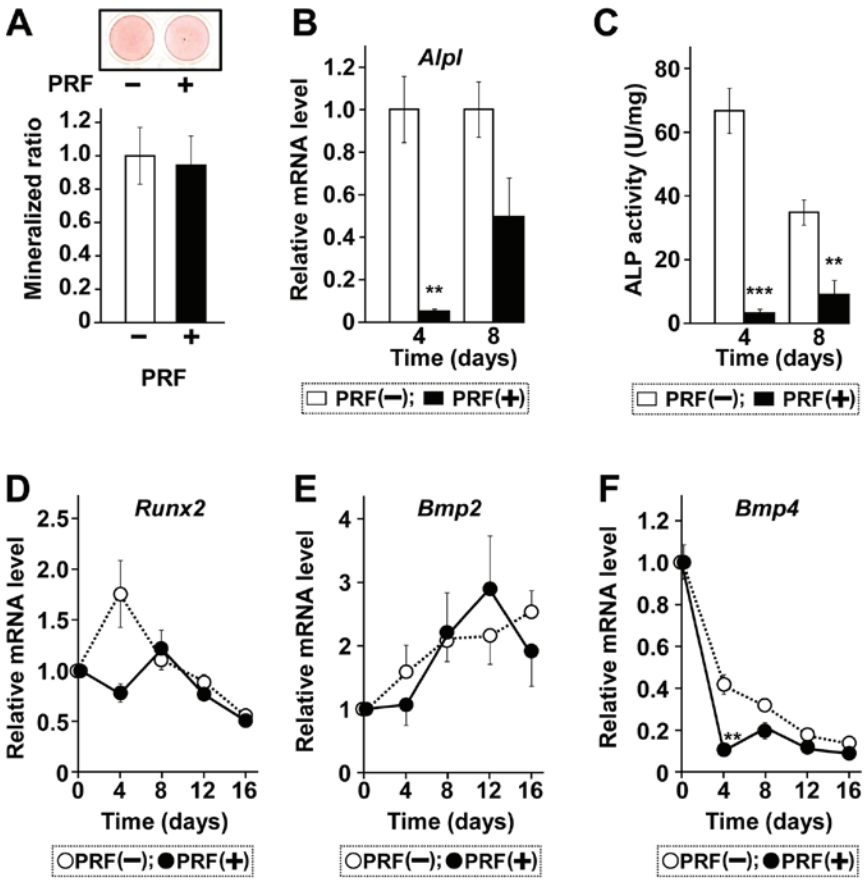

Figure 3. PRF does not affect *in vitro* mineralization or osteoblastic differentiation for mineralization. Cells were treated with (+) or without (-) PRF for 24 days. (A) Calcification determined by AR-S staining. (B) Total RNA was extracted, reverse-transcribed and amplified by qPCR with primer sets for *Alpl* mRNA. (C) Samples from whole-cell extracts were assayed using an ALP kit. Time course of the effect of PRF on expression of (D) *Runx2*, (E) *Bmp2* and (F) *Bmp4* mRNAs, as determined by reverse transcription-quantitative PCR. Cells were treated with or without PRF for the time periods indicated. Representative results were from two independent experiments. Data are presented as the mean ± standard error (n=3). **P<0.01 and ***P<0.001 vs. samples incubated in the absence of PRF at each time point. PRF, platelet rich fibrin; AR-S, alizarin red S; ALP, alkaline phosphatase; CM, conditioned medium; OPG, osteoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand.

whether PRF mediates OPG induction and osteoclastogenesis *in vivo*, a bone defect-healing model in calvaria was adapted for use in mental foramen bone. A bone defect area 2.7 mm in diameter treated with a low-level laser was reported filled with calcium (~180 mg/g-tissue) and phosphorus (~115 mg/g-tissue) within 2 weeks (28). Both values were 1.4 times higher than in control, non-laser treated, bone. After an additional 2 weeks, these increases in calcium and phosphorus deposition were 1.3- and 1.2-fold higher than in control, respectively, suggesting that early stage bone repair process, within 2 weeks after surgery, was extremely important. Therefore, we created a small defect (2.0 mm in diameter) and observed early stage regeneration. After 4 days, OPG-positive cells were 2.7-fold more abundant in the PRF-engrafted than in the control region (Fig. 5A). Conversely, PRF grafting decreased TRAP positivity to 41.7% of that observed in the control region (Fig. 5B). Taken together, these findings indicate that an elevation in OPG/RANKL ratio may be critical during early stage osteoblastic differentiation.

Discussion

Fibrin acts as a scaffold for cells during wound repair (38,39). Insertion of PRF as the sole filling material was found to promote bone regeneration and subsequent sinus elevation (40). Moreover, the combination of PRF and a demineralized freeze-dried bone allograft (DFDBA) accelerated bone formation; although DFDBA-induced maturation required about 8 months, the addition of PRF shortened bone formation to 4 months (41). PRF has been reported to accelerate bone healing both *in vivo* and *in vitro* (8,42,43). The ability of platelet concentrate clots to gradually release PDGFs, TGF-β, IGF-1, and FGF-2 (44) enables them to assist in bone healing in the defect/fracture region, similar to the activity of PRF.

More preclinical and clinical studies have evaluated PRP than PRF, with most studies of PRP showing its benefits for bone repair, although several have reported that PRP has low or limited impact on osteogenesis in bone defect repair (45,46). These discrepancies may be explained by differences among PRP preparations in components and/or their ratios, including differences not only in growth factors but platelets and leukocytes (47,48). Even studies reporting that PRP does not affect bone healing activity did not find that PRP had negative effects on bone healing. Using MC3T3-E1 cell line, which has been widely used as a model for osteoblastic differentiation (14,49,50), here we showed that ALP expression/activity was lower in PRF-treated than in control cells, in which medium contained ascorbate 2-phosphate and

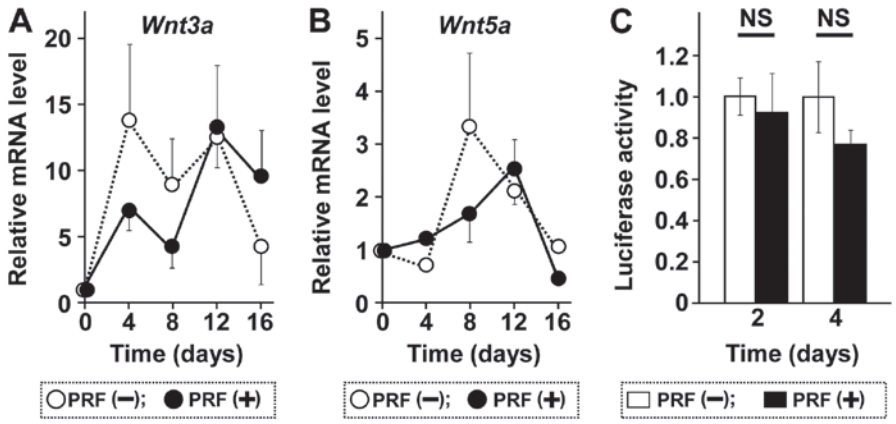

Figure 4. PRF does not affect Wnt signaling. Total RNA was extracted and the levels of expression of (A) *Wnt3a* and (B) *Wnt5a* mRNAs were determined by reverse transcription-quantitative PCR. (C) TCF-4 activity in the presence of PRF was assessed by measuring luciferase activity after transfection of the luciferase vector linked to the TCF-4 binding motif (pGL3-OT) into MC3T3-E1 cells. Data are presented as the mean ± standard error (n=3). Representative results were from two independent experiments. No significant differences were observed by Student's t-tests. PRF, platelet rich fibrin; NS, not significant.

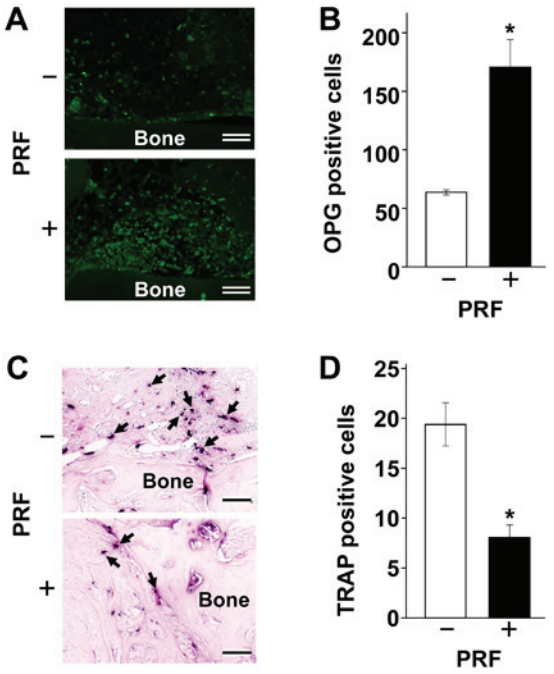

Figure 5. PRF increases the number of OPG-positive cells and reduces the number of TRAP-positive *in vivo* in bone healing regions. Immunohistochemical staining for (A) OPG and (B) number of OPG positive cells. (C) TRAP staining and (D) number of TRAP-positive cells. Arrows indicate TRAP positive cells; scale bars, 10 μm. Representative results were from two independent experiments. Data are presented as the mean ± standard error (n=4). *P<0.05 vs. no PRF treatment. PRF, platelet rich fibrin; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase.

β-glycerophosphate, but no other osteogenic inducers. Because ALP activity is high in mature osteoblasts, PRF did not inhibit osteoblastic differentiation, but rather may have optimized osteoblastic differentiation. Thus, the low ALP mRNA/activity in response to PRF treatment suggests that PRF regulated ALP expression by delaying it, thereby optimizing bone remodeling to an osteogenic state during the early stages of osteoblastic differentiation in our culture system. Indeed, ALP expression/activity gradually increased during the time in culture.

The present study found that OPG was the only molecule induced by PRF stimulation. Many studies have focused on OPG/RANKL ratio and bone resorption, but few have linked these findings to osteoblastic differentiation (51). OPG/RANKL ratio has been reported: Low in immature osteoblasts and high in mature osteoblasts (23,52), suggesting that the PRF-induced increase in OPG/RANKL ratio is associated with bone formation. Our results also showed that PRF affected OPG production, leading to a high OPG/RANKL ratio, suggesting that autologous platelet concentrates may be suitable to balance bone formation (53-55). PRF-induced OPG production by an osteosarcoma cell line was found to peak within 1 day, remaining high up to day 3 and decreasing to baseline level by day 5, while RANKL production remained steady (22). Although our OPG induction peak differed slightly, the two studies, taken together, indicate that PRF-induced increases in OPG/RANKL ratio occur during early stage osteoblastic differentiation, similar to the effects of genestein on early stage osteoblastic differentiation (51).

Clinically, PRF, combined with other materials, has been used in surgical repairs (47,56). Because we found that PRF did not induce markers of osteogenic differentiation, such as *Runx2*, *Bmp2*, or *Bmp4* mRNAs, *in vitro*, PRF may play a role during the early stages *in vivo* to optimize osteoblastic differentiation towards an osteogenic state by inducing OPG production. Similarly, the OPG/RANKL ratio in osteoblastic differentiation was reported to be associated with bone formation during the remodeling process (57).

This study could not determine which factors in PRF are responsible for the induction of OPG. Since OPG expression in osteoblasts was reported to be induced by BMP-2, TGF-β, and PDGF (57,58), it is reasonable to assume that these factors, either alone or in combination in PRF, induce OPG expression. Reverse signaling of RANKL stimulated by RANK was recently reported (59). Although osteoblasts in OPG-deficient mice did not show reduced osteogenic activity (60), studies are needed to elucidate the role of OPG in RANKL-induced reverse signaling during the bone healing process in bone defect/fracture models.

In conclusion, our results showed that PRF increased the OPG/RANKL ratio by inducing OPG production, suggesting that PRF assists in early stage osteogenesis by optimizing osteoblastic differentiation. Because PRF aids in earlier and

better wound healing (61). PRF has advantages in patients undergoing oral surgery, such as sinus lift.

Acknowledgements

The authors would like to thank Dr Keiko Kasai (Jusendo Hospital, Koriyama, Japan) for technical assistance and Dr Satoshi Takada, Dr Hirosi Ito and Dr Yoko Sakurai (Ohu University, Koriyama, Japan) for their critical comments. This work was based on a PhD thesis (Ryuta Sumida), which was published by Ohu University Press (Koriyama, Japan) in 2018.

Funding

This work was supported by JSPS KAKENHI (grant no. JP16K11517).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YK conceived the study and planned the experiments. RS performed all experiments. IK and JY assisted in the animal experiments, and reviewed the manuscript critically for important intellectual content. RS, TM and YK analysed and interpreted the data, and wrote the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Committee of Ohu University (Koriyama, Japan; no. 2017-14).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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