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Original Article

Platelet-rich fibrin as a scaffold in combination with either deciduous or permanent dental pulp cells for bone tissue engineering

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Abstract: Objective: To determine if Choukroun's platelet-rich fibrin (PRF) can increase the osteogenic properties of deciduous and permanent dental pulp cells (DPCs), we evaluated the ability of PRF to stimulate the proliferation and differentiation of these DPCs. Methods: Primary cultured deciduous and permanent DPCs were obtained from 3-month-old and 16-month-old canines, respectively. Blood samples were obtained from the canines and centrifuged to extract PRF. The cell counts and cytotoxicity tests were performed at 1, 4, 7, and 11 days after culture. In addition, deciduous and permanent DPC were cultured under 4 different conditions: control group (without PRF in normal medium), test group (with PRF in normal medium), test group D (without PRF in differentiation medium) and test group D0 (with PRF in differentiation medium). Osteoblast differentiation was analysed using Alizarin Red staining and alkaline phosphatase (ALP) activity. Results: PRF did not have a toxic effect on the cells. It significantly increased the proliferation of both deciduous and permanent DPCs, reaching levels up to 2.5, 3.0, 2.0 and 2.8 times compared to those of controls at 7 and 11 days after inoculation, respectively ($P < 0.01$). In addition, PRF significantly stimulated differentiation of all DPCs as shown by increased calcium nodules ($P < 0.05$), up-regulated ALP levels, and elevated expression of osteogenesis-related genes ($P < 0.05$). These effects were more pronounced in deciduous cells than in permanent cells. Conclusions: PRF has an obvious stimulatory effect on the proliferation and differentiation of both deciduous and permanent DPCs in vitro, suggesting that PRF promotes osteogenesis. Therefore, the combination of PRF with both either deciduous or permanent DPCs could be a promising method for bone tissue engineering.

Keywords: Platelet-rich fibrin, dental pulp cells, bone tissue engineering

Introduction

Cranial and maxillofacial bone defects often cause pain and dysfunction and reduce the patient's quality of life. The goal of bone tissue engineering is to rebuild bone. There are three essential elements to bone tissue engineering: scaffolds, seed cells, and bioactive factors. Platelet-rich fibrin (PRF), a second-generation platelet concentrate, is a type of biological fibre scaffold material [1]. As a kind of network framework, PFR can be used as supporting materials. It is rich in platelets, leukocytes, and various growth factors that can efficiently promote cell proliferation and differentiation [2]. In addition, PRF contains osteogenic cytokines and fibre holders, which can act as a three-dimensional centrifugal mesh stent and form a near-natural polymer that can capture migrating cells and induce the slow release of platelet-derived growth factors, further aiding in osteogenesis. Together with dental pulp cells (DPCs), which can be cultivated and have proliferative and multidirectional differentiation potential, PRF has been used to induce bone repair and regeneration [1, 2]. The aim of this study is to evaluate the proliferation and differentiation capabilities of deciduous and permanent dental pulp cells (DPCs), in the presence of PRF, in order to determine whether the addition of PRF can increase the regenerative and osteogenic properties of deciduous and permanent DPCs for bone tissue engineering.

Material and methods

Cell culture

DPCs were obtained from either exfoliated deciduous teeth from a 3- to 4-month-old canine

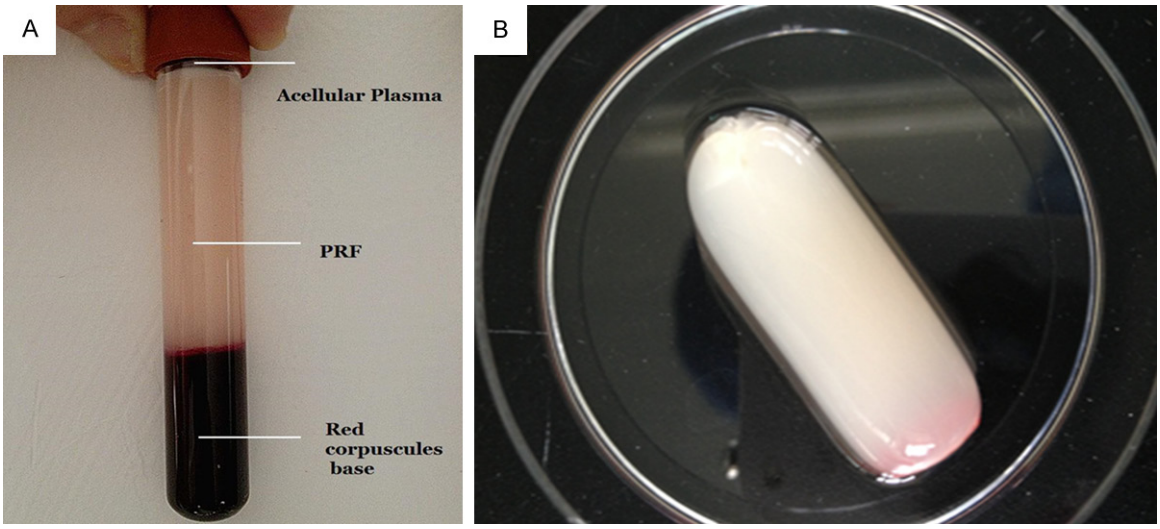

Figure 1. Preparation of PRF. A. PRF formed in the middle of the tube after blood centrifugation. B. PRF was easily separated from the glass tube.

or permanent teeth from a 12- to 16-month-old canine. All cell types were cultured using the explant technique [3]. Teeth were sectioned horizontally below the cement-enamel junction using a high-speed bur with water spray. Pulp tissues were minced with a blade into small fragments and grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell cultures between the third and eighth passages were used.

PRF preparation

Canine blood samples were obtained under the guidelines of the animal house at Shanghai Sixth People's Hospital. Blood was drawn from the internal jugular vein using a 20 ml syringe in 10 mL glass tubes without anticoagulant and immediately centrifuged at 3000 rpm for 10 minutes [4]. After centrifugation, three layers formed in the tube: red blood cells collected at the bottom, a cellular plasma collected at the surface, and a PRF clot collected in the middle (**Figure 1**). The PRF clot was easily separated from the tube (**Figure 1B**) and gently pressed into a membrane with sterile dry gauze for the subsequent experiments.

Scanning electron microscope (SEM) and H&E staining

The morphology of PRF was observed by means of scanning electron microscopy (SEM) (Vega, Tescan, Philadelphia, PA). To prepare the tissue, the PRF was fixed in 2.5% glutaraldehyde for 1 hour, washed with PBS, dehydrated with tertiary butanol, and vacuum dried. All samples were coated with gold using a sputter coater.

The surface structure of PRF was observed using H&E staining. To prepare for staining, PRF tissue was frozen and sectioned on a freezing microtome to 5- to 10-μm-thick sections. Next, the sections were fixed, washed, stained, mounted on slides, and dehydrated.

Cell cytotoxicity

To monitor cell cytotoxicity in vitro, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was developed. Cells were seeded in 96-well plates at a density of 10^3-10^4 cells per well in DMEM containing 10% FCS with PRF for 1, 4, 7, or 11 days as control group and test group. In the last 4 hours of each experiment, 50 μL of the MTT solution was added to each well. The insoluble formazan produced was then dissolved with 150 μL of dimethyl sulfoxide (DMSO). The metabolized MTT was evaluated by optical density (OD) in a UV-visible spectrophotometer microplate reader (Bio-Rad, USA) at 490 nm. For each group, experiments were repeated three times and measurements were performed in triplicate.

Cell proliferation

For cell proliferation studies, each PRF membrane was covered with 5 × 10^4 cells in 60-mm culture dishes for 1, 4, 7, or 11 days. After each

Table 1. Primer sequence and product size of the genes for real-time PCR

Genes	Upstream primer sequences (5'-3')	Downstream primer sequences (5'-3')	Product size (bp)
β-actin	AGAAAATCTGGCACCACACC	GGGGTGTTGAAGGTCTAAA	142
Col I	CCAAGAAGAAGACATCCCACC	CAGATCACGTCATCGCACAA	135
OCN	TCACAGACCCAGACAGAACCG	AGCCCAGAGTCCAGGTAGCG	165
OPN	GGTCACTGATTTTCCCACTGACA	CTATCACCTCGGCCATCATATG	135
RUNX2	AGAATGATGGTGTTGACGCTGAT	GTCGCCAAACAGATTCATCCA	90
ALP	ACTTCAAACCGAGACACAAGCA	GCTCAAAGAGACCCAAGAGGTAGT	102

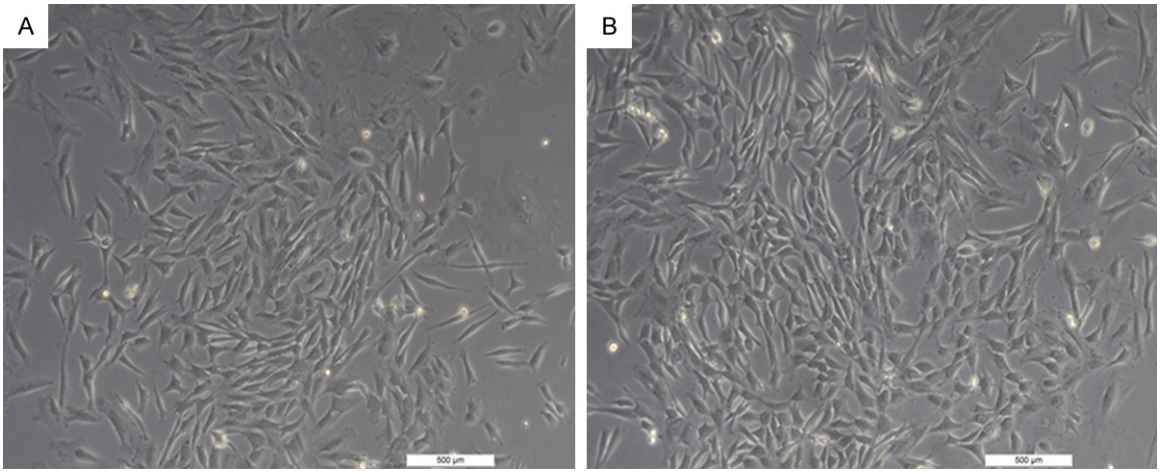

Figure 2. The third generation of two kinds of DPCs observed under inverted microscope. A. Dental pulp cells from exfoliated deciduous teeth (deciduous DPCs); B. Dental pulp cells from permanent teeth (permanent DPCs).

experiment time point, 10 μL trypan blue was added to 10 μL cell suspension and then mixed. Finally, 10 μL of the mix was placed into a hemocytometer chamber, and cell number was determined in a hemocytometer chamber (Cambridge Instruments, Buffalo, NY, USA).

Measurement of ALP activity

Following 1, 4, 7, or 11 days of culture, ALP activity was measured. Cells (without PRF and with PRF in ordinary culture medium and without PRF and with PRF in differentiation medium) were seeded in 96-well plates at a density of 5000 cells per well in DMEM containing 10% FCS. At the end of each treatment time, medium was removed from the wells and replaced by ALP lysis buffer (ALP buffer containing Triton X-100) and the cells were incubated at 37°C for 4-6 hours. The cells were then shocked for 30 minutes and stored at -20°C. A standard curve of 630 nm was obtained by mixing BCA and deionized water. The absorbance of each sample at 405 nm was compared to the standard curve.

Alizarin red staining and quantification of the mineralization nodules

Deciduous and permanent DPCs cultured on 6-well plates for 21 days were stained with Alizarin Red S. Samples were divided into 4 groups: 1) control group (without PRF in normal medium), 2) test group (with PRF in normal medium), 3) test group D (without PRF in differentiation medium), 4) test group D0 (with PRF in differentiation medium). Briefly, cells were stained with Alizarin Red S at room temperature for 20 minutes. After staining, a 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate (pH 7.0) solution was added to each sample and the mixture was incubated at room temperature for 30 minutes. Cells and Alizarin Red were then collected from the 6-well plates, and the mixture was analysed at 405 nm with a spectrophotometric microplate reader.

Quantitative real-time PCR (qRT-PCR)

For each cell type, 16 culture plates (diameter, 60 mm) were used for cultivation for 1, 4, 7, or

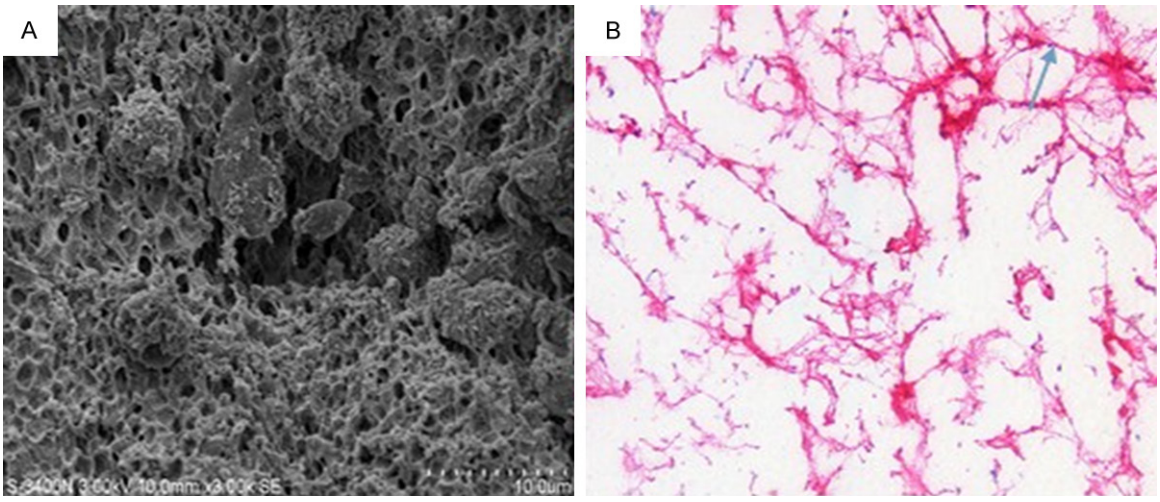

Figure 3. Morphology and structure of PRF. The morphology of PRF was observed by SEM. SEM showed the three-dimensional structure of PRF, densely interwoven into mesh. A. SEM showed that PRF has poor porosity holes and contains lots of white cells. B. HE staining for PRF after the section was frozen, and the arrow showed fibrin network.

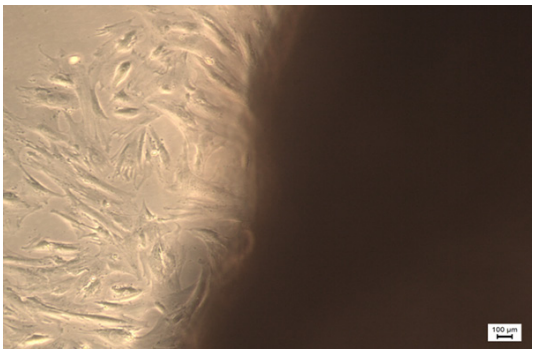

Figure 4. View of co-exist of PRF and DPCs under inverted microscope. When PRF was added to the experimental groups, it was able to co-exist with deciduous and permanent DPCs.

11 days: 4 plates were used as control groups (without PRF in normal medium), 4 were exposed to a PRF membrane, 4 plates were used as test group D (without PRF in differentiation medium) and 4 were exposed to a PRF membrane in differentiation medium. Total RNA was isolated from cultured cells using TRIzol. The RNA samples were then reverse transcribed for first strand cDNA synthesis; the cDNA samples were used for qRT-PCR. PCRs were performed for 22-31 cycles each at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; the primers used in this study are listed in **Table 1**.

Statistical analysis

All assays were repeated 3 times to ensure reproducibility. Data pertaining to the differences in the results for the 2 time intervals were analysed using the paired t-test with the significance set at $P < 0.05$. Statistical analysis was performed using the SPSS software, version 18.0 (SPSS, Chicago, IL, USA).

Results

Cell culture of deciduous and permanent DPCs

Cell fusion can be passaged up to 80%. Deciduous and permanent DPCs are fusiform cells that can proliferate and differentiate at a high rate (**Figure 2**). SEM and H&E staining of PRF clearly demonstrated that a large network of interwoven fibres form a porous support structure, which contains a large number of white blood cells (**Figure 3**). When PRF was added to the experimental groups, it was able to co-exist with deciduous and permanent DPCs, as observed under an inverted microscope (**Figure 4**).

MTT assay for cell proliferation

To examine cell cytotoxicity *in vitro*, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. **Figure 5** demonstrates the effects of PRF on the growth of deciduous and permanent DPCs ($P < 0.05$). PRF showed no toxic effects on these cells. PRF significantly increased the proliferation rate of deciduous DPCs after 7 and 11 days of incubation, which reached 2.0, 2.8, 2.5 and 3.0 times that for controls ($P < 0.01$). Even without PRF

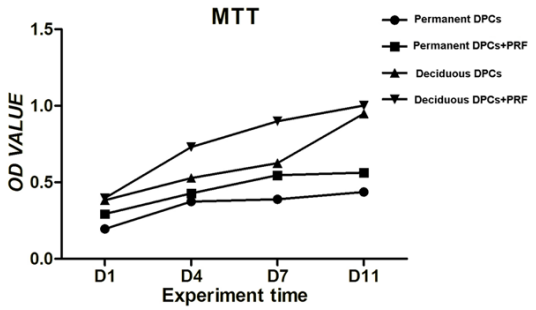

Figure 5. MTT assay for DPCs proliferation. PRF significantly increased the growth of either deciduous or permanent DPCs after 1, 4, 7 and 11 days of incubation.

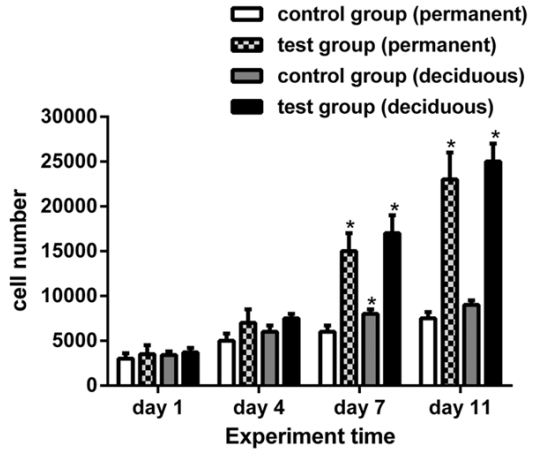

Figure 6. The cell number of DPCs at 4 time points after incubation. PRF significantly increased the proliferation rate of either deciduous or permanent DPCs after 1, 4, 7 and 11 days of incubation.

incubation, the proliferation rate of deciduous DPCs was significantly higher than that of DPCs (**Figure 6**).

Analysis of ALP and calcium nodules

We performed semi-quantitative experiments for ALP. We found that PRF increased ALP activity of deciduous and permanent DPCs compared to that for the control group ($P < 0.05$; **Figure 7A**). In particular, at incubation days 7 and 11, these DPCs showed the most pronounced increase in ALP ($P < 0.01$).

Next, to examine calcium nodules, cells were stained with Alizarin Red S. All DPCs showed significantly higher extraction volumes of Alizarin Red after incubation with PRF as compared to the controls (**Figure 7B**, $P < 0.05$). In addition, deciduous cells developed significantly more calcium nodules than permanent cells, indicative of higher osteogenic activity ($P < 0.01$).

qRT-PCR detection of osteoblast-related genes

Quantitative RT-PCR was performed in cultured cells following 1, 4, 7 and 11 days incubation to further investigate mRNA expression of osteoblast-related genes. We analysed collagen type I (Col I), osteocalcin (OCN), osteopontin (OPN), runt related transcription factor 2 (Runx2), and ALP (**Figure 8**). Overall, deciduous DPCs showed higher mRNA expression of bone differentiation related-genes than permanent DPCs ($P < 0.01$), expressed as a quantitative value higher bone differentiation related genes; experimental group after joining PRF-related gene expression of the osteoblast has raisen and higher. We note that, compared with the experimental group and control group the expression of various genes (except OPN) 7 d of the experiment reached a peak.

Discussion

Tumours, alveolar bone trauma, congenital disorders, and tooth loss often cause absorption of the craniofacial bone, resulting in deformities and dysfunctions. Autogenous bone grafts are used as the gold standard for repair of bone defects. However, depending on the region of bone loss as well as secondary lesions, the application of this technique can pose certain restrictions. Ultimately, the goal of craniofacial reconstruction is to mimic the reconstruction and regeneration characteristics of bone and its morphogenetic and physiological functions [5-7]. Twenty years of unremitting research in bone tissue-engineering technology has allowed us to get closer to this goal.

Platelets contain a large number of autologous growth factors such as TGF-β, PDGF, VEGF, and IGF, which are essential in bone healing. Moreover, platelets secrete fibrinogen, an interwoven protein that promotes the migration of mesenchymal cells when tissue is regenerating [4, 8-10]. PRF is a biological material rich in white blood cells and platelets. Indeed, PRF has been deemed the “second generation platelet concentrate” [11]. Its production process does not require the addition of thrombin and a calcium chloride activator; it is simply extracted by the process of centrifugation to form a suitable three-dimensional fibrin mesh stent. This struc-

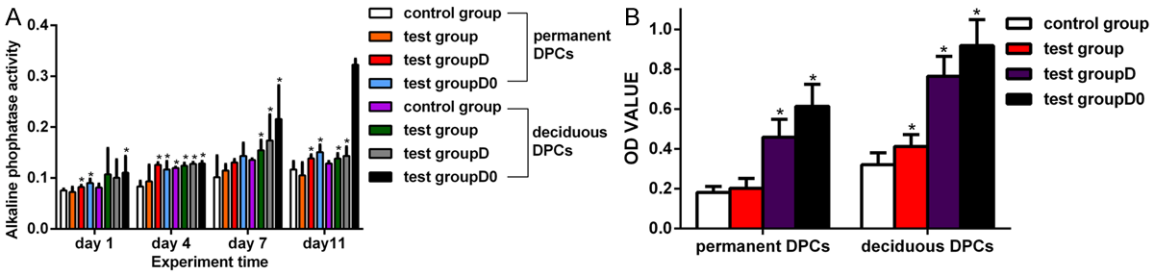

Figure 7. Differentiation of deciduous or permanent DPCs in primary cultures under 4 different conditions: proliferation (control group), proliferation with 1 PRF membrane (test group PRF), differentiation culture conditions (test group D), and differentiation conditions with PRF (test group D0). A. ALP was quantified. B. Mineralization nodules were examined after Alizarin Red S Staining.

ture enables PRF to mitigate cell migration and retain various cytokines to aid in cell growth and tissue restoration [12]. Gassling *et al.* [13] was the first to compare PRF with a collagen membrane scaffold and to show that PRF can improve osteoblast migration, proliferation, and differentiation.

Permanent and deciduous dental pulp cells are coming in the development of dental pulp cells can be used as seed cells for tissue engineering, as they can separate by self-proliferation and have multiple differentiation potentials. Typically, the use of deciduous DPCs is preferred to that of permanent DPCs because they have higher proliferation and differentiation rates and acquiring deciduous DPCs is a simpler clinical process that imposes only minor trauma [14].

In this study, we used centrifugation to polymerize PRF and collect it as a scaffold protein. We examined the interaction of PRF with deciduous and permanent DPCs *in vitro* and demonstrate that, in the presence of PRF, both cell types show increased proliferation after 7 and 11 days of incubation. We also showed that PRF reliably increases the metabolic capacity of both cell types after 4 days of incubation. A significant effect of PRF on DPC proliferation has also been shown by X et al. Therefore, PRF plays a key role in stimulating the proliferation of DPCs. In addition, we show that PRF increases the expression of bone-related differentiation genes, including COL 1, in both cell types, suggesting that PRF stimulates differentiation of both deciduous and permanent DPCs, and may stimulate early osteogenesis. Increased expression of these bone-derived genes as a result of PRF exposure has also been shown by X et al. It is important to note that ALP is a mature osteoblast differentiation marker and reflects the level of bone activity. However, our results show that, in the presence of PRF, alkaline phosphatase activity was generally higher than that for controls, especially on days 7 and 11. We believe that the release cytokines of PRF and osteogenic medium have synergy, in addition, PRF continuing role to maintain at least 11 d. Finally, we showed that PRF exposure increased the number of calcium nodules, especially in deciduous DPCs, suggesting that deciduous cells are more easily induced to mineralization by PRF.

Overall, we found various differences between deciduous and permanent DPCs. When compared to DPCs, deciduous cells showed significantly greater proliferation and cell metabolism as a response to PRF. In addition, they showed greater self-differentiation, suggesting that deciduous DPCs are more responsive to the various cytokines that are slowly released in the presence of PRF (i.e. TGF-β, PDGF, and VEGF). Based on these results, we can propose that deciduous DPCs have increased ability to differentiate into bone tissue and would be a better candidate for bone tissue engineering.

In this study, we provide in depth information demonstrating the use of scaffold composite seed cells for tissue engineering of bone. We show that PRF potently stimulates the proliferation and differentiation of both deciduous and permanent DPCs, albeit deciduous DPCs appear to be a more suitable source of seed cells in bone as they are more responsive to the effects of PRF. Given that the combination of PRF and deciduous DPCs exhibits superior osteogenic capability, future studies should focus on engineering techniques to prepare a composite consisting of PRF and deciduous DPCs,

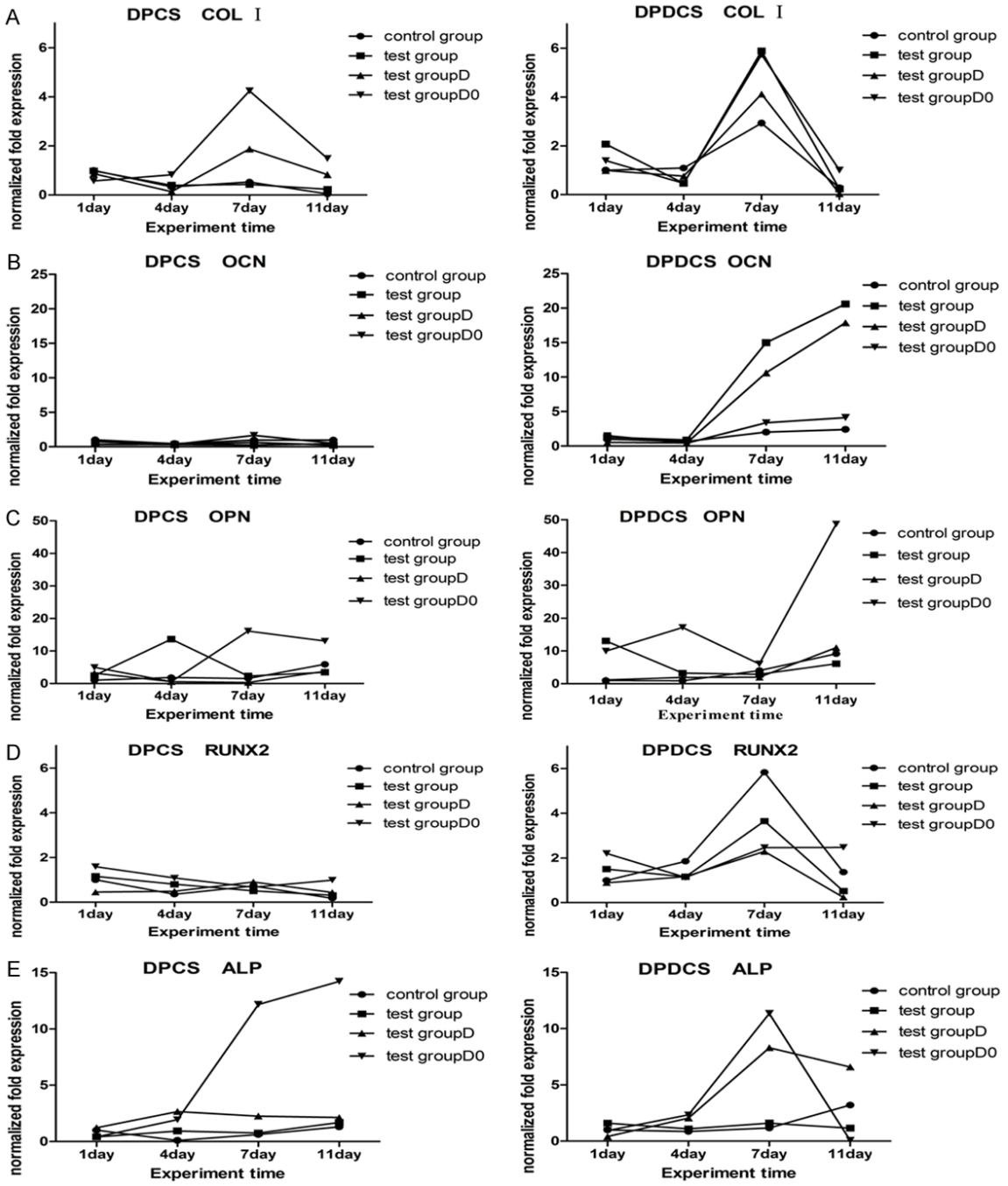

Figure 8. The mRNA expression of osteoblast-related genes and comparisons of gene expression of (A) COL-I, (B) OCN, (C) OPN, (D) Runx2 and (E) ALP after 1, 4, 7, 11 days of incubation.

which can be used to repair complex bone defects and/or in bone tissue regeneration.

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Disclosure of conflict of interest

None.

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