Dentin Phosphophoryn Promotes Odontoblast Differentiation *in vitro* and Induction of Mineralized Matrix *in vivo.*

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Summary

Vital pulp therapy is the method for the treatment of reversible pulpitis**.** The ultimate goal of vital pulp therapy is to rapidly regenerate dentin of excellent quality by using an external agent that possesses novel properties such as biocompatibility and bioactivity.

Dentin phosphophoryn (DPP) is the most abundant of the non-collagenous polyanionic phospho proteins in dentin. The purpose of this study was to examine the effects of DPP on differentiation and mineralization of odontoblasts. MDPC-23, a rat odontoblast-like cell line was used in this study *in vitro* and to investigate mineralized-matrix induction ability of DPP *in vivo*. The cells were cultured with DPP at different concentrations (0, 0.1, 1, and 10 µg/ml). The cell-morphology and proliferation were evaluated. Furthermore, cells were analyzed for mRNA expression of dentin/bone-related proteins by RT-PCR. Moreover, ALPase activity and Alizarin red staining were performed for confirmation of mineralization induced by DPP. The addition of DPP did not affect on proliferation or morphology of MDPC-23. The mRNA expressions of DMP-1 and ALPase were promoted by 0.1, 1 and 10 µg/ml of DPP. Moreover, the mRNA expressions of Osteorix, BSP and OCN were promoted by 1 and 10 µg/ml of DPP but Runx2 and OPN expressions were prominent in case of 10 µg/ml of DPP. The high ALPase activity in MDPC-23 was induced by 1 and 10 µg/ml of DPP. The number of mineralized nodules was higher by addition of 1 and 10 µg/ml of DPP at 7 days. Mineralized –matrix induction was observed after 14 days of implantation of DPP-collagen and RPCs composites on the dorsal side of rat *in vivo.*

This study showed that DPP promotes the differentiation and mineralization of odontoblasts *in vitro* and induction of mineralization *in vivo*. Therefore DPP can be a promising candidate for formulating a new pulp capping material.

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I. Introduction

Preserving natural dental structure is an enormous challenge for contemporary dentistry. Dental structure comprises a complex composition of different specialized tissue and cell types consisting of dentin-producing odontoblasts, ameloblasts and periodontal structures such as cementum, periodontal-ligaments, gingiva and alveolar bone (Mjör et al., 2001). It has been reported that pulp tissue has various functions, such as sensorial reception and communication, nutrition supply to dentin, formation of new (transparent and reparative) dentine and providing immunological defense against bacterial attack (Pashley et al., 1996). However, vital pulp can be exposed by deep seated caries, trauma and tooth preparation procedures. Restorative dentistry relies on the use of medicaments, capping procedures or root canal fillings for exposed or damaged pulp (Murray et al., 2001). Vital pulp therapy is a method for the treatment of reversible pulpitis and the objective of vital pulp therapy is to maintain the vitality of pulpal tissue, including sealed restoration to maintain tooth function (Tziafas et al., 2000). Moreover, the ultimate goal of vital pulp therapy is to rapidly regenerate dentin of excellent quality by using an external agent that possesses novel biocompatibility and bioactivity.

Calcium hydroxide-based agents have been considered to be the gold standard of direct pulp capping materials and numerous studies have shown that $Ca(OH)_2$ should be the material of choice (Iwamoto et al., 2006; Quidement et al., 2007; Tuna and Olmez, 2008) for several decades because of favorable effects such as antibacterial activity and hard tissue stimulation despite its cytotoxic effects in cells (Schrüder et al., 1985). However, due to the high alkalinity (pH 11 to 12) of these agents, they are thought to accelerate the necrosis of pulp tissue. In addition, these agents do not provide close adaptation to dentin or promote consistent odontoblast-differentiation, and it has been demonstrated in cell culture that the cytotoxic effects result in tunnel defects in newly produced reparative dentin (Schrüder et al., 1985; Cox et al., 1996). Tunnel defects in reparative dentine can open a pathway for micro-pathogens to initiate secondary infection of pulp tissue. Studies have shown that mineral trioxide aggregate (MTA) may be used as an alternative to calcium hydroxide for treating damaged or exposed pulp (Min et al*.,* 2008; Eskanderizadeh et al., 2011). Good sealing and excellent marginal adaptation are considered to be the novel properties of this agent (Whiterspoon et al*.,* 2008, Okiji et al*.,* 2009). It has been reported that MTA stimulates formation of dentin bridges faster than

calcium hydroxide, consequently leading to pulp healing, and results in high success rates in clinical procedures (Accorinte et al., 2008; Nair et al*.,* 2008; Whiterspoon et al*.,* 2008; Okiji et al*.,* 2009; Zarrabi et al., 2010), but other publications have reported no significant differences in clinical and histologic results between MTA and calcium hydroxide (Iwamoto et al*.,* 2006). In addition, two randomized controlled studies have shown that MTA may result in similar clinical outcomes as calcium hydroxide after capping caries pulp exposure (Quidement et al*.,* 2007; Tuna and Olmez, 2008). However, it has also been noted to maintain high pH and to require some time to induce reparative dentin formation, similarly to calcium hydroxide-based agents. Moreover, MTA is reportedly difficult to use because of its long setting time, poor handling properties, high material costs and discoloration potentials of dental tissue (Damamaschke et al*.,* 2005; Parirokh and Torabinejad, 2010). Thus, the clinical success rate of direct pulp capping using calcium hydroxide-based agents and MTA does not satisfy dental practitioners. A biocompatible, bioactive agent that induces excellent reparative dentin formation more rapidly is thus required to improve the clinical success of vital pulp therapy.

Dentin phosphophoryn (DPP) is the generic name of a group of compositionally unique compounds; acidic, highly phosphorylated proteins that are a ubiquitous component of the dentin extracellular matrix (Stetler-Stevenson and Veis, 1983; Sabsay et al., 1991). Moreover, DPP is a cleavage product of the larger dentine sialophosphoprotien (DSPP), a member of the SIBLING (Small Integrin Binding Ligand N-linked Glycoproteins) family of proteins, and SIBLING is the major group of non-collagenous proteins in bone and dentin (Feng et al., 1998; George et al., 1999; Yamakoshi et al., 2005; Yamakoshi et al., 2006; Yamakoshi et al., 2008). Members of the SIBLING family share common traits such as RGD integrin-binding domains and casein kinase II phosphorylation sites. DPP contains numerous aspartic acids and phosphorylated serine residues that render this protein the most acidic in nature. The molecule is thus considered to be a “phosphate carrier” (Veis, 1988; Sfeir and Veis, 1995; George et al*.,* 1996). Phosphorylation of all potential serine residues results in a highly negatively charged molecule and is a virtual sink for the binding of calcium ions, an important initiator-modulator of the formation and growth of hydroxyapatite (HA) crystals due to its many negatively charged regions (Boskey et al., 1990; Saito et al., 1997). Apart from its role in

mineralization, DPP has also been shown to actively participate in cell signaling events leading to differentiation of precursor mesenchymal cells (Jadlowiec et al., 2004; Jadlowiec et al., 2006; Eapen et al., 2012). It has recently been reported that the RGD domain in extracellular DPP induces integrin-mediated intracellular signaling events that are propagated to the cell nucleus and thus alter gene activity (Eapen et al., 2012). Other published reports have demonstrated activation of the MAP kinase and SMAD signaling pathway when human mesenchymal cells are stimulated with DPP (Jadlowiec et al., 2004; Jadlowiec et al., 2006). Thus, it can be postulated that DPP induces mineralization as follows (Jadlowiec et al., 2004): (1) DPP independently binds to HA through its highly acidic and anionic residues; (2) DPP directly regulates bone/dentin related genes; or (3) a combination of these two actions.

As mentioned above, DPP is a component of dentin extracellular matrix, and induce osteogenic differentiation. There is, however, little information on induction of dentinogenesis by DPP. Recently, it was demonstrated that DPP promotes-cell migration by acting on integrin on the cell surface of pulp cells via its RGD motif *in vitro* (Yasuda et al., 2008). It is still not known how DPP promote odontoblast differentiation. Therefore, the aims of this study were to investigate

the effects of DPP at different concentrations on odontoblast differentiation *in vitro* and to investigate the DPP on induction of mineralized tissue formation *in vivo*.

II. Materials and Methods

1. DPP collection and purification

DPP was prepared from molar teeth extracted from 8-9-months-old porcine jaws obtained from a local abattoir. In accordance with the method described by Butler (Butler, 1987), porcine dentin was powdered (400-700 μm diameter) after immersion in liquid nitrogen. Powdered dentin was decalcified at 40°C with 0.5 mol/L ethylenediaminetetraacetic acid and 0.05 mol/L Tris-HCl (pH 7.4), containing protease inhibitors (100 mmol/L, 6-aminohexanoic acid, 5 mmol/L benzamidine-HCl, and 1 mM phenylmethylsulnyl fluoride, Sigma Chemical, St. Louis, MO). The extract was dialyzed against distilled water and lyophilized. DPP was extracted with the calcium precipitation method followed by diethylaminoethyl-cellulose column chromatography (Amersham Biosciences, Piscataway, NJ). DPP was mixed with distilled water at 1 mg/ml for this

experiment.

2. *MDPC-23 cell culture*

The MDPC-23 cell line that was provided by Professor Nör, University of Michigan, was used in this study. This cell line was established by removing the dental papillae from 18-19-days fetal rat, and expresses odontoblastic phenotype over passage to passage (Botero et al., 2012). MDPC-23 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Irvine,UK) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37°C until reaching about 80% confluence. Medium was changed at 2-day intervals.

3. Observation of cell morphology

Cells were seeded in 35-mm culture dishes at a density of 5×10^4 cells/dish, and were incubated in the presence of various concentrations of DPP (0.1, 1 and 10 μg/ml, and 0 μg/ml DPP as a control) for 5 days, DPP addition was started from the day after seeding, and was considered to be Day 0. Cell morphology was observed for 1 to 5 days using a microscope (Olympus1X70;

Olympus,Tokyo).

4. Measurement of cell proliferation

Cells were seeded (5×10^4 cells/dish) in 35-mm culture dishes and were incubated in the presence of DPP with various concentrations (0, 0.1, 1 and 10 μg/ml) for 5 days, and DPP addition was started from the day after seeding. Cell proliferation was evaluated by cell number counted using a hemocytometer after trypsinization of cells from day 1 to 5 and statistical analysis was performed.

5. Cell differentiation and calcification

1) Gene expression

Cells were seeded (1×10^5 cells /dish) in 60-mm culture dishes and were incubated. After reaching confluence, DPP with various concentrations (0, 0.1, 1 and 10 μg/ml) was added for 3 days (n=3). After 3 days, cells were collected with TRIzol reagent (Invitrogen). Total cellular RNA was reverse-transcribed using M-MLV (Invitrogen) to synthesize cDNA according to the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out to examine mRNA expression of dentinogenesis/osteogenesis-related genes, such

as dentin matrix protein-1 (DMP-1), alkaline phosphatase (ALPase), osteorix, Runt-related transcription factor2 (Runx2), bone sialoprotein (BSP), osteocalcin (OCN), osteopontin (OPN), and type I collagen (type I col).The housekeeping gene, β-actin, was measured as a control. Primer sequences for all genes are shown in the Table 1. For RT-PCR, PCR conditions are shown in Table 2, and amplification was confirmed in 2% agarose gels. In addition, final PCR products on 2% agarose gels migrated as a single band with a sequence identical to that of the protein.

2) ALPase activity

A total of 5×10^4 cells were seeded in 35-mm culture dishes, and were incubated in the presence of DPP with various concentrations (0, 0.1, 1 and 10 μg/ml) for 7 days. DPP addition was started from the day after seeding, and was considered as Day 0. Medium was changed at 2-day intervals. The experiment was performed three times separately. Samples were collected by mechanically peeling on (day 3, 5 and 7) with 0.1% TritonX (Sigma-Aldrich) in water. Collected cells were sonicated and centrifuged. Supernatants were measured by using a Lab Assay ALP kit (WAKO, Osaka) in accordance with the manufacturer’s

instructions.

3) Alizarin red staining

Cells (5×10^4 cells /well) were seeded in a 24-well culture plate (collagen type I coated; Iwaki, Tokyo) and were incubated with DPP at various concentrations (0, 0.1, 1 and 10 μg/ml) with or without osteogenic agents (10 nM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid) for 7 days under the conditions described. DPP addition was started from the day after seeding and was considered Day 0. Medium was changed at 2-day intervals. After discarding medium, cells were fixed with 10% formalin and stained with 2% Alizarin-red S (WAKO). Staining was observed with a microscope (Olympus1X70). Nodules were counted with image J (Wayne Rashand, MD, USA) software, and statistical analysis was performed.

6. Implantation of composites of DPP-collagen and rat pulp cells into rats

1) Rat pulp cells collection and culture

The protocol of this animal experiment was reviewed and approved by the Health Sciences University of Hokkaido Animal Research Ethics Committee.

A 7-week-old male Wister rat (Hokudo, Sapporo) was sacrificed and lower incisor teeth were collected to obtain pulp tissues. Pulp cells were collected with 3 mg/ml bacterial collagenase (Roche Diagnostics, Basel, Switzerland) digestion (Handa et al., 2002) and were cultured as rat pulp cells (RPCs).

2) DPP-collagen scaffold composite preparation:

Type I atelo-collagen fibrils, from which telopeptides known to be antigens had been removed, were used as a DPP carrier in this study. DPP was cross-linked to porcine-derived type I atelo-collagen fibrils (Collagen sponge, Niitta Gelatin, Osaka) with divinylsulfone (Sigma) (Lihme et al., 1986). To remove any DPP that was not covalently bound, substrates were washed with 0.5 M NaCl and 0.05 M tris-HCl (pH 7.4) ten times, and were washed with distilled water.

3) Implantation into rats

First, composites of 1mg DPP and 5mg collagen were adsorbed with RPCs (1×10^5 cells). After 14 days of incubation, we performed the following implant experiments. We used 10 male Wister rats aged 8 weeks. For

experimental groups, we prepared DPP-collagen scaffold composites with RPCs. As a control, only collagen scaffold with RPCs were used. Rats were anesthetized using an intra-peritoneal injection of pentobarbital (40 mg/kg) and samples were implanted at four sites of the dorsal surface of each rat. Among four sites, two were for experimental samples and other two were for control. Rats were sacrificed at 2 weeks after implantation. After collection, scaffolds were fixed with 10% formalin solution, and were immersed in decalcifying solution A (Wako) for 24 hours. Samples were then embedded in paraffin. We prepared 4-μm sections, and performed hematoxylin-eosin (HE) staining. Histological observations were performed by microscopy.

7. *Statistical analysis*

All numerical data are given as means ± SD. The significance of changes in responses was assessed using Tukey-Kramer test. Statistical significance was set at $P<0.05$.

III. Results

1. MDPC-23 morphology and proliferation

On microscopic observation, it was found that the addition of DPP did not affect the morphology of MDPC-23 (Fig. 1), while cell number data confirmed that DPP did not affect cell proliferation (Fig.2). It was, however, observed in our pilot study that high concentration of DPP such as 50, 100 μg/ml of DPP caused cytotoxic effects to the cell (data not shown).

2. MDPC-23 differentiation and calcification induced by DPP

1) DPP induced osteoblast/odontoblast gene markers

We assessed the expression of several osteoblast/odontoblast gene markers in response to DPP treatment in cells. The mRNA expression of DMP1 and ALPase in MDPC-23 was promoted by 0.1, 1 and 10 μg/ml DPP at 3 days (Fig. 3). The mRNA expression of osteorix, OCN and BSP was promoted by 1 and 10 μg/ml DPP at 3 days (Fig. 3). Moreover, mRNA expression of Runx2 and

OPN was promoted by 10μg/ml of DPP (Fig. 3). There was no change in the mRNA expression of Type I col by the stimulation with DPP (Fig.3).

2) ALPase activity in MDPC-23

In order to further examine the function of DPP in osteogenic and dentinogenic lineage progression, we examined ALPase activity, which is a common phenotypic marker for osteogenesis and dentinogenesis. ALPase activity was measured over a time course of 3, 5 and 7 days. We observed that high ALPase activity in MDPC-23 was induced by 1 and 10 μg/ml of DPP at 3, 5 and 7 days as compared to controls ($p<0.05$) (Fig. 4).

3) DPP increased calcium deposition in MDPC-23

When MDPC-23 cells were cultured with differentiation medium containing 1 and 10 μg/ml DPP, increased Alizarin red S staining (Fig. 5A) and on quantitative analysis, the number of mineralized nodules was increased at 7 days, as compared to controls ($p<0.05$) (Fig. 5B). Moreover, a similar trend was observed for alizarin red S staining, without the addition of differentiation medium containing 0.1, 1 and 10 μg/ml of DPP, increased Alizarin red Staining

(Fig. 5C) and on quantitative analysis, the number of mineralized nodules was Increased at 7 days, as compared to controls ($p<0.05$) (Fig. 5D).

3. Implantation of composites of DPP-collagen and RPCs

In order to investigate mineralized-matrix inducibility for DPP, we performed implantation experiments *in vivo.* Mineralized tissue-like matrix formation was induced by composites of DPP-collagen and RPCs, and rapid degradation of collagen was observed, as compared to controls, at 14 days after implantation into dorsal sites in rats (Fig. 6). Moreover, there was absence of inflammatory cells in the HE staining.

IV. Discussion

In the present study, we firstly demonstrated that the DPP promoted odontoblast differentiation of odontoblastic cell line, MDPC-23. For establishing the exact efficacy of DPP in odontoblast differentiation *in vitro*, stable cell is a pre-condition and for this reason MDPC-23 cell line was used in this study. MDPC-23 line was established by removing the dental papillae from 18-19-days fetal rat. This cell is spontaneously immortalized and expresses odontoblastic

phenotype over passage to passage (Botero et al., 2012). Moreover MDPC-23 is a homogeneous and stable cell line compared to rat dental pulp stem cells that are widely reported as heterogeneous. MDPC-23 cells were cultured in the presence of DPP at various concentrations (0, 0.1, 1 and 10 μg/ml) in order to elucidate the *in vitro* effects of DPP on odontoblast differentiation. Evaluation of osteogenic/dentinogenic marker genes showed that 0.1,1 and 10 μg/ml of DPP enhanced the expression DMP-1. DMP-1 is a member of the SIBLING family, is a specific marker of dentin and is an acidic phosphorylated extracellular protein (George et al., 1993). It is expressed in odontoblasts that secrete matrix protein for the formation of dentin. *In vitro* studies have reported that overexpression of DMP-1 induces differentiation of mesenchymal cells to odontoblast-like cells and enhances mineralization (Narayanan et al., 2001), and that DMP-1 is able to bind to Ca^{2+} to initiate mineral deposition (He et al., 2003), which is crucial for dentin formation. Therefore, DPP may be a signaling molecule involved in the dentin formations.

Next, mRNA expression of osteorix, Runx2, BSP, OCN, OPN and type 1 col was assessed after stimulated by DPP. It was found that 1 μg/ml and 10 μg/ml of DPP promotes mRNA expression of osteorix, BSP and OCN. The

mRNA expressions of Runx2 and OPN were promoted by 10 μg/ml of DPP. DPP binds integrin receptors on the surface via its RGD domain, activating the MAPK signaling pathway that culminate in a mature osteoblast characterized by expression of Osteorix, RUNX2 and OCN (Jadlowiec et al., 2004). Our results suggest that DPP may bind integrin receptor via its RGD domain on the cell surface of MDPC-23. It has been reported that that DPP induces mineralization as follows: (1) by binding to hydroxyapatite (HA) via its highly acidic and anionic residues; (2) by regulating bone/dentin genes via the MAPK and SMAD pathways; or (3) through a combination of these two functions (Jadlowiec et al., 2004, Jadlowiec et al., 2006).Thus, the evidence of significant up-regulation of DMP-1 and high expression of other genes confirms the strong effects of DPP on odontoblast differentiation, which can lead to new dentin formation. ALPase activity is a common phenotypic marker for osteogenesis and dentinogenesis. Both ALPase gene expression and activity were promoted in the presence of 0.1, 1 and10 μg/ml DPP, indicating that DPP also promote odontoblast differentiation at protein level.

It was also shown that DPP increases calcium deposition in MDPC-23 in the presence of differentiation medium and also in the presence of normal

medium with stronger staining and a significantly higher number of nodules in the experimental group when compared to controls. These data reinforce the above questions about the role of DPP in signaling and mineral deposition. It is therefore reasonable to believe that DPP possesses a potent ability for odontoblast differentiation and mineralization consistently *in vitro*.

DPP, a synthetic product of odontoblasts, accumulates at the mineralization front of dentin matrix, and is strongly associated with the mineral phase (Butler, 1995). Our *in vivo* study demonstrated that RPCs/collagen/DPP composites form mineralized tissue-like matrix 2 weeks after implantation without the presence of inflammatory cells. However, it was not known whether the mineralized tissue-like matrix contained dentin matrix. Further experiment is necessary to categorize this formed mineralized tissue-like matrix such as by using different antibody staining assay. High potential of DPP acts as a co-factor of rhBMP-2 in orthotopic hard tissue induction. The DPP/collagen composite was an effective carrier of rhBMP-2 due to its biodegradability (Saito et al., 2004). The present *in vivo* study revealed the rapid biodegradation of collagen fibrils in RPCs/collagen/DPP composites. The rapid biodegradation may be due to the presence of DPP.

The formation of dentin, dentinogenesis, comprises a complex interplay between several factors at both intercellular and extracellular in the tissue. Thus, extracellular stimulation from direct pulp capping agents is crucial for differentiation of exposed pulp cells into odontoblasts in order to initiate the dentinogenesis, while mineralizing potential is vital for extracellular stimulant molecules. The present study indicates that extracellular DPP may be useful for therapeutic agent of direct pulp capping. It is, however, very crucial to explore many unidentified factors before DDP will be applied for human. We used DPP isolated from porcine teeth and the DPP may contain unfavorable factors. Also, it is difficult to obtain huge amount of DPP for therapeutic use. Other methods such as artificial synthesis may be required.

V. Conclusion

The findings of the present study suggest that DPP promotes the differentiation and mineralization of odontoblasts. Therefore, DPP is a promising biocompatible pulp capping material for the future. Further investigations are required in order to elucidate the detailed properties of DPP in order to utilize this molecule both effectively and efficiently.

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Figure legends.

Fig.1 Morphology of MDPC-23 cells.

MDPC-23 cells were incubated with DPP. Representative photograph showing unchanged morphology of MDPC-23 cells after addition of DPP at various concentrations (original magnification ×40) up to 5 days.1:w/o DPP, 2: 0.1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4:10 μg/ml of DPP.

Fig.2 Proliferation of MDPC-23 cells.

MDPC-23 cells were incubated with DPP. Representative figure, showing the unchanged proliferation of MDPC-23 after addition of DPP in different concentration up to 5days.1:w/o DPP, 2: 0.1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4:10 μg/ml of DPP.

Fig.3 Bone/Dentin related-gene expression of MDPC-23 stimulated by DPP.

RT-PCR analysis for DMP-1, ALPase, Osteorix, Runx2, BSP, OPN, OCN, Type I collagen and β-actin were obtained. DMP1 and ALPase expression were prominent incase of 0.1,1, and 10 μg/ml of DPP. Moreover, Osteorix, BSP and OCN, expressions were prominent in case of 1 and 10 μg/ml of DPP but Runx2

and OPN expression was prominent in presence of10 μg/ml of DPP. There was no change in the mRNA expression of Type I col by the stimulation with DPP. 1: w/o DPP, 2: 0.1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4:10 μg/ml of DPP.

Fig.4 ALP activity in MDPC-23 with DPP after 3, 5 and 7 days.

MDPC-23 cells with DPP at various concentrations (0, 0.1, 1 and 10 μg/ml) added from the day after seeding. ALP activity was calculated as units/mg total protein in cell lysates (means ±SD; n=3). a~f indicate significant differences ($P<0.05$) between the same symbols.1: w/o DPP, 2: 1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4:10 μg/ml of DPP.

Fig. 5 Alizarin red staining of MDPC-23 after 7 days.

MDPC-23 cells were cultured with differentiation agents and DPP. A: alizarin red staining for calcium. B: quantification of calcium deposit nodule by using Image J (means ±SD; n=3). * indicates significant differences ($P<0.05$) between the same symbols.MDPC-23 cells were cultured with Normal Medium (10%FBS) and DPP. C: alizarin red staining for calcium. D: quantification of calcium deposit nodule by using Image J (means ±SD; n=3). * indicates significant differences ($P<0.05$)

between the same symbols.1: w/o DPP, 2: 0.1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4: 10 μg/ml of DPP.

Fig.6 Histological observation of mineralized tissue-like matrix formation

A: Photomicrograph of RPC/collagen composites at 2 weeks after implantation. Unchanged (intact) collagen fibril. B: Photomicrograph of the DPP-collagen and RPCs composite showing mineralized tissue-matrix formation (arrow), followed by rapid degradation of collagen fibrils.

Table 1: Polymerase chain reaction (PCR) amplification primer sets.

Gene	Sequence (5’-3’)
DMP-1	Forward: cggctggtggtctctctaag Reward: catcactgtggtggtccttg.
ALPase	Forward: ggaaggaggcaggattgaccac Reward: gggcctggtagttgttgtgagc
Osteorix	Forward: ggaaggaggcaggattgaccac Reward: gggcctggtagttgttgtgagc
Runx2	Forward: ccaccactcactaccacacg Reward: ggacgctgacgaagtaccat
BSP	Forward: ctgctttaatcttgctctg Reward: ccatctccattttcttcc
OPN	Forward: tttccctgtttctgatgaacagtat Reward: ctctgcttatactccttggactgct
OCN	Forward: agctcaaccccaattgtgac Reward: agctgtgccgtccatacttt
Type I Col	Forward: agaatatgtatcaccagacg Reward: cagctgatttctcatcatag
β-actin	Forward: aaccctaaggccaacagtgaaaag Reward: tcatgaggtagtctgtgaggt

Table 2 : Condition of RT- PCR

Target cDNA	PCR conditions and cycles number
DMP-1	94°C 1min, (94°C, 30sec, 52°C 30sec, 72°C 1min): 35 cycles
ALpase	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
Osteorix	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
Runx2	94°C 1min, (94°C, 30sec, 57°C 30sec, 72°C 1min): 30 cycles
BSP	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
OPN	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
OCN	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
Type1 collagen	94°C 1min, (94°C, 30sec, 55°C 30sec, 72°C 1min): 35 cycles
β-actin	94°C 1min, (94°C, 30sec, 48°C 30sec, 72°C 1min): 35 cycles

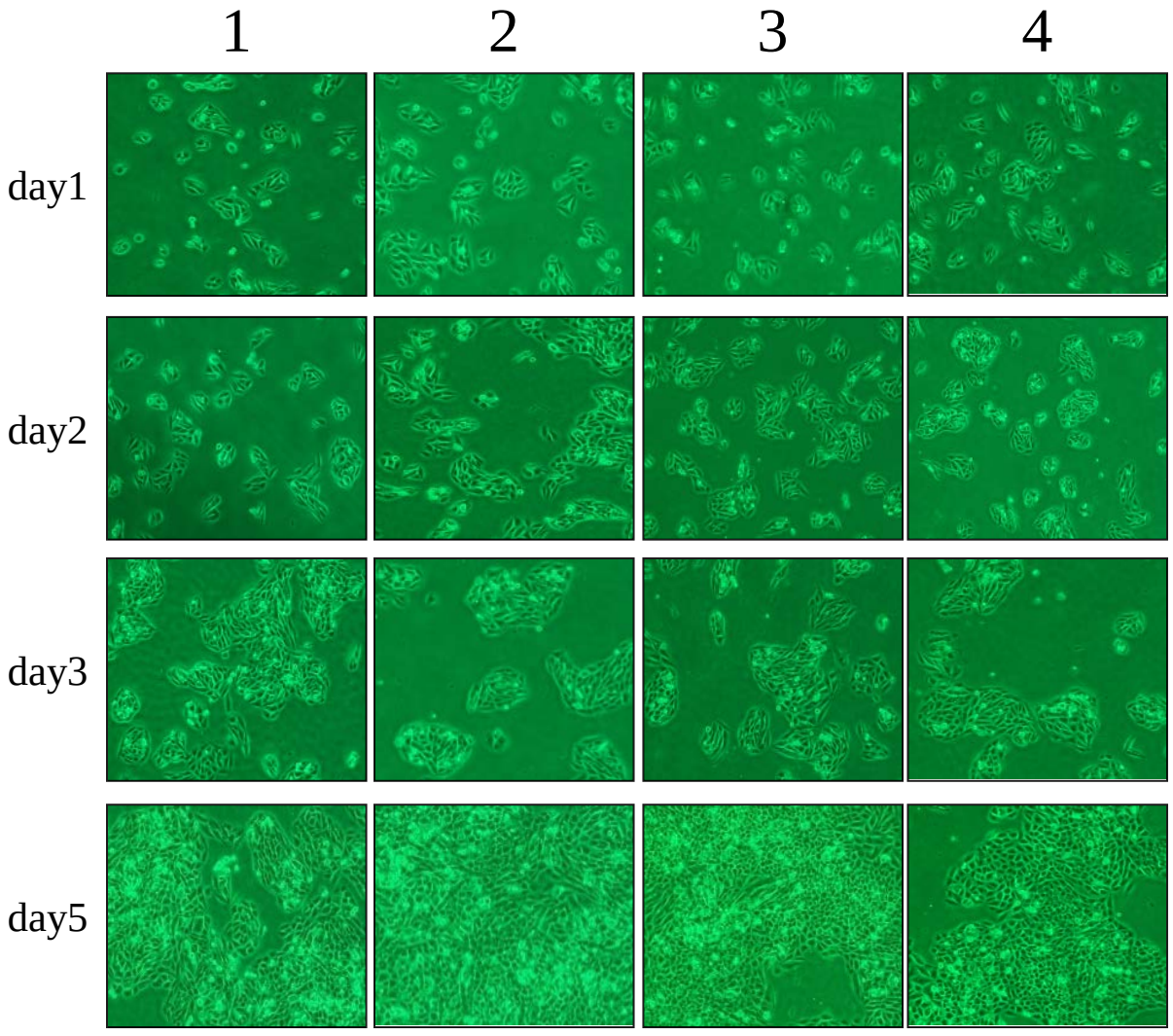


Fig.1 Cell morphology of MDPC-23.
MDPC-23 cells were incubated with DPP. Representative photograph, showing the unchanged morphology of MDPC-23 after addition of DPP in different concentration (Original magnifcationX40) up to 5days.
1:w/o DPP, 2: 0.1μg/ml of DPP, 3 :1.0μg/ml of DPP, 4: 10μg/ml of DPP

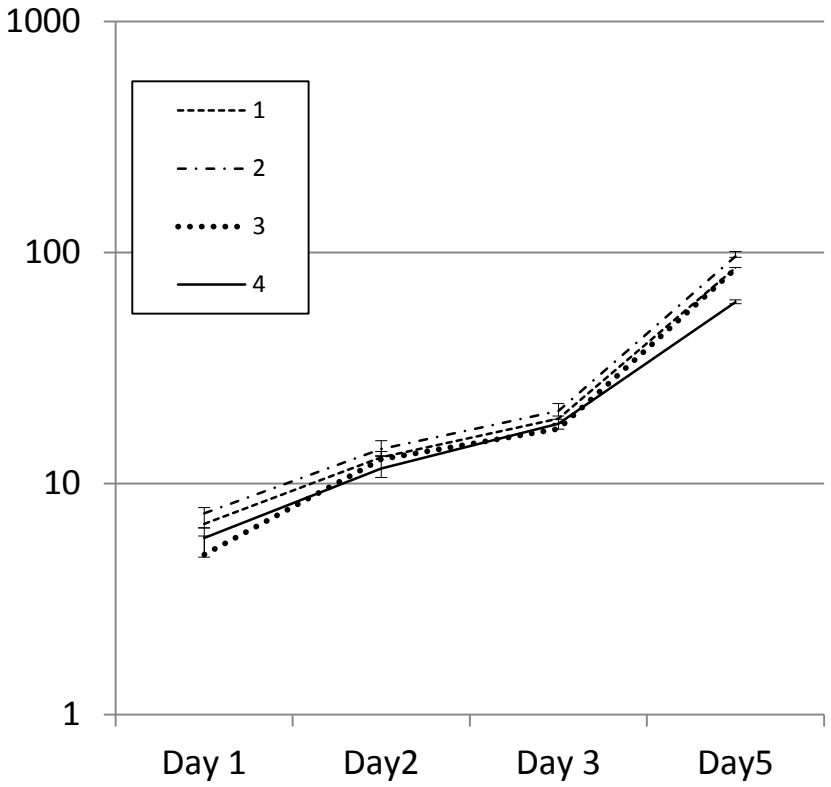
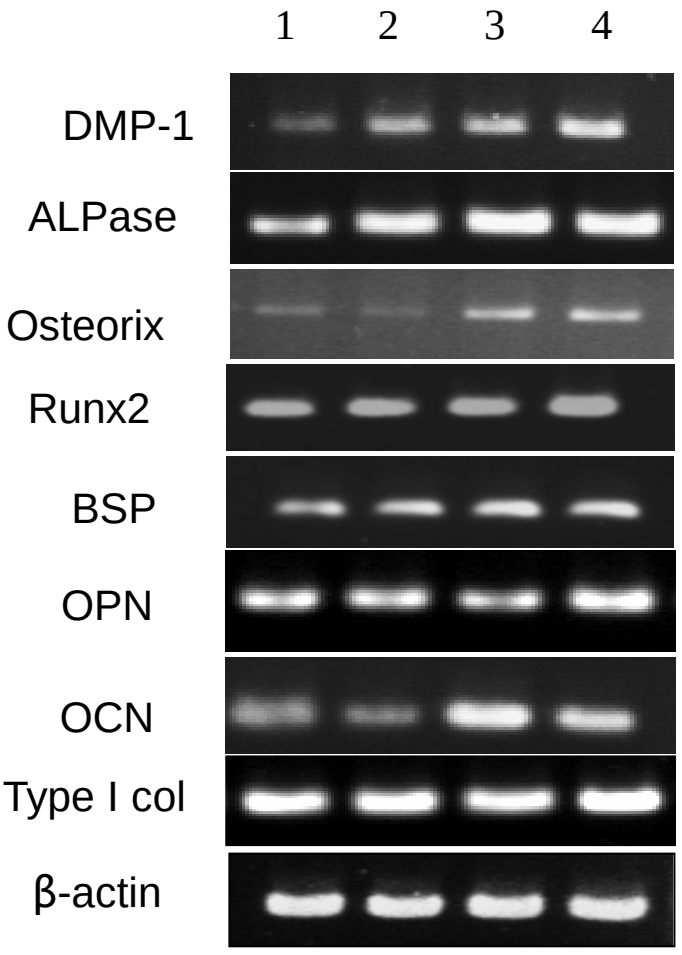


Fig.2 Cell proliferation of MDPC-23.
MDPC-23 cells were incubated with DPP. Representative figure, showing the unchanged proliferation of MDPC-23 after addition of DPP in different concentration up to 5days. 1:w/o DPP, 2: 0.1μg/ml of DPP, 3 :1.0μg/ml of DPP, 4: 10μg/ml of DPP



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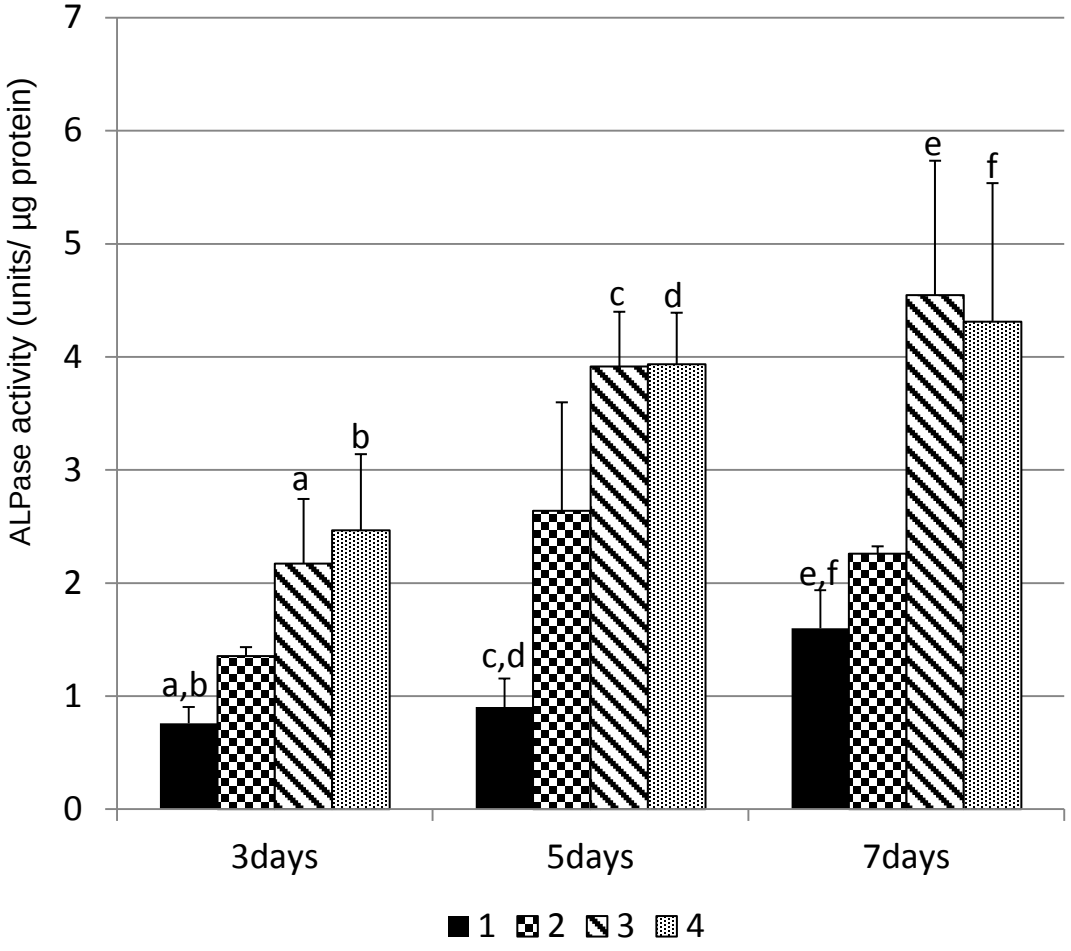
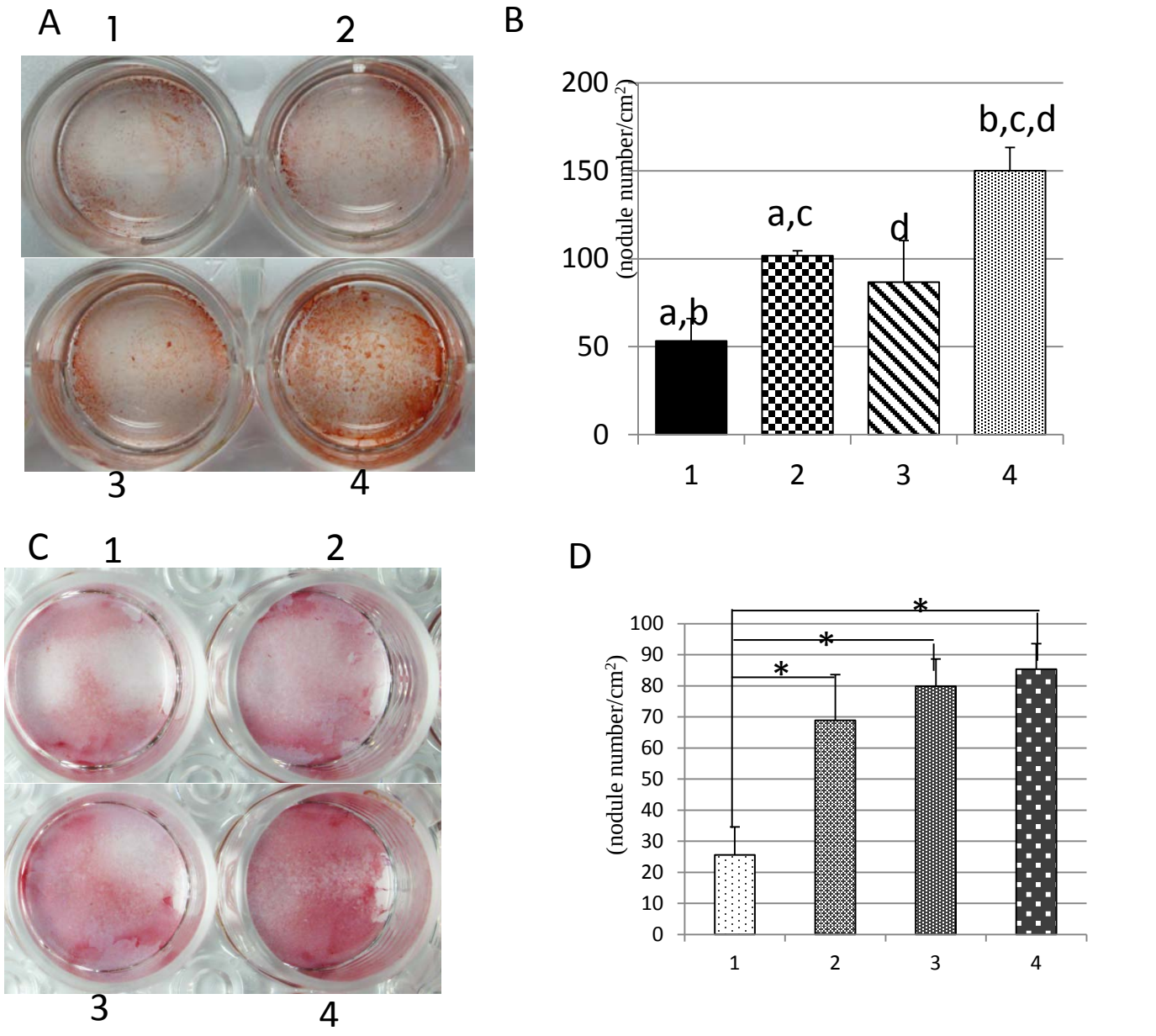


Fig. 4 ALP activity in MDPC-23 with DPP after 3, 5 and 7days.
MDPC-23 cells were various concentration (0.1, 1 and 10μg/ml and without DPP as a control) were added from the next day of seeding. ALP activity was calculated as units/mg total protein of the cell lysate (means ±SD; n=3). a~f, indicates significant differences ($P<0.05$) between the same symbols. 1: w/o DPP, 2: 0.1 μg/ml of DPP, 3: 1 μg/ml of DPP, 4: 10 μg/ml of DPP.



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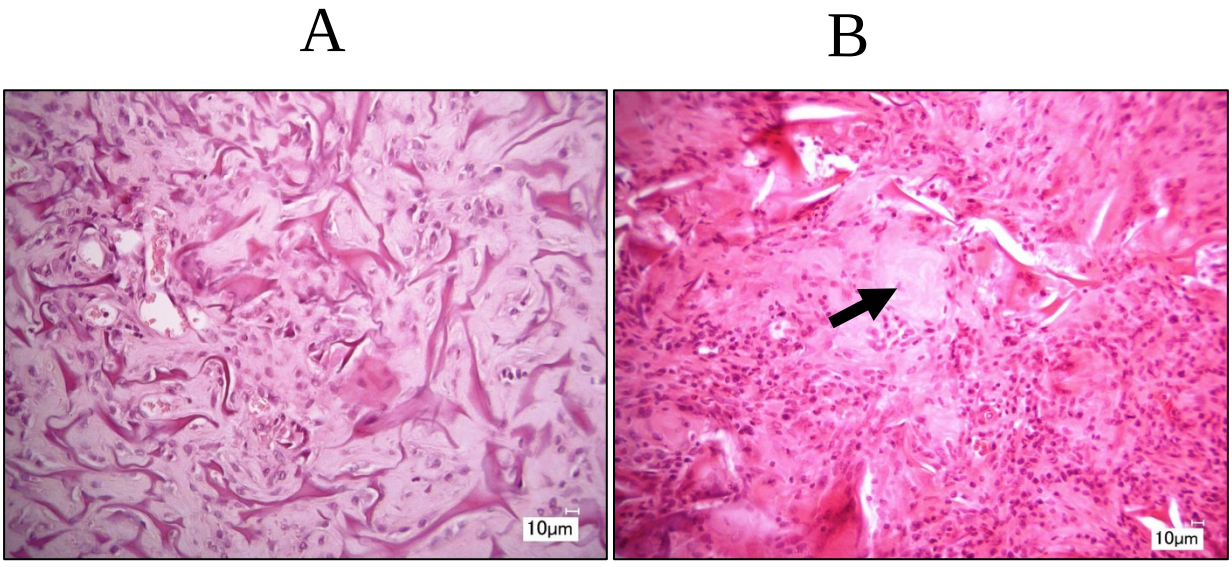

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