

The Behavior of MDPC-23 Cells Modulated by DSPP

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Abstract

Phosphophoryn (PP) and Dentin Sialoprotein (DSP) are two of the most abundant dentin matrix non-collagenous proteins. These two proteins are derived from Dentin Sialophosphoprotein (DSPP) precursor protein cleavage by DMP1 protease. PP was well established as a nucleator to initiate dentin mineralization. The expression of DSPP precursor protein was reported to be required for normal odontoblast lineage differentiation. DSPP protein was also reported to modulate cell migration, cell proliferation and differentiation, leading to dentin formation. To further understand the role of DSPP in odontoblast lineage, we used MDPC23 cells to examine the mineralization process and the effects of DSPP on this process. MDPC23 cell line is an odontoblast-like cells, which exhibits unique features of dental pulp stem cells such as high alkaline phosphatase activity and expression of odontoblast markers: DSP and PP. MDPC23 cells were cultured in Mineralization Medium (MM) containing mineral inducing molecules (i.e., 100 µg/ml ascorbic acid, 10 mM β-glycerol phosphate and 10 nM dexamethasone) to examine MM effects on the cell growth and mineral deposition. Recombinant DSPP protein was used to test its effects on dental pulp cell growth and mineralization. We found that MDPC23 cells cultured with mineralization medium, proliferated slowly and spread widely at 4d. However, MDPC 23 cells in MM group, shrank and gradually detached from the well surface at 6d. Strong mineral deposition was detected with Alizarin Red staining at 7d. In the presence of recombinant DSPP protein (recDSPP), MDPC23 cells cultured with mineralization medium, also proliferated slowly, spread widely but remained vital from 4d, 6d, 7d, 8d, 9d, 10d, 12d through 18d and 20d. Strong mineral deposition was detected with Alizarin Red staining at 18d and 20d. Thus under the combined MM and recDSPP treatment, MDPC23 cells maintained the cell vitality and delayed the mineralization process. This study lent support that DSPP is required for maintaining normal odontoblast lineage.

Keywords: Cell maintenance; Dental pulp cells; Mineralization; Recombinant DSPP

for mineralization [11]. Recently DSP was reported to regulate dental pulp cell differentiation into odontoblasts [12,13].

Introduction

DSP and PP are the most dominant noncollagenous protein in dentin. These two proteins are derived from DSPP mRNA, then translated to a DSPP precursor protein, which is further processed by BMP1 protease to generate DSP and PP proteins [1-3]. Since DSP protein, PP protein and DSPP mRNA were mainly expressed in polarized odontoblasts, DSP and PP were postulated to be associated with dentin mineralization [4]. PP has been well established as a nucleator for dentin mineralization [5-7]. Mutations in DSPP gene have been linked to dentinogenesis II and III [8,9]. DSPP knockout mice displayed symptoms similar to patients with dentinogenesis imperfecta III [10]. Originally, the role of DSP was controversial. For example, DSP was reported as a weak inhibitor

To better understand the role of DSPP in odontoblast lineage differentiation during tooth development, previously we systematically examined teeth from wild-type (wt) and DSPP KO C57BL/6 mice between the ages of post-natal day 1 and 3 months. We found that DSPP Knockout (KO) mice have hypomineralized teeth, thin dentin and a large dental pulp chamber, similar to those from patients with dentinogenesis imperfecta III as reported by Kulkarni et al [10]. Furthermore, we found developmental abnormalities not previously reported, such as circular dentin formation within dental pulp cells and altered odontoblast differentiation in DSPP KO mice, even as early as one day after birth. Surprisingly, we also identified chondrocyte-like cells in the dental pulp from KO mice teeth. Thus we suggested that the expression of DSPP precursor protein is required for normal

odontoblast lineage differentiation and that the absence of DSPP allows dental pulp cells to differentiate into chondrocyte-like cells [14].

The transient expression of DSP protein and DSP-PP transcripts occurs first in the preameloblasts, and next in both preameloblasts and young odontoblasts. Finally, DSP-PP transcripts exhibit sustained expression in odontoblasts [15,16]. PP was reported to promote cell migration and the expression of high levels of Col type I and PP in dental pulp cells. The addition of recombinant DSP/PP proteins affected cell proliferation and differentiation in a dental pulp cell line [17].

Taken together, DSPP does have a major role in dentin mineralization. DSPP also has roles in dental pulp cell migration, proliferation and differentiation. Furthermore, the wider DSPP mRNA expression in preameloblasts, dental pulp cells, bone cells and salivary glands suggest that additional DSPP roles yet to be discovered. MDPC23 cell line was established from fetal mouse molar papillae by Dr. Tom Hank [18,19]. MDPC23 cells express high alkaline phosphatase activity as well as express two odontoblast differentiation markers: dentin phosphoprotein and dentin sialoprotein. In this study, we used MDPC23 cells to investigate the mineralization process *in vitro* and we examined whether DSPP could affect the behavior of MDPC23 cells regarding cell vitality and mineral formation.

Materials and Methods

Cells

An original mouse dental pulp cell line MDPC23 [18,19] was later determined to be a rat odontoblast-like cell line [20]. MDPC23 cell line was obtained from Dr. Jacques Nor (University of Michigan Dental School). MDPC23 cells were used to test the ability to deposit minerals under the mineralization solution containing ascorbic acid, β -glycerol phosphate and dexamethasone (details see Culture medium below). We investigated the effect of DSPP on MDPC23 cell proliferation, differentiation and mineral formation.

Culture medium and cell culture preparation

MDPC23 cells were seeded at 20×10^4 cells/6 well in α -MEM with 0.1% FBS, L-glutamine and 1% penicillin-streptomycin overnight, then their growth medium were replaced with assigned treatments in the next day. For the control group, MDPC23 cells were cultured in growth medium, which was composed of complete α -MEM, supplemented with 15% Fetal Bovine Serum (FBS), L-glutamine and 1% penicillin-streptomycin (GIBCO, USA). MDPC23 cells were changed with fresh growth medium every other day. In the mineralization medium (MM) group, MDPC23 cells were cultured in the same culture medium with additional mineral inducing components 100 μ g/ml ascorbic acid, 10nM Dexamethasone, and 10 mM β -glycerol phosphate. MDPC23 cells in the MM group were changed with fresh growth

medium containing mineralization solution every other day. The third group (MM/recDSPP) group, MDPC 23 cells were cultured with mineralization medium and recombinant DSPP solution (100 μ l recDSPP in insect culture medium/6 well). For each group, the specific culture medium was changed every other day.

Preparation of recombinant DSPP protein from baculovirus system

Rat DSP-PP₂₄₀ cDNA in a baculovirus expression vector pVL1392 was used to generate recombinant DSP₄₃₀/PP₂₄₀ (i.e., DSP protein with 430 amino acid residues and PP protein with 240 amino acid residues) proteins in the insect sf9 cell culture medium. The sf9 cell supernatant from DSP-PP₂₄₀ cDNA in pVL1392 contained DSP₄₃₀/PP₂₄₀ protein mixture, which was used directly in cell culture (100 μ l recDSPP/6 well).

Cell number counting

Cells were treated with 1X trypsin-EDTA. The cell number per 6 well was determined by hemocytometer. Once cells were treated with 1X trypsin-EDTA at Day 4, the 6 wells were discarded. Data are presented as the mean \pm S.D. of triplicate samples.

MDPC 23 Cell Morphology under Different Culture Medium

MDPC23 cells (40×10^4 cells/6 well) were cultured in 2mL of MDPC23 growth medium as described in the previous section. Medium from these cells of three groups was changed every other day for needed days. Triplicates were set up for each group. At d4, d6, d7, d8, d9, d10, d18 and d20, the cells from each group were examined by light microscopy.

Alizarin Red staining for Ca⁺⁺ deposition

Freshly prepared Alizarin Red 40 mM pH4.2. Six wells were washed with cold PBS for 5-10 min and next fixed in cold 70% ethanol for 1hr. Then 6 wells were washed twice with water, and stained with filtered Alizarin Red for 10 min at room temperature. At the final step, 6 wells were washed with water to remove nonspecific staining and photographed.

Statistical Analysis

Results are presented as means \pm Standard Deviation (S.D.). Two-sample t-test for mean difference with unequal variances was carried out using the program Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) by personnel at the Center for Statistical Consultation and Research Center of the University of Michigan.

Results

MDPC23 cells cultured for 4d in regular growth medium showed higher cell proliferation

Culture of MDPC23 cells in three different medium (i.e., Control group, MM group and MM/recDSPP group). All groups of MDPC23 cells were seeded at 20×10^4 cells/6 well in 0.1%

FBS overnight, then next morning were replaced with different treatments (see Table 1). In each group, the cells were changed with specific medium every other day.

	Seeding cell number/6 well	Day 4 cell number/6 well
Control	20x10 ⁴	82x10 ⁴
MM	20x10 ⁴	27x10 ⁴
MM/recDSPP	20x10 ⁴	27x10 ⁴

Table 1: Cell proliferation in the control, MM and MM/recDSPP medium.

After 4d in the culture medium, the MDPC23 cells in the control group had the vigorous growth. Cell proliferation was most pronounced when MDPC23 cells were incubated in the regular growth medium in the control group. The presence of ascorbic acid, β-glycerol phosphate and dexamethasone in the growth medium in MM group reduced MDPC23 cell proliferation. Similarly, MM and recDSPP in the growth medium reduced MDPC23 cell proliferation (see Table 1)

MDPC23 cells in the control group were highly packed with cuboid shape after 4 day and 6 day culture in α-MEM growth medium with 15% FBS, glutamine and 1% penicillin and streptomycin.

Table 1 showed that MDPC23 cells in the control group had a higher cell proliferation number compared to those of MM and MM/recDSPP groups. Figure 1 showed that MDPC23 cells in the control group are highly packed and appeared as cuboid shape after 4 days and 6 days culture (Figure 1).

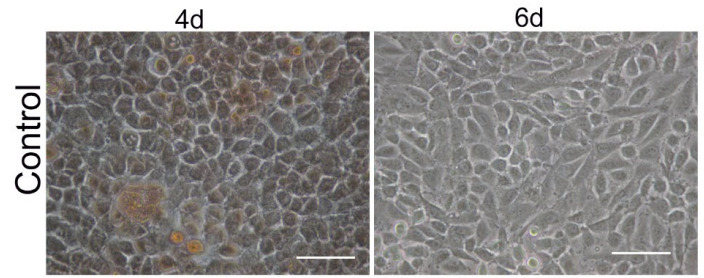


Figure 1: MDPC23 cell images in the control group at 4d and 6d culture. MDPC23 cells were seeded in 40x10⁴ cells/6 well in growth medium with 0.1% FBS overnight. Next day, cells were supplemented with 15% FBS in growth medium and replaced with fresh medium and 15% FBS every other days. After 4 day and 6 day culture, MDPC23 cells showed highly packed cells. Images are at 100x magnification. Bar size = 100μ.

MDPC23 cells in MM group mineralized after 6d culture

Cells of MM group appeared as spindle shape on Day 4. On Day 6, certain cells shrank and detached from the well surface, and more cells detached on Day 7.

MM is well known to stimulate cell differentiation into mineralization tissue in bone cells and dental pulp cells. After 4d MM treatment (Figure 2, 4d), MDPC23 cells showed stretched/attached cells, which is different from MDPC23 cells in control group. As shown in Figure 1, MDPC23 cells without MM treatment, cells were highly packed. Interestingly, after 6d MM treatment, MDPC23 cells shrank and some detached from the well surface (Figure 2, 6d). Under MM treatment at 7d (200x magnification), MDPC23 cells showed less cells on the well surface. Alizarin Red stained the cells read after 7d MM treatment. After MS treatment at 7d, mineral deposition was detected.

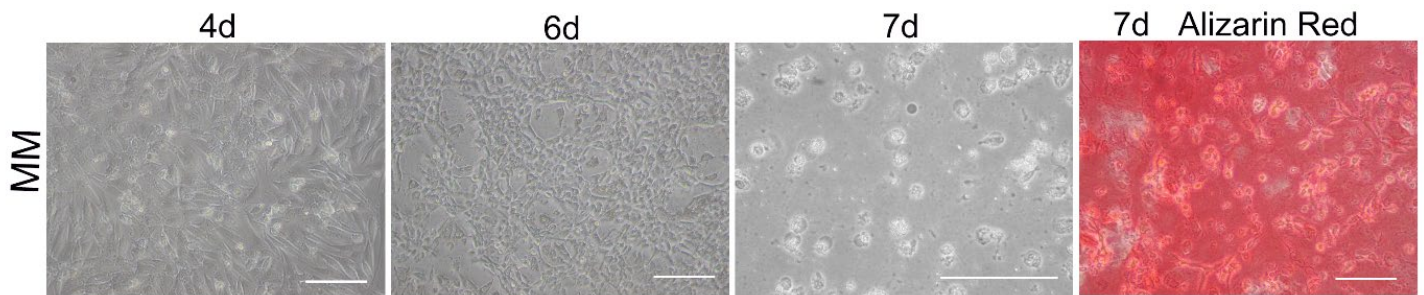


Figure 2: MDPC23 cell images in mineralization medium (MM) group. MDPC23 cells were seeded in 40x10⁴ cells/6 well in growth medium with 0.1% FBS overnight. Next day, cells were replaced with Mineralization Medium (MM). The cells were replaced with fresh medium every other day. MDPC23 cells under MM treatment at 4d, 6d, 7d Alizarin Red were shown at 100x magnification. Cells in MM group at 7d treatment was shown at 200x magnification. Bar size = 100μ.

MDPC23 cells in the MM/recDSPP group maintained the cell vitality and delayed the mineralization process

At 4d treatment, MDPC23 cells were fibroblast like and attached very well to the surface. At 6d, more cells appeared. As shown in Figure 3, 7d, 8d, 9d and 10d, more MDPC 23 cells showed on the well surface. In general, under MM and recDSPP treatment, MDPC23 cells maintained vital appearance from 4d, 6d, 7d, 8d, 9d, 10d, 18d and 20d (Figure 3). MDPC23 cells at 12d (not shown) also displayed vital appearance. At 18d and 20d, Alizarin Red detected mineral deposition in these wells. Vital cell appearance was observed in Alizarin Red stained wells at 18d and 20d (not shown).

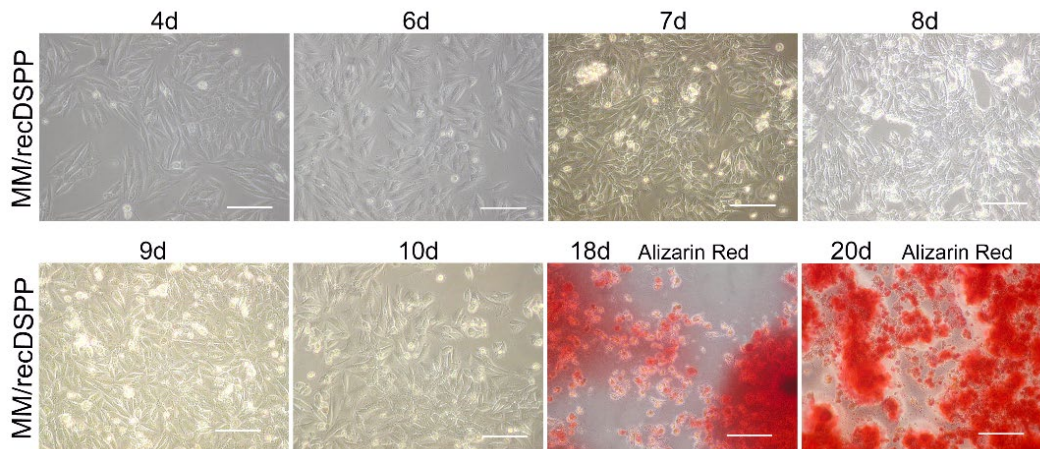


Figure 3: MDPC23 cell images in MM/recDSPP group. MDPC23 cells were seeded in 40×10^4 cells/6 well in growth medium with 0.1% FBS overnight. Next day, cells were replaced with Mineralization Medium (MM) containing recombinant DSPP protein (recDSPP; $100 \mu\text{l}/6$ well). The cells were replaced with this group fresh medium every other day. At 18d and 20d, Alizarin Red staining showed strong mineral deposition. All images are at 100x magnification. Bar size = $100 \mu\text{m}$.

Comparisons of MDPC23 cells in control, MM and MM/recDSPP groups

We would like to emphasize the major difference at 4d and 6d cultures among control, MM and MM/recDSPP groups. Cells in control group showed high packed cuboidal shape. Cells in MM group, showed stretched, spindle shape at 4d and cells shrank and detached at 6d. In contrast, cells in MM/DSPP group at 4d showed less spindle shape cells on the well and at 6d more spindle shape cells on the well (Figure 4).

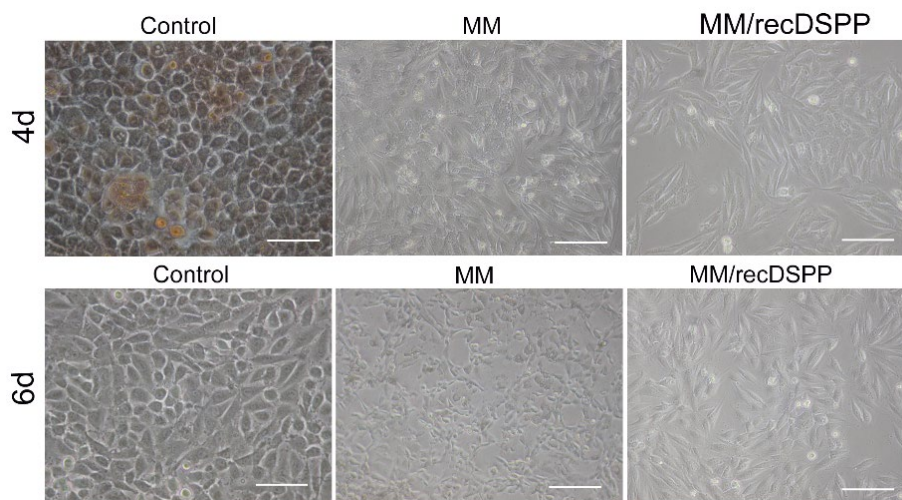


Figure 4: Comparisons of MDPC23 cells grown in control, MM and MM/DSPP groups at 4d and 6d.

Discussion

Mineralization solution is well known to induce bone cell differentiation into osteoblast cells expressing bone related proteins. It is also well known to induce dental pulp cells to differentiate to odontoblast like cells producing dentin matrix proteins, finally leading to dentin mineralization. MDPC23 cells were reported to peak alkaline phosphatase activity at 6d [18]. Likely the MM induced MDPC23 cell differentiation and alkaline phosphatase activity, which lead to the detection of Alizarin Red staining in 7 day culture. At 4d, MDPC23 cells in the Control group were highly packed and MDPC23 cells in MM group were wide spread to the well. At 6d, MDPC23 cells in the Control group were highly packed. However, at 6d MDPC23 cells in the MM group shrank and detached from the well surface. Likely, at 6d, part of the cells in MM group underwent apoptosis. However, strong Alizarin Red staining was detected in 7d cultured wells after extensive washes with water. The whole well at 7d showed red staining, indicated the matrix was stained. Once the collagen was synthesized, alkaline phosphatase and matrix molecules were available, even after cell death, the matrix could undergo mineralization [21]. For example, Marsh, et al. (1995) grew mouse MC3T3-E1 cells grown in medium containing ascorbic acid and β -glycerol phosphate. MC3T3-E1 cells express an osteoblast phenotype and produce a highly mineralized extracellular matrix. They demonstrated that bone-like extracellular matrix mineralized in the absence of functional MC3T3-E1 osteoblasts [21].

It would be interesting to investigate the alkaline phosphatase expression from 2d, 3d, 4d, 5d and 6d and to follow the expression of the proteases in the wells exhibited the cell detachment at 6d in the Future research should be targeted to understand how the relationship between alkaline phosphatase and protease activities, which might shed light to the MDPC23 cell behavior at 6d.

In the mineralization medium group without the availability of recDSPP in the medium, MDPC23 cells cultured for 6d showed shrank cells. In contrast, MDPC23 in MM/recDSPP group did not exhibit shrank cells at 6d. Actually, cells in this group display cell vitality at 6d. The cell vitality lasted from 6d through 7d, 8d, 9d, 10d, 12d, 18d to 20d. Both cells at 18d and 20d showed strong Alizarin Red staining, which indicated high mineral deposition in these wells. In addition, we observed MDPC23 cells at 18d and 20d displayed viable cell appearance in the wells.

How did the MDPC23 cells in MM/recDSPP group display cell vital appearance up to 20d in culture? We would like to briefly describe how previously we proposed a model that DSPP is required for stem cell self-renewal and keeping odontoblast lineage differentiation. When comparing DSPP KO and wild type (wt) mice, DSPP KO mice without DSPP expression show fewer dental pulp cells and thicker dentin molars at early stage as compared to wt animal. Given that there are more differentiated odontoblasts present in KO mice compared to wt animals, there may be a disturbance in stem cell self-renewal such that more stem cells

differentiate and the pool of self-renewing stem cells is depleted. DSPP may participate in dental pulp stem cell self-renewal. The absence of DSPP has altered the dental pulp stem cell fate, such that the stem cells can no longer maintain the odontoblast lineage differentiation program. These data lead us to propose a model to show that in wt animals, the constant supply of odontoblasts needed to sustain a continuous formation of dentin is dependent upon the presence of DSPP [14].

Based on the proposed role of DSPP in stem cell renewal and odontoblast lineage differentiation in an animal model [14], here we proposed a model (Figure 5) to explain how the MDPC23 cells in MM/recDSPP groups display cell vital appearance at d20. A model showing that DSPP expression is likely required for dental pulp stem cell renewal and odontoblast lineage differentiation in MDPC23 cells. When MDPC23 cells were cultured in mineralization medium with DSPP recombinant protein, cells can undergo self-renewal and odontoblast lineage differentiation. In contrast, MDPC23 cells were cultured in mineralization medium without recDSPP, stem cells self-renewal and odontoblast lineage differentiation were gradually depleted.

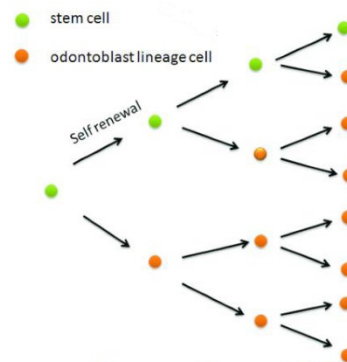


Figure 5: A proposed model for dental pulp stem cell renewals and odontoblast lineage differentiation in MDPC23 cell line. Green cells represent stem cells. Orange cells represent odontoblast lineage cells. In the presence of DSPP protein, stem cells undergo self-renewals and stem cell differentiation into odontoblast lineage cells. In this model, DSPP expression is required for dental pulp stem cell renewal and odontoblast lineage differentiation in MDPC23 cells. When MDPC23 cells are cultured in mineralization medium with DSPP recombinant protein, cells can undergo self-renewal and odontoblast lineage differentiation. In contrast, MDPC23 cells are cultured in mineralization medium without recDSPP, stem cells self-renewal and odontoblast lineage differentiation were gradually depleted

Conclusions

In summary, our cell culture results suggest that (1) regular growth medium promoted MDPC23 cell proliferation, (2) MDPC23 cells cultured in mineralization medium showed active cell spreading at 4d, shrank cell shape and detached cells at 6d,

and mineral deposition (strong Alizarin Red staining) at 7d, (3) MDPC23 cells cultured in mineralization medium and recDSPP, showed active cell spreading from 4d to 20d and exhibited active mineral deposition. Thus under the combined MM and recDSPP treatment, MDPC23 cells maintained the cell vitality and delayed the mineralization process. When MDPC23 cells were cultured in mineralization medium with DSPP recombinant protein, cells undergo self-renewal and odontoblast lineage differentiation. In contrast, MDOC23 cells were cultured in mineralization medium without recDSPP, stem cells self-renewal and odontoblast lineage differentiation was gradually depleted. This study lends support that DSPP is required for maintaining normal odontoblast lineage.

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Author Contributions

Conceptualization, H.H.R.; Methodology, S.-F.C., Y.-H.C., and H.H.R.; Investigation, Y.-H.C. and H.H.R.; Validation, Y.-H.C.; and formal analysis; H.H.R.

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Conflicts of Interest

The authors declare no conflict of interest

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