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The role of dentin matrix protein 1 (DMP1) in regulation of osteogenic differentiation of rat dental follicle stem cells (DFSCs)

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Abstract

Objectives—Primary isolated dental follicle stem cells (DFSCs) possess a strong osteogenesis capability, and such capability is reduced during *in vitro* culture. Because dentin matrix protein 1 (DMP1) is essential in the maturation of osteoblasts, our objectives were to determine (1) the expression of DMP1 in the DFSCs, (2) the correlation between DMP1 expression and osteogenic capability of DFSCs, and (3) the ability of DMP1 to promote osteogenic differentiation of DFSCs.

Methods—DFSCs and their non-stem cell counterpart dental follicle cells (DFC) were established from postnatal rat pups. Expression of DMP1 in the DFSCs and DFC was determined using real-time RT-PCR and western blotting. Different passages of DFSCs were subjected to osteogenic induction. The correlation between osteogenesis and DMP1 expression was analyzed. Then, expression of DMP1 in the DFSCs was knocked-down using siRNA, followed by osteogenic induction to evaluate the effect of DMP1-knockdown. Finally, the late passage DFSCs with reduced DMP1 expression and osteogenic capability were cultured in osteogenic induction medium containing mouse recombinant DMP1 (mrDMP1) to determine if DMP1 can restore osteogenesis of DFSCs.

Results—DFSCs expressed much higher levels of DMP1 than did DFC. DMP1 expression was correlated with the osteogenic capability of DFSCs. Knockdown of DMP1 expression markedly decreased the osteogenesis and osteogenic gene expression in the DFSCs whereas adding mrDMP1 protein to the osteogenic induction medium enhanced osteogenesis.

Conclusions—DMP1 is highly expressed in the DFSCs, but minimally expressed in non-stem cell DFC. DMP1 appears to play an important role for osteogenic differentiation of the DFSCs.

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Keywords

Dentin matrix protein 1; Dental follicle stem cells; Dental follicle cells; Osteogenic differentiation; Gene expression

Introduction

In previous studies, we reported obtaining two distinct populations of cells from the dental follicle (DF) using different cell culture systems. The cells derived from α -MEM +20% Fetal Bovine Serum (FBS) were capable of multipotent differentiation and were designated as dental follicle stem cells (DFSCs). In contrast, cells grown in MEM+10% Newborn Calf Serum (NCS) had no differentiation capability [1], but possessed the characteristics of fibroblasts. These latter cells were designated as dental follicle cells (DFC) [2]. Others have reported the isolation of progenitor or precursor cells from the DF that could be induced to differentiate into calcium-depositing cells [3, 4]. Thus, like other adult tissues, the DF contains adult stem cells that would be valuable for tissue regeneration, such as for bone and craniofacial tissue reconstruction/regeneration [5]. Elucidation of the genes and factors that are involved in regulating the osteogenic capability of DFSCs could facilitate the development of cell-based therapies using DFSCs.

Dentin matrix protein 1 (DMP1) is present in mineralized tissues [6] and is highly expressed in osteoblasts [7] and odontoblasts [8]. Several studies have confirmed that DMP1 can participate in both the intra- and extracellular bio-mineralization process. The acidic domains of DMP1 can function as a nucleator for hydroxyapatite formation in the extracellular matrix [9]. DMP1 protein was also found in the nucleus during early differentiation of odontoblasts and osteoblasts [10, 11]. In addition, it is believed that in undifferentiated preosteoblasts, DMP1 functions as a transcriptional factor to activate osteoblast-specific genes for osteoblast differentiation [10].

A recent study showed the expression of DMP1 in all non-mineralized structures surrounding developing teeth [12]. One such tissue is the dental follicle (DF), a loose connective tissue sac that surrounds the unerupted tooth. Because the DF appears to regulate the osteogenesis required for tooth eruption [13], is DMP1 expressed in its cells? Specifically, one objective was to determine the gene expression of DMP1 in the non-stem cell fibroblast-like dental follicle cells (DFC) versus expression in the stem/progenitor cells (i.e., DFSCs). We also wanted to determine (1) the correlation between DMP1 expression and the osteogenic capability of different passages of DFSCs, and (2) if DMP1 can function to promote osteogenic differentiation of DFSCs.

Materials and Methods

Cell Cultures

Dental follicles (DFs) were surgically isolated from the first mandibular molars of rat pups at postnatal days 5 or 6 from different litters. Use of the animals was approved by the Institutional Animal Care and Use Committee (IACUC) of Louisiana State University (IACUC protocol # 11-011). In each litter, primary cells were obtained by trypsinization of

the DFs collected from 3 littermates. DFSCs and homogeneous non-stem cells DFC were established by growing the primary cells in either a stem cell growth medium (α -MEM + 20% FBS) or a fibroblast growth medium (MEM + 10% NCS + 10 mM sodium pyruvate) as described previously [1, 2]. After establishment of the primary cultures of DFC and DFSCs, the cultures were trypsinized at 80-90% confluency and passaged until the desired passages were reached. Cells used for all experiments were cultured at 37°C and 5% CO₂ in this study.

Expression of Stem Cell Marker Genes in DFSCs and DFC

To assess the differential expression of stem cell-marker genes in the DF derived cells, established DFSCs and DFC were seeded in T-75 flasks and cultured for 7 days. Cells were then collected into RLT buffer (Qiagen, Velenica, CA, USA) for total RNA extraction. Expression of stem cell marker genes [NT5E (CD73), THY1 (CD90), alkaline phosphatase (ALP), BCRP and NOTCH1] was assessed using real-time RT-PCR with gene specific primers (Table 1).

Evaluation of Osteogenic Capability of DFSCs and DFC

The established DFSCs and DFC were seeded in 6-well plates at a cell density of 10⁵ cells/well for evaluation of their differentiation capability under different culture media. DMEM-LG containing 10% FBS were used as the basal medium, and osteogenic induction reagents consisting of 50 µg/mL ascorbate-2 phosphate, 10⁻⁸ M dexamethasone and 10 mM β -glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) were added to the basal medium. This medium was designated as the osteogenic medium because it has been used to induce osteogenesis of stem cells in different laboratories [1, 14, 15, 16 and 17]. Cells cultured in stem cell growth medium (α -MEM+20% FBS) were included as a control. After 14 days of incubation, the cells were fixed with 10% neutral buffered formalin, followed by Alizarin Red staining to reveal calcium depositions. The cells were also collected into RLT buffer (Qiagen, Velenica, CA, USA) for RNA extraction at days 7 and 14 to determine the expression of differentiation marker genes of BSP, OCN and DSPP for evaluation of the cell differentiation using real-time RT-PCR (Table 1). Expression of DMP1 during osteogenic induction was evaluated as well.

Expression of DMP1 in the DFSCs and DFC

For assessment of DMP1 expression in the DF-derived cells under different growth conditions, we conducted two experiments. In the first experiment, DFSCs and DFC were collected from their original culture medium for gene expression analysis; i.e., DFSCs in α -MEM+20% FBS (stem cell growth medium) and DFC in MEM+10% NCS (fibroblast growth medium). In the second experiment, the established DFC were transferred to stem cell growth medium and incubated for 7 days such that both DFSCs and DFC were cultured in the same medium to eliminate the medium effect on DMP1 expression. Cells were then collected for gene expression analysis using real-time RT-PCR and Western blotting.

For real-time RT-PCR, total RNA was extracted with RNeasy® Mini kit (Qiagen, Velenica, CA, USA). RNA concentration was measured with a Nanodrop spectrophotometer. Total RNA of 1 µg from each sample was reverse-transcribed into cDNA. SYBR green real-time

PCR was conducted using primers listed in Table 1 to obtain the C_T values. Relative gene expression (RGE) was calculated with the Delta C_T method with β -actin as an internal control.

In western blotting analysis, cells were lysed with CytoBuster™ protein extraction buffer (EMD Millipore, Billerica, MA, USA) supplemented with 1/50 Protease Inhibitor cocktail (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). After centrifugation of the cell lysate, supernatants containing total protein were transferred to new tubes, and protein concentration was determined with the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Total protein (15 μ g) of each sample was subjected to electrophoresis on 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated with a polyclonal rabbit anti-DMP1 antibody (Abnova Corporation, Walnut, CA, USA) or rabbit anti-actin polyclonal antibody (Abcam, Cambridge, MA, USA). The membrane was subsequently incubated with goat anti-rabbit IgG and then washed with PBST buffer to remove unbound antibody. The membrane was developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology, INC. Dallas, TX, USA).

DMP1 Expression, Osteogenic Differentiation and ALP activity in Different Passages of DFSCs

DFSCs of passages 3, 5, 7 and 9 were collected into RLT buffer (Qiagen, Velenica, CA, USA) for total RNA extraction and real-time RT-PCR was conducted to determine the RGE as described earlier. Different passages of DFSCs were also seeded in 6-well plates at a cell density of 10^5 cells/well. Cells were then incubated in osteogenic medium. After 14 days of induction, the cells were fixed and stained with Alizarin Red. The staining was quantitated by Image-Pro Analyzer version 7.0 (Media Cybernetics, Rockville, MD, USA). In particular, the intensity of Alizarin Red staining was determined by segmentation, followed by counting the number of pixels in the defined range of bright red. ALP activity in different passage of DFSCs during osteogenesis was measured with QuantiFluo™ Alkaline phosphatase Assay kit (BioAssay Systems, Hayward, CA, USA) after 7 days of osteogenic induction. Briefly, DFSCs of passages 3 to 9 were seeded into 12-well plates for osteogenic induction. Cells were collected into Cytobuster buffer for protein extraction after 1 week of induction. Ten μ g of total protein was used for ALP activity assay according to the manufacturer's protocol.

Transfection of DFSCs with DMP1-siRNA

A Dicer substrate siRNA was obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) by annealing two single stranded RNA: sense strand 5'-rCrGrArCrCrArCrArGrUrGrArUrGrArGrGrCrArGrArCrAGC-3' and anti-sense strand 5'-rGrCrUrGrUrCrUrGrCrCrUrCrArUrCrArCrUrGrUrGrGrUrGrGrUrC-3'. The siRNA was designed to target the rat DMP1 mRNA. To knock down gene expression, the DFSCs at passages 3 and 5 were seeded in 6-well plates at a density of 10^5 cells/well and cultured until 70% confluency at the time of transfection. Transfection was carried out using Lipofectamine™ RNAiMAX per manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Briefly, the siRNA and transfection reagent were diluted to 100 nM with Opti MEM I

Reduced Serum Medium (Invitrogen, Grand Island, NY, USA). After 5 min of incubation at room temperature, the siRNA and transfection reagent were mixed and incubated at room temperature for another 25 min to allow the formation of a siRNA- RNAiMAX complex. The resultant complex was added to the DFSC culture to bring the final siRNA concentration to 10 nM. A scrambled siRNA was transfected into the cells as the control. The knockdown efficiency of DMP1 expression was confirmed by real-time RT-PCR.

To assess the effect of DMP1 knockdown on expression of osteogenic related genes, transfected DFSCs were collected after 7 and 14 days of induction for analysis of the expression of selected osteogenic genes (BMP2, BMP5, BMP6, FLT1, MMP13, RUNX2 and BGN) using real-time RT-PCR with the primers listed in Table 1. Briefly, total RNA was extracted from the cells and 1µg total RNA was reverse-transcribed into 20µl cDNA. SYBR green real-time PCR was conducted with 0.5 µL cDNA from each sample for each PCR reaction to obtain the C_T value. RGE was calculated with the Delta C_T method.

Effect of Exogenous DMP1 on Osteogenic Differentiation of DFSCs

The recombinant mouse DMP1 (rmDMP1) was purchased from R & D system (Minneapolis, MN, USA). DFSCs at passage 9 that had reduced endogenous DMP1 expression and reduced differentiation capability were incubated with osteogenic medium plus different concentrations of rmDMP1 (0-250 ng/ml). After 14 days of incubation, Alizarin Red staining was performed to evaluate calcium deposition. The intensity of staining was quantitated by Image-Pro Analyzer version 7.0 as described earlier.

Statistical Analysis

All experiments were repeated a minimum of 3 times with the cells established from different litters. Analysis of variance and Tukey's test were used for multiple comparisons of more than two means. A Student *T*-test was used for comparison of two means. Pearson correlation analysis was performed to determine the correlation between DMP1 expression and osteogenesis in different passages of DFSCs. All of the statistical analyses were performed with SAS 9.3 (SAS, Cary, NC, USA).

Results

Expression of Stem Cell Marker Genes in the DFSCs and DFC

Using real-time RT-PCR, we evaluated the expression of some stem cell marker genes [NT5E (CD73), THY1 (CD90), ALP, BCRP and NOTCH1] in the DFSCs and DFC. The results revealed that DFSCs express significantly higher levels of these genes than do the DFC (Fig. 1). Maximal differential expression was seen in NOTCH1 with the average RGE greater than 50 when comparing the DFSCs to the DFC (Fig. 1E). More than 3-fold increased expression of ALP was seen in the DFSCs (Fig. 1D).

Osteogenic Potential of DF Derived Cells

When DFSCs and DFC were cultured in the medium containing osteogenic induction reagents for 14 days, DFSCs showed strong calcium deposits as determined by Alizarin Red staining, indicating that DFSCs had differentiated into calcium-depositing cells (Fig. 2A). In

contrast, culturing DFC under the same induction conditions did not result in any calcium deposits, as seen by absence of Alizarin Red staining, indicating that the DFC contained no stem/progenitor cells capable of differentiation (Fig. 2B). In addition, when the cells were cultured in the basal DMEM medium containing no osteogenic induction reagents, both DFSCs and DFC could not form calcium deposits. Furthermore, DFSCs maintained in stem cell growth medium did not deposit calcium, suggesting no osteoblasts or other calcium depositing cells in the population.

To evaluate the differentiation of DFSCs at the molecular level, expression of differentiation markers (BSP, OCN and DSPP) was determined before and after osteogenic induction (Fig. 3). After 7 days of induction in osteogenic medium, expression of osteoblast markers, BSP and OCN, was significantly increased in DFSCs with the RGE of 59.69 ± 6.84 for BSP and 307.15 ± 70.64 for OCN, indicating that the DFSCs underwent osteogenic differentiation. Expression of these two markers continued to increase significantly until day 14 (Fig. 3A, B). In contrast to BSP and OCN, minimal changes in expression of the odontogenic marker, DSPP, were seen during differentiation induction of DFSCs. In fact, DSPP expression was decreased after 7 days of induction when compared to the undifferentiated controls. Although an average RGE of 5.48 ± 2.61 was seen after 14 days of induction, the increase was not statistically significant as compared to the controls (Fig. 3C). The C_T values of DSPP were greater than 30 in all treatments, indicating that the expression of this gene was extremely low in the DFSCs (data not shown). Expression of DMP1 was also increased significantly during the osteogenic induction with the RGE of 8.46 ± 0.64 and 19.91 ± 3.39 after 7 days and 14 days of induction (Fig. 3D), respectively.

Gene Expression of DMP1 in the DFSCs and DFC

Expression of DMP1 in the DFSCs and DFC was also examined with real-time RT-PCR in two culture conditions. When the cells at passage 3 were cultured in their original growth medium (i.e., DFSCs in α -MEM + 20% FBS, and DFC in MEM+10% NCS), the RGE of DMP1 was 856 ± 260 in DFSCs vs. 1 in DFC (Fig. 4A); i.e., the DFSCs expressed an average of 856-fold higher of DMP1 than did the DFC. However, when culturing DFSCs and DFC in the stem cell growth medium (α -MEM + 20% FBS), DMP1 expression in the DFSCs was more than 10,000-fold higher than in the DFC (Fig. 4B). The differences were highly significant in both cases. Western blotting was conducted to confirm the RT-PCR results at the protein level where large quantities of DMP1 protein were seen in the DFSCs and almost no DMP1 protein was detected in the DFC (Fig. 4C).

Gene expression of DMP1 in different passages of DFSCs also was determined using real-time RT-PCR. The results revealed an overall decreased gene expression of DMP1 with progression of cell passaging (Fig. 5A). The expression started to decline at passage 7, and by passage 9 there was more than a 50% reduction in expression as compared to the passage 3. This reduction was statistically significant (Fig. 5A).

To determine the correlation between DMP1 expression and osteogenic capability of DFSCs, different passages of DFSCs were induced for osteogenesis for 2 weeks before being stained by Alizarin Red. Osteogenesis of different passage DFSCs was quantitatively measured as shown in Fig. 5B. Pearson correlation analysis showed a strong correlation

between DMP1 expression and osteogenesis in different passages of DFSCs with a correlation coefficient of 0.92503. We also determined ALP activity in different passages of DFSCs after one week of osteogenic induction, and found that passage 3 DFSCs had maximal ALP. Substantial reduction of ALP activity was seen in passages 5 and 7, and further decrease in passages 9 (Fig. 5C).

Effect of DMP1 on Osteogenic Differentiation of DFSCs

Since expression of DMP1 appeared to correlate to osteogenic potential of DFSCs, it was important to determine if there was a cause and effect relationship between DMP1 expression and osteogenesis. In this regard, early passages (P3 and P5) of DFSCs that expressed high levels of DMP1 were transfected with DMP1-siRNA. The knockdown efficiency was about 90% at day 3, 85% at day 5, and then dropped to about 70% at days 7 as determined by real-time RT-PCR. By day 14, the knockdown efficiency was down to about 60% (Fig. 6A). Therefore, the siRNA transfection was repeated at day 7 during the 14 days of osteogenic induction to ensure more than 70% knockdown of DMP1 expression. After 14 days of osteogenic induction, the cells were stained with Alizarin Red to reveal the calcium deposition. When subjecting the transfected DFSCs to osteogenic induction, a significant reduction of osteogenesis was observed in the DMP1-siRNA transfected cells. In contrast, in cells transfected with scrambled siRNA or mock transfected, slight or no reduction of osteogenesis was observed (Fig. 6C). Knockdown of DMP1 also significantly reduced expression of selected osteogenic genes in transfected DFSCs after osteogenesis induction (Fig. 6B). In particular, the reduction was more than 40% for BMP6 and BMP5, and about 30% for BMP2, MMP13 and RUNX 2. A slight reduction of FLT1 and BGN expression also was detected. Statistical analysis determined that the reduction of the expression of these genes was significant as compared to the control (Fig. 6B). To further study the effect of DMP1 on osteogenesis, passage 9 DFSCs that had reduced capability of osteogenesis and expression of DMP1 were incubated in the osteogenic medium supplemented with mrDMP1 protein for 14 days, increase of osteogenesis was observed in a DMP1 concentration-dependent manner. A dramatic increase of osteogenesis was seen at concentrations of 200 and 250 ng/ml (Fig. 7A, B).

Discussion

The DF is a loose connective tissue sac consisting of a majority of fibroblast-like cells. We established two distinct cell populations from the DF, the DFSCs and fibroblast-like DFC. DFSCs were established by culturing the primary DF cells in α -MEM + FBS, whereas DFC were established in MEM + NCS. The major differences of the two culture systems are the basal medium and serum. FBS is collected from the bovine fetus, and NCS is collected from 3 to 10 day old calves. NCS contains a higher proportion of proteins and immunoglobulins, but fewer growth factors than FBS. FBS has been widely used for culturing adult stem cells in different laboratories, but it is not suitable for culturing fibroblast cells. The differential biological effects of NCS and FBS have been described [18, 19]. The basal media, MEM and α -MEM, differ in compositions of salts, vitamins and amino acids. Thus, the cell populations established in these culture systems possess distinct properties regarding their differentiation potential and gene expression.

According to International Society for Cellular Therapy (ISCT), the minimal criteria to define mesenchymal stem cells (MSCs) are: (a) MSCs must be plastic-adherent when maintained in standard culture conditions, (b) MSCs must express CD105, CD73 and CD90, and (c) MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* [20]. We compared the expression of some typical stem cell markers genes [NTSE (CD73), THY1 (CD90), ALP, BCRP and NOTCH1] in the DFSCs and DFC. We found that the DFSCs express significant higher levels of these marker genes than do the DFC. Although both DFSCs and DFC are plastic-adherent, only DFSCs are capable of multi-lineage differentiation into osteoblasts, adipocytes and neural cells [1]. Therefore, the DFSCs meet the criteria for MSCs, but not DFC. We noticed that the terms, DFSCs (dental follicle stem cells) and DFC/DFCs (dental follicle cells) have been used in the literature to denote the DF derived cells that are capable of differentiation. For example, in the publications by Pan et al. and Guo et al. [21, 22], the DFCs were established by culturing the DF derived cells in α -MEM +FBS, which was the same medium used in our study to establish the DFSCs. We use DFSCs to reflect the facts that the cell population possesses the stem cell properties of multi-lineage differentiation.

In this study, we showed that DMP1 was highly expressed in the DFSCs. Gopinathan et al also reported the presence of DMP1 in progenitor cells/stem cells derived from the DF [23], and they focused their study to investigate the differential epigenetic regulation of the DMP expression in the DF and dental pulp derived progenitor cells. Here, we reported that DMP1 was expressed in the DFSCs, but not in their counterpart non-stem cell DFC (Fig. 4A, B and C). This result suggests that DMP1 could be used as a marker to distinguish stem cells/progenitor cells from the majority of fibroblast cells in the DFs. Our studies also provide evidence to demonstrate that DMP1 plays a role in regulating osteogenic differentiation of DFSCs.

It has been reported that DMP1 is expressed in differentiated calcium-depositing cells, including osteoblasts, osteocytes and odontoblasts [6, 7, 8 and 24]. This study showed although DFSCs expressed high level of DMP1, they were not able to deposit calcium without proper osteogenic induction, suggesting that osteoblasts, odontoblasts and other calcium-depositing cells are likely not present in the DFSC population (Fig. 2). Given that the DFSCs express high levels of DMP1 and undifferentiated preosteoblasts also express DMP1 [10], it is possible that the population of DFSCs contains preosteoblast-like cells. However, our previous studies demonstrated that the DFSCs were capable of multipotent differentiation into osteoblasts, adipocytes and neurospheres [1, 25]. It is unclear if the DFSCs are composed of different subpopulations of progenitor cells or only multipotent stem cells. Further studies are needed to clarify this and to determine if the DMP1 is expressed in the given subpopulations of cells.

It is well known that DMP1 plays important roles for osteogenesis. DMP1 is essential in the maturation of odontoblasts and osteoblasts [26, 10]. In this study, we showed that DMP1 was expressed in the established DFSCs without subjecting them to osteogenic induction, and the cells could not spontaneously undergo differentiation into odontoblasts and osteoblasts without a proper induction (Fig. 2). However, when subjecting the DFSCs to osteogenic induction, further increase of DMP1 expression was observed (Fig. 3D). In

addition, DMP1 expression was reduced in long term cultured DFSCs, and when the DFSCs with reduced DMP1 expression were subjected to osteogenic induction, their osteogenic capability was reduced (Fig. 5). These results suggest that DMP1 may function to maintain osteogenic potential of DFSCs at a lower concentration, and increased amount of DMP1 is necessary for osteogenic differentiation of DFSCs. Correlation analysis revealed a strong correlation between DMP1 expression and osteogenic capability of DFSCs during *in vitro* culture. Our DMP1 knockdown study indicated that the DMP1 expression and osteogenic differentiation was not merely correlation, but there was a cause-and- effect relationship (Fig. 6C). This is supported by our observation in the cell culture experiment where addition of mrDMP1 could partially restore osteogenesis in the late passage DFSCs (Fig. 7). Thus, together, the results of this study suggest that DMP1 may function to maintain osteogenic capability of the preosteoblasts, progenitor cells or stem cells. It is worth of future studies to determine if other tissue derived stem cells also express DMP1.

In a recent study, DMP1 has been showed to bind to the promoter of dentin sialophosphoprotein (DSPP) to regulate the expression of that gene [11]. Given that DMP1 possesses a promoter binding property and can function as a transcription factor [10], it would be interesting to determine if DMP1 can regulate expression of osteogenic genes. We found that knockdown of DMP1 resulted in reduction of the expression of osteogenic genes (BMP2, BMP5, BMP6 , FLT1, MMP13, RUNX2 and BGN) during osteogenic differentiation of the DFSCs, suggesting that DMP1 is likely involved in up-regulating the expression of these osteogenic genes necessary for differentiation of DFSCs. This observation is supported by Narayanan et al. who reported that overexpression of DMP1 in mesenchymal stem cells could induce expression of genes involved in early and proliferative stages of mineralization such as RUNX2, BMP2, BMP4, ALP, OCN, DMP2, and DSP [27]. Further studies are needed to determine the regulatory mechanism of DMP1 on these osteogenic genes.

Exogenous DMP1 can exert its effect by entering the cells via endocytosis or by binding to membrane receptors to activate an internal signaling pathway leading to osteogenesis [28, 29]. Recent studies revealed that $\alpha\text{v}\beta 3$ integrin is the cell surface receptor of DMP1 [28]. In this study, we found that adding mrDMP1 to the osteogenic induction medium could recover/ promote the osteogenesis of late passage DFSCs that had reduced osteogenic capability and decreased DMP1 expression (Fig. 7). This finding confirms that DMP1 can also act as an extracellular factor for promoting osteogenesis in the DFSCs.

ALP is a well-known marker of various stem cells including embryonic stem cells and tissue derived stem cells and [30-32]. For example, mesenchymal stem cells (MSCs) could be isolated by selection of ALP-positive cells from human hearts [33]. Present study showed that DFSCs express significantly higher levels of ALP than their counterpart non-stem cells, suggesting that ALP could be a marker for identification and purification of stem cells from dental follicle. We further analyzed ALP activity in different passages of DFSCs during osteogenic induction. The early passages of DFSCs have higher levels of ALP activity than do the late passage cells when subjected to osteogenic induction, and the trend is correlated to DMP1 expression. Because ALP is also known as an early marker of osteogenesis, the reduced ALP activity seen in late passage (P9) DFSCs indicates that the cells have

decreased osteogenic capability confirming the osteogenesis measurement result from alizarin red staining.

In summary, this study has shown that DMP1 is highly expressed in the DFSCs derived from the DF, but not in the non-stem cell fibroblast-like DFC of the same origin. This high level expression of DMP1 is likely necessary to maintain the osteogenic differentiation capability of DFSCs. Moreover, our results indicate that DMP1 participates in regulating expression of osteogenic genes in the DFSCs.

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Highlights

- a.** Determined that DMP1 is highly expressed in the dental follicle stem cells (DFSCs), but not expressed in the counterpart non-stem cell dental follicle cells (DFC).
- b.** Revealed that the DMP1 expression is correlated to osteogenic capability of different passages of DFSCs.
- c.** Determined that DMP1 plays a role in maintaining and promoting osteogenic capability of DFSCs.

Fig. 1.

Comparison of the expression of stem cell marker genes in the DFSCs and DFC using real-time RT-PCR. Note that expression of NT5E (CD73), THY1 (CD90), ALP, BCRP and NOTCH1 are significantly higher in the DFSCs than in the DFC. Single (*) asterisk indicates significant difference at $p < 0.05$.

Fig. 2.

Evaluation of calcium deposition in DFSCs and DFC cultured in different medium as shown by Alizarin Red staining. Note that Alizarin Red staining was detected only in the DFSCs incubated in the medium containing osteogenic induction reagents, but not in either α -MEM + 20% FBS or DMEM + 10% FBS without the induction reagents (A). In contrast, DFC did not form any calcium deposition in all treatments (B).

Fig. 3.

Expression of cell differentiation markers during osteogenic differentiation of the DFSCs as determined by real-time RT-PCR. Note that expression of osteoblast markers, BSP (A) and OCN (B) was greatly enhanced during osteogenic induction of DFSCs, whereas the expression of odontoblast marker (DSPP) showed a minimal increase (C); and expression of DMP1 in the DFSCs was also increased significantly during osteogenic induction (D). Bars with different letters indicate significant difference at $P < 0.05$.

Fig. 4.

Comparison of DMP1 expression in the DFSCs and DFC. Real-time RT-PCR determined that DFSCs expressed high levels of DMP1, whereas non-stem cell DFC had no or an extremely low level of DMP1 expression either in their original growth medium (A) or in α -MEM + 20% FBS (B). Western blotting analysis indicated that DMP1 protein was only seen in the DFSCs, but not in the DFC (C). Double (**) asterisks indicate a significant difference at $p < 0.001$.

Fig. 5.

DMP1 expression, osteogenic differentiation capability and ALP activity in different passages of DFSCs. Note that the DMP1 expression was reduced in passages 7 and 9 as shown by relative gene expression (RGE) determined by Real-time RT-PCR (A).

Osteogenesis was also significantly decreased at passages 7 and 9 based on Alizarin Red staining measured by Image-Pro Analyzer (B). ALP activity was reduced as advancement of cell passages when subjected to osteogenic induction (C). Bars with different letters indicate a significant difference at $P = 0.05$

Fig. 6.

Effect of DMP1 knockdown on osteogenesis and osteogenic gene expression. DMP1 expression was knocked down by transfecting DFSCs with DMP1-siRNA with maximal efficiency at days 3 and 5 posttransfection (A). Real-time RT-PCR determined the decreased relative gene expression (RGE) of selective osteogenic genes in DFSCs after DMP1 knockdown as compared to the control after 7 days of osteogenic induction (B). Alizarin Red staining revealed that knockdown of DMP1 expression in early passages of DFSCs resulted in a notable reduction of calcium deposition after 14 days of osteogenic induction when compared to the mock transfection and scrambled siRNA transfection controls (C). Single (*) and double (**) asterisks indicate significant reduction at $p = 0.05$ and $p = 0.001$, respectively, as compared to the control for the given genes.

Fig. 7. Osteogenesis of late passage DFSCs after addition of exogenous DMP1 to the induction medium. Addition of mouse recombinant DMP1 (mrDMP1) to the osteogenic induction medium increased calcium deposition in passage 9 DFSCs after 14 days of induction as shown by Alizarin Red staining (A). Alizarin Red staining to measure osteogenesis was quantitatively assessed by Image-Pro Analyzer (B). Note that minimal or no staining was seen in the control without addition of mrDMP1 whereas adding mrDMP1 to the osteogenic medium resulted in increase of Alizarin Red staining in a dosage-dependent manner.

Table 1

Primer pairs used in this study

Genes	Abbreviation	Primer sequence	GeneBank accession #
Alkaline phosphatase	ALP	F: 5'-GACAAGAAGCCCTTCACAGC-3' R: 5'-ACTGGGCCTGGTAGTTGTTG-3'	NM_013059.1
Breast cancer resistance protein 1	BCRP	F: 5'-GTTTGGACTCAAGCACAGCA-3' R: 5'-AATACCGAGGCTGGTGAATG-3'	NM181381.2
Biglycan	BGN	F: 5'-AGAATGGGAGCCTGAGTTTCT-3' R: 5'-ACCTTGGTGATGTTGTTGGAGT-3'	NM_017087.1
Bone morphogenetic protein 2	BMP2	F: 5'-CTCAGCGAGTTTGAGTTGAGG-3' R: 5'-GGTACAGGTCGAGCATATAGGG-3'	NM_017178.1
Bone morphogenetic protein 5	BMP5	F: 5'-AATTTGGGCTTACAGCTCTGC-3' R: 5'-AGAAGAACCTCACTTGCCTTGA-3'	NM_001108168.1
Bone morphogenetic protein 6	BMP6	F: 5'-CTTACAGGAGCATCAGCACAGA-3' R: 5'-GTCACCACCCACAGATTGCTA-3'	NM_013107.1
Bone sialoprotein	BSP	F: 5'-ACGCTGGAAAGTTGGTAGCTG-3' R: 5'-TTCCTCTTCTCGTCGCTTTCCTT-3'	NM_012587.2
Dentin matrix protein 1	DMP1	F: 5'-ACCTTTGGAGACGAAGACAATGGC-3' R: 5'-TGTCTTCACTGGACTGTGTGGTGT-3'	NM_203493.3
Dentin Sialophosphoprotein	DSPP	F: 5'-GGGAAGCTCAGTGGAAGTAAAG-3' R: 5'-CTGCTGTGTCCCATGTTGTAT-3'	NM_012790.2
Fms-related tyrosine kinase 1	FLT1	F: 5'-ACAGAAGAGGATGAGGGTGTCT-3' R: 5'-ATCAGCTCCAGGTTTGACTTGT-3'	NM_019306.1
Matrix metalloproteinase 13	MMP13	F: 5'-TTTATTGTTGCTGCCCATGA-3' R: 5'-GAGAGACTGGATTCCCTGAACG-3'	NM_133530.1
Neurogenic locus notch homolog protein 1	NOTCH1	F: 5'-CTCAACACACTGGGCTCTTTC-3' R: 5'-ACACCCTCATAACCTGGCATAAC-3'	NM_001105721.1
Ecto-5'-nucleotidase	NT5E (CD73)	F: 5'-ACTCCACCAAGTGCCTCAAC-3' R: 5'-GTCCTTCCACACCGTTATCAA-3'	NM_021576.2
Osteocalcin	OCN	F: 5'-ACTGCATTCTGCCTCTCTGACCT-3' R: 5'-TATTCACCTCCTTACTGCCCTCCT-3'	NM_013414.1
Runt-related transcription factor 2	RUNX2	F: 5'-TACTTCGTCAGCGTCCTATCAG-3' R: 5'-ATCAGCGTCAACACCATCATT-3'	NM_053470.2
Thymocyte antigen 1	THY1 (CD90)	F: 5'-CAACTTCACCACCAAGGATGAG-3' R: 5'-CCAACCAGTCACAGAGAAATGA-3'	NM_012673.2