DMP1 is a target of let-7 in dental pulp cells

JING YUE, BULING WU, JIE GAO, XIN HUANG, CHANGXIA LI, DANDAN MA and FUCHUN FANG

Department of Stomatology, Nanfang Hospital, College of Stomatology,
Southern Medical University, Guangzhou, P.R. China

Received February 2, 2012; Accepted April 5, 2012

DOI: 10.3892/ijmm.2012.982

Abstract. Members of the let-7 family have been shown to play a critical role in cell differentiation and tumorigenesis. However, potential targets of let-7 are still unclear. In the current study, we used bioinformatic analysis combined with DNA sequence analysis to identify potential let-7 targets. We discovered that dentin matrix protein 1 (DMP1), which is a non-collagenous protein essential in the mineralization of dentin and bone, has a let-7 binding site in its 3'-untranslated region. Furthermore, reporter assays demonstrated that the DMP1 3'-untranslated region can be regulated directly by the members of let-7. Gene expression levels of let-7 and DMP1 were validated by qRT-PCR of dental pulp cells cultured in a mineralizing medium. Our results suggest that DMP1 is regulated post-transcriptionally by let-7 during odontoblast differentiation.

Introduction

Dental pulp cells (DPCs) have the potential to be mineralized, which plays a key role in pulp repair and dentinogenesis. Reparative dentin formation and pulp regeneration after partial degradation are under the control of pulp progenitor cells (1). Dentinogenesis is regulated by tissue interactions associated with complicated signal pathways. Dentin matrix protein 1 (DMP1), an essential noncollagenous and acidic phosphorylated extracellular matrix protein, is highly expressed in tooth odontoblasts and bone osteocytes, with low level expression in osteoblasts and cartilage (2-4). It is a multifunctional protein involved in the biomineralization of bones and dentin (5), phosphate homeostasis, and differentiation of odontoblasts and osteoblasts (6). The DMP1 gene has been mapped to human chromosome 4q21:22 (7) (chromosome 5q21 in mice) (8). Dentinogenesis imperfecta type II, the autosomal dominant disorder of dentin formation, has been also mapped to the same region of the genome, indicating that DMP1 expression is tightly linked genetically to its disease phenotype (9). Accordingly, DMP1 has been shown to play a prime role in dentin mineralization (10,11). Dentin and bone extracellular matrix (ECM) were shown to contain both the full length DMP1 as well as its processed N-terminal (N-ter) (37 kDa) and C-terminal (C-ter) (57 kDa) fragments. It was shown to regulate the transcription of DSPP during early odontoblast differentiation through binding of the promoter region through its carboxyl end (residues 420-489) and was implicated in signaling functions (12). This result led us to the hypothesis that DMP1 may have functions other than regulating mineralization. Recently, it was suggested that DMP1 belongs to the SIBLING (small integrin binding ligand N-linked glycoprotein) family of extracellular matrix proteins (13). The expression of SIBLING family proteins such as osteopontin and bone sialoprotein in soft tissues has been reported. DMP1 gene expression was also detected in fetal bovine brain by northern analysis and in newborn mouse brain by *in situ* hybridization (14,15). These reports raised the possibility of DMP1 expression in other soft tissues, such as liver, muscle, kidney, pancreas, salivary and eccrine sweat glands. Together with results obtained in previous reports, it is suggested that, in addition to its high affinity to calcium or hydroxyapatite due to its acidic character and to its role in the mineralization process, DMP1 may affect various cell activities.

Cells contain a variety of noncoding RNAs, such as microRNAs (miRNAs), which are small (22-nt) endogenous noncoding RNAs that anneal to the 3' untranslated region (3'UTR) of target mRNAs to inhibit translation and lower protein levels. It remains to be established how specific miRNAs contribute to regulate the onset of a tissue-specific phenotype in response to a multifunctional morphogen. Let-7 was one of the first identified miRNA families, consisting of 12 closely related genes in which each isomer is usually located on a different chromosome and which is highly conserved across animal species in sequence and timing of expression (16,17). Let-7 plays a significant role in cell proliferation, differentiation and oncogenesis; identification of the target genes of miRNA may help to characterize its diverse functions (17,18). By bioinformatic analysis, we have found a potential binding site for let-7 within the 3'UTR of DMP1.

It is important to understand the molecular mechanism and specific genes during the developmental process. It has been proven that DMP1 is regulated by many growth

Correspondence to: Professor Buling Wu, Department of Stomatology, Nanfang Hospital, College of Stomatology, Southern Medical University, 1838 N. Guangzhou Ave., Guangzhou 510515, P.R. China
E-mail: bulingwu@yahoo.cn

Key words: dentin matrix protein 1, dual luciferase reporter assay, dental pulp cells, let-7

and transcription factors in previous studies. However, the regulation of DMP1 is not fully understood, particularly the understanding of its post-transcriptional regulation. Here we present the probable miRNA pathway of DMP1 in its post-transcriptional regulation. Sophisticated computer-based prediction approaches of microRNAs and of their targets, and effective biological validation techniques for validating these predictions, now play a central role in discovery of microRNAs and elucidating their functions. In this study, we identified that let-7 may be the potential regulatory miRNA of DMP1 gene through utilization of bioinformatics analysis and a dual luciferase reporter assay. In addition, we assessed the expression levels of let-7 at 10 and 21 days of differentiation in odonto- and osteoblasts in dental pulp cells.

Materials and methods

Subjects and cell culture. Normal human impacted third molars were collected from adults (16-24 years of age) at the Nanfang Hospital in the Southern Medical University. The study protocol was approved by the Institutional Ethics Committee, and informed consent was obtained from all patients. DPCs were isolated and subjected to odontogenic induction as previously reported (19). Tooth surfaces were cleaned and cut around the cementum-enamel junction by using sterilized dental fissure burs to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root and then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 30-60 min at 37˚C. The cells were cultured in a growth medium containing Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 15% fetal bovine serum, 100 U/ml penicillin and 100,000 μg/ml streptomycin, and then cultured at 37˚C in 5% CO_2. DPCs at passage 3 were cultured in DMEM with 15% FBS until they reached 70-80% confluence. Cells were then induced in odontogenic medium consisting of 50 mg/ml ascorbic acid, 10 mM β-glycerophosphate, and 0.01 mM dexamethasone (Sigma) in DMEM with 15% FBS for 7-21 days. Control samples were DPCs grown in DMEM with 15% FBS and harvested at 80% confluence. T293 cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum.

Mineralization staining. Mineralization of cultured DPCs was determined using Alizarin Red (AR) staining. After Day 21, the cell layer was washed with PBS and fixed in 10% formaldehyde (Sigma-Aldrich) at room temperature for 15 min, then washed in duplicate with excess dH_2O prior to the addition of 1 ml of 40 mM AR (pH 4.1). The plates were incubated at room temperature for 30 min under gentle shaking. Following aspiration of the unincorporated dye, the plates were washed twice with dH_2O and visualized using phase microscopy (Nikon).

Bioinformatics. DMP1 was identified as a potential target to search for miRNAs. Three different miRNA target prediction programs were used, TargetScan (http://www.targetscan.org/), the UCSC genome browser tract for PicTar4 (http://genome.ucsc.edu/), and the miRBase for miRanda (http://microrna.sanger.ac.uk/sequences/) (20-22). Each of these programs were searched for complementarity to the miRNA seed region in the 3'UTRs of DMP1. miRNAs were chosen based upon their targeted prediction by all three programs, conservation of the binding region, and strength of the predicted interaction.

Dual luciferase reporter gene construct. A 1041-bp fragment of the DMP1 3'UTR containing the predicted binding site for let-7 was amplified from human genomic DNA using primers with a short extension containing cleavage sites for *Xho*I (5' end) and *Not*I (3' end): DMP1 forward (5'-CCGCTCGAGC ATCAGCTGTCCTAAGAAGCAGTT-3') and DMP1 reverse (5'-ATAAGAATGCGGCCGCTTTCTTCTGGGTATTATAA TCTTTATTAC-3'). Amplicons were cleaved with *Xho*I and *Not*I and cloned in between the *Xho*I and *Not*I cleavage sites of the psi-CHECK2 luciferase vector (Promega) downstream of the Renilla luciferase reporter gene.

Dual luciferase reporter assay. Luciferase assays were performed using the Dual-Luciferase assay kit as previously described (Promega). For let-7 target validation, 293-T cells were grown to a cell density of 60-70% and co-transfected in 24-well plates with the indicated psiCHECK2 luciferase construct (0.5 μg/well) and miRNA (20 μM) using Lipofectamine 2000. Following 48 h, the cells were harvested in passive lysis buffer, and luciferase activity was measured with a GloMax™ 20/20 luminometer (Promega). Renilla luciferase activity was normalized to corresponding firefly luciferase activity and plotted as a percentage of the control between samples. Luciferase experiments were measured in triplicate using independent samples, as indicated.

qRT-PCR. Dental pulp cells cultured in a mineralizing medium were able to differentiate into odontoblast-like cells. Samples were harvested for the isolation of RNA at 10 and 21 days of differentiation to detect quantitative changes in gene DMP1 and three miRNAs. Cells were cultured in a growth medium that served as the control. Total-RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. RNA was analyzed for integrity, purity and concentration by gel electrophoresis and spectrophotometry and stored at -80˚C until analysis.

For quantitative RT-PCR analysis, 0.1 μg of RNA per reaction was used with the QuantiTech SYBR-Green RT-PCR kit and primers specific for DMP1. To quantify miRNA expression, total-RNA was reverse transcribed for use in two-step quantitative RT-PCR using the stem-loop method. The resulting cDNA was subjected to real-time qRT-PCR using the universal reverse primer in conjunction with a sequence-specific forward primer for hsa-let-7a, hsa-let-7c, hsa-let-7d, hsa-let-7f, hsa-let-7g and hsa-let-7i. Each sample was performed in triplicate, and the results were normalized using primers to 18S rRNA (for DMP1) or U6 (for miRNA analysis) (Table I). The reactions were incubated at 95˚C for 5 min for one cycle and then 95˚C/15 sec, 65˚C/15 sec, 72˚C/32 sec for 40 cycles, with a final 10-min extension at 65˚C. Results are expressed as fold change in expression relative to the control sample calculated using the equation $RQ=2^{-\Delta\Delta Ct}$.

Statistical analyses. Statistical analysis was performed with SPSS for Windows (version 13.0) using the ANOVA test. All data are presented as the mean ± SD ($n\geq3$). $P\leq0.01$ was considered statistically significant.

Table I. Oligonucleotides used in this study.

Primer name	Sequence-specific primer
hsa-let-7a-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATATCAA-3'
hsa-let-7c-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACCAA-3'
hsa-let-7d-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTATCAA-3'
hsa-let-7f-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATATCAA-3'
hsa-let-7g-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGTVAA-3'
hsa-let-7i-RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGACAA-3'
U6-RT	5'-AACGCTTCACGAATTTGCGT-3'
hsa-let-7a-F	5'-TGAGGTAGTAGGTTGTATAGTT-3'
hsa-let-7c-F	5'-TGAGGTAGTAGGTTGTATGGTT-3'
hsa-let-7d-F	5'-AGAGGTAGTAGGTTGCATAGTT-3'
hsa-let-7f-F	5'-GCTGAGGTAGTAGATTGTATAGTT-3'
hsa-let-7g-F	5'-TGAGGTAGTAGTTTGTACAGTT-3'
hsa-let-7i-F	5'-GCATGAGGTAGTAGTTTGTGCTGTT-3'
miRNA-R	5'-GTGCAGGGTCCGAGGT-3'
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'
DMP1-F	5'-CCCTTGGAGAGCAGTGAGTC-3'
DMP1-R	5'-CTCCTTTTCCTGTGCTCCTG-3'
18s rRNA-F	5'-CCTGGATACCGCAGCTAGGA-3'
18s rRNA-R	5'-GCGGCGCAATACGAATGCCCC-3'

miRNA-R represents the universal reverse primer in conjunction with a sequence-specific forward primer for hsa-let-7a, hsa-let-7c, hsa-let-7d, hsa-let-7f, hsa-let-7g and hsa-let-7i. RT, step-loop primer; F, forward; R, reverse.

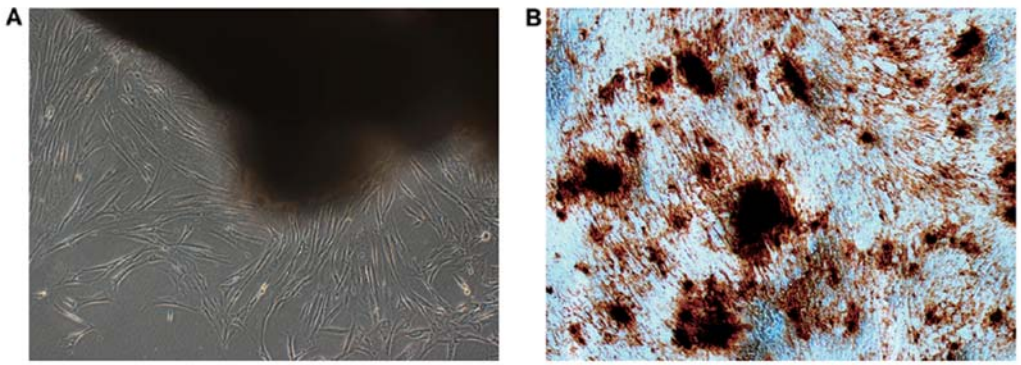

Figure 1. Cultivation and characteristics of dental pulp cells. (A) Dental pulp cells were dispersed from the tissue after about 12 days (magnification, x4). (B) Cells from second passages were used for the mineralization culture with Alizarin Red staining. After about 21 days, mineralized nodules formed and became more condensed (magnification, x4).

Results

Characteristics of dental pulp cells. It has been demonstrated that dental pulp cells can be expanded as a dentin-pulp-like structure. Our results show that DPCs have the potential to differentiate into odontoblast-like cells when cultured in the culture medium contain dexamethasone (Dex) and/or β-glycerophosphate (β-GP) (23). Ten days later, mineralized nodules formed and became more condensed. Alizarin Red staining of mineralized nodules in representative cell cultures is demonstrated at 21 days in Fig. 1B.

Bioinformatic analyses identify let-7 as a regulator of DMP1. With DMP1 being identified as a preferential target, miRNA target prediction was carried out using a combination of the following computational algorithms: TargetScan, miRanda, and PicTar. Based on the stem-loop feature of the miRNA and cross-species comparison, a number of computational algorithms have been developed to predict miRNAs from the genome. Among miRNAs being predicted to target DMP1, let-7 was complementary to multiple sequences of potential miRNAs with a high probability of binding to the 3'UTR of DMP1. To increase our probability of identifying miRNAs capable of regulating DMP1

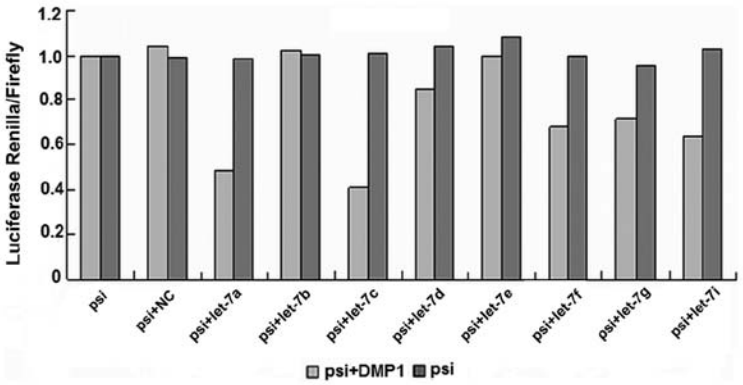

Figure 2. Binding ability of the members of let-7 family. Luciferase assays were performed following co-transfection of the DMP1 3'UTR luciferase reporter and the indicated miRNA-precursors. A luciferase signal is shown after Renilla luciferase readings were normalized to firefly luciferase. psi represents the luciferase vector alone. psiDMP1 represents the luciferase vector with a DMP1 3'UTR fragment. NC does not code for any known miRNA.

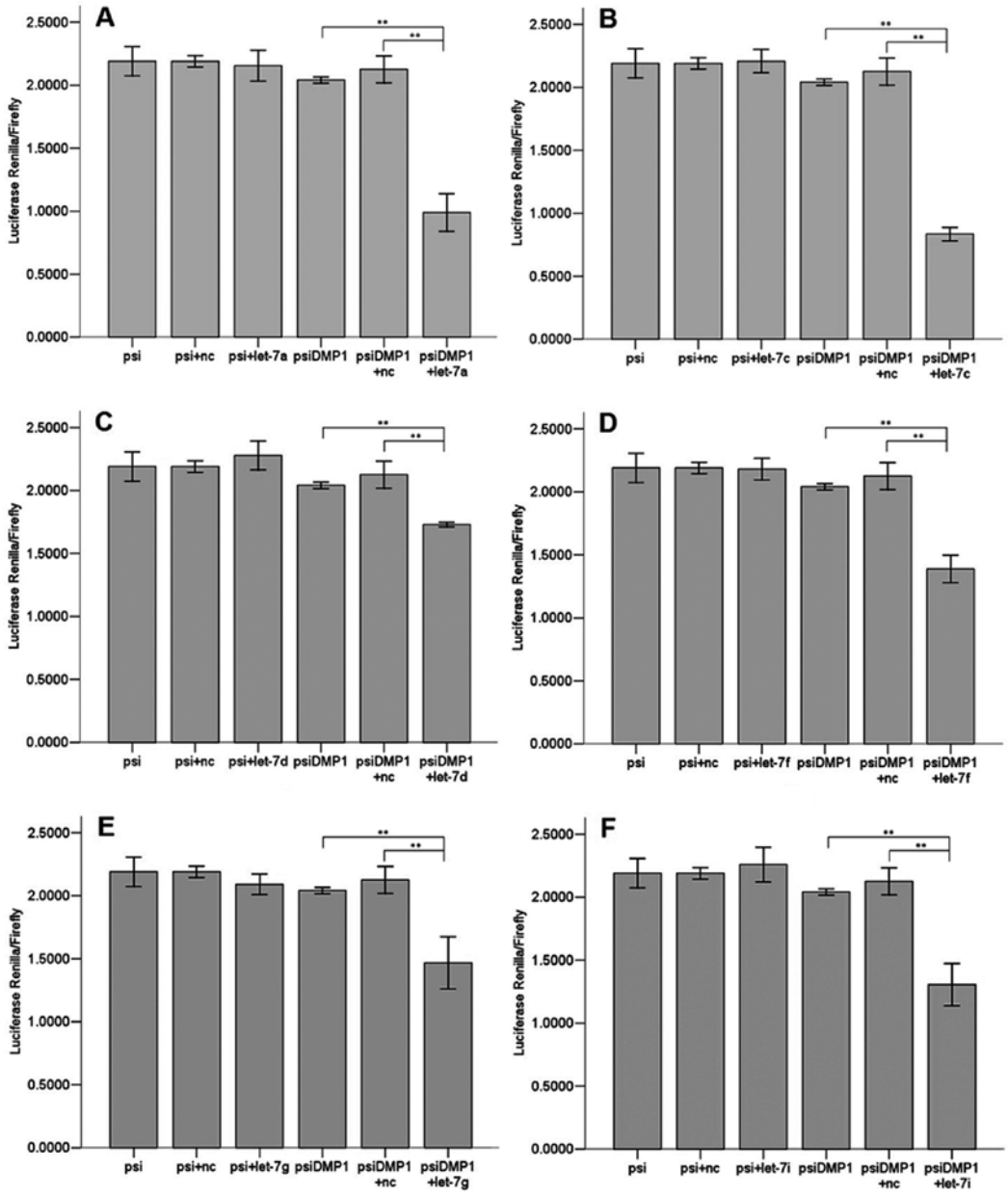

Figure 3. Luciferase reporter assay of (A) let-7a; (B) let-7c; (C) let-7d; (D) let-7f; (E) let-7g and (F) let-7i. Statistical analyses indicates that in the group of cells transfected with the luciferase vector alone, the binding abilities of miRNAs were similar to the negative control (NC) and blank psi luciferase vector alone. Six groups of cells transfected with DMP1 3'UTR vector, let-7a/c/d/f/g/i were able to reduce luciferase activity significantly compared with the blank ($^{**}P<0.01$) and NC ($^{**}P<0.01$) controls. (Error bars, ±2.00 SE).

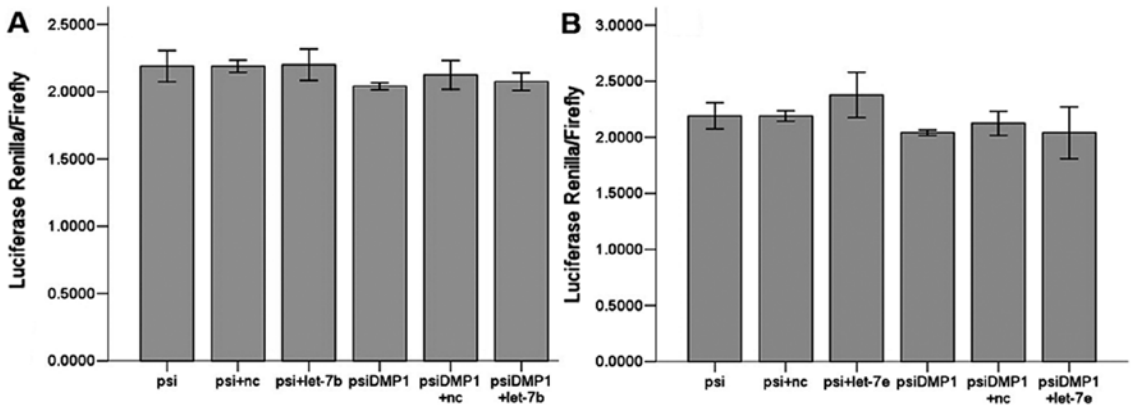

Figure 4. Luciferase reporter assay for (A) let-7b and (B) let-7e. Statistical analyses indicates that in the group of cells transfected with the luciferase vector alone, the binding ability of miRNAs was almost the same to the NC and blank controls. In the group of cells transfected with the DMP1 3'UTR vector, these miRNAs were not able to reduce luciferase activity significantly compared with the blank and NC controls. (Error bars, ±2.00 SE).

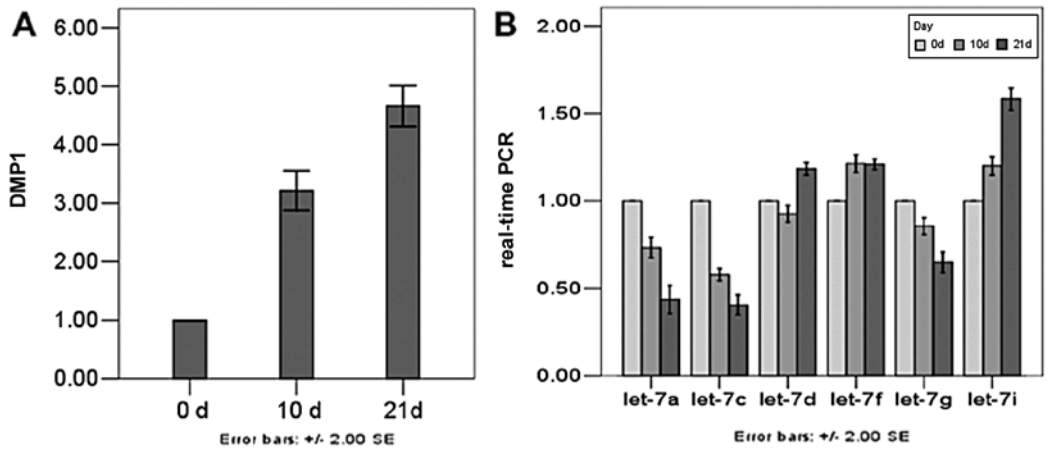

Figure 5. qRT-PCR for (A) DMP1 and (B) let-7a, let-7c, let-7d, let-7f, let-7g and let-7i at Days 0, 10 and 21 during mineralized culture of dental pulp cells. (A) Expression of DMP1 was weak before Day 7, but was increased on Days 10 and 21. (B) The 6 miRNAs existed in undifferentiated and differentiated dental pulp cells. After Day 10, the expression of especially let-7a, let-7c and let-7i were less than that on Day 0.

post-transcriptionally, we selected to further examine eight miRNAs that were identified by all three search algorithms.

Let-7 regulates DMP1. We used a luciferase reporter assay to test whether the 3'UTR of DMP1 contained sequences capable of interacting with let-7. The potential binding sites of let-7 within DMP1 3'UTR were obtained by TargetScan and PicTar. Synthetic oligos including predicted binding sites were annealed then cloned into *Xho*I/*Not*I site of psiCHECK-2. 293-T cells were transiently transfected with the relevant DMP1 3'UTR fragment and microRNA using Lipofectamine 2000 (Invitrogen) at the indicated concentrations.

To test whether the luciferase assay responds to let-7, we then tested reporters in which the 3'UTR of DMP1 was inserted downstream of firefly luciferase. The 3'UTR of DMP1 contains conserved let-7 seed matches at positions 988-994. When adding a miRNA that is able to interact with DMP1 3'UTR, the bioluminescence reaction of *Photinus pyralis* luciferase was annihilated, as miRNA inhibits translation of vector mRNA. This construct allowed us to quickly and quantitatively evaluate the miRNA's effect on the 3'UTR of DMP1. The DMP1 3'UTR fragment was inserted into the psiCHECK2 luciferase vector. The relative luciferase activity in 293-T cells transfected with the luciferase vector alone was set at 100% for comparison. The binding ability of the eight predicted let-7 miRNAs with DMP1 is shown in Fig. 2.

Let-7a, let-7c, let-7d, let-7f, let-7g and let-7i (Fig. 3) all significantly ($P<0.01$) reduced luciferase activity when compared to the negative scrambled miRNA and the luciferase vector alone, while let-7b and let-7e (Fig. 4) did not. There are 6 members of let-7 family miRNAs predicted by the biology software TargetScan and miRanda, and 5 by PicTar4. Let-7 family is considered a potentially important regulator of DMP1 3'UTR.

Expression levels of DMP1, let-7a, let-7c, let-7d, let-7f, let-7g and let-7i miRNA genes determined using qRT-PCR analysis. To validate the above data, the expression of 6 miRNA genes in the process of dental pulp cell differentiation was determined using qRT-PCR analysis. The DMP1 transcript was predominantly expressed in odontoblasts and transiently in preameloblasts along with an involvement with odontoblast differentiation and mineralization, which is used as an indicator of odontoblastic differentiation. Dental pulp cells cultured

in mineralizing medium exhibited odontoblastic features, including increasing DMP1. In our study, expression of DMP1 was weak before Day 0, but the amount increased by Day 10. After Day 21, DMP1 was strongly expressed. As a regulator of DMP1, the members of let-7 family need to exist in undifferentiated and differentiated dental pulp cells. Expression of 3 miRNAs on Day 10 was decreased, especially that of let-7a and let-7c. On Day 21, expression level of the 3 miRNAs was significantly decreased when DMP1 was at its peak. The expression of the other 3 miRNAs was higher in differentiated than undifferentiated cells, but the relative expression of let-7i rose higher than that of the other two miRNAs (Fig. 5).

Discussion

miRNAs constitute a type of endogenous post-transcriptional regulatory gene expression. miRNAs provide important regulatory functions as key negative regulators of diverse biological and pathological processes, including development, organogenesis, apoptosis, cell proliferation, differentiation and in the control of tumorigenesis. Some miRNAs can control gene expression during mesenchymal differentiation and in modulating osteogenic differentiation (24). A group of miRNAs can be co-regulatory when they target genes in common and therefore can involve a complex process. The conserved let-7 miRNA was originally discovered in *Caenorhabditis elegans* as a switch gene induced as cells exit the cell cycle when *C. elegans* reach their adult stage (25-27). In humans and mouse, like *C. elegans*, the expression of let-7 is barely detectable in embryonic developmental stages but increases after differentiation and in mature tissue (28). A previous study has implicated let-7 as a tumor suppressor (29). Let-7 family members may play a role in cancer progression, as they map onto genomic regions altered or deleted, such as HMGA2. This target was identified by a different let-7, which contributes to differentiation during mammary epithelial cell differentiation affecting self-renewal (30,31). Previous studies on the functional effects of let-7 have focused on the targets Ras, HMGA2, and c-Myc (32-35).

Two approaches are used to identify candidate genes targeted by miRNAs: computational target prediction algorithms and experimental target identification strategies. Prediction tools such as TargetScan and miRanda can be used to identify potential target genes for all miRNAs. Current miRNA target prediction tools have the common problem that their false positive rate is unavoidable, so experimental target identification is necessary. A dual luciferase reporter assay is a quick, sensitive and direct method to verify these predicted miRNAs (36). In this study, we predicted let-7 targeting DMP1 using computational analyses and observed various let-7 isoforms identified concurrently, let-7a, let-7c, let-7d, let-7f, let-7g and let-7i being the most predominant. Combined with qRT-PCR, we have shown for the first time that let-7 expression is differentially expressed on the marker gene DMP1 during differentiation of dental pulp cells to odontoblast-like cells. The results showed that on Day 21 the expression levels of DMP1 were highest, and that of let-7a, let-7c and let-7g were lowest. When the level of DMP1 were low, let-7a, let-7c and let-7g were relatively high, which demonstrated that there was some link between DMP1 and miRNAs. But it remains unclear whether let-7 regulates other target genes and what are the key roles of the complex regulatory network between miRNA and mRNA in this molecular pathway for odonto-differentiation.

In a previous study, we used a dual luciferase reporter assay and qRT-PCR to confirm that mir885-5p, mir586 and mir32-targeted DSPP were expressed during differentiation into odontoblast-like cells (37). These findings warrant additional studies to investigate whether miRNA alterations are also involved in the process of differentiation of dental pulp cells to odontoblast-like cells and whether miRNA expression levels would manifest the biological and biochemical consequences in the development of differentiation. The results of the present study suggest the possibility of using miRNAs for the development of cell differentiation. Further experiments will be required to assess the differentiation effect of let-7 miRNA at various stages of the process, and such experiments are currently underway in our laboratory.

Accordingly, future identification of the targets for miRNAs and the regularity for change of miRNAs may provide clues to develop a novel marker during differentiation of dental pulp cells and result in abnormal dentin formation. It is envisaged that such future studies may ultimately provide a foundation for a new paradigm of the involvement of noncoding small RNA species, miRNA, in stem cell differentiation. Our findings suggest a mechanistic link between the let-7 family of miRNAs and DMP1 gene expression in dental pulp cells. We anticipate that unraveling the molecular mechanisms by which miRNAs mediate effects during odontoblast differentiation will allow us to decipher the central regulatory role of miRNAs in many fundamental biological processes.

Acknowledgements

This study was supported by a grant-in-aid for the College intelligences funds from Guangdong province (no. C1030270) and the Events of Science and Technology Program of the Guangdong province (no. 2010B060900053).

References

1. Huang GT, Gronthos S and Shi S: Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88: 792-806, 2009.
2. George A, Sabsay B, Simonian PA and Veis A: Characterization of a novel dentin matrix acidic phosphoprotein. Implications for induction of biomineralization. J Biol Chem 268: 12624-12630, 1993.
3. Hirst KL, Ibaraki-O'Connor K, Young MF and Dixon MJ: Cloning and expression analysis of the bovine dentin matrix acidic phosphoprotein gene. J Dent Res 76: 754-760, 1997.
4. MacDougall M, Gu TT, Luan X, Simmons D and Chen J: Identification of a novel isoform of mouse dentin matrix protein 1: spatial expression in mineralized tissues. J Bone Miner Res 13: 422-431, 1998.
5. Qin C, Brunn JC, Cook RG, Orkiszewski RS, Malone JP, Veis A and Butler WT: Evidence for the proteolytic processing of dentin matrix protein 1. Identification and characterization of processed fragments and cleavage sites. J Biol Chem 278: 34700-34708, 2003.
6. Almushayt A, Narayanan K, Zaki AE and George A: Dentin matrix protein 1 induces cytodifferentiation of dental pulp stem cells into odontoblasts. Gene Ther 13: 611-620, 2006.
7. MacDougall M, DuPont BR, Simmons D and Leach RJ: Assignment of DMP1 to human chromosome 4 band q21 by in situ hybridization. Cytogenet Cell Genet 74: 189, 1996.

8. George A, Gui J, Jenkins NA, Gilbert DJ, Copeland NG and Veis A: In situ localization and chromosomal mapping of the AG1 (Dmp1) gene. J Histochem Cytochem 42: 1527-1531, 1994.
9. Aplin HM, Hirst KL, Crosby AH and Dixon MJ: Mapping of the human dentin matrix acidic phosphoprotein gene (DMP1) to the dentinogenesis imperfecta type II critical region at chromosome 4q21. Genomics 30: 347-349, 1995.
10. He G, Dahl T, Veis A and George A: Dentin matrix protein 1 initiates hydroxyapatite formation in vitro. Connect Tissue Res 44 (Suppl 1): 240-245, 2003.
11. He G, Gajjeraman S, Schultz D, Cookson D, Qin C, Butler WT, Hao J and George A: Spatially and temporally controlled biomineralization is facilitated by interaction between self-assembled dentin matrix protein 1 and calcium phosphate nuclei in solution. Biochemistry 44: 16140-16148, 2005.
12. Narayanan K, Ramachandran A, Hao J, He G, Park KW, Cho M and George A: Dual functional roles of dentin matrix protein 1. Implications in biomineralization and gene transcription by activation of intracellular Ca^{2+} store. J Biol Chem 278: 17500-17508, 2003.
13. Fisher LW and Fedarko NS: Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. Connect Tissue Res 44 (Suppl 1): S33-S40, 2003.
14. D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB and MacDougall M: Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. J Bone Miner Res 12: 2040-2049, 1997.
15. Hirst KL, Ibaraki-O'Connor K, Young MF and Dixon MJ: Cloning and expression analysis of the bovine dentin matrix acidic phosphoprotein gene. J Dent Res 76: 754-760, 1997.
16. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB and Bartel DP: The microRNAs of *Caenorhabditis elegans*. Genes Dev 17: 991-1008, 2003.
17. Roush S and Slack FJ: The let-7 family of microRNAs. Trends Cell Biol 18: 505-516, 2008.
18. He XY, Chen JX, Zhang Z, Li CL, Peng QL and Peng HM: The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice. J Cancer Res Clin Oncol 136: 1023-1028, 2010.
19. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG and Shi S: Stem cell properties of human dental pulp stem cells. J Dent Res 81: 531-535, 2002.
20. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP and Burge CB: Prediction of mammalian microRNA targets. Cell 115: 787-798, 2003.
21. Marin RM and Vanicek J: Efficient use of accessibility in microRNA target prediction. Nucleic Acids Res 39: 19-29, 2011.
22. Madden SF, Carpenter SB, Jeffery IB, Bjorkbacka H, Fitzgerald KA, O'Neill LA and Higgins DG: Detecting microRNA activity from gene expression data. BMC Bioinformatics 11: 257, 2010.
23. Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M and Magloire H: Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif Tissue Int 66: 129-138, 2000.
24. Goff LA, Boucher S, Ricupero CL, Fenstermacher S, Swerdel M, Chase LG, Adams CC, Chesnut J, Lakshmipathy U and Hart RP: Differentiating human multipotent mesenchymal stromal cells regulate microRNAs: prediction of microRNA regulation by PDGF during osteogenesis. Exp Hematol 36: 1354-1369, 2008.
25. Abbott AL: Uncovering new functions for microRNAs in *Caenorhabditis elegans*. Curr Biol 21: R668-R671, 2011.
26. Johnson SM, Lin SY and Slack FJ: The time of appearance of the *C. elegans* let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. Dev Biol 259: 364-379, 2003.
27. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, Horvitz HR and Ambros V: The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in *Caenorhabditis elegans*. Dev Cell 9: 403-414, 2005.
28. Jin H, Lv S, Yang J, Wang X, Hu H, Su C, Zhou C, Li J, Huang Y, Li L, Liu X, Wu M and Qian Q: Use of microRNA Let-7 to control the replication specificity of oncolytic adenovirus in hepatocellular carcinoma cells. PLoS One 6: e21307, 2011.
29. Li Y, VandenBoom TN, Kong D, Wang Z, Ali S, Philip PA and Sarkar FH: Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer Res 69: 6704-6712, 2009.
30. Chen AX, Yu KD, Fan L, Li JY, Yang C, Huang AJ and Shao ZM: Germline genetic variants disturbing the Let-7/LIN28 double-negative feedback loop alter breast cancer susceptibility. PLoS Genet 7: e1002259, 2011.
31. Yun J, Frankenberger CA, Kuo WL, Boelens MC, Eves EM, Cheng N, Liang H, Li WH, Ishwaran H, Minn AJ and Rosner MR: Signalling pathway for RKIP and Let-7 regulates and predicts metastatic breast cancer. EMBO J 30: 4500-4541, 2011.
32. Boominathan L: The guardians of the genome (p53, TA-p73, and TA-p63) are regulators of tumor suppressor miRNAs network. Cancer Metastasis Rev 29: 613-639, 2010.
33. Lee YS and Dutta A: The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21: 1025-1030, 2007.
34. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D and Slack FJ: RAS is regulated by the let-7 microRNA family. Cell 120: 635-647, 2005.
35. Wang S, Tang Y, Cui H, Zhao X, Luo X, Pan W, Huang X and Shen N: Let-7/miR-98 regulate Fas and Fas-mediated apoptosis. Genes Immun 12: 149-154, 2011.
36. Nicolas FE: Experimental validation of microRNA targets using a luciferase reporter system. Methods Mol Biol 732: 139-152, 2011.
37. Huang X, Xu S, Gao J, Liu F, Yue J, Chen T and Wu B: miRNA expression profiling identifies DSPP regulators in cultured dental pulp cells. Int J Mol Med 28: 659-667, 2011.