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Identification of Secretory Odontoblasts Using DMP1-GFP Transgenic Mice

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Abstract

Terminal differentiation of odontoblasts from dental papilla is a long process involving several intermediate steps and changes in the transcriptional profile and expression of proteins secreted by cells in the odontoblast lineage. Transgenic mouse lines in which GFP expression is under the control of tissue- and stage-specific promoters have provided powerful experimental tools for identification and isolation of cells at specific stages of differentiation along a lineage. Our previous studies showed utilization of pOBCol3.6GFP and pOBCol2.3GFP animals for identification of odontoblasts at early and late stages of polarization respectively. In the present study we used the DMP1-GFP transgenic animal as an experimental model to examine its expression during the differentiation of odontoblasts from progenitor cells *in vivo* and *in vitro*. Our observations showed that DMP1-GFP transgene is first activated in secretory/functional odontoblasts engaged in secretion of predentin and then transiently expressed at high levels in newly differentiated odontoblasts. Expression of DMP1-GFP was down-regulated in highly differentiated odontoblasts. The temporal and spatial pattern of expression of DMP1-GFP transgene closely mimics the expression of endogenous DMP1. This transgenic animal will facilitate studies of gene expression and biological functions in secretory/functional odontoblasts.

Keywords

Odontoblast differentiation; dental pulp; progenitors; DMP1; green fluorescent protein

Introduction

Dentinogenesis is regulated by odontoblasts, highly specialized cells originating from the neural crest derived cells of the dental papilla. The differentiation of odontoblasts from the neural crest cells is a long process involving several intermediate steps that are dependent on and regulated by epithelial signals [1–4]. During this process dental papilla differentiates first into pre-odontoblasts then polarized odontoblasts, secretory/functional odontoblasts and finally highly differentiated/terminally-differentiated odontoblasts. In mice the steps between the formation of pre-odontoblasts and highly differentiated odontoblasts are completed within 6–10 hours [5,6].

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Dentin secreted by odontoblasts consists of inorganic and organic components [7]. The inorganic components consist mostly of hydroxyapatite and water [7] and the organic components consist primarily of collagen fibers and non-collagenous proteins (NCPs) including Dentin Matrix Protein-1 (DMP1).

DMP1, an acidic non-collagenous phospho-protein, is a member of the SIBLING (small integrin binding ligand N-linked glycoprotein) family with essential roles in mineralization [8,9]. The essential roles of DMP1 in mineralization have been demonstrated by various *in vitro* and *in vivo* studies. *In vitro* studies showed that MC3T3-E1 cells over expressing DMP1 display accelerated and early onset of mineralization [10]. DMP1 null mutants developed abnormalities in teeth bones and teeth during postnatal development that became more severe with age [11–13]. The abnormalities in bone were linked to osteocyte function and included hypomineralized bone, defects in cartilage formation and hypophosphatemia rickets with elevated levels of FGF23 during postnatal development [11–13]. The abnormalities in teeth included a partial failure of maturation of predentin into dentin, increased width of the unmineralized predentin, reduced expression of osterix in odontoblast, and abnormalities in the dentinal tubules [12,14–16]. The re-expression of DMP1 by the 6 kb fragment of DSPP promoter in mature odontoblasts resulted in only a partial rescue of the defects in dentin, whereas re-expression of DMP1 by the 3.6 kb fragment of Col1a1 promoter in early odontoblasts resulted in complete rescue of dentin defects indicating that DMP1 is a key regulator of odontoblast differentiation and mineralization of dentin [15].

Analysis of bones in transgenic animals in which GFPtpz is under the control of 8 kb upstream regulatory sequences of the DMP-1 (–7892 to +4439) [17–19] showed that DMP1-GFP similar to the DMP1 protein and mRNA [20,21], is expressed by early and mature osteocytes. The 8 kb regulatory region of DMP1 also contains mechanical response elements and DMP1-GFP has provided a useful tool for studying the effects of mechanical loading on osteocytes [18] and analysis of mineralization kinetics in primary cultures from calvaria [19].

Given the important roles of DMP1 in dentinogenesis, in the present study we have used DMP1-GFP transgenic animal as an experimental model to examine the differentiation of odontoblasts from progenitor cells in dental pulp. Therefore, the goal of our studies was not to gain insight into function of DMP1 during dentinogenesis but to determine if the 8 kb fragment can be used to study dentinogenesis.

We analyzed the temporal and spatial expression of this transgene in the developing teeth *in vivo* and during *in vitro* mineralization in primary pulp cultures. Our observations show that the expression of DMP1-GFP transgene mimicked the expression of endogenous *DMP1* in that it was first expressed in secretory/functional odontoblasts engaged in secretion of predentin, prior to the expression of *DSPP*. DMP1-GFP transgene was transiently expressed at high levels in newly differentiated odontoblasts and down-regulated in highly differentiated odontoblasts. The activation of this transgene in secretory/functional odontoblasts will facilitate identification and purification of these cells for further analysis.

Materials and Methods

Expression of DMP1-GFP transgene in developing teeth

Mandibular arches and developing teeth at different stages of embryonic development (E16–19) and postnatal growth (P1 and P16) were isolated from DMP1-GFP hemizygous transgenic mice [17]. In this transgenic animal GFPtpz is under the control of 8 kb upstream regulatory sequences of the DMP-1 (–7892 to +4439) containing approximately 7.9-kb long

promoter region, the first exon, the entire first intron and a small part of second exon (approximately 4.4-kb region of the *DMP1* gene) [17]. Tissue fragments were fixed in 4% paraformaldehyde, and decalcified in 15% EDTA, 0.5% paraformaldehyde for various times (depending on the age), and processed for paraffin embedding. Seven μ m sections were processed for Mallory staining using standard protocols. Adjacent sections were mounted with glycerol/phosphate-buffered saline (PBS), and visualized using a Nikon E600 microscope equipped with FITC and Texas Red dual fluorescent filter cube and photographed using a Spot RT TM digital camera (Diagnostic instruments Inc, Sterling Heights, MI). The digitized images from epifluorescence and dark-field microscopy were assembled with Adobe PhotoShop 6.0 software (Adobe Systems, San Jose, CA, USA).

***In Situ* Hybridization Analysis**

The patterns of expression of *DSPP* and *DMP1* in the developing arches were examined by *in situ* hybridization on tissue sections using 33 P-labeled RNA probes for *DSPP* and *DMP1* as previously described [22]. Serial sections were used so that expression patterns for *DSPP*, *DMP1*, and *DMP1*-GFP could be compared within the same animal.

Primary dental pulp cultures, digital imaging and epifluorescence analysis in cell culture

The coronal portions of the pulps from first molars were isolated from 5–7-day-old hemizygous *DMP1*-GFP and non-transgenic mice and prepared for primary cultures as previously described [23–25]. GFP expression in cell culture was visualized using an Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus America, Inc., Melville, NY, USA). A specific excitation wavelength was obtained using filters for GFPtpz (exciter: D500/20; dichroic: 525DCLP; emitter: D550/40). Images were captured using a SPOTcamera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Detection and Quantification of mineralization in cultures

Mineralized nodules in live cultures were visualized by Xylenol Orange (XO) staining as described previously [24,26]. Mineralization in fixed cultures was also analyzed by von Kossa staining and quantified using Alizarin Red-S staining [24,27]. Values represent mean $\pm$ SE of at least three independent experiments.

RNA extraction and analyses

Total RNA was prepared using TRI Reagent according to the manufacturer's instructions. After DNase treatment, RNA samples were processed for RT-PCR analysis using specific primers as described before [24]. The expression of *Sost* in cultures was also examined by quantitative polymerase chain reaction using 2 ng of cDNA and 2X TaqMan universal PCR master mix (Applied Biosystems, USA) using a one-step program (50°C for 2 min and 95°C for 10 min) followed by 95°C for 30 s, and 59°C for 1 min for 40 cycles. TaqMan gene expression assays for *Sost* (00470479_m1), and *GAPDH* (99999915_g1) were purchased from Applied Biosystems. Gene specific assays were run in quadruplicate in an iCycler thermocycler (BioRad, iCycler, USA) and analyzed using iQv3.1 post-run analysis software. The relative expression level for each target gene, normalized to *GAPDH*, was calculated using the comparative C_T method; amplification efficiency for each TaqMan assay was determined using internal standard curves derived from purified amplicon, diluted 2-fold (2 ng – 0.125 ng) assayed performed in duplicate.

Flow cytometric analysis and Sorting (FACS)

Cultures were prepared for flow cytometric analysis and sorting as previously described [23,25]. Dental pulp cells obtained from non-transgenic mice were used as controls.

Immunocytochemistry and correlation of expression of DMP1-GFP and DSP in mineralized nodules

Cultured cells were first examined and photographed using Zeiss Axiovert 200 microscope for expression of DMP1-GFP. After imaging, live cultures were incubated overnight at 37°C with 30mM of Calcein Blue (Sigma, USA) and then fixed and processed for immunocytochemistry using 1:200 dilution of Anti-DSP [LF-153 (kind gift from Dr. Larry Fisher)] as previously described [24]. Stained cultures were re-examined under Zeiss Axiovert 200 microscope under TRITC red (DSP), TOPAZ (DMP1-GFP) and Sapphire (Calcein Blue) filters. Using AxioVision Rel 4.7 software the expression of DMP1-GFP, Calcein Blue and DSP in the same areas of cultures was compared.

Immunohistochemistry was also performed on paraffin embedded sections of heads and limbs obtained from P1 and P21 mice. Sections were quenched in 1% H₂O₂ and incubated with Target Retrieval Solution (DakoCytomation) for 20 min at 95 °C. Slides were incubated with blocking solution and then with primary antibody (goat Anti-SOST, dilution 1:100) overnight at 4 °C. Labelled Streptavidin-Biotin System, Horseradish Peroxidase (LSAB2 System- HRP, DakoCytomation) kit was used to qualitatively analyze antigen expression, according to manufacturer's protocol. Incubation with DAB Substrate-Chromogen resulted in a brown-colored precipitate at the antigen site.

Cell cycle analysis

The cell cycle and rate of proliferation in DMP1 GFP⁺ and DMP1-GFP⁻ populations were examined in three days old cultures as previously described [25]. The viability and proliferation of sorted cells was examined by MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) as previously described [25].

Statistical analysis

Unpaired, two-tailed t-tests were performed to determine statistically significant differences and $p < 0.05$ was considered statistically significant.

RESULTS

Localization of DMP1-GFP in the developing teeth

The DMP1-GFP transgene was not detected in the dental tissues during the initiation, bud, cap and early bell stages of molar tooth development (E10–18) (Figure 1A and 1B and data not shown). At the late bell stage (E19) DMP1-GFP was expressed at low intensity in odontoblasts at the tip of the cusp of the first mandibular molar (arrowheads in Figure 1E and F). At the secretory stage of crown formation (P1 and P5), DMP1-GFP transgene was expressed at high intensity in the entire layer of odontoblasts covering the dental pulp (Figure 1I and 1J and 2A-C and data not shown). In the cervical loops (Figure 1I and 1J), there was a gradient of expression of DMP1-GFP transgene in that the transgene was expressed at slightly lower intensity in the secretory/functional odontoblasts and at higher intensity in differentiated odontoblasts and odontoblast processes (Figure 1I and J, data not shown). During postnatal growth, DMP1-GFP expression in odontoblasts in the tip of the cusps was lower than in odontoblasts in the remainder of the crown and in the roots and the remainder of the crown (Figure 2A-C & 2D-F). Low levels of DMP1-GFP were also detected in some of the cells in the dental pulp in close vicinity to the cervical loops of the developing molars (Figure 1I and J).

Developing incisors showed an apical to incisal gradient of expression of DMP1-GFP transgene. (Figure 3A and B). DMP1-GFP expression was not detected in the apical end and was detected in the incisal end in the secretory/functional odontoblasts and at higher levels

in fully differentiated odontoblasts (Figure 3A and B) and odontoblast processes (Figure 3E-G). Between E17 and P7, DMP1-GFP was also expressed at high levels in osteocytes within the developing alveolar bone (Figure 1A, 2B and 3A).

Comparison of the patterns of expression of DMP1-GFP transgene with those of *DSPP* and *DMP1* in adjacent sections showed the expression of DMP1-GFP transgene in odontoblasts was well correlated with the expression of *DMP1* and *DSPP* (Figures 1 and 3) indicating its expression in secretory/functional and fully differentiated odontoblasts. In the developing bones DMP1-GFP expression was closely correlated with the expression of *DMP1* (Figures 1D and 3C).

DMP1-GFP expression during mineralization and odontoblasts differentiation *in vitro*

Odontoblast differentiation involves several intermediate steps that occur rapidly (within 6–10 hours) *in vivo*. This rapid transition, and the close proximity of cells in the different stages of differentiation in the developing teeth *in vivo* make it difficult to fully appreciate the stage of activation of DMP1-GFP in odontoblasts. Therefore, the temporal and spatial expression of the DMP1-GFP transgene was examined during *in vivo* mineralization in primary pulp cultures. In these experiments, DMP1-GFP expression was also correlated with the onset and subsequent growth of the mineralized nodules at various time points in real time by Xylenol Orange (XO) staining in the same cultures.

DMP1-GFP expression was detected in a few scattered cells at day 2 and 7 prior to mineralization (Figure 4). After addition of mineralization media at around day 10, DMP1-GFP expression was detected in multilayered areas containing unmineralized and mineralized matrices (detected by XO staining) (Figure 4). At days 14 and 21, after extensive mineralization, expression of DMP1-GFP at high and low intensities was detected in areas of mineralization (stained with XO).

Changes in the percentage of cells expressing DMP1-GFP and the distribution of signal strength within DMP1-GFP+ cells in primary pulp cultures were also examined by flow cytometry. A few cells expressing DMP1-GFP at low intensity were detected at early time points (days 2 and 7). With the onset and subsequent growth of mineralized matrix there was an approximately 18 fold increase in the percent of DMP1-GFP+ cells and a decrease in the percent of DMP1-GFP- cells (Figure 5 and Table 1). There were also continuous increases in the mean intensity of DMP1-GFP between days 2 (75) and day 14 (216).

To determine if the increase in the DMP1-GFP+ population was due to activation of DMP1-GFP in a new population (did not express these transgenes before) and/or proliferation of existing GFP+ population, DNA content/cell cycle analysis was performed. The rate of proliferation in DMP1-GFP- cells was significantly higher (45.7%) than DMP1-GFP+ cells (25.3%) (Table 2) indicating that increases in the number of DMP1-GFP+ cells in cultures were primarily due to activation of DMP1-GFP in new cells.

Comparison of the expression of DMP1-GFP and markers of mineralization

The pattern of expression of DMP1-GFP was also compared to the expression of selected markers for early and late stages of odontoblast differentiation (*Colla1*, *OC*, *DMP1* and *DSPP*) by RT-PCR analysis (Figure 6). In these cultures a low but detectable level of *GFP* was expressed at day 7 (prior to the expression of early and late markers of mineralization) and increased thereafter. The expression of *BSP*, *OC*, *DSPP* and endogenous *DMP1* was not detected at day 7, appeared at day 10 and increased at day 14. It should be noted that the pattern of expression of *DMP1* during mineralization of primary pulp cultures was different from its patterns of expression in primary osteogenic cultures (neonatal calvaria and BMSC) [17]. In osteogenic cultures, *DMP1* was first detected after mineralization and expression of

osteocalcin [17]. We showed in primary pulp cultures the expression of *DMP1* and OC are detected at the same time, just prior to or at the onset of mineralization.

Stage of activation of DMP1-GFP in pulp cultures, mineralization and dentinogenic potentials of DMP1-GFP+ and DMP1-GFP- populations

The presence of a mixture of cells (GFP+ and GFP-) in pulp cultures at day 2 and the expression of DMP1-GFP in pulps of intact teeth made it difficult to study the stage of activation of this transgene during mineralization and dentinogenesis. Therefore, we studied the expression of this transgene in DMP1-GFP- populations.

Primary pulp cultures were prepared from DMP1-GFP transgenic mice and grown in culture conditions supporting their proliferation. At day 7, FACS sorting and re-analysis were used for separation of DMP1-GFP+ and DMP1-GFP- populations with >98% purity (Supplemental Figure 1A). Both populations were re-plated at the same density as unsorted cells (5×10^5 cells/well in 35-mm culture plates) and grown for an additional 7 days in media supporting their proliferation and then in mineralization inducing media. Controls in these experiments included cultures from unsorted cells (primary cultures) and cultures from unsorted cells that were re-plated after 7 days (secondary culture).

Cultures from DMP1-GFP- population remained healthy, proliferated and reached confluence at day 7 (Figure 7). The number of viable cells in these cultures was high and similar to that in secondary cultures (Supplemental Figure 1B). In these cultures, GFP was not detected at day 2 and was detected at low intensity in a very few scattered cells at day 7 (Figure 7). At day 10, three days after the addition of mineralization inducing media, individual and small clusters of cells expressing DMP1-GFP were detected throughout the culture. XO staining showed that mineralization occurred in small spotty areas and were associated with cells expressing DMP1-GFP (Figure 7). At days 14 and 21, these cultures contained an extensive number of multi-layered nodules that stained with XO, and cells expressing DMP1-GFP at low and high intensity. The extent of mineralization in DMP1-GFP- population (Figure 8D) were comparable to those in secondary cultures (Figure 8A and 8D) but lower than that in primary cultures (Figure 8D). DMP1-GFP- population also differentiated into odontoblasts as indicated by the expression of *DSPP* (Figure 8E and 8F). The relative level of *DSPP* in DMP1-GFP- cultures was not significantly different from that in secondary cultures (Figure 8E and 8F).

In cultures from DMP1-GFP+ population only a few cells attached to the culture dish and survived (Supplemental Figure 1C). The attached cells exhibited poor viability and never reached confluence (data not shown). Plating at higher density did not improve their attachment, viability or proliferation (data not shown). Furthermore, the majority of attached cells lost the expression of DMP1-GFP after 2 days of re-plating (Supplemental Figure 1C). Due to poor viability and proliferation, the extent of mineralization and relative levels of *DSPP* expression in DMP1-GFP+ cultures were at background levels. The behavior of the DMP1-GFP+ population suggested that this population might contain cells at a relatively advanced stage of differentiation (i.e., differentiated odontoblasts/late osteoblast/osteocytes). The lack of *DSPP* expression in DMP1-GFP+ cells (data not shown) suggested that this population contains cells in either the osteogenic lineage (late osteoblasts/osteocyte) or odontoblasts that have not initiated *DSPP* expression.

The DMP1-GFP-transgene is expressed by odontoblasts and osteocytes in primary pulp cultures

Our recent immunocytochemical analysis of primary pulp cultures from unerupted molars with anti-DSP antibody showed that in dental pulp cultures approximately half (50%–55%)

of the mineralized tissue was dentin-like matrix secreted by odontoblasts [24]. The lack of DSP expression in the remaining mineralized matrix in these cultures suggested that these cultures contained bone-like matrix deposited by osteoblasts/osteocytes. Previous studies in primary BMSC cultures showed expression of DMP1-GFP at high intensity in late osteoblasts/osteocytes [17]. Therefore, to gain further insight into osteogenic and dentinogenic potentials of the pulp cultures, the expression of DSP in 14 and 21 day-old dental pulp cultures from DMP1-GFP transgenic animals was examined by immunocytochemistry using anti-DSP antibody. Mineralization in these cultures was examined by Calcein Blue staining.

At days 14 and 21, there was a good correlation between the expression of DMP1-GFP and mineralization (Figure 9A and 9C and Supplemental Figure 2). On the other hand, DSP expression was detected only in a fraction of area stained with and DMP1-GFP and Calcein blue (Figure 9B and 9C and Supplemental Figure 2 and data not shown). Cultures stained with Calcein Blue, DMP1-GFP and Anti-DSP showed two distinct areas identified by differential expression of these markers (Figure 9D and Supplemental Figure 2). The first area contained Calcein Blue+/DMP1-GFP+/DSP- matrices (region 1 in Figure 9 and Supplemental Figure 2) representing areas containing osteoblasts/osteocytes secreting bone-like matrix. The osteogenic potential and the formation of osteocytes in pulp cultures was further studied by examining the expression of sclerostin (SOST), an osteocyte specific protein [28]. At P1, SOST was expressed by osteocytes in the calvaria and in the cortical bones but not in the odontoblasts (Supplemental Figure 3). Analysis of pulp cultures showed the expression of low levels of *Sost* only in the 21 day-old pulp cultures. The levels of *Sost* expression in the primary pulp cultures were comparable to those in primary cultures from calvaria and showed the formation of osteocyte-like cells in both cultures, confirming the osteogenic potential of the primary pulp cultures.

The second area contained Calcein Blue+/DMP1-GFP+/DSP+ staining (regions 2 and 3 in Figure 9 and Supplemental Figure 2) representing areas containing odontoblasts secreting dentin-like matrix. More interesting were the patterns of DMP1-GFP expression in areas of cultures containing dentin-like matrices (DSP+ regions) (regions 2 and 3 in Figure 9 and Supplemental Figure 2). Some of the DSP+ regions contained cells expressing DMP1-GFP at both high and low intensities (region 3 in Figure 9 and Supplemental Figure 2) and some of the DSP+ regions contained cells expressing DMP1-GFP at very low intensity (region 2 in Figure 9). There were significant increases in the extent of the DSP+ regions that contained cells expressing DMP1-GFP at very low intensity between days 14 and 21 (Figure 9 and Supplemental Figure 2). The expression of DMP1-GFP at low intensity in DSP+ regions in 21 days old culture is similar to the patterns of expression of DMP1-GFP *in vivo* and represent highly differentiated odontoblasts.

DISCUSSION

In this study, we have examined the expression of DMP1-GFP in developing teeth and during mineralization and odontoblast differentiation in primary pulp cultures. The *in vivo* studies showed that DMP1-GFP was first expressed at E19 in the secretory/functional odontoblasts. The expression of DMP1-GFP intensified in odontoblasts synthesizing primary dentin and was decreased in highly differentiated odontoblasts synthesizing secondary dentin. The patterns of expression of DMP1-GFP in the odontoblasts during embryonic and postnatal stages of development are similar to the patterns of expression of endogenous DMP1 mRNA and protein in mice and rats [29–31] that showed during dentinogenesis DMP1 mRNA and protein are first detected in secretory/functional odontoblasts secreting unmineralized predentin [29–31]. Advancing stages of dentinogenesis are accompanied by gradual decreases in the levels of DMP1 in highly differentiated

odontoblasts [29–31]. These similarities indicated that 8 kb regulatory element of DMP1 driving the expression of GFP have sufficient strength and specificity to monitor the stage-specific changes in DMP1 expression during odontoblast differentiation *in vivo*.

Our *in vitro* studies showed that during mineralization of primary pulp cultures, DMP1-GFP is expressed in cells in odontogenic and osteogenic lineage. The expression of DMP1-GFP and *Sost* in primary pulp cultures provide a strong evidence of osteogenesis and the formation of osteocytes in pulp cultures. In the osteogenic areas of cultures (identified by the lack of DSP expression), DMP1-GFP was expressed at high intensity. On the other hand, in the dentinogenic areas (identified by the expression of DSP), DMP1-GFP was expressed at two different intensities. The expression of DMP1-GFP at high intensity was associated with secretory/functional and newly differentiated odontoblasts whereas the expression at very low intensity was associated with highly differentiated odontoblasts. These results showed the heterogeneity of the pulp cultures with respect to odontoblast differentiation, their capacity to differentiate into odontoblasts at several stages of differentiation and the ability of the 8 kb region of the DMP1 to drive the expression of GFP to odontoblasts in a stage-specific manner *in vitro*. Our observations showed that, unlike during osteogenesis where high levels of DMP1-GFP are maintained in highly differentiated osteocytes, the expression of DMP1-GFP is decreased in the highly differentiated odontoblasts indicating that 8 kb fragment of the DMP1 contains elements that regulate its differential expression in highly differentiated odontoblasts and osteocytes.

Our *in vitro* studies also showed that DMP1-GFP+ population exhibited poor proliferation and survival, suggesting that this population contains post-mitotic cells in advanced stages of differentiation (either the osteogenic and/or dentinogenic lineage). Previous studies in BMSC cultures [17], osteoblasts 2T3 cell line [18] and primary calvarial osteoblasts showed that the expression of DMP1-GFP was not detected at early stages and was detected during later stages of mineralization [17]. The late expression of the DMP1-GFP in these osteogenic cultures [17–19] as compared to its early appearance in dental pulp cultures (in a few cells at day 7 and then at day 10) suggests that the early expression of this transgene in the dental pulp cultures represents its activation in cells in the dentinogenic lineage.

The lack of detectable levels of *DSPP* in DMP1-GFP+ population isolated at day 7 (early time point) from pulp cultures suggested that DMP1-GFP is activated in secretory odontoblasts prior to the expression of *DSPP*. This is consistent with results that suggested roles for DMP1 in regulation of *DSPP* expression [12,14,20,32–34]. Despite the similarities in the abnormalities in the teeth of DMP1 and *DSPP* null mice [32,33], DMP1 null mutants showed significant decreases in the levels of *DSPP* in odontoblasts [12,14,20]. These observations, and the identification of DMP1-response elements in the *DSPP* gene [34], suggested roles for DMP1 in regulation of *DSPP* expression. Further experiments are in progress to examine and compare the expression of *DSPP* and DMP1 during odontoblast differentiation *in vivo* and *in vitro* in dual transgenic mice carrying cyan fluorescent protein under the control of *DSPP* regulatory elements and mCherry fluorescent protein under the control of DMP1 regulatory sequences.

This study complements our recent study [25] using pOBCol3.6GFP and pOBCol2.3GFP transgenic mice that showed that the 3.6-GFP transgene was activated in cells in early stages of polarization whereas the 2.3-GFP transgene was activated at a later stage of polarization just before or at the time of formation of secretory/functional odontoblast [25] (Figure 10). The behaviour of DMP1-GFP+ population is different from 2.3-GFP+ population. Unlike the 2.3-GFP+ population that showed proliferation and mineralization [25], DMP1-GFP+ cells exhibited poor proliferation. Furthermore, the percentage of 2.3-GFP+ population in pulp cultures at all time points was significantly higher than that of DMP1-GFP+

population. In addition, in pulp cultures, DMP1-GFP transgene is activated at later time point than 2.3-GFP transgene [25]. Unlike the 2.3-GFP, DMP1-GFP expression was closely associated with early and late mineralizing nodules. These together provide evidence that during odontoblast differentiation, DMP1-GFP transgene is activated in more advanced stage of differentiation (secretory/functional odontoblasts) than 2.3-GFP transgene that is activated in late stage of polarization [25].

The transition from preodontoblasts to highly differentiated odontoblasts involves many important changes. A recent study showed that changes in the secretory activity of odontoblasts are accompanied by changes in the profile of expression of six genes (PTPRR, NTRK2, MAP2K6, CD14, MAPK13 and FGF1) involved in p38 signaling pathway [35,36]. An earlier report suggested involvement of C-jun and Jun-B in the transcriptional regulation of terminally differentiated odontoblasts [37]. c-Jun and JunB are members of Jun family of proteins and components of AP1 transcriptional complex [38]. These proteins form either homodimers or heterodimers that bind to the consensus sequence TGACTCA in numerous promoters [38] including DMP1 [39] and DSPP [40] promoters.

In summary, our results showed the heterogeneity of pulp cultures and the necessity for development of markers for identification and isolation of more homogenous population for careful lineage analysis. Our data indicated that DMP1-GFP expression is activated in secretory/functional odontoblasts producing predentin (Figure 10). Progression down the odontoblast lineage is followed by a transient increase in the intensity of DMP1-GFP in the young differentiated odontoblasts and then a decrease in highly differentiated odontoblasts (Figure 10). Our analysis of DMP1-GFP transgene during odontoblast differentiation indicates that this transgene can be used for identification and isolation of secretory/functional odontoblasts from the heterogeneous population of dental pulp cells and will allow further characterization of gene expression profiles in this population during normal and abnormal dentinogenesis in mice with various genetic mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of DMP1-GFP transgene in developing molars

Epifluorescence (A, E, I), dark-field (B, F, J) and pseudo-colored bright field (C, D, G, H, K) images of sections through developing molar teeth at E17 (A–D), E19 (E–H), and P1 (I–L). In each stage of development, adjacent sections have been processed for different analyses. (A–D) At E17, DMP1-GFP is not expressed in either the epithelial or mesenchymal components of the tooth germ. DMP1-GFP is expressed in the osteocytes and osteoblasts (indicated by asterisks) of the developing alveolar bone that also express endogenous *DMP1*. (E–H) At E19, DMP1-GFP is expressed in the odontoblasts (indicated by arrowheads) located at the tip of the mesio-lingual cusp of the first mandibular molar. These cells also express *DSPP* and *DMP1*. (I–L) At P1, DMP1-GFP is expressed at high levels in terminally differentiated odontoblasts (Od) expressing *DSPP* (K) and producing mineralized dentin matrix (L). L represents an image of a section stained with Mallory. Low levels of DMP1-GFP expression were observed in the dental pulp cells in the close proximity to the cervical loops. Abbreviations: Am, ameloblast; DP, dental pulp; Od, odontoblasts. Scale bar in all pictures=100 μ m.

Figure 2. Expression of DMP1-GFP transgene in developing molars at P1 and P14
 Stained (A,D), epifluorescence (B,E) and dark-field (C,F), images of sections through postnatal molar teeth at P1 (A–C) and P14 (D–F). In each stage of development, adjacent sections were processed for different analyses. (A–C) At P1 DMP1-GFP is expressed in the entire layer of fully differentiated odontoblasts. At the tip of the each cusp highly differentiated odontoblasts show slight reduction of GFP signal (indicated by arrows). A represents an image of a section stained with Mallory. (D–F) At P14, highly differentiated odontoblasts at the tip of the cusp show DMP1-GFP expression at very low intensity. (D) represents an image of a section stained with H&E. Scale bar in all pictures=100 μ m.

Figure 3. Expression of DMP1-GFP transgene in developing mandibular incisors

Epifluorescence (A, E), dark-field (B) and pseudo-colored bright-field (C, D, F) images of sections through developing incisor teeth at E19 (A–D) and P7 (E–G). In each stage of development, adjacent sections were processed for different analyses. (A–D) At E19 DMP1-GFP is not expressed in apical regions containing pre-odontoblasts and is detected in the incisal region containing secretory/functional and fully differentiated odontoblasts expressing *DSPP* (C) and *DMP1* (D). DMP1-GFP is expressed at higher intensity in fully differentiated as compared to secretory odontoblasts. DMP1-GFP is expressed in the osteoblasts and osteocytes of the developing alveolar bone (indicated as b) that were expressing *DMP1*, but not *DSP*.

(E–G) are images through incisors at P7. Intensity of DMP1-GFP expression increased with further odontoblast differentiation during postnatal life and extended into the odontoblast processes (E–F). G represents an image of a section stained with Mallory. Abbreviations: Am, ameloblast; B, bone; d, dentin; dp, dental pulp; E, enamel; Od, odontoblast; pd, predentin. Scale bar in all images=100mm.

Figure 4. Expression of DMP1-GFP transgene in primary dental pulp cultures during *in vitro* mineralization and dentinogenesis

Images of the same areas in cultures at different time points analyzed under phase contrast, epifluorescent light using filters for GFPtpz for detection of GFP and TRITC Red filter for detection of XO staining and overlay of the two images generated in Photoshop. The bottom row shows representative cultures stained with von Kossa for each time point. In overlay images yellow color represents the areas stained with both XO and DMP1-GFP. Scale bar represents =100µm.

Figure 5. FACS analysis of primary cultures

The X-axis represents the GFP intensity and the y-axis represents the PI staining used to gate only on viable cells. Numbers on the histograms represent the percentage for this representative experiment.

Figure 6. Expression of early and late markers of mineralization in primary pulp cultures and their correlation with DMP1-GFP expression

Low levels of *GFP* expression were detected at day 7, with early marker of mineralization *Col1a1* also expressed. The levels of *GFP* expression increased at days 14 and 21. Markers of mineralization (*BSP*, *OC* and *DMP1*) and dentinogenesis (*DSPP*) were detected at day 14 and increased at day 21. Values represent mean $\pm$ S.E. determined from at least four independent experiments.

Figure 7. *In vitro* differentiation of DMP1-GFP- population

Images of the same areas in live cultures established from DMP1-GFP- population were analyzed at different time points under phase contrast, epifluorescent light. Overlay of the two epifluorescent images generated in Photoshop shows correlation of GFP expression and XO staining. Note that DMP1-GFP is detected in few scattered cells at day 7 and that the number and intensity of GFP increased with time in culture. XO staining was detected at day 10 and increased at day 14 and 21. Note the expression of DMP1-GFP in unmineralized regions at day 10. Also note cells expressing GFP are detected in unmineralized and mineralized nodules. In overlay images yellow color represents the areas stained with both XO and DMP1-GFP. Scale bar=100mm.

Figure 8. Mineralization and dentinogenic potential of DMP1-GFP- and DMP1-GFP+ populations

(A–C) represent images of von Kossa staining of cultures established from unsorted cells (secondary culture) (A), DMP1-GFP- (B) and DMP1-GFP+ (C) cells after 21 days. (D) Histogram showing the amounts of extracted Alizarin Red staining in cultures after 21 days. Values represent the concentration of the extracted Alizarin Red calculated from the mean absorbance $\pm$ S.E. for at least three independent experiments with multiple samples in each experiment (* $p < 0.05$). (E) Histogram showing the relative levels of *DSPP* after 21 days in these cultures. The levels of *DSPP* were normalized to GAPDH. (F) RT-PCR analysis of expression of *DSPP* in these cultures.

Figure 9. Expression of DMP1-GFP, DSP in mineralized matrix *in vitro* at day 21

Images representing expression of DMP1-GFP (A), DSP (B) and Calcein Blue (C) in the same culture at day 21. D represents the Overlay of the two images generated in Photoshop showing the correlation of DMP1-GFP expression and DSP staining. Rectangles indicated by 1, 2, 3 in all images are shown at higher magnifications. Area 1 contains cells expressing DMP1-GFP at high intensity, that is also stained with Calcein Blue but not DSP representing the osteogenic differentiation in this culture. Area 2 contains cells expressing DMP1-GFP at very low intensity, is stained with anti-DSP antibody and Calcein Blue representing the dentinogenic differentiation and the formation of fully highly differentiated odontoblasts. Area 3 contains cells expressing DMP1-GFP at both high and low intensity, is stained with anti-DSP antibody and Calcein Blue characteristic of the dentinogenic differentiation and the formation of secretory/functional odontoblasts and young odontoblasts. Scale bars in all images = 1mm

Figure 10.

Schematic representation of proposed stages of activation of DMP1-GFP during odontoblast differentiation. *DSPP* was used as a marker of early and later stages of mineralization. The expression of 3.6-GFP and 2.3-GFP transgenes during odontoblast differentiation are based on the results reported by Balic et al [25].

Table 1

Expression of GFP in primary pulp cultures obtained from DMP1-GFP animals. FACS analysis of dental pulp cultures at different time points. Values represent means $\pm$ S.E. from at least three individual experiments.

	%DMP1-GFP- cells	%DMP1-GFP+ cells
day2	98 $\pm$ 0.6	1.9 $\pm$ 0.6
day7	94.6 $\pm$ 0.4	5.4 $\pm$ 0.4
day10	86.8 $\pm$ 0.3	13.2 $\pm$ 0.3
day14	62.8 $\pm$ 0.9	37.2 $\pm$ 0.9

Table 2
Analysis of cell proliferation using Hoechst 33342 dye

Three day old primary pulp cultures derived from DMP1-GFP animals were prepared for FACS analysis and results were obtained using ModFit Software. Results represent mean $\pm$ S.E. from at least three experiments. Values represent mean $\pm$ SD of percentage of cells in G₀+G₁, G₂+M and S phases in GFP+ and GFP- populations in at least three independent experiments.

Period in the cell cycle	% DMP1- GFP- cells	% DMP1- GFP+ cells
G ₀ G ₁	54.3 $\pm$ 0.7	77.7 $\pm$ 2.7
S	20.5 $\pm$ 1.1	15.3 $\pm$ 5.0
G ₂ M	25.2 $\pm$ 0.7	10.0 $\pm$ 0.3
S+G ₂ M	~45.7	~25.3