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Research Article

Gene Expression Profiles of Wild Type and DSPP Knockout Mouse Teeth

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ABSTRACT

DSPP knock-out mice exhibited hypomineralized teeth, thin dentin and a large dental pulp chamber, similar to teeth from human patients with dentinogenesis imperfecta III. Systematical examination of teeth from wild-type (wt) and DSPP KO mice revealed developmental abnormalities, such as circular dentin formation within dental pulp cells and altered odontoblast differentiation in DSPP KO mice, even as early as one day after birth. In addition, chondrocyte-like cells were identified in the dental pulp from DSPP KO mice teeth. These studies suggest that the expression of DSPP precursor protein is required for normal odontoblast lineage differentiation and that the absence of DSPP results in the appearance of chondrocyte-like cells. To further understand the undermining mechanisms for the characters of DSPP KO mice, we examined and compared the gene expression profiles between wt and DSPP KO mice via Real Time PCR (RT-PCR) and immunohistochemical images. In DSPP KO mouse incisors at 21-day old, there was no DSPP expression and major odontoblast lineage markers (Dmp1 and Osx) were down-regulated. Runx2 expression was down regulated in differentiated odontoblasts in wt incisors but RT-PCR analyses of DSPP KO mice showed up regulation of Runx2 expression. Furthermore, in DSPP KO incisors, expressing increased amounts of chondrocyte lineage transcription factors Sox9 and Runx2. Immunochemical studies with Runx2 expression and Osx expression support RT-PCR data. Therefore, gene expression profiles support the phenotypes of wt and DSPP KO mice. These findings support that DSPP is required for maintaining normal odontoblast lineage and absence of DSPP promote the development of chondrocyte cell fate.

Keywords

DSPP protein, Odontoblast lineage markers, Dmp1, Chondrocyte lineage markers, Runx2-Sox9-Col II-Col X.

Introduction

During tooth morphogenesis, a series of reciprocal epithelial-mesenchymal (E-M) interactions lead to odontoblast differentiation and dentin mineralization [1-3]. Transcription factor originating from mesenchymal cells, such as Runx2, are well known to be involved in these E-M interactions [4].

DSP and PP are two major noncollagenous protein in dentin. The initial product of the *dspp* transcript is DSPP precursor protein. DSPP precursor by tolloid-related protein 1 or by bone morphogenic protein 1, yields mature dentin sialoprotein (DSP) and phosphophoryn (PP) proteins [5], which are critical for dentin mineralization [6].

Runx2, which is mainly expressed in Bud and Cap stages. When Runx2 begins to diminish, DSPP begins to express. Runx2

knockout (KO) mice show arrested embryonic tooth development at ED 11-13 ("Bud stage") [7,8].

DSPP knock-out mice exhibited hypomineralized teeth, thin dentin and a large dental pulp chamber, similar to teeth from human patients with dentinogenesis imperfecta III [9]. Systematical examination of teeth from wild-type (wt) and DSPP KO C57BL/6 mice. Revealed developmental abnormalities, such as circular dentin formation within dental pulp cells and altered odontoblast differentiation in DSPP KO mice, even as early as one day after birth. In addition, chondrocyte-like cells were identified in the dental pulp from KO mice teeth [10].

These studies suggest that the expression of DSPP precursor protein is required for normal odontoblast lineage differentiation and that the absence of DSPP results in the appearance of chondrocyte-like cells [10].

Differentiated and mature odontoblasts secret collagen type I, Dmp1, osteocalcin (Oc), DSPP, BSP and OPN [11-13]. Thus we examined Dmp1, DSPP, Oc, BSP and OPN expression profile in wt and DSPP KO mouse teeth. Furthermore, we examined transcription factors including Runx2, Osx, Gli1 and Sox9 expression in wt and DSPP KO mouse teeth.

To further understand the undermining mechanisms for the characters of DSPP KO mice, we examined and compared the gene expression profiles between wt and DSPP KO mice via Real Time PCR and immunohistochemical images.

Materials and Methods

Extraction RNA of 21-day old incisors from wt and DSPP KO mice

Wild-type (wt) C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). DSPP KO mice (strain name: B6; 129-Dspptm1Kul/Mmnc) were obtained from MMRRC, UNC (Chapel Hill, NC.) as previously described [10]. 21-day old wt and DSPP KO mice were euthanized and mandibles were collected. Teeth were extracted using a dissecting microscope in order to remove bone, periodontal ligaments, and muscular tissue. RNA quality had to be OD_{260}/OD_{280} ratio greater than 1.7.

Real-time PCR (RT-PCR) analyses of 21-day old incisors from wt and DSPP-ko mice

iSocript product from BioRad was used to perform standard curve. Cycle threshold (Ct) is a value when the fluorescent generated by PCR product distinguishly from background noise. Delta Ct (dCt) presents the difference in Ct value (for the gene of interest) and Ct value for house keeping gene. Each target (primer pair) needs a standard curve to get a PCR Efficiency for ddCt calculations. Each sample run for each primer pair should be within the range of the standard curve. Then normalize with the reference gene (Actin), and compare the experimental sample (KO) with the Control (WT). Primers used in this manuscript are listed in Table1.

Table 1: Primers used for real time PCR (RT-PCR).

mbActin_S	5' AGAGGGAAATCGTGCGTGAC 3‘
mbActin_AS	5' AACCGCTCGTTGCCAATAGT 3‘
mDmp1_S	5’ TTCGCTGAGGTTTTGACCTT 3’
mDmp1_AS	5' CCCAAAGGAACACAAGGAGA 3'
mGli 1_S	5' CCAAGCCAACTTTATGTCAGGG 3'
mGli 1_AS	5' AGCCCGCTTCTTTGTTAATTTGA 3'
mOPN_S	5‘ AGCAAGAAACTCTTCCAAGCAA 3'
mOPN_AS	5‘ GTGAGATTCGTCAGATTCATCCG 3'
mOsx_S	5’ AGCGACCACTTGAGCAAACATG 3’
mOsx_AS	5’ CGGCTGATTGGCTTCTTCTTCC 3’
mRunx2_S	5’ CCTGAACTCTGCACCAAGTC 3'
mRunx2_AS	5’ GAGGTGGCAGTGTCATCATC 3'
mSox9_S	5' AACGATTGCTGGGATTCC 3'
mSox9_AS	5' ACTCTGAAGGAGACAAGCCC 3'

Tissue Preparation and Histological Analyses

Entire head tissues were collected from wt and DSPP KO mice at 1-day and 6-day old. Tissues were fixed with 10% formalin at room temperature for 48 hours, then demineralized with 0.25M ethylenediaminetetraacetic acid (EDTA, pH 7.4) at room temperature for 15 days. Then, tissues were dehydrated, paraffin-embedded, sectioned at 5 μm thickness, stained with Hematoxylin (Fisher Scientific, Waltham, MA)&Eosin (Sigma-Aldrich, St. Louis, MI) (H&E), and mounted with Permount (Fisher Scientific, Waltham, MA). Images were taken using Nikon Eclipse E400 microscope (Tokyo, Japan) and SPOT RT Slider Microscope Camera (Diagnostic Instruments, Sterling Heights, MI).

Immunohistochemical analyses of two transcriptors Runx 2 and Osx expression in wt and DSPP-KO

The sectioned slides from wt and DSPP KO mice were immersed in xylene to remove the paraffin and transferred through a series of diluted alcohols (e.g., 100%, 95%, 70%, and 50%) to water to hydrate the slides. To detect Runx2 expression with anti-Runx2 antibodies (Abcam, Cambridge, MA) at 200X dilution in 1% blocking agent with maleic acid in tooth tissues from wt and DSPP KO next, diluted secondary biotinylated antibody was added and then shed and quenched of endogenous peroxidase activity. Finally, Vectastain Elite ABC reagent was added to the cultures and four drops of DAB stock solution (Vector laboratories) were added to each well for color observation. Following a similar protocol, Similar procedures were used to detect Osx protein expression with anti-Sox antibodies (Abcam, Cambridge, MA).

Statistical Analysis

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

Results and Discussion

Real-time PCR data showed different gene expression profiles between wt and DSPP-KO incisors from 21-day old mice (see Figure 1).

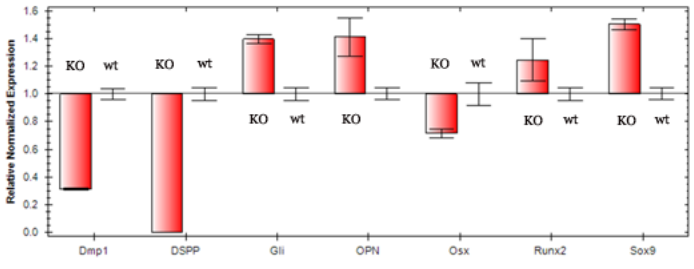

Figure 1: Real-time PCR data of 21-day old wt and DSPP KO incisors. Using Actin mRNA as an internal reference gene, relative mRNA expression in DSPP KO incisors to those of wt controls is shown above. Odontoblast lineage marker genes Dmp1, Osx, OPN, BSP (not shown) and Osteocalcin (Oc) (not shown) and DSPP mRNAs were down-regulated (represented by downward red bar) relative to the wt controls. The wt control gene expression for each gene was set at 1.0 with standard error bars. Chondrocyte related cell markers such as Runx2, Sox9, OPN and Gli were up-regulated in DSPP KO incisors.

Losing odontoblast cell fate in DSPP-KO incisors

Dmp1, DSPP, OPN, Oc and BSP are odontoblast lineage markers. Figure 1 showed that in DSPP KO mice, there was no DSPP expression and major odontoblasts lineage markers (Dmp1, Osx) were down-regulated, These data suggest that dental pulp cells lost odontoblast characteristics. In other words, odontoblasts and dental pulp cells in DSPP-KO incisors changed odontoblast cell fate.

Gaining chondrocyte-like cell fate in DSPP-KO incisors

Sox9 is required for the committement of chondrogen cell leneage and control chondrogenesis [14,15]. Sox9 and Runx2 transcription factors are required for chondrocyte lineage development. Col II and Col X are chondrocyte markers. Interestingly, Sox9 and Runx2 expressions were up-regulated. Furthermore, Col II and Col X (not shown) were immunochemically detected in DSPP KO dental pulp. The expression of Sox9, Runx2, Col II and ColX in DSPP KO mice suggest that dental pulp cells were altered to a chondrocyte cell fate. Gli proteins are the effectors of Hedgehog. The up-regulation of Gli1 in DSPP-KO teeth signaling suggests that Shh in Fgf signal pathway was activated to express Gli1 [16].

Runx2, Sox9, and OPN were chondrocyte related cell markers. In DSPP KO incisors, chondrocyte related cell markers (Runx2, Sox9, and OPN) were up-regulated. The up-regulation of chondrocyte related cell markers suggest the DSPP KO dental pulp cells expressed chondrocyte-like characteristics.

Runx2 protein expression in wt and DSPP KO mouse teeth

Runx2 protein expression in wt mouse teeth

Figure 2A showed wild type (wt) 1d molar 1 (M1) expressed Runx2 protein in alveolar bone (AB) and no Runx2 expression in mesenchymal cells (i.e., odontoblasts and dental pulp). Figure 2D showed wt 6d old M1 also expressed Runx2 protein in alveolar bone (AB) and no Runx2 expression in odontoblasts and dental pulp. It is well known that in wt mice, *Runx2* expression is high in Cap stage, moderate in Bell stage and no expression in late Bell stage. DSPP expression appeared in late Bell stage [7] and continued to express strongly during subsequent developmental stages.

Actually, it was reported that Runx2 expression (i.e., over expression of Runx2) driven by DSPP promoter, inhibited odontoblast terminal differentiation with a decrease in DSPP expression [17]. So high Runx2 expression driven by DSPP promoter did inhibit DSPP expression. This finding suggest that Runx2 protein down-regulates DSPP expression.

Alternatively, the relative expression patterns of Runx2 and DSPP (i.e., Runx2 up and DSPP down) posted the possibility that the higher DSPP expression in wt mice cause down-regulation of Runx2 expression. Future experiments are needed to verify this speculation.

Runx2 protein expression in DSPP KO mouse teeth

DSPP-KO mice provide a unique opportunity to examine whether the absence of DSPP affects Runx2 expression *in vivo*. We examined Runx2 protein expression in 1-day wt and DSPP KO incisor and molars using immunohistochemical analysis with anti-Runx2 antibodies (Abcam, Cambridge, MA). In DSPP KO mice we found Runx2 expression in odontoblasts, dental pulp and alveolar bones in both molars and incisors (Figure 2B and 2C). In 6d old M1, Runx2 expression faded away from odontoblasts. Interestingly, for the first time, Runx2 expression was detected in ameloblast layer. But Runx2 expression was still detected in mesenchymal cells (i.e., dental pulp cells). Also Runx2 expression was present in alveolar bone.

Comparison of RT-PCR of 21-day old incisors from wt and DSPP KO mice showed Runx2 expression is up regulated in DSPP KO mice. These immunochemical data are in agreement with those from RT-PCR that is the absence of DSPP expression in DSPP KO mice resulted in high Runx2 protein expression in incisors and M1 of DSPP KO mice.

Osx protein expression in wt and DSPP KO mouse teeth

DSPP-KO mice provide a unique opportunity to examine whether the absence of DSPP affects Runx2 expression *in vivo*. We examined Runx2 protein expression in 1d wt and DSPP KO incisor and molars using immunohistochemical analysis with anti-Runx2 antibodies (Abcam, Cambridge, MA). In DSPP KO mice we found Runx2 expression in odontoblasts, dental pulp and alveolar bones in both molars and incisors (Figure 2B and 2C). In 6d DSPP KO M1, Runx2 expression faded away from odontoblasts. Interestingly, for the first time, Runx2 expression was detected in ameloblast layer. But Runx2 expression was still detected in mesenchymal cells (i.e., dental pulp cells). Also Runx2 expression was present in alveolar bone.

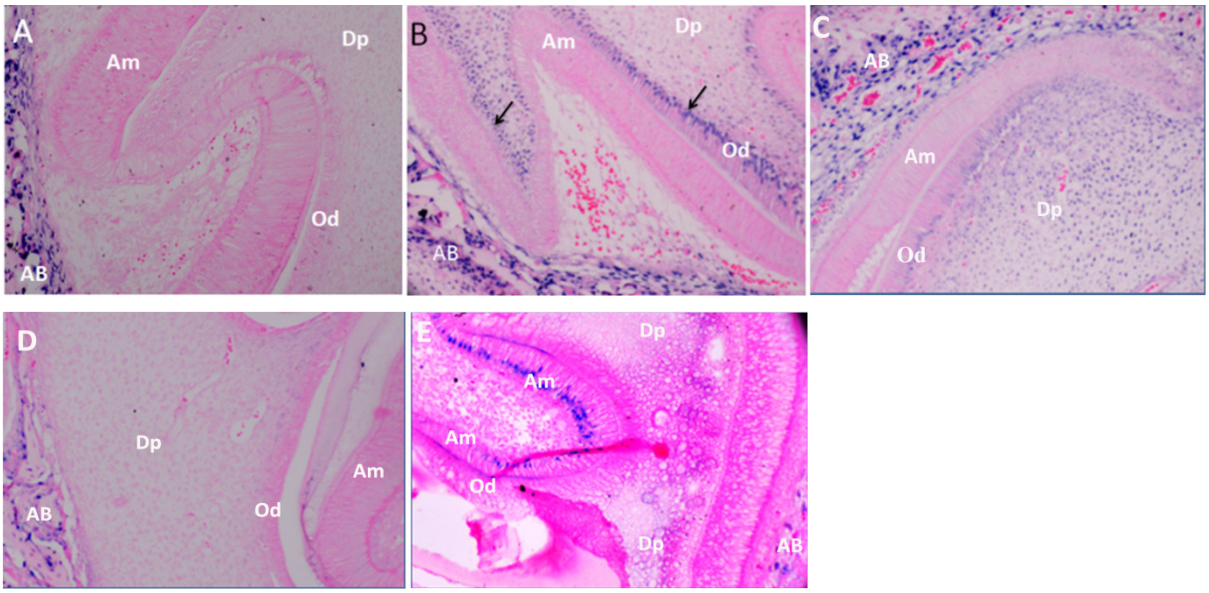

Figure 2: Comparison of Runx2 protein expression in wild type (wt) and DSPP KO teeth on 1d and 6d with Immunochemical stained with Runxz2 antibodies. Upper panel A-C represents 1-day old (1d) teeth. A. 1d wt molar one (M1) section stained with anti-Runx2 antibodies (Abcam, Cambridge, MA) and counterstained with Eosin, 1d wt M1 Runx2 protein expression in alveolar bone (AB) but no Runx2 expression in M1 odontoblasts. B. 1d DSPP KO M1 section stained with anti-Runx2 antibodies and counterstained with Eosin. Runx2 protein was robustly expressed in odontoblasts (as indicated by arrow) and dental pulp. Runx2 protein expression in AB of 1d M1 from DSPP KO mice. C. 1d DSPP KO incisor showed Runx2 protein was expressed in AB, odontoblasts and dental pulp. Lower panel D-E represents 6-day old (6d) teeth. D. wt 6d M1 expressed Runx2 protein in AB and no Runx2 expression in dental pulp and odontoblasts. E. DSPP KO M1 with anti-Runx2 staining showed Runx2 expression in ameloblasts, dental pulp and AB. AB: Alveolar Bone. Am: Ameloblast. Dp: Dental Pulp. Od: Odontoblast.

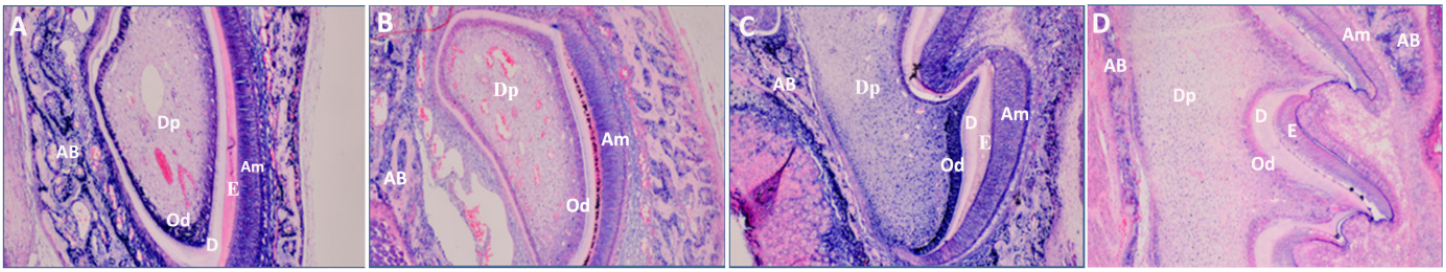

Figure 3: Comparison of Osx expression in 6-day old (6d) incisors and molar 1 (M1) between wt and DSPP KO mice. Osx antibodies was used to examine Osx expression. A. 6d incisor from wt mice showed strong Osx expression in odontoblast layer and ameloblast layer as well as in alveolar bone (AB). B. 6d incisor from DSPP KO mice showed very weak Osx expression in odontoblasts and relatively weak Osx expression in ameloblasts. C. 6d M1 from wt mice showed strong Osx expression in odontoblasts and ameloblasts. It also showed strong Osx expression in alveolar bone (AB). D. 6d M1 from DSPP KO mice showed very weak to almost no Osx expression in odontoblasts and ameloblasts. Osx expression was detected in AB.

Osx2 protein expression in wt mouse teeth

In 1d wt M1, DSPP mRNA expression begins to express DSPP in young odontoblasts and in polarized cells associated in young odontoblasts with early predentin formation [21]. At 3d M1, DSPP was actively expressed in odontoblasts [21]. Because the pattern of Osx expression overlaps with that of DSPP [18]. DSPP and Osx are weakly expressed at the beginning stage (i.e., lower amount of DSPP and Osx expression) in 1d wt teeth, we chose to examine 6d incisors and M1 of wt (with high amount of DSPP and Osx expression in wt). We observed strong Osx protein expression in both odontoblasts and ameloblasts of 6d wt incisor (Figure 3A) and in 6d wt M1 (Figure 3C) as well as strong Osx expression in alveolar bones in wt.

Since RT-PCR showed down-regulation of Osx protein expression in 21-day old DSPP KO incisor compare to that of wt incisor (Fig. 1), we chose to examine whether Osx protein expression patterns are different between incisor and M1 of wt and DSPP KO mice at 6d old.

Osx2 protein expression in DSPP KO mouse teeth

Weak to almost no osx expression was observed in odontoblasts of 6d old incisors (Figure 3B) and M1 (Figure 3D). We speculate that the low amount expression of Osx protein in 6d DSPP KO incisors and M1 is due to Osx's role in odontoblast and ameloblast maturation [18-20]. The absence of DSPP protein in DSPP KO mice could not maintain odontoblast lineage in dental pulp cells. It was reported that the pattern of Osx expression overlaps with that of DSPP. No DSPP expression in DSPP KO mice further resulted in no odontoblast maturation and low or no Osx expression in the presumed odontoblast layer.

RT-PCR of 21-day old incisors from wt and DSPP KO showed down regulation of Osx expression in DSPP KO mice (Figure1). These RT-pCR data are in agreement with the down regulation of Osx expression detected with anti-Osx isimmunochemical staining in incisors and M1 of DSPP KO mice.

Conclusions

Our previous study [10] showed that DSPP knock-out mice exhibited hypomineralized teeth, thin dentin and a large dental pulp chamber, similar to teeth from human patients with dentinogenesis imperfecta III. Systematical examination of teeth from wild-type (wt) and DSPP KO mice revealed developmental abnormalities, such as circular dentin formation within dental pulp cells and altered odontoblast differentiation in DSPP KO mice, even as early as one day after birth. In addition, chondrocyte-like cells were identified in the dental pulp from KO mice teeth. These studies suggest that the expression of DSPP precursor protein is required for normal odontoblast lineage differentiation and that the absence of DSPP results in the appearance of chondrocyte-like cells.

Our current RT-PCR study on gene expression profiles between wt and DSPP KO mouse incisors, indicated that no DSPP in DSPP KO (1) promoted increased Runx2 expression, (2) down regulated Osx expression, (3) decreased Dmp1 and Osteocalcin expression (odontoblast markers), and (4) increased Sox9 and Runx2 expression, which were required for chondrocyte lineage development. In addition, Col II and Col X (not shown) were immunochemically detected in DSPP KO dental pulp. The expression of Sox9, Runx2, Col II and Col X in DSPP KO mice suggest that dental pulp cells were altered to a chondrocyte cell fate [10]. Immunochemical analyses for Runx2 and Osx protein expression in wt and DSPP KO mice lent strong support to the RT-PCR data. In conclusion, DSPP protein is required for maintaining normal odontoblast lineage and absence of DSPP protein could change dental pulp cell fate to chondrocyte.

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