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The role of Dentin Sialophosphoprotein (DSPP) in craniofacial development

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ABSTRACT

DSPP is known to be important in the formation of dentin. In DSPP's absence, a severely hypomineralized dentin is formed, in a condition known as dentinogenesis imperfecta (DGI). DSPP has recently been found in several different tissues, including the mandibular condylar cartilage and craniofacial skeleton. However, there is limited literature on the role of DSPP in these tissues. Therefore, the objective of the present study was to investigate the role of DSPP in craniofacial development.

Two mice strains, DSPP knockout and C57BL/6J wild type, were compared at 1, 3, and 6-months of age. Skulls and condyles were investigated through morphological and histological analyses. Cell culture was also conducted to investigate the potential effects of DSPP absence in osteoblasts from the calvaria.

Mineralization defects were noticed in the structures of skulls and MCC, with the most significant impact at 1 month of age. **Therefore,** DSPP is an essential protein for the normal mineralization of craniofacial tissues.

1. Introduction

There are four types of mineralized tissues in our body: bone, cementum, dentin, and enamel.[1] These tissues are predominantly made of inorganic matter, primarily found in the form of hydroxyapatite (HA) crystals. Organic matter is found in the form of collagen and non-collagenous proteins (NCPs). These proteins function to create the framework for the inorganic matter to mineralize.[2] NCPs play a major role in the conversion of predentin and osteoid to dentin and bone, respectively.[3]

One category of NCPs is the small integrin-binding ligand N-glycosylated (SIBLING) family.[4] This family includes dentin matrix protein 1 (DMP1), osteopontin (OPN), bone sialoprotein (BSP), and dentin sialophosphoprotein (DSPP). The SIBLING family shares a role in regulating the mineralization and interactions with the inorganic matrix (HA) of teeth and bone mineral structure.[4]

During development, DSPP plays an important role in the formation and mineralization of dentin.[5,6] Dentin mineralization, or dentinogenesis, is a result of multiple protein signaling. DSPP products, dentin sialoprotein (DSP), and dentin phosphoprotein (DPP) have been indicated as regulators of the initiation, and of the maturation of dentin mineralization, respectively.[7]

Humans with defective *Dspp* gene express a severe dentin hypomineralization phenotype, a condition known as dentinogenesis imperfecta (DGI).[5,6] This condition causes the teeth to be weaker than normal, prone to rapid wear, breakage, and loss. Animal studies revealed that *Dspp* knockout (*Dspp*−/−) mice manifest hypomineralization defects in dentin, resembling the dentin defects of human DGI.[6]

DSPP was initially thought to be active only during dentinogenesis. This protein, however, has been identified in several other tissues. DSPP has been detected in bone, cementum, and different non-mineralized tissues.[7–9] In the long bone, DSPP is present at a lower expression level than in teeth.[9] Therefore, it has been shown in mice models that DSPP absence causes milder defects in the long bone than in teeth.[10] Also, Gibson et al. showed that DSPP is present in the mandibular condylar cartilage (MCC), however, at a higher expression than in the long bone.[9] DSPP is also expressed in the connective tissues of the craniofacial complex with high expression levels in the extracellular matrix.[11]

Despite its expression level, there have been very limited reports regarding the impact of DSPP's absence in MCC and craniofacial complex during development. Hence, the present data elaborates more investigation of DSPP's expression and phenotypic changes in the MCC and calvarium development of *Dspp*−/−mice.

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2. Materials and methods

2.1. Mice strain

To analyze the role of DSPP in craniofacial development, two mice strains were used. *Dspp*−/−(strain name: B6; 129- Dspp^{tm1Kul}/Mmnc; catalogue number 000047- UNC, MMRRC, UNC, North Carolina, USA) and C57BL/6J (WT) mice from the Jackson Laboratory (Maine, USA) were compared. According to previous literature, the sex of the mice was not considered, as it does not affect the impact of DSPP absence. [9.10][12] The development of the knockout strain has been previously described, as well as the choice of control.[10,12] The animal protocol has been approved by the Animal Care and Committee at the University of Alberta (Study ID AUP00002086).

2.2. Sample preparation

Skulls and hemi-mandibles were removed from 1, 3, and 6 months of age mice after sacrifice. Ten samples of each genotype in each age group were used. Therefore, 60 hemi-mandibles and 60 skulls were analyzed. Samples were subdivided and stored according to the analysis procedure.

2.3. Dual-energy x-ray absorptiometry analysis

Faxtiron (model UltraFocus DXA; Faxitron Bioptics, Arizona, USA) dual-energy x-ray absorptiometry (DXA) system was used to measure bone mineral density (BMD) in regions of interest (ROIs). Samples and detection tray were placed at position 2 of the machine, according to manufacturer instructions. DXA Vision software (Faxitron Bioptics, Arizona, USA) was used to extract BMD results from the scans. ROIs were selected according to previous literature.[13]

2.4. Micro-CT analysis

Three-dimensional micro-CT scans of dissected hemi-mandibles and skulls were taken at 360°, 75 ms, 50 kV, and 0.24 milliamps (Milabs U-CT, Utrecht, Netherlands). Scans were reconstructed in MiLabs Software at 25 μm voxel size and analyzed with Amira software (ThermoFisher Scientific, Ontario, Canada).

2.5. Alizarin red staining

Cell culture protocol was according to Chen et al.[14] Cells were fixed with 1 ml 10% formaldehyde and then stained with 1 ml 2% alizarin red s (A5533, Millipore Sigma, Massachusetts, USA) solution.

2.6. Hematoxylin & eosin (H&E) staining

H&E staining was used to analyze different histological features of *Dspp*−/−and WT samples. Samples were embedded by the Alberta Diabetes Institute at the University of Alberta. Blocks were cut into 5-μm sections with a Leica HistoCore Autocut microtome (Leica, Wetzlar, Germany). Mandibles and long bones were cut in a mesial-distal direction, and skulls were cut from the frontal aspect. H&E staining protocol followed previous literature by Gibson et al.[15]

2.7. Immunohistochemistry (IHC) staining

IHC staining with anti-DSP monoclonal antibody (anti-DSP-2C12.3 Monoclonal – MABT37, EMD Millipore) was used to analyze the difference in expression of DSPP protein in *Dspp*−/−and WT samples. IHC staining protocol followed previous literature by Gibson et al.[9]

2.8. Statistical analysis

Microsoft Excel software (Microsoft Corporation; Redmond, Washington) was used to analyze BMD results. The sample size was calculated based on the power analysis of previous studies on dentin.[16] The paired T-test with two samples for means was used to check the significance of differences between *Dspp*−/−and WT results. The alpha value was set to 0.05. Statistical significance was defined at $p < 0.05$. All descriptive results were assessed by three investigators, one of whom was blinded. The described results were true for all samples analyzed. The images presented in this study were selected by the investigators as the most representatives of their groups.

3. Results

3.1. X-ray results

UltraFocus DXA (Faxitron Bioptics, Tucson, AZ, USA) yielded high-resolution radiographic images used for morphological analyses of the samples under investigation.

In mandibular tissues at 1 month of age *Dspp* −/−mice, radiographic images showed a more radio-opaque head of the condyle than WT mice, most significantly detected in the articular surface. Three-month-old *Dspp* −/− images showed less radio-opaque condylar process compared to the WT images. However, the articular cartilage region of the condyle has a similar appearance to the WT image. At 6-months-old, the condylar process of the *Dspp* −/− mice showed less radiopacity than the WT condylar process (Fig. 1). Radiographic images of calvaria regions of skull samples did not show noticeable differences between *Dspp*−/−and WT mice at 1, 3, or 6 months of age (Fig. 2).

3.2. Faxitron DXA

In WT MCC, the mean average of BMD in mg/cm^2 and standard deviation (SD) results showed higher mineralization in *Dspp*−/−MCC at 1 month of age, followed by higher mineralization in WT at 3 months of age. No significant difference was noticed between the two groups at 6 months of age (Fig. 3). While in skulls results showed higher mineralization in *Dspp*−/−skulls at 1 month of age compared to WT. However, by 3 months of age, WT mice skulls showed higher mineralization. Similar to MCC, no significant difference was noticed at 6 months of age between WT and *Dspp-/-* skulls (Fig. 4).

3.3. Micro-computed tomography (Micro-CT)

Three-dimensional Micro-CT scans were used to compare bone structural surfaces between the WT and *Dspp*−/−mice. At 1 month of age, severe mineralization defects could be noticed in the *Dspp*−/−condyle head. The *DSPP*−/−1-month-old condyle head appears rougher and more porous than the smooth condyle head in the WT image. At 3 months of age, milder mineralization defects could be noticed in the *Dspp*−/− image. The 3 months of age *Dspp-/-* condyle appears rougher than the WT. At 6 months of age, no significant difference could be noticed between the WT and *Dspp*−/− condyles (Fig. 1).

At 1 and 3 months of age, mineralization defects could be noticed in *Dspp*−/−skull surfaces. *Dspp*−/− images showed frontal and parietal bones with significant porosities around the sagittal suture compared to WT samples. At 6 months of age, no significant differences could be noticed between the two skull surfaces (Fig. 2).

3.4. Cell culture

Alizarin red staining was used to investigate calcium deposits at 7, 14, and 21 days of osteoblast culture from WT and *Dspp*−/−mice calvaria. At 7 days, no calcium deposits were visualized in both WT and

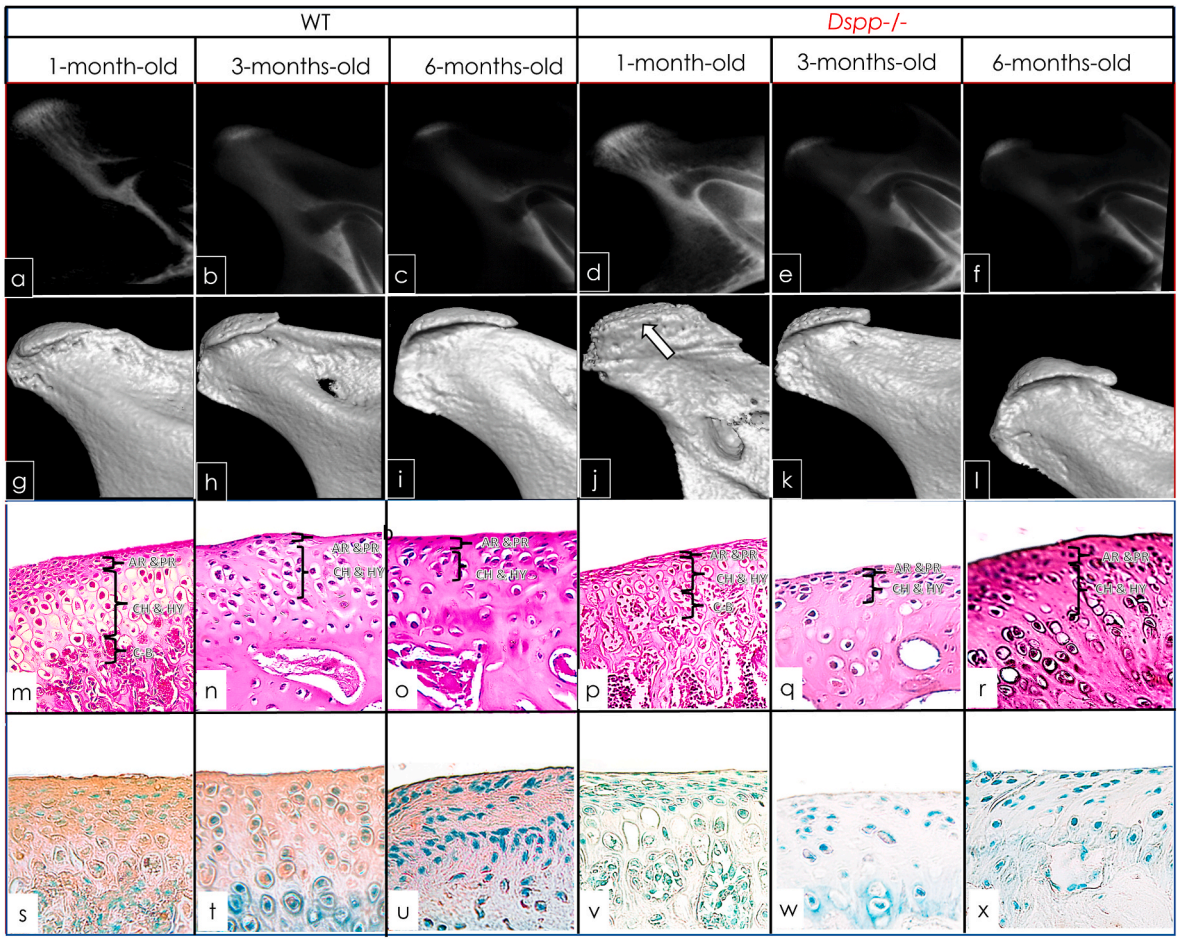


Fig. 1. Images of 1-, 3-, and 6-month-old mice mandibles. Fig. 1(a-c, g-I, m-o, s-u) represent WT mice, and Fig. 1 (d-f, j-l, p-r, v-x) represent *Dspp*−/−mice. AR: Articular. PR: Prechondroblastic. CH: Chondroblastic. HY: Hypertrophic. C–B: Collagen-bone.

Dspp−/−cultured cells. At 14 days, the difference in staining between *Dspp*−/−and WT cultures was detected, with more intense alizarin red staining on WT cultured cells. On day 21, the difference in staining between WT and *Dspp*−/−cultures was obvious, with more intense staining observed on WT cultured cells (Fig. 2).

3.5. Hematoxylin and eosin

The MCC is divided into the following layers: articular layer, prechondroblastic layer, chondroblastic layer, hypertrophic layer, and cartilage-bone interface. It was noticeable at 1 and 3 months of age that the chondroblastic layer was wider in WT images compared to the *Dspp* −/− images. Moderate disorganization of the chondroblasts was also noticed in 1 and 3 months of age *Dspp*−/−images. In the 6 months old *Dspp*−/−image, the chondroblastic layer was wider than in the WT image. There was no significant difference in all other layers between WT and *Dspp*−/−in any of the age groups (Fig. 1).

H&E-stained images of skulls showed noticeable incremental apposition lines in the structure of the bone in WT images at 1, 3, and 6 months of age. *Dspp*−/− images did not show incremental apposition lines in any of the three age groups (Fig. 2).

3.6. Immunohistochemistry staining

Clear differences in immunohistochemistry staining of DSP intensity were noticed between C57BL/6J samples and *Dspp*−/−. Anti-DSP staining was remarkably noticed in C57BL/6J samples at all age groups, contrary to all *Dspp*−/− samples.

In MCC, anti-DSP staining showed strong intensity in the prechondroblastic, chondroblastic, and hypertrophic layers of the WT images at 1 and 3 months of age. At 6 months of age, the WT image showed moderate intensity of anti-DSP staining. *Dspp*−/−images showed negative signals in all age groups (Fig. 1).

In skulls, anti-DSP staining was detected in WT images at 1, 3, and 6 months of age, contrary to the *Dspp*−/−images of the same ages (Fig. 2).

4. Discussion

Bone and dentin are similar in composition and mechanisms of mineralization. They originate from osteoblasts and odontoblasts, respectively. Osteoblasts and odontoblasts secrete unmineralized matrixes rich in type I collagen named osteoid and predentin, respectively.[17] These unmineralized matrixes lie between their originating cells and the mineralization front. In the mineralization front, HA crystals are deposited to form bone and dentin. Mineralization of osteoid and predentin involves an interplay between type I collagen and different NCPs.[17] DSPP is known to be important in controlling the mineralization of predentin, and extensive research has been published outlining the impacts of DSPP's absence in dentin.[3–5] With the recent discovery of DSPP expression in several non-dental tissues, the importance of investigating its role in different tissues came to light.[9]

4.1. MCC

Radiographic images showed clearly defined radiopaque regions in the condyle articular surface and the condylar process of WT samples at

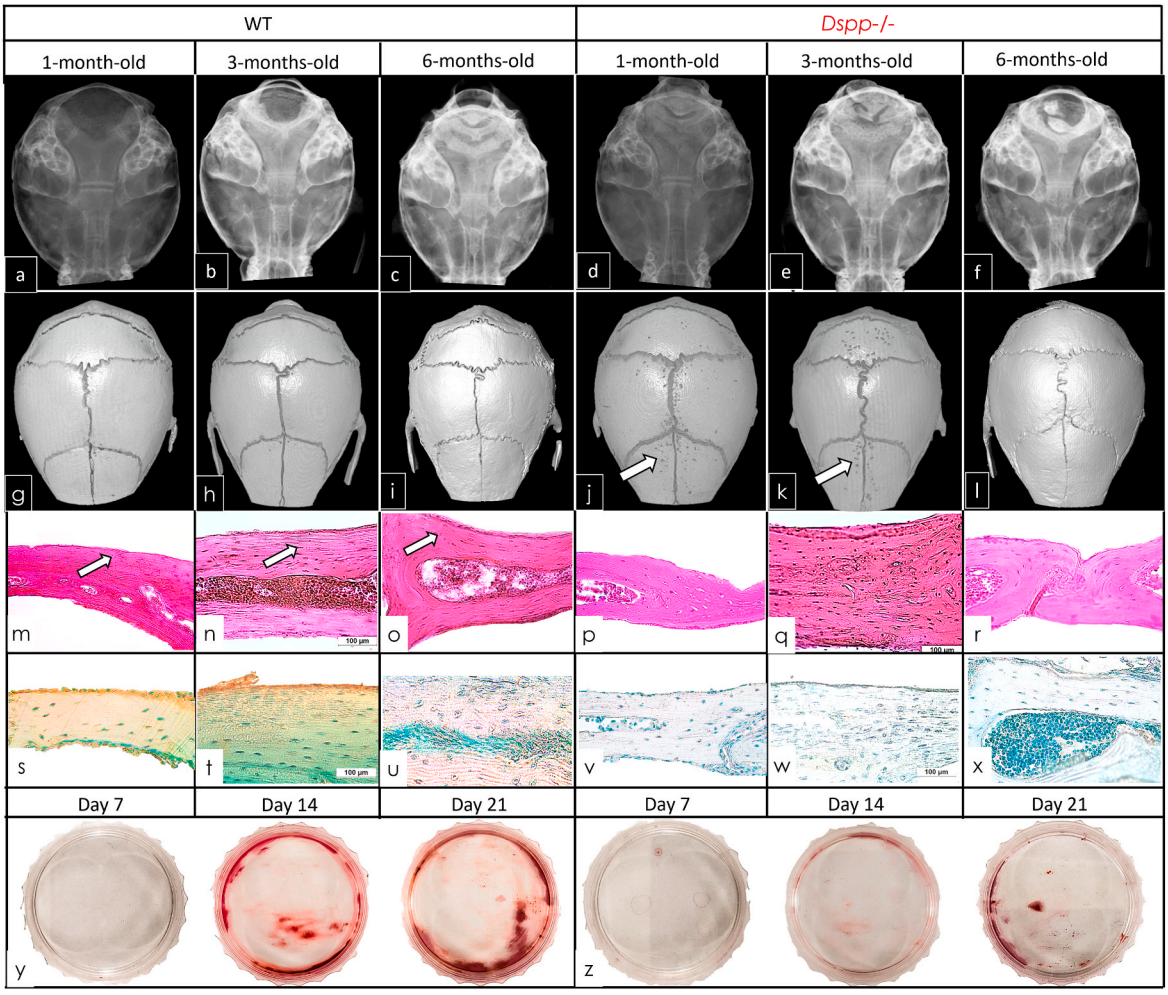
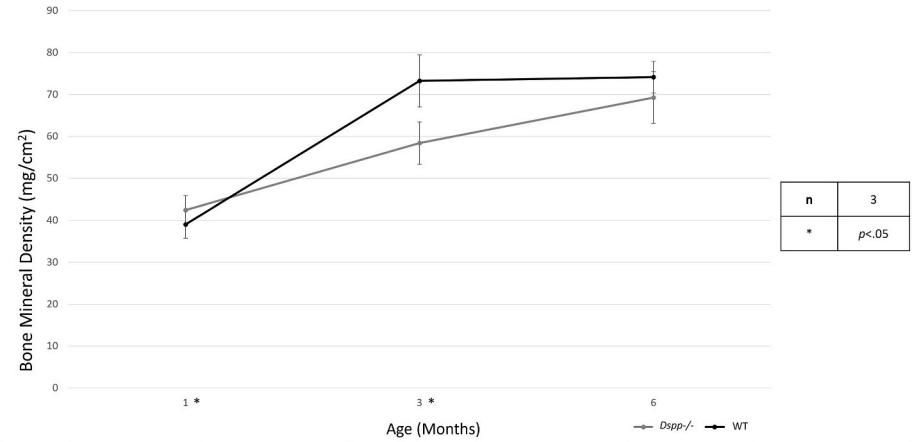


Fig. 2. Images of 1-, 3-, and 6-month-old mice skulls. Fig. 2(a-c, g-i, m-o, s-u, y) represent WT mice, and Fig. 2(d-f, j-l, p-r, v-x, z) represent *Dspp*−/−mice.

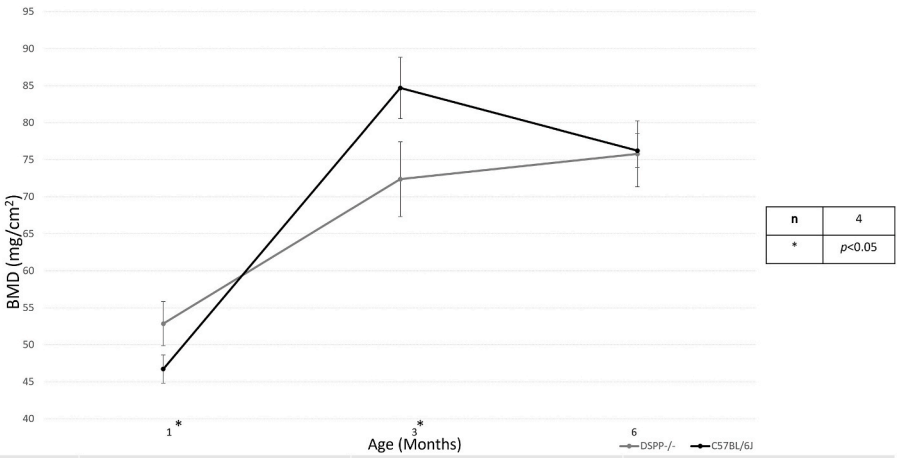


	1 Month			3 Months			6 Months		
	BMD (mg/cm²)	SD	*p* value	BMD (mg/cm²)	SD	*p* value	BMD (mg/cm²)	SD	*p* value
WT	39.0625	3.322695	0.012	73.24625	6.215	0.007	74.13775	3.763882	0.151
Dspp-/-	42.429	3.504421		58.41925	5.036		69.26775	6.167548	

Fig. 3. BMD results of 1-, 3-, and 6-month-old mice MCC ROI. The green line represents WT mice; red line represents *Dspp*−/−mice.

all investigated ages. Meanwhile, higher radiopacity was detected on the condyle articular surface region in *Dspp*−/− images at 1 month of age compared to WT images, suggesting advanced mineralization. According to this result, we theorize that DSPP could have a regulatory function in the mineralization of the articular surface at an early age.

At 3 and 6 months of age, results showed a poorly defined and less radiopaque condylar process in *Dspp*−/−images compared to WT images. This finding suggests a less mineralized condylar process in *Dspp*−/−compared to WT condyles. This could result in a weaker condyle structure more prone to fractures in *Dspp*−/− mice. Expression of DSPP



	1 Month			3 Months			6 Months		
	BMD (mg/cm²)	SD	p value	BMD (mg/cm²)	SD	p value	BMD (mg/cm²)	SD	p value
WT	46.736	1.904	0.005	84.699	4.136	0.003	76.258	2.3	0.436
Dspp-/-	52.853	2.974		72.387	5.058		75.795	4.452	

Fig. 4. BMD results of 1, 3, and 6-months old mice skull ROI. The green line represents WT mice; red line represents *Dspp*−/−mice.

in the mandibular condyle has been evidenced before.[9,18] However, limited reports of DSPP's role in the MCC during development have been published to our knowledge. These results suggest that DSPP could play an active role in the mineralization process of the mandibular condyle.

DXA results showed that MCC had higher BMD in *Dspp*−/− samples at 1-month of age compared to WT. However, at 3 months of age, WT samples had higher BMD compared to *Dspp*−/−, and at 6 months of age, no significant difference was present. For this reason, DSPP might play a role in regulating the mineralization process in the MCC region at the early stages of development.

The absence of DSPP affected early stages of development more severely than advanced stages of development in condyles. This was detected in three-dimensional reconstructions of hemi-mandibles that showed severe mineralization defects in *Dspp*−/−samples at 1 month of age. *Dspp*−/− condyles appeared rougher and more porous compared to the WT condyle. However, at 3 months of age, *Dspp*−/−condyles showed mineralization defects at a milder level than 1 month of age, and at 6 months of age, both groups showed a similar condyle structure.

Although the *DSPP*−/−MCC had higher BMD at 1 month of age and appeared more radiopaque in radiographic images, the micro-CT image shows that the surface of the bone formed was of poor quality. This suggests that in the early stages of development, DSPP might act as a regulator of mineralization in the MCC.

H&E stained image results are following previous literature which has concluded that the loss of DSPP impacts the development of different layers of the MCC.[9,18] DSPP has also been previously found to be mainly expressed in the articular and prechondroblastic layers in neonatal mice, and the loss of function of DSPP affected these layers at the early stages of development.[18] The impacts of DSPP loss of function in the MCC layers might be related to mineralization defects observed in micro-CT results.

4.2. Skull

In the present study, micro-CT images of calvarium surfaces showed clear differences at 1 and 3 months of age. Rougher and more porous calvarium surfaces were observed in *Dspp*−/−samples compared to WT. At 6 months of age, *Dspp*−/−and WT samples did not show clear differences. Suggesting that DSPP might have an active role in the mineralization process of the calvaria during the early stages of development.

The incremental apposition lines seen in H&E-stained images of skulls have been described as a sign of bone remodeling.[19] Micro-CT results also showed that *Dspp*−/−calvaria was more porous than WT calvaria at 1 and 3 months of age. This could be attributed to the fact that bone formation and remodeling might occur with a better organization in WT compared to *Dspp*−/−skulls.

Differentiation of mesenchymal stem cells into osteoblasts and function of osteoblasts might be negatively impacted in the absence of the DSPP protein.[14] Calvaria osteoblast culture showed stronger alizarin red staining intensity in WT cultures compared to *Dspp*−/−cultures at 14 and 21 days.

To our knowledge, this was the first investigation of DSPP expression in the skull calvaria during development. Our results showed a stronger anti-DSP signal at 1 and 3 months compared to 6 months of age in the WT calvaria. This data, along with micro-CT results of the calvaria surface and cell culture of osteoblasts from the region, indicates that DSPP might be important for the normal mineralization of the calvaria during the early stages of development.

Different NCPs might be able to compensate for the absence of DSPP in mature ages, potentially rescuing the defects seen in earlier stages of development. This mechanism is known as genetic compensation. This was seen at 6 months of age when differences were not seen in MCC, alveolar bone, and calvaria between WT and *Dspp*−/− samples regarding BMD results and micro- CT images.

Genetic compensation is defined as changes in protein levels aimed at compensating for the loss of function of another gene. Differences between samples were the most noticeable at 1 month of age, while at 6 months of age, results were similar. There is limited literature on the mechanisms of compensation. It is possible that upregulation of other SIBLING proteins, such as OPN and DMP1, might be occurring to compensate for the DSPP absence.[20]

5. Conclusion

Findings suggest that the knockout of the *Dspp* gene affects early ages more severely than mature ones. We concluded that DSPP is an essential protein for the normal mineralization of craniofacial tissues. Data from the present study showed that DSPP's absence is related to defects in the mineralization of the skull and MCC at the early stages of development. However, a potential genetic compensation mechanism might be able to play a role in partially recovering these defects.

Future work should focus on understanding the mechanisms of genetic compensation. Further investigation of the proteins that are responsible for this compensation would yield a better understanding of the pathways of mineralization in the body.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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