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Research Article

CRISPR/Cas9-Induced Fam83h Knock-out Leads to Impaired Wnt/β-Catenin Pathway and Altered Expression of Tooth Mineralization Genes in Mice

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Background: Dental enamel formation is a complex process that is regulated by various genes. One such gene, Family With Sequence Similarity 83 Member H (Fam83h), has been identified as an essential factor for dental enamel formation. Additionally, Fam83h has been found to be potentially linked to the Wnt/β-catenin pathway.

Objectives: This study aimed to investigate the effects of the Fam83h knockout gene on mineralization and formation of teeth, along with mediators of the Wnt/β-catenin pathway as a development aspect in mice.

Materials and Methods: To confirm the Fam83h-KnockOut mice, both Sanger sequencing and Western blot methods were used. then used qPCR to measure the expression levels of genes related to tooth mineralization and formation of dental root, including Fam20a, Dspp, Dmp1, Enam, Ambn, Sppl2a, Mmp20, and Wnt/β-catenin pathway mediators, in both the Fam83h-Knockout and wild-type mice at 5, 11 and 18 days of age. also the expression level of Fgf10 and mediators of the Wnt/β-catenin pathway was measured in the skin of both Knockout and wild-type mice using qPCR. A histological assessment was then performed to further investigate the results.

Results: A significant reduction in the expression levels of Ambn, Mmp20, Dspp, and Fgf10 in the dental root of Fam83h-Knockout mice compared to their wild-type counterparts was demonstrated by our results, indicating potential disruptions in tooth development. Significant down-regulation of CK1a, CK1e, and β-catenin in the dental root of Fam83h-Knockout mice was associated with a reduction in mineralization and formation-related gene. Additionally, the skin analysis of Fam83h-Knockout mice revealed reduced levels of Fgf10, CK1a, CK1e, and β-catenin. Further histological assessment confirmed that the concurrent reduction of Fgf10 expression level and Wnt/β-catenin genes were associated with alterations in hair follicle maturation.

Conclusions: The concurrent reduction in the expression level of both Wnt/β-catenin mediators and mineralization-related genes, resulting in the disruption of dental mineralization and formation, was caused by the deficiency of Fam83h. Our findings suggest a cumulative effect and multi-factorial interplay between Fam83h, Wnt/B-Catenin signaling, and dental mineralization-related genes subsequently, during the dental formation process.

Keywords: Amelogenesis Imperfecta, CRISPR-Cas Systems, Family with sequence similarity 83 member H protein, Fgf10 protein, Wnt Signaling Pathway

1.Background

It is known that the FAMILY WITH SEQUENCE SIMILARITY 83, MEMBER H (*FAM83H;* OMIM: *611927) is the first gene causing the etiology of Amelogenesis Imperfecta (AI) in humans (1). Interes-tingly, such genetic potentials of *Fam83h* regarding teeth and enamel development in humans have not been applicable uniformly in mice (2, 3). Several studies have shown that mutations in *Fam83h* are related to Autosomal Dominant Hypocalcified Amelogenesis Imperfecta (ADHCAI, OMIM #130900) (4). While *Fam83h* Knockout (KO) mice are identified with their scruffy coat and short lifetime, however, there has been no phenotypic description of teeth formation and development in *Fam83h* KnockOut (KO) mice (2). Our previous studies delineated the *Fam83h* KO outbred mice with scruffy cover, dry eyes-like phenotype, normal life-span, and a specific dental phenotype similar to AI (3). As a non-secretory protein, FAM83H has a domain at its N-terminus that interacts with CK1. CK1 is a mediator of the Wnt/β-catenin pathway which plays an important role in mineralization and tooth growth (5). Mutations in the N-terminus of FAM83H lead to a non-proper subcellular localization and cellular functions of FAM83H and also, prevent binding to Casein kinase 1 (CK1) (6) (Snijders, Lee, *et al.* 2017). Furthermore, activation of the Wnt/β-catenin pathway has been reported to be associated with mutations in the *Fam83h* (7). In this regard, the expression levels of *CK1a*, *CK1e*, and β-catenin in *Fam83h* KO mice were evaluated in a comparative fashion with wild-type mice. In another study, the signaling pathway of Wnt/β-catenin mediators in the skin of scruffy cover of *Fam83h* KO mice as a specific prerequisite factor for hair follicle stem cell was evaluated. Despite all these together, the genes responsible for the mineralization and tooth development were selected based on literature review and STRING: functional protein association networks. the Fam20a, Dspp, Dmp1, Enam, Ambn, Sppl2a, Mmp20, Fgf10, and the mediators of Wnt/β-catenin pathway, as genes responsible for the mineralization and tooth development were evaluated in the absence of the Fam83h.

2.Objective

The molecular mechanisms and interaction network of the Fam83h gene, as a novel important gene responsible for tooth mineralization, hemostasis and development are not well characterized. Also, the companionship of the hair follicle defects and dry eye phenotype in Fam83h KO mice is still unknown. So, the expression levels of mineralization and tooth formation-related genes, as well as mediators of Wnt/β-catenin pathway and Fgf10 which had common effects on the formation of hair follicles were measured in dental root and skin of Fam83h Knockout and wild-type at 5, 11 and 18 days of age.

3. Materials and Methods

3.1. Ethical Compliance

All protocols and experimental procedures were reviewed and approved by the Ethical Committee of Kurdistan University of Medical Sciences (IR.MUK. REC.1396/181).

3.2. Animals and Sample Preparation

The *Fam83h* KO mice (NC_000081:g."7835_7877" del) were generated using Clustered Regularly Interspaced Short Palindromic Repeats(CRISPR) and CRISPR-associated (Cas) (CRISPR\Cas9) and genotypically and phenotypically characterized as described previously(3). These mice were backcross and inbred at two first generations (F2), confirmed by Sanger sequencing and Western blot (3). The F2-genotyped mice were propagated through inbreeding (parental* pups or pups* pups (brother *sister)) until the F6 generation and the *Fam83h* KO mice line were confirmed, and established and rechecked by Sanger sequencing and Western blot (**Supplementary Fig. 1B, C, D**). At each age of 5, 11, and 18 days old, three *Fam83h* KO pups born from homozygous *Fam83h* KO mice showed phenotypes of their parents including discolored teeth, tooth growth retardation, dental disruption in incisors, molars with attrition, smaller size, and less tall were included in the study. Total RNA was extracted from the mandibles to evaluate gene expression levels. The tail biopsy and colon samples were obtained from F4 line homozygous *Fam83h* KO mice to check and confirm the *Fam83h* Knockout by Sanger sequencing and Western blotting. Three skin samples were also taken from 8 weeks-old *Fam83h* KO mice as well as their wild-type counterparts for gene expression level using qPCR. All mice had full access to food and water equally and were kept in IVC cage systems under standard laboratory conditions. Also,

all laboratory works were carried out under standard conditions and in full accordance with the standards for working with laboratory animals approved by the medical ethics committee of Kurdistan University of Medical Sciences.

3.3. Sanger Sequencing

To re-approve the Knockout of *Fam83h* (NC_000081.7), genomic DNA was extracted from tail biopsies of F4 line homozygous KO mice line using a DNeasy Blood and Tissue Kits (Qiagen). PCR was performed using primers designed for Exon 2 of *Fam83h* (3). The PCR product of the targeted region was sequenced and genotyped (**Supplementary Fig. 1**).

3.4. Western-Blotting

To re-approve the *Fam83h* KO mice line, total protein was extracted from colon dissected samples of the F6 generation *Fam83h* KO and normal wild-type mice. Polyacrylamide gel electrophoresis for extracted samples was performed using 10% SDS-PAGE. The isolated protein bonds were then blotted on polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The blotted bonds were incubated with the primary anti-*Fam83h* (127.5 kDa) antibody (1:1000; Bethyl, A304-328A) overnight. After 3-times of washing, the blotted PVDF paper was incubated with HRP-conjugated secondary antibody, (1:10000; A120-101P) for an hour. Bands were finally visualized by an Enhanced Chemiluminescence (ECL) Detection Kit/ System (Sigma). Glycerol-3-phosphate dehydrogenase (GAPDH), was used simultaneously as a reference protein for normalization.

3.5. RNA Isolation and Reverse Transcription

Total RNA was isolated from dental root and skin samples of mice using an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manual of the manufacturer. DNA contamination was removed from RNA samples using RNase-Free DNase I treatment. The concentration and purity of RNAs were then spectrophotometrically assessed using a microplate reader with take3 (Synergy HTX, BioTek, USA). Reverse transcription was performed using 1000 ng of each extracted RNA, random hexamer primers, and 100 U of reverse transcriptase (TaKaRa, Japan) at 42 °C for 70 minutes using Eppendorf Thermal Cycler (Eppendorf, Germany).

3.6. Primers and Quantitative Real-Time PCR (RT-qPCR)

Primers were designed for genes using databases of GenBank. For confirmation of designed primers and to check the other thermodynamic properties of selected primers NCBI primer-blast online tool was used. The sequences of the primers are shown in **Table 1**.

Real-time PCR with SYBR green detection was performed using a RotorGene 6000 machine (Corbett Research, Sydney, Australia). Briefly, for each sample, a reaction mixture containing 100 ng of each sample cDNA, 10 picomoles of each forward and reverse PCR primer, and 12.5 µL of SYBR Premix Ex Taq II (Takara, Japan) was provided in a microtube. A thermal cycle program of incubation at 94 ºC for 10 min for hot start followed by 42 cycles of 94 ºC for 20 sec, 60 ºC for 20 sec, and 72 ºC for 15 sec was applied. Melting curve analysis and 2% agarose gel electrophoresis were performed to verify the qPCR product. For normalization of gene expression, β-Actin was also checked with all samples. To calculate the relative expression levels of genes $2^{-\Delta\Delta Ct}$ method was applied.

3.7. Histological Examination of the Skin Biopsies

A 1 cm^2-sized punch skin biopsy was prepared from three *Fam83h* KO and three normal control mice under local anesthesia. Biopsy specimens were quickly fixed in the formalin solution and consequently embedded in paraffin. Then, the 5-µm thick sections were cut from the embedded tissues, mounted on slides, and stained with hematoxylin and eosin (H&E) to investigate the possible change in hair follicles and other peripheral environments of them.

3.8. Statistical Analysis

The stability of the mRNA expression of beta-actin was evaluated by using the MS Excel application geNorm. All data are presented as mean ± SD of mRNA folds change from three independent experiments for each sample. A one-way ANOVA test was used for calculating the statistical difference between gene expression of *Fam83h* KO and normal wild-type mice. The post-hoc Tukey's test was then used for multiple comparisons of each group. Gene expression differences were calculated using Genex 6 software, and statistical analyses were performed using SPSS 21 software and plotting with GraphPad Prism 7.

Table 1: The sequence of primers used in qRT-PCR.

Gene Symbol	**Sequences**	**Size (bp)**	**Accession number**
Ambn	F:ATGAAGGGCCTGATCCTGTTC R:GTCTCATTGTCTCAAGGCTCAAA	130	NM_001303431.1
Fam20a	F:GATGTGACGCGGGATAAGAAG R:GCTCGGTGGAACAGTAGTAGG	100	NM_001359593.1
Dspp	F:ATTCCGGTTCCCCAGTTAGTA R:CTGTTGCTAGTGGTGCTGTT	128	NM_010080.3
Mmp20	F:GGCGAGATGGTGGCAAGAG R:CTGGGAAGAGGCGGTAGTT	166	NM_013903.2
Ck1 Alpha	F:TCCAAGGCCGAATTTATCGTC R:ACTTCCTCGCCATTGGTGATG	110	NM_001357500.1
Ck1 epsilon	F:GAGCTGCGTGTGGGAAATAAG R:ACATTCGAGCTTGATGGCTACT	120	NM_001359863.1
Sppl2a	F:CATGTCATGCGTGATACTGCT R:ACCCTGATAACTACTGGCAACT	156	NM_023220.2
Fgf10	F:TTTGGTGTCTTCGTTCCCTGT R: TAGCTCCGCACATGCCTTC	132	NM_008002.4
Dmp1	F:CATTCTCCTTGTGTTCCTTTGGG R:TGTGGTCACTATTTGCCTGTG	185	NM_001359013.1
Enam	F:TGCAGAAATCCGACTTCTCCT R:CATCTGGAATGGCATGGCA	114	NM_017468.3
Beta-Catenin	F:ATGGAGCCGGACAGAAAAGC R: CTTGCCACTCAGGGAAGGA	108	NM_001165902.1

4. Result

4.1.The Expression Levels of Fam20a, Dspp, and Dmp1 as Important Genes for Dental Mineralization of the Tooth in the Absence of Fam83h in Tooth Roots

The gene expression level of Family With Sequence Similarity 20, Member A; *(Fam20a)*, Dentin Sialophosphoprotein (*Dspp*), and Dentin Matrix Acidic Phosphoprotein 1 (*Dmp1*) were evaluated as important genes for dental mineralization in *Fam83h* KO compared with normal-wild type mice. *Fam20a* expression level in *Fam83h* KO mice was not significantly different from normal-wild type mice at 5, 11, and 18 days old ($P > 0.05$). The trend of expression of the *Dmp1* gene was increased along with age increased in both normal-wild type and *Fam83h* KO. Also, the expression of *Dmp1* on days 5 and 11 was not statistically significant($P > 0.05$), while in day 18 was significantly decreased in the *Fam83h* KO mice compared with normal-wild type mic($P = 0.0023$). There was a significant decrease in the expression level of *Dspp* in *Fam83h* KO at all three ages 5,11 and 18 days old in comparison with the normal-wild type mice in tooth roots ($P < 0.0001$). The expression pattern of *Dspp* has fluctuated which increased to its highest level on day 11th of birth in both normal-wild types and *Fam83h* KO (**Fig. 1A**).

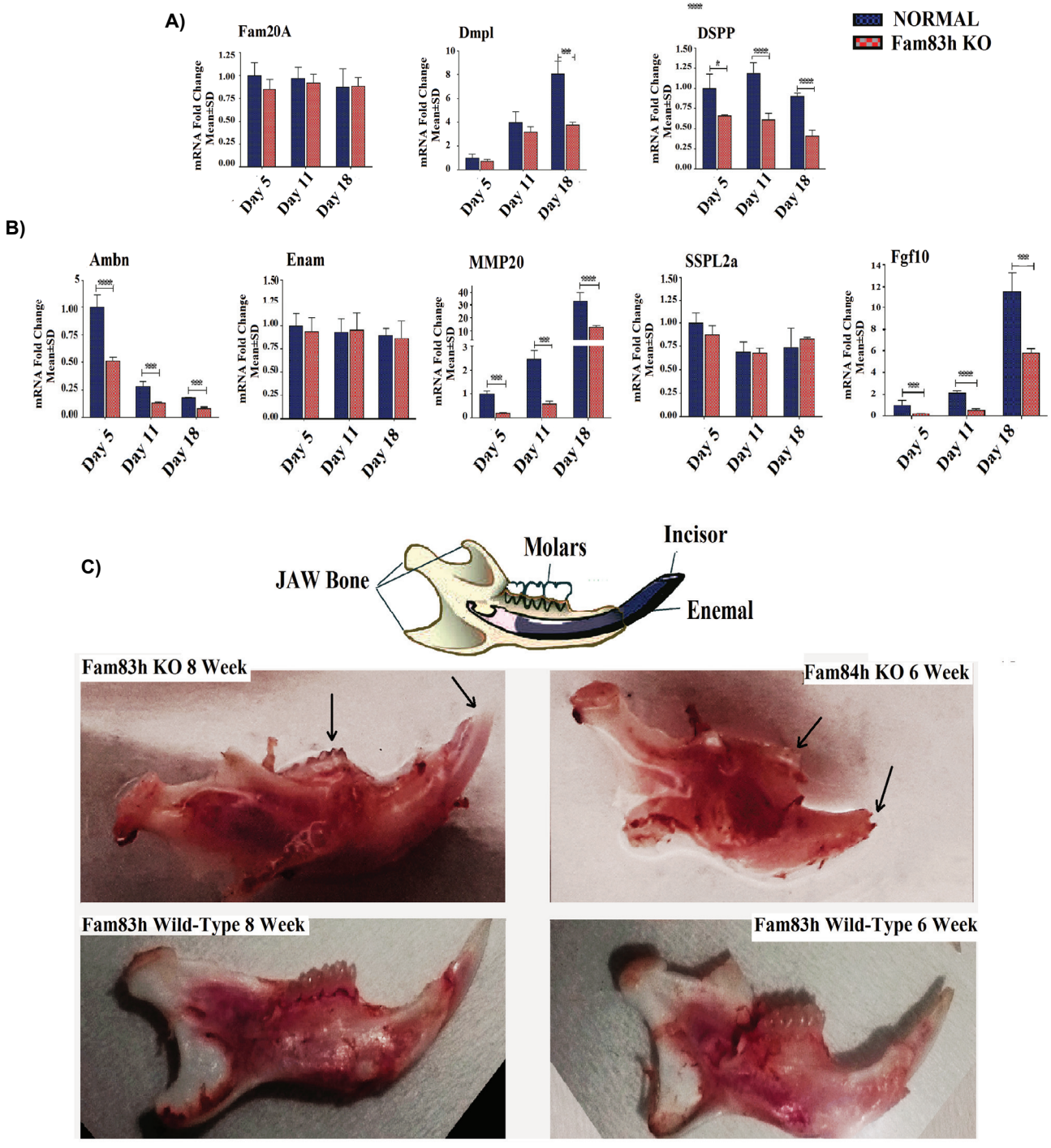

Figure1. Investigation of the expression level of genes related to mineralization, enamel matrix homeostasis and development in dental root in the absence of *Fam83h* gene (NC_000081.7). The comparison expression assessment between *Fam83h* KO mice and normal mice in 5, 11,18 days of birth showed; **A)** a significant decrease in *Dspp* in three examination days, *Dmp1* decreased significantly just in 18th day and *Fam20a* had no significant change as important genes in the dental mineralization. **B**) The expression level of genes related to formation and homeostasis of enamel matrix depicted a significant decrease in *Ambn, Mmp20*, and *Fgf10* and *Sppl2a* and *Enam* had no significant changes. **C)** As shown in the schematic of the normal mandible of an adult mouse and dental tissue extracted from 8 weeks (right) and 6 weeks (left) (arrows), showed despite of natural crown morphology, Fam83h KO mice have underdeveloped and petite incisors, smaller molars and have evidence of attrition. The significant level is indicated by the stars on graph. *P < 0.05, **P < 0.01, ***P < 0.001.

4.2. The Expression Levels of Enam, Ambn, Sppl2a, Mmp20, and Fgf10 as Contributing Genes to the Formation and/or Homeostasis of Enamel Matrix in the Absence of Fam83h in Tooth Roots

The expression level of Enamelin (*Enam*), Ameloblastin (*Ambn*), Signal Peptide Peptidase Like 2A *(Sppl2a)*, Matrix Metallopeptidase 20*) Mmp20 (*, and Fibroblast Growth Factor 10 *)Fgf10 (* were also comparatively assessed between *Fam83h* KO and normal wild-type mice in roots of teeth. *Enam*, *Ambn*, and *Mmp20* are important genes in the formation of the enamel matrix which are related to *Fam83h* gene networks (8). There was no significant changes in the expression levels of *Enam* in *Fam83h* KO mice at days 5, 11, and 18 of birth compared with normal wild-type mice ($P > 0.05$). Also, changes in the expression level of *Enam* and *Sppl2a* (**Fig. 1B**) were similar in *Fam83h* KO and normal wild-type mice at all ages. However, the expression levels of the *Ambn* and *Mmp20* at three different ages (5, 11, and 18 days old) in KO mice showed a significant decrease ($P < 0.0001$) compared with normal wild-type mice. In addition, the expression levels of *Fgf10* as tooth stem cell hemostasis factor demonstrated a significant decrease on days 5, 11 *(P=0.0001* for both), and 18 *(P= 0.0077)* in the *Fam83h* KO mice compared to the normal wild-type mice. Furthermore, the expression level of *Fgf10* showed a rising pattern from day 5 to day 18 of birth in both *Fam83h* KO and normal-wild type mice (**Fig. 2A**).

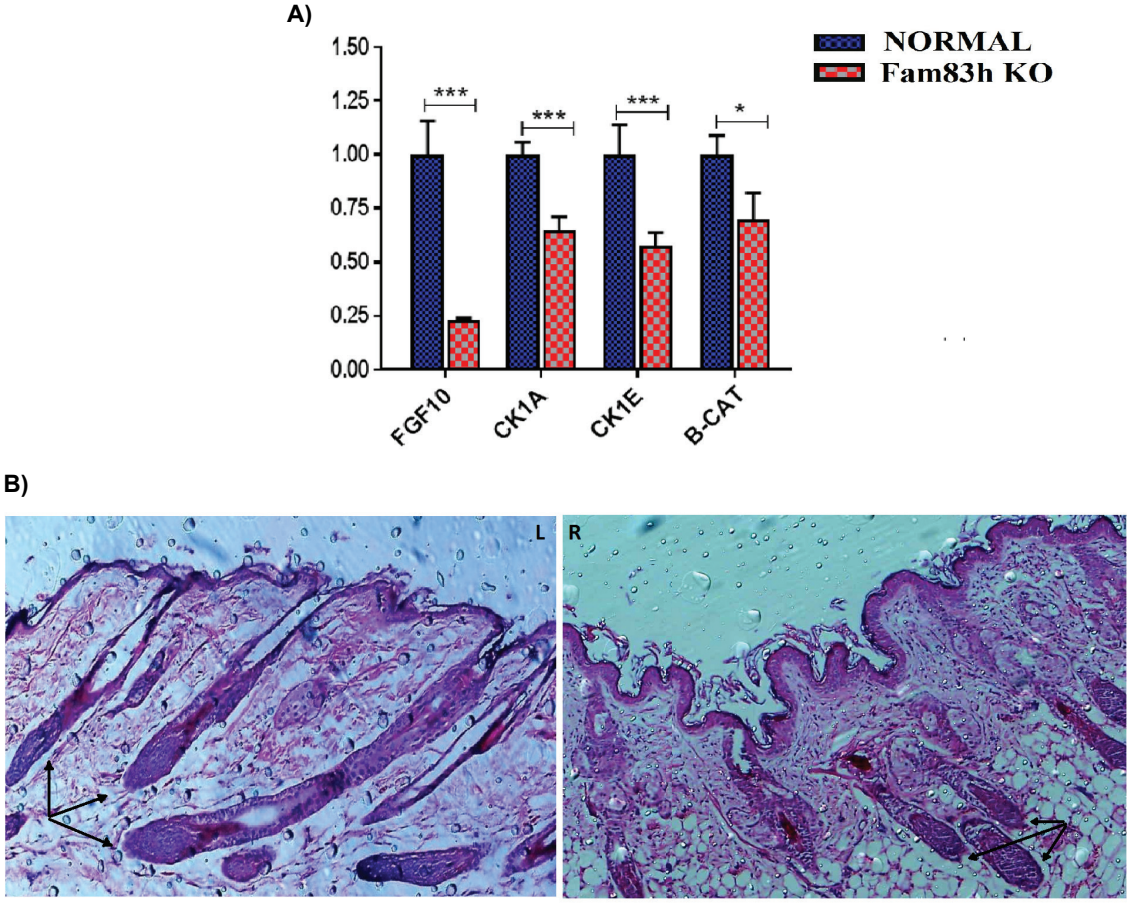

Figure 2. Expression of Fgf10 and Wnt/β-catenin mediators in the skin and histological assessment of hair follicles differentiation in Fam83h KO and normal mice. **A)** Fam83h KO mice have significantly decreased expression of Fgf10, CK1a, CK1e, and β-catenin. **B**) Histological assessment of skin from Fam83h KO mice showed undifferentiated and disrupted hair follicles in the anagen phase, while normal mice showed normal morphology of differentiated hair follicles.Reduced hair density on the skin surface of Fam83h KO mice is due to the decrease of mature follicles. *P < 0.05, **P < 0.01, ***P < 0.001.

4.3. The Expression Levels of the Genes Related to Canonical Wnt/β-Catenin of Fam83h KO Mice Compared with Normal Wild-Type Mice in Tooth Roots

The expression levels of *CK1a* and *CK1e* genes in *Fam83h* KO mice decreased significantly on days 11 and 18 of birth compared with normal wild-type mice (*CK1a*; day11 *P=0.0075* and day18 *P=0.0176*, *CK1e*;day11and 18 *P=0.0001*). This descending pattern was not significant for 5 days-old puppies (*P> 0.05*). Both *CK1a* and *CK1e* showed more expression on day 18. Also, the expression level of the *β-catenin* (*b-CAT*) in *Fam83h* KO mice decreased significantly in all three ages including 5, 11, and 18 days compared with normal wild-type mice (on days 5, 11, and 18 were *P= 0.0332*, *P=0.0474,* and *P= 0.031* respectively). The expression level of this gene reduced over time, from day 5 to 18 (**Fig. 3A**).

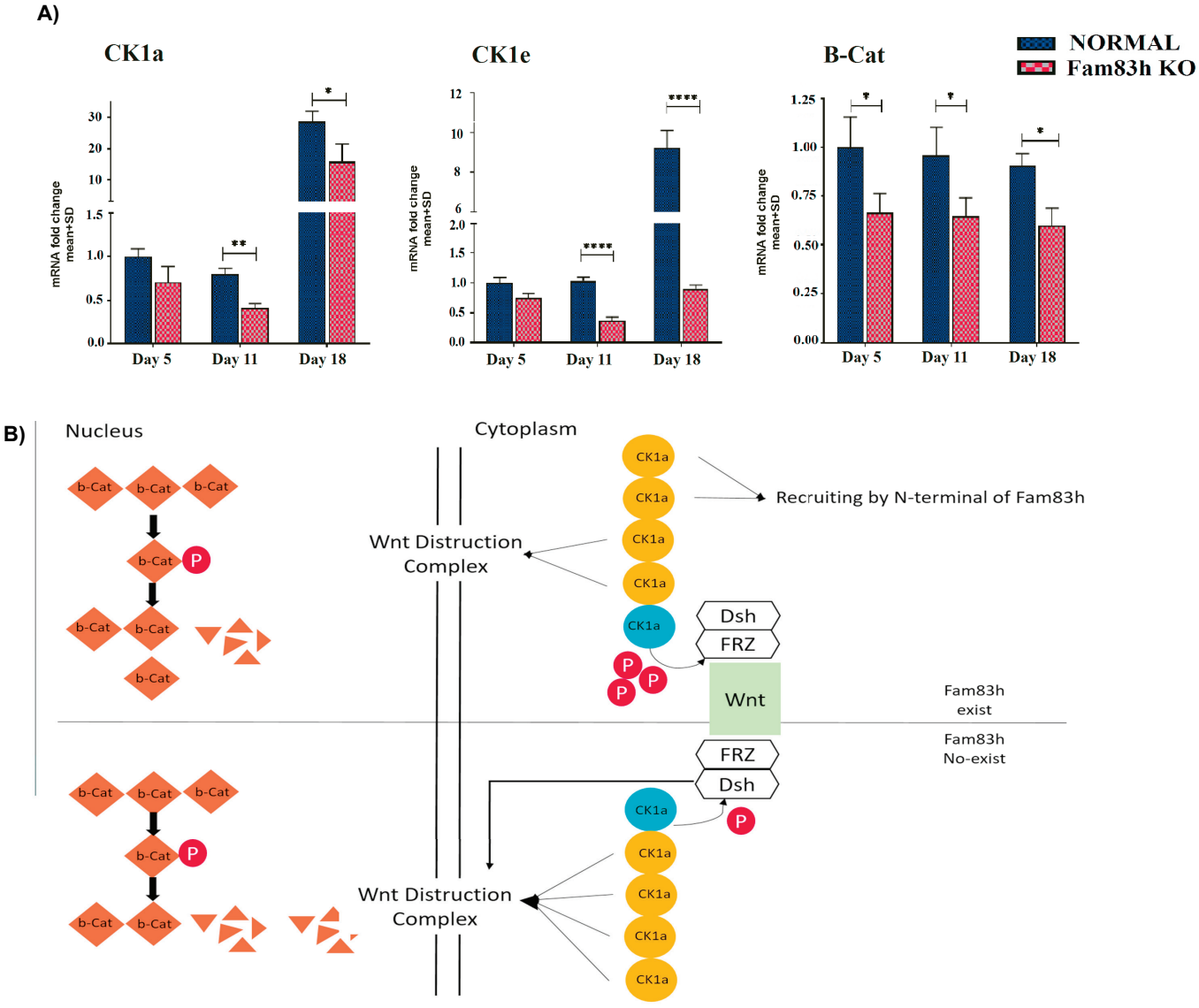

Figure 3.T**he expressional analysis of Wnt/β-catenin mediators and a suggested schematic of the Wnt/β-catenin pathway. A)** In Fam83h KO mice, the expression of Ck1a, Ck1e, and β-catenin as Wnt/β-catenin mediators significantly decreased on days 11 and 18 post-birth compared to normal mice. **B)** The presence of Fam83h protein at physiological state, facilitates the recruitment of Ck1a and regulates the destruction complex, while CK1e acts as a positive regulator. In the absence of Fam83h, Ck1a freely participates in the destruction complex, resulting in enhanced degradation of β-catenin and inhibition of the Wnt/β-catenin pathway..*P < 0.05, **P < 0.01, ***P < 0.001.

4.4. Fgf10 and WNT Mediators' Expression Level and the Histological Assessment in the Absence of Fam83h in the Skin

The Expression level of *Fgf10* in the skin showed a significant decrease (P=0.0010) in *Fam83h* KO mice compared to normal-wild type mice (**Fig. 2B**). Also, the expression level of *CK1a, CK1e*, and *β-catenin* as mediators of the Wnt/β-catenin pathway were measured in the skin sample of both *Fam83h* KO and normal-wild type mice. The results showed a significant reduction of *CK1a* (P=0.0065), *CK1e* (P=0.0075), and *β-catenin* (P=0.0495) in *Fam83h* KO compared with normal-wild type mice (**Fig. 2B**). The histological examination of skin samples was done for both *Fam83h* KO and normal-wild type mice at 8 weeks age of post-natal to determine the possible effect of *Fam83h KO* on the hair follicles. The histological evaluation revealed an obvious difference in the development of hair follicles between these two groups. In normal control mice, the hair follicles presented normal morphology at the anagen phase. In contrast, *Fam83h* knockout mice showed undifferentiated and disrupted hair follicles at the same phase (**Fig. 2C**).

5. Discussion

It is suggested that *Fam83h* correlated with Wnt/β-catenin and could mediate regulation of the organization of cell cytoskeleton and play an important role in ameloblast maturation and differentiation of the enamel matrix (9). The absence of *Fam83h* might increase the potential phenotype of dental deficiency in mice as an important factor that affects dental mineralization and development in mice (3). There is an inconsistency in the phenotypic manifestation of *Fam83h* deletion in outbred and inbred mice as well as the other related phenotypes in humans. Therefore, in the present study, we evaluated the expression levels of Fam83h-related genes and wnt/β-catenin pathway which have an important role in the formation and mineralization of teeth in tooth roots. Furthermore, along with histology assessment, we evaluated *Fgf10* as a gene related to the development of hair follicles and also, the mediators of the Wnt/β-catenin signaling pathway as a prerequisite for hair follicle stem cell specification(10) evaluated in the skin.

Dmp1 is associated with craniofacial abnormalities and Periodontal Breakdown (11). The expression level of this gene is significantly decreased only on the 18th day of birth in *Fam83h* KO mice compared with normal wild-type mice. The deficiency of *Dmp1* does not seem to have a significant effect on tooth formation and mineralization. It is demonstrated that *Dspp*, which is mainly expressed in odontoblasts, is associated with dentinogenesis imperfecta II, dentinogenesis imperfecta III (12, 13), and dentin dysplasia (DD) (14) which the hypo-mineralization are common phenotype among them. The expression level of *Dspp* significantly decreased in 5, 11, and 18 days old mice.

Fam20a is an important factor for dental mineralization and is a critical gene for the mineralization of bone, dentin, and enamel (15). Previously, *Fam20a* deficient mice were associated with a delay in the eruption of molars as well as hyperplasia of the gingival epithelium (16). The expression levels of the *Fam20a* did not change at any of the studied ages.

Previous studies reported *Enam* and *Ambn* as necessary genes for the formation of enamel matrix, which is expressed during the secretory stage of ameloblast (17). The expression level of *Ambn* decreased significantly during the three assessed ages in *Fam83h* KO mice compared with normal wild-type mice. Expression of the *Enam* gene as another gene required for proper enamel formation in *Fam83h* KO mice(18) remained unchanged during the studied ages in *Fam83h* KO mice compared with normal wild-type mice. Ameloblastin (AMBN) is an adhesion molecule synthesized by odontoblasts and ameloblasts. Previous studies, Reported that the enamel layer in *Enam* and *Ambn* KO mice was similarly either a thin or missing enamel layer(19) .

Sppl2a and *Mmp20* are two important genes for the homeostasis of enamel through their association with *Fam83h*. *Sppl2a* as a critical intramembranous protein is essential for maintaining cellular homeostasis in the ameloblasts (20). *Mmp20* is an enamel metalloproteinase that cleaves enamel matrix proteins (21). *MMP20* is one of the genes involved in AI etiology and related to the hypomineralization of dentine(22) that we investigate its expression level in this study. The expression level of *Sppl2a* did not change in the evaluated ages, while the *Mmp20* expression level showed a significant decrease at all ages of 5, 11, and 18 days of birth in *Fam83h* KO mice compared with normal wild-type mice.

The results of the *Fgf10* expression assessment demonstrated a significant decrease on days 5, 11, and 18 of birth in the *Fam83h* KO mice in comparison

with normal wild-type mice. Previous studies have shown an important role for *Fgf10* in the development and maintenance of stem cell compartment during the prenatal (23)and postnatal (24, 25)period in incisor cervical loops.

Based on the expression profile summarized above, there was a significant reduction in the expression level of *Ambn, Mmp20, Dspp*, and *Fgf10* in *Fam83h* KO mice compared with normal wild-type mice. These results were reported for the first time and were consistent with previous reports and showed an association between *Fam83h* KO mice and an abnormal dental phenotype. Significant reduction in the expression level of genes related to dental mineralization such as *Ambn* as a necessary gene for enamel matrix formation, *Mmp20* as a major role in the cleavage of the enamel matrix and essential for normal tooth development, *Dspp* as a gene mainly expressed in odontoblasts and essential for proper mineralization of teeth. *Fgf10*, is an important gene for the maintenance of stem cells in developing mouse tooth roots, largely justifying the phenotype including discolored, fractured, and eroded teeth after breastfeeding in mandible incisors of *Fam83h* KO mice. Based on the results of this study and discussed topics, the phenotype of teeth in *Fam83h* KO mice shows evidence of disrupted, undeveloped, petite incisors and smaller size molars with attrition in comparison with Normal wild-type mice (**Fig. 1**), which might be related to the decrease of mineralization in the absence of *Fam83h*.

The result of the evaluation of the expression of *Ck1a, Ck1e*, and *β-catenin* as mediators of Wnt/β-catenin pathway showed a significant decrease in the expression levels of *Ck1a* and *Ck1e* in *Fam83h* KO mice on days 11 and 18 of birth. The expression level of *β-catenin* also significantly decreased at 5, 11, and 18 days of birth. Previous studies showed a relationship between *Fam83h* mutations and the Wnt/β-catenin pathway. Yang *et al*. reported that mutations of *Fam83h* inhibit the mineralization of ameloblasts by activating the Wnt/β-catenin pathway (7). Fan *et al*. also demonstrated that continuous activation of *β-catenin* leads to incisor enamel hypo-mineralization (26). In another study, Bae *et al*. reported that the excess level of the Wnt/β-catenin signaling pathway disturbs normal process of tooth-root formation (27). To explain these findings, it should be noted that the *β-catenin* is degraded by a multiprotein complex called "destruction complex" in which *CK1a* together with *GSK-3* causes phosphorylation and degradation of *β-catenin* via ubiquitination (28). Kim *et, al*. have shown that nuclear and cytoplasmic localization of *β-catenin* could reduce in response to *Fam83h* knock-down, and ubiquitination and proteasomal degradation of *β-catenin* increased with *Fam83h* knock-down (29). In addition, Kuga *et al* showed that overexpression of *Fam83h* promotes the accumulation of *CK1* in nuclear speckles (30). it can be concluded that the *Fam83h* deletion resulted in the decrease of the Wnt/β-catenin signaling pathway via reducing the expression levels of *Ck1a, Ck1e*, and *β-catenin*. Accordingly, based on our previous and current data, it is plausible to believe that the absence of *Fam83h* would cause the cytoplasmic elevation of *Ck1a*, leading to the formation of "destruction complex". This is basically an intuitive presumption because the lack of *Fam83h* would prevent the employment of *Ck1a* (6, 31), increasing *Ck1a* in the cellular cytoplasmic environment. Further, it can be proposed that increased formation rate of destruction complex would result in degradation of *β-catenin* that subsequently curbes the Wnt/β-catenin signaling pathway. Therefore, as an instinctive possibility, unemployed *Ck1a* maybe considered as a negative regulator of the Wnt/β-catenin signaling pathway (32). Furthermore, decreased level of *Ck1e* expression culminated in reduction of *Dvl-1* phosphorylation. Given the role of *Dvl-1* phosphorylation as a Wnt/β-catenin positive regulator (33), such reduction could be considered as an additional reason for Wnt/β-catenin reduced activity in the absence of *Fam83h*.

Further, the reduced expression level of *Ambn*, *Mmp20*, *Dspp*, and *Fgf10* were compatible with the reduced expression level of Wnt/β-catenin signaling pathway in *Fam83h* KO mice. Zhou *et al*. and Liu *et al*. showed that the continuous signaling activity of the Wnt/β-catenin pathway in the dental epithelium of mice induced ectopic expression of *Dspp* (34, 35). Koizumi *et al*. noted that enhanced expression of *Dspp* and *Dmp1* was completely suppressed by the Wnt antagonist (36). Importantly, it was previously documented that the *Mmp20* plays an important role in the migration of normal ameloblast through tight control of the Wnt/β-catenin signaling pathway (37). Altogether, it is conspicuous that lack of *Fam83h*, along with the reduction of the Wnt/β-catenin signaling pathway, would conduce in the concurrent reduction of

mineral-related genes.

Importantly, in addition to dental development process, the *Fam83h* and Wnt/β-catenin axis are actively involved in the development of hair follicles (38). Our findings demonstrated reduction in the expression level of *Ck1a, Ck1e*, and *β-catenin* genes in the skin tissues of *Fam83h* KO mice compared to their Wild-Type counterparts. Such reduction was also detected in the expression level of *Fgf10* in the skin of *Fam83h* KO mice compared to Wild-Type mice. *Fgf10* is known as a promoter for hair growth-inducing the anagen phase of resting follicles (39, 40). Also, *Fgf10* controls the development of hair follicles in a orchestrated fashion with Wnt/β-catenin signaling pathway (41, 42). Interestingly, the Wnt/β-catenin pathway plays an important role in the initiation, development, and growth of hair follicles (43). It is worth mentioning that several studies have documented that *Fgf10* was the only member of the Fibroblast Growth Factor genes (Fgfs) family, which was selectively expressed in the mesenchyme during the early stage of follicle morphogenesis, contributing to the maintenance of follicle growth (44). It is also, reported that *Fgf10* together with *β-catenin* can induce the development and growth of hair follicles (41). Thus, the reduction of *Fgf10* elucidates the scruffy coat phenotype in *Fam83h* KO mice. Furthermore, it was shown that a novel mutation in *Fgf10* could be responsible for slit-eye mice model having a dry eye (40, 45). Given all above plus targeting *Fgf10* expression as a therapeutic modality in the treatment of dry eyes in a rabbit model (46) supports the identification of dry eye phenotype in *Fam83h* KO mice model. Altogether, It is reasonable to conclude that the absence of *Fam83h* along with decreased *Fgf10* and Wnt/β-catenin expression could be responsible for dry-eye and scruffy coat phenotypes in *Fam83h* KO mice. Figure 3 summarizes the discussion and proposed potentials.

In conclusion, our data provides evidence that the lack of *Fam83h* gene curtails Wnt/β-catenin pathway, causing few potential alterations at the cellular and molecular levels. A potential mechanism for such effects could be based on the accumulation of unemployed *Ck1a*, elevation of destruction complex, and decreased *CK1e* as well as Dvl-1 signaling. Importantly, it is possible that reduction in both mineralization genes and Wnt/β-catenin signaling in the absence of *Fam83h* gene may be responsible for the deficiency of dental formation and mineralization. In addition, our data here indicates that reduction in both *Fgf10* gene and Wnt/β- catenin pathway in the skin may affect hair follicular maturation. Given the central role of Wnt/β- catenin pathway in several pivotal biological processes, it is reasonable to speculate that deficiency in *Fam83h* causes the down-regulation of Wnt/β- catenin signaling, leading to a wide spectrum of phenotypic alterations in a complex and multi-factorial manner, warranting further research. especially evaluating the protein interaction networks, and finding the hub genes which may be common among the Wnt signaling pathway, fam83h, and other related genes can be the future research topics to better understand Fam83h biological manner.

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Competing interests

The authors have no conflict of interest to declare regarding this manuscript.

Author Contributions

SN designed and performed testing as well as drafted the manuscript. SN, ZV, FF, and SP validate the methodology and tests.SN, MBK, BN, MRK, SP, FF, SB preparation of the manuscript.BB, SN, SP, Scientific consultant, and content and writing editor. FF and SN supervised the entire study. All authors revised and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Manifestation

Part of the current article has already been published as a Conference Proceeding, available at :
https://www.imedpub.com/articles/the-lack-of-fam83h-mediated-reduction-of-wntcatenin-signaling-pathway-and-expression-levels-of-dental-mineralization-genes.php?aid=43944

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