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Fibulin-5 deficiency causes developmental defect of premaxillary bone in mice

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ABSTRACT

Craniofacial sutures govern the shape of the craniofacial skeleton during postnatal development. The differentiation of suture mesenchymal cells to osteoblasts is precisely regulated in part by signaling through cell surface receptors that interact with extracellular proteins. Here we report that fibulin-5, a key extracellular matrix protein, is important for craniofacial skeletal development in mice. Fibulin-5 is deposited as a fibrous matrix in cranial neural crest-derived mesenchymal tissues, including craniofacial sutures. Fibulin-5-null mice show decreased premaxillary bone outgrowth during postnatal stages. While premaxillo-maxillary suture mesenchymal cells in fibulin-5-null mice were capable of differentiating into osteoblasts, suture cells in mutant mice were less proliferative. Our study provides the first evidence that fibulin-5 is indispensable for the regulation of facial suture mesenchymal cell proliferation required for craniofacial skeletal morphogenesis.

1. Introduction

Morphogenesis of the craniofacial skeleton begins during embryonic development, and continues after birth. During postnatal development, bone remodeling in sutures at the osteogenic front accounts for the additional size and shape changes of the craniofacial bones [1,2]. The osteoblasts in the sutures are derived from suture mesenchymal stem cells [3]. Proliferation and differentiation of mesenchymal cells are regulated in part by signaling through cell surface receptors that interact with extracellular proteins. However, it remains unclear how the extracellular matrix proteins regulate both proliferation and differentiation in the suture mesenchymal cells.

Fibulin-5 is an extracellular matrix protein secreted by various cell types, including vascular smooth muscle cells [4,5]. Mice deficient for the fibulin-5 exhibit loose skin, tortuous aorta, and emphysematous lung, demonstrating that fibulin-5 is essential for elastic fiber assembly [6–8]. Fibulin-5 is also critical for vaginal stromal tissue homeostasis by suppressing proteases that degrade extracellular matrix [9]. In addition, fibulin-5-null mice exhibit increased angiogenesis after wound healing, suggesting that fibulin-5 functions as an inhibitor of angiogenesis [10]. Thus, fibulin-5 regulates diverse cellular functions in a tissue- and context-specific manner.

Among seven fibulin family proteins, fibulin-1 and fibulin-5 are reported to be expressed in mouse neural crest derivatives, including craniofacial mesenchymal tissues [4,5,11,12]. While fibulin-1 is critical for cranial bone formation [13,14], the importance of fibulin-5 in craniofacial development has not been unraveled. We report here that fibulin-5 is localized in postnatal craniofacial sutures, and contributes to facial skeletal development. This study provides a unique mouse model of midfacial hypoplasia, and the first evidence that fibulin-5 is required for postnatal craniofacial morphogenesis.

2. Materials and methods

2.1. Mice

The *Fbln5*$^{+/-}$ mice [6] were maintained on a C57BL/6J background. Mice from *Fbln5*$^{+/-}$ × *Fbln5*$^{+/-}$ crosses were genotyped by PCR using the forward primers pgk-s1 (5′-CTGCTAAAGCG-CATGCTCCAGACTG-3′) and A55Gs1 (5′-CGCTTTGGGTATCA-GATGGATGAAGG-3′), specific for the mutated and wild-type allele,

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respectively. The common reverse primer A55Ga2 (5′-AATGAGGTTGGTCACCAATGAGATCC-3′) was also used for genotyping. *R26R-EYFP* [15] and *Wnt1-Cre* [16] mice were obtained from The Jackson Laboratory. All mice were maintained in the Animal Facility of The University of Texas Medical School at Houston. The experimental protocol was reviewed and approved by the Animal Welfare Committee, the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston.

2.2. Skeletal preparations and measurement

Staining of bone and cartilage in postnatal mice with alizarin red/alcian blue was carried out as described previously [17,18]. The images were captured by an Olympus DP71 digital camera attached to a SZX16 microscope (Olympus). The lengths between landmarks were measured using cellSence Standard 1.13 (Olympus).

2.3. Histological analysis, immunohistochemistry, and immunofluorescence

Mice were fixed in 10% formalin at 4 °C overnight, followed by decalcification for 2 weeks with 10% EDTA, then embedded in paraffin. For histological studies, mice were serially sectioned (7 μm thickness). Sections were kept on Superfrost Plus glass slides (Fisher Scientific). The paraffin sections were deparaffinized in xylene, and rehydrated in a descending series of ethanol solutions. Hematoxylin-eosin staining was performed according to standard protocols. Elastica van Gieson staining was performed using an Elastin Stain Kit (Sigma–Aldrich, HT25A).

For immunohistochemical staining, sections were subjected to antigen retrieval by the use of Antigen Unmasking Solution (Vector Laboratories, H-3300) for 20 min at 100 °C. Slides were then washed in water, permeabilized with 0.1% TritonX-100 in PBS (PBST) for 15 min, and blocked by incubation with 5% sheep serum in PBST for 30 min. Slides were incubated overnight at 4 °C with antibodies against osterix (1:500, Abcam, ab22552) and Ki67 (1:100, Abcam, ab16667). After washing three times in PBST for 5 min each time, slides were incubated with biotinylated secondary antibodies, followed by the addition of preformed ABC reagent (Santa Cruz, sc-2018). Immunoreactive cells were visualized using 3,3-diaminobenzidine substrate solutioin (Sigma–Aldrich, D4168) as a chromogen, and counterstained with hematoxylin.

For frozen sections, tissues were embedded in Tissue-Plus O.C.T. Compound (Fisher Scientific) and serially sectioned (10 μm thickness). Sections were kept on Superfrost Plus glass slides (Fisher Scientific). Slides were washed in water, permeabilized with PBST for 15 min, and then blocked by incubation with 5% sheep serum in PBST for 30 min. The slides were incubated overnight at 4 °C with antibodies against fibulin-5 (1:100, previously described [8]), and EYFP (1:500, Abcam, ab13970). After washing three times in PBST for 5 min each time, slides were incubated with Alexa Fluor 568- or Alexa Fluor 488-conjugated secondary antibodies (Life Technologies), followed by staining with Hoechst 33342 (Life Technologies). Slides were viewed with an Olympus FluoView FV1000 laser scanning confocal microscope by using the FV10-ASW Viewer (Ver. 3.1).

2.4. RNA in situ hybridization

Paraffin sections were hybridized as previously described [19]. Antisense probes were used for detecting *Col1a1* [20].

2.5. Statistical analysis

A Student's *t*-test was applied for statistical analysis. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Fibulin-5-null mice exhibit postnatal facial anomaly

To study the impact of fibulin-5 deficiency during craniofacial development, we examined the facial phenotypes of wild-type (WT) and fibulin-5-null (KO) mice from postnatal day (P) 0 to P84. While no overt morphological defects were seen until around three weeks of age, KO mice had shortened snouts compared to WT mice at P28 (Fig. 1A). To examine the possibility that the shortened snout is due to smaller stature of mutant mice, we measured body weights. The body weights were comparable between WT and KO mice at P14, P28, and P56 (Fig. 1B). In addition, the facial abnormality of KO mice did not increase in severity during development past P28, suggesting that fibulin-5 may play a critical role in the facial development during the early postnatal period.

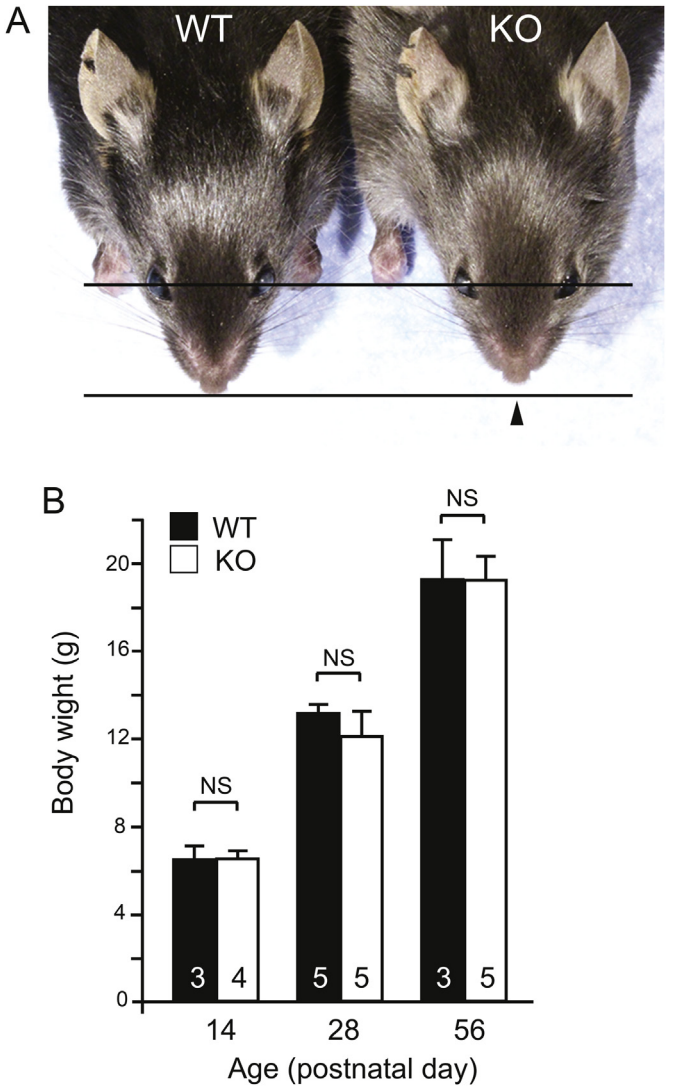

Fig. 1. Fibulin-5-null mice have abnormal facial development. (A) Dorsal views of a male fibulin-5-null (KO) mouse (right) and a wild-type (WT) littermate (left) at P28. Black arrowhead indicates shortened snout in KO mouse. (B) Measurement of body weight of female mice at different time points. Analyzed mouse numbers are indicated in the bars. Error bars indicate standard deviation. NS, not significant.