Biochemical and Biophysical Research Communications 526 (2020) 62–69

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The C-terminal region including the MH6 domain of Msx1 regulates skeletal development

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ARTICLE INFO

Article history:
Received 25 February 2020
Accepted 10 March 2020
Available online 16 March 2020

Keywords:
Msx1
MH6 domain
Bone
Development
Genome editing technology

ABSTRACT

MSX1 is a causative gene for oligodontia in humans. Although conventional *Msx1*-deficient mice die neonatally, a mutant mouse lacking the C-terminus MH6 domain of MSX1 (*Msx1*$^{\Delta MH6/\Delta MH6}$) showed two different phenotypes; newborn homozygotes with cleft palates died neonatally, whereas those with thin palates remained alive and had craniofacial dysplasia and growth retardation compared with wild-type mice, with most mice dying by the age of 4–5 weeks. In a previously reported case of human oligodontia caused by a heterozygous defect of the Msx1 MH6 domain, a small foramen was observed on the occipital bone. The aim of this study was to test the hypothesis that the Msx1 MH6 domain is involved in bone formation *in vivo*. In *Msx1*$^{\Delta MH6/\Delta MH6}$ mice, cranial suture fusion was delayed at embryonic day 18.5, and the anteroposterior cranial diameter was smaller and long bone length was decreased at 3 weeks of age. The femoral epiphysis showed no change in the trabecular number, but decreased bone mass, bone density, and trabecular width in Msx1$^{\Delta MH6/\Delta MH6}$ mice. In addition, cancellous bone mass was reduced and the cartilage layer in the growth plate was thinner in Msx1$^{\Delta MH6/\Delta MH6}$ mice. The mRNA expression levels of major osteoblast and chondrocyte differentiation marker genes were decreased in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice compared with wild-type mice. These findings suggest that the C-terminal region including the MH6 domain of MSX1 plays important roles not only in tooth development and palatal fusion, but also in postnatal bone formation.

1. Introduction

The MSX homeobox gene family encodes transcription factors that play important roles in inductive tissue interaction during vertebrate development [1,2]. *Msx1* is expressed in developing limb buds and craniofacial structures, including the neural crest and branchial arches [3]. *MSX1* was first identified as the causative gene for non-syndromic tooth agenesis [4], and is remarkably expressed in the dental mesenchyme during early tooth development. *Msx1* is also expressed in cranial neural crest (CNC)-derived mesenchymal cells in the frontal bone primordium during calvaria development. Conventional *Msx1* knockout mice with homeodomain disruption resulting in a CNC cell proliferation defect exhibited pleiotropic effects during organogenesis, such as cleft secondary palate leading to death shortly after birth, a mandibular and maxillary alveolar deficiency, and the failure of tooth development [5,6].

Msx family members are known to be required for osteogenesis in the frontal bone primordium in the CNC lineage [6]. Research has focused on Msx2, with the use of knockout or overexpressing transgenic mice [7–10], as *MSX2* mutations in humans affect the cranial sutures, leading to craniosynostosis [11], and viable *Msx2* null mutants exhibit clear skeletal defects with decreased expression of *Runx2*, *Alp*, *Bsp*, *Osc*, and *Pth1r* in long bones [8]. Because *Msx1* null mutants die soon after birth with the developmental arrest of tooth and palate formation [3,12], examination of the

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https://doi.org/10.1016/j.bbrc.2020.03.068

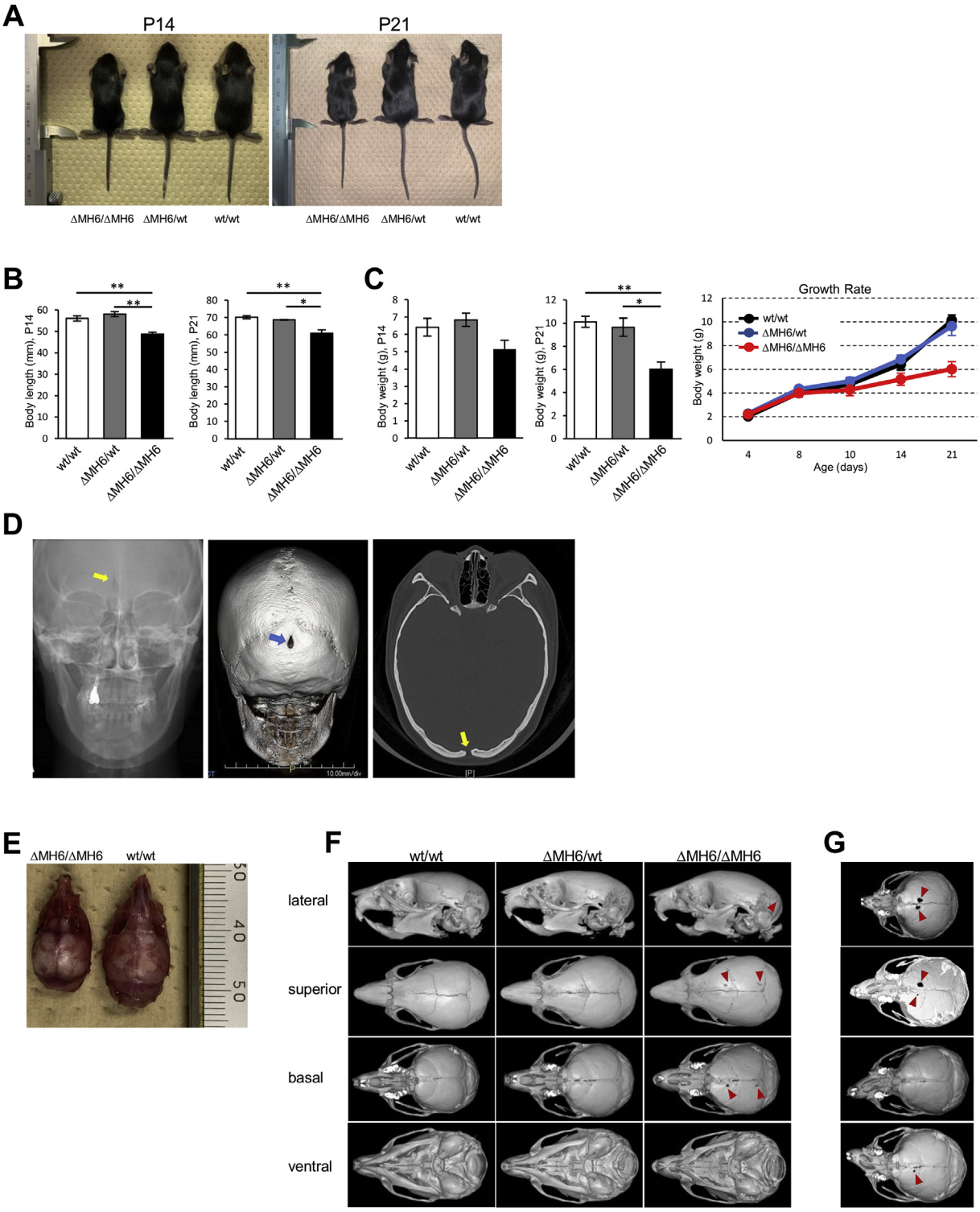

Fig. 1. Impaired postnatal growth in mice lacking the Msx1 MH6 domain. (A–C) Mice lacking the Msx1 MH6 domain ($Msx1^{\Delta MH6/\Delta MH6}$) had significantly lesser body heights and weights compared with wild-type and $Msx1^{\Delta MH6/wt}$ mice, with no difference between wild-type and $Msx1^{\Delta MH6/wt}$ mice, at P21 ($n = 3$ each). *$P < 0.05$, **$P < 0.01$. (D) Posteroanterior cephalometric radiograph, occipital CT view, and bone CT image at the level of the foramen in a patient with oligodontia and Msx1 MH6 disruption. Arrows indicate the bony defect on the occipital bone. (E) The anteroposterior cranial diameter was reduced in $Msx1^{\Delta MH6/\Delta MH6}$ mice compared with the wild type. (F) μCT revealed morphological differences in the lateral, superior, and basal maxilla and ventral cranium between $Msx1^{\Delta MH6/\Delta MH6}$ and wild-type mice at P21. $Msx1^{\Delta MH6/\Delta MH6}$ mice showed foreshortening of the maxilla, slight hypoplasia of the mandible, and small foramens in the cranium (arrowheads). (G) Examples of nasal deviation observed in $Msx1^{\Delta MH6/\Delta MH6}$ mice.

effect of MSX1 on postnatal bone development has been difficult.

Msx1 expressed in the frontal bone primordium plays an essential role in the regulation of osteogenesis during craniofacial development [6]. Abnormalities of the nasal, frontal, and parietal bones, and of the malleus in the middle ear, have been observed in conventional *Msx1* knockout mice [3,12]. Moreover, conditional Msx1 inactivation in the neural crest, from which bone derives, increased frontal foramen size [13].

The MSX1 protein has seven ancient and highly conserved Msx homology (MH) domains that act as binding or functional domains [14]. The homeodomain corresponds to the MH4 domain, and the MH6 domain is located at the farthest extent of the C-terminal end of MSX1. MH6 has been reported to be a requisite domain responsible for the enrichment of polycomb-directed trimethylation of lysine 27 on histone H3 (H3K27me3) to the nuclear periphery for transcriptional repression in myoblasts [15]. We previously reported a heterozygous frameshift mutation disrupting the MH6 domain in affected members of a family with oligodontia [16], and performed target disruption of the MH6 domain of MSX1 in mice ($Msx1^{\Delta MH6/\Delta MH6}$) using genome editing technology. It has enabled *in vivo* functional analysis of individual domains [17], and demonstrated agenesis of the lower incisors with or without cleft palate at embryonic day (E) 16.5 [16]. Agenesis of the upper third molars and the lower second and third molars was observed in 4-week-old $Msx1^{\Delta MH6/\Delta MH6}$ mice, suggesting the importance of the MH6 domain for tooth and palate formation during embryogenesis [16]. Unexpectedly, we observed lesser body lengths in $Msx1^{\Delta MH6/\Delta MH6}$ mice compared with wild-type mice. In addition, a partial defect in the occipital bone had been detected in childhood in the human patient with oligodontia on whom we reported [16]. Because Msx1 has been reported to be expressed in bone even after birth [18], it may contribute to postnatal bone development and its MH6 domain may have an important role in this function.

To test this hypothesis, we analyzed the *in vivo* relevance of the MH6 domain for cranial and long bone development using its deletion mutant. Integrative morphological, histochemical, and quantitative gene expression analyses suggested critical roles of the MH6 domain of the MSX1 protein in postnatal bone physiology.

2. Materials and methods

2.1. Ethical considerations

The Animal Research Ethics Committee of Tokushima University approved all animal experiments conducted in this study (no. 2019-85, T30-122). All animal procedures were conducted in accordance with the Guidelines for Animal Experiments of Tokushima University. The human study was approved by the ethics committee of Tokushima University Hospital, and all investigations were conducted in accordance with the tenets of the Declaration of Helsinki.

2.2. Mouse model and genotyping

The CRISPR/Cas system was applied to generate mutant mice. The protocol has been described in detail elsewhere [16]. Guide RNAs (gRNAs) were synthesized *in vitro* and then co-injected with CRISPR-associated protein 9 messenger RNA into single-cell zygotes. The resulting mosaic mutant was mated with wild-type BDF1 mice. For heterozygous F_1 genotyping, purified PCR amplicons were cloned into plasmids using DynaExpress TA PCR Cloning Kits (BioDynamics Laboratory, Tokyo, Japan). Isolated plasmids from each sample were sequenced with a BigDye terminator sequencing kit (ver. 3.1; Applied Biosystems, Foster City, CA, USA) and an ABI 3500xL genetic analyzer (Applied Biosystems). The mice were maintained in a specific pathogen–free animal facility with food and water provided *ad libitum*. Embryos and mice in the third to fifth generations onward were used for the analysis.

The nucleic and amino acid sequences of $Msx1^{\Delta MH6}$ are represented as those of a frameshift 28-nucleotide deletion (Msx^{-28}) generating a premature stop codon upstream of the MH6 domain (Supplementary Fig. S1), as reported previously [16]. Homozygous mutant ($Msx1^{\Delta MH6/\Delta MH6}$) mice were generated by intercrosses between heterozygous mutant mice ($Msx1^{\Delta MH6/wt}$) with normal palate and tooth development.

The use of gRNAs that simultaneously targeted the upstream and middle regions of MH6 yielded a mosaic mutant (F_0) including a larger-than-expected deletion (ΔMH4–6), which lacked part of the homeodomain (Supplementary Fig. S1). F_1 Msx1 homeodomain-disrupted heterozygous mutant ($Msx1^{\Delta MH4-6/wt}$) mice were generated by mating of F_0 mice with wild-type BDF1 mice to propagate alleles of interest. Homozygous mutant ($Msx1^{\Delta MH4-6/\Delta MH4-6}$) mice were generated by intercrosses between heterozygous mutant mice.

2.3. Micro–computed tomographic analysis

The femurs and skulls from E18.5 embryos ($n = 3$) and 21-day-old male mice ($n = 3$) were carefully harvested and fixed overnight with 70% ethanol, then analyzed using high-resolution micro–computed tomography (μCT; SkyScan 1176 scanner and software; Bruker, Billerica, MA, USA). Images for analysis were acquired at 50 kV and 200 μA, with the samples covered with cotton containing 70% ethanol to prevent movement and dehydration. Then, cross-sectional images were reconstructed and visualized in three dimensions with SkyScan software. For the skull, lateral, superior, basal maxilla and ventral cranium, and mandibular views were provided. For the femur, the region of interest was set directly under the distal growth plate. Microstructural parameters examined were the bone volume, bone volume to trabecular volume ratio, trabecular number, trabecular thickness, and trabecular separation.

2.4. Bone preparation and histological staining

For histological analysis, femurs from 21-day-old male mice ($n = 3$) were isolated and fixed in 4% paraformaldehyde/phosphate-buffered saline overnight at room temperature. The bones were then rinsed several times in water and decalcified with formic acid for 3 days. After dehydration, the samples were embedded in paraffin, sliced 5 μm thick, and stained with hematoxylin and eosin (H&E) for histological assessment and with toluidine blue for proteoglycan visualization.

2.5. Skeletal double-staining

The eyes, skin, internal organs, and adipose tissue were removed from E18.5 embryos ($n = 3$) and fixed in 95% ethanol for 24 h. The skeletal specimens were placed in acetone for 48 h at room temperature to remove residual adipose tissue. They were then double-stained at room temperature with 0.03% w/v Alcian blue stain solution (Nacalai Tesque Inc., Kyoto, Japan) and 0.005% w/v Alizarin red S (Wako Pure Chemical Industries, Osaka, Japan) in a 1:4 mixture of acetic acid and 80% ethanol. The stained skeletons were washed with distilled water and immersed in 50% glycerol:50% (1%) KOH solution for 1 week, then cleaned in increasing concentrations (to 100%) of glycerol over a 1-week period.

2.6. Gene expression analysis

Immediately after isolation of the distal femoral epiphyses,

including the growth plates, perichondria, and trabecular bone, from 14-day-old male mice ($n = 3$), the tissues were frozen individually in liquid nitrogen. The samples were pulverized in a mortar with the Isogen II kit (Nippon Gene, Tokyo, Japan), and total RNA was then isolated according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The expression levels related to osteogenes is and cartilage formation were measured with a real-time quantitative polymerase chain reaction (RT-qPCR) system (model 7500; Applied Biosystems). The *Alp*, *Bsp*, *Col1*, *Oc*, *Opn*, *Osx*, and *Runx2* genes were evaluated as osteoblast differentiation markers, whereas *Acan*, *Col10a*, *Fgf3*, *Ihh*, *Mmp9*, *Pth1r*, and *Sox9* were evaluated as chondrocyte differentiation markers. A gene encoding *18S* ribosomal RNA was used as an internal reference. The primers used are listed in Supplementary Table 1.

2.7. Radiographic examination of a patient with oligodontia

Posteroanterior cephalometric radiographs and cranial CT images were acquired from a 16-year-old Japanese female patient with a heterozygous defect of the Msx1 MH6 domain reported by Mitsui et al. [18], for orthognathic surgical treatment planning.

2.8. Statistical analysis

Each experiment was repeated at least three times independently for each set of conditions. The data are expressed as means ± standard deviations (SDs). Data were analyzed using two-tailed Student's *t* tests or one-way analysis of variance followed by Turkey's multiple comparison test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Lack of the Msx1 MH6 domain caused postnatal growth disorder

One-third of the newborn $Msx1^{\Delta MH6/\Delta MH6}$ mice exhibited thin palate development and were viable; others of these mice had secondary cleft palate and died neonatally. Thus, the phenotypes of $Msx1^{\Delta MH6/\Delta MH6}$ mice with lower incisor agenesis and thin palates were analyzed. No obvious difference in body size was observed in the first week after birth, but $Msx1^{\Delta MH6/\Delta MH6}$ mice had significantly lesser body heights and weights compared with wild-type and $Msx1^{\Delta MH6/wt}$ mice on postnatal day (P) 21 (Fig. 1A–C). The patient with oligodontia and an Msx1 MH6 domain defect reported in previous study [18], had a foramen in the occipital bone (Fig. 1D). $Msx1^{\Delta MH6/\Delta MH6}$ mice had smaller anteroposterior cranial diameters than did $Msx1^{\Delta MH6/wt}$ and wild-type mice at P21 (Fig. 1E). In addition, 50% of $Msx1^{\Delta MH6/\Delta MH6}$ mice exhibited nasal curvature and 75% had small foramens near the cranial suture (Fig. 1F and G).

3.2. Effects of Msx1 MH6 domain deficiency on prenatal bone morphogenesis

It was reported that the conventional *Msx1* knockout mice show several disorders in cranial development, such as delayed closure of the frontal foramen, loss of the mandibular alveolar ridge, and absence of the short process of the malleus, at P0 [3,6,12]. μCT revealed delayed closure of the frontal foramen in $Msx1^{\Delta MH6/\Delta MH6}$ embryos. Although these embryos did not exhibit mandibular

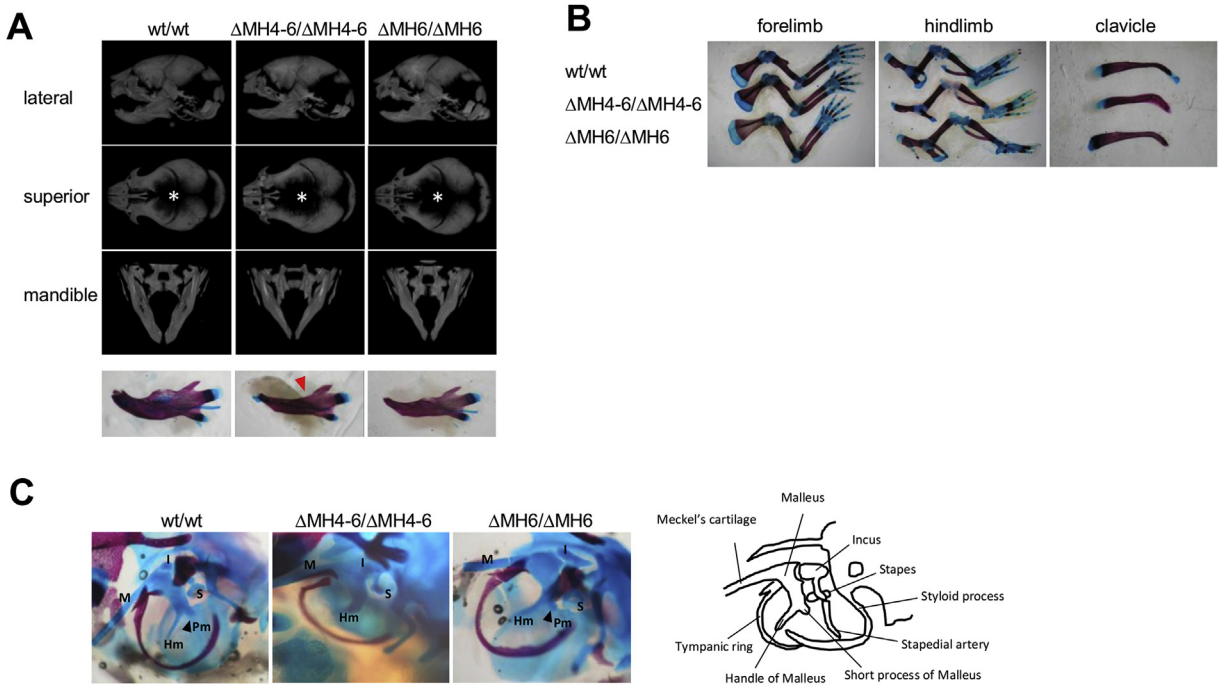

Fig. 2. Defects of skeletal development in $Msx1^{\Delta MH6/\Delta MH6}$ mice. (A) Lateral and superior views of the cranium and view of the mandible obtained by μCT, and lateral views of dissected mandibles stained with Alcian blue and Alizarin red, showing morphological differences among wild-type, $Msx1^{\Delta MH4-6/\Delta MH4-6}$, and $Msx1^{\Delta MH6/\Delta MH6}$ embryo skulls at E18.5. $Msx1^{\Delta MH6/\Delta MH6}$ embryos show foreshortening of the maxilla and slight hypoplasia of the mandible relative to wild-type embryos. The space between the frontal and parietal bones (asterisks) is larger in $Msx1^{\Delta MH6/\Delta MH6}$ embryos than in wild-type embryos, but not as large as in $Msx1^{\Delta MH4-6/\Delta MH4-6}$ embryos. The alveolar ridge is absent in $Msx1^{\Delta MH4-6/\Delta MH4-6}$ embryos (arrowhead), but not in $Msx1^{\Delta MH6/\Delta MH6}$ embryos. Overall mandibular length is decreased in $Msx1^{\Delta MH6/\Delta MH6}$ embryos relative to the wild type, but not as short as in $Msx1^{\Delta MH4-6/\Delta MH4-6}$ embryos. (B) Dissected forelimb, hindlimb, and clavicle specimens from E18.5 mice showing no significant difference in shape or size among embryos. (C) Stained ossicles from E18.5 mice and a schematic representation. The short process (processus brevis) of the malleus in the middle ear is absent in $Msx1^{\Delta MH4-6/\Delta MH4-6}$, but not $Msx1^{\Delta MH6/\Delta MH6}$ embryos (arrowheads). Hm; handle of malleus; I, incus; Pm, process of short malleus; M, Meckel's cartilage; S, stapes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

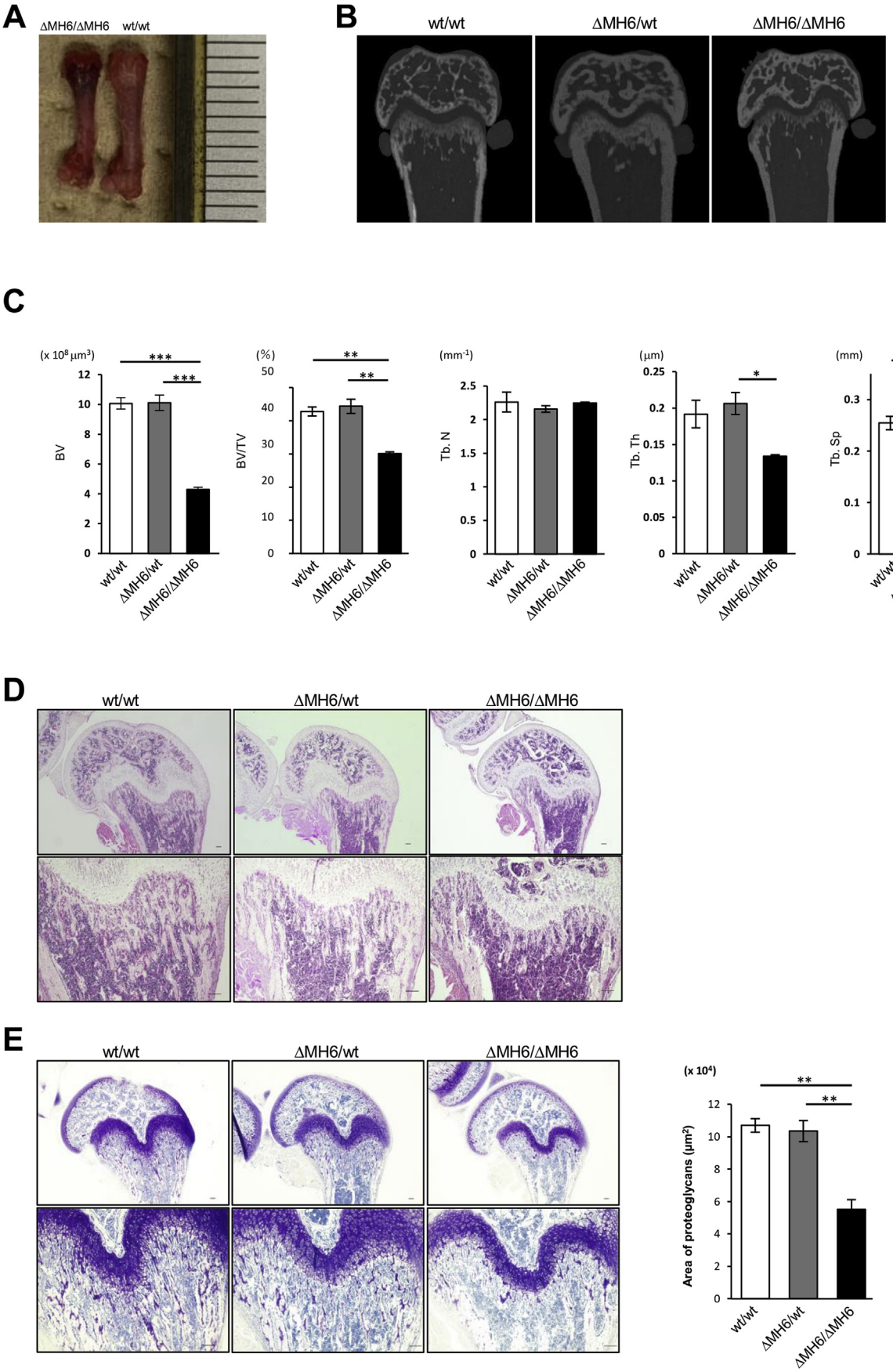
A
ΔMH6/ΔMH6
wt/wt
B
wt/wt
ΔMH6/wt
ΔMH6/ΔMH6
C
(x 10⁸ μm³)
BV
(%)
BV/TV
(mm⁻¹)
Tb. N
(μm)
Tb. Th
(mm)
Tb. Sp
wt/wt
ΔMH6/wt
ΔMH6/ΔMH6
D
wt/wt
ΔMH6/wt
ΔMH6/ΔMH6
E
wt/wt
ΔMH6/wt
ΔMH6/ΔMH6
(x 10⁴)
Area of proteoglycans (μm²)

Fig. 3. The Msx1 MH6 domain affects postnatal long bone development. (A) Reduced femur length in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice compared with the wild type. (B) μCT images of distal femur diaphyses from 21-day-old wild-type, *Msx1*$^{\Delta MH6/wt}$, and *Msx1*$^{\Delta MH6/\Delta MH6}$ mice ($n = 3$ each). (C) Quantification of bone volume (BV), the bone volume/trabecular volume ratio (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular spacing (Tb. Sp). of distal femur diaphyses from 21-day-old wild-type, *Msx1*$^{\Delta MH6/wt}$, and *Msx1*$^{\Delta MH6/\Delta MH6}$ mice ($n = 3$ each). *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$(D) Histomorphometric analysis of *Msx1*$^{\Delta MH6/\Delta MH6}$ long bones revealed a shorter growth plate, shorter primary spongiosa, and reduction of bone trabeculae. Lower panels are higher-magnification excerpts from upper panels. Scale bars = 100 μm. (E) Toluidine blue staining showing narrower and sparse growth plate cartilage, and decreased proteoglycan content in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice. Lower panels are higher-magnification excerpts from upper panels. Proteoglycan areas in wild-type, *Msx1*$^{\Delta MH6/wt}$, and *Msx1*$^{\Delta MH6/\Delta MH6}$ mice. Scale bars = 100 μm **$P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

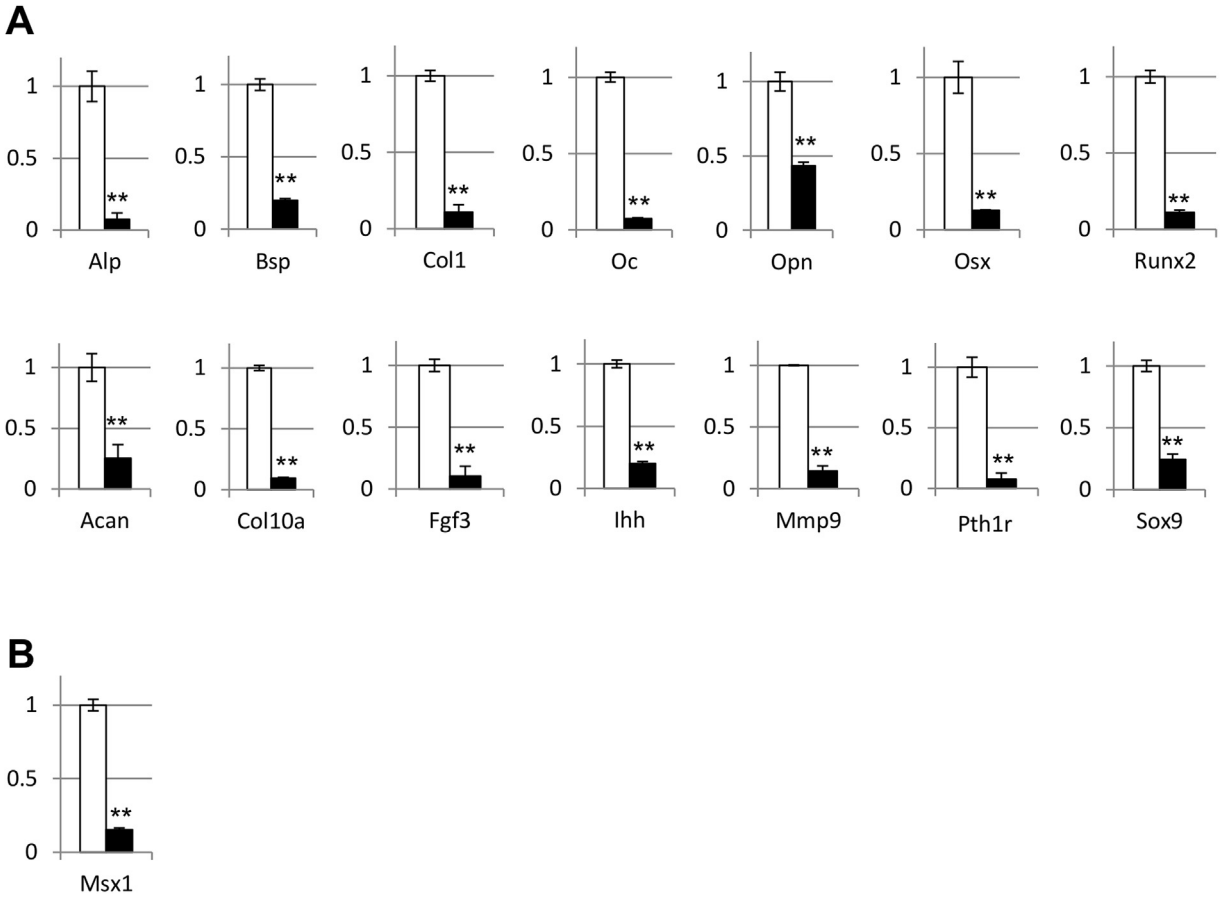

Fig. 4. Deletion of the Msx1 MH6 domain alters gene expression. (A) mRNA expression of genes involved in osteogenesis and chondrogenesis (14-day-old mice, $n = 3$). Dysregulation is apparent, with underexpression of the genes examined in *Msx1*$^{\Delta MH6/\Delta MH6}$ distal femoral epiphyses (black columns) compared with the control wild type (white columns). **$P < 0.01$. (B) Msx1 mRNA expression was also decreased in *Msx1*$^{\Delta MH6/\Delta MH6}$ distal femoral epiphyses (black columns). **$P < 0.01$.

alveolar ridge deficiency on skeletal staining, perhaps because of the presence of the first molar, their mandibles were hypoplastic and reduced in size compared with those of wild-type mice (Fig. 2A). No obvious difference in forelimb, hindlimb, or clavicle size was observed among wild-type, *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$, and *Msx1*$^{\Delta MH6/\Delta MH6}$ embryos at E18.5 (Fig. 2B). Similar to previously reported conventional *Msx1* knockout mice [12], the short process of the malleus in the middle ear was absent in *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$ embryos, whereas all structures, including the incus, stapes, and malleus, were normal in *Msx1*$^{\Delta MH6/\Delta MH6}$ embryos (Fig. 2C).

3.3. The Msx1 MH6 domain affects postnatal bone development

Msx1$^{\Delta MH6/\Delta MH6}$ mice showed a lesser growth rate (as determined by body length) than did wild-type and *Msx1*$^{\Delta MH6/wt}$ mice at P14 and P21, suggesting abnormal bone growth after birth. Therefore, we then performed the morphometric analysis for long bone. Although morphometric analysis determined that femur shape was normal at P21, μCT analysis of the distal femur revealed significant reductions in bone volume and bone density (Fig. 3A and B). Trabecular separation was also significantly increased and trabecular thickness was tended to be decreased in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice, but the trabecular number did not differ among wild-type, *Msx1*$^{\Delta MH6/wt}$, and *Msx1*$^{\Delta MH6/\Delta MH6}$ mice (Fig. 3C). Consistent with the μCT findings, the H&E-stained histological femur sections from *Msx1*$^{\Delta MH6/\Delta MH6}$ mice showed reduced cancellous bone density under the growth plate at P21 (Fig. 3D). The thickness of the growth plate cartilage was also significantly reduced in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice (Fig. 3D). Toluidine blue staining, which was carried out for further analysis of abnormal cartilage and endochondral bone formation, revealed the reduction of the chondrocyte layer and endochondral ossification in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice compared with wild-type and *Msx1*$^{\Delta MH6/wt}$ mice (Fig. 3E). The amount of proteoglycan was also decreased in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice (Fig. 3E). Overall comparison of the femurs revealed more hypoplasia in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice than in wild-type mice.

3.4. The Msx1 MH6 domain is necessary for osteoblast/chondrocyte differentiation

Finally, we confirmed gene expression levels related to osteogenesis and cartilage formation in microdissected distal femoral epiphyses from P14 mice. The expression levels of all genes related to osteoblast and chondrocyte differentiation were significantly lower in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice than in wild-type mice (Fig. 4A), suggesting that the Msx1 MH6 domain is necessary for osteoblast/chondrocyte differentiation. *Msx1* mRNA expression was also decreased in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice (Fig. 4B), suggesting that the Msx1 MH6 domain contributes to the autoregulatory activation of *Msx1* transcription.

4. Discussion

In the present study, we evaluated the *in vivo* function of the MH6 domain of Msx1 using *Msx1*$^{\Delta MH6/\Delta MH6}$ mice lacking the MH6 domain in the most C-terminal region. *Msx1*$^{\Delta MH6/\Delta MH6}$ mice showed abnormal cranial size and shape, with small foramens near the cranial suture, as well as reduced femoral bone density and reduced expression of osteogenic/chondrogenic marker genes compared with wild-type mice. Our findings suggest the importance of the MH6 domain for postnatal bone physiology and the efficacy of genome editing technology for *in vivo* functional domain analysis.

Msx1 controls the proliferation and differentiation of osteoprogenitor cells during bone development [19]. Although *Msx1*$^{\Delta MH6/\Delta MH6}$ mice had cranial developmental defects similar to those of conventional *Msx1* null mice [12] and homeodomain-disrupted *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$ mice at E18.5 in this study, *Msx1*$^{\Delta MH6/\Delta MH6}$ mice showed less enlargement of the space between the frontal and parietal bones and less mandibular hypoplasia compared with *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$ mice. Loss of the short process of the malleus in the inner ear was observed in *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$ mice, but not in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice, suggesting that the MH6 domain of Msx1 is not necessary for the formation of this process. Distinct expression of Msx1 was reported at the level of the periosteal surface of the developing skull bones mainly on suture part at P14 [18]. Fontanelles were closed at P21, even in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice, although these mice had several small foramens along the cranial suture. In addition, a small foramen was observed in the patient with heterozygous Msx1 MH6 domain disruption on a cephalometric radiograph, implying that the lack of the MH6 domain of MSX1 contributes to the formation of such foramens. *Msx1*$^{\Delta MH6/\Delta MH6}$ mice were short in stature at P14 and P21, although the long bone length was not changed at E18.5 compared with wild-type mice. Clear differences in body length and weight between *Msx1*$^{\Delta MH6/\Delta MH6}$ mice and wild-type or *Msx1*$^{\Delta MH6/wt}$ mice became apparent before weaning, indicating that the growth retardation observed in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice was not caused by an eating disorder caused by lower incisor agenesis. Premature ossification was observed in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice with downregulation of osteogenic/chondrogenic marker genes in femur at postnatal stages. As Msx1 is expressed in epiphyseal growth plate cartilage even after birth [18], the Msx1 MH6 domain is likely to have crucial roles in regulating postnatal skeletal development. *Msx1*$^{\Delta MH6/\Delta MH6}$ mice also showed nasal deviation, which is occasionally observed in murine models for systemic bone diseases [20,21]. These findings suggest that the MH6 domain is necessary for postnatal cranial and long bone development regulated by Msx1.

Microdissected distal femoral epiphyses from P14 *Msx1*$^{\Delta MH6/\Delta MH6}$ mice showed lesser mRNA expression of osteoblast and chondrocyte differentiation markers compared with those from P14 wild-type mice. These findings indicate the functional significance of Msx1 in postnatal bone development, and the contribution of the C-terminal MH6 domain to this process. As previous studies have shown that MSX proteins control gene transcription predominantly through repression mechanisms [15], whether MSX1-induced transcriptional activation of osteoblast and chondrocyte differentiation markers is direct or indirect remains unclear. A previous study revealed H3K27me3 enrichment at the nuclear periphery, where MSX1 is expressed, in the developing forelimbs of wild-type mice, but not in those of *Msx1* knockout mice [15]. Moreover, MSX1 lacking the MH6 domain was reported to not co-localize to the nuclear periphery with H3K27me3 due to loss of binding to Ezh2, the functional enzymatic component of the polycomb repressive complex 2 responsible for embryonic development [15]. Thus, the C-terminal region of Msx1 containing MH6 may be necessary for efficient association of MSX1 with histone methyltransferase activity and Ezh2 to repress target genes. On the other hand, recent gene expression studies using gain-of-function and loss-of-function mutants revealed that MSX proteins also can activate transcription of various target genes, possibly mediated by the MSX C-terminal region including the MH6 domain [22]. Notably, strong conservation of a 26-amino-acid sequence in the C-terminus, which includes the MH6 domain, was observed in vertebrate MSX1 and MSX2 proteins [16,22]. Regardless of transcriptional repression and activation activity, therefore, the MSX1 C-terminus appears to regulate transcription at various developmental stages. As we observed a more severe phenotype in our *Msx1*$^{\Delta MH4-6/\Delta MH4-6}$ mice than in *Msx1*$^{\Delta MH6/\Delta MH6}$ mice, the homeodomain may contribute to the magnitude of regulation determined by the MH6 domain. Further studies are required to clarify the mechanisms of the Msx1 MH6 domain's transcriptional regulation for each marker during bone development.

Authors' contributions

A.I., A.Y., and S.M. designed the research. A.I., A.Y., and D.A. performed the research. A.I, A.Y., Y.M., S.O., I.I., and E.T. analyzed the data. A.I., A.Y., and I.I. wrote the paper.

Declaration of competing interest

The authors declare that they have no competing financial interest.

Acknowledgements

This work was supported by JSPS KAKENHI (grant nos. 25463184 and 16H05551 to A.Y., 23390325 and 25560368 to I.I., and 26293436 to E.T.), and the Practical Research Project for Rare/Intractable Diseases of the Japan Agency for Medical Research and Development (AMED) (to Y.M.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.03.068.

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