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MMP-13 is one of the critical mediators of the effect of HDAC4 deletion on the skeleton

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Abstract

Histone deacetylase 4 (Hdac4) regulates chondrocyte hypertrophy. *Hdac4*^{-/-} mice are runted in size and do not survive to weaning. This phenotype is primarily due to the acceleration of onset of chondrocyte hypertrophy and, as a consequence, inappropriate endochondral mineralization. Previously, we reported that Hdac4 is a repressor of matrix metalloproteinase-13 (Mmp13) transcription, and the absence of Hdac4 leads to increased expression of MMP-13 both *in vitro* (osteoblastic cells) and *in vivo* (hypertrophic chondrocytes and trabecular osteoblasts). MMP-13 is thought to be involved in endochondral ossification and bone remodeling. To identify whether the phenotype of *Hdac4*^{-/-} mice is due to up-regulation of MMP-13, we generated *Hdac4/Mmp13* double knockout mice and determined the ability of deletion of MMP-13 to rescue the *Hdac4*^{-/-} mouse phenotype. *Mmp13*^{-/-} mice have normal body size. *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice are significantly heavier and larger than *Hdac4*^{-/-} mice, they survive longer, and they recover the thickness of their growth plate zones. In *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice, alkaline phosphatase (ALP) expression and TRAP-positive osteoclasts were restored (together with an increase in *Mmp9* expression) but osteocalcin (OCN) was not. Micro-CT analysis of the tibiae revealed that *Hdac4*^{-/-} mice have significantly decreased cortical bone area compared with the wild type mice. In addition, the bone architectural parameter, bone porosity, was significantly decreased in *Hdac4*^{-/-} mice. *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice recover these cortical parameters. Likewise, *Hdac4*^{-/-} mice exhibit significantly increased Tb.Th and bone mineral density (BMD) while the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice significantly recovered these parameters toward normal for this age. Taken together, our findings indicate that the phenotype seen in the *Hdac4*^{-/-} mice is partially derived from elevation in MMP-13 and may be due to a bone remodeling disorder caused by overexpression of this enzyme.

Keywords

Mmp13; Hdac4; *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout; cortical bone

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1. Introduction

Histone deacetylase 4 (Hdac4) is a member of the class IIa HDACs and plays a central role in the formation of the skeleton [1, 2]. Hdac4 is expressed in prehypertrophic chondrocytes, and mice with global deletion of HDAC4 are significantly smaller than wild type mice and die during the first week of life. This is mainly due to ectopic ossification of endochondral cartilage, which prevents expansion of the rib cage and leads to an inability to breathe [2]. The formation of cartilaginous templates of endochondral bones occurs normally in the *Hdac4*^{-/-} mice, but the onset of chondrocyte hypertrophy is accelerated and, consequently, endochondral mineralization occurs precociously, leading to ectopic bone formation. [2].

Chondrocyte hypertrophy is the final step in chondrocyte differentiation, in which the cartilage extracellular matrix (ECM) becomes calcified and partially degraded. Ossification begins when hypertrophic chondrocytes undergo programmed cell death and the calcified cartilage is invaded by blood vessels, osteoblasts, osteoclasts and mesenchymal precursors and forms primary ossification centers. Within these centers, the hypertrophic cartilage matrix is degraded, the hypertrophic chondrocytes die, and bone replaces the disappearing cartilage. Studies have shown that chondrocyte apoptosis does not lead to endochondral ossification [3]. Angiogenesis has been implicated as a crucial step in endochondral ossification [4–6], and degradation and remodeling of the cartilage matrix is essential for vascular invasion.

Matrix metalloproteinase 13 (Mmp13, also called collagenase-3) plays an important role in the degradation of components of the cartilage ECM. MMP-13 is expressed in hypertrophic chondrocytes and osteoblasts and promotes the removal of hypertrophic cartilage from the growth plate and the remodeling of newly deposited trabecular bone during long bone development [7,8]. It degrades collagen type II efficiently but also collagens type I, III, and X, which are the major components of cartilage and bone [9]. It has been shown that MMP-13 acts directly during the initial stages of cartilage ECM degradation (the rate-limiting process for chondrocyte programmed cell death) and during angiogenesis prior to invasion by blood vessels and osteoclasts [8]. A mutation in the human *Mmp-13* gene causes the Missouri variant of spondyloepimetaphyseal dysplasia (SEMD), a syndrome with abnormalities in development and growth of endochondral skeletal elements [10]. *Mmp-13* null mice show no overt phenotypic abnormalities, they are fertile and have a normal lifespan but show abnormal growth plates and delayed ossification [7, 8]. Clinical investigation has shown that patients with articular cartilage destruction have high Mmp-13 expression [11, 12], suggesting that increased Mmp-13 may be associated with cartilage degradation. Studies have also shown that Mmp-13-overexpressing transgenic mice develop a spontaneous osteoarthritis (OA)-like articular cartilage destruction phenotype [5].

Recently, our laboratory showed that Hdac4 represses Mmp13 expression through inhibiting the activity of Runx2, and that *Hdac4*^{-/-} mice displayed increased Mmp-13 mRNA and protein levels in hypertrophic chondrocytes and trabecular bone [13]. In the present study, we generated double knockout mice (*Hdac4*^{-/-}/*Mmp13*^{-/-}) to determine whether the elevation of MMP-13 contributes to the phenotype of *Hdac4*^{-/-} mice.

2. Materials and methods

2.1. Mice

Breeding pairs of heterozygous *Hdac4* mice (*Hdac4*^{+/-}) on a C57BL/6 background were generated as described [1]. These heterozygous mice have no phenotypic abnormalities as described previously in [2]. Heterozygous or homozygous *Mmp13*-deficient mice (*Mmp13*^{-/-}) mice on a C57BL/6 background were a kind gift of Dr. Jeanine D'Armiento (Columbia University College of Physicians and Surgeons, New York). We characterized 5 and 8-day-old male and female mice, wild type, *Hdac4*^{-/-}, *Mmp-13*^{-/-} and *Hdac4*^{-/-}/*Mmp-13*^{-/-}. All mice were housed under standard conditions with a 12-hour light/12-hour dark cycle and free access to water and chow. All the experiments using mice were performed following protocols approved by NYU Institutional Animal Care and Use Committee (IACUC).

2.2 Histological Analysis

Tissues were fixed in 10% buffered formalin at 4°C overnight. The samples were then decalcified in 10% EDTA. Formalin-fixed paraffin-embedded femurs and tibiae were cut as 8 µm sections, and stained with hematoxylin and eosin. TRAP staining was performed using the Acid Phosphatase Leukocyte kit (Sigma, St. Louis, MO) following the manufacturer's protocol after deparaffinization and acetate buffer washing. Immunohistochemical analysis of markers for bone formation, alkaline phosphatase, ALP (Abcam) and osteocalcin (Abcam), and a marker of hypertrophic chondrocytes, Type X collagen (COSMO BIO Co., LTD) were carried out with similar sections. Briefly, deparaffinized and hydrated sections were incubated with Proteinase K Solution (20 µg/ml in TE Buffer, pH 8.0) for 15 minutes in a water bath at 37°C. After cooling, the sections were rinsed with PBS, the endogenous peroxidase was removed by incubating sections for 15 minutes at room temperature in 3% H₂O₂ in methanol. After rinsing sections in a BSA solution (3% in PBS), blocking for 1 hour at room temperature (BSA solution containing 1% Triton and 5% FBS) was performed. Primary antibody diluted in PBS with 3% BSA (ALP 1:50, osteocalcin 1:100, α1(X) collagen 1:100) was incubated overnight at 4°C in a humidifying chamber. After three washes in PBS-BSA solution, secondary goat anti-rabbit peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology) was incubated with the sections for 1 hour at room temperature. Sections were developed using Fast 3'3'-Diaminobenzidine (Sigma-Aldrich), prepared following the manufacturer's protocol and, after washing with PBS, counterstained with hematoxylin (Sigma-Aldrich) for 1 minute. Slides were mounted using Permount mounting medium (Thermo Scientific). The staining of surface osteoclasts was quantified using BIOQUANT OSTEO 2015.

2.3 Micro-computed tomography (µCT)

The tibiae and femurs of all four genotypes and pooled sexes (8 day old mice) were fixed in 70% ethanol and prepared for high-resolution µCT (SkyScan 1172). Images were obtained using the following parameters: 40 Kv, 250 uA, pixel size of 8.5 microns, 2000×1332 matrix, 6 averages and a 0.5 mm aluminum filter. Images were reconstructed using a thresholding of 0–0.09, beam hardening correction of 35, ring artefact correction of 7, and Gaussian smoothing (factor=1). From the reconstructed micro-CT images, a 200 micron

volume correlating to 25 slices of the mid-diaphysis that began immediately distal to the third trochanter was examined for total cortical cross-sectional bone area (T.Ar), cross-sectional marrow area (Ma.Ar), cortical bone (tissue) area (B.Ar), polar moment of inertia (MMI) and cortical thickness (Ct.Th). Relative cortical area (RCA) was defined as B.Ar/T.Ar and represented the relative amount of bone tissue in a given bone area. From the reconstructed images of the distal metaphysis, a volume of 637 micron immediately proximal to the growth plate was examined for trabecular bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing (Tb.Sp). BMD was calculated as the ratio of bone mineral content (BMC, mg) to the total volume of distal metaphysis analyzed after removing (masking) marrow space grayscale variability.

2.4 Quantitative real-time RT-PCR

Total RNA was obtained from femurs and tibiae using TRIzol reagent (Invitrogen). cDNA was synthesized from 0.1 µg of total RNA using a TaqMan reverse transcription kit (Applied Biosystems) with hexamer primers following the protocol described by the manufacturers. Gene expression levels were measured using SYBR Green PCR Reagent (Applied Biosystems). Primer pairs used for quantitative detection of gene expression are listed in Table 1. The quantity of mRNA was calculated by normalizing the Ct (threshold cycle value) of specific genes to the Ct of the housekeeping gene β -actin.

2.5. Statistical analysis

Statistical differences were determined using Student's *t* test for survival data of comparison between *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice. Other mean differences were analyzed by two-way ANOVA using IBM SPSS (v22, Armonk, NY). Results are expressed as mean $\pm$ SE and a *p* < 0.05 was considered significant comparing each of the groups.

3. Results

3.1 Mmp13 ablation in HDAC4^{-/-} mice increased their body weight, length and survival rate

To investigate the contribution of MMP-13 elevation to the premature phenotype seen in the *Hdac4*^{-/-} mice, we generated double knockout mice (*Hdac4*^{-/-}/*Mmp13*^{-/-}). *Hdac4*^{-/-} mice were severely runted (Fig. 1A, B) and died during the first week of life (Fig. 1C). *Mmp13*^{-/-} mice showed no gross phenotypic abnormalities (Fig. 1A), were fertile and had a normal lifespan. The *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice were bigger, heavier, and survived longer when compared with *Hdac4*^{-/-} mice (Fig. 1). These results indicate that *Hdac4*^{-/-} mice lacking Mmp13 exhibit partial recovery of their phenotype.

3.2. Mmp13 ablation in HDAC4^{-/-} mice partially restored the skeletal cellularity phenotype of Hdac4^{-/-} mice

Hematoxylin and eosin staining was performed on sections of tibiae isolated from wild type, *Mmp13*^{-/-}, *Hdac4*^{-/-}, and *Hdac4*^{-/-}/*Mmp13*^{-/-} male mice at P8. Overall the histological sections showed that *Hdac4*^{-/-} mice have malformed bones with extremely shortened growth plates. On the other hand, *Mmp13*^{-/-} mice showed a lengthened prehypertrophic and hypertrophic chondrocyte zone. Ablation of Mmp13 in the *Hdac4*^{-/-} mice partially but

significantly recovered the thickness of both the hypertrophic and prehypertrophic chondrocyte zones, as well as the morphology of the cartilaginous zone (Fig. 2). Nevertheless, the bones of the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice were still abnormal. Immunohistochemical (IHC) analysis revealed that ALP and osteocalcin (OCN) expression levels, as early and late osteoblastic markers, respectively, were decreased in *Hdac4*^{-/-} mice compared with wild type mice, whereas in comparison with *Hdac4*^{-/-} mice, ALP expression was increased in the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice (Fig. 3). In the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, the expression of ALP mRNA was significantly recovered from the reduced levels seen in the *Hdac4*^{-/-} mice. But OCN did not recover (Fig. 4A, B). IHC showed that the expression of type X collagen (COL10A1), a marker of hypertrophic chondrocytes, was increased in *Mmp13*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice compared with wild type mice (Fig. 3). In comparison with wild type mice, type X collagen mRNA was very highly increased in *Mmp13*^{-/-} mice (Fig. 4D). Next, we determined if other MMP molecules might compensate for Mmp-13 in *Mmp13*^{-/-} or *Hdac4*^{-/-}/*Mmp13*^{-/-} mice. Indeed *Mmp-9* showed higher mRNA levels in the *Mmp13*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, while *Mmp-2* was unchanged (Fig. 5A, B). *Runx2* expression was unchanged but, interestingly, *Mef2c* levels were increased in *Hdac4*^{-/-} mice and returned to the same levels as wild type in the double knockout mice. Indeed, these results suggest that Mmp-13 may be a regulator of Mmp-9 and Mef2c expression (Fig. 5C, D).

The surface osteoclasts, determined by TRAP staining, a marker of differentiated osteoclasts, was significantly decreased in *Mmp13*^{-/-} mice and slightly decreased in *Hdac4*^{-/-} mice compared with wild type mice. In the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, the TRAP positive osteoclasts returned to the same levels as wild type mice (Fig. 6A), indicating a return to relatively normal bone remodeling. In line with these observations, TRAP mRNA abundance was up-regulated in the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice compared with *Hdac4*^{-/-} mice (Fig. 4C). Taken together these results indicate that MMP-13 in the *Hdac4*^{-/-} mice influences bone formation, especially early stages of osteoblast differentiation, and osteoclast recruitment and differentiation.

3.3 *Hdac4*^{-/-} mice have structurally modified cortical and trabecular bone which was partially restored by deletion of *Mmp13*

Micro-CT analysis of cortical and trabecular areas of the tibiae of male and female P8 mice revealed that *Hdac4*^{-/-} mice had significant decreases in almost all cortical parameters, including bone length (Fig. 8, 9). The *Hdac4*^{-/-} mice had a diminution in porosity and polar moment of inertia (MMI; the index of difficulty to bend), suggesting increased bone rigidity compared with wild type mice. Ablation of *Mmp13* in *Hdac4*^{-/-} mice led to an increase in T.Ar and concomitant increase in M.Ar, but no changes in B.Ar, and significantly recovered porosity. Robustness (T.Ar/Length) increased in the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, reflecting the increase in total area. Lack of *Mmp13* in *Hdac4*^{-/-} mice partially normalized radial bone growth. *Mmp13*^{-/-} mice were largely similar to wild type, except for lower cortical thickness and length and greater porosity.

The trabecular bone compartment was assessed for ~ 600 µm below the proximal growth plate of the tibiae. *Hdac4*^{-/-} mice show reduced BV/TV, however their trabeculae are

thicker, likely due to decreased resorption. Trabecular bone shows differences in structural parameters: ablation of *Hdac4* induced increases in Tb.Th. and BMD. With the deletion of *Mmp13* in *Hdac4*^{-/-} mice, these parameters normalized. The degree of anisotropy (DA), reflecting trabecular structure, decreased in *Hdac4*^{-/-} mice, suggesting more isotropic (organized) bone compared to wild type mice but this was not affected by *Mmp13*. In the *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, Tb.Sp. was unchanged compared to *Hdac4*^{-/-} mice; perhaps influenced by an increase in the marrow area (area inside the endosteal perimeter). Taken together, although Tb.Sp. and DA were not changed, the Tb.Th. and BMD were normalized with deletion of *Mmp13* in *Hdac4*^{-/-} mice. Bone surface density (BS/BV) is a useful parameter to characterize the complexity of trabecular structure. Lower BS/BV in *Hdac4*^{-/-} mice suggests more membranous bone than the other genotypes (wild type, *Mmp13*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice). The micro-CT images of *Hdac4*^{-/-} mice (Fig. 7) show that the surface of the cortical bone of these mice is smoother than the wild type and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, suggesting that *Hdac4*^{-/-} mice had more mature (mineralized) bone than wild type mice. Taken together, our data indicate that increases in MMP-13 in *Hdac4*^{-/-} mice affect cortical bone area and architecture and trabecular bone mineral density and thickness.

4. Discussion

Our initial goal was to determine whether elevations in MMP-13 in *Hdac4*^{-/-} mice contribute to their skeletal phenotype. To that end, we generated *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice. *Hdac4*^{-/-} mice display dwarfism and do not survive to weaning. In the present paper, we show that *Hdac4*^{-/-} mice lacking *Mmp13* have improved body weight, length and survival. In our previous study we showed that *Hdac4* ablation in mice resulted in increased *Mmp13* expression and protein levels *in vivo*. Heterozygous *Hdac4* mice (*Hdac4*^{+/-}) were normal and did not display increased *Mmp13* levels [13]. Previous studies have shown that *Hdac4*^{-/-} mice show impaired chondrocyte hypertrophy leading to ectopic bone formation [2]. On the other hand, *Mmp13*^{-/-} mice display terminally differentiated chondrocytes accumulating in the pre-hypertrophic zone and delayed chondrocyte apoptosis [8] leading to an increase in the height of the hypertrophic zone without changes in overall bone length.

Osteoblast differentiation *in vitro* and *in vivo* can be characterized in three stages: cell proliferation, matrix maturation, and matrix mineralization [14]. The matrix maturation phase is characterized by maximal expression of alkaline phosphatase (ALP). At the beginning of matrix mineralization, the osteocalcin (OCN) gene is expressed. We showed that in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, the expression of ALP mRNA and protein recovered from the reduced levels seen in *Hdac4*^{-/-} mice, suggesting that MMP-13 affects the early stage of osteoblast differentiation. MMP-13 is synthesized by osteoblasts and osteocytes in bone, and hypertrophic chondrocytes in cartilage [15, 16, 17]. An MMP-13 endocytotic-receptor system was identified in osteoblastic cells and chondrocytes [18, 19], and it was shown that extracellular MMP-13 causes activation of ERK1/2 in rabbit chondrocytes [20]. Although there is a possibility that MMP-13 may regulate ALP expression through this receptor system, the mechanism is not clear.

Mmp13^{-/-} mice exhibit significant reductions in the number of activated osteoclasts [21 and Fig. 5]. In contrast, overexpression of MMP-13 resulted in the formation of osteoclasts that were larger in size and displayed a greater resorption capacity [22]. The role of MMP-13 in osteoclastogenesis could be explained as a synergistic effect with MMP-9 [8, 23]. MMP-13 indirectly induces osteoclast differentiation by activating pro-MMP-9, which is known to induce recruitment of osteoclasts, and cleave galectin-3 in a MMP-9-dependent or independent manner, thus blocking its inhibitory effect on osteoclastogenesis [16]. MMP-9 is highly expressed in monocytes (osteoclast precursors) and multinucleated osteoclasts that resorb bone. MMP-9 is not a collagenase, however, the results of this study reveal that MMP-9 may compensate in *Mmp13* null mice. Our data with TRAP staining in *Mmp13*^{-/-} mice showing decreases in osteoclast surface are consistent with previous results. The *Hdac4*^{-/-}/*Mmp13*^{-/-} double knockout mice partially restored the reduction of osteoclast surface in *Hdac4*^{-/-} mice. *Hdac4*^{-/-} mice die at approximately 8 days of age (average 7.3 ± 2.3 days). Nonetheless, based on the H&E staining, it appears that almost all chondrocytes of the growth plate proceed to ossification. Thus, in *Hdac4*^{-/-} mice, the ossification rate is accelerated, so the epiphyses are much smaller. The lack of MMP-13 in *Hdac4*^{-/-} mice appears to have partially restored the ossification and epiphyseal morphology.

Our micro-CT analyses showed that *Hdac4*^{-/-} mice have significantly reduced cortical bone area compared with wild type mice. Elevation in MMP-13 appears to contribute to decreased radial rather than longitudinal growth in *Hdac4*^{-/-} mice. Thus, we assume that in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, bones become wider through periosteal bone apposition [24]. On the other hand, endosteal resorption excavates the marrow cavity [25]. The balance between periosteal bone apposition and endosteal bone resorption is important for advancing overall bone growth. In *Hdac4*^{-/-} mice, it is possible that earlier completion of longitudinal growth and earlier inhibition of periosteal apposition produces a smaller bone. Lack of MMP-13 in *Hdac4*^{-/-} mice appears to partially reverse the inhibition of periosteal bone apposition. It is reported that net endosteal apposition contributes to the final cortical thickness [26, 27]. Since cortical thickness and RCA (B.Ar/T.Ar) are similar in both *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, endosteal resorption may increase in proportion to increase in periosteal apposition. The *Hdac4*^{-/-} mice appear to have more compressed and easily broken cortical bone as suggested by lower cortical bone porosity and MMI, respectively, and these architectural parameters tended to recover in the *Hdac4*^{-/-} mice lacking MMP-13. These results indicate that increases in MMP-13 in *Hdac4*^{-/-} mice affect cortical bone mass and architecture but not length. In conclusion, the premature phenotype seen in *Hdac4*^{-/-} mice is partially derived from MMP-13 elevation and may be due to a bone turnover disorder that influences the whole tibial cortical bone area, trabecular thickness, and BMD. Even though the length of growth plate zones was partially recovered in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice, they may not be able to recover the whole bone length. Although *Mmp13* and *Mmp9* are direct targets of Runx2 in bone tissue [28–32] we did not find any effect of deletion of *Mmp13* or *Hdac4* on Runx2 gene expression. Interestingly, *Mef2c* expression level was increased in *Hdac4*^{-/-} mice and normalized in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice. This suggests that *Mef2c* gene expression is inhibited by HDAC4 and removal of MMP-13 with partial restoration of the bone phenotype in some way leads to normalization of *Mef2c*. It also suggests that the elevated *Mef2c* may have some influence on the premature phenotype seen in *Hdac4*^{-/-}

mice. Mef2c, a MADS (MCM1, Agamous, Deficiens, serum response factor) box factor, was for some time considered only important for regulating muscle and cardiovascular function. In 2007, Amood *et al.* demonstrated that Mef2c is a necessary early regulator of endochondral bone development and deletion of one *Mef2c* allele in *Hdac4*^{-/-} mice normalized ossification of the chondrocostal cartilage and sternum. Ossification of endochondral cartilage depends on the balance between transcriptional activity by Mef2c and repression by HDAC4 [33]. Recently, it has been reported that Mef2c binds to the *SOST* enhancer and it is necessary for transcriptional activation of *Sost* [34, 35]. Sclerostin (encoded by the *SOST* gene) is expressed primarily by osteocytes *in vivo* [36, 37] and is an inhibitor of the Wnt anabolic pathway for bone formation. Osteoblast or late osteoblast/early osteocyte inactivation of *Mef2c* results in increased bone mass [35, 38]. We examined whether *Sost* gene expression is up-regulated in *Hdac4*^{-/-} mice, but found it was not changed (data not shown). Kramer *et al.* revealed that the phenotype seen in late osteoblast/early osteocyte inactivation of *Mef2c* is primarily by a reduction in osteoclast activation (through other mechanisms than reduction of *Sost* gene expression). Mef2c may play an important role in the premature phenotype seen in the *Hdac4*^{-/-} mice but the mechanism of how HDAC4 and MMP-13 regulate *Mef2c* expression and whether and if so, how Mef2c participates in the phenotype that is seen in *Hdac4*^{-/-} mice, is unclear. The *Hdac4*^{-/-}/*Mmp13*^{-/-} mice still have an abnormal phenotype, suggesting the contribution of other factor(s) to the phenotype of *Hdac4*^{-/-} mice and more investigation is needed to completely understand the mechanisms.

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Abbreviations

TRAP	tartrate-acid resistant acid phosphatase
Runx2	Runt-related transcription factor 2
Mmp13	matrix metalloproteinase-13
Hdac4	histone deacetylase 4

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Highlights

- We generated *Hdac4/Mmp13* double knockout mice and determined the ability of deletion of MMP-13 to rescue the *Hdac4*^{-/-} mouse phenotype.
- *Hdac4*^{-/-} mice lacking Mmp13 exhibit partial recovery of the premature phenotype.
- MMP-13 in the *Hdac4*^{-/-} mice influences ALP, Mmp9 and TRAP expression.
- Increases in MMP-13 in *Hdac4*^{-/-} mice affect cortical bone area and architecture and trabecular bone mineral density and thickness.

Fig. 1. Images, body weights and survival of *Hdac4*^{-/-} mice compared with *Hdac4*^{-/-}/*Mmp-13*^{-/-}
 A, Postnatal day 8 (P8) wild type (WT, n=12), *Mmp-13* knockout (*Mmp-13*^{-/-}, n=15), *Hdac4* knockout (*Hdac4*^{-/-}, n=17) and *Hdac4*^{-/-}/*Mmp-13*^{-/-} (DKO, n=11) male mice are shown. B, Body weights of male and female genotypes. Data shown are mean ± SE. Different letters indicate $p < 0.05$ vs. one another. C, Survival of *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp-13*^{-/-} (DKO) male and female mice. * $p < 0.05$ vs. *Hdac4*^{-/-}.

Fig. 2. Histological examination of all four genotypes

A, Hematoxylin and eosin stained tibiae at postnatal day 8 (P8) WT, *Mmp-13*^{-/-}, *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp-13*^{-/-} male mice. 4x. B, Morphometric analysis of the length of the prehypertrophic (ph), and hypertrophic (h) zones in P8 WT (n=3), *Mmp-13*^{-/-} (n=5), *Hdac4*^{-/-} (n=6) and DKO (n=5) female and male mice. Data shown are mean ± SE. Different letters indicate $p < 0.05$ vs. one another. The images were quantified using Image J software.

Fig. 3. Immunohistochemistry of all four genotypes

Tibiae from female mice of postnatal day 8 (P8) WT (n=2), *Mmp-13*^{-/-} (n=3), *Hdac4*^{-/-} (n=4) and *Hdac4*^{-/-}/*Mmp-13*^{-/-} (n=3) were used. 10x

Fig. 4. Gene expression of all four genotypes

Total RNA was extracted from distal femurs of 5-day postnatal male and female WT (n=6), *Mmp-13*^{-/-} (n=6), *Hdac4*^{-/-} (n=5), and DKO mice (n=4). RNAs were measured using real time RT-PCR. The relative levels of mRNAs were normalized to β -actin. Data are shown as -fold change to WT. Data shown are mean $\pm$ SE. Different letters indicate $p < 0.05$ vs. one another.

Fig. 5. Gene expression of all four genotypes

Total RNA was extracted from distal femurs of 5-day postnatal male and female WT (n=6), *Mmp-13*^{-/-} (n=6), *Hdac4*^{-/-} (n=5), and DKO mice (n=4). RNAs were measured using real time RT-PCR. The relative levels of mRNAs were normalized to β -actin. Data are shown as –fold change to WT. Data shown are mean $\pm$ SE. Different letters indicate $p < 0.05$ vs. one another.

Fig. 6. TRAP staining of all four genotypes

A, Tibiae of postnatal day 8 (P8) WT, *Mmp-13*^{-/-}, *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp-13*^{-/-} female mice are shown. 20x. B, Quantification of osteoclast number in postnatal day 8 (P8) WT, *Mmp-13*^{-/-}, *Hdac4*^{-/-}, and DKO mice. Data shown are mean $\pm$ SE. Different letters indicate $p < 0.05$ vs. one another, $n = 4$ in each group.

Fig. 7. Bone images obtained by μ CT analysis

Tibiae from mice at postnatal day 8 (P8) of WT, *Mmp-13*^{-/-}, *Hdac4*^{-/-} and *Hdac4*^{-/-} / *Mmp-13*^{-/-} were used. $n = 7-8$ in each group

Fig. 8. *Hdac4*^{-/-} mice have decreased cortical bone area and structure which were restored in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice

Tibiae from male and female mice of postnatal day 8 (P8) WT, *Mmp13*^{-/-}, *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} were used. Different letters indicate *p* < 0.05 vs. one another, *n* = 7–8 in each group.

Fig. 9. The trabecular thickness, BMD, and bone surface were normalized in *Hdac4*^{-/-}/*Mmp13*^{-/-} mice

Tibiae from mice at postnatal day 8 (P8) of WT, *Mmp13*^{-/-}, *Hdac4*^{-/-} and *Hdac4*^{-/-}/*Mmp13*^{-/-} were used. Different letters indicate *p* < 0.05 vs. one another, *n* = 7–8 in each group.

TABLE 1

Primer sequences for real-time RT-PCR

Gene	Primers (5'-3')
Mouse <i>α1(X)</i>	Forward, TCATGCCTGATGGCTTCATA Reverse, GCACCTACTGCTGGGTAAGC
Mouse <i>ALP</i>	Forward, ATCTTTGGTCTGGCTCCCATG Reverse, TTTCCCGTTCACCGTCCAC
Mouse <i>OCN</i>	Forward, GCAATAAGGTAGTGAACAGAC Reverse, GTTTGTAGGCGGTCTTCAAGC
Mouse <i>TRAP</i>	Forward, CACTCCCACCCTGAGATTTGTG Reverse, ACGGTTCTGGCGATCTCTTTG
Mouse <i>Mmp-9</i>	Forward, CGCTCATGTACCCGCTGTAT Reverse, CCGTGGGAGGTATAGTGGGA
Mouse <i>Mmp-2</i>	Forward, GCCCCATGAAGCCTTGTTT Reverse, GGTCATAGTCCTCGGTGGTG
Mouse <i>Mef2c</i>	Forward, CTTCTGCCACTGGCCAC Reverse, GGGGTTGCCGTATCCATTC
Mouse <i>Mef2c</i>	Forward, CCCAGCCACCTTTACCTACA Reverse, TATGGAGTGCTGCTGGT
Mouse <i>b-Actin</i>	Forward, TCCTCCTGAGCGCAAGTACTCT Reverse, CGGACTCATCGTACTCCTGC