Haploinsufficiency of osterix in chondrocytes impairs skeletal growth in mice

Shaohong Cheng,1 Weirong Xing,1,2 Xin Zhou,3 and Subburaman Mohan1,2

1Musculoskeletal Disease Center, Jerry L. Pettis VA Medical Center, Loma Linda, California; 2Departments of Medicine, Loma Linda University, Loma Linda, California; and 3Department of Genetics, University of Texas, MD Anderson Cancer Center, Houston, Texas

Submitted 10 July 2013; accepted in final form 12 August 2013

Cheng S, Xing W, Zhou X, Mohan S. Haploinsufficiency of osterix in chondrocytes impairs skeletal growth in mice. Physiol Genomics 45: 917–923, 2013. First published August 13, 2013; doi:10.1152/physiolgenomics.00111.2013. —Osterix (Osx) is essential for both intramembranous and endochondral bone formation. Osteoblast-specific ablation of Osx using Col1α1-Cre resulted in osteopenia, because of impaired osteoblast differentiation in adult mice. Since Osx is also known to be expressed in chondrocytes, we evaluated the role of Osx expressed in chondrocytes by examining the skeletal phenotype of mice with conditional disruption of Osx in Col2α1-expressing chondrocytes. Surprisingly, Cre-positive mice that were homozygous for Osx floxed alleles died after birth. Alcan blue and alizarin red staining revealed that the lengths of skeleton, femur, and vertebrae were reduced by 21, 26, and 14% (P < 0.01), respectively, in the knockout (KO) compared with wild-type mice. To determine if haploid insufficiency of Osx in chondrocytes influenced postnatal skeletal growth, we compared skeletal phenotype of floxed heterozygous mice that were postnatal skeletal growth, we compared skeletal phenotype of mice with conditional disruption of Osx in Col2α1-expressing chondrocytes. Micro-CT showed reduced cortical volumetric bone mineral density and trabecular bone volume to total volume in the femurs of Osx<sup>Flox</sup>/Col2α1-Cre mice. Histological analysis revealed that the impairment of longitudinal growth was associated with disrupted growth plates in the Osx<sup>Flox</sup>-positive;Col2α1-Cre mice. Primary chondrocytes isolated from KO embryos showed reduced expression of chondroblast expression markers but elevated expression of chondrogenesis markers. Our findings indicate that Osx expressed in chondrocytes regulates bone growth in part by regulating chondrocyte hypertrophy.

Address for reprint requests and other correspondence: S. Mohan, Musculoskeletal Disease Center (151), Jerry L Pettis VA Medical Center, 11201 Benton St., Loma Linda, CA 92357 (e-mail: Subburaman.mohan@va.gov).
hypertrophy. Our findings demonstrate that both alleles of Osx in chondrocytes are required for postnatal skeletal growth.

MATERIALS AND METHODS

Animals. Chondrocyte-specific Osx knockout mice were generated by crossing Osx floxed mice (Osx\textsuperscript{floxed/floxed}) with a Cre transgenic mouse line driven by the col2α1 promoter (col2α1-Cre) (1, 24). Cre-positive mice with homozygous loxP alleles were referred to as KO (Osx\textsuperscript{floxed/floxed}, col2α1-Cre) and were compared with the corresponding Cre-negative wild-type (WT) mice with homozygous loxP alleles (Osx\textsuperscript{floxed/floxed}). Cre-positive mice with heterozygous loxP alleles were referred to as Het (Osx\textsuperscript{floxed/+}, col2α1-Cre) and were compared with mice with Cre-negative WT mice with heterozygous loxP alleles (Osx\textsuperscript{floxed/+}). All animal rearing and experimental procedures were performed according to approved standards by the Institutional Animal Care and Use Committees of the Jerry L. Pettis Memorial VA Medical Center. Mice were anesthetized with approved anesthetics, isoflurane. For euthanasia, mice were exposed to CO\textsubscript{2} followed by cervical dislocation.

Skeletal staining and measurement. Pregnant mothers were euthanized at 18 days of pregnancy (E18). E18 embryos were fixed in 95\% ethanol for 5 days and acetone for an additional 4 days. The fixed carcasses were then stained with a solution containing 0.1\% alizarin red, 0.3\% alcan blue, acetic acid, and 70\% ethanol at a volumetric ratio of 1:1:1:17. The embryos were then cleared with 1\% KOH in 20% glycerol and stored in glycerol. Whole bone lengths and mineralized bone lengths were measured with a caliper.

Evaluation of bone phenotypes. Bone area, bone mineral content (BMC), and bone mineral density (BMD) of WT and Het mice were measured by dual-energy X-ray absorptiometry (DEXA) with the PXiXus instrument (LunarCorp, Madison, WI) as previously described (10). Femur cortical and trabecular bone microarchitecture from 3 wk old WT and Het mice were assessed using the microcomputed tomography (\textit{mu}CT) (VIVA CT40; SCANCO Medical, Bruttisellen, Switzerland) as previously reported (10). The femurs were scanned by X-ray at 55 and 75 kVp for the distal metaphysis and epiphysis, respectively, with a voxel size of 10.5 μm. Reconstructions were performed with SCANCO software (SCANCO Medical). Sections of ~1 mm (adjusted to lengths of femurs) at the metaphysis were analyzed for cortical bone parameters, and for trabecular bone parameters, sections 0.63 mm (adjust to lengths of femurs) starting from 0.36 mm proximal to the growth plate were analyzed to obtain trabecular bone parameters using the SCANCO software.

Histomorphometric analysis. Tibias of 3 wk old mice were isolated and processed for paraffin sections as previously described (25), followed by Safranin-O staining to visualize cartilage at the growth plates. Control and hypertrophic chondrocytes were identified by Safranin-O staining to visualize cartilage at the growth plates. Columellar and hypertrophic chondrocytes were identified by Safranin-O staining to visualize cartilage at the growth plates. Approximately two fields from primary or secondary spongiosa were measured using the OsteoMeasure software (Ostometrics). Sections of ~1 mm (adjusted to lengths of femurs) at the metaphysis were analyzed for cortical bone parameters, and for trabecular bone parameters, sections 0.63 mm (adjust to lengths of femurs) starting from 0.36 mm proximal to the growth plate were analyzed to obtain trabecular bone parameters using the SCANCO software.

Immunohistochemistry. Immunohistochemistry was carried out according to a previously published procedure (4, 10). Osx antibody (ab22552, Abcam) was diluted at 1:500 in blocking solution and incubated at 4°C overnight. The secondary antibody was detected using the VECTASTAIN ABC-AP kit (AK-5000, Vector Laboratories) followed by color development with the Vector Blue AP substrate (SK-5300, Vector Laboratories).

Primary cell culture. Pregnant mothers were euthanized at E18. E18 embryos were isolated and genotyped. Isolation of primary chondrocytes was performed according to a previously established procedure (8), and the chondrocyte phenotype was confirmed by cell morphology and expression of chondrocyte markers. Briefly, rib cages were deskinned and digested with collagenase type D (3 mg/ml in 1X PBS) at 37°C with shaking (210 rpm) for 1 h. The solution was removed and the ribs were washed with 1X PBS three times followed by additional 3 h of digestion with collagenase type D. Chondrocytes were collected and grown in α-MEM containing 10\% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were grown until 70% confluent and collected for RNA extraction.

Quantitative real-time RT-PCR. RNA was extracted from primary cells with Trizol reagent (Invitrogen) according to manufacturer’s instruction. An aliquot of RNA (400 ng) was reverse-transcribed into cDNA with the oligo(dT)\textsubscript{12-18} primer in a 20 μl reaction volume. Quantitative real-time PCR was performed as previously described (4). Ppia was used as reference gene. Primer sequences used for real-time PCR are listed in Table I.

Statistics. Student’s \textit{t}-test or ANOVA were used for statistical analysis as appropriate. Two-way ANOVA was performed using STATISTICA software (Statsoft, Tulsa, OK).

RESULTS

Chondrocyte-specific ablation of Osx impairs skeletal development and mineralization in mice. To disrupt Osx specifically in chondrocytes, we crossed mice with floxed Osx alleles to mice where \textit{Cre} expression was driven by the chondrocyte-specific col2α1 promoter (Fig. 1A). After two generations of breeding, four genotypes (25\% of each) were obtained: Osx\textsuperscript{floxed/floxed}, col2α1-Cre (homozygous conditional knockout, KO), Osx\textsuperscript{floxed/+}, col2α1-Cre (heterozygous conditional knockout, Het), and their wild-type littermates (Osx\textsuperscript{floxed/floxed} and Osx\textsuperscript{floxed/+}, WT). The conditional KO mice died shortly after birth from a difficulty in breathing. No surviving pups were found to be \textit{Cre} positive, consistent with a recent report (21). To examine whether or not the expression of Osx is ablated in KO chondrocytes, we performed immunohistochemistry with sections of E18 embryos. Osx protein is detected in the pre- and hypertrophic zones of the growth plate in E18 WT embryos. By contrast, Osx expression was sparse in the \textit{Cre}-positive conditional KO chondrocytes (Fig. 1B, arrows). The expression of Osx was also reduced in the hypertrophic chondrocytes of the Het mice compared with WT littermates (Fig. 1C, arrows).

Table 1. Primer sequences used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osx</td>
<td>forward</td>
<td>5′-AGAGGGTGACCTGGCTGGACTAAGA-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-TTGTCAAATTGCGTGGCTTCTC-3′</td>
</tr>
<tr>
<td>Runx2</td>
<td>forward</td>
<td>5′-AAAGCCAGATGAGACCCCTGCA-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-ATTCGACCCTGCACTGAGATTG-3′</td>
</tr>
<tr>
<td>Sox9</td>
<td>forward</td>
<td>5′-CCCCGAGGAGTTGGTGGTAAAG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGCCCTTCCCAAGCTTCGUAGT-3′</td>
</tr>
<tr>
<td>Hes1</td>
<td>forward</td>
<td>5′-GGTCTCTGCCGACAGAGTGAAGG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGTCTCTGCCGACAGAGTGAAGG-3′</td>
</tr>
<tr>
<td>Tgfβ2</td>
<td>forward</td>
<td>5′-GGTCTCTGCCGACAGAGTGAAGG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGTCTCTGCCGACAGAGTGAAGG-3′</td>
</tr>
<tr>
<td>Fgfr3</td>
<td>forward</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td>Col2</td>
<td>forward</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td>Col10</td>
<td>forward</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGTGAGCGAGCACGCGCTGAGTA-3′</td>
</tr>
<tr>
<td>Mmp13</td>
<td>forward</td>
<td>5′-CATCTACTCCCCTGACAGCTTAT-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-CATCTACTCCCCTGACAGCTTAT-3′</td>
</tr>
<tr>
<td>Ppia</td>
<td>forward</td>
<td>5′-CTTCTGGAGCCAGGAACAGGGC-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-CTTCTGGAGCCAGGAACAGGGC-3′</td>
</tr>
</tbody>
</table>

Physiol Genomics • doi:10.1152/physiolgenomics.00111.2013 • www.physiolgenomics.org
expression was reduced in the Het mice compared with WT mice. Bone lengths of skeleton, skull, femur, tibia, and lumbar vertebrae (L1–L6) were reduced by 21, 13, 26, 19, and 14% (Fig. 1D). In addition, mineralized portions of the long bones were significantly reduced in the conditional KO embryos, compared with WT embryos, while the reduction in the Het mice did not reach statistical significance (Fig. 1F).

**Haploinsufficiency of Osx in chondrocytes results in delayed postnatal skeletal growth.** Because conditional KO of Osx in chondrocytes caused neonatal lethality and because Osx expression was significantly reduced in chondrocytes of heterozygous mice, we next investigated the consequence of loss of one copy of the Osx gene in chondrocytes on skeletal growth. At 3 wk of age, the Het mice of Osx are slightly smaller than WT mice. The Het mice exhibited a 16% decrease in body weight (Fig. 2A, \( P < 0.001 \)) and 8% decrease in body length (\( P < 0.001 \)). Bone area, BMC, and BMD were measured at multiple sites by DEXA (Fig. 2B). Bone area of the total body, femur, and tibia was decreased by 19, 15, and 19% (\( P < 0.05 \)), respectively, in the Het mice compared with WT mice. BMC of total body, femur, and tibia were similarly reduced by 22, 18, and 25% (\( P < 0.05 \)), respectively, in the Het mice. BMD of total body, femur, and tibia were also decreased by 5, 7, and 8%, respectively, in the Het mice compared with WT mice. By contrast, lumbar vertebrae showed no statistically significant difference in the bone area, BMC, or BMD between Het and WT mice (Fig. 2B). By 6 wk of age, the bone parameter deficits in the tibia were reduced in the Het mice compared with the WT mice (Fig. 2C). Bone area and BMC of tibia were 11 and 13% (\( P < 0.05 \)) less in the Het mice compared with the WT mice, while BMD showed no difference between the two genotypes. By 14 wk of age, only BMC was significantly less (\( P < 0.05 \)) in Het mice when compared with WT mice. Thus, skeletal growth parameters in Het mice appeared to catch up with WT mice with age.

Changes in cortical and trabecular bone parameters in mice with chondrocyte-specific Osx haploinsufficiency. Next we performed \( \mu \)CT to investigate whether or not the reduced area BMD in Het mice was due to changes of properties in the cortical or trabecular bones. \( \mu \)CT measurements revealed that the volumetric BMD (vBMD) at the femur middiaphysis was reduced by 8% (Fig. 3, A and B) in Het mice compared with WT mice at age of 3 wk. Accordingly, cortical thickness at the
mididiaphysis was not significantly altered (Fig. 3C). Furthermore, loss of a single copy of Osx in chondrocytes did not alter bone size (Fig. 3D), as measured by femur cross-sectional area. μCT at the femur distal metaphysis revealed less developed trabecular bone in mice with chondrocytic Osx haploinsufficiency compared with WT mice (Fig. 3E). Total volume (TV) of trabecular bone was comparable between Het and WT mice (data not shown), but bone volume to total volume (BV/TV) was significantly reduced by 31% (Fig. 3F, P < 0.05). Trabecular number (Tb. N) in the Het mice was reduced by 24% (Fig. 3G, P < 0.05) while trabecular separation (Tb. Sp) was increased in the Het mice compared with WT mice. Tb. Th, trabecular thickness. *P < 0.05, n = 10/group. Data are presented as means ± SE. Bar = 200 μm.

Haploinsufficiency of Osx in chondrocytes impairs chondrocyte hypertrophy in the growth plate. Since Osx is expressed in growth plate chondrocytes and contributes to trabecular bone parameters via endochondral bone formation, we next investigated if lack of single copy of Osx in chondrocytes influenced growth plate morphology. Longitudinal sections of tibia from 3 wk old heterozygous Osx conditional KO and WT mice were stained with Safranin-O. We found that the growth plates in the Het mice were thinner than in the WT mice (Fig. 4, A and B). The heights of columnar and hypertrophic zones were decreased by 13% (P < 0.01) and 15% (P < 0.05), respectively, in the Het mice compared with WT mice (Fig. 4, C and D). In addition, many of the chondrocytes in the hypertrophic zone of the Het mice appeared smaller with atypical hypertrophic morphology, compared with the hypertrophic chondrocytes in the WT mice (Fig. 4, A and B, arrows).

Hypertrophic chondrocytes also produce RANKL and OPG and influence trabecular bone resorption by regulating osteoclastogenesis. Deletion of RANKL expression in mesenchymal lineage abolished osteoclastogenesis in the femur metaphysis. (13, 27, 29). We next analyzed TRAP activity in the WT and Het mice. We found that the TRAP-positive surface to bone surface (Oc.S/BS) is reduced by 23% (P < 0.05) in the primary spongiosa adjacent to the growth plate in the Het tibia (Fig. 4, E–G). However, osteoclast activity in the secondary spongiosa was not altered in the Het mice. The ratios of TRAP-positive Oc.S/BS were similar between WT and Het tibia secondary spongiosa (Fig. 4, E, F, and H).
Since Osx is expressed in the differentiating chondrocytes and Osx haploinsufficiency caused atypical chondrocyte hypertrophy in the growth plate, we next examined gene expression of chondrogenesis and focused on chondral ossification markers in the primary chondrocytes isolated from conditional KO and WT embryos. While the expression of Col2 was not altered in the Het chondrocytes, terminal differentiation markers Col10 and Mmp13 were markedly downregulated in KO chondrocytes compared with WT chondrocytes (Fig. 5A). In contrast, the expression of chondrogenic transcription factor Sox9 and early differentiation marker Runx2 was significantly upregulated in KO chondrocytes (Fig. 5B). As expected, Osx transcript was significantly reduced by 67% in the KO chondrocytes compared with WT chondrocytes (Fig. 5B). Markers for chondrogenic signaling Hes1, Tgfr2, and Fgfr3 were also significantly upregulated in KO chondrocytes (Fig. 5C).

DISCUSSION

In this study, we demonstrate that the expression of Osx in chondrocytes is essential for skeletal growth in mice. Chondrocyte-specific disruption of Osx resulted in severe skeletal defects including a small body, reduced bone length, bent long bones, and reduced bone mineralization in embryos, a phenotype comparable to that of a total bone genotype comparable to that of a total bone.

Osx haploinsufficiency caused atypical chondrocyte hypertrophy in the growth plate, we next examined gene expression of chondrogenesis and focused on chondral ossification markers in the primary chondrocytes isolated from conditional KO and WT embryos. While the expression of Col2 was not altered in the Het chondrocytes, terminal differentiation markers Col10 and Mmp13 were markedly downregulated in KO chondrocytes compared with WT chondrocytes (Fig. 5A). In contrast, the expression of chondrogenic transcription factor Sox9 and early differentiation marker Runx2 was significantly upregulated in KO chondrocytes (Fig. 5B). As expected, Osx transcript was significantly reduced by 67% in the KO chondrocytes compared with WT chondrocytes (Fig. 5B). Markers for chondrogenic signaling Hes1, Tgfr2, and Fgfr3 were also significantly upregulated in KO chondrocytes (Fig. 5C).

Gene expression data further revealed a marked reduction in the expression of hypertrophic chondrocyte markers Col10 and Mmp13. We speculate that the hindrance of chondrocyte hypertrophy could in part contribute to the elevated expression of chondrogenic markers Sox9, Hes1, Tgfr2, and Fgfr3 in the absence of Osx in chondrocytes. In addition to the differentiating chondrocytes at the growth plates, the expression of Osx has also been found in the chondrogenic cell line, ATDC5 (20, 22). In line with our findings, Omotoyama and Takagi (22)
found that knocking down Osx using shRNA impaired differentiation of ATDC5 cells. These data strongly suggest that Osx is important for chondrocyte differentiation. Based on our findings and other recently published findings, we conclude that Osx expressed in chondrocytes plays a much more important role than previously thought in regulating chondrocyte hypertrophy and thereby endochondral bone formation. Future studies will address the molecular mechanism by which Osx exerts its important role on chondral ossification.

Conclusions

By generating heterozygous conditional knockout of Osx in collagen II-expressing chondrocytes, we found that both alleles of Osx are required for skeletal growth in mice. Haploinsufficiency of Osx in chondrocytes resulted in reduced cortical vBMD, trabecular BV/TV, and trabecular number. Disrupted bone growth in Osx haploinsufficient mice was associated reduced heights of columnar and hypertrophic zones of growth plates. The abnormal chondrocyte hypertrophy in Osx haploinsufficient mice was associated with reduced expression of chondrocyte differentiation markers. Our study demonstrates that both alleles of Osx are required for the chondrocyte hypertrophy and endochondral bone formation.

ACKNOWLEDGMENTS

All work was performed at facilities provided by the Veterans’ Administration. The authors thank Catrina Alarcon, Sheila Pourteymour, and Nancy Lowen for technical assistance.

GRANTS

This study was supported by funding from the National Institutes of Arthritis and Musculoskeletal Diseases R01 Grant AR-048139 to S. Mohan and Veterans Administration Biomedical Laboratory Research and Development Merit Review Grant 10826917 to S. Mohan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.C. and S.M. conception and design of research; S.C., W.X., and X.Z. performed experiments; S.C. and S.M. analyzed data; S.C., W.X., and X.Z. manuscript preparation; S.C., W.X., X.Z., and S.M. edited and revised manuscript; S.C., W.X., and S.M. approved final version of manuscript.

REFERENCES


