

Toward an Understanding of the Short Bone Phenotype Associated with Multiple Osteochondromas

Kevin B. Jones,^{1,2} Manasi Datar,^{3,4} Sandhya Ravichandran,¹ Huifeng Jin,^{1,2} Elizabeth Jurrus,³ Ross Whitaker,^{3,4} Mario R. Capecchi²

¹Sarcoma Services, Department of Orthopaedics and Center for Children's Cancer Research, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, Utah, ²Department of Human Genetics, Howard Hughes Medical Institute, University of Utah, 15 North 2030 East Room 5440, Salt Lake City, Utah 84112-5331, ³Scientific Computing and Imaging Institute, University of Utah, Salt Lake City, Utah, ⁴School of Computing, University of Utah, Salt Lake City, Utah

Received 19 June 2012; accepted 29 October 2012

Published online 28 November 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22280

ABSTRACT: Individuals with multiple osteochondromas (MO) demonstrate shortened long bones. *Ext1* or *Ext2* haploinsufficiency cannot recapitulate the phenotype in mice. Loss of heterozygosity for *Ext1* may induce shortening by steal of longitudinal growth into osteochondromas or by a general derangement of physal signaling. We induced osteochondromagenesis at different time points during skeletal growth in a mouse genetic model, then analyzed femora and tibiae at 12 weeks using micro-CT and a point-distribution-based shape analysis. Bone lengths and volumes were compared. Metaphyseal volume deviations from normal, as a measure of phenotypic widening, were tested for correlation with length deviations. Mice with osteochondromas had shorter femora and tibiae than controls, more consistently when osteochondromagenesis was induced earlier during skeletal growth. Volumetric metaphyseal widening did not correlate with longitudinal shortening, although some of the most severe shortening was in bones with abundant osteochondromas. Loss of heterozygosity for *Ext1* was sufficient to drive bone shortening in a mouse model of MO, but shortening did not correlate with osteochondroma volumetric growth. While a steal phenomenon seems apparent in individual cases, some other mechanism must also be capable of contributing to the short bone phenotype, independent of osteochondroma formation. Clones of chondrocytes lacking functional heparan sulfate must blunt physal signaling generally, rather than stealing growth potential focally. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 31:651–657, 2013

Keywords: osteochondroma; exostosis; skeletal dysplasias; shape analysis; mouse genetic models

Multiple osteochondromas (MO), also called hereditary multiple exostoses (HME), multiple hereditary exostoses (MHE), and previously, diaphyseal aclasis, is an autosomal dominant, heritable disorder of connective tissue characterized by the variably penetrant development of multiple cartilage-capped bony excrescences on the metaphyses of long bones, called osteochondromas or exostoses.^{1,2} Individuals with MO and the orthopedic surgeons providing their medical care face three important clinical problems. First, osteochondromas can become symptomatic as mass lesions, pushing on nerves or tendons, or impinging on joint range of motion. Second, very rarely, an osteochondroma will transform into a peripheral chondrosarcoma, requiring aggressive surgical management. Third, individuals with MO variably demonstrate reduced longitudinal growth of the long bones, which leads to some dramatic forearm and leg deformities and mildly reduced stature. The last of these presents the greatest clinical challenges in these patients, but remains poorly understood.

The two genes that associate with the disorder are *EXT1* and *EXT2*, both of which function in a heterooligomeric complex that lengthens heparan sulfate chains on glycoproteins in the endoplasmic reticulum and golgi apparatus en route toward the cell surface and extra-cellular matrix.³ Critical loss of full length heparan sulfate chains results in deranged ligand-receptor interactions as well as abnormal ligand diffusion gradients for a number of signaling pathways.^{4–6}

Until recently, the pathogenesis of osteochondroma formation in the setting of inherited heterozygosity for *EXT1* or *EXT2* was debated to derive either from haploinsufficiency or loss of heterozygosity for the involved gene. The debate was settled by zebrafish and mouse models of the disease that generated chondrocytes with biallelic loss of an *Ext* gene and formed osteochondromas, whereas loss of a single allele showed no clear phenotype.^{7,8}

The short bone phenotype associated with MO has been similarly enigmatic. Mouse models have suggested that haploinsufficiency cannot induce the short-bone phenotype, in that mice inheriting loss of a single functional copy of *Ext1* or *Ext2* in the germline do not have discernibly shortened bones.^{9,10}

It has yet to be confirmed that loss of heterozygosity can drive the short bone phenotype in mice. However, even if loss of heterozygosity drives the short-bone phenotype as it drives osteochondromagenesis, that still leaves two potential mechanistic hypotheses. First, cells that lose both copies of functional *Ext1* across the physis may contribute to a physis-wide reduction in growth signaling. Alternatively, the

The authors have no potential conflicts of interest.

Grant sponsor: National Center for Research Resources; Grant number: 5P41RR012553-14; Grant sponsor: National Institute of General Medical Sciences; Grant number: 8 P41 GM103545-14; Grant sponsor: National Institutes of Health; Grant sponsor: NIH/NCBC National Alliance for Medical Image Computing; Grant number: U54-EB005149; Grant sponsor: National Cancer Institute; Grant number: NIH K08CA138764

Correspondence to: Mario R. Capecchi (T: 801-581-7096; F: 801-585-3425; E-mail: mario.capecchi@genetics.utah.edu)

© 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

formation of osteochondromas might directly lead to a steal phenomenon of sorts, wherein some physal chondrocytes are redirected to grow peripherally rather than contribute to longitudinal growth.

The genetic experiment necessary to decipher rigorously between these models would require a physis bearing a number of *Ext*-null chondrocytes that managed not to form osteochondromas, but might or might not still demonstrate the shortening phenotype. As the formation of osteochondromas is fully penetrant even in our model, which disrupts *Ext1* in only a minority of chondrocytes, such an experiment seemed untenable. Without a clear genetic experiment available, we considered the tools available to address the question. Studying longitudinal growth disturbance in humans with MHE is very difficult, given that these are relatively small disturbances (approximately 10% of length) and population variation in bone lengths is wide. Our mouse model of MO would provide the opportunity for a tightly matched control group of littermates. Because induction of osteochondromas can be timed at different points during growth, we anticipated that a range of severity in osteochondroma formation would be achieved by induction at different ages. Shape analysis of CT scans might measure the bone lengths as well as compute the volumes of bones and localized deviations of that volume by comparing diseased bone to a mean shape from control littermates.

Because we know that the phenotype driven by homozygous *Ext1* loss in a minority of chondrocytes will lead to metaphyseal expansion from osteochondromagenesis and perhaps a general widening, computation of these volumes will permit the assessment of two conditions. First, even shortened bones should demonstrate a consistent volume, if the bone volume generated in the wake of chondrocyte linear growth is merely redirected from a longitudinal to a peripheral direction (Fig. 1). Volumetric loss of length should be matched by volumetric peripheral expansion. Second, the specific volumetric peripheral growth of the bone from the osteochondromas themselves and generally should predict the loss of length observed in the same bone, in the form of a correlation between linear variation and metaphyseal volume variation.

METHODS

Mice

The mouse model of MO was previously described.⁷ For these particular experiments, with the approval of the Institutional Animal Care and Use Committee, mice were induced to generate osteochondromas by administration of doxycycline in the drinking water (4 mg/ml) for a duration of 8 days beginning during the first, second, or fourth week of life. All mice were male and euthanized for imaging at 12 weeks age, as this represents an age well beyond the pubertal growth spurt of mice. While mice do not strictly close their physes, as humans do at skeletal maturity, mice stop growing appreciably shortly after sexual maturity at approximately 8 weeks

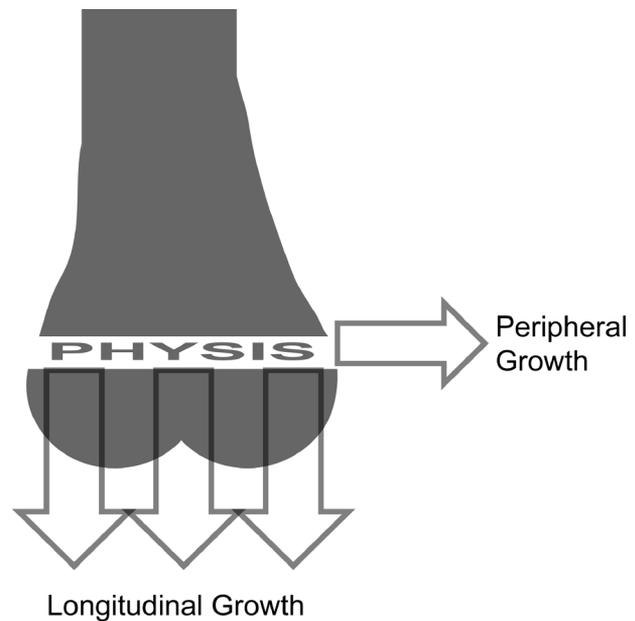


Figure 1. Redirected linear growth of osteochondromagenesis. Schematic representing the peripheral direction in which physal chondrocytes grow while becoming an osteochondroma. As primary spongiosal bone growth fills in behind these peripherally growing chondrocytes, the bone shape expands peripherally. What remains unclear is whether this peripheral expansion directly relates to the reduction in longitudinal growth observed in humans with multiple osteochondromas.

age. Controls were littermates lacking the Cre-recombinase transgene, but otherwise controlled, having similarly received doxycycline.

Computed Tomography (CT) Scans

All scans were obtained at 46 μ m resolution using the 80 KeV EVS-RS9 scanner (General Electric, Fairfield, CT). Files were then exported as DICMs and analyzed.

Statistical Shape Modeling

One popular approach to study shape variation involves the establishment of point *correspondences*, followed by a statistical comparison of the resulting point configurations. Such a statistical shape modeling (SSM) approach can be applied to 3D shapes for an objective comparison of complex morphology without the need to assume ideal geometry. Point distribution models allow representation of a class of shapes by the mean positions of a set of labeled points (called *correspondences*) and a small number of modes of variation about the mean.¹¹ Previously, correspondences for shape statistics were established manually by choosing small sets of anatomically significant landmarks on organs or regions of interest, which would then serve as the basis for shape analysis.¹² The demand for more detailed analyses on ever larger populations of subjects rendered that approach unsatisfactory. A more recent method iteratively distributes *correspondences* across an ensemble of shapes such that their positions result in a geometrically accurate sampling of individual shapes, while computing a statistically simple model of the ensemble.¹³ This method, briefly described below and implemented in the ShapeWorks software (<http://www.sci.utah.edu/>)

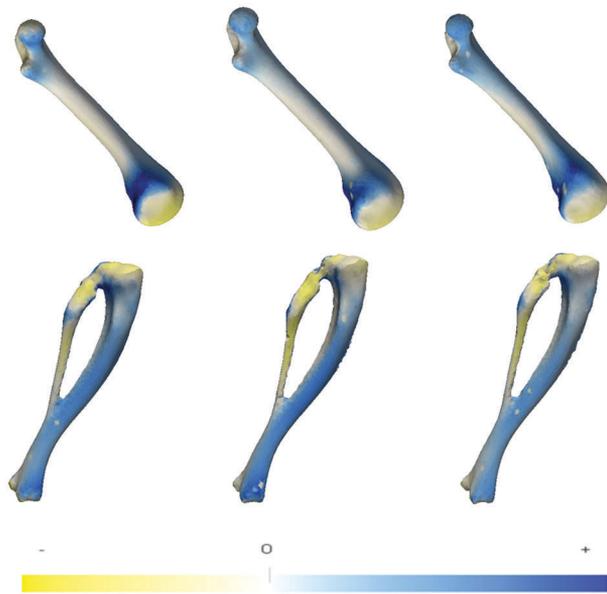


Figure 2. Mean shape of murine femora and tibiae following induction of homozygous loss of *Ext1* in a minority of chondrocytes at variable ages during skeletal growth. All three groups (Doxycycline beginning during week 1 on left, 2 in the middle, and 4 on right) demonstrated similar mean shape changes compared to controls, with shortening demonstrated by bone-end yellow-coloration indicating surface subtraction and metaphyseal widening demonstrated by blue-coloration indicating surface expansion.

software/shapeworks.html), was used to conduct statistical shape analysis.

Binary segmentations of the femora and tibiae, derived from the CT scans were used as input to the SSM process. These segmentations were preprocessed to remove aliasing artifacts, and 2048 correspondences were initialized and optimized on each bone, using the enhanced hierarchical splitting strategy described by Datar et al.¹⁴ The generalized Procrustes algorithm was applied at regular intervals during the optimization, to align shapes with respect to rotation and translation, and to normalize with respect to scale. Group labels were used to separate the point representation of controls and mice with MO, and the mean shape for each group



Figure 3. Volumetric overlay of mean shape of murine femora and tibiae following induction of homozygous loss of *Ext1* in a minority of chondrocytes at variable ages during skeletal growth. Aligned here at the distal femur (upper) and proximal tibia (lower) to demonstrate the overhanging metaphyseal width in each osteochondroma forming group (left, doxycycline at 1 week; middle, doxycycline at 2 weeks; right, doxycycline at 4 weeks) and the overhanging length of the control group mean shape.

Table 1. Length of Femora and Tibia in Mice Experiencing Inactivation of Both Copies of *Ext1* in a Minority of Physical Chondrocytes at Different Ages, Compared to Littermate Controls

	Bone Length in Voxels (Mean ± Standard Deviation)	Mean Percent Shortening	<i>t</i> -Test <i>p</i> -Value
Femur			
Control	244.4 ± 4.8		
Doxycycline at week 1	219.5 ± 7.9	11.3	2.1 × 10 ⁻⁹
Doxycycline at week 2	232.0 ± 8.3	5.3	0.00012
Doxycycline at week 4	233.1 ± 15.2	4.8	0.047 ^a
Tibia			
Control	396.6 ± 6.4		
Doxycycline at week 1	366.2 ± 13.5	7.6	1.4 × 10 ⁻⁵
Doxycycline at week 2	380.6 ± 7.2	4.0	0.00016
Doxycycline at week 4	389.6 ± 9.6	1.8	0.13 ^a

^aThese groups did not strictly reach statistical significance with Bonferroni-modified $\alpha < 0.016$.

was constructed as the mean of the *correspondences* from all shapes belonging to that group.

Statistical Analysis

The femora and the tibiae were analyzed separately, starting with a few common steps. Principal component analysis (PCA) was used to reduce the dimensionality required to examine variation among the different bones. Parallel analysis¹⁵ was then performed to determine the number of principal component modes representing significant variations among the bones. This analysis determined that the first five modes were significant for the femora, while the first four modes should be used for further analysis of the tibiae.

Using the significantly contributing modes designated by parallel analysis, a standard parametric Hotelling t^2 -test was used to test for group differences (control vs. doxycycline at 1, 2, or 4 weeks), with the null hypothesis that the two groups are drawn from the same distribution.

Length of bones and volumes of bones in pixels were compared using Student's t -test and an alpha value of 0.05, modified by a Bonferroni multiplier (for three comparisons in each parameter) to yield a significance at alpha less than 0.016.

RESULTS

Mice bearing Cre-recombinase, and therefore forming osteochondromas, were noted to have shorter femora and tibiae, most consistently among the mice induced to lose both functional copies of *Ext1* in a minority of chondrocytes at a young age, by administration of doxycycline at 1 week, in which approximately 10% shortening was observed (Table 1).

With regard to the mean shape analysis, bones from mice with osteochondromas were both shorter overall and wider in the metaphyses (Figs. 2 and 3). For both the femora and the tibiae, the Hotelling t^2 -test resulted in a significant difference in the group mean shapes with a p -value < 0.01 .

Comparing individual bones from experimental mice to the control mean shape from littermates revealed that more pronounced metaphyseal deviations in shape were not consistently associated with shortening with (Fig. 4).

Plotting length versus total bone volume for each specimen demonstrated the broad range of similar to larger volumes and variably reduced length among the

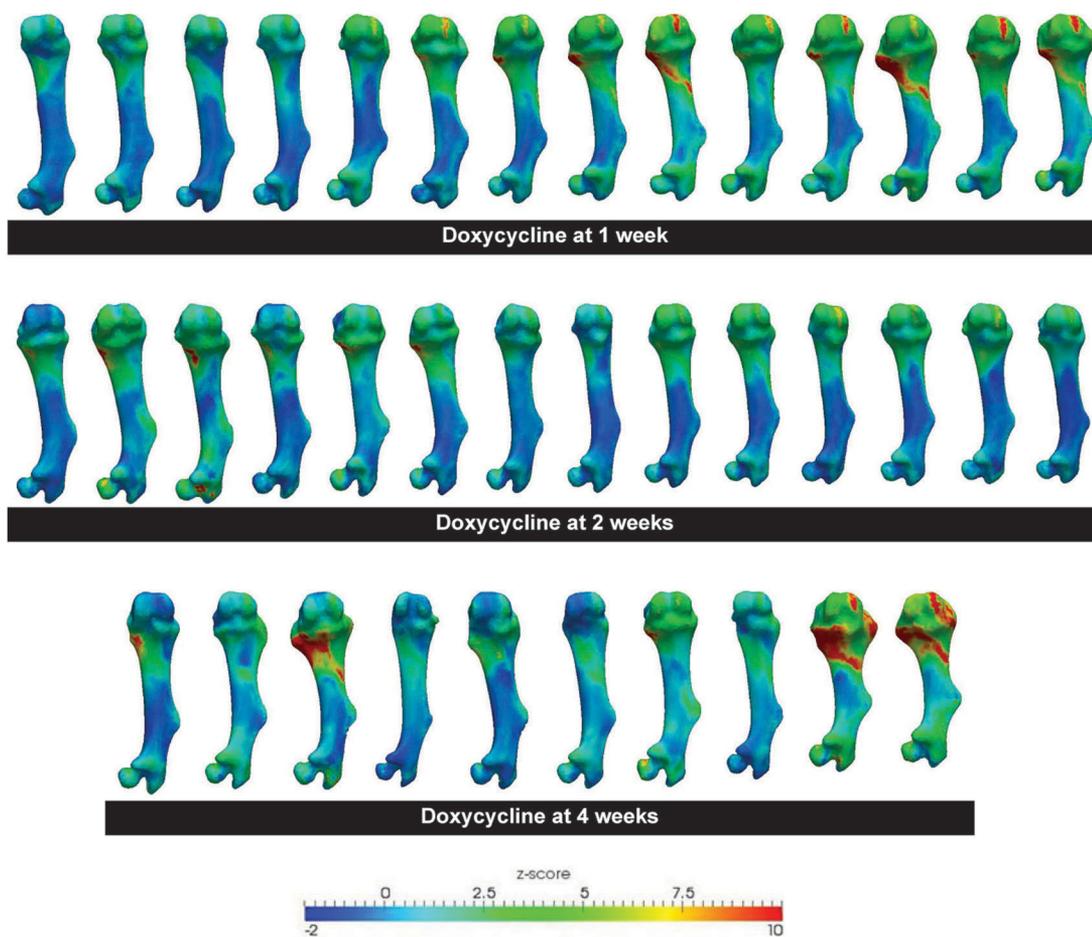


Figure 4. Metaphyseal widening does not consistently increase with decreasing length in the femora of mice that develop osteochondromas. Ordered from longest to shortest in each group, individual femora from mice treated with doxycycline at 1, 2, or 4 weeks age are depicted as rendered shapes, with colors indicating the deviations from the mean shape with regard to volume. While some of the shortest specimens have abundant red showing large deviations in volume, other specimens with large deviations are much longer in length.

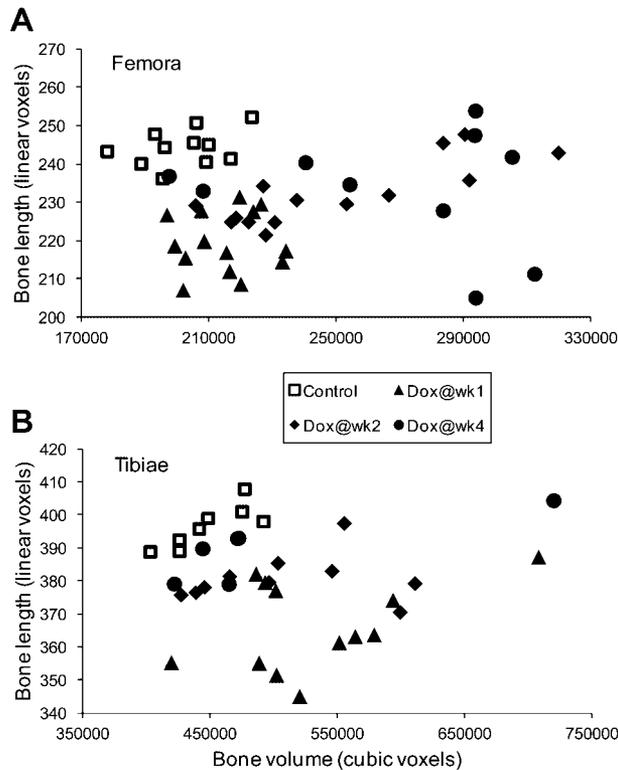


Figure 5. Bone length versus bone volume following induction of homozygous loss of *Ext1* in a minority of chondrocytes at variable ages during skeletal growth. Mice induced with doxycycline beginning during the first week of life demonstrated more consistent shortening. All demonstrated maintained or expanded total volume.

bones from mice with osteochondromas compared to littermate controls (Fig. 5). Notably, no bone volumes in osteochondroma-forming mice were significantly smaller than the control mean volume, suggesting that the volumetric gains of osteochondroma formation compensated for or overcompensated for volume loss due to reduced length.

After aligning femora at the knee and cropping distal femoral volumes to include only the metaphyses in order to remove the volumetric impact of overall bone shortening, deviations of metaphyseal volumes from the mean of the control femora demonstrated no correlation with length (Fig. 6). While there is a severe reduction in length among some femora with large peripheral gains in metaphyseal volumes, others have abundant osteochondromas and metaphyseal volumetric expansion but minimal to no shortening.

DISCUSSION

We report that low-prevalence loss of heterozygosity for *Ext1* in physeal chondrocytes is sufficient to mimic the short-bone phenotype of MO in humans. Because the short-bone phenotype was not previously discernible in mice with germline loss of one allele of *Ext1* or *Ext2*,^{9,10} we conclude that loss of heterozygosity drives this phenotype as it does osteochondromagenesis.

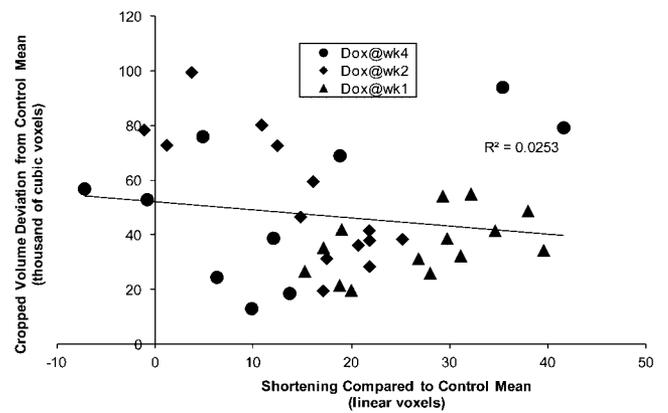


Figure 6. Femur length deviation correlates poorly with metaphyseal volumetric growth. Phenotypic metaphyseal widening, estimated by the deviation from the control mean in cropped volumes correlated poorly with less shortening, rather than more shortening, thus countering a simple steal phenomenon as the mechanism of shortening.

Determining the relationship between the two phenotypes is much more difficult.

When induction of osteochondromagenesis was initiated early in skeletal development, femora, and tibiae were approximately 10% shorter at the end of skeletal growth, but were not smaller in total volume, as the metaphyseal expansion in the form of osteochondromas compensated for and generally even slightly overcompensated for the loss of volume due to shorter length. Induction at later ages resulted in less consistent shortening, but sometimes much greater total bone volumes.

The natural variation in severity of osteochondroma formation did not correlate with reduced length. These data lead us to reject the model of a steal phenomenon at work in the pathogenesis of the short bone phenotype. Although some individual bones with severe metaphyseal expansion secondary to osteochondromas and general widening demonstrated the greatest shortening, there were others with abundant osteochondromas but minimal shortening. Because the mice in all groups were littermates and the control group demonstrated such strong consistency in both length and volume, we must conclude that the wide variation in length and volume relates to the phenotype of low-prevalence biallelic *Ext1* loss in physeal chondrocytes, rather than typical population variation.

We also acknowledge the limitations of this study design. First, we can only claim the absence of a correlation between bone length and a computer analyzed expansion of metaphyseal volume. Not only a weak correlation, but a trend in the opposite direction strengthens our rejection of the steal hypothesis, but we depend on correlation alone, lacking a clear genetic experiment with *Ext1*-null chondrocyte clones shortening a bone without forming osteochondromas. In addition, there may be some other biology at work in this model which does not fit MO precisely. The group that

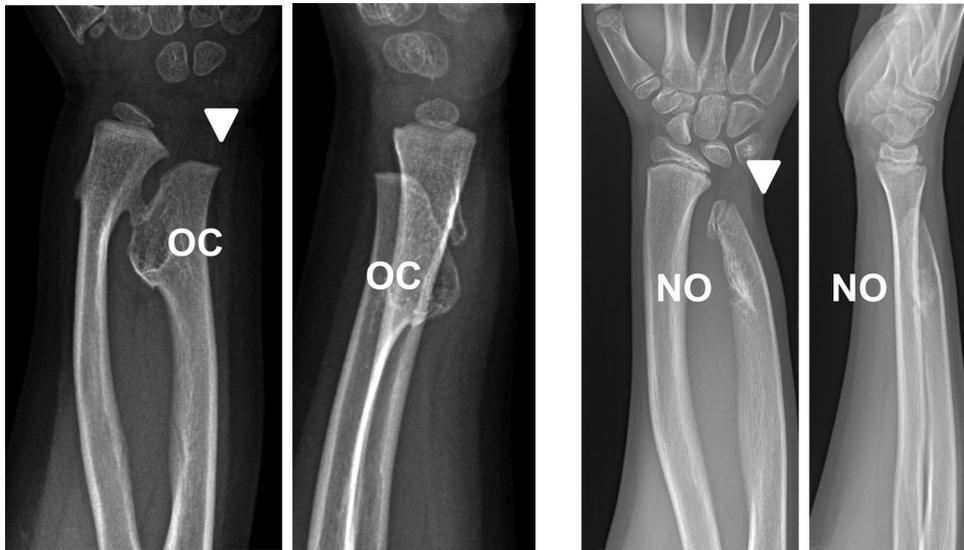


Figure 7. Short-bone phenotype in humans can develop independently from osteochondroma formation. Anteroposterior (left in each pair) and lateral (right in each pair) radiographs of the forearm in two children with MO and severe shortening of the ulna (triangle) relative to the radius, causing radial bowing and wrist deformity. The pair of radiographs to the left shows a large osteochondroma (OC) arising from the shortened ulna. The other forearm (pair to right) shows no significant osteochondroma (NO).

most closely recapitulated the short bone phenotype, those receiving doxycycline during the first week of life, did have a slight correlation between metaphyseal volume expansion and shortening. Possibly, induction of *Ext1* loss in chondrocytes later in skeletal growth has more erratic effects on linear physal growth. Nonetheless, even in this most consistent sub-group, the *R*-squared value of the correlation was less than 0.3, giving comfort to our rejection of the steal phenomenon.

There are apparently inputs into the short bone phenotype other than simply the formation of osteochondromas and metaphyseal widening stealing from longitudinal growth potential. This suggests that some general dysplasia of linear growth in the physis results from the presence of *Ext1*-null chondrocytes as a minority of the physal cell population.

On the clinical level, it has been observed that the short-bone phenotype is not consistently penetrant throughout an individual with MO. This fits well with our rejection of haploinsufficiency as the pathogenetic mechanism, which ought to lead to more consistent phenotypic penetrance. While not always consistent even bilaterally, the ulna and the fibula are the bones most frequently affected by the short-bone phenotype, generating characteristic deformities due to adjacent bones with less shortening. These bones can be anatomically categorized as having physes with the highest ratio of length grown to cross-sectional area. This may be important because in these smaller cross-sectional area physes, disruption of ligand diffusion by a few *EXT*-null chondrocytes is more likely to impact overall growth signals, whereas physes with larger cross sectional areas may be somewhat protected by broader channels for these signals. Further, fitting with our rejection of the steal hypothesis, even in

these bones most prone to shortening in MO, some will have and others will not have large volume osteochondromas associated with the shortening (Fig. 7).

ACKNOWLEDGMENTS

K.B.J. receives career development support from the National Cancer Institute (NIH K08CA138764).

REFERENCES

1. Bovee JV. 2008. Multiple osteochondromas. *Orphanet J Rare Dis* 3:3.
2. Jones KB. 2011. Glycobiology and the growth plate: current concepts in multiple hereditary exostoses. *J Pediatr Orthop* 31:577–586.
3. Busse M, Feta A, Presto J, et al. 2007. Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation. *J Biol Chem* 282:32802–32810.
4. Bornemann DJ, Park S, Phin S, et al. 2008. A translational block to HSPG synthesis permits BMP signaling in the early *Drosophila* embryo. *Development* 135:1039–1047.
5. Bornemann DJ, Duncan JE, Staatz W, et al. 2004. Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* 131:1927–1938.
6. Bishop JR, Schuksz M, Esko JD. 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* 446:1030–1037.
7. Jones KB, Piombo V, Searby C, et al. 2010. A mouse model of osteochondromagenesis from clonal inactivation of *Ext1* in chondrocytes. *Proc Natl Acad Sci USA* 107:2054–2059.
8. Clement A, Wiweger M, von der Hardt S, et al. 2008. Regulation of zebrafish skeletogenesis by *ext2/dackel* and *papst1/pinscher*. *PLoS Genet* 4:e1000136.
9. Stickens D, Zak BM, Rougier N, et al. 2005. Mice deficient in *Ext2* lack heparan sulfate and develop exostoses. *Development* 132:5055–5068.
10. Lin X, Wei G, Shi Z, et al. 2000. Disruption of gastrulation and heparan sulfate biosynthesis in *EXT1*-deficient mice. *Dev Biol* 224:299–311.

11. Cootes TF, Taylor CJ, Cooper DH, et al. 1992. Training models of shape from sets of examples. In: Hogg D, Boyle R, editors. British machine vision conference. London, Great Britain: Springer-Verlag. p 9–18.
12. Cootes TF, Cooper CJ, Taylor CJ, et al. 1995. Active shape models—their training and application. *Comput Vis Image Und* 61:38–59.
13. Cates J, Fletcher PT, Styner M, et al. 2007. Shape modeling and analysis with entropy-based particle systems. In: Karssemeijer N, Lelieveldt B, editors. *Information processing in medical imaging IPMI*. Berlin Heidelberg, Germany: LNCS, Springer-Verlag. p 333–345.
14. Datar M, Gur Y, Paniagua B, et al. 2011. Geometric correspondence for ensembles of nonregular shapes. *Med Image Comput Comput Assist Interv* 14:368–375.
15. Glorfeld L. 1995. An improvement on Horn's parallel analysis methodology for selecting the correct number of factors to retain. *Educ Psychol Meas* 55:377–393.