Bone Response to Loading in Mice With Targeted Disruption of the Cartilage Oligomeric Matrix Protein Gene

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Summary

Exercise induced bone response although established, little is known about the molecular components that mediate bone response to mechanical loading (ML). In our recent QTL study, we identified one such possible molecular component responding to ML: cartilage oligomeric matrix protein (COMP). To address the COMP role in mediating ML effects on bone formation, COMP expression was evaluated as a function of duration and age in response to ML in female B6 mice. A 9N load was applied using a four-point bending device at 2Hz frequency for 36 cycles, once per day for 2-, 4- and 12-days on the right tibia. The left tibia was used as an internal control. Loading caused an increase in COMP expression by 1.3-, 2- and 4-fold respectively after 2-, 4- and 12-days of loading. This increase was also seen in 16 and 36-week old mice. Based on these findings, we next used COMP knockout (KO) mice to evaluate the cause and effect relationship. Quantitative analysis revealed 2 weeks of ML induced changes in vBMD and bone size in the KO mice (5.9 % and 21 % vs. unloaded bones) was not significantly different from control mice (7 % and 24 % vs. unloaded bones). Our results imply that COMP is not a key upstream mediator of the anabolic effects of ML on the skeleton.

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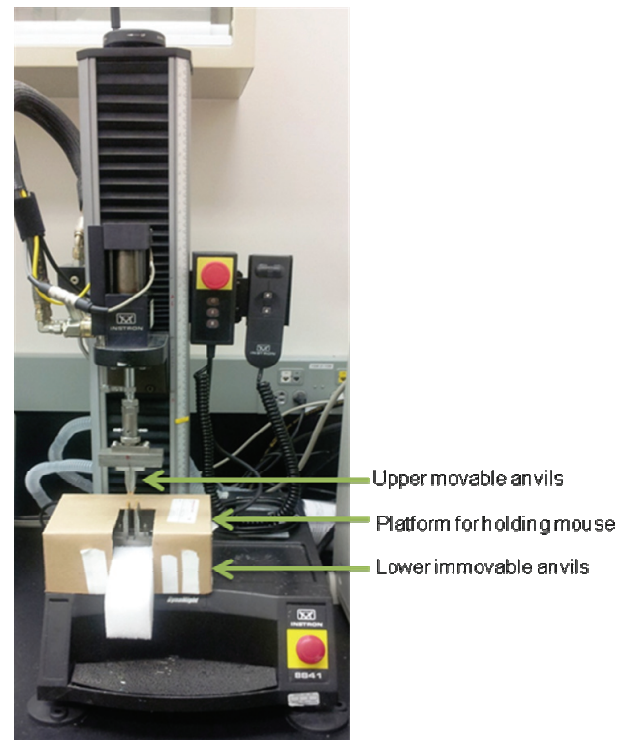C. Kesavan, Musculoskeletal Disease Center (151), Jerry L. Pettis Memorial VA Medical Center, 11201 Benton Street, Loma Linda, CA 92357, USA. Fax: 909-796-1680. E-mail: Chandrasekhar.Kesavan@va.gov

It is well established that mechanical stimulation is essential for bone maintenance and for development of skeletal architecture. We and other's using animal and human models have shown that loading increases bone formation and that lack of stimulation results in reduction of bone mass (Snow-Harter *et al.* 1992, Akhter *et al.* 1998, Kodama *et al.* 1999, Bikle *et al.* 2003, Kesavan *et al.* 2005). Thus, exercise has been used as a strategy to maintain bone mass and prevent bone loss in humans. Although exercise induced increase in bone mass is well documented, little is known about the molecular components that mediate the mechanical signal into anabolic effects on bone *in vivo*. Recently, we identified several novel chromosomal regions that regulate bone adaptive response to loading using a C56Bl/6J (B6) (a good responder) X C3H/HeJ (a poor responder) intercross (Kesavan *et al.* 2006, 2007). Further, narrowing these QTLs, we found a possible candidate: cartilage oligomeric matrix protein that lies within the region of a major QTL (Chr 8, 33-50cM) identified for bone adaptive response to loading.

COMP is a one of five members of the thrombospondin family of extracellular matrix proteins, which have diverse functions, such as cell adhesion, platelet aggregation, cell proliferation, angiogenesis,

vascular smooth muscle growth, and tissue repair (Cho *et al.* 2006). COMP is 435-kDa pentameric protein found predominantly in the extracellular matrix of cartilage, tendons, and ligaments (Muller *et al.* 1998). Reports have shown that COMP binds strongly to vitamin D3, whose interactions have implications in the morphogenesis and mineralization of bone (Ozbek *et al.* 2002). Other studies, in humans, have reported that mutation in the type-3 repeats (calcium binding) of COMP gene leads to skeletal disorders, such as pseudoachondroplasia and multiple epiphyseal dysplasia (Thur *et al.* 2001). Studies in cartilage have shown that COMP gene is primarily revealed in the proliferative zone, but is absent in the resting zone, suggesting that COMP is important for cellular proliferation (Shen *et al.* 1995). It has also been proposed that COMP is involved in signaling between load and cellular response. Furthermore, its bouquet-like structure with five terminal TC domains may, structurally, provide the multivalency necessary for strong interactions with cell surface receptors or other proteins, and, thus, could lead to rapid activation of intracellular secondary messengers. This provides convincing evidence that COMP is involved in signaling, cell adhesion and cell-cell interaction (Smith *et al.* 1997). Taken together, these data illustrate that the functions mediated by the COMP gene are also essential for bone response and formation. Since the COMP gene lies within the region of major QTL identified for bone anabolic response to loading and bone mechanical properties and because *in vitro* studies suggest an important role for COMP in the bone formation process, we propose the hypothesis that COMP is involved in mediating anabolic effects of ML on bone formation. To test this hypothesis, we performed ML using a four-point bending device on mice with disruption of the COMP gene and control mice with intact COMP gene.

A breeding pair of homozygous COMP gene Knockout (mouse) was provided as a gift by Dr. Jacqui Hecht, Department of Pediatrics (Division of Medical Genetics), University of Texas Health Science Center, Houston, USA. COMP knockout mice were crossed with wild type C57BL/6J (B6) mice to generate the heterozygotes. These were crossed with each other to generate 25 % homozygous COMP KO mice on B6 background, 50 % heterozygous and 25 % littermate wild type mice. All mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare

Fig. 1. Image of four-point bending device used to stimulate new bone formation in mouse model.
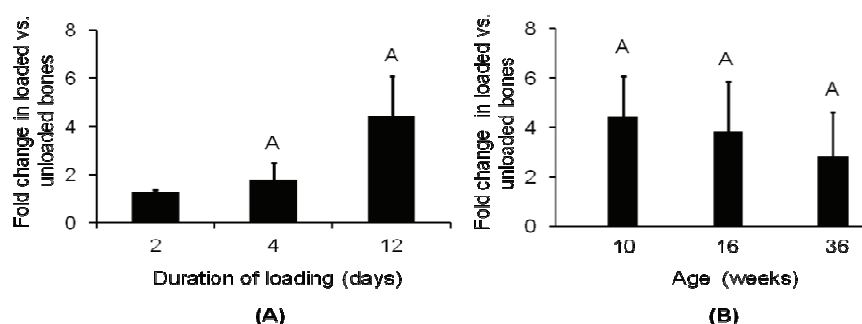
regulations and approved by the local IACUC. At 3 weeks of age, DNA was extracted from tail tissue of female mice, using a PUREGENE DNA purification kit (Gentra System, Inc., Minneapolis, MN) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed to identify COMP KO mice from wild type or heterozygous mice (Svensson *et al.* 2002). Primer specific for COMP gene (forward 5' CAG AAG CAA AGG TCG TGG AA-3') and reverse (5'-TTG CTC GTT AGG GAC TCC AT-3') were used to amplify 380 base pair PCR product to identify WT mice. To identify the KO mice, primer sequence (forward 5' CAG AAG CAA AGG TCG TGG AA-3') and reverse (5'-CGC CTT CTT GAC GAG TTC TT-3') were used to amplify 600 base pair PCR product containing neomycin gene. The following conditions were used to perform the PCR reaction: 95 °C for 2 minutes; 35 cycles at 95 °C for 40 s, 57 °C for 40 s, 72 °C for 40 s, 70 °C for 40 s. The PCR products were run on a 1.5 % agarose gel and the image taken with a ChemiImager 4400 (Alpha Innotech Corp., San Leandro, CA).

ML was performed using a four-point bending device [Instron, Canton, MA] (Fig. 1), as previously reported (Kesavan *et al.* 2005). The mice were loaded using a 9.0 ± 0.2 Newton (N) force at a frequency of 2 Hz

for 36 cycles, once a day under inhalable anesthesia (5 % Isoflurane and 95 % oxygen). The right tibia was used for loading (loading time frame on the tibia per day was 18 seconds) and the left tibia as internal non-loaded control. For the time course study, the loading was performed at 2-, 4- and 12-days on 10-week female B6 mice. After 24 hours of the last loading, mice were euthanized and tibiae were collected for RNA extraction. For varying age groups of female B6 mice, female COMP KO and control mice, the loading was performed for 12 days. After 48 hours of the last loading, four-point bending induced changes in the bone parameters (cortical bone) in the loaded and unloaded tibiae were measured by using Peripheral quantitative computed tomography (pQCT) (Stratec XCT 960M, Norland Medical System, Ft. Atkinson, WI) as described previously (Kesavan *et al.* 2005), followed by mice were euthanized; tibiae (loaded and non-loaded) were collected and stored at –80 °C for further experiments. To measure expression levels of genes in the loaded and unloaded bones, RNA was extracted using a Qiagen lipid extraction kit [Qiagen, Valencia, CA], as previously described (Kesavan *et al.* 2005). Quality and quantity of RNA were analyzed using the 2100 Bio-analyzer (Agilent, Palo Alto, CA) and Nano-drop (Wilmington, DE). Using 200 ng purified total RNA, first strand cDNA was synthesized by iScript cDNA synthesis kit (BIO-RAD, CA, USA), according to the manufacturer's protocol. Quantitative real time PCR

was performed, as previously described (Kesavan *et al.* 2005), in order to analyze the expression levels of COMP and PPIA (peptidylprolyl isomerase A, an endogenous control). The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control (PPIA) to correct for variation in the RNA quality among samples. The normalized Ct values were subjected to a $2^{-\Delta\Delta Ct}$ formula to calculate the fold change between the loaded and non-loaded groups. The formula and its derivations were obtained from the instrument user guide. The data presented are given as mean ± S.D. Standard t-test was used to compare the difference between loaded and non-loaded bones at various time-points, ages and strains using the fold change and percentage data. We used Statistica software (StatSoft, Inc version 7.1, 2005) to perform the analysis and the results were considered significant at $p < 0.05$.

We used the four-point bending method of loading since we and others have previously shown that this method of loading causes robust bone formation at the loaded site of C57BL/6J mice (Kesavan *et al.* 2005). For our study, we used female mice because of the well established role of estrogen in mediating mechanical strain response and because female mice are less aggressive and are more amicable for group housing than male mice.

Fig. 2. Expression levels of COMP gene in response to mechanical loading as a **(A)** function of duration of loading and **(B)** function of age. Values are mentioned as mean ± S.D. The y-axis represents fold increase in COMP gene in response to four-point bending on tibia. $^A p < 0.05$ vs. corresponding unloaded tibiae, N=5-7.
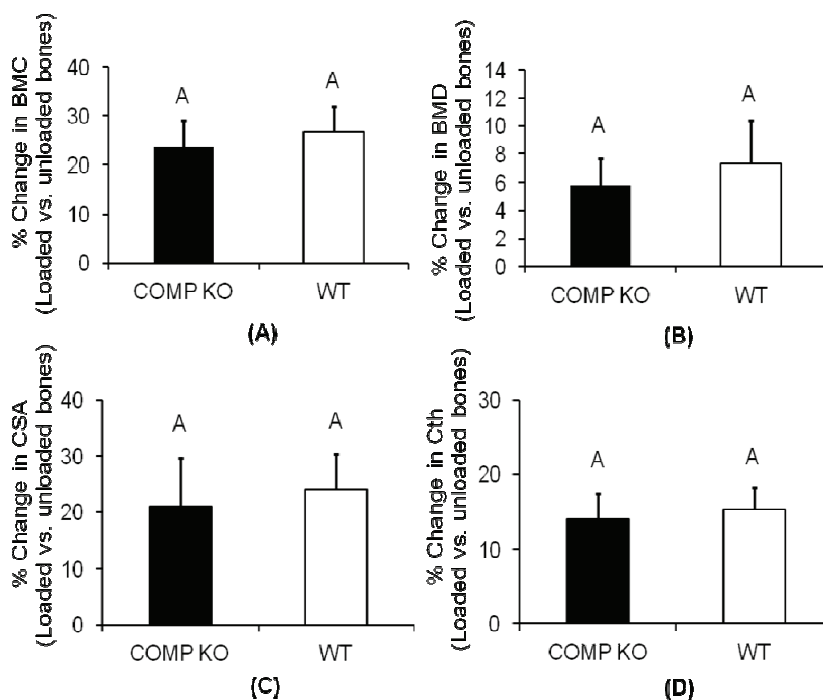
In a previous study, using quantitative trait loci (QTL) approach, we have reported that the variation in bone anabolic response to mechanical loading in the F2 mice of B6XC3H intercross is regulated by multiple genetic loci located in different chromosomes. In these studies, we identified the COMP gene as one of the candidate genes for a major mechanical loading QTL located in chromosome 8. In order to determine, if COMP is in part responsible for increasing bone anabolic response to loading, we evaluated temporal changes in COMP expression during 2 weeks of four-point bending.

We found that ML caused 1.3 to 4- fold increases in COMP expression after 2-, 4- and 12-days of loading (Fig. 2). Two days of loading did not show any significant change while longer duration showed significant change when compared to unloaded bones. Furthermore, ML effect on COMP expression was seen in three different age groups of mice, 10-, 16- and 36-weeks (2-5 fold, $p < 0.05$ vs. unloaded bones, n=5-7). These data demonstrate that COMP is a mechanoresponsive gene in the bones of mice but the cause and effect relationship between increase in COMP

expression and skeletal changes is yet undetermined. To test this, we generated COMP KO and control mice.

It is well established that the amount of mechanical strain exerted by a given load is largely dependent on the cross sectional area (moment of inertia) such that a mouse with a large cross sectional area will experience lower mechanical strain and vice versa. In order to assure that the difference in the bone responsiveness to loading between COMP KO mice and controls is not due to differences in mechanical strain, we measured the bone size by pQCT at tibia mid diaphysis

and calculated the mechanical strain using a mathematical model (Stephen C. Cowin: Bone Mechanics Hand book, 2nd edition, chapter: Techniques from mechanics and imaging, CRC Press, Washington, 2001) for both sets of mice before the loading. We found that there is no significant difference in the bone size (4.63 mm vs. 4.58 mm, p=0.47) as well as in the mechanical strain for 9N (6440 ± 220 µε vs. 6677 ± 213 µε, p=0.71, n=4) between the COMP KO mice and controls. Thus, the applied load was the same for both sets of mice.

Fig. 3. Changes in bone parameters in response to ML on 10-wk female COMP KO and control mice. Values are mentioned as mean ± S.D. The y-axis represents percent increase in bone parameters in response to four-point bending on tibia and x-axis represent COMP KO and WT mice. **(A)** BMC, bone mineral content; **(B)** BMD, bone mineral density; **(C)** CSA, Cross-sectional area and, and **(D)** Cth, cortical thickness. [A]p<0.05 vs. corresponding unloaded tibiae, N=6-8.

To determine if ML induced increase in COMP expression contributes to anabolic effects of ML, we performed four-point bending using a load (9N) that has been shown to exert significant changes in BMD. If COMP is an important mediator of skeletal anabolic response to loading, we anticipated COMP KO mice to show reduced anabolic effects of ML on bone. Quantitative analysis revealed that the loaded bones of COMP KO mice show 5 to 23 % increase in skeletal parameters when compared to unloaded bones in response to loading (Fig. 3). Although there was an increase, the bone response to loading was similar to that control (7 to 24 %, Fig. 3). A possible explanation for the lack of significant differences between the control and KO mice is that COMP disruption could lead to increased expression of other family members (Thrombospondin) which share similar functional properties to compensate

for the loss of COMP. However, earlier reports (Svensson et al. 2002) have shown that mice deficient for COMP were born with no abnormalities and were not compensated for by any other members of Thrombospondin family. This finding and the data from our study suggest that COMP may not be essential for bone formation or bone response to loading but may important for other tissues like cartilage.

In this study, we did not evaluate the extent of bone response to ML in the heterozygous mice. This is because the heterozygous mice appear phenotypically similar to WT and KO mice. Since the KO mice showed loading induced bone response similar to that of WT, it is likely that the response to loading in the heterozygous mice will be same as in WT. In conclusion, we show that COMP is not a key upstream mediator of anabolic effects of ML on the skeleton.

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