mechanosensitive protein may provide a new paradigm for investigating EX-driven bone formation and regulation of osteoblast and osteoclast activity. We propose that FSTL3 may also serve as a target to develop therapeutic drugs for treating bone diseases.

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BONE SECRETED DICKKOPF-RELATED PROTEIN 1 AMELIORATES OSTEOARTHRITIS IN MICE

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Objective: Cartilage loss and subchondral bone changes are hallmarks of osteoarthritis (OA). The family of the Wnt pathway is involved in the regulation of bone and cartilage homeostasis. Our preliminary results suggested that the Wnt/β-catenin pathway is mainly activated in bone during OA, but the effect of the reduction of bone Wnt pathway on cartilage remodeling are unknown. We here investigated the impact of the inhibition of the Wnt pathway specifically in bone tissue during the development of OA.
Methods: Joint instability induced by partial meniscectomy (MNX) was performed in mice to promote OA. We investigated the impact of the inhibition of bone Wnt pathway in OA development using mice overexpressing Dkk1 in bone (2.3 Col1-Dkk1Tg) after MNX. The effects of Dkk-1 in chondrocyte and osteoblast metabolism were further assessed using supernatant transfer and MMP expression.
Results: The Wnt/β-catenin pathway was activated in bone and cartilage, predominantly in osteocytes of subchondral bone and osteophytes, but remained weak in articular cartilage throughout the development of OA. The number of Dkk1 (+) chondrocytes was high at baseline (84.2 ± 3.1%) and decreased markedly during the course of OA from week 4 (14.4 ± 3.8%) to week 6 (5.7 ± 1.6%). At baseline, Dkk1-Tg mice had lower bone volume which was further reduced in MNX knees. Dkk1-Tg experienced a lower OA score than WT mice (5.1 ± 0.63 vs 8.4 ± 0.6, $p = 0.002$) independently of the expression of Dkk1 in chondrocytes. This was accompanied by a reduction of the subchondral bone and osteophyte volume. However, addition of supernatant of osteoblasts derived from Dkk-1-Tg mice or in vitro addition of rhDkk1 in chondrocytes promoted chondrocytic expression of MMP-3, -13 and ADAMTS-4 while the supernatant of pre-exposed osteoblasts with Dkk1 decreased the expression of proteases. Because Dkk1-Tg osteoblasts produced low VEGF, we tested whether VEGF could mediate the anti-catabolic effect observed in vivo. Blocking VEGF in the supernatant of osteoblast cultures reversed the expression of MMPs by chondrocytes.
Conclusion: We confirm that Wnt is mainly activated in bone in OA joints. Inhibition of Wnt pathway by Dkk1 overexpression in bone decreased OA severity by reducing VEGF production. Targeting bone could be a useful approach for the treatment of OA.

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CHIP IS A CRITICAL REGULATOR OF TRAF6 DEGRADATION AND OSTEOCLAST FORMATION

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Purpose: Bone tissue is constantly remodeled. Abnormal osteoclast formation may result in bone disorders, such as osteoporosis, Paget's disease, and rheumatoid arthritis. Nuclear factor of κB (NF-κB) plays a key role in osteoclast formation and bone resorption. A key event in NF-κB signaling is the activation of an adaptor protein TRAF6. Once activated, TRAF6 further activates IKKβ (I-κB kinase), which phosphorylates I-κBα (the inhibitor of NF-κB), leading to its degradation. Carboxyl terminus of Hsp70-interacting protein (CHIP) is an E3 ligase and regulates the stability of several proteins which are involved in tumor growth and metastasis. However, the role of CHIP in bone remodeling *in vivo* has not been reported. The objective of this study is to investigate the role of CHIP in regulation of bone mass and bone remodeling and determine the molecular mechanism of CHIP in regulation of TRAF6 protein stability.
Methods: The bone phenotype of 1-month-old *Chip*$^{-/-}$ mice was examined by histology, histomorphometry, μCT, and gene expression analyses. The regulation of CHIP on TRAF6 degradation and the inhibition of NF-κB signaling was examined by immunoprecipitation, Western blotting, and ubiquitination assay.
Results: In this study, we found that deletion of *Chip* leads to osteopenic phenotype. There was significant reduction in bone volume (%, BV/TV, 66% decrease) and bone mineral density (59% decrease) in *Chip*$^{-/-}$ mice. Trabecular numbers (44% decrease), trabecular thickness (33% decrease) were significantly decreased in *Chip*$^{-/-}$ mice. In contrast, trabecular separation (1.7-fold increase) was significantly increased in *Chip*$^{-/-}$ mice. Higher structure model index (1.4-fold increase) and lower connectivity density (75% decrease) were observed in *Chip*$^{-/-}$ mice, suggesting more fragile bone when *Chip* gene is deleted. Cortical bone volume (40% decrease) and bone mineral density (40% decrease) were also significantly reduced in *Chip*$^{-/-}$ mice. One major reason for the osteopenic phenotype observed in *Chip*$^{-/-}$ mice could be due to the increase in osteoclast formation. TRAP-positive osteoclast numbers (1.6-fold increase) and osteoclast surface (1.5-fold increase) were significantly increased in *Chip*$^{-/-}$ mice. *In vitro* osteoclast formation assay showed that there was 3.5-fold increase in osteoclast formation in *Chip*$^{-/-}$ mice using osteoclast precursor cells isolated from WT and *Chip*$^{-/-}$ mice. Consistently bone resorption area was also increased in *Chip*$^{-/-}$ mice, demonstrated by an *in vitro* bone resorption assay using bone marrow cells derived from WT and *Chip*$^{-/-}$ mice. The expression of osteoclast marker genes, such as *Cathepsin K* (7-fold increase), *Mmp9* (16-fold increase), *Trap* (16-fold increase), and *Nfatc1* (3-fold increase), was significantly increased after RANKL treatment in bone marrow cells derived from *Chip*$^{-/-}$ mice. There was a significant increase in TRAF6 protein levels in *Chip*-deficient bone marrow cells. In contract, no changes in TRAF6 mRNA levels were found in the same *Chip*$^{-/-}$ cells, suggesting that CHIP may regulate TRAF6 protein stability. In a series of *in vitro* studies we found that: 1) Interaction of endogenous CHIP with TRAF6 was detected in RAW264.7 osteoclast-like cells. 2) The ubiquitination of endogenous TRAF6 protein was reduced in *Chip*$^{-/-}$ bone marrow cells. 3) The phosphorylation of IKKα/β and IκBα was significantly increased in *Chip*$^{-/-}$ bone marrow cells after RANKL stimulation. 4) p65 nuclear translocation was enhanced in *Chip*$^{-/-}$ bone marrow cells when the cells were treated with RANKL.
Conclusions: Our studies demonstrated that CHIP interacts with TRAF6 in osteoclast precursor cells and promotes TRAF6 ubiquitination and proteasome degradation; subsequently inhibits NF-κB signaling and regulates osteoclast formation. In *Chip* KO mice TRAF6 protein levels are increased, leading to activation of osteoclast formation and significant bone loss.

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SPATIAL ASSOCIATION OF SUBCHONDRAL OSTEOSCLEROSIS WITH ENHANCED MARROW IMMUNE CELL INFILTRATION, OSTEOCLAST ACTIVITY AND CARTILAGE DEGENERATION IN HUMAN OSTEOARTHRITIS

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Purpose: Osteosclerosis of subchondral bone is a pathological hallmark of osteoarthritis (OA) and increasing evidence supports a pivotal and active role of this tissue in the initiation and progression of disease in experimental and human OA. The cellular and molecular regulation of subchondral osteosclerosis remains poorly understood. Regulation of bone remodeling by immune cells, termed osteoimmunology, has been demonstrated in a number joint disorders including rheumatoid arthritis and osteoporosis. In this study we investigated whether osteoimmunological mechanisms might be involved in regulating subchondral osteosclerosis in human OA.
Methods: Subchondral bone mineralization density (BMD) of explanted OA tibial plateaus was mapped using computed tomography osteoabsorptiometry (CT-OAM) analysis. Areas having a subchondral BMD below 800 or over 1200 Hounsfield units were defined to be non-sclerotic and sclerotic, respectively. Using CT-OAM mapping, histological tissue sections were prepared from both areas ($n = 18$ each). Cartilage degeneration and subchondral bone area fraction were determined using Mankin score and the ImageJ-plugin BoneJ, respectively. Presence of immune cells in subchondral bone marrow tissue was investigated using immunohistological and flow cytometry analyses for expression of CD3 (T-lymphocytes), CD20 (B-lymphocytes) and CD68 (macrophages). Functional osteoclasts were identified by histochemical staining for tartrate-resistant acid phosphatase (TRAP) activity. Outgrowth cultures of nonsclerotic and sclerotic bone were stained for TRAP and alkaline phosphatase (ALP) activity. The effect of conditioned medium from nonsclerotic and sclerotic subchondral bone

pieces on human primary OA osteoblasts was investigated using ALP assay.

Results: Subchondral BMD distribution was heterogeneous and displaying focal areas of relatively high mineralization density that spatially overlapped with areas of severe cartilage degeneration Contralateral tibial plateaus instead showed a homogeneous subchondral BMD of relatively low density. Histomorphometric analysis revealed an increase ($p < 0.001$) in subchondral bone area fraction (B.Ar/T.Ar.) in sclerotic (0.662 ± 0.120) compared with nonsclerotic tissue sections (0.335 ± 0.062). Corresponding with the observations at the macroscopic level, Mankin score and B.Ar/T.Ar were strongly positively correlated at a histological level ($r = 0.61$, $p < 0.001$). Immunohistological and flow cytometry analyses of subchondral bone marrow tissue showed a highly specific ($p < 0.001$) increase in CD68+ mononuclear and multinucleated cells and CD20+ B-lymphocytes in sclerotic compared with nonsclerotic subchondral bone tissue. Corresponding with an increase in multinucleated CD68+ cells, TRAP staining revealed a strong increase in functional osteoclasts that associated with CD34+ vascular structures. An increased number of TRAP+ macrophage-like mononuclear cells was observed in outgrowth cultures from sclerotic bone pieces. Overall, immune and TRAP+ cells were strongly correlated with B.Ar/T.Ar ($r = 0.804$ and $r = 0.670$, $p < 0.001$). Sclerotic OA osteoblasts showed poor in vitro mineralization and increased basal alkaline (ALP) phosphate activity. Lack of osteoblastic ALP induction by conditioned medium from sclerotic subchondral bone pieces suggested a proresorptive milieu in immune cell infiltrated marrow tissue.

Conclusion: We have shown for the first time that osteoimmunological mechanisms, including enhanced immune cell infiltration and osteoclast activity, along phenotypic alterations in osteoblasts are involved in uncoupled and aberrant bone remodeling underlying osteosclerosis in human OA.

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PAPSS2 PROMOTES ALKALINE PHOSPHATES ACTIVITY AND MINERALIZATION OF OSTEOBLASTIC MC3T3-E1 CELLS BY CROSSTALK AND SMADS SIGNAL PATHWAYS

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Several studies have indicated that PAPSS2 (39-phosphoadenosine-59-phosphosulfate synthetase 2) activity is important to normal skeletal development. Mouse PAPSS2 is predominantly expressed during the formation of the skeleton and cartilaginous elements of the mouse embryo and in newborn mice. However, the role and mechanism of PAPSS2 in bone formation remains largely unidentified. By analyzing the expression pattern of the PAPSS2 gene, we have found that PAPSS2 is expressed in bone tissue and bone formation. PAPSS2 transcripts increase during osteoblast differentiation and are in less level in RANKL-induced osteoclast like cells. By using lentivirus-mediated RNA interference (RNAi) technology, we knocked down PAPSS2 expression in MC3T3-E1 osteoblast. Silencing of PAPSS2 expression significantly decreases ALP activity and cell mineralization, inhibits expression of osteoblast marker osteopontin (OPN) and collagen I. Conversely, overexpression of PAPSS2 promotes the MC3T3-E1 to differentiate into osteoblast and mineralization. Moreover, compared to that in the control cells, the mRNA level and protein expression of phosphorylated Smad 2/3, which is a key transcriptional factor in the Smad osteoblast differentiation pathway, showed significant decreases in PAPSS2-silenced cells and increases in PAPSS2- overexpression cells. These results suggest that PAPSS2 might regulate osteoblast ALP activity and cell mineralization, probably through Smads signal pathways.

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VASCULAR PERFUSION OF THE PALMAR CONDYLES OF THE EQUINE THIRD METACARPAL BONE: A POSSIBLE CONTRIBUTING FACTOR TO PALMAR OSTEOCHONDRAL DISEASE?

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Purpose: Palmar osteochondral disease (POD) is an overload arthrosis that commonly affects fetlock joints of racing Thoroughbreds (TB), but the etiopathogenesis of the disease has not been well defined. The aim of this study was to compare India ink perfusion, as a measure of vascular perfusion, in the dorsal and palmar condyles of the third metacarpal bone (MC3) in both passively flexed (control group) and maximally extended (intervention group) fetlock joints from paired normal equine cadaver limbs. We hypothesized that the palmar condyle of MC3 normally has less perfusion than the dorsal condyle, and that loading the fetlock joint in maximal extension would result in further reduction of perfusion to the palmar condyles.

Methods: Pairs of forelimbs were acquired from 5 TB horses euthanized for reasons unrelated to lameness. Limb pairs were perfused intra-arterially with India ink (total of approximately 100 mL) within 1 hour post mortem. One forelimb from each horse was randomly assigned to either the passive flexion ($n = 5$) or maximal extension ($n = 5$) group. For the passive flexion group, limbs were allowed to assume a position of flexion without any external force applied. Limbs in the maximal extension group were placed in a materials testing system and the fetlocks extended to 110°, which has been reported to be maximal extension. Limbs were sectioned sagittally in 3 mm sections through the fetlock, and 12 sections per limb were processed using a modified tissue-clearing technique. Sections were subsequently digitally imaged, and bone perfusion evaluated with image analysis software. Elliptical regions of interest (ROIs) of standard size (3.47 mm x 8.34 mm) were digitally placed on the images in 2 locations: 1) on the subchondral bone just dorsal to the transverse ridge of MC3, and 2) on the subchondral bone just palmar to the transverse ridge (Fig 1). The palmar ROI was in a similar location to what has been reported for POD lesions (5–8 mm palmar to the transverse ridge).

Results: Greater perfusion of the dorsal condyle compared to the palmar condyle was observed in 78% of sections from limbs in passive flexion, and 92% of maximally extended sections (Fig 2). Perfusion to the palmar aspect of the condyle was significantly decreased ($P < 0.0001$) when the limbs were placed in maximal extension compared to passive flexion. The abaxial and mid sagittal ridge regions of MC3 appeared to have dorsal to palmar (D: P) perfusion ratios > 1 in the majority of samples, but did not appear to change based on whether the limb was in flexion or extension. Conversely, the medial axial to medial middle region of the condyle appeared to be the most vulnerable regions to maximal extension and actually had D:P ratios >1.5, thus illustrating the profound effect of maximal extension.

Conclusions: Our indirect evidence of relatively poor perfusion of the palmar condyles compared to the dorsal condyles gives some basis for considering these inherent differences in blood supply as a contributing factor for the pathogenesis of POD. During race training there is repetitive hyperextension of the fetlock joint, which likely further exacerbates this perfusion discrepancy. The locations on the palmar condyle that were most greatly affected by decreased India ink perfusion in our study were those locations where POD has been reported to occur most frequently. Although it was beyond the limits of this study to determine a cause and effect relationship between these observations, it does raise the possibility.

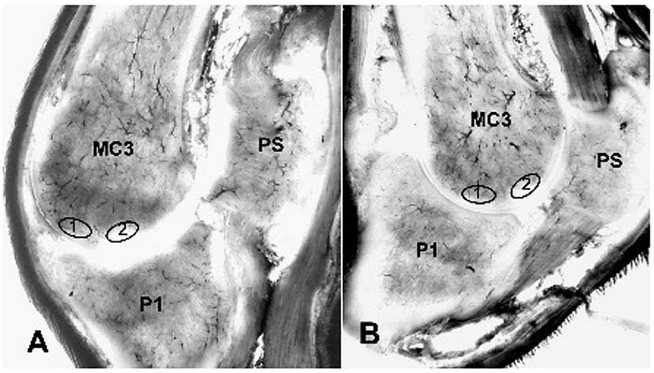

Fig 1. A: Representative sagittal section of the fetlock joint fixed in passive flexion. B: A representative sagittal section of the fetlock fixed in maximal extension. Ellipses illustrate region of interest (1-dorsal condyle; 2-palmar condyle) for calculation of mean pixel density of India ink perfusion. MC3 - third metacarpal bone; P1 - proximal phalanx; PS - proximal sesamoid bone.