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# Meet-the-Professor

# Handout Booklet

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# Genome Editing: From Patients to Mice with CRISPER/Cas

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# Genome Editing: From Patients to Mice with CRISPR/Cas

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## Significance

The development and application of CRISPR/Cas9-based technologies for genome editing are revolutionizing the ability to quickly generate and characterize genetically modified animals and cell lines with gene deletions (knockouts) as well as more precise changes. While the opportunities that this technology offers are numerous, it is also important to recognize what caveats are related to its use.

In this session, we will first present examples of how this system has been utilized to generate different types of genomic changes in rodents. This will include discussions on creating insertions and deletions (indels), the generation of specific point mutations, and attempts at the creation of models with more complex modifications. For each class of modifications, we will discuss our experiences with what have been the most efficient approaches and the challenges that have been encountered in the generation, screening, and validation of the models.

## Learning Objectives

As a result of participating in this session, attendees will gain a greater knowledge of the components of the CRISPR/Cas9 system, its application to genome modification, and the validation necessary to utilize it effectively.

## Outline of Discussion

1. Background of CRISPR/Cas9
2. Testing and validating reagents
3. Utilization to create small deletions and/or insertions
4. Generation of larger genomic deletions (multiple kB)
5. Creation of point mutations or other small modifications (Genome editing)
6. Comments on the feasibility of creating complex changes by targeted genomic editing
7. Discussion of how to screen for changes and validate their transmission
8. Comments on effectively avoiding or identifying off-target effects

Testing & Validation of Reagents

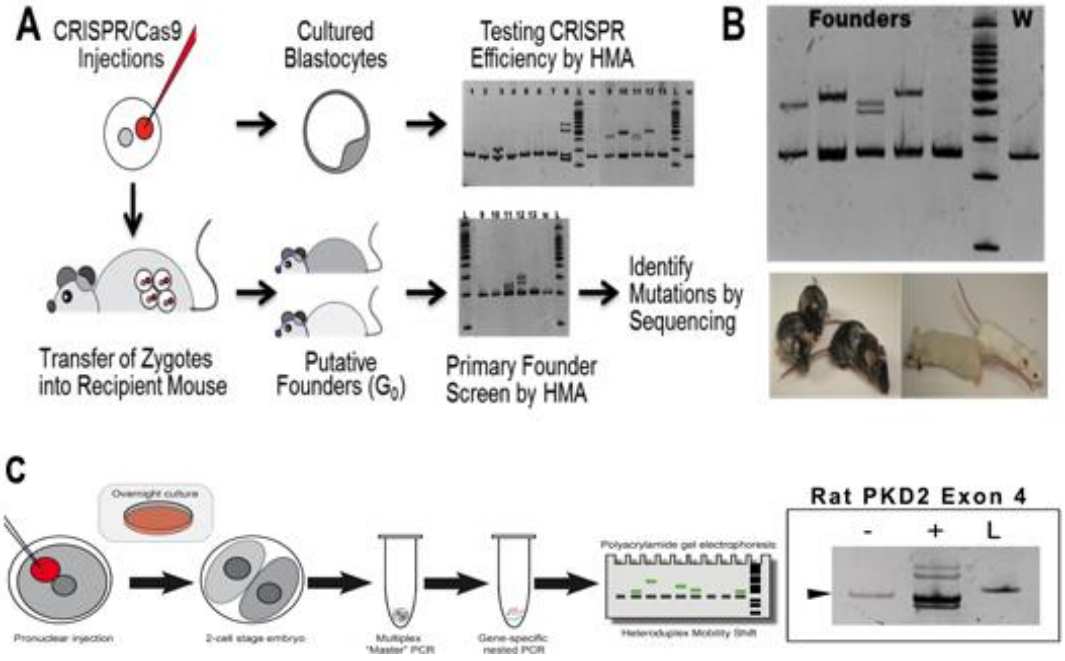


**Figure x. Screening and Generation of CRISPR/Cas9 Induced Mutations in Mice and Rats**. (A) To test the efficacy of guides, CRISPR/Cas9 RNA is injected into the pronucleus of single cell embryo and cultured to the blastocyst stage. DNA is isolated and screened by HMA (shown) or HRM for insertions or deletions. Once efficacy is confirmed, CRISPR/Cas9 injected embryos are transferred into recipient mice to generate (B) potential founders ($G_0$) that are identified by HMA. (B, bottom) The founders are then bred to obtain germ line transmitting alleles. Shown are CRISPR/Cas9 chimeras (left) and germline hypomorphic and null allele mutants for the Tyrosinase gene (right). Schematic diagram (C) illustrating CRISPR-Cas9 nuclease activity assay in cultured 2-cell rat embryos. Gel image shows homo- and heteroduplex mobilities of PCR amplicons of Rat PKD2 gene (exon 4). Lane 1 (-) single band corresponding to an WT PCR amplicon (no heteroduplex HMA); lane 2 (+) shows heteroduplex mobility shift indicating the presence of indels due to CRIPSR-Cas9 nuclease activity. L-ladder

<u>CRISPR/Cas9 Limitations for Discussion</u>

1. Specificity of editing “duplicated” genes
2. Editing of “lethal” genes and bi-allelic conversions
3. Large insertions
4. PAM motif restrictions

# Advances in Osteoarthritis Imaging and Treatment

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# Advances in OA: Imaging and Treatment

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## SIGNIFICANCE OF THE TOPIC

Osteoarthritis is a heterogeneous disease that affects up to 50% individuals greater than 50 years of age. The disease begins with damage or loss of articular cartilage and is followed by outgrowths of new bone. Overtime, the loss of cartilage reduces the ability for individuals to ambulate, and over time progression of OA in weight bearing knee and hip joints can lead to reduced activity, reduced quality of life and need for total joint replacements. Currently there are no treatments approved that can slow the progression of articular cartilage loss. Treatment is limited to analgesia.

A significant challenge in OA research and treatment is that when joint pain from incident OA is present, there is usually a significant loss or damage to the articular cartilage. Traditional imaging with radiographs, is insufficient as the radiograph trails behind the tissue damage. To investigate the natural history of OA, better imaging modalities were needed. Magnetic resonance imaging (MRI) has become a vital technique in imaging of joint abnormalities, especially OA. MRI is a non-invasive, non-ionizing technique which due to its excellent soft tissue contrast images is capable of depicting articular cartilage structure, lesions and providing information with regards to the meniscus, bone, bone marrow and ligaments post-injury[1-3].

**Learning Objectives: As a result of participating in this session, attendees should be able to**

1. Understand advances in the imaging of OA including qualitative and quantitative MRI of bone, and soft tissues.
2. Understand the different grading scales available for semi-quantitative assessments of knee and hip joints.
3. Understand new therapies that have been investigated or are currently under investigation for analgesia and for structure modification in OA subjects.

**IMAGING FOR MORPHOLOGICAL ASSESSMENT OF CARTILAGE:** While the xray based Kellgren Lawrence grading system assigned a single score to the whole joint in OA: with 4 grades focusing on bone changes and has been used for 50 plus years to determine prevent and incident knee OA in both epidemiology studies and clinical trials, with the introduction of MRI, the first imaging modality to assess soft tissue (cartilage, and ligaments), it was determined that significant articular cartilage damage was present while the xray remained normal. Therefore, MRI is now used as the more sensitive tool to assess knee and hip joints. Two-dimensional fast spin-echo (2D FSE) imaging is the method most commonly utilized in clinical settings for imaging of the knee joint and knee cartilage. A combination of proton density and $T_2$ contrast results in higher cartilage contrast than would be seen on a purely-$T_2$-weighted image. 2D FSE images have good signal-to-noise ratio (SNR), contrast between tissues, visibility of cartilage lesions, and visibility of menisci, bone marrow and ligaments [4, 5]. Anisotropic voxels produced by the 2D FSE pose an obstacle to image resolution, and often requires scanning in multiple planes in order to gain high- resolution coverage of the full joint. A 3D version of the FSE sequence has recently been developed, featuring high contrast and isotropic spatial resolution; these developments have resulted in increased accuracy of cartilage imaging. The resulting image data can be reformatted for evaluation of the joint in various planes, and is comparable with multi-planar 2D FSE regarding the evaluation of cartilage, menisci, and ligament. One disadvantage of 3D FSE is that while it has aided the accuracy of cartilage imaging, imaging of the adjacent bone has not similarly improved [6, 7]. Three-dimensional spoiled-gradient-recalled acquisition in steady state (3D SPGR) features higher sensitivity than

2D techniques and is comparable to arthroscopy in the depiction of cartilage defects [8, 9]. These sequences have been primarily used for cartilage volume and thickness quantification [10].

**SEMI-QUANTITATIVE MR GRADING OF KNEE JOINTS FOR OA:** The emerging techniques initially borrowed grading scales used in arthroscopy [11]. Four compartment-based, semi-quantitative systems have been formulated to evaluate MR images of cartilage: the Whole-Organ Magnetic Resonance Imaging Score (WORMS) system was the first. WORMS grading assigns separate scores not only to the various knee cartilage compartments, but also bone, menisci, and ligaments. The system also assesses joint effusion, loose bodies, and periarticular cysts [12]. Like WORMS, the Knee OA Scoring System (KOSS) evaluates cartilage, bone, and menisci, and records the presence and extent of effusion, synovitis, and cysts. KOSS also demonstrated both high inter-observer and intra-observer reproducibility; WORMS made no mention of intra-observer reproducibility [13]. The Boston-Leeds OA Knee Score (BLOKS), assesses the same features as WORMS and KOSS, but describes bone marrow edema-like lesions (BMEL) in further detail [14]. The MRI OA Knee Score (MOAKS) system further refines the rubrics of previous scoring instruments, particularly BLOKS. MOAKS features an altered BMEL scoring method, adds scoring for cartilage sub-regions, and incorporates additional categories of meniscus pathology [15]. Quantitative morphological measures of other features such as bone marrow edema like lesions, meniscal injuries and fractures after anterior cruciate ligament injury have also been quantified, and have been associated with long term evolution into OA.

**QUANTITATIVE IMAGING OF THE ARTICULAR CARTILAGE: *Imaging $T_2$ Relaxation Time:*** The basic premise of MRI is the excitation of protons and their subsequent relaxation back to an equilibrium state; the $T_2$ MRI sequence evaluates the excitation-relaxation phenomenon of water protons with regard to the surrounding proteins. $T_2$ refers to the spin-spin relaxation time. Cartilage is primarily composed of water and proteins such as type-II collagen and proteoglycans (PG). Water protons surrounded by the cartilage matrix undergo interactions with the various macromolecules, which cause faster magnetization decay and a shorter $T_2$. Free water, however, experiences fewer of these interactions, lengthening $T_2$. Using clinically relevant resolutions, $T_2$ studies revealed three laminae in cartilage - a deep layer adjacent to the bone, a superficial layer on the articular surface, and a transitional layer in between [16]. $T_2$ generally increases across cartilage from the bone layer to the articular layer [17, 18]. Thus collagen degradation as seen in OA allows increased movement of free water and $T_2$ has been shown to be elevated in patients with OA [19-21]. Studies using grey level co-occurrence matrix (GLCM) texture analysis of cartilage have shown that $T_2$ is also more heterogeneous in osteoarthritic cartilage than in controls [20]. $T_2$ elevation has also been associated with trabecular bone loss [22] and BMELs [23], and $T_2$ GLCM heterogeneity has been associated with both BMELs and meniscal lesions [20]. $T_2$ also has some degree of predictive power regarding OA. In a cohort with KL and WOMAC pain scores of 0, those determined "at risk" for OA had significantly elevated and heterogeneous cartilage $T_2$ [24]. In addition, $T_2$ at baseline is associated with progression of OA and cartilage defects 2 to 3 years later [23, 25].

**Imaging $T_{1\rho}$ Relaxation Time:** A similar sequence that has recently gained widespread use is $T_{1\rho}$ or spin-lattice relaxation in the rotating frame. The sequence employs a constant, low-power radiofrequency (RF) pulse known as a "spin-lock" in the transverse plane [26-29], which eliminates $T_2$ relaxation. As is the case for $T_2$, $T_{1\rho}$ relaxation is affected when water interacts with large macromolecules. While $T_2$ changes have been associated with collagen concentration and arrangement, biochemical assays suggest that $T_{1\rho}$ is more sensitive to proteoglycan content than to collagen [30, 31] and show that elevated $T_{1\rho}$ is associated with proteoglycan loss [31-34]. Comparisons between $T_{1\rho}$ and $T_2$ have found that $T_{1\rho}$ features superior delineation of cartilage lesions [35], signal-to-noise ratio [35], larger range [36] , higher effect size [36], and greater percentage change with increasing severity of OA [37] as compared to $T_2$. Cartilage $T_{1\rho}$ elevation has also been associated with trabecular bone loss [22], presence and location of BMEL [38] , and higher

WOMAC scores [39, 40]. In addition, elevated baseline $T_{1\rho}$ has been shown to predict OA progression at 2-year follow-up [25].

**QUANTITATIVE MR IMAGING OF TRABECULAR BONE:** MRI is the only imaging method without ionizing radiation to visualize and quantify in-vivo, non-invasively, three dimensional trabecular bone structure. Studies have used MRI to quantify trabecular structure in different regions of the joint to determine whether there are differences in trabecular structure. MR Imaging can be used to quantify apparent bone volume fracture, apparent trabecular thickness, apparent trabecular spacing, and apparent trabecular number, using a spatial resolution on the order of the trabecular thickness. One study by Beuf et al.[41] found differences in trabecular structure between the femur and the tibia in osteoarthritic knees using MRI. It was interesting to note that they also found that the differences in trabecular structure between the two anatomic sites became less pronounced in patients with more severe OA. Lindsey et al.[42] examined patients with OA of the knee using MRI. They found that as cartilage was lost on the medial side of the joint, there was an increase in bone on the medial side of the joint, and a loss of bone on the lateral side of the joint. These results demonstrated the response of bone to OA varies depending on location. The authors suggest that bone responses may be due to joint malalignment. OA can be affected by varus or valgus alignment, which distributes the forces during stance toward the medial and lateral sides of the joint, respectively.

**MR IMAGING OF BONE MARROW EDEMA LESIONS (BMEL)**: In addition to visualizing trabecular bone, MRI is also used to visualize bone marrow edema lesions (BMEL). BMEL do not show up in radiographs or other x-ray based images, but can be visualized using a MRI fat-suppressed SPGR sequence. The water-fat content of the bone marrow edema can also be determined using MR Spectroscopy. Although termed as “edema”, these lesions have shown surprisingly little edema, or accumulation of fluid, based on histopathologic examination as previously reported [43]. Instead, this increase in signal has been attributed to a number of other factors, including abnormal trabeculae, bone marrow necrosis, swelling of fat cells, and marrow hemorrhage. Therefore, it has been recently termed as bone marrow edema pattern (BMEP) or bone marrow edema-like lesions (BMEL). Regarding the association between BMEL and disease severity, Link et al. reported a significant increase of presence of BMEL with increased KL score [44]. Felson et al. discovered a correlation between BMEL and structure deterioration in knee OA, and between BMEL and frontal plane malalignment [45].

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## THE TREATMENT OF OA

OA is a disease that is characterized by loss of the articular cartilage, with remodeling and growth of bone around the joint. As in any chronic disease, changes in the joint biology are usually quite advanced before the patient with OA complains of joint pain with activity. Currently, the only treatments that are available to treat OA include analgesics and anti-inflammatory agents. However, a number of experimental therapies, with many different mechanisms of a action are in development.

This talk with describe the patient population that is targeted to study treatments for knee OA, study methodologies utilized and will review some of the novel agents that are currently in development for the treatment of signs and symptoms of knee OA and for structural modification, disease modifying OA drugs or DMOADs.

**Knee OA (Hip OA study population is similar in inclusion criteria)**

The usual study population for a knee OA study to test an agent that might be useful for either pain or as structure modification

Study population

1 Men and women > 40 years of age
2. Knee pain, most days of the week, > 40 to < 90 on a VAS pain scale or WOMAC pain scale
3. Knee xray Kellgren and Lawrence grade 2 or 3
4. Knee pain despite adequate treatment with analgesic or anti-inflammatory medications

## EXAMPLES OF TREATMENTS THAT ARE IN DEVELOPMENT FOR OA

### Analgesic Compounds

**Anti- nerve growth factor inhibitors:** Arthritis is characterized by pain and inflammation. Recently, attention has been focused on nerve-growth factor (NGF), a neurotrophin that is a key regulator of peripheral nociception because it mediates overexpression of pro-inflammatory neuron-derived molecules such as substance P, serotonin, and calcitonin gene-related peptide. Antibodies have been generated against NGF and its receptor that are effective in reducing pain in preclinical pain models, and clinical trials in patients with advanced knee and hip osteoarthritis and low-back pain. Results show pain reduction is rapid and sustained. Adverse events with anti-NGF included transient paraesthesia, edema, rapidly progressive OA, and, in a small number of patients treated with anti-NGF and nonsteroidal anti-inflammatory drugs, osteonecrosis. Inhibition of the NGF-stimulated nociceptive pathway seems to be effective; however, the adverse effects require further investigation. Recent unpublished Phase 1a and 1b work with oral trka inhibitors will also be discussed.

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## Nutriceuticals (both analgesic and possibly structure modifying)

OA is a disease in which there is no cure, therefore there are many “alternative therapies” that have and are currently being tested. We will review undenatured collagen type II, which is an denatured collagen type II that has been studied for the treatment of painful knee OA and compared to the “gold standard” nutriceutical of glucosamine and chondroitin sulfate.

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## Bisphosphonates for the treatment of knee pain and bone marrow lesions (BMELs) in knee OA

BMELs are known to be associated with knee pain. Interestingly, the presence of BMELs are also associated with juxta-articular cartilage loss. A few studies have evaluated the effects of bisphosphonates, agents that reduce bone turnover, for knee pain, and BMEL size in subjects with knee OA. Although the studies have been of short duration, these potent anti-resorptives agents appear to be effective in reducing knee pain in this subgroup of knee OA subjects. Longitudinal studies are needed to determine if this ant-resorptive agent can alter the course of OA.

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## Wnt Signaling Antagonist.

The Wnt/Beta-catenin signaling pathway is critical for organ formation, repair and homeostasis. A wnt signalling antagonist is in Phase 2 clinical trials for the treatment of signs and symptoms of knee OA. Interestingly, the wnt signaling pathway is critical to both bone and cartilage metabolism. The inhibitor of Wnt now in clinical development, a analog of sfrp3, prevents beta-catenin from translocating to the nucleus of the chondrocyte, and prevents metalloproteinase production, and prevents loss of cartilage. Currently, a molecule, SM04690 is a small molecule Wnt inhibitor in in development for the treatment of OA.

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## FGF18 inhibitor

FGF18 signals through FGF3 to promote chondrogenesis. RCT of rhFGF18 (Sprifermin) found no significant differences between treatments for reduction in central medial FT compartment of cartilage thickness at 12 months. however rhFGF18 related increases in lateral FT cartilage thickness, volume and JSW narrowing were observed. POST-HOC analysis found an increase in Total cartilage thickening sum scores and decreased Total cartilage thinning sum scores vs PLACEBO. These results suggest a need for both subject-specific and location independent analysis of both cartilage thickening and cartilage thinning for assessing effects of DMOADs in the future.

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# Updates on Nutritional Influences on the Musculoskeletal System

Bess Dawson-Hughes, M.D.

**Updates on Nutritional Influences on the Musculoskeletal System**

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**Significance of the topic:**

Osteoporotic fractures result in significant disability and decreased quality of life; hip fractures contribute significantly to long-term disability, institutionalization, and mortality. Public health strategies that address risk factors for and early detection of OP are urgently needed to improve quality of life among aging adults. This session will address the role of diet in preserving bone and muscle mass and function in older adults.

**Learning objectives:**

As a result of participating in this session, attendees should be able to:

1. Identify the intake requirement for calcium and the basis for its selection

2. Understand the effects of vitamin D on muscle performance and on risk of falls and fractures

3. Identify the role of dietary protein in bone and muscle metabolism, its impact on acid-base balance, and the implications of acid-base balance of the diet for bone and muscle.

**Discussion:**

Calcium is required for bone formation. Inadequate intake results in an increase in parathyroid hormone levels and a concomitant increased bone resorption and bone loss. Balance studies are used to identify the intake above which more calcium does not increase total body calcium retention (equivalent to calcium retention in bone). The IOM based intake recommendations [1] are shown in the following table:

| Age | Gender | Calcium RDA (mg/day) | Vitamin D RDA (IU/day) |
|---|---|---|---|
| 51-70 years | Female | 1,200 | 600 |
| | Male | 1,000 | 800 |
| >70 years | Female | 1,200 | 600 |
| | Male | 1,200 | 800 |

The IOM identifies 2,000 mg per day as the safe upper limit. Calcium is integrally involved in muscle contraction, but evidence that calcium intake influences muscle performance is limited. Similarly, evidence that calcium alone lowers fracture rates is limited. It is more commonly used in combination with vitamin D, and the combination is effective (see below).

Vitamin D adequacy is variably defined as a circulating 25-hydroxyvitamin D [25(OH)D] level of ≥ 50 nmol/L (20 ng/ml) and 75 nmol/L (30 ng/ml). The IOM recommends a minimal value of 50 nmol/L for the general population whereas specialty societies that focus on osteoporosis generally recommend a minimal value of 75 nmol/L for their patients. The intakes recommended by the IOM are shown in the table above.

Vitamin D is important for muscle performance and inadequate vitamin D status has been linked to increased risk of falling, although the evidence is not consistent. In a 2,009 meta-analysis of vitamin D intervention trials, intakes of 700 – 1000 IU of vitamin D per day reduced risk of falling whereas lower doses (200 – 600 IU per day) had no effect [2]. Emerging evidence also suggests that the benefit from supplementation is inversely proportional to the starting 25(OH)D level of the individuals.

A recent individual subject level meta-analysis of randomized controlled vitamin D intervention trials in 31,022 men and women, mean age 76 years and 91% women addressed whether vitamin D intake influenced risk of hip or other fracture [3]. In this analysis, there were 1,111 hip fractures and 3,770 nonvertebral fractures. Vitamin D had no significant effect on hip fracture risk overall (HR 0.93; 95% CI 0.87 to o.99). However, when examined by quartile of intake during the trial (calculated as the administered dose x proportion consumed), fracture risk was reduced only in those in the highest intake quartile (median intake 800 IU per day, range 792 to 2,000 IU per day). Findings related to non-vertebral fractures were similar. Sanders and others have reported that higher doses of vitamin D are associated with increased risk of fracture (and falling) [4].

Protein stimulates the production of IGF-1 which promotes bone and muscle growth. It increases calcium excretion, but also increases calcium absorption. The net effect is to improve calcium balance and lower rates of bone loss. Its effect on bone mineral density is enhanced by an adequate calcium intake. The RDAs for protein are 46 gm per day for women and 56 gm per day for men age 51 years and older. Others recommend higher intakes of 1.1 gm/kg of body weight/day.

Acid-base balance is important for muscle and bone. When foods are absorbed and metabolized, the pH of their residue influences overall acid-base balance. Dietary protein and cereal grains add acid in proportion to their content of sulfur, which is metabolized to sulfuric acid. Fruits and vegetables on the other hand are metabolized to the alkali, bicarbonate. A dietary imbalance in favor of acid production has been shown to increase nitrogen excretion (consistent with muscle wasting) and to increase bone turnover (consistent with bone loss) in older adults. Treatment with alkali in the form of potassium bicarbonate or potassium chloride has been shown to reduce bone turnover and calcium excretion. Two trials have assessed the impact of alkaline salts of potassium on change in bone density. One showed a favorable effect on bone mass and architecture, and the other was null. More work is needed to define the role of acid-base balance on bone and muscle.

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# Using Medicare Claims Data to Study Fracture Epidemiology

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# Using Medicare Claims Data to Study Fracture Epidemiology

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Studies with fracture outcomes increasingly rely on Medicare claims to ascertain fracture. For large studies, it may be impractical and cost-prohibitive to ascertain fracture by any other means. Despite the reliance on Medicare claims data to ascertain fracture, there is no one, uniform definition of fracture using Medicare claims data. Published validation studies that have compared a definition of fracture from Medicare claims with a definition of fracture using chart review are largely outdated.

As a result of this session, leaners will be able to:

1. Understand the components of Medicare Claims Data that can be used to define fracture including Medicare Parts A and B, International Classification of Diseases, ninth edition (ICD-9) codes, and Current Procedural Terminology (CPT) codes.
2. Identify current claims-based algorithms to identify fracture and review results of a current validation study.
3. Appreciate the occurrence of misclassification that can be introduced when different definitions of fracture are used and when diagnostic and procedural codes are inconsistent.

**Definitions**

*Medicare Part A* - inpatient hospital claims
*Medicare Part B* - outpatient or provider claims
*Medicare Part C* – Medicare Advantage
*Medicare Part D* – prescription drug plan

*International Classification of Diseases, ninth edition (ICD-9) codes* - diagnosis and procedural codes that can be used to identify fractures in Medicare Part A and B claims; maintained by the World Health Organization

Examples: 820.xx fracture of the proximal femur
81.51 total hip replacement

*Healthcare Common Procedure Coding System (HCPCS)* – system of codes designed to report medical procedures and services by the Centers for Medicare and Medicaid (CMS)

Level 1: Current Procedural Terminology (CPT-4) codes – procedural codes developed by the American Medical Association that can be used to confirm diagnosis of fracture in Part B claims
Example: 27125 – hemiarthoplasty, hip, partial

Level 2: Alphanumeric codes for non-physician services; not used in fracture definition
Example: E0149 - Walker, heavy duty, wheeled, rigid or folding, any type

**Table 1. Example Algorithms to Identify Fractures in Claims Data**

| Fracture site ("case" ICD-9 codes)* | Additional case-qualifying requirements |
|---|---|
| **Hip** - (substr3=820, 73314)<br>V54.13, V54.23, V54.14, V54.24, V54.15, V54.25 | [**Inpatient** primary or secondary diagnosis code in ("case")] [CQ=1 or 2] **OR** [**Carrier line or outpatient claim** with HCPCS in (27230-27248) (Repair code) **OR** diagnosis code in **(**"case") and HCPCS in (27125, 27130) (Hip Replacement) **OR** diagnosis code in **(**"case") and ICD-9 Procedure in (81.51 81.52) (Hip Replacement) **OR** diagnosis code in **(**"case") and ICD-9 Procedure in (78.55 79.05 79.15 79.25 79.35 79.65) (Femur repair)] [CQ=3] |
| **Spine**- (substr3 in (805, 806), 73313)<br>V54.17, V54.27 | [**Inpatient primary** diagnosis code in ("case")] [CQ=1] **OR** [**Carrier line or outpatient** claim with HCPCS in (22520, 22521, 22522, 76012, 76013, 22305, 22310, 22315, 22318, 22319, 22325, 22326, 22327, 22328, 22523, 22524, 22525)] [CQ=3] **OR** [**Carrier line or outpatient** claim with HCPCS in Physician E&M codes† and diagnosis in ("case") plus, up to 10 days earlier, Carrier line or outpatient revenue center claim with HCPCS in (72010-72159, 72240-72295)(Imaging)][CQ=4] |
| **Radius/ulna (forearm)** (substr3(813), 73312)<br>V54.10, V54.20, V54.12, V54.22 | [**Inpatient** primary or secondary diagnosis code in ("case")] [CQ=1 or 2] **OR** [**Carrier line or outpatient** claim with HCPCS in (25600, 25605, 25611, 25620, 25650, 25651, 25652, 24650, 24655, 24665, 24666, 24670, 24675, 24685, 25500, 25505, 25515, 25520, 25525, 25526, 25530, 25535, 25545, 25560, 25565, 25574, 25575 (includes ulnar styloid))] [CQ=3] |

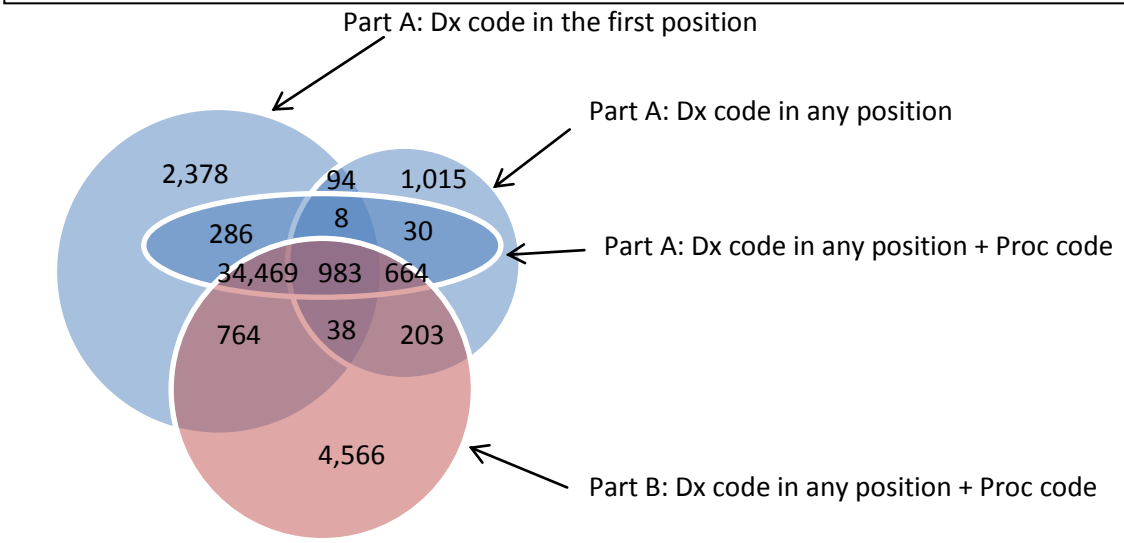
**Table 2. Positive Predictive Value of Medicare Claims-based Fracture Algorithms[1-4]**

| Fracture | PPV (%) |
|---|---|
| Hip | 98 |
| Spine | 61-74 |
| Distal Radius/Ulna | 96 |
| Humerus | 95 |
| Pelvis | 93 |
| Femur | 87 |

## Important Questions and Caveats when Working with Administrative Data Related to Fracture Identification

- What qualifies people for enrollment?
- Does the reason for qualification of enrollment potentially impact ability to ascertain fractures?
- Extent of follow-up and reasons for dis-enrollment
- Missing-ness of potentially important data (e.g. physician speciality; prescriber / provider IDs)

**Figure 1. VIN Diagram illustrating the overlap in fractures identified according to different claims based definitions of fracture**




**Inconsistencies between diagnostic and procedural codes**

We identified 227 fractures with a procedural code for hip fracture and a diagnostic code for a contiguous fracture site (e.g., pelvic and femoral shaft)

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# Utility and Limitations of TBS in Fracture Risk Assessment

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# Trabecular Bone Score (TBS) in Fracture Risk Assessment

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**Significance of the Topic:**

Trabecular bone score (TBS) is a gray-level texture measure extracted from lumbar spine dual energy X-ray absorptiometry (DXA) images, and provides skeletal information beyond standard bone mineral density (BMD) measurement. There has been a flurry of scientific and clinical evidence around the development, validation and clinical application of lumbar spine TBS. Cross-sectional studies, longitudinal studies and a recent meta-analysis of multiple prospective cohorts have demonstrated that reduced TBS identifies increased fracture risk. Moreover, lumbar spine TBS was shown to be a risk factor for osteoporotic fracture and also for risk of death independent of FRAX clinical risk factors and femoral neck bone mineral density (BMD).

Guidance documents from the International Society for Clinical Densitometry (ISCD) and European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) reached similar conclusions that TBS had predictive value for fracture independent of fracture probabilities obtained using the FRAX algorithm (1,2). An expanding potential role in certain forms of secondary osteoporosis was highlighted. Despite many positive reports, there is ongoing confusion around what TBS actually measures, how best to use TBS in clinical practice and for which patients it provides the greatest value. This presentation will highlight recent developments, strengths and known limitations in TBS.

**Learning Objectives:**

<u>As a result of participating in this session, attendees should be able to</u>:
1. Describe the major studies that have examined the ability of TBS to predict fractures.
2. Describe how TBS is used to adjust fracture probability.
3. Describe when TBS has the greatest clinical impact on clinical management.

**Outline:**

*What were the early technical and clinical studies related to TBS?*

TBS arose out of the desire to extract structural information on 3D bone morphology from 2D projection-based images provided by DXA (3). Specifically, TBS is defined as the slope at the origin in the log-log representation of the variogram expressed as variation in gray levels (Y-axis) versus pixel distance per mm (X-axis) as evaluated from least squares regression. The slope at the origin reflects the "roughness" in gray levels: the lower the slope, the greater the regularity (homogeneity) in gray levels. TBS evolved into a commercial product (TBS iNsight®, Med-Imaps, France) and cleared for clinical use in the United States in 2012 with the following labeling: "TBS is derived from the

texture of the DEXA image and has been shown to be related to bone microarchitecture and fracture risk. This data provides information independent of BMD value… The TBS score can assist the health care professional in assessment of fracture risk..."

Cadaveric and ex vivo studies correlations with microstructure: (3-6) (detractors (7-9))

In vivo correlations with microstructure: (10-13) (detractor (14))

Cross-sectional fracture studies: (15-21)

Longitudinal fracture prediction studies: (22-29)

International meta-analysis: (30)

*How has the TBS algorithm changed?*

The importance of soft tissue composition was highlighted in early investigations of TBS. The original TBS algorithm had been optimized for women, and paradoxically gave lower TBS measurements in men than women despite the fact that men have higher BMD, lower fracture risk and would be expected to have a less degraded trabecular structure (31). The presumed mechanism is that image texture tends to degrade (decrease) with increasing adiposity. As adiposity in men tends to be more abdominal/truncal than in women, a simple TBS adjustment based upon BMI would underestimate the effect of abdominal adiposity on the TBS measurement in men. The Manitoba BMD Database was used to confirm that men had lower TBS measurements after age and BMI matching, but not after abdominal tissue thickness and percent fat matching.

The TBS algorithm was modified in version 2.x to address these technical issues, and became applicable to both women and men (32). Under TBS version 2.x for GE/Lunar scanners, the BMD dependency was eliminated (men r = 0.01, women r = -0.01). However, this BMI dependence has not yet been fully addressed with Hologic devices. The manufacturers of TBS software recommend that TBS not be used in individuals with BMI outside of the 15 – 37 $kg/m^2$ range.

*How is TBS accommodated in the FRAX algorithm?*

In 2015, a method for adjusting FRAX probability measurements based upon lumbar spine TBS which also includes the effect of competing mortality was developed (33):

Hip fracture: 10-year probability calculated with TBS is $100 / (1+e^{-W})$

where $W = 15.420 - 12.627 \times TBS - 0.194 \times age + 0.157 \times TBS \times age + 0.920 \times L$, $L = -\ln(100/p - 1)$, p is the 10-year probability calculated without TBS.

Major Osteoporotic Fracture: 10-year probability calculated with TBS is $100 / (1+e^{-W})$

where $W = 5.340 - 4.213 \times TBS - 0.0521 \times age + 0.0393 \times TBS \times age + 0.897 \times L$, $L = -\ln(100/p - 1)$, p is the 10-year probability calculated without TBS.

This adjustment contains a "TBS x age" interaction term which reflects the declining strength of the TBS adjustment on FRAX with increasing age.

*When does TBS have the greatest clinical impact on clinical management?*

Using a population-based DXA registry, we identified 34,316 women with baseline spine and hip DXA, FRAX-based fracture probability measurements (computed with femoral neck bone mineral density), blinded lumbar spine TBS, and minimum 5 years of observation (ASBMR 2015). Population-based health services data were used to identify non-traumatic MOF and HF. MOF and HF probability were estimated using FRAX before and after applying the TBS adjustment. Risk re-categorization was assessed using net reclassification improvement (NRI) for individual FRAX-based intervention criteria and three national clinical practice guidelines (CPGs) (US National Osteoporosis Foundation [NOF], Osteoporosis Canada, UK National Osteoporosis Guideline Group [NOGG]). Overall proportions of women reclassified with the TBS adjustment to FRAX were small (less than 5%), but for women close to an intervention cut-off reclassification rates were much higher (range 17.5% to 25.3%). There was a small but significant improvement in overall NRI for all individual FRAX-based intervention criteria (range 0.007 to 0.018) and all three national CPGs (range 0.005 to 0.011).

**Clinical Pearls (from the ISCD Official Positions (1)):**

- TBS is associated with vertebral, hip and major osteoporotic fracture risk in postmenopausal women.
- TBS is associated with hip fracture risk in men over the age of 50 years.
- TBS is associated with major osteoporotic fracture risk in men over the age of 50 years.
- TBS should not be used alone to determine treatment recommendations in clinical practice.
- TBS can be used in association with FRAX and BMD to adjust FRAX-probability of fracture in postmenopausal women and older men.
- TBS is not useful for monitoring bisphosphonate treatment in postmenopausal women with osteoporosis.
- TBS is associated with major osteoporotic fracture risk in postmenopausal women with type II diabetes.

**Cases with questions:**

For a woman age 75 years with FRAX major fracture probability 15% and hip fracture probability 2%, what level of TBS would be required to exceed the National Osteoporosis Foundation (NOF) treatment thresholds of 20% and 3%, respectively?

How does this change for a woman age 50? Or FRAX major fracture probability 10% and hip fracture probability 1%?

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# Sequential and Combination Therapy for Osteoporosis: Where Are We Now ?

Felicia Cosman, M.D.

**Sequential and Combination Therapy for Osteoporosis: Where are we Now?**

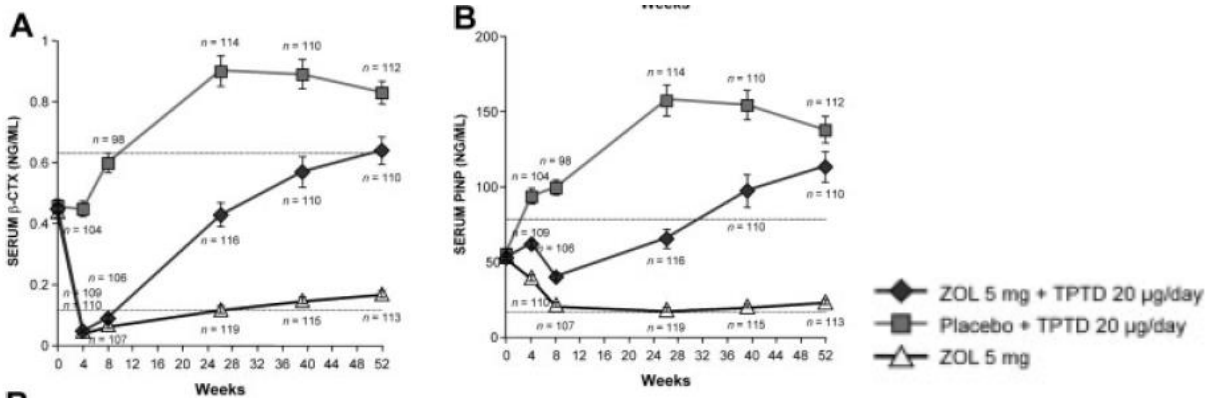
**Felicia Cosman**
Helen Hayes Hospital, West Haverstraw, NY
Columbia University, New York, NY

**Significance:**
The one currently available anabolic agent for osteoporosis (in the US), teriparatide, is used for a maximum of 2 years. All patients with osteoporosis will need treatment for a longer period and gains from teriparatide will be lost in the absence of subsequent antiresorptive therapy. The sequence of anabolic followed by antiresorptive therapy appears to produce a greater BMD response than the sequence of potent antiresorptive followed by anabolic therapy. In patients who have already been treated with potent antiresorptive medications, there may be a role for combination therapy.
For a smaller group of treatment-naïve patients with severe osteoporosis, there may also be a limited role for de novo combination therapy.

**Learning Objectives:**
As a result of participating in this session, attendees should be able to:
1. Cite the major studies investigating treatment sequences and combinations.
2. Understand the major conclusions from the combination and sequential trials.
3. Understand the types of patients where combination therapy could be considered.

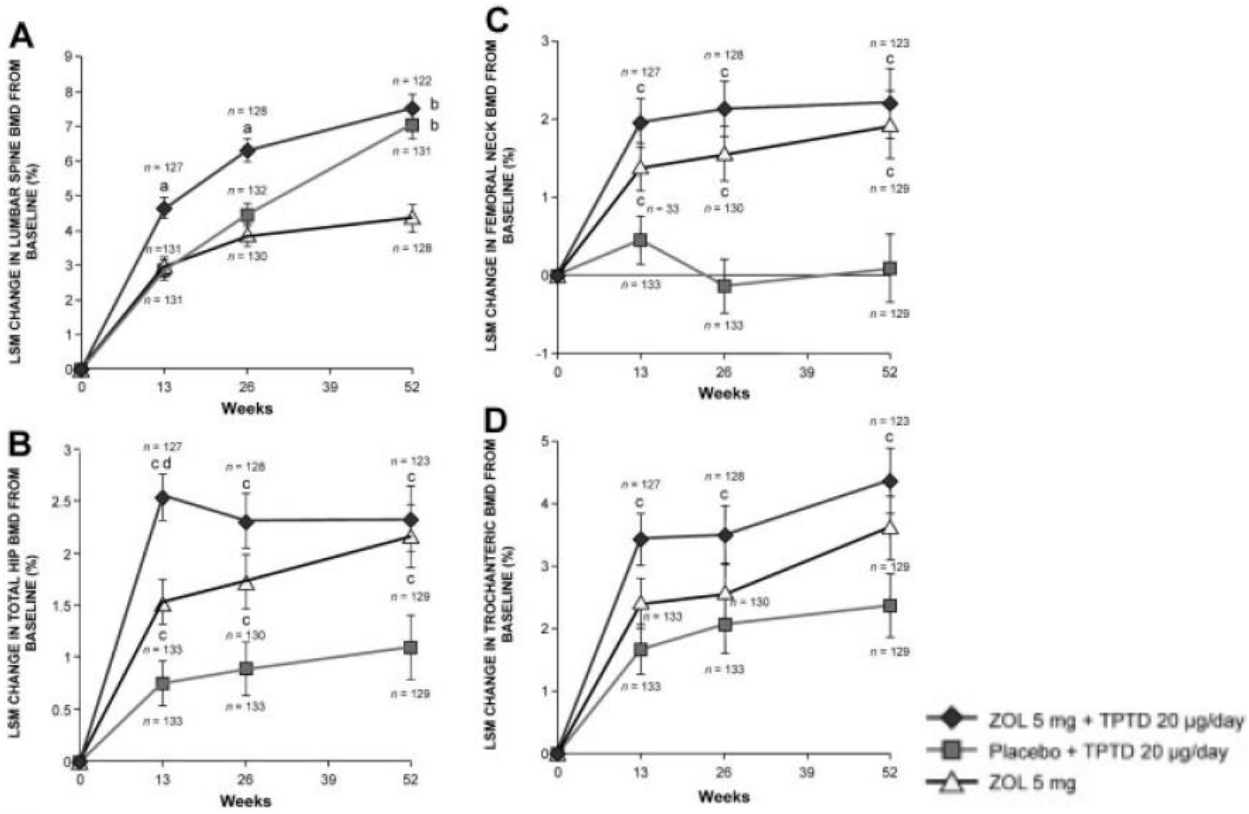
**Key Studies of Combination Therapy in Treatment Naïve Women:**
1. Zoledronic Acid (ZOL) vs Teriparatide (TPTD) vs Combination Therapy[1]
   412 treatment-naïve postmenopausal women randomized to:
   - IV Zoledronic Acid 5 mg
   - IV Placebo + Teriparatide 20 µg SC daily
   - Both Zoledronic Acid and Teriparatide
2. Denosumab (DMAB) vs Teriparatide vs Combination Therapy[2]
   94 postmenopausal women, primarily treatment naïve, randomized to:
   - Teriparatide 20 µg SC daily
   - Denosumab 60 mg SC every 6 months
   - Both Teriparatide and Denosumab

**Serum Levels of Biochemical Turnover Markers (ZOL vs TPTD vs Combination)**



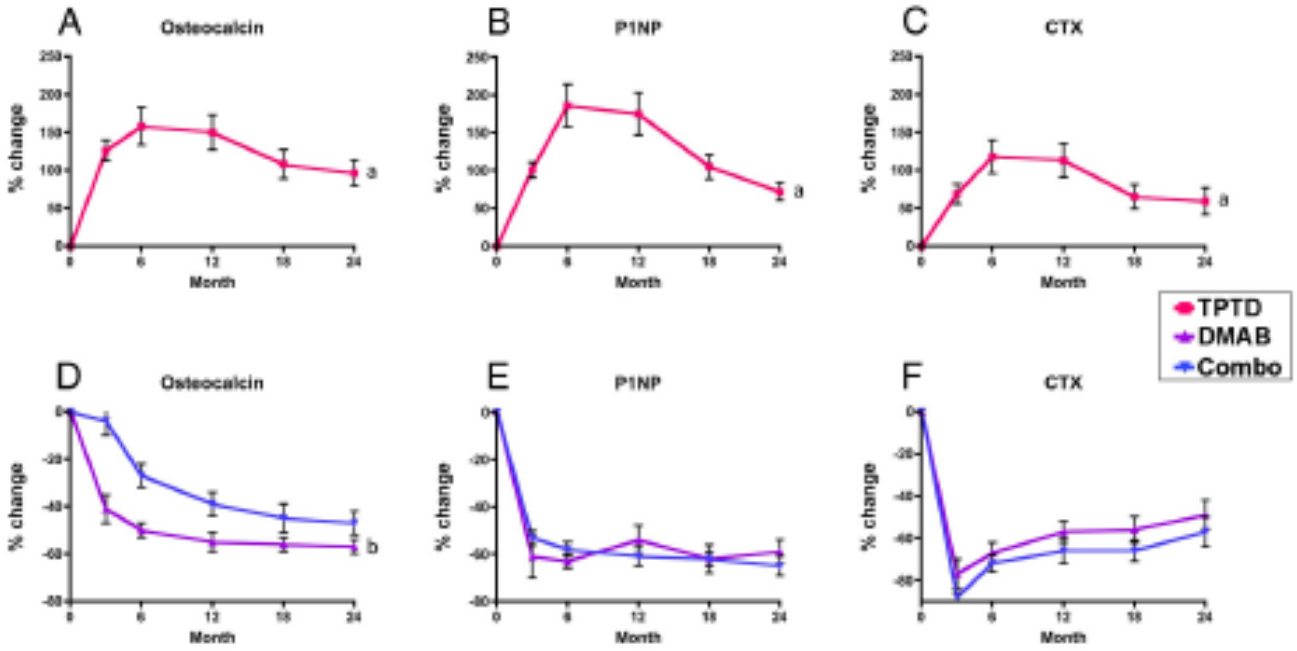

Cosman, et al. JBMR 2011; 26(3):503-511

## Percent Change in Bone Density Spine and Hip (ZOL vs TPTD vs Combination)



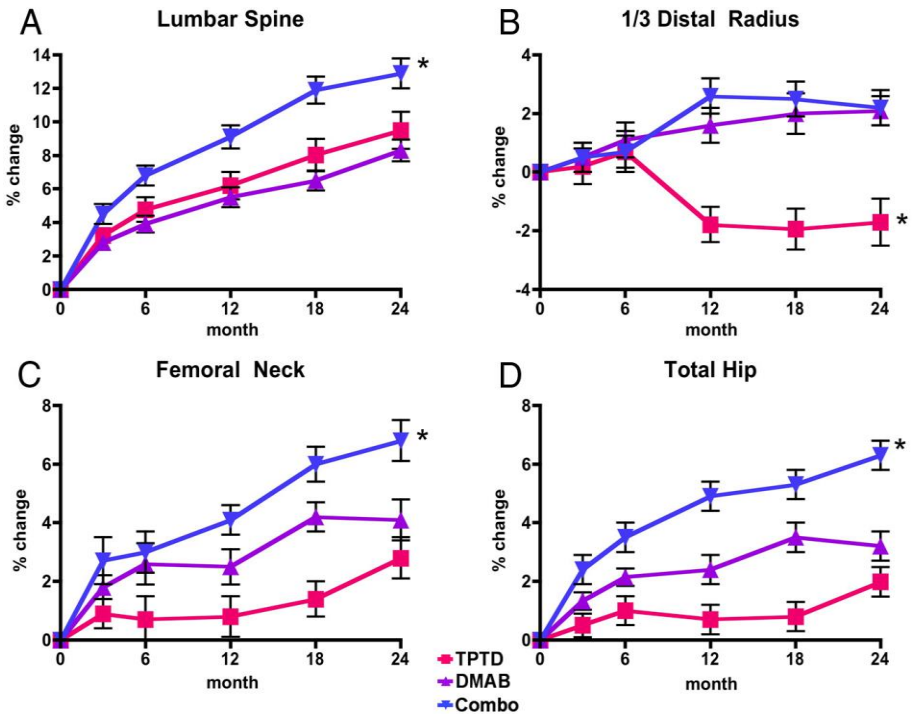

Cosman, et al. JBMR 2011; 26(3):503-511

## Percent Change in Biochemical Turnover Markers (DMAB vs TPTD vs Combination)




Leder, et al. JCEM 2014; 99(5):1694–1700

**Percent Change in BMD of the Spine, Hip and Radius (DMAB vs TPTD vs Combination)**




Leder, et al. JCEM 2014; 99(5):1694–1700

**Summary of Treatment Naïve Combination Therapy Study Findings:**

- Both TPTD+ZOL and TPTD+Dmab produce hip BMD gains greater than TPTD monotherapy
  - Similar conclusions for PTH plus ALN vs PTH alone
- TPTD+Dmab also produces spine BMD gains greater than TPTD alone

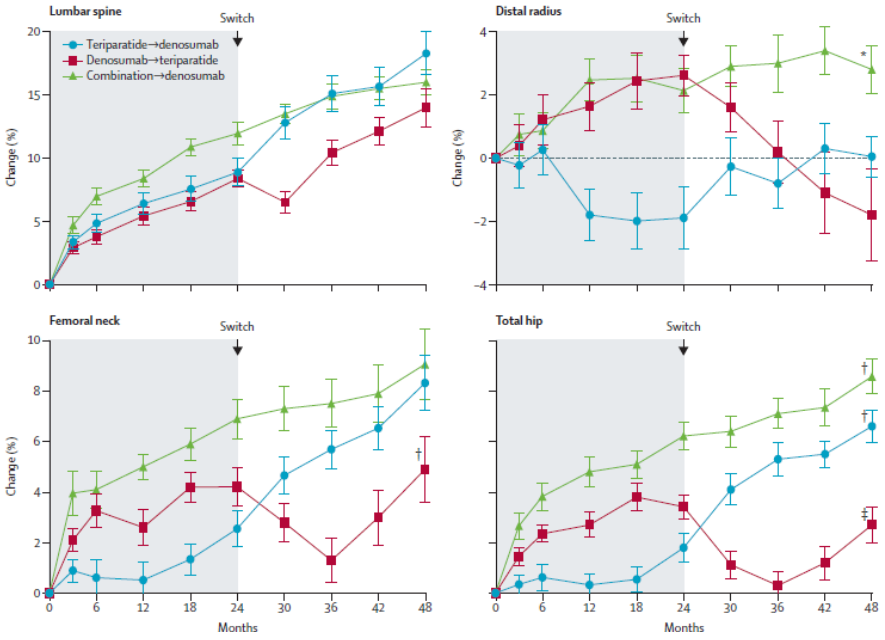
**Key Studies of Combination Therapy in Women on Prior Antiresorptive Therapy:**

In women on prior alendronate or risedronate who switch to teriparatide or PTH:

Hip BMD drops below baseline for at least 12 months.[3-6]

In women on prior denosumab,

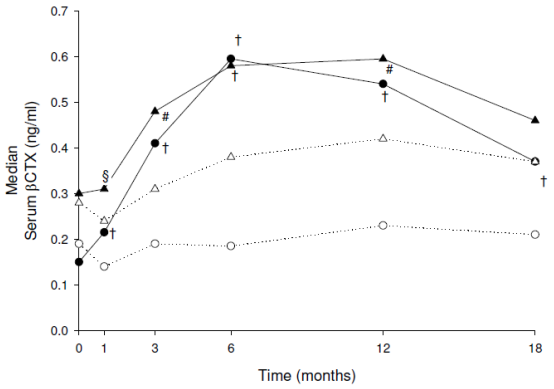
Hip BMD declines and remains below baseline for 2 years (DATA-Switch study).[7]



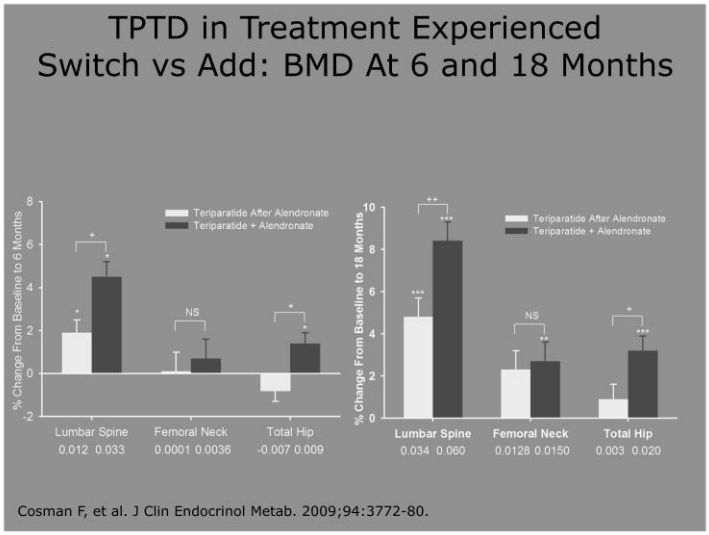

Leder, et al. Lancet 2015; 386: 1147–55

**ADD vs SWITCH Study in Women on prior alendronate**:[6,8]

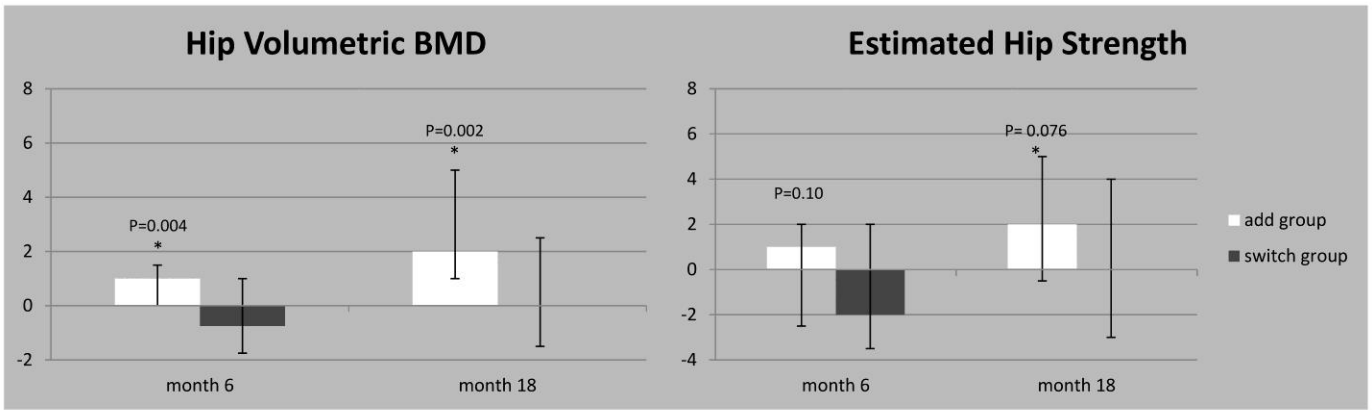
- Compares the effect of Adding vs Switching to TPTD in women on prior ALNin a randomized trial
- Subjects were postmenopausal women ≥ 50 years old
  - On Aln (n=102) for ≥ 18 months (mean > 4 yrs)
- Randomized to continue Aln and ADD TPTD or stop Aln and SWITCH to TPTD



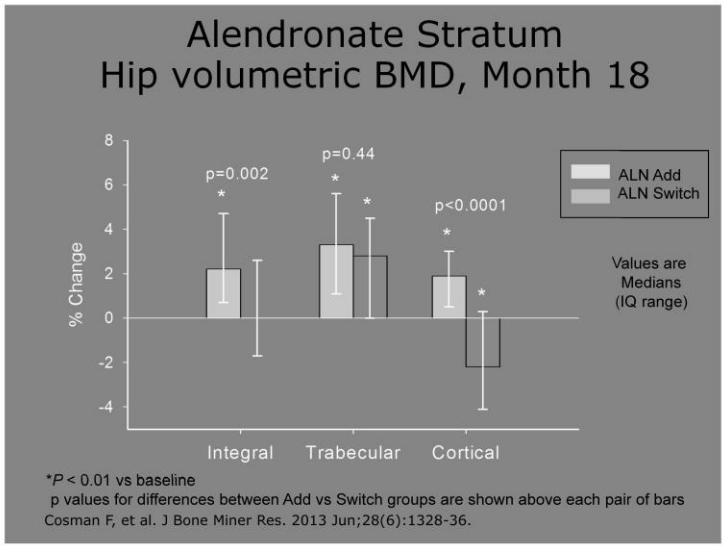

Cosman, et al. JCEM 2009; 94(10): 3772–3780



## Alendronate Stratum Add vs Switch at 6 and 18 months.




*p<0.01 vs baseline p values for differences between add vs switch groups are shown above each pair of bars
Cosman F, et al. J Bone Miner Res. 2013 Jun; 28(6):1328-36




**Summary of Findings in Treatment Established Patients:**

- Switching from potent bisphosphonate therapy to TPTD results in temporary decline in hip BMD
  - Adding TPTD to ongoing bp therapy improves the hip BMD outcome (areal DXA BMD and volumetric BMD by QCT)
- Switching from denosumab therapy to TPTD results in a 2 year decline in hip BMD
  - Adding TPTD to ongoing Dmab therapy might be an effective approach in these patients

**Cases:**

78 year old woman on alendronate for 4 years for osteoporosis by BMD criteria trips and falls, sustaining a left femoral neck fracture.
Spine BMD t-Score -3 (L1-L3), Right FN t-Score -3.2, Right TH t-Score -2.8
What is the best treatment option now?

70 year old woman has her first BMD test.
Vertebral imaging reveals 1 moderate compression fracture at T8
FN t-Score -1.8

70 year old woman has her first BMD test.
Vertebral imaging reveals 1 moderate compression fracture at T8.
FN t-Score -3.5

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# Following up GWAS Findings - From the Dry Lab to the Wet Lab

Brent Richards, M.D., M.Sc.

Matthew Maurano, Ph.D.

# Following up GWAS findings - From the Dry Lab to the Wet Lab
# Meet the Professor Session
# ASBMR 2016
# Saturday, September 17 from 11:00 am - 12:00 pm

Brent Richards, MD, MSc
Associate Professor of Medicine
Departments of Medicine, Human Genetics, Epidemiology and Biostatistics
McGill University
Senior Lecturer, King's College London (Honorary)

Matthew Maurano, PhD
Assistant Professor of Pathology
Institute for Systems Genetics and Department of Pathology
NYU Medical Center

**Significance of the Topic**
Osteoporosis is a highly heritable condition, which is controlled to a large extent by the genetic factors. Bone mineral density (BMD) is the most clinically used risk factor for fracture and is likewise highly heritable.

Understanding the genetic determinants in humans of osteoporosis will provide insights into the pathophysiology of this disease for six main reasons:

1. First, the data derived from human genetics is from humans and does not rely on a model system. This is not to say that model systems are not highly relevant, but beginning with human genetics findings increases the probability that the resultant findings will be relevant to human disease.
2. Second, modern human genetics takes an agnostic approach, by which we mean that results are data-driven and not dependent upon prior assumptions of the relative importance of any gene. This is helpful because unanticipated insights can arise.
3. Third, it is possible to identify *causal* genetic variants.
4. Fourth, human genetic associations are not confounded by other risk factors, except for the potential of confounding by ancestry (also called population stratification). However, this limitation can easily be overcome by ensuring that individuals within a sample are of the same ancestry.
5. Fifth, human genetic variation can be measured with near-perfect accuracy.
6. And last, human genetic findings are not influenced by reverse causation, where the disease itself modifies the risk factor.

These strengths of human genetics have enabled the field to identify dozens of regions of the genome that strongly influence BMD and/or fracture. Importantly, we have shown that these regions contain nearly all of the genes that act as targets for clinically useful osteoporosis therapies and that evidence supporting osteoporosis drug targets from human genetics more than doubles the probability of drug development success. This suggests that amongst the dozens of novel loci identified are high-yield drug targets for clinical care.

Through large-scale international collaboration we have identified dozens of novel regions of the genome that influence BMD, and some of these influence fracture risk. These regions are highly enriched for the known drug targets for osteoporosis therapies and therefore, the novel regions we have identified will likely also contain novel drug targets for the treatment of osteoporosis.

Despite these strengths of human genetic studies major hurdles remain in identifying the causal gene at an associated locus. This Meet The Professor Session will outline some of the challenges of translating human genetic findings by describing limitations of human genetics studies, common pitfalls, and then focus on methods to map associations to causal genetic variation and potentially causal genes.

**Learning Objectives:**
As a result of participating in this session, attendees should be able to:

1. Understand the basic concepts of a genome-wide association study
2. Understand the basic concepts of a whole genome sequencing study
3. Understand the irrelevance of effect size of genetic variants for most purposes
4. Describe common pitfalls of these studies and how they can be overcome
5. Understand the importance of non-coding genetic variation and the relevance of DNase I hypersensitive site maps to mapping causal associations.
6. Understand the importance of cell-specificity of regulatory DNA activity and transcription factor binding.
7. Understand common animal model systems, the utility of large-scale knock out programs and the resources available to the community to use these knock outs.

**To achieve these learning objectives will have host a structured discussion on the following topics with visual slides to demonstrate salient points.**

**1.1 GWAS:**
We will discuss the basic concepts and statistical methods in a GWAS.
We will discuss the relevance of sample size and strict statistical significance thresholds to decrease the probability of false positives.
We will discuss common pitfalls in the interpretation of GWAS findings.
What is imputation and can it be relied upon?

**1.2 Whole-Genome Sequencing**
What is whole-genome sequencing?
What are the metrics that I should use to test whether a genetic variant identified by whole genome-sequencing is real?
What are the anticipated effect sizes that can be identified from low frequency and rare genetic variants in the general population?
What are common pitfalls in WGS?

**1.3 Effect Size of Genetic Variants**
The effect sizes of common genetic variants are small. However, contrary to many study paradigms, the effect size is irrelevant for most of the aims of human genetics. Why is this?
When are large effect sizes helpful?

**1.4 The Importance of Non-Coding Variation**
The majority (95%) of variants associated with common human diseases and traits lie in regions of the genome that do not code for protein. This means that they cannot be analyzed in terms of their effect on protein structure and function, but rather in terms of how they affect gene expression.

**1.5 Regulatory Element Accessibility to DNase I**
All known classes of regulatory elements manifest accessibility to the endonuclease DNase I. Projects including ENCODE and the Roadmap Epigenetics have used high-throughput sequencing to map biochemical signals of regulatory activity genome-wide and generate maps of regulatory DNA for a wide variety of cell types. These maps show that most non-coding associations localize to regulatory elements from specific cell types. We discuss how to access and analyze these maps and integrate them with GWAS results.

**1.6 Genetic Variants and Transcription Factor Binding**
Understanding how non-coding sequence variation affects gene expression is important both for fine mapping an association signal to specific genes as well as for understanding the underlying biology. Non-coding sequence variation likely acts by altering the binding of transcription factors, whose binding is responsible for control of gene expression. We discuss practical methods for analyzing the effect of GWAS variants on TF binding.

**1.8 Credible Sets**
Fine mapping and statistical frameworks for treating uncertainty in attributing association signals to specific variants.

**1.7 Animal models: Highly relevant and Reasonably Available**
We will discuss mouse knock out consortia and the availability of data from these experiments
We will discuss availability of ES cell lines for knock outs.
We will briefly mention appropriate first-pass screening of animal knock outs for bone phenotypes.

**Cases**

**WNT16: A novel gene from a GWAS** (reference: Zheng et al, PLOS Genetics 2012, PMID: 2279207)

**EN1: A novel gene from whole-genome sequencing (reference: Zheng et al, Nature PMID: 26367794)**

## References[1–7]


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# Progenitors for Bone Growth and Repair

Henry Kronenberg, M.D.

Noriaki Ono, D.D.S., Ph.D.

***Progenitors for Bone Growth and Repair***

Noriaki Ono (University of Michigan School of Dentistry, USA)

Henry M. Kronenberg (Massachusetts General Hospital and Harvard Medical School, USA)

**Significance of the Topic:**

Bones grow explosively in early life and maintain their strength throughout life. Bones also possess amazing capabilities to repair – the bone is like new without a scar after complete repair. The current hypothesis is that distinct bone progenitor cells initiate growth and repair; these cells might also contribute to maintenance. In recent years, we have seen a substantial progress in this field – mouse genetic models have been proven as powerful tools to delineate the cell lineage in the native environment. In this session, we will review recent findings in this field, and discuss potential areas requiring further investigations.

**Learning Objectives:**

As a result of participating in this session, attendees should be able to learn up-to-date concepts about bone progenitor cells, and know how current methodologies can be useful for understanding bone biology in a broader context.

1. Bone marrow stromal/stem cells (BMSCs) / mesenchymal stem cells (MSCs) / skeletal stem cells (SSCs) & colony-forming unit fibroblasts (CFU-Fs): A historical background.
2. *In vivo* lineage-tracing experiments using a tamoxifen-inducible *CreER* system & their application to bone cell lineages: Implications, limitations and cautions.
3. How many different types of bone progenitor cells are there in bones? How do they facilitate bone growth and repair?

**Outline – Points of Interest:**

*1. Colony-forming unit fibroblasts (CFU-Fs) assay (or equivalent assays) as a standard for bone progenitor cells.*

*Pros:* Analysis of single cell-derived clones of "stem cells" using culture systems.

*Cons:* Information about the location of these cells *in situ* is lost upon culturing. Impossible to visualize stem cell behaviors in the native setting – these cells can be assayed only upon *ex vivo* cell culture and/or ectopic transplantation.

*Adult:* **CD146$^+$ BM stromal cells** (Sachetti B et al, 2007)

**PDGFRα$^+$Sca1$^+$ non-hematopoietic cells (PαS cells)** (Morikawa S et al, 2009)

***Nestin*-GFP$^+$ BM cells** (Mendez-Ferrer S et al, 2010)

*Growth:* **Mouse skeletal stem cells (mSSCs)** (Chan CK et al, 2015):
CD51$^+$CD90.2$^-$CD105$^-$CD200$^+$

*2. In vivo lineage-tracing studies using CreER system (or equivalent inducible Cre systems).*

*Pros:* Fates of a particular group of cells can be traced in the native setting.

*Cons:* Heterogeneity of cell populations of interest marked by a promoter-based approach complicates overall interpretation. (Is your promoter only marking cells that you want?)

*Adult:* ***Nestin-creER*$^+$ BM cells** (Mendez-Ferrer S et al, 2010)

***Mx1-cre*$^+$ (*) BM cells** (Park D et al, 2012) **pIpC (IFN)-inducible*

***LepR-cre*$^+$ (**) mesenchymal stromal cells** (Zhou BO et al, 2014)

***There is not yet a creER line available to rigorously confirm these findings.*

Growth: ***Grem1-creER*$^+$ BM perisinusoidal cells** (Worthley DL et al, 2015)

***Sox9-creER/Acan-creER/Col2a1-creER*$^+$ cells** (Ono N et al, 2014)

*3. The potential sources/origins of progenitor cells in growth and repair of endochondral bones:*

a. Mesenchymal condensations (development)

***Sox9-cre*$^+$ osteochondroprogenitor cells** (Akiyama H et al, 2005)

b. Cartilage template/growth plate/borderline chondrocytes (development)

***Sox9-creER/Acan-creER/Col2a1-creER*$^+$ cells** (Ono N et al, 2014)

***Col10a1-cre*$^+$ & *Acan-creER*$^+$ chondrocytes** (Zhou X et al, 2014)

***Col10a1-cre*$^+$ & *Col10a1-creER*$^+$ chondrocytes** (Yang L et al, 2014)

c. Perichondrium (development)

***Osx-creER*$^{+}$ osteoblast precursors** (Maes C et al, 2010)

d. Periosteum (adult)

***αSMA-creER*$^{+}$ (***) mesenchymal progenitor cells** (Matthews BG et al, 2014)

****This also marks BM artery pericytes, therefore not specific to the periosteum.*

e. Bone marrow stroma (adult)

***LepR-cre*$^{+}$ mesenchymal stromal cells** (Zhou BO et al, 2014)

Perisinosidal & periarteriolar stromal cells

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# Biomechanics Meets Bone Biology: The Ultimate in Multidisciplinary Translational Research

Clifford Rosen, M.D.

Mary Bouxsein, Ph.D.

# Biomechanics Meets Bone Biology: The Ultimate in Multidisciplinary Translational Research

Clifford J. Rosen, MD
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and
Mary L. Bouxsein, PhD
Center for Advanced Orthopedic Studies, Harvard Medical School, Boston

- **As a result of participating in this session, attendees should be able to:**
  - **Describe the elements that contribute to a successful collaboration**
  - **Consider how and with whom to collaborate to increase scientific productivity**
  - **Appreciate examples of a good collaboration between biomechanics expert and bone biology / clinical expert**

Burroughs Wellcome Fund, Howard Hughes Medical Institute (2006) Making the right move. A practical guide to scientific management for postdocs and new faculty. Chevy Chase. http://www.hhmi.org/ labmanagement

## Why collaborate?

- **Science is complex**
  - **Average number of authors on PNAS publications rose from 3.9 to 8.4 between 1981 and 2001[1]**
- **Collaboration can be productive and fun**

[1] Borner et al, PNAS 2004

## Rules for a successful collaboration

***Before you start…..***

1) **Do not be lured into just any collaboration – learn to say NO when its not right**

2) **Must have shared scientific interest & passion**

3) **Must have <u>complementary</u> skills or knowledge**

References: Smalheiser et al, PLoS Biol 2005; Vincens and Bourne,PLoS Comp Biol 2007

## Rules for a successful collaboration

***At the very beginning…***

4) **Outline who will do what**
   - **memo, email, document phone conversation**
   - **discuss publication strategy**

5) **Be open and sharing -- communicate, communicate, communicate**
   - **share protocols, data, figures, slides**

References: Smalheiser et al, PLoS Biol 2005; Vincens and Bourne,PLoS Comp Biol 2007

## Rules for a successful collaboration

***Throughout the collaboration and beyond…***

6) **Stick to the agree-upon work, be timely**
   -- **discuss first before changing direction**
7) **Acknowledge and cite your collaborators work**
8) **If it works, keep it going!**

References: Smalheiser et al, PLoS Biol 2005; Vincens and Bourne,PLoS Comp Biol 2007

## Rules for a successful collaboration

***And if it doesn't go well….***

9) **Minimize your losses (and the drama) and move on**
   -- **Discuss completing agreed upon tasks and publication (hopefully just one!)**
10) **Reach out to your mentors or other experienced scientists for advice**

References: Smalheiser et al, PLoS Biol 2005; Vincens and Bourne,PLoS Comp Biol 2007

www.nature.com/clinicalpractice/rheum

# Mechanisms of Disease: is osteoporosis the obesity of bone?

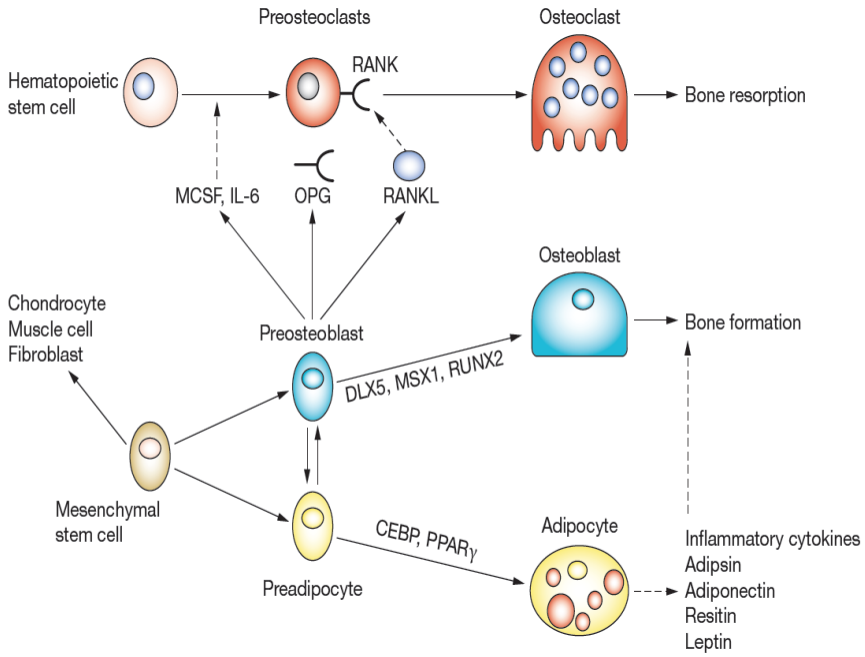
636 Citations

Clifford J Rosen* and Mary L Bouxsein

**Table 1** Knockout and congenic mouse models used to study the interaction between fat, adrenergic signaling and bone.

| Model | Body composition | Skeletal phenotype | Comment |
|---|---|---|---|
| *ob/ob*[12] | ↑ BW, ↑ fat mass | ↑ Trabecular bone volume | Leptin deficiency |
| *db/db*[12,13] | ↑ BW, ↑ fat mass | ↑ Trabecular bone volume | Leptin resistance (leptin-receptor deficiency) |
| βAR2 KO[20] | Normal | ↓ Trabecular bone volume at 6 months | Resistant to deleterious effects of OPX and β-adrenergic agonists on skeleton |
| βAR1,2 KO[20] | ↓ BW | ↓ Total body BMC; normal trabecular bone volume; ↓ mid-diaphyseal cross-sectional area | Resistant to deleterious effects of β-adrenergic agonists on skeleton |
| βAR123 KO (beta-less)[20] | ↑ BW, ↑ fat mass | ↑ Total body BMC, ↑ trabecular bone volume; ↑ mid-diaphyseal cross-sectional area | Not resistant to deleterious effects of OPX on skeleton |
| Congenic 6T[25] | Normal BW<br>↑ fat mass | ↓ Trabecular bone volume; ↓ mid-diaphyseal cross-sectional area; ↑ OB apoptosis, ↑ bone-marrow fat | ↓ Serum/skeletal IGFI; ↑ PPARγ activation |
| PPARγ$^{+/-}$ [22] | ↓ body fat | ↑ Trabecular bone volume; ↑ OB number | PPARγ null lethal |
| SAMP6[25] | ↑ body fat | ↓ Trabecular bone volume; ↑ bone-marrow fat | Accelerated aging model |

BMC, bone mineral content; BW, body weight; βAR, β-adrenergic receptor; IGF, insulin-like growth factor; KO, knockout; OB, osteoblast; OPX, oophorectomy; PPAR, peroxisome proliferative activated receptor; $^{+/-}$, heterozygous.



Endocrinology 2014

# Early-Onset Type 2 Diabetes Impairs Skeletal Acquisition in the Male TALLYHO/JngJ Mouse

M. J. Devlin, M. Van Vliet, K. Motyl, L. Karim, D. J. Brooks, L. Louis, C. Conlon, C. J. Rosen, and M. L. Bouxsein

Department of Anthropology (M.J.D.), University of Michigan, Ann Arbor, Michigan 48104; Center for Advanced Orthopedic Studies (M.J.D., M.V.V., L.K., D.J.B., L.L., C.C., M.L.B.), Beth Israel Deaconess Medical Center, and Harvard Medical School (M.L.B.), Boston, Massachusetts 02215; and Maine Medical Center Research Institute (K.M., C.J.R.), Scarborough, Maine 04074

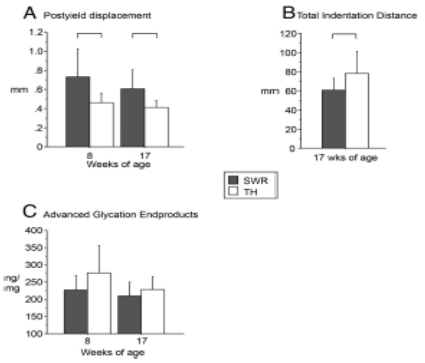



**Figure 4.** A, Tallyho femurs showed low postyield displacement by mechanical testing indicating bone brittleness. B, High total indentation distance (TID) confirmed altered material properties in Tallyho vs SWR. C, Accumulation of nonenzymatic AGEs did not differ in Tallyho vs SWR ($P = .074$). Significant differences ($P < .05$) are indicated by brackets.

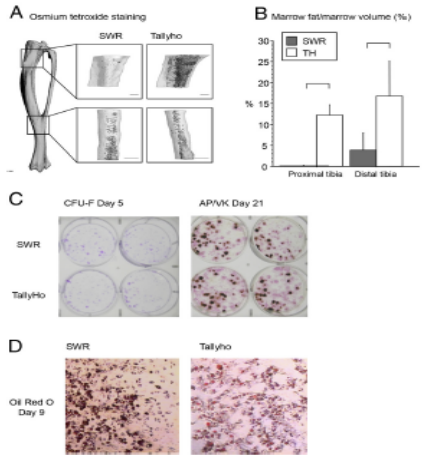


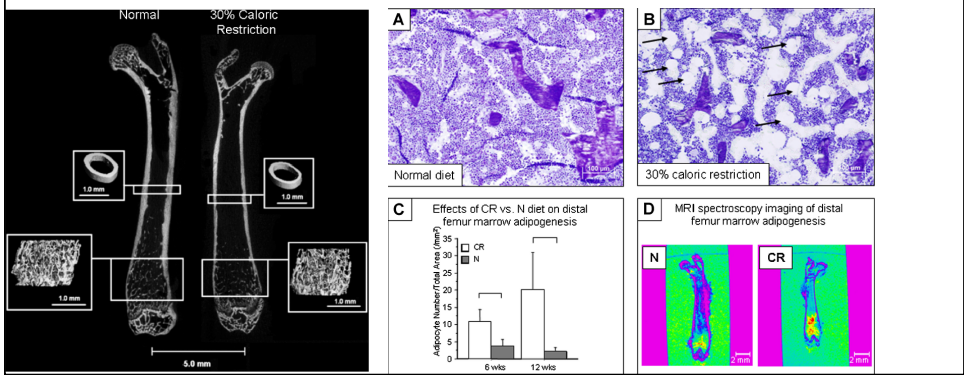

**Figure 5.** A, Osmium tetroxide staining of the tibia showed high

ORIGINAL ARTICLE

JBMR

# Caloric Restriction Leads to High Marrow Adiposity and Low Bone Mass in Growing Mice

Maureen J Devlin,[1] Alison M Cloutier,[1] Nishina A Thomas,[1] David A Panus,[1] Sutada Lotinun,[2] Ilka Pinz,[3] Roland Baron,[2] Clifford J Rosen,[3] and Mary L Bouxsein[1]



# Matricellular Proteins in Bone Remodeling and Repair: Novel Insights

Andrea Alford, Ph.D.

Kurt Hankenson, D.V.M., Ph.D.

# Matricellular Proteins in Bone Remodeling and Repair: Novel Insights

Andrea I. Alford, PhD
University of Michigan, USA

Kurt D. Hankenson, DVM, PhD
Michigan State University, USA

## Significance of Matricellular Proteins

In 1995, Paul Bornstein at the University of Washington proposed the Matricellular protein (MP) concept to help explain characteristics of three novel extracellular matrix (ECM) proteins that his lab, along with the Helene Sage lab, helped discover: Thrombospondin (TSP), SPARC (Secreted Protein Acidic and Rich in Cysteine) and Tenascin (TN). Foundational research on these proteins demonstrated that while the proteins were cell-secreted,ECM components, they were not required for structural integrity of developing connective tissues: mouse models deficient in each of these matrix proteins were viable and apparently normal. Also in contrast to other ECM proteins, these proteins were de-adhesive in cell culture.

Bornstein's original definition of a matricellular protein was **a modular ECM protein that interacts with other matrix proteins, soluble factors and cell surface receptors to achieve its function(s).** As part of this definition, an MP might be intimately associated with a structural ECM component like collagen, but it would not be required for its development into a structurally sound and mechanically competent fibrillar ECM. Also inherent in Bornstein's original definition was that MP function would be highly contextual, depending on the repertoire of ECM components, soluble factors and cell surface receptors available.

Since 1995, MP-biologists have built on Bornstein's original concept and demonstrated that even though they are not required for life, MP are highly expressed in connective tissues during development and diminish with adulthood (with the exception of the skeleton).

MP-scientists have also demonstrated that MP can indeed influence collagen and fibronectin fibrillogenesis, and thereby affect connective tissue quality. MP-experts have also established that MP are re-expressed during wound healing where they play vital roles in controlling inflammation, directing the organization of provisional matrixes and controlling angiogenesis.

Over the two-decade span since Bornstein's original paper, the MP family has grown to include 5 TSPs, R-spondins, 4 TNs, a SPARC relative called hevin, osteopontin, a fibulin, periostin, and the CCN family members. Indeed, the more we learn about the dynamic nature of ECM, the more apparent it becomes that many ECM components have characteristics of MP. For example, structural ECM proteins like fibronectin influence signal transduction directly through integrins. Also, like an MP, some FN-

integrin interactions are contextual, depending on the availability of cryptic integrin binding sites along the FN sequence.

As a tissue that undergoes constant ECM turnover and renewal, the skeleton provides an excellent example of the dynamic nature of ECM and the contextual nature of MP function. MP functions that are critical for normal bone tissue homeostasis, as well as repair and regeneration include facilitation of collagen fibrillogenesis, regulation of growth factor bioavailability, control of angiogenesis and influence on MSC fate determination and lineage progression.

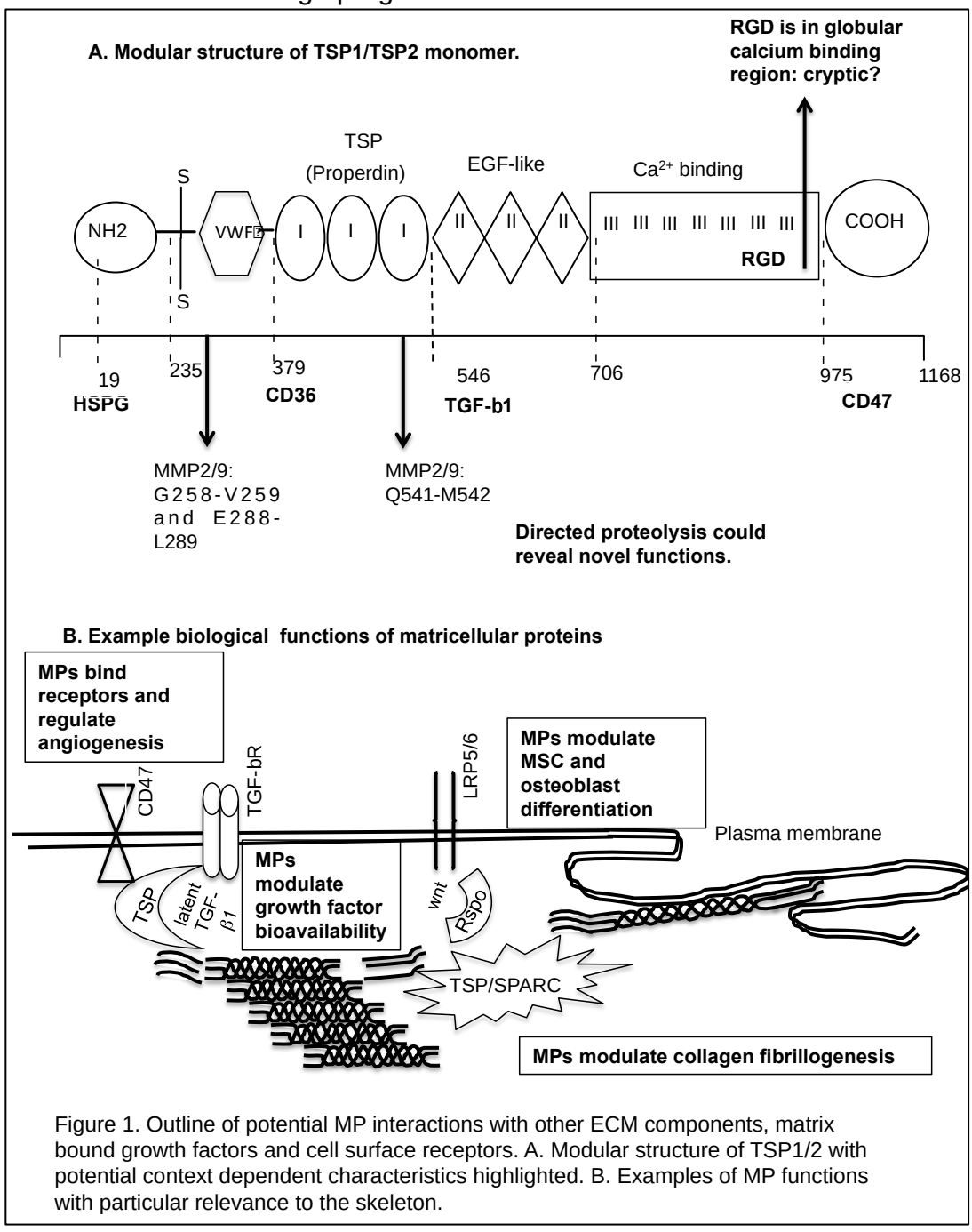



Figure 1. Outline of potential MP interactions with other ECM components, matrix bound growth factors and cell surface receptors. A. Modular structure of TSP1/2 with potential context dependent characteristics highlighted. B. Examples of MP functions with particular relevance to the skeleton.

**Learning Objectives:** As a result of participating in this session, attendees should be able to:

1.Apply the MP concept and appreciate that matricellular proteins are multifunctional and that their ultimate physiologic contributions are contextual.

2.Appreciate the contributions that MP make to the structural and material integrity of the skeleton.

3.Appreciate the contributions of MP to cell and matrix physiology in the skeleton.

**Outline:**

1. Discuss the matricellular protein concept: Is the Matricellular Concept valuable to understand tissue physiology, particularly in bone?

2. Discuss specific examples of Matricellular Proteins:
    a. TSP2 regulation of varying stages of osteoblast differentiation and MSC adipocyte differentiation
    b. Degradation of TSP2 to reveal potentially cryptic functions in bone
    c. Rspo2 regulation of osteoblast differentiation and bone formation
    d. TSP2 regulation of bone vascularization

**Informative Matricellular Protein Literature**

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# Phosphate Sensing: Two Sensors - A Metabolic and an Endocrine One?

Clemens Bergwitz, M.D.

**MEET THE PROFESSOR**

**Title:** Phosphate Sensing: Two Sensors, a Metabolic and an Endocrine One?

**Speaker:** Clemens Bergwitz, M.D., Assistant Professor of Medicine, Section Endocrinology, Yale School of Medicine

**Location:** ASBMR 2016 Annual Meeting, September 16-19, 2016 at the Georgia World Congress Center in Atlanta, Georgia, USA.

**Time:** Saturday, September 17 from 11:00 am - 12:00 pm.

**Significance of the Topic:** How human and other metazoan cells sense inorganic phosphate (Pi) to explain the effects of Pi on cell metabolism ("metabolic" sensing), and how Pi feeds back to regulate the parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and 1,25$(OH)_2$-vitamin D ("homeostatic" sensing) is unknown (5, 18). It is also unknown whether the "metabolic" and the "homeostatic" sensor use the same or different signal transduction cascades. Knowledge of the Pi sensor(s) will improve our understanding of Pi homeostasis and permit us to better treat individuals with acquired and inborn errors of Pi homeostasis.

**Learning Objectives:** As a result of participating in this session, attendees should be able to understand:

1) The role of phosphate in energy metabolism and signal transduction
2) Regulation of Pi homeostasis by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and 1,25$(OH)_2$-vitamin D
3) Current evidence for the existence of metabolic and endocrine sensors for phosphate and approaches that have been pursued thus far to identify theses sensors.

**Outline:**

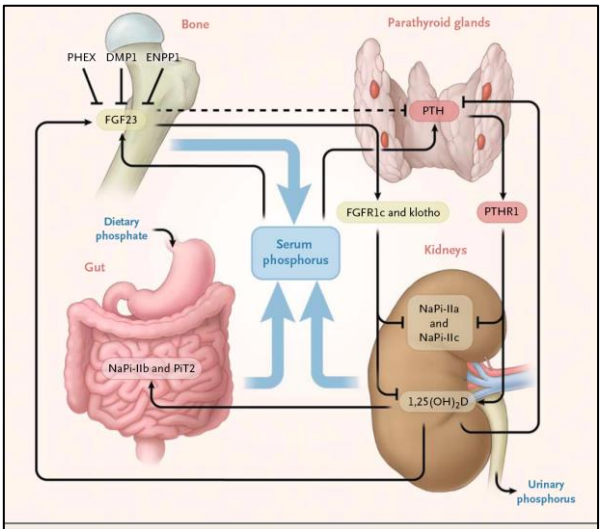
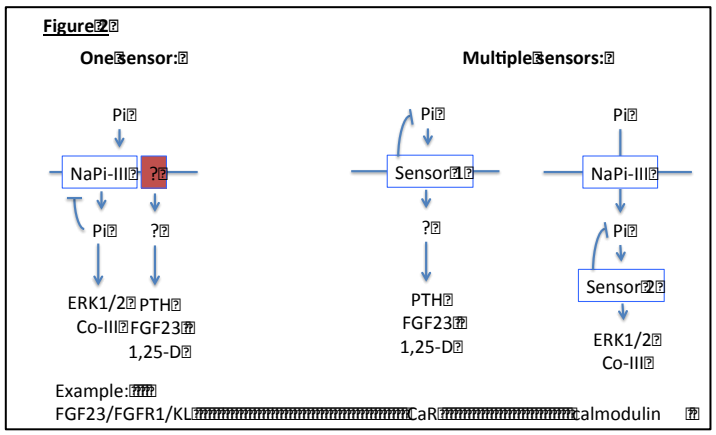


Figure 1. Regulation of Phosphate Homeostasis by Fibroblast Growth Factor 23, Parathyroid Hormone, and 1,25-Dihydroxyvitamin D.

Phosphate (Pi) is absorbed from the diet in the gut and excreted by the kidneys (6). The extracellular (EC) Pi compartment is made up of blood Pi, interstitial Pi and calcium phosphate in the form of hydroxyapatite, which has an important biomechanical role in the skeleton of higher species. Intracellular (IC) Pi is crucial for many processes including energy storage as ATP and phosphocreatine (PCr), signal transduction and acid-base balance (9). Acute hypophosphatemia (low blood Pi) causes muscle cell death (rhabdomyolysis) leading to respiratory and heart failure often complicating the care of patients in the intensive care setting (16). Chronic hypophosphatemia leads to rickets and osteomalacia. Impaired muscle function and early fatigue (hypophosphatemic myopathy) is a feature of rickets or osteomalacia significantly affecting the quality of life in these patients (27, 28).

EC Pi is regulated by Fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH) and 1,25$(OH)_2$D (1,25-dihydroxyvitamin D) (Fig. 1 (3)). FGF23 expression in bone is up-regulated by 1,25$(OH)_2$D (1,25-dihydroxyvitamin D), and it is down-regulated, through unknown mechanisms, by PHEX (encoded by *PHEX*, or phosphate-regulating gene with homologies to endopeptidases on the X chromosome), DMP1 (dentin matrix protein 1), ENPP1 (ectonucleotide- pyrophosphatase phosphodiesterase 1), and probably several additional

proteins that permit cross-talk of iron and Pi-homeostasis (15). FAM20C is an extracellular kinase that was recently shown to cause phosphorylation and degradation of FGF23, while O-glycosylation of the hormone by GALNT3 is required for effective secretion. Blood Pi may feed back at this posttranscriptional level to regulate FGF23 bioactivity (12, 31). FGF23 acts through one or more FGF receptors (e.g., FGFR1c), with the co-receptor klotho, to inhibit renal phosphate reabsorption, to decrease circulating 1,25(OH)2D levels, and possibly to inhibit parathyroid hormone (PTH) secretion by the parathyroid glands (dashed line). Human mutations in all these genes lead to disorders of Pi homeostasis (4). The net effect of these PTH-dependent actions is a decrease in serum phosphate levels and an increase in serum $1,25(OH)_2D$ levels. The $1,25(OH)_2D$ levels are probably linked to regulation of FGF23 by phosphate (6) but might also be regulated directly by the effects of serum phosphorus levels on 1α-hydroxylase or 24-hydroxylase or both (6). The net effect of hypophosphatemia is upregulation of $1,25(OH)_2D$; $1,25(OH)_2D$ is down-regulated by increased serum levels of calcium and phosphorus and by increased FGF23 levels. Vitamin D–receptor and retinoid X–receptor heterodimers facilitate the action of $1,25(OH)_2D$ to enhance the intestinal absorption of phosphate and to stimulate FGF23 synthesis and secretion by osteocytes; furthermore, $1,25(OH)_2D$ inhibits PTH synthesis and secretion by the parathyroid glands. The net $1,25(OH)_2D$ effect is an increase in serum phosphorus levels. NaPi denotes sodium–phosphate cotransporter, PiT2 sodium-dependent phosphate transporter 2, and PTHR1 parathyroid hormone receptor type 1.




Regulation of IC Pi is less well understood in multicellular organisms. Prokaryotic and single cellular eukaryotic organisms such as bacteria and yeast ‘‘sense’’ ambient Pi with a multi-protein complex located in their plasma membrane, which modulates the expression of genes important for Pi uptake and metabolism (pho pathway)(21). Although the type I and III Pi transporter families in higher species are related to the yeast transporters, database searches based on amino acid sequence conservation alone have been unable to identify metazoan orthologs of the intracellular bacterial and yeast Pi sensors. Thus, little is known about how human and other metazoan cells sense IC Pi to regulate the effects of phosphate on cell metabolism (‘‘metabolic’’ sensing). Activation of pERK1/2 mitogen-activated kinase has been reported in many cell types (10) and appears to be evolutionary highly conserved (7, 8). Phosphate also stimulates mitochondrial ATP synthesis for example to support the function of skeletal muscle (23), which may at least in part occur by improving electron flow through complex III of the respiratory chain (poster ASBMR MO-0109). In turn, regulation of type II and III Pi transporter expression appears to depend on pERK1/2 signaling and extracellular calcium (30).

Extracellular and intracellular compartments in multicellular organisms may require separate regulatory mechanisms, since cellular uptake of Pi increases IC Pi, but lowers blood Pi. A single membrane sensor may mediate uptake and cellular homeostasis of Pi in all cells, but in addition may couple to a co-receptor or second messenger pathway in endocrine cells that enables the same sensor to modulate secretion of PTH, FGF23 and 1,25-D (Fig. 2, left panel). An example for this hypothesis is the co-receptor klotho which confers ligand specificity to FGF23

for FGFR1 which permits this universally expressed receptor to regulate Pi homeostasis in proximal tubular cells of the kidneys (19). Alternatively, two or more sensors may exist, one that senses extracellular Pi to regulate endocrine functions, and one that senses intracellular Pi to regulate cellular supply of Pi (Fig. 2, right panel). An example for this hypothesis is the Ca-sensing receptor which mediates endocrine effect of calcium in the parathyroids and distal tubule of the kidneys (26), while calmodulin senses IC Ca to regulate cell metabolism (24).

RNA and protein expression profiling further supported the role of pERK1/2 in metabolic P-sensing (13, 14), promotor analysis identified candidate P-transcription factor responsive elements (10, 22), and genome-wide RNAi screening added to the number of genes possibly contributing to P-sensing (8). RNAi ablation and studies of Pit1 and transport-independent activation of pERK1/2 by a mutant Pit1-transporter may furthermore suggest a role of type III Pi-transporters in Pi-sensing (2, 11), and although *in vivo* evidence for a role of Pit1 as endocrine sensor is (thus far) lacking (1, 20), increased iPTH levels in transgenic rats overexpressing Pit1 (25) and increased cerebrospinal fluid (CSF) Pi after ablation of Pit2 could be consistent with a role of type III transporters as a component of metabolic and possibly endocrine Pi sensors (17, 29).

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# Bone Marrow Microenvironment and Myeloma

Claire Edwards, Ph.D.

G. David Roodman, M.D., Ph.D.

# ASBMR 2016: Meet-the-Professor: Bone Marrow Microenvironment and Myeloma

**Dr. Claire Edwards, Ph.D. University of Oxford &**
**Dr. G. David Roodman, M.D., Ph.D. Indiana University School of Medicine and Roudebush VA Medical Center**

## Significance

Multiple myeloma is a neoplastic disorder of plasma cells in the bone marrow. It is characterised by clonal proliferation within the bone marrow, osteolytic bone disease, and secretion of a monoclonal paraprotein in the blood and/or urine of the patient. Myeloma is the second most common of all haematological cancers (10-15%). It has a global incidence of approximately 120,000 cases per year and accounts for around 1% of all cancers (1). Survival rates have improved in recent years and although myeloma remains incurable, patients are now predicted to have a median survival of approximately 6.1 years(2). The hallmark of myeloma is the osteolytic bone disease that is present in 70-80% of patients (3). It is characterised by the presence of osteolytic lesions accompanied by the suppression of osteoblast differentiation and function (4). Approximately 20% of patients with myeloma will present with a pathological fracture upon diagnosis, and until recent advances in anti-myeloma therapy, almost 60% of patients developed a pathological fracture over the course of their disease (5, 6). Bisphosphonates have been the mainstay of treatment for many years, and an anti-RANK ligand antibody, Denosumab, is in clinical trial for myeloma. However, these agents are limited by their inability to repair existing bone loss. Therefore, research into novel approaches for the treatment of myeloma bone disease is of the utmost importance

## Learning Objectives

**As a result of participating in this session the attendees should**

1. Understand the mechanisms involved in the development of myeloma bone disease
2. Appreciate the importance of the bone marrow microenvironment in distinct stages of disease progression (e.g. MGUS, dormancy, bone disease)
3. Acquire insights into new therapeutic approaches to target myeloma bone disease

## Points of Interest

### i. Myeloma bone disease

Multiple factors produced by myeloma cells and induced by myeloma cells in the bone microenvironment stimulate osteoclasts to resorb bone and inhibit osteoblastic activity. In turn, growth factors released by the increased bone resorption, also increase the growth of myeloma cells, creating a vicious cycle of tumour expansion and bone destruction. The biological pathway of the receptor activator of nuclear factor-kappa B (RANK), its ligand (RANKL) and the soluble decoy receptor osteoprotegerin (OPG), is of major importance for the increased osteoclast activity observed in MM (7). The relationship between the Wnt inhibitors Dickkopf 1 (Dkk1) and sclerostin expression, Activin-A and other inhibitors of osteoblast differentiation have also emerged as a critical route to osteoblast suppression in myeloma (8). However, none of these reversible inhibitors of osteoblast differentiation explained the long-term suppression of osteoblast differentiation that occurs in myeloma patients even when they are in long-term remission. Recent studies have shown that the transcriptional repressor Gfi1 is upregulated in marrow stromal cells in patients with myeloma. Gfi1 in turn induces epigenetic changes in the RUNX2 promoter that suppress RUNX2 activity and block osteoblast differentiation. This upregulation of Gfi1 persists in marrow stromal cells from myeloma patients even when the stromal cells are grown in the absence of myeloma cells for many passages (9).

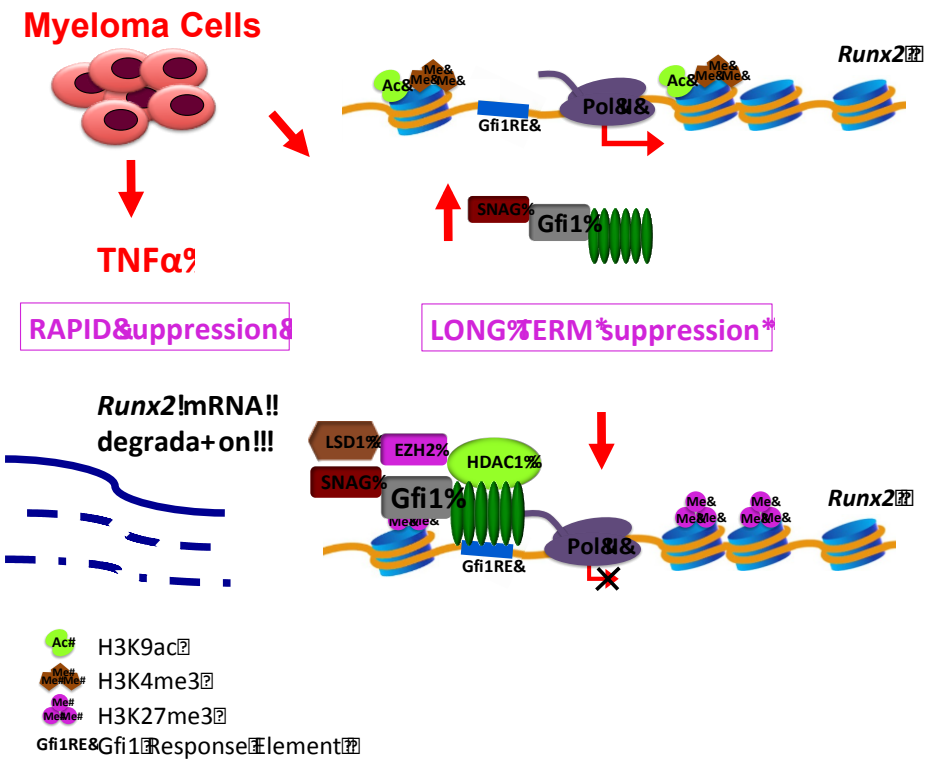


**Figure 1. Mechanism of long-term suppression of osteoblast differentiation in myeloma.** Myeloma cells produce cytokines such as TNF alpha that induce rapid degradation of Runx2 mRNA. Additionally, myeloma cell upregulate expression of Gfi1 in marrow stromal cells. Gfi1 binds to the Runx2 gene and recruits histone modifiers HDAC1, Co-REST, LSD1, and G9a that results in reduction of the transcriptionally permissive euchromatin marks H3K4me3, H3K9ac, H3K12ac, H3K27ac, and H3K36me3. These changes persisted even after removal of MM cells making Runx2 transcription refractory to OB differentiation signals.

**ii.**

**Bone microenvironment**

The bone marrow is a mixed but highly spatially organized tissue comprising bone and fibrous extracellular matrices, mesenchymal stem cells, fibroblasts, cells committed to osteoblast or adipocyte fate, mature osteoblasts, adipocytes and osteocytes, as well as dense vascularity with adapted endothelial cells and innervation, cells of haematopoietic origin including macrophages, osteoclasts, and T cells. All of these cell types are ideally placed to interact with myeloma cells, with such interactions typically supporting tumour growth and/or bone disease within this microenvironment.

Evidence for the importance of the microenvironment comes from a number of key experiments that demonstrate the critical roles that distinct cell populations play in disease pathogenesis. Bone marrow stromal cells have been shown to promote myeloma establishment in mice in which myeloma does not otherwise develop, with Dkk1 identified as a major contributor to the supportive effect of BMSCs(10). Osteoblasts and osteoclasts are known to be important in myeloma bone disease, as described above, but more recently have emerged as key players in myeloma cell dormancy(11). Osteoblasts comprise the endosteal bone niche that is the site in which dormant myeloma cells reside, whereas the activity of osteoclasts to remodel the endosteal niche results in the reactivation of dormant myeloma cells. Adipocytes have been postulated to be important in myeloma progression and the associated bone disease, with hypoadiponectinaemia promoting progression from MGUS to myeloma and the key adipokine adiponectin acting as a potential therapeutic approach to both reduce tumour burden and directly target bone disease(12). Furthermore, using diet-induced obesity to increase host adiposity resulted in a myeloma-like condition in a murine in vivo model(13). Notably tumour burden was reduced upon removal of the high fat diet, suggesting the potential for dietary intervention. Myeloma cells can also employ a number of mechanisms to alter the function of immune cells, and instead enlist the support of immunosuppressive cells such as myeloid-derived suppressor cells.

Recently, osteocytes have been shown to play an important role in myeloma bone disease. These studies found that osteocytes and myeloma cells physically interact in vivo, and these interactions result in bidirectional Notch signaling between the osteocytes and myeloma cells. The bidirectional Notch signaling in turn increases myeloma cell growth and RANK ligand production by myeloma cells and induces osteocyte apoptosis, as well as increased osteocyte production of RANK ligand and sclerostin (14). These studies suggest that osteocytes and sclerostin are potential targets for treating myeloma bone disease.

### iii. Therapeutic Targets

Our increased understanding of mechanisms in disease pathogenesis has resulted in a number of new therapeutic approaches for both treatment of tumour and the associated bone disease (15). Current approaches under investigation for their potential in myeloma bone disease include targeting (i) the RANKL system (16, 17) (ii) the Wnt signalling system via anti-Dkk1(18){Tian, 2003 #3091} or anti-sclerostin(19), (iii) notch signalling (14)and (iv) TGF-b family members, including activin-A(20). In addition, drugs which directly target the tumour have also been found to have activity within the bone microenvironment, e.g. BTK inhibitors which inhibit osteoclast activity and stromal cell secretion of growth factors in addition to exhibiting direct anti-myeloma effects(21).

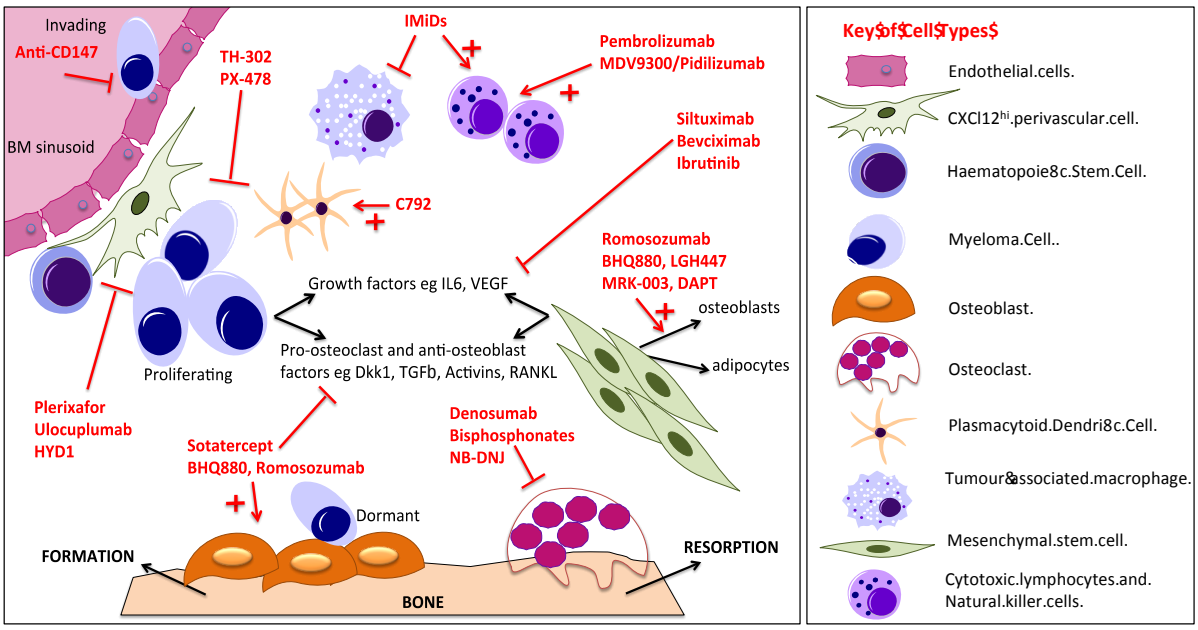


**Figure 2.** The cellular interactions of the bone marrow niche and myeloma, showing currently developed drugs and their target cellular interactions. Drugs labelled in red with indication of site of action. Reproduced from Gooding, S., Edwards, C.M., New approaches to targeting the bone marrow microenvironment in multiple myeloma. (2016) Current Opinions in Pharmacology 28; 43-49. doi:10.1016/j.coph.2016.02.013

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# Pathogenesis and Treatment of Heterotopic Ossification

Benjamin Levi, M.D.

**Meet the Professor "Pathogenesis and Treatment of Heterotopic Ossification"**

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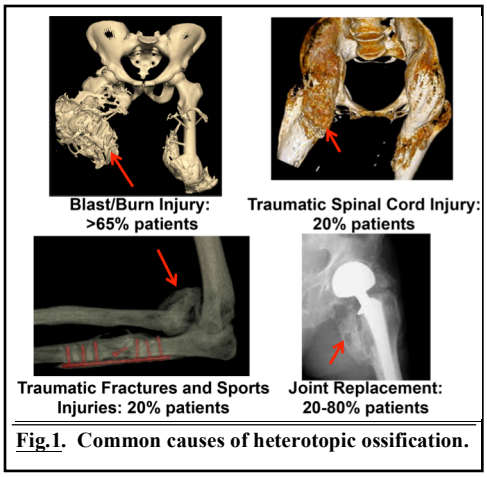


**Fig.1. Common causes of heterotopic ossification.**

## Significance of the Topic:

Heterotopic ossification (HO) is a disabling and frequent complication of burn and post-traumatic musculoskeletal, neurologic, and systemic injuries. HO occurs in over 65% of repeat hip replacement patients and severe combat-injured patients.[1] Advances in critical care medicine have improved the survival of polytrauma and large total body surface area burn patients, causing a concomitant rise in the number of patients at risk for HO and driving a need for diagnostic and prophylactic strategies to prevent its formation. The number of hip arthroplasties continues to rise (300,000 annually) with a 50% increase over the last 10 years.[2] Additionally there are 1,700,000 people living with limb loss in the US alone and 185,000 new amputations are performed each year in the US. A large number of these amputation patients suffer from HO causing severe pain, inability to wear prostheses, wound breakdown, need for further surgery and residual limb osteomyelitis. Once HO forms, patients suffer from limited joint mobility, chronic pain, and open wounds. Surgical HO excision does not improve chronic pain or joint contracture and is frequently complicated by recurrence.[3-6] Therefore, there is a critical need to detect and prevent HO before it occurs, and to eliminate recurrence. Rather than focusing on the ossified lesion, patients would benefit if therapeutics targeted the early stages of cellular differentiation. Although HO causes severe morbidity in a large number of patients, its overall prevalence remains low among a broadly defined population of "at-risk" patients. Therefore, the primary translational gap to prevent this complication of trauma is early diagnosis and access to a prophylactic agent that can be safely administered to appropriate candidates.

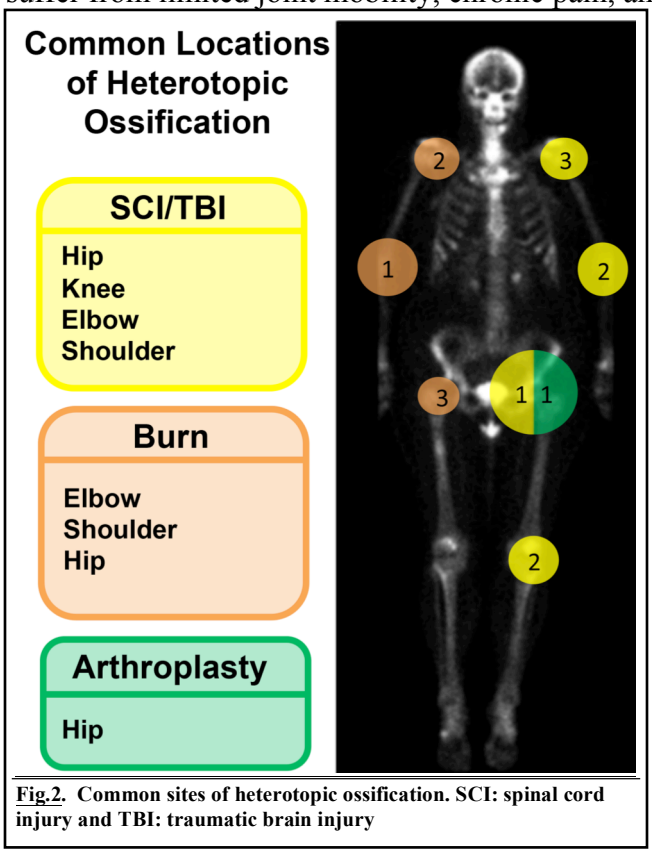


**Fig.2. Common sites of heterotopic ossification. SCI: spinal cord injury and TBI: traumatic brain injury**

A separate group of patients that suffer from debilitating flares of heterotopic ossification are those with Fibrodysplasia ossificans progressiva (FOP). Though trauma also incites HO in these patients, FOP patients can develop HO without direct trauma. FOP is caused by a hyperactivating mutation in the type I bone morphogenetic protein receptor (T1-BMPR) ACVR1/ALK2 (*ACVR1 R206H*), leading to increased SMAD 1/5 phosphorylation and expression of down-stream pro-osteogenic genes.[7-9]

**Learning Objectives:** As a result of participating in this session, attendees should be able to:

1. Identify risk factors for traumatic heterotopic ossification
2. Define current and future diagnostic modalities to assess heterotopic ossification
3. Compare current treatment modalities used to prevent heterotopic ossification
4. Describe surgical options for heterotopic ossification
5. Understand pathways responsible for heterotopic ossification
6. Future treatment strategies for heterotopic ossification
7. Understand animal models available to study heterotopic ossification

**Points of Interest/Clinical Pearls:**

A. Identify risk factors for traumatic heterotopic ossification
   1. Genetic Predisposition: FOP from mutation in ALK2 (*ACVR1 R206H*).[7-9]
   2. Burn Induced HO Risk Score: www.spauldingrehab.org/HOburncalculator
      a. Total body surface area burn
      b. Upper extremity burns
      c. Number of trips to the operating room
      d. Hospital Stay
   3. Extremity trauma risks: Fractures, ligament tears, tendon injuries
   4. Neurologic trauma risks: Traumatic Brain and Spinal Cord Injury
   5. Joint arthroplasty and surgery risks: Hip arthroplasty (posterior approach?), repeat surgery, elbow surgery, spinal surgery with BMP2 recombinant protein

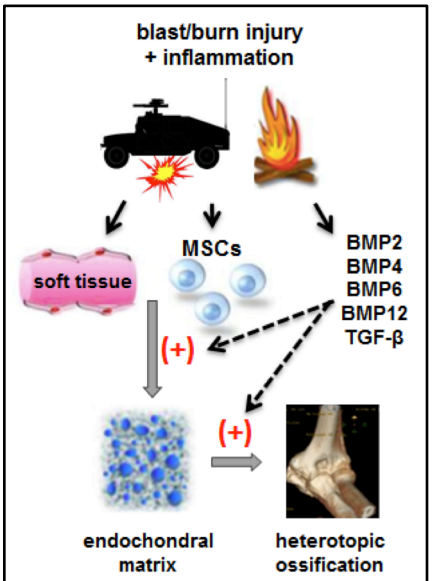

Fig. 3. Schematic of possible pathways involved in traumatic HO.

B. Define current and future diagnostic modalities to assess heterotopic ossification
   1. Current Diagnostics: HO sites elude radiographic detection prior to three weeks post-injury at which time occupational therapy must be halted and joint contractures progress.
      a. X-Ray
      b. CT scan
      c. MRI
      d. SPECT
   2. Future Diagnostics
      a. Ultrasound
      b. Raman Spectroscopy[10,11]
      c. Near Infra-Red[12]
      d. SPECT CT[13 3]
      e. Biomarkers[14]

C. Compare current treatments used to prevent heterotopic ossification
   1. Current Prophylactic Strategies:
      a. NSAIDS[15-22]
      b. Radiation Therapy[23,24]
      c. Bisphosphonates[25]
   2. Future Prophylactic Strategies:
      a. BMP targeted therapies[26,27]
      b. Hypoxia Inducible Factor[28-30]
      c. Anti-inflammatories [17]
      d. Occupational Therapy
   3. Risks of therapies to wound healing

| Study | Treatment Used | Clinically Significant HO | Evidence |
|---|---|---|---|
| Beckmann 2014 | No Treatment<br>Naproxen | 23/92 (25.0%)<br>11/196 (5.6%) | III |
| Brunnerkreef 2013 | Etoricoxib | 0/42 (0.0%) | III |
| Nunley 2011 | Aspirin<br>Warfarin | 1/151/ (0.7%)<br>4/46 (8.7%) | III |
| Saudan 2007 | Celecoxib<br>Ibuprofen | 6/117 (5.1%)<br>16/123 (13.0%) | IB |
| Grohs 2007 | Rofecoxib<br>Indomethacin | 3/46 (6.5%)<br>0/50 (0.0%) | IB |
| van der Heide 1999, 2007 | Placebo<br>Indomethacin<br>Rofecoxib | 2/99 (2.0%)<br>49/170 (28.9%)<br>0/42 (0.0%) | III |
| Karunaker 2006 | Placebo<br>Indomethacin | 13/62 (19.4%)<br>9/59 (15.2%) | IB |
| Fransen 2006 | Placebo<br>Ibuprofen | 26/407 (6.4%)<br>11/391 (2.8%) | IB |
| Burd 2001 | Radiotherapy<br>NSAIDs | 3/78 (3.8%)<br>8/72 (11.1%) | IB |
| Kolbl 1997 | Radiotherapy<br>NSAIDs | 1/188 (0.5%)<br>2/113 (1.8%) | IB |
| Knelles 1997 | Radiotherapy<br>NSAIDs | 0/100 (0.0%)<br>6/183 (3.3%) | IB |

Table 1. Studies of anit-inflammatories

D. Describe surgical options for heterotopic ossification
   1. When to intervene surgically[31,32]: Some surgeons advocate waiting until HO "burns out". Others claim no difference.
   2. What operations to perform and how to provide coverage[33]
   3. How to prevent recurrence[34-36]

E. Understand cells and pathways responsible for heterotopic ossification
   1. Cells involved
      a. Inflammatory Cells: Neutrophils, macrophages[37]

b. Endothelial Cells[38]
c. Mesenchymal Cells[28]
d. Cell Origin: Local vs. Circulating[39]
e. Cell Lineage: Scleraxis, Gli-1, Ve-Cad, NG-2, Prx, Glast,[37,40]

2. Pathways involved
   a. Bone morphogenetic protein ligands (BMP2,4,7) and BMPR1 (ALK2, ALK3, ALK6)[8,26,41,42]
   b. Vascular endothelial growth factor[38]
   c. Transforming growth factor 1 beta[43]
   d. Hypoxic Signaling[28,30]
3. Stages of Development:
   a. Inflammatory Stage
   b. Mesenchymal Condensation
   c. Chondrogenesis
   d. Vascular invasion and osteogenesis

F. Role of occupational therapy and movement: Does early mobilization after trauma or after HO resection help or hurt?

G. Models to Study Heterotopic Ossification
   1. Trauma/Burn induced heterotopic ossification:
      a. Blast/Amputation[44-46]
      b. Burn/Tenotomy[27,28,39]
   2. Neurogenic Heterotopic Ossification[42]
   3. Non-physiologic BMP implantation models[38,47-49]
   4. FOP models: R206 H[9,50] and Q207D[26,51]

H. Future treatment strategies for heterotopic ossification
   1. Anti-Inflammatory Agents: local vs. systemic
   2. Kinase Inhibitors [26]
   3. Ligand Traps[52]
   4. RAR Gamma Agonists[44,53,54]
   5. Tissue Engineering

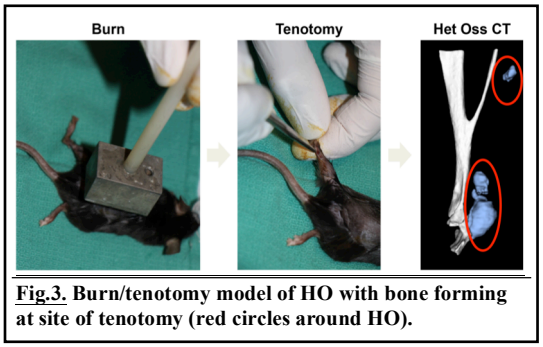

**Fig.3. Burn/tenotomy model of HO with bone forming at site of tenotomy (red circles around HO).**

**Cases:**

1. 21yo male who was working on his boat in a garage inciting an explosion. He suffered 50% total body surface area burns and inhalational injury.
   -What is the patients risk factors?
   -What should you tell occupational therapy?
   -Is there any prophylactic medication the patient can take?
   -Should you alter normal medications given to burn patients?
   -What imaging should be done if you suspect the patient is developing heterotopic ossification?
   -How should you alter occupational therapy after HO diagnosis?
2. 38yo with history of a proximal radial/ulnar fracture who developed heterotopic ossification and is not undergoing resection.
   -What were the patients initial risk factors?
   -What diagnostic workup do you want?
   -How do you decide when to operate?
   -What should you tell the patient he can expect with regards to his range of motion after surgery?
   -What are key principles to operation?
   -What is postop protocol for range of motion?
   -What is the risk of recurrence and what can you do to prevent this?
3. 67yo status post total hip arthroplasty who developed heterotopic ossification
   -What were the likely inciting events?
   -The patient asks if the technique the surgeon used caused this?
   -How do you decide when to operate?
   -What should you tell the patient he can expect with regards to his range of motion after surgery?

-What are key principles to operation?
-What is postop protocol for range of motion?
-What is the risk of recurrence and what can you do to prevent this?

**Discussion:**

1. Intersection of inflammation and differentiation
2. How similar is genetic HO (FOP) to traumatic HO?
3. How does activity alter HO occurrence and recurrence?
4. Is targeting 1 receptor or ligand sufficient?
5. Why not just use an anti-inflammatory?
6. How to balance HO prevention with normal wound healing and fracture repair.

**Additional References:**[1,28,37,55]


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# Fracture Risk Upon Stopping Osteoporosis Therapy

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**Fracture Risk Upon Stopping Osteoporosis Therapy**

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The principal objective of osteoporosis treatment is to reduce the risk of serious fractures. Several drugs of different classes are capable of quickly and substantially reducing the risk of vertebral and hip fracture. (1) With bisphosphonates and denosumab, the effects on bone remodeling and the protection from fractures appears to persist as long as treatment is given.

Like other chronic diseases for which no cure exists, osteoporosis requires long-term management. The unique pharmacology of bisphosphonates provides a potential opportunity to discontinue treatment, at least temporarily, without losing the benefits of therapy. Because of this possibility, studies have evaluated the skeletal effects of discontinuing several bisphosphonates. (2-5) Upon stopping, indices of bone remodeling slowly return toward baseline, and bone mineral density (BMD) values remain stable or decrease very gradually. Information about the persistence of fracture protection have been more difficult to obtain. In the extensions of the alendronate and zoledronic acid fracture prevention trials, the risk of vertebral fracture appeared to double over an interval of 3-5 years in patients who discontinued treatment compared to those who continued. (4-5) The effects of bisphosphonates, compared to placebo, on non-vertebral fracture risk are more modest. No increase in non-vertebral fracture risk has been observed upon stopping bisphosphonates except in a post hoc analysis of patients in the FLEX study whose hip BMD value was less than -2.5 when therapy was discontinued. (6) These results, combined with the possibility that interrupting therapy might reduce the risk of atypical femoral fracture, form the basis of recommendations about bisphosphonates drug holidays from this fascinates after 3-5 years of treatment. (7) While "holidays" can be considered inpatients at modest fracture risk, continuing therapy or switching to another drug, is recommended for patients who remain at high fracture risk after several years of bisphosphonate therapy.

In contrast to the effects of discontinuing bisphosphonates, withdrawing other bone active drugs results in rapid loss of their effects on bone remodeling. (2, 8-10) BMD gains achieved with estrogen, denosumab or teriparatide therapy are lost over 1-2 years. In the cases of estrogen and denosumab, markers of bone turnover rebound to values well above baseline for 1-2 years after stopping therapy, corresponding to the interval of accelerated decrease of BMD. Concern has been raised that this pattern of high bone turnover and rapid bone loss could be associated with a rebound in the risk of fracture during the immediate post treatment interval. Recent reports of five patients who experience multiple and/or severe vertebral fractures within a few months after stopping denosumab has brought this theoretical concern into the clinical arena. (11-13) Details of the 5 patients are summarized in the Table.

| Refer-ence | Age (yrs) | Diagnosis | Denosumab doses | BMD T-score | | | | Outcome: Fractures at | Months since last dose |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Baseline | | After Rx | | | |
| | | | | LS | FN | LS | FN | | |
| 1 | 59 | Rheum arthritis; PMOP | 6 | -3.1 | -2.3 | -2.4 | -2.1 | T10 | 15 |
| 1 | 59 | PMOP | 5 | -3.1 | -2.7 | -2.3 | -2.0 | T10,L3<br>T12,L2, L4 | 10<br>11 |
| 1 | 77 | PMOP | 5 | -4.1 | -3.9 | ? | ? | 9 fractures | 16 |
| 2 | 51 | PMOP | 6 | -2.5 | -1.8 | ? | ? | T12, L1, L2 | 8 |
| 3 | 48 | Premenopausal; breast cancer; AI therapy | 6 | -2.0 | -1.5 | -0.8 | -1.3 | T10 T12, L1-4 | 9 |

*PMOP: Postmenopausal osteoporosis; AI: aromatase inhibitor*

We have very limited information about fracture risk upon stopping denosumab therapy. No increase in fracture incidence after stopping therapy was reported in a small number of patients in the Phase 2 study nor in 256 low risk patents in the denosumab bone loss prevention study. (9,10) Brown and colleagues reported fracture incidence in 797 patients who discontinued denosumab or placebo in the Phase 3 FREEDOM trial. (14) The average duration on therapy before patients discontinued was about 3.4 doses, or less than two years. Clinical fractures occurred after stopping therapy in 9 and 7 % of patients who had received placebo or denosumab, respectively. The rate of vertebral fractures was lower (5.6 per 100 patient-years) in those who had taken denosumab compared to a rate of 9.3 in the previous placebo group. However, the median off-treatment interval was only eight months, and the maximum off-treatment interval was 24 months. Additionally, 28–42 % of these patients had begun other osteoporosis treatments during their off treatment follow-up. An expansion of these data will be presented at ASBMR 2016 by Cummings and colleagues.

It may be pertinent to reflect on the much larger set of information available about fracture risk when estrogen therapy is discontinued. Like with denosumab, estrogen therapy is associated with improvement in bone mineral density and a reduction in fracture risk in both observational and randomized control trials. When treatment is stopped after 2–10 years, relatively rapid bone loss occurs with lumbar spine BMD returning to levels observed in untreated women within 1–2 years. The pattern of bone loss is similar to what is reported in the denosumab discontinuation studies. Markers of bone turnover also quickly return to baseline or untreated levels upon stopping estrogen. In older postmenopausal women with osteoporosis, urinary NTX returned to the levels in untreated patients within three months after withdrawal of conjugated estrogen 0.625 mg daily without evidence of rebound (6). In contrast, when estrogen-progestin therapy was discontinued after four years in younger postmenopausal women without osteoporosis, urinary NTX increased to levels above that seen in

untreated women, returning to the untreated levels two years after withdrawal, a pattern not unlike that observed upon stopping denosumab (2). Unfortunately, fracture incidence after estrogen withdrawal was not reported in those studies. In several observational studies, clinical fracture risk has been observed to increase as much as 50 % in women who stopped estrogen therapy compared to those who continued [15, 16]. Assuming that estrogen reduced fracture risk by about 1/3, this 50 % increase would put the risk back at pretreatment levels. In both Women's Health Initiative (WHI) studies, fracture protection noted on estrogen therapy was lost within 3–5 years of stopping estrogen therapy [17, 18]. There was no evidence of a rebound in clinical fracture risk. This was most clearly shown in Figure 1 of the study by Heiss and colleagues [17]. During the first three years after withdrawal of estrogen-progestin therapy, the cumulative incidence plots of hip fracture were the same in women who had taken estrogen-progestin or placebo. Thus, it appears that fracture protection afforded by estrogen therapy is quickly lost upon stopping therapy but that no "rebound" in fracture risk is observed.

It may be important to note that the patents in the WHI study were younger than most patients who receive treatment for osteoporosis, and very few had osteoporosis as defined by BMD values. Thus, the reassuring observation that stopping estrogen therapy is not associated with an interval of excess fracture risk may not be applicable to older patients with osteoporosis and, likely, more vertebral trabecular deterioration, who discontinue denosumab therapy.

Take home points:

1. There is no justification for a "drug" holiday with non-bisphosphonate drugs
2. If/when non-bisphosphonate therapy is discontinued, it seems prudent to continue therapy with another bone active agent
    a. For patients who remain at high risk for vertebral fracture, teriparatide is an appealing option.
    b. For patients whose BMD has increased to the low normal range and who are deemed to be at low risk of fracture, short-term therapy with a long-active bisphosphonate to prevent the interval of rapid bone loss may be appropriate.

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# Skeletal Development and Mineral Metabolism in the Fetus and Newborn: Insights from Animal Models and Limited Human Data

Christopher Kovacs, M.D.

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**Skeletal Development and Mineral Metabolism in the Fetus and Newborn: Insights from Animal Models and Limited Human Data**

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**Significance of the Topic**:

While a few bones arise directly from mesenchyme, much of the skeleton develops initially as a cartilaginous scaffold that is later sequentially torn down by chondroclasts to make way for osteoblasts that lay down primary spongiosa. A programmed temporospatial sequence of gene expression within chondrocytes and bone cells, combined with influences from hormones, cytokines, and minerals within the bone marrow and circulation, coupled with poorly understood stochastic events, enable coordinated skeletal lengthening and maturation. Understanding how skeletal development is regulated provides insight into normal physiology, improving fracture healing, and potentially limb regeneration.

Adequate delivery of mineral is required for normal cellular function and for the developing skeleton to achieve and maintain appropriate mineral content and strength. Studying fetal mineral metabolism is technically challenging due to the small size of most animal fetuses, while for ethical reasons human data have been largely limited to cord blood samples from normal fetuses, pathological examination of fetuses that died at birth or have succumbed to lethal skeletal disorders. Genetically engineered mouse models have enabled detailed study of the regulation of fetal mineral homeostasis. During fetal development the placenta actively transports calcium, phosphorus, and magnesium. Animal and human data indicate that fetal mineral homeostasis requires parathyroid hormone (PTH) and PTH-related protein (PTHrP) but not vitamin D/calcitriol, FGF23, calcitonin, or sex steroids. It is not until the postnatal period, when intestinal calcium absorption becomes an active process that the organism begins to depend upon vitamin D/calcitriol.

**Learning Objectives:**

As a result of participating in this session, attendees should be able to:

1. Understand how data pertinent to skeletal development and mineralization have been gleaned from animal and human studies
2. Describe key genes involved in regulating skeletal development and how they interact with each other and appreciate how when abnormal have phenotypic consequences.

3. Appreciate how fetal mineral metabolism is regulated differently from in the adult, and the factors that program a switch to adult regulatory mechanisms at birth
4. Contrast the marked difference in role of vitamin D/calcitriol in the fetus vs. the neonate and adult

**References:**

Kovacs CS. Bone Development and Mineral Homeostasis in the Fetus and Neonate: Roles of the Calciotropic and Phosphotropic Hormones. *Physiological Reviews*. 2014; 94(4):1143-1218.

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# What Is the Optimal Dose and Administration of Vitamin D Supplement in Falls and Fractures Preventions?

Kerrie Sanders, Ph.D.

MTP Handout for ASBMR 2016: Professor Kerrie M Sanders

**"What Is the Optimal Dose and Administration of Vitamin D Supplements in Falls and Fracture Prevention"**

Introduction

Vitamin therapies are appealing approaches to decrease the risk of adverse events associated with suboptimal physical functioning and ageing. However history has taught us that inappropriate vitamin therapy can be associated with harm. Twenty years ago results from observational studies led to the widely accepted conclusion that beta carotene (pre vitamin A) is the primary component of a diet high in fruit and vegetables that is associated with the lower risks of cancer and deaths from cardiovascular disease. Findings from subsequent randomized controlled trials (RCTs) concluded that the combination of beta carotene and vitamin A had no benefit and may have had an adverse effect on the incidence of lung cancer and cardiovascular disease in smokers and workers exposed to asbestos[1].

Like vitamin A, vitamin D is a fat soluble vitamin that can be stored in our bodies. However unlike other vitamins, for most of us the main source of vitamin D is not derived from our diet but from a series of reactions commencing with cutaneous production of pre-vitamin D from sunlight. Ageing skin is less efficient in producing pre-vitamin D. This and other factors contribute to the high prevalence of low vitamin D status in many 'ageing' populations consistent with recommendations from many professional organisations to endorse widespread supplementation for older adults.

It is widely accepted that low vitamin D status is associated with suboptimal musculoskeletal outcomes and increased risk of falls and fracture. In principle, agreement exists that vitamin D deficiency should be corrected. However debate continues over the definition of vitamin D deficiency and the target level of serum 25-hydroxy vitamin D. Like research on other vitamins, clinical trials have reported adverse outcomes associated with specific dosing regimens.

As a consequence, the optimal dose and administration of vitamin D supplements is also a controversial issue. With the widespread use of vitamin D supplementation there is an urgency to identify the optimal dose and regimen to ensure our recommendations are consistent with a beneficial effect on musculoskeletal falls and fractures.

Learning objectives

As a result of participating in this workshop, attendees should be able to:

1. Have an increased awareness of the evidence around threshold levels of both deficiency and sufficiency of serum 25-hydroxy vitamin D;
2. Have a better understanding of the published evidence of benefits / harm around different dosing regimens of vitamin $D_2$ and $D_3$;
3. Have a better understanding of the literature to decide if recommendations around initial loading doses of supplemental vitamin D supported by evidence;
4. What doses are needed to achieve significant changes in serum 25-hydroxy vitamin D levels and what is the evidence doses needed to maintain these levels.

Outline of content

With audience participation the workshop will debate that the optimal (equivalent daily) dose is either [A] 800 to 1,000 IU; [B] 2,000 IU or [C] >2,000 IU. Discussion will be focussed on the evidence for white, non-obese adults aged 70+ years. Published data will also be used to debate the optimal administration of vitamin D with a focus on establishing the ideal time interval between doses. Do we need to compromise on persistence rate to ensure our recommendations are conferring musculoskeletal benefits and not harm?

Case studies

Case studies will be used to illustrate variation in individual outcome response of biochemical, physical functioning and falls over time among older women given high dose vitamin D supplements.

Reference

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# miRs and Bone Homeostasis

Anne Delany, Ph.D.

**miRNAs in Bone Homeostasis**

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**Significance:**

A complete understanding of the molecular mechanisms regulating osteoblast and osteoclast biology is the vital basis for designing novel therapies to treat bone loss. microRNAs (miRNAs) are critical post-transcriptional regulators of gene expression, and their importance in regulating osteoblast and osteoclast differentiation and function is now appreciated. MicroRNAs are small, endogenous, single-stranded RNAs that regulate expression of protein encoding genes. miRNAs assembled in the RNA induced silencing complex (RISC) directly bind to the target mRNAs and mediate downregulation of their expression by mRNA degradation and/or translational suppression. miRNAs cause modest changes in the expression of multiple mRNA targets within the same or correlated pathways; the sum of which results in significant changes in key cellular activities. miRNA-based therapeutics have been efficacious in animal models, tempering the deleterious skeletal effects of glucocorticoid excess, estrogen-deprivation and unloading. There are hundreds of miRNAs, and each miRNA can potentially target hundreds of mRNAs. The gap in knowledge regarding the function and regulation of individual miRNAs in the skeleton is massive.

**Learning Objectives:**

As a result of participating in this session, attendees should be able to understand:

- miRNA biogenesis and function
- methods for identifying potential miRNA-target interactions and testing their impact on gene expression
- key miRNA regulated pathways in osteoblasts and osteoclasts
- potential miRNA-based therapeutics and mechanisms of delivery

**Outline:**

I. miRNA biogenesis
   A. miRNA gene organization
   B. miRNA processing
   C. Regulation of miRNA processing

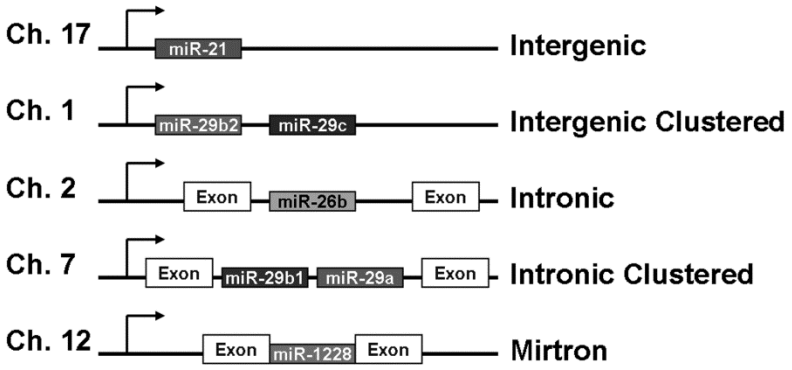
II. miRNA function
   A. Networks, thresholds, noise suppression
   B. Rapid regulation of stable mRNAs
   C. miRNA turnover/half life

III. miRNA-target prediction
   A. Useful websites
   B. Sequence features related to miRNA-target interactions
   C. Non-biased approaches for miRNA-target identification

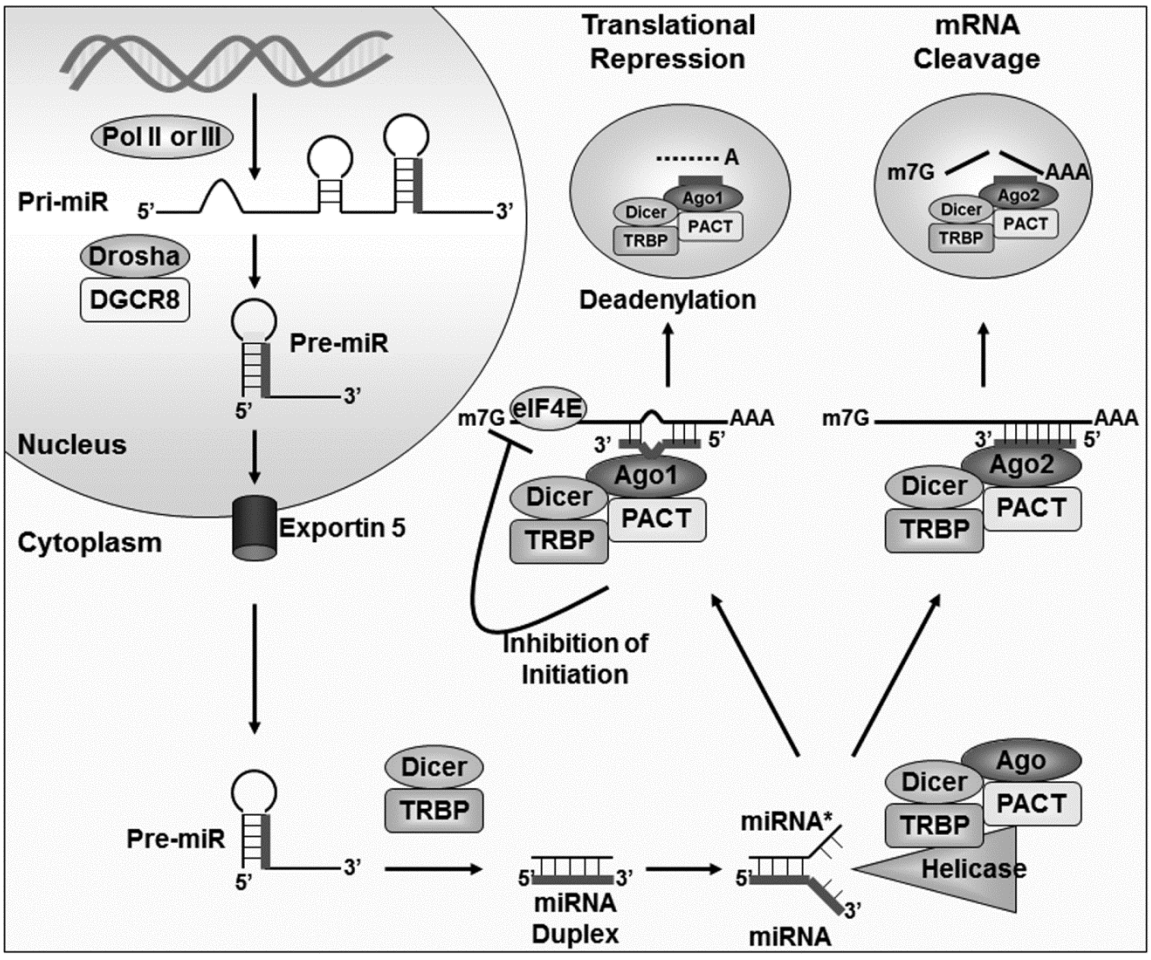
IV. Validation of efficacy
   A. 3’ UTR function
   B. Modulation of cellular function

V. Selected miRNA-target networks
   A. Osteoblast
   B. Osteoclast

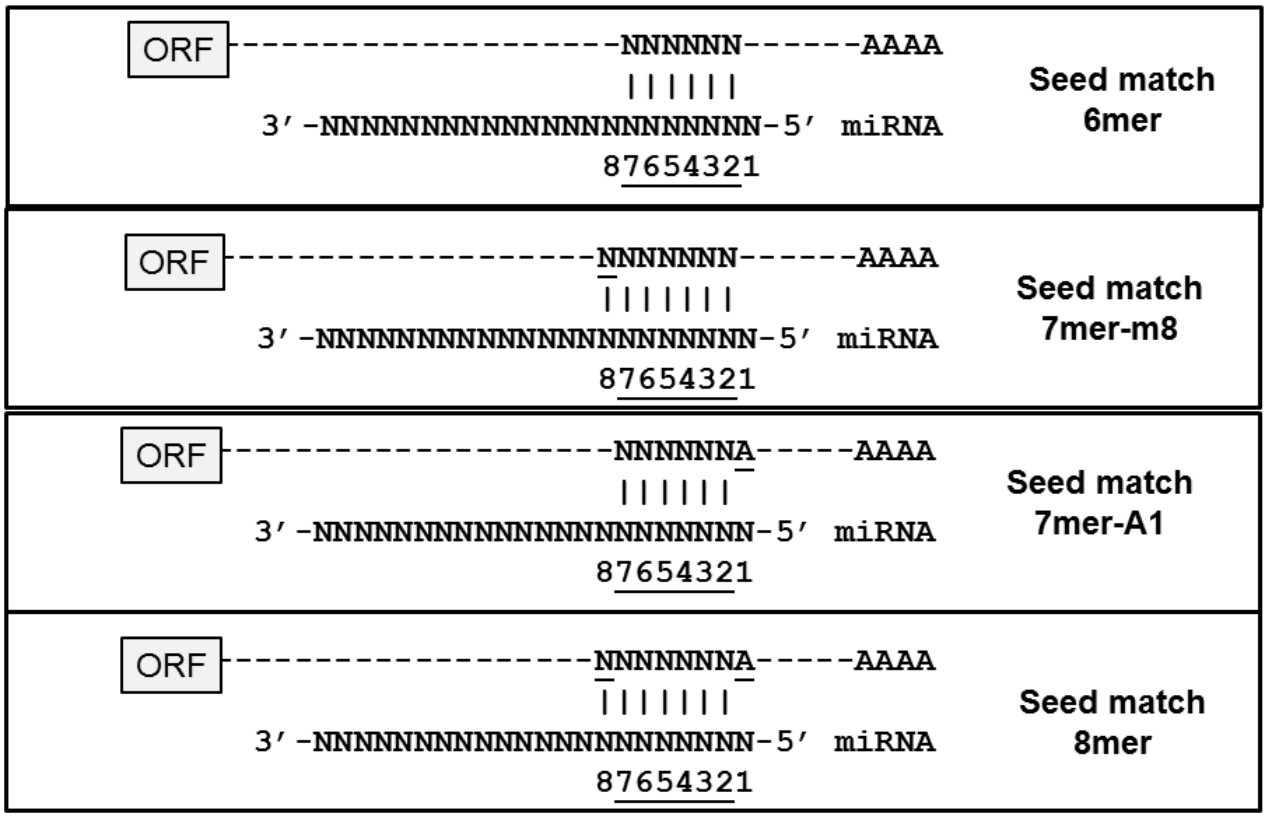
VI. miRNA-based therapeutics
   A. Mouse models - proof of concept
   B. Human trials




**Genomic organization of microRNA genes.** miRNA genes can be intergenic (alone or clustered), in the intron of non-coding RNA or protein-coding genes (alone or clustered), or can be mirtrons (part of a short intron of another gene). From Kapinas & Delany, Arthritis Res Ther 2011




**MicroRNA biogenesis pathway.** miRNAs are transcribed by RNA polymerase into primary (pri)-miRNAs, which are processed by Drosha/DGCR8 (DiGeorge syndrome critical region gene 8) into precursor (pre)-miRNAs. The premiRNA is transported from the nucleus into the cytoplasm by Exportin 5, where it is processed by Dicer/TRBP (Dicer-TAR RNA binding protein) into a miRNA duplex. The duplex is unwound by a helicase and the mature strand is incorporated into the RISC. Depending on miRNA complementarity to a target mRNA, the RISC mediates down-regulation of gene expression by translational repression, destabilization or mRNA cleavage. From Kapinas & Delany, Arthritis Res Ther 2011




**Canonical miRNA-target site complementarity. Seed region is miRNA bases 2-7 (underlined).**

**Characteristics of miRNA binding sites that are more likely to be effective:**

1. Good seed match
2. Binding site conserved across species
3. Complementarity at other miRNA regions, especially miRNA bases 13, 14 or 18, 19
4. Near proximal or distal end of 3' UTR
5. Flanking regions rich in A or U
6. Multiple sites
7. Site not involved in secondary structure

**Useful miRNA-target prediction tools**

Pictar (http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi)

TargetScan (http://www.targetscan.org/vert_71/)

miRanda (http://www.microrna.org/microrna/getDownloads.do)

PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html)

RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid)

rna22 (https://cm.jefferson.edu/rna22/)

Diana Tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software)

| Site | | Features |
|---|---|---|
| Pictar | | Predictions based primarily on evolutionary conservation |
| TargetScan | | |
| miRanda | | Support vector regression (SVR) takes into account miRNA and target features (including site accessibility, conservation) |
| PITA | | Energy of miRNA-target site interaction, site accessibility |
| RNAhybrid | | |
| Diana Tools | micro-CDS: | Trained on positive and negative sets of miRNA Recognition Elements (MREs) located in both the 3'-UTR and CDS regions. |
| | TarBase v7.0 | Manually curated target database. Includes targets from high throughput experiments (i.e. microarrays, proteomics, sequencing [HITS-CLIP, PAR-CLIP]). |
| | mirPath | Performs miRNA pathway analysis. Can utilize predicted miRNA targets and/or experimentally validated miRNA interactions |
| | DIANA-mirExTra | Estimates miRNA effects on expression protein-coding RNAs based on the frequency of hexamers in the 3'UTR sequences of genes. |

Comparing Ago-CLIP (crosslinking immunoprecipitation) and different prediction models, it is thought that the most important predictive features are:

- miRNA-target site seed and flanking region conservation
- miRNA-target site alignment (particularly seed region)

(Wen et al., microRNA transfection and Ago-bound CLIP-seq data sets reveal distinct determinants of miRNA action. RNA 17:820-834, 2011)

# Osteogenesis Imperfecta: Novel Therapeutic Approaches

Joan Marini, M.D., Ph.D.

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# Osteogenesis Imperfecta: Novel Therapeutic Approaches

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**Significance of the Topic:**
Osteogenesis imperfecta is a heritable collagen-related bone disorder, characterized by brittle bones with increased fracture risk that presents most severely in children[1]. Depending on the OI-causing mutation, fracture rates can range from relatively few in the least severe conditions to frequent fractures or intrauterine injury leading to death[2]. OI is widely recognized as a disease of both altered bone quality and high bone turnover, and appropriate treatments that reduce fracture rates by addressing both of these factors remain elusive. A series of therapeutic approaches have been tested over the years in both animal and clinical trials, including pharmacologic (bisphosphonate, denosumab, teriparatide), hormonal (growth hormone), and cellular and molecular (cell engraftment, gene therapy) approaches. Recently, novel techniques such as anti-sclerostin therapy and anti-TGFβ have been used to address the bone fragility in OI models, and are now poised for translation to evaluation in clinical trials.

**Learning Objectives**
As a result of participating in this session, attendees should be able to:

1. Describe the evolving nature of diagnosis of osteogenesis imperfecta, including the classical dominant form, and newly emerging recessive forms of the disease
2. Describe common and proposed treatment options for OI, what mechanism they target, and long-term potential for clinical translation
3. Understand an integrated process of translational collaboration and strategies for identifying opportunities for basic-clinical interactions at their institution.

Osteogenesis Imperfecta: An evolving diagnosis

***Classical OI: A disease of type I collagen***
The great majority of OI cases (80-85%) are caused by mutations in the genes that encode type I collagen, COL1A1 and COL1A2. These conditions have dominant inheritance. Type I OI is caused by null mutations in COL1A1, resulting in a quantitative defect of collagen. However, all of the collagen that is produced is structurally normal and the clinical phenotype is mild.

Types II, III and IV OI have lethal, progressive deforming and moderately severe outcomes, respectively. They are caused by a variety of structural mutations in either alpha chain, most commonly substitutions of a Glycine residue in the Gly-X-Y triplet, but also exon splicing defects, deletions and duplications. In these types, the mutant collagen is secreted into matrix, where it impacts crosslinking of collagen fibrils in ECM, bone mineralization and protein-protein interactions.

***OI in 2006-2016 and beyond: A collagen-related dysplasia***
Beginning in 2006, a series of genetic causes of OI in non-collagenous proteins were identified in genes not previously understood to be critical to bone development. There are now a total of 18 types of OI. The protein products of all these genes relate to collagen in some way.
**Defects in collagen modification**: CRTAP, P3H1 and PPIB
**Defects in collagen folding, processing and crosslinking**:
SERPINH1, FKBP10, PLOD2, BMP1
**Defects in bone mineralization**: IFITM5 and SERPINF1

**Defects in osteoblast differentiation affecting collagen secretion**:
Sp7, TMEM38B, WNT1, CREBL1, SPARC, MBTPS2

Common Features Contributing to OI fragility

1. Matrix-level deficiencies
    a. Collagen alteration in quantity, primary structure and/or modification
    b. Hypermineralization
    c. Altered crosslinking in collagen fibrils
    d. Brittle bone material
2. Cellular balance
    a. Increased bone remodeling
    b. Slow bone apposition
3. Organ level abnormality
    a. Low bone mass
    b. Altered bone geometry: short, gracile, susceptible to deformity

Therapeutic Approaches

Contributors to OI fragility are multifaceted. OI manifests at the level of the extracellular matrix, where alterations in collagen and mineralization impact bone fragility. Moreover, a cellular imbalance that favors increased bone remodeling and slow bone apposition leads to reduced bone mass, contributing to the high level of bone deformity and severe fracture rates observed in the disease. Lastly, short stature, a phenotypic feature usually associated with chondrodystrophies rather than osteodystrophies, occurs in essentially all OI types to varying degrees, and leads to additional challenges.

Correspondingly, multiple layers of targeting the OI phenotype have emerged, each with varying degrees of success. Cellular and genetic targeting seek to correct the fundamental genetic defect responsible for the disease, or mimic the mosaicism or collagen (*COL1A1*) mutant allele silencing that are associated with the mildest phenotypes in patients. Hormone therapy was initiated to ameliorate the growth deficiencies found in OI patients, and was also shown to improve low bone mass in those with a growth response. Established and emerging pharmacologic regulators have been used to correct the cellular imbalance in OI—attempting to increase bone quantity and reduce fragility, even in the absence of alterations in the fundamental genetic cause for the disease.

*Cellular and genetic targeting*

**Cell transplantation**

Proposed mechanism: The underlying hypothesis is that mesenchymal stem cells will be taken up by bone tissue in vivo, where they will differentiate into normal human osteoblasts. The normal cells would then be expected to outgrow cells producing mutant collagen as well as produce matrix with higher efficiency, resulting in a mosaicism situation similar to that seen in some very mildly affected parents of OI children.

Animal model results: Human fetal MSCs and adult MSCs have been transplanted in utero into oim[3] and Brtl mice[4], respectively. In both experimental sets, the uptake of normal cells was quite low. However, surprisingly for a low transplant percentage, transplantation was associated with increased bone strength and thickness. In Brtl mice, the proportion of mutant offspring with perinatal lethality was reduced. Both studies omitted mutant into mutant transplantation that would have controlled for the impact of the cell transplantation process itself.

Human results: Horowitz and colleagues have performed MSC transplantation on a small number of children with OI[5,6] and further studies are now planned in a European consortium. Again, normal cell uptake was low (2-5%). The authors reported transiently increased BMD and growth rates, although there were procedural problems with both measurements.

**Gene Silencing**

Proposed mechanism: This approach is modeled on type I OI, in which individuals have a null COL1A1 allele. They produce a reduced amount of structurally normal collagen and have mild symptoms. In theory, silencing the mutant COL1A1 allele in types II, III or IV OI could convert them to mild type I OI. The catch is that the silencing must be allele specific.

Animal model results: This approach has been investigated using hammerhead ribozymes[7] and siRNA[8,9]. The allele specificity is generally quite high *in vitro.* However, in cells (Brtl mouse and human) the allele-specificity decreases, so that both normal transcripts and protein are also partially suppressed. Cleavage efficiency vs. cycling off efficiency is also an issue.

Human results: Not yet attempted.

*In Clinical Practice*

**Growth Hormone**

Proposed mechanism: Growth hormone stimulates proliferation of resting zone chondrocytes, and stimulates local expression of IGF1, which may increase size of hypertrophic chondrocytes. rGH therapy can nearly restore height in growth hormone deficient children. However, despite short stature, children with OI do not have hormone deficiency, leading to the prediction that the growth plate of OI bone was resistant in some way to normal levels of rGH axis hormones. Administration of standard doses of rGH would supplement the endogenous hormone, and might overcome any tissue level resistance, increasing OI patient stature.

Animal model results: No published studies in OI murine models.

Clinical results: Children with types III and IV OI have been treated with standard doses of rGH[10]. About half of the treated children (mostly type IV OI) had a sustained growth response. Only the children who responded with increased linear growth also had a significant increase in vertebral DXA z-scores and a decrease in long bone fractures. Bone tissue of responders showed increased BV/TV, TBN and BFR, demonstrating a positive anabolic effect on bone that is encouraging for more general bone anabolic drugs.

**Bisphosphonate**

Proposed mechanism: Increased bone turnover in OI patients leads to excessive bone loss and insufficient accumulation of trabecular bone at the growth plates. It was hypothesized that bisphosphonates could be used to reduce high turnover rates in OI, slow the remodeling at the growth plate, and reduce endocortical expansion in growing patients.

Animal model results: Studies in mouse models of OI treated with bisphosphonates have shown strong gains in mass at trabecular sites, with significant retention of calcified cartilage and primary spongiosa in a banding pattern corresponding to each drug dose[11-14]. Cortical bone effects are less well resolved, and biomechanical testing has not demonstrated gains in strength that match modest gains in cortical bone size

Clinical results: Controlled clinical trials and recent meta-analyses have shown data consistent with animal models[15-23]. Significant gains in trabecular bone due to altered growth plate

remodeling are apparent, while gains in long bone cortical bone mass, and reductions in long-bone fracture rate remain unproven.

**Denosumab**

Proposed mechanism: As the molecular mechanisms of increased osteoclast activity have been discovered, the field of osteoporosis has generated more mechanistic means of interrupting the osteoclast activation pathway. Denosumab is a monoclonal antibody to RANKL, disrupting osteoclast activation and resorption by interfering with RANKL-induced activation of RANK. Similarly to bisphosphonates, denosumab is proposed as a means to disrupt high osteoclast activity in OI and reduce high bone turnover and increased endocortical expansion.

Animal model results: OI mice treated with RANK-Fc before, and for up to 6 weeks during fracture repair showed no significant changes in biomechanical properties of contralateral intact femora with treatment[24]. Juvenile oim/oim mice treated with RANK-Fc during growth showed some improved femoral density and geometric features, but no decreases in the rate of spontaneous fractures were observed in this study[25].

Clinical results: Denosumab was used to treat patients with type VI OI, where increased bone resorption results in association with mutations in *SERPINF1*, and patients have traditionally been resistant to bisphosphonate therapy[26]. Two years of denosumab in four type VI patients showed increased bone mineral density and restored vertebral shape. Denosumab has since been tested in patients with COL1A1/COL1A2 mutations, showing positive effects in spine aBMD after 48 weeks of treatment[27].

**Intermittent Parathyroid Hormone**

Proposed mechanism: Currently, the only FDA- approved anabolic bone drug for osteoporosis is intermittent parathyroid hormone/teriparatide/Forteo. Forteo is contraindicated for pediatric use due to pre-clinical trials that showed an increase in osteosarcoma in aged rats treated with long-term PTH. Therefore, its use as an anabolic has been limited to adult therapy.

Animal model results: No published studies in OI murine models.

Clinical results: Postmenopausal OI type I patients were treated with teriparatide for 18 months, and exhibited significant increases in BMD at the lumbar spine, but not in the hip[28]. Bone formation markers were considerably increased in response to therapy, but BMD gains were lower than expected compared to osteoporosis trials. A larger, double-blind, placebo controlled trial showed increased aBMD in the lumbar spine and total hip with treatment over placebo controls[29]. Adults with type I OI showed a much stronger treatment response than patients with more severe forms of type III and IV OI, suggesting that baseline phenotype may influence response to therapy.

*Poised for Clinical Translation*

**TGF-β antibody**

Proposed mechanism: Excessive TGF-β signaling has been proposed as a common feature in OI leading to low bone mass and altered cell signaling via the bone matrix[30]. Reducing excess TGF-β overexpression was hypothesized to alleviate phenotypic features of OI in both dominant and recessive OI mice.

Animal model results: Crtap -/- and G610C mice were both treated with a neutralizing antibody to TGF-β. Treatment was associated with a restoration of trabecular and cortical bone

architecture, and improved biomechanical properties, with associated reductions in osteoclast and osteoblast number. Although it remains unclear to what extent TGF-β is a bystander rather than an effector in recessive OI, an interesting amelioration of lung abnormalities was also noted in Crtap -/- with TGF-β inhibition therapy.

Outlook for clinical trials: No published clinical trials.

**Sclerostin antibody**

Proposed mechanism: Sclerostin antibody is emerging as a potent anabolic bone drug for postmenopausal osteoporosis. Sclerostin antibody reduces inhibition of bone formation by sclerostin, and also appears to downregulate osteoclastic resorption, offering a means to decouple the high turnover present in OI and increase overall bone mass.

Animal model results: Sclerostin antibody significantly improves both the structure and function of cortical and trabecular bone sites in OI mouse models[31-35]. In growing animals, increases in bone mass induced by sclerostin antibody during rapid bone growth and bone modeling coincide spatially with reductions in osteoclast modeling activity. Efficacy of sclerostin antibody may depend on the underlying severity of the disease[36], and likely requires existing bone mass upon which to induce its anabolic actions.

Outlook for clinical trials: No published clinical trials.

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# Bone Marrow Adipose Tissue: Development and Detection

Mark Horowitz, Ph.D.

# *MEET-THE-PROFESSOR*

## Bone Marrow Adipose Tissue: Development and Detection

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**Significance of the Topic:** Marrow adipose tissue (MAT) was identified in the bone marrow (BM) more than a century ago but has recently been associated with age, metabolic disease and low bone volume, highlighting the importance of studying this often neglected adipose tissue depot. White adipose tissue (WAT) and brown adipose tissue (BAT) are found in discrete subcutaneous, visceral or subdermal depots. In contrast, MAT is most commonly located above the growth plate in the secondary center of ossification or just below the growth plate in the primary spongiosa of long bones (tibia and femur). MAT comes in two types, separable by development and physical location within the bone. Constitutive MAT (cMAT) appears early in development of the mouse (detectable by 1 week of age) and is not dependent on strain. As an example, C57BL/6 (B6) and C3H/HeJ (C3H) mice have similar amounts of cMAT (1). In contrast, the amount of inducible MAT (iMAT) is strain dependent as B6 mice have very low levels of iMAT while C3H mice have very high levels of iMAT. Importantly, B6 mice have low bone density while C3H mice have high bone density, indicating that the presence of MAT does not always correlate with bone density. Induction of BM adipogenesis with rosiglitazone feeding, x-irradiation or calorie restriction results in increased iMAT formation. The MAT progenitor has not been characterized although it appears to arise from a cell that is the precursor of both osteoblasts and adipocytes (2). Altering the lineage allocation of this progenitor can change bone density (3, 4). Where this progenitor resides is also unknown. The function of MAT is poorly understood, although it appears to regulate hematopoietic cell development.

Despite the ease of inducing marrow adipogenesis, the study of MAT has been hindered by both the presence of bone (which makes accessing MAT difficult) and the heterogeneous mixture of cells within the BM (which makes lineage tracing difficult). Therefore, new techniques have been developed to circumvent these issues and allow for the study of MAT *in vivo*. First, we have developed a technique to quantitatively measure MAT *in vivo* using osmium tetroxide staining coupled with micro-CT (5). Bones are first fixed in formalin and then decalcified with EDTA, which allows for penetration of the osmium into the medullary canal. Osmium efficiently stains lipid and because it is a heavy metal can be visualized and quantified by mico-CT. The entire bone or regions of interest can be analyzed. Second, our laboratory and others have utilized a variety of fluorescent reporter mice to trace the lineage of marrow adipocytes and bone cells. To determine the origin of BM adipocytes, we have performed lineage tracing using the fluorescent *mT/mG* reporter mouse in concert with various mouse models driving cre-recombinase from lineage specific promoters (6). In *mT/mG* mice, expression of cre-recombinase results in permanent removal of the membrane targeted dTomato (mT) cassette and expression of the membrane targeted eGFP (mG) cassette, resulting in “flipping” from dTomato+ to eGFP+ cells. The *mT/mG* model is extremely useful for lineage tracing because expression of Cre-recombinase in progenitor cells results in the permanent expression of eGFP in both progenitor and daughter cells.

These and other techniques to visualize and quantify MAT will become more important as the relationships between MAT, bone, BM and whole body metabolism are better understood.

**Learning Objectives:** As a result of participating in this session, attendees should be able to…

1. have a sense of where BM adipocytes come from.

2. that BM adipocytes are different than white, brown and beige adipocytes.

3. how to work with BM adipocytes.

4. be able to visualize and quantitate BM adipocytes *in vivo*.

**Points of Interest:**

1. Quantitation of BM adipocytes by staining with osmium tetroxide coupled with micro-CT (5).

2. Technique for Preserving GFP in Bone Marrow Adipocytes (Intact Bone)
(Developed by Ms. Rose Webb, Department of Orthopaedics Histology and Histomorphometry Laboratory), Yale School of Medicine, New Haven, CT.

Green Fluorescent Protein (GFP) is often used to visually observe transgene activity in embryos. Recently, researches have been interested in observing GFP in mature animal tissue using histological techniques. This technique will be discussing specifically how to preserve the GFP in adipocytes in murine bone marrow.

While some GFP may be observed in a sample after routine processing, both solvents and heat are used to achieve ideal paraffin or methacrylate (plastic) sections. So, GPF expression is not preserved. Any evidence of GFP on a paraffin or methacrylate processed slide cannot be used to confidently collect data. To observe the labeling, it is necessary to process the sample for cryotomy.

Immediately after the animal is sacrificed remove as much soft tissue from the bone as possible. Special care must be used to prevent the sample from being exposed to organic solvents at any time. Be very careful to avoid ethanol during the dissection. Ideally the femur is selected, the wide distal portion makes microtomy easier, but any bone can be selected and processed using the same method. If there is a portion of the bone not of interest to the researcher it should be cut off during dissection to quickly expose the marrow to the fixative.

Do not allow the sample to dry, immediately place the sample in 4% paraformaldehyde, prepared in PBS (overnight, at 4$^{o}$C, on a shaker). Wrap the container in aluminum foil to avoid prolonged light exposure. Paraformaldehyde preserves the fluorescents better when compared to neutral buffered formalin. Be sure to use freshly prepared paraformaldehyde. Do not fix the sample longer than overnight, over-fixation causes auto-fluorescence and increases background noise.

Caution: Paraformaldehyde is toxic. Please read the SDS before working with this chemical. Gloves and safety glasses should be worn and the solution should be made in the fume hood. For instruction on preparing 4% paraformaldehyde see a separate protocol.

After overnight fixation the sample should be rinsed well in buffer. Routinly, we use 3-4 15 minute washes in PBS. The paraformaldehyde should be collected in a waste container. Be sure to remove all the paraformaldehyde from the sample, it will take several rinses.

Place the sample in 4% EDTA to decalcify. EDTA takes about 17 days to completely remove the calcium ions from an adult murine femur. Change the EDTA every 3-4 days.

Ethylrndiaminetetracetic acid (EDTA) is the decalcifier of choice. It is a very slow, very gentle way to remove calcium ions. The EDTA must have a pH very close to 7. If EDTA is acidic the decalcification will happen much slower and it's already a slow method so the decalcification will almost stop completely. If the EDTA is basic it will work faster but the GFP will be less intense. So, be sure to pH the EDTA. For instruction on preparing EDTA see a separate protocol.

Rinse the sample in PBS to remove the EDTA

Place the sample in 30% sucrose made in PBS overnight

Place the sample in 50:50 solution of OCT:30% sucrose for 1 hour

Place sample in OCT and freeze down. Ideally isopentane in liquid nitrogen is used. If that is not available, the sample in OCT can be place at -80$^{o}$C

Section in the cryostat at -25$^{o}$C; 5 microns thin

Coverslip at room temperature with 50% glycerin in PBS and view as soon as possible.

3. <u>Collection and Processing of Bone Marrow Adipose Tissue for Fluorescence using Confocal Microscopy (BM Plug)</u>

Dissect out the femur and clean off the majority of soft and connective tissue.

At the proximal end, using a scissors cut off the bone just below the femoral head. At the distal end of the bone cut through the growth plate.

Using a 20-gauge needle slide in through the medullary canal from the proximal to the distal end of the bone. As you advance the needle down the medullary canal rotate the needle to capture as much BM as possible.

Once you have penetrated through the growth plate at the distal end of the bone, affix the needle to a 5 ml syringe (with the plunger already pulled back). Gently push the plunger down, forcing the plug of BM onto a new clean microscope slide.

Surround the plug with a continuous line of Vaseline extruded from a syringe + needle leaving the BM plug in the middle of a small pool surrounded by a wall of Vaseline.
If you want to stain the adipocytes with LipidTox this is the time.

Fill the pool with Fluromount G.

Coverslip the BM plug so that the Vaseline forms a watertight seal with the coverslip.

Seal the coverslip to the slide with nail polish and air dry.

Your BM is ready for the confocal.

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