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Meet-the-Professor

Handout Booklet

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Meet the Professor: Bone Extracellular Matrix Assembly and Mineralization

Marian Young, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 102, Colorado Convention Center in Denver, CO, USA

Bone Extracellular Matrix Assembly and Mineralization

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Significance of the Topic: The skeleton is unique from all other tissues in the body because of its ability to mineralize. The incorporation of mineral into bones and teeth is essential to give them strength and structure for body support and function. For years, researchers have wondered how mineralized tissues form and repair. A major focus in this context has been on the role of the extracellular matrix, which harbors key regulators of the mineralization process. In this introductory "Meet the Professor", key "players" in matrix biology as they relate to mineralized tissues will be outlined. In reviewing key topics in mineralized tissues I hope the attendees will get a broad view of the topic and all of its fascinating complexities.

Introduction

Who are the major players? How were they identified and studied?

Type I collagen

At least 27 different collagen types have been identified so far [1], many of which are found in the skeleton. The most abundant species in mineralized tissue is Type I collagen, long known to have vital roles in regulating skeletal integrity. The production and processing of collagen is highly orchestrated [1] involving a multitude of chaperones and enzymes that modify and crosslink collagen during its assembly into a triple helix and ultimately into fibrils [2]. It is generally believed that collagen orients proteins that serve as a nidus for minerals to localize and accumulate, therefore serving a key function in mineralized tissues [3]. Testimony of the importance of type I collagen in mineralized tissue formation comes from patients with mutations in the type I collagen gene (referred to as the Col1A1 and Col1A2 genes), who are afflicted with severe skeletal deformaties in a condition known as osteogenesis imperfecta (OI, or brittle bone disease)[4]. Interestingly, many lethal mutations in OI are located in the triple helical domain of collagen in a region that aligns with binding sites for other ECM components [5] including proteoglycans [6]. This finding emphasizes the importance of the potential synergy between ECM components where one ECM member can affect the function of another. Further studies are needed to delineate the ECM interplay in mineralized tissue disease.

Non-collagenous proteins: SIBLINGS

The importance of collagen in bone mineralization presents a conundrum: how do tissues that do not make collagen (like enamel) control the mineralization process? In this context, it must further be questioned: why does skin that is rife with type I collagen not mineralize? There must be extracellular matrix components other than collagen that are involved in regulating the mineralization of hard tissues. To address some of these points, a review by Boskey et al. [7] describes the mineralization process and its relationship to a family of proteins called Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs). A biochemical characteristic of the SIBLINGs is that they are highly acidic, which is likely one reason they have affinity for the basic hydroxypapatite that makes up the mineral composition of bone. What is interesting about the SIBLINGs is that they are intrinsically "disordered", meaning that they can adapt to many shapes presumably to give them flexibility and versatility in function. All members of this family have an Arg-Gly-Asp (RGD) cell attachment site that may be used for integrin binding and regulation of cell function. The members include: bone sialoprotein (BSP), dentin matrix protein (DMP), dentin

sialophosphoprotein (DSPP), enamelin, MEPE, and osteopontin (OPN). Each SIBLING has similaries but at the same time unique functions all of which continue to unfold.

ECM and Growth Factor Modulation:
A multitude of studies show that the ECM has important roles in regulating growth factor function. This includes all categoires of the ECM including: collagenous, non-collagenous and proteoglycans discussed further below. One example that demonstrates this paradigm is the proteoglycan biglycan: it binds to numerous factors including wnt 3a, TGF-beta and BMP as well as other ECM components such as the antiangiogenic endostatin thereby influencing down stream effects including bone formation, bone resporption and angiogenesis in bone healing [8,9].

Nice example of ECM affecting bones:
One of the first proteins extracted from bone is SPARC/osteonectin/BM-40, a protein highly expressed in mineralized tissues that was originally believed to be bone-specific with a primary function of linking collagen to mineral [10]. We now know that SPARC is widely expressed in many tissues, making it in some ways like type I collagen: important in both mineralized and non-mineralized tissues. Testimony to the importance of SPARC in bone biology comes from the elegant work from the Delany lab [11,12] that identified a single nucleotide polymorphism (SNP) in the 3' end of the SPARC gene that correlates with the occurrence of osteopenia (osteoporosis) in humans. Further studies revealed this SNP is targeted by the miRNA (miR-433) that down-regulates SPARC levels and subsequently reduces bone mass [12]. The "SPARC story" is a nice demonstration of the broad spectrum approach taken in our field to understand the mineralization process using biochemistry, animal modeling and human genetics to address key questions.

Proteoglycans: Diversity in structure (and function).
Proteoglycans are made of a core protein that has glycoseaminoglycans attached [13]. They are either large and "modular" (ie versican, aggrecan) or relatively small and include the SLRPs (Small Leucine-rich proteoglycans 1-17), the glipicans (1-6) and the syndecans (1-4). The composition of the GAG chains attached can be chondroitin sulfate (CS), dermatan sulfate (DS), heparin sulfate (HS) or keratin sufate (KS). The GAG chains are added to the core as blocks using multiple enzymes that either elongate the GAG chain or degrade the GAG chain (ie Heparanase). For the all the essentials in the current thinking of proteoglycan biochemistry see:
http://www.ncbi.nlm.nih.gov/bookshefl/br.fcgi?book+glyco2. Considering the complexity of the post-translational modifications and their unique core proteins, the task of deciphering the skeletal function each of their structural components is daunting.

Ectopic Mineralization and Tissue Engineering
The majority of this meet the professor session focuses on factors that promote tissue mineralization. A central question still remains: what prevents ossification in soft tissues? In certain conditions such as trauma, soft tissues such as tendons and ligaments, can ectopically ossify (EO). The precise matrix components that regulate EO are not completely known, but could be one or more of the SLRPSs [14]. In recent years, there has been an exponential interest in the role of matrix in tissue engineering. A nice review by Bellis et al. [15] explains how bone mimetic scaffolds can be used as a template for matrix proteins, growth factors and cells that mimic the basic biochemistry and structure of bone and are "inspired" by bones natural composition. A study by the Chen lab [16] shows how devitalized ECM elaborated by bone stem/progenitor cells could be used to retain their "stem-ness". Thus the ECM has potential to either promote or inhibit mineralized tissue formation and is important to consider in in stem cell biology and tissue regeneration.

Summary: There are numerous components in bones and teeth that may directly or indirectly affect mineralization. Considering the complexity of the ECM it's clear there is much more to learn about how they function to control mineralziated tissue function.

Table 1 Major “Players” in the ECM: Collagens and “non-Collagenous” proteins

Collagens/modifying enzymes	**Key features**
Type I	mutated in OI, increased osteoclastogenesis, TGF-beta
Type VI	affects osteoblast shape
LOX, LOX 1-4	regulates collagen processing
Non-Collagenous	
SIBLINGS: Cell attachment and more:	
Dentin sialophosphoprotein (DSPP)	dentinogenesis imperfecta, odontoblast specific
Dentin matrix protein (DMP)	role in FGF-signaling
MEPE	regulation of PHEX (phosphaturic protein)
Bone Sialoprotein (IBSP)	roles in bone, tendon and hematopoesis
Osteopontin (OPN)	roles in immune function, hematopoesis
Glycoproteins	
Osteonectin	bone mass regulator via SNP
Thrombospondin 1 and 2	differential effects on bone and BMSCs
Fibrillin 1 and 2	differential effects on bone, TGF-beta control
Fibronectin	cell attachement
Enzymes	
Alkaline phosphatase	needed for mineralization
MMPs	MT-14 most profound effect on bone
Gamma –Carboxy Glutamic Acid “GLA” containing and others	
Matrix Gla (MGP)	inhibits mineralization
Osteocalcin	many functions outside of bones
Periostin	periodontal calcification, immune functions
Enamel proteins	
Amelogenin (many isoforms)	amelogenesis imperfecta
Ameloblastin	tumor surpressor, promotes osteogenesis
Tuftelin	enamel structure
Proteoglycans (PGs)	
Large:	
Hyaluonanan (HA)	many functions, ie: immune, development
Perlecan	vascularization (in the basement membrane)
Versican	function in bone unclear
Aggrecan	major component of cartilage also in tendon
Small: “SLRPS”	growth factor modulation, collagen fibril organization
Biglycan, decorin, asporin (I)	
Fibromodulin, Lumican, osteoadherin, PRELP, keratocan (II)	
Epiphican, opticin, osteoglycin (III)	
Chondroaderin, nyctalopin, tsukushi (IV)	
Podocan, podocan like, proein 1 (V)	

Matrix effects: direct or indirect?

ECM ⇨ cells ⇨ Bones and teeth

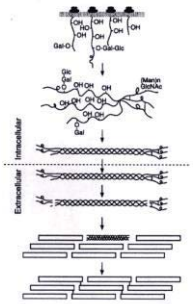
biglycan — Growth factor signaling — mineralization

Collagen Synthesis and Structure

Post-translational modifications

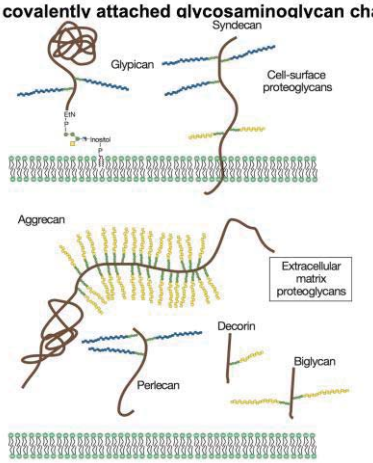
Triple helix formation

Fibril assembly



(Ann Med 2001;33:7-21)

Proteoglycans consist of a protein core and one or more covalently attached glycosaminoglycan chains



Essentials of Glycobiology
Second Edition

Chapter 16, Figure 2

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*For more details on ECM composition of bone See: A.L. Boskey and P.G Robey "The Compostion of Bone" in the Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism 8th addition, Editor, Clifford J. Rosen, Wiley-Blackwell, by the American Society of Bone and Mineral Research, ppps 49-58

Meet the Professor: Bone Marrow Fat in Health and Disease

Beata Lecka-Czernik, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 103, Colorado Convention Center in Denver, CO, USA

ASBMR 2017

Meet-the-Professor Session Handout

"Bone Marrow Fat in Health and Disease"

Beata Lecka-Czernik, PhD

University of Toledo Health Sciences Campus, Toledo, Ohio

Significance of the Topic:

Adipocytes reside in the bone marrow of all mammals and their number increases during both skeletal growth, which is associated with bone acquisition and sexual development, and aging, which is associated with bone loss and decline in gonadal activity. Besides physiological/hormonal regulation of their accumulation (including growth hormone, sex steroids, parathyroid hormone, insulin), environmental (overnutrition, malnutrition, ambient temperature) and pharmacological (TZD, glucocorticoids) factors may also contribute to this process (reviewed in [1]). Marrow adipose tissue or MAT is characterized with high heterogeneity which may suggest both that marrow adipocytes originate from multiple different progenitors and/or their phenotype is determined by skeletal location and environmental cues. Depending on physiological or pathological conditions MAT presence and its activity can be either beneficial or detrimental to bone. Thus, a unique phenotype of MAT and its close ties to bone homeostasis offers an opportunity for therapeutic targeting to treat metabolic bone diseases.

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

- Recognize the complexity of marrow adipocyte origin, phenotype, and function.
- Recognize a beneficial role of MAT in supporting bone homeostasis.
- Recognize MAT contribution to bone diseases including osteoporosis, diabetes, and cancer.

An Outline/Points of Interest

Origin, phenotypes and functions

There is certain controversy on marrow adipocyte origin with some models supporting their common origin with osteoblasts, while others negating it. It appears that both cell lineages originate from a common Myf5-negative progenitor and determination of their fate is under control of retinoblastoma protein (pRB) [2]. Myf5-negative progenitor is also common for peripheral "white" and "beige" adipocytes, with exception of "brown" thermogenic adipocytes which are Myf5-positive and related to muscle cells. Studies by Yue et al. showed that marrow adipocytes are derived from LepR+ skeletal stem cells [3] and Zhou et al. showed that these cells have also potential to differentiate to osteoblasts and can support hematopoietic niche by producing stem cell factor (SCF) [4]. Moreover, Fan et al. showed that MAT contains a population of adipocytes derived from Prx1- and PTHr1-positive osteoblastic progenitors which have a capacity to secrete RANKL cytokine, therefore supporting bone resorption and remodeling [5]. In contrast to the above, studies by Worthley et al. showed that marrow adipocytes do not share the same musculoskeletal ancestor as osteoblasts, chondrocytes, and muscle cells by demonstrating that Gremlin 1-positive mesenchymal progenitors can

differentiate into the above lineages but not into adipocytes [6]. Such diverse evidence for adipocyte phenotype promotes the hypotheses that either marrow adipocytes are derived from several different lineage-specific precursors, some of them closely related to osteoblasts, or they have a common origin but are highly plastic and their terminal phenotype is determined by functional necessity.

In healthy human adults, MAT constitutes up to 10% of total adipose tissues which translates to approximately 1 kg of mass. In humans, marrow adipocytes represent up to 45% of cellular components in hematopoietic or red marrow, and up to 90% of adipocyte-rich yellow marrow. Similarly in rodents, adipocytes are dispersed in epiphysis/metaphysis where bone remodeling and hematopoiesis occur, whereas they are densely packed and resemble yellow marrow in distal tibia and caudal vertebra where hematopoiesis and bone remodeling are absent (Figure 1). It has been recently shown that MAT located in proximal tibia (pMAT) differs from MAT located in the distal part (dMAT) with regard to fatty acids composition and response to low temperature [7]. The dMAT has higher fraction of unsaturated fatty acids as compared to pMAT, which may suggest different metabolic function of these two MAT depots. Moreover, pMAT, but not dMAT, responds to the cold exposure with decreasing in volume. On the other hand, caloric restriction increases MAT volume predominantly in proximal but not in distal location [7].

Structural difference between pMAT and dMAT (dispersed between trabeculae and densely packed, respectively) (Figure 1) correlates with their different phenotype and suggest their function. In healthy young adult C57BL/6 mice, pMAT is characterized with higher expression of beige fat markers than dMAT [8]. The expression of brown/beige markers, such as *Prdm16*, *Tbx1*, and *Dio2*, was relatively high in pMAT *vs* dMAT and in males *vs* females and showed differential regulation by sex steroids. However, an absence of expression of BAT-exclusive *Zic1* marker, and WAT-exclusive *Tcf21* marker, and beige-specific *Tmem26* marker, suggests different phenotype of marrow adipocytes from peripheral adipocytes. The expression of *Ucp1*, although detectable, is relatively low as compared to other fat depot, as well as expression of *Hoxc9*, indicating that marrow adipocytes differ from classical brown and beige adipocytes.

One of the unique features of MAT is simultaneous involvement in the regulation of energy metabolism and bone homeostasis which may, at least in part, explain skeletal response to pathologic changes in energy balance (e.g. obesity, diabetes, caloric restriction, anorexia nervosa). MAT role in the regulation of energy balance comprises production of insulin sensitizing adiponectin at the levels that significantly contribute to the circulating pools of this adipokine especially in conditions of decreased peripheral fat mass [9]. On the other hand, MAT futile metabolic phenotype correlates positively with bone health and negatively with bone loss. BAT-like (energy production and dissipation) characteristics of MAT are compromised with estrogen deficiency, diabetes, and aging despite significant MAT expansion in the bone marrow [8, 10]. This supports a notion that there is a relationship between MAT metabolic profile and bone health. Indeed, marrow adipocytes have a capacity for conversion to beige-like phenotype either upon expression of specific transcriptional regulators, e.g. FoxC2 transcription factor [11], or as a result of manipulation with PPARγ transcriptional activity by either pharmacological use of selective agonists such as telmisartan [12], or by manipulation with PP5 phosphatase activity which controls PPARγ protein phosphorylation [13]. Interestingly, conversion of marrow adipocytes toward beige-like phenotype is associated with increased expression of bone anabolic factors including WNT10b, IGFBP2 and BMP4, and secretion to the growth media of pro-

osteoblastic activities as assessed in co-culture experiments [12, 13]. This provides a strong argument for beige-like MAT possessing beneficial for bone endocrine/paracrine activities.

In this session, we will discuss whether there is a correlation between MAT phenotype and bone health and whether MAT can be targeted to treat bone diseases.

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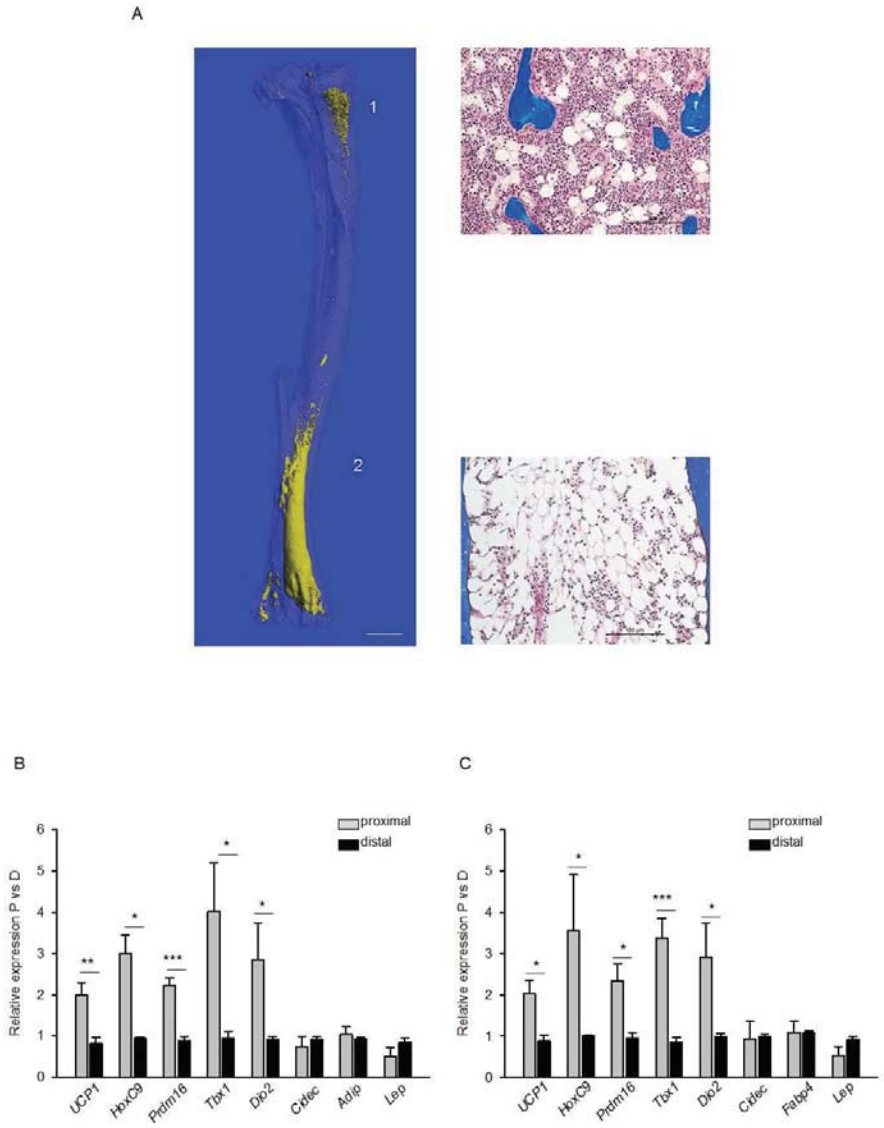

Figure 1. Morphology and polarity of tibia MAT. A. (Left) Longitudinal mCT rendering of decalcified tibia bone stained with osmium tetroxide to visualize lipids (white). Scale bars represent 1 mm. (Right) Representative longitudinal sections of proximal and distal non-decalcified tibia specimens stained with Masson's Trichrome Stain (20× magnification). Scale bars represent 100 µm. B. Gene expression profile of fat metabolic markers in proximal and distal tibia normalized to *Fabp4* expression. C. The same gene expression analysis as in (B) but were normalized to the levels of *Adiponectin* expression. Analysis was performed on 4 tibia bone isolated from 4 males (6 mo old). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Meet the Professor: Diabetes and Bone

Ann Schwartz, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 104, Colorado Convention Center in Denver, CO, USA

Diabetes, Bone, and Fracture Risk

Ann Schwartz PhD
University of California San Francisco, USA
September 8, 2017

SIGNIFICANCE OF THE CLINICAL PROBLEM

Diabetes is associated with higher fracture risk. In type 1 diabetes, hip fracture risk is about 4-5 times higher than for non-diabetic patients [1, 2]. In type 2 diabetes, the increased risk is more modest, about 1.3-1.7 times higher [3, 4]. However, type 2 diabetes affects over a quarter of older adults in the US, resulting in a substantial absolute increase in fracture risk. While fracture risk is increased, bone mineral density in type 2 patients tends to be higher than in those without diabetes. Diabetic patients are less likely to be screened and treated for osteoporosis, in spite of their higher risk. Possible reasons include the difficulties of fracture risk assessment along with the challenges of identifying optimal pharmacological therapy for osteoporosis in diabetic patients.

BARRIERS TO OPTIMAL PRACTICE

Obtaining an accurate assessment of fracture risk in diabetic patients is a challenge. The standard tools, BMD T-score and FRAX, tend to under-estimate risk in this population. Another challenge is identifying the potential impact of specific diabetic medications and of glycemic control on fracture risk. Finally, there are challenges in determining the optimal pharmacological therapy for osteoporosis when this level of treatment is warranted in a diabetic patient.

LEARNING OBJECTIVES:

As a result of participating in this session, learners should be able to:
Identify under-estimation of fracture risk with BMD T-score or FRAX in diabetic patients
Discuss effects of diabetes medications on skeletal health
Describe evidence for optimal pharmacological osteoporosis therapy in diabetic patients

STRATEGIES FOR DIAGNOSIS, THERAPY, AND/OR MANAGEMENT

ASSESSMENT OF FRACTURE RISK

BMD T-score does predict fracture in type 2 diabetes. As shown in Figure 1, among diabetic patients, those with lower BMD have greater fracture risk. However, BMD T-score under estimates absolute fracture risk in diabetic patients compared with non-diabetic patients [5]. As a rough estimate, one can subtract 0.5 from the measured femoral neck BMD T-score to identify the “fracture risk equivalent” T-score in a diabetic patient. For example, an older diabetic woman with femoral neck BMD T-score of -2.0 would have a hip fracture risk similar to an older non-diabetic woman with T-score of -2.5.

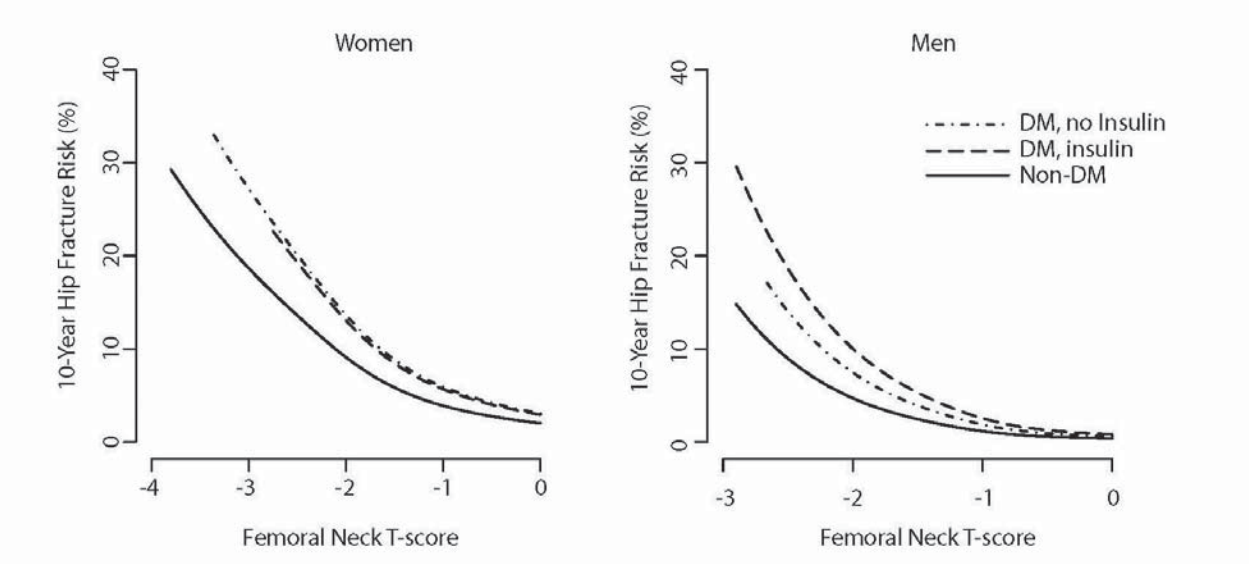

Figure 1. Femoral Neck BMD T Score and 10-Year Fracture Risk at Age 75 Years by DM and Insulin Use Status
Adapted with permission from Schwartz, et. al. [5].

The standard risk factors for fracture that are incorporated into FRAX are also predictive of fracture risk in diabetic patients, such as age, gender and BMI [6]. However, as with T-score, FRAX tends to under-estimate risk in diabetic patients [5, 7]. Diabetes is not currently included in the FRAX algorithm. It may be incorporated into the algorithm in the future but, meanwhile, one can make a crude compensation by checking off “RA (rheumatoid arthritis)” in the FRAX estimator for a diabetic patient.

Similar studies of fracture risk assessment have not been carried out in type 1 diabetes. A meta-analysis of type 1 diabetes, BMD and fracture risk found that the lower BMD associated with type 1 diabetes does not fully account for the substantially increased hip fracture risk in these patients [8]. Based on this finding, it is reasonable to assume that BMD T-score and FRAX will also under-estimate fracture risk in type 1 diabetes. However, without additional studies, it is not known by how much T-score or FRAX might underestimate risk.

FRAX provides a useful method to incorporate traditional risk factors for fracture (age, gender, BMI, etc.) into one score for a patient. But, notably, fall history is not included in the FRAX algorithm. Falls are more common in diabetic patients, and this aspect of patient history should be considered. There are also diabetes-specific factors that are not part of FRAX but could help with a clinical assessment of risk. Key factors to consider: Longer duration of diabetes, Presence of microvascular complications, Insulin therapy, Poor glycemic control.

DIABETES MEDICATIONS

Diabetes medications may affect bone health and fracture risk. Increased fracture risk has been identified with use of thiazolidinediones (TZDs), most definitively in women [9] but also recently in men [10]. One consequence has been greater attention to fracture outcomes in trials of new diabetes medications. The table below summarizes currently available evidence regarding the skeletal effects of different classes of diabetes medications.

Insulin is associated with increased fracture risk which is surprising given evidence that insulin is anabolic for bone. However, insulin use is associated with longer duration of diabetes and higher prevalence of complications. It may therefore be a marker for increased fracture risk rather than a causal factor. Other diabetes medications appear to have a neutral effect on fracture risk with the exception of sodium-glucose cotransporter 2 (SGLT2) inhibitors. The evidence for this class of medications is mixed. Based on analysis of combined smaller RCT's, canagliflozin treatment was associated with higher fracture risk (HR=1.32) compared with placebo/comparator [11]. Recent results for the ADVANCE trial reported at the 2017 ADA meeting confirmed this modest increased fracture risk with canagliflozin [12]. However, an analysis of trials of empagliflozin found no evidence of increased fracture risk [13].

Diabetes Medication	**Bone turnover markers**	**Bone mineral density**	**Fracture risk**
Insulin	??	↑ (2)	↑ (2)
Sulfonylureas	??	??	↔ (1)
Metformin	↓ (2)	↓/↔ (2)	↔ (1)
Thiazolidinediones	↓/↔ formation (1); ↑/↔ resorption (1)	↓/↔ (1)	↑ (1)
GLP-1 receptor agonists	↔ (1)	↔ (1)	??
DPP IV inhibitors	↔ (1)	??	↔ (1)
SGLT2 inhibitors	↔ (1)	↔ (1)	↑/↔ (1)

1 = randomized controlled trials (AE's); 2 = prospective cohort studies

American Diabetes Association in the Standards of Medical Care in Diabetes (2016) recommends: "For patients with type 2 diabetes with fracture risk factors, thiazolidinediones and sodium–glucose cotransporter 2 inhibitors should be avoided as their use has been associated with a higher risk of fractures."

PHARMACOLOGICAL THERAPY FOR OSTEOPOROSIS IN DIABETIC PATIENTS

Bone turnover markers tend to be lower in type 1 and type 2 diabetes {Hygum, 2017 #21094}, leading to concerns that anti-resorptive therapy may not be effective for fracture prevention in these patients. Evidence to date remains limited, but generally indicates that anti-fracture efficacy is similar in diabetic and non-diabetic patients. Studies include subgroup analyses of results from randomized trials of osteoporosis therapies and large observational studies using registry data. A subgroup analysis of the Fracture Intervention Trial found that alendronate increases BMD in diabetic women, similar to its effects in non-diabetic women [16]. Subgroups analyses of the RUTH trial found reduced risk of vertebral fracture in diabetic as well as non-diabetic women [17]. An observational study, using Danish registry data, also found no differences in fracture efficacy for bisphosphonates or raloxifene comparing diabetic and non-diabetic patients [18]. A small observational study of teriparatide found BMD and fracture effects were similar in diabetic and non-diabetic patients [19]. Data are not currently available for strontium or denosumab.

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Meet the Professor: Gene-editing in Cells and Mice

Mark Meyer, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 105, Colorado Convention Center in Denver, CO, USA

Gene-editing in Cells and Mice

Mark B. Meyer, Ph.D.
University of Wisconsin – Madison, USA

Significance of Topic

Genome manipulation and gene editing in mammalian models such as mice have occurred for decades. Traditional knock-outs, CRE-lox, and bacterial recombineering gave genomic researchers the tools to truly investigate at a system-wide level the impact of a deletion, an insertion, or a mutation. In mid and early 2000s, this tool box was improved to include zinc-finger nucleases (ZFN) and then near 2010 TAL effector nucleases (TALENs) were introduced as well. Each method improved the specificity of targeting and speed, but were costly endeavors requiring difficult synthesis and design. However in 2013, a new editing technique arrived born out of a bacterial defense system against invading viruses and foreign DNA [1-3]. Clustered Regularly Interspaced Short Palidromic Repeats (CRISPR) and its CRISPR-associated (Cas) enzyme was adapted and optimized for mammalian cells which dramatically reduced the time, cost, and effort it took with all previous methods to manipulate the genome [4-6].

The premise of CRISPR genome targeting is rather simple and requires only two components: 1) the Cas9 enzyme and 2) an RNA to guide the Cas9 to the specific region of the genome to induce a double strand break (guide RNA or gRNA). The basics of the gRNA include an 18-20 nucleotide targeting sequence followed by a scaffold sequence of RNA to which the Cas9 is recruited and interacts. Once recruited, the Cas9 enzyme can induce a double strand break (DSB) in the DNA. The cell's natural repair machinery will fix this DSB and may do so incorrectly thus mutating your sequence of interest through non-homologous end joining (NHEJ). If the repair machinery is provided a homologous template, then homology directed repair (HDR) will occur to correct or insert a desired mutation. Finally, multiple CRISPR sites may be used to excise a portion of the genome, if no template is provided, the natural repair machinery will join the two ends despite the lack of homology through NHEJ. Since the identification of the Cas9 system, the Cas9 enzyme itself has also been modified to achieve targeting without cutting (nuclease deficient - dCas9) or mutation so only one strand is cut (nickase – nCas9). Furthermore, attaching activator or repressor domains to the dCas9 results in an efficient and effective transcriptional control module, fusion to fluorescent proteins to dCas9 can be used to visualize parts of the genome, insertion of loxP sites for CRE recombination, and many other uses. Through these repair mechanisms and manipulations, researchers can correct, modify, or remove almost any sequence in the genome.

With the great flexibility and ease of targeting of CRISPR, undesired effects at off-target locations is a great concern. As a general guide based on some early work by the Zhang lab (MIT)[6], out of the 18-20 nt of a gRNA targeting sequence, nucleotides 1-12 are more vital to DNA targeting than 13-18. Therefore, a 1 nucleotide mismatch in position 16 may also be recognized and cut by the Cas9. These off-target effects may be minimized by several careful considerations such as bioinformatic comparison across the genome and selection of the gRNA with the least number of potential matches, using the nCas9 for more efficient seamless repair in unintended sites, reducing the amount of Cas9 utilized, and even truncating the gRNA sequences. However, these methods do lower the off-targeting, they may also reduce the targeting efficiency of the gRNA. Several groups and companies are now putting forth "optimized" Cas9 variants that hope to improve targeting efficiency and reducing the off-target mistakes such as Alt-R HiFi Cas9 (IDT), eSpCas9 (Zhang lab, MIT)[7], and SpCas9-HF1 (Joung lab, Harvard)[8]. In addition to off-target optimizations, alternative enzymes are starting to emerge as research progresses, like Cpf1, that have altered protospacer adjacent motif (PAM) requirements as well as utilizing a smaller gRNA[9].

CRISPR/Cas9 editing rapidly advances genomic deletions, mutations, and corrections. How will you use editing to advance your research?

Learning Objectives

As a result of attending this session attendees should be able to:

- Understand the basics of genome manipulation using CRISPR
- Easily find resources online for CRISPR design and creation
- Create a CRISPR strategy for your research
- Plan a genotyping or validation regiment
- Troubleshoot potential pitfalls

Resources and Outline

- CRISPR experimental pathway
    1. Identify genomic region for mutation and which Cas9 enzyme approach is most appropriate
    2. Design gRNA sequences for cloning or direct synthesis
    3. Introduce gRNA sequences with Cas9 protein (either synthesized, mRNA form, or plasmid encoded) into mice zygotes or cultured cells via injection, electroporation, or transfection.
    4. Select cultured cells (if necessary)
    5. Genotype resulting cells or animals including sequencing
    6. Check for off-target mutation or deletions
    7. Answer biological experimental question

- General CRISPR resources
    - AddGene - http://www.addgene.org/crispr/ Particularly helpful is the CRISPR 101 eBook that links many resources at AddGene with the primary literature for each aspect of CRISPR design and function.
- CRISPR design tools
    - CRISPR design tools take into account the complex nature of short guide sequences and the challenges they present when searching for those throughout the entire genome. Much has been learned over the past 5 years since the first design tools were released, however, no algorithm can accurately predict all possible outcomes. It's recommended to use several design tools and cross reference the best possible guides for efficiency and accuracy.
    - An excellent and comprehensive review of the CRISPR design tools can be found from Haeussler M, *et. al*. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 2016. 17(1). 148.
    - CRISPOR - http://crispor.tefor.net/
    - CRISPR-DO - http://cistrome.org/crispr/
    - Cas-OFFinder - http://www.rgenome.net/cas-offinder/
    - CRISPRko - https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
- CRISPR reagents and protocols
    - A staple of the CRISPR engineering in mammalian cells since 2013 have been the pX plasmids from the Feng Zhang lab - http://www.addgene.org/crispr/zhang/ There are many variations of plasmids available with or without nickase, nuclease deficient, GFP tagged Cas9, etc. These are the plasmids my lab still uses today since 2013.
    - Addgene has many varieties of CRISPR reagents to fit all kinds of projects and organisms http://www.addgene.org/crispr/reference/#protocols
- CRISPR transfection
    - Transfection protocols will vary widely and need to be matched to the cell line. For osteoblastic cell lines like MC3T3, UAMS, and MSCs, we have found the FuGene from

Promega works quite well for transfection. However, the efficiency of the pX458 plasmid, for example, is very low and the fluorescence can be pretty weak.

- There are two main strategies for CRISPR transfections. 1) stable cell creation from a single colony and 2) "bulk" transfection and assay.
    1. Stable cell creation
        - We've used this method when we do genomic excisions so we isolate cells with true knockouts and not heterozygous cells.
        - Workflow: Transfection of CRISPR plasmid(s) into larger dish sizes, FACS on cells to identify fluorescent ones, plate single cells into 96 well plates, score wells, genotype, transfer for outgrowth.
        - The problems of clonal isolated cell lines still exist with this method so it is recommended to isolate at least 4-5 knockout cell lines for downstream biological assay.
    2. Bulk transfection and assay
        - If you are looking to knockout or down a gene in a cell population, this method might be adequate to get an understanding of gene function. Gene disruption can be quite simple compared to genomic excision of larger fragments. A good idea is to use several guides to exonic regions of the gene of interest. Transfection of several guides will insure that the gene will undergo mutation and therefore frameshifted into a non-sense protein.
        - Workflow: Transfection of CRISPR plasmid(s) into larger dish sizes, FACS is optional with this method, but will enrich your CRISPR containing cells, plate those cells in larger dish, grow and/or expand, and assay as your research dictates.
- Newer products from companies like IDT have RnP (RNA and Protein) solutions for transfections. These have much higher efficiencies compared to plasmid introduction. More information on their systems can be found here: https://www.idtdna.com/pages/products/genome-editing/genome-editing-overview/crispr-cas9-genome-editing
- There is also the possibility to use viral introduction of the CRISPR plasmids. This, however, is a very permanent solution to your cells. The transient transfection rarely incorporates the CRISPR plasmid in the stable lines, whereas, the viral plasmid will readily remain in the stable lines making it difficult to complete a second round of CRISPR if desired.

- CRISPR mouse injection
    - Our lab has found that making CRISPR mice and isolating the cell line of interest or interrogating the tissue of interest results in a more accurate and reproducible model especially if basal gene levels are the scientific question. The advantage here is that the mouse genome is truly diploid, which is something that cultured cell lines can rarely say. A second advantage is mouse genetics and outbreeding. Any off-target unintended mutations can be bred away from, thus lessening the impact of off-target complications.
    - In this workflow, the CRISPR gRNAs are created either by plasmid cloning and T7 invitro transcription or by direct RNA synthesis. These guides are then injected into 1 day old fertilized zygotes by a reputable mouse facility (still at single cell stage) along with Cas9 protein (purchased from any vendor). Zygotes are implanted into recipient moms and the resulting litters are then genotyped and bred.
    - An advantage to this system is that all components of the CRISPR solution are degraded quite rapidly. Therefore, they are able to complete the desired mutation and are discarded shortly thereafter thus reducing the off-target effects.
- CRISPR donor plasmids or oligos

    - For either the mouse injection or the cell line transfection, single stranded oligo donors (ssODN) can be injected with the gRNA and Cas9 protein.
    - The length needed for homologous recognition and appropriate insert of material depends on your research design. For example, if you are inserting a SNP or a 3-5 bp mutation, the ssODN should typically have 50 nt of sequence homology around the mutated sequence (~105-110 nt total). It is possible to insert very large segments of DNA with the megamers from IDT. It is recommended that single stranded oligos are the best so the DNA material does not randomly incorporate into the genome.
- CRISPR off-target interrogation
    - This is unfortunately a harder thing to confidently test. Next-generation sequencing techniques are still costly and introduce too much noise to truly validate the off-target mutations that may have occurred.
    - The design algorithms usually will output the closest potential matches, although improbable to be mutated, and these can be followed up by PCR, cloning, and sequencing for identification.
    - A good review of some current high-throughput methods for off-target detection are listed in this publication by Martin and colleagues [10]: http://www.mdpi.com/1422-0067/17/9/1507/htm

Good luck in your CRISPR projects!

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Meet the Professor: Stromal Support of Hematopoiesis

Pamela Robey, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 107, Colorado Convention Center in Denver, CO, USA

Stromal support of hematopoiesis

Pamela Gehron Robey, Ph.D., NIDCR/NIH/DHHS, Bethesda, MD, USA

Significance of the topic: It has long been known from the work of Friedenstein and Owen, and others that followed that bone marrow contains a rapidly adherent population of non-hematopoietic fibroblastic cells that have the ability to recreate cartilage (in pellet cultures in vitro), and bone, **hematopoiesis-supportive stroma** and marrow adipocytes (a bone/marrow organ) upon in vivo transplantation [(1)]. When plating bone marrow suspensions at clonal densities, single cells (Colony Forming Unit-Fibroblasts) proliferate to form colonies composed of bone marrow stromal cells [BMSCs, NOT to be called "mesenchymal stem cells" or any iteration thereof [(2)]], one out of five of which are a skeletal stem cells (SSCs, [(3)]) able to form a bone/marrow organ. It is now recognized that in situ, SSCs are pericytes (cells abluminal to marrow sinusoids), and are a component of the hematopoietic stem cell (HSC) niche [(4)], while the vast majority of BMSCs reside in stroma that forms the "bed" upon which hematopoietic differentiation occurs [(5)]. These are **defining features of SSCs/BMSCs** [(6)] that are not displayed by post-natal tissue-specific stem/progenitor from other tissues that have similar cell surface characteristics (and may also be pericytes) in non-pathological conditions. Based on this new understanding of the function of SSCs/BMSCs, it is now apparent that they may also play a role in genetic and acquired hematologic diseases and disorder, and as such, may be therapeutic targets.

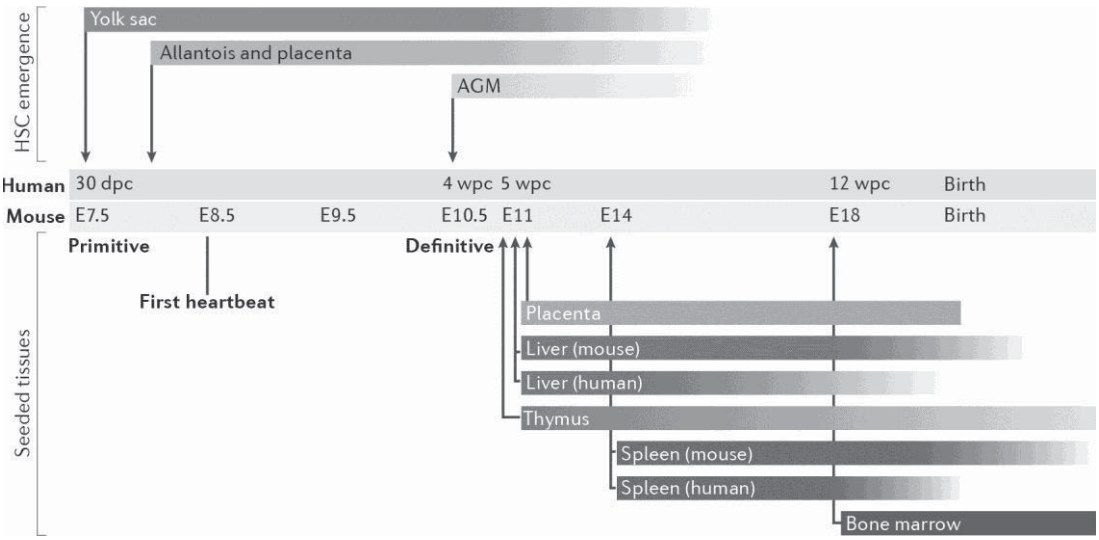
Learning objectives: As a result of participating in this session, attendees should be able to:

1. Have a general idea of how hematopoiesis (HP) is established during development and maintained in bone marrow of the post-natal organism.
2. Recognize the role that SSCs/BMSCs play in post-natal HP.
3. Know what are currently thought to be components of the hematopoietic stem cell (HSC).
4. Have a basic understanding of factors secreted by secreted by niche components that control self-renewal versus differentiation of the HSC.
5. Understand the complexity of the role of BMSCs in controlling differentiation of different hematopoietic cell types.
6. Recognize the role that SSCs/BMSCs play in certain hematological diseases and disorders.

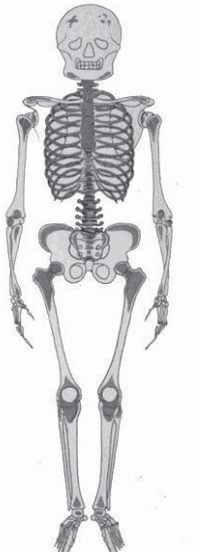
Outline:

Development of hematopoiesis (HP).
The biological nature and activity of bone marrow stromal cells/skeletal stem cells in HP.
Components of the HSC niche
Regulation of hematopoiesis.
The role of bone marrow stroma in hematological disorders – intrinsic (genetic) and extrinsic (microenvironmental) changes.

Embryonic development of HP



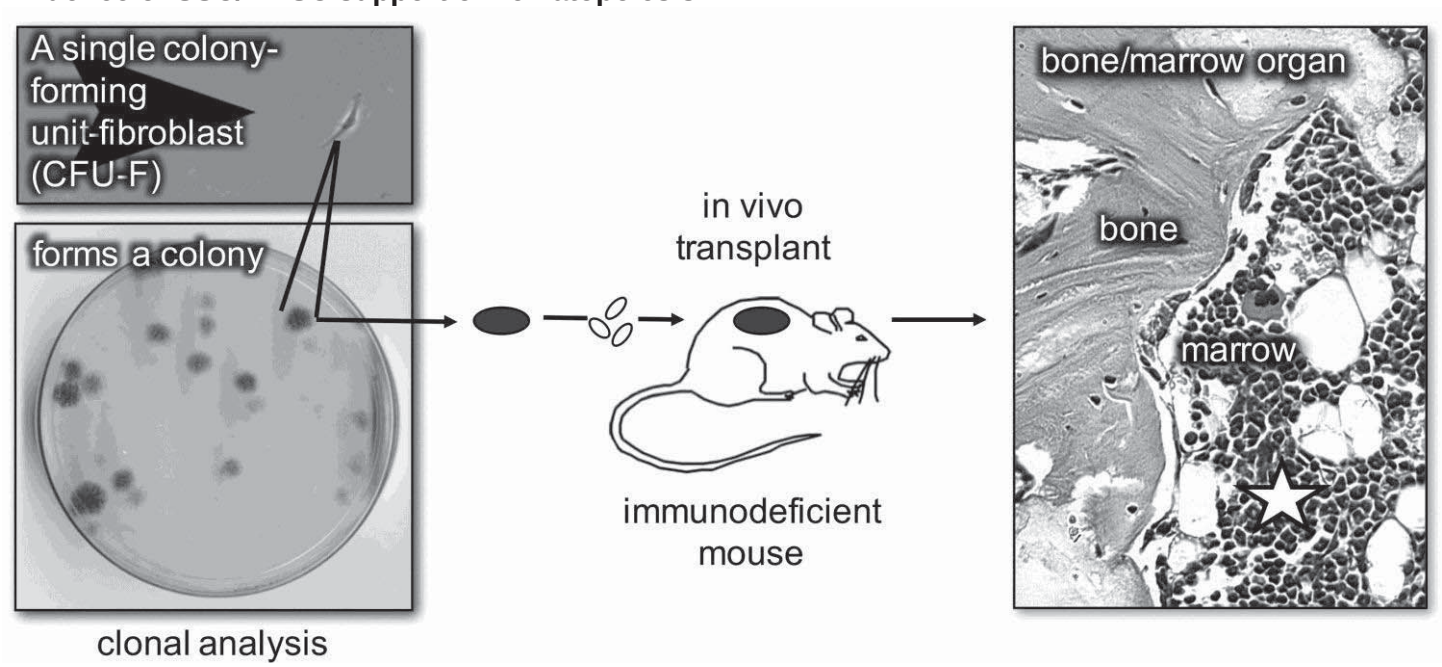
HP in human adults



Hematopoiesis is initially established in extraembryonic mesoderm to rapidly provide HP cells at the onset of circulation. These primitive cells populate a number of tissues, but are not considered to be definitive. Definitive hematopoiesis is currently thought to be established by budding of HSCs from hemangioblasts in the dorsal root of the aorta [the aorta-gonad-mesonephros (AGM) ridge]. This definitive HSC populates fetal liver, followed by

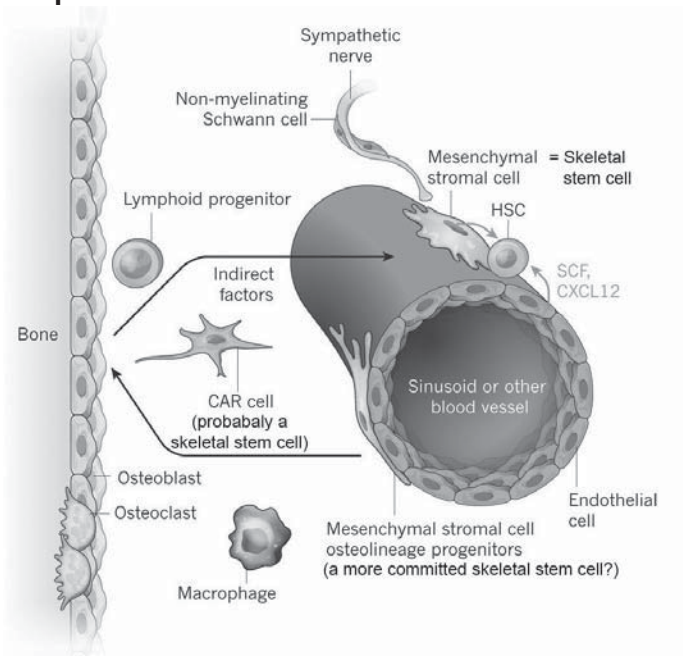
thymus, spleen, and its final home, bone, when its stroma has been established [7,8]. In human adults, HP is restricted to sites in the axial skeleton, and to the proximal and distal regions of long bones [5].

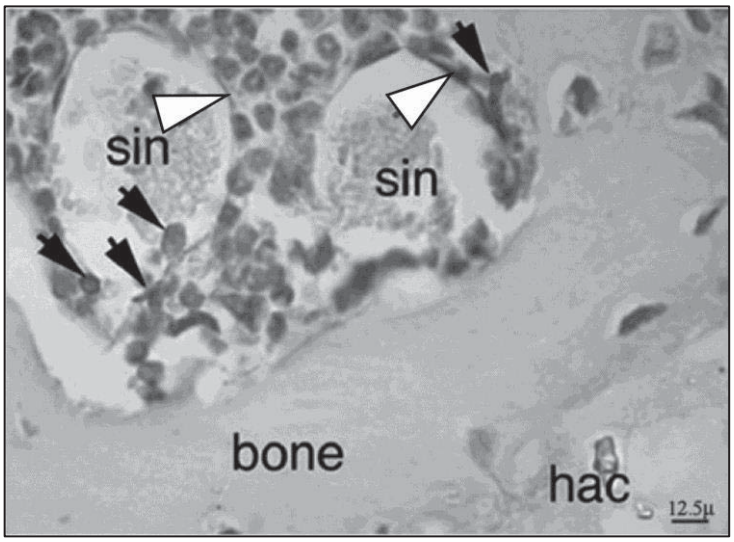
Evidence of SSC/BMSC support of hematopoiesis



Based on previous studies originating from Friedenstein, Owen and others, it was determined by clonal analysis that a subset of bone marrow stromal cells are colony forming unit fibroblasts. When individual colonies are transplanted in conjunction with an appropriate scaffold into immunocompromised mice, a subset are capable of forming a bone/marrow organ, with donor stroma capable of supporting complete hematopoiesis of recipient origin (white star in left panel) reviewed in [9]).

Components of the HSC niche



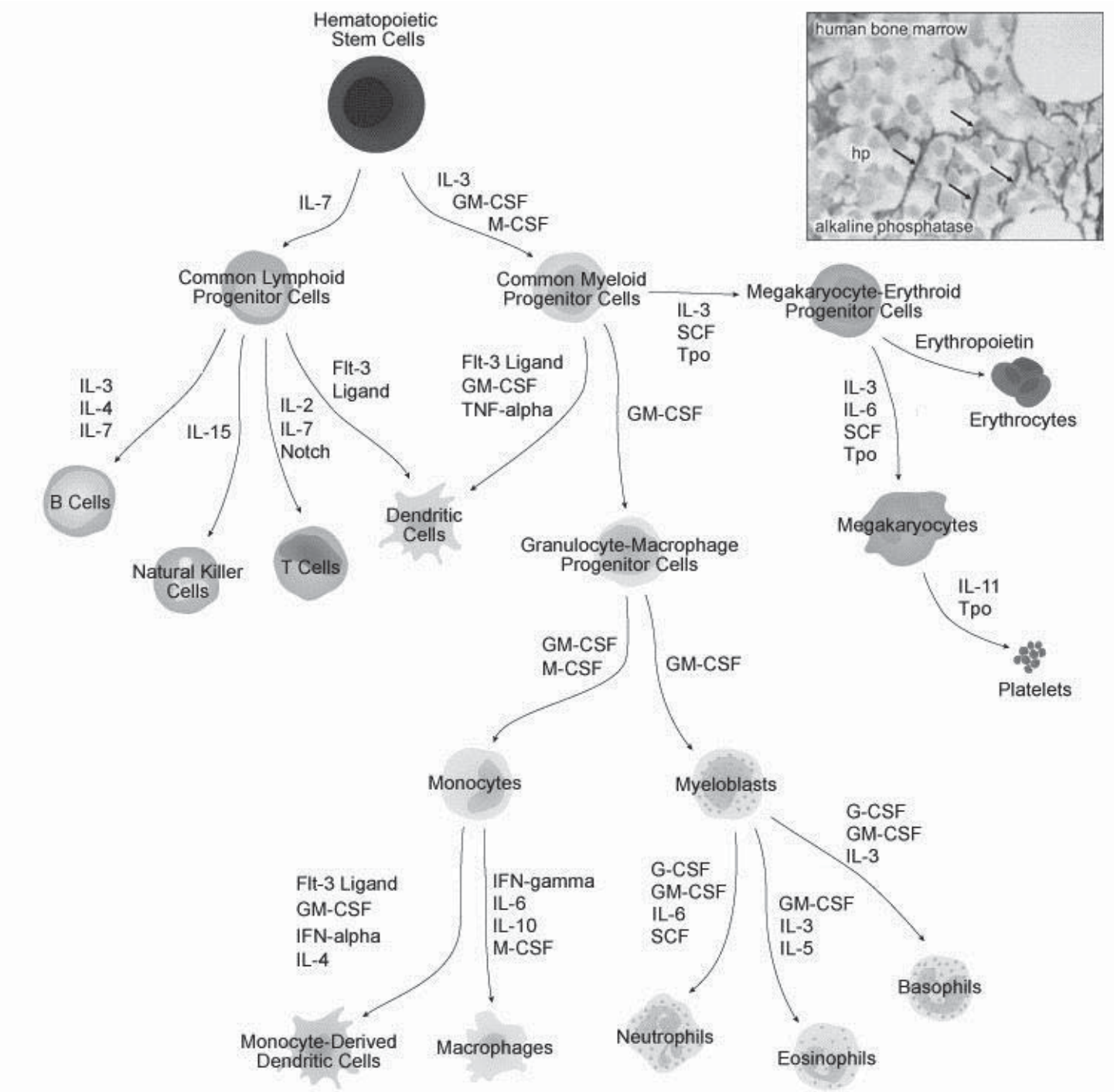


Tie2 positive HSCs (black arrows) are seen in close approximation of perivascular SSCs (white arrow heads), which are $CD45^-/CD34^-/CD146^+$ in human bone marrow.

There is no single cell type that is able to support the HSC on its own (it takes a village). Many cell types have been identified in the niche, including macrophages and lymphoid progenitors, sympathetic nerves with their associated non-myelinating Schwann cells endothelial cells, and two forms of "mesenchymal cells" (both of which are pericytes). Pericytes have been identified as $nestin^+$, and/or $PDGFR\alpha^+$, and/or $Lepr^+$, and/or $Mx1^+$ cells, $VCAM1^+$ cells in mice, and $CD45^-/CD34^-/CD146^+$ cells in human. Maintenance of the HSC appears to be primarily due to secretion of CXCL12, SCF, Angpt1 and FLT3-L by niche cells, which bind to their corresponding receptors

(CXCR4, c-kit, Tie2 and FLT3) on HSCs. Notch, Wnt and TGFβ signaling have also been implicated, but the extent to which they do so is not clear at this time [reviewed in [10-12]].

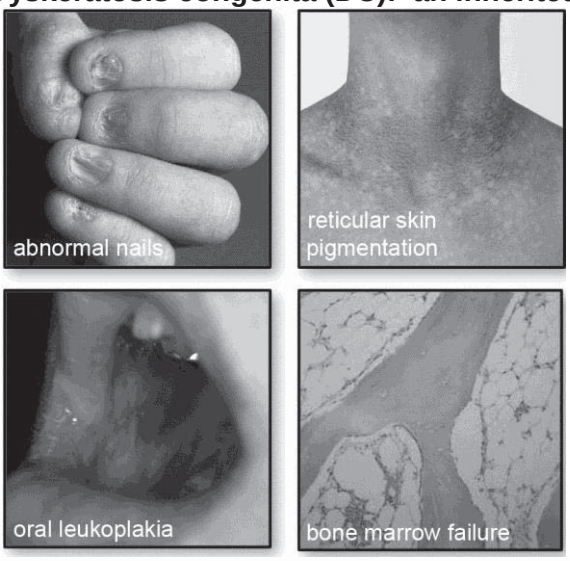
Control of HSC differentiation



(Diagram from R&D Systems as an example – https://www.rndsystems.com/pathways/hematopoietic-stem-cell-differentiation-pathways-lineage-specific-markers. See also http://www.wikipathways.org/index.php/Pathway:WP2849 for transcription factors).

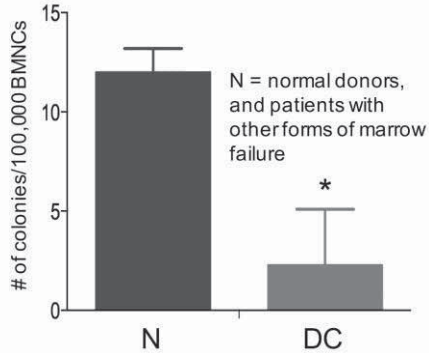
BMSCs, which can be identified by alkaline phosphatase staining (arrows in the upper right hand panel, [13]), form the bed upon which hematopoietic differentiation occurs. Cell-cell interactions undoubted play a role in directing differentiation, with the phenotype of the HP cell being additionally controlled by various mixtures of cytokines and growth factors. The "cocktail" of cytokines for each cell type are often partially overlapping with the mixture required by another HP cell type. Consequently, the control of HP differentiation is most likely regulated by establishment of gradients within the bone marrow microenvironment by BMSCs, endothelial cells, adipocytes and by HP cells themselves, making this a very complex process

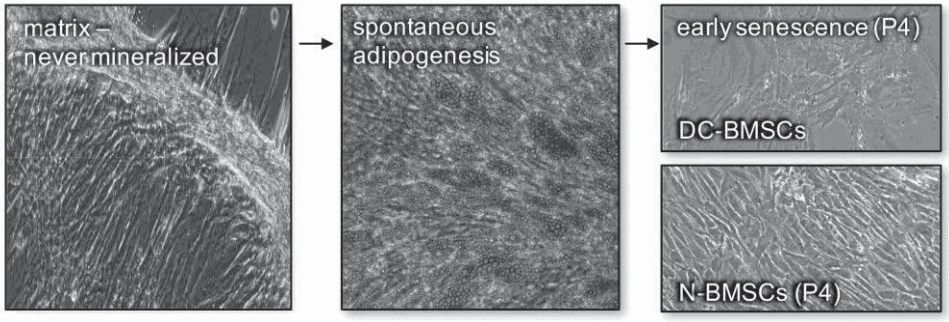
Dyskeratosis congenita (DC): an inherited form of bone marrow failure (intrinsic defect) [14]



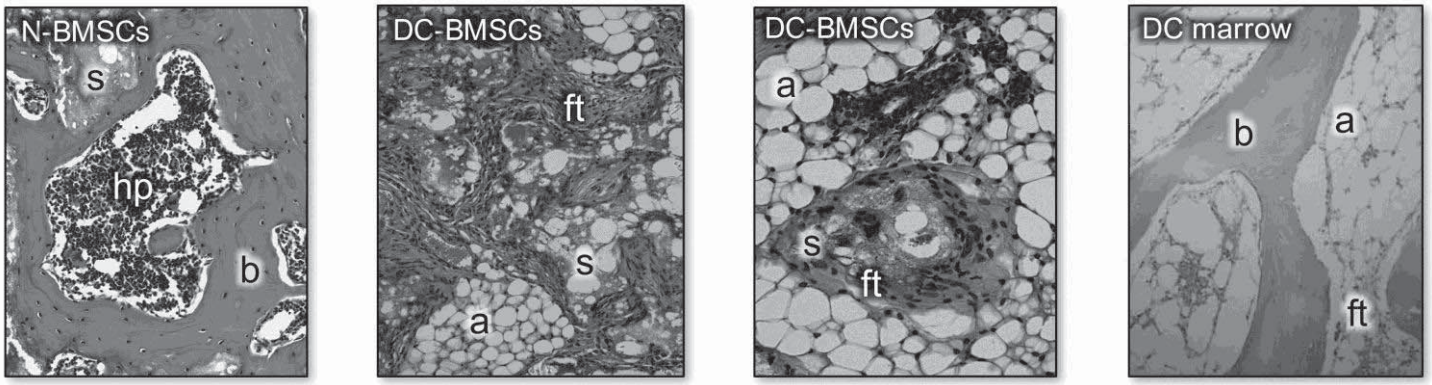
Patients with DC present during early childhood with the clinical triad of nail abnormalities, reticular skin pigmentation and oral leukoplakia. Additionally, these patents exhibit growth retardation, early graying of the hair and osteoporosis, and bone marrow failure. DC, and similar diseases that include bone marrow failure are caused by mutations in the machinery required to maintain telomere length, such as TERT, TERC, shelterins, etc. Collectively, these diseases are classified as "Telomere Biology Diseases" (TBDs) (REFs).

Bone marrow obtained from DC patients had a dramatically decreased colony forming efficiency, the closest approximation of the number of SSCs available. Furthermore, DC-BMSCs produced copious amounts of fibrotic matrix that never mineralized, but did spontaneously differentiate into adipocytes (even in mineralizing conditions). DC-BMSCs also underwent early senescence compared to normal cells.



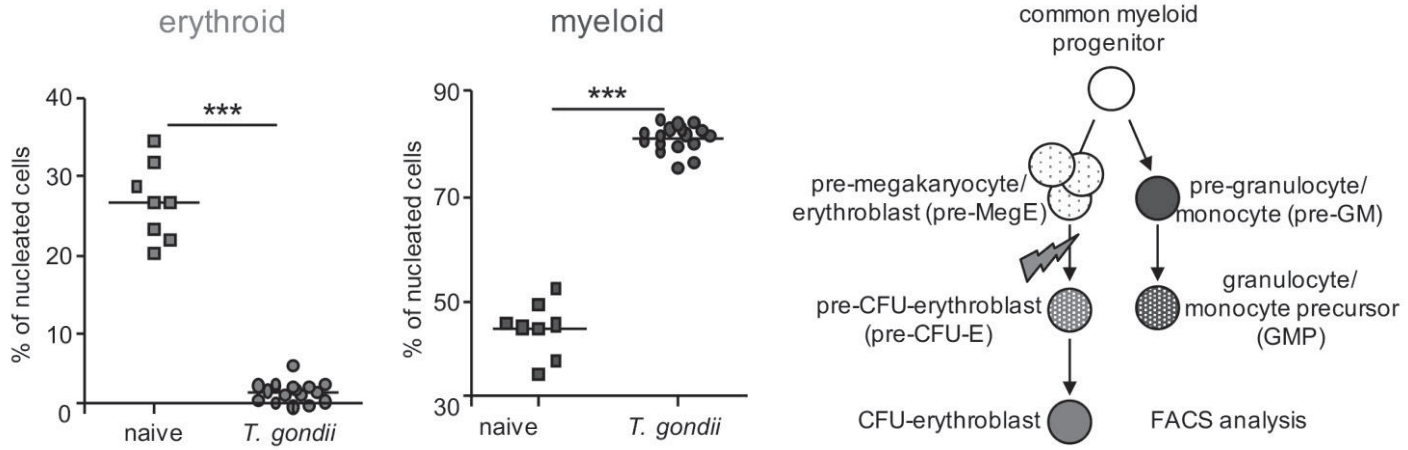


Upon in vivo transplantation, normal BMSCs recreated a bone/marrow organ as expected. However, DC-BMSCs, failed to form bone and did not support hematopoiesis, but instead, formed extensive fields of fibrous tissue and adipocytes, entirely reminiscent of native DC marrow. These results suggest that in addition to a defect in HSCs in Telomere Biology Diseases, SSCs/BMSCs are also defective, and most likely contribute to the bone marrow failure that occurs in these patients [14].

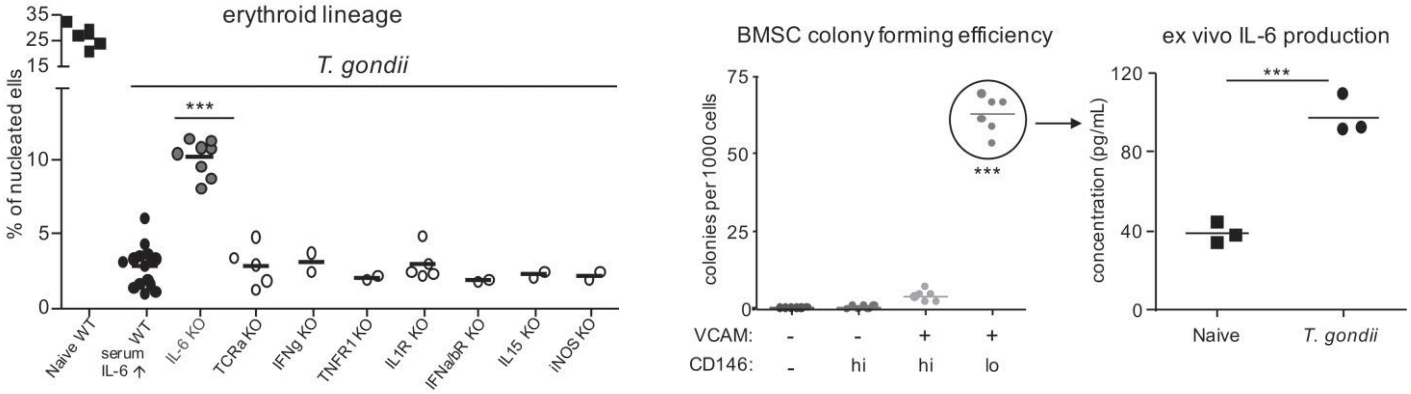


Inflammation induced by *T. gondii* alters BMSC function (extrinsic change) [15]

In addition to playing a role in a genetic hematological disease, it was hypothesized that SSCs/BMSCs may also play a role in acquired (and often transient) hematological disorders, such as *T. gondii*-induced inflammation. After infection, there was a dramatic decrease in erythropoiesis matched by an increase in myelopoiesis. This was due to an inability of pre-MegE cells to differentiate further, which caused the common myeloid progenitor to be diverted into the myeloid lineage (below).



Using KO mice deficient in cytokines and receptors, it was determined that IL-6 was responsible, in part, for the decrease in erythropoiesis, further supported by the increase in serum IL-6 noted in infected wildtype mice (left panel below). By performing different bone marrow transplantation schemes, it was determined that IL-6 was produced by radio-resistant BMSCs (data not shown here). This was confirmed by the isolation of VCAM-1^{+} colonies from infection mice (right panel) that were shown to have increased expression of IL-6.



These data suggest that BMSCs regulate the hematopoietic changes during *T. gondii*-induced inflammation via IL-6. Furthermore, taken together with the DC results, SSCs/BMSCs, a part of the HSC niche and stromal cell network, play a significant role in sculpting hematopoiesis in health and disease.

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Meet the Professor: LRP Receptors in Bone

Mei Wan, Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 108, Colorado Convention Center in Denver, CO, USA

LRP Receptors in Bone

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Significance of the Topic

Close to two decades ago, low-density lipoprotein-related receptor 5 and 6 (LRP5/6) were recognized as Wnt coreceptors for β-catenin signaling activation (1-3). Almost at the same time, causal mutations in the *LRP5* and *SOST* gene were identified to be involved in rare human bone disorders (4-6). Since then, the importance of Wnt signaling for bone has been highlighted, and the pathway is now the target for therapeutic intervention to restore bone mass and strength in osteoporotic patients.

In recent years, the Wnt-independent roles of LRP6 and the involvement of other members of the LRP family (LRP4, LRP8) in the maintenance of bone and their implications in bone diseases have been revealed. Understanding the distinct role of LRP receptors and their involvement in bone diseases will allow us to develop new, more effective therapeutic approaches for skeletal disorders such as age-associated osteoporosis, hyperlipidemia and atherosclerosis-associated osteoporosis, and rare bone disorders.

Learning Objectives

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

1) Understand canonical Wnt signaling pathway and human bone disorders linked to Wnt signaling components (mainly LRP5 and SOST).

2) Appreciate the distinct roles of each LRP family members (LRP4, 5, and 6) in regulating bone homeostasis and the implications in bone disorders.

3) Appreciate Wnt-independent roles of LRP6 and the implications in bone disorders.

An Outline/Points of Interest

LRP5/6-dependent Wnt signaling pathway

Activation of canonical Wnt Signaling (**Figure 1**) (1-3): Wnts binding Frizzled/LRP5/6 receptors induce phosphorylation of Dishevelled (Dsh) and axin leading to inhibition of GSK-3, preventing it from interacting with β-catenin. This allows β-catenin to translocate to the nucleus to activate gene transcription. Inhibition of Wnt signaling: sFRPs sequester Wnt ligands, preventing them from interacting with receptor. DKKs or sclerostin bind LRP5/6 preventing interaction with the Frizzled co-receptor. In these settings, GSK-3 phosphorylates β-catenin, targeting it for degradation. The LRP5/6-independent (non-canonical) Wnt signaling pathways will not be discussed here.

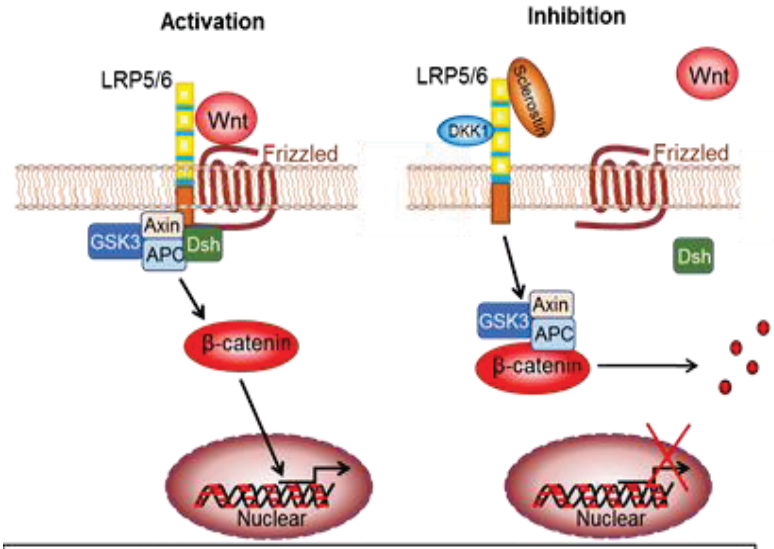

Figure 1. Schematic of the canonical Wnt signaling pathway.

Mutations in LRP5 and SOST associated with human bone disease

In 2001 and 2002, two mutations causing altered bone mass and density were found to occur in *LRP5* with a loss-of-function mutation in this gene being associated with low bone mass in osteoporosis-pseudoglioma syndrome (OPPG; MIM259770) (4) and a gain-of-function mutation being associated with high bone mass in otherwise healthy patients (5, 6). Moreover, two mutations were also found to occur in *SOST*, which encodes for sclerostin, a secreted antagonist to WNT signaling that binds LRP5/6. Lack of sclerostin expression in bone was found to be the cause for high bone mass in sclerosteosis (MIM269500) (7) and Van Buchem disease (VBD; MIM239100) (8, 9). Later, it has been confirmed that the high bone mass–inducing mutations in *LRP5* decrease the binding of sclerostin (10, 11) and another WNT inhibitor, dickkopf 1 (DKK1) (10,12), providing the strong link between WNT signaling and bone homeostasis.

Distinct roles of LRPs in regulating bone homeostasis

Twelve members of the low-density lipoprotein receptor (LDLR) family have been identified so far. Among them, the functions of LRP4, 5, 6, and 8 are highly bone-associated (**Figure 2**). Proteins in this family have unique structural features: their long extracellular domains contain LDL binding repeats, β-propeller motifs, and EGF-like repeats. Their short intracellular domains are responsible for downstream signaling events (13-15). LRP5 and 6 are structurally related proteins and share around 71% homology at the nucleotide level. The cytoplasmic region of LRP5/6 contains five highly conserved PPPSPxS motifs that are critical for β-catenin signaling transduction (1-3). Similar to LRP5/6, LRP4 has four β-propeller motifs and four EGF-like repeats; unlike LRP5/6, LRP4 has a NPxY motif in the cytosolic domain (15). LRP8 is also known as apoE receptor 2, and its structural organization is markedly different from LRP5/6.

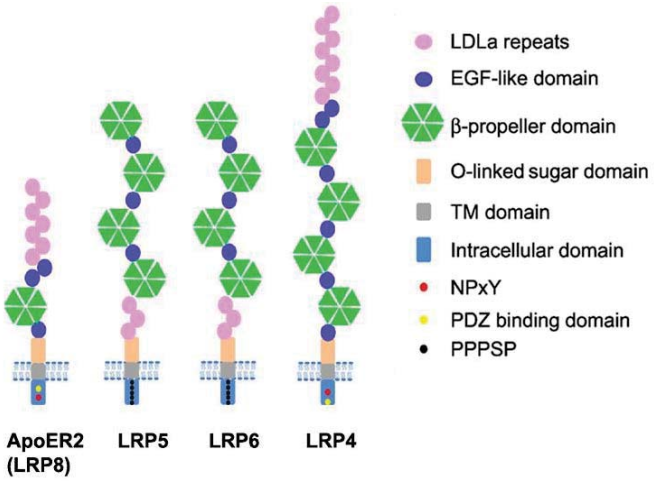

Figure 2. LRP family members that are important in the regulation of bone homeostasis. Adopted from Ref. 15.

Although they have similar structures, the functions of LRP5 and 6 in bone regulation are not fully overlapping. LRP5 has a central role in human bone mass regulation. Mutations in *LRP5* occur in human bone disorders with altered bone mass and density (4-9). *LRP6* mutations have been linked to early onset atherosclerotic coronary artery disease and several features of metabolic syndrome including hyperlipidemia, hypertension, and diabetes (16, 17). All these patients developed premature osteoporosis, indicating that LRP6 is more likely involved in hyperlipidemia/atherosclerosis-associated bone loss. Mutations in *LRP5* or *LRP6* are associated with different phenotypes of skeletal diseases based on genetic mouse studies. *Lrp5* knockout mice are viable but suffer from osteoporosis in adulthood (18). In contrast, *Lrp6* knockout mice are perinatal lethal due to developmental abnormalities like truncations of the axial skeleton, limb defects, and loss of the paraxial mesoderm (19, 20). The analysis of the phenotypes of mice carrying heterozygous mutations of *Lrp6* and either heterozygous or homozygous mutations of *Lrp5* suggest that both affect bone accrual but their actions may occur at nonredundant sites (21). The role of LRP6 in regulating osteoblastic lineage cells in adult mice has been characterized. Three month-old mice with LRP6 specific deletion in mature osteoblasts (OC-Cre model had a significant reduction in bone formation only at femoral secondary spongiosa (i.e. bone remodeling active area), whereas marginal changes were seen in femora of 1 month-old KO mice relative to their WT littermates (22). The results suggest that osteoblast-specific LRP6 is required for bone formation specifically during bone remodeling. Nestin$^+$ cells in bone marrow represent heterogeneous precursor cells mainly in endothelial and mesenchymal lineage (23-25). Mice with homozygous and heterozygous Lrp6-deficiency in nestin$^+$ cells showed normal survival but smaller size and low bone mass at 1 month

of age (26), indicating that LRP6 in mesenchymal/endothelial precursor cells is involved in postnatal skeletal growth and bone accrual. Thus, LRP6 is a key positive regulator for osteoblastogenesis with distinct functions in different differentiation stages of osteoblast lineage cells. Recently, another member of LRP family LRP4 was recognized as a key regulator of bone homeostasis (27, 28). Mutations in LRP4 cause sclerosteosis in human, providing proof for a role of LRP4 in the regulation of bone formation (28-30). Studies in mice demonstrated that global deletion of *Lrp4* is not viable (31). Deletion of *Lrp4* in the osteoblast/osteocyte lineage promotes bone formation by attenuating sclerostin inhibition of Wnt signaling, results in high-bone-mass deficits (32, 33). LRP4 deficiency in osteoblast lineage cells also impairs osteoclast-mediated bone resorption (33, 34). Therefore, LRP4 represents a novel target for future osteoporosis therapies.

Wnt-independent role of LRP6

Accumulating evidence suggest that LRP6 exerts Wnt-independent role in transducing downstream signaling and regulating osteoblastic bone formation. One of the functions of LRP6 in osteoblast lineage cells is mediating parathyroid hormone (PTH) signaling pathways and its bone anabolic effect. In osteoblasts, PTH stimulates the formation of the ternary complex containing PTH, PTH1R, and LRP6, leading to rapid phosphorylation of LRP6, the recruitment of axin to LRP6, and stabilization of β-catenin (35-37). Moreover, LRP6 binds to the $G\alpha_s\beta\gamma$ heterotrimer in response to various ligands of GPCRs such as PTH, resulting in the local accumulation of $G\alpha_s\beta\gamma$ at the plasma membrane to set up a functional GPCR-$G\alpha_s$-AC complex for the rapid production of cAMP and subsequent PKA activation (38, 39). Thus, LRP6 acts as a coreceptor for PTH for the activation of both β-catenin and cAMP/PKA signaling in osteoblasts (**Figure 3A**). In osteocytes, LRP6-mediated activation of cAMP signaling pathway by PTH also inhibits MEF2 transcription factor–stimulated activity of *Sost* transcription and reduces sclerostin production, leading to increased osteoblastic bone formation (40) (**Figure 3B**). This finding adds an additional dimension to the current understanding of LRP6-mediated PTH activity on bone. Importantly, intermittent PTH administration failed to stimulate bone formation in osteoblast-specific *LRP6* deficient mice (22). This *in vivo* evidence and the previous finding that intermittent PTH treatment was fully anabolic in LRP5-deficient mice (41, 42) consistently support the concept that LRP6, but not LRP5, is an essential mediator for PTH-elicited bone anabolic effect during bone remodeling.

In addition to PTH/PTH1R, LRP6 has been found to function as a coreceptor for other signaling pathways such as PDGF-, TGFβ-, and CTGF-stimulated pathways (43-45). Future studies are needed to uncover other Wnt-independent roles of LRP6 in normal bone remodeling and bone disorders.

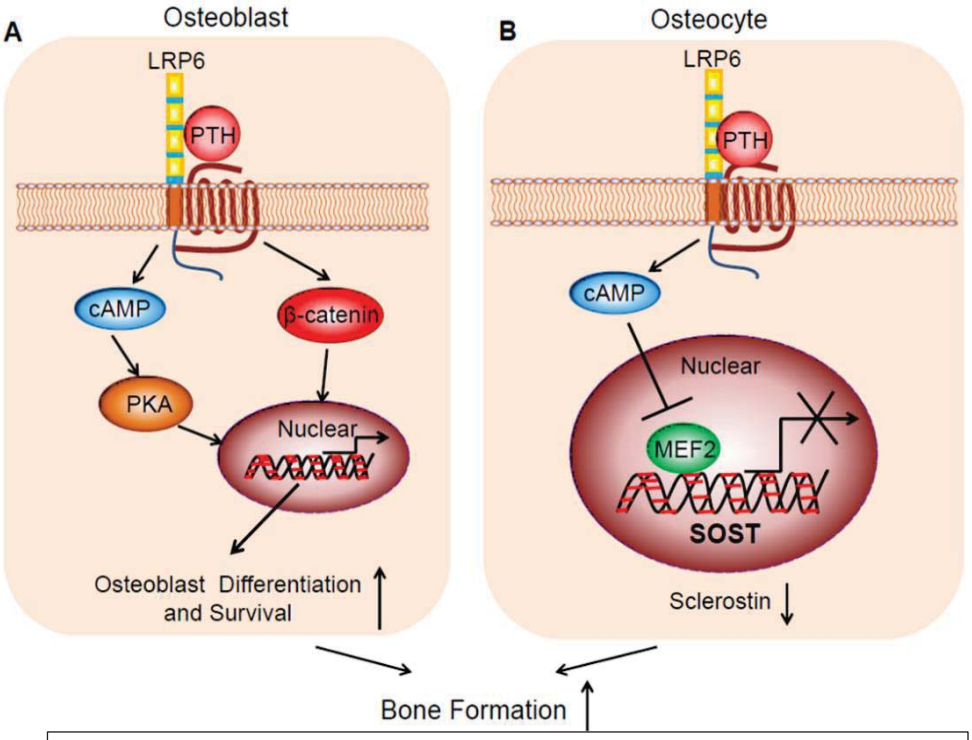

Figure 3. Involvement of LRP6 in PTH-stimulated anabolic bone effect.

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Meet the Professor: Osteocyte and Mechano-transduction

Paola Divieti Pajevic, M.D., Ph.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 109, Colorado Convention Center in Denver, CO, USA

Osteocytes and mechano-transduction.

Paola Divieti Pajevic, MD PhD. School of Dental Medicine, Boston University

Significance of the topic:

Mechanical loading is required for the proper development and maintenance of the musculoskeletal system. Mechanical stimuli regulate numerous cellular functions, including gene expression, protein synthesis, cell proliferation and differentiation and It has been recognized for over a century that loading is fundamental for bone health. Indeed, reduced loading (as in prolonged bed rest, immobilization or space flight) is invariably associated with bone loss. In 1892, Wolff theorized (Wolff's law on bone transformation) that mechanical stress is the driving force for the architecture of bone [1]. His law postulates that the skeleton, through a process known as modeling (i.e. large changes in bone structure driven by independent actions of osteoblasts and osteoclasts), adapts its form to react to mechanical demands. Remodeling, on the other hand, is the continuous and spatially coupled resorption and formation of bone required to preserve its functional integrity. In the late 80's Harold Frost speculated the existence of a mechanism (named the "mechanostat") capable of distinguish between bone modeling (changes in shape) and remodeling (continuous replacement) and he identified the osteocyte as the "mechanostat" of bone [2].

How exactly the skeleton can sense and respond to mechanical forces is still unclear. At the cellular level, bone adaptation to its mechanical environment is orchestrated by osteocytes, the bone cells deeply embedded into the mineralized matrix. These cells play a key role in skeletal mechanosensing whereby they modulate bone modeling and remodeling by orchestrating the activity of both osteoblasts and osteoclasts.

This hand-out summarizes latest research and progress made in understanding osteocyte mechanobiology and critically reviews tools currently available to study these cells.

Learning Objective:

As a result of participating in this session, attendees will learn:

1) Basic osteocyte biology: cell origin, functions and characteristics
2) Principle of mechanobiology, mechano-sensation and mechano-transduction
3) Major signaling pathways involved in skeletal mechanobiology
4) In vitro and in vivo tools available to study the effects of mechanical forces on osteocytes

Osteocyte Biology

Osteocytes derive from mature osteoblasts that, during the process of bone formation, assume a more differentiated morphology and become entrapped in the matrix that they are actively synthesizing. Once embedded in the mineralized matrix, the osteocyte maintains its contacts with adjacent cells (including surrounding osteocytes, osteoblasts, endothelial cells and possibly cells in the marrow cavity) though gap junctions (connexin 43) and receives nutrients via the dendritic processes that lie within the canaliculi of the bone. Osteocytes are terminally differentiated osteoblasts and express many osteoblastic genes such as osteocalcin, osteopontin, parathyroid hormone receptor, RANKL and others (for a comprehensive review on osteocyte biology see [3-5]). These cells also express specific genes, such as Fibroblast-growth factor 23 (FGF-23), dentin-matrix protein1(DMP-1), MEPE, Phex and E11 and, as we have recently demonstrated, carbonic anhydrases (II and III).

It has been calculated that approximately 40% of osteoblasts become osteocytes but the mechanisms and signals controlling the transition from motile osteoblasts to osteocytes are still unknown. Osteocytes are post-mitotic cells and in human, these cells can live up to 20-25 years (as estimated by the rate of bone remodeling). These cells are characterized by a dendritic morphology, a unique localization (well within the mineralized matrix) and the extensive cellular network that allows rapid and efficient cellular communication.

Understanding of the function of osteocytes has expanded dramatically over the last decade primarily due to the identification of osteocytes specific markers, such as DMP-1 and SOST/sclerostin, that has allowed, for the first time a closer look at the biology of these cells. Once new tools became available, surprising functions have been identified for these cells. These functions include regulation of skeletal homeostasis, mechanosensation and mechanotransduction, and as endocrine cells secreting factors that can target distant organs such as kidney and muscle (for comprehensive review of osteocyte signaling see [6]) .

Principle of Mechanobiology

Skeletal adaptation to mechanical stressors is a complex cellular process, which requires the coordinated activity of osteoblastic bone-forming cells and osteoclastic bone-resorbing ones, and entails a biological system capable of sensing and converting applied mechanical cues into biochemical signals. During increased loading, osteoblasts are activated whereas osteoclasts are partially suppressed by the mechanisms described below. Conversely, during reduced loading, bone formation is suppressed and bone resorption is increased. Nonetheless, how the external forces are transmitted at the cellular and molecular levels is still unclear. What is now evident is that osteocytes orchestrate both events[7]. In vertebrates, bone is the tissue best designed to sustain high magnitude of loads (in young human femur the ultimate compressive strength is ~100 MPa). Loading of long bones also increases the intramedullary cavity pressure and generates interstitial fluid flow (IFF) at the endosteal surface as well as within the lacuno-canalicular network [8]. Skeletal responses to loading require a system capable of perceiving the perturbation (mechano-sensation) and then transform this perturbation into a signal (mechano-transduction) and ultimately a cellular response. Below are briefly summarized the mechanisms by which osteocyte perceive and transduce mechanical forces.

<u>Mechano-sensation</u>: Several stressors have been proposed as mechanical stimuli, which include fluid flow shear stress (FFSS), hydrostatic pressure, and direct cellular deformation [9]. These mechanical stresses are driven by micro-deformation or –strain of bone matrix induced by loading and gravitational forces. Moreover, the specific components of these stressors, such as frequency, amplitude and rate, also influence cellular responses. The theory of loading-induced fluid flow shear stress was first proposed by Cowin et al. [10,11] in the late 90's. How are mechanical stimuli sensed by the osteocyte? Several theories have been proposed and experimental studies have identified *integrins, cilia, calcium channels and G-protein coupled receptor*s (GPCRs) as mechano-sensors of bone. Among integrins, $\alpha_v\beta_3$ is highly expressed in osteocytes and connects the intracellular actin cytoskeleton to the extracellular matrix proteins fibronectin, vitronectin, and osteopontin [12]. Another cellular moiety needed to perceive FFSS is the primary cilium, a non-motile structure required for chemo- and mechano-sensation in a variety of tissues, including kidney, liver, cartilage and bone. Initial studies suggested that flow of the canalicular fluid induced bending of the cilium and trigger Ca^{2+} influx via the transient receptor potential vanilloid 4 (TRPV4), leading to suppression of cAMP signaling [13,14]. The TAZ/YAP pathway has also been identified as important for mechano-sensation and its deletion is associated with defective mechano-transduction. In osteocytes, and other cells, biophysical stressors are transmitted to the cells by coupling the extracellular matrix to the actin cytoskeleton through focal adhesions. A major constituent of focal adhesions are focal adhesion kinases (FAK) that are required for osteocytes mechano-sensation and transduction. Recently, spectrin, another structural cytoskeletal protein required for the differentiation of osteoblasts to osteocytes [3], has been identified as a mechanosensitive element within the osteocyte [15]. Disruption of the spectrin network promotes Ca^{2+} influx and nitric oxide (NO) secretion in response to reduces stiffness [15]. Other potential mechano-sensors are ephrins, gap junctions, Connexin 43 (Cx43) hemichannels and ion channels (stretch activated channels). The parathyroid hormone (PTH)-related peptide (PTHrP) and its receptor (PTH1R) have also been shown to be required for skeletal responses to

loading and unloading. Trabecular osteoblasts (TO) isolated from PTHrP-/- animals, flown in space for 6 days, were more sensitive to cell death than control TOs and this effects was reversed by treatment with PTHrP [16]. Surprisingly, cortical osteoblasts (CO) isolated from same animals, were "insensitive" to microgravity. Furthermore, mice with conditional deletion of PTHR in osteocytes were resistant to bone gain induced by axial ulna loading, demonstrating the need of an intact PTH-PTHrP-PTHR axis for proper skelatal mechano-transduction [17]. Finally, recent studies identified plasma membrane disruption (PMD) as a possible initial mechanism of mechano-sensation. PMD are small, reparable tears in the plamsa membrane that can be used as signaling cues.

Mechano-transduction." (the) process of converting physical forces into biochemical signals and integrating these signals into (a) cellular response" is the prerequisite for a functional and healthy skeleton. Once the signal is sensed by the osteocyte, via the mechanisms described above, it needs to be transduced into biological cues. The most studied and best described pathways induced by mechanical forces are intracellular Ca^{2+}, ATP, nitrogen oxide (NO), Prostaglandins (PGE_2) and Wnts. Whereas some of these signals acts exclusively intracellularly (i.e. Ca^{2+}), others are also secreted and affect both osteoblasts and osteoclasts (i.e. NO and PGE_2). Rapid intracellular increase in Ca^{2+} is one of the earliest responses induced by mechanical loading. Pharmacological inhibition of Ca channels impairs osteocyte's ability of respond to mechanical cues and in vivo treatment with Ca channel inhibitors reduces skeletal responses to mechanical forces. ATP quickly increases upon mechanical stimulation and several in vitro studies demonstrated that intracellular Ca^{2+} in required for ATP response. Osteocytes synthesize and release PGE_2 in response to mechanical forces. FFSS stimulates gap junction-mediated intercellular communication, increases Cx43 expression which in turn forms hemichannels allowing the release of prostaglandins [18]. PGE_2 then functions in an autocrine fashion to activate EP_2-EP_4 receptors expressed on osteocytes and in a paracrine fashion to modulate osteoblast and osteoclast activities.

Signaling Pathways

Once the osteocyte perceives the mechanical stimulus, it activates a cascade of events which culminates in gene regulations. Over the past decades, the number of mechano-sensitive genes has expanded quite dramatically. Here we discuss the function and effects of main mechanosensitive genes.

Sclerostin: Sclerostin, the product of the SOST gene, is an osteocyte-specific protein and recently has emerged as an important therapeutic target for bone diseases such as osteoporosis and osteopenia. This osteocyte-specific protein inhibits bone formation, both *in vitro* and *in vivo*, by directly reducing proliferation and differentiation of osteoblasts via the canonical Wnt signaling pathway (**Figure 1**). It has been shown that sclerostin acts by binding the low-density lipoprotein receptor 5 and 6 (LRP5 and 6) and inhibit wnt-βcatenin signaling pathway. Sclerostin is exquisitely regulated by mechanical forces; serum levels increases in humans after immobilization [19,20] and in animals subjected to tail suspension whereas the protein is suppressed by increased mechanical stimuli [21]. These increases in *Sost* /sclerostin likely contribute to the reduced bone formation seen in microgravity. Similarly, mice lacking SOST gene have high bone mass and are resistant to unload-induced bone loss [22] and treatment of tail-suspended mice with sclerostin antibodies prevent unload-induced bone loss [23]

RANKL: Recent findings indicate that osteocytes are a major source of the pro-osteoclastic cytokine RANKL, and that osteocyte-derived RANKL is a key contributor to disuse-induced bone loss in rodent models of unloading [24]. RANKL is required for osteoclasts differentiation and function; in its absence, mice develop severe osteopetrosis whereas its over-expression induces osteopenia. Osteoprotegerin (OPG) is also expressed and secreted by osteocytes and acts as a decoy receptor for RANKL preventing its binding to osteoclast progenitors. In vitro studies using mechanically loaded osteocytic cells, demonstrated that upon FFSS, RANKL is suppressed whereas when cells are subjected to simulated microgravity, this cytokine is increased [25]. Osteocytes also produce matrix extracellular phospho-glycoprotein (MEPE) which upregulate OPG and decrease RANKL/OPG ratio leading to osteoclast inhibition [26].

Fibroblast growth factor 23: Fibroblast growth factor 23 (FGF23) is another secreted factor produced mainly by osteocytes [27]. FGF23, together with PTH, controls phosphate homeostasis by binding to its receptor FGFR1 and the co-receptor Klotho, both in kidney and parathyroid gland. FGF23 prevents phosphate reabsorption and induces hypophosphatemia. The role of FGF23 in osteocyte mechanobiology is controversial and whereas initial studies suggested that this protein was regulated by mechanical forces, subsequent studies failed to corroborate these initial findings. Whether FGF-23 is indeed a mechanosensitive gene and whether its regulation has physiological implications is still unknown.

Besides the factors listed above, a plethora of other genes and molecules are regulated by mechanical forces. DMP1, Phex, MEPE and osteopontin (and others) have all been shown to be mechanosensitive genes although the exact function of these factors in osteocytes' mechanobiology is still unclear.

In vivo and in vitro tools to study osteocyte mechano-transduction

Currently there are several *in vitro* and *in vivo* models routinely used to study osteocyte mechanobiology and their cellular responses. Several osteocytic cell lines are now available and each cell line differs slightly in terms of basal gene expression and skeletal origin. The most studied and characterized one is MLOY-4, [28] a conditionally immortalized cell line derived from long bone of mice in which the SV40 antigen was driven by the osteocalcin promoter. Although these cells possess many of the characteristic of an osteocyte, they do not express high level of Sost/sclerostin or express other osteocyte specific genes, namely FGF23. Other osteocytic cell lines currently available are Oc14 [29], derived from PTHR-/- calvarial bones and two new ones isolated from long bones of conditionally immortalized animals expressing GFP under the Dmp-1 promoter, IDG-SW and Ocy454 [25,30]. IDG-SW cells express basally very low level of *SOST*/sclerostin and require high cell density and two weeks in culture under differentiation conditions to produce detectable *SOST*/sclerostin. Ocy454 ave higher basal Sost/sclerostin expression and, similarly to IDG-SW, are responsive to hormonal (PTH), cytokine (PGE_2), and mechanical stimuli. Ocy454 also showed an enhanced osteocytic phenotype when cultured on a three-dimensional biomaterial, by increasing *FGF23* expression upon PTH stimulation highlighting the importance of optimizing *in-vitro* culture conditions for studying certain aspects of osteocyte biology [25]

These cell lines can be subjected, *in vitro*, to load, as achieved by laminar continuous unidirectional flow or by pulsatile fluid flow. Commercially available systems or investigator custom-made devices have been used to impose FFSS on 2D cultured cells. Alternatively, cells can be grown on flexible-bottom tissue culture plates and exposed to tensile forces or subjected to hypotonic conditions. These systems have been widely used to study the effects of loads on osteocytes and their limitation is that cells are grown in 2D monolayers, not fully recapitulating the physiological relationship and forces present in bone cells *in vivo*. The use of 3D structures, or scaffolds, should be preferred when studying osteocytes (or other bone cells) under altered

mechanical conditions (loading or microgravity). A multitude of scaffolds or inert support are currently available for bone research and they include collagen-based sponges, hydroxyapatite substrates, and synthetic materials, such as polypropylene. The choice of scaffold is often guided by both the experimental end-point (compatibility of the substrate with the end applications) and the culture conditions (geometry of the scaffolds). *In vivo* studies also provided important insights into osteocytes mechano-transduction. Cyclical loads of long bones (tibia or ulna) have been used, *in vivo*, to analyze skeletal responses to increased forces. Animals undergo daily repetitive loading of the tibia or femur utilizing non-invasive loading devices. Recently, vibration platforms have also been used to study bone adaptation. Similarly, several experimental settings have been developed to study cells, or animals, under reduced mechanical cues. Cells can be subjected to simulated microgravity using NASA developed slow-rotating wall vessels (SRWV) or using random positioning machine (RPM) or 3D clinostat. The NASA SRWV bioreactor analog for simulating microgravity operates on the principle of subjecting cells to a rotating fluid environment that randomizes the gravity vector over one revolution. Similar principle of "gravity vector averaging" applies to the RPM. Thus, using the rotating wall analog model alone is not sufficient to fully validate the observed morphological, gene, and hormonal changes of the osteocyte network solely due to unloading conditions. Earth based cell culture unloading analogs (for the study of *in vitro* osteocyte cellular network) cannot separate effects of fluid flow shear stress from the effects of simulated mechanical unloading. Thus, utilizing only earth analogs for osteocyte network mechano-sensing investigations is insufficient to characterize the osteocyte network changes arising from mechanical unloading alone. Real microgravity environment and minimal fluid shear culture conditions available onboard of the International Space Station (ISS) are therefore the gold standard for analyzing osteocytes's responses to unloading. *In vivo* studies, using both animals and humans are also used to gain insights into mechanisms regulating the skeletal response to reduced mechanical loading. In mice and rats, disuse-induced bone loss is achieved by suspending the animal by the tail, so that a coronal rotation of 30° (head-down) is produced, weight bearing by the hindquarters is eliminated, and a cephalad fluid shift occurs. This technique has become one of the most frequently employed for studies of disuse bone loss, with a plethora of data produced. Botox injections, used to paralyze the animal hindlimb, are an alternative approach to hind-limb unloading. Humans studies are far more complex than animal ones and have involved astronauts, spinal cord injured patients or healthy volunteers subjected to prolonged bed-rest (up to 90 days) with a 6° head-down tilt.

Conclusions

Although the past decade has seen an exponential increase of current knowledge on osteocytes mechanobiology, the precise mechanisms by which these cells perceive and transduce mechanical cues are still unclear. What have emerged is the multiplicity and complexity of the signaling systems activated by the mechanical inputs. The unique environment of an osteocyte in vivo, make it difficult to establish in vitro model that faithfully recapitulate it. Recent technological advances have demonstrated an impressive progress in understanding osteocyte biology and functions and further elucidation on the mechanisms of osteocyte mechanobiology holds promises of biological and medical implications.

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Meet the Professor: Interesting Cases in Metabolic Bone Disease

Amy Warriner, M.D.

Friday, September 8, 11:30 AM - 12:30 PM

Room 111/113, Colorado Convention Center in Denver,

CO, USA

Amy H. Warriner, MD CCD

Associate Professor of Medicine, Division of Endocrinology

University of Alabama at Birmingham, U.S.A.

Interesting Cases in Metabolic Bone Disease

Significance: With advancements in imaging techniques and genetic sequencing, our understanding of various metabolic bone diseases continues to progress. As we review cases of recurrent fractures and osteolytic and sclerotic bone cases, we will discuss unique features of each disease in regards to radiologic features, laboratory studies and genetic testing.

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

1. Identify oncogenic osteomalacia in a person presenting with recurrent fractures and/or specific laboratory findings.
2. Differentiate possible causes of osteolysis or vanishing bone, depending on presentation, location of bone changes and underlying pathophysiology.
3. Differentiate causes of elevated bone density and diffuse sclerosis of bone.

Cases:

Case 1

43 year old Caucasian man with a history of hypertension and nephrolithiasis. He developed a stress fracture of his foot 8 months ago and now also has a new non-displaced fracture of calcaneus. Family history is significant for an aunt with Osteogenesis Imperfecta. Physical exam is unremarkable other than pain to palpation of left back. CT of chest shows possible left rib fractures.

- What initial evaluation would you consider for this patient?
- What are the best imaging techniques to consider?

Case 2

A 32 year old Caucasian man with a history of osteogenesis imperfecta (OI) presents with acute-onset intense hip pain. Initially, hip x-ray shows no abnormality. Due to persistent pain, MRI was completed showing moderate marrow edema in the right femoral head and neck extending into the intertrochanteric region. Given concern for stress fracture, he underwent cannulated screw fixation of the right hip. Two weeks post-operatively, x-ray showed almost complete resorption of the femoral head and neck with no significant areas of visible bone in the region.

- What are the potential explanations for these changes?
- Are these changes related to his OI?
- What treatment should be initiated?

Case 3

44 year old African American woman presents to the emergency room due to blurred vision and photophobia associated with facial changes. She had noted pressure at her right eye and facial changes in the past few months along with 67 pounds weight loss over the past year. Physical exam is significant for right enophthalmos, right temporal muscle wasting, wasting of right sided muscles of mastication.

- In addition to bone changes, what are other local changes that occur in this disease process?
- What treatment options exist?
- How should the patient be managed?

Case 4

36 year old African American man with diagnosis of osteopetrosis at age 18 presents for continued pain and facial changes. At age 18, he was having problems with his shoulder and radiographic examination was suggestive of the osteopetrosis. Current ongoing problems of significance: sensorineural hearing loss and cervical spine stenosis. There is no family history of birth defects, bone disorders (fractures, osteopetrosis, osteopenia), deafness, blindness, anemia, mental retardation, and/or learning disabilities. Consanguinity was denied. Physical exam findings of interest: prominence of the parietal regions of the skull and fronto-orbital ridges. There is frontal bossing and the occiput is also prominent and rounded. The eyes are deep set. Dentition is normal. He has no limb asymmetries. The joints have full range of motion.

- What differentiates his disease from osteopetrosis?
- What radiographic features can assist in differentiating diffuse sclerotic bone changes?

Discussion of cases:

Tumor-induced osteomalacia (TIO) is a paraneoplastic syndrome caused by secretion of large amounts of FGF-23 from mesenchymal cell tumors. FGF-23 is a phosphaturic hormone that leads to wasting of phosphorus and altered vitamin D metabolism. If left untreated, it can progress to osteomalacia, fractures, muscle pain. TIO diagnosis is frequently delayed and identification of the source tumor can be difficult. Improvements in imaging techniques and advances in non-surgical treatment options are on the horizon.

Transient osteoporosis is a self-limited disorder associated with acute pain followed by demineralization of bone that goes on to resolve spontaneously. Although it is well described as a complication of pregnancy, it is most commonly seen in middle-aged men. It is thought that an initial insult occurs that leads to localized increased bone turnover. Possible inciting causes include trauma, infection, reduced blood flow, drugs, surgery, and neurological disorders. Risk factors for developing transient osteoporosis include pregnancy, OI, drugs, endocrine disorders. The process can be confused with avascular necrosis. Treatment is usually conservative but case reports have shown response to various treatments, including bisphosphonates and teriparatide.

Gorham Stout disease presents as an osteolytic process with underlying intraosseous lymphangiogenesis. It is unclear whether the increased presence of endothelial lined vessels leads to osteolysis through exposing the region to factors that promote an osteolytic process or by local hypoxia. Many patients have a history of trauma to the area of bone involved in the lytic process but no other clear risk factors have been identified. Various treatments, including bisphosphonates, radiation, and VEGF-inhibitors, have been used with inconsistent results.

“Osteopetrosis” comprises a group of sclerotic bone diseases that result in increased bone density but are frequently associated with increased bone fragility and fractures due to impaired bone remodeling. In adults, elevated bone density on a DXA scan should be recognized as abnormal and alert the clinician to consider underlying bone pathology. The distribution of bone changes and cortical thickening on imaging can help differentiate causes of sclerotic bone disease. Advances in genetic sequencing may help to identify potential genetic variants that could play a role in the pathogenesis of these diseases but continued investigation is needed to clarify the causes of each phenotype.

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Meet the Professor: Aging Bone and MiRs

Johannes Grillari, Ph.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 102, Colorado Convention Center in Denver, CO, USA

Cellular Senescence, miRNAs and bone aging

Johannes Grillari, Ph.D., Christian Doppler Laboratory on Biotechnology of Skin Aging, Department of Biotechnology, BOKU-University of Natural Resources and Life Sciences, Vienna, Austria

Significance of the Topic:

Aging

One of the greatest success stories of human history is the ever increasing life-span due to a huge progress in hygiene measures, nutrition, and medical care. However, this also poses challenges, as with increasing age, the risk to suffer from age-associated diseases is growing and will pose a significant burden to our health care systems. Therefore, we need to aim at increasing the health span, post-pone the onset of age-associated diseases including cardiovascular diseases, cancer, type 2 diabetes, neurodegenerative disorders, non-alcoholic fatty liver disease, and osteoporosis, and thus maximize a self-determined life in dignity for as long as possible without prolonging potential sufferings.

Cellular senescence

Cellular senescence is determined as an irreversible growth arrest of normal cells after a specific number of cell doublings that was discovered in human cell culture experiments. Since the number of doublings correlates to the life span of the species, the age of the donor and the presence of premature aging syndromes (e.g. like Werner sysndrome), such cell culture models are widely used as model systems in biogerontology. By now it is clear that senescent cells accumulate in vivo with age and at sites of age-associated diseases throughout the body. If chronically present, they contribute to deterioriation of tissue functionality and regeneration by (de-)differentiation, loss of tissue specific functions and especially by secretion of pro-inflammatory and tissue remodeling factors, the senescence associated secretory phenotype (SASP). Recently, mouse models have been established in which senescent cells can be specifically removed. A quest for identifying senolytics, substances that specifically remove senescent cells, is therefore ongoing. In consequence to senescent cell removal, the mice have a later onset of age-associated diseases and an increased health span. In view to bone aging, senescent cells have been identified in the bone of mouse models to correlate with osteoporosis and bone aging.

MicroRNAs

MicroRNAs (miRNAs) are short single stranded non-coding RNAs of 20-24 nucleotides length. About 2000 human microRNAs have been identified so far. They are surprisingly well conserved throughout evolution from C. elegans to humans. miRNAs suppress mRNA target translation with low specificity and thus are considered to regulate and fine-tune biological process at a global level. In regard to bone, it is known that interfering with the miRNA

synthesis machinery, also impacts on bone and joint formation and metabolism. In addition, miRNAs have been found to increase with cellular senescence and to be secreted within extracellular vesicles as members of the SASP. Such extracellular vesicle are taken up by mesenchymal stem cells and inhibit osteogenesis.

Learning Objectives:

As a result of participating in this session, attendees should know about:

- current concepts in biogerontology
- the role of cellular senescence as an upcoming 'therapeutic' target
- the role of cellular senescence in bone aging
- current concepts in extracellular vesicles
- the connections between bone aging, cellular senescence and (circulating) miRNAs

Points of Interest:

- Senescent cells accumulate in aging bone
- Telomerase deficient mouse models show increase of cellular senescence and early onset of several age-associated diseases, among them skeletal changes that mimick changes found in human osteoporosis.
- Various senescent cell types secret factors including miRNAs that influence bone and cartilage metabolism

Figures:

Figure 1: Overview on how cellular senescence might contribute to aging of tissues and organisms. (1) Cells are exposed to DNA damage, reactive oxygen species (ROS), high oncogenic signalling or to telomere shortening due to multiple replications. (2) If no repair or cell cycle arrest checkpoints are operative, cells might undergo immortalization and transformation as first steps of tumorigenesis or (3) cells might undergo cellular senescence or apoptosis. The senescent cells then (4) show an altered secretory phenotype and thus influence signalling, or (5a) they might be removed after undergoing senescence by apoptosis or by the immune system. This in turn (5b) leads to replication/transdifferentiation of neighbouring cells or of replication/differentiation of adult stem and progenitor cells, decreasing their proliferative potential. Finally, (6) the senescent cells display an altered behavior and physiology in regard to their "daily" tasks within a tissue. All this in turn leads to (7) changes in the microenvironment of tissues and to their functional decline, which in turn (8) enhances the risk of tumor development and (9) accelerates senescence, thus largely contributing to aging of organisms (Grillari J and Grillari-Voglauer R., Exp. Gerontol 2010).

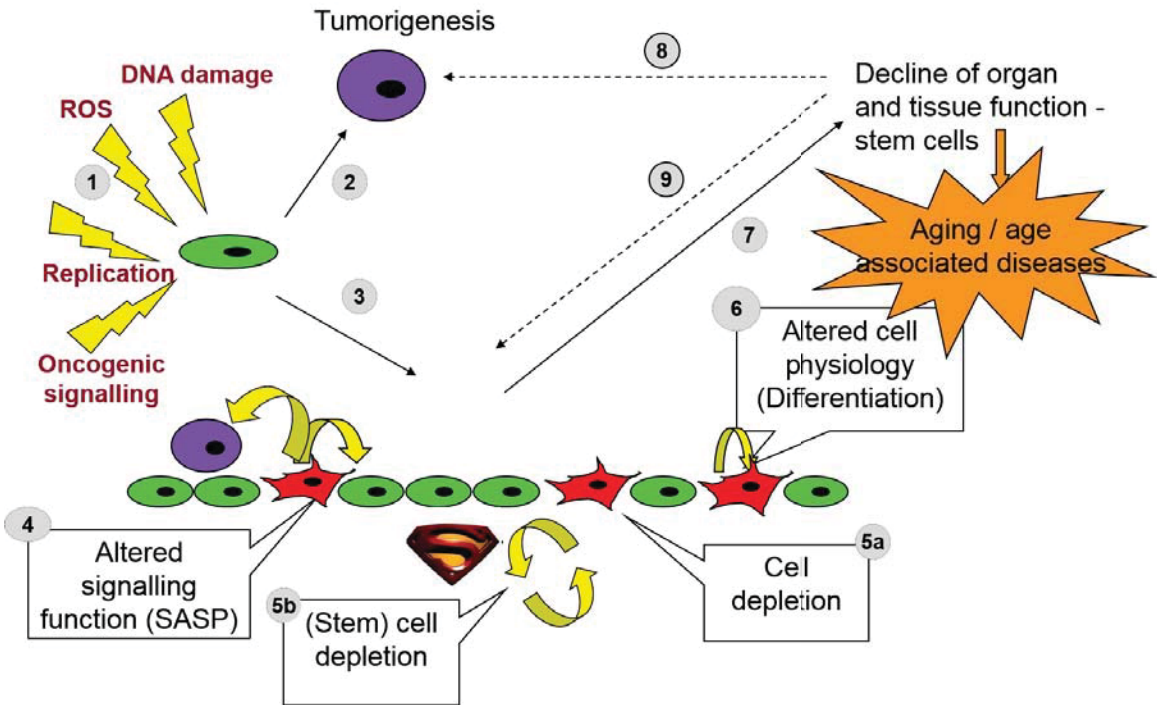
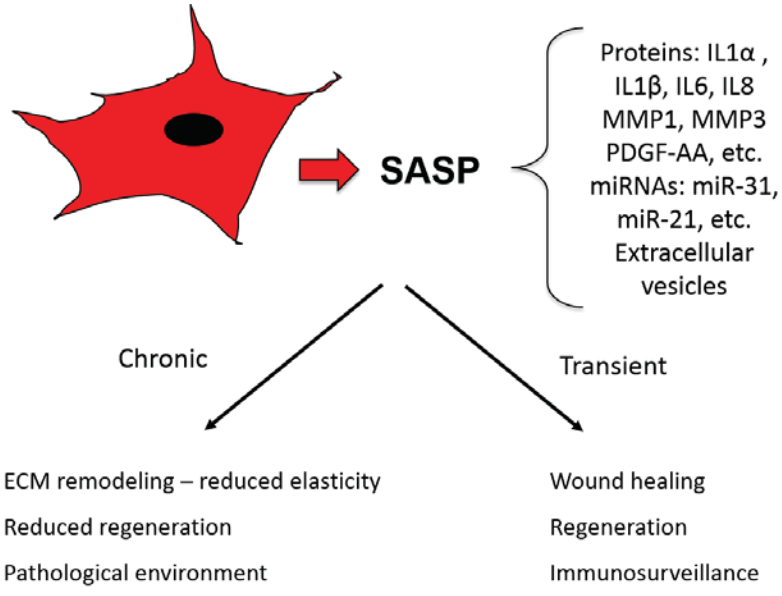


Figure 2: **The concept of SASP as Janus headed factor in tissue homeostasis**. Senescent cells contribute to a pro-inflammatory and tissue remodeling by secreting pro-inflammatory proteins, but also miRNAs packaged into extracellular vesicles. While transiently present senescent cells are considered to have beneficial effects on tissue homeostasis, chronically persistent and accumulating senescent cells contribute to a decline in tissue function and contribute to tumorigenesis by the persistent SASP.



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Meet the Professor: Osteocytes and Bone Resorption

Charles O'Brien, Ph.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 103, Colorado Convention Center in Denver, CO, USA

Osteocytes and Bone Resorption

Charles A. O'Brien

University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System, Little Rock, Arkansas, USA

Osteocytes are now recognized as important regulators of the process of bone resorption by osteoclasts. Much of the evidence supporting this idea comes from genetically-modified mice. In addition, some evidence from such models appears to contradict the idea that osteocytes are involved.

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

1. Describe the evidence for and against the idea that osteocytes control the process of bone resorption by osteoclasts.

2. Understand the strengths and limitations of various mouse genetic models.

3. Identify important questions that remain to be address regarding the role of osteoctyes.

Outline

Historical perspective

Pros and cons of various genetic approaches

Ongoing and future efforts

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Meet the Professor: Chondrocyte Biology and Osteoarthritis

Martine Cohen-Solal, M.D., Ph.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 104, Colorado Convention Center in Denver, CO, USA

Physiopathology of osteoarthritis: promises and pitfalls Pr

Martine Cohen-Solal

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Lariboisière Hospital
2 rue Ambroise Paré, 75010 Paris, France E-
mail: martine.cohen-solal@inserm.fr

Significance of the Topic:

Cartilage damage which characterizes osteoarthritis is accompanied with bone lesions. Joint integrity results from the balance in the physiological interactions between bone, cartilage and synovium. Several local factors regulate physiological remodelling of cartilage, the disequilibrium of these leading to a higher cartilage catabolism. Cytokines and growth factors secreted by bone cells or their precursors can induce chondrocyte differentiation and apoptosis which suggests their role in the dialogue between both tissues.

Several animal models of OA have been developed in order to assess the mechanism of cartilage loss and chondrocyte functions that encompassed surgical, chemical or genetic approaches. Indeed, the animal models are requested to investigate the cartilage changes in the absence of molecules expressed in the cartilage or in the joint tissues. This led to the evidence that cartilage loss is related to different mechanisms such as senescence, increased catabolism or loss of hypoxia. Several molecules have been identified that are potential candidates for new drug targets for osteoarthritis. Effects of each tissue should be approached in an integrative way in animal models in order to better understand the pathophysiology and to limit the side effects.

Learning Objectives:

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

- Know the different animal models in osteoarthritis, their impact and limitations
- Better understand the role of bone molecules in cartilage remodeling
- Better understand the role of hypoxia in cartilage remodeling in osteoarthritis.
- Understand the necessity of including joint assessment in the clinical trials for osteoporosis

Points of Interest

Although the cartilage use is the main hallmark of OA, the disease damages the whole joint including bone, synovial tissues and ligaments. In humans, the characterization of each tissue lesion that leads to cartilage degradation in a longitudinal manner is restricted. Lesions developed in the joints at the early stages of OA. Such evaluations have the advantage of providing the localization and the time-course of the tissues alterations. Synovial inflammation, meniscus and bone marrow lesions are good predictors of OA rapid progression at the knee. However, this approach gives only descriptive information and is not fully contributive to the cause of the disease. Therefore, animal models are valuable tools to fully characterize the kinetics of the changes in the tissues, understand the pathophysiology and mechanism of action and efficacy of new molecules.

The final goal of animal models is to reproduce human OA. Most of them focused in one factor that favors the development of OA such as aging, mechanical stress (surgery), chemical defect (enzyme) or in genetic factors. All of them differ in terms of severity, localization of lesions and pathogenesis. Hence, the choice of the model should be appropriate to the addressed question. The choice should be focused on either the role of tissues or molecules that could trigger OA, the development under a specific genetic background or the use of drugs to prevent the occurrence of OA. Moreover, the necessity of animal models is driven by the need of preclinical studies in order to evaluate the safety, toxicity and effects of drugs.

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Meet the Professor: Notch Signaling

Ernesto Canalis, M.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 105, Colorado Convention Center in Denver, CO, USA

NOTCH SIGNALING
Basic Science for Clinicians

Ernesto Canalis, MD
UConn Health, USA
Saturday, September 9, 2017
11:00 am

Introduction and Significance

Notch Signaling

Notch 1 to 4 receptors are important determinants of cell fate and function, and Notch signaling plays an important role in skeletal development and bone remodeling. Following direct interactions with ligands of the Jagged (Jag 1 and 2) and Delta-like (Dll1, 3 and 4) families, a series of cleavages release the Notch intracellular domain (NICD) which translocates to the nucleus where it induces the transcription of Notch target genes. Classic gene targets of Notch are Hairy and enhancer of split (Hes) and Hes-related with YRPW motif (Hey). In cells of the osteoblastic lineage, Notch activation inhibits cell differentiation and causes cancellous bone osteopenia because of impaired bone formation. However, the effects of Notch are cell context dependent and distinct functions for each Notch receptor have been reported.

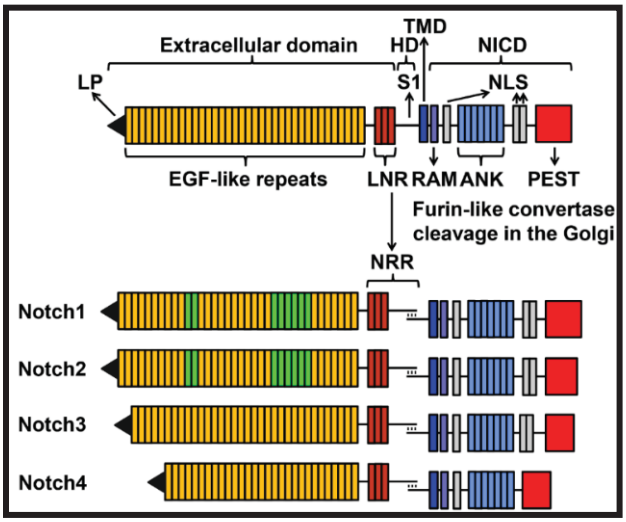
Congenital disorders of loss- and gain-of-Notch function present with severe clinical manifestations, often affecting the skeleton. Enhanced Notch signaling is associated with osteosarcoma and Notch can influence the invasive potential of carcinoma of the breast and prostate. Notch signaling can be controlled by the use of inhibitors of Notch activation, small peptides that interfere with the formation of a transcriptional complex or antibodies to the extracellular domain of specific Notch receptors or to Notch ligands.

Learning Objectives

As a result of participating in this session, attendees should have an understanding of:

1) Notch signaling, basic mechanisms
2) Function of Notch in the skeleton
3) Skeletal diseases associated with altered Notch signaling

Notch Receptors



Notch Signaling

Jag Dll
|
Notch extracellular domain
↓
Notch intracellular domain (NICD) is released
↓
NICD – Rbpjκ – Maml complex
↓
Transcription
Hes, Heys

Function of Notch in the Skeleton

- Maintains mesenchymal stem cells in an undifferentiated state
- Regulates osteoclastogenesis
- Suppresses endochondral bone formation
- Differential effects in osteocytes

Genetic Disorders Associated With Notch Signaling

Disease	Mutation
Loss-of-Function	
Adams Oliver Syndrome	*NOTCH1, EOGT, DLL4, RBPJκ or CSL, ARHGAP31, DOCK6*
Alagille Syndrome	*JAG1, NOTCH2*
Spondylocostal dysostoses	*DLL3, MESP2, HES7, LNF*
Spondylothoracic dysostoses	*MESP2*
Gain-of-Function	
Brachydactyly	*CHSY1*
Hajdu Cheney Syndrome	*NOTCH2*
Lateral Meningocele Syndrome	*NOTCH3*
Uncertain	
CADASIL	*NOTCH3*

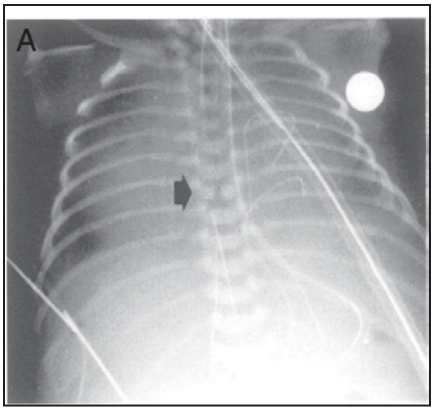
Alagille Syndrome

Mutations
- Jagged1
- Notch2

Heart … Fallot's Vascular

Liver … cholestasis

Skeleton
- Butterfly vertebrae
- Craniosynostosis
- Digit abnormalities
- Short Stature
- Osteoporosis, liver disease



Hajdu Cheney Syndrome

Clinical Features

Craniofacial features
- Facial dysmorphism, microretrognathism, periodontal disease, platysbasia, wormian bones

Skeletal Features
- Acroosteolysis, <u>osteoporosis</u>, fractures

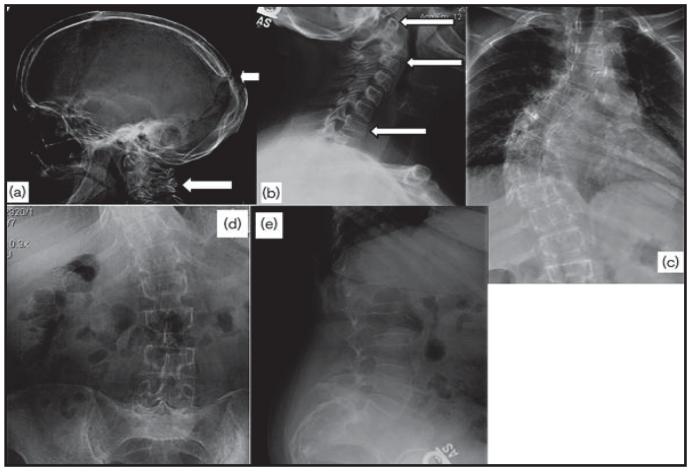
Polycystic kidneys

Neurologic symptoms

Cardiovascular defects

Splenomegaly...(G.Adami et al Bone 2016)

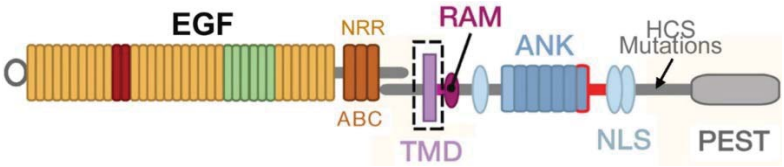
Hajdu Cheney Syndrome Skeletal Features



(a) Wormian bones, C2/C3 fracture, and basilar invagination
(b) Posterior angulation of the dens and C7 fracture
(c) Marked scoliosis
(d) Marked osteoporosis
(e) Osteoporosis and L5 fracture

Hajdu Cheney Syndrome Mutations

HCS is associated with mutations in *NOTCH2* upstream the PEST domain leading to the translation of a truncated protein and gain of NOTCH2 function.

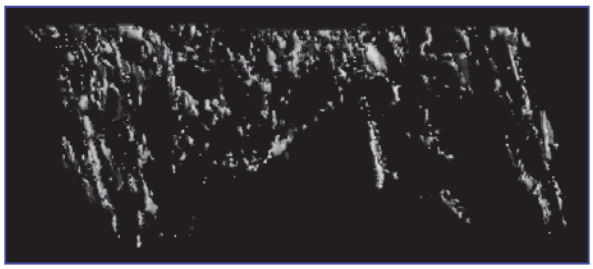


Experimental Models of Hajdu Cheney Syndrome

Notch2HCS mutant mice were created in our laboratory reproducing the mutation found in the human disease. *Notch2HCS* mutant mice are osteopenic and phenocopy the human disease.

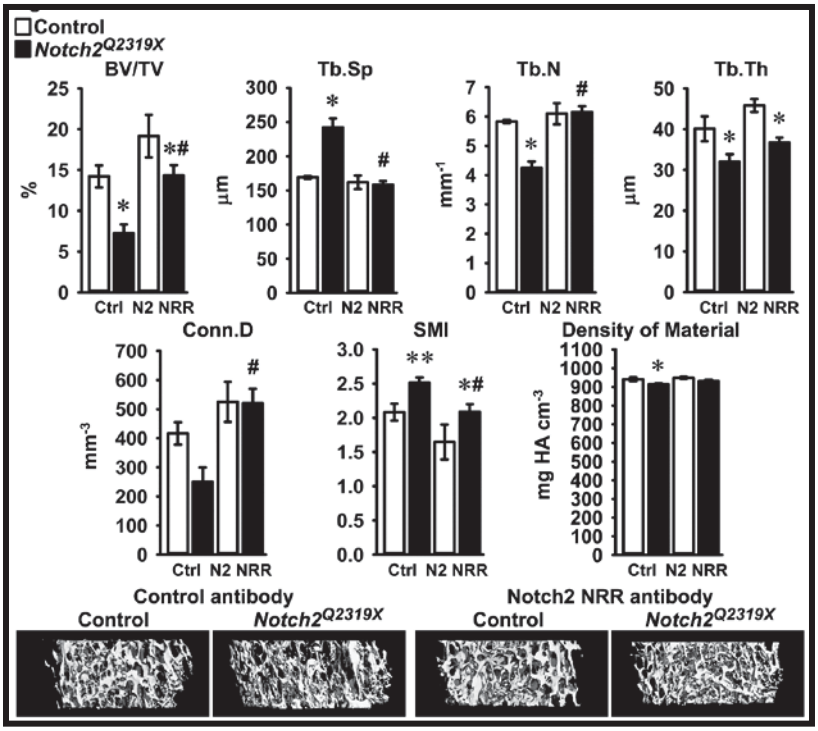
Wild Type

Notch2HCS



Ways to Correct Disorders of Enhanced Notch2 Signaling

Notch2HCS (Notch2^{Q2319X}) mice were treated successfully with anti- Notch2 NRR antibodies, which restored bone structure.



Lateral Meningocele Syndrome
A disease with some similarities to HCS and associated with mutations in *NOTCH3* and gain-of NOTCH3 function.

Classic Craniofacial features
Cognitive and neurologic function
- Developmental delay; intellectual disability
- Hypotonia; decreased muscle mass
- Syringomyelia

Cardiac valve abnormalities

Skeletal features
- Cleft palate
- Short stature
- Scoliosis; pectus
- Wormian bones; thick calvariae
- Thin bones

Mouse model of *Notch3LMS* mutation reproducing the human mutation was created in the laboratory and found to be osteopenic.

Role of Notch in Primary and Metastatic Bone Tumors

1) T cell acute lymphoblastic leukemia associated with *NOTCH1* somatic mutations and gain-of-NOTCH1 function.
2) B cell lymphomas associated with *NOTCH2* mutations and gain-of-NOTCH2 function.
3) Osteosarcoma associated with enhanced NOTCH signaling. Mouse model of Notch overexpression develops osteosarcoma.
4) Tumor invasiveness – breast and prostate tumors are associated with enhanced Notch signaling.

Role of Notch in Fracture Repair

1) Notch is unregulated in fracture callus and inhibition of Notch signaling accelerates fracture healing.
2) However, downregulation of Notch results in non-union fractures.

Role of Notch in Osteoarthritis

1) Notch is detrimental to articular chondrocytes, and NOTCH1 is overexpressed in osteoarthritis.

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Meet the Professor: Osteocytes in Myeloma

G. David Roodman, M.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 106, Colorado Convention Center in Denver, CO, USA

ASBMR 2017 MTP Session: Osteocytes and Multiple Myeloma

Saturday, September 9 at 11:00 AM - 12:00 PM

G. David Roodman, MD PhD

Indiana University School of Medicine, USA

Significance of topic:

Multiple Myeloma (MM) is the second most common hematologic malignancy and the most frequent malignancy to involve bone (1). Approximately 70% of patients present with bone involvement at diagnosis and >85% will have bone involvement over the course of their disease. MM bone disease is characterized by uncoupling of the normal balanced bone remodeling process, in which bone resorption is normally accompanied by new bone formation. In MM, markedly increased osteolytic bone destruction is accompanied by severely suppressed or absent new bone formation that results in pure osteolytic lesions that do not heal. Much is known about the contributions of bone marrow stromal cells, immune cells and osteoclasts (OCL) to myeloma bone disease and their capacity to enhance tumor growth, (2) but until recently, little information was available on the role of osteocytes, which comprise 95% of cells in bone and are the primary regulator of bone homeostasis, in MM.

Learning Objectives:

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

1) Discuss the role of osteocytes in myeloma bone disease
2) Discuss the utility of targeting osteocyte products for myeloma bone disease
3) Discuss the potential of targeting osteocyte apoptosis as a novel therapy for myeloma

Role of Osteocyte Apoptosis/Autophagy in Myeloma

Giuliani and co-workers were the first to identify a role for osteocytes in myeloma (3). They reported that the viable osteocytes numbers were significantly decreased in MM patients compared with healthy controls, and that viable osteocyte numbers negatively correlated with the number of OCLs. Moreover, MM patients with bone lesions had significantly fewer viable osteocytes than those without bone lesions. They found that the decreased osteocyte viability was in part due to increased osteocyte apoptosis. Further, co-culture of human preosteocytes with MM cells increased apoptosis and interleukin (IL)-11 expression in preosteocytes. Importantly, osteocyte expression of IL-11 was higher in the MM patients with bone lesions compared with patients without bone lesions. More recently, these authors reported that MM cells also triggered osteocyte and preosteocyte autophagic death (4). Interestingly, these authors found that patients treated with the proteasome inhibitor Bortezomib had increased numbers of viable osteocytes compared to other therapies. Further, proteasome antagonists decreased osteocyte death induced by MM cells or by high-dose dexamethasone, as well as potentiated the anabolic effect of PTH(1-34). However, the mechanisms responsible for the effects of MM cells on osteocyte apoptosis and/or autophagy are unclear.

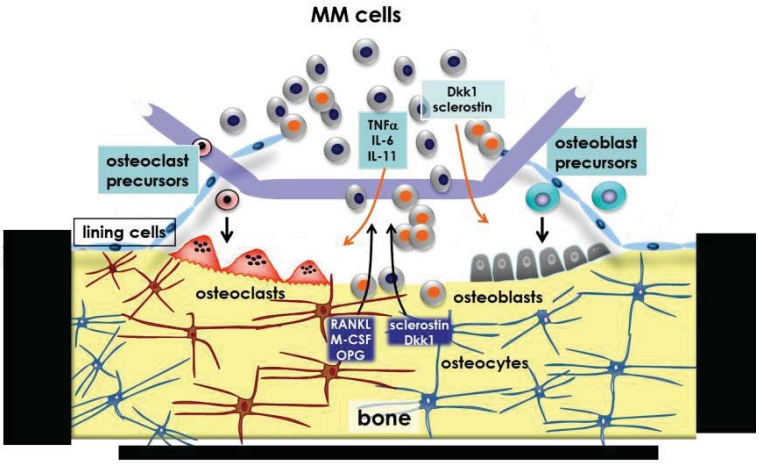

Fig.1. Disruption of the bone remodeling compartment in MM allows cell-to-cell contact and exchange of soluble factors between MM cells and bone cells. Delgado-Calle, Bellido, Roodman. Curr Opin Support Palliat Care. 2014; 8(4):407-13

Previous studies have shown that the bone remodeling compartment is disrupted in MM, (5) and this allows the exchange of soluble factors and direct cell-to-cell contact between MM cells and bone cells. (Figure1). We found that osteocytes in MM-bearing bones physically interact with MM cells *in vivo* in mouse models of MM (6). (Figure 2). We showed that MM cells induce osteocytes to undergo caspase3-dependent apoptosis, and express higher Rankl and Sclerostin levels than osteocytes from control mice. Mechanistic studies revealed that osteocyte apoptosis was initiated by activation of Notch signaling in osteocytes through direct contact with MM cells, and was further amplified by MM cell-secreted TNFα. Apoptotic osteocytes expressed high levels of Rankl and Sclerostin which increased OCL formation and inhibited osteoblast differentiation, respectively. Importantly, direct contact between osteocytes and MM cells also activated Notch signaling in MM cells, increased Notch receptor expression in MM cells and osteocytes, (in particular Notch R3) and enhanced MM cell growth. These studies were the first to identify a previously unknown role for bidirectional Notch signaling between MM cells and osteocytes, and suggest the potential of targeting osteocyte-MM cell interactions as a novel treatment for MM.

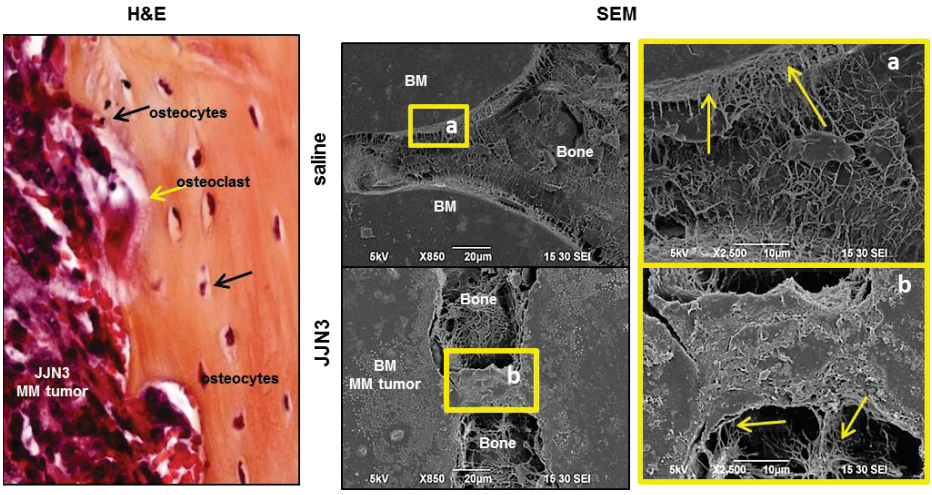

FIG.2. Osteocytes are in direct contact with MM cells in bone. Areas indicated by boxes a and b are magnified on the right. Arrows, osteocytic cytoplasmic projections in contact with the bone marrow compartment.

Osteocytes as Targets for Treatment of Myeloma Bone Disease

As noted above, bidirectional Notch signaling between MM cells and osteocytes increases bone destruction, suppresses bone formation and enhances tumor growth. However, targeting Notch signaling with systemic Notch inhibitors, such as gamma secretase inhibitors. (GSI), can cause significant gastrointestinal toxicity and other unwanted side effects. Therefore, we recently synthesized a bone-targeted Notch inhibitor by linking GSI-XII to an inactive bone-targeting molecule, (BT) (Delgado-Calle et al., ASMR 2017). The BT directs the conjugate to bone where the linker is cleaved by acid produced by osteoclasts, to release the GSI. In vitro, the unconjugated GSI decreased Notch target gene expression (Hes gene family), but BT-GSI had no effect. However, both GSI and BT-GSI pre-incubated at low pH to reproduce acidic conditions in resorption sites, equally inhibited Notch target gene expression. Ex vivo, GSI and BT-GSI (non-preincubated) similarly decreased Hes1/5 expression in whole bone organ cultures that reproduce conditions in the bone microenvironment. When the BT-GSI was given to 4-month old female mice for 2wks, the mice treated with

BT-GSI exhibited decreased Hes7 expression in bone, but not in brain or gut compared to vehicle-treated mice, demonstrating bone specific inhibition of Notch signaling. Further, BT-GSI-treated mice had higher total (3%), femoral (4%), and spinal (7%) BMD compared to control mice. Moreover, BT-GSI decreased serum CTX by 40% and upregulated Opg mRNA expression in bone, decreasing the Rankl/Opg ratio. In contrast, serum P1NP and osteoblast marker expression remained unchanged by BT-GSI treatment. These findings demonstrate that short-term pharmacological inhibition of Notch signaling in skeletally mature mice inhibits bone resorption and favors bone gain. Because BT-GSI does not inhibit Notch signaling in tissues other than bone, it may circumvent the deleterious side effects that limit the use of pharmacological inhibition of Notch in patients.

In addition to targeting Notch signaling, blocking the increased osteocyte production of Sclerostin, Rankl, and FGF23 by osteocytes in MM-involved bone may also be reasonable approaches for treating MM bone disease. Sclerostin (Scl) levels are elevated in MM patient sera and are increased in osteocytes in MM-bearing mice. We recently reported that genetic deletion of Sost, the gene encoding Scl, prevented MM-induced bone disease in an immune-deficient mouse model of early MM. Further, administration of anti-Scl antibody (Scl-Ab) increased bone mass and decreased osteolysis in immune-competent mice with established MM (7). Sost/Scl inhibition increased osteoblast numbers, stimulated new bone formation and decreased osteoclast number in MM-colonized bone. However, Sost/Scl inhibition did not affect tumor growth *in vivo* or anti-myeloma drug efficacy in vitro. These results identify the osteocyte as a major contributor to the deleterious effects of MM in bone and osteocyte-derived Scl as a promising target for the treatment of established MM-induced bone disease. Importantly, Scl did not interfere with efficacy of chemotherapy for MM, suggesting that combined treatment with anti-myeloma drugs and Scl-Ab could be an effective approach to control MM growth and bone disease in patients with active MM. In support of this notion, Eda and co-workers (8) reported that a high-affinity Scl-Ab reversed osteolytic bone disease in a MM xenograft mouse model, but did not demonstrate significant in vitro anti-MM activity. They then combined anti-Scl treatment with the proteasome inhibitor, carfilzomib, and demonstrated that this combination therapy significantly inhibited tumor burden and improved bone disease in their mouse model of MM.

McDonald and co-workers also found that Sclerostin expression was increased in osteocytes from bones of myeloma-bearing mice (9). Mice injected with 5TGM1-eGFP, 5T2MM, or MM1.S myeloma cells had significant bone loss, which was associated with a decrease in fracture resistance in the vertebrae. Treatment with anti-Scl started 24 hours after injection of MM cells increased osteoblast numbers and the bone formation rate, but did not inhibit bone resorption or reduce tumor burden. Anti-sclerostin treatment also prevented myeloma-induced bone loss, reduced osteolytic bone lesions, and increased fracture resistance. Importantly, combined treatment of mice bearing myeloma with anti-sclerostin antibody and zoledronic acid increased bone mass and fracture resistance when compared to treatment with zoledronic acid alone.

Suvannasankha and co-workers recently found that FGF23 levels were also elevated in patients with MM, and that FGF23 increased heparanase expression by MM cells (10). They showed that MM cells express receptors for and respond to FGF23. FGF23 increased the levels of mRNA for EGR1 and its target heparanase, a pro-osteolytic factor in MM. FGF23 signals through a complex of klotho and a classical FGF receptor (FGFR) and both were expressed by MM cell lines and patient samples. Bone marrow plasma cells from 42 MM patients stained positively for klotho, while plasma cells from 8 patients with monoclonal gammopathy of undetermined significance, a premalignant condition that is a precursor for MM, and 6 controls were negative. Intact, active FGF23 was increased 2.9X in sera of MM patients compared to controls. FGF23 was not expressed by human MM cells, but co-culture with mouse bone increased FGF23 mRNA levels in the co-cultures. The FGFR inhibitor, NVP-BGJ398, blocked the heparanase response to FGF23, but did not inhibit MM growth in vitro. NVP-BGJ398 did significantly suppress MM cell growth in bone and induction of RANK ligand, and decreased induction of heparanase mRNA. These results suggest that the FGF23/klotho/heparanase signaling axis may offer another new target for treatment of MM in bone.

Summary:

The role of osteocytes in MM is just being recognized. Osteocytes are an important source of RANKL and Sclerostin in bone and have major effects on MM bone disease and tumor growth. Future studies delineating the mechanisms underlying the crosstalk between MM cells, osteocytes and other cells in the MM microenvironment should provide novel therapeutic approaches for reversing the severe musculoskeletal effects of myeloma, and decreasing tumor progression.

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8. Eda H, Santo L, Wein MN, Hu DZ, Cirstea DD, Nemani N, Tai YT, Raines SE, Kuhstoss SA, Munshi NC, et al. Regulation of Sclerostin Expression in Multiple Myeloma by Dkk-1: A Potential Therapeutic Strategy for Myeloma Bone Disease. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2016;31(6):1225-34.
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Meet the Professor: Paracrine Actions of IL-6 Family Cytokines from Bone, Marrow and Muscle

Natalie Sims, Ph.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 107, Colorado Convention Center in Denver, CO, USA

Cell-specific paracrine actions of IL-6 family cytokines from bone, marrow, and muscle that control bone formation and resorption

Natalie A Sims, St. Vincent's Institute, Melbourne, Australia
Email: nsims@svi.edu.au
Twitter: @NatalieASims

Significance of the topic: The IL-6 family comprises a range of cytokines required for normal bone mass, strength, response to anabolic and catabolic stimuli. They are also involved in a range of skeletal pathologies due to local or systemic inflammation, cancer metastasis, or genetic modifications.

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

1. Identify the members of the IL-6 family and know that the receptor complexes are, well, complex.
2. Identify cell types in bone that produce these cytokines and express receptors
3. Describe a paracrine pathway of an IL-6 family cytokine in bone physiology
4. Describe a paracrine pathway of an IL-6 family cytokine in bone pathology
5. Describe a human mutation in an IL-6 family cytokine member that leads to skeletal defects

An outline and some references:

1. **Meet the family**
   - Sims, N.A., Cell-specific paracrine actions of IL-6 family cytokines from bone, marrow and muscle that control bone formation and resorption. *Int J Biochem Cell Biol*, **79**:14-23, 2016.
   - Sims, N.A., Cardiotrophin-like cytokine factor 1 (CLCF1) and neuropoietin (NP) signalling and their roles in development, adulthood, cancer and degenerative disorders. *Cytokine Growth Factor Rev*, **26**:517-22, 2015.
2. **Which bone cells express family members**
   - Sims, N.A. and N.C. Walsh, GP130 cytokines and bone remodelling in health and disease. *BMB Rep*, **43**:513-23, 2010
3. **Some examples of paracrine pathways: CT-1 in osteoclasts / CNTF in muscle / OSM in osteocytes & macrophages**
   - Walker, E.C., N.E. McGregor, I.J. Poulton, S. Pompolo, E.H. Allan, J.M. Quinn, M.T. Gillespie, T.J. Martin, and N.A. Sims, Cardiotrophin-1 is an osteoclast-derived stimulus of bone formation required for normal bone remodeling. *J Bone Miner Res*, **23**:2025-32, 2008.
   - Johnson, R.W., J.D. White, E.C. Walker, T.J. Martin, and N.A. Sims, Myokines (muscle-derived cytokines and chemokines) including ciliary neurotrophic factor (CNTF) inhibit osteoblast differentiation. *Bone*, **64C**:47-56, 2014.
   - Walker, E.C., N.E. McGregor, I.J. Poulton, M. Solano, S. Pompolo, T.J. Fernandes, M.J. Constable, G.C. Nicholson, J.G. Zhang, N.A. Nicola, M.T. Gillespie, T.J. Martin, and N.A. Sims, Oncostatin M promotes bone formation independently of resorption when signaling through leukemia inhibitory factor receptor in mice. *J Clin Invest*, **120**:582-92, 2010.
   - Sims, N.A. and J.M. Quinn, Osteoimmunology: oncostatin M as a pleiotropic regulator of bone formation and resorption in health and disease. *Bonekey Rep*, **3**:527, 2014.
4. **Pathological paracrine pathways: LIFR and metastasis / IL-6 and inflammatory arthritis**
   - Johnson, R.W., E.C. Finger, M.M. Olcina, M. Vilalta, T. Aguilera, Y. Miao, A.R. Merkel, J.R. Johnson, J.A. Sterling, J.Y. Wu, and A.J. Giaccia, Induction of LIFR confers a dormancy phenotype in breast cancer cells disseminated to the bone marrow. *Nat Cell Biol*, **18**:1078-1089, 2016.
   - Wong, P.K., J.M. Quinn, N.A. Sims, A. van Nieuwenhuijze, I.K. Campbell, and I.P. Wicks, Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis. *Arthritis Rheum*, **54**:158-68, 2006.
5. **Some examples of human mutations: LIFR / IL-11R / CRLF1:**
   - **HUMAN:** Dagoneau, N., D. Scheffer, C. Huber, L.I. Al-Gazali, M. Di Rocco, A. Godard, J. Martinovic, A. Raas- Rothschild, S. Sigaudy, S. Unger, S. Nicole, B. Fontaine, J.L. Taupin, J.F. Moreau, A. Superti-Furga, M. Le Merrer, J. Bonaventure, A. Munnich, L. Legeai-Mallet, and V. Cormier-Daire, Null leukemia inhibitory factor receptor (LIFR) mutations in Stuve-Wiedemann/Schwartz-Jampel type 2 syndrome. *Am J Hum Genet*, **74**:298-305, 2004.
   - **MOUSE MODEL:** Poulton, I.J., N.E. McGregor, S. Pompolo, E.C. Walker, and N.A. Sims, Contrasting roles of leukemia inhibitory factor in murine bone development and remodeling involve region-specific changes in vascularization. *J Bone Miner Res*, **27**:586-95, 2012.
   - **HUMAN:** Nieminen, P., Morgan, N. V., Fenwick, A. L., Parmanen, S., Veistinen, L., Mikkola, M. L., van der Spek, P. J., Giraud, A., Judd, L., Arte, S., Brueton, L. A., Wall, S. A., Mathijssen, I. M. J., Maher, E. R., Wilkie, A. O. M., Kreiborg, S., Thesleff, I. Inactivation of IL11 signaling causes craniosynostosis, delayed tooth eruption, and supernumerary teeth. Am. J. Hum. Genet. 89: 67-81, 2011.
   - **MOUSE MODEL:** Sims, N.A., B.J. Jenkins, A. Nakamura, J.M. Quinn, R. Li, M.T. Gillespie, M. Ernst, L. Robb, and T.J. Martin, Interleukin-11 receptor signaling is required for normal bone remodeling. *J Bone Miner Res*, **20**:1093-102, 2005.

The IL-6 cytokine super-family and the known receptor complexes

This is how the cytokines can have specific effects… all induce JAK/STAT phosphorylation, but different patterns & cells.

From left to right:
gp130: glycoprotein 130 (gene name *Il6st*)

Interleukin 6: IL-6, IL-6R (x2), gp130	homodimeric
Interleukin 11: IL-11, IL-11R (x2), gp130	homodimeric
Oncostatin M (type I): OSM, OSMR, gp130	heterodimeric
Leukemia inhibitory factor: LIF, LIFR, gp130	heterodimeric
Cardiotrophin-1 (gene name *Ctf1*)**:** CT-1, LIFR, gp130	heterodimeric
Oncostatin M (type II): OSM, LIFR, gp130	heterodimeric
Ciliary neurotrophic factor: CNTF, CNTFR, LIFR, gp130	multimeric
Neuropoietin: NP, CNTFR, LIFR, gp130	multimeric
Cardiotrophin-like cytokine factor: CLCF1, CNTFR (x2), LIFR, gp130	crazy **CLCF1 /**
cytokine receptor-like factor 1: CLCF1, CRLF1, CNTFR, LIFR, gp130	insanity
Humanin: humanin (hum), CNTFR, IL-27R, gp130	insanity
cytokine receptor-like factor 1: p28, CRLF1, IL-6R, IL-27R, gp130	insanity
Interleukin 27: p28, EBI3, IL-27R, gp130:	multimeric

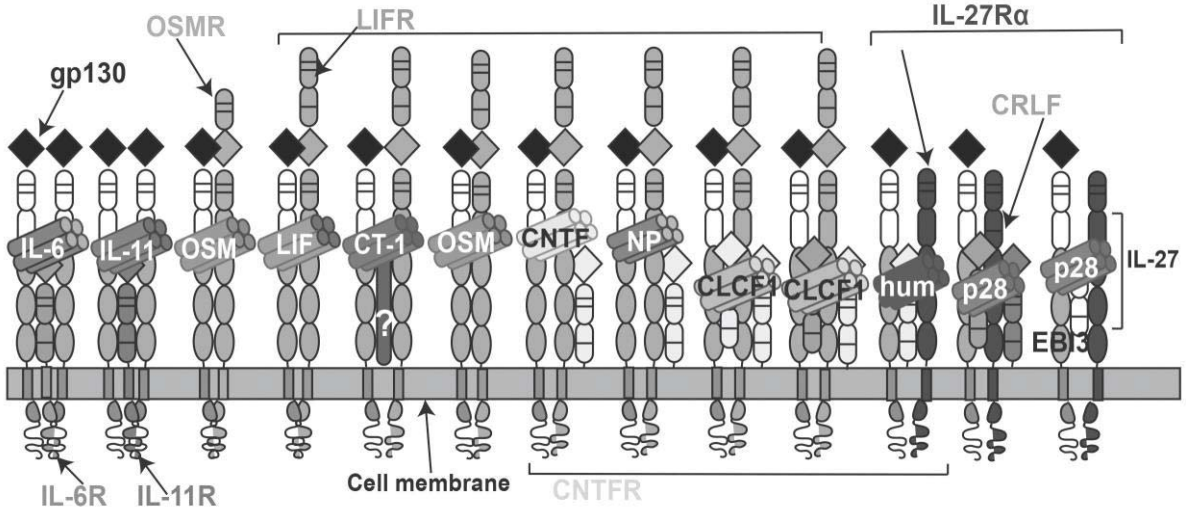


Figure from: Sims, N.A., Cell-specific paracrine actions of IL-6 family cytokines from bone, marrow and muscle that control bone formation and resorption. *Int J Biochem Cell Biol*, **79**:14-23, 2016.

Note: there are also soluble receptor isoforms, as well as amplifying complex members such as sortilin. For

more discussion of the complexity of CLCF1 secretion and signalling complexes:
Sims, N.A., Cardiotrophin-like cytokine factor 1 (CLCF1) and neuropoietin (NP) signalling and their roles in development, adulthood, cancer and degenerative disorders. *Cytokine Growth Factor Rev*, **26**:517-22, 2015.

Ligand and receptor expression in bone

Table 1. Expression patterns of gp130 cytokines and receptor subunits in bone and joint cells. +indicates a report of positive expression in this cell type, - indicates a confirmed report of expression lacking in this cell type

	Osteoclast	Osteoblast	Osteocyte	Chondrocyte	Synovial fibroblast
Receptors:					
gp130	+(105)	+(22)	+(43)	+(104)	+(111)
IL-6R	+(105)	+(107)	+(109)	+(110)	−(112)
IL-11R	+(22)	+(22)	Not reported	Not reported	−(111)
LIFR	−(106)	+(106)	+(43)	−(104)	−/+(111)
CNTFR	Not reported	+(108)	Not reported	Not reported	Not reported
OSMR	−(43)	+(43)	+(43)	+(104)	+(111)
WSX-1	Not reported	+(37)	Not reported	Not reported	+(113)
Ligands:					
IL-6	Occasional (25)	+(114)	Occasional (25)	+(117)	+(70)
IL-11	Not reported	+(22)	Not reported	+(118)	+(22)
LIF	Not reported	+(115)	Not reported	+(119)	+(70)
OSM	−(43)	+(43)	+(43)	Not reported	−(70)
CT-1	+(14)	−(14)	−(14)	+(120)	Not reported
CNTF	+(28)	+(116)	+(28)	+(28)	Not reported
IL-27	Not reported	Not reported	Not reported	Not reported	Not reported

Table from: Sims, N.A. and N.C. Walsh, GP130 cytokines and bone remodelling in health and disease. *BMB Rep*, **43**:513- 23, 2010
Numbers in brackets indicate references available from the above review;
N.B. This is accurate for 2010.

Also note ligand expression from cells within the marrow, and other cells known to influence the skeleton – some examples (not at all exhaustive):

T lymphocytes: IL-6
Macrophages: OSM, IL-6
Adipocytes: CT-1
Mast cells: IL-6, LIF, OSM
Granulocytes: IL-11, OSM
Muscle: CNTF

Probably many others!

Some examples of IL-6 family paracrine pathways within the skeleton:

Five examples of cell specific actions of IL-6 family cytokines acting on bone:

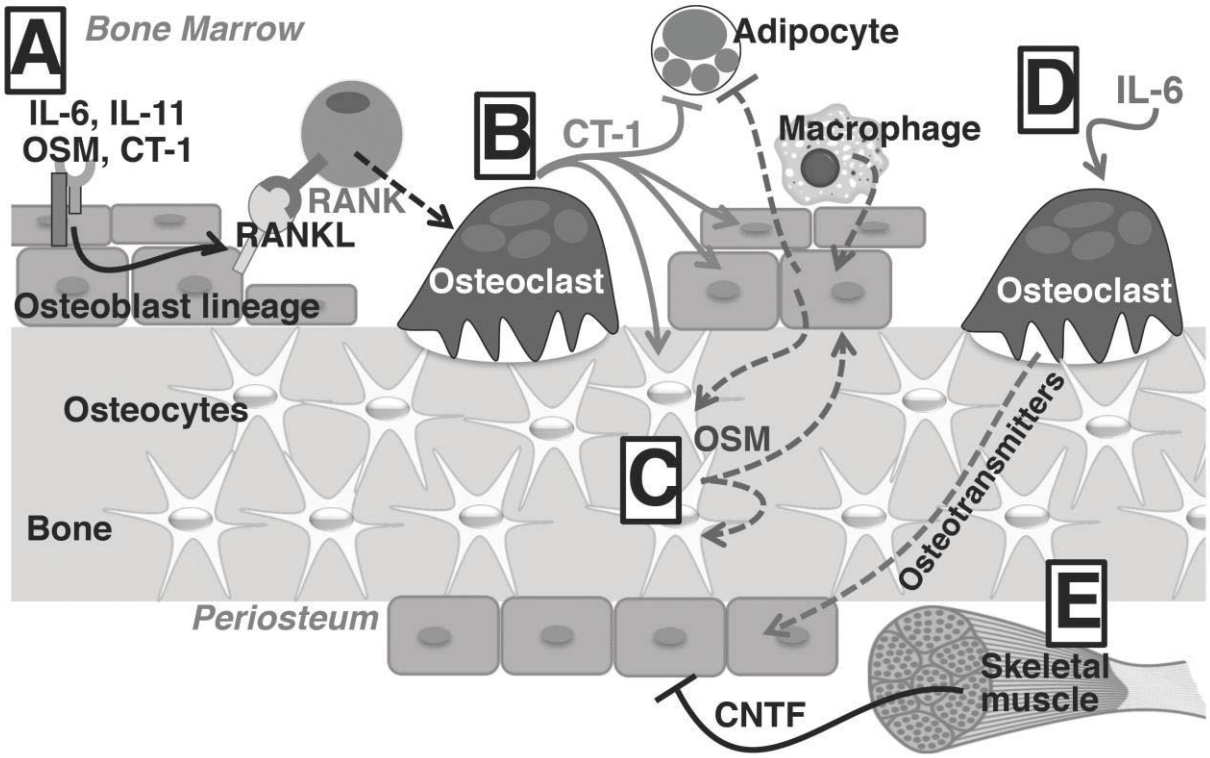
A: Osteoblast lineage cells (which may include precursors, canopy cells, lining cells, or osteocytes) support osteoclast formation by expressing RANKL in response to IL-6, IL-11, Oncostatin M (OSM) and Cardiotrophin-1 (CT-1). The interaction of RANKL with RANK on osteoclast precursors promotes osteoclast differentiation (dotted line).

B: Osteoclasts secrete Cardiotrophin-1 (CT-1), which acts on osteocytes, osteoblasts, and their precursors to stimulate bone formation, as a **coupling factor**, and to suppress adipogenesis.

C: Oncostatin M, produced by osteoblast lineage cells, including osteocytes and by macrophages (dashed orange lines), stimulates bone formation via osteocytes and suppresses adipogenesis.

D: IL-6 acts on osteoclasts to stimulate release of **osteotransmitters** (dashed green line) that pass through, or act through the osteocyte lacuno-canalicular network to stimulate osteoblasts on the periosteum.

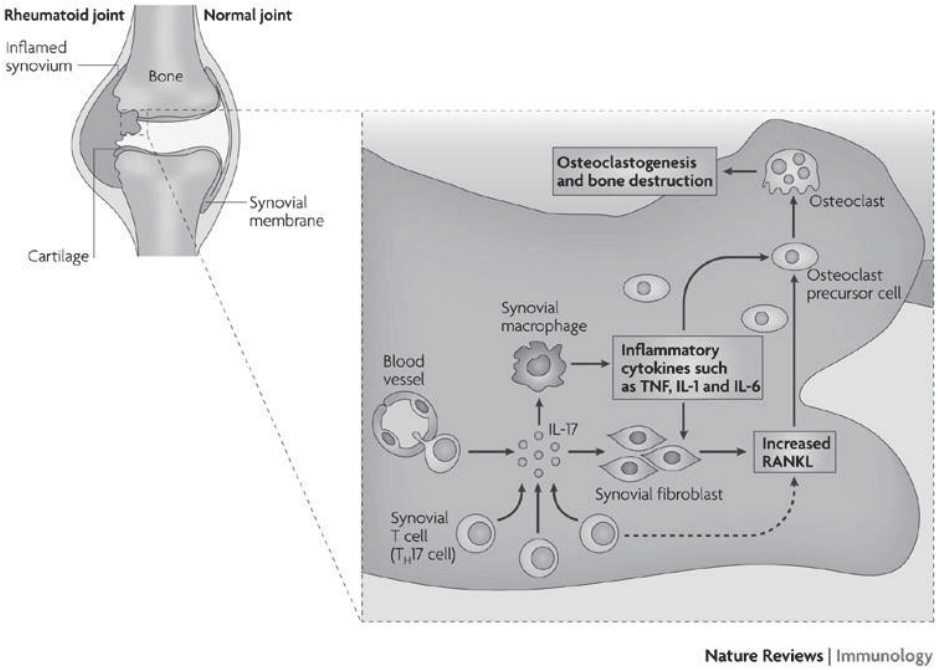
E: Ciliary Neurotrophic Factor (CNTF), released as a **myokine** from skeletal muscle suppresses bone formation on the periosteum.



Adapted from: Sims, N.A., Cell-specific paracrine actions of IL-6 family cytokines from bone, marrow and muscle that

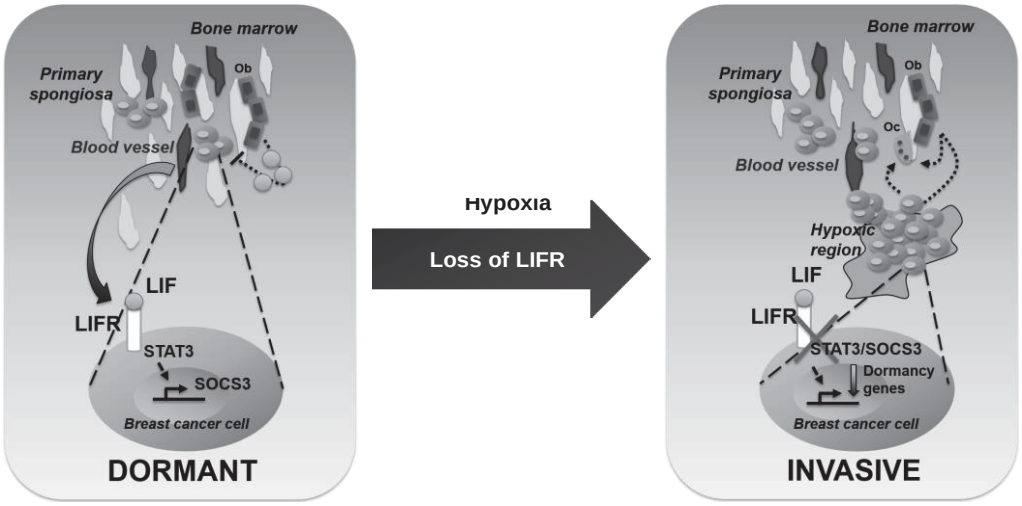
control bone formation and resorption. *Int J Biochem Cell Biol*, **79**:14-23, 2016.

Rheumatoid Arthritis: Systemic and local elevation of IL-6 and IL-6R levels. Targeted by Tocilizumab for inflammation; also provides benefit for the skeleton.
Collagen-induced arthritis model: less focal and systemic bone destruction in IL-6 KOs. Less osteoclastogenesis by BMM from arthritic IL-6 KO mice than from controls.
From: Wong, P.K., J.M. Quinn, N.A. Sims, A. van Nieuwenhuijze, I.K. Campbell, and I.P. Wicks, Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis. *Arthritis Rheum*, **54**:158-68, 2006.



This figure showing focal erosion events from: Takayanagi H, Nature Reviews Immunology 7, 292-304 (April 2007)

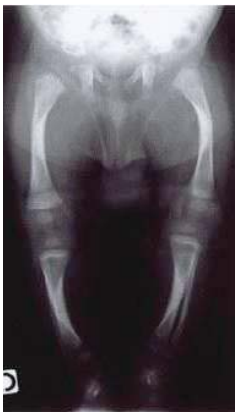
Breast Cancer Metastasis

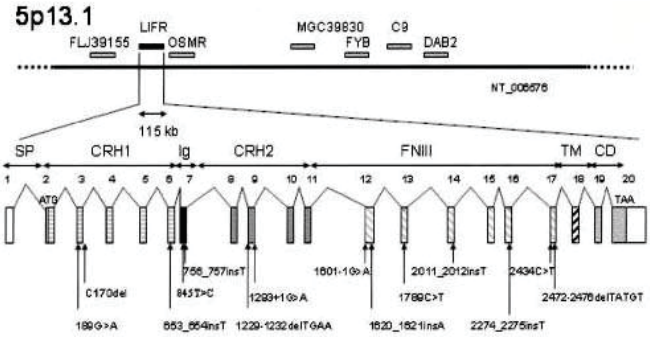


Hypoxia differentially regulates LIFR and PTHrP, which signal via STAT3 and SOCS3 to regulate dormancy-associated genes and bone colonization. Working model for LIFR:STAT3 signaling in disseminated breast cancer cells transitioning from a dormant to invasive phenotype in strongly hypoxic regions of the bone marrow.
From: Johnson, R.W., E.C. Finger, M.M. Olcina, M. Vilalta, T. Aguilera, Y. Miao, A.R. Merkel, J.R. Johnson, J.A. Sterling, J.Y. Wu, and A.J. Giaccia, Induction of LIFR confers a dormancy phenotype in breast cancer cells disseminated to the bone marrow. *Nat Cell Biol*, **18**:1078-1089, 2016.

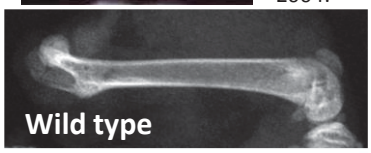
IL-6 family mutations in human skeletal conditions:

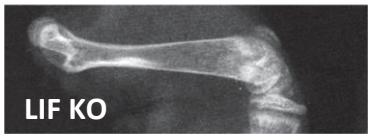
Stüve-Wiedemann Syndrome: LIFR mutations





From: Dagoneau, N., et al, and V. Cormier-Daire, Null leukemia inhibitory factor receptor (LIFR) mutations in Stuve-Wiedemann/Schwartz-Jampel type 2 syndrome. *Am J Hum Genet*, **74**:298- 305, 2004.





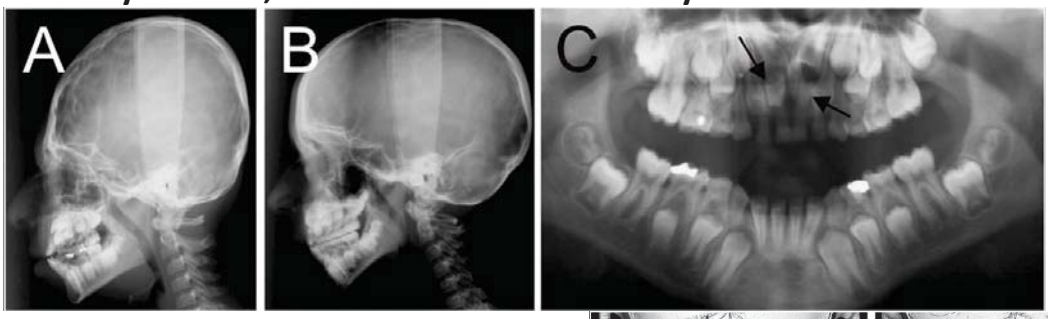
LIF ligand knockout has the same skeletal phenotype:
Limb shortening, disrupted primary spongiosa

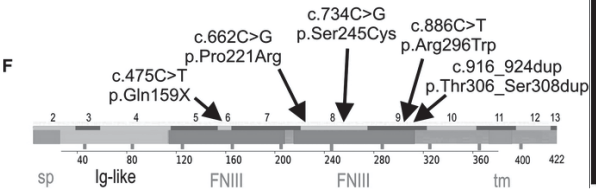
Poulton, I.J., N.E. McGregor, S. Pompolo, E.C. Walker, and N.A. Sims, Contrasting roles of leukemia inhibitory factor in murine bone development and remodeling involve region-specific changes in vascularization. *J Bone Miner Res*, **27**:586-95, 2012.

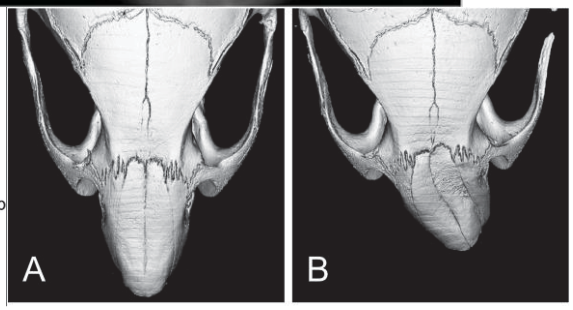
LIFR knockout similar, but neonate lethal.

Knockouts for the other ligands of LIFR do not recapitulate the Stüve-Wiedemann phenotype.

Craniosynostosis, reduced osteoclast activity - IL11R mutations







HUMAN & MOUSE: Nieminen, P., et al, Thesleff, I. Inactivation of IL-11 signaling causes craniosynostosis, delayed tooth eruption, and supernumerary teeth. Am. J. Hum. Genet. 89: 67-81, 2011;

MOUSE (long bone phenotype): Sims, N.A., B.J. Jenkins, A. Nakamura, J.M. Quinn, R. Li, M.T. Gillespie, M. Ernst, L. Robb, and T.J. Martin, Interleukin-11 receptor signaling is required for normal bone remodeling. *J Bone Miner Res*, **20**:1093-102, 2005.

Mouse model showed lower levels of bone formation, and reduced osteoclast numbers, and the reduction in osteoclast numbers was cell lineage autonomous: either mediated by loss of RANKL production by osteoblasts in the absence of IL-11 signalling, or a change in the marrow population – less osteoclast precursors present.

Meet the Professor: Managing an Osteoporosis Practice in an Era of Healthcare Reform

Robin Dore, M.D.

Saturday, September 9, 11:00 AM - 12:00 PM

Room 110/112, Colorado Convention Center in Denver, CO, USA

Managing an Osteoporosis Center in the Era of Health Care Reform

Robin K. Dore, MD

Rheumatology

Private Practice Tustin, CA

Clinical Professor of Medicine, David Geffen School of Medicine, UCLA, Los Angeles, CA

Introduction

I have a rheumatology practice that has offered DXA for my patients and those of the community physicians since 1989. Currently I lose $50 on each DXA that is performed in my office. Today we will discuss how I manage my practice in today's health care environment.

Networking

I meet with my referring HCPs several times per year and we discuss cases. Many of them are regarding referrals that I have received where the consultation is to treat a patient whose BMD has decreased significantly when in fact the decrease is due to poor positioning of the patient so that the studies cannot be accurately compared. The community then realizes that my center performs DXAs of good quality and increases their referrals to my center away from the hospital and other DXA centers where the studies are of poor quality

I am a professional member of the NOF. This allows me to receive referrals from the NOF for osteoporosis consultation as well as performing DXAs

Adding Value

If the practice is in an area that can financially support it, offering TBS would add value that most radiology centers and hospitals can't offer. If weight loss clinics are in the area, providing total body and tissue composition can add value to the practice.

Clinical Trials

I reach out to pharma companies and clinical research organizations (CROs) to notify them that I perform clinical trials and that I have a center that offers DXAs so that I can perform osteoporosis clinical trials. Notify the CROs that our center has multiple DXA capabilities including total body and tissue composition, LVA, forearm/wrist and hand in addition to lumbar spine and hip/femur. Some of my clinical trial experience has been providing total body composition and fat composition for trials of weight loss agents. Some trials have been interested in hand DXA which very few hospitals and radiology centers offer

MACRA

National Quality Strategy Domain: Effective Clinical Care Measure 39 gives credit for "screening for osteoporosis in women aged 65-85 years of age". Over 80% of the new rheumatology consultations that I perform are post-menopausal women who have never had a DXA performed or have not had a DXA for

over 3 years. In addition to performing the consultation, I also perform a DXA on the patient. By using the modifier -25, I can code for the consultation for the separate rheumatology problem 99203 and the DXA 90770. Thus, I can charge for the DXA in addition to the consultation and meet a quality measure

Meet the Professor: Anti-sclerostin and Multiple Myeloma

Peter Croucher, Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 102, Colorado Convention Center in Denver, CO, USA

Sclerostin and Multiple Myeloma

Peter Croucher, Garvan Institute of Medical Research, Sydney, Australia

Significance of the Topic

A number of cancers either grow predominantly in the skeleton, such as the haematological malignancy multiple myeloma, or metastasize to bone, including the common solid tumours, breast and prostate cancer. Once present in the skeleton these cancers have profound effects causing the development of osteolytic and/or osteosclerotic disease. Multiple myeloma represents one such example.

Multiple myeloma is characterized by the clonal expansion of malignant B-cells in the bone marrow. More than 95% of individuals with myeloma develop bone disease. This is characterized by both generalized osteoporosis and focal osteolytic bone lesions, which lead to a 16-fold increase in pathological fractures (Melton et al., 2005). This leads to considerable bone pain and for many this remains one of the most important causes of morbidity. The focus of treatment has been the development and introduction of anti- resorptive treatments, particularly bisphosphonates, which include clodronate and zoledronic acid. However, despite the fact that these agents slow the development of skeletal related events, patients with myeloma continue to experience fractures. There is therefore an urgent need to develop strategies to replace bone lost from the skeleton in order to increase bone strength and reduce the likelihood of further fractures. The development and introduction of bone anabolic approaches for the treatment of myeloma also offers the potential to repair osteolytic bone lesions, which may impact further on the future fractures.

Learning Objectives

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

1. The importance of inhibition of bone formation in the development of osteolytic bone disease in myeloma.

2. The impact of myeloma on sclerostin expression in the bone marrow microenvironment.

3. The impact of inhibiting sclerostin on the development of myeloma bone disease.

4. The impact of combining anti-sclerostin antibody treatment with bisphosphonate treatment on myeloma bone disease.

5. The impact of anti-sclerostin treatment on myeloma burden.

Points of interest

1. Osteoblast inhibition and the development of osteolytic bone disease in myeloma

Early histomorphometric studies revealed that the osteolytic bone disease in myeloma is mediated by an increase in osteoclastic bone resorption. This is associated with an early increase in bone formation, which likely reflects the coupling of resorption and formation, but is then associated with a reduction in bone formation. This increase in bone resorption and suppression of bone formation is mediated by the production of local factors by the myeloma cells themselves (Guiliani et al 2006). Whilst soluble factors are produced by myeloma cells that can suppress bone formation the Wnt family of osteoblast regulators has been shown to play a particularly important role. For example, the soluble Wnt antagonist dickkopf-1 (Dkk1) has been shown to be produced by myeloma cells, serum concentrations are elevated in patients with myeloma and inhibiting Dkk1 with antibody treatment prevents the suppression of bone formation and development of bone disease in experimental models of myeloma (eg Tian et al 2003, Politou et al 2006, Heath et al 2009). Interestingly, in these studies anti-dkk1 treatment often has little effect on bone resorption suggesting that stopping the inhibition of osteoblast suppression is sufficient to prevent myeloma bone disease. However, such strategies

have yet to be translated into the clinic, which may reflect the challenges associated with targeting tumour products that can show heterogenous expression. Mechanisms to target molecules such as sclerostin that is present in the bone microenvironment, offer an alternative approach.

2. The impact of myeloma on sclerostin expression in the bone marrow microenvironment

The discovery of sclerostin as an osteocyte product that could control bone formation led to studies exploring whether sclerostin is abnormally regulated in patients with myeloma. Serum levels of sclerostin are increased in patients with myeloma (Terpos 2012). Furthermore, like Dkk1, plasma cells from patients with myeloma have been reported to express sclerostin (Brunetti et al 2011). However, this is not seen in all studies. For example McDonald et al (2017) have analysed plasma cells isolated from a large cohort of patients with myeloma and myeloma cell lines by either microarray or RNAseq analysis and shown expression of Dkk1 in myeloma cells but not sclerostin. The reasons for the discrepancy between studies is unclear but may reflect the sensitivity of the various analytical techniques. More recently, Delgado-Calle (2016) have shown that myeloma cells can interact locally with osteocytes within developing bone lesions and can increase sclerostin expression within osteocytes. Together these data argue that myeloma cells can either directly or indirectly regulated expression of sclerostin in the bone microenvironment. This has led to studies investigating the effect of inhibiting the actions of sclerostin on myeloma bone disease in experimental models.

3. The impact of inhibiting sclerostin on the development of myeloma bone disease

Delgado-Calle (2017) has shown that genetic deletion of Sost , the gene encoding sclerostin, can prevent the development of myeloma bone disease in experimental models of myeloma suggesting this molecule has a pivotal role in regulating development of the bone disease. In support of this, three recent studies have investigated the ability of different anti-sclerostin antibodies to prevent development of myeloma bone disease (Delgado-Calle et al 2017, Eda et al 2016, McDonald et al 2017). In these studies anti-sclerostin treatment was shown to prevent generalized bone loss as well as the development of focal osteolytic bone lesions (eg Figure 1). The prevention of bone loss was seen in the long bones and the vertebra, which is an important site clinically. Detailed histomorphometric analysis showed that this was mediated by preventing osteoblast suppression and increasing bone formation. Importantly, these structural changes were associated with increases in bone strength and resistance to fracture (eg Fig 2). This argues that in experimental models targeting sclerostin may be at least as effective as bisphosphonates in treating myeloma bone disease. Whether anti-sclerostin treatment can repair existing bone lesions remains to be determined.

4. The impact of combining anti-sclerostin antibody treatment with bisphosphonate treatment on myeloma bone disease

Whilst inhibiting sclerostin is effective in preventing myeloma bone disease in experimental models, developing a translational strategy poses a number of challenges. One of these challenges is that bisphosphonates remain the current standard of care and potential new treatments would need to be considered in this context. To address this McDonald et al (2017) have investigated the effect of combining zoledronic acid, which is used in the clinic to treat myeloma bone disease, with anti-sclerostin antibody treatment. In these studies, both agents alone prevented myeloma bone disease. However, when used in combination, the two agents were better able to increase bone volume as either agent alone. Importantly, the combination increased bone strength when compared to zoledronic acid treatment alone (eg Fig 3). This supports the notion that anti-sclerostin antibody treatment could be added to the existing standard of care to improve skeletal outcomes for patients with myeloma.

5. The impact of anti-sclerostin treatment on myeloma burden

There is a complex interdependence between tumour cells and the local bone microenvironment and therefore targeting environmental products may affect myeloma burden. Furthermore, blocking an inhibitor of wnt signaling may be expected to have stimulatory effects on tumour development. However, to date, the studies that have investigated the effect of inhibiting sclerostin in experimental models of myeloma have not observed effects on tumour burden (Delgado-Calle et al 2017, Eda et al 2016, McDonald et al 2017).

Considerations Going Forward

Significant progress has been made in understanding the role of the sclerostin in myeloma. However, further work is required to:

- Understand how sclerostin is controlled in myeloma
- Understand whether targeting sclerostin can be used to repair bone lesions
- Understand whether using anti-sclerostin strategies to alter the bone microenvironment impact on the temporal development of tumour growth in the skeleton
- Develop an approach to see anti-sclerostin antibody treatment transitioned into the clinic

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Graphs

Figure 1. The effect of anti-sclerostin antibody treatment on bone volume in the lumbar vertebra of mice bearing 5TGM1 murine myeloma cells

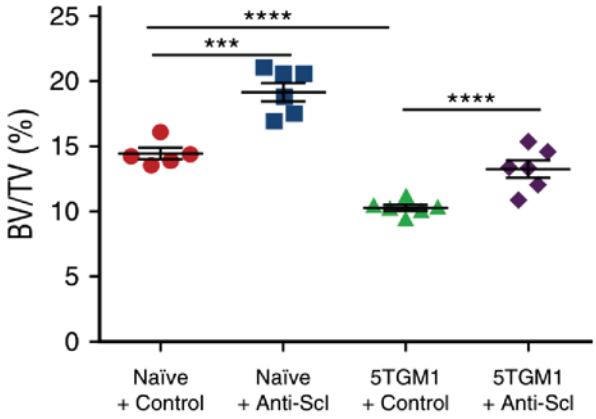


Figure 2. The effect of anti-sclerostin antibody treatment on bone strength in the lumbar vertebra of mice bearing 5TGM1 murine myeloma cells

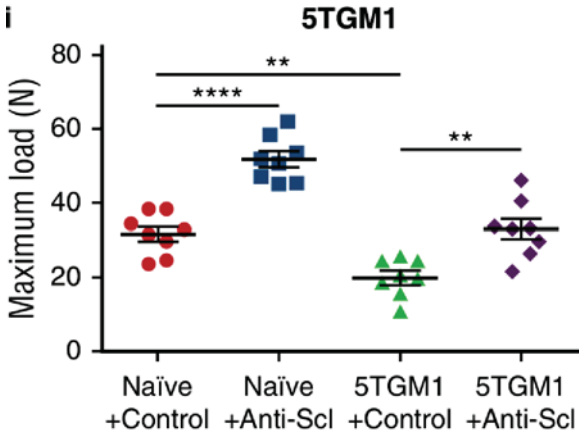
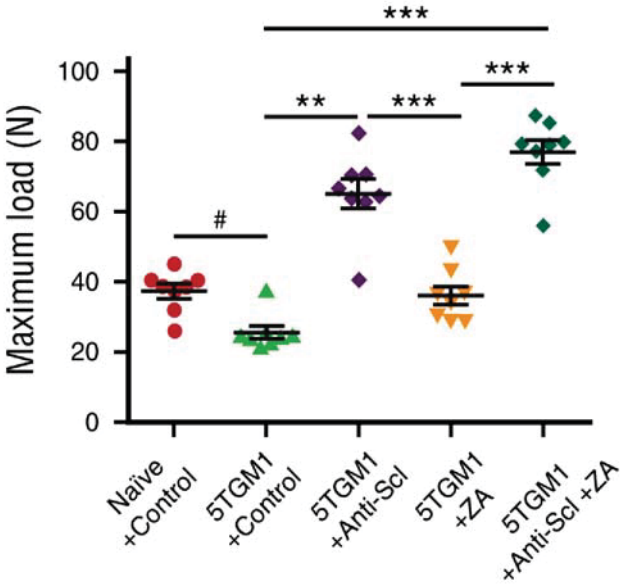


Figure 3. The effect of combining anti- sclerostin antibody treatment with zoledronic acid on bone strength in the lumbar vertebra of mice bearing 5TGM1 murine myeloma cells



Meet the Professor: Glucocorticoids and Bone

Mark Cooper, M.D., Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 103, Colorado Convention Center in Denver, CO, USA

Glucocorticoids and Bone – for clinicians

Mark S Cooper

ANZAC Research Institute, University of Sydney, Concord Repatriation General Hospital, Sydney

Significance of the topic:

Glucocorticoid-induced osteoporosis (GIOP) is the commonest secondary form of osteoporosis. It is characterised by a greatly increased risk of fracture, particularly for fractures of the spine. Glucocorticoids also decrease bone mineral density (BMD) but the risk of fracture appears to be greater than expected for the decrease in BMD. The pathophysiology of GIOP appears to be distinct to that of post-menopausal or age-related osteoporosis. The cellular targets and mechanisms of disease appear to be different between these conditions. Additionally, long-term glucocorticoid use is usually seen in the context of a significant underlying inflammatory condition which itself could contribute to the development of bone disease. In many people taking glucocorticoids bone disease could be as a result of pre-existing osteoporosis, disease associated inflammation or disease related impairment of nutrient absorption or kidney dysfunction. The role of adjunctive treatments such as calcium and vitamin supplements and the use of glucocorticoid sparing approaches need to be considered.

This interactive session will combine what is known about the pathophysiology of GIOP with the clinical evidence and experience relating to the management of the condition. Many of the management decisions relating to the treatment of GIOP are based on an established evidence base from randomised trials but an understanding of the pathophysiology involved is needed when deciding between treatments or in situations where the evidence base is limited.

Learning objectives:

1 To understand the pathophysiological mechanisms underlying the adverse effects of glucocorticoids on bone. This would involve knowing the effects of glucocorticoids and osteoporosis treatments on bone turnover, bone quality and cellular homeostasis. This would also involve an understanding of the dynamics of changes which will inform decisions regarding the timing of initiation and of cessation of protective therapies.

2 To appreciate the relationships between glucocorticoid effects and underlying disease. In particular the way that inflammation and glucocorticoids independently and collectively influence bone health.

3 To apply this knowledge to the management of patients treated with glucocorticoids.

Overview of pathophysiology:

A wide range of mechanisms have been proposed to account for the detrimental effects of glucocorticoids on bone. The most important of these are outlined in the figure below. The major contrast to other forms of osteoporosis is that bone formation is considerably reduced in GIOP whereas increased bone resorption dominates in most other situations. Both reduced bone formation and increased bone resorption can lead to an imbalance in bone remodelling favouring loss of bone.

Clinical Scenarios: Case 1:

A 75 year old woman is diagnosed with polymyalgia rheumatic. She was commenced on 20mg prednisone per day. It is intended to continue the glucocorticoids for 12 months. She has never had a fracture. At baseline her DXA scan shows a BMD T-score of -2 at both hip and spine.

What pathophysiological mechanisms impact on her fracture risk and bone density?

What is the onset and offset of effects on risk and what are the mechanisms underlying this?

Which treatments reduce risk and how do they do it? Are there differences in effectiveness between treatments based on how they work?

What role is there for calcium and vitamin D supplementation?

Case 2:

A 65 year old man has recently been diagnosed with a systemic vasculitis with renal involvement. His current eGFR is 25 ml/min. He is currently treated with prednisone 15 mg/day and cycles of cyclophosphamide.

What pathophysiological mechanisms input on fracture risk and BMD in this case? How does his glucocorticoid treatment impact on his fracture risk?

What is the role for calcium and vitamin D supplementation?

Case 3:

A 30 year old woman has a 5 year history of systemic lupus. Her current treatment is 10mg prednisone per day and hydroxychloroquine. Her BMD T score is -1.6 at hip and spine. Her periods were regular.

What pathophysiological mechanisms impact her fracture risk and bone density? Which treatments reduce fracture risk in this setting?

Case 4:

A 55 year old woman is referred for assessment of bone health. She has a 35 year history of Addison's disease currently treated with hydrocortisone 20mg in the morning and 10mg in the afternoon. She is also taking levothyroxine replacement therapy following radioactive iodine treatment for Graves' disease. On examination her blood pressure was 150/90. She was mildly overweight but had no overt features of Cushing's syndrome.

What pathophysiological mechanisms impact her fracture risk and bone density?

Do physiological levels of glucocorticoids (either endogenous or as replacement) impact on

bone? doses?

Does the choice of glucocorticoid influence bone health in replacement or in treatment

References:

Guidelines for treatment:

Buckley et al., 2017 American College of Rheumatology Guideline for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis Arthritis and Rheumatology, 2017;69:1521-37

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Impact of endogenous glucocorticoids on bone:

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Meet the Professor: Muscle Power vs. Muscle Strength: Impact for Falls and Mobility

Elsa Strotmeyer, Ph.D., M.P.H.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 104, Colorado Convention Center in Denver, CO, USA

Muscle power vs. muscle strength: Impact for falls and mobility

Elsa S. Strotmeyer, PhD, MPH
Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, USA

• Significance of the Topic: Provide a 1-2 paragraph introduction highlighting the importance and need for your topic/presentation.

Lower-extremity muscle power (force*velocity, or rate of performing work) may be a differential or independent determinant of physical function and falls compared to traditional lower-extremity muscle strength[1,2,3,4] and predicts mortality independently of strength and muscle mass.[5] Typically, population studies measure strength (maximum force). Age-related strength decline is partially explained by a decrease in muscle mass;[6,7] however declines in strength are three times greater than lean mass declines[7] implying that muscle function loss is more critical. Numerous muscle (e.g. fiber type) and neural (e.g. motor unit firing frequency, innervation) properties impact development of power, which likely contributing to a more marked age-related decline in power compared to strength,[8] particularly in the context of certain age-related diseases and conditions.[9] Peak power has not been investigated, or compared to strength, as a sensitive predictor of future geriatric outcomes; though is cross-sectionally related to falls and physical function.[1,2,3,4,10,11,12,13]

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

1. Understand definitions of muscle power and muscle strength.

2. Describe common measures of muscle power and muscle strength.

3. Appreciate how age-related decline in muscle power vs. strength may differentially affect falls and mobility.

• **Outline/Points of Interest**

1. Definitions of muscle power and muscle strength

a. Muscle power

b. Muscle strength

2. Common measures of muscle power and muscle strength

a. Muscle power
    i. Leg press
    ii. Task-based

b. Muscle strength
    i. Grip strength
    ii. Leg extension or press

3. Age-related decline in muscle power vs. strength may differentially affect falls and mobility

a. Falls and fall injuries

b. Physical function, including mobility and disability

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Meet the Professor: Non-Coding RNAs

Matthias Hackl, Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 106, Colorado Convention Center in Denver, CO, USA

Non-coding RNAs

Matthias Hackl, Ph.D., TAmiRNA GmbH, Austria,

Significance of the Topic:

Recent studies have revealed that about 90% of the eukaryotic genome is transcribed, but only 1–2% of these transcripts encode for proteins. In fact, the vast majority of transcribe regions encode for so-called non-coding RNAs (ncRNAs)[1]. NcRNAs play an important role in the epigenetic regulation of gene expression. This regulation mainly occurs on transcriptional and post-transcriptional level, resulting in the "titration" of protein levels in a cell in response to external or internal stimuli.
NcRNAs can be divided into two main types: infrastructural and regulatory ncRNAs. Infrastructural ncRNAs are responsible for key processes in cells, which are always turned on: for example protein translation and RNA splicing, which depend on protein/ncRNA complexes that consist of ribosomal (rRNA), transfer (tRNA) and small nuclear RNAs (snRNAs).

Regulatory ncRNAs are not constantly expressed but highly regulated via RNA polymerase II promoters (in contrast to RNA polymerase III promoters that control expression of most infrastructural ncRNAs). Regulatory ncRNAs can be divided based on their length in short and long non-coding RNAs.

MicroRNAs

MicroRNAs (miRNAs) are a relatively well characterized class of short regulatory ncRNAs. Approximately 2000 human microRNAs exist, of which many are highly (often perfectly) conserved in other mammalian species. Mature miRNAs are single-stranded oligonucleotides (20–24 nt) that derive from longer RNA transcripts (primary miRNA, pri-miR) that form distinctive secondary structures called "hairpins" or "stemloops". Primary miRNA transcripts are enzymatically processed by Drosha/Dgcr8 and then Dicer to give rise to either one or two mature miRNAs, which are loaded into the RNA induced silencing complex (RISC), which contains miRNA-interacting proteins such as Dicer, Ago2, or GW182. Within the RISC, mature miRNAs act as a guide that enables interaction of RISC with messengerRNA (mRNA) transcripts that harbor complementary RNA sequence patch in their 3'UTR region. Pairing of RISC to an mRNA results represses translation either because the mRNA becomes degraded or by inhibiting the initiation of translation (Figure 1). This mechanism is referred to as RNA interference (RNAi). RNAi works even if there is an incomplete match between a miRNA and its mRNA target, as long as the first 6-8 basepairs ("seed region") at the 5' end of the miRNA match the target. This relatively low specificity of miRNA/mRNA interaction allows miRNAs to hybridize with hundreds of different mRNAs, which harbor partially complementary sites. Interestingly, the mRNA targets of a single miRNA often fall into the same or similar biological process. Consequently, miRNAs represent an important layer in the regulation of gene expression and their regulatory power is

often compared to that of transcription factors[2].

Changes in miRNA transcription (and therefore changes in protein levels of their targets) can have severe impact on cell phenotypes, and in fact, cell differentiation or redifferentiation drastically affects cellular miRNA levels. However, not only differentiation is facilitated by miRNA regulation, also processes such as proliferation/growth arrest, and apoptosis are controlled by specific miRNAs. The progression of a cell or tissue from a physiologic to a pathophysiologic state (for example characterized by uncontrolled proliferation, cell damage, oxidative stress, etc.) may be caused and/or result in abnormal miRNA expression. **By determining miRNA expression we can gain a better understanding of disease mechanisms, and use this knowledge to develop novel pharmaceutical strategies to combat disease.** One of the biggest challenges in developing (micro)RNA-based therapeutics is the target identification and the concomitant design of a delivery vehicle that confers high stability to the therapeutic candidate and tissue-specific uptake[3], while avoiding potential toxicities and off-target effects[4].

In 2008, after 8 years of intense miRNA research, Lawrie et. al[5] described for the first time stable presence of extracellular ("circulating") miRNAs. By now, significant amounts of circulating miRNAs were verified in 12 biofluids such as plasma, serum, cerebrospinal fluid, saliva, and urine[6]. They are remarkably stable, due to the fact that they are either encapsulated in extracellular vesicles (EV) or they are associated with proteins, mainly Ago2 or apolipoproteins[7] (Figure 2). Based on i) the stability of miRNAs in the circulation, ii) the availability of validated technologies to quantify miRNAs, and iii) the validated biological function of miRNAs, **circulating miRNAs represent a very promising tool for diagnosis and prognosis of disease and predicting/monitoring the response to therapy.**

Learning Objectives:

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

- know the landscape of regulatory non-coding RNAs
- understand the biogenesis and biological function of microRNAs
- get insights regarding the biological function of non-coding RNAs during bone formation, resorption, and development of bone-related diseases such as osteoporosis
- understand the concept of therapeutic and diagnostic application of non-coding RNAs, especially microRNAs
- understand the utility of including circulating microRNAs as biomarkers in clinical trials for osteoporosis and other age associated diseases

Points of Interest:

MiRNAs as diagnostic tool:

From a diagnostic point of view, parallel analysis of multiple circulating miRNAs (so-called "signatures") is highly promising. This is because microRNA signatures allow to integrate information about pathophysiologic processes from different tissues. This is especially useful for the diagnosis of multifactorial diseases that involve multiple tissues, but to a varying degree depending on the individual patient.

Fracture-risk due to osteoporosis falls under the category "multifactorial disease". Novel diagnostic concepts are required to personalize disease management: for example, fracture- risk driven by bone fragility should be treated using medications that increase bone quality, i.e. anti-resorptive or anabolic drugs. Fracture-risk due to frequent falling, however, might be better mitigated using exercise and dietary intervention in addition to improving bone quality.

To this stage, cross-sectional and prospective studies involving more than 700 patients have been conducted to identify circulating microRNAs that can predict fracture-risk in postmenopausal women accurately[8]. There is strong evidence that fracture patients show characteristic miRNAs profiles in serum, which have potential as biomarkers for osteoporotic fractures and for the early detection of osteoporosis[9–12]. In total, 11 miRNAs were identified, which are significantly associated with the risk for osteoporotic fractures, referred to as "osteomiRs". Interestingly, it seems that some osteomiRs are also significantly regulated in patients with secondary forms of osteoporosis, who currently cannot be identified with routine diagnostic procedures. An important example is diabetic osteopathy, which cannot be detected by means of routine bone densitometry[13]. The biological and clinical relevance of this new biomarker candidates is currently investigated on the basis of model systems for osteoporosis in the laboratory, and clinical studies.

MiRNAs as potential therapeutic target:

MiRNA-based therapeutics can be divided into miRNA mimics and inhibitors of miRNAs (also known as anti-sense oligonucleotides (ASO) or "antimiRs"). MiRNA mimics are synthetic double-stranded small RNA molecules that match the corresponding miRNA sequence and therefore functionally aim to replenish the lost miRNA expression in diseases. By contrast, antimiRs are single stranded and based on first-generation antisense oligonucleotides (ASOs), which had been designed to target mRNAs, or modified with locked nucleic acids (LNAs)[14].

One example is miR-122, which reached phase II trials for treating hepatitis. Inhibition of miR-122 using LNAs resulted in a significant reduction in infection load and reduced liver damage in mouse models of HCV infection[15, 16]. The first report of using miR-122-targeted LNAs to treat HCV infection demonstrated reduced viral titres in mice[15] and in non-human primates[16]. Subsequently, LNAs against miR-122 achieved a significant reduction in viral titres in clinical trials of HCV-infected patients. Currently, there are two companies (Roche/Santaris

and Regulus Therapeutics) engaged in clinical trials using antimiR-122 LNAs as a therapy against HCV infections.

Figures:

Figure 1: MicroRNA biogenesis (Adapted from Barron, Hackl et al. 2012, Elsevier)

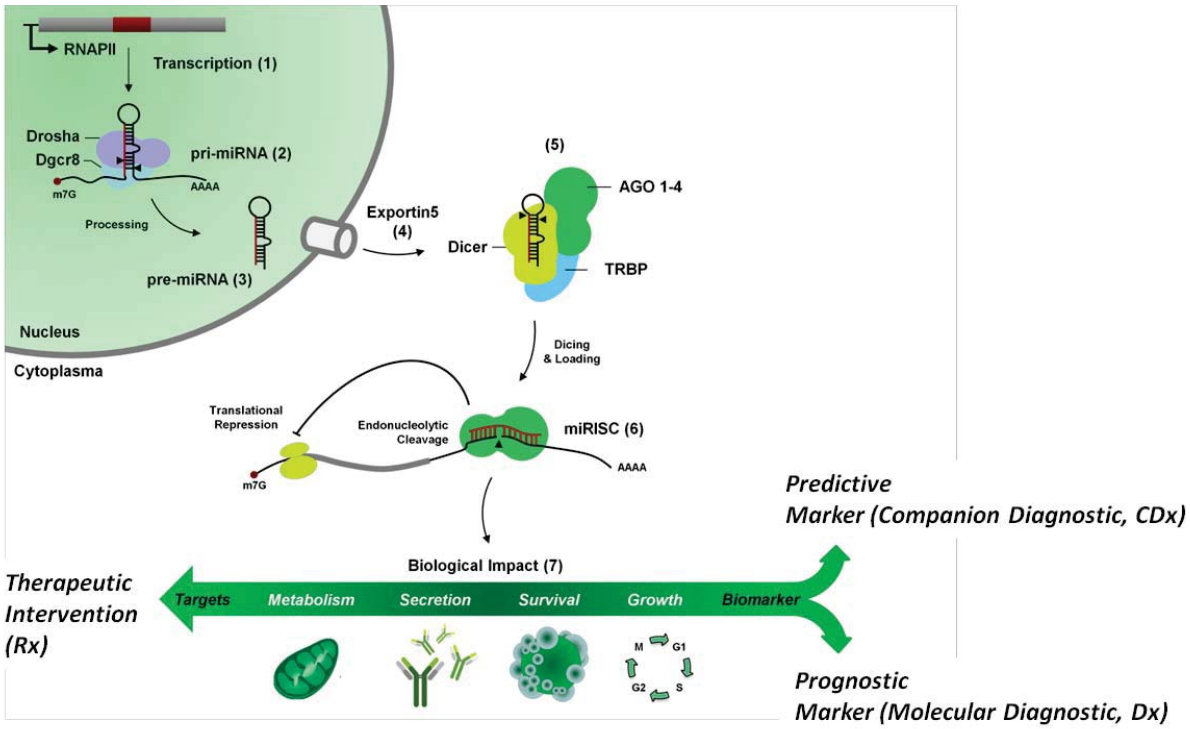
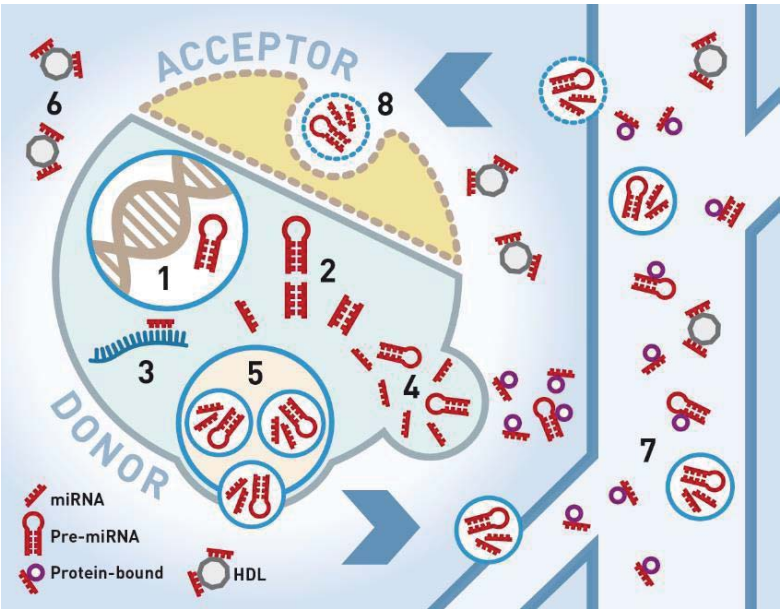


Figure 2: **The concept of circulating microRNAs as novel source of minimal-invasive biomarkers**. miRNAs are produced in the nucleus and processed in the cytoplasma where they regulate mRNA transcription. A significant fraction of microRNAs is packaged in small or large vesicles and exported into the supernatant. Uptake of this messages by other cells is achieved by receptor mediated endocytosis.


ACCEPTOR
DONOR
1
2
3
4
5
6
7
8
miRNA
Pre-miRNA
Protein-bound
HDL

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Meet the Professor: ROS Signaling in Bone Cells

Maria Jose Almeida, Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 107, Colorado Convention Center in Denver, CO, USA

Meet-the-Professor: ROS Signaling in Bone Cells

Maria Almeida, Ph.D., Center for Osteoporosis and Metabolic Bone Diseases, University Arkansas for Medical Sciences, Little Rock, Arkansas, USA.

Significance:

The mitochondrial electron transport chain leaks electrons and generates reactive oxygen species (ROS) during normal respiration. The gradual mitochondrial dysfunction that is observed to accompany aging has been proposed to cause aging. Genetic mouse models have elucidated that mitochondrial ROS is implicated in various diseases of aging, including metabolic syndrome and atherosclerosis, cardiac aging, skeletal muscle pathology, sensory defect, neurodegenerative diseases, and cancer (1). ROS inhibit osteoblastogenesis and promote osteoblast and osteocyte apopotosis. However, mitochondrial ROS are not always deleterious and can even stimulate pro-differentiation pathways. For example, both RANKL and M-CSF increase the levels of ROS in osteoclast progenitors and this event potentiates osteoclast formation. These findings indicate that there are fundamental differences in the way osteoblasts and osteoclasts handle ROS. The levels of ROS increase in bone with sex steroid deficiency and old age. Attenuation of ROS using pharmacological or genetic tools has elucidated that ROS contributes to the loss of bone mass under these conditions.

Learning Objectives:

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

- Describe the most critical sources of intracellular ROS and antioxidant mechanisms.
- Identify the actions of ROS in different bone cells.
- Identify the approaches that have been pursued thus far to elucidate actions of ROS on the skeleton.
- Describe the contribution of ROS to the bone loss that occurs with old age or sex steroid deficiency.

Points of Interest:

<u>Sources of ROS and antioxidants</u>

Generation of oxygen-derived free radicals is an inescapable consequence of aerobic metabolism and occurs primarily in mitochondria due to the escape of electrons passing through the electron transport chain (2, 3). This process generates superoxide, which is highly reactive and short-lived. Superoxide is rapidly converted to the more stable and less reactive H_2O_2, the most abundant form of ROS that diffuses freely through the mitochondrial membranes into the cytosol (4-6). In addition to mitochondria, ROS, including H_2O_2, are generated at the plasma membrane by the NADPH oxidases Nox1 and Nox2 (7). ROS can cause harm by damaging proteins, lipids, and DNA leading to cell demise and have, therefore, been

implicated in the biology of aging and aging-related diseases for over sixty years (8). ROS, however, also function as propagators of intracellular signaling for physiological cell function (9-11). These seemingly different actions may depend on the levels of ROS generated during signaling (lower) as opposed to stress (higher), and perhaps fundamentally different ways by which different cell types handle or compartmentalize ROS (2, 12). Several antioxidant enzymes, including peroxiredoxins, glutathione peroxidases, and catalase, prevent H_2O_2 accumulation and cell damage (5).

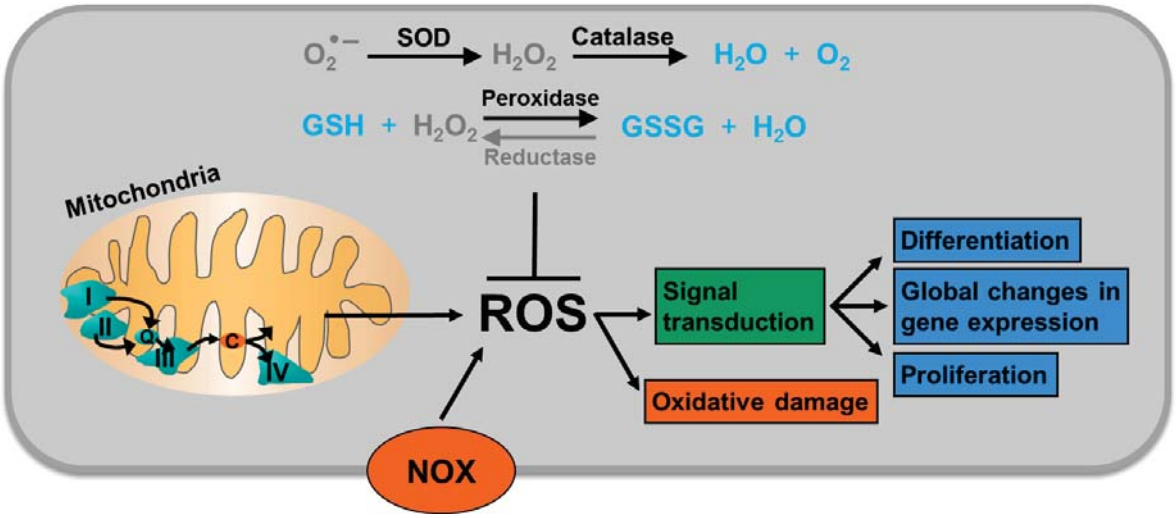

Figure 1. Cells utilize several mechanisms to prevent oxidative stress which involve both enzymatic reactions and altered gene transcription. Of the most important antioxidant enzymes, various forms of superoxide dismutase (SOD) catalyze the conversion of the superoxide anion to H2O2 and catalase converts H2O2 to water and oxygen. Alternative mechanisms of ROS detoxification involve reactions with thiol-containing oligopeptides, the most abundant of which are glutathione (GSH) and thioredoxin. These oligopeptides are continually regenerated by glutathione and thioredoxin reductases.

Actions of ROS in bone cells

ROS stimulate osteoclastogenesis (13) and are, most likely, indispensable for the transition of osteoclast progenitors to mature osteoclasts, as evidenced by the finding that anti-oxidants completely prevent osteoclast generation in vitro (14, 15). ROS produced by NADPH oxidases or the mitochondria promotes osteoclast differentiation triggered by RANKL signaling (14, 16, 17). Furthermore, mitochondrial ROS in myeloid progenitors potentiates osteoclast formation and bone resorption in mice under physiological conditions (16).

In contrast, ROS promotes osteoblast and osteocyte apoptosis in vitro (18, 19). Furthermore, administration of the antioxidants NAC or catalase abrogates osteoblast apoptosis in ovariectomized or aged mice (19, 20); and osteoblasts from Sod1-null mice exhibit decreased lifespan (21). ROS also decreases Wnt-induced osteoblastogenesis. Specifically, ROS decreases the association of beta-catenin with TCF/lef transcription factors via a FoxO dependent mechanism (22, 23).

An increase in ROS in the long–lived osteocytes, caused by targeted deletion of the antioxidant enzyme MnSOD, decreases bone formation and increases bone resorption leading to low bone mass in young mice

(24). The changes in bone formation and resorption are associated with increased expression of sclerostin and RANKL.

ROS in skeletal physiology and pathophysiology

The levels of ROS increase in bone with estrogen or androgen deficiency as well as with old age (15, 19). Evidence from pharmacological and genetic studies in mice has provided support for a deleterious effect of ROS in the skeleton under these conditions. Specifically, the loss of bone caused by gonadectomy in males or females is attenuated by administration of antioxidants (15, 19), or elimination of mitochondrial H2O2 specifically in cells of the myeloid lineage by targeting catalase to the mitochondria of myeloid cells (16). In contrast, elimination of mitochondrial H2O2 in myeloid cells does not alter the loss of bone mass with aging (25).

Importantly, attenuation of H_2O_2 produced in the mitochondria of mesenchymal progenitors, osteoblast and osteocytes attenuates the cortical thinning and the expansion of the medullary cavity caused by old age (25); this effect is associated with attenuation of the age-dependent decline in mineralizing surfaces. These observations suggest that part of the mechanisms of aging leading to the decline in bone mass is an increase in H_2O_2 levels in cells of the mesenchymal lineage.

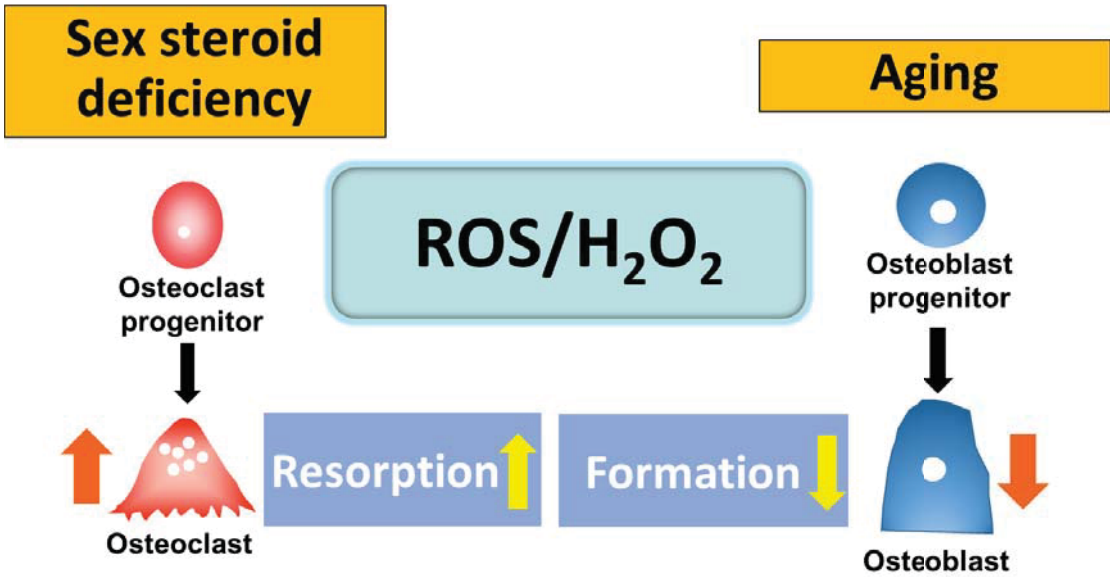

Figure 2. ROS in osteoclasts or in osteoblast lineage cells is required for the increased resorption that occurs with sex steroid deficiency or for the decline in bone formation that occurs with old age, respectively.

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Meet the Professor: Rigor and Reproducibility in Animal Experiments

Robert Jilka, Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 108, Colorado Convention Center in Denver, CO, USA

Rigor and Reproducibility in Animal Experiments

Robert L. Jilka, Ph.D.
University of Arkansas for Medical Sciences
USA

Objectives

The recognition that a significant proportion of published research cannot be independently confirmed has prompted scientific societies and funding agencies to identify factors that ensure the highest possible reproducibility of research findings. In addition, journals are increasingly demanding more methodologic details to promote transparency, and more rigorous statistical reporting. The purpose of this session is to discuss some of the most commonly encountered issues, particularly as they apply to murine models of musculoskeletal health and disease (1=3).

A. Rigorous experimental design (4)

- Use of inbred mouse strains like C57BL/6 eliminates a source of phenotypic variation, but they suffer from a lack of genetic diversity. The use of outbred strains moderate this problem, but they have only limited genetic diversity compared to humans. Recently, diversity outbred (5) mice bred from classical inbred and wild-caught strains have been developed that comprise populations of genetically unique individuals of high diversity.
- Animals must be assigned to treatment groups using a defined approach.
- Use enough animals to permit detection of an expected effect size, should there be one, i.e. do a power analysis before starting the experiment.
- Cre recombinase alone can affect phenotype, and most Cre models exhibit deletion of floxed genes in more than one cell type (6).
- Only "littermates" can control for effects of litter size, gang caging, diet and water, microbiome, different animal handlers.

B. Rigorous data reporting and appropriate use of statistics

- Use the appropriate statistical tool; see the Nature Methods series (7).
- The *P* value……
    - combines information on effect size and how precisely it is measured.
    - represents the "long-run frequency of getting the same result or one more extreme if the null hypothesis is true" (8).
    - IS NOT the probability that the test hypothesis is true; or the probability that chance alone produced the result; or the probability that result will be replicated in a second experiment.
- Mis-understanding and mis-use of dichotomous *P* value thresholds (e.g. 0.05) to establish "statistical significance" promotes non-reproducibility, as well as publication bias (9). Findings that meet the threshold tend to be published, often at the expense of the findings that do not meet the threshold. This can create the illusion that the published data are "true", when in reality the null hypothesis is true.

C. Transparent reporting

- Report all features of the animals, treatment and analytical procedures in sufficient detail to permit exact replication by an independent laboratory. A checklist called ARRIVE has been developed by NC3R to assist investigators (1,2).

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Meet the Professor: Therapeutic Approach of Osteogenesis Imperfecta in the Adult

Bente Langdahl, M.D., Ph.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 109, Colorado Convention Center in Denver, CO, USA

Therapeutic approach of Osteogenesis Imperfecta in the adult

Professor Bente L Langdahl, MD, PhD, DMSc
Aarhus University Hospital
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Aarhus, Denmark

Osteogenetic imperfecta (OI) is a rare disorder (1:10,000) caused by mutations in collagen type I or related genes. A hallmark of the disease is recurrent fractures and therefore prevention of fractures is a cornerstone in management of patients with OI.

Learning objectives:

1. Evaluate fracture risk in adult patients with OI
2. Treatment options and choice of treatment in adult patients with OI

Outline of the MTP session:

Osteogenesis imperfecta (OI) is the collective name given to a group of rare inherited disorders characterised by low bone mass, abnormal bone matrix and a greatly increased risk of fragility fractures. Four major clinical subtypes of OI have traditionally been recognised forming the basis of the Sillence Classification, although further subtypes have been recognised with a distinct molecular basis. The majority of patients with OI carry mutations in the genes encoding type I collagen, but more recently mutations in other genes encoding proteins primarily responsible for post-translational modification of collagen have been described which can also cause this disorder.

The consequences of these mutations vary considerably, but clinical characteristics are bone deformities, scoliosis, dentinogenesis imperfecta, impaired hearing, coloured sclerae and fractures.

The recurrent fractures cause pain, further bone deformities including deformities of the thoracic cage with impairment of the cardiopulmonary functions and disability. Prevention of fractures has therefore been the focus of the management of patients with OI since drugs for preventing osteoporotic fractures became available.

Bisphosphonates are widely used in the treatment of OI with the intention of reducing fracture risk. There is good evidence that bisphosphonates increase bone mineral density (BMD) in OI patients when compared with placebo but inconsistent results have been

reported with regard to the effect on fractures. These inconsistencies may be explained by the fact that the clinical trials of bisphosphonate therapy in OI have been small in size and not adequately powered to detect a reduction in fracture occurrence. We conducted a meta-analysis which demonstrated that the proportion of patients who experienced a fracture was not significantly reduced by bisphosphonate therapy (odds ratio = 0.83 [0.69- 1.01], p=0.06). The fracture rate was reduced by bisphosphonate treatment when all studies were considered (odds ratio 0.71 [0.52-0.96] p=0.02), but with considerable heterogeneity (I^2=36%) explained by one study where a small number of patients in the placebo group experienced a large number of fractures. When this study was excluded, the effects of bisphosphonates on fracture rate was not significant (odds ratio 0.79 (0.61- 1.02), p=0.07, I^2=0%). The effects of bisphosphonates on fracture prevention in osteogenesis imperfecta are therefore inconclusive. This meta-analysis has revealed several limitations in the evidence base for using bisphosphonates to reduce fracture risk in osteogenesis imperfecta. Few eligible placebo-controlled randomised trials were identified and the studies that were performed were primarily designed to detect effects on BMD rather than fractures. Furthermore, most studies have been performed in children.

The single study that was performed in adults with OI had only 64 patients and therefore insufficient power to investigate the effect on fractures. Therefore, trials investigating the effect of bisphosphonates on fracture risk in adults with OI are urgently needed.

It has been previously speculated that intravenous bisphosphonates may be preferable to oral bisphosphonates in the management of OI because of the very encouraging effects that have been observed with this treatment modality in observational studies. Although one randomised study with intravenous neridronate in children with OI has been reported which showed beneficial effects on fracture, this study was not placebo controlled. The neridronate study demonstrated no significant reduction in the proportion of patients with incident fractures during the controlled phase over the first year of treatment (relative risk 0.60 [0.21-1.59]). Although it is possible that intravenous bisphosphonates may be more effective than oral bisphosphonates in OI this remains to be proven in the context of a properly designed randomised controlled trial.

The effect of denosumab in patients with OI has only been investigated in small studies in children with OI.

The effect of the bone anabolic treatment, teriparatide in adults with OI has been investigated in a single trial. Compared with placebo, teriparatide increased lumbar spine and total hip BMD and increased biochemical markers of bone formation and resorption. However, it was noticed that the teriparatide-induced elevation of P1NP levels was less pronounced in severe forms of OI (type III/IV) compared with the milder form (type I). Type I OI patients exhibited robust BMD increases with teriparatide; however, there was no observed benefit for those with type III/IV OI. There was no difference in self-reported fractures between the teriparatide and placebo treated patients. The study was relatively small (n=79) and the study duration was only 18 months. From studies of teriparatide in patients with osteoporosis, it is known that the full effect of teriparatide on bone mass is not seen until the treatment has been followed by an antiresorptive for a couple of years. The evidence to support the use of antiresorptives and bone anabolic treatments for fracture prevention in OI is sparse and emphasises the importance of performing adequately powered randomised trials with a fracture endpoint in order to evaluate the risks and benefits of bisphosphonates in this condition. Until such evidence becomes available, patients with OI and their carers need to be made aware of this very limited evidence for fracture prevention. This is especially relevant in adults with OI, given the fact that patients are often on long term treatment and as a result may be at increased risk of serious adverse effects such as atypical subtrochanteric fractures and osteonecrosis of the jaw (ONJ). Although current evidence suggests that ONJ does not pose a risk in children with OI on long-term bisphosphonates, there is evidence to suggest that the risk of subtrochanteric fractures is increased in this subgroup of patients.

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Meet the Professor: High Throughput Screening of KO Mouse Lines with Automated µCT and Histomorphometry

David Rowe, M.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 110/112, Colorado Convention Center in Denver, CO, USA

High throughput screening of KO mouse lines with automated µCT and histomorphometry

Sunday, September 10 at 11:00 AM - 12:00 PM

David W. Rowe, University of Connecticut Health, USA

Significance of the Topic:

Genetics is a major contributor to susceptibility or resistance to osteoporotic bone disease. Human GWAS and mouse KO screening programs estimate between 500 and 2000+ genes can influence adult bone mass. This reality poses a major bioinformatics challenge to our field to identify, organize and classify bone phenotyping information in a big data format in a way that is meaningful to the skeletal biologist and can be integrated with other genetic, proteomic and drug interaction databases.

The presentation will describe our efforts to develop an experimental and bioinformatics platform that could be a functional beginning towards a big data solution for genes impacting skeletal variation. It is based on our experience of screening mice from the International Mouse Phenotyping Consortium (IMPC) in which individual genes have been inactivated within the same genetic background, and the resulting knock out lines (KOs) are characterized at multiple tissue levels. Our unselecting screen of KO mice is based on µCT of the distal femur and vertebra, which proved to identify significantly more variant KO lines than the DXA used by the IMPC process. The bones of the KO lines with a major variation in trabecular or cortical bone volume were further characterize by a histomorphometric analysis. How these methods were employed, what has been learned to date and how the skeletal biology community can participate in this effort will be discussed.

Learning Objectives:

As a result of participating in this session, attendees should be able to understand the strategy and methodology used for automated bone phenotyping, how well these methods perform and the manner that the results are presented on the webportal. The session will be designed to invite participants to register to the website and suggest improvements in the analysis and presentation on a continuing basis.

Experimental Design: Cost vs. Depth of Analysis; Consistency in microbiome and mouse manipulation.

•Homozygous KO – We expanded breeding pairs of viable KO lines from the Jackson Laboratory IMPC production facility (in the US, it is called the Knock Out Phenotyping Project, KOMP). We elected not to study heterozygotes of embryonic lethal or poor breeding lines because of the extra expense of breeding and genotyping.

•Controls – Each month a set of C57BL6/NJ mice were generated for analysis by the same workflow as the KO lines.

•Controlled breeding and harvesting - The off springs of each lines were grown to 12 weeks of age. They were injected with calcein 7 days and alizarine complexone 2 days prior to sacrifice. All the breeding, cage management and tissue harvesting was performed in the same room at the Jackson Laboratory. Tissue samples were shipped to UCONN for analysis.

Tissue analysis: Controlled work flow, observer-independent data accumulation.

•Sample collection and distribution: Each KO or control line was collected over the course of 1-4 months of breeding and growing depending on the fecundity of the line. The collection was considered complete after 8 males and 8 females were obtained. A laboratory information management system (LIMS) controlled the flow by generating bar codes for each sample and each hands-on step in the process.

•Screening by µCT – The distal femur and 2nd lumbar vertebra were scanned at 16µ resolution in using a carousel containing multi-sample holders. Custom software determined the ROI of each tissue from which the architectural features were determined. The calculated output was uploaded into the LIMS, the data was inspected by the team and the initial interpretation was released for distribution on the webportal.

•Bone histomorphology – KO lines with a significant variation by µCT were processed using a tape-transfer cryohistological protocol optimized for non-decalcified tissues. Either 4 distal femur or 4 lumbar vertebra (#4-6) were embedded together in a parallel orientation so that the tape captured 4 samples collected from 3 depths separated by ~50µ. A full analysis of a femur or vertebra is collected on 6 slides each containing 8 sections at 3 section depths from the male and female KO line. The sections are processed for 4 rounds of staining and automated imaging using the Zeiss Axioscan. (1) Accumulated mineral and mineralization lines. (2) Demineralization and TRAP staining using the Elf97 substrate. (3) Alkaline phosphatase staining using fast red substrate. (4) toluidine blue. All steps are aqueous to prevent tissue shrinkage.

•Image analysis of histological images – The 10 grayscale image files that are produced by the Axioscan are overlayed, vertically aligned, background corrected and binarized. The region of interest is determined and each fluorescent signal is projected to the surface of the trabecular bone. The extent of each fluorescent signal, which represents a specific biological activity, is expressed as the percentage of trabecular surface. The primary measurements are: BV/TV, TbTh, MS/BS (% labeling surfaces), MAR (distance between mineralizing lines), AP/BS (% AP positive surfaces) and TRAP/BS (% TRAP positive surfaces. The analysis allows the AP and TRAP to be subdivided into signals that are overlying a mineralizing bone surface (MBS) on non

mineralizing bone surface (nMBS). This is interpreted to distinguish active osteoblasts (AP/MBS) from bone lining cells (AP/nMLBS), and remodeling (TRAP/MBS) and resorbing (TRAP/nMBS) bone surface. It also identifies sites where AP, TRAP and MBS coincide, which is interpreted as a bone remodeling unit. The resulting calculated values are uploaded into the LIMS.

Data Presentation: Navigating the web portal, www.bonebase.org.

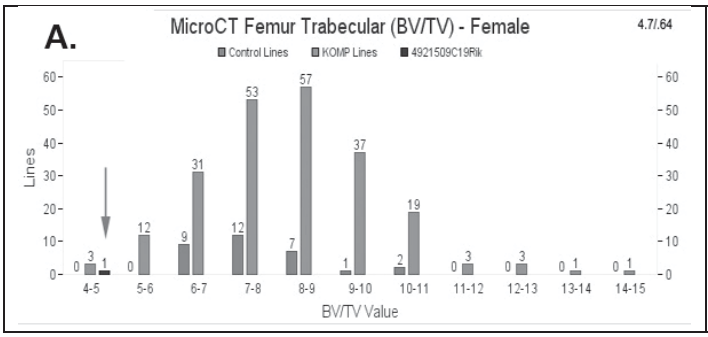
The web site has two components, static and dynamic. On the left panel are links to static presentations of the details of the µCT and histological methods, definitions used and how to navigate the dynamic database. Most important is the explanation on how calls are made that a KO has a meaningful variation in bone architecture or cellular composition. It is from this information that the dynamic database organizes the different phenotypes of the KO mouse lines.

•Use of control data – The monthly set of control mice (37 sets) provided to opportunity to observe the consistency of µCT and histological measures over time. For µCT, it clearly demonstrated the greater variance in male vs female animals as well as the month-to-month changes that cannot be explained by seasonal conditions. For histomorphometry, the controls were very useful for identifying errors in the histology and image analysis that once corrected significantly improve the variance of the measurements. A number of control steps were introduced to ensure that the enzymatic stains for AP and TRAP remained constant across control and KO lines.

•Making abnormal calls of KO lines – Based on the significance test using the IMPC-developed statistical package (Phenstat), the LIMS identifies measurements that are regarded as meaningful to the bone biologist. For example, a BV/TV of trabecular bone of distal femur of a test to control ratio >1.35 or <0.75 is considered to be significant. These criteria place the called KO line beyond the body of the distribution curve of the controls and the majority of the KO lines (fig 1A).

•Search page – Upon entering the hyperlinked KOMP Experimental Data, the screen presents the range of skeletal and body size variation of the 220 lines that were analyzed. The selection boxes that are scored as low, normal or high based on the µCT values for BV/TV of the distal femur or vertebra, the total volume of the trabecular space of the distal femur or vertebra, the total volume for the femoral cortex and the body weight. KOs that fit any of these measurements alone or in combination will be found. Clicking the male or female link will sort based on the test to control ratio value.

•Detail pages – Clicking the gene Id hyperlink will present a table that provides all of the call measurements for µCT, histomorphometry and body composition. Hyperlink titles over the µCT and histomorphometry lead to tables that provide the mean group data and individual mouse data. Tabs above the table provide:(1) a frequency distribution graph of the BV/TV measurement relative to the control and other KOMP lines; (2) horizontal slider graphs of the µCT and histomorphometry



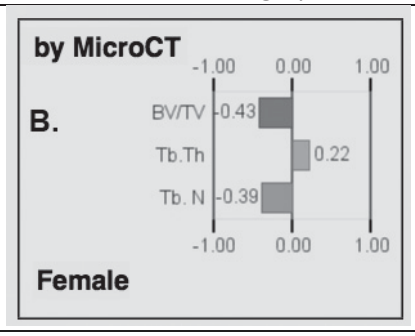


Figure 1:
A. Frequency distribution graph of BV/TV that are binned into 1% intervals. Arrow points to KO.
B. Slider graphs of the ratio of test vs control -1 of the primary µCT measurements of trabecular bone. Values to the right are >1 while bars to the less are <1.

test/control data in which a ratio of 1 is ploted as unchanged (0.00) One tab is for the femur and the other for the vertebra; (3) Pubmed. Articles related to the KO related gene that have been selected by us can be viewed; (4) links to supporting information. This page provides links to other sites including the IMPC findings for the KO line, OMIN, GWAS, MGI, gene expression (EMBL and Visigene) and text associations (iHOP); (5) Classification and interpretation (not currently active). This is a work in progress in which the webportal staff, external experts and registered users can provide their interpretation of the phenotype its genetic/molecular basis. Three levels of classification are being developed that bin the phenotypes based on the architectural, cellular and molecular findings, again reflecting the interpretation of these classification objectives.

Surveying the KO lines – Screening by µCT and body weight places KO in different groupings.

The search screen provides entry into specific lines for greater detail. The µCT data, presented as the ratio of test vs control, can be selected based on the 4 primary measures (BV/TV and total volume of the ROI of the femur and vertebra, the

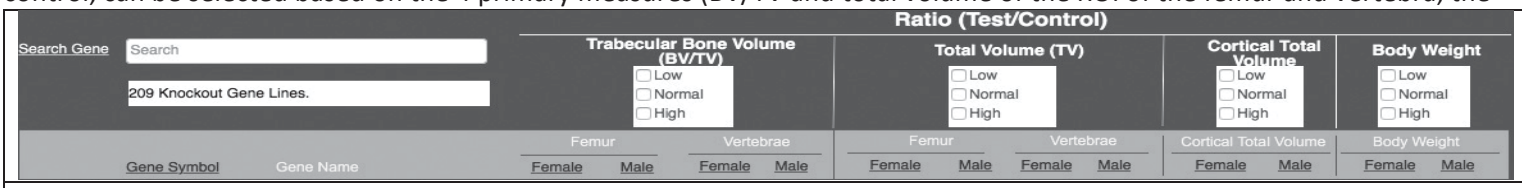


Figure 2: Screen shot of the seach page for selecting KO mice for further analysis. The check boxes under the 4 selection options will call up those KO lines that meet a single or "and" combinatorial selection.

cortical volume of femur and body weight) by clicking the range of values. By select one value, all KO lines that meet the single critera are shown, while selecting multiple values pull up only the KOs that meet all of the criteria. Figure 3 shows the top 10

A. 22 Knockout Gene Lines. Trabecular Bone Volume (BV/TV) ☑ Low

	Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male
1	Irf8 ←	interferon regulatory factor 8	.57	.32	.73	.67
2	Ceacam16	CEA-related cell adhesion molecule 16	1.03	.62	1	.81
3	Dnajb3	DnaJ (Hsp40) homolog, subfamily B, member 3	.81	.62	.9	.81
4	Hspb3	heat shock protein 3	.89	.62	.94	.96
5	Tmem136	transmembrane protein 136	.84	.62	.87	.8
6	Cp	ceruloplasmin	.86	.63	.81	.72
7	4921509C19Rik	RIKEN cDNA 4921509C19 gene	.64	.92	.74	.9
8	Htr1d	5-hydroxytryptamine (serotonin) receptor 1D	.66	.75	.79	.93
9	Moxd1	monooxygenase, DBH-like 1	.68	.8	.85	.87
10	Arrb1	arrestin, beta 1	.77	.69	.73	.78

B. 33 Knockout Gene Lines. Trabecular Bone Volume (BV/TV) ☑ High

Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male
Rin3	Ras and Rab interactor 3	1.95	1.43	1.04	1.05
R3hcc1l	R3hcc1l<tm1b(KOMP)Wtsi>/J	1.78	1.46	1.01	1.12
Fam186b	family with sequence similarity 186, member B	1.69	1.23	1.07	1.02
Osm ←	oncostatin M	1.66	1.2	1.19	1.38
Lipn	lipase, family member N	1.62	1.29	1.18	1.12
Elk1	ELK1, member of ETS oncogene family	.99	1.58	1.08	1.23
Akap11	A kinase (PRKA) anchor protein 11	1.26	1.51	.82	1.22
Ocstamp ←	Ocstamp, osteoclast stimulatory transmembrane protein	1.5	.86	1.18	.87
Spp1 ←	secreted phosphoprotein 1	1.5	1.32	1.02	1.13
Try4	trypsin 4	1.5	1.28	1	1.08

Figure 3: Selected screen shot of the 10 most affects hits for either low (A) or high (B) trabecular bone mass. Not shown are the associated values for total bone volume, cortical volume and body weight. The arrows identify KOs that have be previously published. The yellow and purple boxes highlight the value that was sorted by the search algorithms.

results of a low or high BV/TV only request. This search is places all the examined KOs in the same context of genetic background and experimental technique allowing the relative magnitude of one KO to the entire population of KOs to be appreciated.

When additional selection criteria are applied, groups of KOs with similar features of bone size (TV and cortical volume) and body size (weight) emerge. Figure 4 shows the list of mice with a low BV/TV but otherwise with a normal bone size and body weight. Most of these KOs were not detected by the IMPC screen that is based on whole body DXA. Note that the abnormal BV/TV can be in the femur or vertebra or both, and can be observed in females or males or both. Presumably, these mutations affect trabecular bone directly without having widespread metabolic or basic housekeeping effects.

LTBV, normal bone size, normal body weight

	Gene Symb	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Cortical Total Volume Female	Cortical Total Volume Male	Body Weight Female	Body Weight Male
1	Irf8	interferon regulatory factor 8	.57	.32	.73	.67	1.12	1.09	1.11	1.06	1.14	1.08	.94	.92
2	Ceacam16	CEA-related cell adhesion molecule 16	1.03	.62	1	.81	.98	.89	1.01	.91	1.01	.93	.92	.87
5	Tmem136	transmembrane protein 136	.84	.62	.87	.8	.93	.94	1.03	1.04	.96	.96	.98	1.03
6	Cp	ceruloplasmin	.86	.63	.81	.72	1	.95	1.17	1.12	.98	.91	.99	.96
10	Dnajc28	DnaJ (Hsp40) homolog, subfamily C, member 28	.81	.71	.96	.85	1.06	1.03	1.13	.95	1.03	.98	1.13	.97
11	Dnajb7	DnaJ (Hsp40) homolog, subfamily B, member 7	1.15	.73	.86	.81	1.09	1.03	1.12	.97	1.06	.97	1.06	.9
12	Dnajc5g	DnaJ (Hsp40) homolog, subfamily C, member 5 gamma	.73	.86	.91	.97	1.02	1.05	1.1	1.08	1.03	1.01	1.04	1.03
13	Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal	.74	.96	.86	.96	.97	.96	.96	1.06	.99	.97	.98	1.03
14	Gpnmb	glycoprotein (transmembrane) nmb	.74	.95	.96	.94	.9	.95	1.04	1.08	.92	.95	.95	1.04
15	Hfe2	hemochromatosis type 2 (juvenile) (human homolog)	.84	.74	1.03	.94	1.01	.95	1.09	1.03	1.06	1.01	1	1.03
17	Rab3c	RAB3C, member RAS oncogene family	.97	1.25	.77	1.08	1	1.04	.78	.97	.98	1.06	.9	1
18	Tprn	taperin	.93	.78	.93	.79	.98	.9	1.1	1.04	1	.9	.98	.92
19	Ahrr	aryl-hydrocarbon receptor repressor	.88	1.25	.79	1.08	1.07	1.07	1.02	1.06	1.04	1.08	1.05	1.08
20	Ffar2	free fatty acid receptor 2	1.15	1.18	.8	.89	.96	.98	.86	.79	1.01	1.01	.96	.95

Figure 4: Results of a search of all KO lines with a low BV/TV in either the femur or vertebra or both and not have an abnormal measure of bone size or body weight. To be included in the list, the BV/TV has to be less than 0.75 in the femur and 0.80 in the vertebra. Note that gene numbers 2,10,11,15 and 18 are male restricted, while 12, 13, 14 and 19 are female restricted. Also the gene numbers 12, 14,15 are femur restricted while 17, 19, 20 are vertebra restricted.

In contrast, other grouping can be generated in which low BV/TV is accompanied by a low bone size and or low body weight or both (Figure 5). Some members of this category probably represent mutations that have multiple metabolic or basic

LTBV, normal bone size, low body weight

	Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Cortical Total Volume Female	Cortical Total Volume Male	Body Weight Female	Body Weight Male
4	Hspb3	heat shock protein 3	.89	.62	.94	.96	.95	.89	1.07	1.18	.97	.91	.88	.82
9	Ghsr	growth hormone secretagogue receptor	1.11	.7	1.15	.9	.94	.87	1.16	1.04	.98	.89	1	.83
19	Nmrk2	nicotinamide riboside kinase 2	1.01	.74	1	.89	.89	.89	.86	.88	.94	.91	.83	.82

LTBV, Low bone size, normal body weight

	Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Cortical Total Volume Female	Cortical Total Volume Male	Body Weight Female	Body Weight Male
21	Eif1b	eukaryotic translation initiation factor 1B	1.27	.95	.89	.75	.98	.96	.73	.78	.96	.99	.93	.89

LTBV, Low bone size, low body weight

	Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Cortical Total Volume Female	Cortical Total Volume Male	Body Weight Female	Body Weight Male
7	4921509C19Rik	RIKEN cDNA 4921509C19 gene	.64	.92	.74	.9	.85	.9	.67	.86	.92	.96	.83	.89
8	Htr1d	5-hydroxytryptamine (serotonin) receptor 1D	.66	.75	.79	.93	.85	.84	.75	.79	.88	.9	.85	.8
10	Arrb1	arrestin, beta 1	.77	.69	.73	.78	.84	.8	.75	.78	.87	.85	.78	.79
20	Rab36	RAB36, member RAS oncogene family	.74	.79	.77	.91	.92	.86	.8	.77	.94	.91	.92	.85

LTBV, high body weight

	Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Cortical Total Volume Female	Cortical Total Volume Male	Body Weight Female	Body Weight Male
3	Dnajb3	DnaJ (Hsp40) homolog, subfamily B, member 3	.81	.62	.9	.81	1.06	.98	1.08	1.08	1.06	.97	1.2	1.02

Figure 5: Classification of low trabecular bone volume in association with measures of bone size and body weight. A similar classification can be constructed based on high trabecular bone volume (HTBV).

housekeeping functions. Another possibility is that some represent genes that could be classified as frailty associated genes. The goal of this architectural classification is to provide some context that will assist the viewer to focus on genes that fit their research interest.

Examining a KO line in detail

Interferon regulatory factor-8 (IRF-8), a transcription factor expressed in immune cells, is a key regulatory molecule for osteoclastogenesis. IRF-8 expression in osteoclast precursors is down-regulated during the initial phase of osteoclast differentiation. Previously published KO mice have a very low trabecular bone mass due to activated osteoclast lineage.

Allele - Irf8$^{tm1b(KOMP)Wtsi}$

Gene Symbol	Gene Name	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Femur Female	Femur Male	Vertebrae Female	Vertebrae Male	Female	Male
Irf8	interferon regulatory factor 8	.57	.32	.73	.67	1.12	1.09	1.11	1.06	.94	.92

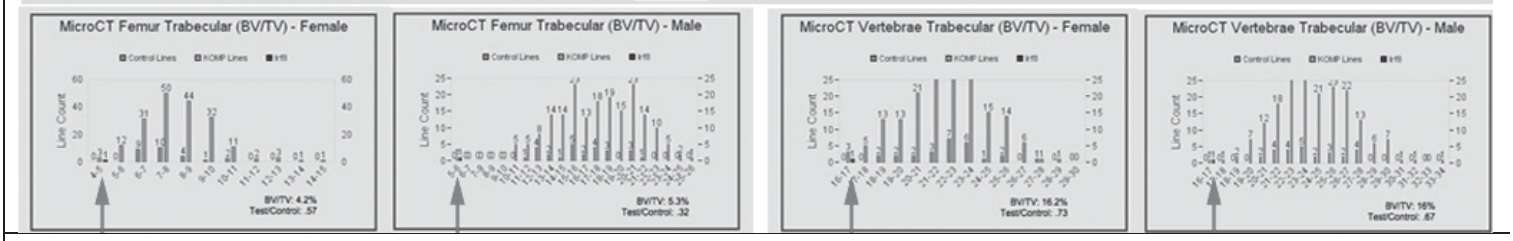


Figure 6: Composite image of Irf8 values show from the search page and the frequency distribution graphs that place the values of Irf8 relative to the monthly controls and the studied KO lines. The arrow is added to emphasize the Irf8 values.

It is the most severe LTBV KO that we have encountered. To examine the details of our analysis, click the gene abbreviation on the search page and the frequency distribution graphs of the BV/TV in the femur and vertebra in both sexes are shown (figure 6). The arrow points to the Irf8 values to show its position relative to the running controls and the other KO lines.

summary tab brings the summary table in which specific measurements in µCT, histomorphometry and body composition are call as being either low, normal or high (figure 7). The header for each table section is a hyperlink to the primary group and individual mouse data. In the case of histomorphometry, a third level of detail is provided in which the histological images and calculated measurement for the three sections from each bone sample can be viewed.

BV/TV Distribution | Summary | Femur | Vertebra | Links

A. Micro CT

Summary data	Femur Trabecular F	Femur Trabecular M	Vertebra Trabecular F	Vertebra Trabecular M	Femur Cortical	F	M		F	M
Bone Volume (BV)	Low	Low	Low	Low	Total Area	High	Normal	Marrow Area	High	High
Total Volume (TV)	Normal	Normal	Normal	Normal	Bone Area	Normal	Low	Femur Length	Normal	Normal

B. Histo

	Femur Trabecular F	Femur Trabecular M	Vertebra Trabecular F	Vertebra Trabecular M
Matrix Formation (F)	High	Normal	High	High
Osteoblast Surfaces (T)	High	High	Normal	High
Bone Forming Surfaces	Normal	Normal	Normal	Normal
Bone Lining Surfaces	High	High	High	High
Osteoclast Surfaces(R)	High	High	High	High
Remodeling Surfaces	High	High	Normal	Normal
Resorbing Surfaces	Normal	Normal	Normal	Normal
Remodeling Unit	High	High	High	High

C.

IMPC Info	F	M
Body Weight	Normal	Normal
Body LBM	Normal	Normal
Body Fat	Normal	Normal
Body Activity	Normal	Normal
Weight at Sac. Date	Normal	Normal

Figure 7: Summary table. A. Data from µCT analysis including BV/TV and TV of femur and vertebra, and the cortical bone cross sectional measures and femur length. B. Data from histo-morphometry for both bones that includes the 8 categorical measure of formation, resorption and remodeling. C. body composition obtained by DXA during the IMPC phenotyping. The weight at sacrifice tab is a measurement made by our group. The data supporting these outcome calls are hyperlinked.

The measurements that support these calls are graphically presented in separate tabs for the femur and vertebra (figure 8, femur slider graph). The A grouping is µCT and histological measured of trabecular bone while the B grouping is the bone size including TV of femur and cortical volume of femur and the body size as assessed by weight and femur length. The

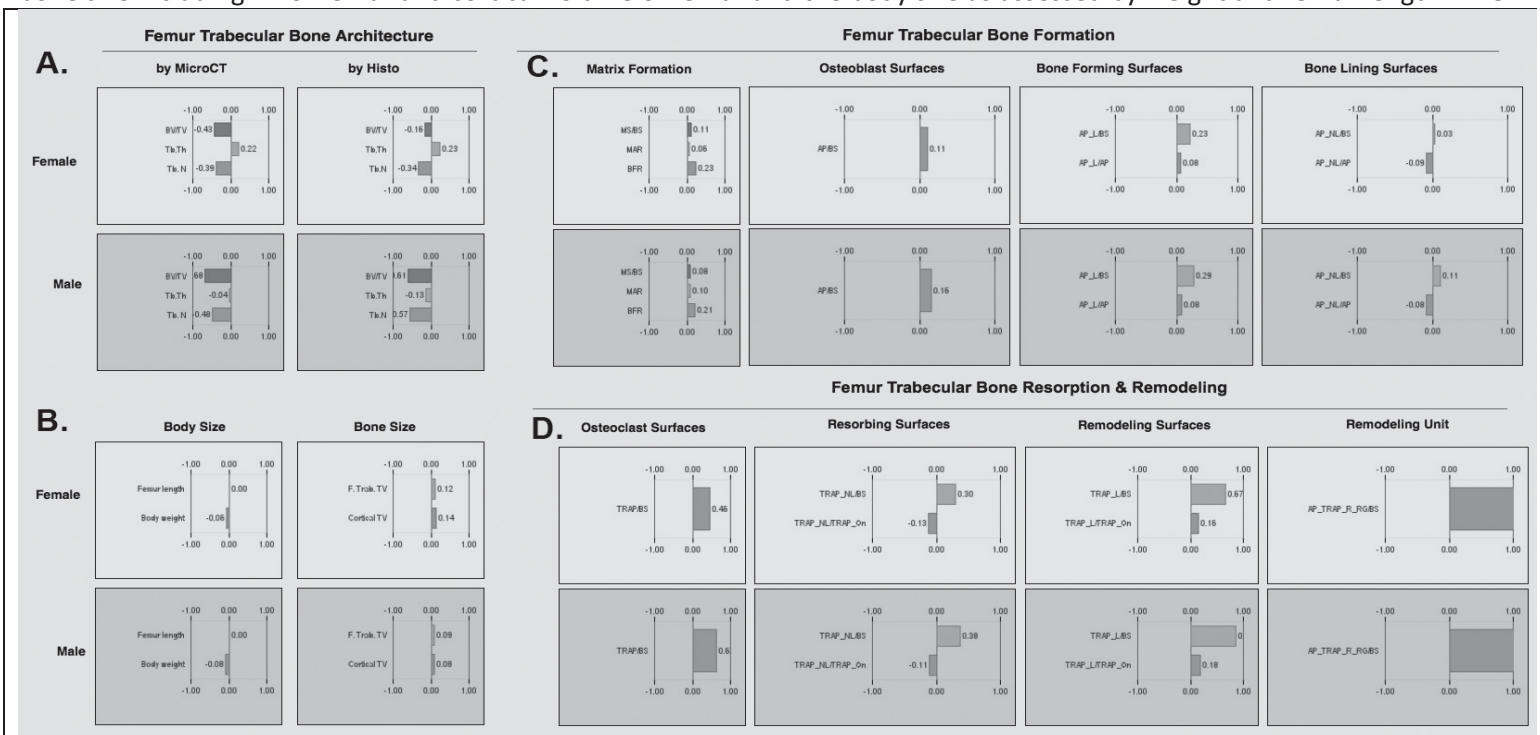


Figure 8. Slider graphs of all the µCT and histomorphmetric measurement of the femur (male and female). The horizontal bars reflect the fractional increase (right side) or decrease (left side) of the 0.00 line of each measurement. See text for the interpretation of the data.

graphs show that both µCT and histology agree that the low BV/TV is primarily a loss in trabecular number. Bone and body size are in the normal range. Panel C presents the bone forming measures from the histomorphology. A relatively modest increase in bone formation rate (BFR) primarily due to an increase in mineralizing surfaces was obtained from the dynamic labeling

study. Total osteoblast surfaces was also moderately increased and it was distributed primarily to bone forming vs bone lining surfaces. The most dramatic measures come from the TRAP study (panel D). Total osteoclast surface was increased and a greater proportion of the activity was located on bone forming (remodeling) rather than inactive surfaces. However the TRAP was primarily located at sites where the AP and mineralization coincided which we regard as a remodeling unit. This is one of the highest values we have observed to date. The same finding was observed in the vertebra. The conclusion from the histomorphometry study is a state of high bone turnover that is localized at the confluence of matrix-forming osteoblasts.

The link tab presents the results of searches of various websites for information regarding this KO line. A comment on the finding and a direct link to the web page or pub med document is provided.

Other dramatic and informative KOs to examine include:

Ras and Rab interactor 3 (Rin) – This is the highest BV/TV phenotype and the histology shows a low bone formation/low osteoclast number/low resorption process. The link page points to articles indicating that it is a GWAS hit for Paget's disease.

Ocstamp – The KO produces a previously described defect in osteoclast fusion. However these publications did not identify an abnormality in trabecular bone. Our µCT screen did identify the expected high BV/TV phenotype in both the femur and vertebra, but it was restricted to females. The previous study examined males! The histology shows a high osteoclast number, but no increase in bone formation or remodeling suggesting the osteoclasts are defective in bone resorption.

Future Plans for the Web Portal

Going forward, some of the new features that will be added to our phenotyping workflow will include:

- Breed the IMPC mice in house – By obtaining breeders from an external source and establishing the breeding/growing lines at the same location that the samples are processed, a consistency in the microbiome, housing and harvesting is maintained. This allows obtaining the IMPC mice from different production facilities using one set of control mice rather than a control for each peripheral breeding site. This will increase the number and type of KO lines that we can evaluate.
- Rapid identification of a KO with an exceptional architectural phenotype - We want to extend the breeding of KO lines with a major bone/body variance for production of additional animals, embryo cryopreservation and potentially for transfer to an external investigator.
- Deeper investigation of KO lines with evidence suggestive of a frailty or fitness phenotype - The IMPC uses DXA to measure lean and fat body mass composition. Tissue will be taken from KO lines with an abnormal DXA value for either value to assess skeletal muscle for fiber size and number and bone for µCT assessment of bone marrow fat.
- Classification – The concept of a classification system for the architectural and body composition measurements was introduced in figures 4 and 5. The KO lines were identified manually but in the future can be produced as a defined group. The criteria for the groupings will need to be defined, and subsequently modified as more KO are encountered. A similar classification is envisioned for the histomorphological findings. This will allow identification of KO lines with a defined cellular basis for the architectural phenotype, a step that will require developing criteria for defined groupings. Finally, the molecular pathways that interconnect with the KO line need to be developed to understand why the KO exerts its architectural and cellular phenotype. Classification based on pathway needs to be developed to identify different KO lines that map to similar pathways.
- External Input – Our eventual goal is to make this site evolve into a community resource for genes that impact the mineralized skeleton. We need to elicit input from experts in different cell and molecular aspects of skeletal biology to help develop meaningful classification systems and to provide their interpretation of the assembled information. We have invited a limited number of experts to begin this process, but will extend the invitation to others who wish to participate. A registered user list will be developed who will be segmented on specific area of expertise/interest, e.g. osteoclast or osteoblast biology, coupling, frailty, fitness, a specific molecular pathway. When a new KO line is identified that may fit one of these interest areas, the interested individuals will be invited to a video conference to inspect the data and to contribute to the interpretation. Registered users can request a KO line that is currently active and shows preliminary µCT data of an abnormal architectural/somatic phenotype. If they receive the mice, they will be requested to share the results of their study on the website.
- Use of the workflow for non-IMPC mice – Because the workflow is highly automated, we can perform a comprehensive µCT and histomorphometric analysis at a significant cost and time advantage from traditional methods. However unlike the IMPC mice, each investigator-derived line will require its own set of controls. A mechanism for producing and shipping the bone samples that integrate with our workflow has been developed, or an investigator can send the breeders to our site to have the entire process performed in house. Histomorphometry cores that wish to establish the methods in their institution and use the image analysis platform are welcomed to visit and have a hands-on learning experience.

Meet the Professor: Screening Strategies for Young Postmenopausal Women (50-64)

Carolyn Crandall, M.D.

Sunday, September 10, 11:00 AM - 12:00 PM

Room 111/113, Colorado Convention Center in Denver, CO, USA

Screening Strategies for Young Postmenopausal women (50-64)

Carolyn J. Crandall, MD, MS
David Geffen School of Medicine at the University of California, Los Angeles
USA

Significance of the Topic

1 in 2 postmenopausal women and 1 in 5 older men will have an osteoporosis-related fracture in their lifetimes.[1] The United States Preventive Services Task Force highlights the evidence gap regarding the accuracy of risk assessment tools for predicting fractures in younger postmenopausal women as a key research gap.[2]

The goal of osteoporosis screening is to identify women with bone mineral density (BMD) T-score ≤-2.5 because they are candidates for osteoporosis pharmacotherapy to prevent fractures. Clinical osteoporosis guideline recommendations vary regarding the best approach to screening for osteoporosis in young postmenopausal women. Newly-emerging evidence will help to inform decisions about which risk assessment strategies may be reasonable in this age group. Key challenges regarding screening in this age group are related to 1) the lower absolute fracture risk at a given BMD value in this age group compared with women ≥65 years-old, and 2) the paucity of evidence regarding anti-fracture efficacy of pharmacotherapy in this age group.

Learning Objectives

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

1. Describe guideline recommendations regarding screening for osteoporosis in young postmenopausal women
2. Describe evidence from recent prospective studies regarding identifying young postmenopausal women who have bone density T-score ≤-2.5.
3. Describe critical evidence gaps regarding osteoporosis screening in young postmenopausal women

<u>Case 1.</u> ID is a 53-year-old women had her final menstrual period 15 months ago. She wonders if she needs BMD testing. How should we make this decision?

<u>Case 2.</u> A 57-year-old women, no prior fractures, healthy except for controlled hypertension. On baseline BMD report from outside MD, T-scores at hip and spine are >-1. When to repeat?

Points of Interest/Clinical Pearls

Objective 1: Guidelines regarding selecting postmenopausal women <65 y/o for BMD testing

Age group	When to order BMD testing	Organization
<65	10-year risk of osteoporotic fracture ≥ that of a 65-year-old white woman who has no additional risk factors (≥ 9.3%)	USPSTF (Grade B) [1]
50-64	fracture during adulthood (after age 50), or condition (e.g. rheumatoid arthritis) or medication associated with low bone mass or bone loss	NOF [3]
<65	Based on clinical risk factor profile: Fracture without major trauma, radiographic osteopenia, initiating or taking long-term systemic glucocorticoid therapy ≥3 months, low body weight (<127 lb or body mass index <20 kg/m^2), family history of osteoporotic fracture, early menopause (<40 years-old), current smoking, excessive alcohol consumption, secondary osteoporosis	AACE (Grade B)[4]
≥50	Based on FRAX. In individuals at intermediate risk, bone mineral density (BMD) measurement should be performed using dual-energy X-ray absorptiometry and fracture probability re-estimated using FRAX.	UK NOGG [5]

Objective 2. Recent evidence on FRAX for screening decisions in women aged 50-64 years

Two large studies used data from the Women's Health Initiative to compared FRAX with other risk assessment tools in women aged 50-64 years-old:

- Crandall CJ, et al Comparison of fracture risk prediction by the US Preventive Services Task Force strategy and two alternative strategies in women 50-64 years old in the Women's Health Initiative. J Clin Endocrinol Metab. 2014 Dec;99(12):4514-22.
- Crandall CJ, et al. Osteoporosis screening in postmenopausal women 50 to 64 years old: comparison of US Preventive Services Task Force strategy and two traditional strategies in the Women's Health Initiative. J Bone Miner Res. 2014 Jul;29(7):1661-6.

Prior to advent of FRAX:

- Osteoporosis Self-Assessment Tool (OST):
  - OST = (wt kg – age years)/5 Truncate to integer (Cadarette 2004, Geusens 2002, Gourlay 2005, Lydick 1998)
- Simple Calculated Osteoporosis Risk Estimation Tool (SCORE):
  - non-black race (5 points) + rheumatoid arthritis (4 points) + non-traumatic fracture after age 45 years (4 points for each type of fracture—hip, wrist, rib-with maximum score of 12) + age (3 * first digit of age in years) + prior estrogen therapy (1 point for never) + weight ([-1 * weight/ 10], truncated to integer) (Lydick 1998)

Identifying major osteoporotic fracture during 10 years of follow-up, by age group

	Osteoporotic Fracture		
	Sensitivity (95% CI)	**Specificity (95% CI)**	**AUC (95% CI)**
Aged 50-54 years (n=14679)			
USPSTF (FRAX ≥9.3%)	4.7 (3.3-6.0)	97.0 (96.8-97.3)	0.54 (0.52-0.55)
SCORE (>7)	18.5 (16.0-21.0)	78.8 (78.1-79.5)	0.54 (0.52-0.56)
OST (<2)	22.9 (20.1-25.6)	74.2 (73.5-74.9)	0.54 (0.52-0.56)
Aged 55-59 years (n=22363)			
USPSTF (FRAX ≥9.3%)	20.5 (18.6-22.3)	86.3 (85.8-86.7)	0.55 (0.53-0.56)
SCORE (>7)	22.1 (20.2-24.0)	81.1 (80.5-81.6)	0.53 (0.51-0.54)
OST (<2)	36.7 (34.5-39.0)	63.9 (63.3-64.6)	0.52 (0.51-0.53)
Aged 60-64 years (n=25450)			
USPSTF (FRAX ≥9.3%)	37.3 (35.4-39.1)	72.3 (71.7-72.9)	0.56 (0.55-0.57)
SCORE (>7)	57.6 (55.7-59.5)	44.4 (43.7-45.0)	0.53 (0.52-0.54)
OST (<2)	48.1 (46.2-50.1)	49.6 (48.9-50.2)	0.54 (0.52-0.55)

Crandall CJ, et al Comparison of fracture risk prediction by the US Preventive Services Task Force strategy and two alternative strategies in women 50-64 years old in the Women's Health Initiative. J Clin Endocrinol Metab. 2014 Dec;99(12):4514-22 by permission of Oxford University Press.

Summary:

- Among women aged 50-64, none of the 3 strategies performed better than chance alone in discriminating between women who did and did not have a subsequent fracture.
- These findings suggest that fracture risk prediction in young postmenopausal women requires assessment of risk factors not included in currently available strategies.
- The USPSTF and the UK/NOGG strategies which use FRAX-predicted fracture risk to guide which young postmenopausal should receive BMD testing is not optimal.
    - AACE and NOF approach (using clinical risk factors) may be superior
    - But they are harder to examine in large cohort studies- long list of risk factors

Identifying BMD T-score ≤-2.5 at Femoral Neck

Nonusers of menopausal HT (n=2163)	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**AUC (95% CI)**
USPSTF (FRAX ≥ 9.3)	**33.3 (26.3–40.4)**	86.4 (85.1–87.7)	13.7 (10.4–17.0)	**0.60 (0.56–0.63)**
SCORE (>7)	74.1 (67.6–80.7)	70.8 (69.1–72.5)	14.1 (11.9–16.4)	0.72 (0.69–0.76)
OST (<2)	79.3 (73.2–85.4)	70.1 (68.4–71.8)	14.7 (12.4–16.9)	0.75 (0.72–0.78)

Crandall CJ, et al. Osteoporosis screening in postmenopausal women 50 to 64 years old: comparison of US Preventive Services Task Force strategy and two traditional strategies in the Women's Health Initiative. J Bone Miner Res. 2014 Jul;29(7):1661-6

Also: In the youngest women 50-54 years-old, USPSTF FRAX-based strategy was no better than chance alone in youngest women aged 50-54 (AUC 0.50).

Summary:

- USPSTF FRAX-based strategy was inferior to simpler OST strategy to identify T-score ≤-2.5, which is the goal of screening. (T-score ≤-2.5 indicates treatment candidates).

Other studies confirm higher sensitivity and higher AUC for OST than for FRAX in identification of BMD T-score ≤-2.5:

- Canada: Manitoba Bone Density Program (Leslie et al J Clin Densitom 2013)
- US: Smaller studies (Pecina et al J Am Board Fam Med 2016, Jiang et al Maturitas 2016)

<u>Objective 3. Evidence gaps in selecting postmenopausal women 50-64 y/o for BMD testing</u>

Key knowledge gap	**Consequences of knowledge gap**

No clinical trials specifically evaluated safety and efficacy of pharmacotherapy for *screen-detected* T-scores ≤-2.5 in this age group.	• No clinical guideline is specific to this age group.
	• We extrapolate treatment recommendations from clinical trials that enrolled older women and/or women with pre-existing fractures (not routine screening setting)
	• Treatment may cause more harm than benefit by initiating when women are 50-64 years-old and continuing long-term
	  o Guidelines recommend continuing therapy if BMD T-scores remain ≤-2.5 after initial treatment period
	  o Risk for severe AEs increases with prolonged use of bisphosphonates. (ACP)
	  o Atypical subtrochanteric and diaphyseal femoral fractures: absolute risk with BPs is 3.2 to 50 cases per 100,000 person-years but long-term use associated with higher risk (~100 per 100,000 person-years for ~ 8-9 years of use)(Shane et al *J Bone Miner Res.* 2014)

No clinical trial was conducted to primarily assess the effects of fracture prevention in women with osteopenia. (ACP) Osteopenia common in this age group. (Wright et al J Bone Miner Res 2014 29 (11): 2520-6)	• We treat osteopenia in women <64 years-old the same as we would in women ≥65 years-old, which may not be appropriate. • Women younger than 65 years with osteopenia will benefit less from treatment than women 65 years of age or older with osteopenia. (ACP) • In women with low BMD (T-score >-2.5) but without vertebral fractures, 4 years of alendronate did not reduce the risk of clinical fractures (non-traumatic non-face non-skull). (Cummings et al JAMA 1998)

How often to screen untreated younger postmenopausal women 50-64 y/o?

Women 50-64 y/o without osteoporosis on 1st BMD test are unlikely to benefit from frequent rescreening before age 65.

- Women 50-54 years-old in Women's Health Initiative study who had no osteoporosis at baseline (N = 4068): 12 years were required for 1% of women to experience hip or clinical vertebral fracture. (Gourlay et al Menopause 2015)

Australian clinical guideline incorporated this recent evidence: If BMD is stable and/or individual is at low risk of fracture (T-score > –1.5), less-frequent monitoring, up to an interval of 5–15 years, can be considered. (2017 Royal Australian College of General Practitioners & Osteoporosis Australia)

Discussion of cases

Summary:

- Screen if disease/condition associated with bone loss.
- Reasonable to use OST score <2 to select screening candidates
    - Also simpler than FRAX to use
- FRAX does not work well in this age group for identifying pharmacotherapy candidates, i.e. T-score ≤-2.5, which is goal of screening.
- Despite rapid bone loss during menopausal transition, *absolute* fracture risk is much lower for a given BMD if younger than older (Kanis et al Osteoporos Int 2001), no evidence re long-term safety of long-term treatment started at ages 50-64 years-old.

References

1. Screening for osteoporosis: U.S. preventive services task force recommendation statement. *Ann Intern Med.* 2011;154(5):356-364.
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3. National Osteoporosis Foundation. *Clinician's Guide to Prevention and Treatment of Osteoporosis.* Washington, DC: National Osteoporosis Foundation;2014.
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5. Compston J, Cooper A, Cooper C, et al. UK clinical guideline for the prevention and treatment of osteoporosis. *Arch Osteoporos.* 2017;12(1):43.