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Meet-the-Professor
Handout Booklet

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This booklet contains handouts supplied by the professors by the printing date of 9/13/10 and are intended to be a supplement to the material being presented in the session. Please be sure to complete an evaluation form of the Meet-the-Professor sessions and provide feedback and suggestions for the Meet-the-Professor Handout Booklets for the future.

Biglycan Signaling Regulates Chondrogenesis In Disease

Marian Young, Ph.D.

Proteoglycans and the Skeleton: Diversity in Function

Biglycan Regulates Chondrogenesis in Disease

Marian Young, PhD, Chief, Molecular Biology of Bones and Teeth Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, USA

Significance: The extra-cellular matrix (ECM) of skeletal tissues is massive and complex and yet, it remains largely understudied. For many years the role of the ECM including proteoglycans was considered to be structural in nature and that the ECM served as an inert scaffold. Some ECM components have unique biochemical properties in bone and for that reason have also served as "Markers" of bone activities including bone formation and turnover. The objective of this review is to explain the complex nature of the ECM and show that it has important functional roles including the regulation of differentiation, proliferation, apoptosis. The components of the ECM can work by direct interaction with cells or by regulating growth factor activation and modulation.

Objectives: As a result of participating in this session attendees should understand the complexity of the ECM of bone and understand the nature of the proteoglycans that are found there. The attendee will learn how proteoglycans can affect the skeleton with particular emphasis on the role of biglycan in regulating chondrogenesis in the temporomandibular joint.

Outline:

1) Major components of the ECM in bone: Collagen and non-collagenous

2) Proteoglycans large and small: diversity in structure

3) Proteoglycans: Functions in numerous tissues besides the skeleton

4) Proteoglycans: How they are made and removed

5) Proteoglycans: The SLRP family

6) SLRP knockouts: Compensation

7) ECM and the Interplay with Growth factors

8) ECM and stem cells

9) General Concepts/messages to take home

10) References

1) Composition of the extracellular matrix (ECM) in bone

-Collagen-over 20 types (type I (Col1A1), II, X etc)

-Non-Collagenous Proteins (NCP)-acidic (BSP, osteopontin, COMP)

-Proteoglycans-large and small (aggrecan, SLRP's)

2) Proteoglycans

-Heavily glycosylated

-Polysaccharides attached are called glycsosaminoglycans or "GAGs"

-This makes them negatively charged

The proteoglycans are extremely diverse in size and composition.

-Aggrecan, perlecan, versican cores >200 kD, a million kD with their GAGs

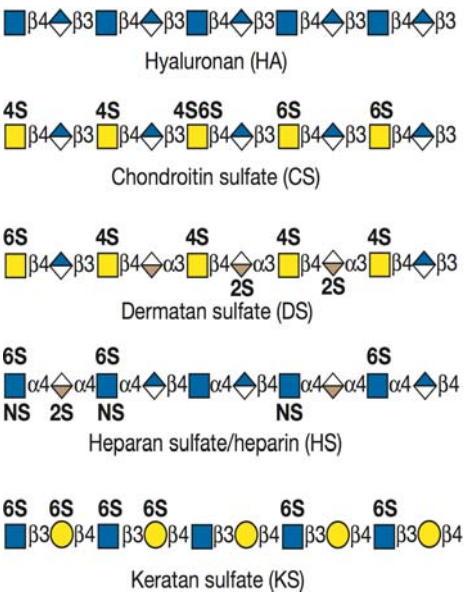
-Glipicans (1-6) cores 60-70 kD, contain HS chains

-Syndecans (1-4), cores 20-45 kD, contain 3-5 HS chains

- SLRP/ Small leucine-rich proteoglycans (1-17) cores ~45 Kd, have CS, DS and KS GAGs

GAGs (Glycosaminoglycans) consist of repeating disaccharide units

-Chondrointin sulfate (CS) and Dermatin sulfate (DS) derived from epimerization, Heparin sulfate (HS), Keratin sulfate (KS), Hyaluronic Acid (HA)



3) Proteoglycans are studied extensively in other systems including

-Development (ES Cells and more)

-Cardiovascular system

-Neural, eye, and oral systems (teeth, jaw, TMJ, SG)

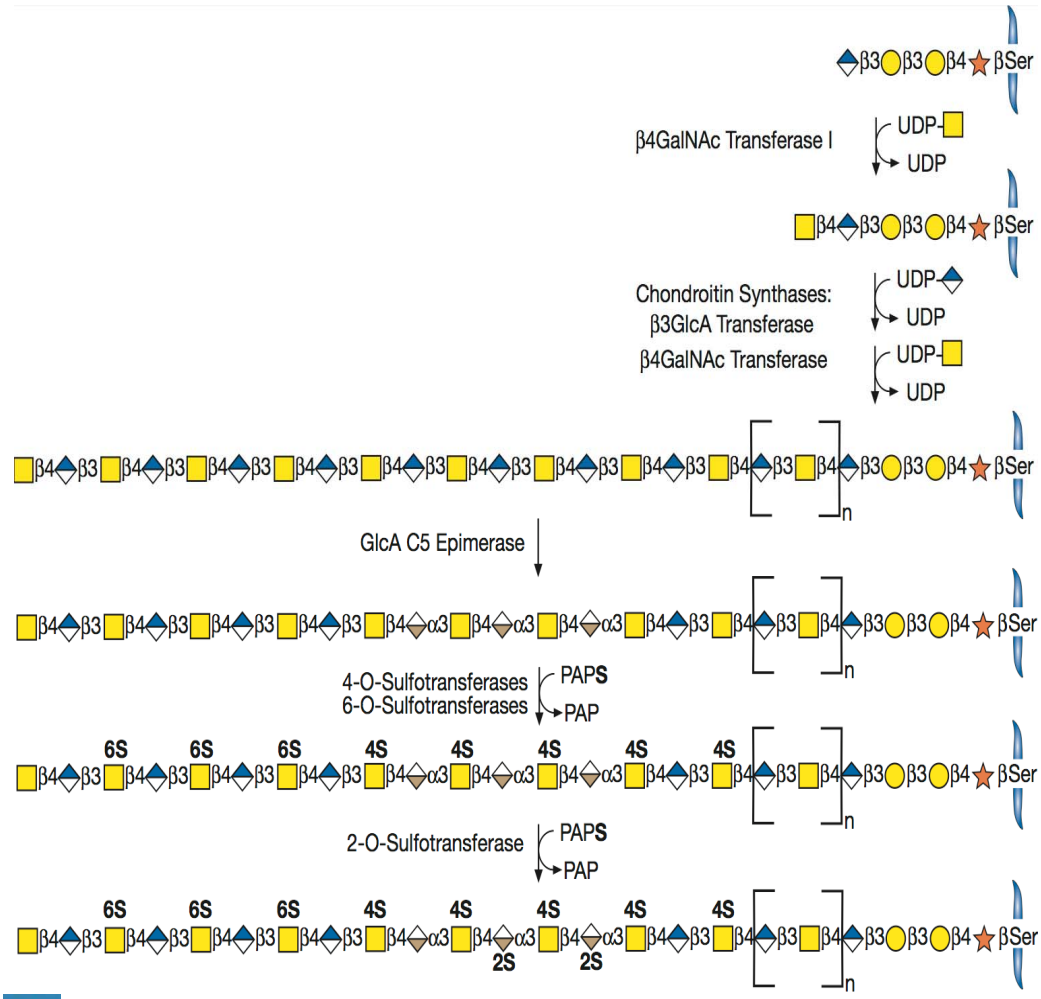
-Immunology/inflammation

-Regenerative Medicine

-Cancer

-The role of proteoglycans in skeletal formation and aging is understudied

4) Proteoglycans: how they are made: an example is the biosynthesis of chondroitin sulfate/dermatan sulfate

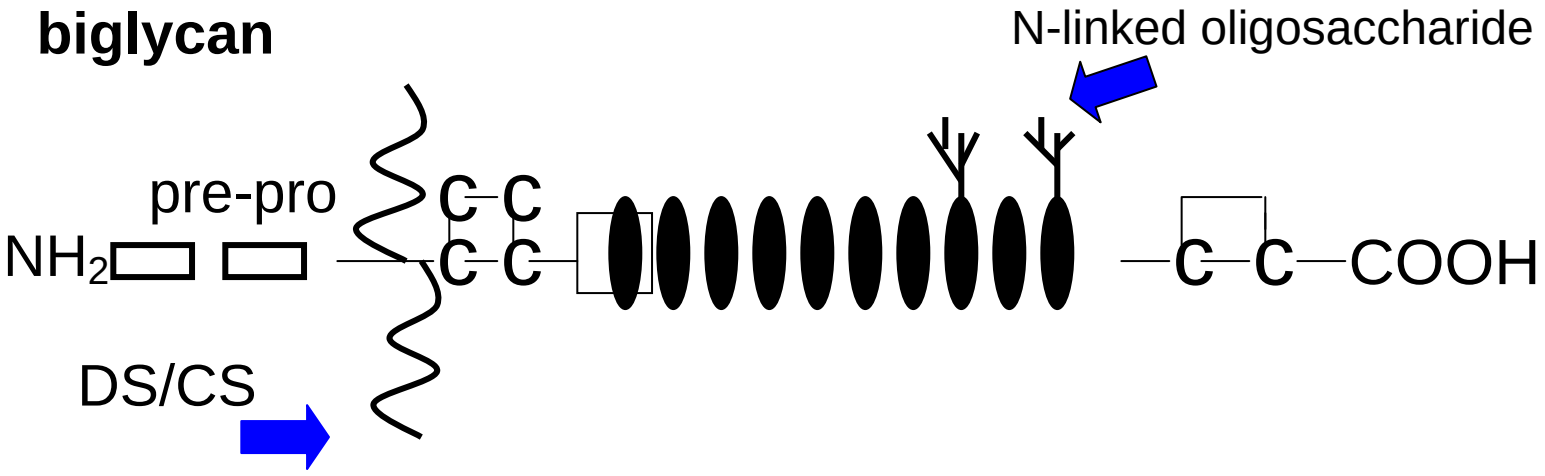


5) The Small Leucine-Rich Proteoglycan (SLRP) family:

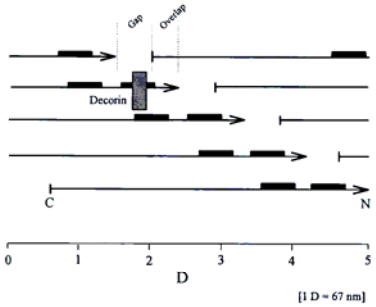
The genes are located in tandem on separate chromosomes

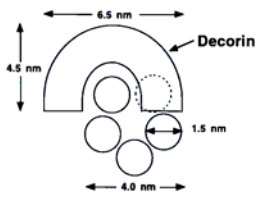
X	**1q32**	**9q21-23**	**12q21**
biglycan (I) (Xq28)	fibromodulin (II) PRELP (II) optican (III)	asporin (I) osteoadherin (II) osteoglycin/ mimecan (III)	decorin (I) lumican (II) keratocan (II) epiphican/ PG-Lb/DSPG3 (III)

SLRPs are divided into classes depending on gene and core protein structure (ie Class I contains biglycan, asporin and decorin, etc). The family is “growing” and may have up to 17 members. They have CS, DS and KS GAG chains attached. Nearly all of the SLRPs have been detected in skeletal tissues.



6) SLRP knockouts: Compensation: Biglycan and Decorin





Structural Defects in BGN/DCN Double Knockout Mice

	Skin	Bone
DCN-KO	Thin dermis Larger irregular fibrils	Normal Mass Small Fibrils
BGN-KO	Thin dermis Larger irregular fibrils	Lower Mass Larger irregular fibrils
BGN/DCN-KO	Loose Disorganized dermis Larger,highly irregular fibrils	Severe osteopenia Larger,more irregular fibrils than BGN-KO

Additional examples of SLRP compensation and its outcome:

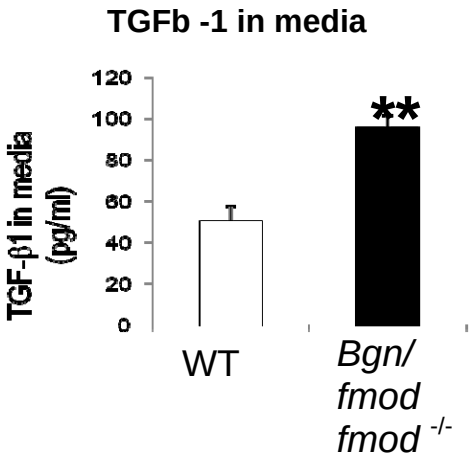
-Biglycan and decorin deficiency causes extreme osteopenia, (not able to breed)

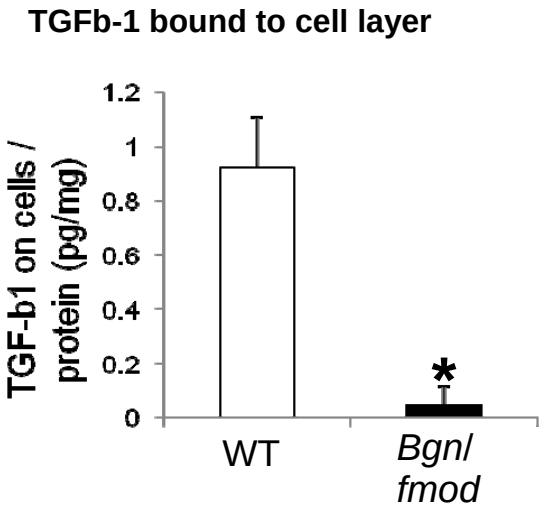
-Biglycan and fibromodulin deficiency causes
osteopenia (from too much bone resporption)
ectopic ossification of tendon (from stem cell abnormality)
osteoarthrtitis (OA). These mice are able to breed and age

-Biglycan/fibromodulin deficient cartilage has:
Increased proliferation
Increased differentiation
Increased apoptosis
Increased degradation of aggrecan and type II collagen
Increased activity of ADAMTS4/5 and MMP's

7) Proteoglycans/ECM and the Interplay with Growth factors

-Sequestration of TGF-beta is not normal in biglycan/fibromodulin KO cartilage
-Proven using cartilage cells cultured and then incubated with TGF-beta





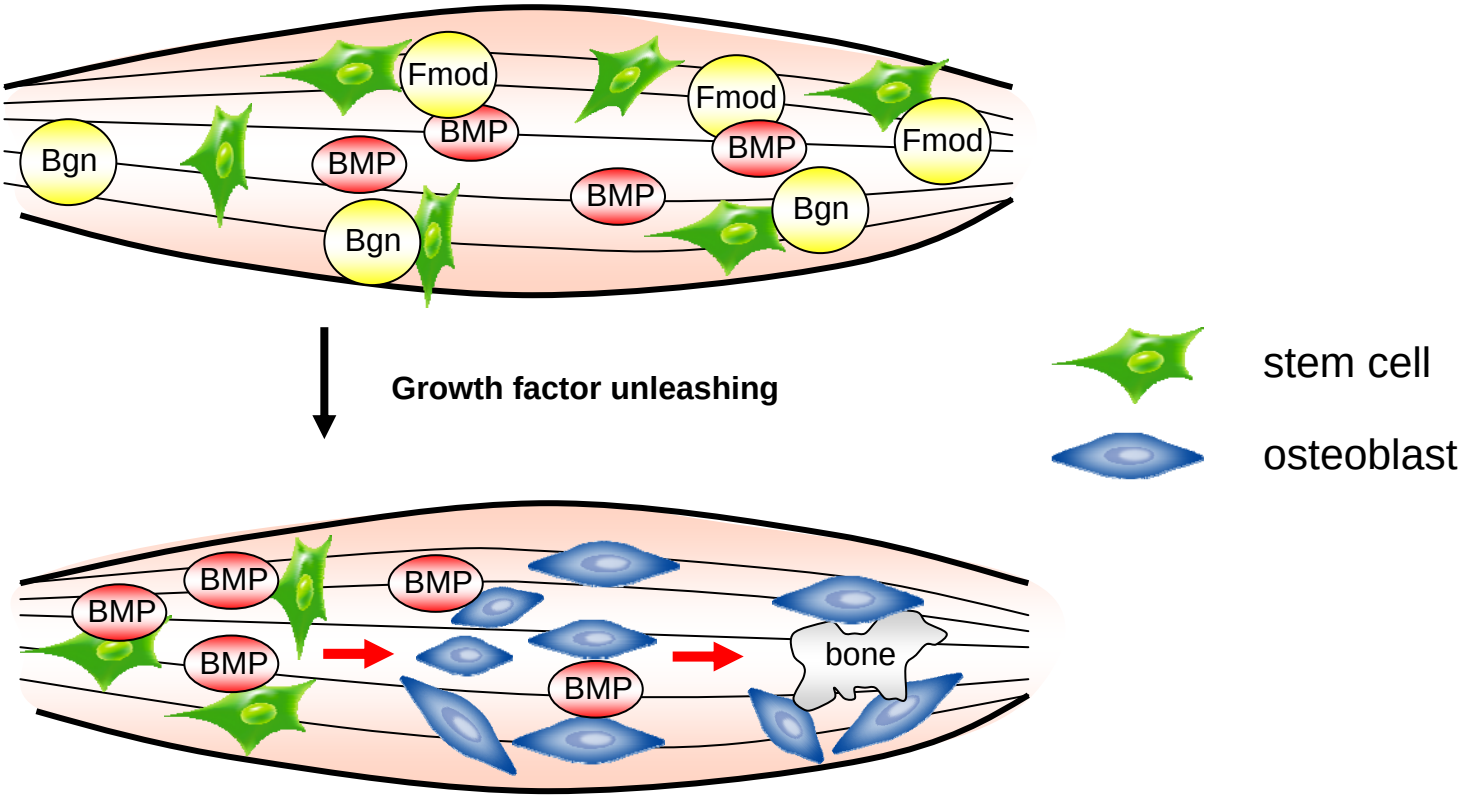
This unleashing of TGF-beta causes overactive growth factor signaling leading to changes in:

-Differentiation-"a switch in fate"
-Proliferation-too much expansion
-Premature cell death via apotosis

Overall phenotype: accelerated aging

Growth Factor regulation is **context dependent.** For example: loss of SLRPS causes low bone mass and, at the same time, ectopic ossification of tendon. This may involve dys-regulation of stem cells

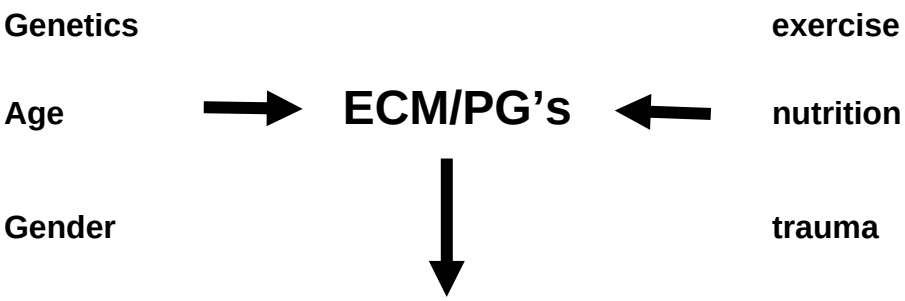
8) Proteoglycans/ECM and the interplay with stem cells



Stem cell fate is pushed towards osteogenesis rather than tendon formation due to overactive BMP-2 signaling when biglycan (Bgn) and fibromodulin (fmod) are absent. The ECM therefore provides the correct stem cell niche that can control the balance of growth factors which in turn controls the function and activation of the tendon stem/progenitors**.**

The combined role of biglycan and fibromodulin in normal skeletal function is still not proven but appears to involve control osteoclastogenesis. A SLRP family member called PRELP impairs osteoclastogenesis by inhibiting NF-kBeta (Rucci et al, J Cell Biol 187(5):669-83, 2009)

Factors affecting the ECM interface



Cell proliferation, differentiation, apoptosis, cell fate

9) General Comments/concepts to take home

ECM, Growth Factors and Stem Cells: a Football Paradigm

-synergism and cooperation

-compensation and substitution (with a limit)

-"placement in the goal" (ie growth factor)

Summary for Proteoglycans/ECM:

- Extremely diverse in structure and function: core and GAG production, modification and turnover

- Regulate numerous processes including differentiation, proliferation, attachment, migration, apoptisis (and more)

-Work either directly in and on cells, through growth factor modulation or by controlling tissue integrity and by affecting ECM assembly

-As major components of the bone cell microenviroment could be therapeutic targets

10) Reference Materials

http://www.ncbi.nlm.nih.gov/bookshefl/br.fcgi?book+glyco2

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Merline F, Schaefer R, Schaefer L, The matricellular functions of small leucine-rich proteoglycans (SLRPs) J Cell Commun. Signal 3:323-335, 2009

Bi Y, Shi S, Tina Kilts T, Wadhwa S, Xu T, Iozzo RV, Young MF and Chen X-D. Extracellular matrix proteoglycans control the fate of bone marrow stromal stem cells. J Biol Chem 280(34):30481-30489, 2005

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Ameye L, Aria D, Chen, X-D, Gehron Robey P, Oldberg A, Young MF Biglcyan and fibromodulin double-knockout mice develop ectopic sesamoid bones and premature arthritis in the knee joint, FASEB J 16:673-680, 2002

Embree M, Kilts T, Syed-Picard F, Karsdal M, Oldberg Å, Inkson C, Bi Y and Young MF TMJ Osteoarthritis: Potential Role of the ECM in Regulating Chondrogenesis and Cartilage Degradation Am J Pathol. 176(2): 812-26, 2010

Eph Signaling in Bone

Natalie Sims, Ph.D.

EPH Signaling in Bone

Natalie A Sims, St. Vincent's Institute of Medical Research, Australia

Significance of the topic:

Signalling between Ephrins and their receptors (EPHs) has been shown to be important for cell function in a range of tissues, most recently in bone and in cancer metastasis. The field is complicated by virtue of the size of this family (the largest family of receptor tyrosine kinases currently known) and the promiscuous nature of each member. In the skeleton, EPH signalling has been noted to regulate both osteoblast and osteoclast function, and may function not only within each cell lineage, but also between osteoblasts and osteoclasts. Currently, research in EPH signalling in bone is in its infancy compared to what is known in other organ systems.

Learning objectives:

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

Recognise the difference between forward and reverse signalling
Understand the promiscuity of EPH and Ephrin signalling
Understand which cells in bone may interact by EPH:Ephrin interactions

Useful references:

1. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell*. 2008 Apr 4;133(1):38-52.
2. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, Suda T, Matsuo K. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab*. 2006 Aug;4(2):111-21.
3. Allan EH, Häusler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B, Pompolo S, Sims NA, Gillespie MT, Onyia JE, Martin TJ. EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts. *J Bone Miner Res*. 2008 Aug;23(8):1170-81.
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5. Martin TJ, Allan EH, Ho PW, Gooi JH, Quinn JM, Gillespie MT, Krasnoperov V, Sims NA. Communication between ephrinB2 and EphB4 within the osteoblast lineage. *Adv Exp Med Biol*. 2010;658:51-60.
6. Davy A, Bush JO, Soriano P. Inhibition of gap junction communication at ectopic Eph/ephrin boundaries underlies craniofrontonasal syndrome. *PLoS Biol*. 2006 Oct;4(10):e315.
7. Xing W, Kim J, Wergedal J, Chen ST, Mohan S. Ephrin B1 regulates bone marrow stromal cell differentiation and bone formation by influencing TAZ transactivation via complex formation with NHERF1. *Mol Cell Biol*. 2010 Feb;30(3):711-21
8. Sims NA "EPHs and Ephrins: Many pathways to regulate osteoblasts and osteoclasts" IBMS BoneKEy September 2010.

Model of EPH and Ephrins.
From Pasquale EB. *Nat Rev Cancer*. 2010 Mar;10(3):165-80.

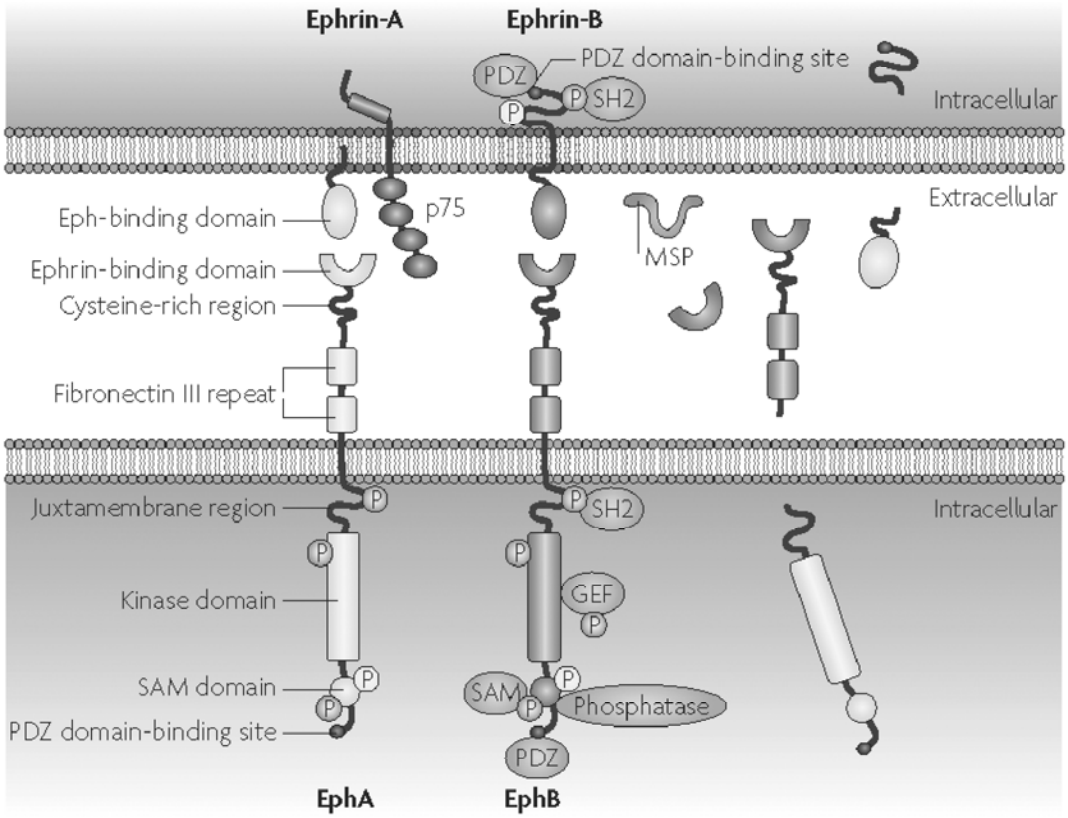
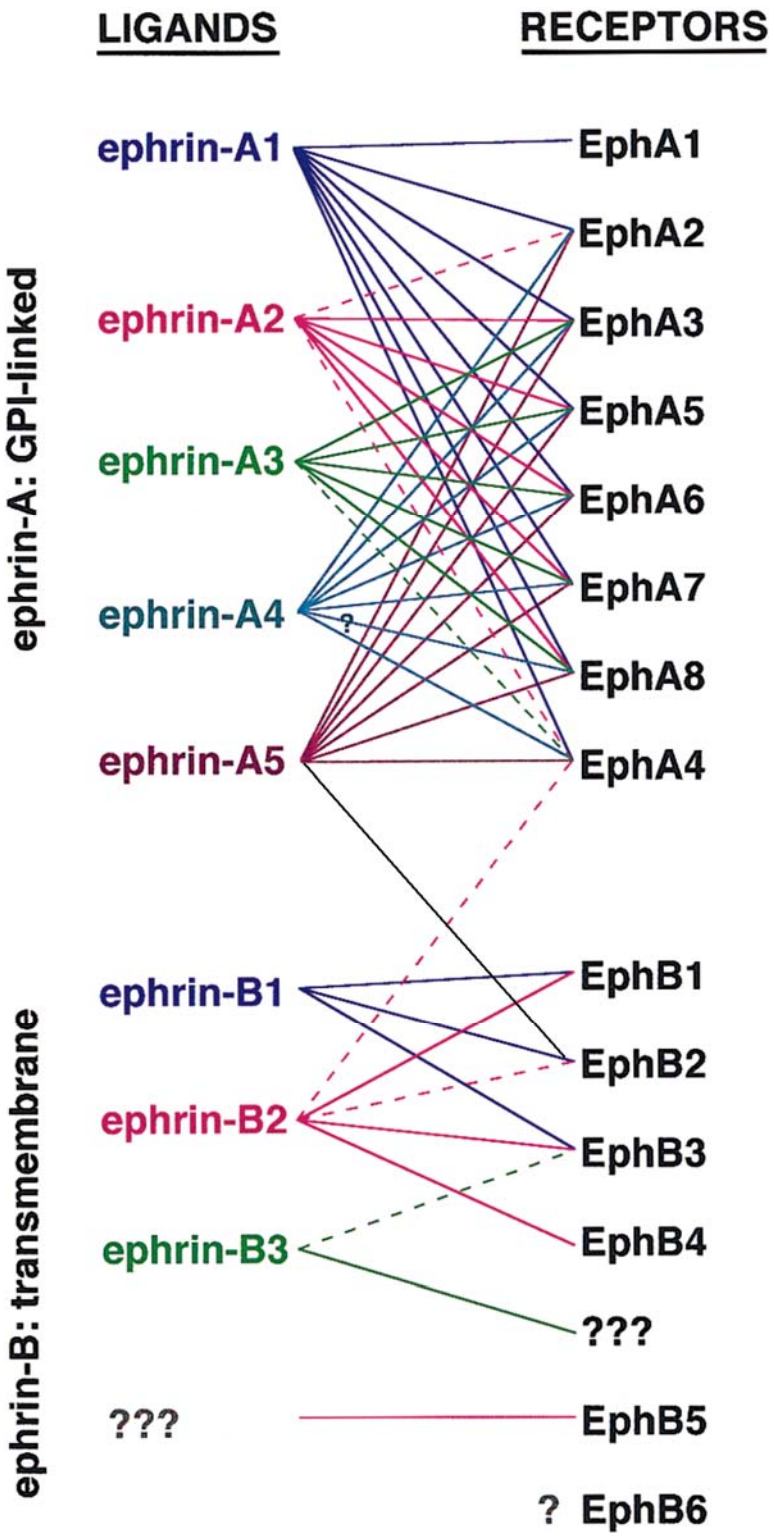
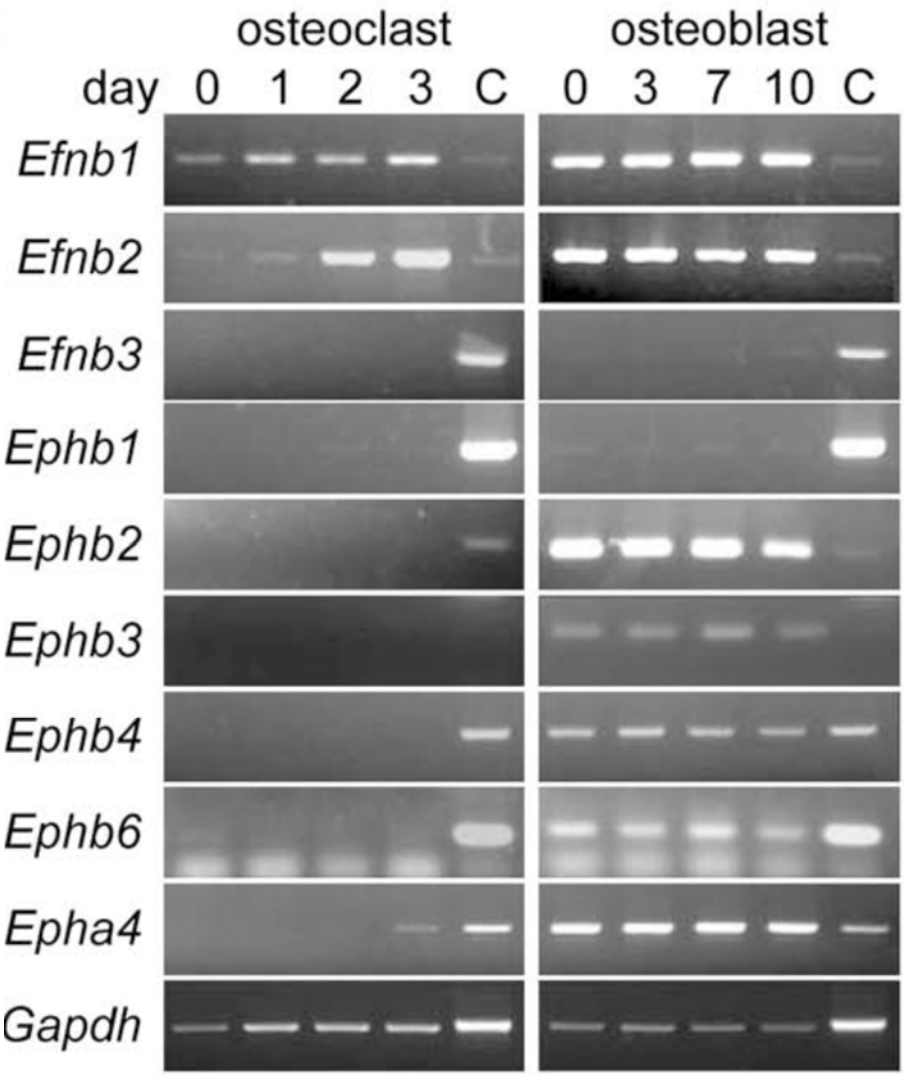


Figure 1 | **Eph receptor and ephrin domain structure and signalling interactions.**

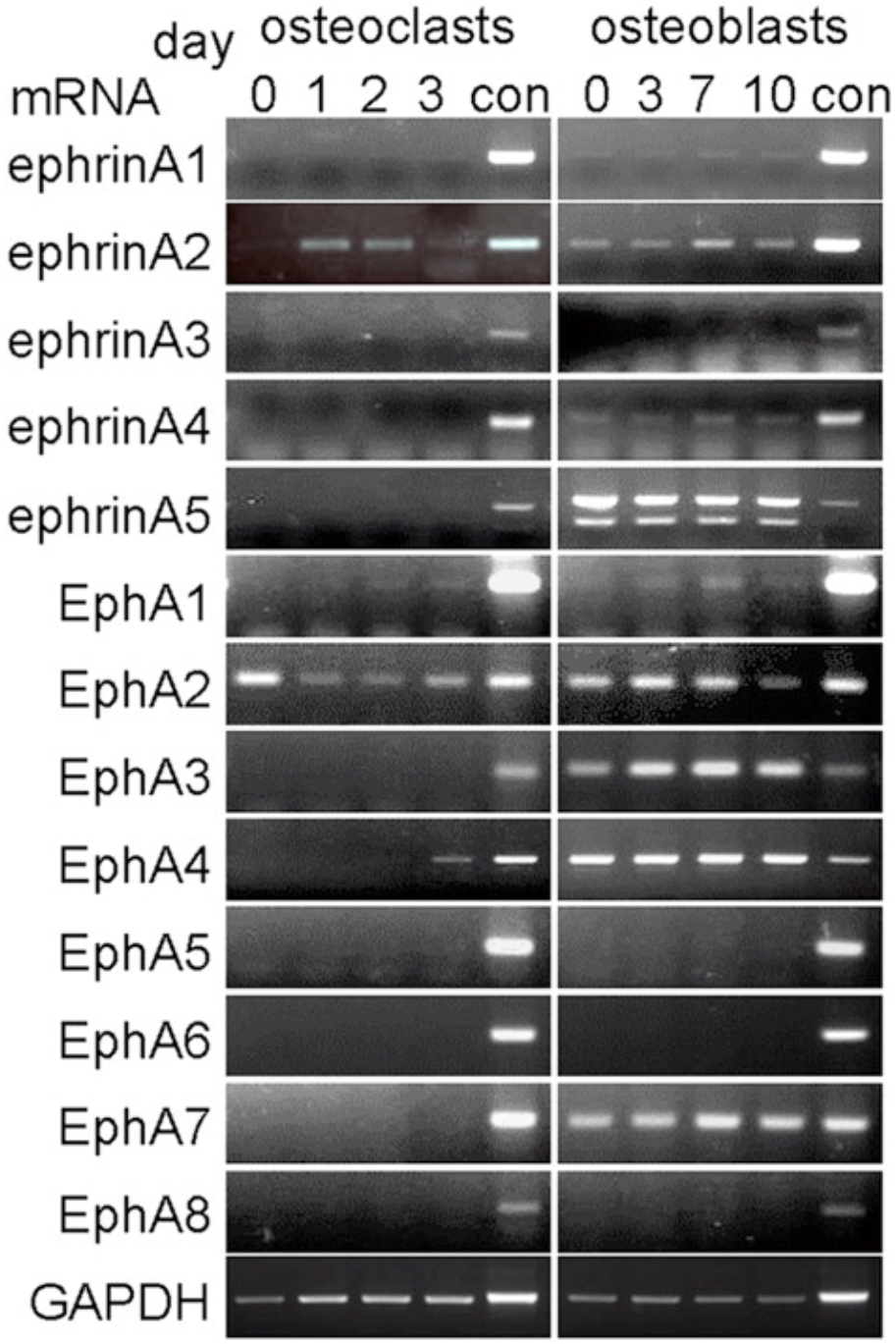
EPH: Ephrin promiscuity (reproduced from Zhou R. The Eph family receptors and ligands. *Pharmacol Ther*. 1998 Mar;77(3):151-81; interaction of Ephrin A5 with EPHB2 added from Himanen et. al., Nature Neurosci 2004 May;7(5):501-9.):



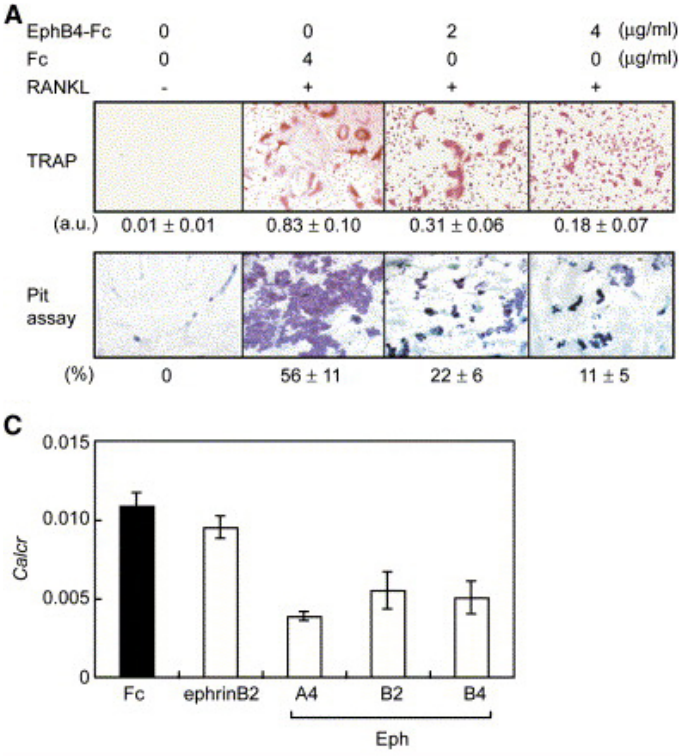
EPHB and EphrinB interacting family members in cultured murine bone cells by PCR – reproduced from Zhao et al *Cell Metab*. 2006 Aug;4(2):111-21.

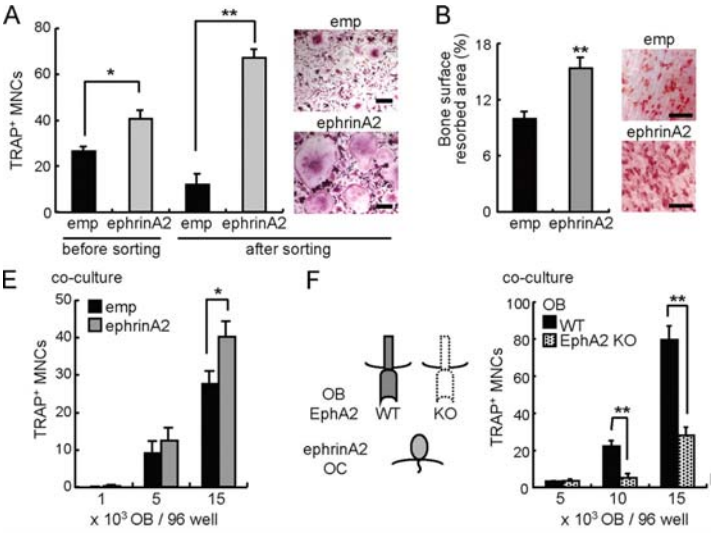


EPHA and EphrinA family members in cultured murine bone cells by PCR – reproduced from Irie et al *J Biol Chem*. 2009 May 22;284(21):14637-44.

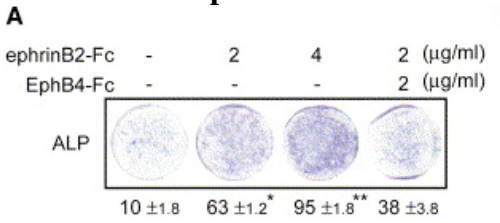


Effects of Ephrins on osteoclast formation:
Data from Zhao et al *Cell Metab*. 2006 Aug;4(2):111-21
and Irie et al *J Biol Chem*. 2009 May 22;284(21):14637-44.

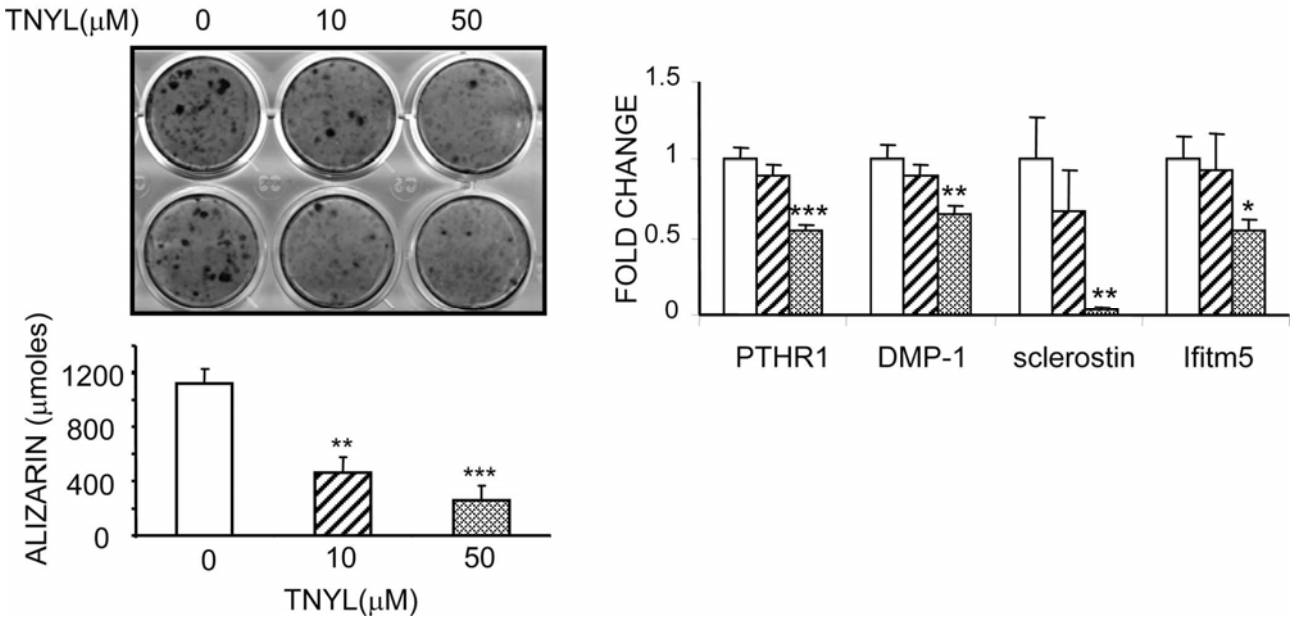




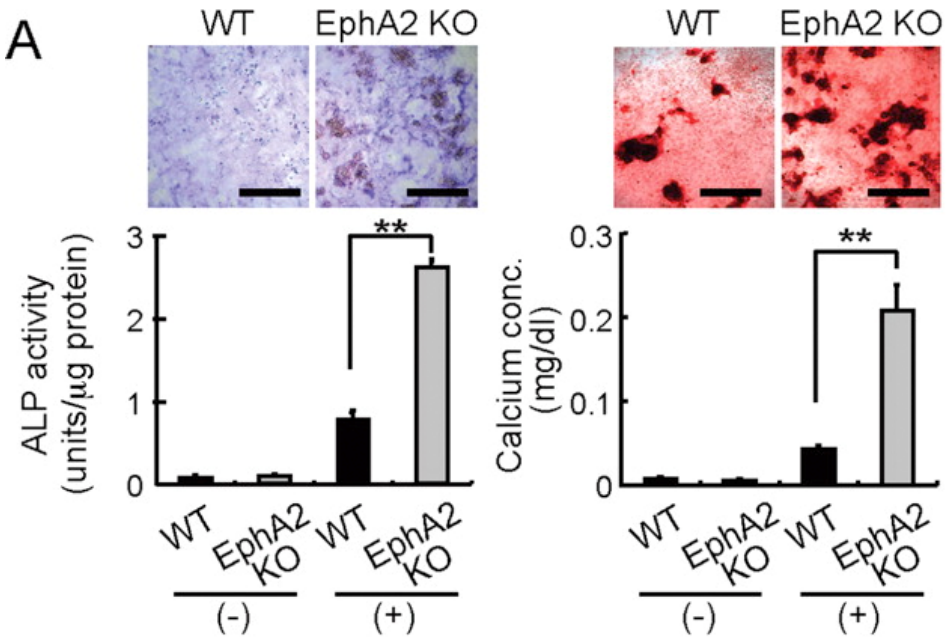
Influences of Ephrins on osteoblast differentiation



Zhao et al *Cell Metab*. 2006 Aug;4(2):111-21

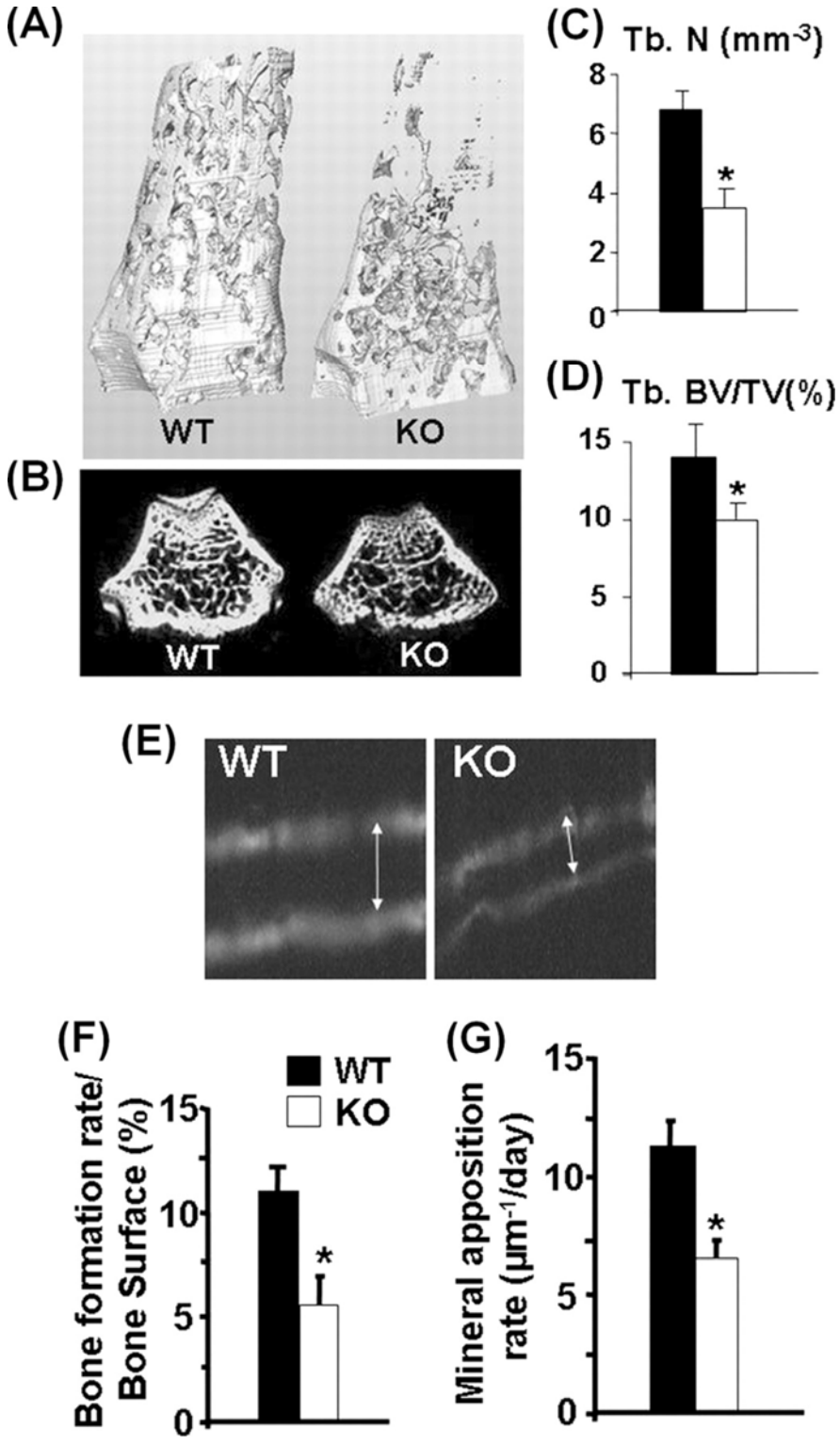


Allan et al *J Bone Miner Res*. 2008 Aug;23(8):1170-81.



Irie et al *J Biol Chem*. 2009 May 22;284(21):14637-44.

Bone phenotype of Osteoblast specific (Col1{alpha}2-iCre) EphrinB1 knockout mouse.
From Xing W et al *Mol Cell Biol*. 2010 Feb;30(3):711-21



Model of EPH:Ephrin interactions within bone.
Reproduced from Sims NA, IBMS BoneKEy September 2010.

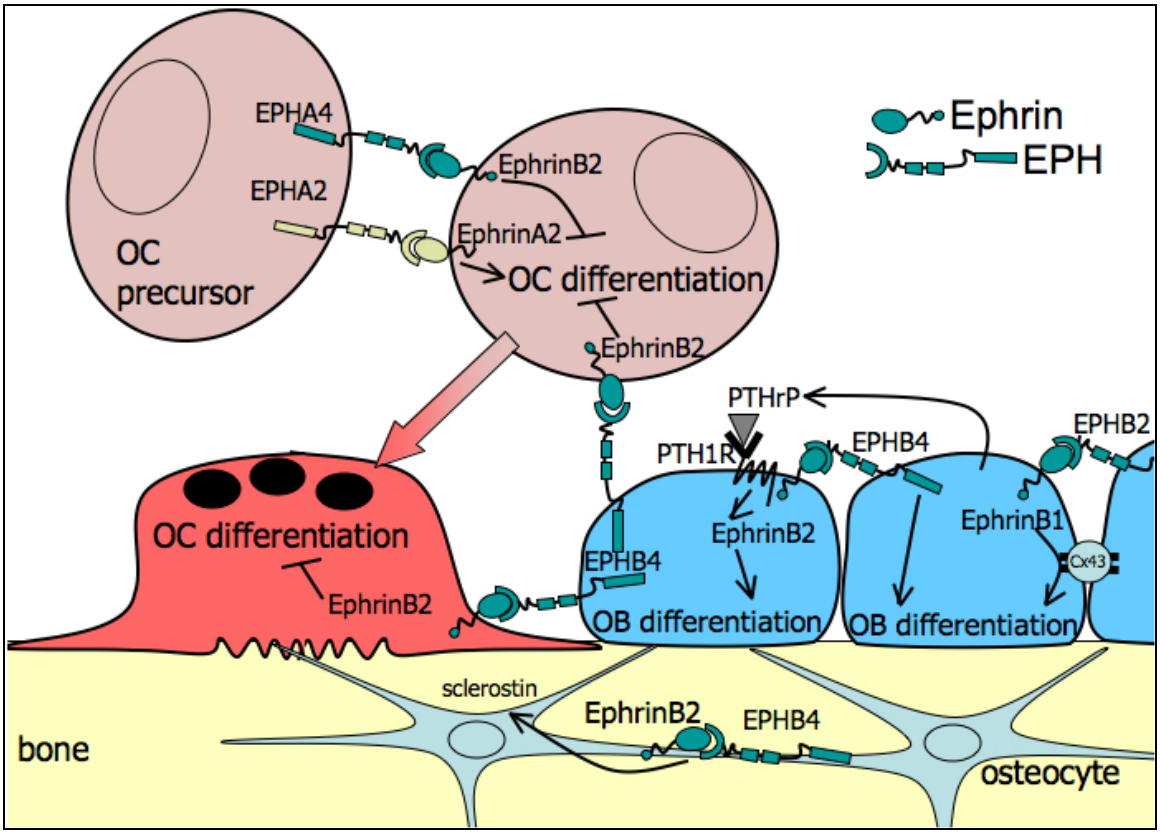


Fig. 1. Postulated EPH/Ephrin interactions in the bone remodeling unit. EphrinB2 signaling within osteoclast precursors (an EPHA4 interaction) and interaction of osteoclast precursors and osteoclasts with EPHB4-expressing osteoblasts limits osteoclast differentiation. In osteoblasts, paracrine PTHrP, acting through the PTH receptor (PTH1R), enhances EphrinB2 production. EphrinB2/EPHB4 interaction within the osteoblast lineage enhances osteoblast differentiation. EphrinB1/EPHB2 interaction within the osteoblast lineage also enhances osteoblast differentiation through a direct interaction of EphrinB2 with connexin 43 that promotes gap junction formation. Both EphrinB2 and EPHB4 are expressed by osteocytes embedded in the bone matrix, and interfering with this interaction inhibits sclerostin expression.

Genetics of Bone Loss, Determinants of BMD and Fractures

Doug Kiel, M.D., MPH

Genetics of Bone Loss, Determinants of BMD and Fractures

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Significance of the topic:

Twin and family studies have shown that between 50 and 85% of the variance in peak BMD is genetically determined. Twin studies have confirmed heritability of bone loss with aging but the genetic contribution seems to be weaker than for peak bone mass. Heritability of fracture has been less consistently reported due to the complexity of this phenotype. Also there are some data to suggest that earlier occurring fractures (e.g., hip) may be more heritable than those occurring in advanced age.

Identifying genetic elements that are important to bone health will improve the understanding of the pathophysiology of osteoporosis and may lead to novel treatments and prevention strategies.

Learning Objectives:

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

- Understand different approaches to studying the genes contributing to osteoporosis
- Be familiar with genome-wide association studies of skeletal traits
- Understand the next steps following genome-wide association studies

Outline:

I. Heritability of bone phenotypes

A. High heritability twin and family studies h^2>0.5 for cross-sectional traits such as BMD, BUA, bone geometry, fracture (early)

B. Heritability of longitudinal change in BMD not well studied.

C. Peak BMD and BMD in older age heritable

D. Limited support for heritability of fracture (see table 1 from Duncan review)

TABLE 1. Genetic epidemiology studies of fracture risk

First author (Ref.)	Ethnicity	No. of relative pairs	Findings
Kannus (88)	Finnish	15,098 twins	No increase in MZ concordance for fracture. Reanalysis by MacGregor suggested 35% heritability (89).
Andrew (17)	British	6,750 twins	Heritability of Colles' fracture, 54%
Michaelsson (90)	Swedish	33,432 twins	Heritability of any fracture of 16%, osteoporotic fractures of 27%, and hip fracture of 46%. Heritability of fracture 68% if age at fracture <69 yr, but only 3% if age >79 yr.
Deng (16)	American	6,274 sisters or mothers	Heritability of Colles' fracture, 25.4%
Deng (18)	American	50 families	Hip fracture heritability, 53%, but marginally significant ($P = 0.048$)

MZ, Monozygotic twin.

From Duncan JCEM 2010

E. Limited shared genetics between BMD and fracture but premenopausal daughters of mothers with fracture history have low BMD and all genes associated with fracture are associated with BMD. Thus, still need more information about shared genes between BMD and fracture.

F. Collectively, genes explain only small amount of variance in BMD and genes that have been associated with BMD have very small effects. This "missing heritability" has been the rule for many genetic studies of complex traits.

Disease	Number of loci	Proportion of heritability explained	Heritability measure
Age-related macular degeneration[72]	5	50%	Sibling recurrence risk
Crohn's disease[21]	32	20%	Genetic risk (liability)
Systemic lupus erythematosus[73]	6	15%	Sibling recurrence risk
Type 2 diabetes[74]	18	6%	Sibling recurrence risk
HDL cholesterol[75]	7	5.2%	Residual* phenotypic variance
Height[15]	40	5%	Phenotypic variance
Early onset myocardial infarction[76]	9	2.8%	Phenotypic variance
Fasting glucose[77]	4	1.5%	Phenotypic variance

* Residual is after adjustment for age, gender, diabetes.

From Manolio Nature 2009

G. Missing heritability of complex traits

Many explanations for this missing heritability have been suggested, including much larger numbers of variants of smaller effect yet to be found; rarer variants (possibly with larger effects) that are poorly detected by available enotyping arrays that focus on variants present in 5% or more of the population; structural variants poorly captured by existing arrays; low power to detect gene–gene interactions; and inadequate accounting for shared environment among relatives. Consensus is lacking, however, on approaches and priorities for research to examine what has been termed 'dark matter' of genome-wide association—dark matter in the sense that one is sure it exists, can detect its influence, but simply cannot 'see' it (yet). (Manolio Nature 2009)

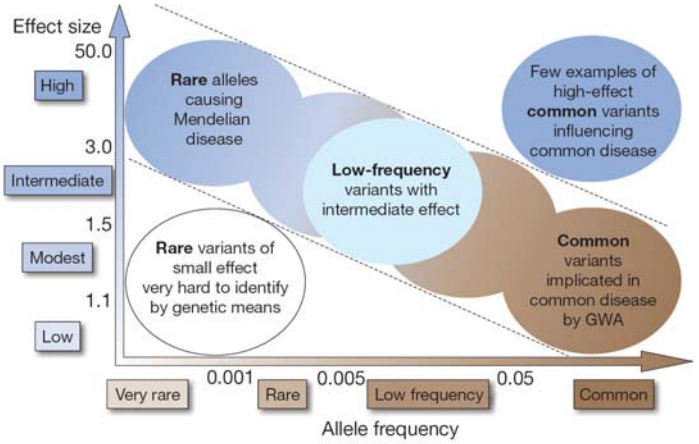


Figure 1 | Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio). Most emphasis and interest lies in identifying associations with characteristics shown within diagonal dotted lines. Adapted from ref. 42.

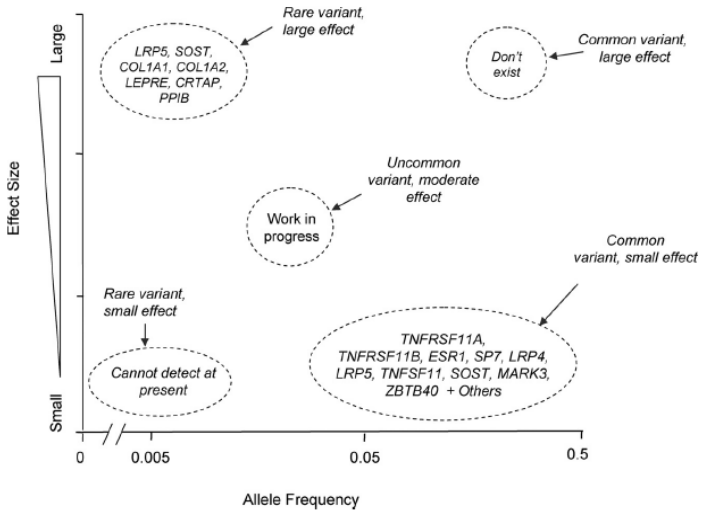
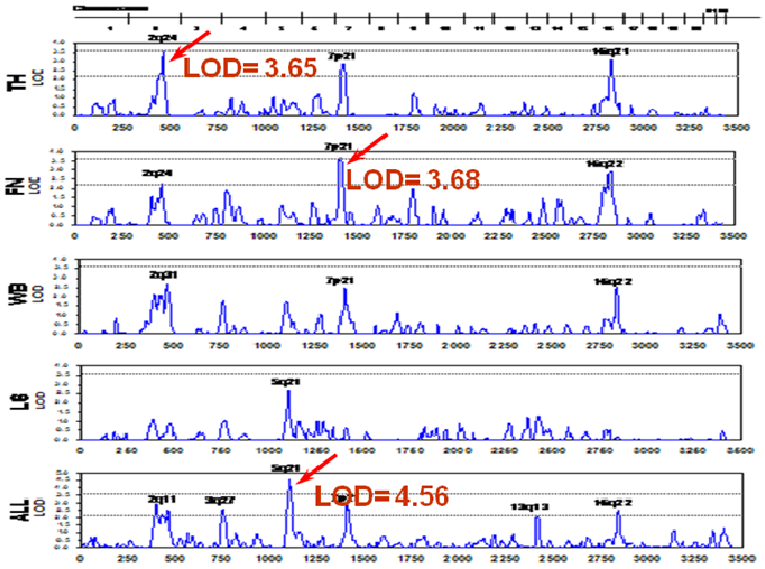


Figure 2: Allelic architecture of susceptibility to osteoporosis (from Ralston and Uitterlinden JCEM 2010)

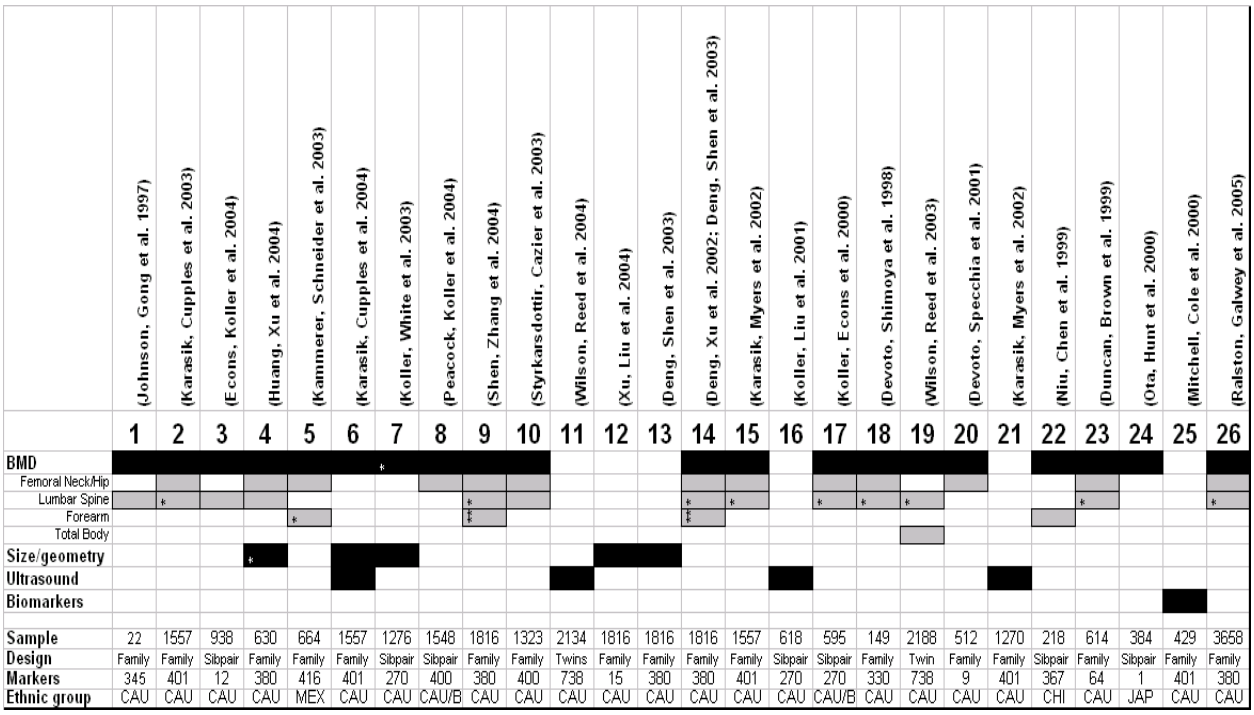
II. Genetic Approaches to Finding Osteoporosis Genes

A. Linkage

1. Used for classic Mendelian diseases but has not been as successful in quantitative bone traits or fracture

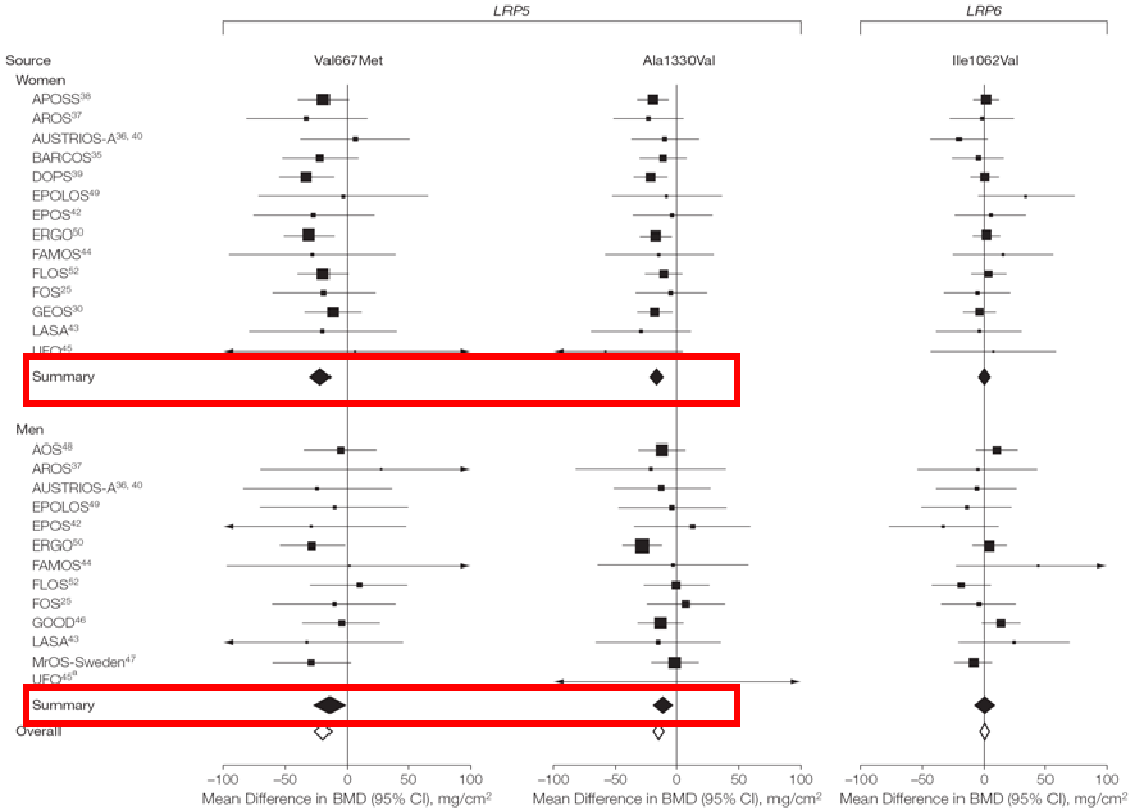


Whole-genome linkage study by genotyping 400 Microsatellite markers across whole genome in 4000 siblings with extreme high low concordance of their hip BMD. As showed in this figure, X-axis is the genetic map by cM from chromosome 1 to chr22. The y-axis is Multiple-point LOD score. LOD score large than 3.6 means its p-value less than 10 to -5 which reaches the genome-wide significance level. From top to bottom panels are for different skeletal sites. 3 genome-wide significant QTL regions including 2Q24 FOR TH, 7P21 FOR FN AND 5Q21 FOR combined multiple-skeletal sites (Hsu JBMR 2007)



From Uitterlinden 2009

B. Candidate gene association studies
    1. Multiple, small, underpowered studies
    2. Meta-analyses



vanMeurs, et al., JAMA 2008

Table 4. Unadjusted Odds for Fracture Risk per Minor Allele for *LRP5* Val667Met, *LRP5* Ala1330Val, *LRP6* Ile1062Val, and *LRP5* Haplotype Alleles[a]

SNP, Subgroup	Any Fracture			Vertebral Fractures (All Types)		
	No.[b]	OR (95% CI)	*P* Value	No.[b]	OR (95% CI)	*P* Value
LRP5 Val667Met (per Met copy)						
Men	10975	1.09 (0.93-1.27)	.28	4782	1.14 (0.84-1.57)	.40
Women	20460	1.17 (1.06-1.30)	.003	15706	1.29 (1.08-1.54)	.004
All	31435	1.14 (1.05-1.24)	.002	20488	1.26 (1.08-1.47)	.004
LRP5 Ala1330Val (per Val copy)						
Men	11035	1.07 (0.97-1.17)	.20	4786	1.11 (0.91-1.36)	.30
Women	20164	1.06 (1.00-1.14)	.06	15310	1.12 (1.00-1.26)	.049
All	31199	1.06 (1.01-1.12)	.02	20096	1.12 (1.01-1.24)	.03
LRP6 Ile1062Val (per Val copy)						
Men	11102	0.98 (0.90-1.07)	.71	4849	1.23 (1.04-1.46)	.02
Women	20704	1.01 (0.94-1.09)	.69	15838	1.01 (0.91-1.12)	.84
All	31806	1.00 (0.95-1.06)	.95	20687	1.07 (0.98-1.17)	.15
Haplotype (Val667Met-Ala1330Val)						
1 (Val667-Ala1330)	30227	1 [Reference]	NA	19737	1 [Reference]	NA
2 (Val667-Val1330)		1.06 (0.95-1.19)	.30		1.04 (0.91-1.19)	.59
3 (Met667-Val1330)		1.18 (1.02-1.37)	.02		1.28 (1.08-1.55)	.006

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a]Results on single SNPs are based on inverse-variance random-effects analysis of individual-level data. Results on haplotypes are based on linear mixed models.
[b]Number of individuals in these analyses.

vanMeurs, et al., JAMA 2008

C. GWAS

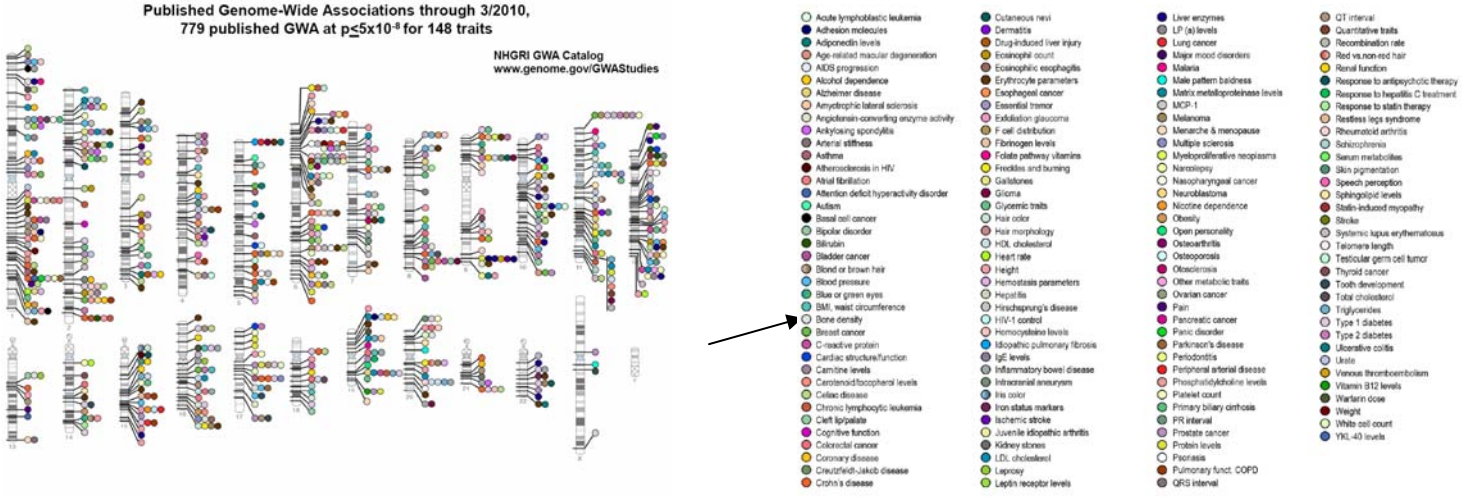


TABLE 4. Genes and loci with genome-wide significant evidence for association with BMD

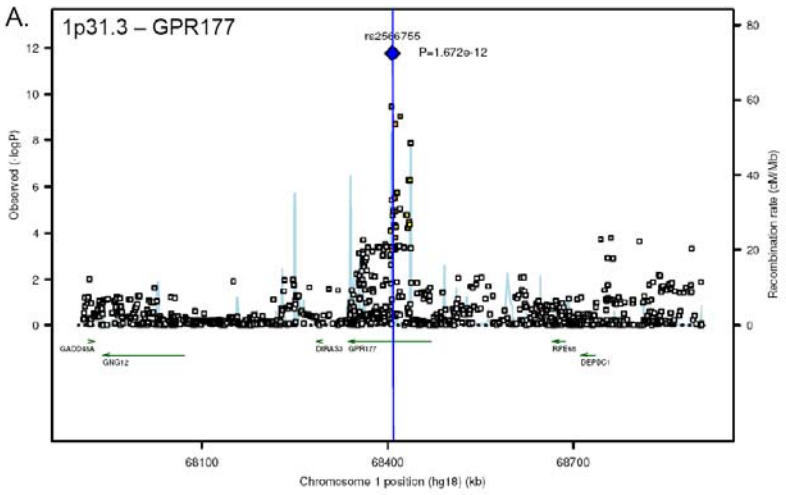
No.	Gene(s)	Locus	Novel[a]	Spine BMD	Hip BMD	Fracture[b]	Mode of id
1	*ADAMTS18*	16q23.1	Yes	−	+	+	
2	*CRHR1*	17q21	Yes	+	−	−	GWAS meta-analysis
3	*CTNNB1*	3p22	No	−	+	−	GWAS meta-analysis
4	*DCDC1/DCDC5*	11p14.1	Yes	+	−	−	GWAS meta-analysis
5	*ESR1*	6q25	No	+	+	+	GWAS
6	*FLJ42280*	7q21.3	Yes	+	+	−	GWAS meta-analysis
7	*FOXL1/FOXC2*	16q24	No	+	−	−	GWAS meta-analysis
8	*GPR177*	1p31.3	Yes	+	+	−	GWAS meta-analysis
9	*HDAC5*	17q21	Yes	−	+	−	GWAS meta-analysis
10	*MARK3*	14q32	Yes	−	+	−	GWAS
11	*MEF2C*	5q14	No	−	+	−	GWAS meta-analysis
12	*LRP4/ARHGAP1/F2*	11p11.2	Yes	−	+	+	GWAS
13	*LRP5*	11q13.4	No	+	+	−	Candidate gene; GWA
14	*MEPE/IBSP/OPN*	4q21.1	No	+	−	+	GWAS meta-analysis
15	*MHC*	6p21	Yes	+	−	+	GWAS
16	*SOST*	17q21	No	−	−	+	GWAS
17	*SOX6*	11p15	Yes	−	+	−	GWAS meta-analysis
18	*SPTBN1*	2p16	Yes	+	−	+	GWAS meta-analysis
19	*SP7*	12q13	No	+	−	−	GWAS
20	*STARD3NL*	7p14	Yes	+	−	−	GWAS meta-analysis
21	*TNFRS11B*	8q24	No	+	+	+	GWAS
22	*TNFRS11A*	18q21	No	+	+	+	GWAS
23	*TNFSF11*	13q14	No	−	+	−	GWAS meta-analysis
24	*ZBTB40*	1p36	Yes	+	+	+	GWAS
	Total	24	12 (50%)	15 (63%)	15 (63%)	10 (42%)	

[a] Not previously known to play a role in bone metabolism.

[b] None of the genes shown demonstrate genome-significant evidence for an association with fracture.

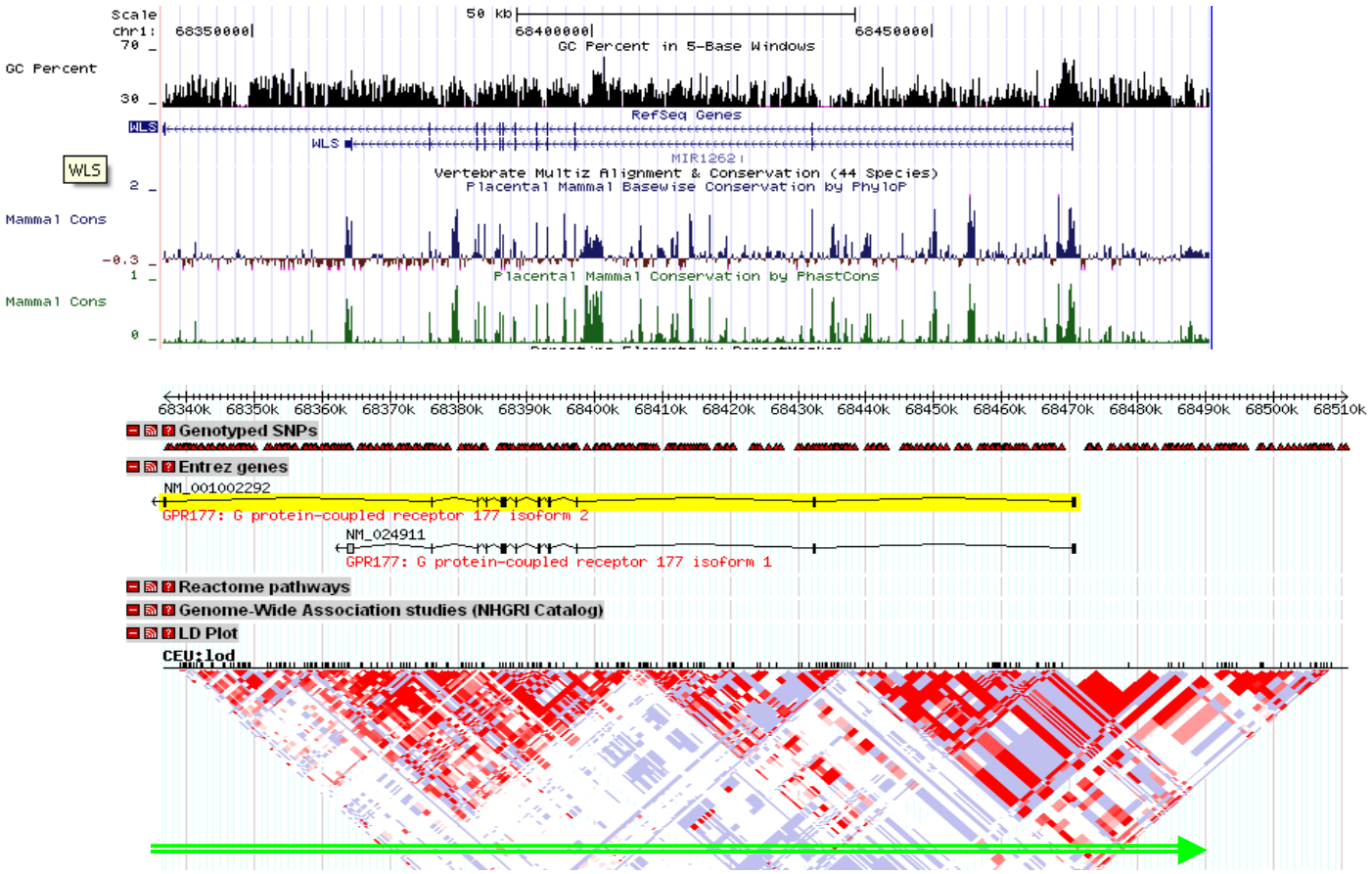
[c] Primary route through which genome-wide significant association with BMD was attained.

From Ralston and Uitterlinden Endocrine Reviews 2010



WLS (*formerly GPR177*) is part of the highly evolutionarily conserved Wnt signaling pathway, which is involved in bone cell differentiation and development. The gene is a positive regulator of the I-κB kinase–NF-κB cascade, part of the receptor activator of nuclear factor kappa B("RANK" system), which itself is a critical regulator of osteoclastogenesis. Cross-talk between the NF-κB and mitogen-activated protein kinase (MAPK) pathways has been indicated by the identification of genes that are expressed in both pathways, including several G protein–coupled receptors. Recently, a transgenic disruption of the *WLS* gene was reported to result in death in the early embryonic stage and there was a lack of Wnt3 (important pathway for embryogenesis) signaling detected in early embryogenesis. Thus *WLS* was established to have an essential role in the establishment of the antero-posterior axis of the mouse embryo, and in this same study Wnt proteins were found to modulate the cellular level and the expression of *WLS*. In another meta-analysis carried out by our group, SNPs in *WLS* were also found to be significantly associated with BMD and to have significant expression in the developing osteoblast as well as in osteoblast cell cultures stimulated with PTH. Thus, this candidate from our previous work was selected for resequencing.

GPR177/WLS gene (1p31.3)



Abstract 1243

"Meta-analysis of Genome-wide Association Studies Identifies 34 loci that Regulate BMD with Evidence of Both Site Specific and Generalized Effects: the GEFOS Consortium"

Newest GWAS meta-analysis from GEFOS Consortium
Also 49 suggestive loci

…These 34 GWS hits map within or nearby genes involved in pathways relevant to bone biology; i.e. genes in WNT pathway (*WNT16, AXIN1, WNT4, LRP5, CTNNB1, GPR177, FOXC2*), NF-kB pathway (*AKAP11, TNSFR11B, RANK, OPG*) and/or part of the SOX family of transcription factors (*SOX4, SOX6, SOX9*). Site specificity was observed in six loci based on significant evidence for heterogeneity (Phet): four had significantly stronger effect on LS-BMD (*AKAP11, STARD3NL, KCNMA1, MPP7* with Phet<0.0002) and two loci on FN-BMD (*MEF2C, XKR9* with Phet<0.0006). Such site specificity in ~18% of the GWS loci is consistent with the ~70±6% genetic correlation observed between BMD sites. The 34 GWS loci accounted for ~5% of the phenotypic (~8% of the genetic) variance in BMD.

Approaches to identify causal variants in genetic association studies

1) Bioinformatic studies to identify:
   Transcription factor binding sites
   MicroRNA coding sites
   Conservation across species
   Protein coding changes
   Alterations in splicing
2) Refinement of linkage disequilibrium blocks by studies in different ethnic groups
3) EMSAs and promoter-reporter assays
4) Cell biology-based studies:
   Cell culture from subjects of different genotype
   Expression of different variants *in vitro*
   "Knock-in" or ethylnitrosourea-based studies of model organisms with variant alleles
5) Studies on the effect of alleles on gene expression *in vivo*:
   Levels of mRNA expression
   Allele-specific transcription in heterozygotes

From Ralston and Uitterlinden Endocrine Rev 2010

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Osteoporosis and Hip Fracture in Asia-How to Rise up to this Challenge?

Edith Lau, M.D.

Osteoporosis and hip fracture in Asia-How to rise up to this challenge

Dr Edith Lau, MD, FRCP, MSc, FFPHM, FFCM, FHKAM (Community Medicine)
Director, Center for Health and Medical Research, Hong Kong

Significance of the topic

Osteoporosis is rapidly becoming an epidemic problem in Asia. By the year 2050, there will be a total of 3.2 million hip fractures in Asia. The epidemic of hip fracture in Hong Kong was first described by Lau et al in 1985. Since then, attention begins to be focused on how the epidemic of osteoporosis can be prevented in Asia.
The International Osteoporosis Foundation recently published the Asian Audit, which is the first compendium of data on osteoporosis in Asia. This forms a basis for plans to rise up to the epidemic of osteoporosis in Asia.
In this session, reflections on how the epidemic of osteoporosis in Asia can be prevented will be shared with participants. It is hope that the interactive discussion will result in a published plan on preventing osteoporosis in Asia.

Learning objectives

As a result of participating in this session, attendees should be able to :-
1. Gain an understanding into the epidemiology of osteoporosis in Asia
2. Develop insights into the programmes currently being implemented in various Asian countries.
3. Develop ideas into how to prevent osteoporosis in their own country
4. Plan research into osteoporosis in their country

Points of interests

1. Incidence and trends of hip fractures in Asia
2. Prevalence of vertebral fracture in Asia
3. The industrial scene-distribution of DEXA machines around Asia and the drugs available for treating osteoporosis
4. The barrier to preventing the osteoporosis epidemic in Asia
5. First hand experience from Hong Kong, where the control of osteoporosis has been a success

Reference

The Asian Audit-Epidemiology, cost and burden of osteoporosis in Asia in 2009, Mithal and Lau on behalf of IOF. Available on the IOF website.
A full reference list is available within the document.

Stem Cells: What Are they and What is Their Potential for Treating Skeletal Disorders

Pamela Robey, Ph.D.

Stem cells: what are they?
And how can they be used to treat skeletal disorders?

Meet the Professor
American Society for Bone and Mineral Research
October 15th, 2010
11:00 AM - 12:00 PM

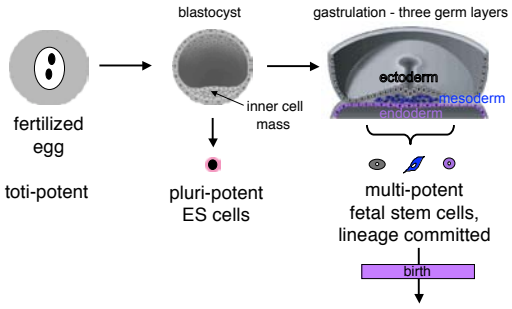
Pamela Gehron Robey, Ph.D.
Craniofacial and Skeletal Diseases Branch
National Institutes of Dental and Craniofacial Research
National Institutes of Health
Department of Health and Human Services

I am a US Government employee. I have no conflicts of interest

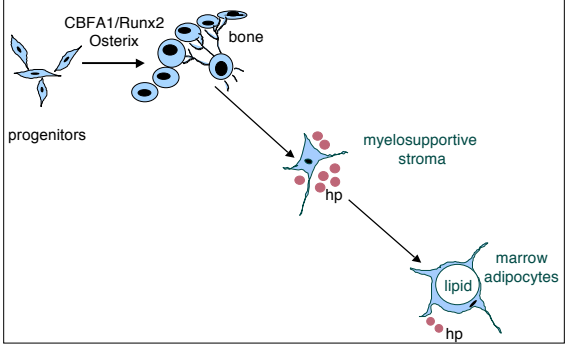
Points to talk about:

- embryonic development (and bone formation as an example)
- stem cell definitions properties
- how skeletal stem cells can be used in regenerative medicine
- "comparative" stem cell biology

Development



Stages of maturation



Post-natal stem cell hypothesis:
(Regaud, Weidenreich, Dantschakoff, Maximow)

- after birth, there are tissues that continuously regenerate
- regeneration must depend on the existence of a post-natal stem cell (may vary in numbers and properties as a function of age)

Hierarchy of tissue turnover & stem cells

- tissues with a high rate of replacement
  - blood
  - skin
  - gastro-intestinal tract
- tissues with a slow rate of replacement
  - bone
- tissues with limited ability to repair
  - muscle
  - teeth (dentin)
- tissues never thought to repair
  - nervous tissue

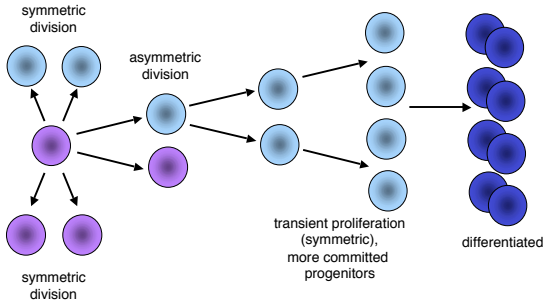
Properties of stem cells

in the body:
- offspring of a single cell are able to reconstitute an entire tissue
- able to self-renew

in the culture dish (assumptions):
- "clonogenic"
- self-renewal = unlimited proliferation
- stemness = undifferentiated

(not necessarily true for all stem cells!)

Self-renewal - post-natal stem cell kinetics



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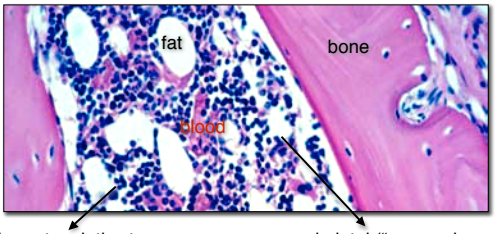
in the culture dish (assumptions):
- "clonogenic"
- self-renewal = unlimited proliferation
- stemness = undifferentiated

(not necessarily true for all stem cells!)

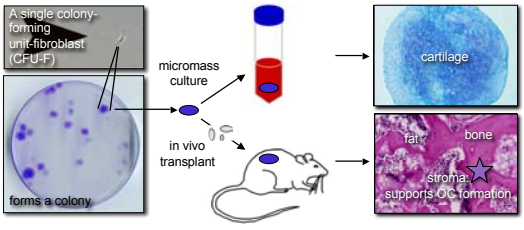
Bone marrow: home of a dual system of stem cells



hematopoietic stem cells

skeletal ("mesenchymal") stem cells

A single stromal stem cell (SSC, "MSC") regenerates all types of skeletal tissue



10-20% of the CFU-F are multipotent

Bianco and Robey, JCI, 2000

Other sources of skeletal ("mesenchymal") stem cells:

dental pulp
(baby teeth and wisdom teeth)
periodontal ligament

Shi et al., Gronthos et al.

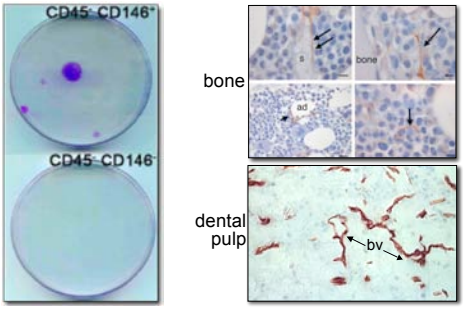
every tissue in the body?

Markers of "mesenchymal" stem cells: cell sorting

$CD34^-/CD45^-/CD14^-$ (blood)

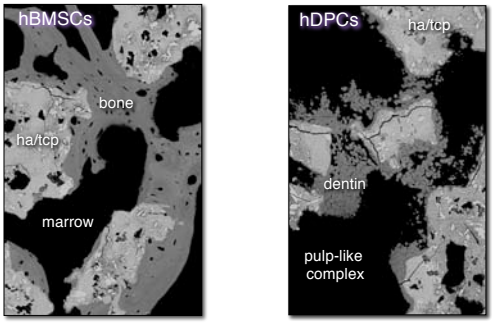
$CD13^+/CD29^+/CD44^+/CD49a^+/CD63^+/CD90^+$
$CD105^+/CD106^+/CD166^+/Stro1^+$
$CD146^+$
(CT, connective tissue markers)

$CD146^+$ cells are CFU-Fs and are pericytes



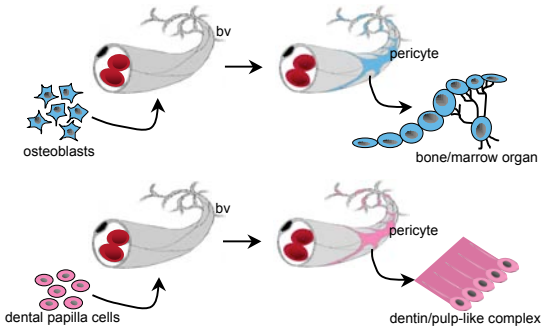
Shi & Gronthos, JBMR, 2003; Sacchetti et al, Cell, 2007

Tissue-specific differentiation in vivo



Gronthos et al., JDR, 2001

Evolution of pericytes as local progenitors

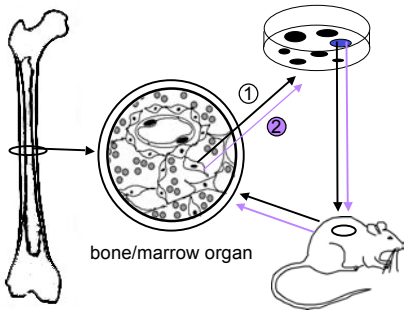


modified from Bianco, et al., Cell Stem Cell, 2008

"Mesenchymal" stem cell: what's in a name?

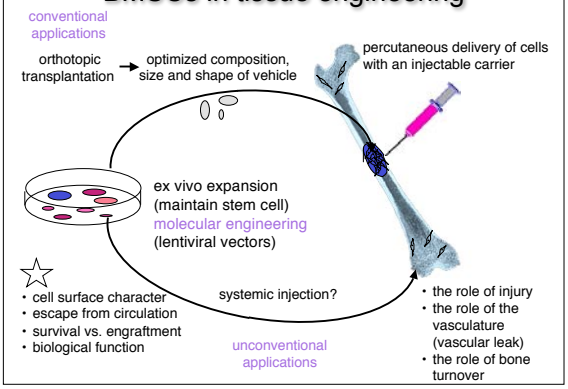
- during development, there is no one single mesenchymal stem cell that gives rise to all connective tissues.
- while clonogenic cells from different tissues are $CD146^+$ pericytes, they are not equivalent in their differentiation potential in vivo.
- therefore, there is no common "mesenchymal" stem cell distributed throughout the body.
- terminology should be based on tissue of origin.

Clonal analysis and self-renewal

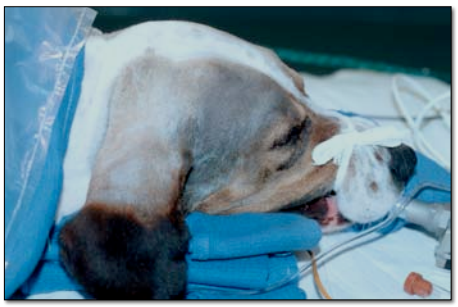


(same assay for other "mesenchymal" stem cells is required)

BMSCs in tissue engineering

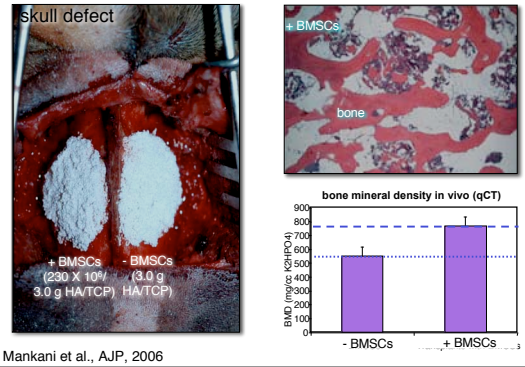


Critical size defects in canines



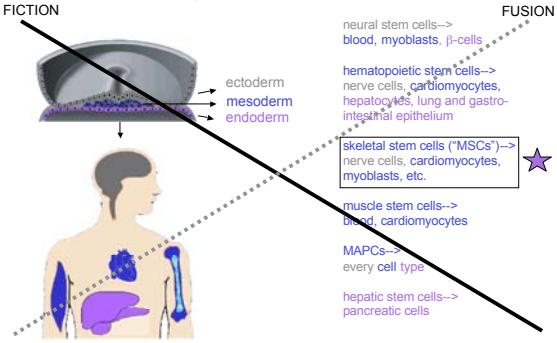
Mankani et al., AJP, 2006

Defects that will never heal on their own

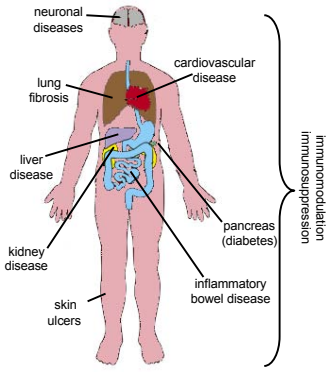


Mankani et al., AJP, 2006

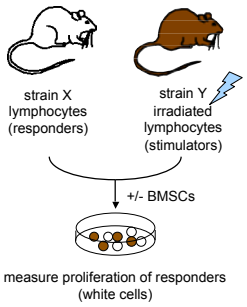
"Transgermal" differentiation: fact, fiction or fusion?

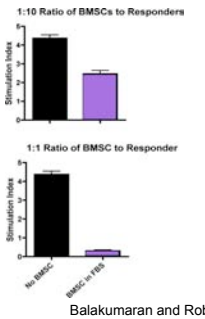


- BMSCs do not persist for long periods of time in sites of disease or injury.
- there is a beneficial effect.
- BMSCs may "nurse" endogenous cells to begin repair ("paracrine bioreactors").
- "stemness" may not be required for the beneficial effect of BMSCs on injured tissues.



Immunosuppression: mixed lymphocyte reaction





Balakumaran and Robey

NIH Bone Marrow Stromal Cell Transplantation Center

Established October, 2008

Mission:

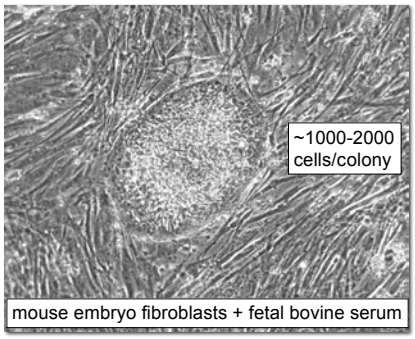
1. To provide investigators with high quality clinical grade BMSCs that are prepared using procedures known to maintain their biological activities.

2. To assist investigators in the preparation of clinical protocols through the development of boilerplate language for the cell portion of the protocol, and in the preparation of FDA INDs.

What we MUST know about stem cells to use them in regenerative medicine

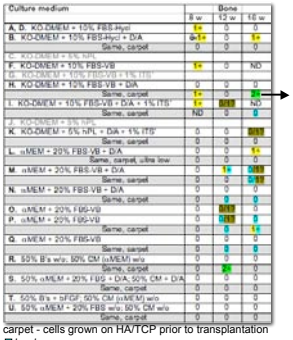
embryonic stem cells, iPSCs	fetal stem cells	post-natal stem cells
• unlimited replication • pluripotency	• extensive replication • multipotency • commitment	• controlled and predictable differentiation

HSF6 hES cell line

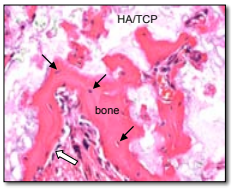


mouse embryo fibroblasts + fetal bovine serum

Osteogenesis of hES cells in vivo



carpet - cells grown on HA/TCP prior to transplantation
teratoma



- need to improve osteogenic differentiation conditions.
- can apply to inducible pluripotent cells (IPCs) in the future.

Kuznetsov

Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors

Cell

Kazutoshi Takahashi,[1] Koji Tanabe,[1] Mari Ohnuki,[1] Megumi Narita,[1,2] Tomoko Ichisaka,[1,2] Kiichiro Tomoda,[3] and Shinya Yamanaka[1,2,3,4,*]

Oct3/4, Sox2, Klf4, cMyc

Sciencexpress Report

Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells

Junying Yu,[1,2]* Maxim A. Vodyanik,[2] Kim Smuga-Otto,[1,2] Jessica Antosiewicz-Bourget,[1,2] Jennifer L. Frane,[1] Shulan Tian,[3] Jeff Nie,[3] Gudrun A. Jonsdottir,[3] Victor Ruotti,[3] Ron Stewart,[3] Igor I. Slukvin,[2,4] James A. Thomson[1,2,3]*

Oct3/4, Sox2, Nanog, Lin28

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BAC Recombineering – Gene Engineering

Douglas Mortlock, Ph.D.

BAC Recombineering – Gene Engineering

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With contributions from Dr. Mark Magnuson, Dr. Trish Labosky, and Jennifer Skelton, Center for Stem Cell Biology, Vanderbilt University School of Medicine

Much of this document was adapted from guidelines created for the Vanderbilt Transgenic Mouse / Embryonic Stem Cell Shared Resource core facility. The TMESC-SR website can be found through these links:

www.vanderbiltresearch.org/
www.vcscb.org/shared_resource/

Significance:

Bacterial Artificial Chromosomes (BACs) are particularly useful substrates for engineering transgenes involving vertebrate genomic DNA. The development of "recombineering" methods for BACs has been crucial to allow this. "Recombineering" refers to any of several similar methods that use homologous recombination in *E.coli* cells in order to engineer desired modifications within BAC clones.

The most widely used applications of this technology are to quickly build (1) targeting constructs that can be used for directing homologous recombination ("knockout") manipulations in mouse ES cells; (2) reporter or "driver" constructs to express desired gene products in transgenic animals or cells; (3) deletion, insertion or point mutation engineering in BACs for sophisticated gene expression/function studies.

The need for BAC engineering is now entrenched. Although BACs are the most practical, readily available source of mammalian DNA clones, their large size makes them impractical to be modified with traditional ligation-based methods. On the other hand, recombineering can be done on clones of any size and allows precise modifications at virtually any location within the BAC. For example, because it has been generally so laborious to build ES-cell targeting vectors by sequential ligation steps, BAC recombineering is now the method of choice to build these constructs (Liu et al. 2003).

Utility of BACs for bone and mineral research: The increased efficiency of BACs for building gene targeting vectors has obvious advantages for those who are interested in creating new genetic mouse models for skeletal research. Since BAC transgenes themselves can often drive gene expression that closely matches in vivo regulation of the gene in question on the BAC, they are particularly useful for driving cell-type specific gene expression with high fidelity. Examples of how BAC transgenes have been used in bone research include the mapping of distant regulatory elements for BMP2, RANKL and SOST (Chandler, R. L. et al. 2007b; Kim et al. 2007; Loots et al. 2005) and creation of fluorescent reporters to tag specific cell types in bone with different colors simultaneously (Maye et al. 2009).

LEARNING OBJECTIVES

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

1. Understand what BACs are and how BAC recombineering can be used.

2. Understand basic workflow and practical issues with using BACs.

A. Bacterial Artificial Chromosomes (BACs) and BAC "Recombineering": A primer

1. What are BACs?

BACs are bacterial cloning vectors that can carry large (150-250 kb or more) inserts of foreign genomic DNA ligated to a 7-12 kb vector. They are propagated in bacteria as circular molecules at 1-2 copies per cell.

2. What is BAC recombineering?

Recombineering is the engineering of BAC clones by homologous recombination. This is performed inside bacterial cells, using *E.coli* cells that have been engineered to permit homologous recombination, via controlled expression of the analogous Red or ET protein systems. This enables insertion, deletion or replacement of DNA sequences in the BAC, or subcloning (retrieval) of fragments out of the BAC into plasmids. Since homologous recombination is used, the engineering is not limited by location of restriction sites used for traditional ligation steps.

3. Why use BACs?

a. BAC library resources: Excellent BAC libraries exist for the genomes of mouse, human and other species. BAC clones that span a gene of interest

can usually be quickly identified on the web, using genome browsers (see below). BACs containing mouse DNA from the C57/BL6 strain or the 129/Sv strain can be identified this way. If you can identify a BAC clone on a genome browser, you can usually buy a bacterial glycerol stock of the clone from an outside vendor. BAC DNA can be engineered purified using methods that are accessible to most labs that use routine molecular cloning methods.

b. Specific uses of BACs: BACs are primarily useful for **(1) building targeting vectors** for ESC-mediated gene targeting experiments, or **(2)** generating transgenes intended for **pronuclear injection**. In the case of building targeting vectors, BACs can be very useful for obtaining the homology arms that are needed for proper targeting of vectors in ESCs. Also, by using recombineering methods many steps in building the targeting vector can be done using the BAC itself as a scaffold. This can eliminate many of the often-difficult ligation steps needed to make targeting vectors.

c. Regulatory fidelity. In the case of pronuclear injection, BACs can be very useful for making transgenes that drive expression of desired genes (e.g. CRE, LacZ, GFP). This is primarily due to the large size of BACs as compared to traditional plasmid-based transgenes. Because of the large size of BAC inserts, they are more likely to contain all cis-regulatory elements for the gene in question. Unlike smaller transgenes, which are fairly prone to position-mediated silencing and ectopic expression following transgene integration, BAC transgenes tend to be more resistant to these problems.

4. **What are key issues to know about using BACs?**

BACs are large as compared to most standard plasmid cloning vectors (150-250 kb vs. 3-15 kb). Therefore, purified BAC DNA is more susceptible to shearing and enzymatic degradation than most plasmids, so not all methods and prep kits that work for plasmids can be used for BACs. However, standard methods and kits for BAC preps are widely available. They are low copy vectors, so the DNA yield is low compared to high-copy plasmids.

B. How to use BACs

1. Recombineering methodologies: an overview.

"Recombineering" refers to various methods that all use homologous recombination in *E.coli* to engineer modifications into vectors (primarily, BACs). Of these, the most widely used systems are based on controlled expression of proteins that permit homologous recombination at reasonable efficiencies. These allow recombination through homologies

as small as 42 bp. Since most *E.coli* strains do not have this capability, the methods require either the use of engineered strains in which the recombination genes have been inserted into the *E.coli* chromosome (e.g. Neal Copeland method), or alternatively, the recombination genes are temporarily transferred into the cells via a plasmid vector (e.g. Francis Stewart method). In either case, the recombination proteins are under inducible control so they are not constitutively expressed. They are induced transiently to permit recombination, and the desired recombinant clones are usually identified with antibiotic selection. For the Copeland method, a very useful website exists that decribes available protocols, reagents and instructions for requesting reagents:
http://recombineering.ncifcrf.gov/
Descriptions have been published on how to use these tools to engineer BACs (Copeland et al. 2001; Lee et al. 2001; Warming et al. 2005) and for making knockout vectors (Liu et al. 2003).

2. **Constructing gene targeting vectors using BACs.**

Vectors for ESC-mediated gene targeting need to have several components: sufficiently long homology arms to the target locus, positive and negative selection cassettes, and often other components such as LoxP and FRT sites. Recombineering frees the researcher from needing to depend on restriction sites and ligations for cutting and pasting these together in vitro. Essentially, recombineering allows direct subcloning of a targeting fragment from a BAC into the plasmid in order to build the targeting vector. Recombineering can be used either before or after this step (that is, to modify the BAC first, or the plasmid subclone) to insert selection cassettes, LoxP sites, or other modifications into the targeting fragment, depending on your specific design goal. Articles describing these approaches have been published (Chan et al. 2007; Liu et al. 2003).

A typical work-flow for building targeting vector from a BAC might be as follows:

a. Transfer BAC into strain permitting homologous recombination.

b. Subclone a segment of the BAC containing the targeting cassette into a plasmid that contains a negative selection vector (e.g. HSV-Tk).

c. Recombine other components as needed, e.g. PGK-NeoR, FRT or LoxP sites, into desired locations in the BAC. LoxP-flanked and FRT-flanked Neo cassettes exist to facilitate building many types of vectors.

Note, the precise order of recombineering steps and whether they occur before or after subcloning from the BAC may vary depending on your design strategy.

3. **Pronuclear injection of BACs**

BAC DNA can be successfully used for pronuclear injection. BAC DNAs are usually injected at 0.5-1.5 ng/µl concentration, which is slightly lower than what is typically used for plasmid DNAs (~3 ng/µl). The lower concentration helps reduce needle clogging problems that are more common with injecting BAC DNA.

BAC DNAs can be injected as uncut, circular molecules or linearized and both have been used successfully to make transgenic mice. For both BACs and plasmid DNAs, a rapid process of breakage, religation and recombination between transgene isomers occurs that tends to generate concatamers of transgene DNA before integration into a random genomic location (Bishop and Smith 1989). This is often sufficient for the scientific goals of the project. While plasmid transgenes are almost always injected as linear fragments, BACs may be injected as circular or linear molecules with similar integration frequencies. Most BAC vectors have *Not*I sites near the vector/insert junctions, so many mammalian BAC inserts can be cut free of the BAC vector using NotI. For projects where the insertion of single, linear, intact BACs is absolutely required, linearization and purification steps may be needed.

A typical workflow of recombineering and injecting BACs might be as follows:

a. Transfer BAC into strain permitting homologous recombination. Alternatively, using the BAC host cells, introduce a plasmid that confers inducible homologous recombination activity.

b. Recombineer the BAC as desired.

c. Purify BAC DNA by cesium chloride gradient or BAC column kit (CsCl method is strongly recommended.)

d. Verify concentration and integrity of purified BAC DNA by agarose gel analysis.

e. Dilute BAC DNA prior to injection.

The Mortlock lab has published a BAC DNA purification protocol that has been reliably used to generate transgenic mice (Chandler, K. J. et al. 2007a; Chandler, K. J. et al. 2009; Chandler, R. L. et al. 2007b).

C. Considerations for selecting BACs and handling BAC DNA

1. **Gene structure issues:** Most, but not all mammalian genes can fit entirely within BACs. Existing mouse and human BAC libraries have fairly deep

coverage and at least some clones exist for almost every region of the genome without gaps. However, individual BAC clones will vary in the extent of 5' and 3' coverage around a gene of interest. Genome browsers (see below) are invaluable for selecting BAC clones that best match your needs. BACs can contain multiple genes, so if the BAC is intended for pronuclear injection, over-expression of linked genes may have undesired phenotypic effects or even lethality. In this case, recombineering may be used to trim other genes out of the BAC. Alternatively, a BAC with a closer break-point might be a better choice.

2. **Fragility:** DNA molecules that are over 30-50 kilobases are much more prone to shearing and degradation than smaller molecules. Standard techniques for plasmid DNA handling such as vortexing or repeated pipetting to dissolve DNA pellets are not appropriate for BACs and will shear the DNA. Minimize pipetting steps and use wide-bore tips when possible. Keep purified BAC DNAs on ice or at 4˚C. Store at 4˚C and avoid freeze-thawing, which will cause shearing.

3. **Validation:** BACs can usually be propagated very stably. However, keep in mind you are trying to engineer the BAC clone. PCR is usually not sufficient to verify completely that successful engineering has occurred for two reasons: first, false positives can occur if the clone is not truly clonally pure (i.e. a mixture of recombineered and unmodified cells); second, PCR usually can't tell you if your BAC is still intact. Restriction digest of BAC DNA to observe predicted alterations, and lack of unwanted rearrangements, is highly recommended. Prior to pronuclear injection, BAC DNA should be run on a pulsed-field gel to verify it is intact and of correct size. Standard agarose gel electrophoresis cannot distinguish well between intact and sheared BAC DNA.

4. **Purification Kits:** Standard plasmid DNA prep kits usually cannot purify intact BAC DNA. This is because almost all kits use DNA-binding columns with wash buffers optimized for low-molecular-weight plasmid DNAs. Different buffers are needed for large molecules such as BACs, so use BAC purification kits made for this purpose. However, standard alkaline lysis / ethanol precipitation is fine for isolating BAC DNA in sufficient amounts for simple PCR and gel analysis.

5. **Direct BAC sequencing** is best performed on BAC DNA made by cesium chloride method or BAC prep kits. Note, for sequencing, shearing is not as big a concern as purity.

D. How to locate BACs on genome browsers:

Several mouse, human and other BAC libraries have been deeply end-sequenced, meaning that single sequence reads were generated from both ends of the insert for most clones. If both end sequences for one clone can be aligned to unique positions in the genome so that they are "facing each other" and within a few hundred kilobases, their aligned positions probably reflect the actual ends of the BAC insert. These predictions can be displayed on genome browsers. Most of them are right, but when you get your BAC it is important to check that it contains your gene by PCR.

1. **C57BL/6 strain BACS:** The **UCSC genome browser** has extensive BAC maps for the *Mus musculus domesticus* (C57BL/6 strain) BAC libraries RPCI-23 and RPCI-24. To visualize them on the browser:

   a. Log on to UCSC genome browser gateway page
   http://genome.ucsc.edu/cgi-bin/hgGateway

   b. Select mouse genome and search for your gene or genomic region of interest

   c. On the browser window page, scroll down below window to "Mapping and sequencing Tracks". Click and change the BAC end pairs pulldown tab setting to "full".

   d. Click the "refresh" button. BAC positions should be visible. BACs from the C57BL/6 strain will have the prefix "RP23" or "RP24". Note, BACs with the prefix "MSMg01" are from a subspecies *Mus musculus molossinus* BAC library.

2. **129S7 strain BACs:** The **Ensembl genome browser** has BAC maps for the bMQ library that was made from this strain. See part I.2.b above for a detailed description on how to locate a BAC for your gene.

3. **Where to get BACs**

a. *Mus musculus domesticus* (C57/BL6 strain) BAC libraries RPCI-23 and RPCI-24:
Weblink to: BACPAC Resources Center (BPRC)
http://bacpac.chori.org/
(The BPRC is at the Children's Hospital Oakland Research Institute (CHORI), Oakland, CA.)

b. Mus musculus domesticus (129S7 strain) BAC library bMQ
Weblink to: *Geneservice, Ltd.*

http://www.geneservice.co.uk

c. *Mus musculus domesticus* (129S6 strain) BAC library RPCI-22: Clones and library filters are available from the BACPAC Resources Center (BPRC). Clones are also available from Invitrogen (call to inquire about availability of pooled plate DNAs to facilitate screening the library by PCR).

Note: Invitrogen and Open Biosystems also provide clones from the mouse CITB/CTB BAC libraries; however, these are derived from the 129SV substrain, which has been shown to be genetically contaminated with DNA from a non-129 strain. In other words, clones from this strain may or may not be isogenic to 129S6 or 129S7 ESCs. Therefore it is risky to use these to make targeting vectors.

4. **A description of BAC clone nomenclature can be found at**:

http://www.ncbi.nlm.nih.gov/genome/clone/nomen.html

E. Web Links

Very useful NCI web site with information, protocols and instructions for requesting reagents:

http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx

UCSC genome browser gateway page:

http://genome.ucsc.edu/cgi-bin/hgGateway

Ensembl genome browser:

http://www.ensembl.org

BACPAC Resources Center (BPRC)

http://bacpac.chori.org/

Geneservice, Ltd.

http://www.geneservice.co.uk/home/

F. Protocols and Reagents

Protocols and reagent request forms for Copeland method strains can be found at http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx

A kit with reagents for the Stewart recombineering method can be obtained from Genebridges at http://www.genebridges.com/

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How To Best Define Mineral Properties

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How to Best Define Mineral Properties

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Significance of the Topic:
Bones and teeth and many pathologic mineralized deposits in soft tissue are composites of a mineral phase (hydroxyapatite) and an organic matrix (consisting predominately of collagen). The mineral increases the strength of the composite, and the way it does so depends on how much mineral is present, how the mineral crystals are arranged and distributed, how many impurities the mineral contains, and how the mineral crystal composition differs from site to site. There are numerous methods available to determine each of these mineral properties, but the choice of method must be determined by the question being asked, the material available for assay, and the accessibility of these techniques.

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

(1) Know what the mineral phase in bones, teeth, and pathologic deposits have in common, and how they differ.

(2) Recognize why defining mineral properties is important.

(3) Decide what techniques for mineral analysis would be most suited to their questions and the materials they have available, and where to go to get such analyses performed.

Outline:

I. What is the mineral of bones, teeth, and pathologic deposits? How do the properties of the mineral in these tissues compare?

II. How mineral properties affect fracture risk? Density, Geometry, Composition, Size, Orientation

A. Osteopetrosis – fragile bones of high BMD and small crystal size

B. Osteogenesis Imperfecta – fragile bones with low BMD and smaller crystals

C. Osteoporosis – fragile bones associated with altered crystal size and loss of heterogeneity

D. Kidney disease

– fragile bones associated with oxalate crystal accumulation in bone and kidney

- renal osteodystrophy

– decreased carbonate content reflects high turnover

III. What properties of the mineral are important?

A. To Clinicians

B. To Engineers

C. To Biologists

IV. Methods appropriate for different research questions – Advantages, Limitations, Sources

A. Radiographic methods

Densitometry

Micro-Computed Tomography

pQCT, HR-QCT, ex vivo & in vivo micro-CT

B. Diffraction Methods

XRD, SAD, TEM, dark field TEM, SAXS

C. Chemical methods
    Ash density
    Compositional analysis
D. Imaging Methods
    qBEI NMR
    SEM – HR field emission SEM
    FTIRI Raman
E. Modeling and Prediction
    FEM or HR-CT or microCT

Disclosures

Dr. Boskey is the director of the NIH-Sponsored Musculoskeletal Repair and Regeneration Core Center at the Hospital for Special Surgery which provides micro-CT, mechanical testing, infrared- and in vivo molecular imaging. She also serves on the Scientific Advisory Board of Skelscan – which is developing P31-NMR for clinical applications.

CHARACTERIZATION OF BONE MINERAL PROPERTIES

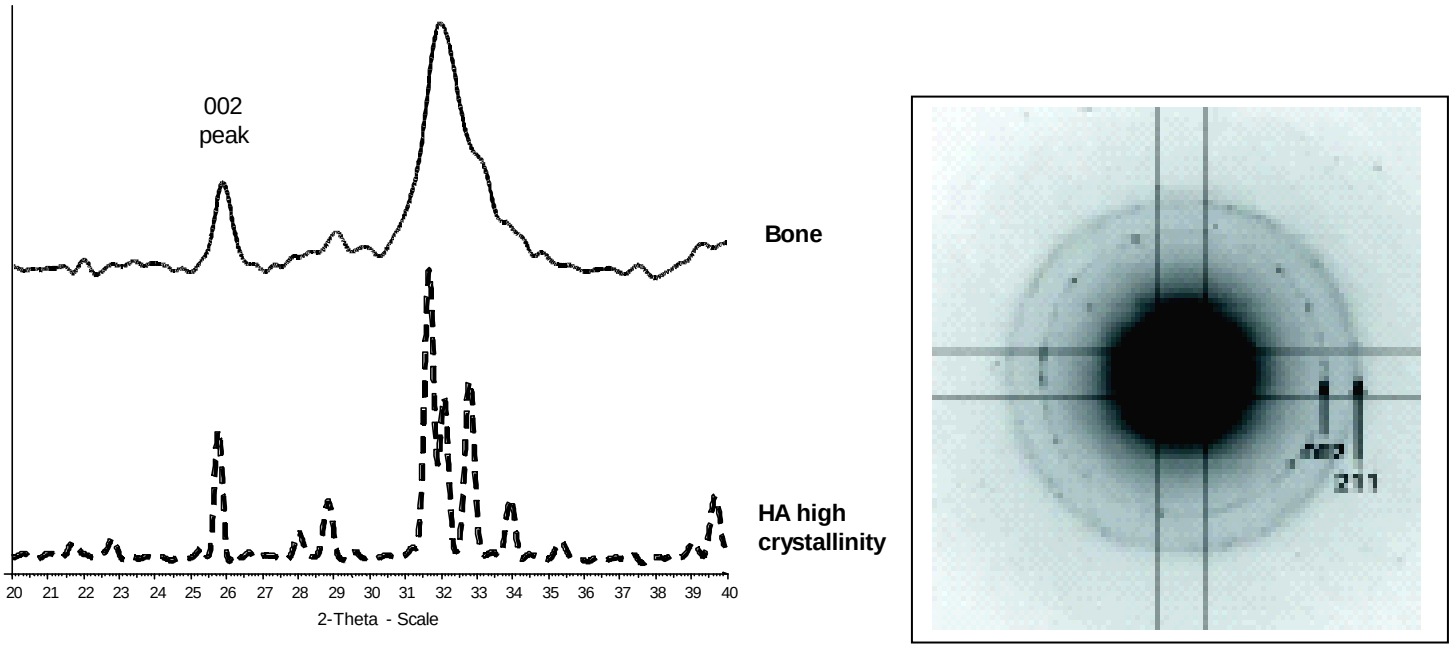


Fig 1: Diffraction Methods - Powder Xray diffraction –width of peaks inversely proportional to crystal size and Selected Area Diffraction (TEM)

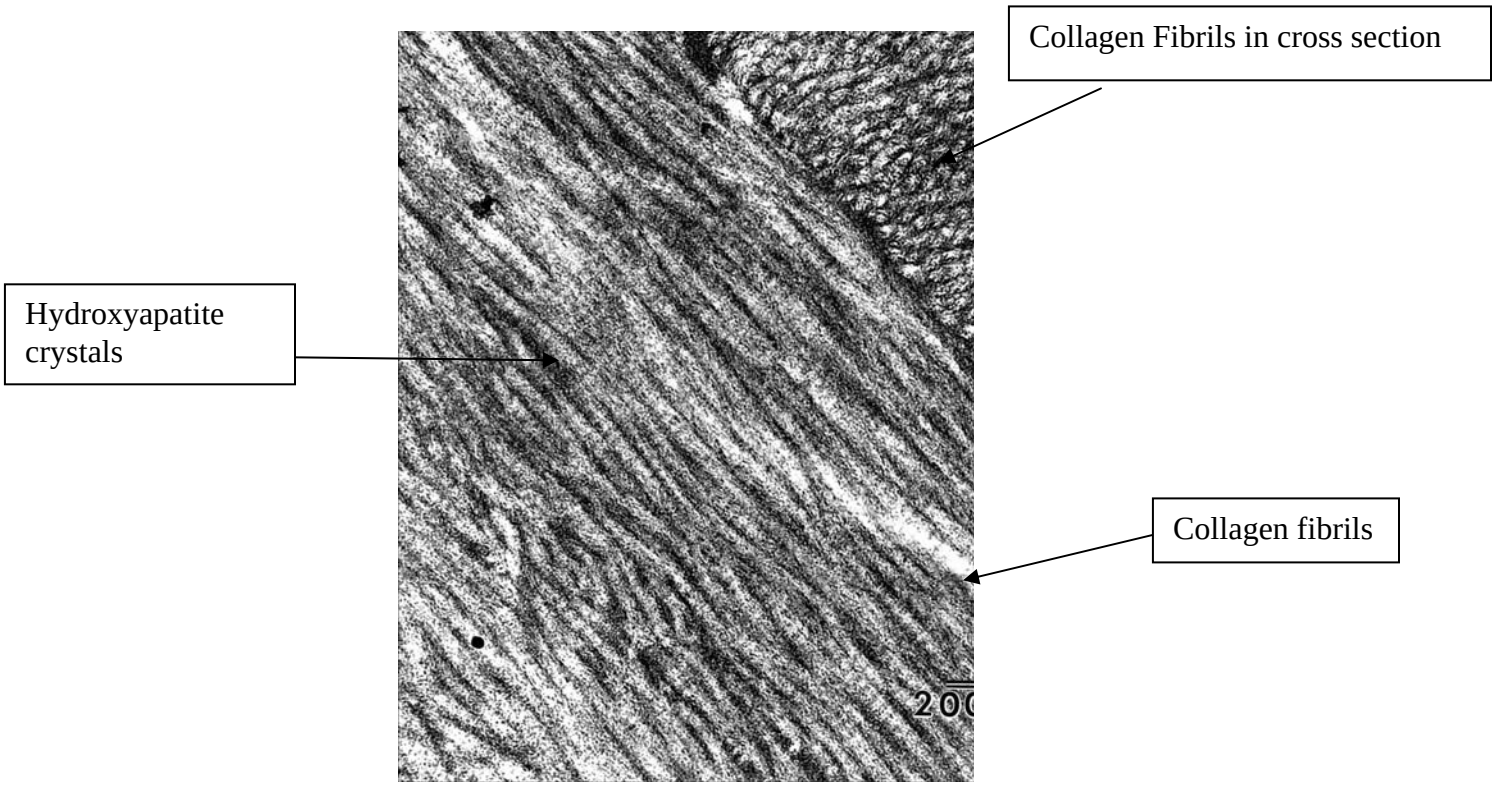


Figure 2: TEM shows alignment of HA crystals – dark field could be used to measure sizes

AFM measurements of mature bovine bone – Eppell et al, JOR, 2001*

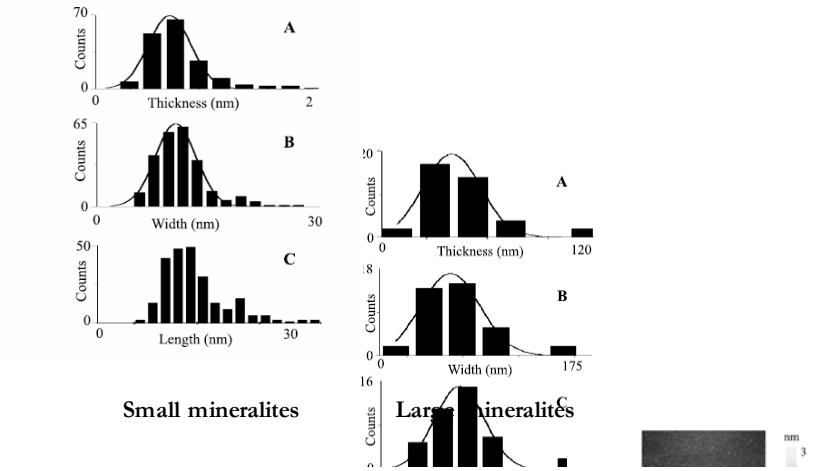
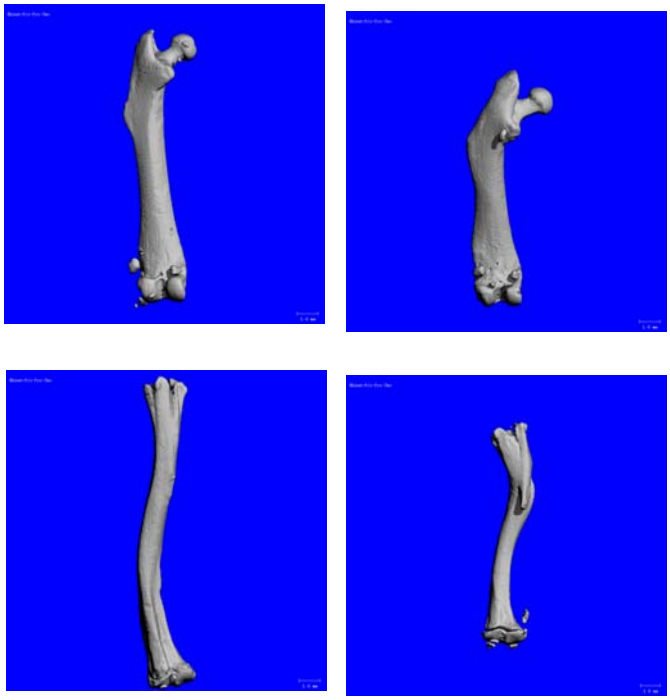
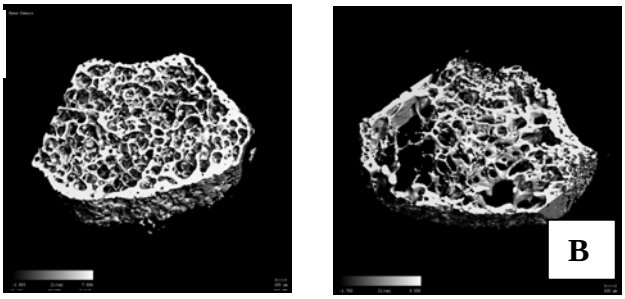


Figure 3: actual crystallite sizes and their distribution can be measured. Reprinted with permission from Journal of Orthopaedic Research, Wiley, License Number: 2470351229627

Figure 4 – Micro-computed tomography analysis of bone from mouse models of osteogenesis imperfecta



Femur and Tibia of WT (left) and fro/fro Mice (6 mo)



Effects of alendronate treatment in oim/oim mice cancellous bone. A) WT , B) oim/oim; oim/oim + ALN (Raggio et al., unpublished)

u-CT parameters for Cancellous bone:

BV/TV; BMD;
Tb.N (trabecular number); Tb.Th (trabecular thickness); Tb.Sp (trabecular separation); BS/BV (bone surface/bone volume)

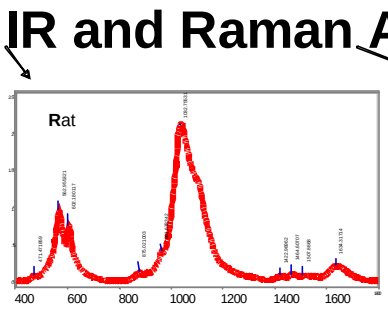
Additional: **Connectivity density; SMI (structural model index)**

u-CT parameters for Cortical bone:

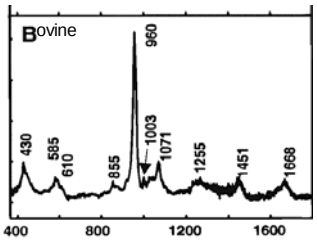
BV/TV (bone volume fraction);
BMD (bone mineral density);
Ct.Th (cortical thickness);
Moments of inertia:
pMOI (polar moment of inertia);
Imin; Imax;
BA(bone area); TA(total area); BA/TA;
Porosity=(1-BV/TV)

Additional : **Outer P and Inner P (P - perimeter)**

Figure 5 – Spectroscopic Analysis

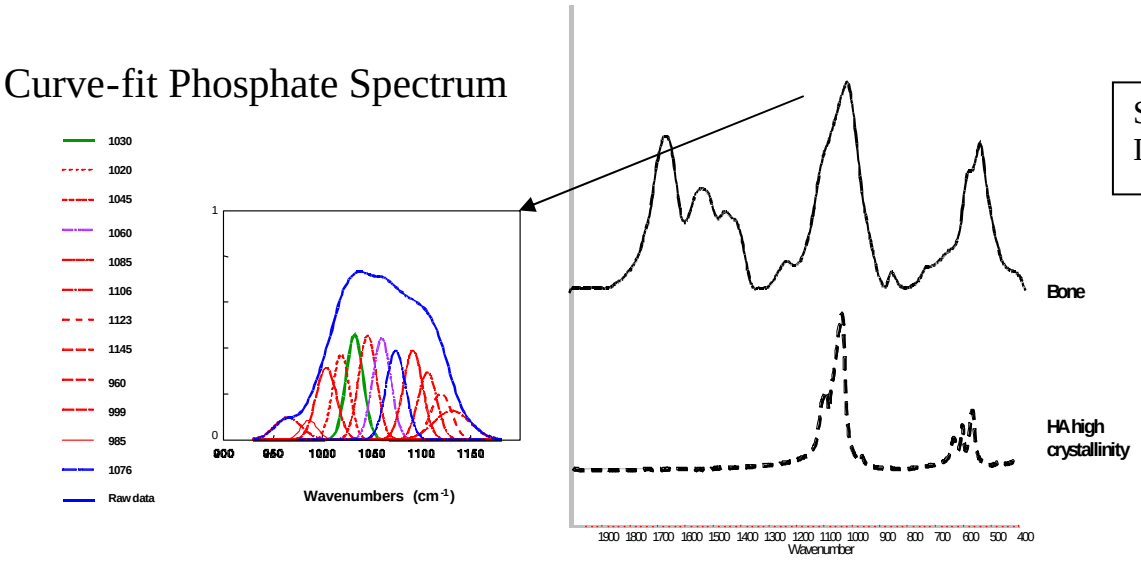


960 ν_1 PO_4 symmetric stretch
982,985 PO_4 in HA
999-1004 ν_3 PO_4 asymmetric stretch
1020 ν_3 PO_4 asymmetric stretch non -stoichiometricHA
1030 ν_3 PO_4 asymmetric stretch stoichiometric HA
1045 ν_3 PO_4 asymmetric stretch - CO3 substitution
1055-60 ν_3 PO_4 asymmetric stretch
1075-76 ν_3 PO_4 asymmetric stretch
1096 ν_3 PO_4 asymmetric stretch
1112 ν_3 PO_4 asymmetric stretch, Immature HA (HPO_4)
1116 ν_3 PO_4 asymmetric stretch, mature HA
1123-27 ν_3 PO_4 asymmetric stretch
1145 Acid phosphate

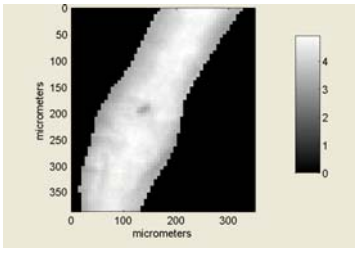


Raman frequency shift (cm^{-1})	*Assignment (intensity)*
580	PO_4^{3-} ν_4, (m)[a]
855	Proline ring (w)
876	Hydroxyproline ring (w)
950–964	PO_4^{3-} ν_1 (P—O symmetric stretch; vs)[a]
1001–1003	Ring breathing mode of phenlyalanine (m)
1030	PO_4^{3-} ν_3 (P—O asymmetric stretch; m)[a]
1065–1070	CO_3^{2-} ν_1 (C—O in plane stretch; m)[a]
1245, 1270	Amide III, C—N—H stretch (m)
1445	CH_2 wag (m)
1665	Amide I, C—C—N stretch (m)

FTIR spectra



Spatially resolved FTIR Image of Mineral/matrix



Defining Bone Mineral Properties				
Method	**Information Obtained**	**Bone Sample Needed**	**Limitations**	**Ref**
Ash weight	Mineral and water contents	~10 mg	Destructive; no information on other properties	2,5
AFM	Crystal size & distribution Surface Topology With chemical probe can identify species	Thick section	High resolution - analyses of multiple regions needed to obtain global information	19
Elemental Analysis Chemical Electron probe micro analysis	Elemental composition	Powder or ash (thick section for EPMA)	No phase information, no crystal size or orientation information	3,19
DEXA	2D bone mineral content /Geometry	In situ	Low resolution; no information on other properties	1
FTIRI	Mineral content, collagen maturity, carbonate and HPO_4 substitution, crystallinity at ~ 7um spatial resolution	Thin section	Cannot be done without biopsy	3,4,6, 9,10,11,20
Histochemistry von Kossa Alizarin Red Fluorescent dyes	Distribution of phosphate (and other anionic) and calcium and other cationic species, respectively.	Cultures, histology slides	No information on phase or crystal size Artifacts due to nature of reaction Dyes expensive	19,20
QCT pQCT HR-QCT	Mineral content, architecture & geometry	In situ	No information on composition, affected by presence of heavy metal ions	7,18
NMR Proton P31	Bone density, water content, architecture Distribution of phosphate species	In situ	Resolution low	1,12,13
Micro-CT	Tissue Mineral content, geometric and	(small sample for	No information on nature of mineral	8,18

	architectural properties	ex vivo measures; small animals for in vivo)	phase or size of mineral crystals	
qBEI (BSI)	BMDD – bone mineral density distribution – mean Ca content	Thick Section	Data affected by presence of heavy metal ions	3
Raman Microscopy	Mineral content, collagen maturity & orientation, carbonate substitution, at ~1um spatial resolution	Thin or thick section –in situ in development	Difficult to characterize mineral crystal size ; sensitive to orientation (polarization)	9,17
SAXS	Mineral particle sizes/ particle orientation	Thick section	Requires assumptions about particle distributions	14,15
TEM	Crystal size; crystal orientation ; phase composition (selected area diffraction)	Thin section	Small fields characterized – need multiple sections	16,19
XRD	Phase composition; crystal and particle size; presence of impurity phases	Powdered bone	No information on organic material, destructive	5

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Tutorial on Using Human Tissue for Research

Julie Glowacki, Ph.D.

TUTORIAL ON USING HUMAN DISCARDED BONE, MUSCLE, AND MARROW FOR BASIC AND TRANSLATIONAL RESEARCH

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There are no conflicts-of-interest in this topic; research was supported by the NIH.

Significance of the Topic: Some experimental models are limited by having un-validated or poor "connectivity" to human physiology or pathophysiology. Hypotheses generated with animal models, mutant mice, or immortalized cells *in vitro* can be tested for applicability with non-malignant human tissues, available as surgical discarded tissues. Unfixed fresh discarded tissues can be considered a precious resource for novel research; proper safeguards must be in place to avoid violating patient privacy. With care taken to include demographic information, comorbidities, and medications, discarded surgical tissue can be used for bone research.

Learning Objectives: This tutorial concerns research with excess human materials (discarded tissue) with either de-identified (anonymized) or enrolled (consented) protocols. Guidance will be given on teamwork needed for access to clinical material and data. Examples of discarded tissues that have been studied include bone, knee cartilage, marrow from joint arthroplasty, muscle, skin and gingival fibroblasts, and pediatric ribs and iliac crest. Tissues can be obtained for research if discarded during surgery, if consented to, e.g. a biopsy or blood, or if encountered during autopsy from preconsented subjects. We have shown that properties of discarded tissue reflect many clinical features of the subject from whom the tissue and cells were obtained, for example, age, gender, vitamin D status, comorbidities, and medications.

Research with precious human biospecimens should involve

- "Connectivity" to an important clinical question
- Feasibility
- Need for genuine collaboration
    - Bedside-to-bench-to-bedside teamwork
    - Assistance from Operating Room staff for researcher to procure specimens
    - Some hospitals have Biorepositories that release blood, sera, plasma that is in excess of that needed for diagnostics.
- Access to clinical material and data
    - Age, gender, diagnoses, medications.
    - Discarded tissue or specimens obtained solely for research
    - Logistics
- Protection of Privacy: Informed consent, waiver of consent

Levels of Research with Excess Human Materials, IRB Requirements, and Research Limitations

Levels of Research/Review	***Excess Materials: Tissue, Blood***	***Human Subject Research***	***Consent***	***Access to Clinical Information***
1	Yes	No	No	No
2	Yes	Exempt from IRB review (Exemption #4)	No	Limited access; deidentified; can never go back to the record
3	Yes	Yes Annual IRB review	Waiver of Consent (8 criteria must be met)	Can access, record, and reaccess medical data; coded to protect identification
4	Yes	Yes Annual IRB review	Informed consent	Interact with patient: questionnaires; additional tests, blood, tissue; follow-up

Two types of studies with discarded human tissue

Deidentified	**Consented**
Limited amount of clinical information: age, gender, all Dxs, Meds	**Additional tests, questionnaires, follow-up**

To get started~

Research study staff are required to have training and regular retraining on the protection of human research subjects. Many institutions accept the web-based Collaborative Institutional Training Initiative (CITI) program. https://www.citiprogram.org/

Advise one global protocol for surgical discarded, deidentified tissue. Each protocol requires submission documents and approvals, amendments to the protocol, changes in study staff, changes in funding, due dates for annual reports, etc. Example of a protocol title: "Biology of Human Skeletal Cells in Vitro".

How to identify specimens for research. Someone must have authority/password to access and scan the OR schedule on a regular basis. Must have a way to request that the OR staff transfer the discarded tissue from specific subject to research refrigerator for pickup by research lab. Research refrigerator should be distinct from Pathology refrigerator. Someone must have authority/cardkey to access the OR research refrigerator.

Inclusion Criteria Discarded marrow from joint arthroplasty for specific condition, age range, gender.

Exclusion Criteria Exclude infectious diseases; inflammatory diseases; aseptic necrosis (avascular necrosis). Consider whether you wish to exclude (or include) subjects with features that may influence skeletal metabolism, for example:

~ premature menopause, primary hyperparathyroidism, hypercalciuria, hyperthyroidism, Paget's Disease of bone, rheumatoid arthritis, cancer within 5 years, diabetes, fracture due to high-impact trauma, renal insufficiency, alcoholism, active liver disease, malabsorption, ankylosing spondylitis, morbid obesity,

~ the use of medications such as anti-osteoporosis drugs, >2000 IU/day vitamin D, or glucocorticoids.

Dr. Glowacki's Experience with Discarded Biospecimens

Cell Type	Surgical Procedure	Selected References
Chondrocyte	*Pectus excavatum*	1, 2
Chondrocyte	Knee Arthroplasty	3
Bone marrow; Bone	Hip, Shoulder arthroplasty; Iliac crest graft leftover from cleft palate repair	Osteoclastogenesis 4-7 Osteoblastogenesis 8,9 Adipocytogenesis 10,11
Dermal fibroblast	Circumcision (newborn and adult); Gingiva from molar extraction	12,13
Joint ligament	Arthroscopic surgery	
Muscle	Shoulder arthroplasty	

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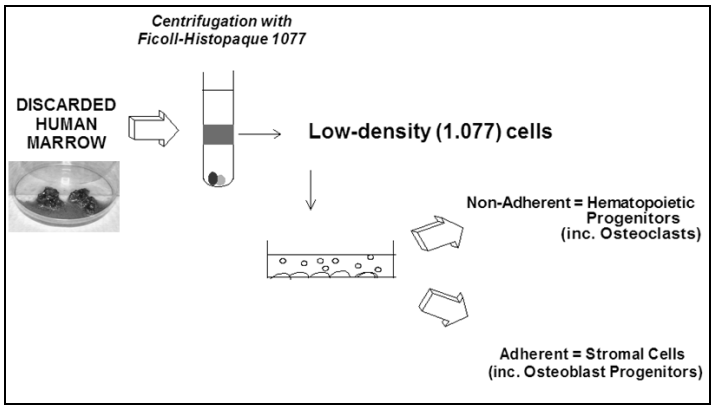
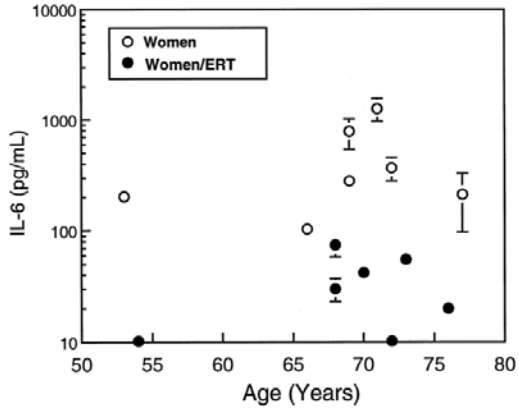
Human marrow was obtained as discarded tissue during the course of orthopedic surgery. We enrich for low-density, undifferentiated cells by centrifugation with a solution that has density of 1.077.

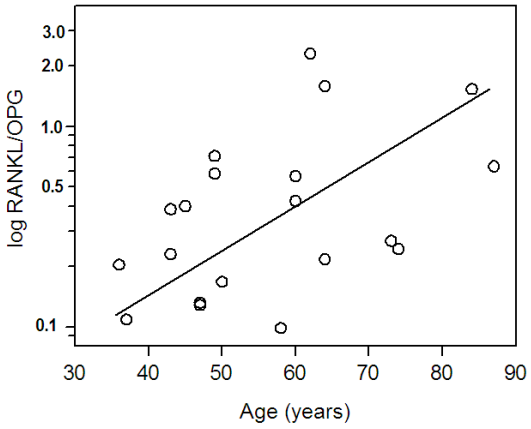


Examples of *in vivo/in vitro* studies.

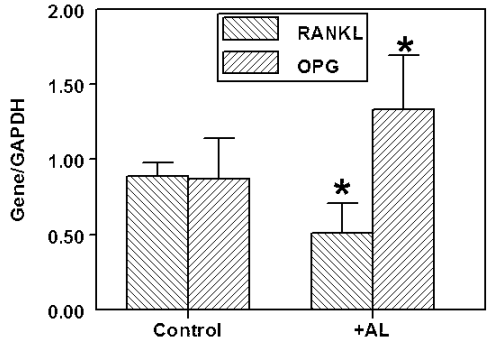
In vitro IL-6 secretion by MSCs from women receiving Estrogen Replacement Therapy was 7.5% of that by MSCs from age-matched controls. [4]



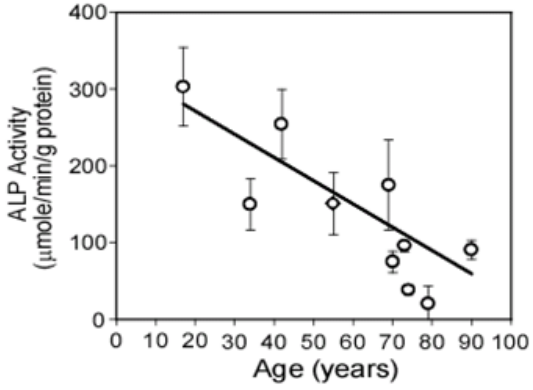
Effect of subject age on ratio of RANKL-to-OPG expression by hMSCs. [6]



Comparison of RANKL and OPG expression in marrow obtained from 5 post-menopausal women taking alendronate (+AL) and 5 age-matched controls. [8]



Effect of subject age on osteoblast differentiation of hMSCs, as measured by alkaline phosphatase activity after 2 w in osteogenic medium. [9]



Chronic Kidney Disease and Osteoporosis: How to Diagnose It, How to Treat It and When Is Bone Biopsy Indicated?

Sophie Jamal, M.D., Ph.D.

Osteoporosis in Patients with Impaired Renal Function: To Treat or Not to Treat?

Meet The Professor ASBMR 2010
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Objectives

- Review the epidemiology of fractures in patients with CKD – a specific focus on osteoporosis
- Review the etiology of fractures
- Review the methods to assess fracture risk
- Outline treatment options for patients with fracture and CKD

Fractures in Patients with CKD

- Increased hip fracture risk with worsening renal function:
  - Data NHANES: eGFR < 60 ml/min: OR = 2.12 (1.18 to 3.8) **(Nickolas TL et al J Am Soc Nephrol 2006)**
  - Data from SOF: eGFR 45-59 ml/min: HR = 1.57
  eGFR < 45 ml/min: HR = 2.32 **(Ensrud et al Arch Int Med 2007)**
- Patients with stage 5 CKD:
  - Up to 50% prevalence of fractures
  - Up to 50% excess mortality
  - Fractures occur at least 10 years earlier

The Dilemma

Q: I have a dialysis patient with a **hip fracture** and a **T-score of - 4.0**. What drug should I prescribe?

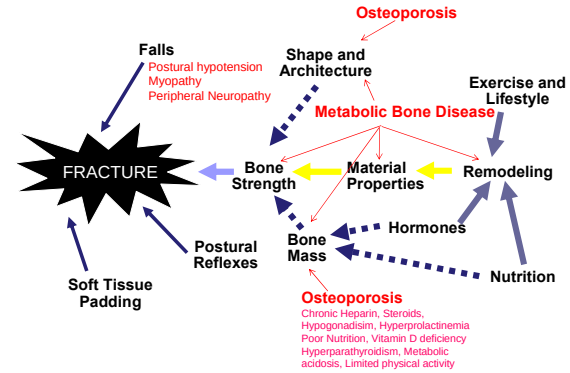
A: What disease do they have?

KDIGO: CKD – Mineral and Bone Disorder

- A systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following:
  - Vascular of other soft tissue calcification
  - Abnormalities of calcium, phosphorus, PTH or vitamin D metabolism
  - Abnormalities in bone turnover, mineralization, volume, linear growth or strength

Moe et al. KI. 2006.

Fractures are Multifactorial



Adapted from Heaney RP, *Bone.* 2003;33:457-465.

Diagnosing Renal Bone Disease

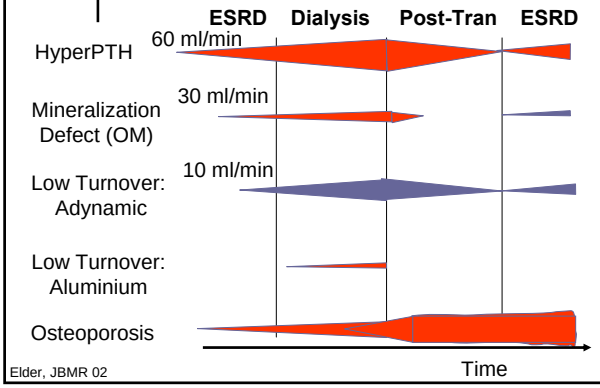
- Quantitative bone histomorphometry
- Classification based on turnover and mineralization:
  - *Hyperparathyroid bone disease*
  - *Osteomalacia*
  - *Adynamic bone disease*
  - *Mixed bone disease*

NKF K/DOQI Guidelines AJKD 2002

Adynamic Bone Disease

- Increasing prevalence
  - 1973: 0%
  - 1993: 49%
- Consequences of ABD
  - Hyperphosphatemia - mortality
  - Fractures ?
- Etiology
  - Vigorous suppression of PTH
  - CKD decreases anabolic activity at skeleton
    - Decreased BMP-7, Wnt
    - Decreased calcitriol
    - Increased phosphate, increased FGF23

Bone and Mineral Disorders in ESRD and After Transplantation



Limitations of Histomorphometry

- Invasive
- Specialized expertise
- Costly
- Histology may be “fluid”

Identifying the Type of Bone Disease is Critical

- Different bone diseases have different treatments
- BP can make adynamic bone disease worse

Exceptions to the Biopsy Rule?

- When certain there is no adynamic bone disease
  - Prevalence is felt to be low before stage 4 CKD
- If ruled out hyperparathyroidism/osteomalacia
  - 25 hydroxy vitamin D level
  - Serum PTH
- Then WHO criteria and/or the fragility fractures can be used to diagnose osteoporosis in Stages 1-3 CKD

What Do We Do in Stages 4/5 CKD?

- Markers of mineral metabolism - PTH and alkaline phosphatase
- Bone mineral density (BMD) by DXA
- Peripheral quantitative computed tomography (pQCT)

Profiling by PTH Levels

Disorder	Serum intact PTH Levels (pg/mL)
Hyperparathyroidism	
Mild	200-400
Moderate	350-800
Severe	> 700
Aluminum Bone	10-500 (mostly < 100)
Adynamic Bone	< 100-150
Osteomalacia	Normal/mildly elevated

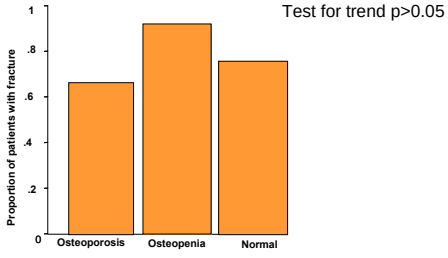
Miller P and Shane E 2004

Profiling by BSAP Levels

Disorder	Serum intact BSAP Levels
Hyperparathyroidism	
Mild	Normal
Moderate	Normal to elevated
Severe	Elevated
Aluminum Bone	Normal
Adynamic Bone	Normal to low
Osteomalacia	Mildly elevated

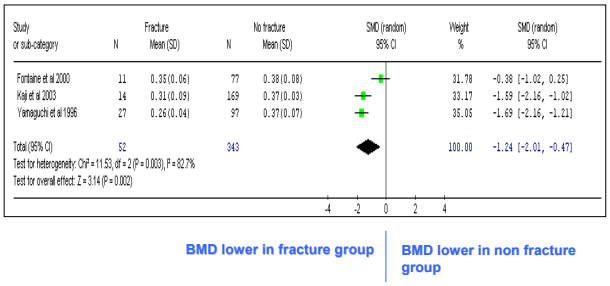
Miller P and Shane E 2004

Stage 5 CKD - No Difference in BMD by Fracture



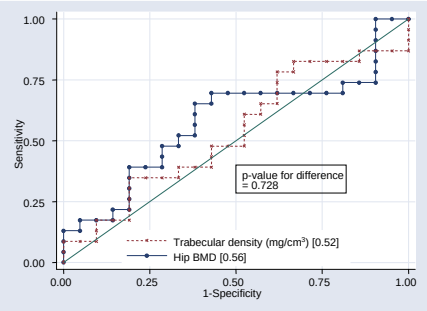
Jamal SA et al. AJKD 2002

Association between Forearm BMD and Fracture



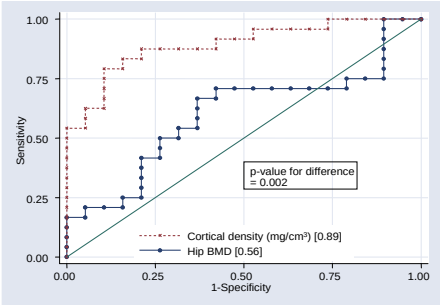
Jamal SA et al. AJKD 2007

Trabecular Density by pQCT does NOT Identify Patients with Fractures



Jamal SA et al JBMR, 2006

Cortical Density by pQCT Identifies Patients With Fractures



Jamal SA et al JBMR, 2006

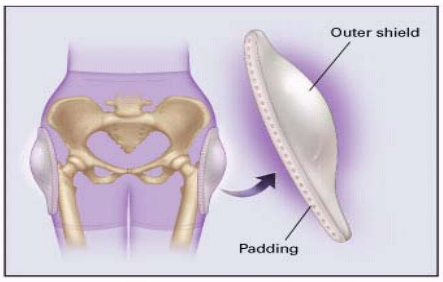
What We Know

- Patients with CKD have fractures
- Cause of fractures is multifactorial
- Stages 1 to 3 can likely use T scores, fragility fractures, PTH and vitamin D to impute cause
- Stage 4 and 5 – consider bone biopsy

How Can We Prevent Fractures

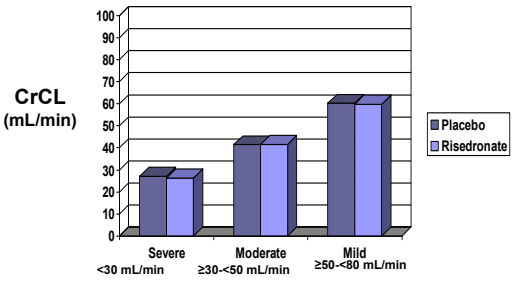
- Prevent fall related injuries
- Control PTH
  - Lower phosphate
  - Replace Vitamin D
  - Calcimimetics
- Drugs that act directly on bone
  - Bisphosphonates
  - PTH
  - Raloxifene, ERT, Calcitonin

Hip protectors-Prevent Fall Related Injuries



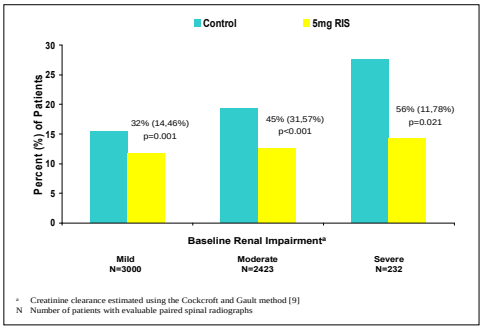
Kannus et al, NEJM 2000; 343:1506-13

Mean baseline eGFR
(determined by the Cockcroft-Gault Method)



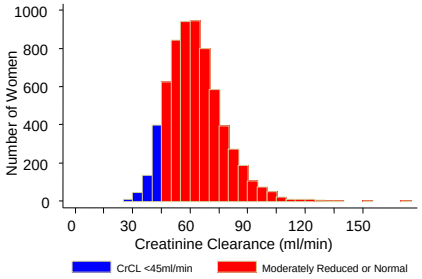
Miller PD et al. JBMR 2005

Vertebral Fracture Risk Reduction with Risedronate



Miller PD et al. JBMR 2005

Women with Normal Creatinine, Decreased Clearance (n = 581) Participating in FIT (n = 6458)



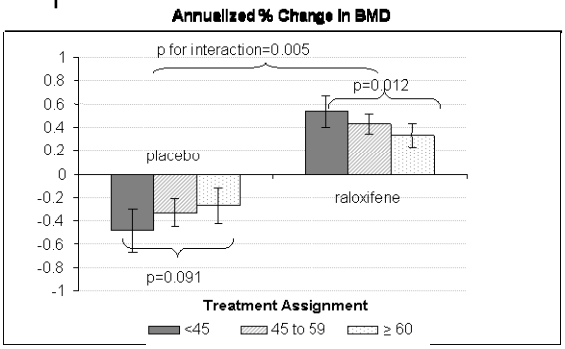
Jamal SA et al. JBMR 2007

Fracture Risk with Alendronate by Creatinine Clearance

Site	CrCl	OR (95%CI)	Interaction p value
Clinical Fractures	<45 ml/min	0.78 (0.51 to 1.2)	0.90
	45 ml/min or higher	0.81 (0.70 to 0.94)	
Spine Fractures	<45 ml/min	0.72 (0.31 to 1.7)	0.44
	45 ml/min or higher	0.50 (0.32 to 0.76)	

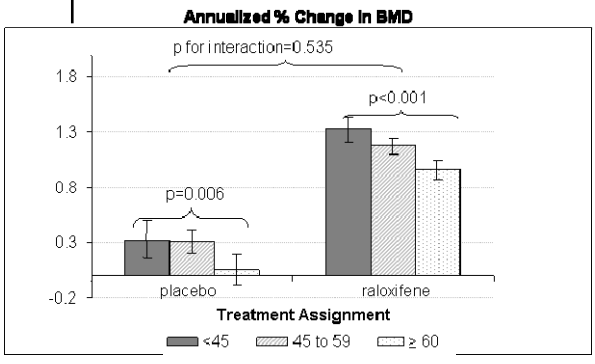
Jamal SA et al. JBMR 2007

Change in Femoral Neck BMD by Creatinine Clearance



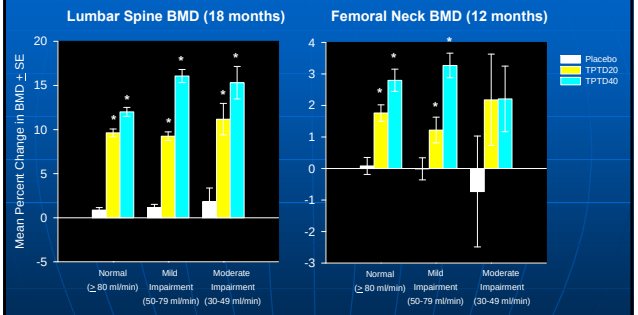
Ishani et al, JASN 2008

Change in Lumbar Spine BMD by Creatinine Clearance



Ishani et al, JASN 2008

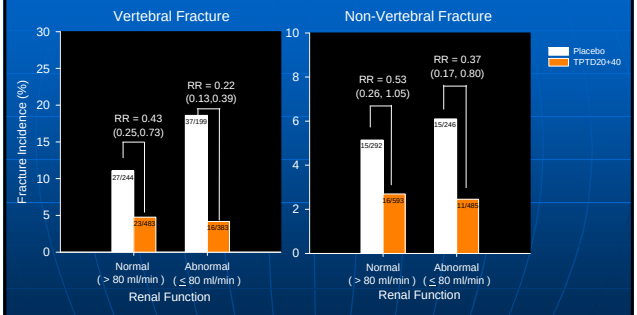
Effect of Renal Function on Changes In Bone Mineral Density with Teriparatide



* P<0.05 from Placebo

Miller P, et al. *Osteopor Int* (in press)

Effect of Renal Function on Fracture Efficacy of Teriparatide



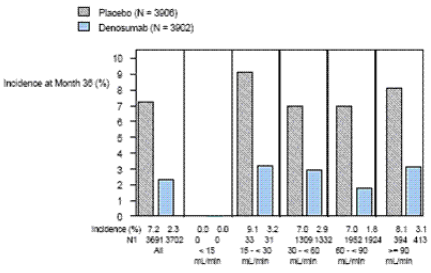
Miller P, et al. *Osteopor Int* (in press)

Table 1. Effect of Denosumab, compared with placebo on incident vertebral and nonvertebral fractures and BMD over 36 months, by stage of renal function.

Outcome	Stage 4 CKD eGFR 15-29ml/min (N= 73)	Stage 3 CKD eGFR 30-59ml/min (N= 2817)	Stage 2 CKD eGFR 60-89ml/min (N=4069)	Stage 1 CKD/Normal eGFR ? 90ml/min (N=842)
Vertebral Fractures; Odds Ratio (95%CI)	0.31 (0.02 to 5.08)	0.38 (0.26 to 0.57)	0.23 (0.15 to 0.34)	0.33 (0.16 to 0.66)
Nonvertebral Fractures; Odds Ratio (95%CI)	0.51 (0.04 to 7.26)	0.88 (0.66 to 1.16)	0.69 (0.54 to 0.89)	0.89 (0.51 to 1.52)
Lumbar Spine BMD; %change	5.0 (-0.8 to 10.8)	8.9 (8.4 to 9.3)	9.0 (8.6 to 9.4)	8.1 (7.2 to 8.9)
Femoral Neck BMD; %change	5.9 (3.3 to 8.5)	5.1 (4.7 to 5.5)	5.2 (4.9 to 5.5)	5.6 (4.9 to 6.3)
Total Hip BMD; % change	5.9 (3.0 to 8.7)	6.4 (6.1 to 6.7)	6.4 (6.2 to 6.7)	5.8 (5.2 to 6.3)

Note: Odds ratio < 1 in fracture risk or difference in BMD % change >0 in favor of denosumab.

Jamal SA, Oral Presentation ASBMR 2010



Jamal SA, Oral Presentation ASBMR 2

Treatment of Osteoporosis in CKD

- Stages 1-3 CKD
  - Treatment not different from PMO since clinical trials randomized patients down to "GFR" of 30 ml/min
- Stage 4 CKD
  - Post-hoc analysis show efficacy and safety through 3 years of risedronate, alendronate raloxifene, and denosumab down to GFR of 15 ml/min
- Stage 5 CKD:
  - Off-label consideration for fracturing patients (very high risk with established osteoporosis)

Therapies in Stage 5 CKD Patients: Caution Still Advised

- No data on benefit or harm in patients with stage 5 chronic kidney disease (GFR <15 mL/min).
- Use only in very specific circumstances
  - Specific fragility fractures
  - Clear diagnosis
- Bone retention over time with bisphosphonates in patients with low GFR unknown
  - For BP: ½ of registered dose for PMO /men or GIOP for no longer than 2-3 years
- Bone retention might not be an issue with denosumab

The Dilemma… Revisited

Q: I have a dialysis patient with a **hip fracture** and a **T-score of - 4.0**.

A: What disease do they have?

If bone biopsy available – just do it!

If not – first do no harm

Summary

- Fractures are common in patients with CKD
- Fractures are multifactorial
- Patients with stages 1-3 CKD and low T-scores/fragility fractures likely to have OP
- CKD stages 4,5 may require a bone biopsy especially in those that fracture and in treatments are being considered
- Bisphosphonates/Denosumab are safe and effective to treat high risk patients down to stage 4 CKD for a short time
- Teriparatide down to stage 3 CKD

Diagnosing and Treating Vitamin D Deficiency and Insufficiency

John Adams, M.D.

Diagnosing and Treating Vitamin D Deficiency and Insufficiency

Meet-the-Professor
American Society for Bone and Mineral Research
Toronto, Canada
October 16 & 17, 2010

John S. Adams, M.D.
Director, Orthopaedic Hospital Research Center
Departments of Orthopaedic Surgery, Medicine and Molecular, Cell & Developmental Biology
UCLA

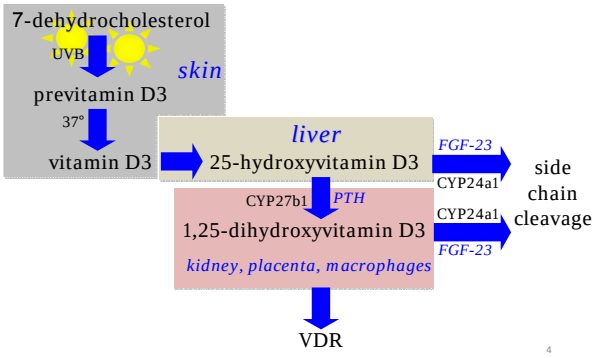
Significance of Topic

- There exists a "pandemic" of vitamin D insufficiency/deficiency
- Vitamin D insufficiency/deficiency associated adverse outcomes extend beyond the musculoskeletal system
- Human fat accumulation, particularly in the abdominal cavity, is major driver of vitamin D deficiency worldwide
- Speculation on a vitamin D
- Treatment
  - Weight reduction
  - Supplemental vitamin D
    - "Natural" or "artificial"?
    - How much?
    - How often?
    - D2 or D3?

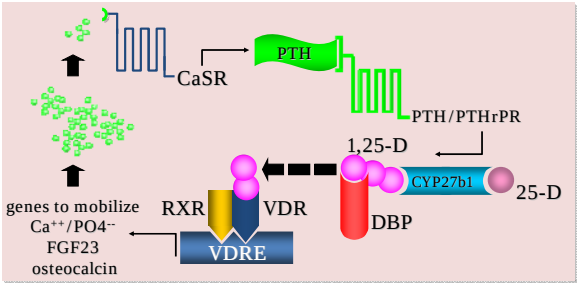
Learning Objectives

- Understand the rationale for diagnosing vitamin D deficiency/insufficiency
- Comprehend the etiology of vitamin D deficiency/insufficiency in modern times
- Gain insight into the endocrine crosstalk among the kidney, fat, bone and gut
- Understand option for treating vitamin D deficiency/insufficiency

Vitamin D Synthesis and Metabolism



Defense of Normocalcemia in the Face of Vitamin D-Deficient Intestinal Calcium Absorption



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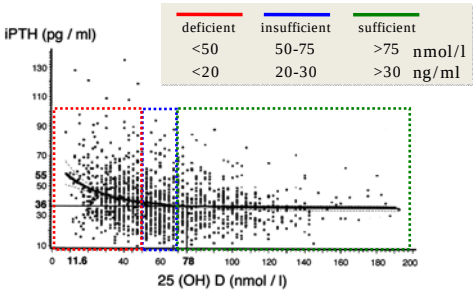
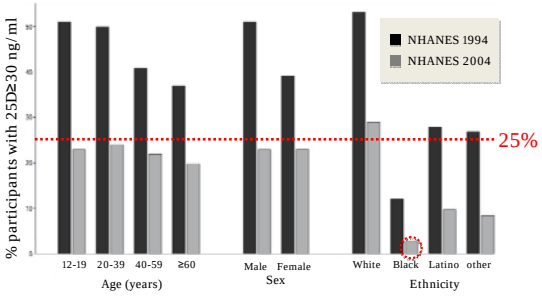
Determinants of Endocrine Vitamin D Insufficiency



Chapuy et al. Osteoporosis Int. 1997

6

Increase in Prevalence of Vitamin D Sufficiency in the U.S. in the last 10 Years



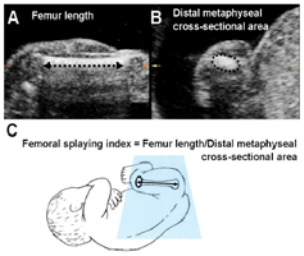
Ginde et al. Arch Intern Med. 169:626, 2009.

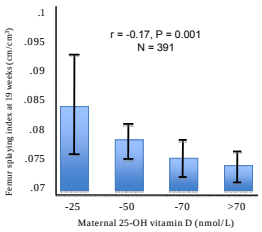
Relation of Race and Season with Cord Blood 25D Levels

Population	All year	April 1–October 31[2]	November 1–March 31[2]
		nmol/L	
All	33.7 ± 20.7[2] [100]	48.7 ± 24.0 [15]	30.7 ± 19.2 [83]
African American[3]	26.2 ± 15.0 [67]	32.7 ± 10.0 [9]	25.2 ± 14.2 [58]
White[3]	48.7 ± 24.0 [33][4]	72.4 ± 10.0 [6]	44.2 ± 22.9 [25]

Greer. Am J Clin Nutr. 88:529S-533S, 2008.

Femoral Metaphyseal Splaying in Fetuses of Vitamin D-Deficient Mothers Apparent *in Utero*



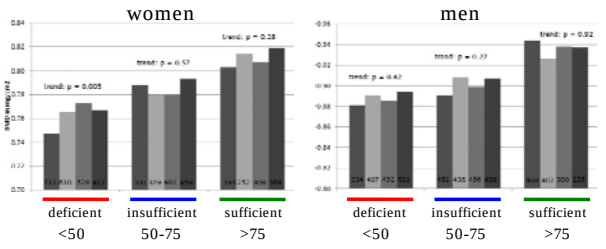


Mahon et al. J Bone Miner Res. 25:14, 2010.

Adverse Musculoskeletal Outcomes Directly Related to a Low 25D Level

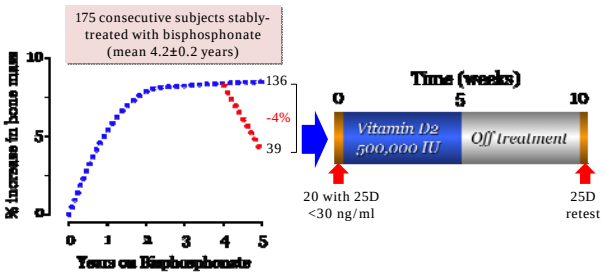
▪ low bone density	Bishoff-Ferrari, *JBMR* 24:935, 2008
▪ nonvertebral fractures	Bishoff-Ferrari, *Arch Int Med* 169:551, 2008
▪ risk of hip fracture	Cauley, *Ann Intern Med* 149:242, 2008
▪ slowed walking speed	Annweiler *JBMR* 25:1856, 2010

Total Hip BMD by Oral Calcium Intake and 25D Level; NHANES III (1988-94)



Bischoff-Ferrari et al. J Bone Miner Res. 24:935, 2009

Significant Decrease in Bone Density in 22% of Bisphosphonate-Treated Patients *2005-2006*



Geller et al. Endo Pract. 14:293, 2008

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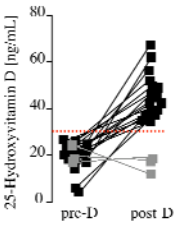
Vitamin D-Responders Significantly Increased Bone Density



Post-Vitamin D	lumbar spine	femoral neck
Responders (17)	+3.0±1.3%*	+2.7±1.8%*
Nonresponders (3)	-3.8±3.0%	-4.6±3.2%

*p<0.02

Geller et al. Endo Pract. 14:293, 2008

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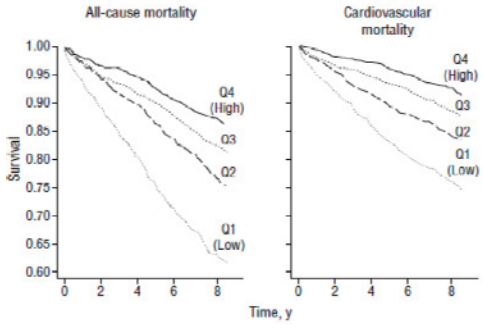
Adverse Metabolic Outcomes Related to a Low 25D Level

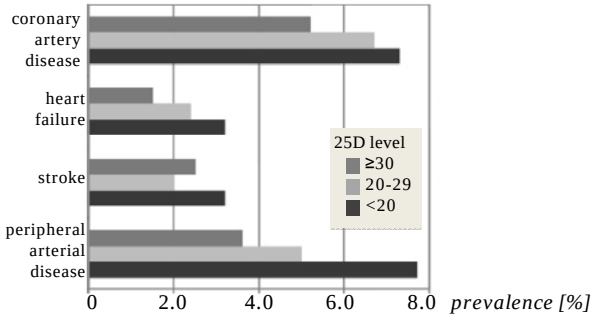
✓ all cause mortality	Melamed, *Arch Intern Med* 168:1340, 2008 Dobing, *Arch Intern Med* 168:1629, 2008
✓ cardiovascular disease	Kim, *Am J Cardiol* 102:1540, 2008 Wang, *Circulation* 117:503, 2008 Kendrick, *Atherosclerosis* 205:255, 2009
✓ systolic blood pressure	Judd, Am J Clin Nutr 87:136, 2008
✓ body mass index	Looker, *Am J Clin Nutr* 88:1519, 2008
✓ insulin resistance	Liu, *J Nutr* 139:329, 2009
✓ circulating C-peptide	Wu, *J Nutr* 139:547, 2009
✓ gestational diabetes	Zhang, *PLOS one* 3:3753, 2008

Kaplan-Meier Plots for All-Cause and Cardiovascular Mortality in 25D Quartiles



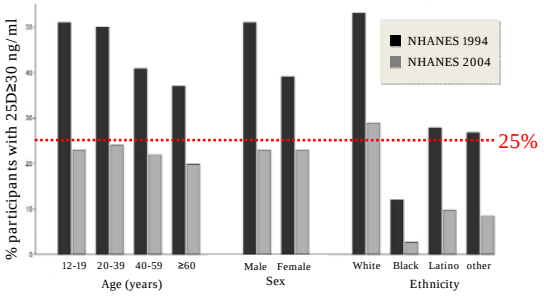
Dobnig et al. Arch Intern Med. 168:1340, 2008.

Prevalence of Cardiovascular Disease by 25D Level: NHANES 2001-2004



Kim et al. Am J Cardiol. 102:1540, 2008.

Increase in Prevalence of Vitamin D Sufficiency in the U.S. in the last 10 Years



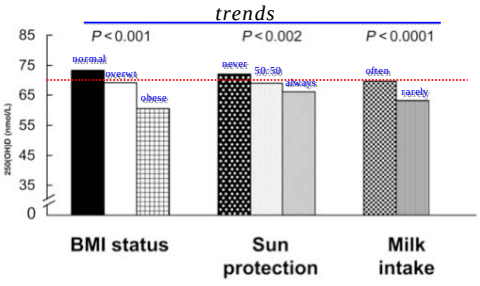
Ginde et al. Arch Intern Med. 169:626, 2009.

Age-adjusted Prevalence of Overweight, Obese and Extremely Obese U.S. Adults

	NHANES III 1988-94 n=16,679	NHANES 1999-2000 n=4,117	NHANES 2001-02 n=4,413	NHANES** 2003-04 n=4,431	NHANES** 2005-06 n=4,356
Overweight (BMI greater than or equal to 25.0 and less than 30.0)	33.1	34.0	35.1	34.1	32.7
Obese (BMI greater than or equal to 30.0)	22.9	30.5	30.6	32.2	34.3
Extremely obese (BMI greater than or equal to 40.0)	2.9	4.7	5.1	4.8	5.9

National Center for Health Statistics, 2009.

Mean Age- and Sex-Adjusted 25D Levels for Whites According to BMI, Sunscreen Use and Milk Intake (NHANES III; 1988-94)



Looker et al. Am J Clin Nutr. 88:1519, 2008.

BMI Inversely Related to 25D in:

- Children, adolescents and adults
- In subjects ranging from normal weight to extreme obesity
- Both sexes
- All ethnic groups

Hypothesis #1
The Vitamin D-Fat Axis

- Accelerated decrease in populational serum 25D levels caused by:
  - decreased cutaneous synthesis of vitamin D3
  - decreased ingestion of vitamin D-fortified foods
  - increased storage of vitamin D in fat stores
  - failure to mobilize stored vitamin D from fat

Serum Micronutrient Levels, Including 25D Levels, Pre- and Post-Intestinal Bypass Extremely Obese Humans (n=64)

Characteristics	Basal time	6 months	9 months	1 year	2 years	3 years
Ferritin, ng/ml	74.5 ± 50.1	87.4 ± 70.3*	97.4 ± 94*	51.4 ± 59*	33.7 ± 24*	26.9 ± 23*
Vitamin A, µmol/l	2.9 ± 3.8	1.6 ± 0.5*	1.6 ± 0.4*	1.8 ± 0.6*	2.0 ± 0.9*	1.3 ± 0.8*
Vitamin D, nmol/l	32.9 ± 15.8	43 ± 50*	42 ± 49*	65 ± 27*	72 ± 34*	46.7 ± 54*
Vitamin E, µmol/l	29.6 ± 9.6	19.2 ± 5.3*	20 ± 5.8*	19.8 ± 6.5*	19.3 ± 5.7*	21 ± 4.4*
Vitamin K, µmol/l	1.4 ± 1.1	1.1 ± 0.8	1 ± 0.8	1.1 ± 1.3	2.3 ± 1.9	2 ± 1.1
Calcium, mg/dl	9.0 ± 0.6	9.1 ± 0.9	8.8 ± 0.9	8.9 ± 0.4	9.3 ± 0.8	8.8 ± 0.5
Phosphorus, mg/dl	4.1 ± 0.3	3.9 ± 0.7	4.0 ± 0.6	3.8 ± 0.6	3.9 ± 0.8	4.0 ± 0.5
PTH, pg/ml	70.1 ± 34	86.5 ± 49*	99.7 ± 46.2*	113.7 ± 57*	101 ± 44*	95 ± 49*
Vitamin B_{12}, pg/ml	420.6 ± 201	430 ± 303	313 ± 119	387 ± 227	377 ± 214	344 ± 152
Folic acid, ng/ml	4.4 ± 2.1	5.9 ± 2.8	9.1 ± 4.7*	7.7 ± 3.7*	9.9 ± 4.7*	7.14 ± 4*
Zinc, µmol/l	17.2 ± 19	11.3 ± 2.7*	9.5 ± 2.2*	8.8 ± 2.1*	9.8 ± 2.2*	9.1 ± 1.4*
Copper, µmol/l	20.4 ± 4.9	15.6 ± 6.3*	16.4 ± 3.1*	15.1 ± 5.5*	17.5 ± 1.9*	17.8 ± 5.3*

* $p < 0.05$ compared to basal data.

De Luis et al. Ann Nutr Metab. 53:234, 2008

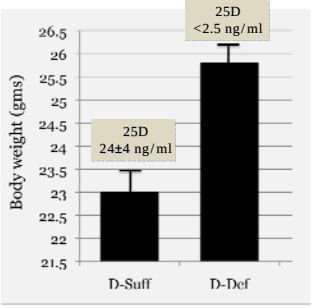
Which Comes First?

Decrease in 25D?

or

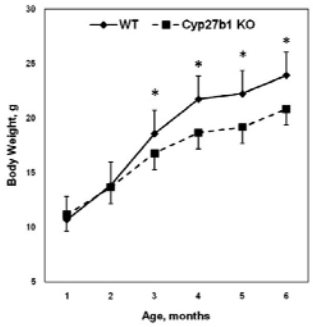
Increase in fat?

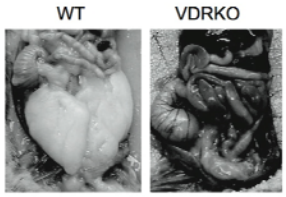
Adolescent C57BL/6 Mice (n=10/grp) Gain Weight on a D-Deficient Diet



Hewison et al, *unpublished*

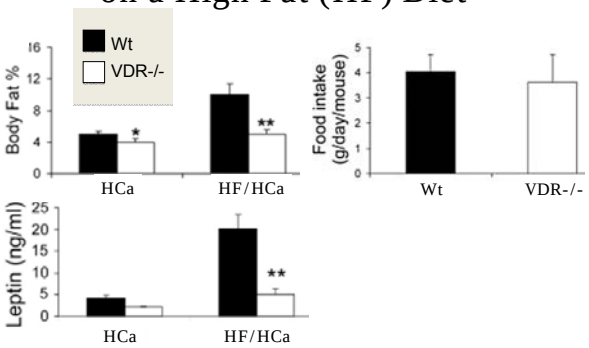
Compared to Wild-type, CYP27b1-Hydroxylase$^{-/-}$ and VDR$^{-/-}$ Mice are Thin





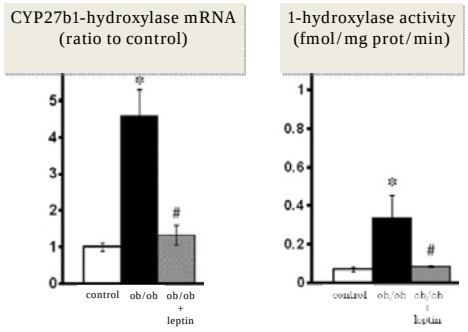
Narvaez. Endocrinology. 150:651, 2009

VDR-/- Mice Fail to Gain Body Fat on a High Fat (HF) Diet



Wong et al. Am J Physiol Endocrinol Metab. 296:E820, 2009.

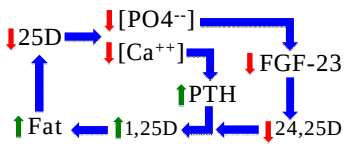
Leptin Squelches the CYP27b1-Hydroxylase



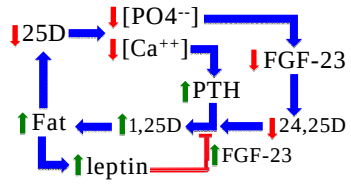
Matsunuma and Horiuchi, Arch Biochem Biophys. 463:118,2007.

Hypothesis #2

Vitamin D Deficiency Drives Fat Accumulation in a Mouse

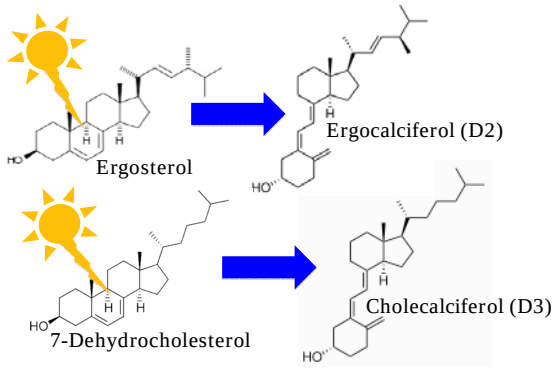


Leptin-Mediated Feedback Inhibition of 1,25D Synthesis

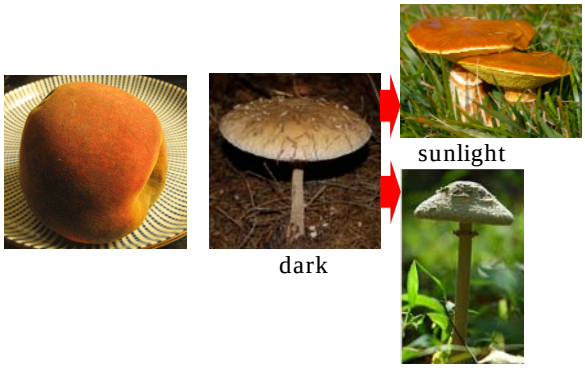


Horiuchi et al. J Bone Min Res. *in press*, 2010.

Photosynthesis of Vitamin D2 and D3



Eat Fungus for a Natural Source of D2



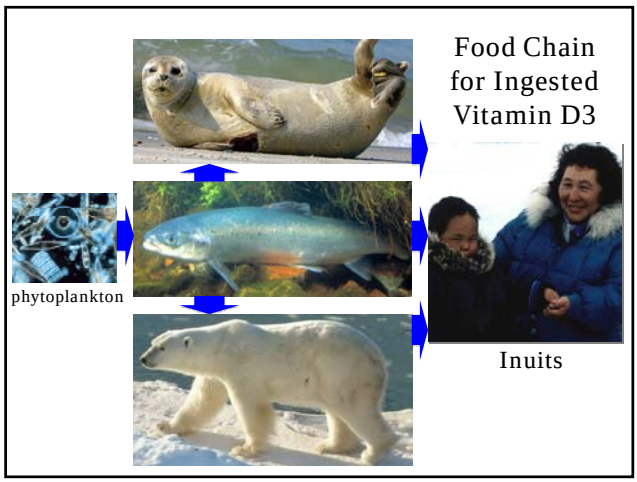
Vitamin D3 Content of Fish Flanks

	IU/3.5 oz (N)
Farmed salmon	249 ± 40 (24)
Wild salmon	981 ± 89 (20)
Bluefish	419 ± 112 (12)
Mahi	342 ± 96 (13)
Farmed trout	371 ± 63 (12)
Swordfish	447 ± 126 (12)
Tuna Ahi-YT	164 ± 42 (9)
Cod	80 ± 14 (9)
Gray sole	45 ± 9 (13)
Haddock	78 ± 22 (13)
Clam	59 ± 21 (4)
Mussels	33 ± 4 (3)
Squid	8 ± 0 (2)
Whitting	48 (1)

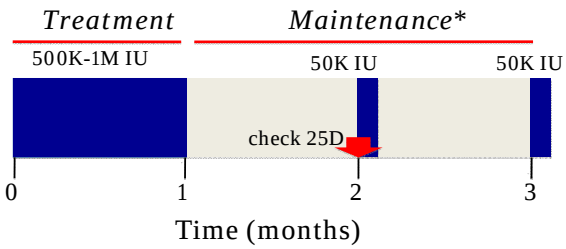
Effect of cooking on vitamin D content in fish

	IU/3.5 oz (N)
Farmed salmon, raw	274 ± 16 (6)
Farmed salmon, microwaved	272 ± 1 (2)
Farmed salmon, baked	248 ± 3 (2)
Farmed salmon, fried	142 ± 21 (2)
Boston Mackerel, raw	10 ± 2 (2)
Boston Mackerel, microwaved	8 ± 1 (2)

Chen et al. Arch Biochem Biophys. 460:213, 2007



Vitamin D Replacement Regimen



** Equivalent to ~1600 IU per day*

Commercial Measurement of 25D

- Immunoassay
  - Gold standard; used in NHANES and WHI
  - Measures total 25D
  - Measures 25D2 and 25D3 equally well
- LC:MS:MS
  - Measures 25D2 and 25D3 individually
  - Normative standards not yet developed
- HPLC
  - Measures 25D2 and 25D3 individually
  - Time-consuming and expensive

Energy Metabolism In Bone

Beata Lecka-Czernik, Ph.D.

Energy metabolism in bone

Recent advances in understanding the role of bone in the systemic regulation of energy metabolism indicate that bone marrow cells, osteoblasts and adipocytes, are involved in this process. Marrow adipocytes store significant quantities of fat and produce adipokines (cytokines produced by adipose tissue), leptin and adiponectin, which play a role in the regulation of energy metabolism, whereas osteoblasts produce osteocalcin, a bone-specific hormone that has potential to regulate insulin production in the pancreas and adiponectin production in fat tissue. Both osteoblasts and marrow adipocytes express insulin receptor and respond to insulin in a manner, which links bone tightly with the energy metabolism system.

The marrow adipocyte phenotype is similar to that of adipocytes present in white and brown fat, but the unique location of these cells in bone presumably directs their more specialized functions. For years, marrow fat was merely considered a cellular component of bone that served a passive role by occupying space no longer needed for hematopoiesis. However, recent developments suggesting that marrow fat plays an essential role as an endocrine organ involved in energy metabolism, places marrow fat under a new research spotlight.

Marrow fat tissue may acquire following physiologic functions:

1) an energy storage in a form of fat;

2) an energy and a heat provider for hematopoiesis, bone healing, and bone remodeling;

3) a protective role in pathologic conditions of lipid metabolism (e.g. diabetes and obesity) by serving as a “sink” for circulating triglycerides;

4) a sensitive to insulin endocrine tissue of local and perhaps systemic functions.

Marrow fat functions

A relatively well-characterized role of marrow adipocytes is in the support of hematopoiesis by producing the necessary cytokines (e.g., IL-6) and heat for hematopoietic cell development. In addition, marrow fat may participate in lipid metabolism by clearing and storing circulating triglycerides thereby providing a localized energy reservoir for emergency situations affecting, for example, osteogenesis (e.g., bone remodeling and fracture healing). Interestingly, a distribution of fat in marrow cavity is not even. As showed with computed tomography, it preferentially localizes to the trabecular area of metabolically active bone, which has high energy demands to support extensive bone remodeling process (Fig. 1). Marrow fat accumulates also at the site of bone injury during the early stages of bone healing process. These suggest the role of marrow fat in providing energy for bone formation and for the maintenance of bone mass.

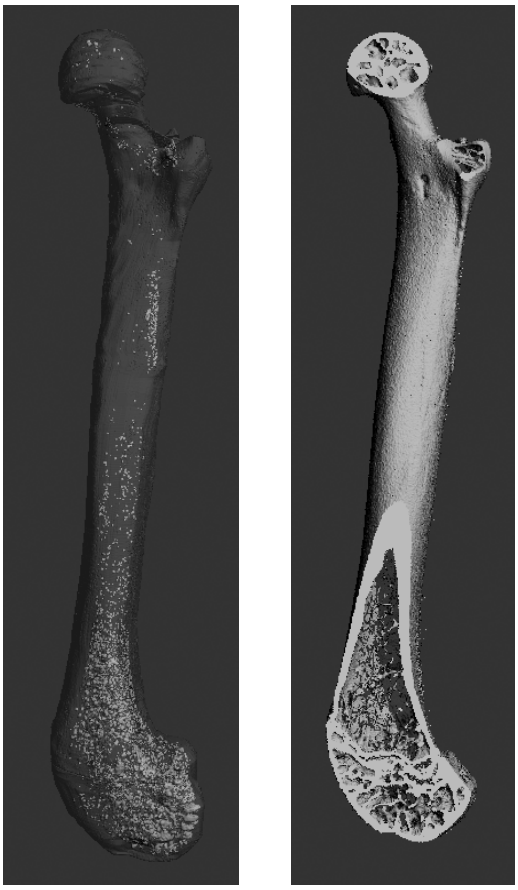


Fig.1. Colocalization of fat (left panel) and trabecular bone (right panel) in murine distal femur.

Marrow fat and energy metabolism

Marrow fat is highly sensitive to environmental and systemic changes which modify energy balance. The following examples provide discussion points for marrow fat versatile phenotype and function.

Energy metabolism diseases such as obesity and diabetes lead to increased fat infiltration in bone. Changes in the hormonal controls resulting from a lack of sex steroids lead to increased fat quantities in bone. On the other hand, diseases associated with a significant reduction in peripheral fat, such as lipodystrophy or anorexia nervosa, are also associated with accumulation of fat in bone. Upon intensive chemotherapy and bone marrow ablation, the grafting of hematopoietic compartment is preceded by populating bone cavity with fat cells.

Bone loss with aging is associated with increased quantities of fat in marrow. It has even been suggested that osteoporosis is an obesity-like disease of bone. Interestingly, with aging a profile of cytokine expression in bone fat changes toward more inflammatory type, which resembles the profile of visceral fat known for its role in development of diabetes. Thus, it is possible that with aging, marrow fat undergoes metabolic changes similar to visceral fat leading to changes in insulin-dependent glucose and fatty acid metabolism.

From the energy metabolism perspective there are two types of peripheral fat; white fat or WAT, which main function is energy storage, and brown fat or BAT, which function is energy dissipation. Marrow fat is often referred as yellow fat (YAT) due to its mixed white and brown phenotype. Indeed, an analyzes of YAT for changes in gene expression profile in murine models of altered energy metabolism, such as 1) estrogen deficiency, 2) diabetes, and 3) improved insulin signaling due to administration of rosiglitazone, demonstrated YAT remarkable plasticity. The profile of YAT was compared to that of WAT and BAT derived from the same animal. We compared levels of expression of genes regulating energy dissipation (UCP1, β3 adrenergic receptor), insulin sensitivity (leptin and adiponectin), and brown adipocyte differentiation (PRDM16 and FoxC2). Estrogen deficiency increased weight of all analyzed fat depots, however the expression of UCP1 was increased only in YAT and BAT, and decreased in WAT. Interestingly, lack of estrogen increased expression of adiponectin and PRDM16 only in YAT, but not in WAT and BAT. In contrast, insulin resistance did not have an effect on UCP1 expression, but led to increased adiponectin expression in all fat depots, despite the fact that circulating levels of this adipokine were decreased in this animal model. Administration of rosiglitazone for 4 weeks to either estrogen deficient or diabetic animals increased expression of UCP1 in YAT and WAT by more than 10-fold. Consistent with insulin sensitizing activity of rosiglitazone, the expression of adiponectin was increased in all fat depots, however only YAT responded to rosiglitazone with increased expression of leptin and PRDM16. These data indicate that YAT is metabolically active tissue and responds to changes in the energy metabolism with either WAT- or BAT-like phenotype.

Marrow fat endocrine function

Marrow adipocytes produce leptin and adiponectin, which regulate caloric intake and insulin sensitivity. These adipokines are the focus of increased attention as possible mediators of marrow fat endocrine function. Based on the fact that cells of osteoblastic lineage express receptors for leptin and adiponectin and the evidence that these adipokines may modulate osteoblast differentiation and function, it is reasonable to believe that bone fat has a local endocrine function and that it modulates the marrow environment supporting bone remodeling. Indeed, based on the quantity of bone fat, which by the third decade of human life occupies almost the entire cavity of long bones, one can speculate that adipokines produced in bone may enter the circulation and contribute to the systemic energy metabolism. Thus, it is reasonable to propose that marrow fat serves a number of endocrine functions not only of local, but also systemic, significance.

Role of PPARs in energy metabolism in bone

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the superfamily of nuclear receptors. Other members of this family include retinoic acid, estrogen, thyroid, vitamin D, and glucocorticoid receptors, and several other proteins involved in metabolism of xenobiotics. PPARs act on DNA response elements as heterodimers with the retinoid X receptor. Their natural activating ligands are lipid-derived substrates. The family of PPARs is represented by three members: PPAR-α, PPAR-δ, and PPAR-γ. They play an essential role in energy metabolism; however, they differ in the

spectrum of their activity—PPAR-γ regulates energy storage, whereas PPAR-α and PPAR-δ regulate energy expenditure through β-oxidation of fatty acids. Although, all three PPARs are expressed in bone, only PPAR-γ appears to be directly involved in both, regulation of bone mass and regulation of energy metabolism in bone. The role of PPAR-α and PPAR-δ in bone energy metabolism is largely unknown.

PPAR-γ is an essential regulator of lipid, glucose, and insulin metabolism. PPAR-γ is a key transcription factor for adipocyte differentiation and for the maintenance of the adipogenic phenotype. This nuclear receptor is also a target for a class of anti-diabetic drugs, thiazolidinediones (TZDs), which regulate adipose tissue capabilities to store fat and produce endocrine factors sensitizing peripheral tissues to insulin. Perhaps not surprisingly, PPAR-γ plays an important role in the maintenance of bone mass. PPAR-γ regulates the lineage commitment of both marrow mesenchymal stem cells (MSCs) towards adipocytes and away from osteoblasts, and of hematopoietic stem cells (HSCs) towards osteoclasts. As a result, PPAR-γ controls both components of the bone remodeling process, bone formation and bone resorption.

In adipocytes, PPAR-γ regulates energy storage and insulin signaling. The dietary abundance of fatty acids increases the activity of PPAR-γ and leads to the activation of lipogenesis, a program designed for energy storage in the form of accumulated lipids in fat tissue. A scarcity of nutrients results in a decrease in PPAR-γ activity and allows for lipolysis, a process that mobilizes energy stored in fat tissue. Continuous over-nutrition leads to excessive upregulation of PPAR-γ activity, increased lipid storage, the development of obesity and insulin resistance.

An analysis of the marrow fat response to antidiabetic TZDs, which improve energy metabolism, presents a paradigm that may unravel additional function(s) of marrow fat. PPAR-γ transcriptome upon rosiglitazone treatment shows significant increase in the expression of genes involved in carbohydrate and fat metabolism, and inhibition of the proinflammatory gene expression. Furthermore, the response of marrow fat to TZDs is strikingly similar and distinctive from that of extramedullary fat. There is a significant upregulation of genes essential for fatty acid metabolism, including fatty acid synthase, fatty acid-binding proteins, hormone-sensitive lipase, uncoupling protein 2, and cholesterol transporter CD36. Interestingly, although a large number of genes involved in carbohydrate metabolism are upregulated, there is no change in the expression of any of the important insulin-dependent glucose transporters, including GLUT4, suggesting that marrow fat functions in lipid not glucose metabolism. Most importantly, in marrow adipocytes, rosiglitazone induces the expression of genes involved in insulin signaling, among them the insulin receptor, insulin receptor substrate-1 and FoxO1, while suppressing the expression of negative regulators of this signaling network such as Socs3. This profile suggests that upon rosiglitazone activation, marrow fat is sensitized to insulin and has increased fatty acid metabolism.

Concluding remarks

Osteoporosis, obesity, and diabetes are the most common pathologies occurring in highly industrialized countries. Since PPAR-γ is positioned at the crossroads of the control of bone mass and energy expenditure, the therapeutic manipulation of its activities may affect the skeleton, in both a positive and a negative fashion. On the other hand, there is an increasing interest in the function of marrow fat including its capabilities to contribute to insulin-dependent fatty acid metabolism. If marrow fat plays such a role than pharmacologic harnessing of the metabolic properties of this fat depot is an attractive possibility for increasing our armamentarium to fight metabolic diseases including diabetes and osteoporosis.

Management of Bone Health in Patients Undergoing Bariatric Surgery

Shonni Silverberg, M.D.

Bariatric surgery and the Skeleton

Shonni J. Silverberg, M.D., Columbia University, New York, New York, USA

Significance of the Topic: Bariatric surgery reverses many of the complications of obesity, and is associated with decreased mortality in obese individuals. This has led to a dramatic increase in the number of such procedures performed worldwide. In the face of this, there is an increasing body of data suggesting that bariatric surgery may have deleterious effects on bone and mineral metabolism.

It is first important to understand preoperative bone and mineral metabolism in those who undergo bariatric procedures. Bone density is increased in the morbidly obese, despite the fact that the vast majority of them are vitamin D deficient. Preoperative 25-hydroxyvitamin D levels are indeed best predicted by BMI. Those who are most overweight, African American and those who have limited sun exposure are most likely to be vitamin D deficient. These factors can be used to identify patients at highest risk for vitamin D deficiency, who may benefit from repletion prior to bariatric surgery. The effect of bariatric surgery itself on mineral metabolism and the skeleton depends on the specific procedure undertaken. Data on biliopancreatic diversion, jejuno-ileal bypass, gastric bypass and gastric banding procedures will be reviewed. The evolution of bariatric surgery has favored procedures that tend to diminish severe malabsorption, with its attendant marked vitamin D deficiency and secondary hyperparathyroid state. However, malabsorption of calcium and vitamin D are still seen after Roux-en-Y gastric bypass, which remains the gold standard procedure today. Furthermore, bone loss in both malabsorptive and non-malabsorptive (banding) procedures may be most closely associated with the extent of weight loss itself. Bone loss is most prominently recognized at the hip site on bone mineral density testing.

In conclusion, it is important to recognize and replete preoperative vitamin D deficiency in those who are about to undergo bariatric surgery. Bariatric procedures are associated with bone loss, particularly at weight bearing sites. The effect may be worse in those who have malabsorptive procedures, in which vitamin D deficiency and hyperparathyroidism may contribute to the decline. Available data highlight directions for future research, including: 1) what is the clinical significance of the reduction in BMD with weight loss in patients in whom baseline BMD is frequently high; 2) what regimens best prevent vitamin D deficiency after gastric bypass; and 3) can the reduction in BMD after bariatric surgery be prevented.

Learning Objectives:

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

1. **Understand the importance of preoperative assessment and management of abnormalities of mineral metabolism in the morbidly obese patient undergoing bariatric surgery.**
2. **Understand the expected consequences of bariatric surgery based upon the particular bariatric procedure undertaken.**
3. **Understand the limitations of current knowledge and important directions for future research in this area.**

Bariatric Surgery: a Response to the Epidemic of Obesity

- Meta analysis 621 studies 1/90-4/06
- N=135,246
- Mean age 40 yrs; 80% female; BMI 48
- Wt Loss: 38.5 kg = 56% excess body wt
- Diabetes: Resolved 78% Improved 87%
  - Confirmed by measures insulin, FBS, HbA1c
  - Maintained for 2 yrs

Buchwald H et al., Am J Med, 2009

Fat Mass

- Independently related to areal BMD
  - Multiple regression models including lean body mass
- Independently related to volumetric BMD
  - Metacarpal cortical thickness, pQCT radius/tibia, ultrasound (not just response to wt bearing)
- Change predictive of change in BMD
  - Over 2 and 10 yrs
- Association stronger in
  - Women, Postmenopause, Sedentary
- High BMI protective against fracture
  - All, osteoporotic
  - Hip, vertebral

Possible Links: Weight Loss & Bone Loss

- Decreased mechanical load
  - Changes greatest at wt bearing sites
  - Changes in local bone factors (ie PG's)
  - Changes in the mechanostat
- Artifact
  - Variability of BMD increases @ depths > 25 cm
  - Fat around bone falsely increases BMD
  - Few subjects below DXA wt limits
- Nutrition related
  - Calcium intake inversely related to adiposity (likely a confounder)
  - Calciotropic hormones
    - Vitamin D low/PTH high

Possible Links: Weight Loss & Bone Loss

- Adipocyte hormones
  - Estrogen, Leptin, Adiponectin, Resistin, IL-6
- Beta cell hormones
  - Insulin, Amylin, Preptin
- Etc: IGF-1, cortisol, calcitonin, glucose-dependent insulinotropic peptide, glucagon-like peptide-2

Mineral Metabolism in the Morbidly Obese

Important to recognize abnormalities that exist PRIOR to bariatric surgery

Inverse Association: 25(OH)D and Adiposity

- Inadequate intake
  - Foods
  - Supplements
- Limited sun exposure
- Decreased bioavailability
  - Sequestration in excess adipose tissue

Significant Predictors of 25(OH)D Levels in Obesity

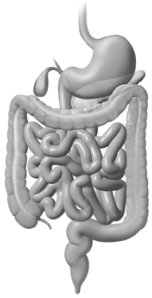
- BMI: Increase of 1 kg/m^2 associated with decline in 25OHD of 1.3 nmol/L
- African American race
- Sun exposure
- PTH
  - Associated with BMI
  - Relationship mediated by vitamin D

Explained 40% of variance in 25(OH)D

Assess vitamin D status BEFORE surgery!

Stein et al., Clin Endocrinol, 2009

Bariatric Surgery: NIH Criteria



BMI > 40kg/m^2

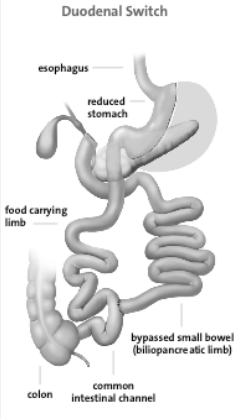
or

BMI > 35 kg/m^2 with Co-morbid Conditions

Jejuno-Ileal Bypass:
Traditional Malabsorptive Procedure

- 5 yr follow-up N=100
  - mean wt loss 33% (50 kg)
- Skeletal Consequences
  - Calcium absorption decrease by 50%
  - Vitamin D malabsorption
    - Vitamin D deficiency, Secondary HPT
  - Osteomalacia
- Additional consequences:
  - Diarrhea (60%); Electrolyte disturbances
  - B12 & Folate Deficiency (88%)
  - Hepatic stuctural abnormalities including cirrhosis (30%)
  - Oxalate kidney stones

Hocking et al., NEJM '83

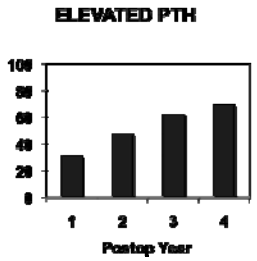


Biliopancreatic Diversion with Duodenal Switch

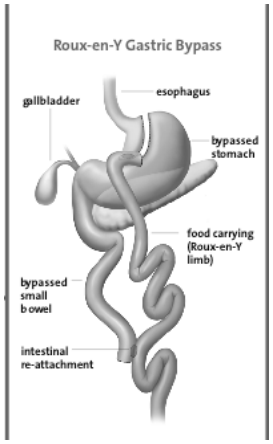
- Portion of stomach removed
- Remaining stomach attached to duodenum
- Duodenum connected to distal small intestine

Biliopancreatic Diversion

- 170 Patients
- NY and Australia
- 69% had hi PTH
- Vitamin D deficiency in 63% @ Yr 4



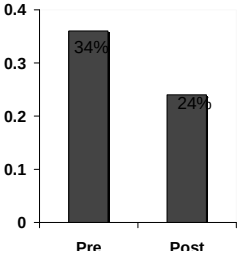
Slater et al., J GI Surg, 2004



- Stomach pouch restricts intake
- Pouch attached to jejunum

  bypasses distal stomach, duodenum, proximal jejunum
- Alters secretion of hormones affecting glucose regulation hunger/satiety

True Fractional Calcium Absorption

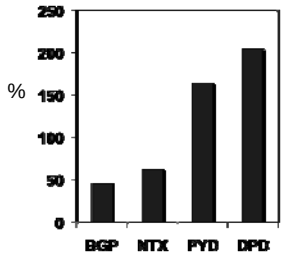


- Declines Postop:
  - Wt: 140 to 101 kg
  - BMI: 53 to 38
  - TFCA:
    - HIGH AT BASELINE
    - REMAINS WITHIN NORMAL RANGE

Riedt et al., Obesity, 2006

Increased Bone Turnover Following RYGB

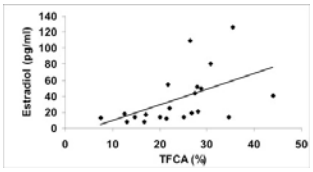
- No change:
  - 25OHD
    - 25 to 29 ng/ml
  - PTH
    - 81 to 77 pg/ml
  - 1,25(OH)2D
  - Cortisol
  - Estradiol
  - Estrone



Riedt et al., Obesity, 2006

Fractional Calcium Absorption Change with RYGB

- Baseline
  - No hormonal regulators
  - Calcium intake explains variance
- After RYGB
  - Association with E2
    - R= 0.512; p <0.02
  - Estradiol explains 62% of variance (p<0.01)

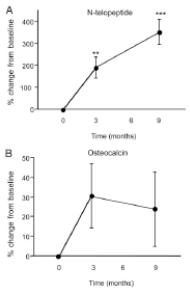


Riedt et al., Obesity, 2006

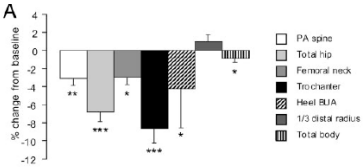
Summary

- Decline in TFCA
  - Less dramatic than Jejuno-Ileal bypass
  - Remains in normal range
  - If intake high, can absorb adequate amounts
- Estrogen regulates calcium absorption
- Markers: Resorption outpaces formation

RYGB Study: Prospective F/U 9 months (N=15)

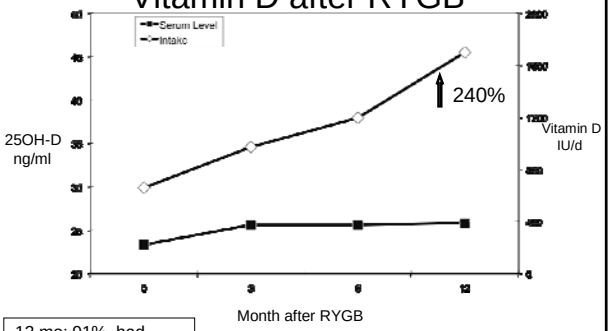


BMD & Broadband Ultrasound Attenuation



Coates et al. JCEM, 2004

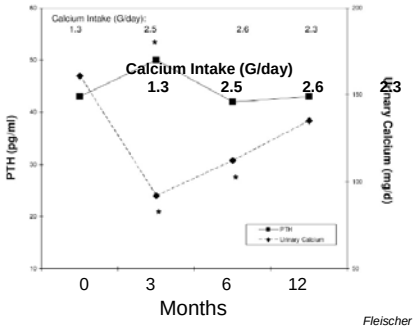
Prospective Follow Up Vitamin D after RYGB



12 mo: 91% had 25OHD < 30 ng/ml

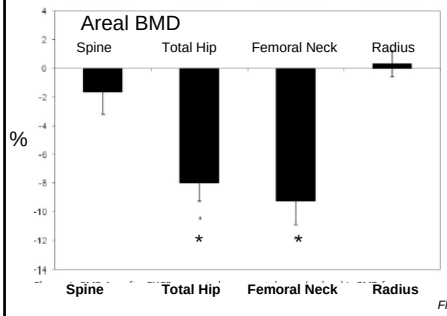
Fleischer et al., JCEM, 2008

Changes in Calcium Intake, PTH & Urinary Calcium



Fleischer et al., JCEM, 2008

BMD 1 Yr after RYGB



BMaD=

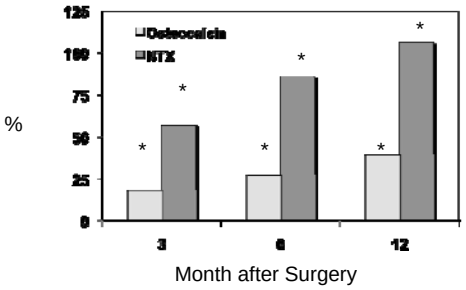
(BMD/ √ bone area)

5% ↓ @ FN

LS: no change

Fleischer et al., JCEM, 2008

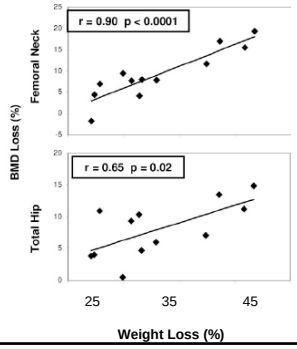
Bone Turnover Markers Rise after RYGB



•p<0.01 vs baseline

Fleischer et al., JCEM, 2008

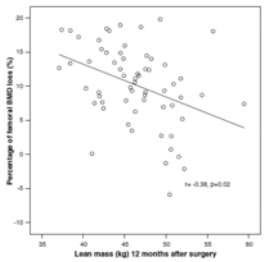
Extent of Weight Loss Associated with Decline in BMD



Fleischer et al., JCEM, 2008

Related to Lean Body Mass?

- 62 pts
- BMD 10% FN 3% LS
- Osteopenia @ 12 mo: 19% LS; 16% FN
- Odds related to
  - Menopause
    - RR 9.13 (1.04-80.1)
  - Lean Body Mass
    - RR 0.82 (0.70-0.97)



Vilarrasa et al., Obes Surg 2009

Changes Associated with RYGB

	Holdstock JCEM '03	Cummings NEJM '02	Coates JCEM '04	Shak Obes Surg '08	Bruno JCEM '10
Ghrelin ↓	62%	77%	51%*	No Δ	
Adiponectin	98% ↓				52% 6 mo ↑
Leptin ↓	64%			65%	76% 6 mo 87% 18 mo

*@ 3 mo; back to baseline @ 9 mo

Coincident with, but not clearly causally related to bone loss

Change in NTX Associated with Leptin Over 18 Months

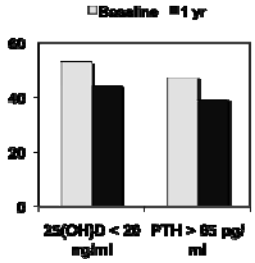
- RYGB n=20 @ 6 m; 19@ 18 m
- NTX Increased 80% @ 6 m; Still up by 56% @ 18 m ANOVA p<.001
- NTX rise associated with
  - ↓BMI, ↓Leptin, ↑25OHD
- Multiple regression model: Only ↓Leptin Predicted ↑NTX

Bruno et al., JCEM, 2010

Vitamin D Repletion after RYGB

- Observational Study
- 108 patients
- 1500 mg Ca/d
- 800 IU D/d

Routine Supplementation Insufficient



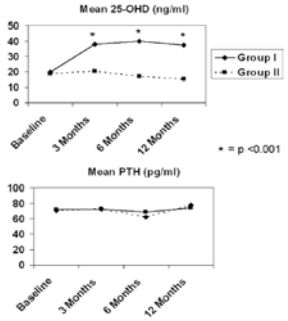
Carlin AM, Rao DS et al., Surg Obes Rel Dis., 2006

RCT of Vitamin D in RYGB

- 60 women, all received 1500 mg calcium
- Baseline
  - BMI: 50 (wt 294 lbs)
  - 25(OH)D: 19 ng/ml
  - PTH: 71 pg/ml
- 30 randomized to 50,000 IU D2/wk
- 30 randomized to 800 IU D/day

Carlin AM, Rao DS et al., Surg Obes Rel Dis., 2009

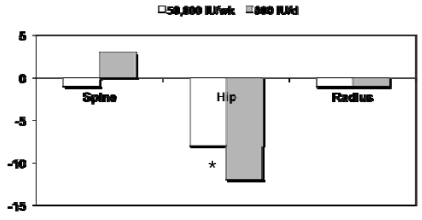
25(OH)D Levels after RYGB



- 50K D/wk (group 1)
  - 19.7 to 37.8 ng/ml
  - Range: 7-61 ng/ml
- 800 IU D/d (group 2)
  - 18.5 to 15.2 ng/ml
- Secondary HPT
  - Persisted in 40% of both groups @ 12 mo

Carlin AM, Rao DS et al., Surg Obes Rel Dis., 2009

Hip BMD Declined Less in Vitamin D Replete Patients

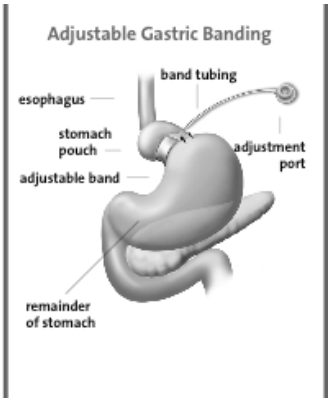


Despite Identical Declines in

%Fat: 33%

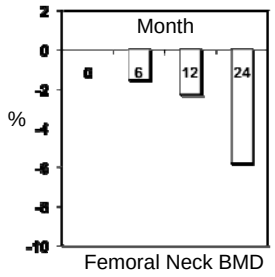
Body Weight: 35%

Carlin AM, Rao DS et al., Surg Obes Rel Dis., 2009

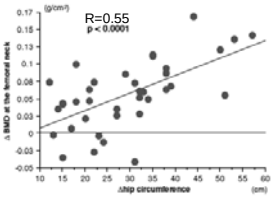


- Inflatable tube around stomach @ GE junction
- Adjustable via addition/removal saline (SQ port)
- Reversible
- Less invasive
- No bypass
- No re-anastamosis

Gastric Banding
Femoral Neck BMD Declines

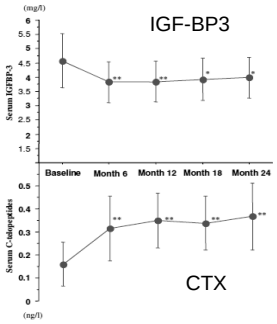


Associated with Decreased Hip Circumference



Giusti et al., Intl J Obesity, 2005

Gastric Banding Study
No Secondary Hyperparathyroidism



6 mo:
- CTX ↑
- IGF-1 unchanged
- IGFBP3 ↓
  - High Baseline in Obesity

Indices stable 6-24 mo

Giusti et al., Intl J Obesity, 2005

What We Know

- Bariatric surgery is associated with
  - Increased bone turnover
  - Decline in areal and volumetric BMD
- Procedures other than gastric banding are associated with calcium and vitamin D malabsorption, but data suggest that increased intake can overcome
- Bone loss most closely associated with extent of wt loss, but affected by vitamin D status, estrogen levels and perhaps changes in adipocyte and satiety regulating hormones
- Skeletal response is procedure specific

Limitations of available studies

Design/Study Populations:
- Small number of subjects
- Short duration of studies
- Limited data on African Americans
- No data in adolescent patients

DIRECTIONS FOR FUTURE RESEARCH

- Clinical significance of findings regarding decrease in BMD unclear
- No assessment of bone quality

Stein et al. Abstract SU0464
Reduced cortical density may influence bone fragility in morbidly obese women

- Longer term impact on skeleton
- What happens with regaining of weight
- Optimal regimen for vitamin D repletion

DIRECTIONS FOR FUTURE RESEARCH

MECHANISTIC QUESTIONS
- Interaction of weight loss & vitamin D treatment on proinflammatory cytokines
- Significance of reported associations?

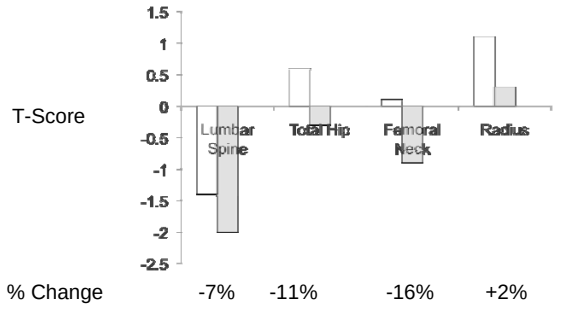
LABS
Longitudinal Assessment of Bariatric Surgery

- NIDDK funded
- 6 Clinical centers
- >5000 enrolled (LABS 1 & 2)
- Teen LABS begun 2007
  - N= 200

56 yo Postmenopausal Woman S/P RYGB

Month	0	3	6	12
BMI	**45**	**37**	**31**	**27**
Ca Intake	1400 mg	3949	3415	3595
Vit D Intake	**400 IU**	**1331**	**1077**	**7383**
25OHD	**22 ng/ml**	**20**	**23**	**42**
PTH	33 pg/ml	63	42	43
Urine Ca	169 mg/24 h	99	103	103
NTX	**27**	**48**	**76**	**75**

Change in BMD T-Score 1 Yr After RYGB



Role of HDAC in Regulating Skeletogenesis

Jennifer Westendorf, Ph.D.

ROLE OF HDACS IN REGULATING SKELETOGENESIS

Jennifer J Westendorf, PhD
Mayo Clinic
Rochester, MN USA

Significance

Histone deacetylases (Hdacs) are intracellular enzymes that directly affect chromatin structure and transcription factor activity. Key roles for several Hdacs in bone development and biology have been elucidated though *in vitro* and *in vivo* models. Recent findings suggest that clinical usage of small molecule Hdac inhibitors, for treating conditions like epilepsy, bipolar disorder, cancer, and a multitude of other conditions, may have unintended effects on bone.

Learning Objectives

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

1) Explain structural and functional features of mammalian Hdacs,
2) Describe the effects of altering Hdac expression on bone formation and osteoblast function,
3) Discuss the consequences of inhibiting Hdac enzymatic activity on bone structure and osteoblast and osteoclast activity, and
4) Identify gaps in existing knowledge of the roles of Hdacs in bone biology.

Outline

1. Hdac proteins and protein complexes
2. Hdac interactions with bone cell-specific transcription factors
3. Hdac inhibition in osteoblast cell lines
4. Hdac suppression in knockout mice
5. Hdac inhibitors
6. Hdac inhibitors & rodent bone density
7. Hdac inhibitors & human skeletal health

References

1. Jensen, E.D., Nair, A.K., and Westendorf, J.J. Histone deacetylase co-repressor complex control of Runx2 and bone formation. *Critical Reviews in Eukaryotic Gene Expression*. 17(3): 187-196; 2007.
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3. Jensen, E.D., Gopalakrishnan, R., Westendorf, J.J. Regulation of Gene Expression in Osteoblasts, *Biofactors*, 36:25-32; 2010. PMC2820584.
4. Razidlo D.F., Whitney T.J., Casper M.E., McGee-Lawrence M.E., Stensgard B.A., Secreto F.J., Li X., Knutson S.K., Hiebert S.W., and Westendorf J.J. Histone deacetylase 3 depletion in osteo/chondro-progenitor cells decreases bone density and increases marrow fat. *PLoS ONE*. 5(7) e11492; 2010. PMC2901996.
5. McGee-Lawrence M.E. and Westendorf J.J. Histone Deacetylases in Skeletal Development and Bone Mass Maintenance. *Journal of Biomedicine and Biotechnology*. In press.

Figures

Figure 1: Structure and classification of the 18 mammalian Hdacs.
(*From: McGee-Lawrence and Westendorf. J. Biomed Biotech. 2010. In press.*)

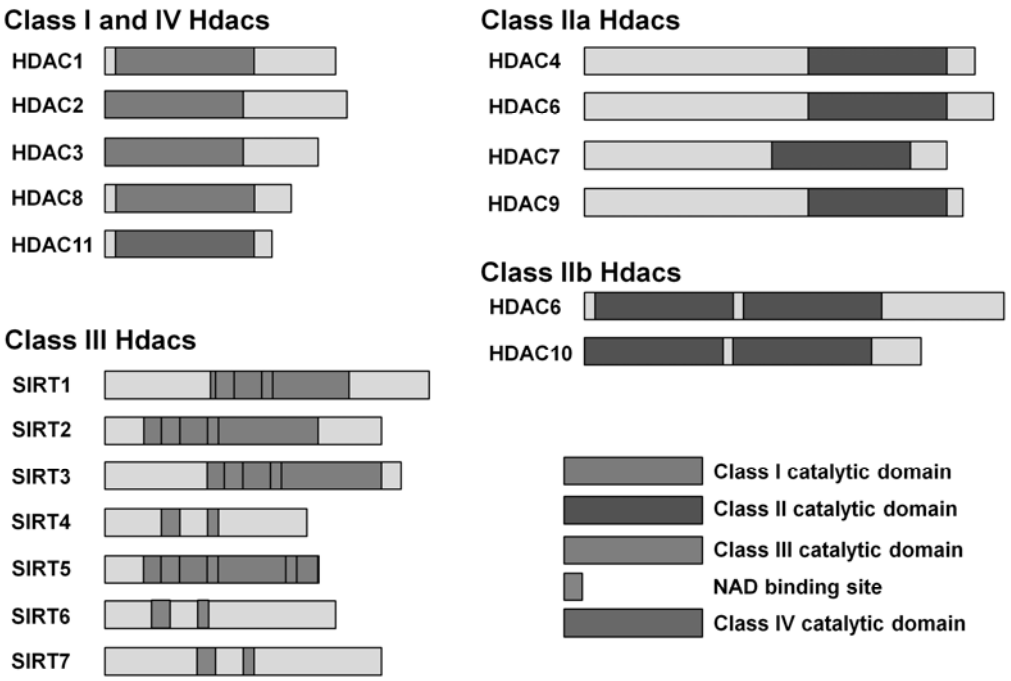

Figure 2. Hdac Binding Domains in Runx2
(*From: Jensen et al. Crit Rev Gene Exp, 2007.*)

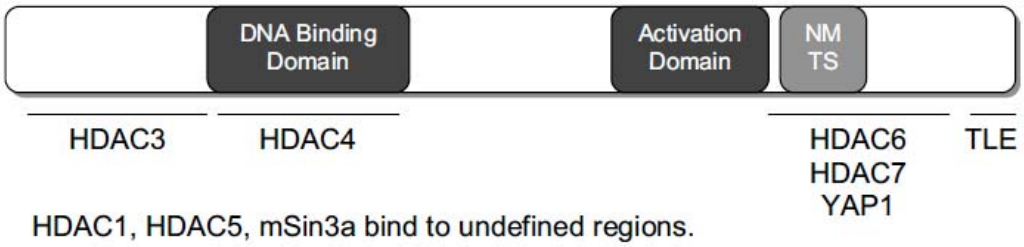

Figure 3. Interactions between Runx2 and Class IIa Hdacs
(*From: Jensen et al. Biofactors 2009*)

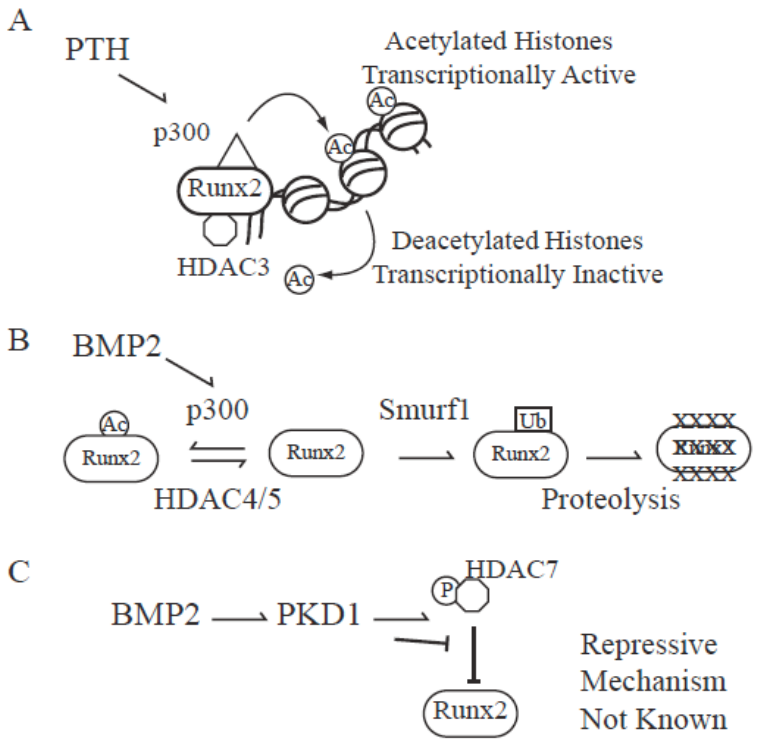

Figure 4: Effects of HDAC Inhibitors on Mesenchymal Progenitors. Hdac inhibitors (HDIs) can have detrimental effects on mesenchymal stem cell (MSC) proliferation, survival, and pluripotency while simultaneously stimulating osteoblast differentiation and activity. These deleterious effects on stem cells, which could ultimately deplete the osteoblast progenitor population, may help explain why HDIs have a stimulatory effect on osteoblastic cell lines *in vitro* but can cause bone loss *in vivo*. (*From: McGee-Lawrence and Westendorf. J. Biomed Biotech 2010. In press.*)

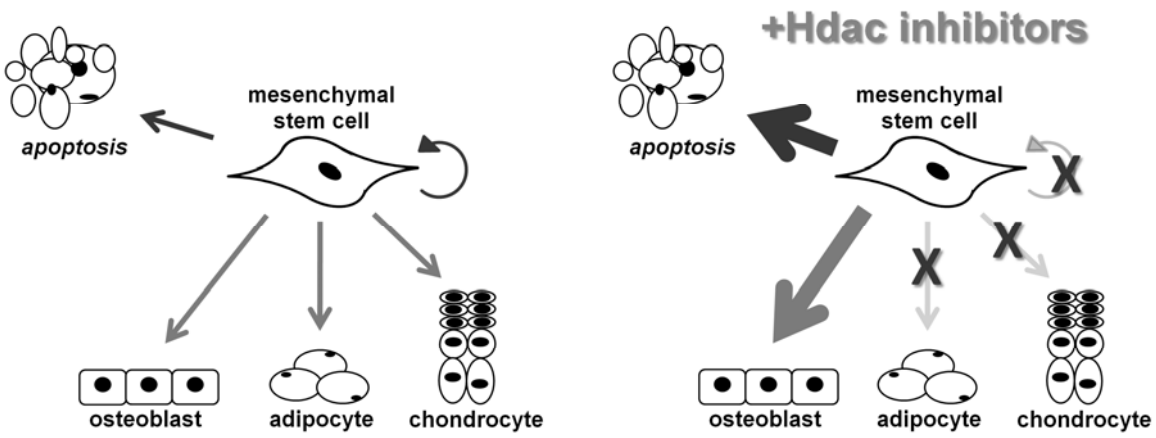

Figure 5. Structure of Common Hdac Inhibitors and Acetylated Lysine
(*From: Westendorf, J Cell Biochem 2007*)

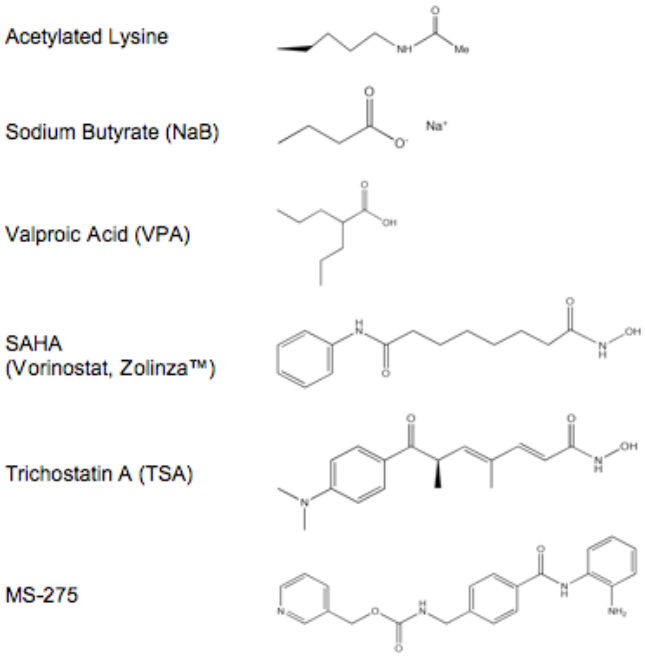

Table 1. Animal Models of Hdac Overexpression or Depletion

Class	Hdac	KO/CKO/Tg	Cells affected	Result
I	1	KO	All (germline deletion)	• Embryonic lethal
I	2	KO	All (germline deletion)	• Reduced body size (↓ vertebrae/pelvis size)
I	3	KO	All (germline deletion)	• Embryonic lethal
		CKO	Osteochondral lineage (*Osterix : Cre* deletion)	• cortical bone loss • trabecular bone loss • decreased bone formation rate • decreased Ob.N • increased marrow Ad.N
I	8	• KO	• All (germline deletion)	• ossification defects in frontal and interparietal bones
				• No effect
		• CKO	• Pre-osteoblasts (*Twist1:Cre* deletion)	• No effect
		• CKO	• Osteoblasts (*Col1a1: Cre* deletion)	• No effect
		• CKO	• Chondrocytes (*Col2a1: Cre* deletion)	• ossification defects in frontal and interparietal bones
		• CKO	• Neural crest cells (*Wnt1: Cre* driven deletion)	
II	4	• KO	• All (germline deletion)	• ectopic chondrocyte hypertrophy and enhanced endochondral ossification • no endochondral ossification
		• Tg	• Chondrocytes (*Col2a1* promoter driven)	
II	5	Tg	All (antagonization of Hdac5 repressor)	• trabecular bone loss • decreased cortical bone strength • decreased bone formation rate • decreased Ob.N
II	6	KO	All (germline deletion)	• increased trabecular bone mineral density
III	SIRT 1	KO	All (Germline deletion)	• trabecular bone loss • Increased Oc.N • Decreased Ob.N

From: McGee-Lawrence and Westendorf. J. Biomed Biotech. 2010. In press.

Table 2. Summary of *in vitro* and *in vivo* effects of Hdac inhibitors on bone

Cell/animal model		Effect
In vitro	Osteoclasts	• Promote apoptosis • Suppress differentiation/maturation
	Osteoblasts	• Promote differentiation • Increase alkaline phosphatase production • Increase osteoblastic gene expression • Increase mineralized matrix production • Increase Runx2 transcriptional activity
	Mesenchymal stem cells (MSC)	• Promote apoptosis • Promote cell cycle arrest • Decrease proliferation • Decrease pluripotency • Increase osteoblast lineage differentiation • Decrease adipocyte lineage differentiation • Decrease neural lineage differentiation • Decrease chondrocyte lineage differentiation
In vivo	Humans	• Decrease bone mineral density • Increase fracture risk • Cause teratogenic craniofacial defects
	Rats	• Decrease bone mineral content
	Mice	• Decrease trabecular bone mass (strain-dependent)

From: McGee-Lawrence and Westendorf. J. Biomed Biotech. 2010. In press.

Targeting PTH Gene Expression as a Potential Treatment for Osteoporosis

Tally Naveh-Many, Ph.D.

Targeting PTH Gene Expression as a Potential Treatment for Osteoporosis

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Significance of the Topic: Parathyroid hormone (PTH) regulates serum calcium and phosphate levels and bone strength. The parathyroid senses small changes in serum calcium through the G-protein coupled calcium receptor (CaR) to alter PTH secretion, gene expression and if prolonged parathyroid cell proliferation. Parathyroid cells have few secretory granules as compared to other endocrine cells and therefore PTH production is regulated largely at the levels of PTH gene expression and parathyroid cell proliferation.

Secondary hyperparathyroidism is a common disorder in patients with chronic kidney disease and is associated with an increased risk of morbidity and mortality. In experimental models of secondary hyperparathyroidism due to chronic kidney failure or a calcium depleted diet there are marked increases in serum PTH and PTH mRNA levels and if prolonged also parathyroid cell proliferation The regulation of PTH gene expression in these models is post-transcriptional due to difference in PTH mRNA stability. This is mediated by the regulated binding of *trans*-acting parathyroid proteins to a defined evolutionarily conserved *cis*-acting element in the PTH mRNA 3'-untranslated region (UTR). We shall discuss the molecular mechanisms that regulate PTH mRNA stability and thereby determine serum PTH levels and mineral metabolism. A better understanding of the regulation of PTH gene expression may highlight novel targets to control secondary hyperparathyroidism. It may also elucidate ways to modulate endogenous serum PTH levels for the prevention and treatment of postmenopausal osteoporosis.

Learning Objectives: As a result of participating in this session participants will be able to:

1. Recognize the post-transcriptional mechanisms that determine PTH mRNA stability and levels of relevance to gene expression in general.
2. Become familiar with the PTH mRNA 3'-untranslated region (UTR) *cis*-acting element that determines PTH mRNA stability.
3. Explain the role of the PTH mRNA *trans*-acting stabilizing and decay promoting proteins that bind to the *cis*-acting element thereby controlling PTH gene expression in secondary hyper- and hypoparathyroidism.
4. Consider the possibility of applying these principles therapeutically.

Introduction

The parathyroid is unique in that the trigger for PTH secretion is a low extra-cellular calcium rather than high calcium as for other hormones. Small decreases in serum calcium and more prolonged increases in serum phosphate stimulate the parathyroid to secrete PTH. PTH then acts on its target organs the kidney and bone to correct serum calcium and phosphate levels. Parathyroid cells have few secretory granules as compared to other endocrine cells and consequently PTH production is regulated largely at the levels of PTH gene expression and parathyroid cell proliferation. It is therefore important to understand the mechanisms that regulate PTH gene expression and consequently serum PTH levels in both health and disease.

Patients with chronic kidney disease often develop secondary hyperparathyroidism which involves increases in PTH secretion and parathyroid cell proliferation. In experimental models of secondary hyperparathyroidism due to renal failure or a calcium depleted diet, there are marked increases in serum PTH, PTH mRNA levels and parathyroid hyperplasia (1). A phosphate depleted diet has opposite effects (2). There is a ~60-fold difference in PTH mRNA levels between hypocalcemic and hypophosphatemic rats and we have used these dietary models as tools to define the mechanisms that control PTH gene expression (1;3). Changes in serum calcium or phosphate levels and chronic kidney failure affect PTH gene expression post-

transcriptionally through regulated protein-PTH mRNA interactions that determine PTH mRNA stability (4). This regulation is in contrast to the effect of $1,25(OH)_2$ vitamin D (1,25D) which decreases PTH gene transcription (5).

mRNA stability plays an important role in gene expression. For many mRNAs decay rates are determined by critical *cis*-acting elements, mostly in the untranslated regions (UTRs) that are targets for *trans*-acting proteins regulating mRNA stability, translation and or cytoplasmic localization. Adenine- and uridine-rich elements (AREs) are a well defined family of *cis*-acting elements critical for the expression of various mRNAs (6). A number of ARE binding proteins have been identified. Some are mRNA decay-promoting factors while others are stabilizing factors. Among these, K-homology (KH) -type splicing regulatory protein (KSRP) is an example of a decay promoting protein (7). KSRP recruits the exosome, a multi-subunit protein complex mediating mRNA degradation, to ARE-containing mRNAs thereby promoting their decay (7). AU-rich binding factor 1 (AUF1) promotes either decay or stabilization, depending on the mRNA and cell type (8). Both AUF1 and KSRP interact with a defined *cis*-acting ARE in the PTH mRNA 3'-UTR and have opposite effects on PTH mRNA stability (4) (Fig 1).

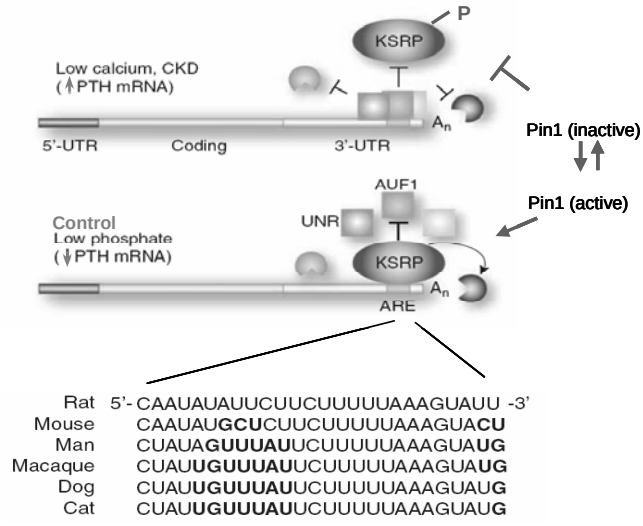

Figure 1. Model for the regulation of PTH mRNA stability by changes in serum calcium and phosphate levels and kidney failure. Schematic representation of the PTH mRNA and binding proteins. AUF1 and Unr stabilize and KSRP destabilizes PTH mRNA upon binding to its 3'- untranslated region (UTR). The peptidyl-prolyl *cis/trans* isomerase Pin1 is upstream of KSRP and leads to KSRP dephosphorylation and activation. In hypocalcemic and experimental chronic kidney disease (CKD) rat parathyroids, Pin1 enzymatic activity is reduced and KSRP is phosphorylated and hence less active. The stabilizing proteins AUF1 and Unr then bind the PTH mRNA 3'-UTR ARE with a greater affinity leading to increased PTH mRNA stability and levels. In control and more so in phosphorus depleted rats, there is increased association of PTH mRNA with KSRP and decreased association with AUF1 and Unr. KSRP

then recruits the multi-protein complex exosome (pac-man) leading to PTH mRNA decay. The nucleotide sequence of the 26 nucleotide *cis*-acting element in different species is presented. Nucleotides that differ from the rat sequence are in bold. From (4) *with permission from the Endocrine Society*.

The PTH mRNA 3'-UTR *cis*-acting protein binding element

PTH mRNA consists of three exons coding for the 5'-untranslated region (UTR) (exon I), the prepro region (exon II) and the structural PTH hormone together with the 3'-UTR (exon III) (Fig 1). Protein-PTH mRNA binding experiments showed specific interaction of rat and human parathyroid extracts with the rat and human PTH mRNA 3'-UTR terminal regions (9;10). In rat parathyroids this binding was regulated by calcium or phosphate depletion and correlated with PTH mRNA levels and stability in vivo (1;3). A 26 nucleotide sequence at the 3' end of the PTH mRNA 3'-UTR was the minimal protein binding region and is conserved in rat, mouse, dog, cat, macaque monkey and man (9;10) (Fig. 1). The conservation of sequences within a region that does not code for protein (UTR) suggests that the binding element is a functional unit that has been evolutionarily preserved. This specific sequence and flanking nucleotides at the 3' end of the rat PTH mRNA 3'-UTR is rich in A and U nucleotides and was identified as a *cis*-acting ARE that determines PTH mRNA stability (Fig. 1).

There is no functional parathyroid cell line, therefore a cell-free mRNA in vitro degradation assay (IVDA) was utilized to identify the factors involved in PTH mRNA decay. The IVDAs have been shown to reproduce differences in mRNA stability that occur in vivo and specifically the differences in PTH mRNA stability induced by calcium, phosphate and chronic kidney failure (1;11;12). In the IVDAs, parathyroid extracts from hypocalcemic and chronic kidney failure rats stabilized and extracts from hypophosphatemic rats destabilized transcripts for the PTH mRNA compared to parathyroid extracts from control rats, correlating with steady state PTH mRNA levels in vivo. These effects were dependent upon the terminal 60 nucleotides of the PTH mRNA 3'-UTR that include the protein binding ARE (1;12). This element, containing the conserved 26 nucleotides and flanking regions, was both necessary and sufficient to regulate PTH mRNA stability and to confer responsiveness of reporter mRNAs to changes in serum calcium and phosphate levels and chronic kidney failure (3;9) (Fig. 1).

The PTH mRNA *trans*-acting stabilizing and destabilizing proteins

Two PTH mRNA binding and stabilizing proteins have been identified by PTH 3'-UTR RNA affinity chromatography. These *trans*-acting proteins are AUF1 and Up-stream of N-*ras* (Unr) (13;14). Addition of recombinant AUF1 to parathyroid extracts increased PTH RNA half-life in IVDAs (10;13). Over-expression of AUF1 or Unr in human embryonic kidney (HEK) 293 cells stabilized co-transfected rat or human full-length PTH mRNA but not a PTH mRNA lacking the terminal *cis*- acting element (10;14). Knock-down of each of these proteins by siRNAs led to the opposite effects, decreasing PTH mRNA levels (14;15). These results identified AUF1 and Unr as PTH mRNA binding and stabilizing proteins (Fig 1).

The half-life of most mRNAs is determined by the coordinate association of both stabilizing and destabilizing factors with the specific mRNA. We showed that the decay promoting protein KSRP specifically binds to the PTH mRNA 3'-UTR ARE. In transfected cells, KSRP over-expression and knock-down experiments showed that KSRP decreases co-transfected PTH mRNA levels through the PTH mRNA ARE. Over-expression of the PTH mRNA stabilizing protein AUF1 blocked KSRP-PTH mRNA binding and partially prevented the KSRP mediated decrease in PTH mRNA levels (3). KSRP and AUF1 protein-PTH mRNA interactions in the rat parathyroid gland were studied using a RNA immunoprecipitation (RIP) assay which provides a measure of protein-mRNA interactions at a specific time point in vivo. In this assay the parathyroid glands were cross-linked, AUF1 or KSRP immunoprecipitated and the amount of PTH mRNA associated with each of the proteins determined by real-time qRT-PCR analysis. KSRP-PTH mRNA interaction was decreased in glands from calcium depleted or chronic kidney failure rats, where PTH mRNA is more stable, and increased in parathyroids from phosphate depleted rats, where PTH mRNA is less stable. In contrast, AUF1-PTH mRNA interactions were increased in parathyroids from rats fed a low calcium diet and decreased in parathyroids from rats fed a low phosphorus diet compared to rats fed a control diet (3). Therefore, PTH mRNA interactions with AUF1 and KSRP are inversely regulated by changes in serum calcium or phosphate concentrations and chronic kidney failure (Fig 1). This differential interaction of KSRP and AUF1 suggests that these proteins have opposing roles in the regulation of PTH gene expression in vivo, and that PTH mRNA half life is mediated by the balanced activities of AUF1 functioning as part

of a stabilizing complex and the destabilizing protein, KSRP which recruits the degradation machinery to PTH mRNA (4) (Fig 1).

The peptidyl-prolyl isomerase Pin1 determines PTH mRNA levels and stability in secondary hyperparathyroidism

As discussed above, KSRP and AUF1, directly or indirectly, respond to changes in serum calcium and phosphate concentrations and kidney failure by altering their association with the PTH mRNA 3'-UTR ARE leading to differences in PTH mRNA stability and levels. RNA-binding proteins are often subjected to post-translational modifications that link extracellular signals to various gene regulation pathways (16;17). Such post-translational modifications could alter the binding affinity of the ARE binding proteins to the PTH mRNA. Indeed, AUF1 is post-translationaly modified in the parathyroids of secondary hyperparathyroidism rats (15;18). KSRP is a phospho-protein with two identified phosphorylation sites involved in ARE mediated mRNA decay (17).

The peptidyl-prolyl *cis/trans* isomerase Pin1 specifically binds phosphorylated Ser/Thr-Pro protein motifs and catalyzes the *cis/trans* isomerization of the peptide bonds thereby changing the biological activity, phosphorylation and turn-over of its target proteins (19). Pin1-catalysed conformational regulation has a profound impact on many key proteins involved in various cell functions (19;20). We have identified Pin1 as a PTH mRNA destabilizing protein in rat parathyroids and in transfected cells (21). The regulation of PTH mRNA stability by Pin1 was mediated by the PTH mRNA 3'-UTR ARE and the PTH mRNA destabilizing protein KSRP. We showed for the first time that KSRP is a Pin1 target protein. Pin1 interacts with KSRP and induces KSRP dephosphorylation leading to activation of KSRP-dependent PTH mRNA decay. Pharmacological inhibition of Pin1 increased PTH mRNA levels and stability and decreased KSRP-PTH mRNA interaction in the parathyroid. Importantly, Pin1 enzymatic activity was decreased in parathyroid extracts from rats with secondary hyperparathyroidism due to either a calcium depleted diet or kidney failure. Furthermore, *Pin1*$^{-/-}$ mice displayed increased serum PTH and PTH mRNA levels (21). Therefore, Pin1 determines basal PTH expression in vivo and in vitro and decreased Pin1 activity correlates with increased PTH mRNA levels in rats with secondary hyperparathyroidism (Fig 1).

Our data suggest that phosphorylated KSRP is inactive. Upon interaction with Pin1, *cis/trans* isomerization of KSRP leads to KSRP dephosphorylation and activation resulting in enhanced PTH mRNA decay. In hypocalcemic and experimental chronic kidney failure rat parathyroids, the enzymatic activity of Pin1 is reduced and KSRP is phosphorylated and hence less active. The stabilizing proteins AUF1 and Unr then bind the PTH mRNA 3'-UTR ARE with a greater affinity leading to increased PTH mRNA stability and levels (Fig 1). These results indicate that Pin1 is a key mediator of PTH mRNA stability and suggest a role for Pin1 in the pathogenesis of secondary hyperparathyroidism (21). Therefore, it may be possible to modify PTH mRNA stability and levels by altering the enzymatic activity of Pin1, thus changing KSRP phosphorylation status and KSRP/AUF1-PTH mRNA interactions in the parathyroid cell.

Summary

The parathyroid is regulated at the levels of PTH secretion, gene expression and parathyroid cell proliferation (Fig 2). Changes in serum calcium and phosphate levels as well as chronic kidney failure regulate PTH gene expression post-transcriptionally by the interaction of RNA binding proteins with the PTH mRNA 3'-UTR ARE. These protein-PTH mRNA interactions are orchestrated by the enzymatic activity of the peptidyl-prolyl isomerase Pin1. Understanding these mechanisms may enhance the development of parathyroid specific modulators of ARE binding proteins which would result in drugs effective to control of the high PTH mRNA stability and serum PTH levels of secondary hypeparthyoridism and other conditions were PTH levels are impaired.

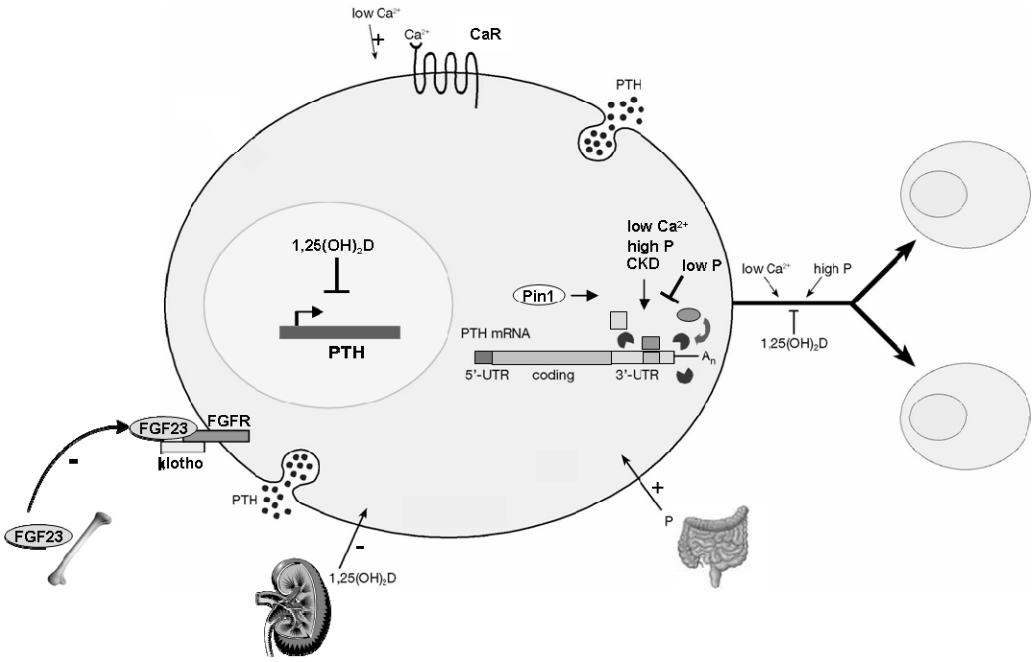

Figure 2. Regulation of PTH gene expression, secretion and parathyroid cell proliferation. The parathyroid cells synthesize and secrete PTH unless they are restrained by the parathyroid calcium receptor (CaR) which senses serum calcium levels. A low serum calcium leads to increased PTH mRNA stability and levels, PTH secretion, and parathyroid cell proliferation. A high serum phosphate and chronic kidney disease (CKD) lead to similar changes in all these parameters. Changes in serum calcium and phosphate levels as well as chronic kidney failure regulate PTH gene expression post-transcriptionally by altering PTH mRNA half-life. PTH mRNA stability is determined by the balanced interactions of protective and decay promoting parathyroid *trans*-acting proteins that bind to a defined *cis*-acting element in the PTH mRNA 3'-UTR. These interactions are orchestrated by the peptidyl-prolyl *cis/trans* isomerase Pin1. 1,25D acts though the vitamin D receptor (VDR) to decrease PTH gene transcription and parathyroid cell proliferation. Fibroblast growth factor 23 (FGF23) is a bone derived hormone that decreases PTH gene expression and secretion. From (4) *with permission from the Endocrine Society*.

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Imaging the Mechanisms of Osteoclast Migration, Differentiation and Function

Masaru Ishii, Ph.D.

Imaging the Mechanisms of Osteoclast Migration, Differentiation and Function.

Masaru Ishii

Laboratory of Biological Imaging, Immunology Frontier Research Center (IFReC), Osaka University, Osaka, Japan.

Significance of the Topic:

During the last decade, multi-photon or “two-photon” excitation microscopy has launched a new era in the field of biological imaging. The near-infrared excitation laser for two-photon microscopy can penetrate thicker specimens, enabling the visualization of living cell behaviors deep within tissues and organs without thin sectioning. The minimized photo-bleaching and toxicity enables the visualization of live and intact specimens for extended observation periods. By using this advanced imaging technique I have established a new system for visualizing *in situ* behavior of osteoclasts and their precursors within intact bone tissues (**Figure 1**). This approach enables us to grasp the mode of action (migration, differentiation and function) of osteoclasts and their precursor cells.

In this MTP session, I will present the latest data on this new concept in the field of bone and mineral researches, and will also show, with plenty of video datas, the detailed methodology of intravital bone imaging and discuss its further application in this field.

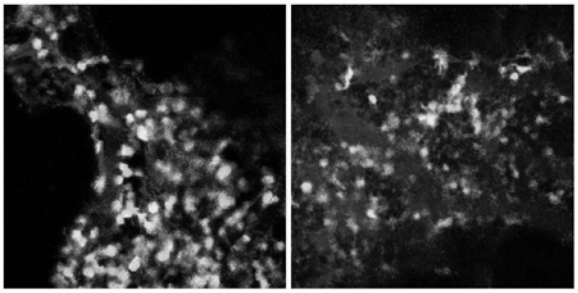


Figure 1. Imaging of live bones by using intravital two-photon microscopy. Granulocytes (LysM-EGFP) (left) and monocytes (CX3CR1-EGFP) (right) are fluorescently labelled, and bone marrow vasculatures are visualized by injecting Rhodamine-conjugated high molecular dextrans.

Learning Objectives:

As a result of participating in this session, attendees should be able to understand the detailed methodology on intravital two-photon imaging of bone tissues, and current and future application of this novel technique in the field of bone and mineral researches. The bone remodeling system configures highly dynamic networks, where various kinds of cell types, such as osteoclast, osteoblast/osteocyte, and bone-resident immune cells, interact with each other in specific areas called as ‘niches’. Conventional methodologies, such as flow cytometry, cell- or tissue culture, biochemistry, and histology, have brought tremendous achievement within this field, although the dynamics of bone cells in a living animal remain less clear.

Technological progress of fluorescence microscopy has enabled us to visualize the intact biological phenomenon that has been uninvestigated. Among the advancements, the recent emergence and prevalence of two-photon excitation-based laser microscopy has revolutionized the research field, such

that the dynamic behaviors of cells deep inside living organs can be visualized and analyzed. Although there were limitations to visualizing the deep tissue of bone, because of the crystallized calcium phosphate in the bone matrix scattering lasers for excitation, we have developed a novel imaging system for visualizing the living bone marrow cavity with high spatiotemporal resolution. We chose the skull of a mouse as the observation site because it is about 100 μm thick, which is can be passed through by two-photon excitation. By using this system, we have revealed a mode of regulation of osteoclast precursor migration by several chemoattractants. We also show this techniques is further usable for elucidating the modes of differentiation and function of osteoclasts *in situ*.

Outline:

Osteoclasts are bone-resorbing multinuclear giant cells that differentiate from mononuclear macrophage/monocyte-lineage hematopoietic precursors. They play critical roles not only in normal bone homeostasis (“remodeling"), but also in the pathogenesis of bone destructive disorders such osteoporosis, rheumatoid arthritis, periodontosis, and bone metastatic cancers. Although many molecules, M-CSF and RANKL chief among them, are known to play critical contributions in osteoclast differentiation, a critical process that has been less well documented is the trafficking of osteoclast precursors to and from the bone surface, where they undergo cell fusion to form the fully differentiated multinucleated cells that actually mediate bone resorption.

By means of the specialized imaging technique with two-photon microscopy we have established a new imaging system for revealing the dynamics of osteoclasts and their precursors within intact bone tissues (**Figure 1**), and found that sphingosine-1-phosphate (S1P), a lipid mediator enriched in blood, controls the movement of osteoclast precursors between the blood and endosteum (their site of final differentiation). Osteoclast precursor monocytes in bone tissues express functional S1P receptor, and potent S1P receptor agonist stimulated their mobilization *in vivo* (**Figure 2**). Because S1P concentration in blood is always higher than that in tissues, S1P-mediated chemotaxis of osteoclast precursors contributes to their recirculation from bone tissues to systemic blood flow.

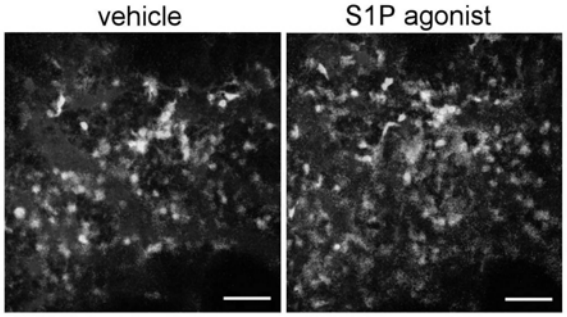


Figure 2. S1P agonist mobilizes monocyte/osteoclast precursors *in vivo* bone tissues, visualized by intraviral two-photon microscopy.

Further examinations are revealing the possible role of several bone-enriched chemokines on the migration control of osteoclast precursors *in vivo* bone tissues (**Figure 3**). The bulk of these results

support the hypothesis that fine tuning of osteoclast migration mediated by various chemokines dynamically modulates bone homeostasis, suggesting a unique point of action on osteoclastogenesis that may be promising as a future therapeutic target.

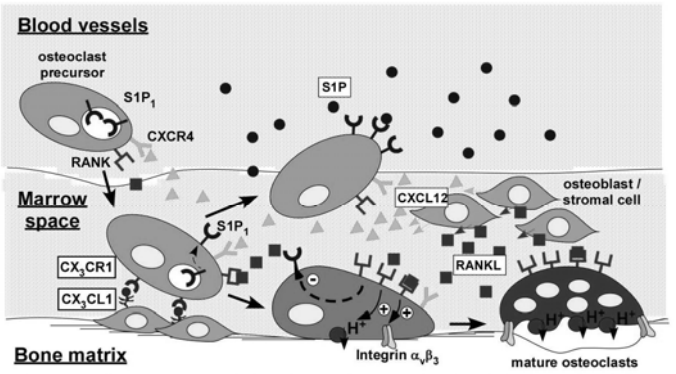
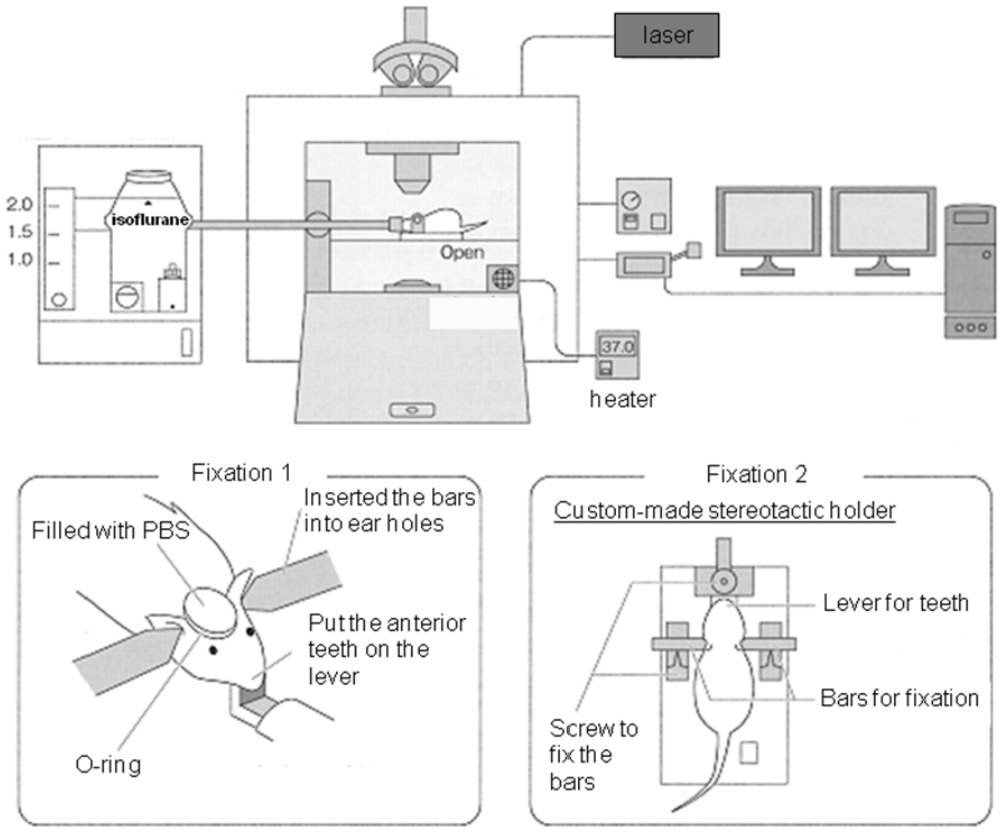


Figure 3. Schematic representation of chemokine-mediated control of migratory behavior of monocyte/osteoclast precursors.

This imaging technique has been enabling us to grasp the in vivo mode of action, such as differentiation and function, of osteoclasts, combined with several genetically fluorescence labeling techniques. In this MTP session, I will present the latest data showing osteoclast dynamics *in vivo* visualized with intravital two-photon microscopy and discuss the further application of this technique.

<Schematic illustration of intravital two-photon microscopy of mouse skull bone tissues>



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Premenopausal Bone Loss: Etiology and Management

Robert Recker, M.D.

Session: Meet-the-Professor

Moderator: Robert R. Recker, M.D.

Institution: Creighton University

Country: USA

Handout Title: Premenopausal Bone Loss: Etiology and Management

This is a significant issue, though it is not commonly recognized. The most frequent cause is bone loss due to associated co-morbidities. Education of clinicians on the co-morbidities that result in secondary loss of bone in the premenopausal years is important. Thus, it is important: 1) to recognize that premenopausal bone loss does occur in some situations, 2) to know that currently available osteoporosis drugs, in particular the anti-remodeling drugs, are effective in halting and/or reversing premenopausal bone loss, and 3) to recognize that bone loss at any time in life is generally nearly completely irreversible. Once bone loss has occurred, it is very difficult with current technology to restore it! Thus, the goal is to anticipate it, and prevent it. Clinicians need to understand that while bone loss of one T-score in premenopausal years does not necessarily confer significant immediate increase in fracture risk, but adds to fracture risk late in life since it cannot be fully restored.

Learning objectives:

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

1) Learn about clinical circumstances causing secondary bone loss in premenopausal female population.
2) Understand the irreversible nature of premenopausal bone loss.
3) Understand an approach to treating and preventing premenopausal bone loss.

Points of interest:

- Premenopausal bone loss confers disproportionate increase in risk of fracture in postmenopausal years.
- In a healthy population of women, bone gain continues throughout the third decade of life and remains stable or declines slightly from then until menopause.
- It is important to counsel patients on lifestyle factors such as calcium intake and vitamin D so that deficiencies in them, do not impede second and third decade bone gain, nor result in bone loss in premenopausal life.
- A large number of diseases and/or treatments can cause premenopausal bone loss and it is necessary to anticipate them so that bone loss can be eliminated or minimized. The following is an incomplete list that includes common ones: corticosteroid administration, inflammatory arthritis, generalized inflammatory diseases such as lupus, dietary calcium deficiency, early surgical menopause,

inflammatory bowel disease, GI surgery of any kind, asthma, chronic infectious disease, celiac disease and more. Discussion at the session will broaden this list.

It is important to realize that nearly any clinical circumstance that might result in bone loss in premenopausal women is amenable to treatment and/or prevention!

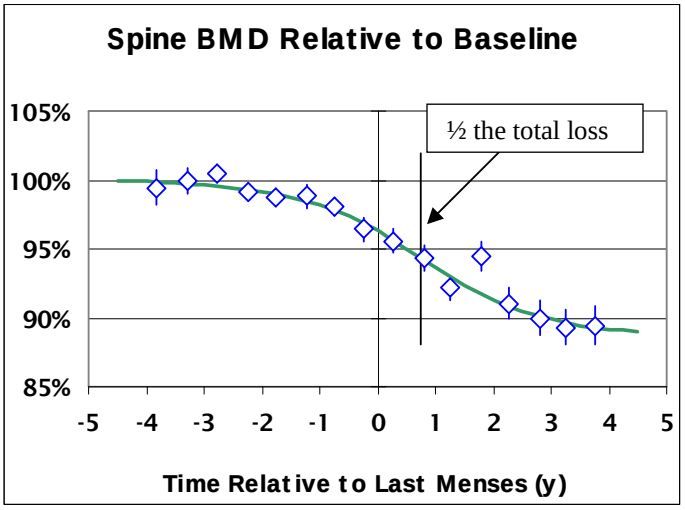

Figure 1. Course of spine BMD in 51 healthy women not on hormone replacement studied over a period of time encompassing the transition from premenopausal years to nearly 5 years after last menses. DXA measurements were made at intervals of 6 months. Each DXA value was expressed as % of baseline. The total transmenopausal loss averaged about 10 percent. (Recker, et. Al, J Bone Miner Res 2000; 15:1965-1973)

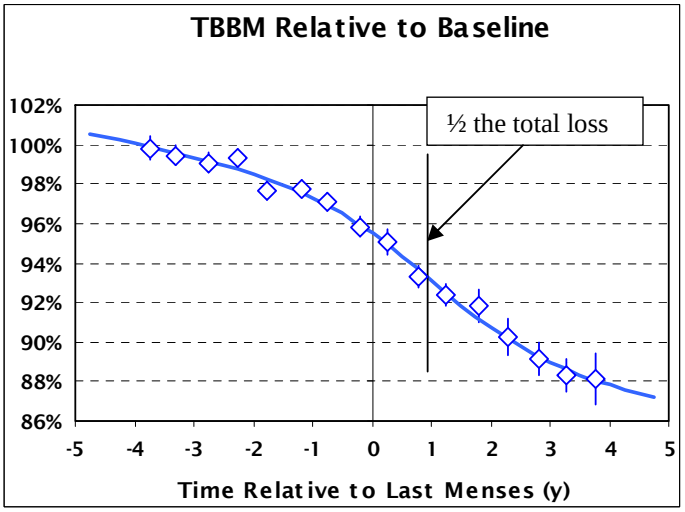

Figure 2. Course of total body bone mineral content in 51 healthy women not on hormone replacement studied over a period of time encompassing the transition from premenopausal years to nearly 5 years after last menses. DXA measurements were made at intervals of 6 months. Each DXA value was expressed as % of baseline. The total transmenopausal loss averaged about 12 percent. (Recker, et. Al, J Bone Miner Res 2000; 15:1965-1973)

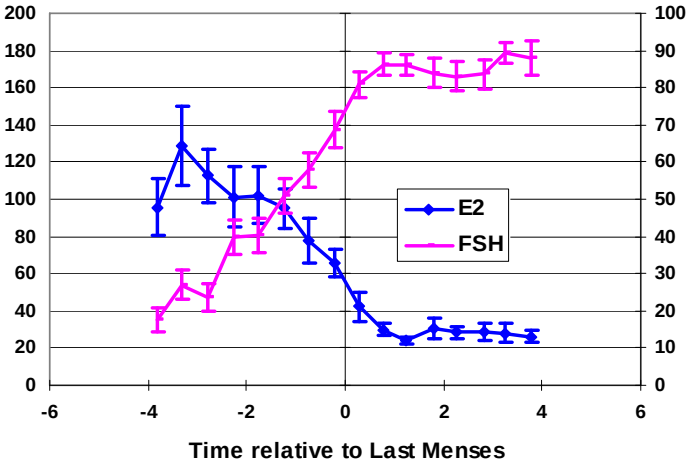

Figure 3. Serum levels of estradiol and follicle stimulating hormone in 51 healthy women not on hormone replacement studied over a period of time encompassing the transition from premenopausal years to nearly 5 years after last menses. Measurements were made at intervals of 6 months. (Recker, et.al, J Bone Miner Res 2000; 15:1965-1973)

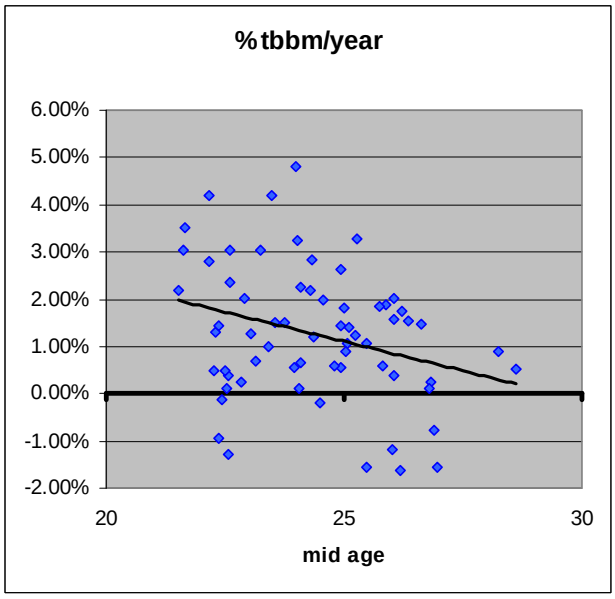

Figure 4. Total body bone gain in 156 healthy young college women during the third decade of life. Gain occured at a declining rate until nearly age 30 when further gain no longer occurred. The total gain during the third decade averaged 12.5%. (Recker RR, Davies KM, Hinders SM, Heaney RP, Stegman MR, Kimmel DB. Bone gain in young adult women. JAMA 1992; 268:2403-2408.)

Foxo1 and Osteoblast Physiology

Stavroula Kousteni, Ph.D.

FoxO1 and Osteoblast Physiology

Meet-the-Professor
Monday, October 18, 1:30 p.m. - 2:30 p.m.

Stavroula Kousteni
Division of Endocrinology
Department of Medicine
College of Physicians & Surgeons
Columbia University
USA

The FoxO isoforms constitute an ancient family of Forkhead transcription factors which throughout evolution have conserved their function and their cellular targets. FoxOs are ubiquitously expressed and their signal transduction pathways have been preserved in differentiated tissues for a variety of specialized functions such as differentiation, proliferation and survival in cells as diverse as adipocytes, hepatocytes, β-cells, myoblasts, thymocytes and cancer cells (1-5). There are 4 FoxO molecules, FoxO1, FoxO3, FoxO4 and FoxO6 encoded by different genes, with FoxO6 being structurally and functionally distinct from the other 3 isoforms. Among them, FoxO1 regulates the most diverse array of biological activities which include 4 major aspects of physiology and disease: organismal growth, insulin action, tumorigenesis and angiogenesis. The 4 latter properties are specific to (glucose metabolism) or mainly regulated by FoxO1.

In all its functions, FoxO1 regulates whole organism physiology by regulating homeostasis at many different levels. It was thus, not a surprise that similar to its effect in other organs, FoxO1 was found to be required for the maintenance of bone homeostasis (6). FoxO1 in osteoblasts utilizes a previously unrecognized mechanism to organize anti-oxidant responses and maintain osteoblast function and proliferation. This mechanism promotes protein synthesis through interaction of FoxO1 with ATF4 and downstream inhibition of a p53-dependent signaling pathway which mediates ROS-induced anti-proliferative actions and early senescence. These results established that FoxO1 is a transcriptional determinant of redox balance and of normal function of osteoblasts.

Comparing the many actions of FoxO1 in different organs, what became apparent was that its function in the skeleton has a unique characteristic in the sense that it affects other organs as well. Indeed FoxO1 is one of the transcription factors determining the endocrine function of the skeleton in regulating energy metabolism (7-9). Osteoblast-expressed FoxO1 acts through promoting carboxylation of osteocalcin to suppress insulin production by the pancreas as well as insulin sensitivity in liver, muscle and white adipose tissue (7). Osteocalcin, when uncarboxylated, acts as a hormone favoring insulin secretion and sensitivity (8;10-14). Osteocalcin decarboxylation is induced by the acidic environment generated during osteoclastic bone resorption and it is controlled by insulin signaling in osteoblasts (9).

What do these observations tell us about the role of osteoblast-expressed FoxO1 in the biology of the whole organism? At one end FoxO1 is beneficial for the skeleton. By organizing a response of bone to continuous, low-intensity physiologic stress signals it is essential for normal bone cell function, preservation of bone mass and skeletal regeneration. On the other hand, it suppresses glucose metabolism and decreases insulin sensitivity. In addition FoxO1 activity in osteoblasts is under the opposing control of oxidative stress and insulin. The first stimulates, whereas the second suppresses FoxO1 activity. It is possible that these opposing actions and the dual mode of regulation may serve as a dual rescue mechanism. This hypothesis may be particularly relevant within the context of aging. It is possible that under conditions where both the skeleton and glucose handling are deteriorating, such as in aging, FoxO1 may confer a rescuing signal of energy supply from the wasting skeleton to the energy demanding organs that control glucose metabolism.

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Treatment and Prevention of Skeletal Complications in Men with Prostate Cancer

Matthew Smith, M.D., Ph.D.

Treatment and Prevention of Skeletal Complications in Men with Prostate Cancer

Matthew R. Smith, M.D., Ph.D.
Massachusetts General Hospital Cancer

Skeletal complications are a major cause of morbidity in men with prostate cancer. In men with advanced stage prostate cancer, bone is dominant site of metastases and most men have bone-only or bone-predominant pattern of metastatic disease. Bone metastases are associated with a variety of clinical complications including pain, pathological fractures, spinal cord compression, and myelophthisis. Androgen deprivation therapy, the mainstay of systemic therapy for prostate cancer, increases bone turnover, decreases bone mineral density, and increases fracture risk. Due to the adverse effects of androgen deprivation therapy on bone, men without bone metastases are also at risk for skeletal complications. Recent clinical research has evaluated the role of bisphosphonates and RANKL-targeted therapy for prevention of disease and treatment-releated skeletal morbidity.

As a result of participating in this session, attendees should be able to understand the clinical complications and pathophysiology of bone metastases, recognize the problem of treatment-related fractures, and describe the result of recent clinical trials designed to prevent disease and treatment-related skeletal morbidity.

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Review

Treatment and prevention of bone complications from prostate cancer

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ABSTRACT

Bone metastases and skeletal complications are major causes of morbidity in prostate cancer patients. Despite the osteoblastic appearance of bone metastases on imaging studies, patients have elevated serum and urinary markers of bone resorption, indicative of high osteoclast activity. Increased osteoclast activity is independently associated with higher risk of subsequent skeletal complications, disease progression, and death. Osteoclast-targeted therapies are therefore a rational approach to reduction of risk for disease-related skeletal complications, bone metastases, and treatment-related fractures. This review focuses on recent advances in osteoclast-targeted therapy in prostate cancer. Bisphosphonates have been extensively studied in men with prostate cancer. Zoledronic acid significantly decreased the risk of skeletal complications in men with castration-resistant prostate cancer and bone metastases, and it is FDA-approved for this indication. Denosumab is a human monoclonal antibody that binds and inactivates RANKL, a critical mediator of osteoclast differentiation, activation, and survival. Recent global phase 3 clinic trials demonstrated an emerging role for denosumab in the treatment of prostate cancer bone metastases and prevention of fractures associated with androgen deprivation therapy.

Contents

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Clinical manifestations of bone complications in prostate cancer

The American Cancer Society estimates for 2009 included over 192,000 new cases of prostate cancer in the United States, accounting for 25% of cancer diagnoses in men and over 27,000 deaths from metastatic disease [1]. The major site of hematogenous spread of prostate cancer is bone, seen in 80–90% of men with castration-resistant metastatic prostate cancer undergoing therapy [2,3], and 90% of patients at autopsy [4]. The most common sites of bone metastasis are the vertebral column, pelvis, ribs, long bones, and skull. These are areas of active hematopoiesis in adults and are hypothesized to provide tumor cells with a rich growth environment. Unlike other cancers that commonly metastasize to bone and cause osteolytic lesions, prostate cancer causes predominantly osteoblastic lesions.

Bone metastases from prostate cancer are a major cause of morbidity. Pain is the most common symptom. Vertebral metastases may cause compression fractures, spinal cord compression, nerve root compression, and cauda equina syndrome. Pathologic fractures of proximal long bones occur at lower rates compared with vertebral fractures [5]. Hypocalcemia, as a result of excessive bone formation, and subsequent secondary hyperparathyroidism is common[6]. Ineffective erythropoiesis due to bone metastases and cancer therapies contributes to a high prevalence of anemia among men with advanced prostate cancer.

Fragility fractures due to osteoporosis are common in older men [7]. The common risk factors for developing osteoporosis in men include hypogonadism, chronic glucocorticoid therapy, and excessive alcohol intake [8]. Androgen deprivation therapy (ADT) with a gonadotropin-releasing hormone (GnRH) agonist is the standard initial hormonal treatment for metastatic prostate cancer. ADT causes severe hypogonadism and significantly increases the risk of fracture [9,10]. Thus, the biology and treatment of advanced prostate cancer cause important changes in bone integrity with significant clinical consequences. Opportunities for therapeutic intervention and clinical trial data on the treatment and prevention of bone complications from prostate cancer are reviewed here.

Normal bone physiology

In adults, normal bone homeostasis is the product of constant remodeling, maintained by a balance of formation by osteoblasts and resorption by osteoclasts. Osteoclasts arise from the monocyte lineage. Osteoclasts are activated by local and systemic factors to resorb bone during normal bone remodeling and in pathologic states. Bone resorption by activated osteoclasts is accomplished by the release of proteases that dissolve the matrix. This process releases immobilized growth factors, further enriching the marrow's growth factor milieu [11,12].

RANK signaling plays a central role in osteoclast differentiation, activation, and survival. Osteoclasts express receptor activator of nuclear factor-κB (RANK), a member of the tumor necrosis factor (TNF) receptor superfamily. RANK is activated by RANK ligand (RANKL). RANK expression is required for osteoclast differentiation and activation. RANKL is expressed by bone marrow stromal cells (also called mesenchymal stem cells) and osteoblasts, and the binding of RANKL to RANK induces the differentiation of osteoclasts from their precursors. Osteoprotegerin (OPG) is a decoy receptor for RANKL and thereby protects bone from resorption [13]. OPG is expressed by osteoblasts and other tissues and is itself a member of the TNF receptor superfamily. The ratio of RANKL to OPG regulates the activity of osteoclasts. Animal studies highlight the importance of the RANKL/OPG ratio: overexpression of OPG in transgenic mice causes osteopetrosis [14], whereas targeted deletion of OPG causes osteopenia [15].

Pathophysiology of bone metastasis from prostate cancer

Numerous factors may account for the propensity for cancer to metastasize to bone, including high blood flow to bone marrow, a rich growth factor milieu in areas of active hematopoiesis, and a large repository of immobilized growth factors in the matrix [12]. Despite having a dense radiographic appearance, the woven bone of osteoblastic metastases from prostate cancer is structurally weak and associated with increased fracture risk [16].

Osteoclast number and activity are increased in osteoblastic metastases and in adjacent bone, consistent with the high turnover state of prostate cancer bone metastasis [5,16,17]. Markers of bone resorption, such as urine N-telopeptide (NTx) and bone-specific alkaline phosphatase, are higher in patients with bone metastasis compared to those without, indicative of elevated bone turnover despite the osteoblastic radiographic appearance [18,19]. Increased osteoclast activity is independently associated with risk for subsequent skeletal complications, disease progression, and death [5,20]. These studies underscore the contribution of cancer-mediated osteoclast activation to the clinical complications of metastatic disease.

Parathyroid hormone (PTH) stimulates osteoclast formation by inducing RANKL expression in bone marrow stromal cells and osteoblasts. Hypocalcemia caused by osteoblast-driven calcium–phosphate deposition may stimulate PTH production, with resultant secondary hyperparathyroidism. This creates a vicious cycle of osteoclast activation, growth factor liberation from the bone matrix, tumor cell proliferation in the bone, osteoblast activation, calcium–phosphate deposition, and secondary hyperparathyroidism.

Osteoclast modulation has been examined in animal models. In a murine model of prostate cancer, inhibition of osteoclast activity by zoledronic acid did not inhibit the development of osteoblastic metastases [21]. Therefore, an unanswered question in bone metastasis biology is whether bone resorption precedes osteoblastic metastatic development or is a consequence of increased bone formation.

Osteoblast activity is another rational target for therapeutic inhibition in prostate cancer. Atrasentan is an investigational agent that inhibits the endothelin receptor A, resulting in decreased osteoblast activity. The development of atrasentan and other endothelin antagonists in prostate cancer is reviewed elsewhere [22].

Pathophysiology of prostate cancer therapy-induced osteoporosis

Therapy for prostate cancer influences osteoclast activity. Androgen deprivation therapy (ADT) is the cornerstone of treatment for metastatic prostate cancer. ADT is also a routine part of the management for many men with intermediate or high risk, early stage prostate cancer undergoing radiation therapy, as well as those with recurrent disease following surgery or radiation therapy for early stage disease. ADT can be accomplished by either bilateral orchiectomies or chronic therapy with a GnRH agonist or antagonist. The intended therapeutic effect of ADT is severe hypogonadism. Although

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castrate testosterone has traditionally been defined as a serum testosterone level of less than 50 ng/mL, modern assays have shown that surgical or medical castration generally lowers serum testosterone to under 20 ng/mL [23,24].

The profound hypogonadal state is associated with metabolic changes including decreased bone mineral density (BMD) [25]. ADT with GnRH agonists increases PTH-mediated osteoclast activation, as well as biochemical markers of osteoblast and osteoclast activity [26,27]. In prostate cancer patients, ADT raises the risk of fracture [9,10]. In a review of over 50,000 patients in the Surveillance, Epidemiology, and End Results (SEER) program and Medicare database, 19.4% of patients who received ADT experienced a fracture compared with 12.6% in those who did not receive ADT [10]. Fracture prevention strategies for those at high risk for fragility fractures are important opportunities for intervention.

Osteoclast-targeted therapies

Several commercially available and investigational agents inhibit osteoclast function, leading to decreased bone resorption and potentially less skeletal morbidity due to metastatic prostate cancer or ADT-induced osteoporosis. Most of these agents have also been explored as treatments for osteoporosis or for other neoplasms that spread to bone. Other potential targets of osteoclast signaling in prostate cancer (e.g., Src tyrosine kinase, integrins, matrix metalloproteinases) that are in preclinical or early stage clinical development will not be reviewed here.

Bisphosphonates

Bisphosphonates are synthetic analogues of pyrophosphate, a normal component of bone matrix. They bind to areas of exposed bone mineral and are rapidly cleared from the circulation. By binding hydroxyapatite crystals, bisphosphonates diminish the availability of those crystals for osteoclast-mediated resorption. Bisphosphonates can directly inhibit the activities of osteoclasts and their precursors, including recruitment, differentiation, attachment, and survival [28]. Bisphosphonates indirectly inhibit osteoclast differentiation and activation via effects on osteoblasts [28]. Bisphosphonates can induce apoptosis and inhibit RANKL expression in prostate cancer cells, which may further diminish osteoclast activity [29].

The potency of bisphosphonates is determined by the R2 side chain. Those that contain a primary amino group at R2 (e.g., pamidronate) are more potent than non-amino-group containing bisphosphonates, such as clodronate or etidronate. The most potent bisphosphonates, including zoledronic acid, contain a secondary or tertiary amino group, with activity 100 times more potent than clodronate or pamidronate, and at least 1000 times more potent than etidronate.

Bisphosphonates are an established and important component of care for patients with bone metastasis. In 1995, intravenous pamidronate was approved to treat patients with multiple myeloma or metastatic breast cancer based on evidence from randomized controlled trials that pamidronate decreases risk of skeletal complications [30,31]. In 2002, intravenous zoledronic acid was approved to treat patients with multiple myeloma and bone metastases from any solid tumor including prostate cancer. This approval was based on the results of three randomized controlled trials involving more than 3000 patients [32–34].

The ability of bisphosphonates to delay the appearance and progression of visceral and skeletal metastasis is unclear. Preclinical data in mouse models of metastatic breast cancer indicate a significant reduction in bone and visceral metastases with zoledronic acid treatment [35]. Diel et al. [36] randomized 302 women with primary breast cancer considered to be at high risk for bone metastasis (due to the presence of tumor cells in the bone marrow) to receive oral adjuvant clodronate or standard follow-up, for 2 years. Patients who received clodronate had a significantly lower incidence of osseous and visceral metastases, as well as fewer bone metastases per patient. However, subsequent similarly designed studies failed to demonstrate a significant reduction in the occurrence of bone metastasis with adjuvant clodronate in breast cancer patients, although the inclusion criteria were slightly different [37,38]. The addition of zoledronic acid to adjuvant endocrine therapy in localized breast cancer patients resulted in a 36% relative reduction of the risk of disease progression (the ABCSG-12 trial) [39]. Additional ongoing phase 3 trials in breast cancer will examine the effect of the addition of bisphosphonates to adjuvant therapy on disease-free survival (NSABP B-34, BIG 1-04 AZURE). The role of bisphosphonates, especially the more potent amino-bisphosphonates, in prevention of metastasis in prostate cancer patients remains undefined.

Denosumab

Denosumab (AMG 162; Amgen Inc., Thousand Oaks, CA) is a fully human monoclonal IgG_2 antibody directed against RANKL, with an extremely high affinity for human RANKL (K_d approximately 10^{-12} M) [40]. In contrast to bisphosphonates, denosumab does not accumulate in bone and has a long circulatory half life (>30 days). Denosumab has been examined in postmenopausal osteoporosis, rheumatoid arthritis, multiple myeloma, breast cancer, prostate cancer, and other solid tumors. In postmenopausal women, a single administration of denosumab resulted in rapid (within 12 hours), marked (>80%), and sustained (6 months) suppression of osteoclast activity [41]. In patients with multiple myeloma or bone metastasis from breast cancer, denosumab was well tolerated and achieved rapid and sustained suppression of osteoclast activity [42,43].

Osteoprotegerin

RANKL inhibition using recombinant osteoprotegerin has also been tested. The Fc portion of the immunoglobulin heavy chain was fused to the amino-terminus of OPG to generate recombinant Fc-OPG. In postmenopausal women with osteoporosis, Fc-OPG decreased markers of osteoclast activity by 80% after 4 days, with significant effects lasting 45 days; markers of osteoblast activity were not changed [44]. No serious adverse effects were reported. A different formulation of OPG, AMGN-0007, was examined in patients with multiple myeloma or breast cancer with lytic bone lesions [45]. AMGN-0007 was well tolerated and exhibited comparable effects on bone metabolism to pamidronate.

When the two forms of RANKL inhibition were compared, denosumab was more potent than recombinant OPG, with greater decreases in bone turnover markers and longer duration of action [44]. Further, two theoretical risks inherent to recombinant OPG are not applicable to denosumab: (1) the generation of anti-Fc-OPG antibodies which may cross-react and interfere with endogenous OPG function, and (2) the binding of OPG to TNF-related apoptosis-inducing ligand (TRAIL), which may interfere with its role in the normal defense mechanism against tumorigenesis [44]. Despite its promise, recombinant OPG has therefore given way to denosumab in strategies to inhibit RANKL activity and osteoclast activation.

Clinical settings for osteoclast-targeted therapies

How does inhibition of osteoclast activity and bone remodeling influence disease progression? Four common clinical questions have been studied. (1) Does inhibition of bone remodeling reduce skeletal-related events (SREs; including pathologic fracture, need for radiation therapy or surgery to bone, spinal cord compression) in prostate cancer patients with bone metastases and castration-resistant disease? (2) Can SREs be reduced in patients with bone metastases and castration-sensitive disease? (3) Does osteoclast-targeted

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Table 1
Randomized controlled trials of osteoclast-targeted therapies in bone metastatic prostate cancer.

Study	*n*	Study population	Arms	Outcome
Zometa 039 [34,46]	643	Castration-resistant, asymptomatic or minimally symptomatic	4 mg zoledronic acid vs. placebo, every 3 weeks for 15 months	Significant decrease in skeletal related events (SREs) (33.2% vs. 44.2%); trend toward improved survival; established zoledronic acid as standard of care in this setting
CGP 032/INT 05 [47]	350	Castration-resistant, symptomatic	90 mg pamidronate vs. placebo, every 3 weeks for 27 weeks	No significant difference in pain, analgesic use, or SREs
NCIC CTG Pr.6 [48]	209	Castration-resistant, symptomatic	Mitoxantrone and prednisone ± 1500 mg clodronate, every 3 weeks until progression	No significant difference in palliative response, duration of response, progression-free survival, overall survival, overall quality of life
Denosumab protocol 20050103 [49,50]	1,901	Castration-resistant	120 mg denosumab vs. 4 mg zoledronic acid, every 4 weeks	Primary end point: denosumab was noninferior to zoledronic acid in time to first on-study SRE; secondary end point: denosumab was superior to zoledronic acid; no difference in overall survival or adverse event rates
MRC PR05 [50,51]	311	Castration-sensitive	2080 mg daily oral clodronate vs. placebo, for 3 years maximum	Trend toward improved bone progression-free survival ($P = 0.066$); significantly improved 8-year overall survival (22% vs. 14%; HR = 0.077, $P = 0.032$)
CALGB/CTSU 90202	680[a]	Castration-sensitive	4 mg zoledronic acid vs. placebo, every 4 weeks until progression to CRPC or first SRE, then cross-over to open label	*Ongoing* Primary end point: SRE or prostate cancer death

[a] Targeted accrual.

therapy prolong bone metastasis-free survival in men with castration-resistant disease and no bone metastases at baseline? (4) Can such therapies reduce fracture rates due to ADT-induced osteoporosis? The clinical trials addressing these questions are presented below and are summarized in Tables 1–3.

Metastatic castration-resistant prostate cancer

Three contemporary randomized controlled trials have evaluated the efficacy of bisphosphonates for patients with bone metastases and disease progression despite first-line hormonal therapy (castration). One recently completed study compared denosumab with zoledronic acid in this setting. These patients are contemporarily described as having castration-resistant prostate cancer (CRPC, previously termed "hormone-refractory prostate cancer"). At present, zoledronic acid is the Food and Drug Administration (FDA)-approved standard of care for the prevention of SREs in this patient population. The demonstrated superiority of denosumab over zoledronic acid may alter this standard.

Zometa 039

In the Zometa 039 study, 643 men with CRPC and asymptomatic or minimally symptomatic bone metastases were assigned randomly to intravenous zoledronic acid (4 or 8 mg every 3 weeks) or placebo [34]. All men continued ADT (bilateral orchiectomies or treatment with a GnRH agonist) throughout the study and received additional antineoplastic therapy at the discretion of the treating physicians. The primary study end point was the proportion of men who experienced one or more SRE (pathological fracture, spinal cord compression, surgery or radiation therapy to bone, or change in antineoplastic treatment to treat bone pain) by 15 months.

Adverse renal events (grade 3 increases in serum creatinine) prompted two study amendments. First, the infusion time for zoledronic acid was increased from 5 to 15 minutes, with an increase in infusate volume from 50 to 100 mL. Second, the zoledronic acid dose in the 8 mg treatment group was reduced to 4 mg, serum creatinine monitoring was implemented prior to each dose, and the primary efficacy assessment became the comparison of the 4 mg group versus placebo. After these amendments, the rates of deterioration in renal function between the zoledronic acid 4 mg and placebo groups were similar.

At 15 months, fewer men in the zoledronic acid treatment group had SREs than men in the placebo group (33.2% versus 44.2%, $P = 0.021$). Zoledronic acid also increased the median time to first SRE (488 days versus 321 days, $P = 0.009$) [46]. Median survival was longer in the zoledronic 4 mg group than in the placebo group (546 days versus 464 days, $P = 0.091$). Notably, the study was not designed to evaluate the effect of zoledronic acid on survival and the observed difference in overall survival was not statistically significant. Based on the results of this study, zoledronic acid (4 mg intravenously every 3–4 weeks) was approved to treat men with bone metastatic prostate cancer and disease progression despite first-line hormonal therapy.

CGP 032/INT 05

In two similarly designed, multicenter trials, CGP 032 and INT 05, 350 men with CRPC and symptomatic bone metastases were assigned randomly to either intravenous pamidronate (90 mg) or placebo every 3 weeks for 27 weeks [47]. Primary end points were self-

Table 2
Randomized controlled trials of osteoclast-targeted therapies in prevention of bone metastasis in nonmetastatic prostate cancer.

Study	*n*	Study population	Arms	Outcome
MRC PR04 [52]	508	Clinical stage T2–T4	2080 mg daily oral clodronate vs. placebo, for 5 years	No difference in development of bone metastasis or overall survival
ZEUS [54]	1433	High-risk disease (PSA ≥ 20, lymph node-positive disease, or Gleason sum 8–10)	Standard prostate cancer therapy ± zoledronic acid 4 mg intravenously every 3 months for 48 months (open label)	*Ongoing* Primary objective: evaluate superiority of zoledronic acid over control in incidence of bone metastasis
Denosumab protocol 20050147	1400[a]	Castration-resistant, high-risk by PSA criteria	120 mg denosumab vs. placebo, every 4 weeks	*Ongoing* Primary end point: bone metastasis-free survival Final results expected, 2010.

[a] Targeted accrual.

Table 3
Randomized controlled trials of fracture prevention in prostate cancer.

Study	*n*	Study population	Arms	Outcome
Denosumab HALT 138 [62]	1468	Current androgen-deprivation therapy; no metastases; high risk for fracture	60 mg denosumab vs. placebo, every 6 months for 3 years	Significant increase in bone mineral density; significant 62% reduction in 3-year incidence of new vertebral fractures
Toremifene protocol G300203 [63]	1389	Current androgen-deprivation therapy; high risk for fracture	80 mg daily oral toremifene vs. placebo	Fewer new vertebral *f*ractures (2.5% vs. 4.9%, P<0.05); increased bone mineral density, decreased breast pain, decreased hot flashes, favorable lipid profile changes

reported pain, analgesic use, and SREs (defined as pathologic fracture, radiation or surgery to bone, spinal cord compression, or hypercalcemia). Results from the two studies were pooled. Pain scores, analgesic use, proportion of men with at least one SRE by 27 weeks, and survival did not differ between the groups.

Pamidronate decreased urinary NTx markers of osteoclast activity by approximately 50%. In contrast, zoledronic acid decreases urinary markers of osteoclast activity by 70–80% [34]. Less potent suppression of osteoclast activity by pamidronate may have contributed to its lack of efficacy in CGP 032/INT 05. Inclusion of subjects with more advanced disease and use of less precise study end points may have also contributed to the negative results.

NCIC CTG PR.6

The National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) PR.6 study evaluated the palliative benefit of intravenous clodronate in patients with symptomatic, bone metastatic CRPC. Two-hundred and nine men were treated with mitoxantrone (12 mg/m^2 intravenously every 3 weeks) and prednisone, and were randomly assigned to receive either intravenous clodronate (1500 mg every 3 weeks) or placebo [48]. Subjects completed pain index and quality of life questionnaires at each visit and recorded analgesic use in daily diaries. The primary end point was palliative response, defined as a two-point decrease in the pain index (or reduction to zero) or a 50% decrease in analgesic intake, without increase in the other outcome.

Palliative responses were achieved in 46 (46%) of 104 patients on the clodronate arm and in 41 (39%) of 105 patients on the placebo arm ($P=0.54$). The median duration of response, symptomatic disease progression-free survival, overall survival, and overall quality of life were similar between the arms. Subgroup analysis suggested a possible benefit in men with more severe pain.

Together, the results of Zometa 039, CGP 032/INT 05, and NCIC CTG PR.6 show that zoledronic acid, but not other less-potent bisphosphonates, decreases the risk of skeletal complications in men with CRPC and bone metastases.

Denosumab protocol 20050103

In Amgen Inc., protocol 20050103 (NCT 00321620), a randomized, double-blind multicenter study, 1901 men with CRPC and bone metastases were assigned to denosumab (120 mg subcutaneously every 4 weeks) or zoledronic acid (4 mg intravenously every 4 weeks). The primary end point was time to first on-study SRE (pathological fracture, radiation to bone, surgery to bone, or spinal cord compression). The primary objective was to demonstrate noninferiority of denosumab compared with zoledronic acid. The secondary objective was to demonstrate the superiority of denosumab and comparative safety and tolerability of the two drugs.

In a preliminary report of the study, denosumab was superior to zoledronic acid in delaying the time to first on-study SRE (hazard ratio (HR) = 0.82, 95% confidence interval (CI) = 0.71–0.95) and reducing rates of multiple SREs (HR = 0.82, 95% CI = 0.71–0.94) [49,50]. Overall survival and time to disease progression were similar between the groups. Adverse event rates were similar, without a significant difference in osteonecrosis of the jaw (22 in the denosumab arm and 12 in the zoledronic acid arm). Based on these results, denosumab may become the new standard of care for prevention of SREs in men with castration-resistant metastatic prostate cancer.

Castration-sensitive metastatic prostate cancer

MRC PR05 is the only completed, randomized, controlled trial to evaluate the efficacy of a bisphosphonate in metastatic prostate cancer patients receiving first-line hormone therapy (castration). CALGB/CTSU 90202, an ongoing study, is designed to evaluate the efficacy of zoledronic acid in this setting.

MRC PR05

In the Medical Research Council PR05 study, 311 men with prostate and bone metastases who were either initiating or responding to primary ADT were assigned randomly to either oral clodronate (2080 mg daily) or placebo [50]. All men continued primary ADT. The primary study end point was symptomatic skeletal disease progression or prostate cancer death. Overall survival was a secondary end point. After a median follow-up of 59 months, the clodronate group had nonsignificant improvements in bone progression-free survival (HR = 0.79, 95% CI = 0.61–1.02, $P=0.066$) and overall survival (HR = 0.80, 95% CI = 0.62–1.03, $P=0.082$). Men in the clodronate group reported more gastrointestinal problems and required more frequent dose modification of study drug (HR for any adverse event = 1.71, 95% CI = 1.21–2.41, $P=0.002$). In exploratory analyses, a short interval between diagnosis of bone metastases and initiation of investigational treatment was associated with better outcomes. Long-term overall survival data after 258 deaths confirmed a significant benefit in the clodronate group compared with placebo (8-year OS 22 vs. 14%; HR = 0.77, 95% CI = 0.60–0.98, $P=0.032$) [51]. As the survival benefit of early use of clodronate in castration-sensitive metastatic disease has only recently been reported, the clinical impact of this study is as yet unknown.

CALGB/CTSU 90202

An ongoing randomized controlled trial will help define the role of zoledronic acid in castration-sensitive metastatic prostate cancer. CALGB/CTSU 90202 (NCT00079001) will enroll 680 men with prostate cancer and bone metastases who have initiated ADT within 3 months. Subjects are assigned to zoledronic acid (4 mg intravenously every 4 weeks) or placebo. Subjects will crossover to open-label zoledronic acid at either progression to castration-resistant disease or first SRE. The primary study end point is SRE or prostate cancer death. Accrual is ongoing.

Prevention of bone metastasis in nonmetastatic prostate cancer

Two randomized controlled trials to evaluate the efficacy of bisphosphonates for prevention of bone metastases in men with

nonmetastatic prostate cancer have been reported. A randomized, placebo-controlled study evaluating denosumab in this setting is underway. Currently, there is no evidence that osteoclast-targeted therapy prevents the subsequent development of symptomatic bone metastasis.

MRC PR04

MRC PR04 evaluated the efficacy of clodronate to prevent symptomatic bone metastases in patients considered to be at high risk of developing bone metastases. The study included 508 men receiving standard treatment for clinical stage T2–T4 prostate cancer with no evidence of bone metastases and WHO performance status 0–2 [52]. Men were randomly assigned to either oral clodronate (2080 mg daily) or placebo for 5 years. Most of the subjects received external beam radiation therapy, external beam radiation therapy with hormone therapy, or primary hormone therapy as standard treatment. The primary end point was time to development of symptomatic bone metastases or prostate cancer death. At a median follow-up of 10 years, there were 148 events total, with no significant difference between the groups. The overall 5-year survival was 78% for the entire study population. Prostate cancer death event rates were similar in both groups (HR = 1.07, 95% CI = 0.76–1.49, $P = 0.71$). In long-term follow-up after 281 deaths (60%), there was no difference in overall survival between the groups (HR = 1.12, 95% CI = 0.89–1.42, $P = 0.94$). These results are in contrast with the survival benefit seen with clodronate in castration-sensitive metastatic disease (MRC PR05, described above) [51].

Zometa 704

Zometa 704 was designed to evaluate the effects of zoledronic acid on time to first bone metastasis in men with nonmetastatic CRPC. The study included prostate cancer patients with no radiographic evidence of metastases and PSA progression despite ADT. PSA progression was defined as three consecutive rises in serum PSA (measured at least 2 weeks apart), initial PSA rise within 10 months of study entry, and last PSA ≥150% of nadir value. Subjects were assigned randomly to zoledronic acid (4 mg intravenously every 4 weeks) or placebo. Bone scans were performed every 4 months. The primary study end point was time to first bone metastasis. Target accrual was 991 subjects.

Between September 1999 and September 2002, 398 subjects were enrolled. In December 2001, the Data and Safety Monitoring Board placed the study on hold because the observed event rate was lower than expected. In September 2002, the study was terminated. Time to first bone metastasis was similar for both groups, although the low event rate and early termination of the study preclude evaluation of efficacy.

Analyses of the placebo group from the study have helped characterize the natural history of a rising PSA in men with castrate nonmetastatic prostate cancer [53]. One-third of subjects developed bone metastases at 2 years. Median bone metastasis-free survival was 30 months. Median time to first bone metastasis and overall survival were not reached. Baseline PSA and PSA velocity independently predicted shorter time to first bone metastasis, metastasis-free survival, and overall survival. Other covariates did not consistently predict clinical outcomes. These observations facilitate the identification of men at high risk for development of bone metastases and informed the design of subsequent clinical trials in this setting.

ZEUS

The Zometa European Study (ZEUS) is an ongoing, open-label, randomized controlled trial evaluating the effect of zoledronic acid on prevention of bone metastasis in patients with high risk prostate cancer [54]. Eligible patients were required to have at least one high-risk prognostic factor: PSA ≥20 ng/mL, lymph node-positive disease, or Gleason sum of 8–10. As of 2008, 1433 patients were randomly assigned to standard prostate cancer therapy with or without zoledronic acid (4 mg intravenously every 3 months for 48 months). The primary objective is to demonstrate superiority of zoledronic acid over control in the proportion of patients with at least one bone metastasis after 48 months of treatment. Secondary objectives will evaluate the effects of zoledronic acid on overall survival, symptomatic disease progression, PSA doubling time, and biochemical markers of bone turnover. The study is ongoing.

Denosumab protocol 20050147

Amgen Inc., protocol 20050147 (NCT 00286091) will accrue 1400 men with prostate cancer, no bone metastases, and rising PSA despite current ADT. It will include only subjects at high risk for development of bone metastases based on PSA ≥8 ng/dL and/or PSA doubling time ≤10 months. Subjects will be randomly assigned to denosumab (120 mg subcutaneously every 4 weeks) or placebo. The primary end point is bone metastasis-free survival. Final study results are expected in 2010.

Treatment-related fragility fractures

The hypogonadal state caused by ADT accelerates loss of BMD with an associated increase in risk of fractures. Several bisphosphonates have been demonstrated to improve BMD in men receiving ADT, including alendronate [55], pamidronate [26,56], zoledronic acid [57,58], and neridronate [59]. Use of selective estrogen receptor modulators (SERMs) such as raloxifene [60] and toremifene [61] constitutes a second strategy to improve BMD in this clinical setting.

To date, there are no FDA-approved medications for fracture prevention in prostate cancer patients. Two positive phase 3 fracture prevention trials including prostate cancer patients on ADT with a high risk of fracture have recently been reported. The denosumab and toremifene studies are the first trials to demonstrate significant reductions in fracture risk in this patient population. Therefore, these studies are likely to change clinical practice in the near future. Whether such interventions alter the natural history of disease progression is unclear at this time.

Denosumab HALT 138

Denosumab protocol 20040138, or HALT 138, enrolled 1468 patients receiving ADT who were considered high risk for fracture based on age ≥70, low baseline BMD, or prior history of osteoporotic fracture [62]. Patients were randomly assigned to receive denosumab (60 mg subcutaneously every 6 months) or placebo for three years. The primary end point was percent change in BMD in the lumbar spine, with secondary end points including change in BMD at other sites and incidence of new vertebral fractures. Compared to placebo, denosumab significantly increased BMD of the lumbar spine (6.7%), total hip (4.8%), femoral neck (3.9%), and distal third of radius (5.5%). Further, denosumab therapy reduced the 3-year incidence of new vertebral fractures by 62% (relative risk (RR) = 0.38, 95% CI = 0.19–0.78, $P = 0.006$). Fractures at any site were reduced in the denosumab group by 28% ($P = 0.10$). Multiple fractures were significantly reduced by 72% ($P = 0.006$). Rates of adverse event were similar in the treatment groups.

Toremifene protocol G300203

Toremifene protocol G300203 (NCT00129142) enrolled 1389 patients receiving ADT who were considered high risk for fracture due to age ≥70 or low BMD [63]. Subjects were assigned randomly

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either toremifene (80 mg by mouth daily) or placebo for 2 years. The primary end point was incidence of morphometric vertebral fractures. Secondary end points included BMD at hip and lumbar spine, breast pain, hot flashes, and lipid profile.

Men treated with toremifene had significantly fewer new vertebral fractures compared with the placebo group (2.5% vs. 4.9%; RR = 0.50, $P<0.05$). Toremifene also significantly increased BMD at the lumbar spine by 2% and at the hip by 1.6%, and decreased breast pain and hot flashes. Lipid profile changes in the toremifene group were favorable (i.e., increased high-density lipoprotein and decreased low-density lipoprotein and triglycerides).

Conclusions

Bone metastases and skeletal complications are major causes of morbidity in men with advanced or metastatic prostate cancer. Taken together, the aforementioned studies support the use of zoledronic acid (4 mg every 3–4 weeks) in one setting: to reduce skeletal complications in men with CRPC and bone metastases. Other less-potent bisphosphonates did not prevent SREs in similar studies. Additional studies are needed to determine the optimal timing, schedule, and duration of bisphosphonate treatment in men with bone metastases.

Recent studies of denosumab in prostate cancer and other diseases (breast cancer, osteoporosis, multiple myeloma) have demonstrated the central role of RANKL signaling in bone metastatic disease. Denosumab was superior to zoledronic acid in treatment of bone metastatic disease in CRPC, the one setting in which a bisphosphonate is approved for prostate cancer. Furthermore, denosumab has proven efficacy in fracture prevention in men on initial ADT. The SERM toremifene also significantly decreases fracture risk in patients on ADT. Ongoing phase 3 studies will address other important unmet medical needs including metastasis prevention.

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Vitamin D and Immunity - Why Do We Need More?

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Vitamin D and Immunity - Why Do We Need More?

Meet the Professor session: Chantal Mathieu, KULeuven, Belgium

Significance of the Topic:

The title of the present 'Meet the Professor session' suggests that we need more vitamin D for immune health and that in the present session reasons for this statement will be given. However, the debate on this issue is still very much open, although in many places in lay and professional press conclusions seem to be clear.

In the present session, the evidence on the issue of impact of vitamin D insufficiency on the immune system and of vitamin D (or derivatives) supplementation on the immune system and immune diseases will be discussed.

Learning Objectives:

In this session we will discuss the available evidence *in vitro* as well as *in vivo*, in preclinical models and in humans for a possible role for vitamin D in the immune system and as a result of participating in this session, attendees should be able to more critically evaluate the whole discussion on vitamin D supplementation and the prevention or intervention in immune mediated diseases. The participants will be able to formulate advice on how we should further approach this issue in a scientific manner. Participants should be able to contribute to the design of relevant clinical intervention trials using vitamin D (derivatives) in immune models (*in vitro* and *in vivo*) of human disease.

Overview: Vitamin D and the immune system

In humans, vitamin D can be obtained from two distinct sources, either from diet or by UV-mediated synthesis in the epidermal layer of the skin. Therefore, by definition, vitamin D cannot be considered as a true vitamin but rather as a pro-hormone. Two forms of vitamin D can be obtained by nutritional intake: vitamin D_2 (also known as ergocalciferol) is present in fungi/yeast, while vitamin D_3 (also known as cholecalciferol) is found in foods from animal origin. Only few foods naturally contain significant amounts of vitamin D. For example cod liver oil and oily fish are considered as rich sources, whereas butter, cream and egg yolk contain only small amounts. Human and cow´s milk, on the other hand, are poor sources of vitamin D. Despite the fact that vitamin D_3 can be obtained by nutrition, the most important source of this pro-hormone is the skin, which has a great capacity to produce vitamin D_3 upon sunlight exposure. In the skin, UV rays promote photolytic cleavage of 7-dihydrocholesterol (7-DHC) into pre-vitamin D_3, which is

subsequently converted by a spontaneous thermal isomerisation into vitamin D_3. After synthesis, vitamin D and its metabolites are bound to a carrier molecule, known as the vitamin D binding protein (DBP), for systemic transport. Regardless of the source of vitamin D, it needs to be hydroxylated twice in order to become biologically active: first in liver (at the carbon 25-position by 25-hydroxylases) and second typically in kidney (at the carbon 1 by the 1a hydroxylase CYP27B1), yielding $1,25(OH)_2D_3$. The action of vitamin D on cells is mediated by $1,25(OH)_2D_3$ through a nuclear receptor, the vitamin D receptor (or VDR).

Vitamin D insufficiency is a term used to describe the biochemical evidence of deficiency without obvious clinical bone signs or symptoms, such as rickets or osteomalacia. Although there is continuing discussion about the precise levels of 25-hydroxyvitamin D_3 [$25(OH)D_3$], which define the different categories of vitamin D status, there is general agreement that vitamin D insufficiency or hypovitaminosis D is prevalent in many populations across the globe. Vitamin D insufficiency is most commonly diagnosed by a serum concentration of $25(OH)D_3$ between 15 and 30 ng/ml; levels <15 ng/ml are defined as vitamin D deficiency, with concentrations <5 ng/ml being severely deficient and associated with presentation of osteomalacia and rickets. Currently, serum concentrations of 30–50 ng/ml $25(OH)D_3$ are accepted to meet the requirements for vitamin D sufficiency; however, recent evidence suggests that the optimal serum $25(OH)D_3$ levels may be much higher.

Expression of the VDR and vitamin D metabolizing enzymes is not restricted to cells involved in calcium and bone metabolism. Within the immune system, VDR is expressed by almost all immune cells, including activated CD4+ and CD8+ T cells, B cells, neutrophils, and antigenpresenting cells (APCs), such as macrophages and dendritic cells (DCs) . In addition, the key enzymes needed for vitamin D activation and degradation are expressed by certain immune cells. 1-α-hydroxylase has been detected in macrophages, DCs, and even B- and T cells. Although identical to the renal enzyme, expression of 1-α-hydroxylase in macrophages is upregulated by immune signals such as interferon (IFN)-γ and lipopolysaccharide (LPS) or viral infections. In DCs, upregulation of 1-α-hydroxylase is associated with the maturation process of these cells, suggesting that local production of $1,25(OH)_2D_3$ might serve as a negative feedback loop to prevent excessive inflammation .
Both in humans and experimental animal models, insufficient levels of vitamin D have been linked to important immune defects in the context of autoimmune disorders. Multiple epidemiological studies show a correlation between areas with low vitamin D supplies (due to insufficient sunlight exposure time or nutritional vitamin D uptake) and incidences of different autoimmune diseases (e.g. type 1 diabetes, multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis). Also in animal models of various autoimmune diseases, vitamin D deficiency has been shown to accelerate disease development.

In vitro effects of vitamin D on immune cells

Effects on T cells

The active form of vitamin D, the twice hydroxylated form, $1,25(OH)_2D_3$, inhibits activation and antigen-induced T cell proliferation, in addition to altering their cytokine expression profile. It inhibits expression of interleukin (IL)-2, an autocrine growth factor secreted by T lymphocytes to maintain their survival and induce further activation and proliferation. Moreover, IFNγ expression, the major macrophage activator and Th1-recruiting cytokine, is also reduced in $1,25(OH)_2D_3$-treated T lymphocytes. By inhibiting Th1 differentiation and recruitment, $1,25(OH)_2D_3$ promotes an indirect shift in the immune response to a Th2 profile. Direct effects on Th2 lymphocytes are less clear, with one study showing increased GATA-3 and c-maf levels, resulting in an induction of the Th2 cytokines IL-4, IL-5 and IL-10. In Th17 lymphocytes, $1,25(OH)_2D_3$ appears to directly inhibit IL-17 production, although expression of the Th17 lineage-specific transcription factor RORγt is not directly impaired, but is down-regulated in an indirect manner mediated by modulated DCs. More recently, $1,25(OH)_2D_3$ in combination with IL-2, was shown to directly inhibit pro-inflammatory T cell production of cytokines and promote the development of Tregs expressing CTLA-4 and Foxp3, in the absence of DCs.

Effects on antigen-presenting cells

$1,25(OH)_2D_3$ is able to induce differentiation of monocytes and monocytic cell lines to a macrophage-like phenotype. In addition, exposure of macrophages and monocytes to $1,25(OH)_2D_3$ stimulates phagocytosis and mycobacteria killing ability, in contrast to impairing their T cell stimulatory capacity by reducing the expression of co-stimulatory molecules (e.g. CD80, CD86, CD40) and production of IL-12.

Among APCs, DCs are the main target of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ was extensively shown to impair *in vitro* and *in vivo* DC differentiation and maturation from either human peripheral blood monocytes or mouse bone marrow precursors, inducing the appearance of a different kind of DC, with a more tolerogenic or semi-mature phenotype. As for monocytes and macrophages, $1,25(OH)_2D_3$ profoundly alters DC surface marker expression phenotype, down-regulating co-stimulatory molecules (CD40, CD80, CD86), as well as CD83 and MHCII. Interestingly, $1,25(OH)_2D_3$ up-regulates the expression of the monocytic marker CD14, without driving DC differentiation towards a macrophage-like phenotype, as the endocytic capacity of $1,25(OH)_2D_3$-treated DCs is not enhanced to the level of macrophages and iDCs. In human DCs, the up-regulation of T cell inhibitory molecules, such as programmed death-1 ligand (PD-L1) and immunoglobulin-like transcript 3 (ILT3), are increased upon 1,25(OH)2D3 treatment. Although up-regulated in $1,25(OH)_2D_3$- treated DCs, ILT3 is indispensable for the induction of CD4+FoxP3+ Tregs. On the contrary, PD-L1 was proven to be an essential molecule for the induction of antigen-specific Tregs by modulated DCs and its blockade during priming redirects T cells to an effector phenotype with production of IFNγ, T-bet and low IL-10 expression.

The production of IL-12, the major cytokine driving Th1 responses and inhibiting Th2 differentiation, is also affected by treatment of $1,25(OH)_2D_3$ on DCs. The hormone exerts direct effects on nuclear factor kappa B (NF-κB) transcription factor family members (e.g. relB, c-Rel), preventing activation, nuclear

translocation and binding of NF-κB to its binding site on the promoter of IL-12p40 subunit gene. Moreover, a VDRE has been found in the promoter of the relB gene, contributing to the $1,25(OH)_2D_3$-mediated inhibition of NFκB-mediated maturation of DCs, in a process controlled by chromatin remodeling by means of histone deacetylase 3 activity. On the other hand, IL-10 production is enhanced by 1,25(OH)2D3-treatment in DCs, although only when the hormone was added during the maturation phase of these cells. Interestingly, the effects on surface marker expression and IL-12 secretion by DCs are only seen on the myeloid and not the plamacytoid population. In addition, $1,25(OH)_2D_3$ was shown to impair migration of type I IFN-differentiated DCs in response to the inflammatory and lymph node-homing chemokines CCL4 and CCL19, without affecting the expression of their respective receptors, CCR5 and CCR7. $1,25(OH)_2D_3$-modulated DCs are thus unable to fully activate T cells and initiate an immune response, as in the absence or low levels of co-stimulatory signals, stimulation of T cell receptors induces T cell anergy, unresponsiveness (decreased proliferation and cytokine secretion) and increased apoptosis levels.

Innate immune system

Monocytes and macrophages are key players in mounting innate immune responses against various infectious agents, including bacteria, viruses, fungi, and parasites, as they rapidly detect pathogen-associated molecular patterns (PAMPs) of dangerous microbial invaders by means of pattern-recognition receptors, such as toll-like receptors (TLRs). We and others identified various defects in macrophage functions indispensable for antimicrobial activity in vitamin-D-deficient mice, including defects in chemotaxis, phagocytosis, and proinflammatory cytokine production. In accordance with these observations, experimental data demonstrate the ability of $1,25(OH)_2D_3$ to stimulate innate immune responses. $1,25(OH)_2D_3$ exerts prodifferentiating effects on monocytes and monocyte-derived cell lines, driving them toward a macrophage-like phenotype. In addition, exposing macrophages to $1,25(OH)_2D_3$ enhances their chemotactic and phagocytic capacity, which is indispensable for their tumor-cell cytotoxicity and antimicrobial activity. $1,25(OH)_2D_3$ is known to protect cultured human monocytes and macrophages against tubercle bacilli. Recently, a central mechanism underlying the antimicrobial effects of $1,25(OH)_2D_3$ was identified: $1,25(OH)_2D_3$ induces expression of cathelicidin antimicrobial peptide (CAMP) in various cell types, including myeloid cells, keratinocytes, neutrophils, and bronchial epithelial cells, directly leading to enhanced antimicrobial activity. Interestingly, $1,25(OH)_2D_3$-mediated induction of CAMP was found to be an integral component of human TLR-mediated immune responses: TLR2/1-triggering of human monocytes selectively induces expression of VDR and 1-α-hydroxylase, making the cells able to convert $25(OH)D_3$ into active $1,25(OH)_2D_3$ and allowing the $1,25(OH)_2D_3$-mediated induction of CAMP. Therefore, adequate functioning of this antimicrobial mechanism critically depends on the presence of $25(OH)D_3$ in the medium: TLR2/1-stimulated production of CAMP was severely impaired when monocytes were cultured with serum from dark-skinned African Americans, as this serum contains low levels of $25(OH)D_3$ due to lower vitamin D production in dark skin. Addition of $25(OH)D_3$ to the medium could indeed restore impaired CAMP production. It is suggested that IL-15 is responsible for TLR2/1-mediated induction of VDR and 1-α- hydroxylase in monocytes. Remarkably,

induction of CAMP is not the only mechanism linking vitamin D signaling to TLR-mediated antimicrobial responses: TLRinduced expression of another antimicrobial peptide, defensin beta 4, by monocytes requires convergence of both VDR- and IL1β-activation pathways. Together, vitamin-D-mediated induction of antimicrobial peptides (at least partially) explains the success of UV therapy as a way to improve or even cure disease in tuberculosis patients. Nevertheless, additional mechanisms are likely to further complement the antimicrobial effects of $1,25(OH)_2D_3$, as $1,25(OH)_2D_3$ mediates generation of reactive oxygen species in human monocytes and macrophages. Induction of inducible nitric oxide synthase (iNOS) has been proposed as a potential mechanism by which $1,25(OH)_2D_3$ mediates bacterial killing. However, controversial data have been obtained concerning regulation of iNOS expression by 1,25(OH)2D3. In a human macrophage-like cell line, induction of iNOS expression by the hormone was observed, whereas others reported inhibitory actions of $1,25(OH)_2D_3$ on this enzyme. Remarkably, while participating in TLR-induced antimicrobial responses, $1,25(OH)_2D_3$ induces hyporesponsiveness to PAMPS by down-regulating the expression of TLR2 and TLR4 on monocytes, probably providing a negative feedback mechanism to prevent excessive TLR activation and to shut down the inflammatory response at a later stage of infection.

In vivo effects of vitamin D in animal models of immune disease

Also *in vivo*, $1,25(OH)_2D_3$ administration has important immune effects, such as prolongation of graft survival in different experimental animal models (e.g. heart, liver, pancreatic islets, skin, small-bowel allografts), and prevention or attenuation of autoimmune disorders, such as type 1 diabetes (T1D), inflammatory bowel disease, collagen-induced arthritis and experimental autoimmune encephalomyelitis. For instance, in the non-obese diabetic (NOD) mouse, an animal model used to study T1D, treatment with $1,25(OH)_2D_3$ from weaning until 200 days of age prevents insulitis and delays diabetes onset. In addition, $1,25(OH)_2D_3$ restores defective regulator cell population, enhances defective apoptosis sensitivity of diabetogenic T lymphocytes in the thymus through DCs and inhibits recurrence of diabetes after syngeneic islet transplantation, with enhanced protective effects after combination with a subtherapeutical dose of classical immunomodulators (e.g. cyclosporin A). Moreover, it induces an autoantigen-specific immune shift towards a Th2 response in NOD mice, but not to disease-irrelevant antigens. Finally, in a model of murine islet transplantation, a combination of $1,25(OH)_2D_3$ with mycophenolate mofetil prevented graft rejection and induced the frequency of a CD4+CD25+ Treg population, which was able to confer protection to untreated mice upon transfer.

One of the major drawbacks for the use of $1,25(OH)_2D_3$ in therapeutic interventions for growth arrest, cell differentiation and immune modulation is the accompanying dose-dependent calcemic side effects, such as hypercalcemia, hypercalcinuria and increased bone resorption in vivo, induced by the administration of high concentrations of this compound needed to achieve its non-classical effects. In view of exploiting the non-classical effects of $1,25(OH)_2D_3$ in the clinic, more than 2000 of its structural analogues have been

designed and synthesized, presenting a clear dissociation between undesired calcemic and beneficial non-classical effects.

Vitamin D deficiency and the immune system *in vivo*

The physiological importance of vitamin D and its active metabolite in the immune system is further emphasized by multiple studies linking vitamin D insufficiency/deficiency to aberrant immune responses. Multiple groups have reported a relation between vitamin D deficiency and susceptibility to infections, especially in the context of infection by Mycobacterium tuberculosis. A higher susceptibility to tuberculosis is seen in patients with relatively low serum vitamin D levels, such as the elderly, uremic patients, and dark-skinned people. Different epidemiological studies report an inverse correlation between vitamin D status and the incidence of autoimmune diseases, such as T1D, SLE, multiple sclerosis (MS), inflammatory bowel disease (IBD), and rheumatoid arthritis (RA). For example, a considerable percentage of the population living in more northern areas of the northern hemisphere (and thus receiving less UV radiation) is vitamin D deficient and this deficiency positively correlated with higher incidences of autoimmune diseases. Also, similar to the serum levels of $25(OH)D_3$, the onset and exacerbations of different autoimmune diseases have been documented to vary with seasonality. Furthermore, patients suffering from different autoimmune diseases such as MS, SLE, RA, and T1D display lower serum $25(OH)D_3$ levels in comparison to healthy individuals. In the context of T1D, a Finnish birth cohort study revealed a three-fold increased disease incidence in individuals that were vitamin D deficient during early life.

Interventions using vitamin D in immune diseases *in vivo*

Although promising results were obtained in a few small clinical trials of immune disease (see reviews), there is still a lack of non-biased large-cohort studies that can sustain the proposed benefits of vitamin D supplementation for optimal immune function. Small sample sizes, short follow-up duration and lack of control groups constitute major limitations of the reported studies. In addition, different doses of vitamin D have been applied, and the initial vitamin D status of the individuals included was not always known, making it unclear whether the administered vitamin D supplements restored existing deficiencies or augmented circulating vitamin D in already sufficient individuals.

Conclusion

Besides its well-established role in the maintenance of mineral homeostasis and bone health, the active vitamin D metabolite, $1,25(OH)_2D_3$, has been identified as a central regulator of normal immune functioning. In addition, the discovery of vitamin-D-activating enzymes expressed in certain immune cells, allowing a local extrarenal activation of vitamin D, has led to the emerging viewpoint of an auto or paracrine role for vitamin D metabolites in the immune system. This concept is supported by various

studies linking vitamin D deficiency or insufficiency to conditions related to dysregulated immune responses, including various autoimmune diseases and infections.

Interesting recent reviews

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