Citation: IBMS BoneKEy **10,** Article number: 333 (2013) | doi:10.1038/bonekey.2013.67

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Oral Presentations

Oral Presentations 1

OC01

Effects of Odanacatib on Bmd and Safety in the Treatment of Osteoporosis in Postmenopausal Women Previously Treated with Alendronate-a Randomized Placebo-Controlled Trial

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Odanacatib (ODN) is an orally-active cathepsin K inhibitor being developed for the treatment of postmenopausal osteoporosis. This study evaluated the effects of ODN 50mg once weekly on BMD, bone turnover markers and safety in patients previously treated with alendronate (ALN). This was a randomized, double-blind, placebo-controlled, 24-month study. The primary endpoint was % change from baseline at month 24 of femoral neck (FN) BMD. Postmenopausal women (*n*=243) ≥60 years of age with low BMD T-score at the total hip, FN or trochanter but no history of hip fracture and who have taken ALN for ≥3years were randomized to receive ODN or placebo. Patients received vitamin D3 and calcium supplementation.
In the ODN group, BMD changes from baseline at 24 months were significantly increased from placebo at the femoral neck, trochanter, total hip and lumbar spine (1.7%, 1.8%, 0.8%, and 2.3%, respectively). In the placebo group, BMD at the femoral neck, trochanter and total hip declined significantly from baseline by month 24 (–0.9%, –1.4%, and –1.9% respectively).ODN significantly decreased bone resorption marker, u-NTx/Cr, and significantly increased bone formation markers, s-P1NP and s-BSAP, vs. placebo. The increase observed for the bone resorption marker s-CTx with ODN treatment was unexpected. The overall safety profile appeared similar between ODN and placebo.
In this study ODN provided incremental BMD gains in osteoporotic women following ALN treatment.

OC02

Effect of Blosozumab on Bone Mineral Density in Japanese and Non-Japanese Postmenopausal Women with Low Bone Mineral Density

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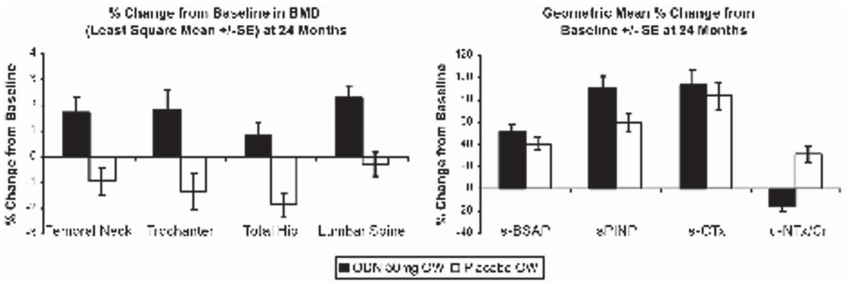
Administration of antibodies that neutralize sclerostin has been shown to increase bone mass. We report findings of a phase 2 study of the human sclerostin antibody, blosozumab (bmab). Study GSDB was a randomized, double-blind, placebo-controlled study, designed to assess the dose-response relationship of bmab in postmenopausal women with low bone mineral density (BMD) (lumbar spine [LS] T score, –3.5 to –2.0). Participants were randomized to one of 3 subcutaneous (SC) bmab treatment regimens (180 mg every 2 weeks [Q2W]; 180 mg every 4 weeks [Q4W], 270 mg Q2W) or placebo for 52 weeks. In a study addendum, additional participants were randomized to bmab 270 mg SC every 12 weeks (Q12W) or placebo. Overall, 154 postmenopausal women (Japanese 66; Non-Japanese 88) were enrolled, with mean age 65 years. There was a significant increase in BMD at all bmab doses (Table); the frequency of adverse events was similar across all treatment groups. For Japanese and non-Japanese respectively, mean LS BMDs at baseline were 0.71 and 0.83 g cm^{-2}; mean total hip (TH) BMDs were 0.66 and 0.81 g cm^{-2}, respectively. Absolute BMD increases were similar in Japanese and non-Japanese in all active treatment groups (table). The numerically higher percent increases in BMD in the Japanese were a function of the lower baseline BMD values in this subgroup. In conclusion, both in the overall treatment population

[OC01]



[OC02]

Table Least square mean BMD change from baseline at 52 weeks (g cm^{-2}) (least square mean % change)

	Placebo (N=37)	Blosozumab 270mg Q12W (N=26)	Blosozumab 180mg Q4W (N=31)	Blosozumab 180mg Q2W (N=30)	Blosozumab 270mg Q2W (N=30)
LUMBAR SPINE	–0.012 (-1.52)	0.054* (6.72)	0.065* (8.39)	0.114* (14.86)	0.141* (17.75)
Japanese	–0.015 (-2.20)	0.056* (7.72)	0.071* (10.00)	0.131* (18.64)	0.141* (19.41)
Non-Japanese	–0.012 (-1.34)	0.055* (6.49)	0.059* (6.94)	0.102* (12.08)	0.142* (16.61)
TOTAL HIP	–0.005 (-0.69)	0.018* (2.44)	0.016* (2.17)	0.031* (4.57)	0.051* (6.70)
Japanese	–0.003 (-0.84)	0.019 (2.89)	0.015 (2.13)	0.034* (5.62)	0.044* (6.63)
Non-Japanese	–0.007 (-0.93)	0.019* (2.45)	0.017* (2.10)	0.029* (3.78)	0.058* (7.06)

*p<0.05 vs placebo

and the Japanese subgroup, bmab treatment resulted in significant increases in LS and TH BMD at 52 weeks.

OC03

Calcium Supplements and Cardiovascular Risk: a Subgroup Analysis

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Background: Calcium supplements have been reported to increase the risk of myocardial infarction. We wished to determine whether the effects of calcium supplements on cardiovascular risk vary across different population groups.
Methods: We modelled the effect of calcium (with or without vitamin D) on the time to incident cardiovascular events in prespecified subgroups for age, dietary calcium intake, body mass index (BMI), smoking history, history of hypertension, diabetes, and previous cardiovascular disease, using interaction terms in Cox proportional hazards models in two datasets- our re-analysis of the Women's Health Initiative Calcium and Vitamin D study (WHI CaD), and our pooled patient-level meta-analysis of trials of calcium supplements with or without vitamin D.
Results: For women in WHI CaD not taking calcium supplements at randomization (*n*=16718), we found no significant interactions between treatment allocation, the risk of myocardial infarction, stroke, or coronary revascularization, and any of the baseline variables. In the pooled patient level dataset of six trials of calcium with or without vitamin D (*n*=24869), there were also no significant interactions between treatment allocation, risk of myocardial infarction or stroke, and any of the baseline variables.
Conclusion: We found no evidence that the increased cardiovascular risk from calcium supplements differs across varying patient populations.

OC04

What Makes Bones Of Polynesian People Strong? Comparison of Gene Expression in Osteoblasts from Polynesian and Caucasian Patients

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Polynesians have higher bone mineral density and lower rate of hip fracture compared to age-matched Caucasian in New Zealand, and anecdotal evidence suggests that bones of Polynesian patients heal much faster than those of Caucasians. We compared gene expression in osteoblasts cultured form bone samples taken from patients of Polynesian and Caucasian origin, in order to identify genes and pathways that contribute to the greater bone strength, density and enhanced healing of Polynesian bones. RNA was extracted from primary osteoblasts cultured from bone samples obtained during orthopaedic surgery from 30 Polynesian and 30 Caucasian patients. Global gene expression was determined in 10 samples from each group using PrimeView GeneChip microarrays (Affymetrix). The samples were age, sex, and BMI matched. Of the >20000 genes represented on the arrays, 171 genes had a two-fold or greater difference in expression levels between the two groups, with about half of the genes showing higher levels of expression in each group. A number of the genes identified by the microarrays were further investigated by real-time PCR in the larger group of samples. So far, the levels of expression of NOV, EFNB2 and EFHD1 were found to be significantly lower in the Polynesian group. Significant differences have been identified between osteoblasts of the two ethnic groups and hypotheses about the contribution of the candidate genes to the better healing of Polynesian bone can be formulated and tested.

OC05

C165R Mutant Sclerostin Retained a Comparable Inhibitory Potency to the Wild Type on Wnt/ Beta Catenin Signaling Despite of Inappropriate Folding

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Sclerosteosis is a rare recessive high-bone-mass disorder. Recently, the first missense mutation in the SOST gene causing sclerosteosis was reported, in this mutation Cys167 (Cys 165 in mice) in the cystine-knot motif was substituted by Arg. However, the importance of cystine knot function in Sclerostin has not been studied thoroughly. Secretion of cysteine mutant proteins including C165R was partially impaired shown by western analysis, furthermore confocal microscopic images

and FACS analysis showed the retained C165R Sclerostin in ER. Meanwhile the cysteine mutants expressed in E-coli revealed a comparable activity to the wild type Sclerostin on inhibition of Wnt signaling unexpectedly. The binding of C165R mutant to MC3T3-E1 cells impaired significantly compared to the wild type. Furthermore, we also observed the increase in the XBP1 splicing, an ER stress marker. Taken together, C165R mutant Sclerostin retained a comparable inhibitory potency on Wnt/b-catenin signaling, despite of inappropriate folding. The breaking of single disulfide bond in the cysteine knot motif of Sclerostin leads to partial impairment of secretion with ER retention by miss-folding. In addition chronic ER stress followed by retention of C165R mutant by miss-folding, and failure of keeping high concentration of Sclerostin at the surface of the target cells could lead to craniotubular hyperosteosis.

OC06

Interleukin (Il)-11 Controls Bone and Adipose Tissue Mass by Regulating Osteoblastic and Adipogenic Differentiation

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We have demonstrated that IL-11 enhances osteogenesis with a reduction in adipogenesis from bone marrow stromal cells, without affecting osteoclastogenesis. Although IL-11 knockout (KO) mice exhibit low bone mass with reduced bone formation, it is unclear whether adipogenesis is affected in the adipose tissue as well as in the bone marrow of IL-11KO mice. The present study was undertaken to clarify the role of IL-11 in the regulation of cell fate switch between osteoblasts and adipocytes, and to examine whether there is any change in the adipose tissue mass.
IL-11KO mice exhibited reduced BMD and increased fat mass not only in the bone marrow but also in the adipose tissue measured by microCT. Subcutaneous, visceral and total adipose tissue mass was higher in IL-11KO than in wild-type (WT) mice. Expression of adipogenic transcription factors including PPARγ and C/EBPα was enhanced in both the bone marrow and the adipose tissue of IL-11KO mice. When bone marrow stromal cells from WT mice were cultured in adipogenic medium, IL-11 expression sharply declined with adipogenic differentiation. There was no significant difference in body weight, food intake, oxygen consumption or carbon dioxide production between IL-11KO and WT mice. These results demonstrate that IL-11 plays an important role not only in the regulation of the switch between osteogenesis and adipogenesis in the bone marrow, but also in the control of adipogenic differentiation and fat mass in the adipose tissue.

OC07

The NXI Motif and its Neighboring Residues of Sclerostin Play an Important Role for the Inhibition of Wnt/Beta-Catenin Signaling

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Sclerostin has been spotlighted as a potential target for osteoporosis because of its specific expression in osteocytes. Recent studies on anti-Sclerostin Ab treatment in mouse showed dual effects on bone such as enhancement of bone formation and inhibition of bone resorption. To identify the functional residues of Sclerostin contributing for the inhibition of Wnt/beta-catenin signaling, Sclerostin mutant proteins were expressed in E. coli and the inhibitory activity was assessed with special reference on the finger 2 and the loop 2. The mutations of the positively charged residues of finger 2 region did not affect Wnt signaling. Meanwhile, the inhibitory potency of Sclerostin on Wnt signaling was reduced significantly by the substitution of Asn115 with Ala and/or Ile117 with Glu of the NXI motif in the mouse Sclerostin, or deletion of VKWW residues. A newly engineered cystine knot protein inhibited the Wnt signaling efficiently. R124A, P125A, and N126A mutants also revealed a significant reduction of the inhibitory activity. We conclude that the active domain of Sclerostin is the loop 2 rather than finger 2 region for the interaction with Lrp5/6, and the NXI motif and its neighboring residues play an important role for the inhibition of Sclerostin on Wnt signaling. This study on Sclerostin mutants will enhance our understanding of the structure-activity relationships for Sclerostin-LRP5/6 interaction, and also provide some clue for the development of Sclerostin antagonist.

OC08

Effects of Odanacatib on the Distal Radius and Tibia in Postmenopausal Women: Improvements in Cortical Geometry and Estimated Bone Strength

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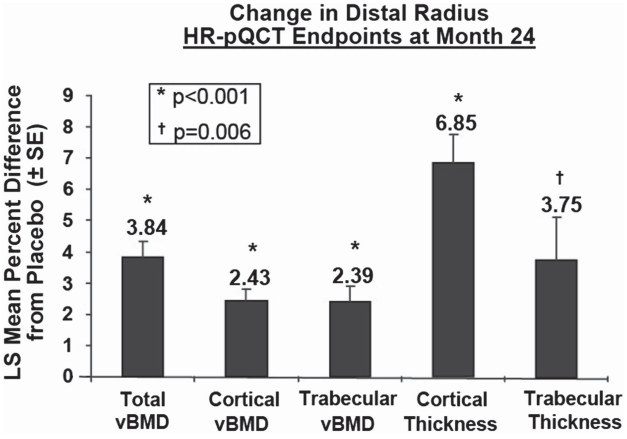
The cathepsin K inhibitor odanacatib (ODN) preserves bone formation while reducing bone resorption. In phase 2, 5 years of ODN 50 mg weekly progressively increased areal BMD at the lumbar spine (LS) and total hip (11.9 % and 8.5% from baseline). In OVX primates, ODN increased cortical thickness and periosteal bone formation at the femur. This was a randomized, double-blind, placebo-controlled trial, using high resolution quantitative computerized tomography (HR-pQCT) of the

distal radius and tibia. 214 postmenopausal women (mean age 64 ±6 years, baseline LS T-score –1.8 ±0.8) were randomized to oral ODN 50 mg or PBO weekly for 2 years. LS areal BMD % change from baseline at 1 year (primary endpoint) was greater for ODN than PBO (3.5% treatment difference, $p<0.001$). At 2 years, there were significantly greater improvements with ODN than PBO in total, trabecular, and cortical volumetric BMD; cortical thickness; and strength estimated by finite element analysis at the distal radius (exploratory endpoints, Figure). At the radius, ODN attenuated an increase in cortical porosity seen with placebo (treatment difference in mean % change from baseline –7.7, $p=0.066$). At the distal tibia, changes in volumetric BMD, cortical thickness, and strength were similar to the radius. Safety was similar between treatment groups. In conclusion, odanacatib increased estimated strength, cortical and trabecular density, and cortical thickness of the distal radius and tibia compared to placebo.



OC09

The Endoplasmic Reticulum Stress Sensor BBF2H7 Suppresses Apoptosis by Activating the ATF5-MCL1 Pathway in Chondrocytes

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A number of cellular stress conditions lead to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen. This type of the stress is called ER stress. Previously, we identified the novel ER stress sensor BBF2H7. This molecule is a basic leucine zipper transcription factor and is activated in response to ER stress. *Bbf2h7*-deficient (*Bbf2h7–/–*) mice exhibited the severe chondrodysplasia caused by down-regulation of Sec23a, which is responsible for protein transport from the ER to Golgi and one of the BBF2H7 target genes in chondrocytes. In the *Bbf2h7–/–* mice cartilage, the number of proliferating chondrocytes was decreased compared with wild type, due to increased apoptosis. In this study, we found that BBF2H7 directly acts on the promoter region of a transcription factor ATF5 and facilitates its transcription. Furthermore, ATF5 which is induced by BBF2H7 activated the transcription of MCL1, which belongs to anti-apoptotic Bcl-2 family members. Finally, we demonstrated that the BBF2H7-ATF5-MCL1 pathway specifically suppressed the ER stress-induced apoptosis in chondrocytes. These results suggested that BBF2H7 plays crucial roles not only in acceleration of extracellular matrix proteins secretion mediated by Sec23a but also in suppression of ER stress-induced apoptosis by activating the ATF5-MCL1 pathway during chondrocyte differentiation.

Oral Presentations 2

OC10

Increased Insulin Sensitivity and Improved Glucose Metabolism in Mice Expressing ΔFOSB in the Ventral Hypothalamus

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The hypothalamus (HT) plays a pivotal role in bone homeostasis as well as energy balance and glucose metabolism through endocrine and neuronal pathways. We have previously shown that targeted injections of adeno-associated virus (AAV) encoding ΔFosB, a truncated form of FosB that opposes FosB AP-1 transcriptional activity, to the ventral HT (VHT) in mice results in increased bone mass and energy expenditure. To examine the role of ΔFosB in the VHT in glucose metabolism, we performed intraperitoneal (IP) glucose (GTT) and insulin (ITT) tolerance tests in C57BL/6 mice stereotaxically injected in the VHT with AAV-ΔFosB or AAV-GFP.

AAV-ΔFosB mice had markedly improved glucose tolerance with lower insulin response to an IP glucose bolus compared to AAV-GFP mice during GTT. Pancreatic islets of AAV-ΔFosB mice were smaller and had decreased insulin gene expression. Despite lower insulin response, blood glucose levels during GTT were significantly lower in AAV-ΔFosB mice suggesting increased insulin sensitivity. ITT indeed revealed that AAV-ΔFosB mice were more insulin sensitive with elevated insulin signaling in periphery, an effect observed even before their body and fat mass fell behind those of AAV-GFP mice. Taken together, these results demonstrate that hypothalamic signaling downstream of ΔFosB, which regulates both bone and energy homeostasis, affects positively glucose metabolism by increasing insulin sensitivity, lowering blood glucose despite low insulin secretion.

OC11

***In Vivo* Imaging of Bone Remodeling in Bone Tissue of the Osteoblast-Osteoclast Dual Labeled Mice**

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Bone remodeling is a continuous process which involves a removal of old bone mediated by bone-resorbing osteoclasts

and a subsequent synthesis of new bone mediated by bone-forming osteoblasts. It has been speculated that the reciprocal interaction between osteoblasts and osteoclasts either by cell-cell interactions or by secreting factors plays an important role in the transition of sequential phase of bone remodeling. However, little is known about the behavior of osteoblasts and osteoclasts *in vivo*. To investigate the dynamics of bone remodeling, we performed intravital two-photon imaging of osteoblasts and osteoclasts in the mouse calvarial bone by using the newly established dual fluorescent-labeled mice. The osteoblast-osteoclast dual labeled mice were generated by crossing the osteoblast-labeled mice (Col2.3-ECFP) with the osteoclast-labeled mice (TRAP-tdTomato). By using intravital imaging technique, we found that Col2.3-ECFP-labeled osteoblasts spatially localized away from the population of TRAP-tdTomato-labeled mature osteoclasts in the endosteum. In addition, we found that the number of osteoblasts and osteoclasts interacting with each other was limited but was increased at the area where the transition from bone resorption and bone formation can occur. The intravital imaging of the osteoblast-osteoclast dual labeled mice can be a useful tool to evaluate bone remodeling by comparing the cell motility parameters between physiological and pathological condition.

OC12

Microrna miR-29a Protects Against Glucocorticoid-Induced Bone Loss by Regulating RunX2 Acetylation

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Long-term glucocorticoid administration increases the risk of osteoporosis. MicroRNAs reportedly regulate osteoblast differentiation. Herein, we created miR-29a transgenic mice and investigated the biological role of miR-29a signaling in glucocorticoid-mediated bone loss. Excessive glucocorticoid treatment deteriorated skeletal integrity in association with loss of miR-29a expression. Interestingly, miR-29a transgenic mice with glucocorticoid treatment had improved trabecular bone micro-architecture and reduced cortical bone porosity compared to the glucocorticoid-treated wild type mice. Gain of miR-29a signaling reduced the inhibitory actions of glucocorticoid on *ex vivo* osteoblast differentiation capacity in bone-marrow mesenchymal cells. Loss of miR-29a signaling by lentivirus-shuttled miR-29a inhibitor accelerated loss of mineral acquisition in bone tissue and mineralized matrix accumulation in primary bone-marrow mesesnchymal cells. miR-29a reduced the glucocorticoid-mediated HDAC4 expression and attenuated osteogenic transcription factor Runx2 deacetylation and polyubiquitination. *In vitro*, inhibition of HDAC4 signaling restored the adverse action of glucocorticoid on histone acetylation (H3K4 and H3K7) and miR-29a transcription. Taken together, miR-29a reciprocally regulates HDAC4 and stabilizes Runx2 signaling. Control of miR-29a actions has therapeutic potential for reducing glucocorticoid-induced bone mass loss.

OC13

Ex Vivo Expanded Allogeneic Mesenchymal Stem Cells (MSCS) Improved Osteogenesis in Patients with Severe Hypophosphatasia- Three Case Reports of MSC Infusions Followed by Bone Marrow Transplantation

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Hypophosphatasia (HPP) one of the bone metabolic disorders caused by mutations of the tissue-nonspecific alkaline phosphatase (TNSALP) gene. The disease is characterized by the disturbance of bone and tooth mineralization. Patients with severe forms have ventilator-dependent respiratory disturbance and TNSALP mutations which induce extremely low ALP activity, resulting in having a fatal course. However, there has been no curative treatment for HPP. Mesenchymal stem cells (MSCs) have multipotency that differentiates into various mesenchymal lineages including bone, and cartilage. Herein, we performed transplantation of ex vivo expanded MSCs for 3 patients with severe HPP that underwent preceding bone marrow transplantation (BMT) to determine the effects of MSCs on bone mineralization. Their donors of MSCs and BM were asymptomatic patients' relatives harboring heterozygous mutation of the TNSALP gene. We performed BMT, and then infused MSCs sequentially from 3 to 7 times. Their physical development and respiratory status improved whenever MSCs were infused. The bone mineral density and roentgenologic analyses revealed that the mineralization has gradually ameliorated. Chimerism analysis of bone, teeth and BM elucidated the existence of donor cells. Adverse events of this treatment were tolerated. Our data suggests that multiple MSC infusions followed by BMT can be considered as an effective treatment modalities to facilitate bone mineralization in patients with severe HPP.

OC14

The Role of Autophagy in Critical Illness-Induced Bone Loss

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Critically ill patients display low circulating levels of bone formation markers, and an increase in markers of bone resorption, suggesting uncoupling between bone formation and degradation. We have previously shown that increased circulating osteoclast precursors in critically ill patients result in increased osteoclast differentiation *in vitro*. Such aberrant osteoclast formation is also observed in Paget's Disease of Bone (PDB), and recently, a link has been made between autophagy and PDB,

as increased osteoclast activity is linked to mutations in p62, a scaffold protein that is important in the sequestration of protein aggregates during autophagy. We have previously shown that autophagy is deficient in critically ill patients, and in the current study, p62 protein expression was increased in osteoclasts isolated from critically ill patients compared to matched healthy controls, suggesting deficient autophagy. This was supported by a decrease in Atg5 and LC3-II protein levels in patient osteoclasts. Interestingly, culturing patient osteoclasts in autophagy inducers Rapamycin or Spermidine reduced osteoclast formation and resorption on dentine slices. *In vivo*, critically ill rabbits displayed a reduction in trabecular and cortical bone area after only 3 days of illness, which was rescued with admission of Rapamycin or Spermidine. These results give important indications for potential therapeutic targets in the treatment of critical illness-induced bone loss.

OC15

Wnt5a-Ror2 Signal Regulates Osteoclast Polarization Through Daam2 and Rho

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We have shown that Wnt5a enhances RANKL-induced osteoclast formation through Ror2-JNK signaling. To clarify roles of Wnt5a-Ror2 signals in the bone-resorbing activity of osteoclasts, we generated osteoclast-specific Ror2 conditional knock-out mice (Ror2 cKO) by crossing $Ror2^{fl/fl}$ mice with Cathepsin K-Cre ($Ctsk^{Cre/+}$) mice and analyzed their bone phenotypes. Bone volume in Ror2 cKO was higher than that in control ($Ror2^{+/+}$: $Ctsk^{Cre/+}$) mice. Serum CTX, a bone resorption marker, was lower in Ror2 cKO. However, histomorphometric analysis showed that osteoclast number was normal in femurs from Ror2 cKO. These results suggested that the bone-resorbing activity of osteoclasts was impaired in Ror2 cKO. Osteoclasts formed from bone marrow cells of Ror2 cKO failed to resorb bone due to a defect of actin ring formation. Wnt5a activates both Rho and Rac in control osteoclasts, but not in Ror2 cKO osteoclasts. Overexpression of constitutively active (CA)-RhoA, but not CA-Rac1, rescued the bone-resorbing activity in Ror2 cKO osteoclasts. We examined how Wnt5a-Ror2 signals regulate osteoclast function through Rho activity. Disheveled-associated activator of morphogenesis (Daam) 1 is reported to be involved in Wnt5a-induced RhoA activation. Daam2, but not Daam1, was highly expressed in osteoclasts. shRNA-mediated knockdown of Daam2 in osteoclasts inhibited their bone-resorbing activity. Taken together, Wnt5a-Ror2 signals regulate the function of osteoclasts through the Daam2-Rho pathway.

Oral Presentations 3

OC16

A Direct Computer-Assisted Method for the Spatial 3D Mapping of Trabecular Termini in the Spine

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Bone loss with age is insidious, leaving the skeleton fracture-prone. Development of an accurate method of cancellous connectivity assessment (a significant structural strength factor), in 2D and 3D is challenging. Current histological methods providing topographical data from microarchitecture are limited as they usually indirectly assess 3D structural quality by using 8μm sections. Aaron *et al* (2000) developed a thick (300μm) slicing and superficial staining method whereby 'real' trabecular termini (ReTm) are identified directly in their 3D context. To extrapolate from and automate this previous manual method embalmed vertebral bodies were μCT (micro-computerised tomography) scanned, plastic embedded, sliced (300μm), superficially stained and ReTm mapped using light microscopy. Coordinates were assigned corresponding to topographical regions for spatial 3D mapping of ReTm as loci of structural weakness. A transparent 3D cortical shell enclosing ReTm was generated (Matlab 7.3, Mathworks, USA), and then refined and validated using the μCT data with overlaid coordinate data. ReTm distribution was shown to be heterogeneous and independent of bone volume ($P<0.05$) with preliminary evidence for central endplate disconnection. ReTm automated

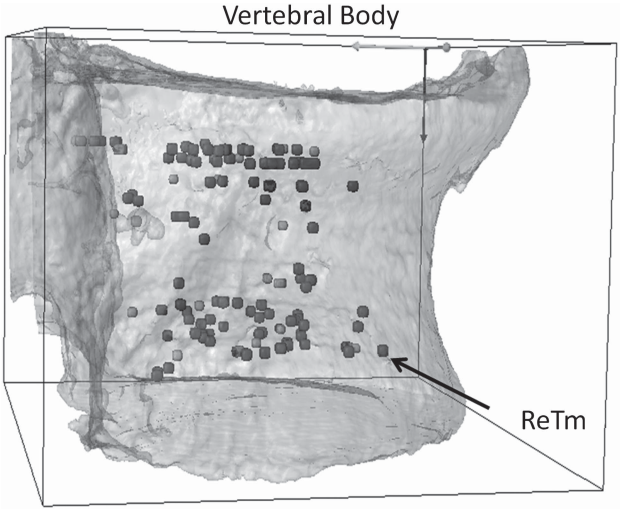

Figure 1 Computer generated construct of a human vertebral body showing the spatial mapping of structural weakness in the form of 'real' trabecular termini (ReTm).

visualisation spatially in a 3D framework overcomes the constraints of established 2D histology. Trabecular disconnection patterns in the spine are now being mapped for further insight into intransigent atrophy as a major cause of disability

OC17

Fetal Stage-Specific Mineral Metabolism in *Hyp* Mice Implies the Role of FGF23 in Placenta

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We have previously reported that placenta expresses αKlotho, which is required for FGF23 signaling. Here we investigated the roles of FGF23 in mineral metabolism of fetal stage, using *Hyp* mice with high level of serum FGF23. *Hyp* and wild-type (WT) female mice were mated with WT male mice, and the mothers and their male fetuses were analyzed at E18.5. Despite the hypophosphatemia of *Hyp* mothers, Pi levels in E18.5 fetuses were comparable among the 3 groups; *Hyp* and WT fetuses from *Hyp* mothers and WT fetuses from WT mothers. FGF23 levels in *Hyp* mothers were elevated compared to those in WT mothers. Interestingly, FGF23 levels in *Hyp* fetuses were about 20-fold higher than those in *Hyp* mothers. On the other hand, WT fetuses from *Hyp* mothers exhibited low levels of FGF23, as did the fetuses from WT mothers, suggesting that FGF23 does not go across the placenta. In kidneys of *Hyp* fetuses, the expressions of *Npt2a* and *Npt2c* were decreased and that of *24OHase* was increased, which seemed to be the effects of their high FGF23 levels. *24OHase* expression was elevated in the placenta also in *Hyp* fetuses. Finally, to further investigate the effect of FGF23 in placenta, we injected recombinant FGF23 into placenta of WT fetuses of WT mothers. The injection of FGF23 induced the placental expression of *Egr1* and *24OHase in vivo*. These results indicate that FGF23 exerts its effects on placenta as well as fetal kidney and plays a role in mineral metabolism in *Hyp* mice of fetal stage.

OC18

Functional Activity Of Calcium-sensing Receptor Determines Clinical Presentations In Patients With Autosomal Dominant Hypocalcemia

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Objective: Autosomal dominant hypocalcemia (ADH) is caused by activating mutations in the calcium-sensing receptor (CASR) gene. We recruited 12 ADH patients to analyze the relationship between functional activity of mutant CaSRs and clinical presentations of the patients.

Design and Methods: We studied 2 sporadic and 10 familial cases of ADH. A luciferase reporter activity in response to various concentrations of extracellular Ca was assessed using HEK293 cells transfected with wild-type or mutant CaSRs.

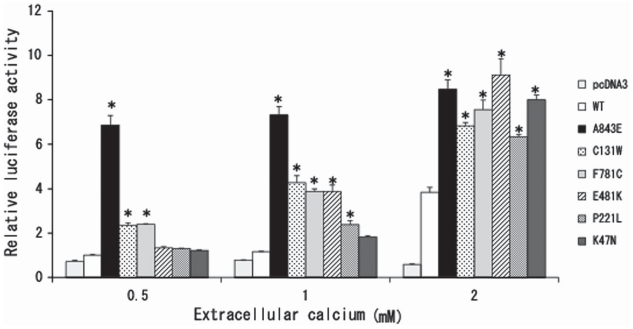

Figure 1 Functional analysis shows the order of activity of mutant CaSRs as follows: A843E > C131W ≒ F788C > P221L ≒ E481K > K47N.

Results: Hypomagnesemia with elevated fractional excretion of Mg (FEMg) was observed in patients with A843E, C131W, and F788C mutations. On the contrary, serum Mg was within the reference range in patients with other three mutations. Patients with A843E and C131W mutations also presented with Bartter syndrome type 5. An increase in intact PTH in response to severe hypocalcemia was observed only in patients with P221L, K47N, and E481K mutations. Functional analysis showed the order of activity of mutant CaSRs as follows: A843E > C131W ≒ F788C > P221L ≒ E481K > K47N.

Conclusions: Functional activity of the mutant CaSR determines clinical and biochemical presentations of ADH. The most active mutant CaSRs cause Bartter syndrome type 5. Hypomagnesemia with elevated FEMg and the loss of PTH response to hypocalcemia are observed in patient with more active CaSRs than those without these features.

OC19

Selective Knockout Of The Parathyroid Cyp27b1 Gene Blunts The Ability Of Hyperparathyroidism To Increase Serum Calcium, 1,25-dihydroxyvitamin D_3, And FGF23 Levels In Mice

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Current thoughts on mineral balance specify that the renal 25-hydroxyvitamin D_3 1-α-hydroxylase (Cyp27b1) is the major source of circulating $1,25(OH)_2$-vitamin D_3 (1,25D) and a key basis for producing hypercalcemia in primary hyperparathyroidism (1° HPT). To address whether the Cyp27b1, expressed in parathyroid cells (PTCs), also produces 1,25D to participate in the development of hypercalcemia in 1° HPT, we compared serum PTH (sPTH), Ca (sCa), 1,25D (s1,25D), FGF23 (sFGF23), and phosphate (sPi) levels in mice with heterozygous knockout (KO) of the calcium-sensing receptor (CaSR) (CaSR-Het), homozygous Cyp27b1 KO (Cyp-KO) or both targeted specifically to PTCs. PTC-specific gene deletion was confirmed by PCR analyses of genomic DNA and RNA from various tissues. sPTH, sCa, s1,25D, and sFGF23 levels were significantly (P<0.01) increased in CaSR-Het vs control (Cont) mice (Table below), indicating a 1° HPT phenotype. In contrast, Cyp-KO

[OC19]

Table 1 Serum Hormones and Chemistry

Genotype	sPTH, ng ml^{-1}	sCa, mg dl^{-1}	s1,25D, pM	sFGF23, pM	sPi, mg dl^{-1}
Cont	112 ± 8	9.78 ± 0.04	109.1 ± 14.7	78.6 ± 9.8	6.39 ± 0.13
CaSR-Het	218 ± 27	11.01 ± 0.20	219.4 ± 13.9	183.2 ± 16.5	6.77 ± 0.28
Cyp-KO	369 ± 71	9.45 ± 0.08	42.0 ± 3.3	37.5 ± 6.5	7.34 ± 0.21
CaSR-Het//Cyp-KO	380 ± 49	10.22 ± 0.08	31.7 ± 1.0	55.1 ± 9.2	6.06 ± 0.16

mice developed 2º HPT as indicated by reduced s1,25D, sCa, and sFGF23 levels and increased sPTH and sPi levels, compared to Cont mice. In CaSR-Het//Cyp-KO mice, ablating parathyroid Cyp27b1 significantly (P<0.01) decreased sCa, s1,25D, sFGF23, and sPi despite the presence of higher levels of sPTH compared to the CaSR-Het mice, suggesting the development of both 1º and 2º HPT in the former mice. Our data suggest that parathyroid Cyp27b1 is a critical source of circulating 1,25D essential for maintaining mineral metabolism in basal and HPT states.

OC20

Impaired Response Of FGF23 To Oral Phosphate In Patients With T2DM: A Possible Mechanism Of Atherosclerosis

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Fibroblast growth factor (FGF)-23, which is released from osteocyte/osteoblast, plays a major role in the regulation of Pi, a major cardiovascular risk factor. Because of osteocyte/osteoblast dysfunction in type 2 diabetes mellitus (DM), we examine whether incremental response of serum FGF-23 and PTH after oral Pi stimulation is impaired in type 2 DM patients. Serum FGF-23, intact parathyroid hormone (iPTH) and Pi were measured serially in type 2 DM (n=10) and non-DM patients (n=10) after oral Pi administration at the daily dose of 2.0 g. Serum FGF-23 significantly increased by 2h after the start of Pi stimulation test and iPTH significantly by 4h in non-DM patients, but not in DM patients. Serum FGF-23 and iPTH increased in non-DM patients after 2 days of Pi stimulation, but not in DM counterparts. In all patients, initial changes of serum FGF-23 (0-2 h) and iPTH (0-4 h), which correlated positively with each other (r =0.528), showed a significant and negative correlation with later change of serum Pi (2-4 h) (r= –0.457; r= –0.673). Consistent with the impaired response of serum FGF-23 and iPTH in DM patients, serum Pi (2-4 h) significantly increased, in contrast with insignificant rise in non-DM counterparts. It was demonstrated that incremental response of serum FGF-23 and PTH by Pi stimulation was impaired in type 2 DM patients with serum Pi increased thereafter, suggesting it one of the mechanism for the advanced atherosclerosis in type 2 DM patients.

OC21

The C-type Natriuretic Peptide (CNP)/ Guanylyl Cyclase-B (GC-B) System In Growth Plate Promotes Endochondral Bone Growth In An Autocrine/paracrine Manner

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C-type natriuretic peptide (CNP) is an endogenous bioactive peptide, which exerts its biological actions by binding a subtype of receptor guanylyl cyclase B (GC-B). As the CNP/GC-B system exists in wide variety of tissues, in order to elucidate its physiological role in the growth plate cartilage, we performed targeted depletion of CNP or GC-B in cartilage using Cre-loxP system. The cartilage-specific CNP or GC-B knockout mice showed prominent short stature phenotype due to impaired endchondral bone growth: naso-tail lengths of cartilage-specific CNP knockout mice and cartilage-specific GC-B knockout mice were 75.6% and 62.1%, respectively, compared to those of their control mice. Histological examination revealed that the hypertrophic chondrocyte layer of the growth plate was drastically reduced and the nonhypertrophic chondrocyte layer was moderately reduced both in cartilage-specific CNP knockout mice and in cartilage-specific GC-B knockout mice. Furthermore, BrdU-labeling assay revealed that the proliferation of growth plate chondrocytes of specific CNP or GC-B knockout mice was inhibited. The extent of impaired endochondral bone growth observed in cartilage specific CNP or GC-B knockout mice was almost the same as that in total CNP or GC-B knockout mice, respectively. These results indicate that the local CNP/GC-B system in growth plate is responsible for physiological endochondral bone growth.

Oral Presentations 4

OC22
The Docking Protein NEDD9 Is A Novel Regulator Of Vicious Cycle Between Breast Cancer And Bone In Bone Metastasis

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Establishment of vicious cycle between cancer cells and bone microenvironment is a unique pathophysiology of bone metastasis. To identify a molecule involved in the regulation of the vicious cycle, microarray analysis between GFP labeled MDA-MB-231 human breast cancer cells isolated from bone metastases and mammary tumors using FACS Aria was conducted. We identified the CAS family docking protein NEDD9 (neural precursor cell expressed developmentally down-regulated protein 9) that is shown to be up-regulated in anti-estrogen-resistant human breast cancers. Immunohistochemical examination revealed increased NEDD9 expression in MDA-MB-231 cells in bone metastases. TGFβ increased NEDD9 mRNA in conjunction with elevated Snail and N-cadherin and reduced E-cadherin. MDA-MB-231 cells overexpressing NEDD9 exhibited increased bone metastases with promoted osteoclastogenesis, while shRNA knockdown of NEDD9 decreased bone metastases. Interestingly, NEDD9 up-regulated Smad-dependent TGF-β signaling, resulting in an increase in PTH-rP expression. NEDD9 converted the cell shape from epithelial to migratory mesenchymal with reduced E-cadherin expression in culture.
In conclusion, our results suggest that NEDD9 plays a critical role in driving the vicious cycle in bone metastasis via enhancing aggressiveness of metastatic breast cancer cells. It is also suggested NEDD9 is involved in the regulation of epithelial-mesenchymal transition in breast cancer metastasized in bone.

OC23
Heparanase Suppresses Osteoblast Differentiation And Stimulates Adipocyte Differentiation In Multiple Myeloma

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Heparanase (HPSE) is an enzyme that degrades heparan sulfate molecules both at the cell-surface and within the extracellular matrix. Multiple myeloma (MM) cells expressing high HPSE levels (HPSE-high cells) stimulate osteolysis by upregulating RANKL and MMP-9 compared to HPSE-low cells. However, little is known about the effect of HPSE on mesenchymal osteoblastic cells and bone formation. In this study, tumors harvested from animal models of MM and bone marrow specimens from 40 MM patients were immunostained for HPSE and osteocalcin (OC). Detailed analysis revealed a significant negative correlation between HPSE expression by MM cells and the number of OC-positive osteoblasts, suggesting that HPSE suppresses bone formation. *In vitro*, both conditioned medium from HPSE-high cells and recombinant HPSE inhibited the osteoblastic differentiation and mineralization of primary mesenchymal progenitors, while concomitantly stimulating adipogenic differentiation. Western blot and ELISA demonstrated that HPSE treatment enhanced DKK1 secretion in both myeloma cells and mesenchymal progenitors, resulting in the inhibition of canonical Wnt/β-Catenin signaling. Thus, the characteristic absence of osteoblasts observed in MM bone disease mediated by the Wnt/β-Catenin pathway is a target of HPSE action. Collectively, these data suggest that HPSE inhibitors are valid therapeutic targets for the treatment of MM bone disease, which could inhibit osteolysis and enhance bone formation.

OC24
Functional Roles Of The Cancer Stem Cell Marker CD44 In The Development Of Bone Metastasis

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CD44, an adhesion molecule that binds to the extracellular matrix, primarily to hyaluronan (HA), has been implicated in cancer cell migration, invasion, and metastasis. CD44 has also recently been recognized as a marker for cancer stem cells. However, the functional roles of CD44 in the development of bone metastasis remain unclear. Here, we addressed this issue by using bone metastatic cancer cell lines, in which CD44 was stably knocked down. Tumor sphere formation and cell migration and invasion were significantly inhibited by CD44 knockdown *in vitro*. Furthermore, the downregulation of CD44 markedly suppressed tumorigenicity and bone metastasis in nude mice. Of note, the number of osteoclasts was decreased in the bone metastases. Microarray analysis revealed that the expression of HA synthase 2 was downregulated in CD44-knockdown cells. The localization of HA in the bone metastatic tumors was also reduced. We then examined the roles of CD44-HA interaction in bone metastasis using 4-methylumbelliferone (4-MU), an inhibitor of HA synthesis. 4-MU decreased tumor sphere and osteoclast formation *in vitro*. Moreover, 4-MU inhibited bone metastases *in vivo* with reduced number of osteoclasts. These results suggest that CD44 expression in cancer cells promotes bone metastases by enhancing tumorigenicity, cell migration and invasion, and HA production. Our results also suggest the possible involvement of CD44-expressing cancer stem cells in the development of bone metastases.

OC25

Indian Hedgehog And Runx2 Are Required For Generation Of Non-endothelial Nestin-positive Cells In The Perichondrium During Early Endochondral Bone Development

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[1]Massachusetts General Hospital, Boston, MA, USA; [2]Albert Einstein College of Medicine, Bronx, NY, USA

Nestin-positive (Nes+) cells are putative mesenchymal stem cells in adult bone marrow. However, how these cells develop during endochondral bone formation is unknown. To answer this question, we studied mice carrying Nes-GFP and various mutant alleles. In the limb bud at embryonic day 10.5 (E10.5), 7.7±1.8% of Nes+ cells expressed the endothelial marker, CD31. When the growth cartilage appeared at E12.5, Nes+ cells were found only in the perichondrium (PC) with large numbers expressing CD31+. Non-endothelial Nes+ cells appeared in the innermost portions of the PC adjacent the incipient hypertrophic chondrocytes (HC). The number of both endothelial and non-endothelial Nes+ cells continued to increase as the PC developed toward E14.5. When vascular invasion occurred at E15.5, non-endothelial Nes+ cells increased in the primary spongiosa. Indian hedgehog (Ihh) expressed by HC is a critical regulator of the PC development. Robust Ptch1-LacZ reporter activities were observed in E13.5 PC. The absence of Ihh significantly reduced the number of Nes+CD31+ cells and led to complete loss of non-endothelial Nes+ cells. Runx2 is an essential regulator of osteoblast differentiation downstream of Ihh. The absence of Runx2 also reduced the number of Nes+CD31+ cells and led to complete loss of non-endothelial Nes+ cells in the E13.5, 16.5 and 18.5 PC. These data indicate that Ihh and Runx2 are required for generating non-endothelial Nes+ cells in the PC during embryonic bone development.

OC26

Possible Regulatory Role Of γδ T Cells In Osteoclastogenesis

Kazuaki Nishimura[1], *Hani Mawardi*[1,2], *Isao Ichimonji*[1], *Shinji Matsuda*[1], *Yusuke Matsuda*[1], *Yoshinori Shinohara*[1], *Wichaya Wisitrasameewong*[1], *Toshihisa Kawai*[1,2]
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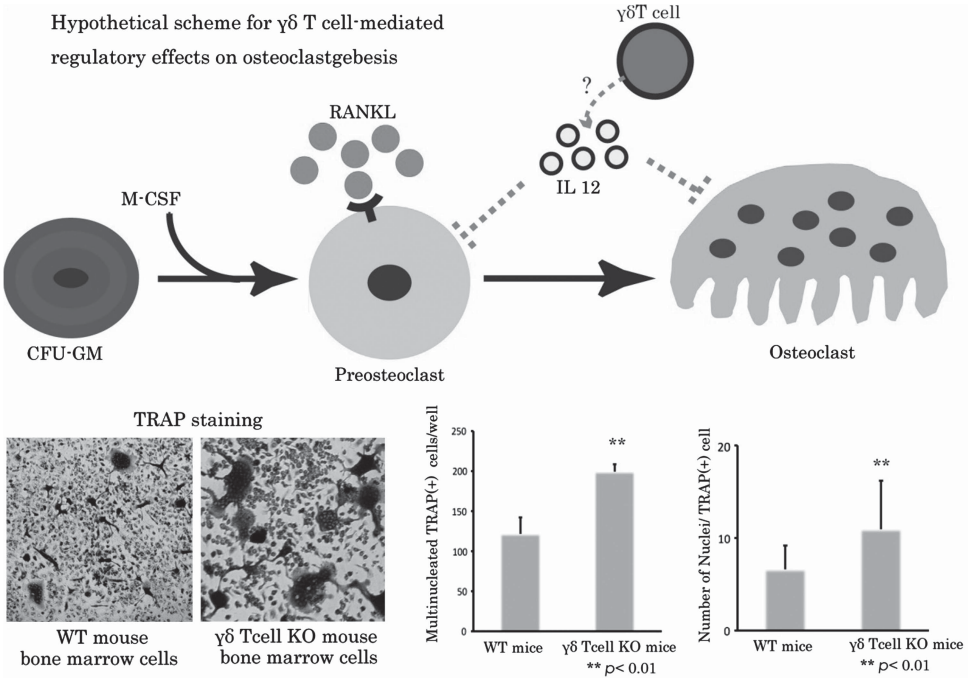
Osteoimmunology is an interdisciplinary research field combining the fields of bonebiology and immunology. It is reported that subsets of αβ T cell receptor (TCR) T cells, such as, Th17, Th1 or Treg, affect bone remodeling by the production of osteoclastogenesis (OCgenesis) up- or down-regulatory factors, RANKL, IFN-γ or IL-10, respectively. On the other hand, possible enrolment of γδ TCR T cells which consist only 1-5% of whole T cell population in bone remodeling process remains unclear. The present study investigated the possible regulatory roles of γδ T cells in the OCgenesis. Adult γδ TCR knockout (γδ KO) mice (12-w, female) demonstrated significantly lower bone mineral density in femur than age/sex-matched wild type (WT) mice (C57BL/6J). In response to *in vitro* stimulation with RANKL and M-CSF, mononuclear cells isolated from bone marrow (BM) of γδ KO mice developed significantly more TRAP+ multinucleated cells than WT-BM cells. Furthermore, the number of nuclei per TRAP+ cell was significantly higher in γδ KO-BM than WT-BM cells, indicating that cell-cell fusion process may be suppressed by the presence of γδ T cells. Among the multiple cytokines examined, only IL-12 concentration was significantly higher in WT-BM cell culture than γδ KO-BM suggesting that IL-12, a known OCgenesis-inhibitor, appears to be responsible for down-regulation of OCgenesis mediated by γδ T cells. These findings suggest that γδ T cells may play a regulatory role in RANKL-induced OCgenesis.

[OC26]



OC27

Dynamic Visualization Of Rankl And Th17-Mediated Control Of Osteoclast Function

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Osteoclasts are bone-resorbing multinucleate cells that differentiate from mononuclear macrophage/monocyte-lineage hematopoietic precursor cells. Although previous studies have revealed key molecular signals, how the bone-resorptive functions of such cells are controlled *in vivo* remains less well characterized. Here, we have visualized fluorescently-labeled mature osteoclasts in intact bone tissues by using intravital multiphoton microscopy, identifying their characteristic movements on the bone surface. Within this mature population were cells with distinct motility behaviors and function, with the relative proportion of 'static - bone-resorptive (R)' to 'moving - non-resorptive (N)' varying in accord with the pathophysiological conditions of bones. We also found that rapid RANKL application converted many moving (N) osteoclasts to static (R) ones, suggesting a novel point of action of RANKL in controlling mature osteoclast function. Furthermore, we showed that Th17, a CD4+ T cell subset expressing RANKL, could induce rapid N to R conversion of mature osteoclasts via cell-cell contact, revealing one mechanism by which Th17 cells have a potent effect on controlling bone resorption *in vivo*. These findings provide new insights into the activities of mature osteoclasts in situ and identify novel actions of RANKL expressing Th17 that may be promising as a new therapeutic target in bone-resorptive diseases.

Oral Presentations 5

OC28

Testosterone Is As Effective As Bone Morphologic Protein-2 In Promoting The Repair Of Critical-Size Segmental Defect Of Femoral Bone In Mice

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Loss of large bone segments due to fracture is a common clinical problem. The goal of this study was to evaluate the use of scaffolds containing testosterone, bone morphogenetic protein-2 (BMP-2), or a combination both for the treatment of critical-size segmental bone defects in mice. A 2.5-mm wide osteotomy was created on the left femur of wild type and androgen receptor knockout (ARKO) mice, The aforementioned drugs were delivered locally using a scaffold that bridged the fracture. Results of X-ray imaging showed that in both wild type and ARKO mice, BMP-2 treatment induced callus formation within 14 days after initiation of the treatment. Testosterone treatment induced callus formation within 14 days in wild type but not in ARKO mice. Callus formation was observed 7 days earlier in the fractures treated with both BMP-2 and testosterone than in those treated with either testosterone or BMP-2 alone in the wild type. Micro-computed tomography revealed that testosterone treatment caused similar degrees of callus formation as BMP-2 treatment in wild type mice, but had no such effect in ARKO mice, suggesting that the androgen receptor is required for testosterone to initiate fracture healing. These results demonstrate thatcombination therapy with testosterone and BMP-2 is superior to single therapy. Results of this study may provide a foundation to develop a cost effective and efficient therapeutic modality for the treatment of bone fractures with segmental defects.

OC29

Tieg Regulates Estrogen And Canonical Wnt Signaling In Bone

Anne Gingery[1]*, John Hawse*[1]*, Muzzafer Cicek*[1]*, Kevin Pitel*[1]*, Sarah Grygo*[1]*, Urszula Iwaniec*[2]*, Russell Turner*[2]*, Malayannan Subramaniam*[1]*, Thomas Spelsberg*[1]
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TGF-β Inducible Early Gene-1 (TIEG) plays important roles in regulating bone physiology. TIEG knockout (KO) mice exhibit a female-specific osteopenic phenotype. Ovariectomy and estrogen replacement studies in these animals demonstrate that TIEG expression is essential for maximal estrogenic activity in the skeleton as the response to estrogen was halved in KO mice. Gene expression studies on the cortical shells of long bones revealed that sclerostin exhibited a 12-fold increase in expression in KO relative to WT littermates. Serum sclerostin levels were also significantly elevated in KO mice. TIEG over-expression was shown to suppress sclerostin promoter activity in bone cells. Use of the TOPGAL reporter mouse demonstrated that loss of TIEG expression decreased canonical Wnt pathway activity by 40% in the skeleton relative to WT mice. Finally, treatment of MLOA5 osteocyte cells with estrogen resulted in increased expression of TIEG with a concomitant decrease in sclerostin mRNA levels. These effects were blocked by the potent anti-estrogen, ICI 182-780, suggesting a direct role for estrogen receptors in regulating both TIEG and sclerostin expression. Taken together, these data support important roles for TIEG mediating the estrogen action on the skeleton via estrogen and sclerostin/wnt signaling demonstrating that TIEG serves as a crosstalk molecule between estrogen and Wnt pathways in the skeleton.

OC30

Thyroid Hormones Decrease Plasma 1α,25-dihydroxyvitamin D Levels Through Transcriptional Repression Of The Renal 25-hydroxyvitamin D_3 1α-hydroxylase Gene (CYP27B1)

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Hyperthyroid patients have been reported to have low levels of plasma 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D), however, its detailed mechanism is still poorly understood. The present study determined whether renal 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) gene expression was negatively regulated by thyroid hormones. 3,3′,5-tri-iodothyronine (T_3)-induced hyperthyroid mice showed marked decreases in plasma 1,25$(OH)_2$D levels and in renal expression of CYP27B1 mRNA. In addition, we observed that T_3 administration significantly decreased plasma 1,25$(OH)_2$D and renal CYP27B1 mRNA levels that were increased by low calcium diet, and induced hypocalcemia in mice fed a low calcium diet. Promoter analysis revealed that T_3 decreases the basal transcriptional activity of the CYP27B1 gene through thyroid hormone receptors (TRα, TRβ1) and the retinoid X receptor α (RXRα) in renal proximal tubular cells. Interestingly, we identified an everted repeat negative thyroid hormone response element (1α-nTRE) overlapping the sterol response element (SRE) and the TATA-box -50 to -20 bp from the human CYP27B1 gene transcription start site. Finally, we established that CYP27B1 gene transcription is positively and negatively regulated by SRE-binding proteins (SREBPs) and T_3-bound TRβ1/RXRα via the 1α-nTRE. These results suggest that transcriptional repression of the CYP27B1 gene by T_3-bound TRs/RXRα, acting through the 1α-nTRE, results in decreased renal CYP27B1 expression and plasma 1,25$(OH)_2$D) levels.

OC31

Dullard Gene Regulates Endochondral Bone Formation Via Suppression Of Tgf-β Signaling During Skeletal Development

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The transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling pathways play important roles in endochondral bone formation, an essential process for skeletal growth. However, it has also been shown in mouse models that limit of TGF-β and BMP signaling is also essential for normal skeletal development. We report that an intracellular factor Dullard, which can function as an inhibitor of both TGF-β and BMP signaling, controls endochondral bone formation by supressing TGF-β signaling. Genetic deletion of Dullard in mesenchye in early limb bud and sternum by Prx1-Cre impaired ossification and longitudinal bone growth. Approximately 70% of the Dullard (Prx1) mutant mice died 1 day after birth and the rest of them displayed dwarfism and defects in locomotion and died before weaning. Histological analysis showed occupation of hypertrophic chondrocytes in sternum of the Dullard (Prx1) mutant mice. Deletion of Dullard gene in osteoprogenitors using Osx1-Cre did not cause impaired ossification, suggesting that Dullard deficiency in osteoprogenitors is not responsible for impaired ossification observed in Dullard (Prx1) mutant mice. Primary chondrocyte from Dullard (Prx1) mutant mice showed enhanced response to TGF-β rather than BMP based on luciferase reporter assay. These results suggest that Dullard regulates endochondral bone formation via suppression of TGF-β signaling in chondrocytes.

OC32

Systemic Circulation And Bone Recruitment Of Osteoclast Precursors Tracked By Using Fluorescent Imaging Techniques

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Osteoclasts are bone-resorbing polykaryons differentiated from monocyte/macrophage-lineage hematopoietic precursors. It remains unclear whether osteoclasts originate from circulating blood monocytes or from bone tissue-resident precursors. To address this question, we combined two different experimental procedures: (1) shared blood circulation 'parabiosis' with fluorescently labeled osteoclast precursors, and (2) photoconversion-based cell tracking with a Kikume Green-Red protein (KikGR). In parabiosis, CX3CR1-EGFP knock-in mice in which osteoclast precursors were labeled with EGFP were surgically connected with wild-type mice to establish a shared circulation. Mature EGFP+ osteoclasts were found in the bones of the wild-type mice, indicating the mobilization of EGFP+ osteoclast precursors into bones from systemic circulation. RANKL stimulation increased the number of EGFP+ osteoclasts in wild-type mice, suggesting that this mobilization depends on the bone resorption state. Additionally, KikGR+ monocytes (including osteoclast precursors) in the spleen were exposed to violet light, and 2 days later we detected photoconverted 'red' KikGR+ osteoclasts along the bone surfaces. These results indicate that circulating monocytes from the spleen entered the bone spaces and differentiated into mature osteoclasts during a certain period. In conclusion, this study first clearly demonstrates that osteoclasts can be generated from circulating monocytes once they home to bone tissues.

OC33

The Role Of Osteocytes In Bone Resorption During Orthodontic Tooth Movement

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We investigated the roles of osteocytes in osteoclastic bone resorption during orthodontic tooth movement using the transgenic mice in which osteocytes can be specifically ablated. Because these transgenic mice express the receptor for diphtheria toxin on the cell surface of osteocytes, the injection of diphtheria toxin can ablate their osteocytes *in vivo*. Injection of diphtheria toxin into the transgenic mice significantly increased the number of the ablated osteocytes in alveolar bone compared to that in wild-type mice with or without diphtheria toxin injection. An increased number of the ablated osteocytes was observed from day 4 to day 12 after the injection in alveolar bones as well as cortical bone of tibiae. We applied the orthodontic force 4 days after the injection of diphtheria toxin, and the distance of tooth movement on day 12 was significantly smaller in transgenic mice than that in control mice. The number of osteoclasts and the eroded bone surface at the compression site were significantly lesser in the transgenic mice injected with diphtheria toxin than that in control mice. These results are the first *in vivo* demonstration of osteocyte involvement in osteoclastic bone resorption during orthodontic tooth movement.

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OC35

Profiling SNX Proteins In Bone Identifies Sorting Nexin 27 (Snx27) As A Crucial Modulator Of Skeletal Homeostasis

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During bone growth, resident cells are required to synthesize and transport proteins and other endocytosed material between membrane-delimited organelles to preserve skeletal homeostasis. Protein trafficking is governed by sets of conserved protein families of which members of the Phox (PX) domain-containing sorting nexins (SNXs) are rapidly emerging as key regulators of endocytic transport. However, the importance of SNXs in skeletal development remains largely unknown. To address this, we have systematically screened the expression and localization profiles of all SNXs in major bone and cartilage cells. By combining quantitative-PCR with immunoanalyses we demonstrate that SNXs are differentially expressed in osteoclasts, osteoblasts, osteocytes and chondrocytes. Consistent with their role in facilitating endosomal trafficking, SNXs co-localize with prototypical markers of early endosomes and the retromer. Moreover, genetic ablation of SNX27 in mice results in severe growth retardation attesting to the vital importance of this protein family in skeletal development. Micro-computed tomography scans and histological assessment of SNX27–/– mice revealed drastic reductions in bone mineral density, total bone volume, growth-plate thickening, chondrocyte hyperproliferation and reduced proteoglycan levels as revealed by safranin-O staining, all consistent with a phenotype of chondrodysplasia. Collectively, these data posit an essential role for SNXs in skeletal homeostasis.

OC36

Bone Healing By The Combination Of SAG- And TH-Loaded Artificial Bones

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In this study, we developed osteoinductive artificial bones by loading a smoothened agonist (SAG) and a helioxiantin derivative (TH), an osteogenic compound, on tetrapod-shaped calcium phosphate granules (TetraBone).
To confirm osteogenic activities of SAG and TH, we cultured C3H10T1/2 cells for 14 days with either of them or both. SAG and TH induced expression of alkaline phosphatase (Alp) and osteocalcin (Oc), respectively, suggesting that the two compounds promoted different stages of osteogenesis. The combination of SAG and TH induced expression of both Alp and Oc more strongly than either of them; von Kossa staining revealed extensive calcification. The effect of SAG+TH was confirmed in rat primary bone marrow stromal cells and further verified by increased ossification in the mouse metatarsal organ culture. We then monitored release of SAG or TH from SAG- or TH-loaded TetraBones. SAG-TetraBones induced Alp expression for 12 days in C3H10T1/2 cells, and TH-TetraBones induced Oc expression for more than 35 days in MC3T3-E1 cells. The prolonged-releases were observed even after ethylene oxide gas sterilization. To evaluate bone healings by the combinatorial use of SAG- and TH-loaded TetraBones, we implanted them into bone defects created in rat femurs. Histological and quantitative analyses on micro CT-scanned images showed bone healings were significantly increased in the combination compared to either of them at 14 days after implantation.

OC37

Expression Of BMP3 By Osteoblasts Is Regulated By Canonical Wnt Signaling

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Bone morphogenetic protein 3 (BMP3) is a negative regulator of bone formation. Targeted disruption of Bmp3 in mice results in high bone mass, while transgenic overexpression of BMP3 in osteoblasts leads to delayed mineral deposition and

spontaneous rib fractures. In the adult skeleton, BMP3 is produced by osteoblasts and osteocytes and once secreted, it is able to suppress BMP-induced osteoblast differentiation and maturation through interaction with the BMP/activin receptor type 2b. Here we report that canonical Wnt signaling stimulates BMP3 expression in osteoblasts. We observed elevated BMP3 expression by calvarial osteoblasts isolated from DKK1 heterozygous knockout mice, a model of increased Wnt signaling. In addition when calvarial osteoblasts harvested from wt mice were treated with Wnt3A, mRNA for BMP3 was strongly increased. By using primary osteoblasts obtained from mice carrying a LacZ knock-in allele to the Bmp3 locus as a system to measure BMP3 levels, we determined that Wnt3A greatly increased BMP3 production, as did treatment with other canonical Wnts, and with LiCl, an inhibitor of GSK3β. In contrast, treatment with Wnt5A, an activator of noncanonical Wnt signaling, had no effect on BMP3 expression. Taken together, these data suggest that the BMP and Wnt signaling pathways interact at the level of BMP3. Based on these results, we hypothesize Wnt signaling not only induces bone formation but also regulates osteogenic BMP activity *in vivo* via induction of BMP3.

OC38

The Transcription Factor FoxC1 Up-regulates PTHrP Expression Together With Gli2 In Chondrocytes

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Endochondral ossification is regulated by various transcription factors in a temporo-spatial manner. Identification of novel transcription factors involved in chondrogenesis would facilitate to uncover the molecular basis of endochondral ossification. To approach this, we generated transgenic mice carrying Venus driven by Col2a1 promoter. Venus-positive chondrogenic cells dissociated from E12.5 limb buds were sorted using the FACS Aria, followed by differential microarray between Venus-positive and -negative cells. We identified FoxC1 (Forkhead Box C1) as a candidate transcription factor highly expressed in chondrocytes. Mutations of FOXC1 cause Axenfeld-Rieger malformations (AR) characterized by skeletal abnormalities. Immunohistochemical analysis revealed strong expression of FoxC1 in the perichondrial cells where PTHrP is synthesized. FoxC1 increased PTHrP expression induced by Gli2, a transcriptional mediator of Ihh. Co-IP analysis showed a physical interaction between FoxC1 and Gli2. DNA pull-down and ChIP assays showed direct binding of FoxC1 to the PTHrP gene promoter. Interestingly, pathogenic missense mutation of FoxC1 (F112S) suppressed PTHrP expression and failed to interact with Gli2. In conclusion, we identified FoxC1 as a novel transcriptional regulator of PTHrP expression. Given the critical role of Gli2 and PTHrP in chondrogenesis and AR due to FoxC1 mutations, FoxC1 appears to be involved in the regulation of chondrogenesis via PTHrP.

OC39

Menin Is Required To Maintain Bone Mass In Older Mice

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Although previous *in vitro* studies have shown that multiple endocrine neoplasia type 1 (*Men1*) gene encoding menin has important roles in the osteoblast lineage, little is known about the *in vivo* role of menin in bone. We conditionally inactivated *Men1* in postnatal mature osteoblasts driven by *osteocalcin*-Cre ($Men1_{ob}^{-/-}$). Nine-month-old $Men1_{ob}^{-/-}$ mice displayed reduction in BMD by DXA and in trabecular and cortical bone by micro-CT analysis. By histomorphometric analysis bone volume/total volume, osteoblast and osteoclast number, as well as mineral apposition rate (MAR) were reduced in $Men1_{ob}^{-/-}$ mice. The mRNA expression of osteoblast genes, OPG, RANKL, BMP-2, Runx2, Osterix, Dlx2, Dlx5, and cyclin-dependent kinase inhibitors, p15, p18, p21 and p27, were all reduced, whereas that of cyclin dependent kinases, CDK2 and CDK4, were increased in isolated osteoblasts from $Men1_{ob}^{-/-}$ mice. In contrast, 12-month-old transgenic mice overexpressing menin in osteoblasts ($Men1_{ob}^{TG/+}$), showed a gain of bone mass. Osteoblast number and MAR were increased in $Men1_{ob}^{TG/+}$ mice. Taken together, depletion of menin in the osteoblast leads to decreased osteoblast and osteoclast numbers as well as impaired bone remodeling, resulting in a reduction in bone volume whereas overexpression increases bone mass by enhancing bone formation. Therefore, maintenance of menin expression and function in the osteoblast is important to avoid decreased bone mass.

Oral Presentations 7

OC40

Selective Deletion Of Superoxide Dismutase 2 In Osteocytes Causes Bone Fragility In Mice

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Aging causes bone loss and fragility associated with oxidative damages. Superoxide dismutase 2 (*Sod2*) is a mitochondrial antioxidant enzyme that plays a pivotal role in maintenance of redox balance. In osteocytes, however, the physiological function of *Sod2* is still unelucidiated. To address this question,

mice lacking *Sod2* in osteocytes were generated by crossing mice harboring a *Sod2* conditional allele with Dmp1-Cre transgenic mice. *Sod2* cKO femur showed significantly reduced BMD (–20%), BV/TV (–29%), cortical thickness (–12%) as well as bone stiffness (–23%) by pQCT, micro-CT, and three-point bending analysis. Histochemical analysis revealed that *Sod2* cKO mice significantly increased empty lacunae and osteocyte loss. *Sod2* cKO mice also showed reduced MS/BS (–24%), MAR (–32%), and BFR/BS (–41%) in trabecular bone by calcein labeling and increased osteoclast number (+35%) and surface (+37%) in trabecular bone by TRAP staining. *In vitro* experiment revealed that immature *Sod2*-deficient osteocytes showed suppressed the formation of mineralized nodule, suggesting decreased bone formation. In bone marrow cells culture, *Sod2* deletion in osteocytes did not impair differentiation and proliferation of osteoclasts. Our results indicate that mitochondrial *Sod2*-deficiency induced osteocytes loss associated with impaired bone metabolism, suggesting that *Sod2* in osteocytes plays a crucial role for the maintenance of bone mass.

OC41

Selective Inhibition Of AP-1 In Cart Or AgRP Hypothalamic Neurons Simultaneously Increases Energy Expenditure And Bone Density In Mice

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The regulation of skeletal homeostasis and energy metabolism, once thought to be self-governing, was suggested to be coordinated by common neuronal relays. We have previously shown that ΔFosB, a splice isoform of FosB with AP1 antagonistic properties, increases both energy expenditure and bone formation in a transgenic mouse model or following viral-mediated gene delivery in the ventral hypothalamus (VHT). The aim of the present study was to identify the AP-1 responsive neuronal circuits mediating the metabolic and/or skeletal effects.
For this purpose, we generated Cre-inducible lentiviral vectors expressing transcription factors sharing some AP1 antagonist activity: ΔFosB, Δ2ΔFosB, DNJunD, and Fra1. The expression of these antagonists was restricted to two specific VHT neuronal types - CART and AgRP- via stereotaxic delivery into the VHT of transgenic mice expressing Cre-recombinase under control of neuron-specific promoters. Overexpression of any of these AP-1 antagonists in either anorexigenic CART or orexigenic AgRP neurons resulted in a consistent increase in energy expenditure and was associated with marked increases in bone density, despite the classically divergent functions of these neurons in energy balance. This study demonstrates that selective inhibition of AP-1 transcriptional machinery in one hypothalamic neuron subtype is sufficient to stimulate both total body metabolism and bone density, suggesting the two are under common neuronal regulatory control.

OC42

FGF23 Suppresses Chondrocyte Proliferation In The Presence Of Soluble α-klotho

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X-linked hypophosphatemic rickets (XLH) is characterized by impaired mineralization and growth retardation associated with elevated serum FGF23. The clinical fact that administration of phosphate and calcitriol is not sufficient to fully reverse impaired growth may suggest the existence of a disease-specific mechanism in the development of growth retardation. Here, we showed that FGF23 suppressed chondrocyte proliferation in the presence of soluble α-Klotho (sKL). *In vitro* studies revealed that FGF23 formed a protein complex with sKL and that FGF23 binding to FGFR3 was enhanced in the presence of sKL. Ex vivo metatarsal culture showed that FGF23/sKL suppressed the linear growth of metatarsals, which was antagonized by co-incubation with neutralizing antibodies against FGF23 or by knocking-down FGFR3 expression. Histologically, the length of the proliferating zone (PZ) was diminished and was associated with decreased chondrocyte proliferation. FGF23/sKL suppressed Indian hedgehog (Ihh) expression and Ihh protein partially rescued the impaired metatarsal growth. Administration of sKL in Hyp mice, a murine model for XLH, caused a decrease in the length of the PZ associated with decreased chondrocyte proliferation without altering serum phosphate. These findings suggest that suppression of chondrocyte proliferation by FGF23 could have a causative role in the development of growth retardation in XLH.

OC43

Osteocalcin Signals Within The Hypothalamus To Reduce Bone Mass: Completing The Bone/Brain Circuit

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Several pathways have been identified that signal from the brain to bone. For these pathways to regulate bone in a controlled manner, a feedback signal must exist. We hypothesised that osteocalcin (Ocn) may form a feedback loop to the brain, to regulate bone mass.
We first examined whether Ocn could signal in the brain. An i.p. (1ug) bolus of Ocn induced c-fos in neurons of the arcuate nucleus, demonstrating that serum Ocn activates hypothalamic neurons. Injection of Ocn directly into the CSF circulation also activated neurons in the arcuate, indicating direct Ocn signalling in the brain.
To model chronic Ocn supply to the hypothalamus, we injected a viral vector expressing Ocn (AAV-Ocn) into the arcuate of 10 week old mice and examined bone 12 weeks later. AAV-Ocn reduced cancellous bone volume of the distal femur (40%),

with reduced mineral apposition rate (33%) and increased osteoclast surface (35%). These changes occur despite a fall in serum Ocn (AAV-empty 137±7 ng ml^{-1} vs AAV-Ocn 115±6 P<0.05), highlighting the importance of central signalling to the resultant net bone loss.
Neuropeptide Y (NPY), which acts in the arcuate to control bone mass, is required for this central Ocn effect on bone; as AAV-Ocn injection did not alter bone in NPY KO mice Ocn signals directly in the arcuate to reduce bone mass. This is the first study to define a central feedback mechanism for bone mass, thereby completing the bone-brain circuit.

OC44
Alpha Klotho Enables The Distinct Interactions By Recognition Of A Specific Sugar Motif To Mediate Mineral Homeostasis
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Alpha Klotho is 130kDa membrane-bound protein that plays a critical role in mineral homeostasis by regulating hormones, PTH and vitamin D. alpha Klotho's extracellular region consists of repeated sugar hydrolase-like domains. So far, it has been known that alpha Klotho binds at least two target molecules: One is Na,K-ATPase to induce PTH in response to fluctuation of extracelluar calcium concentration, and another is FGF23 to suppress NaPi (phosphate transporter) translocation and CYP27B1 (vitamin D 1α-hydroxylase) expression in kidney tubules. However, it remained unclear how alpha Klotho is capable of recognizing multiple molecules. We purified the target motif from FGF23 using MSpec and revealed that alpha Klotho directly binds sulfated glucuronide (GlcA). Consequently, alpha Klotho binds through the common motif to Na,K-ATPase beta subunit and FGFR1 as well. These facts demonstrated that alpha Klotho is the first GlcA-recognizing lectin. Moreover, we found that vitamin D induces molecular association of αKlotho to FGFR1 *in vivo*. Together with ligand concentration, the tethering mechanism for receptor complex determines biological signal.

OC45
Advanced Glycation End Product 3 (Age3) Suppresses The Mineralization Of Mouse Stromal ST2 Cells By Increasing TGF-β Expression And Secretion
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Advanced glycation end products (AGEs) cause bone fragility due to quality deterioration in diabetic patients. We previously showed that AGEs suppressed the mineralization of mouse stromal ST2 cells. It has been reported that TGF-β is especially abundant in bone, and that enhancement of its signal deteriorates bone quality. However, it is still unclear whether or not TGF-β signal is involved in the AGEs-induced suppression of mineralization in the osteoblast lineage. We thus examined the roles of TGF-β in the suppression of mineralization of ST2 cells induced by AGE3 (200μg ml^{-1}), which was made by incubating BSA with glycolaldehyde. Treatments of the cells with AGE3 significantly inhibited mineralization by 71.2% on experimental day 21 (P<0.001). The transfection of siRNA of the receptor for AGEs (RAGE) significantly recovered this process (P<0.05). AGE3 significantly increased the mRNA expression and protein level of TGF-β by real-time PCR and ELISA of whole cell lysates, respectively, on days 3, 5, and 7 (P<0.001). Moreover, Alizarin red staining showed that AGE3-induced suppression of mineralization was completely recovered by the treatment of TGF-β type I receptor kinase inhibitors (2.5μM SD208 and 3.0μM SB431542). These findings indicate that AGEs-RAGE pathway inhibits the mineralization of ST2 cells by increasing TGF-β expression and secretion, suggesting that TGF-β adversely affects not only primary osteoporosis but also diabetes-related bone fragility.

Young Investigator Seminar

YIS01
Relaxin Affects Differentiation And Mineralization Of MC3T3-E1 Cells Through Rxfp2
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Relaxin is a peptide hormone that binds to Rxfp1 and Rxfp2. Reduced bone mineral density in hypogonadism is related to the Rxfp2 mutation T222P, still, no effect has been reported of relaxin on bone formation. We examined relaxin's effect on osteoblastic cells as well as that of Rxfp1 and Rxfp2 individually.
Method: We established stable MC3T3-E1 clones lacking Rxfp1 (MCsiRxfp1) and Rxfp2 (MCsiRxfp2), and cultured them in differentiation medium with 20ng/ml relaxin. We examined cell proliferation by MTT assay, osteoblast differentiation by ALP activity, matrix mineralization by Alizarin Red S staining, MMP proteolytic activity by zymography and cell signalling by Western blot.
Results: Administration of 20ng ml^{-1} relaxin did not significantly inhibit proliferation, while increasing osteoblast mineralization and matrix degradation of MC3T3-E1 cells. Relaxin application increased Erk1/2 phosphorylation on all cell clones but more markedly on MCsiRxfp1 cells. MCsiRxfp1 cells showed increased cell proliferation and enhanced osteoblast differentiation and mineralization. In contrast, MCsiRxfp2 showed increased cell proliferation and enhanced matrix degradation.
Conclusion: Relaxin can affect mineralization and matrix degradation of MC3T3-E1 cells. Individual expression of Rxfp1 and 2 can alter the proliferation and biological function of

MC3T3-E1 cells. Rxfp1 seems to relate to extracellular matrix metabolism while Rxfp2 relates to osteoblast differentiation and mineralization.

YIS02

L51p, A Novel Bmp-2 Antagonist Inhibitor, Enhances Bone Formation Potential Of Bmp-2

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Objectives: Bone morphogenetic protein 2 is a well known osteoinductive protein. The high BMP dosage in clinical use may induce adverse effects, such as ectopic bone formation. One of the possible ways to improve BMP-2 efficacy is to suppress activities of BMP antagonists. Our group generated the BMP-2 mutant (L51P) lacking in BMP receptor binding site and previously reported that L51P can bind to noggin and interfere with its binding to BMP-2. In our study, we examined the biological functions of L51P *in vitro* and *in vivo*.
Methods: C2C12 cells stably expressing the BMP-responsive mouse Id promoter were cultured with BMP-2 and/or L51P, and luciferase activity was measured. Osteogenic differentiation of MC3T3E1 cells was assessed by Alizarin red staining and quantitative RT-PCR for Alp and Ocn mRNAs. The levels of the p-Smad 1/5/8 was evaluated by western blots. *In vivo*, the effect of L51P on bone formation was examined. The BMP-2 and/or L51P-containing gelatin-hydrogels were implanted in the full-thickness calvarial defects of rats. After 4 weeks, bone formation was evaluated.
Results: Addition of L51P to BMP-2 induced the p-Smad 1/5/8, and accelerated the luciferase activity (3.1 fold) and mRNA levels of Alp (1.6 fold) and Ocn (2.6 fold) compared to BMP-2 alone. The radiographic examination of calvarial bone defect showed enhanced bone formation in combination of L51P and BMP-2.
Conclusion: Addition of L51P to BMP-2 enhances BMP-2 osteoinductive effect *in vitro* and *in vivo*.

YIS03

EphrinB2 Signaling In Osteoblasts Is Required For Normal And Pth-induced Bone Formation

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EphrinB2 expression by osteoblasts is stimulated by parathyroid hormone (PTH), and its interaction with EphB4 regulates osteoblast and osteoclast differentiation through both ephrinB2 and EphB4 signaling. This study examined the role of ephrinB2 (reverse) signaling in PTH anabolic action *in vivo*. 8-week-old male mice with ephrinB2 deleted in osteoblasts (Osx1Cre.efnB2fl/fl) and controls were treated with PTH (30μg kg^{-1}; 5 per week) or vehicle for 4 weeks. Femora were analyzed by microCT, qPCR and reference point indentation, and tibiae by dynamic histomorphometry. Osteoblast number was 30% greater ($P<0.01$), and collagen1α1 and runx2 levels were significantly increased in efnB2 null mice compared to controls. Despite this, efnB2 null mice showed reduced mineral apposition rate (MAR) ($P<0.05$), cortical thickness ($P<0.01$), cortical tissue mineral density ($P<0.05$), bone material stiffness ($P<0.05$), sclerostin and osteocalcin mRNA levels compared to controls, suggesting impaired osteoblast function in the absence of efnB2. PTH treatment of control mice increased osteoblast surface, trabecular and periosteal MAR and decreased sclerostin levels. In efnB2 null mice, despite a significant PTH-induced increase in osteoblast surface, neither trabecular nor periosteal MAR were increased compared to vehicle. These results demonstrate that ephrinB2 signalling within the osteoblast lineage is required for normal and PTH-induced bone formation in both cortical and trabecular bone.

YIS04

The Development Of Culture Method For Chondrogenic Differentiation Of Human Ips Cells

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Human induced pluripotent stem cells (hiPSCs) form cartilaginous tissues in teratomas *in vivo*. However, no reliable system for *in vitro* chondrogenic differentiation of hiPSCs has yet been reported. Here, we examined the chondrogenic differentiation capability of hiPSCs using a multistep culture method consisting of embryoid body (EB) formation, cell outgrowth from EBs, monolayer culture of sprouted cells from EBs, and three-dimensional pellet culture. Monolayer-cultured cells expressed markers for mesenchymal stem cells. After two to three weeks of pellet culture, cells in pellets exhibited a spherical morphology typical of chondrocytes and were surrounded by extracellular matrix that contained acidic proteoglycans. The expression of type II collagen and aggrecan in pellets progressively increased. Histological analysis revealed that over 70% of pellets successfully underwent chondrogenic differentiation. However, immunohistochemistry (IHC) could not detect the expression of type II collagen. Then, we examined the effect of BMP2 and c-type natriuretic peptide (CNP) on chondrogenic differentiation by addition to pellet culture medium. The expression of type II collagen was partially detected both in BMP2- and CNP-treated hiPSC pellets by IHC. Our study demonstrates that hiPSCs can be efficiently differentiated into the chondrogenic lineage *in vitro* via generation of mesenchymal progenitor cells and that BMP2 and CNP can promote the chondrogenic differentiation of hiPSCs.

YIS05

Dynamic Changes In Chromatin Accessibility During Early Osteoclastogenesis

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Osteoclastgenesis is strictly controlled by several transcription factors (TFs) and dynamic changes of chromatin states in response to various environmental signals. Chromatin accessibility is altered at cis-regulatory regions by the de novo binding of sequence-specific DNA binding TFs, resulting in increased hypersensitivity of local chromatin to DNase I attack. Therefore, delineation of DNase I hypersensitivity sites (DHS) by using a deep sequence approach (DNase-seq) is a powerful strategy to identify regulatory regions important for osteoclastogenesis. To identify *cis*-regulatory elements important for early stage of osteoclastogenesis, we performed *in vivo* DNase-seq by using RANKL-stimulated Raw264 cells. Analysis of DNase-seq data from RANKL-stimulated Raw264 cells revealed 13545 DHS regions in the whole genome, and 5411 regions of the whole DHS were unique in RANKL-stimulated Raw264 cells. Some of these RANKL-stimulation specific DHS were nearly located at the osteoclastgenic genes. 23.2% of these DHS are mapped to promoter regions, and DNase footprints by binding transcription factors were detected within 0.5 kb from transcription start site. We searched the putative TFs bound to each footprint using SeqPos, and identified motifs of oteoclastogenic TFs. These results provide a global view of the dynamic changes of chromatin structure during osteoclastogenesis, and we will find novel TFs regulating cell fate by DNase-seq.

YIS06

Role Of Lectin-like Oxidized Low-density Of Lipoprotein Receptor-1 In Regulating Osteoclastogenesis And Inflammatory Bone Destruction

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We have demonstrated that osteoclastogenesis is associated with the extracellular level of low-density lipoprotein (LDL). Here, we used scavenger receptor class A (SRA) and lectin-like oxidized LDL recptor-1 (LOX-1) knockout (KO) mice to elucidate the role of oxidized LDL in regulating osteoclastogenesis and inflammatory bone destruction. Although RANKL-induced osteoclast formation from SRA KO osteoclast precursors was equivalent to that from wild-type cells, the osteoclastogenesis from LOX-1 KO precursors was increased and the multinucleated cells formed in culture contained more nuclei than wild-type cells. However, RANKL-induced expression of osteoclastogenesis-related proteins such as NFATc1 and TRAP did not alter between LOX-1 KO and wild-type osteoclasts. In contrast, RANKL-induced Akt phosphorylation in LOX-1 KO osteoclast precursors was greater than that in wild-type cells, suggesting a pro-apoptotic receptor of LOX-1. When inflammation was induced by subperiosteal injection of lipopolysaccharide on calvaria *in vivo*, gene expressions of TRAP and RANKL in the injected region were elevated equivalently in both WT and SRA KO mice. In contrast, the elevation in LOX-1 KO mice was reduced compared to WT mice. In conclusion, although LOX-1 is potentially a negative regulator in osteoclastogenesis, LOX-1 may contribute to the inflammatory bone destruction.

YIS07

Quantification Of Cell Networks: Computer-Assisted Method For 3D Mapping Of Osteocyte Populations In The Ageing Human Femur

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Osteocytes create a pervasive syncitium of processes and no part of the bone matrix is more than a few microns from a cell. A role in mechanotransduction has been proposed for the network whereby it directs remodelling and repair. To assess regional variability and the morphological influence of stress input, a novel method has been developed that combines undecalcified histology, confocal microscopy (CLSM) and image analysis software to enable reliable and convenient 3D quantitative characterisation with special reference to cancellous bone. Ageing femoral heads were used to compare the network in traditionally low stress (osteoporosis, OP) and high stress (osteoarthritis, OA) conditions. Segments were en-bloc stained in calcein fluorochrome before embedding in resin. Slices, 300μm thick, were examined by CLSM. Individual 2D Tiff images were imported into software (ScanIP, Simpleware, UK) that generated complementary 3D binary masks specifically representing cell body and process components. Corresponding in-house code (Matlab, Mathworks, USA) was written to quantitate the complementary paired masked aspects including the number of cells, their length, and interconnection. In OP, cells were sparse with fewer processes, forming a poorer interconnected syncitium than that found in OA. This novel method apparently enables topographic appraisal of the syncitium, and the prospect of a more precise evaluation of its potential for biomechanical exchange in ageing and disease.

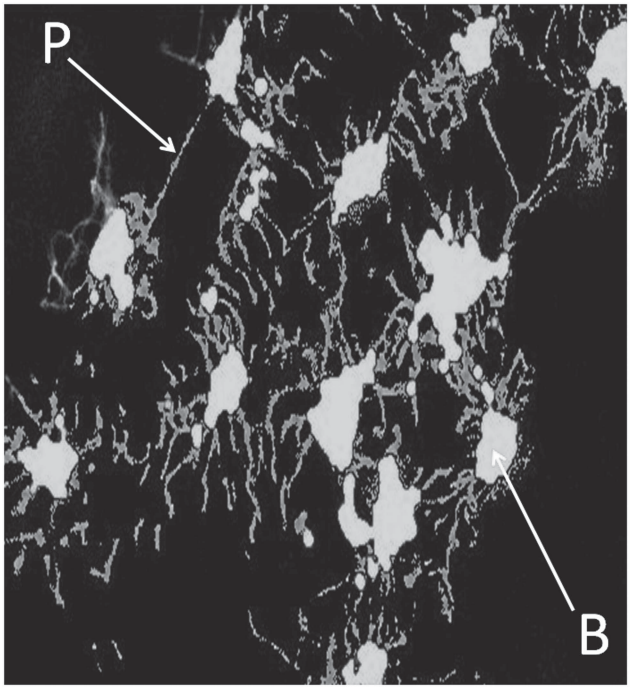

Figure A 2D TIFF image of an osteocyte syncitium, from within osteoarthritic bone, showing the application of two binary masks for the quantification of the network, one representing the osteocyte cell body elements (B, blue), and the other the cytoplasmic processes (P, green).

YIS08

Induction Of Bone Formation In Myeloma Osteolytic Lesions By Cathepsink Inhibition

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Multiple myeloma (MM) enhances osteoclastogenesis while suppressing osteoblastogensis to develop devastating bone destruction. Unlike other anti-resorptive agents, cathepsin K inhibitors potently suppress bone resorption while sparing cytotoxic damage in osteoclasts (OCs). In the present study, we explored the effects of cathepsin K inhibition on bone destruction in MM. The cathepsin K inhibitor KK1-300-01 (KK1) potently suppressed pit formation enhanced in the cocultures of rabbit bone cells with MM cells. However, KK1 did not affect osteoclastogenesis, and allowed OCs to facilitate *in vitro* mineralized nodule formation by MC3T3-E1 cells, suggesting the preservation of OC-driven osteoblastogenesis by KK1. We next examined the *in vivo* effects of KK1 using human INA6 MM-bearing SCID-rab models, which exhibit tumor progression with osteolytic lesions in implanted rabbit bones. Oral dosing of KK1 prevented bone destruction with marked increase in bone trabecular size and BMD in the rabbit bones and tumor reduction within their bone marrow cavity. Histological analyses showed increased bone volume/total volume with a marginal change in OC numbers in the treated mice. Given OC-derived 'coupling', KK1 is suggested to spare the damage in OCs while inhibiting bone resorption to retain the 'coupling' for bone formation together with reducing the release from bone of anti-anabolic factors such as TGF-beta, leading to robust bone formation and thereby MM contraction in bone.

YIS09

Bone Malignant Melanoma Induces Angiogenesis With The Production Of Prostaglandin E2 By Host Stromal Cells

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Bone metastasis of malignant melanoma is accompanied by severe bone destruction with increased bone resorption. When B16 cells were subcutaneously implanted into both wild-type mice (Wt) and *mPges-1* null mice (*mPges1–/–*), *mPges1–/–* showed the suppression of B16 solid tumor formation. In the fluorescent imaging for *in vivo* analysis, the angiogenesis was detected by Angiosense as fluorescent signals, and dramatically suppressed in the group of *mPges1–/–*. By the intravein injection of B16 cells, the bone metastasis accompanied with angiogenesis was detected in the femur and tibia. In *mPges1–/–*, both metastasis and angiogenesis were perfectly suppressed compared with Wt. When dermal fibroblasts derived from Wt were co-cultured with fixed-B16 cells, PGE2 production was markedly increased in the culture medium. Dermal fibroblasts collected from *mPges1–/–* produced less amounts of PGE2. The increased production of vascular endothelial cell growth factor (VEGF)-A and basic fibroblast growth factor (bFGF) was detected in the dermal fibroblasts collected from Wt, but not from *mPges1–/–*. These results suggest that PGE2 produced by host stromal cells promotes VEGF-A and bFGF production, which leads the angiogenesis at the site of melanoma metastasis. The blockage of PGE2 signaling such as PGE receptor antagonist could be a possible candidate for the therapy of bone cancer associated with angiogenesis.