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5B (Basic) - Cell Biology

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O51

The expression of chloride channel 7 (Clcn7) and Ostm1 in osteoclasts is co-regulated by Microphthalmia transcription factor

N. Meadows^{1,2}, S. Sharma³, G. Faulkner¹, M. Ostrowski³, D. Hume^{1,2} and A.I. Cassady^{1,2}

¹ Institute for Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia;

² Co-operative Research Centre for Chronic Inflammatory Diseases, Australia;

³ Department of Molecular Genetics, The Ohio State University, Columbus, Ohio 43210, USA

Microphthalmia transcription factor (Mitf) regulates osteoclast function by regulating expression of genes including TRAP and Cathepsin K in response to RANKL-induced RANK signaling. To identify novel Mitf target genes we have overexpressed Mitf in the murine macrophage cell line RAW264.7 subclone 4 (RAW/C4) and examined the gene expression profile after RANKL-stimulated osteoclastogenesis. Microarray analysis identified a set of genes superinduced by Mitf overexpression, including chloride channel 7 (Clcn7) and

osteopetrosis-associated transmembrane protein 1 (Ostm1). Electrophoretic mobility shift assays identified two Mitf binding sites (M-boxes) in the Clcn7 promoter and a single M-box in the Ostm1 promoter. Anti-Mitf antibody supershifted the DNA-protein complex for the promoter sites in both genes while Mitf binding was abolished by mutation of these sites. The Clcn7 promoter was transactivated by co-expression of Mitf in reporter gene assays but mutation of only one proximal M-box prevented Mitf transactivation while mutation of the other site reduced basal promoter activity. Chromatin immunoprecipitation assays confirmed that the two Mitf-binding and responsive regions *in vitro* also bind Mitf in genomic DNA. The expression of Clcn7 was repressed in the dominant negative mutant Mitf mouse, *mi/mi*, indicating that the dysregulated bone resorption seen in these mice can be attributed in part to transcriptional repression of Clcn7. Mitf regulation of expression of TRAP, cathepsin K, Clcn7 and Ostm1, which are critical for osteoclast resorption, suggests the role of Mitf is more significant than previously perceived and that Mitf is a master regulator of osteoclast function and bone resorption.

O52

Ex vivo and in vivo evidence for anabolic and catabolic bone defects in *Nfl*^{+/-} mice

A. Schindeler^{1,2}, M. Ramachandran¹, C. Godfrey¹, M. McDonald¹ and D.G. Little^{1,2}

¹ Department of Orthopaedic Research & Biotechnology, the Children's Hospital at Westmead, Sydney, Australia.

² Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia.

Type I neurofibromatosis (NFI) is a common autosomal dominant disorder that affects 1 in 3500 individuals. The orthopaedic manifestations of NFI include scoliosis and congenital pseudarthrosis of the tibia (CPT), and patients with these conditions respond poorly to conventional treatments. NFI patients also show a higher incidence of osteopenia. We have used *Nfl*^{+/-} mice to investigate the underlying bone deficiencies caused by this genetic deficit.

Ex vivo culture studies revealed that primary *Nfl*^{+/-} osteoblasts possess a reduced capacity for osteogenic differentiation and mineralisation. This anabolic deficiency was confirmed *in vivo* using a bone morphogenic protein 2 (BMP-2) induced heterotopic ossification assay. *Nfl*^{+/-} mice consistently developed less BMP-2 induced bone than *Nfl*^{+/+} controls.

Nfl^{+/-} heterotopic bone samples showed increased numbers of osteoclasts, suggesting catabolic excess may also contribute to the phenotype. Increased osteoclastogenesis was not observed in RANKL-induced osteoclast cultures, implying that *Nfl*^{+/-} osteoblasts may mediate increased catabolism. Indeed, *Nfl*^{+/-} osteoblasts were found to secrete increased levels of RANKL at the early stages of culture.

To further study the faulty bone repair associated with NFI, we have started to examine tibial fracture healing in *Nfl*^{+/-} mice. Analogous to emerging orthopaedic techniques, we have used BMP-2 to augment the healing of proximal and distal tibial fractures. Pilot results again show decreased BMP-2 induced bone formation in the *Nfl*^{+/-} mice.

Taken together, these data suggest that a combination of anabolic and catabolic defects may contribute to the difficulties associated with treating the orthopaedic complications of NFI.

O53

Vascular smooth muscle cells support functional osteoclast formation from bone marrow and spleen cells in a RANK-dependent manner

I. Endo^{1,2}, D. Inoue¹, T. Doherty¹, P.K. Shah², T.B. Rajavashisth² and T. Matsumoto¹

1. Department of Medicine and Bioregulatory Sciences, The University of Tokushima Graduate School, Institute of Health Biosciences, Tokushima Japan

2. The Atherosclerosis Research Center, Division of Cardiology, Department of Medicine, Cedars-Sinai Medical Center and David Geffen School of Medicine at UCLA, Los Angeles, California

Arterial calcification has been shown to share fundamental mechanistic properties with developmental osteogenesis. Besides osteoblastic cells forming bone-like tissues, TRAP-positive multinucleated cells (TPMNCs) are present at the site of arterial calcification. However, neither origin nor function of these cells is understood. In the present study, we found that mouse bone marrow cells (BMCs), co-cultured with

primary VSMCs in the presence of 1 α ,25-dihydroxyvitaminD3 (1,25D), dexamethasone (DEX) and PTH, produced TPMNCs, which expressed cathepsin K, calcitonin receptor and carbonic anhydrase II, and which formed resorption pits. Spleen cells, when co-cultured with VSMCs, also produced functional TPMNCs, but not at all in the absence of VSMCs. RT-PCR analysis revealed mRNA expression of M-CSF, RANKL, OPG, ICAM-1 and VCAM-1 by VSMCs. Expression of RANKL and M-CSF was confirmed at the protein level. Treatment of VSMCs with 1,25D, DEX or PTH increased mRNA expression of RANKL and ICAM-1. ALP mRNA was also induced by 1,25D or PTH. TPMNCs were formed in co-cultures of RANK^{-/-} VSMCs and RANK^{+/+} BMCs, but not at all in co-cultures of RANK^{+/+} VSMCs and RANK^{-/-} BMCs, indicating a critical role of RANK on BMCs. In summary, mesenchymally derived VSMCs can partially obtain osteoblastic phenotype expressing RANKL, M-CSF, ICAM-1 and VCAM-1 to support functional osteoclast-like cell formation in a RANK-dependent manner in vitro. Our results further imply that thus formed osteoclasts in vascular environment may participate in the pathogenesis of arterial calcification, which can be further explored using the novel co-culture system established here.

O54

PTH regulates subcellular localization of type IIa sodium-dependent phosphate transporter (NaPi-IIa) through phosphorylation of ezrin

Y. Taketani, A. Nakamura, A. Tanimura, K. Nashiki, H. Segawa, H. Yamamoto, K. Miyamoto and E. Takeda
Department of Clinical Nutrition, and Department of Molecular Nutrition, University of Tokushima, Tokushima 770-8503, Japan

Type IIa sodium-dependent phosphate (Pi) transporter (NaPi-IIa) plays a crucial role in the regulation of serum Pi homeostasis. NaPi-IIa forms a macromolecular complex (called NaPi-IIa transportsome) with NHERF-1, ezrin, actin, and signal transduction molecules in the membrane microdomains including caveolae and lipid-rafts in the apical plasma membrane. We have reported that ezrin is a target molecule for PTH signal leading rapid NaPi-IIa endocytosis⁽¹⁾. Here, we investigated that the role of phosphorylation of ezrin in the regulation of NaPi-IIa by PTH. To determine the role of ezrin in the sub-cellular localization of NaPi-IIa, we transiently expressed mutant ezrin at phosphorylation site or C-terminal truncated dominant-negative ezrin (ezrin-DN) in OK-N2 cells (1), and analyzed the NaPi-IIa localization by using surface biotin labeling assay and isolated membrane microdomains. Overexpression of ezrin-DN in OK-N2 cells reduced the amount of NaPi-IIa in the isolated membrane microdomain fraction. Surface biotin labeling assay revealed that expression of ezrin-DN decreased the amount of PTH receptor (PTH-R) in the plasma membrane as well as NaPi-IIa. Furthermore, PTH-mediated reduction of NaPi-IIa was also inhibited by expressing ezrin-DN. Amino acid substitution analysis demonstrated that T149D, can mimic phosphorylated residue, diminished the plasma membrane localization of NaPi-IIa. These results suggest that phosphorylation of ezrin at T149 may affect the membrane localization of NaPi-IIa and that would be a key event for PTH signal leading the rapid endocytosis of NaPi-IIa.

(1) Nashiki K, et al. *Kidney Int.* 68:1137-1147, 2005

O55

Trabecular bone volume in young female mice and adult male mice is dependent on calcitonin receptor expression in osteoclasts

R.A. Davey¹, W.S.M. Chiu¹, A.J. Moore², C. Ma¹, E. Doust¹, J.F. McManus¹, H.A. Morris², D.M. Findlay³ and J.D.Zajac¹.

¹Department of Medicine AH/INH, University of Melbourne, Heidelberg, Victoria, ²Hanson Institute, IMVS, Adelaide, ³Orthopaedics and Trauma, University of Adelaide.

We have previously demonstrated that global knockdown (KD) of the calcitonin receptor (CTR) by greater than 95% results in trabecular bone loss in the distal femur and vertebra in female mice at 6 weeks of age. In contrast, in males, global KD of the CTR results in increased labeled surface ($P < 0.05$) and bone formation rate ($P = 0.09$). Despite this increase in bone formation it was not of sufficient magnitude to result in a net increase in trabecular bone volume (BV/TV).

To extend these studies to investigate the physiological control of bone turnover by the CTR on osteoclasts (OCLs), we have generated a genetically modified mouse model in which the CTR is knocked-out specifically in OCLs (OCL-CTR KO) using the Cre/loxP system.

BV/TV was decreased by 20% in the femur of female OCL-CTR KO mice at 6 weeks of age ($P<0.05$). This was associated with a decrease in trabecular number (TbN) ($P<0.05$), while trabecular thickness (TbTh) was unaffected. In contrast, BV/TV was decreased by 35% in males at 12 weeks of age as a result of decreased TbN ($P=0.08$) and TbTh ($P<0.05$). The numbers of OCLs per bone surface and serum X-Laps were unchanged in female and male OCL-CTR KO mice at 6 and 12 weeks of age respectively. We are further assessing the effect of CTR deletion on OCL activity in these mice in vitro and on bone formation in vivo.

In conclusion, BV/TV in young female mice and in adult males is dependent on the expression of the CTR on osteoclasts.

O56

O-linked glycosylation by ppGaNTase-T3 prevents processing of fibroblast growth factor (FGF)23

N. Ito¹, S. Fukumoto¹, T. Yamashita², Y. Frishberg^{*3}, T. Fujita^{*1}.

¹Division of Nephrology & Endocrinology, Department of Medicine, University of Tokyo Hospital, Tokyo, Japan,

²Pharmaceutical Research Laboratories, KIRIN Brewery Co., Ltd., Takasaki, Japan,

³Division of Pediatric Nephrology, Shaare Zedek Medical Center, Jerusalem, Israel.

FGF23 with phosphaturic activity is partly processed into inactive fragments. Tumoral calcinosis (TC) is characterized by hyperphosphatemia and high 1,25-dihydroxyvitamin D levels. This disease is a mirror image of hypophosphatemic diseases caused by excess FGF23 activity. GALNT3 encoding a peptide (ppGaNTase-T3) involved in the initiation of mucin-type O-glycosylation was identified to be responsible for TC. Patients with TC by GALNT3 mutations have rather low levels of biologically active full-length FGF23 while the amount of C-terminal fragments is markedly elevated. Therefore, the processing and production of FGF23 is enhanced in these patients. Although FGF23 has several O-linked glycans, it has been unknown whether mutations in GALNT3 actually affect processing of FGF23. We therefore investigated the effect of silencing of GALNT3 expression. HOS-TE85 cells that express ppGaNTase-T3 were treated with siRNA for GALNT3 and then transfected with expression vector for FGF23. Immunoblotting confirmed that the siRNA decreased the expression of ppGaNTase-T3. Western blotting demonstrated that the amount of full-length FGF23 decreased while the processed C-terminal fragment increased by the siRNA. Measurement of FGF23 by full-length assay indicated the amount of FGF23 in the conditioned media decreased to 60.9 +/- 6.1 % by the siRNA treatment. In contrast, C-terminal assay, which detects both full-length and processed C-terminal fragment, demonstrated that FGF23 by cells with siRNA for GALNT3 was 96.2 +/- 17.5 % of cells with control siRNA. These results indicate the inhibition of ppGaNTase-T3 expression enhances the processing of FGF23. Therefore, ppGaNTase-T3 activity and therefore O-glycosylation is necessary for maintenance of full-length FGF23.

O57

Chromosome 13 locus for peak femoral bone mineral density modulates bone formation in mice

R. Nakanishi, M. Shimizu, M. Mori, B. Otsuki, S. Okudaira, K. Higuchi, T. Nakamura and T. Tsuboyama

Department of Orthopaedic Surgery, Graduate school of Medicine, Kyoto University, Kyoto, Japan

In the previous genome wide linkage study, using SAM (senescence-accelerated mouse)P6 and SAMP2, we identified two significant QTLs (quantitative trait loci) for femoral bone mineral density (BMD) on Chr (chromosome) 11 and 13, and we constructed a congenic strain P6.P2-Pbd2, which carried a 15cM SAMP2 interval of Chr13 QTL on the SAMP6 derived background. The aim of the present study was to further narrow the interval and to characterize its effect on bone.

We mated SAMP6 and P6.P2-Pbd2^b and developed sub-lines which have different segregated QTL regions. Then the bone fraction area (BA/TA%) at the femoral mid-shaft was measured in sub-lines, and we constructed P6-P2-13 which has the shortest QTL interval and a relatively higher BA/TA than others. Using

P6.P2-13 we investigated the effects of this locus on morphology by micro-CT and on bone formation ability by dynamic histomorphometry. Furthermore, we examined osteoblastic activity of calvaria cells harvested from SAMP6 and P6.P2-13.

P6.P2-13 had a segregated 2.4Mb QTL region. In the morphometrical analysis, P6.P2-13 showed increased BA/TA by 6.6% at the diaphysial cortex, and increased trabecular bone volume (BV/TV) by 54.2% at metaphysis when compared to SAMP6. In the dynamic histomorphometrical study, P6.P2-13 exhibited a 40% higher bone formation rate in the femoral mid shaft than that of SAMP6. In vitro, osteoblasts harvested from P6.P2-13 showed a higher osteoblastic activity than those from SAMP6. In conclusion, the locus on Chr13 directly stimulates bone formation and consequently, affects peak femoral BMD.

O58

Functional interaction of the focal adhesion protein testin with the calcium-sensing receptor

A.L. Magno^{1,2}, B.K. Ward^{1,2}, A.D. Conigrave³ and T. Ratajczak^{1,2}

¹Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands Western Australia, 6009.

²Western Australian Institute for Medical Research and UWA Centre For Medical Research, University of Western Australia, Nedlands, Western Australia, 6009. ³School of Molecular and Microbial Biosciences, University of Sydney, Sydney, New South Wales, 2006.

The calcium-sensing receptor (CaR) is a G protein coupled receptor that plays an integral role in calcium homeostasis. To elucidate the mechanisms that convert the various extracellular stimuli for the CaR to specific intracellular responses we used the CaR intracellular tail as bait in a yeast two-hybrid screen of a mouse haemopoietic cell line library. Three distinct clones of testin containing a 61 amino acid overlapping region were identified. The interaction between the CaR and full-length testin was confirmed in coimmunoprecipitation studies using lysates from HEK293 cells transiently expressing FLAG-tagged CaR and EGFP-tagged testin. Direct interaction between the two proteins is being investigated in pulldown assays using purified His-tagged CaR tail and GST-testin fusion proteins. Testin is a triple LIM domain protein that localises to focal adhesions and actin stress fibres. LIM domains are composed of two zinc fingers that mediate protein interactions important in coordinating signalling pathways. The overlapping region of the identified clones incorporates the second zinc finger of the first LIM domain. Yeast two-hybrid based mapping studies revealed that testin binds to the membrane-proximal region of the CaR tail, which is critical for activation of the ERK pathway and other signalling cascades. Preliminary experiments indicate that overexpression of testin in HEK293 cells stably expressing the CaR inhibits the receptor's ability to mediate ERK activity at physiological levels of extracellular Ca²⁺ but not at higher concentrations. Testin then may have a modulating role in CaR-mediated signalling linked to focal adhesions and actin stress fibres.