findings highlight the importance of heparin in controlling growth factor mediated signaling and Wnt-stimulated bone formation.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.115

OP27

Development of a novel animal model for the syndrome of inclusion body myopathy, Paget's disease and fronto-temporal dementia

A. Daroszewska[a,*], L. Rose[a], K. Rose[a], R. Sedlmeier[b], R.J. van 't Hof[a], S.H. Ralston[a]
[a]*Rheumatic Diseases Unit, University of Edinburgh, Edinburgh, UK*
[b]*Ingenium Pharmaceuticals AG, Martinsried, Germany*

The hereditary syndrome of inclusion body myopathy, Paget's disease of bone and fronto-temporal dementia (IBMPFD) is an incurable disorder caused by mutations in the *VCP* gene. Progress in understanding the pathogenesis of this disease has been hampered by the lack of a suitable disease model. Here we describe the development of a mouse model for VCP using ENU mutagenesis.

In order to develop a disease model for IBMPFD, we screened a DNA archive from >16,000 ENU mutagenised C3HeB/FeJ male mice for point mutations in VCP by PCR amplification of the respective chromosomal regions followed by heteroduplex analysis of the generated fragments using a temperature gradient capillary electrophoresis system. The hindlimbs of VCP-mutant and wild type mice were scanned using a Skyscan 1076 in vivo μCT scanner at 18 μm resolution at 2 monthly intervals from the age of 6 months. Limbs were scored for severity of lesions on a scale from 0 to 3, and the scores for both limbs added.

Nine different VCP mutations were detected of which one, the L96P mutation, is in a highly conserved residue of VCP and is predicted to mimic the effects of the R95G mutation which is known to cause IBMPFD in humans. Sperm from the heterozygous mutant carrier animal identified in the archive screening, was used for in vitro fertilization of C3HeB/FeJ wild type oocytes to generate generation 2 (G2) animals, and heterozygotes from the G2 were crossed to provide G3 which includes wild type (WT), heterozygote (HET) and homozygous mutant (HOM) animals. The HET animals from G2 had all developed lytic lesions, of predominantly mild severity (score 2–3) by 12 months. By 6 months of age, almost all HET and HOM animals from G3 had developed lesions, with the HOM animals showing more severe lesions than the HET animals (average score 3.0 ± 1.1 for HET and 5.2 ± 1.1 for HOM; $p < 0.01$). In addition, 2 mice (both female HET) developed behavioral abnormalities such as hyperactivity and head bobbing, indicating that these mice may be developing neuronal abnormalities as well as bone lesions.

In summary, our preliminary results indicate that the L96P-VCP mice develop Pagetic-like bone lesions. The VCP-L96P mouse may be an important resource with which to gain greater understanding of disease mechanisms in IBMPFD and classical Paget's disease.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.116

OP28

Identification of calpain-6 as a new target involved in cell death of bone cancer cells

A. Marion[a,*], F. Dieudonné[a], A. Patiño[b], F. Lecanda[b], P. Marie[a], D. Modrowski[a]
[a]*Unit 606, INSERM, Paris, France*
[b]*Oncology Division, Center for Applied Medical Research, University of Navarra, Pamplona, Spain*

Calpain-6 is a microtubule stabilizer during cytokinesis which is mainly expressed during embryogenesis and down-regulated after birth. We have previously shown that syndecan-2 acts as a key regulator of cell death promoting apoptosis and increasing chemosensitivity of osteosarcoma cells. To gain insight into the mechanism of syndecan-2-mediated apoptosis, we performed global transcriptomic analysis in human osteosarcoma cells. Calpain-6 was identified as one of the genes that were down-regulated upon syndecan-2 overexpression. This was validated by qPCR analysis in a panel of osteosarcoma cell lines. Interestingly, calpain-6 expression was also found to be decreased in vivo in bone tumours obtained by injection of murine osteosarcoma K7M2 cells transduced to overexpress syndecan-2 as compared to tumours obtained with control K7M2 cells. Furthermore, rescuing calpain-6 expression using a lentiviral vector abolished syndecan-2-dependent cell death induced by low doses of Doxorubicin in syndecan-2-overexpressing cells. Consistently, calpain-6 inhibition by specific shRNA resulted in a striking increase in the number of apoptotic cells as shown by Apopercentage dye uptake and TUNEL assays. Inhibition of calpain-6 by shRNA also decreased cell proliferation as assessed by BrdU incorporation. These results indicate that the alteration of calpain-6 expression contributes to syndecan-2-induced apoptosis and suggest a role of calpain-6 as a survival factor in bone cancer cells. To further explore the relevance of these findings in clinical samples, we investigated the expression of calpain-6 in human osteosarcoma tissues and normal bone using a tissue microarray constructed with osteosarcoma, metastasis and normal bone samples. Immunohistochemistry analysis revealed almost complete absence of calpain-6 staining in osteoblasts and osteocytes in normal bone. In contrast, 75% of primary osteosarcomas ($n = 24$) showed a positive staining, including 25% of tumour samples displaying a strong and widespread staining. Interestingly, calpain-6 was also detected in bone and lung metastasis. There was a striking strong expression of calpain-6 in most cells of recurrent tumours examined ($n = 9$). These results indicate that calpain-6 may be a selective factor contributing to the survival of bone cancer cells and hence to the mechanism of recurrence. Furthermore, our data suggest that inhibition of calpain-6 may represent a new pharmacological target to overcome chemoresistance in osteosarcoma cells.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.117

OP29

Opposing effects of nutrition hormones ghrelin and leptin in osteoblastogenesis

M. van der Velde[a,*], B.C.J. van der Eerden[a], Y. Sun[b], P.J.D. Delhanty[a], A. van der Lely[a], H. Weinans[c], R.G. Smith[b], J.P.T.M. van Leeuwen[a]
[a]*Internal Medicine, Erasmus MC, Rotterdam, Netherlands*
[b]*Huffington Centre of Aging, Baylor College of Medicine, Houston, USA*
[c]*Orthopedics, Erasmus MC, Rotterdam, Netherlands*

There exists and intriguing and complex relation between fat and bone cells with respect to aging and osteoporosis. Leptin is proposed as a signal from fat to bone. Besides its role in bone metabolism, leptin is most well known for its anorexigenic properties. Opposed to leptin is ghrelin, an orexigenic peptide derived from the stomach. In other systems, like the gonadal and immune systems, ghrelin and leptin also act as each other's antagonists. We therefore asked the following question: do ghrelin and leptin also act as antagonists on bone? We cultured bone marrow from 3 months old male WT, leptin deficient (ob/ob), ghrelin receptor deficient (Ghsr −/−), and ob/ob.Ghsr −/− mice under osteogenic conditions. After 9 days we counted the number of alkaline phosphatase (ALP)-positive colonies, which are a measure or the number of osteogenic progenitors in the bone marrow.

We found that the cultures of ob/ob mice had less ALP-positive colonies than those of WT mice. There was no difference between the cultures of WT and Ghsr −/− mice. However, additional deletion of Ghsr in ob/ob mice compensated for the lower number of ALP-positive colonies in the ob/ob mice bone marrow cultures and brought it back to WT levels. Ghrelin- and leptin receptors are expressed on osteoblasts implicating direct effects of ghrelin and leptin on osteoblast differentiation and function. This prompted us to investigate whether the observations in the knock out models could by explained by direct effects of these peptides on osteoblasts. We therefore cultured bone marrow of 3 months old ob/ob mice under osteogenic conditions and added ghrelin and leptin to the cultures. Consistently, leptin treatment increased the number of ALP-positive colonies. Ghrelin treatment did not affect the number of ALP-positive colonies, but was able to block the leptin effect.

We have shown that ghrelin and leptin have opposing effects on the number of ALP-positive colonies in ex vivo bone marrow cultures both by knock out approaches and direct effects of ghrelin and leptin treatment. These results show that osteogenic precursors in the bone marrow are direct targets of these two peptide hormones that also regulate food intake and nutritional status. It further shows that ghrelin and leptin interaction plays a role in the link between nutrition and bone health.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.118

OP30

The role of calcium phosphate crystals in the phosphate-dependent activation of osteoblasts

S. Khoshniat[a,*], M. Julien[a], A. Bourgine[a], M. Petit[b], T. Rouillon[a], M. Masson[a], M. Gatius[a], P. Weiss[a], L. Beck[c], J. Guicheux[a]
[a]*Physiopathology of Skeletal Tissues and Cartilage Engineering, INSERM U791 LIOAD, France*
[b]*Laboratoire de Synthèse Organique, CNRS UMR 6513, Nantes, France*
[c]*Centre de Recherche Croissance et Signalisation, INSERM, U 845, Paris, France*

Inorganic phosphate (Pi) acts as a signalling molecule in bone-forming cells, affecting cell functions and gene expressions (1). Particularly, Pi stimulates the expression of mineralization-associated genes such as osteopontin (OPN) and matrix gla protein (MGP) through the ERK1/2 pathway (2,3). With respect to the concomitant presence of elevated extracellular calcium and phosphate levels during bone remodelling, we questioned whether calcium may play a role in the phosphate-dependent effects in osteoblasts. As evidenced by real-time PCR and Western blot, we first confirmed that ion pair (10 mM Pi; 1.8 mM calcium) stimulates MGP and OPN expression through ERK1/2 phosphorylation in MC3T3-E1 osteoblastic cells. The use of increasing phosphate and calcium concentrations showed that at least 1.8 mM calcium is required for Pi-dependent ERK1/2 phosphorylation and MGP/OPN up-regulation. In addition, ion pair-dependent cellular effects were blocked using foscarnet and EDTA, a Pi transporter inhibitor and a calcium chelating agent, respectively. Furthermore, because the exact mechanisms by which Ca and Pi act on osteoblasts remain unclear, we then questioned whether ion pair-dependent cellular effects may be mediated through the formation of calcium phosphate crystals. By transmission electron microscopy and elemental microanalysis (EDX), we demonstrated that adding Pi (10 mM) in the culture medium containing 1.8 mM calcium led to the formation of apatitic crystals. Interestingly, phosphocitrate, an inhibitor of crystal formation, inhibited ion pair-induced cellular effects. In order to strengthen these data, we precipitate phosphate and calcium by addition of 10 mM Pi in a medium containing 1.8 mM calcium. We next collected the crystals in a medium containing saturating concentrations of Pi and calcium (1.8 mM calcium and 2.8 mM Pi), and treated MC3T3-E1 cells with these crystals. Our results indicate that calcium phosphate crystals induced osteoblast activation at both the ERK 1/2 signaling and mRNA levels. Our data thus strongly suggest that calcium is required for Pi-dependent ERK1/2 phosphorylation as well as regulation of mineralization-associated genes in osteoblasts and that phosphate–calcium crystals seems to be involved in this osteoblastic activation. Whether the cellular effects of calcium and phosphate are mediated by specific sensing receptors, ion transporters or by crystal-mediated mechanisms would be paid further investigation.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.119

OP31

Plekhm1 is involved in trafficking of cathepsin K-containing endosomal vesicles in osteoclasts

F.P. Coxon[a,*], A. Taylor[a], L. Van Wesenbeeck[b], W. Van Hul[b]
[a]*Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*
[b]*Medical Genetics, University of Antwerp, Antwerp, Belgium*

Plekhm1 is an essential protein for osteoclast function, since loss-of-function mutations underlie the osteopetrotic phenotype of the incisors absent rat as well as an intermediate type of human osteopetrosis. Plekhm1 is a cytosolic protein that is also present on endosomal/lysosomal vesicles, where it colocalises with Rab7, a small GTPase that is essential for the trafficking of these vesicles to the ruffled border in osteoclasts. This suggests that Plekhm1 may also play a role in this process, and that the lack of ruffled border formation in osteoclasts from patients bearing the PLEKHM1 mutation results from defective trafficking of endosomes/lysosomes. Trafficking of TRAP containing vesicles also appears to be defective, since osteoclasts from the patients accumulate high levels of vesicular TRAP. Using overexpression of GFP-tagged forms of Plekhm1 and Rab7, we have now further characterized the vesicles to which these proteins localize (by detecting TRAP, acidic vesicles, and cathepsin K activity) in both HEK293 cells and human osteoclasts using confocal microscopy in fixed cells. In addition, we have studied the effect of Rab7/Plekhm1 overexpression on trafficking of these vesicles by live cell imaging, using a Deltavision Core system with deconvolution. As expected, cathepsin K activity was largely confined to acidic vesicles in osteoclasts. Plekhm1 and Rab7 also localised mainly to vesicles that are acidic and contain abundant cathepsin K. Conversely, there was little localisation of either Rab7 or Plekhm1 to vesicles containing TRAP in osteoclasts, detected by immunostaining or using a fluorescent phosphatase substrate. Live cell imaging demonstrated that in HEK293 cells, vesicles overexpressing either Plekhm1 or Rab7 undergo rapid bidirectional transport on microtubules, with a tendency to accumulate in the perinuclear region that is more apparent in Plekhm1-transfected cells. Moreover, vesicles expressing Rab7 or Plekhm1 became larger and less motile with longer incubation periods, suggesting that both these proteins are also involved in vesicular fusion. Similar results were found in pre-fusion osteoclasts cultured on glass. These results provide further evidence that Plekhm1 is an important mediator of endosomal trafficking to the ruffled border in osteoclasts, and suggest that the accumulation of TRAP in osteopetrotic patients bearing the PLEKHM1 mutation is an indirect consequence of disruption of endosomal transport.

Conflict of interest: None declared.

doi:10.1016/j.bone.2009.03.120