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Program Chairmen D.B. Burr and W.S.S. Jee

The presentation of the Sun Valley Hard Tissue Workshop abstracts in two parts: Oral presentations (OR) and posters (P). Specifically:

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OR-1

OSTEOPOROSIS IN WOMEN

R.R. Recker
Creighton University, Omaha, NE
rrecker@creighton.edu

The Sun Valley Workshop this year opens with a session on osteoporosis in women, to be followed by a session on osteoporosis in men. The sessions will characterize the "state-of-the-art" in both genders with attention paid to the differences between them and the insights those differences may give in understanding skeletal biology and biomechanics.

The first session will be given by Dr. Robert Marcus and will give an overview of current questions in osteoporosis. He will present his view of the status of the questions, and in the process give background that introduces the topics of subsequent speakers.

The subsequent speakers will be Drs. Cummings, Burr, Keaveny, Martin and Ambling.

Dr. Cummings will examine the question whether osteoporotic fractures in women are on the increase. The first problem here is whether the absolute number of fractures is increasing and the answer to this is almost certainly yes. The real question, however, is whether the increase is due to the increasing numbers of our aging population or whether it is due to change intrinsic to skeletons of people as they are aging. Dr. Cummings will examine the current evidence on both of those. He will also discuss in some detail how and why does fracture risk change during drug treatments. This topic has created considerable controversy in the field, and exploration of the reasons for the apparent disconnect between antifracture efficacy and bone mass changes during anti-resorptive treatment may give insights into the biology and biomechanics of the skeleton.

Dr. Burr will examine bone material properties with an eye toward examining mineral matrix contributions to fracture risk with age and contrast these with what is known about the differences in them between women and men. While the incidence of fractures in men is increasing, it is still much below the incidence in women. Is there a difference in bone material properties between women and men?

Dr. Keaveny will examine differences in the micro and macro structure of the skeleton between men and women. The first question is whether there is a difference and the second is whether the difference accounts for the sex differences in fracture risk between women and men, and finally, if there are differences, what might be the mechanisms for the differences?

Dr. Martin will examine the issue about whether skeletal size and structure have anything to do with fracture risk. Further, whether differences in size and structure allow generation of conclusions about the reasons for the difference in fracture risk between men and women, and whether they are due to differences in morphology.

Finally, Michael Ambling will discuss unique osteoporosis models with an eye toward exploring the mechanisms behind skeletal fragility.

The first session will be the starting point for subsequent discussions of the mechanism of skeletal fragility in both men and women, ranging from submicroscopic through microscopic and macroscopic morphology and extending to dynamic function. For example, we will have discussions regarding mechanosensing and transduction, genetics of mechanical loading sensitivity, the role of osteocytes in mechanosensing, skeletal development, and neurotransmitter functions.

OR-2

EXPANDING OUR VIEW OF POSTMENOPAUSAL OSTEOPOROSIS

R. Marcus
Stanford University and Veterans Affairs Medical Center, Palo Alto, CA.
marcuse@leland.stanford.edu

Why is the incidence of osteoporotic fracture so much higher in women than in men? The dominant medical view holds that the exaggerated skeletal fragility and fracture risk of postmenopausal women solely reflects the loss of bone following withdrawal of endogenous estrogen. Indeed, an enormous amount of research in this area has attempted to understand the rise in fractures after menopause in terms of the impact of estrogen lack on bone remodeling. Recent insights suggest that this simple view does not offer an adequate explanation for the greater susceptibility of older women to fracture compared to that of men. It seems more reasonable to view bone

health as a lifelong process, reflecting the contributions and influences of myriad events occurring throughout life to skeletal acquisition and maintenance. Only recently has the medical community recognized that the amount of bone present at skeletal maturity makes a powerful contribution to lifelong skeletal status. A second area that must be incorporated into discussions of this topic relates to bone size and geometry. Women's bones are inherently smaller than those of men. A bone's strength is determined by its size as well as by its material properties. In boys, pubertal increases in the cortical thickness of long bones are achieved by (testosterone-dependent) periosteal apposition. By contrast, increased cortical thickness in girls reflects bone expansion into the medullary space, with little or no periosteal apposition, suggesting an inhibitory effect of estrogen on the latter process. Consequently, at skeletal maturity, men have wider bones of greater mechanical competence. Although estrogen is generally held to be skeletally protective, this aspect of its actions may actually render women more susceptible to some fractures. In later life, men may lose even more bone from appendicular sites than do women, but men show much greater concomitant increases in periosteal apposition than women, permitting them to maintain a relatively favorable mechanical profile. These several findings are based on cross-sectional observations of relatively few individuals and therefore require confirmation in prospective longitudinal studies. The degree to which gender-related differences in later life skeletal adaptation reflects a bone's mechanical or metabolic environment has been frequently discussed but still awaits experimental confirmation.

OR-3

HOW DRUGS DECREASE FRACTURE RISK: LESSONS FROM TRIALS

S.R. Cummings
University of California, San Francisco, CA
scummings@psg.ucsf.edu

In women with osteoporosis, each 1% improvement in spine BMD (by DXA) is expected to reduce vertebral fracture risk about 4%. However, randomized trials of antiresorptive agents show that 1 to 6% improvements in spine BMD reduce vertebral fracture risk by 35 to 50%. Less 20% of the decreased spine fracture risk produced by alendronate or raloxifene be explained by improvement in spine BMD. The discrepancy is even greater during the first year or two of treatment when 1 to 4% improvements in BMD are associated with 65-68% decreases in spine fracture risk. Bisphosphonates continue to increase BMD but the reduction in fracture risk wanes to 20 to 45%.

DXA underestimates change in bone density of spinal trabecular bone and this might explain part of the discrepancy between expected and observed reductions in spine fracture risk. Even more accurate measurement of BMD would not explain the rapid onset and later waning of effect despite gradually increasing BMD. The biomechanical effects inhibiting bone resorption could explain the early onset but not the waning effectiveness. The waning effectiveness of antiresorptives raises concerns that prolonged inhibition of remodeling may weaken bone by allowing microdamage to accumulate.

The effect of drugs on nonspine fracture risk is more complex and cannot be predicted from changes in DXA BMD. For example, Beck showed that long-term users of estrogen increase section modulus vs. nonusers with a net increase in section modulus and predicted femoral neck strength despite losing about 0.4% per year in femoral neck BMD. PTH reduces spine fracture risk and this effect is more completely explained by improvement in spine BMD. This suggests that sustaining the increased BMD produced by PTH may maintain long-term reductions in fracture risk.

OR-4

BONE MATERIAL PROPERTIES AND MINERAL MATRIX CONTRIBUTIONS TO FRACTURE RISK OR AGE IN WOMEN AND MEN

D.B. Burr
Indiana University School of Medicine, Indianapolis, IN
dburr@iupui.edu

The strength of bone is related to its mass and geometry, but also to the physical properties of the tissue itself. Bone tissue is composed primarily of collagen and mineral, each of which changes with age, and each of which can be affected by pharmaceutical treatments designed to prevent or reverse the loss of bone.

With age, there is a decrease in collagen content, which is associated with an increased mean tissue mineralization, but there is no difference in cross-link levels compared to younger adult bone. In osteoporosis, however, there is a decrease in the reducible collagen cross-links without an alteration in collagen concentration; this would tend to increase bone fragility. In older people, the mean tissue age (MTA) increases, causing the tissue to become more highly mineralized. The increased bone turnover following menopause may reduce global MTA, and would reduce overall tissue mineralization.

Bone strength and toughness are positively correlated to bone mineral content, but when bone tissue becomes too highly mineralized, it tends to become brittle. This reduces its toughness, and makes it more prone to fracture from repeated loads and accumulated microcracking. Most approved pharmaceutical treatments for osteoporosis suppress bone turnover, increasing MTA and mineralization of the tissue. This might have either or both of two effects. It could increase bone volume from refilling of the remodeling space, reducing the risk for fracture. Alternatively, the increased MTA could increase the propensity to develop microcracks, and reduce the toughness of bone, making it more likely to fracture. There may also be changes in the morphology of the mineral crystals that could affect the homogeneity of the tissue and impact mechanical properties. These changes might have large positive or negative effects on fracture incidence, and could contribute to the paradox that both large and small increases in density have about the same effect on fracture risk.

Bone mineral density measured by DXA does not discriminate between density differences caused by volume changes, and those caused by changes in mineralization. As such, it does not entirely reflect material property changes in aging or osteoporotic bone that contribute to bone's risk for fracture.

OR-5

ARCHITECTURE AND TRABECULAR BONE—TOWARD AN IMPROVED UNDERSTANDING OF THE BIOMECHANICAL EFFECTS OF AGE, GENDER, AND OSTEOPOROSIS

T.M. Keaveny, O.C. Yeh
University of California, Berkeley, CA
tmk@me.berkeley.edu

From an engineering perspective, trabecular bone is a highly complex material, being anisotropic with different strengths in tension, compression, and shear and with mechanical properties that vary widely across anatomic sites, and with aging and disease. While mechanical properties depend very much on volume fraction, the role of architecture and tissue material properties remain uncertain. In the context of osteoporosis, there is wide interest in the biomechanical role of architecture since this should lead to improved understanding of the disease and ultimately better diagnosis and drug treatment assessment.

This study reviews what is known about architectural changes in trabecular bone associated with age, gender, and osteoporosis and the role of these changes in mechanical properties of the bone. Recent development of three-dimensional high-resolution imaging technologies has provided more accurate measures of quantitative metrics of architecture, thereby providing new data and raising questions about earlier conclusions. Focusing on the hip and spine, this literature is synthesized and outstanding issues are identified.

In addition, the changing paradigm of biomechanical research on trabecular architecture is addressed. Because of the complexity of the trabecular micromechanics, the prevailing approach to date can be classified as an inverse one, whereby candidate metrics of architecture are developed and tested for efficacy in an empirical trial-and-error fashion. In this approach, the biomechanics is treated only as an assay since it is not used to guide development of the candidate metrics. By contrast, a more forward approach is to study the associated micromechanics using engineering analysis and from that identify the metrics that in theory most affect mechanical properties. The latter approach, facilitated by the new high-resolution imaging techniques and increased computational power, is discussed in an attempt to direct attention to new types of architectural metrics that are independent of bone density and that should improve the ability to explain how age, gender, and osteoporosis affect the mechanical properties of trabecular bone.

OR-6

Size, Structure, and Gender: Lessons about Fracture Risk

R.B. Martin
University of California at Davis, Sacramento, CA
rbmartin@ucdavis.edu

The differences in age-related fracture risks among men and women must reflect gender differences in the relevant variables. We are concerned here with gender differences in structural variables that relate to the size and shape of bones. As children grow, their bones grow in diameter through periosteal modeling. Studies show that radial growth is driven by mechanical forces and is not just "genetically programmed." Moving bone mass farther from the center of the diaphysis makes it more effective in resisting bending and twisting forces, and disproportionately so in comparison to changes in bone mass. Gender differences in long bone structure appear to arise because the bone cells of males and females function in different hormonal environments which affect their responses to mechanical loading. In girls bone formation on the metacarpal periosteal surface essentially stops at puberty, and is replaced by formation on the endosteal surface, reducing endosteal diameter until about age 20. Bone strength is 60% greater in male metacarpals than in those of females because bone is added periosteally in boys and endosteally in girls. At menopause endosteal resorption resumes, accompanied by slow periosteal apposition, weakening cortical structure. Similar phenomena occur in such critical regions as the femoral neck.

Another fundamental gender difference in skeletal development is that whole body bone mineral content increases in linear proportion to lean body mass throughout skeletal maturation in boys, but in girls there is a distinct increase in the slope of this relationship at puberty, when estrogen rises. Frost's hypothesis is that this reflects an effect of estrogen on bone's mechanostat set point, and this is increasingly supported by data showing that estrogen and mechanical strain act through a common pathway in osteoblast-like cells. If Frost's hypothesis is correct, the mechanostat is set for maximal effect of mechanical loading on bone gain during the 2-3 years preceding menarche. During the child-bearing years, the set point is at an intermediate level, and at menopause, it shifts again to place the skeleton into the metabolic equivalent of a disuse state. The most direct approach to resolving this problem would be to simulate the putative effect of estrogen on the set point itself.

OR-7

Mouse-models for Osteoporosis

M. Amling, M. Priemel, A.F. Schilling, M. Haberland
Hamburg University, Hamburg, GERMANY
amling@uke.uni-hamburg.de

While our understanding of the developmental biology of the skeleton, like that of virtually every other subject in biology, has been transformed by recent advances in human and mouse genetics, we still know very little, in molecular and genetic terms, about skeletal physiology. Thus, among the many questions that are largely unexplained are the following: why is osteoporosis mainly a women's disease? How is bone mass maintained nearly constant between the end of puberty and the arrest of gonadal functions? Molecular genetics has emerged as a powerful tool to study previously unexplored aspects of the physiology of the skeleton. Among mammals, mice are the most promising animals for this experimental work. This has been previously demonstrated e.g. through the tremendous impact of the different osteopetrotic models on our molecular understanding of osteoclastic bone resorption. Until recently the only way of studying bone loss situations and osteoporosis in mice was by using ovariectomy with all its limitations. Today, however, we have access to more sophisticated osteoporotic mouse-models from four different origins: Transgenic mice (HSV-TK), knock-out mice (OPG), inbred-strains (SamP6), and through physiological modulation (icv application). These new models have already taught us several important lessons. The first is, that bone remodeling is more than just an autocrine/paracrine process. Multiple experimental evidence has demonstrated that the latter regulation exists, but genetics proves that there is no functional cross-control between resorption and formation. The second lesson is, that remodeling is, at least in part, subject to central regulation. Thus, osteoporosis is partly a central or hypothalamic disease. However, the most dramatic change and the most important advantage we feel is, that today we have models to test a new hypothesis regarding the etiology of osteoporosis before it turns to dogma. Taken together, mouse-studies may lead to a shift in our physiological understanding of skeleton biology and to the emergence of novel paradigms. These, in turn, should help us to devise new treatments for degenerative diseases of the skeleton such as osteoporosis and its associated clinical problems.

OR-8

Osteoporosis in Men

E.S. Orwoll
Oregon Health Sciences University, Portland, OR
orwoll@ohsu.edu

The second session is a companion to the first. The first session is devoted primarily to gender differences in the skeleton and especially to how gender affects the material properties, structure and size of bone. To compliment the discussion of gender, and to focus on men and osteoporosis, the second session will consider the character of osteoporosis in men and new developments in the interaction of sex steroids and bone. Speakers include Drs. Cauley, Kousteni and Negro-Vilar.

Dr. Cauley will consider the issue of the determinants of osteoporosis and fracture risk in men. Although the data available are fewer than those concerning women, recent information provides a much greater understanding of the field, Are there gender differences in the character or causation of osteoporosis? Dr. Cauley will also use ethnicity to highlight the potential reasons for group differences in fracture rates.

Dr. Kousteni will discuss the cellular and molecular mechanisms of sex steroid action in bone. In addition to classical sex steroid receptor mediated regulation of gene transcription, there may be other signal transduction pathways that are affected by sex steroids. Can the combination of transcriptional and non transcriptional events help explain the similarities and differences in sex steroid effects on bone in men and women? Are androgen and estrogen effects distinct in bone?

Dr. Negro-Vilar will focus on the new field of selective androgen receptor modulators. Androgens are thought to be an essential part of the development of gender differences in the skeleton as well as in the maintenance of skeletal integrity in adults. Can the effects be used to advantage in a manner that selectively preserves the positive actions of androgens but minimizes disadvantages?

OR-9

The Determinants of Fracture in Men

J.A. Cauley
University of Pittsburgh, Pittsburgh, PA
jcauley@vms.cis.pitt.edu

Osteoporosis represents an increasingly important clinical and public health problem among older men. Estimates indicated that 1-2 million (3-6%) men aged 50 years and over in the United States have osteoporosis and 8-13 million (28-47%) have osteopenia. The lifetime risk of suffering a hip, spine or forearm fracture for a 50-year-old man is 13%, similar to the risk for prostate cancer. The number of osteoporotic fractures in men is expected to increase dramatically due to aging of the population and secular increases in fracture rates. Identification of men who are at greatest risk of osteoporosis and the risk factors, which predispose men to fracture, are essential so that preventive steps can be taken. Data on risk factors are emerging but many questions remain. Men may fracture at a higher bone mineral density (BMD) level than women. However, estimates of volumetric BMD, which correct in part for gender differences in bone size, and risk of fracture, may actually show similar relationships in men and women. Fracture rates are similar in older African American women and Caucasian men. Improved understanding of ethnic differences in fracture could identify potential reasons for gender differences. Family history and genetic factors are also important risk factors for fractures but the specific candidate genes are not known and whether gender modifies the effects of these genetic polymorphisms on BMD and the risk of fracture is also not

known. In general, lifestyle factors and anthropometric measurements show similar relationships with fractures in men and women although few comprehensive prospective studies have been conducted. Current data will be reviewed on the relationships between markers of skeletal health, genetic polymorphisms, lifestyle and anthropometric factors and fracture.

OR-10

The Molecular Biology of Sex Steroid Effects in Bone

S. Kousteni, J.R. Chen, S.C. Manolagas
University of Arkansas for Medical Sciences, Little Rock, AR
koustenistavroula@uams.edu

The anti-apoptotic effects of estrogens and androgens on osteoblasts and osteocytes are mediated by a nongenotropic, yet receptor-dependent mechanism of action, which involves activation of a Src/Shc/ERK signaling cascade. Estrogen receptor (ER) a or b or the androgen receptor (AR) can transmit the anti-apoptotic signal with similar efficiency irrespective of whether the ligand is an estrogen or an androgen. This signal is transmitted by the ligand binding domain of the receptor protein, is preserved when targeting this protein to the membrane, but is lost when targeting it to the nucleus. These nongenotropic effects can be dissociated from the transcriptional actions of the receptors with synthetic ligands. These observations along with evidence that several members of the MAP kinase signaling pathway, including Src, Shc and ERKs, are clustered in caveolae (also found in osteoblastic cells), suggest that nongenotropic activation of signaling pathways by sex steroids is mediated via the classical receptors, or perhaps a shortened spliced variant, that is localized in the membrane, and in particular within caveolae. More recently we have found that rapid activation of MAP kinase cascades by estrogens or androgens also leads to potent downstream regulation of transcriptional events. These effects also require membrane localization of the receptor and are lost when the receptor is targeted to the nucleus. These results indicate that rapid signals originating from membrane-associated receptors influence transcription as well. Therefore, the response of a target cell to sex steroids may be determined by the balance between membrane- and nucleus-associated receptor actions. We have also found that as in the case of the anti-apoptotic effects of sex steroids on osteoblasts, both estrogens and androgens are equally effective in the induction of murine osteoclast apoptosis in a gender-independent and sex-nonspecific manner, strongly suggesting that the opposite effects of sex steroids on osteoclast and osteoblast apoptosis are mediated via a similar mechanism of action. Sex-nonspecific signaling by estrogen or androgen through the ER or the AR may account, at least in part, for the puzzling efficacy of either class of sex steroids in the adult skeleton of females and males and the equivocal skeletal phenotype of ER or aromatase deletions or mutations in rodents and humans.

OR-11

Novel, Non-Steroidal, Selective Androgen Receptor Modulator (SARMs) with Anabolic Activity in Bone and Muscle and Improved Safety Profile

A. Negro-Vilar
Ligand Pharmaceuticals, San Diego, CA
anegrovilar@ligand.com

A novel approach to the treatment of osteoporosis in men, and possibly women, is the development of selective androgen receptor modulators (SARMs) that can stimulate formation of new bone with substantially diminished proliferative activity in the prostate, as well as reduced virilizing activity in women. Over the last several years, we have developed a program to discover and develop novel, non-steroidal, orally-active selective androgen receptor modulators (SARMs) that provide improved therapeutic benefits and reduce risk and side effects. In recent studies, we have used a skeletally mature orchiectomized (ORDX) male rat as an animal model of male hypogonadism for assessing the efficacy of LGD2226, a non-steroidal, non-aromatizable, and non-5alpha-reducible SARM. We assessed the activity of LGD2226 on bone turnover, bone mass and bone strength, and also evaluated the effects exerted on classic androgen-dependent targets, such as prostate, seminal vesicles and muscle. A substantial loss of bone density was observed in ORX animals, and this loss was prevented by SARMs, as well as standard androgens. Biochemical markers of bone turnover revealed an early increase of bone resorption in androgen-deficient rats that was repressed in ORX animals treated with the oral SARM, LGD2226, during a 4-month treatment period. Differences in architectural properties and bone strength were detected by histomorphometric and mechanical analyses, demonstrating beneficial effects of LGD2226 on bone quality in androgen-deficient rats. Histomorphometric analysis of cortical bone revealed distinct anabolic activity of LGD2226 in periosteal bone. LGD2226 was able to prevent bone loss and maintain bone quality in ORX rats by stimulating bone formation, while also inhibiting bone turnover. LGD2226 also exerted anabolic activity on the levator ani muscle. Taken together, these results suggest that orally-active, non-steroidal SARMs may be useful therapeutics for both muscle and bone in elderly hypogonadal men through their anabolic activities. Since SARMs both prevent bone loss, and also stimulate formation of new bone, they may have significant advantages relative to currently used anti-resorptive therapies. Coupled with their activity in muscle and their ability to maintain or restore libido, they offer new therapeutic approaches for male and female hormone replacement.

OR-12

Genetic Studies of Peak BMD and Bone Structure in Young Women

M.J. Econs
Indiana University School of Medicine, Indianapolis, IN
mecons@iupui.edu

Osteoporotic fractures are largely due to an increased propensity to fall and an increase in bone fragility due to a reduction in bone strength with aging. Several factors contribute to bone strength including bone mineral density (BMD), bone geometry, bone quality and bone turnover, all four of which have phenotypes that are highly heritable. The long-term goal of our study is to identify genes that underlie bone fragility using the positional cloning/candidate approach. The first step to attaining this goal is to identify chromosomal regions that harbor genes that affect the components of bone fragility and predisposition to fracture. To date, our studies have concentrated on the genetic determinants of peak bone density and femoral geometry in young, premenopausal women. Although the final genome wide screen will be performed in 1,000 sister pairs, we have performed non parametric linkage analysis in a subset of 429 sister pairs to identify chromosomal regions that contain genes that affect peak BMD in women. We have identified several chromosomal regions with significant or suggestive evidence of linkage to peak BMD. We made measurements of several structural variables from femoral radiographs and performed an autosomal genome screen in 309 Caucasian sister pairs. Analysis of these data demonstrate several chromosomal regions with significant evidence of linkage to femoral geometry phenotypes.

These studies represent important steps toward identifying genes contributing to osteoporosis in the general population. Identification of these genes may: 1) lead to molecular tests that predict the risk of osteoporosis, thereby allowing the early institution of preventive measures; 2) provide insight into the basic skeletal biology that underlies bone fragility and the predisposition to fracture; and 3) identify molecular targets for the development of therapeutic agents aimed at increasing bone strength.

OR-13

Utilizing Inbred and Congenic Strains of Mice for Skeletal Analyses

W.G. Beamer, L.R. Donahue, C.J. Rosen
The Jackson Laboratory, Bar Harbor, ME
wgb@aretha.jax.org

The rationale for use of inbred strains of mice in bone research is well recognized and includes: a) practical factors (economics of scale, rapid development of adult status, pre-existing knowledge, down-sized technologies) and b) proven methodologies for genetic studies (polygenic trait analyses,

mapping tools, genomic sequencing, methods for gene manipulation).

Initial investigations of inbred strains of mice showed that femoral and lumbar vertebral volumetric bone mineral density (BMD, mg/mm^3) by pQCT varied in excess of 50% for femurs and 9% in vertebral BMD. Two strains - low BMD C57BL/6J (B6) mice and high BMD C3H/HeJ (C3H) – were investigated for insights to their BMD diversity. B6C3F2 females derived from intercrossing B6C3F1s were raised to adult skeletal status at 4 months, then necropsied for phenotyping of bone and genotyping of genomic DNA. 1000 F2 females were genotyped for PCR product polymorphisms on all 19 autosomes at ~15 cM. Genome wide analyses for genotype-phenotype correlations showed 10 chromosomes (Chrs) carried genes for femoral and 7 Chrs for vertebral BMD. LOD scores ranged from 2.90 to 24.4, and percent of F2 variance accounted for ranged from 1 to 10%. Analyses of main effects revealed both dominant-recessive and additive inheritance patterns. Both progenitor strains carried alleles with positive and negative effects on BMD of each bone sites. A remarkable array of additonal skeletal phenotypes (femur and vertebral geometry, strength measures, serum markers) also proved polygenic in nature, with complex segregation patterns.

Verification of BMD quantitative trait loci (QTLs) was undertaken by creating congenic B6 strains carrying individual QTL regions from C3H. Following 6 cycles of backcrossing a QTL-containing region from C3H to the B6 strain, N6F2 congenic strain mice were aged to 4 months, then genotyped for the QTL region and phenotyped for skeletal traits. Comparison of mice homozygous for C3H alleles versus homozygous for B6 alleles in the QTL regions showed that femoral BMD increased or decreased significantly in congenic strains, as was predicted from F2 data. Gender differences specific to BMD QTLs have been revealed, as have more than 30 additional phenotypes associated with cortical and trabecular structural parameters and biomechanical properties.

OR-14

Genetic Regulation of Bone Mineral Density in Mice

R.F. Klein
Oregon Health & Science University and Portland VA Medical Center, Portland, OR
kleinro@ohsu.edu

Peak bone mass is a major determinant of risk of osteoporotic fracture. Family and twin studies have found a strong genetic component to the determination of bone mineral density (BMD). However, BMD is a complex trait whose expression is confounded by environmental influences and polygenic inheritance. The number, locations and effects of the individual genes contributing to natural variation in this trait are all unknown. The extreme difficulty of dissecting out environmental factors from genetic ones in humans has motivated the investigation of animal models. Genetically distinct animal strains raised under strict environmental control are critical tools for defining genetic regulation. The availability of inbred strains, combined with its relative fecundity, has established the mouse as the best model system for the study of mammalian genetics and physiology. Importantly, genes identified in murine analyses can usually be readily mapped to particular human chromosomal regions because of the high degree of synteny that exists between the mouse and human genomes. We employed quantitative trait locus (QTL) analysis to examine peak BMD in 24 recombinant inbred (RI) mouse strains, derived from a cross between C57BL/6 (B6) and DBA/2 (D2) progenitors (BXD RI). The distribution of BMD values among these strains clearly indicated the presence of strong genetic influences, with an estimated narrow sense heritability of 35%. The differences in peak whole body BMD in the BXD strains were integrated with a large database of genetic markers previously defined in the RI BXD strains to generate chromosome map sites for QTL locations. This QTL analysis provisionally identified a number of chromosomal sites linked to BMD. In the second phase of our BMD QTL mapping efforts, we used three independent mouse populations (all derived from B6 and D2 progenitor strains) to confirm and narrow the genetic locations of 4 QTLs (on chromosomes 1, 2, 4, and 11) that strongly influence the acquisition of peak BMD in mice. Using a novel, fine-mapping approach (recombinant inbred segregation testing), we have succeeded in narrowing two of the BMD-related chromosomal regions and in the process eliminated a number of candidate genes. The homologous regions in the human genome for each of these murine QTLs have been identified in recent human genetic studies. In light of this, we believe that findings in mice should aid in the identification of specific candidate genes for study in humans.

OR-15

Genetic Determinants of Skeletal Strength, Composition, and Morphology

N.A. Sharkey, D.H. Lang, G.P. Vogler, D.J. Vandenbergh, L.G. Larsson, D.A. Blizard, J.T. Stout, G.E. McClearn
Pennsylvania State University, University Park, PA
nas9@psu.edu

Twenty C57BL/6 X DBA/2 (BXD) recombinant inbred (RI) strains of mice, as well as F_1 and F_2 cohorts derived from the same parental strains, were used to elucidate the genetic regulation of musculoskeletal strength and architecture. Measures of behavior and activity were made at approximately 150 days (young adulthood) after which the femur, tibia, and several muscles were harvested from right hindlimbs. Muscle masses were recorded and dimensional, compositional, and mechanical measurements were made on the bones. Physical measurements were normalized to body mass index. The influence of age and sex on phenotypic variation in the parental strains was examined to determine the suitability of the model for skeletal studies. Phenotypic measures were made on approximately 20 mice from each RI strain, 20 F_1 mice, and 400 F_2 mice (over 800 animals in all, equally divided between male and female). F_1 and F_2 mice were used to estimate the heritability of each phenotypic variable. Co-variation of genetically determined traits, such as animal activity, muscle mass, and bone strength, was explored in the RI strains and F_2 cohort. Separate sex-specific Quantitative Trait Loci (QTL) analyses were performed on both the RI strains and F_2 cohort to locate chromosomal regions (QTLs) influencing each phenotypic variable. Definitive QTLs were determined by nominating loci using the RI strains and then replicating these analyses in F_2 animals (using in-house genotyping with 96 anonymous microsatellite markers).

Preliminary analyses of the parental strains revealed many strain-, sex-, and age-dependent phenotypic variables, indicating the utility of the BXD series for genetic studies of skeletal health. Heritabilities calculated from F_1 and F_2 variances fell between 0.34 and 0.91 for many of these variables. Genetically controlled correlations (with coefficients often exceeding 0.60) were found between skeletal strength, composition, morphology, muscle mass, and activity level. Many sex-dependent QTLs were identified in both the RI and F_2 analyses. Often times the same locus influenced activity, muscle mass, skeletal dimensions, and skeletal mechanics, suggesting that the same gene or group of genes exerted its effects on behavior (activity), muscle, and bone simultaneously or that its action on one was transmitted to the others through a cascade of events. One reasonable hypothesis is that bone strength and size are dictated by muscle mass, which, in turn, is indirectly dictated by genes that influence behavior. Irrespective of mechanistic pathways, definitive QTLs (those nominated in the RI analyses and confirmed in the F_2 cohort) influencing femoral strength have been found on chromosomes 2, 15, and 17.

OR-16

Genetic Regulation of Bone Geometry and Adaptation

C.H. Turner
Indiana University School of Medicine, Indianapolis, IN
turnerch@iupui.edu

Studies of twins have established that peak bone mass is about 70% heritable. The skeletal response to exercise contributes to peak bone mass, as mechanical loading increases skeletal mass during growth and development. It is possible that the skeletal responsiveness to mechanical loading is under genetic control, so that some individuals will build stronger bones with exercise. This appears to be the case in mice. Long bones in mice of the C3H/He strain are largely unresponsive to mechanical loading. Ironically, this strain of mice has very high bone density. Perhaps the genes that regulate BMD are not the same as those that regulate mechanical loading response. Studies of recombinant inbred and congenic strains derived from C3H mice will help to identify genes influencing bone size, density and responsiveness to mechanical loading.

OR-17

OSTEOCYTES: A PROPOSED MULTIFUNCTIONAL BONE CELL

L.F. Bonewald
University of Texas Health Science Center, San Antonio, TX
bonewald@uthscsa.edu

Most cell types are ascribed a single function. The osteoclast holds the unique distinction of performing only one function in the body-that of resorbing bone. The osteoblast has been ascribed the major function of bone matrix production. Other less well-defined cell types include progenitor cells and the nebulous cell type that can support osteoclast formation upon stimulation with various bone resorbing cytokines. Obviously, these cells could have other functions. The definition of an osteocyte is descriptive of its location-cells surrounded by mineralized matrix-not its function. For this year's Sun Valley Workshop on osteocytes, several proposed functions will be presented. First, a general consensus exists that osteocytes are most likely sensitive to mechanotransduction and translate mechanical strain into biochemical signals. Consensus does not exist on the nature of the mechanical strain, the form of the biochemical signals, the target cell(s), or the viability status of the osteocyte. Second, it is also proposed that this cell is incredibly adaptable and expresses plasticity in response to mechanical stimuli. In other words, this cell can readjust its responses to strain in the presence of other bone agents such as hormones and bone factors. Thirdly, it will also be presented that osteocytes maintain systemic mineral homeostasis by regulating mineral release and deposition over the enormous surface area over which these cells interface with the surrounding matrix.

Although osteocytes are terminally differentiated osteoblasts, they appear to have separate and distinct properties from their predecessors. Bone cell biologists loaded with an arsenal of bone anabolic and catabolic factors are examining the expression and effects of these factors on osteocytes. Engineers trained in mathematical modeling have generated new models of strain and connectivity to be tested. The unique morphology of osteocytes suggests that the cytoskeleton in these cells may function differently from osteoblasts and other cell types. Osteocytes may consist of different subpopulations; some that possess receptors for PTH and others that only express receptors for carboxyl terminal PTH suggesting different functions and responses. Osteocytes may respond rapidly to strain through glutamate receptor-like mechanisms, through calcium influxes, through gap junctions, and less rapidly through the production of small molecules and factors. Strain may take the form of substrate stretching and/or fluid flow. Osteocytes may communicate with other osteocytes and/or bone surface cells such as lining cells, stromal cells, osteoblasts, and/or osteoclasts and their precursors. The viability status of the osteocyte may determine the type of signals sent from these cells. If the cells are deprived of oxygen or nutrients, the apoptotic cells may send signals for initiation of resorption. If the cells and/or their dendritic process are ripped or torn by microdamage, they may send signals of both resorption and formation. If the majority of these theories are correct, then the osteocyte is the 'smart' cell that can direct or orchestrate the bone resorbing and bone forming cells even in its death and dying.

OR-18

THE ROLE OF OSTEOCYTES IN BONE REGULATION: MINERAL HOMEOSTASIS VERSUS MECHANORECEPTION

D.M. Cullinane
Boston University Medical Center, Boston, MA
bones@bu.edu

Early work on the role of osteocytes in bone regulation suggested that the primary function of these cells was osteolysis. This lytic function was not precisely defined but included mineral homeostasis and at least the initiation of matrix remodeling, if not a primary role in remodeling. This paper is an attempt to promote the concept of osteocytic osteolysis as a method of systemic mineral homeostasis and to separate it from bone remodeling. Although recent investigations have pointed to mechano-transduction as a primary function of osteocytes, resulting in a general abandonment of the osteocytic osteolysis concept, the corpus of evidence suggests that osteocytes likely have a multipurpose role in the biology of bone. The osteocyte network represents an enormous surface area over which the cells interface with the surrounding matrix, useful for both strain detection and matrix mineral access. Osteocytes have been found to possess receptors for PTH, a known regulator of mineral ion homeostasis. Cultured osteocytes placed on dentin slices demonstrated no capacity to pit the dentin, but they were not treated with a regulating factor such as PTH, nor does mineral homeostasis require substantial bone volume removal. Scaling relationships suggest that osteocyte density is inversely proportional to body mass, $R^2 = 0.86$, and thus directly proportional to metabolic rate. Thus, species with higher metabolic rates (and therefore a greater demand for immediate access to minerals) have more osteocytes per bone volume. Finally, osteocytes express molecules typically associated with nerve cells and which are involved with glutamate neuro-transmission. By this system, almost instantaneous messages may be transmitted throughout the network, an important feature in cells whose homeostatic function would be utilized on a scale of seconds, rather than hours or days.

Experimental procedures for determining the role of the osteocyte in mineral homeostasis would require calcium mobilization from the bone matrix on a relatively immediate time scale. The experimental procedure would then be coupled with a high resolution histomorphometric analysis of lacunar radiographic area and mineral density. Added to this would be an in vitro study of mineral activation capacity via cultured osteocytes treated with PTH. Osteocytic osteolysis would be confirmed by an increase in the demineralized volume of osteocytic lacunae and the identification of a chemical mechanism by which osteocytes can readily access the mineral portion of their immediate bone matrix. It should also be true that a reverse capacity exists by which osteocytes can remineralize their immediate matrix utilizing alkaline phosphatase for example, a chemical which they, like osteoblasts, are known to generate. It is thus proposed that osteocytes are both mechanoreceptors and systemic mineral homeostasis regulators.

OR-19

PTH RECEPTORS AND APOPTOSIS IN OSTEOCYTES

P. Divieti, F.R. Bringhurst
Massachusetts General Hospital and Harvard Medical School, Boston, MA
rbinghurst@partners.org

Osteocytes comprise a heterogenous population of terminally differentiated osteoblasts that direct bone remodeling in response to applied mechanical loading of bone. Increased osteocyte density accompanies the anabolic effect of PTH *in vivo*, whereas accelerated osteocyte death may be precipitated by estrogen deficiency or excess glucocorticoid exposure (conditions benefitted by intermittent PTH therapy) and by renal failure (where circulating intact PTH and, especially, PTH carboxyl-fragments are elevated). Osteocytes express type-1 PTH/PTHrP receptors (PTH1Rs), which are fully activated by amino-terminal PTH fragments and couple to multiple signal transducers, including adenylyl cyclase and phospholipase C. Activation of PTH1Rs in osteocytes promotes gap junction-mediated intercellular coupling, increases expression of MMP-9, potentiates calcium influx via stretch-activated cation channels, amplifies the osteogenic response to mechanical loading *in vivo*, and regulates apoptosis. Control of osteocyte apoptosis by PTH1Rs is complex, in that intermittent PTH(1-34) administration reduces the fraction of vertebral apoptotic osteocytes at 1 month in adult mice but increases femoral metaphyseal osteocyte apoptosis at 1-2 weeks in young rats. In MLO-Y4 cells, PTH(1-34) prevents apoptosis otherwise induced within 6 hr by dexamethasone. In older studies, large doses of intact PTH(1-84) caused rapid "degenerative" morphologic changes in osteocytes, similar to those described in renal osteodystrophy.

We isolated clonal conditionally immortalized osteocytic (OC) cell lines from mice homozygous for targeted ablation of the PTH1R gene. OC cells express abundant (2-3 x 10^6 per cell) receptors specific for the carboxyl(C)-terminus of intact PTH(1-84) ("CPTHRs") but, as expected, do not express PTH1Rs or respond to PTH(1-34). CPTHRs are expressed at much lower levels by other skeletally-derived cell lines. Several highly conserved ligand determinants of CPTHR binding have been identified, including PTH(24-27), PTH(53-54) and the sequence PTH(55-84), loss of which reduces binding affinity by over 100-fold. Human PTH(53-84), like PTH(1-84), PTH(24-84), and PTH(39-84), increases OC cell apoptosis. Ala-scanning

mutagenesis to define sequences within PTH(55-84) important for binding and bioactivity is underway. We conclude that osteocytes may be important targets for CPTH fragments that are secreted by the parathyroid glands or generated by peripheral metabolism of intact PTH and that accumulate in blood, especially in renal failure. Studies of functional interplay between responses to CPTHRs and (transfected) PTH1Rs, using receptor-specific ligands in OC cells, should provide new insight into PTH regulation of osteocyte function and survival.

OR-20

THE ROLE OF CALCIUM CHANNELS IN OSTEOCYTE FUNCTION

Y. Mikuni-Takagaki, K. Naruse, Y. Azuma and A. Miyauchi
Kanagawa Dental College, Kitasato University School of Medicine, Teijin Limited and National Hyogo Chuo Hospital, JAPAN
yukomtak@kdcnet.ac.jp

Cells in bone are equipped with mechanisms to sense diverse physical forces and transduce signals so that they adjust themselves to their mechanical environment. Studying osteocytic stretch-sensing mechanisms showed that certain types of mechanical stress are received only by certain stages of osteogenic cells. In cell processes of primary young osteocytes, we have reported PTH-potentiated Ca influxes, which utilize PkA signaling pathways for the downstream anabolic responses such as production of IGF-I and osteocalcin. The upregulation of mRNA levels of these molecules occurred in a manner similar to that of typical immediate early genes, c-fos or cox 2. In search of mechanotransduction pathways unique to osteoblasts and of crosstalk among signaling pathways, we studied the anabolic response of osteogenic cells to low-intensity, pulsed ultrasound, a non-invasive therapeutic treatment of use for fracture repair and distraction osteogenesis. Effects of 20-min exposure to 200-μs burst of pressure pulses (sine wave of 1.5 MHz repeated at a frequency of 11.0kHz), which was repeated every millisecond, were examined in mouse bone marrow-derived ST2 cells, and primary rat bone- and bone marrow-derived cells. The intensity was 30 mW/cm^2, same as that of clinical fracture healing devices (Exogen Inc.). By using conventional and semi-quantitative RT-PCR analyses, ST2 cells cultured in the presence of ascorbate and exposed to the ultrasound showed that steady state levels of immediate early genes such as c-fos or cox 2 were upregulated most in a less differentiated population of osteoblasts. IGF-I, osteocalcin and other bone protein messages as well as c-fos and cox 2 were upregulated to some extent in the more differentiated population. Mature osteoblasts and osteocytes derived from newborn rat tibia, on the other hand, were insensitive to the pulsed ultrasound. Compared to the stretched osteocytes, none of these cells showed any Ca influxes even when cells were responding to the ultrasound. Inhibition of MAPK and other upstream effectors also resulted in distinct modulation profiles of the anabolic responses to ultrasound and stretching. Our findings support the notion that strain is sensed by the osteocytic cells through Ca channels while other mechanical stress is sensed by other types of cells in the osteogenic lineage, through different machinery.

OR-21

OXYGEN SENSING AND OSTEOCYTE MECHANOTRANSDUCTION

T.S. Gross, N.A. Rabaia, S. Srinivasan
University of Washington, Seattle, WA
tgross@u.washington.edu

The rapid removal of bone tissue that ensues when the skeleton is exposed to disuse, paralysis, or space flight suggests that mechanical loading is essential to maintain homeostasis of bone cell populations. We have been using a complementary *in vivo* and *in vitro* approach to examine one potential mediator of this process, osteocyte hypoxia. Previously, we have found that osteocytes rapidly become hypoxic when bone is unloaded, and that this physiologic response is inhibited by brief daily loading. We therefore hypothesized that disuse would upregulate the hypoxia dependent transcription factor, HIF-1a, a 'master' regulator of cellular response to low oxygen environments. Using immunohistochemistry with the avian ulna model of disuse osteopenia, we found that acute disuse (1 to 6 d) results in a significant increase in the percentage of osteocytes staining positive for HIF-1a versus normal bone (30.9 ± 6.1% versus 14.1 ± 3.8%, $p < 0.001$). The percentage of osteocytes staining positive for HIF-1a was consistent at 6 equidistant sites around the cortex (range: 28.3 ± 5.9% versus 34.4 ± 5.7%). No differences in HIF-1a expression in response to disuse were observed between the endocortical (30.8 ± 4.4%), intracortical (26.8 ± 4.1%) and periosteal surfaces (33.7 ± 5.6%). *In vitro*, Western blot analysis revealed that acute oxygen deprivation (4 to 12 hr of 2% O_2) resulted in a 2.1 to 3.7 fold upregulation of HIF-1a protein expression in MLO-Y4 osteocyte-like cells as compared to cells cultured in parallel under normal oxygen conditions. Although these data are the first observation of HIF-1a regulation by osteocytes, the ability to upregulate HIF-1a in response to oxygen deprivation has been observed in numerous cell types. Given known HIF-1a target genes such as VEGF, we suggest that osteocyte hypoxia and subsequent upregulation of hypoxia dependent pathways may serve to initiate and/or mediate disuse induced bone resorption. Viewing bone mechanotransduction from this physiological perspective holds the potential to provide new insights into challenging musculoskeletal problems such as healing of fracture non-unions and tissue engineering of bone substrates.

OR-22

MECHANOSENSATION AND FLUID TRANSPORT IN LIVING BONE

S.C. Cowin
City College of NewYork, City University of New York, New York, NY
scccc@cunyvm.cuny.edu

The mechanosensory mechanisms in bone include (i) the cell system that is stimulated by external mechanical loading applied to the bone; (ii) the system that transduces that mechanical loading to a communicable signal; and (iii) the systems that transmit that signal to the effector cells for the maintenance of bone homeostasis and for strain adaptation of the bone structure. The effector cells are the osteoblasts and the osteoclasts. These systems and the mechanisms that they employ have not yet been unambiguously identified. The candidate systems will be reviewed. It will be argued that the current theoretical and experimental evidence suggests that osteocytes are the principal mechanosensory cells of bone, that they are activated by shear stress from fluid flowing through the osteocyte canaliculi, and that the electrically coupled three-dimensional network of osteocytes and lining cells is a communications system for the control of bone homeostasis and structural strain adaptation.

The movement of bone fluid from the region of the bone vasculature through the canaliculi and the lacunae of the surrounding mineralized tissue accomplishes three important tasks. First, it transports nutrients to the osteocytes in the lacunae buried in the mineralized matrix. Second, it carries away the cell waste. Third, the bone fluid exerts a force on the cell process, a force that is large enough for the cell to sense. This is probably the basic mechanotransduction mechanism in bone, the way in which bone senses the mechanical load to which it is subjected. The mechanisms of bone fluid flow are described with particular emphasis on mechanotransduction. Also described is the cell to cell communication by which higher frequency signals might be transferred, a potential mechanism in bone by which the small whole tissue strain is amplified so the bone cells can respond to it. One of the conclusions is that higher frequency low amplitude strains can maintain bone as effectively as low frequency high amplitude strains. This conclusion leads to a paradigm shift in how to treat osteoporosis and how to cope with microgravity.

OR-23

MICROSTRUCTURAL STRAIN NEAR OSTEOCYTE LACUNA IN CORTICAL BONE *IN VITRO*

D.P. Nicolella, J. Lankford, L. Bonewald, E. Sprague
Southwest Research and University of Texas Health Science Center at San Antonio, San Antonio, TX
dnicolella@swri.edu

Mechanical factors affect bone remodeling such that increased mechanical demand results in net bone formation, whereas decreased demand results in net bone resorption. Two proposed mechanical signals are stress-generated fluid flow forces acting on cells and bone matrix deformation itself. A prominent current theory is that bone cells are more

responsive to fluid flow than to mechanical strain. Recent experiments support this conclusion: bone cells increase their production of osteopontin (OPN) mRNA, prostaglandin (PGE_2), and nitric oxide (NO) in response to fluid flow in contrast to cells stimulated by mechanical strain levels similar to those measured *in vivo*. However, when cells are subjected to substrate strains levels many times greater than those measured *in vivo*, increased biological activity again results. We assert that it is neither fluid flow nor matrix deformation per se, but rather the resulting cell deformation that causes cell biological response.

Machined specimens of undamaged bovine cortical bone were subjected to increasing levels of macroscopic strain while observed under an optical microscope at 220X. Continuum level strain was measured using a standard foil strain gauge attached to the back of the specimen and ranged from 500 to 6,000 microstrain. Images of the specimen surface at each strain level were captured. To determine the level of osteocyte deformation that results from fluid flow *in vitro*, MLO-Y4 cells were cultured on collagen coated 190 cm^2 plastic sheets and subjected to steady fluid flow at 16 dynes/cm^2. Images representing the initial undisturbed cell configuration and the configuration of the cells after ten minutes of fluid flow were acquired from a videotape of the flow experiment. The captured unloaded vs. loaded image pairs were analyzed to determine the local deformation and strain fields using a digital stereoimaging system.

When subjected to a nominal continuum strain level approximately equal to that measured in humans *in vivo* during rigorous activity (2,000 microstrain), the local, osteocyte level strains can be as high as 12,000 to 15,000 microstrain (1.2% to 1.5%). Average osteocyte strains due to fluid flow *in vitro* increase from 7,972 microstrains after 16 seconds of flow to 22,856 microstrains after 64 seconds of flow. In contrast, maximum strains measured *in vivo* are approximately 1,800 microstrain in humans and up to 3,000 microstrain in other species. These data may help to explain why bone cells are more sensitive to fluid flow than substrate strain; fluid forces result in cell deformations much higher than those considered to be "physiological."

OR-24

Skeletal Adaptation to Mechanical Stimuli in the Absence of Formation or Resorption of Bone

C. Rubin, S. Judex, M. Hadjiargyrou
State University of New York at Stony Brook, Stony Brook, NY
clinton.rubin@sunysb.edu

Too often, unique loading environments fail to alter bone mass and morphology, calling to question the validity of Wolff's Law; the skeleton's plasticity to mechanical signals. We propose that a critical aspect of bone tissue's ability to adapt to mechanical stimuli is achieved via functional processes of the osteocyte, and that accommodating new loading environments can be achieved without the need to form or resorb tissue. We suggest that the osteocyte is capable of "normalizing" its local mechanical environment by modulating its cytoskeletal architecture, attachment to the matrix, configuration of the periosteocytic space, and communication channels to surrounding cells. We believe that through this local adaptive mechanism the osteocyte can accommodate the majority of changes in the mechanical milieu without altering the tissue architecture. It is only when bone tissue is subject to more severe (albeit rare) increases or decreases in the functional environment, the osteocyte participates in the formation and/or resorption of bone by coordinating site-specific recruitment of osteoblasts and/or osteoclasts. *In vivo* models of bone adaptation, combined with *in situ* reverse transcriptase-PCR, semi-quantitative RT-PCR, Northern analysis, immuno-cytochemistry and histomorphometry, can demonstrate how distinct mechanical stimuli influence the osteocyte's cytoskeletal and lacunar architecture, coupling (and uncoupling) of the osteocyte to the matrix and neighboring cells, and the osteocyte's participation in the recruitment and differentiation of osteoblasts and osteoclasts. Thus, the osteocyte controls three strategies to modulate its local and global environment in response to three distinct functional stimuli: 1) exogenous mechanical stimuli which are distinct from normal but sufficient to maintain bone mass, 2) mechanical stimuli which are osteogenic, and 3) disuse. If it is true that the resident cell population is capable of accommodating subtle changes in the functional milieu before modification of tissue morphology is deemed necessary, a novel strategy for the development of prophylaxes for osteopenia, osseointegration and fracture healing may become apparent.

OR-25

Principles of Developmental Biology

G.C. Schoenwolf
University of Utah School of Medicine, Salt Lake City, UT
schoenwolf@hsc.utah.edu

The field of developmental biology has a history that spans the last 500 years. Within the last 10 years, our understanding of developmental mechanisms has grown exponentially by employing modern techniques of genetics and molecular biology, frequently combined with experimental embryology and the use of molecular markers, rather than solely morphology, to identify critical populations of cells and their state of differentiation. Three main principles have emerged. First, mechanisms of development are highly conserved, both among developing rudiments of a variety of organ systems and among diverse organisms. This conservation occurs both at the level of tissue and cellular mechanisms, and at the molecular level. Second, the development of organ rudiments is influenced by surrounding tissues through interactions called inductive interactions. Such interactions are mediated by highly conserved growth factors and signaling systems. Third, development is a life-long process and can be reawakened in events such as wound healing and regeneration, and in certain diseases. Advances in understanding normal development provide hope that diseases in which development runs amuck, such as cancer, may soon be preventable and fully treatable. Supported by NS 18112 and DC 04185 from the NIH.

OR-26

Regenerative Biology and Medicine

D.L. Stocum
Indiana University-Purdue University, Indianapolis, IN
dstocum@iupui.edu

The replacement of damaged tissues and organs with tissue and organ transplants or bionic implants has serious drawbacks. There is now emerging a new approach to tissue and organ replacement, regenerative biology and medicine. Regenerative biology seeks to understand the cellular and molecular differences between regenerating and non-regenerating tissues. Regenerative medicine seeks to apply this understanding to restore tissue structure and function in damaged, non-regenerating tissues. Regeneration is accomplished by three mechanisms, each of which uses or produces a different kind of regeneration-competent cell. Compensatory hyperplasia is regeneration by the proliferation of cells which maintain all or most of their differentiated functions (e.g., liver). The urodele amphibians regenerate a variety of tissues by the dedifferentiation of mature cells to produce progenitor cells capable of division. Many tissues contain reserve stem or progenitor cells that are activated by injury to restore the tissue while simultaneously renewing themselves. All regeneration-competent cells have two features in common. First, they are not terminally differentiated and can re-enter the cell cycle in response to signals in the injury environment. Second, their activation is invariably followed by the dissolution of the ECM surrounding the cells, suggesting that the ECM is an important regulator of their state of differentiation. Regenerative medicine uses three approaches. First is the transplantation of cells into the damaged area. Second is the construction of bioartificial tissues by seeding cells into a biodegradable scaffold where they produce a normal matrix. Third is the use of a biomaterial scaffold or drug delivery system to stimulate regeneration *in vivo* from regeneration-competent cells. There is substantial evidence that non-regenerating mammalian tissues harbor regeneration-competent cells that are forced into a pathway of scar tissue formation. Regeneration can be induced if the factors leading to scar formation are inhibited and the appropriate signaling environment is supplied. An overview of regenerative mechanisms, approaches of regenerative medicine, research directions, and research issues will be given.

OR-27

WNT-SIGNALING AND SKELETOGENESIS

C. Hartmann, C. Tabin
Harvard Medical School, Boston, MA
hartmann@rascal.med.harvard.edu

Members of the Wnt gene family, encoding secreted cystein-rich glycoproteins, have been isolated from a variety of organisms. They serve as important developmental signaling molecules and have been implicated to play crucial roles in such diverse processes as cancer, organogensis and pattern formation.

Experiments by Zakany and Duboule (1996), and Rudnicki and Brown (1997) have suggested a role for Wnt molecules in negatively regulating chondrogenesis. However, neither of the two Wnt genes used in these studies is endogenously expressed in chondrogenic regions. We and others have found that in the chick limb at least four members of the Wnt gene family, Wnt-4, Wnt-5a, Wnt-5b, and Wnt-14, are expressed in defined regions of the developing chondrogenic elements. With the exception of Wnt-5b, which is expressed in perichondrial cells and prehypertrophic chondrocytes, the expression of the three other Wnt genes is restricted to the perichondrium surrounding the cartilage element. Viral misexpression studies in chick suggested that Wnt-4 acts as a positive signal originating from the joint region and when misexpressed accelerates chondrocyte maturation, while Wnt-5a and Wnt-5b both negatively regulate chondrocyte maturation. We have further shown that they utilize different signaling pathways; while Wnt-4 signals through the canonical Wnt-pathway, Wnt-5a and Wnt-5b do not. Interestingly, the delay in chondrocyte maturation due to Wnt-5a misexpression is associated with an up regulation of Wnt-5b expression in the prehypertropic chondrocytes. Concomitantly, Wnt-5b misexpression also delays chondrocyte maturation. However, preliminary studies suggest that the two Wnt genes affect different steps in the maturation process.

Wnt signaling, however, is not only regulating chondrogenesis but is also involved in the segmentation process of the appendicular skeleton. Localized misexpression of the fourth Wnt gene, Wnt-14, which is expressed early in the presumptive joint region, induces morphological and molecular changes indicative of an early joint interzone, suggesting that Wnt-14 plays a pivotal role in the induction of the joint interzone.

OR-28

NOVEL MECHANISMS OF STEROID HORMONE ACTION IN MUSCULOSKELETAL CELLS

B.D. Boyan, D.D. Dean, V.L. Sylvia, Z. Schwartz
University of Texas Health Science Center, San Antonio, TX, and Hebrew University, Jerusalem, ISRAEL
boyanb@uthscsa.edu

Recent studies on the rapid actions of steroid hormones have caused us to re-examine our traditional concepts of how they exert their effects on chondrocytes and osteoblasts.

Our laboratory has focused its efforts on the mechanisms used by the vitamin D metabolites 1a,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ and the sex steroids estrogen and testosterone in eliciting rapid nongenomic responses, as well as how these rapid effects are related to gene expression and the action of classic steroid hormone receptors. Our experiments and those of other groups support the hypotheses that growth plate chondrocytes and osteoblasts possess unique membrane receptors for 1a,25$(OH)_2D_3$, 24R,25$(OH)_2D_3$, and 17β-estradiol.

The expression and activity of these receptors are cell maturation-dependent, and in the case of 17β-estradiol, sex-specific. Activation of the membrane receptors leads to increased protein kinase C (PKC) activity, but different upstream signaling pathways are involved.

Activation of the membrane receptor also stimulates activity of the ERK family of MAP kinases, providing a mechanism for regulating gene expression.

Studies are presently underway to examine the relationship between this new class of membrane receptors and the nuclear receptors for 1a,25$(OH)_2D_3$ and estrogen. (Supported by NIH grants DE-08603 and DE-05937)

OR-29

MECHANICAL EFFECTS ON SKELETAL GROWTH

I.A.F. Stokes
University of Vermont, Burlington, VT
stokes@med.uvm.edu

The growth (i.e. increase of external dimensions) of long bones and vertebrae occurs longitudinally by endochondral ossification at the growth plates, and radially by apposition of bone at the periosteum. It is thought that mechanical loading influences the rate of longitudinal growth. The 'Hueter-Volkmann Law' proposes that growth is retarded by increased mechanical compression, and accelerated by reduced loading in comparison with normal values. The present understanding of this mechanism of bone growth modulation comes from a combination of clinical observation (where altered loading and growth is implicated in some skeletal deformities) and animal experiments in which growth plates of growing animals have been loaded.

The gross effect of growth modulation has been demonstrated qualitatively and semi-quantitatively. Sustained compression of physiological magnitude inhibits growth by 40% or more. Distraction increases growth rate by a much smaller amount. Experimental studies are underway to determine how data from animal studies can be scaled to other growth plates. Variables include: differing sizes of growth plate, different anatomical locations, different species and variable growth rate at different stages of skeletal maturity. The two major determinants of longitudinal growth are the rate of chondrocytic proliferation and the amount of chondrocytic enlargement (hypertrophy) in the growth direction. It is largely unknown what are the relative changes in these key variables in mechanically modulated growth, and what are the signaling pathways that produce these changes.

OR-30

NEUROTRANSMITTER FUNCTIONS IN BONE REMODELING

T. Skerry
University of York, UNITED KINGDOM
tms3@york.ac.uk

Bone strength and therefore its resistance to fracture are strongly correlated with the mass and orientation of the load-bearing extracellular matrix. The matrix is in turn the result of the orchestrated activity of osteoblasts and osteoclasts that form and model/remodel the tissue. Changes in bone mass and architecture are therefore linked directly to the regulated activity of those cells, and therefore the endocrine, paracrine and autocrine influences on them. An ability to influence intercellular communication would provide the basis for novel therapeutic strategies for bone diseases, but conventional approaches to the discovery of novel targets, and the search for and development of compounds capable of influencing them can be protracted.

One way in which this process can be shortened is by the identification of a signaling pathway in bone that is known in another tissue, in which case, agents already developed for that tissue could have utility in bone. Such approaches have one drawback though, in that side effects of treatment of bone diseases may arise in the original target organ or tissue, limiting the usefulness of putative new osteotropic drugs. In this respect, the central nervous system has a major advantage in that the blood-brain barrier exists to protect the brain from numerous circulating factors that would be deleterious to its function. The identification of signaling systems in bone that are known to have functions in the CNS may therefore present exciting therapeutic opportunities, as drugs that regulate bone and CNS cell function but are unable to cross the blood-brain barrier would have innate tissue specificity.

For some years it has been known that neurotransmitters such as bradykinin CGRP and VIP influence osteoblast activity, but recent studies focusing on the roles of glutamate, dopamine and serotonin in bone are the subject of this session. While it is not simple to move from basic studies to new drugs, the vast array of agents that modulate neurotransmission already includes some that are incapable of entering the brain, and could therefore regulate bone mass. Modification of others by addition of charged groupings for example could decrease their ability to enter the CNS, so

reducing the normal scale of drug development time considerably. Whether this approach becomes a clinical reality is not yet clear, but the data of the three presenters provide rational targets for further work.

OR-31

Regulation of Bone Resorption by Glutamate: A Pathway for the Neural Control of Bone Metabolism?

C. Chenu, C. Itzstein, L. Espinosa, B. Merle, B. Burt-Pichat
INSERM Unit 403, Hôpital E. Herriot, Lyon, FRANCE
chenu@lyon151.inserm.fr

L-glutamate (Glu) is the predominant neuromediator in the mammalian central nervous system (CNS). Bone is highly innervated and there is growing evidence of a neural control of bone cell metabolism. The recent discovery of Glu-containing nerve fibers in bone and Glu receptors (GluR) and transporters in bone cells suggest that this neuromediator may also act as a signalling molecule in bone and regulate bone cell function. Our previous studies have demonstrated that ionotropic N-Methyl-D-Aspartate (NMDA) GluR are highly expressed by mammalian osteoclasts. NMDA receptors (NMDAR) are heteromers associating the NR1 subunit and one of the four types of NR2 subunits (NR2A to D). We showed that osteoclasts express NR1, NR2B and NR2D subunits, suggesting a molecular diversity of NMDAR in these cells. Electrophysiological studies have confirmed that NMDAR are functional in mature osteoclasts, and features of Glu-induced current recorded in these cells indicate a major NR2D subunit composition. Using an *in vitro* assay of bone resorption, we showed that several antagonists of NMDAR binding to different sites of the receptor inhibit bone resorption. In particular, the specific NMDAR channel blocker MK801 had no effect on osteoclast attachment to bone and survival while it rapidly decreased the percentage of osteoclasts with actin ring structures that are associated with actively resorbing osteoclasts. NMDAR may thus be involved in adhesion-induced formation of the sealing zone required for bone resorption. NMDAR are also expressed by osteoclast precursors isolated from mouse bone marrow. We recently confirmed the presence of NR1, NR2B and NR2D in these cells and demonstrated their expression at all differentiation stages from osteoclast precursors to mature resorbing osteoclasts. No regulation of these subunits mRNA expression levels was observed throughout the osteoclastic differentiation sequence. Activation of NMDAR may therefore represent a new mechanism for regulating osteoclast formation and activity. While the origin of Glu in bone is still unknown, the possibility of a glutamatergic neurotransmission in this tissue is suggested by the detection of Glu in nerve fibers in close contact to bone cells. Furthermore, we recently demonstrated that sciatic neurectomy in growing rats induces a bone loss associated with a reduction of nerve profiles immunostained for Glu. These results suggest that Glu may be released from glutamatergic nerve profiles present in bone and therefore contribute to the local regulation of bone cell function.

OR-32

Osteoblastic Glutamate Receptor Function Regulates Bone Formation and Resorption

A.F. Taylor
University of York, UNITED KINGDOM
aft102@york.ac.uk

Previous studies showed that a variety of bone cells express protein components necessary for neuronal-like glutamatergic signaling and implicated glutamate as having a role in mechanically induced bone remodeling. Initial functional studies concentrated on the role of glutamate signaling in bone resorption and provided compelling evidence to suggest that glutamate signaling through functional NMDA type ionotropic glutamate receptors (iGluRs) is a prerequisite for *in vitro* osteoclastogenesis. Originally, effects of iGluR antagonists seen in co-cultures were attributed to antagonists acting directly on osteoclast precursors. However, in the light of recent osteoblast studies it now seems likely that the observed effects on osteoclasogenesis are an indirect effect of modulating the function of pre-osteoblast present within these cultures.

The presence of iGluRs in osteoblasts suggests a role for them in bone formation and this paper reviews and discusses the emerging data relating to the role of glutamate signalling in osteoblasts. A number of recently published studies have shown that osteoblasts not only express a wide number of 'pre-synaptic' glutamatergic proteins but also possess the ability to both regulate glutamate release and actively recycle extracellular glutamate. The functionality of osteoblastic 'post-synaptic' glutamatergic components has also been shown as both primary and clonal osteoblasts express electrophysiologically active iGluRs, metabotropic type glutamate receptors (mGluRs) along with a variety of glutamate receptor associated signaling proteins.

There is however little published data regarding the actual role of glutamatergic signaling in osteoblastic bone formation.

In vivo and *in vitro* studies performed in our laboratory provide evidence that glutamatergic signaling is a necessity for normal osteoblast function.

In a number of different models of *in vitro* bone formation the addition of non-competitive antagonists of iGluRs prevents the formation of mineralized bone, moreover antagonizing some sub-types of iGluR mediates the differentiation of pre-osteoblasts. iGluR antagonist modulate osteoblast function in a manner that correlates with the previously reported data regarding in vitro osteoclastogensis.

Interestingly iGluR mediated glutamate signaling appears to function differently in osteoblasts derived from flat and long bones. This implies the components of osteoblastic glutamatergic signaling may be adapted *in vivo* possibly to reflect the differential function of osteoblasts in those regions of the skeleton.

OR-33

The Role of Dopamine and Serotonin in Regulating Bone Mass and Strength: Studies on Dopamine and Serotonin Transporter Null Mice

M. Bliziotes, M. Gunness, A. Eshleman, K. Wiren
Oregon Health and Science University and Portland VAMC, Portland, OR
mmb@teleport.co

Neurotransmitter regulation of bone metabolism has been a subject of increasing interest and investigation. Dopamine (DA) has been reported to have effects on calcium and phosphorus metabolism. The dopamine transporter (DAT) is believed to control the temporal and spatial activity of released DA by rapid uptake of the neurotransmitter into presynaptic terminals. We have evaluated the histologic and biomechanical properties of the skeleton in mice homozygous for deletion of the DA transporter gene (DAT) to help delineate the role of DA in bone biology. We have demonstrated that DAT $^{-/-}$ mice have reduced bone mass and strength. DAT $^{-/-}$ animals have shorter femur length and dry weight, and lower ash calcium content. Cancellous bone volume in the DAT $^{-/-}$ proximal tibial metaphysis is significantly decreased with reduced trabecular thickness. DAT $^{-/-}$ vertebrae have lower cancellous bone volume as a consequence of increased trabecular spacing and reduced trabecular number, and cortical thickness and bone area in the femoral diaphysis are reduced. The ultimate bending load (femoral strength) for the DAT $^{-/-}$ mice is 30% lower than the wild-type mice. Thus, deletion of the DAT gene results in deficiencies in skeletal structure and integrity.

Since serotonin (5-HT) plays a role as a regulator of craniofacial morphogenesis, we explored the expression and function of 5-HT receptors and the 5-HT transporter (5-HTT) in bone. Primary cultures of rat osteoblasts (rOB) and a variety of clonal osteoblastic cell lines including ROS 17/2.8, UMR 106-H5 and Py1a show mRNA expression for the 5-HTT, and the 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A} and 5-HT_{2B} receptors by RT-PCR analysis and immunoblot. A relatively high density of nanomolar affinity 5-HTT binding sites is present in ROS 17/2.8 and UMR 106-H5 cells. The maximal [^{3}H]5-HT uptake rate in ROS cells was 110 pmol/10 min/well, with a K_m value of 1.13 µM. In normal differentiating rOB cultures, 5-HTT functional activity was observed initially at day 25, and activity increased by almost eight-fold at day 31. In mature rOB cultures, the estimated density of [^{125}I]RTI-55 binding sites was 600 fmol/mg protein. PMA treatment caused a significant 40% reduction in the maximal uptake rate of [^{3}H]5-HT, an effect prevented by pretreatment with staurosporine. 5-HT potentiates the PTH-induced increase in AP-1 activity in UMR 106-H5 cells. In 5HTT $^{-/-}$ animals, cancellous bone volume (BV/TV) in the lumbar vertebrae is reduced, with a trend toward decreased trabecular thickness and trabecular

number. These results demonstrate that osteoblastic cells express a functional serotonin system, with mechanisms for responding to and regulating uptake of 5-HT, and disruption of the 5-HTT gene may cause osteopenia.

P-1

Periosteal Resorption at the Proximal Tibia Metaphysis Due to Mechanical Unloading: Evidence from Longitudinal pQCT Measurements

M.R. Allen*, S.A. Bloomfield
Texas A & M University, College Station, TX
matt@hlkn.tamu.edu

Periosteal resorption has been measured to evaluate alterations in bone remodeling in response to various treatments (long-term mechanical loading, ovariectomy, disuse). These data were collected using pre-treatment fluorochrome labeling techniques in mid-diaphysis cortical bone and metaphyseal cancellous bone. To our knowledge, no published data exists concerning periosteal resorption at long bone metaphyseal regions (e.g. proximal tibia (PT)). Loss of periosteal bone at the PT could compromise structural strength, a deleterious effect at a site of attachment for numerous ankle extensor and flexor muscles. We present an approach to evaluating periosteal resorption at the PT using peripheral quantitative computed tomography (pQCT, Research M), citing results from *in vivo* longitudinal studies in our laboratory using skeletally mature (6-mo.-old) Sprague-Dawley rats subjected to hindlimb-unloading (HU), a commonly used animal model for skeletal changes due to disuse/space flight. *In vivo* reproducibility for total cross-sectional area by pQCT at the PT in our laboratory is 1.94% using three consecutive 0.5mm thick slices at 5.5, 6.0, 6.5mm from PT plateau and a voxel size of 0.10mm. Study 1 utilized retired breeder female rats (n=8/group) exposed to either 28d HU or normal cage activity. After 28d, control animals gained 1.54 ± 2.14% in total area at the PT compared to a loss of 3.5 ± 2.66% in HU animals ($p<0.10$). These longitudinal data, suggesting periosteal resorption, are supported by data from pilot studies in male rats after 65d HU as well as virgin female rats after 28d HU. In Study 2, *in vivo* data were collected to examine the effects of estrogen (E_2), raloxifene (RAL; a selective estrogen receptor modulator), or vehicle (VEH) in an ovariectomized (OVX) and HU model. Using virgin females, PT were scanned *in vivo* at baseline, after 4 weeks of OVX, and then after 28d of HU during which time animals were given either E_2 (n=9), RAL (n=10), or VEH (n=8) via implanted time-release pellets. Four weeks of OVX resulted in an increase (13%, $p<0.001$) in PT total area (pre: 12.31 ± 0.23mm^2; post: 13.98 ± 0.29), an observation common in the OVX model. Over 28d, both E_2 and RAL animals lost significant total area at the PT (-6.6% and -10.2% respectively, $p<0.05$) while VEH animal declines were non-significant (-5.5%, $p=0.29$). These collective data provide strong evidence that periosteal resorption occurs at the proximal tibial metaphysis region in response to long-term HU. If endocortical bone formation was increased, thus maintaining cortical thickness, the compromised structure that results from the loss of total area could be partially compensated for, yet this has not been shown to occur in the HU model. The loss of endocortical bone, as well as cancellous bone, at the PT that occurs with HU would likely further compromise the structural strength caused by decreasing total area. Further studies should utilize pre-treatment fluorochrome labeling techniques to help confirm these findings. In addition, novel mechanical testing techniques should be developed to test the functional consequences of this periosteal bone loss at a metaphyseal bone site that contains numerous muscle attachments.

P-2

HDAC-1 Mediates the Repressive Function of Smad6 in BMP Signaling

S. Bai*, W. Shi, X. Cao
University of Alabama, Birmingham, AL
sbai@path.uab.edu

Smad proteins transduce BMP signaling from the cell membrane to the nucleus. Smad6 is one of the inhibitory Smads that preferably inhibits BMP signaling. Smad6 interacts with the activated type I receptors and blocks the phosphorylation of the receptor regulated Smads. Smad6 was also identified as interacting with the phosphorylated Smad1, forming an inactive Smad1-Smad6 complex in the cytoplasm. Previously, we identified that Smad6 acts as a transcriptional co-repressor in BMP signaling through interacting with Hoxc-8. Here we report that Smad6 represses gene transcription by directly recruiting histone deacetylases (HDACs). HDACs are involved in chromatin modification and are recruited to specific gene promoters by transcriptional repressors or co-repressors that silence gene expression. Transfection studies demonstrated that the HDAC inhibitor, trichostatin A (TSA) partially blocks Smad6-mediated osteopontin promoter activity, indicating HDAC is involved. In immuno-precipitation assays, both Smad6 and Hoxc-8 were co-precipitated with HDAC-1. Furthermore, over expression of HDAC-1 and Smad6 completely inhibited the BMP-induced osteopontin promoter activity. We also performed the HDAC activity assay with immuno-precipitated Smad6 protein complexes, in which 3H-labled acetylated histones were incubated with Smad6 complex. The result showed that the histones deacetylation rate with Smad6 complex is 25% comparing with the 1% in the control. In addition, we found that Smad6 MH1 domain directly binds to DNA, and the MH2 domain of Smad6 masks this binding activity, suggesting that Smad6 MH1 and MH2 domains associate reciprocally and inhibit each other's function. Interestingly, the interaction of Hoxc-8 with Smad6 induced Smad6 binding to DNA in gel-shift assay. Our data indicate that Smad6 and Hoxc-8 interact with HDAC-1, and recruit HDAC-1 to the osteopontin promoter, leading to inhibited BMP-induced transcriptional activity as the negative feedback loop in the BMP signaling pathway.

P-3

Mature Rat Skeletal Changes in Response to Simulated Microgravity:A Gender Comparison

S.A. Bloomfield, M.R. Allen
Texas A & M University, College Station, TX
sbloom@tamu.edu

Previous studies have documented decrements in tibial bone density and bone formation rate with long-term hindlimb unloading (HU) in mature adult male rats, yet have not examined the response of adult female rats to HU. Therefore, the purpose of this study was to compare the bone response to 28-d HU in male and female Sprague-Dawley rats (6-mo-old). Bone mineral density (BMD) and area were determined by *ex vivo* peripheral quantitative computed tomography (pQCT; Stratec XCT-M, Norland Corp) at the proximal tibia, a site composed of both cortical and cancellous bone. Percent labeled periosteal surface (%LABEL), a measure of bone formation, was assessed near the tibia-fibula junction (TFJ; a pure cortical bone site) using fluorochrome labeling. Two-way analyses of variance were performed within gender between 28-d HU (HU-males, n=8; HU-females, n=7) and 28-d cage activity controls (CON-males, n=8; CON-females, n=8) using a significance level of $p<0.05$. Male body mass was significantly higher compared to females (447 ± 12g and 280 ± 5g respectively) with no difference between HU and CON groups within gender. Over 28d, with food provided ad libitum, CON-males body mass significantly increased (+5%) while all other groups were unchanged. Both male and female HU groups had significantly reduced soleus weight (-52% and -53%, respectively, vs. controls) confirming effective HU. Proximal tibia pQCT showed that HU-males had significantly lower cancellous BMD (-19%), as well as total (-11%), cortical, and marrow area compared to CON-males. Interestingly, only differences in total BMD and cortical area at the proximal tibia were found in the HU-females group vs. CON-females. A significantly lower %LABEL was observed after 28-d in both HU-males (-55%) and HU-females (-68%) compared to their controls, suggesting declines in bone mineralization rate during after 28-d HU. These data suggest that there are gender-specific responses to HU in adult rats at sites composed of both cortical and cancellous bone, with female rats maintaining cancellous BMD and exhibiting changes in bone geometry that differ from those in males. Both genders experience similar declines in mineralization at the purely cortical site we studied. Further studies are necessary to determine if adult female rats continue to show resistance to change at cortical/cancellous sites beyond 28-d of HU, and the impact of differing food intakes during HU.

P-4

The Role of Fatigue Damage in Postmenopausal Bone Fragility

A.B. Castillo*, R.B. Martin
University of California at Davis, Sacramento, CA
abcastillo@ucdavis.edu

The purpose of our studies is to develop the ferret model to investigate the temporal and spatial interactions between fatigue damage, osteocyte apoptosis, and bone remodeling in estrogen-replete and estrogen-deficient animals. The ferret model may be a feasible alternative to the dog and rat models because it is small and, unlike the rat, it normally undergoes osteonal remodeling of its cortical bone. To this end we performed preliminary studies to determine (1) serum estrogen concentrations (2) resorption cavity density and (3) yield strength of tibiae in intact-estrous and spayed female ferrets. We collected blood samples from intact-estrous animals (n=2) and from spayed animals (n=4) on a biweekly basis. The estrus period in intact animals was distinguished by an enlarged vulva. Serum estrogen concentrations for each blood sample were determined using a radioimmunoassay (RIA). Estrogen concentrations were averaged to give a mean value for intact-estrous and spayed animal groups. Animals were euthanized and tibiae were harvested. Euthanasia of spayed animals occurred at 34 to 52 days post-spaying. Six tibiae from spayed animals and 5 tibiae from intact-estrous animals were tested monotonically to failure in three-point bending. Tibiae were serial sectioned at the midshaft and resorption cavity densities for each animal group were calculated.

Mean serum estrogen concentrations between intact-estrous (67.26 ± 2.62 pg/ml) and spayed (17.78 ± 2.02 pg/ml) (mean ± SD) animals were significantly different ($p < .0001$). Mean resorption cavity density of tibiae from spayed animals (.14 ± .09, *.01-.22* #/mm^2) was greater than, but not significantly different from, intact-estrous animals (.08 ± .04, *.02-.11* #/mm^2) (mean ± SD, range). Mean tibial yield strength for spayed (146 ± 45 MPa) and intact-estrous (198 ± 34 MPa) (mean ± SD) animals were not significantly different.

The data suggest that spaying activates intracortical remodeling in the tibia of female ferrets and the effect is highly specific to individual animals. That is, all but one spayed animal had a greater resorption cavity density than each of the intact-estrous animals, and excluding this animal from the analysis resulted in a significant difference in mean values between animal groups. Furthermore, longer periods following spaying will allow more time for estrogen loss to take effect and may result in greater resorption cavity densities. Mean tibial yield strength tended to be lower in the spayed animals and differences in both resorption cavity density and yield strength may reach significance with a larger sample size. Future studies with a greater number of animals and of longer duration are planned.

P-5

Bisphosphonate Therapy for Osteogenesis Imperfecta

L.A. DiMeglio*, L. Ford, G. Liu, R. McClintock, M. Peacock
Indiana University School of Medicine, Indianapolis, IN
dimeglio@iupui.edu

Osteogenesis imperfecta (OI) is a genetic disease of collagen synthesis, which manifests primarily as increased bone fragility. Affected persons have low bone mineral density (BMD), recurrent fractures, and progressive bony deformity. In its most severe form, the disorder is lethal in infancy. Improvements in BMD and in fracture rates in children with OI treated with intravenous (IV) bisphosphonates have been reported. The efficacy of oral bisphosphonates has not been established. Oral treatment for these children would clearly be advantageous in terms of ease of use and cost.

We hypothesize that both IV and oral bisphosphonates will increase BMD in children with OI. We have instituted an open-label, prospective, randomized clinical trial of the efficacy and safety of oral compared to IV bisphosphonate in children with OI aged 4-18 years in order to determine the relative efficacy of these regimens. Children receive either IV pamidronate, 3 mg/kg over 3 days every 4 months or oral alendronate 1 mg/kg, from a minimum of 10 mg to a maximum of 40 mg daily. The primary efficacy outcomes are total body and lumbar spine BMD, measured at 4-month intervals. Secondary outcomes include: ultrasound characteristics at the heel, fracture incidence, calcium biochemistry, biomarkers of bone formation and resorption, anthropometric measures, effect on dental anomalies, audiologic evaluations, assessment of quality of life, pain measures, and evaluations of gross motor function.

Eight children have been enrolled into the randomized study. Six other children who were ineligible for oral therapy (because of age or gastrointestinal problems) have also been started on IV bisphosphonate. No adverse effects have been noted. On follow-up, all children have had an increase in BMD, beyond that expected with normal growth. They have also had a concomitant decrease in bone resorption as assessed by monthly urine biochemistry.

Our preliminary data suggest that oral bisphosphonate therapy is as safe and effective for children with OI as IV bisphosphonate therapy. Further, the response in BMD is substantial, well beyond what would be expected with normal growth. If maintained, this increase will likely reduce the fracture rate.

P-6

In Vitro Inhibition of Bone Resorption by Human PTH(7-84)

P. Divieti*, M.R. John, H. Jüppner, F.R. Bringhurst
Massachusetts General Hospital, Boston, MA
divieti@helix.mgh.harvard.edu

The linear sequence of intact mammalian PTH consists of 84 amino acids, of which only the most amino(N)-terminal portion, i.e. PTH(1-34), is required for the classical actions of the hormone on mineral ion homeostasis mediated by the type 1 PTH/PTHrP receptor (PTH1R). Like the N-terminus, the carboxyl(C)-terminal sequence of PTH is highly conserved among species, and various circulating PTH C- fragments are generated by peripheral metabolism of intact PTH or are directly secreted, in a calcium-dependent manner, by the parathyroid glands. Certain synthetic PTH C-fragments exert actions on bone and cartilage cells that are not shared by PTH(1-34), and specific binding of C-PTH peptides has been demonstrated in bone cells in which PTH1R expression was eliminated by gene targeting. The peptide hPTH(7-84) recently was shown to inhibit the calcemic actions of hPTH(1-34) or hPTH(1-84) in parathyroidectomized animals. To determine if this anti-calcemic effect of hPTH(7-84) *in vivo* might result from direct actions upon bone, we studied its effects upon both resorption of intact bone *in vitro* and formation of osteoclasts in primary cultures of murine bone marrow. Human PTH(7-84) (300 nM) reduced basal 72-hr release of preincorporated ^{45}Ca from neonatal mouse calvariae by 50% (9.6 ± 1.9% vs. 17.8 ± 5.7%; $p<0.001$) and similarly inhibited resorption induced by hPTH(1-84), hPTH(1-34), 1,25$(OH)_2D_3$, prostaglandin E_2 or interleukin-11. In 12-day murine marrow cultures, hPTH(7-84) (300 nM) lowered 1,25$(OH)_2D_3$-dependent formation of osteoclast-like cells by 70%. These actions of hPTH(7-84) were not observed with the PTH1R antagonists hPTH(3-34)NH2 or [L^{11},D-W^{12},W^{23},Y^{36}]hPTHrP(7-36)NH_2, which, unlike hPTH(7-84), did inhibit PTH1R-dependent cAMP accumulation in ROS 17/2.8 cells. We conclude that hPTH(7-84), acting via receptors distinct from the PTH1R and presumably specific for PTH C-fragments, exerts a direct antiresorptive effect upon bone that may be partly due to impaired osteoclast differentiation.

P-7

Disuse Osteopenia in Hibernating Bears

S.W. Donahue*, M.R. Vaughan, L.M. Demers, H.J. Donahue
Michigan Technological University, Houghton, MI, Pennsylvania State University, Hershey, PA, Virginia Tech, Blacksburg, VA
sdonahue@mrl.hmc.psu.edu

Disuse osteopenia occurs in patients with spinal cord injuries, those confined to prolonged bedrest, immobilized bones following surgery, total arthroplasty patients, and astronauts. Recovery of lost bone with remobilization is slow and incomplete, which may increase the risk for osteoporotic fractures. It has been proposed that hibernating black bears (*Ursus americanus*) posses a biologic mechanism to prevent disuse osteoporosis.

We isolated serum from wild black bears during active and denning periods, for biochemical analysis of cortisol, carboxy-terminal propeptide of type I collagen (PICP) and cross-linked C-telopeptide of type I collagen (ICTP). Serum PICP positively correlates with bone formation rate, and serum ICTP positively correlates with resorption rate and negatively with BMD, making

these markers useful for assessing bone turnover in osteoporosis patients.

Cortisol and ICTP significantly ($p<0.04$) increased and PICP decreased during disuse in males and females (with and without cubs), suggesting that bone formation was uncoupled from resorption during disuse, resulting in net bone loss. The uncoupling was most pronounced in lactating bears.

Glucocorticoids induce osteoporosis by increasing resorption and decreasing formation. Thus, serum cortisol may regulate immobilization induced bone loss in bears. Remobilization only partially restores bone lost by disuse in dogs. Since bears hibernate annually and may live for 30 years or more, the unique feature of bear bone metabolism may not be the ability to prevent disuse osteopenia, but rather the ability to completely recover during remobilization. Understanding how black bears recover from disuse osteopenia may provide insight for other osteopenias (e.g., age-related and postmenopausal), and provide a rationale for the development of pharmacologic therapies for osteoporosis.

P-8

Temporal Aspects of Fluid Flow Induced Calcium Signaling in Osteoblastic Cells

S.W. Donahue*, H.J. Donahue, C.R. Jacobs
Michigan Technological University, Houghton, MI, Pennsylvania State University, Hershey, PA, Stanford University, Stanford, CA
sdonahue@mrl.hmc.psu.edu

When a daily mechanical stimulus is partitioned into discrete loading bouts, bone formation in rat tibiae is enhanced. This suggests that bone cells have a refractory period, during which they are insensitive to additional mechanical stimuli. Mechanically induced fluid flow in bone may contribute to adaptation by providing cells with physical stimulation and enhancing molecular transport. We hypothesized that osteoblastic cells have a refractory period, during which oscillations in cytosolic calcium concentrations are insensitive to further bouts of oscillating fluid flow.

Intracellular calcium concentrations were measured in osteoblastic cells isolated from rat long bones using Fura-2 and ratiometric imaging techniques. Sub-confluent cells were exposed to a two-minute bout of oscillating fluid flow, during which there were oscillations in intracellular calcium, which lasted approximately 60 seconds before returning to baseline values. After a rest period of 5, 30, 60, 300, 600, 900, 1800, or 2700 seconds, the cells were exposed to a second bout of fluid flow.

Some cells could respond to the second bout after only a 30-second rest period. However, the magnitude of the second calcium oscillation was significantly ($p<0.01$) lower than the first oscillation when the rest period was less than 1800 seconds. This finding suggests that the refractory period for mechanically induced calcium oscillations may be as short as 30 seconds for some cells, but 900 to 1800 seconds is required to regain the magnitude of the oscillation. With the onset of one hour of continuous oscillating fluid flow, there was an immediate oscillation in intracellular calcium, but no subsequent oscillations occurred during the duration of the one-hour loading period. When cells were given a 2700 second rest period between loading bouts, they could respond with oscillations to as many as four different bouts of fluid flow.

These findings suggest that fluid flow induced intracellular calcium oscillations in bone cells may play a role in bone adaptation to mechanical loading. Fluid flow induced calcium signaling has been linked with osteoblastic gene expression *in vitro*. However, the temporal aspects of intracellular calcium signaling that control downstream events are yet to be elucidated.

P-9

Planning a Longitudinal Training Intervention to Increase Bone Formation in Humans: How Can We Use Results from Animal Studies?

E.W. Helge*
University of Copenhagen, Copenhagen, DENMARK
ewhelge@ifi.ku.dk

Background: Animal studies have shown that bone formation is dependent on a high and diverse osteogenic strain with a high strain rate. However, it has been shown that the osteogenic response adapts very fast, so that the duration of strain can be relatively short (CT Rubin & LE Lanyon 1984, 1985). The Mechanostat Theory suggests that bone strain is perceived in relation to a certain set point for bone formation. An increased set point can be caused by changes in the biochemical milieu (e.g. low concentrations of sex hormones), which will lead to a net bone loss unless the mechanical strain is increased accordingly (HM Frost 1992).

In premenopausal elite athletes with depressed concentrations of female sex hormones it has been found that distance runners suffer from low bone mineral density (BMD), whereas gymnasts exhibit a high BMD (EW Helge, in press). With reference to The Mechanostat Theory the different effect of low concentrations of sex hormones in runners and gymnasts could be explained by the different strain in the two activities. While running can be characterized as a low strain/repetitive activity, gymnastics include higher and more diverse strain that might be sufficient to reach the higher set point and thus lead to bone formation.

Several human studies have studied the impact of physical activity including weight-bearing exercise and resistance training on bone formation in both pre- and postmenopausal females (OM Rutherford 1999). However, longitudinal studies have found only minor increments in BMD following a training program: 0%-6% (BA Wallace 2000), and the dose-response relationship is largely unsolved. The major part of these studies has applied very general training regimes that were not based on the results from the animal studies mentioned above. The question is therefore how we can apply results and theories from animal studies to optimize a human training intervention study? "Bridging" that gap will be the topic of this presentation.

Hypothesis of future study: In the presence of exercise-induced menstrual dysfunction and low BMD in female runners BMD can be increased through specific resistance training if the strain is sufficiently high to compensate for the increased bone formation set point.

Subjects: Female distance runners (20-40 years) with menstrual dysfunction and low BMD. *Controls:* Female distance runners (20-40 years) with normal menstrual function and normal BMD.

Based on hypotheses generated from animal studies this presentation will discuss: duration of training intervention, duration and frequency of training sessions (in relation to response adaptation), type of muscle contractions and relative training load (in relation to strain magnitude and rate), type of movement (weight-bearing or non-weight-bearing), variables in question (e.g. BMD (g/cm^2), maximal muscle strength (N), maximal muscle power (N/ms), biological bone markers (nmol/l), sex hormones (nmol/l), DHEAS (nmol/l)).

P-10

The Relationship Between Bone Mechanical Properties and BMD is Not Modified by Increased Mineralization

C.J. Hernandez*, G.S. Beaupré, D.R. Carter
VA Palo Alto, HCS, Palo Alto CA and Stanford University, Stanford, CA
hernandez@post.harvard.edu

Bone volume fraction (BV/TV) and the degree of mineralization (expressed here as the ash fraction, a, ash mass/total mass) are both known to influence the mechanical properties of bone. Meunier and Boivin (Bone 1997, 21: 373-7) have suggested that variation in ash fraction and not bone volume fraction may explain how small increases in areal bone mineral density (BMD) are correlated with unexpectedly large decreases in fracture incidence in patients taking bisphosphonates. In a recent analysis we derived predictive models to describe the separate contributions of bone volume fraction and ash fraction on bone strength and elastic modulus (Hernandez et al. Trans. ORS 2001, 529). We found that variation in ash fraction can change the bone strength much more than similar variations in bone volume fraction. The relationship between BMD and mechanical properties was not addressed. In this analysis we determine whether increased mineralization may modify the relationship between BMD and bone strength, suggesting whether it could contribute to unexpectedly large decreases in fracture incidence seen in patients taking bisphosphonates.

A method is derived to calculate changes in BMD caused by modification of bone volume or ash fraction. The changes in bone strength caused by increased BMD are evaluated when 1) BMD increases are caused entirely by bone volume fraction and 2) BMD increases are caused entirely by ash fraction.

The increase in bone strength predicted from an increase in bone mineral density was the same whether the BMD increase was caused by bone volume fraction or ash fraction (see figure P-10). The relationship between

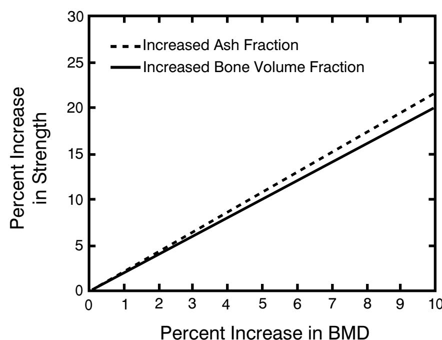

Figure P-10

bone strength and BMD therefore does not appear to be modified by increased ash fraction. Our previous study suggested that small increases in ash fraction improve bone strength more than similar increases in bone volume fraction. This analysis suggests that the separate effects of bone volume fraction and ash fraction on bone strength are accounted for by BMD measures. We suggest that it is unlikely that mineralization induced increases in bone strength are responsible for any unexpectedly large changes in fracture incidence during bisphosphonate treatment.

P-11

Implications for Understanding Fluid Flow Dynamics During Functional Loading: Application of Regional Microstructural Heterogeneity in the Turkey Ulna

K.J. Hunt*, J.G. Skedros
University of Utah School of Medicine, Salt Lake City, UT
kenneth.hunt@hsc.utah.edu

It has been suggested that the mechano-sensitivity of osteocytes is mediated by fluid-flow through bone's lacunar-canalicular porosity. This idea has been examined in an analytical model of the turkey ulna [Srinivasan & Gross, Med. Eng. & Phys., 2000]. During normal loading, this bone experiences circumferential strain gradients that are highest along the neutral axis, which typically traverses the cranial-caudal cortices. Regional differences in fluid-flow dynamics within the turkey ulna have also been described. Intercortical and transcortical pressure gradients and fluid flux are largely dependent on matrix porosity. We speculate that heterogeneities in osteocyte lacuna density and non-lacuna porosity, in addition to other material characteristics, might be important considerations in understanding fluid-flow and related strain dynamics. A transverse segment was cut at mid-diaphysis of 11 skeletally mature domestic turkeys, and four 200X backscattered electron images (two endocortical and two pericortical; excluding circumferential lamellae) were obtained from cortical octants: D, D-Cr, Cr, D-Cd, Cd, V-Cd, V, V-Cr (D = dorsal, Cr = cranial, Cd = caudal, V=ventral). These images were examined for osteocyte lacuna population densities and non-lacuna porosity (primary and secondary canals, vascular channels). Secondary osteon population densities were quantified in cortical quadrants (D, V, Cr, Cd). Octant comparisons demonstrated more lacunae in the Cr and Cd cortices compared to the other locations ($p<0.001$) [Means: Cr 1,316.6/mm^2; Cd 1,388.0; range in other regions: D-Cd 966.7 to V-Cr 1,100.1]. There was relatively greater porosity in Cd, V-Cd, and D-Cd regions ($p<0.05$). However, non-lacuna porosity and lacuna density were not correlated ($r=0.008$). Quadrant comparisons showed significantly more secondary osteons in the caudal cortex. Previous data have shown that this region has significantly greater thickness and lower mineralization (%ash). Pericortical-endocortical comparisons showed more lacunae in the pericortical region (1,234.4 vs. 1,170.1, $p=0.05$) and greater non-lacunar porosity in the endocortical region ($p=0.06$). These data demonstrate significant regional microstructural heterogeneity. In the context of fluid-flow analyses, it is important to recognize that regional variations in lacuna and non-lacuna porosities might not be correlated. These are important considerations in analytical models examining strains and fluid flow. An important clinical challenge is to understand fluid-flow dynamics so that we can ultimately comprehend the mechanisms that mediate bone maintenance and adaptation for applications in disease prevention.

P-12

A Murine Model of Postmenopausal Osteoporosis and Estrogen Replacement Therapy: Assessments of Volumetric BMD Using pQCT and of Three-Dimensional Trabecular Microstructure Using μCT

Y. Jiang*, J. Zhao, V. Shen, S. Bain, H.K. Genant
University of California, San Francisco, CA, and Skeletech, Inc., Bothell, WA

Because of the recent wide availability of various genetically altered mice in genomics research and for drug discovery and development, there is an unprecedented new interest in developing a murine model for investigating osteoporosis. The purpose of this study was to characterize volumetric BMD and the three-dimensional (3D) trabecular bone and microstructure of a murine model of osteoporosis induced by estrogen deprivation and effects of hormone replacement therapy (HRT) on the model using computed tomography (CT), a non-destructive advanced image technique. Seventy 3-month-old Swiss Webster mice were equally divided into 7 groups: baseline, sham surgery received placebo (sham, 2 groups), ovariectomy received placebo (OVX, 2 groups), ovariectomy received 17β-estradiol at 250 μg/kg/week s.c. (HRT, 2 groups). One group of the animals from sham, OVX, and HRT were sacrificed at 5 weeks post-surgery, and the rest were sacrificed at 13 weeks post-surgery. The distal femur was scanned using a pQCT (Stratec, Germany), and scanned with a μCT scanner (Scanco, Switzerland) with isotropic resolution of 9 μm^3. 3D μCT trabecular structure, including structure model index (SMI) and degree of anisotropy (DA) in the secondary spiongiosa were directly measured without stereological model assumption. Serum osteocalcin and NTx were measured as indicators of bone turnover. Compared to sham animals, OVX led to significant reductions in BMD at 5 and 13 weeks post-OVX. These reductions were apparent at 5 weeks in the distal femur by pQCT (-16%). μCT showed that at 5 weeks post-surgery, there was a significant change in BV (-50%), Tb.N (-29%), Tb.Th (-8%), Tb.Sp (+54.38%), SMI (+14%), and DA (-10%) in OVX compared with those in sham. These changes were similar at 13 weeks post-surgery, and no further bone loss was observed. HRT prevented OVX-induced changes up to the sham level. OVX increased bone turnover at 5 weeks post-surgery. These data indicate that OVX induces short-term high-turnover accelerated deterioration of 3D trabecular structure in the Swiss Webster mouse. The trabeculae become more rod-like and more isotropic after OVX. HRT only prevented OVX-induced bone loss without anabolic effect at this dose.

P-13

L-Type Calcium Channels Mediate Mechanically Induced Bone Adaptation *In Vivo*

J. Li*, R.L. Duncan, D.B. Burr, C.H. Turner
Indiana University School of Medicine, Indianapolis, IN
jli@anatomy.iupui.edu

Introduction: Calcium channel antagonists have been found to alter the intracellular calcium response to a variety of stimuli in bone cells *in vitro*. It has been hypothesized that activation of mechano-sensitive calcium channels activate Long-lasting (L-) type voltage sensitive calcium channels (VSCC) that, in turn, trigger chemical signals in the signal cascade from applied load to cell response in bone tissue. In the present study, we investigated whether L-type VSCC mediate mechanically induced bone adaptation *in vivo* using two L-type VSCC antagonists, verapamil and nifedipine.

Materials and Methods: Twenty-four adult rats were divided into three groups: control, verapamil treated and nifedipine treated. Verapamil and nifedipine were orally administered by using gavage once at a dose of 100mg/kg. One bout of loading was carried out 90 minutes after administration of verapamil or 30 minutes after administration of nifedipine. The control animals were loaded 30 minutes after administration of polyethylene glycerol vehicle. The right tibia of each animal was externally loaded with 64 N peak force for 360 cycles at 2 Hz in a four-point bending device, and the left tibia was used as a nonloaded control.

Histomorphometric measurements were performed at the endocortical surface on midshaft cross sections of tibiae.

Results: Paired t-tests showed higher mineralizing surface on the endocortical surface in loaded limbs of control animals than in nonloaded limbs. However, no significant differences between right and left limbs were found in either verapamil or nifedipine treated animals ($p=0.05$ and $p=0.17$). Verapamil and nifedipine suppressed the load-induced bone formation found in controls by 53% ($p<0.05$) and 75% ($p<0.01$) (ANOVA) respectively.

Discussion: This study demonstrates that preventing the influx of calcium into the cell by blocking the L-type calcium channel *in vivo* prevents the mechanically-induced increase in bone formation that could normally occur. This suggests that the L-type calcium channel is important in mediating the mechanical signals for bone adaptation. Inhibition of this channel prevents the load-induced increase in bone formation found in control animals.

P-14

Prostaglandin E2 and Bipedal Stance "Exercise" had an Additive Effect on the Augmentation of Cancellous Bone Mass in Aged Sham and Ovariectomized Rats

Y.F. Ling, W.S.S. Jee, C.Y. Li, A. Mo, X.Y. Tian, M. Su, R.B. Setterberg, W. Yao
University of Utah School of Medicine, Salt Lake City, UT
yfling2001@yahoo.com

Previous reports showed that bone loss was only partially prevented by bipedal stance "exercise" following ovariectomy (OVX), and that it was well documented that Prostaglandin E_2 (PGE_2) had an anabolic effect on the rat skeleton. The aim of this study was to determine whether low doses of PGE_2 could prevent OVX-induced cancellous bone loss and whether a combination of PGE_2 plus bipedal stance "exercise" would be more effective than using PGE_2 alone. Seventy-eight 12-month-old female Sprague-Dawley rats were either OVX or sham-operated (Sham) on day 0 and then treated with PGE_2 (0,0.3 or 1 mg/kg/d) and/or housed in normal height cages (NC, 28 cm) or raised cages (RC, 33 cm) for 8 weeks. Bone histomorphometry was performed on the double fluorescent-labeled proximal tibial metaphyses. We found that in Sham rats, combination treatment had additive effects in increasing bone area, trabecular width and number by having additive effects in stimulating mineral apposition rate, bone formation rate but decreased bone turnover. As expected, cancellous bone loss was 34% following OVX, accompanied by elevated bone turnover. Without "exercise", PGE_2 alone prevented OVX-induced bone loss at a dose of 1 mg/kg/d while this completed prevention effect was observed at the dose of 0.3 mg/kg/d when combined with RC. Like their effects in Sham rats, PGE_2 and RC had additive effects in augmenting cancellous bone mass and architecture and maintaining the elevated bone formation but depressing bone resorption and bone turnover. We concluded that with bipedal stance "exercise" the PGE_2 dose required to achieve complete prevention of OVX-induced cancellous bone loss in the proximal tibial metaphyses could be lower than PGE_2 alone.

P-15

Molecular Cloning and Functional Characterization of the Canine Androgen Receptor

B. Lu*, S.L. Smock, T.A. Castleberry, T.A. Owen
Pfizer Global Research and Development, Groton, CT
bihong_lu@groton.pfizer.com

Sex steroids, including testosterone, play a major role in determining peak bone mass and the subsequent loss of total bone mass with age. Testosterone and its active metabolite dihydrotestosterone (DHT) bind with high affinity to the androgen receptor (AR), a member of the nuclear hormone receptor superfamily. These receptors function as transcription factors, binding, together with accessory proteins, specific DNA response elements in the promoters of androgen responsive genes. Cloning of the cDNAs encoding the AR from several species has revealed a highly conserved, modular molecule with transactivation, DNA binding, and ligand binding domains. To further study AR function in a model species of relevance to bone, we cloned the canine AR by first screening a canine kidney cDNA library and then by cloning the remaining 5' segment by PCR from canine ventral prostate cDNA. The complete sequence obtained was 3596 bp. This sequence contained a single open reading frame of 2724 bp, potentially encoding a protein of 907 amino acids with a predicted molecular weight of 98.7 kD. Sequence analysis of the protein encoded by this open reading frame reveals that the modular domains providing the DNA binding and ligand binding functions are identical to those reported for the human, mouse, and rat ARs. Northern analysis of poly-A^+ RNA from kidney and ventral prostate revealed two very low abundance transcripts of approximately 9 kb. RT-PCR analysis of canine ventral prostate, spleen, skeletal muscle, heart, testis, liver and kidney demonstrated that while AR mRNA was detectable in all tissues, it appeared to be of higher abundance in prostate, testis, and kidney. Competition binding studies using ^{3}H-DHT as ligand demonstrated specific displacement by DHT, testosterone, and the anabolic steroid stanozolol, with IC_{50} values of 1.3 nM, 2.5 nM, and 3.8 nM, respectively. Binding of ^{3}H-DHT was weakly displaced by dexamethasone, a ligand for the glucocorticoid receptor, with an IC50 of 6 uM. Following cotransfection of the canine AR into 293 cells, treatment with these active ligands also resulted in the stimulation of a luciferase reporter under the control of a glucocorticoid-androgen responsive element. Immunohistochemistry on canine ventral prostate using an antibody directed to the N-terminal 21 amino acids showed strong staining of the secretory epithelial cells as has been reported in other species. Together, these data indicate that we have cloned the canine androgen receptor and that its functional DNA binding and ligand binding domains are absolutely conserved with those found in the human AR.

P-16

Catabolic Effects of Continuous Parathyroid Hormone (hPTH 1-38) *in vivo* is Associated with Sustained Stimulation of RANK Ligand and Inhibition of Osteoprotegerin

Y.F. Ma, R. Cain, D.L. Halladay, X. Yang, Q.Q. Zeng, J. Hoover, R.R. Miles, S. Chandrasekhar, T.J. Martin, J.E. Onyia
Eli Lilly Company, Indianapolis, IN, and St. Vincent's Institute of Medical Research, Fitzroy, Victoria, AUSTRALIA
ma_linda@lilly.com

Continuous infusion of PTH *in vivo* results in active bone resorption. To investigate the molecular basis of the catabolic effect of PTH *in vivo*, we evaluated the role of osteoprotegerin (OPG) and RANK ligand (RANKL) which are known to influence osteoclast formation and function. Weanling rats fed a calcium free diet were parathyroidectomized (PX) and infused with PTH via Alzet pump to examine i) the changes of serum calcium and osteoclast number, ii) the expression of OPG/RANKL mRNA and protein, (iii) the expression of osteoblast phenotype bone formation-associated genes such as osteoblast specific transcription factor (cbfa1), osteocalcin (OC), bone sialoprotein (BSP) and type I collagen (COL1A1). PTH (1-38) (0.01-20ug/100gms) continuous infusion for 1-24 hours resulted in a dose dependent increase in serum ionized calcium in PX rats, and a corresponding dose dependent increase in osteoclast number, indicating an increased bone resorption. At 20ug/100gm PTH dose level, serum ionized calcium was 2.1-fold of the vehicle control and not different from Sham-PX, while osteoclast number was 3-fold of the vehicle control and 1.7-fold of the Sham-PX. Circulating OPG protein level was also decreased by 30%. Immunohistochemical evaluation of bone sections confirmed that OPG level was reduced in proximal tibial metaphysis upon PTH infusion. In the distal femur, RANKL mRNA expression was increased (27-fold) while OPG mRNA expression was decreased (4.6-fold). The changes in RANKL and OPG mRNA levels were rapid (as early as 1h), dose dependent, and sustained over a 24h period that was examined. The expression of genes that mark the osteoblast phenotype was significantly decreased [cbfa1 (2.3-fold), OC (3-fold), BSP (2.8-fold) and COL1A1 (5-fold)]. In contrast to the effects of continuous PTH infusion, acute exposure to PTH1-38 resulted in a rapid but transient change in OPG and RANKL expression. The decrease in OPG mRNA (3.4-fold) and increase of RANKL mRNA (3.8-fold) were observed at 1 hour and they both recovered to near the control levels by 3 hours. These results suggest that the catabolic effect of PTH infusion *in vivo* is associated with a reciprocal expression of OPG/RANKL and a co-ordinate decrease in the expression of bone formation related genes. We propose

that the rapid and sustained increase in RANKL and decrease in OPG initiate, maintain and favor the cascade of events in the differentiation/ recruitment and activation of osteoclasts.

P-17

Distal and Mid-Shaft Tibia Bone Mass Assessment Using Peripheral Quantitative Computed Tomography (pQCT) in Healthy Children and Adolescents

L.J. Moyer-Mileur, R. Roberts*, S.D. Ball
University of Utah School of Medicine, Salt Lake City, UT
ryan.roberts@hsc.utah.edu

This cross-sectional study used pQCT to evaluate the influences of age, gender, body size, and puberty on bone acquisition in healthy children and adolescents. The pQCT technique provides analyses of volumetric bone mineral density (vBMD, gm/cm^3) for total bone and bone compartments and bone strength expressed as polar strength strain index (PSSI, mm^2). Bone mass of the non-dominant tibia by pQCT (XCT 2000, Norland Medical Systems, Inc) was measured in 287 healthy children and adolescents (166 girls; 121 boys; 4 to 19 y). Measurements were obtained at 4% and 66% from the distal end plate. The distal site (4%) assessed trabecular bone, and the mid-shaft site (66%) assessed total bone, cortical bone, and muscle cross-sectional area (MCSA). Age, gender, weight, height, body mass index (kg/m^2), and pubertal stage were recorded. Mean age, weight, and height were 12.2 ± 4.2 y, 46.7 ± 21.7 kg, and 1.46 ± 0.2 m, respectively. Pubertal stage distribution (Tanner 1-5) was 47%, 10%, 15%, 11%, and 17%, respectively. Age, gender, weight, and height correlated with total, cortical, and trabecular bone mineral content (BMC, mg), bone area (BA, cm2), vBMD, PSSI, and MCSA. Only cortical vBMD was influenced by puberty. Multiple regression found 69% of the variance in cortical vBMD explained by pubertal stage and age, 23.5% of the variance in trabecular vBMD explained by age and gender, and >75% of the variance in PSSI and MCSA explained by body weight ($p<0.001$). Boys had greater trabecular vBMD values than girls (317.9 vs 284.8 mg/cm^3; $p=0.001$) while total and cortical vBMD values were similar. Both genders had a 10% decrease in trabecular vBMD and 14% increase in total and cortical vBMD with age. We conclude that bone measurements by the pQCT technique provides information on bone acquisition, architecture, and strength that may prove useful in the assessment of normal bone growth and development as well as pediatric bone disease.

P-18

Subchondral Bone Failure: An SEM Study in Horses

RW. Norrdin, S.M. Stover
Colorado State University, Fort Collins, CO and University of California, Davis, CA
robert.norrdin@colostate.edu

Introduction: Subchondral bone stiffening with sclerotic thickening of the trabeculae is important in the pathogenesis of arthrosis. Subchondral sclerosis and areas of bone devitalization are common in the overload arthrosis of equine athletes. Actual subchondral bone failure occurs focally in the palmar metacarpal condyle of the fetlock joint where indentation and collapse of the articular cartilage is seen. This is referred to as “traumatic osteochondrosis”. It is primarily a disease of racehorses and is very common in the U.S. It is often symptomless but more severe forms are associated with fetlock lameness. The consistency of the site makes it a useful model in which to study the bone changes leading to failure. The objective of this work was to study morphologic changes at the site in condyles with various stages of disease using scanning electron microscopy.

Material and Methods: Fetlock condyles from 23 racehorses with mild, moderate and severe subchondral bone sclerosis, subgross cracks beneath the calcified cartilage, and flattening or indentation of the cartilage were studied. Parasagittal slices 2mm thick were photographed, radiographed and digested free of soft tissue. They were hand ground, sputter-coated with gold and examined with a Phillips XL30 scanning electron microscope.

Results: Fine intralamellar cracks between collagen fibers and short radiating fine cracks in the matrix were seen in most samples and may have been a drying artifact. This change was more prominent in the layer of bone immediately beneath the calcified cartilage and in the more sclerotic bone at the failure site. Consistent changes in osteocyte lacunae and canaliculi were not appreciated. The earliest subgross cracks developed within 1-2 mm of the calcified cartilage layer and radiated parallel to it for several mm in irregular branching lines. There was fragmentation of crack margins as a gap developed. In one sample, smoothly ground fragments were seen within the gap along with entrapped soft tissue. In other samples, loss or fragmentation of bone at the crack margins had led to collapse and compaction of the subchondral layer. Here there was flattening or indentation of the intact overlying cartilage layers. There was little evidence of reactive change at the site but evidence of osteoclastic enlargement of vascular canals was often seen deep to the lesion site and could be recognized radiographically.

Conclusions: Focal failure of subchondral bone involves the development of cracks, fragmentation along crack margins, compaction of the matrix, and collapse of articular cartilage.

Interestingly, none of the samples in this study, even those with more severe or obviously chronic lesions, were known to be responsible for clinical lameness.

P-19

Bisphosphonate-Induced, Hemichannel-Mediated, Anti-Apoptosis through the ERK Pathway: A Gap Junction-Independent Action of Connexin43

L.I. Plotkin*, T. Bellido
University of Arkansas for Medical Sciences, Little Rock, AR
plotkinliliani@exchange.uams.edu

Osteocytes buried throughout the mineralized matrix of bone communicate with each other and with cells of the bone surface via gap junctions. This network provides the means for detecting microdamage and transmitting signals leading to bone repair. We have recently demonstrated that bisphosphonates (BPs) inhibit osteocyte apoptosis through activation of the extracellular signal regulated kinases (ERKs) (JCI, 104:1363-1374, 1999), suggesting that preservation of the osteocyte network contributes to the anti-fracture efficacy of these agents. To probe into the mechanistic basis of the phenomenon and, in particular, the means by which BPs interact with osteocytes, we investigated the requirement of gap junctions for the anti-apoptotic effects of the drugs. BP-induced ERK activation and prevention of MLO-Y4 osteocytic cell apoptosis in response to different pro-apoptotic stimuli was independent of cell-to-cell contact. However, these effects were abolished by 18 a-glycyrrhetinic acid (AGA) – an agent that disassembles connexin (Cx) channels – but not by its inactive analog glycyrrhizic acid (GA), suggesting that while Cx channel integrity may be required, gap junctions are not. Consistent with this contention, either addition of BPs or removal of calcium – an established maneuver that opens Cx hemichannels – increased lucifer yellow uptake in adherent or MLO-Y4 cells maintained in suspension; and, AGA, but not GA, abolished this effect. Moreover, Cx43 – the main Cx expressed in osteocytic cells – was detected in non-junctional osteocyte membranes by cell surface biotinylation, establishing that osteocytic cells do indeed express functional Cx43 hemichannels that are open by BPs. Furthermore, embryonic fibroblasts and osteoblastic cells derived from Cx43 deficient mice or osteoblastic cell lines lacking Cx43, unlike control cells expressing Cx43, did not exhibit the anti-apoptotic effect of BPs. This effect was specific for Cx43, as transfection of Cx43 – but not Cxs 26, 31, 32, 37, 40, or 45 – to Cx-deficient HeLa cells rendered them responsive to the anti-apoptotic effect of BPs. These results demonstrate that Cx43 hemichannels are required for the prevention of apoptosis by bisphosphonates, and reveal a novel and gap junction-independent function of Cx43 in the regulation of survival signaling pathways.

P-20

Hoxa-9 Represses TGF-b-Induced Osteopontin Gene Transcription

W. Shi*, X. Shi, X. Cao
University of Alabama, Birmingham, AL
wshi@path.uab.edu

Smad2 and Smad3 are down-stream TGF-β signaling molecules. Upon phosphorylation by its type-I receptor, Smad2 or Smad3 forms a complex

with Smad4 and translocates to the nucleus where the complex activates target gene transcription. In the present study, we report that Smad3 binds directly to the osteopontin (OPN) promoter, and that Smad4 interacts with the Hox protein and displaces it from its cognate DNA binding site in response to TGF-b stimulation. In gel shift assays, the GST-Smad3 fusion protein was found to bind to a 50-bp DNA element (-179 to -229) from the OPN promoter. Also, we found that both Hoxc-8 and Hoxa-9 bound to a Hox binding site adjacent to Smad3 binding sequence.

Interestingly, Smad4, the common partner for both BMP and TGF-β signaling pathways, inhibited the binding of Hox protein to DNA. FLAG-tagged Smad4 co-immunoprecipitated with HA-tagged Hoxa-9 from cotransfected COS-1 cells, demonstrating an interaction between Smad4 and Hoxa-9. Transfection studies showed that Hoxa-9 is a strong transcriptional repressor; it suppresses the transcription of the luciferase reporter gene driven by a 124-bp OPN promoter fragment containing both Smad3 and Hox binding sites.

Taken together, these data demonstrate a unique TGF-β-induced transcription mechanism. Smad3 and Smad4 exhibit different functions in activation of OPN transcription. Smad3 binds directly to the OPN promoter as a sequence-specific activator, and Smad4 displaces the transcription repressor, Hoxa-9, by formation of Smad4/Hox complex as part of the transcription mechanism in response to TGF-β stimulation.

The abbreviations used are: HOX, homeobox; HA, hemagglutinin; GST, glutathione S-transferase; SMAD, a merger term of Sma and Mad, vertebrate mediators of TGF-β family signals homologous to Sma and Mad (Cell, Vol. 87, 173, October 18, 1996).

P-21

In Vivo Fatigue Loading of the Rat Ulna Induces Both Bone Formation and Resorption and Leads to Time-Related Changes in Bone Mechanical Properties and Density

M.J. Silva*, Y-F Hsieh
Barnes-Jewish Hospital at Washington University, Saint Louis, MO
silvam@msnotes.wustl.edu

Fatigue loading of bone triggers intracortical resorption and is associated with stress fractures. Bone fatigue may also play a role in osteoporotic fractures. Despite the widely accepted view that bone fatigue is relevant to skeletal health, neither the osteogenic response nor the changes in bone mechanical properties have been quantified following *in vivo* fatigue loading. To further characterize the skeletal response to fatigue loading, we assessed bone formation, mechanical properties, density and resorption in the ulnae of 72 adult, female Fisher rats subjected to a single bout of *in vivo* fatigue loading followed by recovery periods of 0, 6, 12 or 18 days. Axial, compressive loading (peak force 13.3 N, 2 Hz) was applied to the right forelimb using a materials testing machine until the ulna was fatigued to a pre-determined level (a 60% increase in actuator displacement). The left forelimb served as a contralateral control. The primary osteogenic response to fatigue loading (determined by dynamic histomorphometry) was woven bone formation that occurred exclusively on the periosteal surface of the ulnar diaphysis. Woven bone increased significantly with recovery time from 6-18 days ($p<0.05$). Ultimate force of the ulna (determined by three-point bending) decreased by 50% and stiffness decreased by 70% on day 0 ($p<0.01$ vs. control), indicative of acute fatigue damage. By day 12, ultimate force and stiffness had returned to control levels ($p>0.05$) and by day 18 had increased to approximately 20% beyond control levels ($p<0.01$). Consistent with the increases in woven bone formation and mechanical properties, bone tissue cross-sectional area, moment of inertia, and mineral content (based on peripheral quantitative computed tomography, pQCT) increased with recovery time following fatigue loading ($p<0.01$). Intracortical resorption space density and osteoclast density (determined by tartrate resistant acid phosphatase staining) also increased significantly with recovery time ($p<0.05$), indicating a remodeling response triggered by fatigue loading.

In summary, our findings demonstrate the remarkable ability of the adult skeleton to rapidly form periosteal woven bone and thereby offset the negative structural effects of acute fatigue damage and subsequent intracortical resorption.

P-22

Bipedal Stance "Exercise" Completely Prevents Cancellous Bone Loss and In Combination with Estrogen has a Synergetic Effect on Inhibiting Bone Resorption in Lumbar Vertebrae of OVX Rats

M. Su, W.S.S. Jee, C.Y. Li, J. Chen, A. Mo, R.B. Setterberg, W. Yao
University of Utah School of Medicine, Salt Lake City, UT
mingsu98@yahoo.com

This study aimed to observe the individual or combination effects of bipedal stance “exercise” and estrogen on the cancellous bone of lumbar vertebrae in 8-month-old Sham or ovariectomized (OVX) rats. Specially designed raised cages (RC) were employed making rats rise to erect bipedal stance for feeding. At the age of 6 months, six rats were sacrificed as baseline control; all the others were either bilateral sham or ovariectomized. The rats were housed in normal height cages or RC and injected s.c. biweekly with 10 μg/kg of 17 β-estradiol (E_2) or vehicle for 4 and 8 weeks. Histomorphometric measurements were performed on the undecalcified mid-transverse sections in the cancellous bone of the 4th lumbar vertebrae body. We found that 1) After OVX, bone formation, turnover and resorption significantly increased throughout the study period and cancellous bone area decreased by 21% in 8 weeks; 2) E_2 alone completely prevented this bone loss at week 8 by inhibiting bone formation and turnover as well as bone resorption; 3) Similarly, RC alone completely preserved the cancellous bone mainly by the inhibiting of bone resorption and decreasing activation frequency but maintained the elevated bone formation; 4) E_2 plus RC completely prevented OVX-induced bone loss starting from week 4 and had a synergetic effect on reducing bone resorption especially at the early stage after ovariectomy. In conclusion, both estrogen and raised cage alone can completely prevent cancellous bone loss of lumbar vertebrae in OVX rats. Combination regimen has a synergetic effect on depressing bone resorption, especially at the early stage after estrogen depletion.

P-23

Combined Low Dose Estrogen and Exercise Completely Prevented Marrow Cancellous Bone Loss and the Elevated Intracortical Bone Remodeling Following Ovariectomy in the Femoral Neck

X.Y. Tian*, W. Yao, J. Chen, C.Y. Li, A. Mo, M. Su, R.B. Setterberg, W.S.S. Jee
University of Utah School of Medicine, Salt Lake City, UT
xyt65@yahoo.com

The current study was designed to investigate the effects of estrogen and exercise on the femoral neck (FN) of the ovariectomized (OVX) rat. Exercise was conducted by employing a raised cage model so as to make the rats rise to bipedal stance for feeding. Six-month-old female Sprague-Dawley rats were bilateral sham-ovariectomized or ovariectomized at day 0. They were housed in normal height or raised cages (RC) and injected twice a week with 10 μg/kg of 17β-estradiol (E_2) or vehicle for 8 weeks. We found that 1) Marrow cancellous bone lost by 39% and intracortical porosity area increased by 108% while total bone area did not change significantly due to the periosteal expansion following OVX. 2) E_2 alone partially prevented the decrease of marrow cancellous bone and the increases of porosity area by inhibiting endosteal bone erosion. It decreased the periosteal bone formation. 3) Bipedal stance exercise alone also partially prevented the decrease of marrow cancellous bone by preventing the increased endosteal bone erosion but did not affect periosteal bone formation compared to OVX’d animals. 4) E_2 plus RC completely preserved the marrow cancellous bone and prevented the intracortical remodeling by having an additive effect on endosteal bone resorption. RC helped to prevent a decrease of periosteal bone formation after estrogen administration (Table P-23); as a result, total bone area increased. In conclusion, apart from inducing marrow cancellous bone loss, OVX also increased intracortical remodeling in the FN. Both E_2 and RC partially prevented these changes. Combination treatment completely prevented OVX-induced bone loss by having an additive effect on endosteal bone erosion and RC counteracted with E_2 by inhibiting the decrease of bone formation after E_2 administration.

Parameters	Ct.Ar %	Po.Ar %	TB.Ar %	B.Ar %	Ps-MAR µm/d	Ps-BFR um/d×100	Es-MAR µm/d	Es-BFR µm	Es-E.Pm %
Baseline	70.29	1.47*	80.47	11.21*	0.72*	19.09*	0.51*	1.83*	3.97*
SD	5.20	0.32	3.92	1.57	0.19	8.36	0.11	1.18	1.50
Sham	72.52	1.38*	82.58	11.06*	0.73*	19.78	1.19	2.30	3.14*
SD	3.56	0.25	2.61	2.15	0.19	9.74	0.24	0.84	1.99
OVX	75.77	2.87	80.30	6.72	1.02	60.24	1.25	3.99	5.60
SD	3.56	0.80	4.02	1.35	0.10	8.20	0.21	0.78	1.43
E	70.17	1.59*	77.68	8.62	0.61 *	16.38 *	1.41	3.47	2.95 *
SD	4.47	0.36	2.94	1.74	0.06	4.88	0.40	2.02	0.76
RC	72.05	1.84	80.61	9.88 *	0.93	50.28	1.22	2.43	3.02 *
SD	3.29	0.93	2.43	2.88	0.32	23.13	0.45	1.12	0.89
E+RC	80.25*	1.40*	90.92*	11.79*	0.84	32.15*	0.95	1.91 *	1.23*
SD	9.56	0.39	6.06	4.96	0.16	14.99	0.41	0.80	0.49
	Two-way ANOVA								
E	0.666	0.001	0.440	0.122	0.000	0.000	0.017	0.188	0.000
RC	0.008	0.571	0.000	0.058	0.001	0.011	0.772	0.018	0.000
E × RC	0.054	0.040	0.001	0.109	0.522	0.958	0.308	0.273	0.609

Note: * $p<0.05$; E, estrogen; RC, raised cage; Ct.Ar, cortical area; Po.Ar, porosity area; TB.Ar, total bone area; B.Ar, trabecular area inside marrow; Tb.Wi, trabecular width; Tb.N, trabecular number; MAR, mineral apposition rate; BFR, bone formation rate.

Table P-23

P-24

SWIM EXERCISE AND SOY DIET INCREASES TIBIAL CORTICAL BONE FORMATION IN OVARIECTOMIZED RATS

S.E.Warner*, K.J. Hart, J.M. Shaw, S. Heaton, M. Hegsted, S.C. Miller
University of Utah, Salt Lake City, UT
sew1.utah.edu

The purpose of this study was to examine the influence of a three month intervention composed of both a soy protein diet and swim exercise on the tibial cortical bone parameters in forty ovariectomized retired breeder rats. Following two weeks of a standardized rat chow, rats were ovariectomized and then randomly assigned to one of four groups: standard casein diet (C), soy diet (S), casein swim (CS), and soy swim (SS). The soy diet contained 14% soy protein with 1.2 mg genistein per gram protein. Swim exercise was progressively increased over the first 6 weeks and then was maintained at one hour per day five days a week for a total of 12 weeks. Fluorescent labels were injected 12 and 2 days prior to sacrifice for histomorphometric analysis by fluorescent microscopy. Tibias were excised, dehydrated, and embedded in methyl-methacrylate prior to slide preparation of cross-sections sampled proximal to the tibia-fibular junction. Cortical area (CA) and cortical width (CW) was greater in the swim groups (CS and SS; $p<0.05$) indicating increased bone mass. Soy fed rats exhibited greater percent double label (%dL), mineral apposition rate (MAR), and bone formation rate (BFR) compared to rats fed the casein diet ($p<0.05$). CS had greater percent single label (%sL) and % mineralizing surface (%MS) than non-exercised casein fed rats ($p<0.05$). The combination of swim exercise and soy diet achieved the highest histomorphometric measures of %dL, %MS, CA, CW, and BFR of all four groups.

These results demonstrate positive independent and combined effects of a soy diet and swim exercise on bone mass and indices of bone formation in a model of estrogen deficiency.

P-25

OSTEOCONDUCTIVE BIOMIMETIC SCAFFOLDS AND HUMAN MESENCHYMAL STEM CELLS - CELL-MATRIX INTERACTIONS

X.B. Yang*, H.I. Roach, N.M.P. Clarke, S.M. Howdle, K.M Shakesheff, R.O.C. Oreffo
University of Southampton, UNITED KINGDOM, and University of Nottingham, UNITED KINGDOM
x.yang@soton.ac.uk

The ability to augment bone formation remains a key clinical need. The advent of techniques for the generation of inductive/conductive biomaterial scaffolds in combination with the isolation and culture of stem cells of connective tissue offers tremendous potential for the fabrication of bone and cartilage structures for skeletal use. We have examined the efficacy of the osteotropic factor, osteoblast stimulating factor-1, in combination with biodegradeable scaffolds to modulate human bone marrow stromal cell adhesion, chemotaxis, proliferation, differentiation and colony formation (colony forming unit-fibroblastic, CFU-F). Osteoblast stimulating factor-1 (osf-1), also known as pleiotrophin or HB-GAM, is an extracellular matrix-associated protein, which is present in those matrices that act as targets for the deposition of new bone.

Human bone marrow cells were cultured with or without addition of recombinant human osf-1 (10pg-50ng) in basal and osteogenic conditions either on tissue culture plastic or seeded onto 3-D porous biodegradable scaffolds, generated from poly (-lactic acid coHglycolic acid) (PLGA) (75:25) and poly (D, L lactic acid) using a novel supercritical fluid method. Cell adhesion and spreading were examined by confocal microscopy following incorporation of fluorescent labels as well as by scanning electron microscopy. Proliferation was assessed by colony formation (colony forming unit-fibroblastic, CFU-F). Osteogenic differentiation was determined by alkaline phosphatase activity as well as type I collagen, osteocalcin and osf-1 by immunocytochemistry. The chemotactic ability of osf-1 was examined on patterned surfaces generated using EM grids on tissue culture plastic coated with osf-1 and irradiated with UV light. Osf-1 was chemotactic to human osteoprogenitors, which migrated to the areas of intact osf-1. Osf-1 significantly stimulated total and alkaline phosphatase-positive colony formation, as well as increased alkaline phosphatase specific activity in basal and osteogenic conditions by around 30% compared to controls. The concentrations required were extremely low (10 pg/ml), whereas rhBMPs alone are known to require 1000-fold greater concentrations for similar effects. On 3-D scaffolds adsorbed with osf-1, alkaline phosphatase activity, type I collagen formation, synthesis of cbfa-1, osteocalcin and osf-1 were observed in the attached cells. In contrast, negligible cellular growth was observed on PLGA and PLA scaffolds alone.

In summary, Osf-1 has the ability to promote human osteoprogenitor adhesion, migration, expansion and differentiation. The successful generation of 3-D biomimetic structures incorporating osf-1, which maintain the ability to modulate human osteoprogenitor activity and differentiation through exploitation of cell-matrix interactions, indicates the potential for the augmentation of *de novo* bone formation for skeletal repair.

P-26

Annexin V Contributes to a Mechanism by which Oscillating Fluid Flow Stimulates Ca^{2+} Transients in Osteoblast-like Cells

C.E. Yellowley*, T. Haut-Donahue, C.R Jacobs, H.J. Donahue
Pennsylvania State University College of Medicine, Hershey, PA, and Stanford University, Stanford, CA
cyellowley@psu.edu

Ca^{2+}_i is a second messenger that has been implicated in the mechanism by which physical signals exert biological effects on bone cells, however the mechanism by which Ca^{2+}_i signals are generated is unclear. The aim of this study was to examine the role of Annexin V (AnxV), a Ca^{2+} dependent phospholipid binding protein, in the Ca^{2+} response to oscillating fluid flow (OFF) in human osteoblastic MG 63 cells. AnxV has been shown to function as a Ca^{2+} selective ion channel and possesses a number of attributes that suggest it is ideally suited for a role as a mechanoreceptor, including its ability to interact with both extracellular matrix and cytoskeletal elements. In addition, the cellular location of AnxV has been shown to be sensitive to Ca^{2+}_i levels. Since OFF stimulates Ca^{2+}_i transients, we also looked at the effects of OFF on the cellular relocation of AnxV. Ca^{2+}_i imaging: MG 63 cells cultured in monolayer were loaded with Fura-2AM and placed on a parallel plate flow chamber. Following a 1 minute no flow period, cells were exposed to OFF at 1 Hz and a peak shear stress of 20 dynes/cm^2 for 3 minutes. Prior to OFF, cells were exposed to anti-Anx V antibody (40 ug/ml for 24 hours) to disrupt Anx V activity, anti-cFOS antibody (40 ug/ml for 24 hours) as a control, or standard media for 24 hours. Ca^{2+}_i transients of 80 nM or greater were considered responses. Anx V relocation: MG63 cells were exposed to OFF at 1 Hz and a peak shear stress of 20 dynes/cm^2 for at least 1hr, after which plasma membrane, cytosol, nuclear extract and nuclear membrane fractions were isolated. Expression of Anx V in each fraction was assessed by Western blot. Densitometry was used to quantitate differences in Anx V expression between control and OFF stimulated cell fractions.

The percent of cells responding to fluid flow with an increase in Ca^{2+}_i was significantly attenuated in cells exposed to anti-Anx V (27.3±11.3 %) compared to either control (75.5±3.9 %, p=0.002) or anti-cFOS (63.6±12.8 %, p=0.024) treated cells (n=6). AnxV expression increased 37±13%, 2±6%, 136±69% and 73±48% in the plasma membrane, cytosol, nuclear membrane and nuclear extract respectively in cells stimulated with OFF compared to no flow controls (n=7). These data suggest that Anx V may be involved in the mechanism by which mechanical signals are detected by bone cells, and that OFF may modulate the cellular location of Anx V. Future studies will examine whether Anx V is functioning as a Ca^{2+} selective channel in bone cells and whether its activity is influenced by cellular location.

P-27

Collagen-Bridged Microcrack Model For Cortical Bone Tensile Strength

Y.N. Yeni*, D.P. Fyhrie
Henry Ford Hospital, Detroit, MI
yeni@bjc.hfh.edu

Bone microcracks have attracted much interest because they accumulate with age and loading, and they are targeted by bone remodeling. While the presence of microcracks can degrade the mechanical properties of the tissue, the process of microcracking can provide extra strength by dissipating energy before and during failure. The microcracks typically found in human cortical bone are approximately 50 micrometers long and stain with dyes such as basic fuchsin that attach to the organic matrix. The appearance of the microcracks under light microscopy and evidence from examination of the torn surfaces of larger cracks is consistent with a mechanism of *fiber bridging* across the cracks. Fiber bridging is a means of toughening a material where the tendency of small cracks to propagate is reduced by the presence of fibers that interconnect the faces of the crack. The fiber connection (the bridge) causes a stress that resists the opening of the crack that is called a *crack closure stress*. This stress reduces the chance that the crack will grow larger. Differences in the resistance to microcrack growth between bones may result in differences in the number and size of microcracks between bones subjected to similar loads, having significant effects on the macro-fracture behavior and biological response of bone to damage. Bone has a hierarchical microstructural organization where constituent size ranges between the order of nanometers and several hundred micrometers. Due to this microstructural organization, it is possible that the constituent that predominantly affects the resistance of bone to crack growth depends on the size of the crack of interest. We propose that the growth of microcracks are affected by the collagenous structures at the fibril to fiber levels. We have developed an analytical fracture mechanics model of a microcrack bridged by collagen fibers. Using this model and data from the literature on tensile testing of normal and demineralized human femoral cortical bone, we predicted that the length of bridging fibers that participate in bridging is 3-10 μm for a range of failure strain values obtained from demineralized cortical bone. This length is consistent with the experimentally measured minimum length of Type I collagen fibrils from tendon and about 6 μm of longer fibrils being stretched during crack opening. The consistency of the prediction with observed fibril lengths supports the likelihood that fiber bridging is active across real microcracks. Our prediction supports the idea that any condition or treatment that changes the bonding of one fibril to another or to the mineralized matrix will change the microcracking behavior and the apparent strength of the tissue.

P-28

DNA Microarray Analysis of Fusing Macrophages Reveals Clues about the Functional Consequences of Multinucleation

J. Zhang*, J. Li, H. Sterling, R. Barton, A. Vignery
Yale University School of Medicine, New Haven, CT, and Boehringer Ingelheim Parmaceutical Inc. Ridgefield, CT
juanzhang_2000@yahoo.com

Osteoclasts are characterized by multinucleation, a powerful ability to resorb bone, and differentiate by fusion of precursor cells that belong to the monocyte-macrophage lineage. While it is well established that cytokines, chemokines and growth factors that are produced by cells located in the osteoclast microenvironment control osteoclast differentiation and activation, the question as to whether osteoclasts themselves produce soluble molecules that control their differentiation and their activity has not been investigated. We have reported previously that freshly isolated rat alveolar macrophages cultured at high density spontaneously fuse to differentiate into multinucleated macrophages that express osteoclast functional markers, in the absence of exogenously added cytokine or factor (Int J Exp Pathol 81:291, 00). This suggested that macrophages control their multinucleation by means of factors that they produce. To investigate this possibility, we subjected fusing rat alveolar macrophages to DNA microarray analyses using Affymetrix technology which employs oligonucleotide hybridization, and includes mismatched control oligos. RNA was extracted from freshly isolated macrophages, and macrophages cultured at high density for one hour, one day or five days, when multinucleation reaches 99%. Experiments were repeated thrice. Our results indicate that macrophage multinucleation was accompanied by a differentially regulated statement of transcripts coding for the canonical osteoclast markers, for signaling molecules and for transcription factors. Multinucleation was also accompanied by a highly significant accumulation of transcripts coding for MMP proteases such as MMP-9 which increased over 100 fold in multinucleated cells when compared to freshly isolated cells. Most interesting was the strongly induced accumulation of transcripts coding for extracellular matrix proteins and growth factors, suggesting that multinucleated macrophages control their own microenvironment. This analysis revealed that macrophage multinucleation is accompanied by the regulated statement of a specific set of genes and suggested a sophisticated cross talk between osteoclasts and their microenvironment via a network of growth factors.

P-29

Monoclonal Antibody-induced Arthritis in Mice and its Prevention by Dexamethasone

C. Lin, C. Liu, H. Chen, R. Reife, M. Bailey, M. Heggem, R. Velasco, S. Bain, V. Shen
SkeleTech, Inc., Bothell, WA

In humans, the incidence and severity of rheumatoid arthritis (RA) increases with age and is characterized by joint inflammation and stiffness. While a number of preclinical models have been used to evaluate putative arthritis therapies (e.g. collagen and adjuvant induced RA), a murine model

employing type II collagen antibodies as an arthritis-inducing agent has yet to be fully characterized. The present study was therefore undertaken to assess the extent and severity of RA induced in mice by type II collagen monoclonal antibodies (MoAb). In addition, the ability of the co-administration of dexamethasone (Dex) to alleviate the RA was also studied. Experimentally, 18 female Balb/c SPF mice (6-8 weeks old) were divided into three groups (6 mice/group) and treated as follows for two weeks: control (saline), MoAB RA (MoAb) and RA+Dex (MoAb+Dex). MoAb (Arthrogen-CIA MoAb, Chondrex, Seattle, WA) was given by a single tail i.v. injection (2 mg/mouse), while Dex by daily s.c. injection (0.15 mg/kg/day). In addition, in mice treated with MoAb, a single dose of LPS (25 mg/mouse) was given on day 3. RA was induced in all mice treated with MoAb as indicated by persistent swelling of paws starting from day 8. Histological examination of the navicular and 2nd distal tarsal joint of the hind paws showed marked infiltration of mononuclear cells and proliferative invasion of synovial tissues into the joint space. Joint damage was further characterized by the presence of fibrin deposits in the joint space, the loss of proteoglycans staining in the articular cartilage, and by the erosion of articular cartilage. Destruction of the joint and articular cartilage were accompanied by marked bone effects as evidenced by microradiographic examination of the joints and as well as significant increases in the activation and extent of osteoclastic bone resorption that was revealed by TRAP-stained histological sections. The co-administration of Dex prevented the cartilage and bone destruction. When these results are combined with previously published work we conclude that MoAB-induced RA in mice is: 1) more rapid and extensive than other methods of RA induction; 2) non-strain dependent 3) characterized by osteoclastic mediated bone destruction; and 4) a useful model for rapid *in vivo* screening of putative RA therapies.

P-30

Differential Diagnosis between "Physiological" and "True" Osteopenias Employing DXA References

G.R. Cointry, J.L. Ferretti, S.L. Garc a, R.F. Capozza, H. Plotkin, E.J.A. Rold n, J.R. Zanchetta
Universities of Rosario (Rosario) and El Salvador USAL), and IDIM/FIM, Buenos Aires, ARGENTINA

The strength (and indirectly the mass) of bones is determined by the stiffness and the spatial distribution of the mineralized matrix. Both these determinants are inversely related through a feedback mechanism (bone mechanostat theory). Bone mass/strength losses can only come from a disturb in that biomechanical regulation. The stimulus for the mechanostat is the strain history resulting from the mechanical usage of the skeleton (contractions of the regional muscles). As evidence of that, the bone/muscle masses are linealy related, showing the same slope for any gender, age or body habitus, and are mutually affected by physical activity. The setpoint of the system, genetically determined, is sensitive to.systemic (disturbing) factors as hormones, drugs, etc. As evidence of that, the intercepts of that relationship vary with the gender and reproductive status. Therefore, the bone mass can only be lost (osteopenia) because of a prolonged inactivity or weightlessness (reduced input, "physiologic" osteopenia), or a genetic or systemic disorder (shifted setpoint, "true" osteopenia). Only when that osteopenia involves a biomechanical compromise (bone fragility) the condition can be regarded as a "disuse" or a "true" osteoporosis, respectively. The DXA-BMC is the best resource for measuring bone mass and diagnosing an osteopenia; not so for diagnosing an osteoporosis, because it does not provide any information on bone tissue quality or distribution. However, DXA is able to assess all bone (BMC), fat, and lean (proportional to muscle) masses. Thus, DXA allows correlating the whole-body or regional BMC and "muscle" mass and approaching a differential diagnosis between "physiologic" and "true" osteopenias (appropriate or inappropriate bone/muscle mass proportion) as pre-conditions for diagnosing a "disuse" or a "true" osteoporosis, respectively.

We have developed adequate reference BMC/lean-mass charts for whole-body DXA data (*XR-26, Norland, Wisconsin*) in normal Argentine boys and girls (n=545), men (n=228), and pre- and post-menopausal women (n=330, 347) showing the 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 99 percentiles. The same were also performed for BMC values adjusted to a common, 18-kg fat mass according to the corresponding regression equations. A very simple, graphic procedure estimates any percentile and would approach a differential diagnosis between physiologic and true osteopenias according to a suitable reference limit. Appropriate factors allow the data transformation for use with different densitometers.

P-31

Bone/Lean mass Interaction are Altered in Obese, Euglycemic, Hyperinsulinemic Women

M.R. Ulla, M. Stivala, R. Noriega, G. Cointry, J.L. Ferretti
Centro de Estudios de Osteopatías Médicas, Córdoba, and CEMFoC, Universidad Nacional de Rosario, ARGENTINA

The aim of this study was to describe and correlate the changes in fat, lean, and mineral masses (FM, LM, BMC) in euglycemic women showing both, higher than normal basal serum insulin levels and the typical distribution of abdominal fat.

We measured the whole-body BMC, FM and LM (DEXA, Norland XR-26) and the serum glucose and insulin levels both in basal conditions and after a stimulation test by glucose administration in 24 women pre- and post-menopausal. The BMC was expressed either in crude form or statistically adjusted to a common, 18-kg FM (FA-BMC) according to the natural, logarithmic association between those variables. Both the BMC/LM and the FA-BMC/LM ratios were calculated. For comparison purposes, patients were classified into 3 groups according to their basal insulin levels (I : <19, II: 19-26, and III: >26 .../dl).

Positive correlations between basal serum insulin and body weight, fat mass or lean mass were observed. No correlation was found between the BMC and the basal serum insulin. The BMC or FA-BMC/LBM ratio decreased exponentially with the basal serum insulin level or the body weight. Correlations between the BMC or FA-BMC and the LBM were lineal for all groups I, II and III, and parallel to those shown by 400 age- and sex-matched, normal controls. However, significant differences (ANCOVA, always $p<0.001$) were observed between the intercepts for the different groups, showing the decreasing order I > II > III. The intercept difference with respect to the control subjects was slightly positive for group I and negative for group II, and significantly negative for group III. The significance of these results was unaffected by the pre- or post-menopausal condition of the women. No associations were observed between any of the variables studied and the magnitude of the difference between the basal- and glucose-stimulated serum insulin levels.

Results show that, despite to show no apparent effect on serum glucose in these patients, the increased basal insulin activity enhanced their body weight and fat and lean masses. In addition, should the LM be proportional to the muscle mass, these patients should have a disproportionate enhancement of the muscle mass with respect to the relatively unaffected bone mass. If so, then the excess of insulin would have reduced the natural, biomechanical influence of muscles on the skeleton, proposedly changing the setpoint of the biomechanical control of bone mass and structure according to the bone "mechanostat" theory [CTI 62:1,1998]).

P-32

Mineral, Lean, and Fat Masses in Pan-Hypopituitary Men and Women before and after GH treatment

H.C. Hermberg, G.R. Cointry, J.L. Ferretti
Hospital Alemán, Buenos Aires; Centro de Estudios de Metabolismo Fosfocálcico, Universidad Nacional de Rosario, ARGENTINA

The GH effects on the muscle/bone relationships have been scarcely investigated. This study analyzes the relationships between mineral, lean, and fat masses (BMC, LM, FM) in pan-hypopituitary men (n=14) and women (n=15,), both before and after sc treatment with GH during 1 year. Whole-body measurements were made by DEXA and compared with data from 600 age-matched, normal men and post-menopausal women. The BMC data were analyzed both in crude form and statistically adjusted to a common, 18-kg FM (FA-BMC) according to the natural, logarithmic association involved [Bone 22:683,1998].

Concerning the whole-body measurements, the slopes of the correlations between BMC or FA-BMC (y) and LM (x) were similar to those shown by controls in men and women, both before and after treatment. The intercepts were similar to those of controls for the crude BMC in both pre- and post-treated men, and lower than that in all other instances. Treatment enhanced all, BMC or FA-BMC and LM correlatively in men and women,

but failed to improve the intercepts of any of the impaired correlations between BMC and LM. However, the slopes of the significant, linear correlations between the changes induced in BMC or FA-BMC (y) and in LM (x) were slightly higher for men than women. The number of patients was insufficient to detect any effect of HRT on those differences.

Assuming a direct proportionality between LM and muscle masses, results suggest that the affected men tended to maintain the normal muscle-bone relationships but had a disproportionately high FM, while women seemed to show a low bone mass independently of the FM. Nevertheless, the anthropometrical correlation between BMC and LM remained positive and parallel to normal in all patients, both before and after treatment, and was also respected by the treatment effects on both kinds of variables. Therefore, GH treatment would have been generally useful to enhance both bone and muscle masses following the physiological proportions, but either unable or much less effective than that to improve the bone/muscle proportion. The slightly more positive effects shown by men, if confirmed in further studies, would indicate 1. a more effective stimulation of bones by the men's more massive muscles, and/or 2. a gender-related effect of GH perhaps in connection with the particular, negative influence of the lack of estrogen in the affected women.

P-33

Effects of Peritoneal Dialyses and Hemodialyses on Bone/Lean Mass Relationships in Men and Women

A.L. Negri, G.R. Cointry, D. Salica, J.R. Zanchetta, J.L. Ferretti
Metabolic Research Institute/Foundation (IDIM/FIM) and Faculty of Medicine, Salvador University (USAL), Buenos Aires; CEMFoC, Natl. Univ. of Rosario, ARGENTINA

We had shown that the whole-body mineral content (BMC), either crude or statistically adjusted to a common, 18-kg fat mass (FA-BMC) was linearly correlated to lean mass (LM), showing the same slopes but different intercepts in decreasing order for pre-menopausal (pre-MP) women > men > postmenopausal (post-MP) women > boys and girls. On regarding LM as linearly proportional to muscle mass, this would indicate that bone mass is determined by the mechanical usage of the skeleton the same way in the species (bone "mechanostat" theory) but the proportionality of that relationship is normally affected by nonmechanical factors related to gender and reproductive status.

This study aims to compare the whole-body BMC or FA-BMC and LM (DEXA, Norland XR-26) in stable chronic peritoneally-dialysed (CAPD) and hemodialysed (HD) men and pre- and post-MP women, in which a different metabolic interference with the mechanical control of bone mass can be proposed to further affect the bone/muscle relationship. Data were compared with those from 600 sex- and age-matched controls.

The dialysed patients had a lower bone mass than the mean values shown for the corresponding sex and age. The linear correlations between the BMC or FA-BMC (y) and LM (x) showed that both CAPD and HD patients plotted significantly lower than their respective controls. The curves for men and pre-MP women were similar in slope but showed significantly lower intercepts than those of their controls. Distinctly, post-MP women showed a significant tendency to have lower BMC or FA-BMC per unit of LM than their controls in proportion with the reduction observed in LM, reaching very low BMC or FA-BMC values in the extreme cases.

Results suggest that 1. dialysed men and pre-MP women follow the normal biomechanical laws concerning the control of bone mass by muscle mass, regardless of the fat mass status; 2. however, the metabolic interference from either CAPD or HD reduces the proportionality of the consequent, BMC/LM relationship, and 3. this situation is further affected by menopause, after which the skeletal mass control seems to be impaired in proportion with the reduction in mechanical usage resulting from the deterioration of muscle mass. This would confirm the hypothesis that metabolic (nonmechanical) factors induced by the disease would have affected the bone "mechanostat" setpoint over the natural endocrine influences in these patients.

P-34

The Role of Connective Tissue Growth Factor (CTGF) in Bone Formation *in Vitro* and *in Vivo*

F.F. Safadi*, S.L. Smock, A-H. Selim, M. Rico, T.A. Owen, S.N. Popoff
Temple University, Philadelphia, PA
fsafadi@astro.temple.edu

Connective tissue growth factor (CTGF) is a secreted, extracellular matrix-associated protein that regulates diverse cellular functions including proliferation, adhesion, migration, differentiation, matrix production and survival. CTGF mRNA expression and protein production has been demonstrated in various cell types including chondrocytes, and most recently by our lab, in osteoblasts. In a study of differential gene expression, we showed that CTGF mRNA is expressed in normal bone and highly over-expressed in bone from osteopetrotic mutants. Subsequent *in situ* hybridization and immunohistochemical localization demonstrated the presence of CTGF mRNA and protein in osteoblasts. In primary cultures of osteoblasts, CTGF mRNA levels exhibit a bimodal pattern of expression that is high during proliferation and increases again as the cells terminally differentiate. Furthermore, the protein is synthesized by osteoblasts and secreted into the medium. For this study we generated recombinant rat CTGF (rCTGF) and examined its effects in primary rat osteoblast cultures. Since the mitogenic effect of CTGF has been universally demonstrated in various cell types, we first examined its effect on cell proliferation and, as expected, rCTGF showed a dose-dependent increase in cell proliferation with peak activity at 50ng/ml. Next we examined the effects of CTGF on various functional parameters associated with osteoblast differentiation. Treatment of primary osteoblast cultures with rCTGF caused an increase in alkaline phosphatase activity, osteocalcin gene expression and calcium deposition/matrix mineralization. Based on the results from osteoblast cultures, we tested its capacity to induce bone formation *in vivo* using a local delivery system. Adult male rats (12-16 weeks of age) were anesthetized, the distal femur was surgically exposed and 1 μg of rCTGF in 20 μl saline was injected into the marrow cavity; control femurs were injected with the same volume of saline or 1% BSA in saline. After 1 week, the animals were euthanized and femurs removed or radiographic of histological analyses. Radiographic analysis of rCTGF-injected femurs showed increased radiodensity within the marrow cavity compared with control-injected femurs. Histologically, the rCTGF-injected femurs had island of newly formed woven bone within the marrow cavity; the bony trabeculae were lined with rows of active, cuboidal osteoblasts and labeled intensely with calcein. There was no evidence of an osteogenic response in any of the control-injected femurs. Collectively, data from these experiments establish that CTGF is a multifunctional protein involved in the development of the osteoblast phenotype and functions associated with the differentiated osteoblast. We propose that the principle role of CTGF is that of osteoblast differentiation, while its role in osteoblast proliferation may be secondary. Clearly, the association between CTGF and bone is an interesting one that requires further investigation.

P-35

Micro-Computed Tomography

B. Koller, A. Laib
Scanco Medical, AG, Bassersdorf, SWITZERLAND

Microtomographic imaging for specimens was introduced a decade ago in different research laboratories, and meanwhile commercial desktop systems have become available. Microtomography allows to study the internal three-dimensional structures of objects with very high resolution up to 10 μm in a non-destructive way. Also, in contrast to many microscopic and SEM/TEM/AFM techniques, absolutely no sample preparation is needed.

Typically, specimens are scanned with an isotropic resolution, allowing an unbiased assessment of structural features such as porosity, mean diameter of structural elements, spacing, connectivity, and degree of anisotropy. Most of these measures have previously only been available with stereological methods based on 2-D images, where the extrapolation of the results to the third dimension needed some model assumption, or induced additional scatter in the results. These things can now be altogether avoided with the comprehensive assessment of the complete three-dimensional object.

For newly developed *in vivo* systems, the radiation exposure and acquisition time are the most limiting factors. Thus a high quantum detection efficiency is essential for a low radiation exposure imposed on the scanned animal, and powerful x-ray tubes together with fast read-out electronics are needed to allow scan times of the order of minutes to avoid motion artifacts. The images show applications of *in vitro* and *in vivo* micro computed tomography for applications in bone research.

P-36

EVIDENCE OF A HYPERMINERALIZED CALCIFIED FIBROCARTILAGE ON THE HUMAN FEMORAL NECK

J. Shea, E. Vajda, R. Bloebaum
VA Salt Lake City Health Care System, University of Utah, Salt Lake City UT
j.shea@m.cc.utah.edu

Introduction: Femoral neck fractures are significant cause of morbidity and mortality in the elderly. Recent investigations have identified a hypermineralized tissue on the femoral neck that increases in fractional area with age and it has been hypothesized that the tissue is correlated with tendon and ligament insertions sites.

Materials and Methods: Seven femurs were embedded in methylmethacrylate and coronal sections were taken from the midsection of the neck. The same regions were imaged with backscattered electron imaging and light microscopy to determine if the hypermineralized tissue was located at a tendon or ligament insertion, and if it had a similar morphological appearance as the calcified fibrocartilage of the insertion.

Results: The light microscopy results found that the areas that contained hypermineralized tissue corresponded to the calcified fibrocartilage zone of the insertion site. Regions of the neck that did not contain calcified fibrocartilage had a layer of connective tissue that resembled the periosteum found on the diaphysis.

Discussion: The hypermineralized tissue previously observed on the neck of the femur appears to be calcified fibrocartilage. Calcified fibrocartilage may be less capable of repairing microdamage due to its low cell density, lack of gap junctions, and reduced vascularity. This, in combination with the high degree of mineralization may influence the susceptibility of this region to crack initiation and subsequent fracture when an individual falls.