Abstract

PTH-CBD, a Long-Acting Parathyroid Hormone Analog, restores normal bone formation after cyclophosphamide therapy in mice.

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PTH-CBD is a hybrid protein of PTH(1-33) and a bacterial collagen binding domain designed to accumulate in collagen rich areas such as bone. We have previously shown that PTH-CBD can improve bone mineral density (BMD) in chemotherapy osteoporosis. We now investigate the time course of this response, examining effects on bone formation and bone removal evident in serology and histological analysis, paired with measurements of BMD, in a mouse model with chemotherapy-induced impairments in bone formation. 8 week old C57BL/6 mice were treated with cyclophosphamide (50 mg/kg/wk IP x 3 weeks), cyclophosphamide plus PTH-CBD (320 mcg/kg SQ x1), or vehicle alone. BMD was measured every 2 weeks. Animals were sacrificed at baseline, 1 month, 2 months, or 3 months to provide blood and tissue samples throughout the expected time of the treatment response. BMD measurements confirm that cyclophosphamide treatment prevented the normal increases in BMD expected in young mice (BMD at 6 weeks: 68.2+/-1.2 chemo vs. 74.4+/-1.1 mg/cm2 no chemo, p<0.05), presumably because of impairments in bone formation. PTH-CBD therapy restored the normal pattern of increases in BMD in cyclophosphamide-treated mice (BMD at 6 weeks: 72.9+/-1.2 PTH-CBD + Chemo vs. 68.2+/-1.2 mg/cm2 chemo, p<0.05), such that there was no difference between animals which did not receive chemotherapy. Serum samples will be processed for alkaline phosphatase, osteoproteregin, TRAP to assess effects of cyclophosphamide and PTH-CBD on bone turnover. Calcium will also be measured to assess for potential side effects. Histological samples will be processed for histomorphometry and TRAP staining. Together, these data will provide confirmation of the effects of cyclophosphamide on bone formation and bone removal, and will define which of these processes is most important in mediating the restoration of normal BMD seen with PTH-CBD treatment.
Measuring musculoskeletal function and pain in rodents

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For all studies of repair, regeneration and pain, the most critical standard for success is a change in functional outcome. There are numerous quantitative, validated and reproducible ways to assess neurological and musculoskeletal function (including pain and analgesia) in rodents. These can be broadly grouped into several classes of behavior: A) spontaneous behaviors (activity, exploration, etc) B) sensorimotor tests (motor coordination etc) C) neurological tests (reflexes etc) and D) evoked responses (sensory thresholds etc) and E) tests of cognition and emotion that are sensitive to neuromuscular defects and pain. In this presentation we show a sample of common assays in each of these behavioral domains and include representative data to illustrate the application and sensitivity of each of the tests.
Glia, gap junctions and pain: Role of sensory ganglia in tactile hypersensitivity

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Pain modulating sites provide valuable information for the study and treatment of chronic pain. Dorsal root ganglia and dorsal horn or trigeminal ganglia act as relays for appropriate pain signal amplification and suppression; however, in pathologic states the ability to control nociceptive activation thresholds is lost and this may lead to chronic pain. The arrangement of neurons in sensory ganglia is unusual, in that cell bodies of peripheral mechano-, thermo- and noxious receptors are separated from one another by sheaths of satellite glial cells (SGCs). SGCs have properties similar to those of astrocytes in the central nervous system, including expression of cell specific enzymes, membrane receptors and cytoskeletal proteins and gap junctions that connect the four-six SGCs that surround a single neuron. Our interest has been in properties of the SGCs and their interactions with the sensory neurons and how these properties change in several mouse models of peripheral pain. One phenomenon that appears to be shared by many of the mouse pain models (temporomandibular joint injury, submandibular inflammation, nerve section, chemotherapy-induced pain, pelvic pain, systemic inflammation induced by LPS injection) is increased coupling among the SGCs in the peripheral ganglia. We will present studies in which we have used a variety of techniques to examine inflammatory orofacial pain in the trigeminal ganglia to illustrate techniques that are available as well as some of the probable underlying mechanisms.

For these studies, we injected Freund’s adjuvant into the submandibular skin, causing transient inflammation but triggering a chronic orofacial tactile hypersensitivity as evaluated by von Frey filament threshold testing. Hypersensitivity was reversed by systemic injection of the Panx1/gap junction blockers mefloquine or carbenoxolone at 1 wk (peak inflammation) and at 4 wks after CFA-injection, suggesting the contribution of these channels to hypersensitivity. The functional presence of the P2X7R-Panx1 complex in TG was demonstrated by BzATP-induced YoPro uptake into neurons and glia of TG cultures, which was prevented by mefloquine. Moreover, intact TG of CFA-injected mice showed more ATP release. Immunostaining of TG revealed higher Panx1 expression at 1 wk after CFA-injection compared to controls. Development of hypersensitivity was prevented in P2X7R-null and Panx1-null mice, and was attenuated in mice with glia-specific deletion of Panx1 (GFAPcre:Panx1f/f) but not with neuron-specific deletion (NFHcre:Panx1f/f), emphasizing the importance of glial Panx1 signaling in pain. Further, Ca2+ imaging studies using GCaMP3 mice demonstrated increased glial-neuronal Ca2+ signaling in TG under orofacial pain, which was mediated by P2Rs and Panx1, and to some extent by gap junctions. Our results show that the P2X7R-Panx1 complex likely plays a major role in signaling events contributing to tactile hypersensitivity.
Endoplasmic Reticulum Calcium Handling in Osteocyte Mechanobiology

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Osteocytes exhibit spike-like oscillations in cytosolic calcium in response to mechanical stimuli both in vitro and ex vivo, whereas osteoblasts demonstrate fewer, weaker responses. Previous inhibitor studies have implicated release of Ca\(^{2+}\) from endoplasmic reticulum (ER) stores as being critical to these multiple responses. Thus, the mechanisms of Ca\(^{2+}\) release and reuptake by the ER may be important to osteocyte mechanobiology. We sought to visualize the dynamics of calcium signaling within bone cells under fluid flow by simultaneously monitoring Ca\(^{2+}\) separately in the cytosolic ([Ca\(^{2+}\)\(_{\text{cyt}}\)]) and endoplasmic ([Ca\(^{2+}\)\(_{\text{ER}}\)]) spaces using an ER-targeted FRET calcium sensor, intracellular calcium indicator, and multi-emission microscopy (Fig.1). In osteocytes, elevations of [Ca\(^{2+}\)\(_{\text{cyt}}\)] coincided with depression of [Ca\(^{2+}\)\(_{\text{ER}}\)], with subsequent peaks occurring after recovery of [Ca\(^{2+}\)\(_{\text{ER}}\)] levels (Fig.2A). In osteoblasts, only a single [Ca\(^{2+}\)\(_{\text{cyt}}\)] response was observed, and while the ER contributed to this response, it did not refill in the time course of the experiment (Fig.2B). Previous studies also implicated T-type voltage sensitive channels (VSCC) in regulating osteocyte calcium oscillations. In osteocytes, T-type inhibition caused the ER to deplete faster than untreated controls, suggesting T-type channels are involved in ER calcium release. Under flow, OCY pretreated with the inhibitor demonstrated fewer [Ca\(^{2+}\)\(_{\text{cyt}}\)] spikes, corresponding to delayed recovery of [Ca\(^{2+}\)\(_{\text{ER}}\)] (Fig.2C). These studies demonstrate the ER is more intimately involved in osteocyte calcium signaling than previously considered and support the hypothesis that osteocytes are capable of generating characteristic multiple responses by an ability to refill the ER stores, possibly through interaction with T-type VSCC.

Figure 2. Quadview beamsplitter allows for simultaneous imaging of intracellular indicator (red) and FRET sensor (YFP, CFP)

Figure 1. Simultaneous monitoring of cytosolic and ER Ca\(^{2+}\) levels under flow in (A) osteocytes, (B) osteoblasts, and (C) osteocytes pre-treated with the T-type inhibitor NNC 55-0396
Dynamic Fluid Flow Stimulation Induced Mechanobiological Modulation of
In Situ Osteocytic Ca\textsuperscript{2+} Oscillations in an Intact Mouse Femur

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Current clinical evidences point to the critical role of mechanotransduction in various bone processes. As osteocytes are suggested as the major mechanosensors in bone, this study aimed to visualize and quantify calcium responses of in situ osteocytes in response to dynamic fluid flow stimulation into intact ex vivo mouse femurs. Fresh mouse femurs were stained with Fluo-8 AM. Dynamic fluid flow loading of 1Hz, 5Hz, 10Hz, and 20Hz was introduced to each sample via a 24-gauge catheter connected to a fluid-filled syringe pump that was controlled by a function generator set at a constant voltage. Real-time confocal imaging was performed to capture the osteocytic calcium signals. Dynamic fluid flow stimulation at 1Hz did not induce any calcium response within the osteocytes. However, 5Hz, 10Hz, and 20Hz of loading lead to 25%, 93%, and 52% of responsive cells, respectively. Loading at 5 Hz, 10 Hz, and 20Hz lead to 1.4, 2.6, and 3.0 spikes, respectively. Loading at 10Hz lead to 5% and 7% greater calcium spike magnitude compared to 5Hz and 20Hz of loading, respectively, and exhibited 65% and 11% shorter calcium initiation time compared to 5 Hz and 20 Hz of loading, respectively. Osteocytes displayed calcium spikes in response to in situ dynamic fluid flow stimulation into intact mouse femur in a frequency dependent manner, where optimized at 10Hz of loading. This study advances our better understanding of bone mechanotransduction in response to dynamic fluid flow stimulation, which may lead to great insights into current clinical challenges.
Abstract

* [Ca\textsuperscript{2+}]\textsubscript{i} and Actin Dynamics in Osteocytes in Intact Mouse Tibiae under Cyclic Mechanical Loading

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Osteocytes are regarded as the major mechanosensors in bone due to their arrangement as an extensive cell network in the lacunar-canalicular system. Previous studies of [Ca\textsuperscript{2+}]\textsubscript{i} signaling in bone cell networks in vitro have revealed that osteocytes exhibit robust and repetitive [Ca\textsuperscript{2+}]\textsubscript{i} spikes under fluid flow stimulation [1], which have been confirmed in ex vivo osteocytes in response to cyclic mechanical loading [2]. Single-cell studies demonstrate that [Ca\textsuperscript{2+}]\textsubscript{i} peaks are accompanied by contraction of the actin network [3]. Thus, we aim to examine the role of [Ca\textsuperscript{2+}]\textsubscript{i} in actin contractions in ex vivo osteocytes under cyclic mechanical loading. Bilateral tibiae from Lifeact-mRFPruby mice [4,5] were dissected immediately after euthanasia, incubated in cell culture medium for 2-4 hours, then dyed with 15 µM Fluo-8 AM. Both [Ca\textsuperscript{2+}]\textsubscript{i} and actin network dynamics of the ex vivo osteocytes were recorded simultaneously in response to ATP, ionomycin, and cyclic mechanical loading using confocal microscopy. A custom-built mechanical loading system applied a load ramp of 0.5-s duration to a peak load of 8-N, followed by a symmetric unload ramp. A dwell time of 5s was applied between cycles for image acquisition. A decrease in actin network strain was observed immediately following [Ca\textsuperscript{2+}]\textsubscript{i} responses to ATP and ionomycin, which are interpreted as whole-cell contractions. Most osteocytes demonstrated repetitive [Ca\textsuperscript{2+}]\textsubscript{i} responses under cyclic mechanical loading. Of these responsive cells, 41.2% also exhibited a decrease in strain. On average, the magnitude of incremental contractile strain was 1.8%. Our findings confirm that oscillatory [Ca\textsuperscript{2+}]\textsubscript{i} responses in osteocytes under mechanical loading precede whole-cell contractions. These two processes may serve as a mechanotransduction mechanism of osteocytes with relevance in bone remodeling and clinically related bone diseases, such as osteoporosis and age-related bone fractures.


Figure 1. (A) Ex vivo osteocytes expressing Lifeact-mRFP (red) and dyed with Fluo-8 (green); (B) Custom ex vivo mechanical loading system

Figure 2. Actin cytoskeleton strains and [Ca\textsuperscript{2+}]\textsubscript{i} over time in response to ATP (top), ionomycin (middle), and mechanical loading (bottom).
Elevation of Glucose to Levels Associated with Type 1 Diabetes Profoundly Diminished Bone Cell Mechanosignaling

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Patients with insulin dependent diabetes mellitus (IDDM; Type 1 diabetes) manifest a range of bone problems, including increased risk for bone fractures and defective bone healing. However, the underlying mechanisms are still not well understood. Given that the maintenance of skeletal integrity in response to daily physical activity relies on the regulation of the bone forming cells, osteoblasts, and the primary load sensing cells, osteocytes, we hypothesized that bone loss in Type 1 diabetes is primarily triggered by exposure to high glucose levels which in turn compromises ability of bone cells to perceive and respond to mechanical loading as a result of altered expression of mechanosignaling mediators: specific purinergic receptor (P2Rs) and pannexin 1 (Panx1) channels. To simulate the extracellular glucose levels to which bone cells are exposed in healthy vs. diabetic bones in vitro, we cultured MOB-C osteoblasts and MLO-Y4 osteocytes for 10 days in medium (α-MEM) containing 1 or 4.5 g/L glucose. Changes in the expression of P2Rs and Panx1 were determined using Western Blot analysis. Cells were subjected to oscillatory fluid shear stress (OFSS; τ = ± 10 dyne/cm²) at 1 Hz in μ-Slide VI0.4 chambers (ibidi). We used the ratiometric Ca²⁺ indicator (Fura-2 AM) to image OFSS-induced changes in intracellular Ca²⁺ levels. ATP concentration in each flow sample was measured using the Luciferin-luciferase bioluminescence assay. Cells were also exposed to selective agonists (BzATP, UTP) to evaluate the effects of high glucose (HG) on function of each P2R subtype. The Akita mouse model of Type 1 diabetes (C57BL/6J-Ins2Akita) was used in parallel to evaluate the effects of HG on bone integrity and mechanosignaling system in situ. Micro-CT analysis from male Akita (16-week old) and age- and sex-matched wildtype (WT) femurs revealed reduced diaphyseal cross sectional size in Akita compared to WT mice. Immunoblots of protein harvested from long bones of Akita (8-week old) and age-matched WT mice and in vitro cultures indicated altered expression of P2R (specifically P2Y₂R, P2Y₄R and P2X₇R) and Panx1 expression in diabetic compared to controls. Moreover, HG exposure blunted normal OFSS-induced Ca²⁺ and ATP signaling. Our findings indicate that Type 1 diabetes impairs the proper response of bone cells to mechanical loads, which is required to maintain bone health.
Remodeling of Distribution of Elastic Modulus Gradients as Predictors of Early Stage Osteopenia

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The objective of this study is to investigate remodeling in cortical bone due to functional unloading. Left tibial bone samples were obtained from 5-month old virgin female Sprague Dawley rats, including 1) baseline control (n=9), and 2) hind limb suspended (HLS) (4 weeks, n=9). 2mm segments were cut from the mid-shaft in transverse plane and elastic modulus measured by Nano indentation (Hysitron, Minneapolis). Three additional control rats were sacrificed and hind limbs axially loaded, from knee to the foot joint, (Bose, Minneapolis) using 6-10N at 1Hz. Strains at three rosette strain gauge sites were measured. Bones were then scanned in micro-CT scanner at 36μm resolution. Using linear beam theory MATLAB codes were built to find planar strain distribution. Results: Elastic moduli and strain gradients from periosteum to endosteum are much higher in the anterior and posterior regions (2.6GPa, 45µε) than the medial region (0.2GPa, 5µε), in control group (Fig1, 2). Correlation between elastic modulus and strain gradient is significant (r^2>0.95), Fig 3. In disuse group, however, the elastic modulus gradient in the anterior posterior regions reduced to 1.2GPa and increased in the medial, 2.7GPa (Fig 4). Presence of elastic modulus gradient in direction of strain gradient (r^2>0.95) in control shows that material property of bone is strongly influenced by overall strain magnitude. Under disuse condition the modulus gradient is decreased between different regions, implying progressive bone remodeling. It is suggested that variations in micro-mechanical gradients among different regions can serve as a predictor for early stage of osteopenia.
Creep Properties of Bone and its Relation to Bone Mineral Content

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Introduction: Bone is known to be viscoelastic, however, there is very little data showing how the viscoelastic properties vary at different levels of mineralization. In order to optimize analytical and numerical models of bone tissue, this study aims to characterize the creep properties of bone as a function of bone mineral content (BMC). Methods: Creep tests were performed on cortical bone specimens that were obtained from the proximal diaphysis of fresh human tibiae. After failure, the BMC was calculated from the ratio of the mineral weight to the dry weight. The steady-state creep rates were calculated from the slope of the central linear region in the creep curves. A power relationship was fit to the steady-state creep rate \( \dot{\varepsilon}_{ss} = \alpha \sigma^\beta \), \( \dot{\varepsilon}_{ss} \) is the steady-state creep rate, \( \alpha \) and \( \beta \) are the exponential parameters, \( \sigma \) is the applied stress) and a regression analysis was performed to see how the mineral content affects the exponential parameters. Results: Viscoelastic creep properties were studied as a function of bone mineral content. There was a positive correlation between the steady-state creep parameter \( \beta \) (n=5, R=0.9106, p<0.05) and a negative correlation between the steady-state creep parameter log(\( \alpha \)) (n=5, R= -0.9504, p<0.02) as a function of mineral content (Figure 1). Discussion: These viscoelastic parameters can be used in finite element models to predict the response of bone tissue and orthopedic implants in a wide variety of applications that involve situations where the bone is subjected to loads for long durations.

Figure 1: The steady-state creep parameters show a significant change with BMC.
Abstract

Local Bone Tissue Mechanical Properties Change Without Remodeling: A Study Of Lactating Mice

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Introduction: Osteocytes can dynamically remodel their pericellular bone matrix through osteocytic osteolysis, leading to increases in lacunar and canalicular size [1,2]. Furthermore, these increases are reversible. It is well established that cortical bone modulus is strongly and inversely coupled to its vascular (Harvesian) porosity [3,4], but whether changes in osteocyte pericellular void space can also alter local material properties of bone is unknown. In the current study, we tested whether bone elastic modulus and lacunar-canaliculal space (LCS) change at the microscopic level in response to lactation and post-lactation recovery. Methods: Experimental design: Under IACUC approval, C57/B6 mice (n=15, 3 m.o.) were mated and allowed to nurse their pups. After 2 wks of lactation (Lac), mice were divided into three groups. Group 1 was sacrificed after 2 wks Lac; Groups 2 and 3 were allowed to recover for 1 wk and 4 wk after forced-weaning, respectively. Age-matched controls were also examined. Femurs were stored at -20C until testing. MECHANICAL TESTING: We measured the elastic modulus of bone at the microscopic level to eliminate potential effects of changes in the larger vascular spaces. Three 1 mm thick transverse sections were cut from the mid-diaphysis, affixed to plastic slides and surface polished with graded carborundum paper and diamond abrasives to achieve a 0.25 µm finish. Bones were kept hydrated using Ca2+ supplemented PBS during sectioning, polishing and microindentation testing. Anterior (A) and posterior (P) quadrants of tibial cross-sections were analyzed. Indentation Hardness (Hv) and Elastic Modulus (Ei) were measured by using a Wilson microindenter with Vickers diamond micro-indenter tip. Indent tests were performed only within the mid-cortex to avoid edge effects from the periosteal and endocortical surfaces. Six indents (deadweight = 50gf applied, dwell time = 10s) were applied within each anatomical region of a bone section. Hv values were measured directly from individual indent profiles at 400x magnification. Ei values were calculated by using the linear regression approach described by Currey and Brear [5]. OSTEOCYTE LCS: Whole femurs were stained with basic fuchsin and embedded into PMMA. Femoral 90 µm thick cross-sections were imaged using super resolution microscopy (100 nm resolution fluorescence imaging) and lacunar and canalicular areas measured using Image J. Statistical analysis: Differences in Ei values among groups were tested with a Kruskal-Wallis ANOVA test and post-hoc comparisons were performed with the Mann-Whitney U test. Results: Two weeks of Lac caused marked reductions in bone Ei (10% and 15% at Posterior and Anterior regions, respectively) compared to controls. After 1 wk post-Lac, Ei returned to pre-Lac levels and did not increase further at 4 wks post-Lac. (Fig 1). Lac did not cause intracortical resorption or increases in vascular spaces. Lacunar and canalicular areas increased by 2 wks Lac (19% and 15%, respectively) and returned to baseline levels after 1 wk post-Lac (Fig 2). Conclusion: These results reveal that tissue-level cortical bone material properties are reversibly modulated in response to physiological challenge without bone remodeling. Small changes in LCS appear sufficient to alter local material. In addition, these studies demonstrate that Lac-induced reductions in Ei and increased in LCS void space reverse quickly once Lac stops. The precise mechanisms underlying osteocytic osteolysis and lacunar/canalicular are as yet unknown. Nevertheless, our studies reveal that bone possesses an intrinsic ability for rapid and dynamic regulation of its material properties, accomplished by the action of osteocytes. References: 1) Qing et al, J Bone Miner Res 27:1018, 2012; 2)Wysolmerski, Bone 54:230,2013; 3)Martin, Burr, Sharkey, Skeletal Tissue Biomechanics, 1998; 4)Cardoso et al, J Biomech 46:253-65,2013; 5) Seref-Ferlengez et al, J Bone Miner Res 2014
Characterization of Damage Mechanisms Associated with Reference Point Indentation in Human Bone

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Measurement of bone mineral density (BMD) is the clinical gold standard in situations of compromised skeletal integrity, such as osteoporosis. BMD, however, is somewhat limited since it cannot directly assess post-yield properties, an ability that would be a significant advance in the field. Reference point indentation (RPI) is a recently-developed technology which has been designed to achieve this goal. While RPI appears to be useful in detecting altered tissue properties, the underlying physical mechanism has not been fully characterized. We designed an experiment whereby the contribution of test cycle number and test load level to RPI test-induced damage were characterized and quantified. Twenty-four standardized rectangular specimens were prepared from cadaveric human tibiae. A custom-built rig was fabricated to allow for accurate positioning and mapping of indentation sites. For testing, cycle number was increased from 0–20 in increments of 5 cycles (8 N, 2 Hz), and, in a second study, load level was increased from 0–10 N in increments of 2 N (20 cycles, 2 Hz each). Specimens were subsequently processed for histological analysis and damage quantification. These revealed a consistent microdamage pattern underneath the indentation site with evidence of damage via compaction. Quantification of damage zones indicate that radial damage area increased with cycle number while damage in the direction of loading was established early in the test, and progressed relatively little. RPI forms predictable mechanical and histological patterns in human cortical tibial bone. These data help to facilitate RPI’s use as a clinical diagnostic tool.
In Situ Osteocyte Modulation of β₃ Integrin in Response to Hindlimb Unloading

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β₃ integrin (β₃) adhesions linking osteocyte (OCY) processes to canalicular walls appear to be critical sites of mechanotransduction. However, the structure and behavior of these complexes in situ are incompletely understood. We used Structured Illumination Microscopy (SIM), providing 100nm fluorescence resolution, to i) characterize the number and distribution of β₃ foci along OCY processes in situ, ii) test whether the foci contained vinculin (Vin), as a marker for the cytoskeletal linking proteins found in typical focal adhesions, and iii) determine whether the foci are altered due to changes in mechanical loading. Bones of C57Bl/6 mice (4 m.o. male, N=6/grp) subjected to hindlimb unloading (HLU) for 5 or 14 days or left as cage controls were studied. Fixed and demineralized femoral mid-diaphyseal cross sections (5mm) were double stained for β₃ and Vin, and images acquired via SIM. 10 OCYS/animal were analyzed with ImageJ for β₃ cluster number & size in 3 concentric areas (L1-L3) extending 0.5, 2.5 and 3.5mm from the OCY lacunar margins. ANOVA & post hoc Mann-Whitney U-test were used to compare data by distance from cell bodies and by unloading group. In control animals, β₃ staining was punctate and localized in cell processes as previously observed (Fig 1). Focal number and size were highest in L1, closest to the OCY bodies, and 70% and 50%, respectively, in L3 (Fig 2). Vin staining was also punctate, but located exclusively in OCY cell bodies, consistent with earlier findings for paxillin (Fig 1). HLU reduced the number of β₃ foci after 5 and 14 days (by over 50% in L2, p<0.05), but did not alter their size. In contrast, the area stained by Vin was unchanged with HLU, though Vin focal size was reduced at 14 days (Fig3). These results show that β₃ adhesion foci in OCY processes are not typical focal adhesions, and suggest that β₃ mechanotransduction in these sites must be mediated by non-cytoskeletal pathways like ion channels. In addition, β₃ foci in situ are dynamic, changing markedly in response to mechanical unloading while Vin foci in the cell body do not. Whether these changes are reversible with remobilization remain to be determined. Acknowledgements: Grants AR041210, AR057139 and AR060445 from NIAMS (MBS) & NASA NNX08BA35G (SJ)
Sclerostin Antibody Administration Activated Bone Formation in Ovariectomized Rats with Concurrent Mechanical Unloading

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Severe bone loss occurs when postmenopausal osteoporosis patients had concurrent immobilization and it is challenging condition to manage. Sclerostin antibody (Scl-Ab) increased bone formation and bone mass, and decreased bone resorption in postmenopausal osteoporosis. The current study was to examine the effect of Scl-Ab in mitigating the conditions in ovariectomized (OVX) rats with concurrent hindlimb suspension (HLS). 4-month-old female SD rats were used (n=11). HLS was introduced 2 weeks after sham and OVX. Scl-Ab (25 mg/kg) or vehicle was injected sc twice weekly for 5 weeks starting at the time of HLS. Histomorphometry analyses were performed at metaphyseal trabecular bone of the distal femurs. OVX alone, but not HLS resulted in significant decrease in BV/TV (-36%) vs control. HLS+OVX showed more bone loss than OVX alone. Scl-Ab significantly increased bone mass in all three conditions (HLS, OVX alone and OVX with concurrent HLS) with relative BV/TV increase of 37%, 71% and 87% compared with respective controls (Fig. 1 & 2a). Similar trend was observed in Tb.Th (Fig. 1 & 2b). Trabecular mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) were significantly increased in Scl-Ab treated rats (HLS alone, OVX alone and HLS+OVX) compared with respective controls. In summary, Scl-Ab prevented trabecular bone loss in OVX rats with concurrent HLS. Histomorphometric analyses reveal that Scl-Ab promoted bone formation in OVX rat model with concurrent HLS. The data suggest that sclerostin antibody represents a promising therapeutic approach for osteoporosis induced by estrogen deficiency with concurrent mechanical unloading.

Fig. 1. Representative images of calcein labeled trabecular bone histomorphometry in distal region of femur. a) sham control, b) Sham+HLS+Veh, c) Sham+HLS+Scl-Ab, d) OVX+Veh, e) OVX+Scl-Ab, f) OVX+HLS+Veh and g) OVX+HLS+Scl-Ab.

Fig. 2. a) Graphs show mean+SD values for histomorphometric bone volume fraction (aBV/TV). OVX, and HLS plus OVX showed significant decrease of aBV/TV in comparison to sham control. Scl-Ab significantly increased bone mass vs. the respective controls, i.e., vs. HLS alone(*), OVX alone(^), and HLS+OVX(&). b) Similar trend in (a) was found in the mean+SD values for trabecular thickness (aTb.Th). c), d), e) mean+SD values for Histomorphometric MS/BS, MAR, BFR/BS respectively. Scl-Ab increased bone formation rate against respective controls, i.e., vs. HLS alone(*), OVX alone(^), and HLS+OVX(&). (Overall, *p<0.05 vs. Sham+Veh; ^p<0.05 vs. Sham+HLS+Veh; &p<0.05 vs. OVX+Veh; #:p<0.05 vs. OVX+HLS+Veh)
Abstract

Green tea polyphenol treatment is chondroprotective, anti-inflammatory and palliative in a mouse post-traumatic osteoarthritis model

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Osteoarthritis (OA) is a joint disease that affects more than 27 million Americans and a leading cause of pain and disability that currently has no cure. EGCG (epigallocatechin 3-gallate), a major bioactive polyphenol present in green tea, exerts numerous health promoting effects to counteract inflammation, aging, and cancer. Of note, EGCG has been reported to attenuate inflammation and catabolic activity in chondrocytes in vitro, and exert chondroprotection in rheumatoid arthritis animal models. However, whether EGCG may alter OA progression in vivo and improve OA-related symptoms, especially pain, has not been reported. In this study, we used a post-traumatic OA mouse model (destabilization of the medial meniscus, DMM) to test whether daily EGCG administration could slow the progression of OA and relieve OA-associated pain and to examine possible mechanisms underlying its chondroprotective actions. Eight weeks after DMM surgery, articular cartilage in EGCG-treated mice exhibited less Safranin O loss and cartilage erosion, and a lower OA histologic score (2.1±1.6) compared to vehicle-treated controls (6.5±3.2, p<0.05), which was associated with reduced immunohistochemical staining for cleavage of cartilage extracellular matrix components aggrecan and type II collagen, and reduced staining for proteolytic enzymes MMP-13 and ADAMTS5. Compared to vehicle controls, mice treated with EGCG exhibited reduced OA-associated pain, as indicated by higher locomotor behavior (i.e., distance traveled and rearing), and lower sensitivity towards mechanical stimuli. This study provides the first evidence in an OA animal model that EGCG significantly slows OA disease progression and exerts a palliative effect.
**Abstract**

CITED2 mediates a novel chondroprotective pathway involving cross-talk between mechanical loading and IL-4 to suppress MMP-13

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The biomechanical environment is critical for cartilage homeostasis. Previous studies suggest that anti-inflammatory cytokine IL-4 expressed in chondrocytes exerts a chondroprotective effect, although high doses may result in adverse effects. CITED2 is a multiple stimuli response transcriptional regulator, which was originally identified as an IL-4-inducible molecule in T-lymphocytes and a critical mediator that down-regulates MMPs during moderate loading. By testing the hypothesis that CITED2 in response to IL-4 and mechanical loading would result in a synergistic effect on chondroprotection, we found that while both moderate loading (2.5% uniaxial strain, 1Hz, 1 hr) and IL-4 (1ng/ml) induces CITED2 expression, combined treatment of IL-4 (1ng/ml) and loading elevated CITED2 expression to a level similar to that achieved with a high dose of IL-4 (10ng/ml). As a chondroprotective outcome, expression of proteolytic enzyme MMP-13 was downregulated most with this combination of treatment compared to each individual treatment. In vivo, moderate treadmill running (10m/min for 45 min) reduced Mmp-13 mRNA levels in knee articular cartilage of wild type mice. This reduction was partially abolished in IL-4 knockout mice and completely abolished in cartilage when Cited2 expression was knocked down by intra-articular injection of Cited2 siRNA in knee joints. Our studies suggest: 1) IL-4 and mechanical loading collaboratively induce CITED2. 2) A synergetic anti-inflammatory effect (i.e., suppressing MMP-13) in chondrocytes can be reached by combined treatment of IL-4 at a low dose and moderate mechanical loading, a potential new intervention strategy in cartilage protection while avoiding adverse effects of high doses of IL-4.
Infrapatellar fat pad (IPFP)-derived inflammatory response and articular cartilage degradation: a novel pathway for chondroprotective effects of CITED2 and exercise

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Obesity generates a chronic, low-grade inflammation that contributes to cartilage degeneration. The infrapatellar fat pad (IPFP) is an adipose tissue located within the synovial capsule of the knee joint. Recent studies suggested IPFP exerts catabolic effects to cartilage homeostasis by secretion of pro-inflammatory adipokines/cytokine. CITED2, a mechanical-inducible chondroprotective transcriptional regulator, is also known to interact with several adipogenesis-related transcription factors, and its deficiency (i.e. during aging) is associated with altered adipogenesis of mesenchymal stem cells. In this study, we found 1) Cited2+/− mice gained 34% more weight over wild-type littermates after 10 weeks on a high fat diet, suggesting a regulatory role of Cited2 in fat metabolism. 2) Cited2+/− mice exhibited an altered adipo-related gene expression profile in the IPFP, with similar trends to those seen in wild-type WT mice on a high fat diet. We further found treadmill running led to suppressed expression of adipokines, adipo-regulatory transcription factors, and proteolytic enzymes including adipsin, leptin, C/EBPα, MMP-13, and ADAMTS5 in the IPFP in WT mice, while the suppression was largely not observed in IPFP of Cited2+/− mice. Furthermore, IPFP from WT, but not Cited2+/− mice subjected to treadmill running markedly inhibited the expression of proteolytic enzymes in co-cultured articular cartilage from separate naïve WT mice. The finding of a novel role that CITED2 plays in adipokine regulation and in mediating loading exerted anti-inflammatory effects in fat tissue such as IPFP may provide a potential new concept and intervention strategy for controlling obesity, inflammation and for the maintenance of cartilage integrity.
CITED2 is a principal regulator of telomerase expression, a representative feature of a functionally active tendon stem progenitor cell population

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Adult stem cells are cellular populations that reside in specified niches of a given tissue, with regenerative capacities to repair tissue damage. Efficient stem cell self-renewal is dependent on proper telomere maintenance mechanisms, regulated by telomerase activity. Telomerase reverse transcriptase (TERT), one of the two components of telomerase, has recently been detected in adult stem cell populations within high-turnover tissues. However, whether tendon, a low-turnover tissue, contains a stem/progenitor cell population expressing TERT, and whether the function of such stem cells are altered during aging are unknown. Real-time PCR analysis of TSPCs isolated from laser captured stem cells in human tendon tissue \textit{in situ} sections revealed: 1) TERT expresses in human tendon stem/progenitor cells (TSPCs), 2) expression of TERT was significantly lower in aged TSPCs compared to young TSPCs, which was associated with the down-regulation of transcription regulator CITED2, stem cell marker Oct4, and stem cell renewal regulator Bmi1, and associated with the up-regulation of stem cell proliferation inhibitor gene p16\textsuperscript{INK4a} and senescence-associated gene FucA1. Furthermore, we found TERT expression in TSPCs derived from haploinsufficient Cited+/- mice was also significantly reduced compared to that in the wild-type littermates and the expression levels of Bmi1, Oct4, p16\textsuperscript{INK4a} and FucA1 in wild-type vs. Cited2 +/- all mirrored those observed between young and aged TSPCs in human. These findings provide new insight regarding reparability of tendon tissue and may shed light into novel strategies for tendon repair and regeneration by enhancing the pool and activity of this innate population of stem cells.
Objective: Cervical disc arthroplasty (CDA) was developed to treat cervical degenerated disc diseases with the advantages of preserving the kinematics of the functional spinal unit. However, the safety and reliability of multi-level CDA are still debated. It has shown unclear benefits in terms of clinical results, functional recovery, and incidence of complications. The purpose of this study is to estimate the effectiveness of multi-level cervical arthroplasty over single-level CDA for the treatment of cervical disc diseases. Methods: To compare the studies of multi-level CDA versus single-level CDA in patients with cervical spondylosis that reported at least one of the following outcomes: functionality, neck pain, arm pain, quality of life, reoperation and incidence of heterotopic ossification, electronic databases (Medline, Embase, Pubmed, Cochrane Central Register of Controlled Trials) were searched. No language restriction was used. Results: Out of eight studies that were included in the study, four were prospective clinical trials and the other four were retrospective. The results of the meta-analysis indicated that there was no significant difference in Neck Disability Index (NDI) scores, neck visual analog scale (VAS), arm VAS, morbidity of reoperation, heterotopic ossification, and parameters of living quality when comparing multi-level CDA with single-level CDA at one or two years follow-up postoperatively (p > 0.05). Conclusions: The meta-analysis revealed that the outcomes and functional recovery of patients performed with multi-level CDA are equivalent to those with single-level CDA, which suggests the multi-level CDA is as effective and safe as single-level invention for the treatment of cervical spondylosis.
Progranulin Inhibits Intervertebral Disc Degeneration in Aging Mice

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**Objective:** The study aimed examining progranulin’s (PGRN) expression in intervertebral disc (IVD) under physiological and pathological degenerative conditions, defining the role of PGRN in IVD degeneration in aging, and elucidating the signaling pathways involved. **Methods:** The samples of IVD came from murine and human subjects. We evaluated the expression of PGRN in IVD tissues using immunohistochemistry and western blotting. The degeneration of the IVD samples were analyzed by HE staining, Safranin O staining, TRAP staining, immunohistochemistry and µCT. The expressions of genes associated with cartilage degeneration, osteoblastogenesis and osteoclastogenesis were analyzed. We also analyzed the IVD samples from wildtype and PGRN-/- mice for the NF-κB and Wnt/β-catenin signaling pathways. **Results:** PGRN was detectable in both human and murine IVD. PGRN’s level was upregulated in murine IVD in the course of aging. PGRN-/- mice exhibited an accelerated formation of bony tissue in end plate and elevated activity of bone resorption in vertebra with aging. More severe destruction of cartilage was observed in IVD of PGRN-/- mice. Furthermore, deletion of PGRN resulted in altered expressions of the molecules known to be involved in cartilage degeneration, osteoblastogenesis and osteoclastogenesis. Additionally, the acceleration of IVD degeneration was probably due to the enhanced NF-κB and Wnt/β-catenin signaling seen in PGRN-deficient mice. **Conclusion:** PGRN plays a critical role in homeostasis of IVD, and the deficiency of PGRN leads to an acceleration in disc degeneration with aging. These findings show that PGRN is a potential molecular target for prevention and treatment of disc degenerative diseases.
Predicting Direction-Dependent Failure Properties of Human Trabecular Bone: Effects of Fabric Anisotropy, Poroelastic Ultrasound and Individual Trabecular Morphology

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Introduction: A decline in Bone Mineral Density (BMD) is a major factor indicative of possible bone fracture. BMD accounts for ~60% of the total variability in bone strength1, and predicts 40-70% of all fractures2. However, BMD does not fully correlate with improvements in fracture risk by anabolic or anti-resorptive drugs3-4. Additionally, BMD cannot explain why some patients with low BMD do not fracture and other patients with normal BMD do5-4. To more accurately determine fracture risk, we 1) systematically evaluated the effects of anisotropy, as explained by the fabric tensor, on the yield properties of trabecular bone. To precisely characterize fracture risk we 2) evaluated direction-dependent ultrasound to predict trabecular yield properties by considering poroelastic effects and fabric-anisotropy. Finally, we 3) investigated direction-dependent morphology to better understand individual trabeculae contribution to bone mass anisotropy. Methods: To evaluate the capability of fabric anisotropy and poroelastic ultrasound in predicting direction-dependent yield behavior in the human calcaneus trabecular bone, 651 voxel-based micro finite element (μFE) models in compression (n=7) and shear (n=7) were completed, and 217 finite difference time domain (FDTD) ultrasound simulations in transmission were evaluated (n=7). From μFE, compressive and shear stiffness, apparent yield stress and yield strain were calculated. From FDTD ultrasound, fast-wave (FW) and slow-wave (SW) speeds (SOS), velocities (VEL), and broad band attenuations (BUA) were assessed. Direction-dependent morphology was evaluated by decomposing 50 trabecular bone structures (n=10) into individual elements. Then, individual length (Tb.L), thickness (Tb.Th), volume (Tb.V) and number were integrated to create tensors representing apparent level anisotropy. Results: μFE indicated that fabric anisotropy was correlated with compressive stiffness (R²= 0.68) and compressive yield stress (R²= 0.66), but poorly correlated to compressive yield strain (R²= -0.33). Similarly, fabric anisotropy was correlated with shear-stiffness (R²=0.67) and the shear yield stress (R²=0.68), but poorly correlated to shear yield strain (R²=0.01). Ultrasound tests indicate that FW-SOS, SW-SOS, FW-VEL, SW-VEL, FW-BUA and SW-BUA strongly correlate with compressive stiffness and compressive yield stress (R²=0.83-0.95). Moreover, all ultrasound parameters weakly predicted yield strain (R²=0.30-0.45). μFE and FDTD shows that fabric and ultrasound strongly predicted anisotropic stiffness and yield stress, however, yield strains are independent of direction of loading or wave propagation. Morphological tensors indicated that Tb.L, Tb.Th, and Tb.V are transversely-anisotropic, and trabecular number is orthotropic. Results show that at specific direction morphology might have a unique mechanical response (e.g., buckling), independent of mass. Conclusion: For the first time, we demonstrated systematic correlations between fabric anisotropy and the direction-dependent failure of trabecular bone. Similarly, our results verified the ability of ultrasound to predict direction-dependent failure. These results allows for a more accurate bone-fracture assessment in the clinic via non-invasive methods.

Focal Injury in Pannexin 1 Knockout Mouse Yields Reduced RANKL Expression: Possible Mechanism Involving Pannexin 1 Channels for Osteoclast Recruitment?

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Osteocyte apoptosis is necessary to trigger osteoclast recruitment and resorption at bone microdamage sites (1). Recently, we found that healthy osteocytes nearby apoptotic osteocytes at a microdamage site express RANKL to promote osteoclastogenesis (2). How dying cells communicate to neighboring healthy osteocytes to produce osteoclastogenic signals is unknown. Recently, Pannexin 1 (PANX1) channels have been shown to mediate release of “find-me” signals from apoptotic cells to recruit phagocytes to clear apoptotic debris in ischemic heart and brain tissue (5, 6). We hypothesize that apoptotic osteocytes communicate to neighboring healthy osteocytes via PANX1 channels.

PANX1−/− mice (n=4) and Panx1+/+ mice (C57/Bl6, WT) were subjected to focal indent microinjury (7) to examine the effect of PANX1 deficiency on osteocyte RANKL expression. Tibiae were subjected to 5 N load for 10 cycles with a test probe to create a single focal microcrack at the indent apex. After 24 hours, bones were isolated, fixed and decalcified. Immunohistochemistry was performed on 5 µm sections, and were stained for RANKL and cleaved caspase-3 to assess RANKL expression and osteocyte apoptosis respectively. Number of RANKL and cleaved caspase-3 positive osteocytes was counted as a function of distance from the indent apex. Mann-Whitney U tests were used for statistical significance (p<0.05). We found increased number of apoptotic osteocytes near the focal microinjury site and increased RANKL expression in healthy osteocytes adjacent to microdamage in WT mice, consistent with previous studies (2). PANX1 deficiency abolished osteocyte RANKL expression in mice subjected to the same focal microinjury (p<0.05), despite no differences in number of apoptotic osteocytes between WT and PANX1−/− mice. These results indicate that the lack of osteocyte RANKL expression near microdamage sites in PANX1−/− mice is not due to differences in osteocyte death, but is mediated by communication between healthy and dying osteocytes via PANX1. The precise mechanism by which PANX1 channels mediate osteoclastogenic signaling associated with osteocyte apoptosis is unknown. However, PANX1 channels are implicated in nucleotide release during apoptosis (6,9), and ATP can upregulate RANKL expression in osteoclasts (10,11). These data suggest that PANX1 channels play a critical role in relaying “find-me” signals in bone tissue, which are necessary to activate osteoclast recruitment.
Knockdown of Heat Shock Protein 70 impairs Osteogenic Differentiation in Human Mesenchymal Stem Cell

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Introduction: Bone marrow derived human mesenchymal stem cells (hMSCs) have shown great potential for bone tissue-engineering applications. Heat shock protein (HSP) 70 is one of the highly conserved HSP family members in mammalian cells and plays an important role during development and repair under stress conditions via their chaperon functions. However the role of HSP70 in hMSC differentiation for tissue regeneration is not well studied and the mechanism for these multiple function is not entirely understood. Materials and Methods: Considering the potential toxicity and non-specificity of chemical inhibitor, we used target-specific and non-toxic HSP70 shRNAs, HSPA1A and HSPA1B, to knock down HSP70 expression in hMSCs and attempted to clarify the role of HSP70 during hMSC osteogenesis. Human MSCs with knocked down HSP70 in osteogenic cultures were exposed to mild heat shock at 39°C for one hour once every other day and a scramble shRNA was used for a control. Osteogenesis was assessed by alkaline phosphatase (ALP) activities, quantitative calcium deposition, and gene expression of osteogenic markers. Results and Discussion: Interruption of HSP70 expression in hMSCs significantly reduced ALP activities and calcium deposition during osteogenesis with or without heat shock. We further showed that significantly decreased Runx2 and Osterix expression levels during the osteogenic differentiation of hMSCs accompanied this inhibition. Conclusions: These data suggests that HSP70 plays a key role in hMSCs osteogenesis and this study would help elucidate the mechanism of thermal-enhanced bone growth.
In Vitro Growth Trajectory and In Vivo Implantation of a Cell-Based Disc-like Angle Ply Structure for Total Disc Replacement

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To treat end stage disc disease, we have developed an engineered disc for total disc replacement that replicates the hierarchical structure of the native tissue. This engineered disc consists of an aligned electrospun nanofibrous scaffold annulus fibrosus (AF) and a hydrogel-based nucleus pulposus (NP); combined they form a disc-like angle ply structure (DAPS). When seeded with cells, these composites increase in compositional and functional properties with time in \textit{in vitro} culture [1]. In this study we evaluated the long-term \textit{in vitro} maturation of DAPS seeded with either native AF and NP cells or with mesenchymal stem cells (MSCs) to establish a growth trajectory and used a rat tail disc replacement model [1] to determine if a cell-seeded DAPS can integrate into the rat caudal disc space.

DAPS seeded with either native disc cells or MSCs mature compositionally with time in \textit{in vitro} culture and approached rat caudal and human lumbar disc compositional and functional benchmarks by 15 weeks. When pre-matured DAPS seeded with native disc cells were implanted into the rat caudal spine, they remained in the disc space, retained their morphological features, and showed signs of integration with surrounding native tissue structures. Loss of proteoglycan in the NP region was evident, suggesting that it may be necessary to deliver factors \textit{in vivo} to sustain the phenotypic production of ECM in this region. Taken together, our data support the continued translation of a cell-based disc-like angle ply structure for the replacement of severely degenerated intervertebral discs.
TLR4 Inhibition Mitigates Inflammatory Induced Biophysical Changes of Nucleus Pulposus Cells

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Nucleus pulposus (NP) cells within the intervertebral disc (IVD) are mechano-sensitive. We have previously shown that inflammatory stimulation associated with degeneration, including activation of the Toll-Like Receptor 4 (TLR4) pathway increases cell size and biophysical properties of NP cells. In the current study, we examined the effect of TLR4 inhibition on mitigation of inflammatory induced biophysical changes of NP cells using the small molecule inhibitor TAK-242. We hypothesize that inhibition of the TLR4 pathway by TAK-242 will prevent changes in mechanobiology of NP cells induced by inflammatory stimulation with LPS or TNFα. TAK-242 was found to decrease LPS- and TNF-induced inflammatory mediators in a dose dependant manner. Inflammatory stimulation was once again confirmed to increase the hydraulic permeability (Lp) of cells. Treatment of cells with TAK-242 inhibited the inflammatory induced changes in Lp, returning Lp down to baseline levels. We also examined changes in cytoskeleton elements (F- and G-actin, vimentin, tubulin), and cell nucleus morphology to further explore the mechanobiological implications. Our findings indicate that LPS stimulation alters cell and nuclear size, and alters the cytoskeleton, via actin depolymerisation and thickening of the vimentin network. The understanding of the NP cellular behaviour under inflammation is crucial to determine the effect on their biomechanical properties and response to loading. Alterations in the cytoskeleton architecture directly affect cell morphology and capacity for load bearing. Our findings also suggest that blocking pathways of the innate immune system may protect the mechanobiological function of NP cells from inflammatory induced changes associated with degeneration.
Effects of Depth of Annular Injury and Tumor Necrosis Factor-alpha on Disc Degeneration and Pain

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Disc injury and inflammation may play an important role in discogenic pain, and this study aimed to determine the effects of depth of annular injury and TNFα on disc degeneration and pain. Degenerative changes were induced in rat lumbar discs via complete (3mm) or incomplete (1.5mm) annular tear followed by saline or TNFα injections. Sham surgery without puncture or injection and naïve (without surgery) groups were included. Pain in rats was assessed using hindpaw mechanical hyperalgesia tests, while severity of disc degeneration was determined using radiographic disc height, postmortem MRI and histology. Annular injury with intradiscal injections induced continuous decreases in paw withdrawal threshold and disc height. Complete annular tear induced more obvious change in disc height, and also exhibited more degenerative changes in MRI and histology. However, there was no association between pain sensitivity and severity of disc degeneration. Lower withdrawal threshold was found after TNFα injections compared to PBS injections. The findings show that annular puncture with intradiscal injection induced behavioral and structural changes representative of discogenic pain. The extent of disc injury is the most important determinant of disc degeneration, which might result from loss of annular integrity and reduced GAG. The inflammatory state of the spine may be more relevant to discogenic back pain, and pro-inflammatory cytokines (e.g. TNFα) may play an important role in the initiation of discogenic pain. These discogenic pain models with different severities of degeneration have significance for their use understanding mechanisms of discogenic pain and for screening future treatment modalities.

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ADAMTS 18 is Important Regulator of Post-Natal Skeletal Development and Bone Remodeling.

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ADAMTS 18 (a distintegrin and metalloproteinase with a thrombospondin type motif, AD18) is a member of a secreted Zn-metalloproteinase ADAMTS family. AD18 was identified as bone mass determination factor and associated with hip bone fractures in major human ethnic groups in a genome-wide association study (GWAS). The purpose of this project is to determine the role of ADAMTS 18 in skeletal development and remodeling. The growth and weight curve for AD18−/− mice were recorded for 3 months and injected with calcein and alizarin red for histomorphometry analysis. Mice were euthanized at age of 3 months, trabecular bone at proximal tibia was analyzed using microCT and mechanical properties were accessed with four-point bending at femoral mid-diaphysis. To understand the underlying mechanism microarray studies were conducted and confirmed using real time PCR. The phenotypic analysis of AD18−/− confirmed smaller structure of KO mice relative to litter mates at 14 weeks. AD18−/− mice exhibit smaller tibia and growth plates with significantly compromised trabecular bone quality and quantity. Cortical bone does not show significant difference but mechanical testing show significant reduction in bone strength indicating potential disorientation of collagen fibers in bone matrix. AD18 deletion significantly inhibited genes associated with osteogenesis and activated genes associated osteoclastogensis. Over-expression of AD18 confirmed increased Ssh1, Prrx 1, Runx 2 and Osterix and inhibited osteoclastogensis associated genes Lair 1 and siglec 15 in RANK ligand. In summary, the data from current study strongly implies that AD18 is an important and novel regulator of bone remodeling.
Are Trabecular Length and Trabecular Thickness Able to Predict Fabric and Anisotropic Elastic and Inelastic Mechanical Behavior of Trabecular Bone In Compression and Shear Tests?

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Trabecular bone adapts its porosity, microarchitecture and tissue composition in response to its mechanical environment. Such variations are related to changes in bone mechanical function and competence to resist fracture. Analytical relationships between orthotropic elastic constants and bone architecture (bone volume fraction and fabric) have been previously defined [1, 2]. However, the effect of microarchitectural parameters such as trabecular thickness (Tb.Th) and length (Tb.L) on the mechanical properties of trabecular bone remains to be fully examined. Specifically, our hypothesis is that Tb.Th and Tb.L can explain directional-dependent changes in fabric and anisotropic trabecular bone elastic and inelastic behaviors. Tridimensional equivalent trabecular models were built by using for Tb.Th and Tb.L values obtained from µCT images [3, 4]. The parametric structures were tested in pure compression and shear using finite element method. The apparent module of elasticity and the yield stresses and strains in compression and shear were quantified from the stress-strain curve [5]. Results confirm porosity as the main predictor of trabecular bone mechanical behavior. However, the correlation coefficient between anisotropic elastic constants and yield stress and strain components increases when porosity is supplemented with Tb.Th and Tb.L (12% increase in R^2). Moreover, Tb.L is a good predictor of fabric components (55% larger R^2 value). In summary, the models provide exhaustive information about the dependence of fabric and anisotropic mechanical behavior of trabecular bone on microarchitectural parameters. Clinically the results prove how the combination of porosity and shape parameters could be useful for the overall prediction of trabecular bone fragility.

Regional Variations in Density and Shear Strength of Human Lumbar Vertebral Endplate and Trabecular Bone

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Thoracic vertebrae (T10) were harvested from fifteen embalmed human cadaveric spines. A coring drill was used to obtain identical cylindrical cores of 7.24 mm (diameter) by 3.15 mm (height). The samples represented 8 different anatomical regions: vertebral endplate (central Vs. lateral, cranial Vs. caudal) and cancellous (superior Vs. median, central Vs. lateral). The samples were radiographed for optical density measurements. After mechanical shear testing, apparent, material, and ash densities were measured. From female to male, cancellous specimens, elemental shear, global, and maximum load to failure increased by 117%, 102%, and 102% respectively. Endplate elemental shear increased by 58% whereas maximum load increased by 87% (from female to male). We also observed an average decrease in endplate maximum load of 29 % from the inferior to the superior and of 56 % from the lateral to the central regions. Maximum load carrying capacity in males was eight times higher in the lateral endplates compared to the anterior ones (p = 0.035).

Figure 1 - Density values for cancellous bone specimens.

Table 1 - Units of shear (elemental and global) strength and maximum load to failure are in MPa and N, respectively. Values represent the mean (SE) of cancellous (median and superior combined) and endplate (inferior and superior combined). Changes are given as percentage of decrease from lateral to central and from lateral to anterior. Statistical significance (*: p < 0.05).
Distal Tibia Bone Density and Biomechanical Strength of Interlocking Screws for Intramedullary Nails

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Treatment of distal metaphyseal tibia fractures is often challenging. Newer tibial intramedullary (IM) nails are designed to offer greater stability with a wider variety of distal interlocking options. In this study we evaluated the biomechanical properties of the distal tibia and their effects on the load carrying capacity of three different configurations of interlocking screws. Twenty four human cadaveric tibiae (twelve individuals) were nailed using a tibia nail (T2, Stryker). Distal locking was performed in one of the three configurations: Group I: two parallel medio-lateral (ML) screws; Group II: two orthogonal screws, 1 ML and 1 antero-posterior (AP); Group III: three screws, 2 ML and 1 AP. Our results showed that a configuration with three orthogonal screws could provide higher load carrying capacity than the two screws although not statistically significant (Fig. 1). Cancellous bone density had a strong positive correlation with load at 3 mm displacement for specimens with two parallel screws ($R^2 = 0.92$) (Fig. 2). Group II had a fair relationship ($R^2 = 0.72$) whereas Group III showed a very poor relationship with cancellous bone density ($R^2 = 0.05$). Cortical thickness showed mild but more uniform correlations with load carrying capacity at 3 mm displacement. Load carrying capacity was significantly higher for male than for female ($p = 0.00804$). Samples with highest values for thickness and density were older than 90 year old.

![Figure 1. Average load carrying capacity of the 3 Groups.](image1)

![Figure 2. Load vs. Density graph for the 3 Groups at 3 mm displacement.](image2)
A New Osseointegrated TMJ Implant Design.

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TMJ implants have been on the market for the last 20 years. However, most of the previous implant models have faced several problems of design and long-term performance [1, 2]. TMJ implants demand has increased because of the high prevalence (~45%)(3) of Temporo-mandibular disorders (TMDs), a pathological entity affecting any of the multiple components of the TMJ system and its associated structures. There are three existing designs currently available for TMJ prosthetics and they follow a similar replacement system as a “ball and socket” type prosthetic mimicking a hip implant. The designs of these implants are relatively similar in components. They provide a plate for the mandibular component attached to a fossa component. The mandibular component is fixed to the mandibular ramus using screws to attach the device and support most of the stress generated by the implant function (4). We propose a new design of an osseointegrated TMJ implant, with an intramedullary fixation. The new design presents a Ferrule ring component. The Ferrule ring helps to keep the von Misses stresses inside the implant, not the mandible, which may reduce the risk of failure of the device. The intramedullary design allows a more physiological adaptation to the anatomy and a better distribution of the loads in the bone.

References
Injectable, Redox-Polymerized Carboxymethylcellulose Hydrogels for Stem Cell-Based Nucleus Pulposus Tissue Engineering.

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Intervertebral disc degeneration (IVDD) is strongly associated with back pain, a debilitating condition that annually affects close 15-30% of the United States population, leading to annual costs of $194 billion [1]. IVDD is characterized with drastic loss of proteoglycans and collagens, matrix molecules important for disc function, which results in a dehydrated nucleus pulposus (NP) [2,3]. Tissue engineering is a promising approach that may potentially restore biological and functional properties of damaged NP. Carboxymethylcellulose (CMC) is a water soluble derivative of cellulose particularly useful for this application due to its large water absorbing capacity and structural similarity to glycosaminoglycans. Photocrosslinked CMC hydrogels seeded with human mesenchymal stem cells (hMSCs) were recently shown to support cell viability and functional extracellular matrix production in the presence of transforming growth factor-β (TGF-β3) [4]. However, an injectable hydrogel system that does not rely upon the application of UV light for polymerization may be more readily translated to the clinic. This study investigated the potential of redox crosslinked CMC hydrogel as an injectable vehicle for stem cell-based NP tissue formation. Specifically, the effects of seeding density (i.e. 20x10^6 cells/ml (20M) and 40x10^6 cells/ml (40M)) cell viability and NP-like differentiation capability of hMSCs in these gels were examined. A higher seeding density was expected to produce superior constructs with respect to biochemical composition and mechanical functionality [5-7]. After 35 days of culture in chondrogenic media with TGF-β3, both groups showed an increase in matrix deposition, and a resulting decrease in swelling ratio (Qw) over time, with significantly lower values at day 35 (21.54±0.80 and 18.18±0.47, respectively) compared to the earlier time points. The GAG content was significantly higher in the 40M than the 20M group on days 21 and 35, while the 20M group produced significantly more Col II than the 40M group on days 21 and 35. As a result, the GAG:Col II ratio in both 20M and 40M groups (~5:1 and ~16:1, respectively) fell within the range reported for native human NP (2.7:1) [8]. Histological staining confirmed the biochemistry results, with more extensive interterritorial alcian blue staining (GAG), and less Col II and Col VI staining in the 40M gels. In particular, GAG and collagen staining was less intense in the center of the 40M gels, while the 20M gels had a more homogeneous matrix distribution. This discrepancy in matrix deposition in the 40M group might be indicative of a negative feedback mechanism due to greater pericellular localization of collagens in the outer regions of the construct that has more exposure to growth factors and nutrients. Limited nutrient diffusion into the gel interior of the 40M gels could have also contributed to this result, as also indicated in previous reports of similar hydrogel systems [6, 7]. However, the overall greater GAG deposition in the 40M group may have contributed to its superior compressive properties. The E, of both 20M and 40M groups by day 35 (~18 kPa and ~28 kPa, respectively) exceeded reported values for native human NP (~5 kPa) [9], but may be beneficial in the harsh in vivo environment associated with IVD degeneration [10]. Cell proliferation and viability quantified via DNA content and mitochondrial activity, respectively, remained constant over time in both groups. These results were consistent with the Live/Dead staining images, which indicated minimal cell death at D35. In terms of ease of delivery, rheological analysis measured a gelation completion time of ~11 min which provides sufficient time for surgical manipulation and is on the order specified by ISO standard 5833/1-1999 E for injectable materials. Overall, the 40M group displayed the highest mechanical properties, consistent with our hypothesis, but the 20M constructs retained more Col II and may be sufficient to produce functional NP-like tissue in redox-polymerized hydrogels. Moreover, the results suggest that an optimal density is required to maintain functionality and that cell densities above the threshold might disrupt the matrix distribution and differentiation capacity [5]. The lack of cytotoxicity in both groups further supports the utility of these gels as an injectable material for NP replacement, given that prior studies have indicated cytotoxic effects of other redox-initiated hydrogel formulations [11]. Future work will test the efficacy of this hydrogel system in vivo.


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Contrast-Enhanced μCT Arthrography (CE-μCTA) of Hard and Soft Tissues in Mice Knee Joints

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Morphological changes in small animal joints are often assessed using 2D histomorphometry, or with the help of modern imaging modalities such as micro-Computed Tomography (μCT). However, quantitative assessment of changes in morphology is limited by soft/hard tissue contrast in μCT. Recently, the use of μCT has been extended beyond the imaging of hard tissues by the use of contrast agents, such as asparagin sulfate in oil (7), Microfil, a lead-chromate based silicon contrast agent(3), and Hexabrix (1,4-6,8-9). In this study we tested the ability of Hexabrix and Iodine potassium Iodine under different scanning conditions, to provide improved images of both soft and hard tissues in the intact whole knee joint of normal mice, as well as the imaging of articular tissues in an OA mice model induced via surgical destabilization of the medial meniscus (DMM). Hind limbs from thirty-nine male C57BL/6 mice (Charles River Laboratories, Sparks, NV) were harvested after mice were euthanized using CO₂ in accordance to IACUC procedures at City College of New York and imaged using a μCT scanner (1172 SkyScan, Belgium). Diffusion kinetics of iodine-based contrast agents was investigated at different concentrations using twelve knee joints scanned at 4.3-µm resolution. Also, the effect of scanning resolution was investigated by imaging mice knee joints at 2, 3, 4, 5, 6, 7, 8, 10, 12 or 14-µm voxel size to assess the ability of μCT to detect soft tissue changes due to surgical destabilization of the medial meniscus (DMM). Newly formed osteophytes (Oph) and cartilage ulcerations were analyzed based on morphological changes and appearance of the underlying bone and cartilage contours in μCT images. CE-μCTA with IKI and HEX was able to readily image both hard and soft tissues, including bone, cartilage, ligaments, tendons, fat, synovial capsule, blood vessels, and muscle. The ability of μCT to depict the morphology and composition of tissues being scanned depend on several factors, including images resolution, type of contrast agent, scanning media, contrast agent concentration, scanning settings, etc. Our results indicate that for optimal imaging of muscle, researchers may select the use of IKI over HEX, since IKI permits clear depiction of muscle fibers. On the other hand, studies aimed to quantify changes in adipose tissue should select the use of Hexabrix, since the contrast between fat and any other soft tissue is superior to the one provided by IKI. Most other soft tissues, including ligaments and tendons can be readily observed when using any of these two contrast agents. High resolution is however needed to image cartilage in mice (ideally 2 to 5µm). The use of CE-μCTA also allowed the simultaneous characterization of trabecular bone microarchitecture, as well as the development of osteophytes and cartilage ulcerations 4 weeks after surgical DMM.

References:
Effects of Mild Heating on the Osteogenesis of Mesenchymal Stem Cells During Inflammation

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Joint regeneration in vitro under inflammatory conditions has not been well studied. In this study, human MSCs (hMSCs) were cultured with pro-inflammatory cytokines Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) in osteogenic medium and growth medium. Cells were heat shocked at 39°C at different intervals, then alkaline phosphatase (AP), calcium deposition, and expression of osteogenic genes Runx2 and Osterix were evaluated on different days and compared to controls. TNF-α was observed to inhibit proliferation of MSCs in the early days of differentiation, but cell mass was significantly higher in heat shocked cultures containing IL-6 and TNF-α compared to respective controls at 37°C, indicating that periodic hyperthermia mitigates the inhibited growth effects of inflammatory cytokines. Maximal cell mass was observed after 12 days following heat shock once every two days. AP activity was higher in differentiated cultures containing TNF-α than no-cytokine controls or cultures with IL-6 by day 6 at 37°C. But by day 12, AP activity was similar in all differentiated conditions except for cells cultured with high-dose TNF-α (1ng/mL) which showed significantly higher AP activity. Maximal AP activity was observed in cultures heated at 39°C for 1 hour three times a day, but the same thermal dosing also produced significantly lower cell mass. Calcium mass was highest in cultures with TNF-α, though there was no significant difference between the heating patterns. Results of osteogenic gene expression by RT-PCR are currently pending. Based on these data, a thermal treatment may be a potential therapy to repair bone and cartilage degradation in osteoarthritis.
Thermal Regulation of Human Mesenchmal Stem Cell Differentiation Toward Bone and Cartilage Lineages

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Osteoarthritis (OA) is one of diseases that seriously affect elderly people's quality of life. Human mesenchymal stem cells (hMSCs) offer a potential promise for the joint repair in OA patients. The methods used for enhancing osteogenic and chondrogenic differentiation from hMSCs have been intensively studied. However, the differentiated cells are still not as functionally mature as primary adult cells. Thermal regulation of hMSC differentiation may be one missing factor to be investigated in terms of further maturation and optimization. In this study, the direct effects of mild heat shock (HS) on the differentiation of hMSCs into osteoblasts in self-assembling peptide hydrogel (PuraMatrix) and chondrocytes in 3D pellet culture were investigated. Periodic HS at 41°C for 1 hr significantly increased the alkaline phosphatase (ALP) activity and calcium deposition in osteogenic cultures and upregulated osteo-specific genes such as osterix, osteopontin, BMP2 and Runx2. For chondrogenic cultures, HS significantly increased sulfated GAG synthesis. IHC and Western blot analyses revealed an increased expression of collagen type II and aggrecan by HS. In addition, HS also upregulated the expression of collagen type I and X. HSP70 expression was upregulated via HS in both osteogenic and chondrogenic cultures. In summary, these results demonstrate that HS accelerated the differentiation of hMSCs and enhanced the maturation of osteoblasts and chondrocytes differentiated from hMSCs in the early stage. The results of this study will guide the design of future protocols using thermal treatments to facilitate bone and cartilage regeneration with human mesenchymal stem cells.
Abstract

Minimally Invasive Stereotactic Separation Surgery for Resection of Metastatic Spine Lesion: A Feasibility Study

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Introduction: The current treatment of spinal metastasis consists of algorithms combining reconstructive surgical and radiation modalities. Recently the concept of separation surgery followed by adjuvant stereotactic radiosurgery (SRS) was shown to be a safe and effective treatment to achieve long-term local tumor control. We examined the possibility of a minimally invasive approach to separation surgery first in a cadaveric feasibility study and then in a patient cohort with spinal metastasis in conjunction with intra-operative computer-assisted navigation. Methods: A cadaveric study using standard minimally invasive access systems examined the feasibility of spinal cord decompression in the thoracic and lumbar spine. Ten patients (7M and 3F) with spinal metastasis underwent MISS and percutaneous pedicle screw fixation using intraoperative navigation or fluoroscopy. All patients were at least 3/5 strength pre-operatively. Endpoints included neurological function, operative time, estimated blood loss, incision length, hospital stay duration, complications, and degree of decompression. Results: The cadaveric study demonstrated a proof of principle with a wide decompression of the spinal cord. Postoperative imaging demonstrated excellent separation that meets the requirements for safe SRS. All patients remained at or improved their neurological baseline with excellent pain control. One patient incurred a perioperative complication (pulmonary embolism). The mean estimated blood loss was 290 cc. The mean incision length was 4.9 cm. Operative time mean was 5.2 hours and the mean length of stay was 7.5 days. Conclusions: MISS for spinal metastasis allows for a circumferential decompression of the spinal cord and safe post-operative SRS. In addition we demonstrated the efficacy of intra-operative navigation in guiding the resection. Future prospective enrollment of patients can help to determine which patients would be ideal candidates for MISS and if the smaller incision and an ensuing faster healing process can allow a faster start of radiation and chemotherapy.
Regulation of PTH-induced Bone Loss: A Role for Monocyte Chemoattractant Protein-1.

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Parathyroid hormone (PTH) stimulates bone resorption as well as bone formation in vivo. The catabolic actions of PTH have been recognized in patients with hyperparathyroidism, or with acute infusion of hPTH(1-34). Our microarrays of bone from rats injected with hPTH-(1-34) daily for 14 days showed a number of cytokines and chemokines were highly induced, in particular, RANKL, IL-6, and CCL2 (Monocyte Chemoattractant Protein-1, MCP-1). We also found that transient MCP-1 is essential for the anabolic effects of hPTH(1-34) on bone. In this study we investigated the role of MCP-1 in vivo as a mediator of the catabolic effects of PTH. To determine the role of MCP-1 in prolonged continuous PTH, as seen in hyperparathyroidism, we treated 8-week-old female WT and MCP-1/-/- mice with continuous infusion of hPTH(1–34) or vehicle for 14 days, using Alzet osmotic pumps.

To assess the differential effects of hPTH-(1-34) on cortical and trabecular bone, microcomputed tomography was utilized to analyze femurs harvested at death. Cortical bone showed that infusion with hPTH induced significant bone loss in WT mice with decreased bone volume/total volume (BV/TV), bone mineral density (BMD), mean crosssectional area (B.Ar) and mean polar moment of inertia (MMI) when compared with the saline treated group. In addition, cortical thickness (Cs.Th) and total cross sectional area (T.Ar) were also found to be significantly lower in PTH-infused WT mice. In contrast, hPTH did not cause significant cortical bone loss in MCP-1/-/- mice. Further, µCT analysis of trabecular bone showed that compared with the saline group, the hPTH group had reduced trabecular thickness (Tb.Th), and structure model index in WT mice. Again, the MCP-1/-/- mice were protected against PTH induced bone loss. To establish whether MCP-1 is required for PTH to induce osteoclast formation in vitro, bone marrow macrophages (BMMs) from MCP-1/-/- and WT mice were cultured with M-CSF and RANKL for 7 days and osteoclasts were counted. Data revealed that BMMs from MCP-1/-/- mice showed decreased multinucleated osteoclast formation compared to WT mice. Further, mRNA analysis of the distal femurs of hPTH(1-34) infused mice showed that PTH induced the expression of NFAT, TRAP, Carbonic anhydrase and Cathepsin-K in WT mice but failed to demonstrate such changes in MCP-1/-/- mice, indicating that MCP-1 is necessary for the recruitment of monocytes and pre-osteoclastic cells and assists in the formation of mature osteoclasts. Together these are the first data to show that MCP-1 is required for the catabolic response to PTH.
Utilizing Cell Surface Markers to Define the Stages of Osteoblast Differentiation

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Osteosarcoma is the most common primary malignant bone tumor in children. Despite advances in osteosarcoma treatment, survival rates have remained stagnant over the past three decades. A comprehensive understanding of the osteosarcoma “cell of origin” and its place in osteoblast differentiation would shed light on the pathogenesis of osteosarcoma. Elucidating the behavior of osteosarcoma cells may lead to novel molecular targets for drug discovery. In this study we seek to delineate the stages of mesenchymal stem cell (MSC) differentiation into osteoblasts by assessing cell surface markers. A human bone marrow derived MSC line was differentiated using Osteoblast Differentiation Media. Cells were harvested at various time-points, stained with antibodies specific for cell surface markers, and analyzed using flow cytometry. The markers CD44, CD105, GD2, CD49b, CD325 and CD54 were differentially expressed during MSC to osteoblast differentiation. This antibody panel was further tested and validated in four osteosarcoma cell lines. The expression of these cell markers varied in a defined pattern at multiple stages during MSC to osteoblast differentiation, indicating the existence of distinct cell subsets. These distinct cell populations will be further assessed in both genetic and functional studies to assess their pluripotent potential. Furthermore the ability of these cell populations to recapitulate an osteosarcoma phenotype may provide insight into the “cell of origin”.