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#2 Multiple myeloma regulates bone marrow adipocyte number, localisation and adipokine secretion

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Objectives: Multiple myeloma (MM) is a fatal haematological malignancy where tumour growth and bone disease are dependent upon cellular interactions within the bone marrow. Bone marrow adipocytes (BMAs) have an emerging role in bone physiology and are a major source of adiponectin, an adipokine negatively associated with MM. Our goal was to elucidate the reciprocal relationship between MM cells, adiponectin and BMAs in vitro and in vivo.

Methods: We have combined in vivo studies using the 5TGM1 murine model of MM and imaging of BMAs with in vitro cellular and molecular biology. Studies have used a panel of MM cell lines, BMAs differentiated from ST2 bone marrow stromal cells (BMSCs) and primary MM cells, BMSCs and BMAs derived from patients with MM.

Results: We have examined the number and localisation of BMAs during development of myeloma in vivo, using perilipin to identify BMAs. A significant negative association between tumour burden and BMA number was demonstrated ($p < 0.05$). Further analysis revealed a 40% increase in BMAs closely associated with tumour, and an 83% reduction in BMAs in areas of non-tumour bone marrow, suggesting a differential response of BMAs within the myeloma-bone microenvironment. Coculture of MM cells with BMAs or BMSCs increased MM cell viability by up to 95%, induced a 4-fold increase in migration and decreased apoptosis, with no significant difference between BMSCs or BMAs. A significant increase in adiponectin mRNA and protein was detected in BMAs as compared to BMSCs, in both cell lines and primary cells. An adiponectin receptor agonist induced MM cell apoptosis, however coculture of MM cells with BMAs significant decreased adiponectin mRNA expression and protein expression and secretion ($p < 0.05$), providing a mechanism by which MM cells can down-regulate adiponectin to avoid the tumour-suppressive effect of this adipokine.

Conclusions: BMAs are closely associated with MM cells in vivo. Our studies suggest a supportive effect of BMAs on MM growth and survival, mediated in part by a reduction in adiponectin. Elucidating the BMA-MM relationship could reveal new therapeutic approaches for the treatment of MM.

#3 Expansion of bone marrow adipose content in response to irradiation or high fat diet

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Changes in the bone microenvironment have the potential to impact tumor cell homing, colonization, and growth. Compared to the contributions of other cell types in the bone and marrow, relatively little is known about how marrow adipocytes influence bone metastasis. The goal of this work was to establish clinically relevant models of rapid marrow adipose tissue (MAT) expansion. To this end, we have characterized changes in the bone and MAT in mouse models of obesity and therapeutic single-site radiation. To model obesity, female C57Bl/6 mice were fed a high fat (60 kcal% fat) or nutrient-matched control diet (10 kcal% fat) starting at 20 weeks of age. Mice receiving high fat diet (HFD) had significantly higher body weight and showed impaired glucose tolerance after only 1 week. Circulating leptin levels were significantly higher after 2 weeks of HFD. MAT in the tibia was evaluated *ex vivo* by osmium tetroxide staining and microCT and expressed as a percentage of total marrow volume. One week of HFD was sufficient to significantly increase MAT compared to mice fed a control diet ($p = 0.0001$). There were no changes in bone mineral density or trabecular bone volume at either the 1 or 2 week time points. In our second model of rapid MAT induction, female 20 week old C57Bl/6 mice received a single- limb radiation dose of 2Gy. We have previously shown that changes in bone can occur as early as 7 days post-irradiation. Bone parameters and MAT were evaluated at 3 and 7 days post-irradiation and irradiated bones were compared to the contralateral limb as well as sham-irradiated controls. While no changes were observed at 3 days post-irradiation, we found significantly increased MAT ($p = 0.004$) and decreased trabecular bone volume ($p = 0.012$) in the irradiated limb at 7 days post-irradiation. Interestingly, MAT values in the contralateral limb were not significantly different from sham-irradiated controls, suggesting that induction of MAT in response to radiation is likely a local effect rather than one induced by circulating factors. Overall, these results indicate that MAT expansion can occur rapidly in response to clinically-relevant conditions such as obesity and therapeutic levels of radiation. Characterizing additional changes that

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take place within the bone microenvironment of these non-tumor models will not only give valuable insight into marrow adipocyte biology, but may also reveal mechanisms by which MAT can influence cancer cell behavior in the bone.

#4 Bone Marrow Adipocyte-Induced Oxidative Stress Enhances HO-1 Expression in Metastatic Prostate Cancer

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Bone is a preferential site of metastasis from prostate cancer (PCa). Age and obesity, conditions that increase adipocyte numbers in bone marrow, are risk factors for skeletal metastases from PCa. Research in our laboratory focuses on understanding the interactions between adipocytes and tumor cells that have infiltrated the bone marrow. We are examining how the secretion, transport, and uptake of adipocyte-supplied factors promote metastatic progression in bone. One factor with strong links to tumor progression and survival is Heme Oxygenase 1 (HO-1), an inducible, anti-oxidant enzyme capable of promoting growth through a regulation of Reactive Oxygen Species (ROS). We have shown previously that HO-1 levels are induced in PCa cells upon exposure to marrow adipocytes. Here, we hypothesized that adipocyte-induced HO-1 expression protects the cells from oxidative stress and promotes survival.

Through the Oncomine database analyses we demonstrate that HO-1 levels are significantly increased in human metastatic PCa tumors as compared to primary tumors. We also show significant HO-1 upregulation in PCa bone tumors from mice with diet-induced marrow adiposity. We confirm that exposure to bone marrow adipocytes *in vitro* increases HO-1 protein and mRNA levels, and we demonstrate adipocyte-induced nuclear translocation of HO-1 in a panel of PCa cell lines. We also show that stable HO-1 overexpression in PC3 and ARCaP(M) cells results in increased invasiveness *in vitro* and results in accelerated growth and progression of bone tumors *in vivo*. Our additional data reveal that coincident with HO-1 induction, exposure to adipocytes results in elevated ROS levels in PCa cells, whereas treatment with antioxidant N-acetylcysteine reduces HO-1 expression to baseline levels. This suggests marrow adipocytes play a role in driving oxidative stress response in tumor cells. Associated with adipocyte-induced oxidative stress and HO-1 overexpression, there is an induction in markers of endoplasmic reticulum (ER) stress, elevated expression of pro-survival markers, and enhanced clonogenic growth.

Studies are currently underway to determine the significance of HO-1 expression and activity in tumor cells survival and response to chemotherapy. Collectively, the results of our studies demonstrate that adipocyte-induced oxidative stress may be playing a significant role in promoting prostate tumor aggressiveness, invasiveness, and survival in bone.

Genomics and immunoncology

#5 Cytokines from the dura induce proliferation of bone marrow macrophages and may promote an immunosuppressive phenotype: possible role in spinal metastatic disease.

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The spine represents the most common and debilitating site of metastatic spread of malignant disease. The cause of this preferential tumor growth in the spine is not understood. Current theories propose that the presence of red marrow in adult vertebrae and the existence of vertebral venous plexuses, devoid of valves, explain the high incidence of spinal metastases. Nonetheless, molecular mechanisms responsible for the initiation and growth of spinal metastases are still unknown. We hypothesized that the dura mater, the outer layer of the meninges, that surrounds and protects the spinal cord, situated in close contact with the vertebrae, may contribute to create favorable conditions for spinal metastases. To begin to understand the role of the dura in modulating the spinal pre-metastatic niche, we cultured primary mouse dura fibroblasts and analyzed their transcriptome by RNA Seq. This analysis showed high expression of secreted factors including numerous chemokines (*Cxcl12, Cxcl5, Cxcl1, Cxcl2, Ccl2, Ccl5*), members of the *Tgfβ* family (*Tgfβ3, Tgfb1, Tgfβi*), ligands of receptor tyrosine kinases (*Igf2, Fgf2, Hgf, Vegfα, Vegfβ, Pdgfα)* and factors with role in bone remodeling (*Ctgf, Csf1, Rankl*). We treated bone marrow (BM) from the spine and long bones with dura conditioned media (DCM) and show that DCM induced substantial proliferation of BM cells (9.9 and 13.37-fold, $p < 0.0001$). Many of the chemokines identified in the transcriptome of the dura are known to induce immunosuppression and accumulation within tumors of myeloid derived suppressive cells (MDSCs). These cells are recognized in the mouse by the co- expression of CD11b and Gr1. We used flow cytometry to analyze BM cells treated with DCM and show that dura secreted factors induce the expansion of CD11b+ cells, most prominently of the population of $CD11b^{+}Gr1^{neg}$ macrophages. It is known that within tumors, infiltrating MDSCs gradually lose expression of Gr1 and become tumor associated macrophages (TAM) with strong immune-suppressive capacity. Future experiments will test the immunosuppressive activity of the cell populations expanded by secreted factors from the dura and their contribution to early and late phases of spinal metastatic disease. We propose that secreted factors from the dura create an immunosuppressive environment, beneficial in preventing damaging inflammation of the CNS, creating however a tumor friendly environment which promotes immune evasion and spinal metastatic disease.

#6 A humanized mouse model of breast cancer-related bone metastasis for immuno- oncology drug discovery

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Immunotherapies have proved efficacy on many primary tumors in preclinical studies, but bone metastases have been omitted in the field of immunotherapy. Bone marrow is a common site for metastasis, but it is also where hematopoietic stem cells (HSC) reside. HSC are the precursors of the human immune system, and the presence of human HSC is a necessity in studying immune therapy in animal models for bone metastasis. The bone marrow provides a natural site for studying tumor-immune cell interactions and efficacy of new immunotherapies on bone metastases that are currently incurable.

Intratibial injections of $1x10^{6}$ of BT-474 (ER+, PR+, HER2+) human breast cancer cells were given to CIEA NOG® and humanized NOG mice (huNOG: HSCFTL-NOG-F, provided by Taconic Biosciences) stably engrafted with hCD34+ HSCs. Tumor-induced bone changes were monitored by radiography for 8 weeks and analyzed at endpoint by dual x-ray absorptiometry (DXA) and micro-computed tomography (μCT). Bone turnover changes were monitored by measuring the bone resorption and formation markers, serum CTX and PINP, respectively. Immune-related organs and tumor-bearing tibias were analyzed for a cascade of differentiated human immune cells, CTLA-4 and PD-L1.

Tumor-induced osteoblastic new bone growth was observed in all tumor-bearing tibias. Bone lesions were larger in huNOG mice compared to NOG mice. Osteoblastic bone growth increased bone mineral density in huNOG mice, which correlated with increased cortical and trabecular bone volumes. Serum CTX values were lower in huNOG mice than in NOG mice, but PINP values were similar. Strong expression of CD3, CD4, CD8, CD20, and CD45 was observed in huNOG mice, indicating a high prevalence of active human immune cells. CD45-positive tumor-infiltrating lymphocytes (TILs) and CD4-positive T-helper cells were observed in 80% of the huNOG mice. PD-L1 was expressed in 50% of the tibias and no CTLA-4 expression was observed.

This study describes the first characterization of tumor growth in bone of humanized mice. As a result of regulation of tumor growth and bone formation by the human immune cells, the huNOG mice showed more severe phenotypic data than NOG mice. These findings highlight the importance of the microenvironment on tumor-immune cell interactions, and demonstrate that humanized mouse models provide an improved new platform for preclinical testing of cancer immunotherapies, particularly for therapies targeting cancers that often metastasize to bone.

#7 TGF-β's Role in Myeloid Regulation of Tumor-Induced Bone Disease (TIBD)

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Bone is one of the most common metastatic sites for breast cancer, where nearly 70% of patients that die from disease develop skeletal metastases. TGF-β has a complex role in the regulation of tumor growth and is critical for the initiation and continuation of bone destruction. Our lab has published that myeloid cells ($CD11b^+Gr\text{-}1^+$) from tumor bearing mice have a 2-fold increase in expression of TGF-β. Previous publications have demonstrated that TGF-β can affect myeloid cells by increasing their expansion in the tumor microenvironment and modulating host immune surveillance, thus supporting a tumor promoting environment. Therefore, we hypothesize that inhibition of TGF-β will decrease TIBD by reducing myeloid expansion and tumor growth in bone. To address this we injected mice with the TGF-β inhibitory antibody, 1D11, one week prior to tumor inoculation and continuously treated until sacrifice where myeloid expansion and parameters of TIBD were measured. An immunodeficient model inoculated with human breast cancer cells (MDA-MB-231) and an immunocompetent model using murine breast cancer cells (4T1) were used for these studies. Flow cytometry analysis revealed that TGF-β inhibition decreased the $F4/80^+$ myeloid population by six percent. *In vitro* qPCR analysis revealed that macrophages had a decreased expression of both interleukin 10 and arginase, factors known to be immune suppressive, after treatment with 1D11. This suggests that inhibiting TGF-β not only reduces the expansion of this pro-tumorigenic myeloid population but also decreases the immune-suppressive profile in bone. Micro-CT analysis demonstrated that tumor bearing 1D11 treated mice had a ten percent increase in bone volume in both mouse models. X-ray analysis demonstrated a two-fold reduction in bone destruction in tumor bearing mice treated 1D11 compared to isotype control in both mouse models. Furthermore, histomorphometric analyses demonstrated that TGF-β inhibition reduced tumor burden in both mouse models. Together these results suggest that inhibiting TGF-β prior to tumor inoculation and subsequently after may prevent expansion of tumor-promoting myeloid populations and progression of TIBD, thus altering the bone microenvironment such that tumors can no longer grow in bone. Understanding how TGF-β responsive myeloid cells contribute to the progression of TIBD will further our ability to create novel therapies for patients.

#8 Temporal Dynamics of Macrophage Plasticity in Bone Metastatic Prostate Cancer

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Bone metastatic prostate cancer generates incurable lesions. Understanding how metastatic prostate cancer (mPC) manipulates the bone microenvironment (BME) is crucial for therapy development. Macrophage (M0) plasticity has well described roles in pro-/anti-tumor progression, and separately in normal bone healing. However, temporal dynamics of M0 infiltration and plasticity is unexplored to date in bone mPC, especially in the context of bone microenvironmental cells such as osteoblasts and osteoclasts. We hypothesize that M0 polarization affects cancer-bone interaction and ultimately the pathophysiology of bone mPC. To test our hypothesis, we used a mathematical model of the bone microenvironment since it allows for the analysis of multiple parallel cellular interactions in a temporal manner. We therefore parameterized ordinary differential equation (ODE)-based models with what is empirically known about M0 in normal bone repair. Inflammation follows bone injury, marked by inflammatory monocyte recruitment, which readily polarize to pro-inflammatory (M_i) status when exposed to factors such as nitric oxide. Once the M_i clear the stromal debris (24-48 hours), osteoblast (OBL) precursors expand (Days 1 to 3) and secrete osteoclastogenic factors including receptor activator of nuclear kappa B ligand (RANKL). Osteoclasts resorb bone (Days 1-4), releasing growth factors like transforming growth factor β (TGFβ). TGFβ drives anti-inflammatory (M_α) polarization to suppress inflammation and facilitate OBL-driven bone formation (Week 1 to 3). The M_α recedes, returning the bone to homeostasis. To validate the accuracy of the ODE model, we profiled macrophage plasticity *in vivo* subsequent to intratibial injury (percutaneous injection). Bone marrows were isolated at time 0, 24, 48, 72, 168 and 360hrs (n = 3/group) and profiled by flow cytometry for CD11b, F480, ARG-1, iNOS. Results confirm the accuracy of the ODE model and demonstrate a significant rapid influx of M_i cells that dissipate within 48 hours and give rise to M_α cells that eventually recede over a 2 week period as the bone returns to homeostasis. We are currently using an integrated ODE/ immunocompetent biological model approach to interrogate how interfering with macrophage polarization can impact bone repair and how metastatic prostate cancer cells impact macrophage behavior over time. We believe this approach will reveal how M0 contribute to the progression of bone metastatic PC and will identify key circuits that can be therapeutically targeted to treat the disease.

#9 Toll-like receptor 9 as a marker for zoledronate effect in breast cancer *in vivo*

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Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer and generally represents 10-24% of invasive cancers. Less than 30% of patients with metastatic TNBC survive 5 years.

Toll-like receptor 9 (TLR9) is a cellular innate immunity receptor for microbial and vertebrate DNA. We first demonstrated that TLR9 is widely expressed also in cancer. We further discovered that TLR9 has prognostic value in TNBC patients. Specifically, TNBC patients with low tumor TLR9 expression upon diagnosis have a dramatically worse prognosis than patients with high tumor TLR9 expression. We further showed that in the absence of TLR9, TNBC cells become highly invasive in hypoxia. Currently, there are no targeted therapies for TNBC patients, and the cornerstones of their treatment (surgery, radiation, chemotherapy) have essentially remained unchanged for the past 40 years. Given their dismal survival rates, especially the low-TLR9 TNBC group urgently needs improved treatments.

Bisphosphonates (BPs) are osteoporosis drugs that inhibit osteoclast-mediated bone resorption and prevent cancer-induced skeletal events. Due to their unique pharmacokinetics, high affinity to bone hydroxyapatite and short half-life in blood, BPs were initially thought only to affect cells that reside in bone. It is, however, now well established that BPs have anti-cancer effects also in visceral tissues. We discovered that breast cancer cells with low TLR9 expression are significantly more sensitive than cells with high TLR9 expression to the growth inhibitory effects of BPs *in vitro* and *in vivo*. These findings were not limited to TNBC cells as similar results were also seen in estrogen receptor-positive breast cancer cells. The effects were most profound with the BP zoledronate.

We encapsulated zoledronate into mesoporous silica nanocarriers to overcome the low bioavailability. The nanocarriers were further functionalized with folic acid to boost the affinity for breast cancer cells *in vivo*. The developed nanocarriers were biocompatible *in vivo*, and no non-specific accumulation within bone was observed. Further, the intravenously administered zoledronate-loaded nanocarriers showed clear tumor growth suppression in breast cancer xenograft-bearing mice.

Based on these results, we hypothesize that low-TLR9 TNBC patients could represent the group that benefits from adjuvant BP treatments. To study this further, clinical BP patient cohorts need to be studied.

#10 HTLV-1 Viral Oncogene HBZ Induces Lymphoproliferative and Osteolytic Bone Disease in Transgenic and Humanized Mouse Models

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Adult T-cell leukemia/lymphoma (ATL) develops in a subset of patients infected with the retrovirus HTLV-1 after a 40+ year latency. In addition, 80% of ATL patients develop osteolytic lesions and hypercalcemia of malignancy, causing severe skeletal complications. The HTLV-1 virus encodes 2 viral oncogenes, Tax and HBZ. The transcriptional activator Tax is critical to ATL development and tumor growth. In a transgenic mouse model, Granzyme B driven Tax expression is sufficient for the development of leukemia and osteolytic lesions. HBZ promotes tumor cell proliferation and disrupts Wnt pathway modulators, but its role in tumor progression and osteolytic bone destruction is undefined. To determine if HBZ is sufficient for the development of hematopoietic disease and bone loss, we established a transgenic mouse model with Granzyme B driven HBZ expression (Gzmb-HBZ). Lymphoproliferative disease developed in 40% of Gzmb-HBZ mice, with tumors primarily localized to mesenteric and lymphoid tissues by 18 months. A subset of mice had reduced white cell counts, and tumors and spleen expressed HBZ by immunohistochemistry. Pathologic bone loss and hypercalcemia were observed at 18 months compared to wild type (WT) mice. Finally, T-cells from Gzmb-HBZ mice have increased expression of osteoclast (OC) activating factors RANKL and PTHrP and osteoblast inhibitor DKK1, demonstrating that Gzmb-HBZ mice model the bone loss present in ATL patients.

We next evaluated if HBZ causes bone loss in the context of the entire HTLV-1 genome, utilizing the human immune system (HIS) mouse model. In this model, a human immune system is established in neonatal NSG mice. Previous work has shown that HTLV-1 infected HIS mice (HTLV-1 HIS) recapitulates the disease course of human ATL. HTLV-1 HIS mice developed lymphoproliferative disease and had a median survival of 6 weeks. To evaluate whether HTLV-1 HIS mice also developed HTLV-1 associated bone loss, long bones were evaluated by microCT at different stages of disease progression. As early as 3 weeks post-infection, HTLV-1 HIS mice had significant and continuous bone loss, and increased OC activation, which negatively correlated with spleen weight and human CD4 T cell number in peripheral blood. Together, this data suggests HTLV-1 HIS mice are a model for studying ATL tumorigenesis and bone loss, and demonstrate that HBZ mediates HTLV-1-associated bone loss in transgenic and humanized mouse models of ATL.

New models for cancer in bone and biomechanics

#11 A Method to Analyze the Development of Osteolytic Lesions In Mice Using *In Vivo* μCT and Rigid Image Registration

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Osteolytic lesions are a key feature of diseases such as multiple myeloma and metastatic breast and prostate cancer; and the development of micro-CT has allowed these lesions to be visualised and measured in murine models of cancer. More recently, the development of *in vivo* micro-CT has made it possible to detect and map the progress of bone lesions over time. However, currently there is no established method to analyse their growth. Here, using a myeloma xenograft model, we have developed a method to longitudinally image and analyse bone lesions. NOD/SCID-γ mice were injected intravenously with $1x10^6$ human myeloma U266 cells, where bone lesions are known to increase over time. After 6 and 9 weeks post- tumour cell injection the right tibia of each tumour-bearing mouse was imaged using an *in vivo* micro-CT scanner. The resulting longitudinal datasets (N = 6) were then analysed for bone lesion growth over time using volume rendering software. Images were initially manual orientated, before we next examined the suitability of using rigid image registration to improve the robustness of the method. We found that image registration improved reproducibility, with analysis of repeated datasets resulting in 1.3% variation compared to 21.1% when repeatedly analysing the same manually orientated dataset. Furthermore, we found that image registration led to better accuracy, with bone lesion area of unregistered datasets varying from that of registered datasets by an average of

10.1%, and that image registration had the potential to improve trabecular bone measurement accuracy. In summary, *in vivo* micro-CT offers a novel way of monitoring the development of bone disease over time in *in vivo* murine models of cancer, and importantly, image registration improves the robustness of analysing bone lesion growth.

#12 Modelling the plateau phase in multiple myeloma *in vivo*

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Multiple myeloma (MM) is a bone marrow plasma cell cancer accounting for 114,000 diagnoses yearly worldwide. MM is largely incurable and $<50\%$ of patients survive >5 years. $>85\%$ of patients exhibit a bone disease that can cause bones to fracture, increasing MM fatality. Currently no treatments exist to repair bone lesions and chemotherapy does not fully eradicate tumour. After chemotherapy, most patients enter a 'plateau phase' of clinical stability for >3-6 months with minimal residual disease (MRD). The plateau phase provides an auspicious opportunity to test promising anabolics and study MRD to identify new ways to eradicate tumour.

We hypothesised that incorporating chemotherapy into current *in vivo* models would yield a model(s) with MRD, mimicking the plateau phase seen clinically. Thus we aimed to optimise xenograft models to mimic the plateau phase.

Human MM cell lines that are sensitive to bortezomib (bort) *in vitro* (JJN3, OPM2 and U266) were transduced with GFP and luciferase and purified by fluorescence- activated cell sorting. 0.5-1x10^6 cells were i.v. injected into NSG mice. After tumour development, 0.75 mg/kg bort was administered twice weekly for ≥ 3 weeks. Tumour and bone lesions were monitored *in vivo* by bioluminescence imaging (BLI), serum paraprotein ELISAs and μCT. Flow cytometry (FC), histomorphometry, μCT and QPCR were performed for endpoint analyses.

Bort increased survival in the JJN3 model by 4 days ($p<0.01$), but did not alter tumour. Bort had no effect on OPM2 survival or tumourigenesis. However in the U266 model, 2 weeks of bort reduced tumour, indicated by an 80% reduction in paraprotein ($p<0.0001$) and a 100-fold decrease in BLI signal ($p<0.001$). The low tumour load remained stable throughout bort therapy and for 1 week post-therapy. 2 weeks after treatment was ceased, tumour load remained 10-fold lower by BLI and 70% lower by FC ($p<0.001$) compared to endpoint controls. All models exhibited bone disease having lytic lesions and expression of osteoblast inhibitory factors *Dkk1* and *Tgfb1*. In the U266 model, lesions developed 6-8 weeks after tumour inoculation and bort reduced TRAP+ osteoclasts and increased osteoblasts ($p<0.01$), likely due to lower tumour load.

To our knowledge, this is the first mouse model that reliably mimics the plateau phase. This model will aid optimal selection of anabolics for translation to clinical trials and investigation of how bone repair affects MRD and tumour eradication to find curative treatments for MM.

#13 Effects of LIV Pre-treatment on Musculoskeletal Endpoints in Mice Following Complete Estrogen Deprivation

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Post-menopausal, estrogen receptor-positive breast cancer patients treated with aromatase inhibitor experience increased fracture risk [1]. Pharmacological agents to inhibit osteoclastic bone resorption are effective to prevent bone loss but may have adverse effects. Low intensity vibrations(LIV), a mild mechanical signal reintroducing high-frequency elements of physical activity[2], have been demonstrated to safely preserve bone in two murine models of cancer[3, 4] without compromising animal safety, as well as upregulate muscle-bound satellite cell populations in ovariectomied(OVX) mice[5]. In fact, marrow mesenchymal stem cells were directed towards an osteogenic rather than adipogenic state. We hypothesized that LIV could preserve bone mass and muscle strength following complete estrogen deprivation. Twenty weight-matched, 4w female C57BL/6 mice were treated (LIV: 90Hz, 0.35g, 1x/d) or sham-treated (SH) 4w prior to and following complete estrogen deprivation by OVX and treatment with aromatase inhibitor letrozole. Longitudinal bone measurements were quantified via DXA imaging (1x/3w) and high-resolution *in vivo* μCT scanning (1x/4w; resolution = 19μm). Evaluations of the tibia performed 1mm distal to the metaphyseal growth plate assessed trabecular (Tb) microarchitecture. No significant differences were observed in mice weights or chow consumption after 19w of LIV-treatment. DXA measurements showed that, while total bone mineral density was identical in both groups, total lean mass had decreased by 7% and 3% in SH and LIV, respectively(not statistically different). Conversely, total fat mass had increased by 56% in SH relative to baseline, while increasing by only 16% ($p<0.0001$) in LIVtreated mice. Further, LIV-treated mice showed a 32% increase from baseline) in grip strength, compared to a 19% increase in SH; 13% (not statistically significant) difference between groups. *In vivo* μCT revealed a 7% increase in Tb bone volume fraction in LIV compared to SH, not yet statistically significant. Tissue mineral density in this region decreased by 40% in SH relative to baseline but decreased by only 30% in LIV, 25% ($p<0.01$) greater than the mineral content quantified in SH. The studies are ongoing; bone mass, body composition and grip strength will be followed until stabilized. However, the current data suggest that LIV may be a novel means to prevent the accrual of visceral fat while mitigating deficiencies in muscle and bone that are associated with cancer treatment.

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#14 EVOCA (*Ex Vivo* Organ Coculture Assay) for High Throughput Screening for Inhibitors of Tumor Growth in Bone

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Several solid tumor types and multiple myeloma (MM) preferentially colonize the skeleton, where they resist treatment. Testing agents to block tumor growth in bone has been limited on one hand by the slowness, cost and complexity of animal models and on the other by the difficulty of reproducing the bone microenvironment in vitro.

We developed an ex vivo organ co-culture assay (EVOCA) where human tumor cells (2,000-10,000) are added to 5mm bone discs from immune-naïve 14d mouse calvariae ($n=4$). Tumor growth is followed noninvasively as *Gaussia* luciferase secreted into conditioned media. After one week, cultures are homogenized and expression of tumor and bone genes ssayed by species-specific Q-PCR. EVOCA supported bone colonization by breast (MDA-MB-231 & MCF7), prostate cancer (PC-3 &

LNCaP) and MM (RPMI-8226 & JJN3) cell lines. Histology showed osteolysis. EVOCAs with PC-3 or LNCaP prostate cancer (PC) cells showed increased Wnt inhibitor expression (Dkk1 & Sost) at 5d, which decreased at 7d, paralleling changes in Dkk1 in PC bone metastases described by Hall & Keller (Cancer Metastasis Rev, 2016). Both cell lines similarly increased bone RANKL and suppressed Opg and Col1a1, a marker of osteoblast activity. Tumor cell expression of human RANKL was increased in PC-3s, but decreased in LNCaPs, by co-culture with bone.

EVOCA was validated with human MDA-MB-231 breast cancer cells and the osteoclast inhibitor zoledronic acid (ZA, 50nM), which was effective when administered as pretreatment (single dose) and throughout (prevention model) and less so when used to treat established tumor (treatment model). ZA suppressed osteolysis-associated mRNAs, such as RANKL and TRAP, and increased markers of tumor apoptosis. A mechanistically different osteoclast inhibitor odanacatib, which targets cathepsin K enzymatic activity, was ineffective. EVOCAs with MDA-MB-231 were used to test the hydrogen sulfide donor, GYY4137, and an inhibitor of plasminogen activator receptor, IPR-803. While the former was inactive, the latter effectively decreased tumor growth in bone.

EVOCA is compatible with a variety of human tumor cells including multiple myeloma, can be used with genetically modified mouse bones, permits rapid screening of compounds for which animal dosing is unknown, and facilitates assessment of specific responses of tumor and bone cell types, including, osteoblasts, osteocytes, and osteoclasts.

#15 Comparative oncology: Naturally-occurring bone cancer in pet dogs as a model of human osteosarcoma

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Despite being the most common primary bone tumor, osteosarcoma is diagnosed much more frequently in canine populations than humans, and pet owners increasingly seek veterinary treatment for this disease. Naturally-occurring canine osteosarcoma shares many characteristics with the human disease, including sites of occurrence, histologic and molecular features, metastatic propensity, and response to treatment. Clinical trials of investigational agents in canine osteosarcoma may inform drug development in human oncology, where patient recruitment into these trials is more challenging. The Comparative Oncology Trials Consortium of the NCI/NIH was established to link veterinary colleges to perform these types of clinical trials in a coordinated manner and to the same standard as human oncology trials. For example, a randomized controlled trial of standard of care adjuvant carboplatin chemotherapy with or without rapamycin will conclude enrolment in 2017. At the Ontario Veterinary College, a multidisciplinary research group focused on osteosarcoma was recently established within the University of Guelph's Institute for Comparative Cancer Investigation. Research in this group is directed at many clinical and basic features of osteosarcoma, including molecular characterization of metastasis, biomarkers of tumor progression, radiation and chemotherapy sensitizers, bisphosphonate treatment, an immunotherapy with a focus on oncolytic virus treatment. Infrastructure, including a large tumor bank, tissue microarrays, and dedicated clinical trials coordinator, have been created to facilitate translational studies using dogs that are referred for treatment to the OVC Animal Cancer Centre.

#16 Low Magnitude Mechanical Signals Suppress Expression of Osteolytic Genes in MDA- MD-231 Breast Cancer Cells

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Bone is a preferred site of breast cancer metastasis, with subsequent complications including pain, pathological fractures, spinal cord compression, muscle weakness, and ultimately death. The growth factor rich bone microenvironment supports cancer growth and invasion, while states of high bone turnover, such as estrogen depletion, perpetuate metastatic potential and bone lysis. Low magnitude mechanical signals, delivered in the form of low-intensity vibration (LIV), stimulate bone formation. Recent work shows that LIV restricts bone loss and tumor progression in mouse models of multiple myeloma and ovarian cancer. Furthermore, transmission of LIV signals is accomplished by direct connection of the actin cytoskeleton and the cell nucleus via the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. This study examined direct effects of LIV on human breast cancer cells, hypothesizing that LIV suppresses catabolic gene expression, which exacerbates bone lysis. Human MDA-MB-231 cells were exposed to LIV (90Hz, 0.3g) in twenty minute bouts, either once or twice a day in the presence or absence of TGFβ1. Of the genes surveyed, only expression of PTHrP significantly decreased with once-daily LIV (2.54-fold, $p < 0.01$). In contrast, exposure to LIV twice-daily resulted in significant reduction of PTHrP (6-fold, $p < 0.001$), CTGF (2.3-fold, $p < 0.05$), IL-11 (1.76-fold, $p < 0.001$), and RANKL (2-fold, $p < 0.05$) mRNA. With the exception of RANKL, significant ($p < 0.05$) reductions were also seen in the presence of TGFβ1. Notably, with respect to PTHrP, twice-daily LIV resulted in over 2-fold greater reduction compared to once-daily LIV. Using transwell assays, decreased invasion of MDA-MB-231 cells was observed following LIV, but cell death was unaffected. As previous work demonstrated that nucleo-cytoskeletal connectivity enabled transmission of LIV signals, we examined expression of LINC complex genes in response to LIV. Expression of Syne1 (1.83-fold, $p < 0.001$), Syne2 (2.63-fold, $p < 0.05$), Sun1 (2.28-fold, $p < 0.001$), and Sun2 (4.33-fold, $p < 0.05$) were significantly increased in MDA-MB-231 cells with twice-daily LIV. These data show that direct application of LIV to human breast cancer cells suppressed invasion and expression of genes that promote osteolysis. Furthermore, increased expression of LINC complex genes suggests that LIV enhances nucleo-cytoskeletal connectivity, a mechanism through which mechanical signals may be transmitted to inhibit metastasis and osteolysis.

#17 3D *in vitro* Bone-templated Model of Tumor-induced Bone Disease

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When metastatic tumors establish in bone, patients experience bone pain, increased risk of fractures, and reduction in mobility. Unfortunately, it is not possible to predict which tumors will induce bone disease or how tumors will respond to therapeutics. The objective of this study is to develop an in vitro model of tumor establishment in bone to predict the course of disease progression and response to drug treatments in individual patients. Using microCT imaging in tandem with 3D inkjet printing technology, we have designed 3D Tissue Engineered Bone Constructs (TEBCs) that recapitulate the mechanical and topological properties of trabecular bone. MicroCT images of human bone samples of the femoral head, proximal tibia, and vertebral

body were used to 3D-print wax templates. TEBCs fabricated from these templates showed no significant differences in bone morphometric parameters (BV/TV, SMI) or elastic modulus compared to the human bone templates. Furthermore, the TEBCs exhibit similar surface roughness to dentin and also contain over 50% hydroxyapatite. Culture of human mesenchymal stem cells (hMSCs) on the TEBCs promoted osteogenic differentiation, and degree of matrix mineralization was dependent on anatomical site. Gene expression of osteogenic markers alkaline phosphatase (ALP) and osteopontin (OPN) of hMSCs cultured on the TEBCs also demonstrated anatomical site dependence. When MDA-MB-231 cells were treated with the integrin inhibitor Cilengitide or the TGF-β receptor kinase inhibitor SD208 in 2D culture, expression of the bone-metastatic factors Gli2 and PTHrP decreased two-fold ($p < 0.01$), however this effect was not apparent when cultured on the 3D TEBCs. In contrast, treatment with the Gli2 inhibitor GANT58 reduced both Gli2 and PTHrP expression three-fold ($p < 0.01$) in both 3D and 2D. In vitro 3D TEBC models with bone-like mechanical and topological properties may be an effective high-throughput approach for predicting the tumor cell drug response.

New therapeutics for clinical management

#18 Modified Herpes Simplex Virus (HSV1716 (SEPREHVIR)) induces potent myeloma onocoylis *in vitro* and *in vivo* and prevents cell line regrowth.

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Background: Multiple myeloma remains a largely incurable disease and despite current therapies achieving good initial responses, disease usually re-accumulates often resulting in death. Therefore, new approaches are required that not only reduce the tumour load, but also prevent the regrowth of residual disease. One such approach is the use of modified oncolytic viruses that have shown efficacy in several clinical trials. We hypothesised that the modified Herpes Simplex Virus HSV1716 (SEPREHVIR®), that replicates only in transformed cells, would induce myeloma cell death and prevent tumour regrowth.

Methods: Myeloma cell lines (JJN3, U266 and 5TGM1) or normal healthy cells were infected with HSV1716 (MOI of 0.5-100) and cell death was monitored over time by flow cytometry using PI staining. Apoptosis and autophagy were assessed following viral infection using qPCR and annexin V staining. Viral efficacy *in vivo* was tested in the JJN3 xenograft and the 5TGM1 syngeneic models of myeloma, where 10^6 myeloma cells were injected *i.v.,* then 1 week later mice were treated with HSV1716 (10^7 pfu, *i.v.* twice a week) or control (PBS) for 2 weeks. Tumor burden was measured *ex vivo* in the long bones by flow cytometric and histological analyses.

Results: Infection of myeloma cells resulted in pronounced cell death by 3 days post viral infection, reaching greater than 90% ($p < 0.001$) in human (JJN3 and U266) and 60% ($p = 0.0125$) in murine (5TGM1) cells, respectively. The remaining myeloma cells in culture did not increase in number for up to 35 days after viral exposure, suggesting re-infection. There was a marked increase in the expression of FASL (an apoptotic marker) in all HSV1716 infected myeloma cells compared to control cells and an increase in annexin V positivity 24 h after infection. Interestingly in preliminary experiments, HSV1716 prevented regrowth of myeloma cell lines following treatment with bortezomib. No impact on control cells such as primary lymphocytes or osteoblasts was seen. Tumour load was significantly reduced (greater than 50%, $p < 0.05$) in JJN3 xenograft mice treated with HSV1716 compared to control treated mice.

Conclusions: HSV1716 has potent anti-myeloma effects that are meditated via apoptosis of myeloma cells *in vitro*. We are currently investigating combination treatment of HSV1716 with standard therapies and are assessing the potential for HSV1716 as a therapy to prevent myeloma relapse.

#19 ARQ-197, a small-molecule inhibitor of c-Met, reduces tumor burden and prevents tumor-associated bone disease in a murine model of myeloma

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The receptor tyrosine kinase c-Met, its ligand HGF, and components of the downstream signaling pathway, have all been implicated in the pathogenesis of myeloma, both as a modulator of plasma cell proliferation and as an agent driving osteoclast differentiation and osteoblast inhibition, thus contributing substantially to the bone destruction typically caused by myeloma. Patients with elevated levels of HGF have a poor prognosis. Therefore, targeting these entities in such patients may be of great benefit. We hypothesized that ARQ-197 (Tivantinib), a small molecule c- Met inhibitor, would reduce myeloma cell growth and prevent myeloma-associated bone disease in a murine model. *In vitro* we assessed the effects of HGF on osteoblast differentiation, ARQ-197 (0.1563 μM - 5 μM) on myeloma cell proliferation, cytotoxicity and c-Met protein expression in the JJN3 human cell line. *In vivo* we intravenously injected NOD/SCID-γ mice with 10^6 JJN3 cells and 1 week later treated mice with either ARQ-197 (200 mg/kg/day, 5 times per week by oral gavage) or vehicle for 2 weeks. *In vitro* exposure of JJN3 cells to ARQ-197 (0.625 μM - 5 μM) resulted in a significant inhibition of cell proliferation ($p < 0.0001$) and an induction of cell death ($p < 0.001$), probably caused by significantly reduced levels of phosphorylated c-Met. *In vivo* ARQ-197 treatment of JJN3 tumor-bearing mice resulted in a significant reduction in tumor burden ($p < 0.001$), where tumor infiltration of the bone marrow was reduced by approximately 43% ($96 \pm 4.9\%$ vehicle vs $55 \pm 20\%$ ARQ-197 treatment). ARQ-197 treatment also significantly prevented the formation of myeloma-induced bone lesions ($P < 0.001$) and the loss of trabecular bone ($p < 0.01$) compared to vehicle treated JJN3-tumor bearing mice. Dynamic histomorphometry showed ARQ-197 treatment prevented significant decreases in the mineralizing bone surface ($p < 0.001$), the mineral apposition rate ($p < 0.01$), the Bone formation rate ($p < 0.01$), and prevented complete loss of osteoblasts on the cortico-endosteal bone surface compared to the vehicle group. In summary, these results suggest that ARQ-197 is a promising therapeutic in myeloma patients who express high levels of HGF, leading to both a reduction in tumor burden and an inhibition of myeloma-induced bone disease.

#20 Temporal and Spatial Characteristics of Bone Metastasis of Breast and Prostate Cancer

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Purpose: Recent developments in the treatment for early bone metastases (BM) requires better understanding of the metastatic distribution, incidence, and timing of bone metastases, as it may be possible to employ these therapies early in the course of treatment to avoid clinical development of bone metastases.

Methods: Patients with BM who also had breast or prostate cancer were included. A total of 347 patients, including 204 prostate cancer patients and 143 breast cancer patients, were evaluated. The date of diagnosis of primary cancer, date of development of BM, and available histological and treatment data were recorded. The distribution of BM at diagnosis was recorded from imaging reports, and the time to development of BM were recorded and analyzed.

Results: 34% of patients with prostate cancer and 24% of patients with breast cancer were diagnosed at initial presentation. The mean time to development of BM if not diagnosed at staging was 82 months and 81 months for prostate and breast cancer, respectively. Higher Gleason Scores were associated with decreased time to diagnosis of bone metastases ($p = < 0.01$) as well as risk of bone metastases at staging ($p = < 0.08$). For breast cancer there was no significant difference in the time to diagnosis of bone metastases based on Nottingham grade ($p = < 0.76$), histological subtype ($p = 0.62$), or receptor status ($p = 0.76$, 0.17, and 0.24 for estrogen receptor, progesterone receptor, and Her2/Neu expression, respectively). The distribution of bone metastases was similar between breast and prostate cancer, with increased rates of lesions in the pelvis for prostate cancer, and the sternum and calvarium for breast cancer. The number of regions of bone metastases discovered on imaging was higher in prostate compared to breast cancer.

Conclusion: Our data show that the time to development of bone metastases is decreased with increasing Gleason score. Furthermore, our data suggest that individual Gleason scores, particularly scores 8-10, may sub-stratify the risk of metastases, which is in contrast to traditional categorization of “high risk” as Gleason score 8-10 and “low risk” as Gleason score 7 or less. Our data did not find a similar correlation with Nottingham grade, histological subtype, or receptor status for breast cancer, suggesting these parameters have much less value for predicting the metastatic potential of breast cancer subtypes.

#21 Targeting Multiple Roles of EZH2 Methyltransferase in Myeloma-Induced Abnormal Bone Remodeling

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Multiple myeloma (MM) patients develop osteolytic bone lesions due to hyperactivation of osteoclast precursors (OCLp). The lesions rarely heal even after therapeutic remission due to MM-induced suppression of bone marrow stromal cell (BMSC) differentiation into osteoblasts (OB). To date, there are no agents that can reliably repair MM lesions.

EZH2, the methyltransferase subunit of Polycomb Repressive Complex 2 (PRC2), catalyzes tri-methylation of histone-3 lysine-27 (H3K27me3), which induces gene repression. We reported that MM cells induce increased EZH2 recruitment to the *Runx2* gene in MM patient-derived BMSC and the murine pre- OB cell line MC4, resulting in H3K27me3-mediated repression of *Runx2*, thus leading to suppression of OB differentiation. Using GSK126, a small molecule inhibitor of EZH2 activity, we show that MM-induced epigenetic repression of *Runx2* is reversible in both BMSC from MM patients and MM-treated MC4 cells, resulting in increased expression of *Runx2* and RUNX2 target OB genes *Ocn*, *Bsp*, and *Alpl*, and rescued mineralization.

In contrast, EZH2 and H3K27me3 levels are upregulated during the initial 24h of RANKL-induction, thus epigenetically silencing several OCL inhibitory factors such as *MafB*, *Irf8* and *Arg1,* and permitting OCL formation. EZH2 inhibition with GSK126 or *Ezh2* knockdown prevented OCL formation. The presence of MM1.S-conditioned media (MMCM) only during the 3-day M-CSF expansion phase of bone marrow OCLp significantly enhanced multinucleated OCL formation. MMCM enhanced RANKL-induction of *Ezh2*, OCL marker genes and the MM-supporting genes *Anxa2*, *Il6* and *MMP9* expression was blocked when GSK126 was present at addition of RANKL. However, when GSK126 was present during the M-CSF expansion and removed before RANKL addition, only MMCM amplification of OCL differentiation was inhibited. A novel cytoplasmic role of EZH2 methyltransferase activity during early RANKL signaling was revealed. EZH2 is required for (and GSK126 blocks) RANKL activation of the pAKT-pmTOR-pS6RP signaling axis affecting the translation ratio of the C/EBPβ- LAP and LIP isoforms, resulting in increased binding of the repressive LIP isoform to the *MafB* promoter, which is necessary for OCL formation.

Our *in vitro* studies suggest that *in vivo* GSK126 inhibition of the epigenetic modifier EZH2 may improve the osteogenic potential of MM-exposed BMSC and suppress OCL formation, and thus may prove a valuable therapeutic strategy to repair bone disease in MM patients.

#22 Delivery of Nanoparticle-Encapsulated Gli Inhibitor Blocks Tumor-Induced Bone Disease

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Advanced solid cancers originating in the breast, prostate, and lung frequently metastasize to bone. Once established in bone, these tumors initiate a vicious cycle of bone destruction known as tumor-induced bone disease (TIBD). While there are many factors in the bone microenvironment that promote tumor-induced bone destruction, our group has shown that Gli2, a Hedgehog (Hh) transcriptional activator, is overexpressed in metastatic tumor cells that stimulate osteoclast-mediated bone resorption. Moreover, genetic inhibition of Gli2 significantly reduces both tumor burden ($p < 0.001$) and bone destruction ($p < 0.05$). Therefore, Gli2 is a promising therapeutic target for the treatment of TIBD. Several studies have reported efficacious small-molecules which inhibit Gli downstream of the Hh receptors Patched and Smoothened; this is critical since Gli2 expression is not always regulated by canonical Hh signaling. Our own investigations show that treatment with the Gli antagonist GANT58 significantly reduces ($p < 0.001$) expression of the bone-destructive gene parathyroid hormone-related protein (PTHrP) in bony-invasive MDA-MB-231 breast cancer cells. Unfortunately, in vivo delivery of this drug to tumors in bone has been challenging due to its hydrophobicity. We hypothesized that encapsulating Gli inhibitors within nanoparticles would facilitate delivery of these agents to tumors residing in bone and cause a significant reduction in TIBD. Accordingly, we loaded GANT58 ($\leq$10 mg/kg) into reactive oxygen species (ROS)-responsive nanoparticles made from poly (propylene sulfide-co-oligoethylene glycol acrylate) (PPS-POEGA) to inhibit Gli2 expression in

bone-tropic MDA-MB-231 cells that were injected into the tibia of athymic nude mice. The GANT58 nanoparticles (GANT58-NPs) were fabricated using an oil-in-water solvent evaporation method and characterized for particle size (d = 170 nm), drug loading (13%), and encapsulation efficiency (90%). Our preliminary data shows a preferential localization of GANT58-NPs at the tumor site in bone ($p < 0.05$) compared to non-tumor bone. Furthermore, mice treated intravenously with GANT58-NPs had a significant reduction in bone lesion area ($p < 0.05$) and significantly improved trabecular bone volume ($p < 0.001$). In summary, delivery of nanoparticle-encapsulated Gli inhibitors is a promising therapy for TIBD as it allows for targeted inhibition of Gli2 to decrease PTHrP expression and bone destruction.

#23 Anti-TGFβ therapy in combination with zoledronic acid repairs osteolytic lesions in multiple myeloma

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Rationale and hypothesis: Over 90% of patients with myeloma develop lytic bone disease and frequently present with a catastrophic skeletal event. Current treatment for myeloma bone disease (MBD) relies on bisphosphonates (BP), which effectively prevent further bone destruction but do not repair existing lesions. Therefore, the use of a bone anabolic agent in combination with a BP has potential to substantially improve outcomes of MBD. We hypothesised that anti-TGFβ (1D11) (a bone anabolic) given in combination with the BP zoledronic acid (ZOL) would treat MBD and repair osteolytic lesions in murine models of myeloma.

Object: To confirm the bone anabolic effect of 1D11 on osteoblasts *in vitro* and *in vivo*, and to determine whether the combination of 1D11 with ZOL prevents myeloma induced bone loss and repairs osteolytic lesions in models of myeloma.

Methodology: Primary osteoblasts and SAOS-2 cells were treated with rTGFβ and isotype control or 1D11, and alkaline phosphatase (Alp) and mineralisation were assessed. NSG mice were injected with PBS or JJN3 cells *i.v*. After 7 days mice were treated with 1D11 (20 mg/kg *i.p* every 3 days) and culled after 14 days. U266 cells were injected into NSG mice *i.v* and after 6 weeks were treated with PBS, ZOL (125 μg/kg *s.c* day 0 and 3 days) or ZOL and 1D11 (as above) for 3 weeks. Bone parameters were analysed using *ex vivo/ in vivo* micro-CT and histology.

Results: 1D11 restored Alp production and mineralisation in primary osteoblasts and Alp production in SAOS- 2 cells following rTGFβ treatment. 1D11 therapy when given alone resulted in a significant increase in BV/TV in both naïve ($p = < 0.05$) and NSG-JJN3 mice ($p = < 0.001$), which was a direct effect upon bone formation, not a result of reduced tumour burden. Most importantly, when 1D11 was given in combination with ZOL in NSG-U266 mice, lesions were significantly smaller and in 6/8 mice osteolytic lesions were completely repaired, whereas in the ZOL only group lesion area remained the same or increased.

Conclusion: 1D11 has a potent bone anabolic effect on primary osteoblasts and in naïve and NSG mice bearing the aggressive JJN3 myeloma cell line. Most importantly, when given in combination with ZOL it prevented myeloma-induced bone loss compared with ZOL monotherapy and repaired osteolytic lesions in NSG mice bearing U266 myeloma cells. This therefore demonstrates substantial potential for enhanced outcomes in the future treatment of MBD in patients.

#24 HDAC inhibition: A novel therapeutic approach for the treatment of primary and lung metastatic osteosarcoma

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Osteosarcoma (OS) is the most common primary skeletal malignancy. Disappointingly, mortality rates have remained static for the past two decades. Therefore, therapies that can prevent OS progression are of urgent clinical need. Recently, we reported that panobinostat, a histone deacetalyase (HDAC) inhibitor, limits the viability of a wide range of OS cell lines *in-vitro*. Here, we explore whether panobinostat is effective in preventing OS growth and metastasis *in vivo* using the immunocompetent K7M2 OS model.

Objective 1. Primary OS growth and metastasis. Mice were intratibially inoculated with K7M2-Luc cells and then treated with vehicle (n = 11) or panobinostat (n = 10; 10 mg/kg for all *in vivo* studies). Tumor growth was measured by bioluminescence (RLU). The clinical end point was $1x10^6$ RLU (IVIS-200). Primary OS growth was significantly slower in the panobinostat treated group ($p < 0.05$ by day 17) with the median time to clinical endpoint being 2.5 fold longer than the controls (control median: 21 days vs. panobinostat median: 53 days, $p < 0.05$). μCT analysis demonstrated panobinostat limited OS induced bone disease. Panobinostat also impacted spontaneous lung metastasis (Time to detection; control, 10 days vs. panobinostat, 21 days). Histological analyses showed significantly lower metastatic burden and growth rates (pHistone-H3/cleaved caspase-3) in the panobinostat group. Analysis of acetylated histone H3 in isolated tissues confirmed HDAC inhibition by panobinostat. **Objective 2.** Pretreatment to prevent OS lung seeding. Mice were pretreated with vehicle (n = 9) or panobinostat (n = 5) for 5 days prior to tail vein inoculation of $1x10^6$ K7M2-Luc cells. Bioluminescence demonstrated that panobinostat significantly ($p < 0.05$) delayed tumor growth compared to control animals. This translated into higher overall survival rates (control median: 54 days vs. panobinostat 93 days, $p < 0.05$). Despite halting panobinostat treatment at day 38, 40% (2/5) of the mice remained alive at day 100. **Objective 3.** Treatment of established lung metastases. Mice were tail-vein inoculated with $1x10^6$ K7M2-Luc cells and randomized after three days into control (n = 12) and panobinostat (n = 15) treated groups. Bioluminescence readings demonstrated that panobinostat significantly reduced the growth of established OS lung metastases ($p < 0.05$).

Taken together panobinostat is effective for the treatment of OS and our findings provide rationale for the initiation of HDAC inhibitor focused clinical trials.

#25 Selective inhibition of matrix metalloproteinase-2 in the multiple myeloma-bone microenvironment

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Multiple myeloma is plasma cell malignancy that homes aberrantly to bone causing extensive skeletal destruction. Despite the development

of novel therapeutic agents that have significantly improved overall survival, multiple myeloma remains an incurable disease. Matrix metalloproteinase-2 (MMP-2) is associated with cancer progression and is significantly overexpressed in the bone marrow of myeloma patients. Immunofluorescent staining of human myeloma biopsies demonstrated MMP-2 expression to be widespread throughout the bone microenvironment (including CD138+ myeloma cells.) These data provide rationale for selectively inhibiting MMP-2 activity as a multiple myeloma treatment strategy. Given that MMP-2 is systemically expressed, we used novel "bone-seeking" bisphosphonate based MMP-2 specific inhibitors (BMMPIs) to target the skeletal tissue thereby circumventing potential off-target effects of MMP-2 inhibition outside the bone marrow-tumor microenvironment. *In vitro* analysis, demonstrate BMMPIs to inhibit MMP-2 activity at nanomolar concentrations with high specificity. Using the U266 mouse model of multiple myeloma, we examined the efficacy of BMMPIs for the specific inhibition of MMP-2 in the bone microenvironment. Our data demonstrate that BMMPIs can decrease multiple myeloma burden (~41% reduction at week 10.) as measured by circulating myeloma specific IgE and bioluminescense. Further, our studies show that BMMPIs protect against cancer- induced osteolysis as measured by X-ray (TuV/BV 0.15 vs. 0.06; <0.05), μCT (BV/TV 0.01 vs. 0.08; $p<0.05$) and histomorphometry (BV/TV 0.01 vs. 0.12; $p<0.05$.) Additionally, using an ex vivo MMP-2 assay we demonstrated a 55% reduction in MMP-2 activity in the multiple myeloma-bone microenvironment. Analysis with a broad-spectrum MMP probe, revealed no significant difference in MMP activity. These data underscore the feasibility of developing targeted and tissue selective MMP inhibitors. We posit that this strategy could be adapted for the inhibition of other MMPs known to be important in skeletal malignancies thereby limiting potential toxicities associated with systemic MMP inhibition. Given that bisphosphonates are well tolerated in humans, we anticipate that BMMPIs could be rapidly translated to the clinical setting where they would ultimately extend the overall survival of multiple myeloma patients.

#26 Phase 1 study of the bone-targeting cytotoxic conjugate, etidronate- cytosine arabinoside (MBC-11), in cancer patients with bone metastases

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Background: MBC-11 is a first-in-class therapeutic conjugate of the bone targeting bisphosphonate etidronate covalently linked to the antimetabolite cytosine arabinoside (Ara-C). In preclinical studies, MBC-11 localizes at the site of cancer-induced bone disease (CIBD) where it demonstrates both antiresorptive and antitumor activities following local release of Ara-C. Robust efficacy was observed in several rodent models of CIBD, as well as in spontaneous osteosarcoma in dogs. Herein, the results of the first-in- human study of MBC-11 are reported.

Methods: Patients with advanced solid cancers and CIBD were treated with escalating doses (0.5-10 mg/kg/day) of MBC-11 administered as an intravenous infusion daily for 5 days every 4 weeks for up to 4 cycles. Fifteen patients (prostate cancer [PC; 7], breast cancer [BC; 7], cervical cancer [1]) received 38 total cycles. The study sought to characterize the safety, pharmacokinetics, and the effects of MBC-11 on bone turnover, and tumor response by ^{18}F-FDG-PET/CT imaging and tumor biomarkers.

Results: Myelosuppression was generally grade 1-2, involved all lineages, and was the principal toxicity of MBC-11. Two of three patients treated at the 10 mg/kg dose level had dose-limiting toxicity (DLT), each with both grade 4 neutropenia and thrombocytopenia, the maximum tolerated dose (MTD) was 5 mg/kg. Four of 5 patients with pretreatment elevations of the bone resorption marker Trap5b had persistent decrements. ^{18}F-FDG- PET/CT imaging demonstrated partial metabolic responses in 3 patients; one BC patient treated at the 0.5 mg/kg and two CRPC patients treated at 1.0 mg/kg dose levels. An additional 3 patients had stable metabolic responses according to PERSIST. SUV values were reduced by at least 25% in 111 (53.8%) of 206 measurable bone lesions; significant activity was noted at all doses.

Conclusions: At doses that were well tolerated and even much lower than the MTD, MBC-11 treatment resulted in substantial reductions in metabolic activity in CIBD patients, providing a foundation for further disease-directed studies to further assess efficacy.

#27 CaMKK2 Inhibition as a "Dual-Hit" Strategy against ADT-Induced Osteoporosis and Bone-Metastatic Prostate Cancer

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Prostate cancer (PCa), the most frequently diagnosed cancer in men, will have an estimated 26,730 deaths in the US during 2017. Androgen deprivation therapy (ADT), the standard treatment for PCa contributes to osteoporosis and increased fracture risk, drastically reducing the patient's quality of life. Established clinical anti-resorption therapies slow the loss of bone, but are unable to promote bone formation. In contrast, anabolic therapies are vastly underdeveloped and form the greatest clinical need in the treatment of osteoporosis.

Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) plays a role in both the anabolic and catabolic pathways of bone remodeling. Pharmacological inhibition of CaMKK2 using STO-609 protects against ovariectomy-mediated and reverses age-associated bone loss in mice. In PCa, CaMKK2 is highly over-expressed and regulated by the androgen receptor. *In vitro* studies have shown its inhibition suppresses the growth and migration of PCa cells. Moreover, *in vivo* treatment with STO-609 significantly impaired growth of xenografted PCa cells. We also observed that CaMKK2 inhibition suppresses *in vitro* 3-D spheroid formation by the androgen independent cell line, C4-2 but does not affect spheroid numbers or size of an androgen dependent cell line PC3, potentially revealing a role for CaMKK2 in PCa cell growth and adhesion.

Based on these preliminary data, we hypothesize that inhibition of a single target, CaMKK2; will result in the therapeutic alleviation of two major complications in advanced-stage PCa, i.e., bone metastatic tumor burden and ADT-induced bone loss. To replicate ADT-induced bone loss we performed sham or bilateral orchiectomy (ORX) on pretreated (saline/STO-609) 5-week-old male athymic nude mice ($n=10$ per cohort). Tri-weekly intraperitoneal (i.p.) injections continued for 6 weeks (saline/STO-609). Micro-computed tomography analysis indicated a prevention of ORX-induced bone loss in STO 609 treated mice compared to saline treated controls (3-fold, $p<0.05$). Additionally, two weeks after surgery, sham and ORX mice were intra-tibially injected with C4-2B cells. Radiographic and histomorphomteric analyses reveal a decrease in PCa cell-initiated bone lesions in STO-609 treated mice compared to the saline treated cohorts. Taken together these studies a role for CaMKK2 in PCa growth in the bone and in the prevention of ADT-induced bone loss.

#28 Therapeutic impact of TAK1 inhibition on myeloma tumor progression and bone destruction

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Multiple myeloma (MM) has a unique propensity to develop and expand almost exclusively in the bone marrow and generates destructive bone disease. MM cells constitutively overexpress Pim-2; cocultures with bone marrow stromal cells (BMSCs) or osteoclasts (OCs) were found to further upregulate Pim-2 as an anti-apoptotic mediator in MM cells and induce it also in BMSCs and OCs to progress bone destruction (Leukemia, 2011, 2015), indicating the critical role of Pim-2 in MM tumor growth and bone destruction. We recently identified TGF-β-activated kinase-1 (TAK1) as an upstream mediator responsible for Pim-2 up-regulation in these cells. In this study, we aimed to clarify the role of TAK1 in MM growth and bone destruction and therapeutic impact of TAK1 inhibition. TAK1 was constitutively overexpressed and phosphorylated in MM cells. The TAK1 inhibitor LLZ1640-2 abolished TNF-α-induced NF-κB, p38MAPK and ERK activation and IL-6-induced STAT3 phosphorylation in MM cells. Importantly, LLZ1640-2 suppressed Pim-2 up-regulation and induced apoptosis in MM cells even in cocultures with BMSCs or OCs, while reducing VEGF production and the expression of BCMA and TACI, receptors for BAFF and APRIL. LLZ1640-2 as well as TAK1 knockdown impaired adhesive interactions between MM cells and BMSCs along with reducing VCAM-1 and RANK ligand expression and IL-6 production by BMSCs, which blunted protective activity of BMSCs for MM cells against anti-MM agents. The known inhibitors for osteoblastogenesis in MM, including IL-3, IL-7, TNF-α, TGF-β and activinA, as well as MM cell conditioned media all induced TAK1 phosphorylation and thereby Pim-2 up-regulation in MC3T3-E1 preosteoblastic cells; however, LLZ1640-2 abolished the Pim-2 up-regulation and restored the osteoblastogenesis. TAK1 inhibition potentiated BMP-2-mediated Smad signaling in MC3T3-E1 cells. Furthermore, RANK ligand induced the TAK1-Pim-2 signaling in RAW264.7 cells; LLZ1640-2 suppressed their osteoclastogenesis by RANK ligand. Finally, treatment with LLZ1640-2 markedly suppressed MM tumor growth and prevented bone destruction in mouse MM models with intra-tibial injection of 5TGM1 MM cells. From these results, TAK1 appears to play a pivotal role in tumor progression and bone destruction in MM. TAK1 inhibition may become a unique anti-MM therapeutic option with bone-modifying activity.

#29 Anti-sclerostin antibody prevents myeloma bone disease and increases bone strength

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Multiple myeloma is a plasma cell cancer that causes bone destruction and fractures, which result from increased bone resorption and decreased bone formation. Anti-resorptive drugs prevent further bone loss but fail to influence bone formation, so patients continue to fracture. Development of drugs that stimulate bone formation and prevent fracture are a priority. Sclerostin is a soluble Wnt antagonist that inhibits bone formation with expression restricted to osteocytes. We hypothesized that anti-sclerostin antibody would prevent bone disease and increase fracture resistance in myeloma. Sclerostin was not expressed in plasma cells isolated from 630 patients with myeloma, or 54 human myeloma cell lines. Mice injected with 5TGM1-eGFP, 5T2MM or MM1.S myeloma cells, demonstrated significant bone loss (32%, 36% and 69%, $p < 0.01$, respectively) caused by increased osteoclastic bone resorption (182%, $p < 0.01$) and suppressed bone formation (92%, $p < 0.001$), when compared to non-tumour bearing mice. Bone loss in the vertebrae resulted in decreased bone strength in 5TGM1 (37%, $p < 0.01$), 5T2MM (30%, $p < 0.05$) and MM1.S (51%, $p < 0.01$) bearing mice. Anti-sclerostin treatment (100mg/kg weekly) increased bone volume in 5TGM1 (54%, $p < 0.01$), 5T2MM (516%, $p < 0.01$) and MM1.S (110%, $p < 0.05$) tumour- bearing mice, returning bone volume to control levels. Anti-sclerostin treatment also reduced the number of osteolytic bone lesions ($p < 0.02$). Anti-sclerostin treatment increased osteoblast numbers (663% $p < 0.05$) and bone formation rate (91%, $p < 0.01$), but did not alter osteoclastic resorption or tumour burden. Anti-sclerostin antibody increased vertebral strength in mice bearing 5TGM1, 5T2MM and MM1.S cells to levels in control naïve mice. Combining anti-sclerostin antibody, to stimulate bone formation, with zoledronic acid, to suppress bone resorption, led to increases in bone volume (112% $p < 0.001$) and bone strength (114% $p < 0.001$) beyond zoledronic acid treatment alone. This study defines a therapeutic strategy superior to current approaches, which will reduce fractures, enhance quality of life and ultimately improve survival in patients with myeloma.

#30 Bone-induced expression of integrin β3 on breast cancer metastases enables targeted nanotherapy

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Bone metastases occur in approximately 70% of metastatic breast cancer (BC) patients, often leading to the development of severe skeletal injuries. Current treatments are largely palliative, highlighting the unmet clinical need for improved therapies. Here, we report for the first time that the bone microenvironment strongly induces integrin β3 expression on murine BC bone metastases, as compared to weak β3 expression on primary mammary tumors or visceral metastases.

Evaluation of tumor tissue from metastatic BC patients demonstrated high β3 expression on bone metastases, compared to significantly lower expression on primary tumors from the same patient (n = 42). We identified TGF-β signaling through SMAD2/SMAD3 as necessary for BC induction of β3 within the bone. We next asked whether targeting integrin αvβ3 could improve drug delivery to bone metastases and found that αvβ3-targeted micelle nanoparticles (αvβ3-MPs, ~12.5nm) specifically colocalized with BC bone metastases. αvβ3-MP-mediated delivery of the chemotherapeutic docetaxel significantly reduced bone tumor burden and bone destruction as compared to free-docetaxel, with less hepatotoxicity. Furthermore, we observed a significant decrease in bone-residing tumor cell proliferation with αvβ3-MP/docetaxel treatment as compared free-docetaxel. Collectively, we demonstrate that integrin αvβ3 is a promising target for nanoparticle-mediated drug delivery against BC bone metastases and provide support for αvβ3 as a molecular target in patients with breast cancer bone metastases.

#31 Dual BET domain-kinase Inhibitors for the treatment of Multiple Myeloma

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Bromodomain protein 4 (BRD4) is a histone acetyl transferase (HAT) that has been associated with the activity of oncogenic transcriptional programs in many malignancies including multiple myeloma. BRD4 inhibitors such as JQ1, effectively prevent myeloma progression. However, studies have shown that resistance to BET bromodomain inhibitors can evolve, indicating that single agent therapies targeting BRD4 may not provide durable therapeutic responses.

To this end we have developed SG3-14, a compound that is capable of inhibiting BRD4 activity with an IC_{50} in the nM range (27nM) similar to that of JQ1 (20nM), but additionally can inhibit the activity of kinases that are often highly expressed in cancer namely, JAK2, FLT3 and RET also in the nM range. *In vitro*, we observed that SG3-14 limited the viability of multiple myeloma cell lines at IC_{50}s an order of magnitude above that of JQ1 (5TGM1; SG3-14, 0.85μM vs. JQ1, 4.7μM and U266; SG3-14, 0.99μM vs. JQ1, 16μM). Molecular analyses demonstrated that SG3-14 can block c-Myc expression similarly to that observed with JQ1.

To test the efficacy of SG3-14 *in vivo*, we used the immunocompetent luciferase expressing 5TGM1 model of multiple myeloma. Mice (n = 10/group) were inoculated via tail vein with 5TGM1 cells ($1x10^6$) and treated with either vehicle (15% (2-Hydroxypropyl)-β-cyclodextrin), SG3-14 (25mg/Kg) or JQ1 (25mg/Kg). We observed that SG3-14 and JQ1 significantly delayed tumor growth rate compared to control as measured by bioluminescence and that SG3-14 was superior to JQ1 ($p < 0.05$). Slower myeloma growth rates in the SG3-14 group translated into significantly better overall survival compared to control (SG3-14 median; 50.5 days, JQ1 median; 46 days; Control; 40.5 days). Importantly, no toxicities were noted in the SG3-14 group at the chosen dose. Immunohistochemistry analyses of tibias containing myeloma-bearing bones for IgG2b, cleaved caspase-3 and phospho-Histone H3 confirmed the efficacy of SG3-14. microCT/Xray/histomorphometry analyses also illustrated the beneficial effects of SG3-014 in protecting against myeloma induced bone disease. We are currently exploring the key kinases inhibited by SG3-014 in the context of multiple myeloma.

Collectively, our data show that a dual BET domain-kinase inhibitor approach is superior to BET inhibition alone and this strategy may prove more resilient to drug resistance in multiple myeloma.

Other cells affecting tumor growth in bone and bone pain

#32 Prostate cancer promotes a pro-metastatic bone marrow niche through inducing osteocytes to release GDF15.

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Advanced prostate cancer frequently metastasizes to bone. The interaction between tumor cells and their bone microenvironment is critical for malignant progression. Although studies have evaluated both osteoclasts and osteoblasts in promoting bone metastasis, the role of osteocytes in regulating metastatic development are poorly understood. The goal of this study was to better define the role of osteocytes in prostate cancer progression. Prostate cancer cell line conditioned-medium (CM) (VCaP and C4-2B prostate cancer cell lines) induced the osteocyte cell line MLO-Y4 to promote prostate cancer cell proliferation, migration and invasion. To identify soluble factors that could mediate this effect, we performed a screen of cytokines modulated in MLO-Y4 by prostate cancer cell CM. We found that prostate cancer CM induced MLO-Y4 to produce growth-derived factor 15 (GDF15). Knockdown of GDF15 in MLO-Y4 cells prevented the ability of the osteocytes to induce prostate cancer tumor promoting activity. C4-2B and PC-3 prostate cancer cells exposed to CM from MLO-Y4 cells with knockdown of GDF15 had decreased EGR1 expression compared to cells exposed to CM with GDF15. Inhibition of EGR1 in C4-2B and PC3 abrogated the ability of CM from prostate cancer CM-treated MLO-Y4 cells to induce their proliferation. These data demonstrate that prostate cancer cells modulate MLO-Y4 cells to promote PCa pro-metastatic phenotype through GDF15 and EGR1. These results suggest that prostate cancer educates the bone microenvironment through osteocytes to promote metastasi

#34 Hypoxia and HIF Signaling in the Bone Microenvironment is Associated with Metastatic Colonization of the Bone

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The cellular and molecular mechanisms that regulate tissue specific metastatic organ tropism are incompletely understood. One unique feature of the bone microenvironment (BME) is that this region provides the niche for hematopoietic stem cells (HSCs). Interestingly, within the BME, metastatic tumor cells localize to niches occupied by HSCs and pharmacologic expansion of the HSC niche leads to an increase of tumor cell engraftment to this region. These observations led to the hypothesis that metastasizing tumor cells have the ability to "hijack and parasitize" the HSC niche. These discoveries highlight the importance of understanding the molecular underpinnings that drive metastatic engraftment and colonization of this shared region. The BME contains areas of vascular heterogeneity which contribute to regional areas of low oxygen tension, or hypoxia. HSCs localize to hypoxic regions within the BME as hypoxia inducible factor (HIF) signaling is required for HSC function and metabolism. In addition, HIF signaling regulates expression of genes that contribute to the initial homing and engraftment of malignant cells to the BME. However, the function of hypoxia and HIF signaling in regulating the later stages of bone metastasis is less clear. We hypothesize that hypoxia and HIF signaling within the BME contributes to metastatic colonization of the bone by malignant cells. Our studies demonstrate that components of the HIF signaling pathway are expressed and HIF signaling is active in both bone marrow mesenchymal stromal cell and cancer cells. Furthermore,

our data demonstrates that upon intra-cardiac delivery, breast carcinoma and melanoma cell lines colonize to hypoxic regions in the BME. Given these observations, we have begun to further explore the role of HIF signaling in the bone metastatic niche through the use of genetic mouse models in which HIF signaling is conditionally ablated in cells of osteoblastic and vascular HSC niche. Our studies will reveal the contribution of hypoxia and HIF signaling within the BME for bone colonization by metastatic tumor cells.

#35 Effects of Anti-Estrogen Therapy on the Musculoskeletal System and Implications for the Tumor Microenvironment

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Adjuvant endocrine therapy is a standard treatment for postmenopausal women with estrogen receptor (ER)-positive breast cancer. Unfortunately, up to 50% of women treated with an aromatase inhibitor (AI) develop muscle weakness, bone loss, and joint pain that result in treatment discontinuation. Previous studies in our laboratory demonstrated that AI treatment induced bone loss and skeletal muscle weakness, recapitulating effects observed in cancer patients. We also demonstrated that prevention of AI-induced osteoclastic bone resorption using a bisphosphonate attenuated the development of ER- negative breast cancer bone metastases and improved muscle function in mice. These findings highlight the bone microenvironment as a modulator of tumor growth locally and muscle function systemically.

Because muscle weakness and arthralgia are also commonly reported in women treated with selective estrogen receptor modulators (SERMs), a follow-up study was designed to compare the musculoskeletal effects of AI with a bone-sparing SERM endoxifen (Endx) in a non-tumor model. Female C57BL/6 mice underwent OVX and were treated daily with vehicle, the AI letrozole (Let), or Endx, and changes in bone volume and microarchitecture were assessed over time at the proximal tibia by μCT. After eight weeks of treatment, trabecular bone volume fraction (BV/TV) decreased in OVX-vehicle and OVX-AI mice (-53% and -32%, respectively) whereas BV/TV increased threefold in Endx mice (+303%). At the termination of the study, muscle-specific force was significantly lower in OVX-Endx mice relative to both OVX-vehicle and OVX-AI mice indicating that SERM-induced muscle weakness is likely independent of osteoclastic bone resorption. Histological and biochemical assessment of skeletal muscle will be performed in order to determine a mechanism for muscle weakness in Endx-treated mice, and ongoing preclinical studies will evaluate how Endx-driven changes to bone may impact the tumor microenvironment. Identification of the mechanism(s) of endocrine therapy-induced muscle weakness could lead to the development of non-estrogenic therapeutics that improve compliance for these life-prolonging therapies.

#36 Mesenchymal stem cells promote the evolution of apoptotic resistant prostate cancer in the bone microenvironment.

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Bone metastatic prostate cancer is hallmarked by excess bone formation. Mesenchymal stem cells (MSCs) that reside in the bone marrow can differentiate into bone forming osteoblasts and are thought to promote cancer cell growth. We therefore hypothesized that prostate cancer MSC interactions could lead to increased osteogenesis and tumor growth. To this end, primary murine MSCs were isolated from donor C57BLl/6 mice and characterized by flow cytometry (CD29+, SCA1+, CD45-) and differentiation assays. Rag-2 immunocompromized mice (n = 8/group) were then intratibially inoculated with MSCs alone ($2x10^4$), PAIII cells alone ($2x10^4$)-prostate cancer cells capable of inducing mixed osteolytic and osteoblastic lesions or a 1:1 ratio of MSCs:PAIII cells ($4x10^4$ total). Using bioluminescence as a correlate of tumor growth, we observed that MSCs initially suppressed PAIII growth up to day 11 ($p < 0.05$) but after this point, PAIII cells in this group grew at a significantly ($p < 0.05$) faster rate than the PAIII group alone. Post study analyses also revealed, as expected, that MSCs significantly reduced tumor induced osteolysis (X-ray) while promoting an osteogenic phenotype (μCT and histomorphometry). *In vitro* studies revealed that the co-culture of PAIIIs with varying concentrations of MSC conditioned media (CM) significantly promoted PAIII apoptosis within 5-hours as measured by cleaved caspase-3.Successive exposures of PAIIIs to MSC CM *in vitro* yielded PAIIIs (F2 PAIII) that were resistant to apoptosis induced by MSC CM, etoposide and docetaxel. Subsequent *in vivo* studies (n = 10/group) demonstrated that co-injection of F2-PAIII with MSCs, grew at significantly faster rates than the F2-PAIII group alone or the parental PAIII cells indicating MSCs were capable of promoting the evolution of more aggressive prostate cancer cells. We next explored the mechanism by which MSCs promoted the outgrowth of faster growing PAIII cells. Cytokine array analysis of MSC CM revealed apoptotic factors including interleukin 28 (IL-28). Depletion of IL-28 from MSC CM prevented PAIII apoptosis. These observations were confirmed using independent cell lines (DU145 and Myc-CaP). We are currently exploring whether IL-28 receptor depletion in parental PAIII cells can promote apoptosis resistance. Understanding how altered downstream effectors of IL-28 receptors mediate this effect may reveal potential therapeutic targets to effectively treat bone metastatic prostate cancer.

#37 Host-derived matrix metalloproteinase-13 contributes to the progression of multiple myeloma.

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Multiple myeloma promotes systemic skeletal bone disease that greatly impact the patient's quality of life. Processing of the type I collagen rich bone matrix results in the release of sequestered growth factors that can drive progression of the disease. Matrix metalloproteinase 13 (MMP-13) is a type-I collagenase that is expressed predominantly in the skeleton by mesenchymal stromal cells (MSCs). Analysis of public datasets demonstrate that myeloma significantly induces the expression of MMP-13 in MSCs (1.81 LogFC, $p < 0.05$.) Consistent, with this observation, immunofluorescent analyses of human myeloma biopsies also demonstrated MMP-13 expression in bone lining osteoblasts but interestingly, not in bone resorbing osteoclasts. We therefore determined whether host-derived MMP-13 could contribute to myeloma progression and overall survival. Immunocompromised mice, wild type or null for MMP-13, were inoculated with luciferase expressing 5TGM1. Using bioluminescence, IgG2b and immunohistochemistry as a readout for tumor burden, we detected no difference in myeloma growth rate. Surprisingly, we did observe a significant increase in overall survival in the MMP-13 null group (mean 39 vs. 43 days; $p < 0.05$.) *Ex vivo* analysis by high resolution μCT, normalized to age-matched control mice, demonstrated that myeloma-induced bone resorption was significantly reduced in the MMP-13 null mice (0.5 vs. 0.23 ratio; $p < 0.05$). Analysis of stromal co-cultures identified that MMP-13 null osteoclast formed more slowly than their wild type counterparts with reduced functionality (24% reduction in resorption of a bone mimetic, $P < 0.05$.) Further exploration of conditioned media derived from wild type and MMP-13 null MSCs indicate a number of potential substrates through which MMP-13 may

be contributing to osteoclast formation and function. Taken together with a recent report demonstrating myeloma- derived MMP-13 can also impact osteoclast behavior in a non-catalytic manner, we propose that MMP-13 plays multiple roles in myeloma induced bone disease and that selective exosite inhibitors that limit catalytic and non-catalytic roles would be beneficial for the treatment of the disease.

#38 Role of Dura in Pathogenesis of Prostate Cancer Spine Metastases.

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Background:

In the US there are approximately 63,000 men with bone metastases from prostate carcinoma (PCa), the majority of which are found in the spine. Spine metastases effect stability of the spinal column, can damage spinal nerves and cause spinal cord compression, leading to immense pain, neurologic deficits, paralysis, poor quality of life and enormous health care costs. The mechanisms of preferential tumor growth in the spine are not fully understood.

The “seed and soil” theory posits that differences in the local microenvironment lead to differential tumor dissemination and growth. In close proximity to vertebral metastases, lies the dura mater, the outer layer of the meninges, which forms a protective tube around the spinal cord inside the vertebral canal. During development, dura mater regulates morphogenesis in the CNS and in the overlying bone by actively modulating the surrounding environment, secreting factors like CXCL12, FGF2, and TGFβ, which guide proliferation, migration and differentiation of precursor cells.

We hypothesize that the dura continues to shape its environment into adulthood, and interact with tumor cells to contribute to the preferential metastatic growth in the spine. No published studies to date have investigated the role of the dura in promoting tumor growth.

Methods/Results:

To test this hypothesis, we analyzed the transcriptome of mouse dural fibroblasts by RNA-Seq and found abundant expression of secreted growth factors including CXCL12, TGFb and 3 ligands of the CXCR2 receptor: CXCL1, CXCL2 and CXCL5. We than utilized co- cultured experiments with conditioned media from the dura (DCM) and observed significantly increased proliferation of DU145 PCa cells (2-fold, $p = 0.0003$). This proliferation was abrogated by a specific CXCR2 small molecule inhibitor. DCM also promoted increase in migration of DU145 cells ($p = 0.0374$). To examine the cross-talk between dura and prostate cancer cells we performed co-culture experiments with dura and PC3 or DU145 revealing that many of the secreted factors initially seen in dura cultures are strongly up-regulated in the presence of tumor cells (e.g. a 70-fold increase in expression of CXCL5).

Conclusion:

These data demonstrate the bio-active nature of dura and show that dura has a direct effect on prostate cancer cell proliferation and migration. In addition, we show paracrine crosstalk between tumor and dura that increases factors known to promote cancer growth and metastases.

#39 Gfi1 Transcription Factor Modulates SphK1 to Maintain Growth and Survival of Myeloma Cells

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Multiple myeloma (MM) is an incurable hematologic malignancy caused by the accumulation of malignant plasma cells in the bone marrow featuring osteolytic lesions in the majority of patients. We previously reported that Growth independent factor 1 (Gfi1) upregulation in bone marrow stromal cells (BMSC) causes suppression of osteoblast differentiation. Our recent findings show that Gfi1 is also increased in the majority of $CD138^+$ cells from MM patients and cell lines. We hypothesizes that Gfi1 plays a central role in MM regulating cell growth and survival in part by modulating SphK1 levels, a known modulator of cancer progression. Therefore, we determine the effect of knock down (KD) or overexpression (o/e) of Gfi1 in MM cell growth and survival and the contribution of SphK1 to these effects.

The effects of Gfi1 KD on MM cell survival were assessed by transduction with pLKO.1-puro lentivirus encoding Gfi1 or non-mammalian shRNA. The anti-apoptotic effects of Gfi1 o/e were tested by transduction with pUC2 lentivirus encoding Gfi1 or the empty vector (EV).

We found that SphK1 mRNA is highly expressed in $CD138^+$ cells from MM patients and cell lines compared with normal donors and that Gfi1 protein levels correlate with the expression of active SphK1. Microenvironmental soluble factors (IL-6 and S1P), hypoxia and adhesive interactions with BMSC further increased Gfi1 and SphK1 mRNA and protein levels in MM cells. KD of Gfi1 displayed a profound decrease of SphK1 mRNA levels and protein activity in MM cells while inhibiting the growth and inducing apoptosis of these cells. Enforced expression of Gfi1 had opposite effects on SphK1 levels and conferred a survival advantage to MM cells over control cells. These data suggest that Gfi1 regulates MM growth in part via enhancing the expression and the activity of SphK1. SphK1 inhibitor (SKI2) profoundly reduced cell viability in MM cells harboring p53 wt. In p53 null MM cells, although SKI2 treatment induced caspase 3 activation, even in the presence of stromal cells, the pro-survival Mcl-1 levels were unchanged possibly due to the activation of Gfi1.

Taken together, our results suggest that Gfi1 may act as a key regulator of MM growth and survival at least partially through modulation of SphK1. Therefore, targeting Gfi1 may be a novel therapeutic strategy for MM patients.

#40 Sympathetic Activation Alters the Bone Vasculature: Implication for Osteotropic Breast Cancer Metastasis

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The skeleton is a common site for breast cancer metastasis. Although significant progress has been made to manage osteolytic bone lesions, patients still die of the disease. Thus, there is a need to identify the early determinants of the breast cancer bone metastatic process in order to treat the disease more effectively. Progression and recurrence of breast cancer, as well as reduced survival of patients with breast cancer, are associated with chronic stress, a condition known to stimulate sympathetic nerve outflow. In this study, we show that stimulation of the beta 2-adrenergic receptor (β2AR) by isoproterenol, used as a pharmacological surrogate of sympathetic nerve activation, leads to increased blood vessel density and *Vegf-a* expression in bone. It also raised levels of secreted Vegf-a in osteoblast cultures, and accordingly, the conditioned media from isoproterenol-treated osteoblast cultures promoted new vessel formation in two *ex vivo* models of angiogenesis. Blocking the interaction between Vegf-a and its receptor, Vegfr2, blunted the increase in vessel density induced by isoproterenol. Genetic loss of the β2AR globally, or specifically in type 1 collagen-expressing osteoblasts, diminished the increase in bone vessel density induced by isoproterenol and the higher incidence of bone metastatic lesions

following intracardiac injection of an osteotropic variant of MDA-MB-231 triple negative breast cancer cells. Inhibition of the interaction between Vegf-a and Vegfr2 with the blocking antibody mcr84, in mice treated with isoproterenol, also prevented the increase in bone vascular density and bone metastasis triggered by isoproterenol treatment. Together, these results indicate that stimulation of the β2AR in osteoblasts triggers a Vegf-a-dependent neo-angiogenic switch that promotes bone vascular density and the colonization of the bone microenvironment by metastatic breast cancer cells. This work suggests that potential clinical benefits of beta-blockers and anti-angiogenic agents for breast cancer metastases need to be investigated further.

#41 Targeting bone pain: Multiple myeloma increases nociceptive markers through interactions with the bone microenvironment

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Multiple myeloma (MM) is a haematological malignancy that causes osteolytic bone disease arising from interactions within the bone microenvironment. At diagnosis, the most common symptom of MM is bone pain, either in the form of chronic pain, breakthrough pain, or peripheral neuropathy. The aim of our study was to determine how MM dysregulates nerve function, and whether current models of MM disease recapitulate this common symptom. C57Bl/KaLwRij mice were inoculated with 5TGM1-MM cells intravenously, resulting in tumour growth within bone marrow and osteolytic disease. Mouse Grimace Scoring did not reveal acute pain at any time during the experiment, however movement tracking detected reduced locomotion 25 days after tumour inoculation (39.7% reduction vs. non- tumour mice, $p < 0.01$), before development of paraplegia. This associated with increased spinal cord astrocyte activation, visualised by GFAP IHC and indicating chronic pain (23.4% increase vs. non- tumour mice, $p < 0.05$; Pearson correlation with movement r = -0.5802, $p < 0.05$). In contrast, tumours formed from direct intratibial injection of cells did not affect locomotion. Bone marrow CGRP+ nerves were detectable in control and myeloma-bearing bone marrow, yet neuroma formation was not observed, in contrast to other bone cancer models in the literature.

Nerve Growth Factor (NGF) is a major pain mediator in osteoarthritis and sarcoma, so we hypothesised that NGF may be dysregulated in MM. NGF-β is the active subunit of NGF, and myeloma growth in mice was associated with a significant increase in serum NGF-β (baseline 486.8pg/mL, post-tumour 2461pg/mL, $p < 0.001$). 5TGM1-MM cells expressed TNFα, IL1β and IL6, but did not express NGF-β mRNA. Conversely, *in vitro* bone stromal cell lines and *ex-vivo* KaLwRij osteoblasts expressed high levels of NGF-β, suggesting the bone microenvironment as the source of NGF. Coculture of osteoblasts with MM cells induced a 1.8-fold increase in osteoblast NGF-β protein and mRNA ($p < 0.05$), while treatment with TNFα resulted in a 3.8-fold NGF-β increase ($p < 0.001$). Conversely, dexamethasone and the adiponectin receptors AdipoR1 and AdipoR2 reduced NGF-β transcription. Taken together, our results suggest that chronic pain in the 5T model is separate from tumour growth alone, and may depend upon interactions with the bone microenvironment, including an upregulation of NGF. Identifying such cellular and molecular mechanisms may reveal new approaches for control of bone pain in MM.

#42 A novel osteolinage-derived cancer-associated fibroblast population in primary tumors expresses DKK1 and enhances tumor growth

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We recently demonstrated that the Wnt/β-catenin inhibitor DKK1 is a strong immune suppressive factor, inducing the expansion of immature myeloid suppressor cells, potent inhibitors of T cell responses during tumor progression. Mice bearing extra-skeletal tumors (B16, LLC and PyMT) have increased DKK1 in the circulation and in the bone marrow fluid; and DKK1 neutralization reduces tumor growth by restoring anti-tumor immune responses. We find that DKK1 is expressed in the tumor stroma, but also and at higher levels in the bone of mice with extra-skeletal tumors. We confirmed that both osteoblasts (OBs) and osteocytes of tumor bearing mice produce DKK1, whereas bone marrow cells do not. To identify the cell population within the tumor stroma responsible for DKK1 production at tumor site, we separated the tumor adherent fraction, enriched in cancer-associated fibroblasts (CAFs) and analyzed DKK1 mRNA levels relative to the rest of the tumor. CAFs express abundant DKK1, but also, unexpectedly, two OB-specific markers Osterix (Osx) and osteocalcin. To determine whether these previously unidentified Osx+ cells among CAFs are the DKK1-producing cells, we tracked Osx+ cells in vivo using the doxycycline-sensitive Osx-Cre to activate the TdTomato (TdT) reporter. Doxycycline was administered to dams and pups until weaning to prevent Osx-Cre activation in perinatal mesenchymal stem cells. At 8 weeks of age, mice were inoculated with B16, LLC or PyMT tumors. We found that 3-9% cells in the tumor mass were TdT^{Osx+}, and also expressed the fibroblast markers, Fsp-1 and Vimentin. Importantly, DKK1 mRNA was significantly higher in TdT^{Osx+} than in TdT^{Osx-} cells, suggesting that this sub-population is involved in DKK1 production at tumor site. TdT^{Osx+} cells were also present in the bone marrow (15.3 ± 5.06% of total bone marrow cells) and, interestingly, we further detected TdT^{Osx+} cells in the circulation of tumor bearing mice (16 ± 4.48% of total blood cells), suggesting that Osx+ CAFs might be mobilized from the bone microenvironment. Finally, co-injection of TdT^{Osx+} cells isolated from primary B16 tumors with B16 cells into WT mice resulted in enhanced tumor growth relative to mice injected with tumor cells alone. In summary, we have identified a novel population of CAFs that express osteolineage markers and contribute to DKK1 production and tumor growth. Osx+ cells may represent a new platform for targeting the stroma with the purpose of inhibiting cancer growth.

#43 Decreased JMJD3 Expression in Mesenchymal Stem Cells Contributes to Long-term Suppression of Osteoblast Differentiation in Multiple Myeloma

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Multiple myeloma (MM) is the most frequent cancer to involve the skeleton, with over 80% of myeloma patients developing lytic bone disease (MMBD). Importantly, MM-associated bone lesions rarely heal even when patients are in complete remission. Mesenchymal stem cells (MSCs) isolated from MM patients have a distinct genetic profile and an impaired osteoblast (OB) differentiation capacity when compared to healthy donor MSCs. Utilizing an *in vivo* model of MMBD and patient samples, we showed that MSCs from tumor-bearing bones failed to differentiate into OBs weeks after removal of MM cells. Both Runx2 and Osterix, the master transcription factors for OB differentiation, remained suppressed in these MSCs. However, the molecular mechanisms for MM-induced long-term OB suppression are poorly understood. We characterized both Runx2 and Osterix promoters in murine pre-osteoblast MC4 cells by chromatin immunoprecipitation (ChIP). The Runx2 and Osterix transcriptional start sites (TSSs) in untreated MC4 cells

were co-occupied by transcriptionally active histone 3 lysine 4 tri-methylation (H3K4me3) and transcriptionally repressive histone 3 lysine 27 tri-methylation (H3K27me3), termed the "bivalent domain". These bivalent domains became transcriptionally silent with increasing H3K27me3 levels when MC4 cells were co-cultured with MM cells or treated with TNF-α, an inflammatory cytokine enriched in MM bone marrow. The increasing H3K27me3 levels induced by MM cells and TNF-α were associated with the downregulation of the H3K27 demethylase JMJD3 in MC4 cells and MSCs. As expected, the pre-treatment of MC4 cells with NF-kB inhibitors and not MAPK inhibitors effectively prevented the downregulation of JMJD3 induced by TNF-α. Consistent with these results, knockdown of JMJD3 in MC4 cells inhibited OB differentiation. Importantly, MSCs from MM patients had markedly decreased JMJD3 expression compared to healthy MSCs.

Our findings demonstrated that MM cells induce changes of histone modifiers in MSCs in the MM bone marrow microenvironment and suggests that MM cells resolve the bivalent domains at the Runx2 and Osterix TSSs to transcriptionally silent domains in part by decreasing JMJD3 expression in MSCs. These changes make MSC refractory to osteogenesis. Thus, targeting epigenetic changes on the Runx2 and Osterix TSSs should increase bone formation and possibly decrease tumor burden in MM.

Skeletal effects of cancer in bone

#44 Development of a simple selection-free method for detecting disseminated tumor cells (DTCs) in murine bone marrow

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Approximately 30% of prostate cancer patients develop disease recurrence and metastasis after initial therapy. This is thought to be largely due to the presence of minimal residual disease (MRD), which may be present at the primary site, in lymph nodes, or in bone. Because 90-100% of men who die of prostate cancer have bone lesions at autopsy, our group's goal is to develop technology to not only detect and quantify, but to understand the biology of individual disseminated tumor cells (DTCs) within bone of human patients as well as mouse models of the disease. Several techniques currently exist with which to detect and quantify circulating tumor cells (CTCs) in the blood of cancer patients, but no reliable techniques exist to do so with DTCs in the bone marrow. We are the first group to have developed a method to detect and quantify DTCs in the bone marrow of mice. The procedure entails removal of the bone marrow via centrifugation from the long bones (femur and tibia) of mice that contain cancer cells. The bone marrow then undergoes RBC lysis and is spread onto glass adhesion slides. The cells are then fixed and stained (IF and/or RNA-FISH can be used). The staining can include any type of marker, including epithelial, mesenchymal, disease-specific, or species-specific. Stained slides are scanned using an automated microscope, and accompanying software generates galleries of candidate DTCs based on fluorescent intensity. The resulting images are manually reviewed, and DTCs are enumerated based on specific criteria. We consistently detected bone marrow DTCs from xenograft models using cells from prostate, breast, lung, and kidney cancer lines (intracardiac, subcutaneous, and intratibial), syngeneic models (mouse cancer cells injected into mice), and transgenic models (prostate cancer model). Based on spiking experiments, our protocol detects upwards of 70% of spiked tumor cells. This protocol is simple and efficient, removes potential selection bias, and is flexible for use in any type of mouse model. We have further adapted this protocol to include the ability to measure proliferation, detect rare proteins, and stain slides in multiple rounds so as to expand the number of proteins we can detect. Our group is also optimizing this protocol for use in human prostate cancer patients, which could have a sizable impact on the ability to detect MRD in patients and predict patients that might recur, particularly with metastatic disease.

#45 Suppression of Tumor Growth in Bone by Novel Small-Molecule uPAR Antagonists

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The urokinase plasminogen activator receptor (uPAR) increases with disease progression in several solid tumors and in multiple myeloma (MM). It contributes to invasion and metastasis to bone in breast (BC) and prostate cancers (PC). A uPAR-neutralizing antibody decreased BC bone metastases in a mouse model (Rabbani et al, Neoplasia, 2010). Starting from a small molecule that we previously had found to bind to uPAR, we developed a series of 12 derivatives that possess a novel heterocyclic aromatic chemical scaffold with different substituents. The compounds were tested for binding to uPAR using biochemical assays. In cell culture, they showed strong inhibition of cancer cell invasion with single-digit micromolar IC50s, without exhibiting cell toxicity. Two of these compounds, XHS-32 and XHS-34, were tested for their ability to inhibit tumor colonization of bone in a novel *ex vivo* organ coculture assay (EVOCA) using both BC and MM cells lines. Human tumor cell lines were grown for 1 week on 5 mm calvarial bone discs from 14-day old mice. Tumor growth was assayed by *Gaussia* luciferase secreted from stably transduced cancer cells. Expression of mouse bone and human tumor genes was determined at day 7 by Q-PCR with species-specific primers. Compounds were added to bone cultures prior to the addition of tumor cells to test the agents in a prevention model.

Both XHS-32 and XHS-34 effectively inhibited growth in bone of the MDA-MB-231 BC line. At day 7 XHS- 32 effectively decreased markers of osteolysis (RANKL and TRAP), with little effect on osteoblast markers (Col1a1 and osteocalcin). PTHrP and BAX, a marker of tumor apoptosis, were significantly increased. XHS-34 caused similar changes, except for increasing osteocalcin. Both agents decreased tumor uPAR mRNA. XHS-32 also effectively decreased growth in bone of the JJN3 osteolytic MM cell line but did not block RPMI-8226 MM cells. When bone alone was incubated with XHS-32 for 7 days, there were no changes in TRAP, RANKL, cathepsin K or type 1 collagen mRNAs, while the early osteoblast marker alkaline phosphatase was increased.

Small-molecule uPAR inhibitors may effectively reduce growth in bone of metastatic solid tumors and multiple myeloma, while potentially lacking deleterious skeletal effects, warranting development of more potent derivatives of the XHS compounds from our limited structure-activity results, followed by testing in animal xenograft models.

#46 CXCL14 is a Marker of Prostate Cancer Bone Metastasis: A Pro-Metastatic Chemokine with CXCR4 Dependent and Independent Actions

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Chemokines function as crucial links between tumor cells and stromal cells, promoting proliferation, migration and invasion through multiple signaling pathways. The chemokine CXCL14 was identified in a screen for prostate cancer bone metastatic factors. The expression of CXCL14 in prostate cancer bone metastasis was investigated using a human prostate cancer tissue microarray. CXCL14 expression was significantly higher in bone metastases compared to normal prostate, benign prostatic hypertrophy, primary prostate cancer, and metastases to lymph nodes and soft tissues (H-score $p < 0.0001$). Investigations were performed to understand the biology of CXCL14 as a pro-metastatic prostate cancer marker. CXCL14 stimulated *in vitro* migration and invasion of the human prostate cancer cell line $ARCaP_M$, and promoted expression of epithelial-to-mesenchymal transition-associated genes (*SLUG*, *SNAIL*, *TWIST*) in $ARCaP_M$ and PC-3 prostate cancer cells. Moreover, CXCL14 activated MAPK and PI3K/AKT signaling pathway proteins. CXCL14 signaling activation was not due to direct interaction with the chemokine receptor CXCR4, addressing controversy as to the identity of the CXCL14 chemokine receptor. CXCL14 did not activate CXCR4 signaling in a luminescent complementation reporter assay that detects CXCR4 recruitment of β-arrestin 2. Furthermore, an interaction of CXCL14 with CXCR4 or CXCR7 was not detected using a sensitive luminescent complementation assay. Despite the lack of a CXCL14/CXCR4 interaction, CXCL14-activated migration was dependent on CXCR4 as demonstrated using a novel microfluidic cell migration assay. Together, these data suggest that CXCL14 interacts with an unidentified chemokine receptor that may have cooperative actions with CXCR4. This investigation thus identified CXCL14 as a novel prostate cancer pro-metastatic chemokine and may represent an appealing target for the treatment of prostate cancer bone metastasis.

#47 Novel Cytoplasmic Functions of the Vitamin D Receptor in Breast and Prostate Cancer

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Previous reports show that vitamin D deficiency promotes human breast and prostate cancer growth in bone. While this effect appeared to be mediated indirectly via changes in the bone microenvironment, vitamin D and the vitamin D receptor (VDR) may also exert direct actions on cancer growth in bone. We hypothesized that VDR knockdown enhances human breast and prostate cancer cell growth. To investigate the role of VDR in cancer cell growth, VDR expression was knocked down by stable expression of shRNA in breast cancer cell lines MDA-MB-231-TxSA (MDA-VDR-KD) and MCF-7 (MCF7-VDR-KD), and prostate cancer cell line PC-3 (PC-3-VDR-KD), with non-target (NT) cells generated as control cells. Using single cell clonal selection two stable VDR knockdown clones were generated from VDR knockdown MCF-7 cells.

We demonstrate that knockdown of VDR expression in human breast and prostate cancer cells reduced cancer cell proliferation and increased apoptosis *in-vitro*, both in the presence and absence of its cognate ligand, 1,25-dihydroxyvitamin D. To investigate VDR-KD tumor growth *in-vivo*, VDR knockdown cells were implanted into the mammary fat pad of nude mice. Compared to NT cells, VDR knockdown was associated with significantly smaller tumor size and reduced tumor mass in the three cell lines used. In the intra-tibial xenograft model, MDA- VDR-KD and PC-3-VDR-KD cells resulted in reduced tumor growth in bone compared controls, while VDR knockdown greatly reduced the ability of MCF-7 cells to form tumors in the bone.

To elucidate the mechanisms for these ligand-independent effects of the VDR, we used a mutated VDR in which the nuclear localization signal is dysfunctional, leading to the accumulation of the VDR in the cytoplasm. Stable expression of the mutant VDR in VDR knockdown cells restored cell growth to levels similar to wild type cells both *in-vitro* and *in-vivo*, indicating that the cytoplasmic VDR is able to promote breast and prostate cancer cell growth.

We conclude that the VDR in the cytoplasm promotes breast and prostate cancer cell growth both outside and within the bone microenvironment. This effect strongly contrasts with the well- established anti-proliferative and pro-apoptotic nuclear actions of the VDR-vitamin D ligand complex. This discovery adds to our understanding of VDR signaling in breast and prostate cancer. In future, further investigations of cytoplasmic VDR signaling may open new avenues of therapeutic strategy for breast and prostate cancers.

#48 MMP Processing of PTHrP Yields a Selective Regulator of Osteogenesis, $PTHrP_{1\text{-}17}$

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Parathyroid hormone-related protein (PTHrP) is a critical mediator of bone resorption and augments osteolysis in skeletal malignancies via regulation osteoblasts and osteoclasts. Mature PTHrP consists of 36 amino acids and signals through the type I PTH receptor (PTH1R), however the protein is also susceptible to proteolytic cleavage. Here, we demonstrate that matrix metalloproteinases (MMP-2, -3, -7, and -9) can rapidly process $PTHrP_{1\text{-}36}$ into novel peptides ($PTHrP_{1\text{-}17}$, $PTHrP_{18\text{-}26}$, and $PTHrP_{27\text{-}36}$), and we investigate the effects of these PTHrP peptides on osteoblast and osteoclast behavior.

In our signaling assays, we observed rapid ERK phosphorylation in osteoblasts treated with $PTHrP_{1\text{-}17}$. $PTHrP_{1\text{-}17}$ also stimulated intracellular calcium flux, but, unlike $PTHrP_{1\text{-}36}$, we did not observe any impact on cAMP production. $PTHrP_{18\text{-}26}$ and $PTHrP_{27\text{-}36}$ did not show activity. Additionally, ERK phosphorylation and calcium flux stimulation by $PTHrP_{1\text{-}17}$ was abrogated when PTH1R expression was silenced by shRNA, suggesting that $PTHrP_{1\text{-}17}$ mediates its cellular effects via PTH1R. Focusing further on $PTHrP_{1\text{-}17}$, we compared its biological activity to $PTHrP_{1\text{-}36}$ and found that $PTHrP_{1\text{-}17}$ significantly stimulated osteoblast differentiation (*in vitro*) compared to control (1.6-fold, $p < 0.05$) as well as stimulated bone formation in *ex vivo* calvaria cultures and *in vivo* ectopic ossicle assays. *In vitro* we observed that unlike $PTHrP_{1\text{-}36}$, $PTHrP_{1\text{-}17}$ did not induce RANKL expression or osteoclastogenesis. This was supported by *in vivo* data demonstrating that $PTHrP_{1\text{-}17}$, when injected over the calvaria, failed to induce an osteolytic response in comparison to $PTHrP_{1\text{-}36}$. Using $PTHrP_{1\text{-}17}$ specific antibodies coupled with quantitative mass spectrometry, we found that $PTHrP_{1\text{-}17}$ is present in prostate cancer (PaIII) and osteosarcoma (Saos-

2) conditioned media, and the amount of detectable $PTHrP_{1-17}$ can be reduced by adding an MMP inhibitor (GM6001) or enhanced by treating with recombinant MMPs.

Our data demonstrate that MMPs generate a distinct PTHrP peptide, $PTHrP_{1-17}$, that retains the ability to stimulate osteoblast differentiation without impacting osteoclast activity and bone resorption. Collectively, our data suggest a novel role for MMPs in regulating the osteolytic-osteogenic responses in normal bone turnover and in the context of skeletal malignancies.

#49 Notch3 promotes Prostate Cancer-Induced Osteoblastic Bone Metastasis

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Background: Notch signaling is dysregulated in bone metastatic prostate cancer (PCa), but how it contributes to bone metastasis is unknown. PCa bone metastasis is typically osteoblastic. The molecular basis for osteoblastic lesion formation remains poorly understood. In this this study, we demonstrate that Notch3 activity in PCa tumor cells is responsible for driving an osteoblastic phenotype.

Methods: Several PCa cell lines, in which Notch3 signaling was suppressed by Tet-inducible shRNA or enhanced by expression of Tet-inducible NCID3, were injected into the tibiae of SCID mice. X-ray was used to monitor bone lesion development and osteolytic lesion area measured using Metamorph software. Harvested tibiae were subjected to histological analyses, qRT-PCR, or immunoblotting. We used cultured bone marrow from naïve mice to differentiate osteoblasts or osteoclast *in vitro* in the presence or absence of conditioned medium from Notch3 expressing cancer cells. The proliferation of osteoblasts or osteoclasts were measured by Crystal violet staining or MTT assays and differentiation monitored by ALP or TRAP staining, respectively.

Results: PCa cell lines that promote mixed osteoblastic bone lesions (C42B and 22RV1) express more Notch3 after intra-tibia injection relative to cell lines that promote osteolytic bone lesions (PC3). Overexpression of active Notch3 (NICD3) in PC3 cells decreased osteolytic lesions and decreased the number of osteoclasts in the tumor-bone microenvironment. Conversely, inhibition of Notch3 in PC3, 22rv1, or C42B cells with shRNA, promoted osteolytic lesions. Conditioned medium from PC3-NICD3 cells increased osteoblast proliferation in vitro, while conditioned medium from PC3-NICD3 cell inhibited osteoclastgenesis, but had no effect on osteoclast proliferation or apoptosis. Human MMP3 levels were elevated in tibia injected with PC3-NICD3 cells, whereas Notch3 shRNA tibia tumors expressed less MMP-3. Recombinant MMP3 blocked osteoclastogenesis and stimulated osteoblast proliferation in vitro.

Conclusions: Notch signaling in PCa tumors favors osteoblastic metastasis by stimulating the production of MMP3 and release into the tumor microenvironment to inhibit osteoclastogenesis while also inducing osteoblast proliferation.

#50 TBX2 and RANKL: Factors that mediate prostate cancer bone and visceral metastases

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We found that TBX2, a T-box family transcription factor that negatively regulates p21 cell cycle inhibitor during embryonic development, is also overexpressed in: *a)* human PCa specimens, and b) Mouse xenograft bone metastases from PC3, $ARCaP_M$, and RANKL overexpressing LNCaP human prostate cancer cells. Blocking endogenous TBX2 using a dominant negative construct (TBX2 DN) in PC3 and $ARCaP_M$ human PCa cell lines - that show reduced WNT3A expression upon blocking TBX2 - abrogated the bone metastasis capability of these cell lines in an experimental-metastasis xenograft mouse model; in addition to dramatically reducing the tumor growth and bone remodeling capacity of these cells in an intra-tibial xenograft mouse model. We identified a novel mechanism where TBX2 acts in *trans* to promote transcription of the canonical WNT (WNT3A) promoter. Genetically rescuing WNT3A levels in PC3-TBX2 DN cells partially restored the PCa metastatic capability of these cells in mice; conversely, WNT3A neutralizing antibodies or WNT antagonist SFRP-2 blocked TBX2-induced invasion. Our findings suggest that TBX2 is an independent factor that drives PCa metastasis. TBX2 may be a novel therapeutic target upstream of WNT3A, and WNT3A antagonists could be a novel treatment for PCa metastasis.

We also extended our earlier findings that RANKL drives PCa metastases in immune-deficient mice (Chu et al., 2014) to immune-intact C57/Bl6 mice. MPC3 mouse PCa cells with RANKL overexpression (MPC3-Luc-GFP-RANKL) develop 70-80% limb and jaw within 4 weeks of intra-cardiac injection in immune-intact mice. Control MPC3 cells had no bone metastasis. Bone lesions visualized by luciferase imaging and X-ray were confirmed by micro CT and immunohistochemistry. RANKL signaling drove bone and visceral metastases via the downstream CXCL12/CXCR4 signaling axis. MPC3-Luc-GFP-RANKL cells showed increased CXCR4 protein levels by immunohistochemistry. Metastatic bone marrow flush showed dramatically increased levels of CXCL12 mRNA compared with control mice (MPC3-Luc-GFP- EV). In sum, *1)* circulating PCa cells induce a marked CXCL12 elevation after colonizing bone, triggering chemotaxis and recruiting CXCR4-positive PCa cells to migrate to bone; and *2)* osteomimetic PCa cells with increased RANKL expression interact with osteoclasts to enhance bone resorption and turnover, releasing additional growth factors and chemokines for PCa cell growth and survival in bone.

#51 Blocking IL-1b signaling alters blood vessels in the bone microenvironment

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Background: We have recently identified interleukin 1B (IL-1B) as a potential biomarker for predicting breast cancer patients at increased risk for developing bone metastasis. In addition we have shown that blocking IL-1B activity inhibits development of bone metastases from breast cancer cells disseminated in bone and reduces tumour angiogenesis. We hypothesise that interactions between IL- 1B and Il-1R also promotes formation of new blood vessels in the bone microenvironment stimulating development of metastases at this site.

Objectives: Investigate the effects of blocking IL-1B activity on blood vessel formation within bone.

Methodology: The effects of IL-1R inhibition on vasculature in trabecular bone were determined in mice treated with 1mg/kg of the IL-1R antagonist (anakinra) for 21/31 days, the IL-1B antibody (Ilaris) for 0-96 hours or in genetically engineered IL-1R1 KO mice. Vasculature was visualised following CD34 and endomucin immunohistochemistry and the concentration of VEGF and endothelin-1 in serum and/or bone marrow was determined by ELISA. Effects on bone volume were measured by uCT.

Results: Ilaris caused a significant decrease in the length of new blood vessels from 0.09mm (control) to 0.06mm (24 hours Ilaris) ($P = 0.0319$). IL-1R1 KO mice and mice treated with anakinra

demonstrated a downwards trend in the average length of new blood vessels. Inhibition of IL-1R resulted in increased trabecular bone volume. Anakinra caused a 69% decrease in the concentration of endothelin-1 in mice treated for 31 days (P = 0.0269) and a 22% decrease in VEGF concentration in mice treated for 21 days (P = 0.0104). Ilaris caused a 46% reduction in VEGF concentration and a 47% reduction in endothelin-1 concentration in mice treated for 96 hours.

Conclusions: These data demonstrate that IL-1R activity plays an important role in the formation of new vasculature in bone and inhibiting its activity pharmacologically has potential as a novel treatment for breast cancer bone metastasis.

#52 *In vitro* and *in vivo* evidence that RANK/RANKL promotes early breast cancer dissemination to the bone marrow

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Denosumab is a fully human monoclonal antibody to human RANKL, currently approved in the management of established bone metastases, mainly due to its anti-resorptive activity which blocks tumour-induced osteoclastogenesis and osteolysis. AMG161 is an IgG1 equivalent to Denosumab (an IgG2) and suitable for preclinical studies.

Our aims are to determine the role of RANK expression in BC bone metastasis and the efficacy of RANKL blocking by AMG161. AMG161 does not recognize murine RANKL, so we use a knock-in (KI) mouse model, which expresses a chimeric murine/human RANKL.

Long-term studies showed that intra-arterial (i.a.) injection of MDA-MB231 RANK *versus* MDA-MB231 leads to a poorer overall survival (OS), with the most striking differences seen in the earlier time-points. In this respect, the extent of radiographic osteolytic lesions was higher in the RANK overexpressing group than in the control group. By contrast, at later time points, bone and lung metastatic burden was similar between the two groups, suggesting that the poor OS of the RANK overexpressing group was due to a faster tumour progression owing to enhanced bone homing and colonization. Indeed, using short-term studies, i.a. injection of MDA-MB231 RANK led to higher incidence and extension of bone marrow micrometastases than MDA-MB231, whereas there was no difference for lung micrometastases.

We therefore hypothesized that RANK overexpressing BC cells interact with RANKL produced by osteocytes, osteoblasts and other cells in the osteogenic niche, favouring homing, survival and colonization of BC cells in bone. To test this hypothesis we made use of heterotypic mammosphere assays in which BC cells were cultured with or without cells of the osteogenic niche (e.g. pre-osteoblasts), recombinant RANKL and anti-RANK antibody or AMG161. Co-culture with recombinant RANKL or a cellular source of RANKL increased BC cell mammosphere formation, and blocking RANK/RANKL signaling impaired it. Preliminary *in vivo* studies of i.a. injection of B02 cells onto KI mice and subsequent AMG161 treatment (2x a week, 3mg/kg) inhibited bone marrow micrometastases formation.

In conclusion, RANK/RANKL signalling promotes the early engraftment of BC cells in the bone marrow. Blocking this signalling with AMG161 decreases bone marrow micrometastasis. This strategy could be equally useful in pre-metastatic early breast cancer patients.

#53 Molecular Connections Underlying Co-Incidence of Breast Cancer and Thyroid Cancer

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Background: Recent studies have indicated that women with breast cancer have a higher risk for development of thyroid cancer and women with thyroid cancer may be at increased risk of breast cancer suggesting a common etiology. The transcription factor thyroid hormone receptor β (TRβ) can slow the growth of both thyroid and breast tumors. Despite a recognized tumor suppressor role for TRβ in these cancers, the molecular mechanisms by which TRβ blocks tumor growth are not understood. We recently identified a novel signaling pathway in thyroid cancer cells, where TRβ suppressed RUNX2 (runt-related transcription factor) (Carr et al Endocrinology 2016). RUNX2 in breast and thyroid cancer cells is highly oncogenic, driving tumor progression and is known activator of metastatic bone disease. In thyroid cancer cells, we established that loss of TRβ correlated with increased levels of RUNX2 and RUNX2-regulated prometastatic genes. A compelling question is whether and by what mechanism(s) does the tumor suppressor TRβ modulate RUNX2 directed tumor growth and metastasis in breast cancer.

Results: We find the same reciprocal relationship of high levels of TRβ and absence of RUNX2 in normal thyroid and breast epithelial cells, and the converse in aggressive SW1736 thyroid and MDA-MB-231 breast cancer cells (very low TRβ and high RUNX2). A functional relationship was established between TRβ and RUNX2. Addition of TRβ directly suppressed RUNX2 gene promoter activity in breast cancer cells. Chip–Seq revealed direct binding of TRβ to three TR motifs in the RUNX2P1 promoter and mutation of the TRβ sites reduced RUNX2 promoter activity (luciferase-reporter expression assay). Further, addition of T3 was found to activate endogenous TRβ resulting in decreased RUNX2 levels, strongly implicating hormone status as a contributing component to this dysregulated TRβ-RUNX2 pathway in breast cancer. Of clinical importance, higher levels of non-mutated TRβ are correlated with improved outcomes in triple negative breast cancer (TCGA data base).

Conclusions: Our findings indicate that pharmaceutical interventions with TRβ can enhance tumor suppression in thyroid and breast cancer and potentially inhibit the development of a secondary primary tumor from thyroid cancer to breast and breast cancer to thyroid with accompanying metastasis to bone by both cell types. In vivo xenograft studies of thyroid and breast cancer cells with modified levels of TRβ are in progress.

#54 Runx2 facilitates autophagy in bone metastatic breast cancer cells

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# Equal contribution

Bone metastasis of breast cancer causes significant patient mortality. Recent studies suggest that metastatic cancer cells induce autophagy to survive metabolic stress including starvation and hypoxia. During autophagy, cytoplasmic components and damaged organelles are captured by autophagosomes followed by fusion and degradation by the lysosome resulting in release of metabolites as energy sources to meet metabolic demands. Although the components of autophagy have been well characterized, the regulatory mechanism of autophagy in metastatic cancer cells in the bone microenvironment is still unknown. To examine whether autophagy changes during bone metastasis, we used a bone metastatic isogenic variant of breast cancer MDA-MB-231 cells isolated from a xenograft tumor mouse model of metastasis. We examined autophagy by monitoring microtubule-associated protein

light chain (LC3B-II) levels, an autophagy-specific marker. Electron and confocal microscopic analyses were utilized to identify alterations in autophagic vesicles. We found increased autophagic flux in the bone metastatic variant compared to parental MDA-MB-231 cells. Previously, we and others have shown that Runt-related transcription factor-2 (Runx2) promotes cell survival, metastasis of mammary tumors to bones and associated osteolysis. To define the regulatory mechanisms of autophagy during bone metastasis, we examined whether Runx2 regulates autophagy for increased cell survival in the bone microenvironment. Our results show that Runx2 enhances autophagy in metastatic breast cancer cells. The Runx2 knockdown cells accumulate LC3B-II protein and autophagic vesicles due to reduced turnover. Interestingly, Runx2 promotes autophagy by enhancing trafficking of autophagic vesicles. Our mechanistic studies revealed that Runx2 promotes autophagy by increasing acetylation of α-tubulin sub-units of microtubules. Inhibiting autophagy decreased cell adhesion and survival of Runx2 knockdown cells. Furthermore, analysis of LC3B protein in clinical breast cancer specimens and tumor xenografts revealed significant association between high Runx2 and low LC3B protein levels. Our studies reveal a novel regulatory mechanism of autophagy via Runx2 and provide molecular insights into the role of autophagy in bone metastatic cancer cells.

Tumor dormancy

#55 RANKL-induced bone remodelling controls prostate cancer cell dormancy in an *ex vivo* calvaria model

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Skeletal metastasis is a hallmark of advanced prostate cancer (PCa), with lack of effective treatments. Rare, dormant PCa cells residing in 'niches' in the skeleton are thought to initiate bone metastasis and contribute to disease recurrence. Previously we demonstrated that androgen withdrawal through castration caused bone loss and activated growth of disseminated PCa cells in bone. Bone remodelling has also been shown to regulate reactivation of tumour cells in other models of cancer development in the skeleton including multiple myeloma and breast cancer, however our understanding of this is limited. To address this we are developing approaches to model the cancer cell-bone interactions *in vivo* and *ex vivo*, including the effect of RANKL-induced bone remodelling on tumour cell dormancy.

To identify dormant tumour cells, PC3-GFP human PCa cells were labelled with a fluorescent membrane-bound dye (vybrant-DiD®). DiD is lost as the dye is shared between daughter cells (reactivated; DiD-ve) and retained in non-dividing or dormant cells (DiD+). A progressive loss of DiD-label is observed in cultured PC3-GFP cells over 14 days, as all cells proliferate. However, in vivo only a small number of label-retaining dormant tumour cells can be detected in the femurs of mice (visualised by intravital imaging) 6-weeks post intra-cardiac injection, even in the presence of growing tumour. This phenomenon was recapitulated *ex* vivo using organ cultures of bone; dormant PCa cells were visualised, even in the presence of proliferating cells, up to 21 days after seeding onto the endocranial surface of excised calvaria from neonatal mice. To activate bone-resorbing osteoclasts, RANKL was added to cultures 7 days after seeding PCa cells (to allow initial tumour cell dormancy on bone, visualised by 2-photon microscopy). RANKL treatment resulted in a 4-fold decrease in the number of dormant PCa cells on calvaria and an increase in the number of proliferating (DiD-ve) PCa cells, quantified by flow cytometry. This suggests that increased osteoclastic bone resorption can cause reactivation of dormant PCa tumour cells.

While further verification is required *in vivo,* these studies support the concept that remodelling of the bone microenvironment can control the fate of dormant tumour cells in the skeleton. Furthermore, treatments that enhance local or systemic bone remodelling could promote PCa disease recurrence, through tumour cell-extrinsic mechanisms.

#56 Investigating the mechanisms that drive chemotherapy-induced bone loss

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Breast cancer is the second leading cause of cancer-related deaths among women in the United States. About 70% of metastatic patients harbor bone metastases. Most patients diagnosed at an early stage without evidence of overt metastases undergo "curative" resection. Despite the fact that the majority of breast cancer cases are diagnosed early, approximately 10-20% of patients recur with metastatic disease within 10 years. Among metastatic sites, bone metastasis in particular correlates negatively with survival and quality of life for these patients. Significantly, these cases arise in patients previously "cured", demonstrating that primary tumor cells metastasize early and remain dormant in distant sites only to awaken years later. Despite tremendous advances, it remains impossible to predict which patients will relapse. Thus to prevent recurrence most patients receive adjuvant chemotherapy (chemoRx) following resection despite the fact that most will never recur. Unfortunately, the side effects of chemoRx severely impacts quality of life by driving bone loss in these patients leaving them susceptible to catastrophic fractures. Investigation into the mechanisms responsible for chemoRx-induced bone loss has revealed that loss of ovarian function and subsequent reductions in estrogen contribute bone loss. However, the rapidity of this loss and the fact that post-menopausal women and women on aromatase inhibitors lose bone raise the provocative possibility that additional factors contribute to bone loss. Paradoxically, while chemotherapeutic agents robustly kill significant numbers of tumor cells, I find that it also induces the activation of stromal cells and secretion of protumorigenic factors (often pro-resorptive) collectively referred to as senescence-associated secretory phenotype (SASP). Preliminary data indicates that mice treated with Doxorubicin, a clinically relevant chemoRx, experience drastic bone loss and upregulation of SASP factors, such as IL-6, in their bones. I also found that chemo-treated mice drove significantly more bone loss compared to those that were ovarectomized (OVX), a commonly used model of estrogen deficiency. These data mirror the clinical scenario observed when post-menopausal women who, despite the already low levels of estrogen in their bodies, experience significant bone loss upon chemotherapy. Our current work is focused on elucidating a novel mechanism that contributes to chemoRx-induced bone loss.

#57 Epigenetic regulation of the LIFR and its role in breast cancer dormancy

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Breast cancer cells often metastasize to the bone, where they may enter a dormant state. Our laboratory previously found that leukemia inhibitor factor receptor (LIFR) maintains breast cancer cells in dormancy and that when LIFR is lost, breast cancer patient survival is reduced and tumor cells down-regulate a number of pro-dormancy genes and become proliferative and bone destructive *in vivo*. Interestingly, LIFR expression on breast cancer cells can be stimulated (>7-fold, $p < 0.01$) using the FDA-approved pan-histone deacetylase (HDAC)

inhibitor valproic acid (1-10mM), leading to significant upregulation of pro-dormancy genes (TSP1, TPM1, p27, AMOT, P4HA1, miR-190, PDCD4, SELENBP1, QSOX1; up to 3.8-fold, $p < 0.05$-0.0001). In addition, HDAC2 is predicted to bind to the LIFR in the UCSC genome browser, suggesting that HDACs may directly regulate LIFR expression. Based on these findings, we hypothesized that LIFR is epigenetically down-regulated in breast cancer and that HDAC inhibitors (HDACi) could be used to induce LIFR expression and maintain tumor cells in a dormant state. To test this, we treated MCF7 breast cancer cells, which we and others have proposed as a model of tumor cell dormancy *in vivo,* with HDACi (romidepsin, panobinostat, entinostat, vorinostat) belonging to various structural classes and with proposed differential specificities for the HDACs. These HDACi are currently FDA-approved or in phase II clinical trials (entinostat) for treatment of various malignancies, including breast cancer and multiple myeloma. All HDACi significantly induced LIFR mRNA levels (2.9 - 3.9-fold, $p < 0.05$-0.01) after 6-12 hours of treatment and dramatically increased LIFR protein levels by 24 hours. Further investigation of romidepsin and panobinostat, which most potently induced LIFR mRNA and protein levels, revealed increased mRNA levels of several dormancy associated genes including IGFBP5, p27, and AMOT (4-5-fold, $p < 0.01$-0.0001). Together, these data indicate that HDACi induce LIFR expression and stimulate a pro-dormancy gene signature. Studies to elucidate the epigenetic mechanism by which LIFR is regulated and to determine whether HDACi can prevent outgrowth of dormant tumor cells *in vivo* and/or revert aggressive breast cancer cells into a dormant state are currently in progress. Importantly, these studies may have clinical implications for the use of HDACi as a means to activate a pro-dormancy program and maintain breast cancer cells in a dormant state.

#58 Analysis of Bone Marrow Disseminated Tumor Cells from Patients with Localized Prostate Cancer

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Bone marrow disseminated tumor cells (DTCs) are thought to be the source of many prostate cancer recurrences. However, isolation and analysis of these cells from bone marrow aspirates has remained problematic for decades. Immuno-magnetic bead based isolation followed by single cell picking has yielded useful data, but contamination with erythroid precursors and other non-malignant cells has remained a problem. Therefore, we improved and tested our fluorescence activated cell sorting (FACS) based, dual marker EPCAM+ / CD45- methods for DTC isolation and analysis with addition of three negative selection markers; CD235a (erythroids), alkaline phosphatase (osteoblasts) and CD34 (HSCs). Using these techniques, we selected a putative DTC population of viable, single cells with high EPCAM expression and no expression of the four negative markers. This multiple marker selected population was present in 10 of 20 patients with localized prostate cancer, one patient with metastatic prostate cancer and 1 of 7 normal donors. Cells selected by the prior methods of selecting CD45- cells with dim or strong EPCAM expression yielded populations present in all patients with localized or metastatic PCa and all normal donors – suggesting that these less stringent methods yield many non-malignant cells. As further validation, we found that the frequency of the multiple marker selected DTCs in marrow from localized prostate cancer patients significantly correlated with the risk of recurrence predicted by the CAPRA-S score. Finally, we analyzed samples from two patients with multiplex qRT-PCR. The new putative DTC population had expression of prostate cancer markers NKX3.1, KRT8, KRT18, PSA and PCA3, much higher than EPCAM+ / CD45- cells isolated by existing methods. We conclude that FACS based prostate cancer bone marrow DTC isolation using high EPCAM expression and negative selection for CD45, CD235a, CD34, and alkaline phosphatase is an improved technique and will continue validation of this promising approach.

#59 Single-Cell RNA Sequencing of Dormant Myeloma Cells Identifies New Therapeutic Targets

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Multiple myeloma is a neoplasm of plasma cells which develops in the skeleton. The bone microenvironment supports long-term survival of dormant myeloma cells and myeloma growth. Despite targeted treatments, patients relapse and survival is poor. Dormant cells are resistant to conventional chemotherapy and contribute to disease relapse and therefore represent potential therapeutic targets. However, the molecular pathways that control dormancy are unclear. We developed a novel method to identify dormant cells *in vivo* and used single-cell RNA sequencing (scRNA seq) to define their transcriptome profile and identify targetable pathways that control dormancy.

5TGM1 murine myeloma cells expressing eGFP (5TGM1eGFP) were labelled with a fluorescent membrane dye, (Vybrant DiD). Cell division leads to loss of the DiD label, hence reactivated cells become DiDneg, whereas, non-dividing dormant cells retain DiD (DiDhigh). DiD-labelled cells were injected (i.v.) into C57BLKalwRij mice. After 21 days, individual DiDhigh and DiDneg cells were sorted into 384 well plates by FACS. ScRNA seq libraries were generated and sequenced to a depth of >2 million reads per cell. Each individual 5TGM1 cell was validated by the reconstruction of the unique clonal idiotypic sequence and the expression of specific Ig genes. A mean of ~4000 unique transcripts were identified with over 900 genes up-regulated in DiDhigh cells compared to DiDneg cells. Ten transcription factors, including *Irf7*, *Stat1*, *Spic,* were expressed in DiDhigh cells and predicted to control 50% of the 1493 differentially expressed genes. Functional annotation of differentially expressed genes confirmed the dormant status of DiDhigh cells with down-regulation of metabolic pathways and up-regulation of immune system-related pathways. We identified the receptor tyrosine kinase, *Axl,* as a potential therapeutic target. FACS analysis confirmed expression of Axl in dormant but not reactivated cells and analysis of CD138+/CD38+ plasma cells from patients with myeloma identified a small population of cells express AXL. Treatment of mice bearing 5TGM1eGFP cells with BMS-777607, a selective Axl inhibitor, decreased dormant cell numbers and increased tumour burden suggesting Axl is important in controlling dormancy.

These data demonstrate that dormant myeloma cells persist in the skeleton, have a unique transcript profile and by targeting novel dormant gene signature pathways we can alter the behavior of myeloma growth in bone.

#60 Comparative evaluation of EGFR and HSP family proteins and cancer stem cell genes in primary and metastatic cell lines isolated from genetically engineered mouse models of osteosarcoma

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Osteosarcoma is the most common primary bone tumor in humans and dogs. While the tumor may be removed successfully in the majority of cases, the development of metastatic disease limits survival. Unfortunately, tumor biology used to inform treatment decisions aimed at suppression of metastatic disease are largely based on information gained from the primary tumor. The development of genetically engineered mouse models of osteosarcoma that develop metastatic disease has resulted in an opportunity to compare potential treatment targets between primary and metastatic lesions that develop in these mice. Using models of both fibroblastic and osteoblastic osteosarcoma, we compared protein expression of EGFR and HSP family proteins in cell lines established from paired primary and metastatic lesions. These targets were chosen due to previous gene expression studies that demonstrated differential expression of EGFR and HSP70 genes between the two osteosarcoma subtypes. Additionally, as osteosarcoma has been demonstrated to conform to a cancer stem cell model of tumorigenesis and progression, we also compared sarcosphere formation and gene expression of the stem cell markers Nanog, Oct4, STAT3, Sox2, and CD133 in these cell lines. Finally, to assess comparative differentiation of cells in both monolayer and sarcosphere conditions between primary and metastatic cell lines of both subtypes, gene expression of DMP-1 and osteocalcin was quantified.

Protein expression of EGFR, HER2, HSP70 and GRP78 was increased in metastatic cell lines compared to primary cell lines in both fibroblastic and osteoblastic osteosarcoma subtypes. Significant differences in sphere forming ability in serum-starved non-adherent conditions was observed, but sphere formation was not consistently higher in the metastatic cell lines.

Sarcosphere cell populations had increased expression of Nanog, Oct4, CD133 and Sox2 compared to adherent cultures. Primary osteosarcoma cell lines had higher expression of DMP- 1 than metastatic cell lines, and sarcospheres displayed increased expression of both DMP-1 and osteocalcin compared to the same cells grown in monolayer culture.

Ongoing work is focused on validating these results in a larger sample size, to confirm the possibility that potentially treatable targets may have higher expression in metastases compared to the primary tumor.

#61 PTHrP promotes breast cancer exit from dormancy in a PTHR1-independent

manner

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Parathyroid hormone-related protein (PTHrP) is expressed at high levels in breast cancer bone metastases compared to primary tumors. Human MCF7 breast cancer cells home to the bones of immune deficient mice following intracardiac inoculation, but do not grow well and stain negatively for Ki67, thus serving as a model of breast cancer dormancy *in vivo*. We have previously shown that PTHrP overexpression in MCF7s overcomes this dormant phenotype, causing them to grow as osteolytic deposits, and that MCF7 PTHrP-overexpressing cells showed significantly lower expression of genes associated with dormancy compared to vector controls (e.g. *AMOT, P4HA1, H2BK, SELENBP1*) by RNAseq and qPCR. Since early work showed a lack of cyclic AMP (cAMP) response to parathyroid hormone (PTH) in MCF7 cells, and cAMP is activated by PTH/PTHrP receptor (PTHR1) signaling, we hypothesized that the effects of PTHrP on dormancy in MCF7 cells occurs through non-canonical PTHR1-independent signaling. The data presented here confirms the lack of cAMP response in MCF7 cells to full length PTHrP and PTH(1-34) in a wide range of doses, while response to two known activators of adenylyl cyclase was present: calcitonin (100-fold max) and prostaglandin E_2 (PGE_2) (30-fold max). Moreover, whereas both calcitonin and PGE_2 activate a cAMP Cre-luciferase reporter construct transfected into MCF7 cells, PTHrP and PTH had no effect. PTHR1 mRNA was detectable in MCF7 cells at a low level (30-50-fold lower than in osteoblastic cells with functional PTHR1), and was similarly found in 8 other human breast and murine mammary carcinoma cell lines. Although PTHrP overexpression in MCF7 cells changed expression levels of many genes (>2500 genes with >1-$\log_2$fold change, $p<0.05$), only 2/32 PTH1R-responsive cAMP / CREB genes (e.g. *NRP1, AREG*) were significantly upregulated and PTHR1 was unaltered. We conclude that MCF7 breast cancer cells have no functional PTHR1. Thus changes in gene expression in response to PTHrP overexpression must therefore result from autocrine or intracrine actions of PTHrP independent of PTHR1, through signals emanating from other domains within the molecule, such as the nuclear localization sequence. These cells therefore offer a model with which to investigate the significance of non-canonical PTHrP actions.

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