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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.