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Speaker Abstracts

GREG MUNDY MEMORIAL LECTURE

Mundy

The Cancer Diaspora: Bone Metastasis beyond the Seed and Soil Hypothesis

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The term “metastasis” is derived from the Greek “methistemi”, meaning to remo1ve or set free. In its simplest form, metastasis is the movement of cancer cells from one part of the body to another. Paget’s “seed and soil” theory suggested there are factors within the metastatic site that promoted growth similar to the tendency of seeds to grow in fertile soil, i.e. factors in the environment of the metastatic site could contribute to the proliferation of cancer cells. James Ewing later proposed cancer cells grew at a particular site because they were directed to that site by the direction of blood flow and lymphatics. Both of these theories are correct. In the strictest sense, Paget focused on the process of immigration; Ewing that of migration. Isaiah Fidler defined the modern “seed and soil” hypothesis as consisting of three principles: cancerous tissues contain heterogeneous subpopulations of cells with different angiogenic, invasive and metastatic properties, the metastatic process is selective for cells that have survived the long journey to the distal organ, and the success of the metastatic cells depends on the ability of these cells to interact and utilize the “soil” provided in their microenvironment. We have sought to understand metastasis/migration in terms of an evolutionary complex adaptive ecosystem to develop a better understanding of the mechanisms underlying the metastatic process and identify new targets for prevention and treatment. But even describing metastasis in terms of migration is not a rich enough concept to cover the nuances of cancer spread. A diaspora, from the Greek διασπορα, meaning scattering or dispersion is the movement, migration, or scattering of people away from an established homeland. Central to the concept of the diaspora is ancestral memory – the dispersed population does not assimilate completely into the new environment, keeping an identity that has roots in their original home. It is now clear that metastasis is not just a one-way street, not a simple migration, but rather a diaspora of cells that are inherently linked to the primary site from which they came as well each other. Utilizing ecological principles, the cancer diaspora can be targeted at multiple levels to disrupt tumor growth at multiple sites. We have termed this “scaled network” disruption.

PLENARY 1: THE TUMOR MICROENVIRONMENT AS MODEL OF AN ECOSYSTEM FAILURE

S01

Bone Marrow Derived Cells are Critical to Metastatic Progression of Human Breast Cancer

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Metastatic breast cancer is overwhelmingly lethal. Most breast cancer research has focused on the genetic and epigenetic changes that occur in cancer cells themselves. We have focused on stromal changes induced by breast cancers which may increase the likelihood of malignant spread. Bone marrow-derived myeloid immune cells are known to infiltrate malignant tumor sites in large numbers and are a prominent feature in the stroma surrounding tumors. Their presence in the latter and in the blood stream of breast cancer patients is associated with poor prognosis. Specific attention has been drawn to myeloid-derived suppressor cells (MDSCs), a heterogeneous mixture of immature myeloid cells as linkers of inflammation, immunosuppression, and metastatic progression. In mice, two subpopulations of MDSCs have been described based on the expression of the two Gr-1 epitopes. In humans, however, specific markers to fully define human MDSC subpopulationsCs, remain to be further identified. Our group has shown that human breast cancer cells injected into the mammary fat pad of NOD scid gamma (NSG) mice, compared to other immunosuppressed mice consistently develop metastases. In NSG mice, MDA-MB-231 cells and dissociated cultures of primary human breast tumors [DT16, DT25 and DT28] are metastatic to sites such as lung, liver and lymph nodes. We compared the gene expression of stromal (mouse) genes in cancer-bearing tissue -including the primary tumor and metastatic lesion sites- to that of healthy, non-tumor bearing tissue. In these xenograft models, stromal gene expression analysis of all the cancer-bearing tissue -including primary site and metastasis- in all of our models, showed differential expression of a group of genes consistent with myeloid cell infiltration. Importantly, even though NSG mice lack T, B and NK cells, MDSCs isolated from tumors and spleens of tumor-bearing NSG mice retain their suppressive function as confirmed by T cell proliferation suppression assay and gene expression analysis. Moreover, *in vivo* targeting of these cells delayed tumor growth and lowered metastatic burden. Our data support an additional mechanism through which MDSCs may favor tumor progression which could ultimately translate into viable, novel therapeutic interventions.

S02
The Participation and Contribution of Mesenchymal Stem Cells(MSC) in the Tumor Microenvironment

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To meet the requirements for rapid tumor growth, a complex array of non-neoplastic cells are recruited to the tumor microenvironme nt. These cells facilitate tumor development by providing matrices, cytokines, growth factors, as well as vascular networks, however their precise origins remain unclear. Understanding the origin and composition of these stromal components will help to identify targets for therapeutic intervention. We unraveled the tumor microenvironment through a series of multicolored cellular transplant procedures, and have quantitatively assessed the contribution of bone marrow-derived, local tissue-derived, and adipose-derived cells to stroma within tumors using a novel multispectral detection system. Our results indicate that tumor-associated fibroblasts (TAF) are recruited from two distinct sources. The majority of fibroblast specific protein (FSP) positive and fibroblast activation protein (FAP) positive TAF originate from MSC located in bone marrow, whereas most vascular and fibrovascular stroma (pericytes, a-SMA myofibroblasts, and endothelial cells) originates from neighboring MSC sources, primarily neighboring adipose. Additionally, the majority of endothelial cells and fibrovascular element all derived from neighboring adipose.
Next, as we sought to identify the key mediator of the MSC-> TAF transition, we investigated the criticality of CD44 on MSC-a key inflammatory sensing receptor thru blocking MSC-expressed CD44 with neutralizing antibody, soluble CD44 decoy receptor, siRNA knockdown, or utilizing a CD44KO mouse. We report that the tumor-supportive aggressive phenotype of the MSC derived tumor associated fibroblast (TAF) is repressed with the removal of CD44 expression. Moreover, MSC deficient in CD44 are migratory deficient and incapable of supporting angiogenesis.
These results highlight the capacity for tumors to utilize multiple sources of structural cells in a systematic and discriminative manner, and that the tumor microenvironment generates potent modulators of MSC recruitment, engraftment, and activation.

S03
Musculoskeletal Effects of Cancer and Bone

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Theresa A. Guise, M.D.
Bone metastases cause significant morbidity and once housed in bone, the tumors are incurable. Tumors produce factors which stimulate osteoclasts and osteoblasts to dysregulate normal bone remodeling. The bone microenvironment alters the behavior of metastatic tumor cells, driving a feed-forward cycle that makes skeletal metastases refractory to treatment and cure. Transforming growth factor beta (TGFbeta) is a central factor in this vicious cycle. It is deposited into mineralized bone matrix by osteoblasts, released and activated by osteoclastic bone resorption, and changes the phenotype of tumor cells. In mouse models, TGFbeta blockade inhibits osteolytic bone metastases due to breast cancer prostate cancer and melanomas by blocking tumor-produced1 osteolytic and prometastatic factors (PTHrP, IL-11, CTGF). It also increases bone mass, independent of effects on cancer cells, by increasing osteoblast activity and reducing osteoclast activity. These effects are potentiated with the use of a bisphosphonate, zoledronic acid. Muscle weakness frequently occurs in patients with advanced cancer and many of these patients have bone metastases. Preclinical data indicate that cancer-associated muscle weakness occurs in mice with bone metastases due to breast and prostate cancer, and not in mice with primary tumors alone. A strong correlation exists between osteolytic bone destruction and muscle weakness, which indicates that products from the bone microenvironment as a consequence of bone destruction can act systemically to promote muscle weakness. The molecular mechanisms of the muscle weakness will be discussed as well as potential for molecularly targeted therapy.

S04
The Functional Contribution of Fibrosis in the Emergence and Progression of Cancer and Metastasis

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Tumors are unorganized organs that contain many different cell types and non-cellular constituents that are associated with cancer cells. The central goal of our laboratory is to evaluate the functional role of these non-cancer cells/constituents in cancer progression and metastasis. Cancer progression significantly depends on the influence of many different host cells on the genetically unstable cancer cells. Whether such host responses are recruited to control cancer progression or further aid in tumor growth (or both) is still unclear. Additionally, chronic tissue fibrosis involves fibroblast activation and inflammation that leads to deposition of type I collagen and eventual organ failure. There is a strong experimental and clinical correlation between tissue fibrosis and incidence of cancer. But it remains unclear how fibrosis may contribute to the emergence of cancer. This lecture will discuss the role of extra-cellular matrix, angiogenesis, and mesenchymal cells, organ fibrosis and tumor immunity in cancer progression and metastasis.

S05
Blocking Syndecan-4 Inhibits Bone Metastasis Formation: Potential Involvement in the Prometastatic Activity of Autotaxin

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Autotaxin (ATX/NPP2) is a secreted glycoprotein that generates Lysophosphatidic acid (LPA) due to its lysophospholipase D activity. We have shown that ATX controls the progression of osteolytic bone metastases through the production of LPA in the tumor microenvironment. However, the molecular mechanisms

involved in the local production of LPA at the bone metastatic site are still not well characterized. Binding of ATX to beta3 integrins has been proposed for LPA delivery to its receptors present at the surface of tumor cells. However, we found *in vitro* that the treatment of tumor cells with LM609 monoclonal antibody only partially inhibited ATX interaction with tumor cells indicating the involvement of other partners than alphavbeta3 integrins. Recent studies suggested that ATX could potentially interact with cell surface Heparan-sulfate proteoglycans (HSPs). Among the HSP family members, we found that syndecan-4 (SDC4) was commonly highly expressed in different cell lines. Silencing of SDC4 expression by synthetic siRNAs in MG-63 osteosarcoma cells and in 4T1 murine breast cancer cells, highly decreased cell adhesion on ATX. Additionally, pretreatment of MG-63 cells with an anti-human SDC4 antibody (5G9) and pre-treatment of 4T1 cells with an anti-mouse SDC4 antibody (KY/8.2), but not with the isotypic control antibody, also decreased cell adhesion to ATX. We have already shown that 4T1 cells that induce the formation of osteolytic bone metastases in immunocompetent BALB/c mice, express active ATX and that stable silencing of ATX in these cells inhibited the extent of osteolytic lesions. Here, we showed that pre-treatment of 4T1 cells with the anti-mouse SDC4 antibody (KY/8.2) but not with the isotypic control antibody, before intravenous injection to BALB/c mice significantly decreased the number of medullar disseminated tumor cells. Moreover, pre-treatment of 4T1 cells with anti-mouse SDC4 antibody reduced the extent of osteolytic lesions after intra-osseous injection. Altogether, these results demonstrated for the first time the role of SDC4 in bone metastasis formation and strongly suggested close interaction of SDC4/ATX at the cell surface during this process. These results may have important implications in the development of new therapies for patients with bone metastases.

S06

Role of the Tumor-Bone Microenvironment in Muscle Weakness and Cachexia

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Cancer cachexia is a devastating paraneoplastic syndrome that frequently occurs with breast cancer bone metastases and is characterized by weight loss and muscle weakness. Using a model of human breast cancer metastatic to bone (MDA-MB-231) we show that skeletal muscle dysfunction is independent of weight loss and not present in mice with primary breast cancer (no bone metastases).

Mice with bone metastases, compared to age-matched control mice, lost significant weight by 4wks (20.5 g±0.6 v. 23.2 g±0.4; $p<0.001$) due to loss of lean and fat mass. *Ex vivo* contractility of the extensor digitorum longus (EDL) muscle showed a significant reduction in specific force (corrected for muscle size) in tumor mice (213.2 kN/m^2±17 v. 361.1 kN/m^2±10; $p<0.001$) that correlated with greater osteolysis ($p<0.05$). To determine if muscle weakness was due to weight loss associated with reduced food consumption, we monitored food and water intake in mice with bone metastases. These mice had a 40% reduction in food intake during the last week before death. We restricted food of healthy mice by 40% for one week and despite a significant decrease in body weight, mice with caloric restriction exhibited no decrease in forelimb grip strength or EDL specific force, indicating that loss of muscle mass alone cannot account for muscle weakness associated with cancer. It is well established that cancer is associated with oxidative overload. We examined oxidation of muscle proteins and found that sarcomeric proteins (tropomyosin/myosin) and excitation-contraction coupling proteins (ryanodine receptor/Ca2+ release channel (RyR1) on the sarcoplasmic reticulum) were oxidized in mice with bone metastases. Oxidation of RyR1 causes depletion of the stabilizing subunit calstabin1 resulting in intracellular Ca2+ leak and muscle weakness. This was not observed in muscle from mice with primary breast cancer. Preventing oxidation-mediate loss of calstabin1 from RyR1 with a Rycal (S107) restored muscle function (431.0 kN/m^2±19.4 v. 362.8 kN/m^2±7.2; $p<0.0001$) with no affect on body weight. S107 treatment also increased intracellular Ca2+ release during tetanic stimulation.

Thus, there is a primary loss of muscle function in addition to weight loss in mice with bone metastases. These results demonstrate a mechanism for skeletal muscle dysfunction in cancer cachexia: leaky RyR1 channels. Moreover, these data show that the bone microenvironment plays a critical role in intracellular Ca2+ leak via remodeled RyR1.

S07

The Participation and Contribution of Mesenchymal Stem Cells(MSC) in the Tumor Microenvironment

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Significant progress has been made in defining the molecular mechanisms through which tumor cells resculpt with the bone microenvironment to promote tumor growth and bone invasion, including tumoral and host cell misregulation of genes such as PTHrp, TGFbeta, RANKL, Beta 3 Integrins, HIF1alpha and growth factor/cytokine signaling pathway genes.Initial studies and therapies focused on tumor induced bone destruction particular since this is largely responsible for the significant skeletal complications of bone metastasis including: pathologic fractures, severe bone pain, symptomatic hypercalcemia and spinal cord compression. Therapies targeted to inhibit osteoclastic bone resorption have been highly effective in reducing these skeletal complications but have not resulted in improved survival for patients with bone metastases, despite preclinical evidence for such survival benefits. Promising studies preclinically and clinically suggest that antiresorptive therapies may enhance survival when administered earlier in the disease. The molecular mechanisms through which tumor cell home to bone, and localize to bone vascular and stromal compartments particularly within osteoblastic hematopoietic stem cell niches represent promising targets including: CXCR4 and VLA4, Annexin II and GAS6. The role of tumor initiating cells (cancer stem cells) through EMT pathways and their localization

in stem cell niches particularly through the abundant TGFb and BMPs within bone are also intriguing therapeutic candidates. The role of mesenchymal stem cells, osteoblasts and osteocytes upregulation (prostate cancer) and disruption (multiple myeloma) have been targeted with the osteoblast promoting c-Met/VEG-FR2 inhibitor, Cabozantanib, and DKK1 antagonist in prostate cancer and MM respectively. Finally, the bone marrow derived pro-tumor myeloid derived cells and VEGFR+ prevascular cells that form the premetastic niche and immunosculpting of tumor microenvironments provide yet another important role for the bone microenvironment in promoting tumor progression.

S08

Multiple Myeloma Bone Marrow Derived Mesenchymal Stem Cells (MSCs) Show Decreased Osteogenesis in Part Due to Decreased Expression of MicroRNA HSA-MIR-199A-3P, MIR-15A-5P and MIR-16-5P

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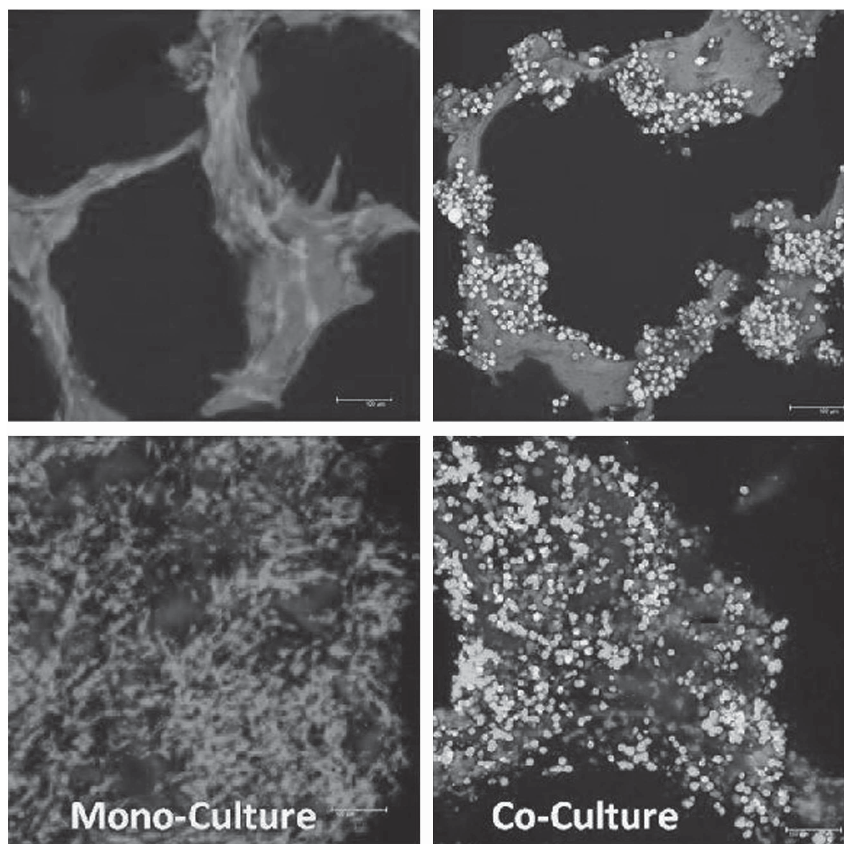
Multiple Myeloma (MM) involves clonal proliferation of malignant plasma cells within the bone marrow leading to a deterioration of bone structure and function. The disease creates a forward feedback system with local mesenchymal stem cells (MSCs) within the bone marrow that causes abnormalities in MSC function, but the exact mechanisms, cellular changes, roles of miRNAs, and downstream consequences within the stroma are largely unknown.

Proliferation and osteogenic differentiation of MSCs from myeloma patients (MM-MSCs) and normal donors (ND-MSCs) cultured with or without MM1S cells were characterized in 2D culture and in 3D porous silk scaffolds (Figure 1). MM-MSCs demonstrated decreased osteogenesis assessed by alizarin red staining (ARS) and qRT-PCR for osteopontin, osteocalcin, and RUNX2. Three-dimensional *in vitro* co-cultures assessed cell proliferation, cell-cell interactions and ECM production using confocal microscopy, IHC and histology, and demonstrated MM inhibition of MSC growth and osteogenesis. qRT-PCR and Nanostring were used to examine 800 miRNAs and 230 cancer-related mRNAs in clinical samples and *in vitro* co-cultures. Thirty-seven microRNAs were significantly different between MM- and ND-MSCs, including a down-regulation of miR-15a, miR-16, Let-f and miR-199a-3p, (>1.2 fold, $p<0.05$). Unbiased gene expression profiling showed discrete clustering of MM-MSCs distinct from ND-MSCs and 51 mRNAs significantly different between the groups (>1.2 fold, $p<0.05$). Higher expression of CDKN1A and CDKN2A was observed in MM-MSCs, suggesting a novel mechanism to explain their inhibited proliferation.

Of the miRs with decreased expressed in MM vs ND-MSCs and in the 3D model (MM.1S-co-cultured MSCs vs MSCs alone), 5 were investigated for potential osteoinductive effects using miRvana mimics. Overexpression of hsa-miR-199a-3p, miR-15a-5p and miR-16-5p increased MM-MSC expression of osteogenic markers (OPN, BGLAP, and RUNX2) and 199a-3p increased mineralization based on ARS.



Our 3D platform provides a simple, flexible, clinically relevant tool to model myeloma growth within bone. We utilized the 3D scaffold model system to investigate bone and cancer interactions during osteogenesis with non-destructive imaging techniques. The model recapitulated decreased bone formation as found in MM patients and proposed miR-199a-3p, miR-15a-5p and miR-16-5p as novel bone anabolic agents, which were confirmed to induce osteogenesis *in vitro* in clinical samples.

PLENARY 2: DORMANCY/QUIESCENCE-WHAT MAKES THE MONSTER SLEEP AND WAKE UP

S09

Inactivation of Neogenin Drives Bone Metastasis in Prostate Cancer

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In spite of significant progress toward early detection and treatment, once Prostate Cancer (PCa) has become refractory to anti-androgen therapy and metastatic, it cannot be effectively cured. The mechanisms by which oncogenic lesions cause transition from Prostate Intraepithelial Neoplasia to invasive and metastatic PCa and eventually enable prostate cancer cells to become refractory to hormonal therapy remain unclear. Neogenin functions as a neuronal guidance receptor by binding to netrins in the extracellular matrix or on the surface of other cells, but recent studies have revealed that it also participates in epithelial morphogenesis. Although it is not known if neogenin plays a role in cancer, we have observed that hormone-refractory metastatic prostate tumors and small cell Neuroendocrine (NE) prostate tumors express severely diminished levels of Neogenin. We will present evidence that loss of Neogenin promotes prostate tumor progression to castration resistance and bone metastasis and describe the underlying mechanisms.

S10

Bone Micro-Environmental Signals that Regulate Tumor Cell Dormancy

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In cancers like breast and prostate, bone metastases are preceded by an asymptomatic phase where disseminated tumor cells (DTCs) remain dormant. Interestingly, in many cancers bone metastasis never develop but DTCs are detectable for years in the bone marrow despite growth never ensuing in this site. This suggested that before becoming hospitable for some cancer metastasis, the bone marrow microenvironment might encode dormancy inducing signals that may be exploited to maintain DTCs dormant. This is important because if DTCs are quiescent, current therapies are virtually useless but also because inducing or maintaining dormancy might be a therapeutic option. Thus, there is a need to identify these mechanisms to design therapies that target the quiescent tumor cells. We discovered that tumor cells that enter dormancy upregulate TGFβ2, a growth suppressive cytokine that regulates hematopoietic stem cell quiescence in the bone marrow. Inspection of the lung microenvironment that in mouse models and human cancer is usually metastasis permissive vs. the bone marrow that is metastasis restrictive, showed that the bone marrow had higher levels of TGFβ2 than the lung. This led to the findings in an HNSCC model showing that strong TGFβ2, but not TGFβ1 signaling in the bone marrow induces a paracrine loop that activates p38, inducing a [ERK/p38]low signaling ratio. This results in a DEC2/SHARP1-dependent induction of p27, downregulation of CDK4 and dormancy of malignant DTCs. In lungs, a metastasis "permissive" site, TGFβ2 signals are weaker and only a short-term dormancy ensues. Importantly, systemic inhibition of TGFβ-receptor-I or p38α/β activities awakens dormant DTCs fueling multi-organ metastasis. The ability of TGFβ2, but not TGFβ1 to induce quiescence via TGFβ-receptor-I was dependent on the engagement of TGFβ-receptor-III, which sustained SMAD1/5 activation and p27 induction. We propose that DTC dormancy in a metastasis "restrictive" or "permissive" target organ microenvironment is controlled by the intensity of at least TGFβ2 signaling through canonical and non-canonical pathways. We also propose that therapies that mimic TGFβ2 signaling may induce and maintain residual disease dormant and asymptomatic.

S11

Prostate Cancer Disseminated Tumor Cells, Dormancy and Bone Metastases

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The majority of prostate cancer (PCa) related deaths are due to metastases, with PCa metastasizing to the bone in approximately 90% of patients who die of PCa. Growth of tumor cells in the bone can lead to replacement of bone marrow, anemia, spinal cord compression, severe bone pain, cachexia and death. While chemotherapeutic strategies show some promise, there is no effective therapy that substantially prolongs survival for castration resistant PCa. Prostate cancer cells can disseminate early in the disease process. Disseminated tumor cells (DTC) in the bone marrow can remain dormant for prolonged periods before recurrence. Our aim was to isolate and characterize individual DTC, analyze tumor cell heterogeneity, and identify markers of dormancy. The focus of this seminar centers on the isolation and characterization of DTC, dormancy and the development and character of PCa metastases in the bone.

S12

Antibody-Based Therapy Targeting Integrin α5 is an Effective Strategy to Treat Experimental Breast Cancer Bone Metastasis

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Integrin α5β1 is a specific fibronectin receptor that is often upregulated in breast cancer cells undergoing epithelial-to-mesenchymal transition and in tumor-associated endothelial cells. Indeed, the disruption of α5β1 binding to fibronectin

leads to tumor growth reduction through inhibition of angiogenesis. Additionally, $\alpha5\beta1$-fibronectin interaction promotes *in vitro* the survival of growth-arrested breast cancer cells in the bone marrow microenvironment. This is in line with the observation that disseminated tumor cells (DTCs) in the bone marrow express $\alpha5\beta1$ integrin. DTCs in the bone marrow represents the earliest sign of development of metastatic disease in patients. However, the involvement of tumor-derived $\alpha5\beta1$ integrin in bone marrow metastasis formation remains poorly documented. Using a cohort of 427 radically resected breast cancer patients, we have found that a high $\alpha5$ expression in primary tumors detected by qRT-PCR is a negative independent predictive factor of bone relapse. Moreover, compared with tumor-bearing animals treated with the vehicle, a preventive treatment of BALB/c immunodeficient mice bearing human breast MDA-MB-231/B02 tumors with a chimeric IgG4 monoclonal antibody that specifically binds to human integrin subunit $\alpha5$ (M200;15 mg/kg three times per week starting from the day before intra-arterial tumor cell inoculation) significantly delayed the onset and reduced the extent of osteolytic skeletal lesions, as detected by bioluminescence and radiography.

Histomorphometric analysis of metastatic legs after 28 days of treatment confirmed that M200 substantially decreased skeletal tumor burden and increased the bone volume. When the bone marrow was flushed from the hind limbs of animals on day 7 after tumor cell inoculation, at which time there was no evidence of metastases, and placed in culture under puromycin selection, the growth of antibiotic-resistant DTCs colonies in the bone marrow from M200-treated mice was dramatically decreased compared with vehicle-treated animals. *In vitro*, M200 antibody did not affect MDA-MB-231/B02 cell survival. By contrast, it specifically inhibited MDA-MB-231/B02 cell adhesion to fibronectin and cell invasion.

Overall, our results suggest that $\alpha5\beta1$ integrin expression in breast cancer cells facilitates bone marrow micrometastasis formation and the subsequent development of osteolytic lesions.

S13

Visualization of Tumor Cell Dormancy and Activation in the Skeleton by Two-Photon, Intra-Vital Imaging

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Cancer cells can exist in a dormant state in the skeleton and can be activated to form overt tumours. However, our understanding of these events is limited due to an inability to identify dormant cells, to follow their activation in bone and to purify and define their phenotype. Using multiple myeloma as a model, we developed novel labeling and intra-vital imaging techniques to visualize individual dormant cancer cells and their activation in live mice and performed transcriptome analysis to define their phenotype.

5TGM1eGFP murine myeloma cells were labeled with a membrane dye (Vibryant DiD), which is retained by dormant, non-dividing, cells (DiDHigh), but lost on sharing with daughter cells as they divide (DiDNeg). Cells were injected (i.v.) into C57BL-KalwRij mice and visualized after 1, 6, 14, 21, or 28 days, in the bone marrow microenvironment, of intact tibia of live mice by two-photon, intra-vital, microscopy. DiDHigh and DiDNeg cells were isolated for flow cytometric analysis and ST2.0 whole mouse genome array analysis, whereas CD138+ve cells myeloma cells and colonies were identified by immunohistochemistry.

Intra-vital microscopy identified limited numbers of individual, DiDHigh, cells directly opposed to endosteal bone surfaces at each time point, which was confirmed by flow cytometry ($171\pm31/10^6$ total cells). Individual CD138+ve cells were seen by immunohistochemistry. DiDNeg/GFP+ve cells could be identified from day 14, which increased through to day 28. This was associated with formation of a limited number of overt DiDNeg/GFP+ve colonies and CD138+ve colonies at day 21 (14.8 ± 1.1). Individual DiDHigh tumour cells remained evident at day 28. Microarray analysis identified a distinct transcriptome profile of DiDHigh cells when compared to DiDNeg cells. A panel of long non-coding RNAs were the most strongly up-regulated transcripts in DiDHigh dormant cells, consistent with the regulatory architecture of the genome playing a role in maintenance of dormancy.

These data demonstrate that two-photon, intra-vital microscopy can be used to visualize dormant cancer cells and their activation in the skeleton in live mice. These data also show that only a limited number of the dormant cells present in bone are activated to form tumour colonies. Furthermore, the DiDHigh cells have a unique transcriptome profile, which may be critical in retention of the dormant phenotype in bone.

S14

Myeloma Cell Dormancy is an Acquired State *In Vivo*

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Despite continually improving treatments in myeloma, patients eventually relapse. It has been suggested that some myeloma cells can evade chemotherapy by residing within protective niches in the bone marrow. We hypothesise myeloma cells reside within specialised niches close to bone and remain in a dormant state until activated. In this study we aimed to establish if myeloma cell dormancy is restricted to an intrinsic population of tumour cells or if it is an acquired state *in vivo*.

5TGM1-eGFP cells were labelled with a long-chain dialkylcarbocyanine membrane probe (Vybrant DiD) to monitor cell proliferation or dormancy and injected into C57BLkaLwRij mice. Tumour burden was measured by fluorescent activated cell sorting (FACS), immunohistochemistry and multiphoton microscopy; bone disease was measured by microCT and static histomorphometry. Sub-populations of proliferating (GFP/DID^{Neg}) or dormant 5TGM1 cells (GFP/DiD^{High}) were isolated from *in vitro* cultures or *ex vivo* from bone marrow flushes of 5TGM1 tumour-bearing mice by FACS. Cells were then

characterised *in vitro* in osteoblast conditioned media assays or *in vivo* by injection into C57BLkaLwRij mice.
In a preclinical model of myeloma, we identified key stages in myeloma disease development. At the end stage of disease, both proliferating and dormant sub-populations of 5TGM1 cells were successfully isolated by FACS and characterised. *In vitro*, cells were cultured in osteoblast-conditioned media, and the proliferation of both populations was inhibited. Injection of these cells *in vivo* showed proportions of dormant cells could be activated to form tumour colonies; and proliferating cells could become dormant.
We have identified, isolated and characterised populations of dormant and proliferating myeloma cells, when cultured *in vitro* or *in vivo* both populations behaved in a similar manner. These findings suggest myeloma cell dormancy is an acquired state and targeting such cells in patients may prevent relapse.

S15

Identifying Haematopoietic Stem Cell (HSC) Niche Markers in Human Prostate Cancer Cells: Possible Mediators of Dormancy in Metastatic Cancer. Freyja Docherty, Ning Wang, Anne Fowles, Julia Hough, Clive Buckle, Ingunn Holen, Colby Eaton

Freyja Docherty[1], *Ning Wang*[1], *Anne Fowles*[1], *Julia Hough*[1], *Clive Buckle*[2], *Ingunn Holen*[1,2], *Colby Eaton*[1]
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Background/Rationale: Our recent studies with a human prostate cancer xenograft model show that tumour cells homing to bone are mitotically dormant. Others have suggested that tumour cells hijack HSC niches in this model[1]. In this study we have identified dormant cells in a prostate cancer cell line grown *in vitro* and tested the hypothesis that deployment of HSC niche components is a characteristic of dormant prostate cancer cells.
Methods: FACS was used to evaluate the following interacting pairs of HSC niche molecules in the human prostate cancer cell line PC3NW1 (PC3 expressing luciferase): CXCR4/CXCL12, Jagged/Notch, Tie 2/Ang1 and N-Cadherin. These cells were stained with Vybrant DiD cell membrane dye and dye retention over 14–21 days used to identify dormant cells. These were separated by FACS and HSC niche molecules expressed/present in dormant and non-dormant populations were compared by RT-PCR/immunofluorescence and for growth, in colony assays.
Results: FACS analysis of the entire PC3 population showed that N-Cadherin and Notch were present on >50% of cells. CXCR4 and Jagged were present on a minority population (<5%). We identified and isolated a slow growing, potentially mitotically dormant, cell sub-population from PC3-NW1 cells *in vitro* based on DiD retention for 14 days. Isolated dormant cells expressed significantly higher levels of CXCR4 and Jagged than the rapidly dividing population. Microscopically, DiD retaining cells were morphologically distinct being ~ twice the size of rapidly growing cells and immunofluorescence analysis confirmed the presence of high levels of CXCR4 in DiD+ve cells. Once separated both DiD+ve and –ve population formed growing colonies in monolayer.
Conclusions: These results suggest that the PC3 cell line contains a mitotically dormant phenotype with distinct characteristics and HSC niche factor profile. This may confer an increased ability to home to the skeleton in these cells. The study also shows that once isolated, this population is able to proliferate after long periods of dormancy. The factors that control entry/exit from a dormant state are potentially relevant to understanding homing to bone and the initiation of growing lesions *in vivo*.
Reference
1. Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bonemarrow. Shiozawa Y. et al. J Clin Invest. 2011 Apr;121(4):1298–312.

PLENARY 3: STEM CELLS

S16

The HSC Niche Regulates Metastasis and CSC Phenotype of Solid Tumors

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Recently we demonstrated that the metastatic process of solid tumors is similar to the 'homing' behavior of hematopoietic stem cells (HSC). As both HSCs and prostate cancer (PCa) cells utilize the CXCL12/CXCR4 chemokine axis and annexin II, an adhesion molecule expressed by osteoblasts and endothelial cells, and its receptor to gain access to the bone. In the marrow HSC homing, quiescence and self-renewal are dependent on a region called the HSC "niche". As solid tumor metastases use the HSC homing pathways to establish footholds in the marrow, we hypothesized that solid tumor cells would also target the HSC niche during the dissemination.
To determine where HSCs and disseminated solid tumor cells are in the marrow, pre-labeled HSCs and PCa cells were injected into SCID mice. Imaging visualized that two cell types localize within a few microns of each other within a 24 h period. *In vitro* adhesion assays demonstrated that PCa cells are able to compete with HSCs for binding to niche constituents, whereas non-metastatic cells are not able to prevent HSC binding. In functional assays the presence of disseminated PCa cells prevented the long-term reconstitution of HSCs in the niche, when animals were implanted with PCa cells subcutaneously several weeks prior to the bone marrow transplantation.
We next explored the extent to which binding to the niche alters the expression of the PCa stem cell phenotype. To understand more fully how DTC-niche interactions regulate metastases, we injected PCa cells into SCID mice and examined the phenotype of DTCs 24 h after they entered the niche. Surprisingly, DTCs recovered from the niche are highly enriched in the CD133+/CD44+ population which have been shown to have tumor initiating capacity. Moreover, these cells express genes associated with a "stem-like" phenotype. The shift in phenotype from a largely CD133-/CD44- population (99.5%) to CD133+/CD44+ (10–40%) is not seen in cells recovered from other tissues. We have shown that this phenomenon is not due to proliferation or to selective survival of the population in

the blood stream. Collectively, these findings suggest that the HSC niche itself is central to the shift in DTC phenotype towards a less mature, more cancer stem-like cell–a phenotype that is resistant to chemotherapy and may persist in a dormant state for years following primary treatment.

S17

Novel Experimental Systems for Modeling Dormancy of Metastatic Breast Cancer Cells in the Bone Marrow

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Mortality of breast cancer patients is due overwhelmingly to metastatic spread of the disease. Although dissemination is an early event in breast cancer, extended periods of cancer cell dormancy can result in long latency of metastasis development. Deciphering the mechanisms underlying cancer cell dormancy and subsequent growth at the metastatic site would facilitate development of strategies to interfere with these processes. A challenge in this undertaking has been the lack of models for cancer cell dormancy. We have established novel experimental systems that model the bone microenvironment of the breast cancer metastatic niche. These systems are based on 3D co-cultures of breast cancer cells with cell types predominant in bone marrow. We identified conditions in which cancer cells are dormant, and in which they proliferate. Dormant cancer cells were able to proliferate upon transfer into supportive microenvironment or upon manipulation of signaling pathways that control dormancy. These experimental systems will be instrumental for metastasis studies, particularly the study of cellular dormancy.

S18

MIR25 as a Regulator of Cancer Stem Cells Maintenance and Bone Metastasis in Human Prostate Cancer via Modulation of Notch Signaling and Integrin Expression

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Experimental and clinical evidence highlight the importance of prostate cancer stem/progenitor cells in carcinogenesis, metastasis and therapy resistance. Previously, we reported that the ALDHhigh cellular subpopulation in human prostate cancer is phenotypically enriched for cancer stem/progenitor cells (CSCs). ALDHhigh cells display strong clonogenic and migratory potential when compared to the more differentiated ALDHlow subpopulation (transit amplifying and differentiated luminal cells). In this study, transcriptional profiling was performed on ALDHhigh vs ALDHlow cells revealing multiple, differentially expressed microRNAs (miRs). Here we investigated the involvement of miR-25 in the acquisition of an invasive phenotype and epithelial plasticity in osteotropic human prostate cancer cell lines (PC-3M-Pro4, C4-2B). We found that ALDHhigh subpopulation of human prostate cancer cells is enriched in stem/progenitor-like cells that show strong tumor- and metastasis-initiating properties in preclinical models of bone metastasis *in vivo*. Our miR profiling revealed that miR-25 was strongly downregulated in ALDHhigh CSCs compared to ALDHlow CSC-depleted prostate cancer cells. Similarly, in primary tumors from prostate cancer patients, the expression of miR25 strongly increased in the epithelial subpopulations during differentiation (stem cells -> transit amplifying cells -> luminal epithelial cells). When overexpressed, miR-25 induced dramatic changes in cell morphology and reduced the mesenchymal, invasive phenotype. Our target analysis reveals that miR25 directly targets multiple steps along the Notch pathway and blocks the acquisition of an invasive phenotype via suppression of integrin-αv (ITGAV) and integrin-α6 (ITGA6). We demonstrated, for the first time, that miR-25 directly targets the 3´UTR of Notch1 and validated this interaction at protein level. In conclusion, our data suggest that miR25 seems to be a master regulator of stem cell maintenance, invasiveness and epithelial plasticity in human prostate cancer through its interaction with Notch signaling and integrin expression. Our data are supported by published studies on the critical role for the Notch pathway and integrins in skeletal metastasis. The identification of miRs and functional validation of their target genes, as we have shown here for Notch, is crucial for the elucidation of the mechanisms involved in CSC maintenance, tumor progression and therapy resistance in human prostate cancer.

S19

How Bone Marrow Microenvironment Prepares the Bone Pre-Metastatic Niche for Breast Cancer Cells?

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Bone metastasis is an incurable complication of breast cancer (BC) affecting approximately 70% of advanced patients. Although novel findings demonstrate the bone marrow (BM)-microenvironment significance in BC progression, the majority of studies have focused on end-stage disease, but little is known

about the how is the BM preparing the bone pre-metastatic niche. In this study, we demonstrated that BC induces substantial changes in peripheral blood (PB) and BM-microenvironments of untreated advanced patients without bone metastasis compared with healthy volunteers (HV). Data suggest that high RANKL, MIF and OPG levels in BC patient (BCP)-PB could play a role in the intravasation, angiogenesis, survival and epithelial-to-mesenchymal transition (EMT) phenotypes of circulating BC cells (BCCs). Interestingly, ICAM-1, VCAM-1 and PDGF-AB levels in BCP-BM plasma were significantly higher than HV-values, suggesting that they could be involved in the BCC escape from the blood vessels into the BM. We demonstrated that BCP-BM-mesenchymal stem cells could control the recruitment of the BCCs modifying the MCF-7 and MDA-MB231 cell migration. In addition to its angiogenic and EMT properties, PDGF-AB could be responsible for the higher proliferation of MDA-MB231 cells when we used BCP-BM plasma compared with HV-plasma. Finally, the high PDGF-AB, ICAM-1 and VCAM-1 levels in the BM plasma would increase bone resorption, leading to BCC invasion and proliferation. Taken together, the BM of untreated advanced BCP without bone metastasis provides an ideal environment for the development of the pre-metastatic niche.

S21

The Bone Marrow Niche Controls the “Stemness” of Disseminated Tumor Cells

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Background: Despite improvements in local treatments of prostate cancer (PCa), bone metastasis remains a major cause of death in PCa patients. The presence of disseminated prostate cancer cells in the bone marrow represents a major therapeutic challenge. The development of methods to identify and characterize disseminated tumor cells (DTCs) is therefore of critical importance to understanding the biology of DTCs and identifying therapeutic targets.
Methods & Results: Since it has been reported that the expression of the cytokeratin and EpCAM may vary considerably *in vivo* and *in vitro*, we explored the extent to which human specific HLA cell surface antigens could serve as alternative targets for the detection of human cells in murine tissues. We found that HLA-ABC antigen was highly identified on human PCa cell lines (PC3, DU145, LNCaP, and C4-2B). To determine if human PCa cells can be isolated from murine marrow with anti-HLA-ABC antibodies, intracardiac injections of PCa cells into SCID mice were performed. Intriguingly, disseminated PCa cells recovered from marrow were highly enriched in CD133+/CD44+ stem-like population. Compared to CD133+/CD44+ cells isolated from culture, the mRNA expression of the self-renewal genes Bmi1, KLF, and Nanog were dramatically increased in CD133+/CD44+ DTCs recovered from the marrow. Importantly, the CD133+/CD44+ DTCs recovered from the marrow of mice form sphere-like structures. Interestingly, the enrichment of CD133+/CD44+ population was not due to the effects of survival within the circulation, proliferation, or homing. The enrichment of CD133+/CD44+ population occurred only in the PCa cells spread to the bone marrow, but not lung or spleen. In addition, the enrichment of CD133+/CD44+ population was inhibited when mTOR signaling pathway was blocked prior to the injection. Moreover, when the CD133-/CD44- population was inoculated into the mice, the conversion into CD133+/CD44+ population was observed in PCa cells recovered from bone marrow.
Conclusions: In this study we developed an *in vivo* murine model in which the disseminated human PCa cells are recovered from murine marrow using flow cytometry. Our findings suggest that the bone marrow microenvironment, or niche, may play a potential role in the accumulation of tumor initiating PCa cells in the bone marrow. The pre-clinical model and strategy described will facilitate deeper insights into the nature and phenotype of DTCs.

S22

Tumor-Initiating Stem Cells are Regulated by α-CAMKII-Induced VEGF in Human Osteosarcoma

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Osteosarcoma (OS) is among the most frequently occurring primary bone tumors, primarily affecting adolescents and young adults. Chemoresistance and disease recurrence are major challenges in the clinical management of OS and are thought to be caused by a small subpopulation of tumor-initiating stem cells (TISCs). Human OS TISCs are characterized by their expression of surface antigens CD117+ and Stro-1+, and the stem cell regulating transcription factors Sox2, Nanog, and Oct4. These OS TISCs are known to express high levels of receptors for vascular endothelial growth factor (VEGF). We have previously demonstrated that alpha-Ca2+/Calmodulin kinase two (α-CaMKII) regulates VEGF and its autocrine signaling functions in human OS. Here, we examine whether OS TISCs are regulated by α-CaMKII-induced VEGF. Using fluorescence-activated cell sorting, we discovered that the pharmacologic inhibition of α-CaMKII or VEGF in 143B OS cells by tamoxifen (1 μM) or bevacizumab (1 μM), respectively, decreases the population of CD117+ and Stro-1+ TISCs, and the gene expression (60%) and protein levels (80%) of Sox2, NANOG, and Oct4. Additionally, we developed a novel pre-clinical xenograft mouse model to examine the recurrence and metastasis of human OS. 143B OS cells were intratibially injected into mice, and tumors were allowed to grow for 2 weeks. Hind limbs-containing tumors were then amputated, and mice were confirmed to be tumor free by bioluminescent imaging 7 days post-surgery. Mice were randomized into four treatment groups: saline, tamoxifen (500 μg/kg/day), and/or bevacizumab (5 μg/kg twice weekly) and monitored monthly by bioluminescent imaging for the development of metastasis. The incidence of pulmonary metastasis/recurrence in saline treated mice was 100% two months after amputation. However, the incidence decreased to 38% in bevacizumab-treated mice, 12% in tamoxifen-treated mice, and 0% when both drugs were used. The levels of the TISCs subpopulation in pulmonary metastasis were determined by immunohistochemistry for Sox2, NANOG, and Oct4. We show that the number of TISCs were significantly increased in the recurrent metastatic pulmonary

tumors when compared to primary amputated tumors. Furthermore, we show that treatment with tamoxifen and/or bevacizumab significantly decreases the number of TISCs when compared with saline treated mice. Taken together, our results demonstrate that α-CaMKII-induced VEGF controls the levels of TISCs both *in vitro* and *in vivo*.

S23

Primary Craniofacial Osteosarcomas Show Expression Patterns Distinct from Appendicular Osteosarcoma and Consistent with a Persistent Embryonic Neural Crest Cell Origin

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Osteosarcoma (OS) is the most common primary tumor of bone and the third most common malignancy in adolescents. OS accounts for approximately 40–60% of all primary malignant tumors of bone. Approximately 6–13% of OS tumors occur in the craniofacial skeleton. In patients with craniofacial osteosarcoma, the jaw is the primary site: 55% of tumors occur in the mandible and 31% occur in the maxilla. Chondroblastic osteosarcoma is the predominant histological phenotype of craniofacial osteosarcoma. Based on phenotypic differences including median age of onset, degree of cellular atypia, frequency of local versus distant recurrence and time until metastasis, there is strong evidence that primary osteosarcoma of the appendicular skeleton and primary osteosarcoma of the craniofacial skeleton represent separate and distinct diseases.

Primary craniofacial OS tumors arise in the parts of the skull that are derived from the neural crest. The neural crest is a multipotent population of cells that arises at the neural plate border in the vertebrate embryo. The neural crest contributes to numerous tissues throughout the body including progenitor tooth mesenchymal cells, osteoblasts and chondroblasts of the craniofacial skeleton. Neural crest stem cells have been found to persist into adulthood as a population of dormant multipotent stem cells with a high capacity for self-renewal and an extraordinary degree of plasticity. Indeed, their plasticity and capacity for self-renewal is surpassed only by pluripotent embryonic stem cells.

A comparison of the expression data from primary craniofacial OS with appendicular OS revealed that they had distinct expression patterns and that the craniofacial OS expression pattern had a high degree of similarity to expression patterns of neural crest stem cells as well as other tumors derived from neural crest cells suggesting that primary craniofacial OS tumors arise from the skeletogenic neural crest stem cells. Our data are thus consistent with a model in which primary craniofacial OS tumors represent a malignant transformation of the persistent neural crest-derived stem cells resident in the craniofacial skeleton and that these malignantly transformed stem cells may also function as cancer stem cells in the craniofacial OS tumors. Craniofacial OS tumors thus represent an opportunity to analyze a tumor that arises from embryonic rests of neural crest stem cells.

S24

Redefining the Prostate Cancer Stem Cell

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In order to characterize cancer stem cells (CSC) researchers have co-opted the principles of hematopoietic stem cells (HSC). While many facets of HSC are reflected in CSCs, there is not always a clear correlation. To better understand biology of prostate CSC, we have explored various aspects of HSC and how they are reflected in CSC. We identified that some markers of many normal stem cells and cancer stem cells such as aldehyde dehydrogenase (ALDH) do not appear to be a clear CSC in PCa, although it does indicate tumor aggression. In contrast, CXCR1, the IL-8 receptor, shown to identify breast CSCs also identified prostate CSCs. Asymmetric replication is the property in which the stem cell divides to create an identical copy of the original parental stem cell (self-renewal) and a daughter cell that will differentiate along a non-stem cell path. However, we have found that prostate CSCs, originally derived from the same parental CSC, when isolated from different tissue sites (e.g, bone versus lung versus liver) gain different metastatic properties indicating that in vivo selection changes the properties of prostate CSC. Chemotherapeutics target the bulk of the non-CSC tumor component; whereas, the remaining CSC form a chemoresistant reservoir. We have found that the bone microenvironment is chemoprotective for prostate cancer cells. It appears that the proportion of prostate CSC is larger in the bone microenvironment compared to soft tissue sites prior to chemotherapy which is consistent with the observed chemoprotective bone microenvironment. Murine models are often used to identify the presence of CSC phenotype. However, results can be highly dependent on the immune status of the model used. We have identified that in highly immunocompromised mice, that some markers would identify a CSC phenotype; whereas, they would not have the CSC phenotype in more immune intact mice. In summary, the identity and properties of the prostate CSC is constantly in flux and being revised. Part of the challenge is the definitions and models used to assess for CSC properties. There are similarities between CSC and normal stem cells, but clearly there are unique properties of CSC. Defining these properties will assist towards enhancing cancer therapies.

PLENARY 4: PLASTICITY

S25

Mammary Gland Cell Fate Determinants as Regulators of Breast Cancer Metastasis

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Increasing evidence suggests that the normal genetic programs underlying various developmental processes can often be usurped in pathological conditions such as cancer metastasis. Genes that play crucial role in regulating adult stem cell renewal and differentiation into different lineages, have been recognized as important regulators of cancer progression and metastasis. We found that ΔNp63, a master regulator of mammary gland stem cell, is frequently overexpressed in basal-like

breast cancer, and control stem cell activities in normal mammary gland and cancer stem cells in malignant tissues. Another transcriptional factor, Elf5, a key regulator of alveologenesis in the mammary gland, suppresses epithelial-mesenchymal transition in both mammary gland development and metastasis by direct transcriptional repression of Snail2/Slug, a master regulator of mammary stem cells and a known inducer of EMT. These findings establish a paradigm that cell lineage regulators during normal mammary gland development can serve as important regulator of cellular plasticity and metastasis in breast cancer.

S26 Abstract presentation declined.
Identification of Novel Tumor Initiating genes, RPL39 and MLF2 that Mediate Lung Metastasis Through Nitric Oxide Signaling

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Development of resistance to a wide variety of drugs and metastatic spread of cancer cells to distant organs pose major challenge in successful treatment of breast cancer. Virtually all the annual breast cancer related deaths in the U.S. can be said to have occurred because chemotherapy failed. Therefore, understanding the molecular factors responsible for drug resistance and metastasis of especially triple negative breast cancer is urgently needed to develop novel therapeutic approaches. We previously determined breast cancer stem cells (BCSCs) are intrinsically resistant to treatment. We generated a 477-gene tumorigenic gene expression signature from patient BCSCs. Using shRNA to knockdown all genes from the tumorigenic signature in a high throughput mammosphere formation efficiency screen for stem cell self-renewal, we determined the top candidate genes. Of these, two targets, RPL39 and MLF2, were tested further in patient-derived breast tumorgrafts using siRNA. Our stringent screens identified RPL39 and MLF2 as the top two candidates that affect BCSC self-renewal. Selective siRNA knockdown of RPL39 and MLF2 in patient-derived breast tumorgrafts showed reduced tumor volume and lung metastases with a concomitant decrease in BCSC markers. Thus, targeting BCSCs in combination with chemotherapy should eliminate the heterogeneous populations within a tumor. Additionally, next generation RNA-seq confirmed mutations in RPL39 and MLF2 in 50% of lung metastases from breast cancer patients. Allele specific PCR confirmed damaging mutations in RPL39 and MLF2. Gain-of-function of these mutations was demonstrated by increase in proliferation, invasion, and self-renewal assays. *In vitro* and *in vivo* siRNA knockdown of RPL39 and MLF2 showed decrease in nitric oxide synthase, suggesting these genes are driven by nitric oxide signaling. Our findings enhance the understanding of treatment-resistant BCSCs, the mutations that cause metastases and also lay foundation for developing new therapies for such cancers with poor prognosis.

S27
Development of a Circulating Tumor Cell Capture Method Based on Ob-Cadherin Expression in Metastatic Prostate Cancer

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Epithelial tumor cells can reversibly transition their phenotype into mesenchymal stem-like cells and vice versa, a process known as epithelial plasticity. We hypothesize that during the process of metastasis, circulating tumor cells (CTCs) lose their epithelial phenotype and acquire a mesenchymal phenotype that may not be sufficiently captured by existing epithelial-based CTC technologies. Prostate cancer cells are known to exhibit properties of osteomimicry and up-regulate molecules important in bone homing and formation. We report here on the development of a novel CTC capture method, based on the biology of epithelial plasticity, which isolates cells based on osteoblast (OB)-cadherin cell surface expression. Cells are then characterized using beta-catenin, rather than cytokeratin expression, and CD45 in order to detect non-leukocytes with loss of cytokeratin. Using this mesenchymal-based assay, OB-cadherin cellular events are detectable in men with metastatic prostate cancer and are less common in healthy volunteers. This method may complement existing epithelial-based methods and may be particularly useful in patients with bone metastases.

S28
Activation of the WNT Pathway Through use of AR79, a Glycogen Synthase Kinase 3β Inhibitor, Promotes Prostate Cancer Growth in Soft Tissue and Bone

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Due to its bone anabolic activity, multiple methods to increase Wnt activity, such as inhibitors of dickkopf-1 and sclerostin, are being clinically explored for bone-related diseases. Glycogen synthase kinase (GSK3β) inhibits Wnt signaling through inducing β-catenin degradation. Therefore, AR79, an inhibitor of GSK3β, is being evaluated as a bone anabolic agent. However, a concern of Wnt activation is its potential ability to promote tumor growth. Accordingly, the goal of this study was to determine if AR79 impacted progression of prostate cancer (PCa) tumors in soft tissue and bone. PCa tumors were established in subcutaneous and bone sites of mice followed by AR79 administration. Tumor growth, activation of β-catenin, cell proliferation (Ki67 expression) and apoptotic activity (caspase 3 activity) were measured. Additionally, PCa and osteoblast cells lines were treated with AR79 and β-catenin status, proliferation and proportion of the ALDH+CD133+ stem-like cells was determined. AR79 promoted PCa growth, decreased phospho-β-catenin expression and increased total and nuclear β-catenin expression in tumors and increased tumor-induced bone remodeling. Additionally, it decreased caspase 3 and increased Ki67 expression. In addition, AR79

increased bone formation in normal mouse tibiae. AR79 inhibited β-catenin phosphorylation, increased nuclear β-catenin accumulation in PCa and osteoblast cell lines and increased proliferation of PCa cells *in vitro*. Furthermore, AR79 increased the ALDH+CD133+ cancer stem cell-like proportion of the PCa cell lines. We conclude that AR79, while being bone anabolic, promotes PCa cell growth through Wnt pathway activation. This suggests caution should be used with the various Wnt pathway-activating agents, such as sclerostin antibody, being currently evaluated for therapy of bone disease.

S29

Twist1 Expression in Breast Cancer Promotes Bone Metastasis Formation

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Background: The basic helix-loop-helix transcription factor Twist1 initially identified as a major regulator of tissue organization in early embryogenesis is aberrantly reactivated in human cancer. Twist1 expression in primary breast tumors is associated with disease aggressiveness and poor survival mainly by conferring growth advantage to tumor cells and by facilitating their intravasation in the circulation and their dissemination to the lungs. During these processes Twist1 promotes epithelial-to-mesenchymal transition of circulating and disseminating tumor cells (DTC). In this respect, Twist1 is expressed in breast cancer DTC that persist in the bone marrow after chemotherapy. However its involvement in breast cancer bone metastasis formation is unknown.
Results: To address this question, we chose the human osteotropic MDA-MB-231/B02 breast cancer cell line which has a mesenchymal phenotype, but does not express Twist1. Twist1 ectopic expression in B02 cells substantially increased the extent of osteolytic lesions in animals, being 50% larger than that of animals bearing mock-transfected tumors, as determined by radiography. This difference was accompanied with a sharp reduction of the bone volume (indicating a higher bone destruction) and a 2-fold increase in the tumor volume compared with mice bearing mock-transfected tumors, as determined by histomorphometry. Osteoclast activity was not alterated *in vivo*, neither *in vitro* by Twist1 expression pointing to its specific role in regulating skeletal tumor outgrowth. Additionally, the repression of Twist1 expression in B02 breast cancer cells in the presence of doxycycline (dox) abolished the stimulatory effect of tumor-derived Twist1 on bone metastasis formation *in vivo*. Importantly, examination of the bone marrow from untreated and dox-treated animals on day 7 after tumor cell inoculation, at which time there is no evidence of radiographic osteolytic lesions, revealed that the number of DTC colonies in the bone marrow from untreated mice was dramatically increased compared with that of dox-fed animals. *In vitro*, Twist1 expression promoted tumor cell invasion and enhanced microRNA-10b expression, a pro-invasive factor, but was dispensable for growth of breast cancer cells.
Conclusion: Overall, these results establish that Twist1 in breast cancer is important for the engraftment of tumor cells to the bone marrow, facilitating the subsequent formation of osteolytic bone metastases.

S30

Integrin Beta-3 and TGF-Beta Receptor Type II Cross-Talk Induces Osteolysis in Bone Metastatic Breast Cancer Cells

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The importance of the microenvironment in the regulation of tumor cell behavior and bone turnover is increasingly recognized. While many aspects of this environment have been explored, we have shown that the physical rigidity of bone can regulate gene expression of PTHrP and Gli2 in bone metastatic cells. Since studies in soft tissue tumors have demonstrated that αvβ3 integrin mediates tumor response to rigidity, we hypothesized that the tumor response to bone rigidity was also integrin-dependent. It was found that this response was reliant on the cross-talk of integrins and TGF-β Receptor type II (RII). Limiting integrins αvβ3, by utilizing a dominant negative Rho associated protein kinase (ROCK) construct, blocked the rigidity-mediated increase in PTHrP expression, while a dominant active construct increased PTHrP expression. Additionally, direct αvβ3 inhibition by LM609, a αvβ3 inhibitory antibody, reduced the expression of PTHrP and Gli2 by 2.8 and 10-fold respectively ($p \leq .01$), and reduced PTHrP expression in the ROCK dominant active cells by 9-fold ($p \leq .005$). To more precisely determine the importance of integrin β3, we stably transfected MDA-231 cells with β3 shRNA and measured the cells ability to respond to rigidity using 2D polyurethane (PUR) films. Integrin β3 knock-down reduced PTHrP expression by 54-fold in cells grown on rigid PUR films ($p \leq .001$), but not on compliant films. Since we have previously shown that RII is required for the mechanotransduction response, we investigated whether integrin and TGF-β pathways interacted, finding that β3 and RII co-localized by immunoprecipitation, suggesting a physical interaction between the receptors. Finally, since p38MAPK is known to play an important role in mechanotransduction and the MAPK inhibitor SB202190 reduced PTHrP expression on rigid films by 4-fold ($p \leq .01$), we investigated whether rigidity alone, in the absence of exogenous TGF-β, could upregulate p38 MAPK phosphorylation. It was found that p38 MAPK phosphorylation was increased on rigid films by Western blot, while no changes were observed in the expression of non-phosphorylated p38MAPK. No increase in p38 MAPK phosphorylation was observed in the ROCK dominant negative cells. Taken together these data indicate that integrin β3 is required for tumor cells to sense the rigidity of bone and cross-talks with the TGF-β pathway, suggesting that integrin inhibition may be a valuable target for inhibiting osteolytic tumor establishment in bone.

S31
Rank Expression on Circulating Tumor Cells (CTCs) in Metastatic Breast Cancer (MBC): Clinical Implications

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Background: It has recently been demonstrated that many solid tumors express RANK both in primary sites and in metastatic bone. Moreover RANK expression on cancer cells is associated with bone homing mechanism in early bone metastasis formation. The CTCs represent the “liquid” phase of metastasis and are considered an appealing biomarker for investigating the phenotype and the role of these migrating tumor cells. These evidence allow us to consider the RANK presence on the CTCs surface and its modulation during denosumab therapy as a potential predictor of treatment response in MBC or as a prognostic factor of skeletal outcome.
Experimental Design: An automated sample preparation and analysis system for enumerating CTCs (CellSearch) was integrated with a specific mAb for detecting RANK-positive CTCs. CTCs from blood were analyzed at baseline and at day2, 7, 14, 28 after the first denosumab administration. Time-to-first-SRE was estimated by Kaplan-Meier analysis and the p-value calculated by log-rank test according to CTC and RANK-positive CTC counts at each time point of the blood draw calendar. A companion algorithm (ΔAUC) was developed to express the difference between RANK-positive and RANK-negative CTC concentration-Time Area (AUC), as calculated according to the following formula:
ΔAUC= RANK-positiveCTC AUC - RANK-negativeCTC AUC
Results: 34 bone MBC patients (age 32–89) are currently included. At the first blood draw 5 MBC were CTC positive and RANK-negative and 16 out of 34 MBC were CTC positive and RANK-positive (RANK-positive CTCs from 3.6% to 100%). The detection of RANK-positive CTCs at day2 and day7 were associated with significant longer Time-to-first-SRE (p=0,008 and p=0,019 respectively). Categorizing the patients for positive ΔAUC value (that is expression of persistence of RANK-positive CTCs over the first 28 days of treatment) vs negative or 0 ΔAUC (that is expression of RANK-negative CTCs over the same period or balanced numbers of RANK-positive and RANK-negative CTCs) a median of 6 months of delay in terms of Time-to-first-SRE was observed in the positive ΔAUC group of MBC that retain RANK-positive CTCs.
Conclusion: For the first time we demonstrated that RANK expression is detectable by immunofluorescence on CTCs in MBC. The RANK-integrated test has potential for monitoring dynamic changes, in addition to CTC count, to evaluate SRE risk in patients under denosumab therapy. Accrual is ongoing, updated data will be presented at the meeting.

PLENARY 5: CHEMORESISTANCE

S32
Molecular Characterization of Chemoresistance in Breast Cancer

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Resistance of breast cancer to chemotherapy can be divided into 3 types: 1) intrinsic resistance due to molecular characteristics present before chemotherapy is initiated; 2) adaptive resistance due to molecular changes soon after chemotherapy is initiated; and 3) acquired resistance, indicated by residual or recurrent disease after prolonged chemotherapy exposure. Most likely all 3 types of resistance contribute when patients with breast cancer do not achieve a pathologic complete response (pCR) to preoperative chemotherapy.
Understanding the 3 types of resistance will lead to development of novel targeted therapy that may be incorporated into neoadjuvant chemotherapy. There are many exciting targeted therapies on the horizon (e.g., inhibitors of PI3K, ALK, MET, MEK, mTOR, Akt, ALK, IGFR), but which targets are related to the different types of chemoresistance and how best to incorporate these exciting new targeted therapies into current multidisciplinary breast cancer treatment to overcome chemoresistance remain unanswered.
We can identify intrinsic molecular characteristics that determine pCR rates after neoadjuvant chemotherapy. For example, by gene profiling, Lehmann et al. (J Clin Invest 121:2750–2767, 2011) found that triple-negative breast cancer (TNBC) can be classified into 6 clusters—basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor—plus an unstable cluster. In our dataset, the BL1 subtype had the highest pCR rate (52%); the BL2 and luminal androgen receptor subtypes had the lowest (0% and 10%, respectively). TNBC subtype and pCR status were significantly associated (P=0.044), and TNBC subtype was an independent predictor of pCR status (P=0.022) by a likelihood ratio test. We speculate that luminal androgen receptor subtype is driven by an androgen receptor pathway. For BL2, the drive could be due to EGFR or MAPK pathways. These findings can help investigators develop innovative, personalized medicine strategies for patients with TNBC.
However, although we can identify molecular characteristics underlying intrinsic chemoresistance, for adaptive and acquired resistance, data on clinically relevant molecular characteristics are very limited.

S33
Exploring the Role of the Extracellular Matrix in Tumor Progression

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The extracellular matrix (ECM) is a complex meshwork of proteins providing architectural support for cells. In addition, ECM proteins bind and present to cells growth factors, thus

providing both biophysical and biochemical cues that are major regulators of cell behavior.

The ECM exerts many roles during tumor progression: it supports proliferation and survival of tumor cells; it participates by its nature and/or architecture to the formation of a pro-invasive environment; and finally, it contributes to the invasion of distant sites by providing a fertile soil that will support tumor cell seeding and growth. Classical pathology has used excessive ECM deposition (desmoplasia) as a marker of tumors with poor prognosis long before the complexity of the ECM was even deciphered. And yet, very few ECM genes are mutated in tumors. Recent studies have also suggested that the ECM could act as a barrier to drug delivery and confers chemo-resistance to tumors.

To date, no studies have systematically addressed the question of tumor ECM composition *in vivo*. We have developed an innovative proteomic method to characterize the composition of tumor ECMs. Our approach allows the comprehensive identification of 100+ ECM proteins from any given tumor or tissue sample (Naba et al., 2012). Using human melanoma and mammary carcinoma xenograft models, we have demonstrated that tumors of different metastatic ability differ in both tumor- and stroma-derived ECM components (Naba et al., 2012 and in preparation). Moreover, we showed that several tumor-derived ECM proteins, characteristic of highly metastatic tumors, played a causal role in metastatic dissemination (Naba et al., in preparation). Furthermore, using colorectal cancer patient samples, we have demonstrated that the composition of the ECM of metastases differs from the primary tumor ECM in characteristic ways.

We now wish to evaluate whether we can predict, given its ECM if a tumor 1) will metastasize and, if so, to which organ and 2) will respond to treatment or not. We anticipate that the characterization of ECM signatures of primary tumors and their metastases will allow us to identify novel biomarkers that will serve as prognostic and diagnostic tools for cancer patients and possibly as future targets for therapeutic intervention.

S34

Pim Inhibition Preferentially Induces Myeloma Cell Death and Restores Drug Sensitivity in BCRP1-Expressing Myeloma Cells in Acidic Bone Lesions in Myeloma

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Bone marrow microenvironment in acidic bone lesions in myeloma (MM) confers drug resistance in MM cells. We recently demonstrated that Pim-2 kinase is up-regulated in both MM cells and bone marrow stromal cells (BMSCs) through their mutual interaction in MM bone lesions, and plays a pivotal role in tumor survival and bone loss in MM. The present study was undertaken to clarify the role of Pim inhibition in MM cell drug resistance in acidic bone lesions in MM. Acidic conditions as well as BMSCs enhanced Pim-2 expression and the phosphorylation of 4EBP1, a regulator of protein translation known as the substrate of Pim kinases, in MM cells. Although BMSCs enhance the survival of MM cells, acidic conditions rather enhanced the cytotoxic effects of the Pim inhibitor SMI-16a on MM cells even in cocultures with BMSCs. Furthermore, treatment with SMI-16a resumed and potentiated the cytotoxic activity of doxorubicin against MM cells blunted in acidic conditions. Cocultures with BMSCs or acidic conditions significantly reduced the intracellular retention of auto-fluorescence emitting mitoxantrone and doxorubicin, substrates of the ABC transporter BCRP1, in BCRP1-expressing RPMI8226 cells as determined in flow cytometry. However, treatment with the Pim inhibitor SMI-16a substantially restored the intracellular levels of these drugs, which were more prominent in cocultures with BMSCs in acidic conditions. Consistently, the Pim inhibition minimized the size of a "side population", a highly drug-resistant fraction with enhanced BCRP1 activity, in RPMI8226 and KMS11 MM cells in acidic conditions. Interestingly, the Pim inhibition reduced BCRP1 expression at protein levels in MM cells in acidic conditions, which may be in part due to the suppression of protein translation by 4EBP1. Taken together, Pim-2 may become an important therapeutic target of MM cells which preferentially gain drug resistance in acidic bone lesions. Combinations of anti-cancer agents with Pim inhibitors warrant further study to overcome drug resistance in MM cells in bone lesions.

S35

Differential Anti-Tumour Effects of Zoledronic Acid in Breast Cancer According to ER Status and Levels of Female Hormones

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Background: Breast cancer clinical trials, pre- (neo-adjuvant) and post-surgery (adjuvant), have demonstrated enhanced anti-tumour efficacy of Zoledronic acid (Zol) in patients with oestrogen receptor (ER) negative disease (neo-adjuvant), and low levels of female hormones such as inhibin (adjuvant). The tumour suppressor activin is inhibited by both inhibin, and its binding protein follistatin. We hypothesize that Zol can differentially affect tumour activin/follistatin interactions according to ER status and presence of inhibin.

Objectives: 1. Determine effect of follistatin and activin on breast cancer cell proliferation

2. Establish effect of Zol and inhibin on breast cancer cell secretion of follistatin.

3. Compare serum levels of follistatin in breast cancer patients treated +/− neo-adjuvant Zol.

Methods;*In vitro*;Effect of follistatin and activin on proliferation of ER-negative and ER-positive breast cancer cell lines was assessed using an MTS assay, following treatment with follistatin (240–2400pg/ml) and/or activin (60–6000pg/ml) for 1, 3 and 5 days. Effect of Zol on secretion of follistatin into supernatant, was evaluated in both cell lines with 25 µM Zol for 48 hours +/−10 ng/ml inhibin A.

Clinical samples; Serum follistatin (baseline, day 5 and day 21) was retrospectively analysed according to ER status (ER-positive n=28, ER-negative n=9) from patients with operable or locally advanced breast cancer who received neoadjuvant FE100C chemotherapy (CT) +/− Zol (4 mg i.v.) with first cycle.

Results: *In vitro*: Follistatin stimulated tumour cell proliferation, negating the tumour suppressive action of activin in both cell lines. Zol decreased secretion of follistatin from ER-negative cells (~57.2% of control) but not ER-positive cells. The presence of inhibin in the medium diminished the decline in follistatin secretion seen with Zol (~30.8% of control).
Clinical samples: Percentage change from baseline in serum follistatin was significantly different at day 5 in ER-negative patients only (median % change; CT+Zol –38.9% ± 10.62, CT +5.33% ±7.34, p=0.0159 Mann Whitney).
Conclusions: The anti-tumour effect of Zol in ER-negative breast cancer cells may be linked to its ability to decrease follistatin secretion, and inhibin diminishes this effect of Zol. The ER dependent effect of Zol on follistatin secretion *in vitro* is mirrored in neo-adjuvant clinical serum samples, suggesting Zol's effect on follistatin is influenced by ER status.

S36

Contribution of the Marrow Microenvironment to Chemoresistance in Myeloma

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The marrow microenvironment plays a key role in chemoresistance of Multiple Myeloma (MM). Multiple components of the microenvironment contribute to this process. Marrow stromal cells (BMSCs) play a major role in the development of resistance of MM cells to chemotherapy and novel agents. BMSCs express increased levels of IL-6, VEGF and IGF when MM cells bind BMSCs. XBP1s is induced in the BMSCs and XBP1s overexpression in BMSCs increases expression of VCAM-1 and IL-6, enhancing BMSC support of MM cell growth *in vitro* and *in vivo*. Knock-down of XBP1 in BMSCs greatly compromises increased VCAM-1 protein expression and IL-6 secretion and reversed their enhanced support of MM-cell growth, suggesting an important role for XBP1s in the chemoresistance of myeloma cells. Further, BMSCs secretion of IL-6 and VEGF, significantly decreased the sensitivity of MM cells to bortezomib. This resulted from BMSC inhibiting the up-regulation of miRNA-15a in MM cells treated with bortezomib. The extracellular matrix (ECM) also contributes to chemoresistance in MM. Adhesive interactions between MM cells and hyaluronan and fibronectin in bone marrow ECM increase IL-6 production and enhance STAT3 and Notch signaling in MM cells, thereby enhancing their survival. Recently, marrow macrophages have also been implicated in drug resistance in MM. Macrophage infiltration is increased in the marrow of MM patients. PSGL-1/selectin and ICAM-1/CD18 interactions that activate ERK1/2, Src and the c-myc pathways and suppress caspase activation in MM cells by chemotherapy drugs appear to be responsible for these effects. Several mechanisms have also been identified that are involved in lenalidomide resistance. Cereblon is a direct target for IMids who's down regulation results in IMid resistance. IRF4, which is required for MM cell survival, is a downstream target of Cereblon. Activation of the Wnt/b-catenin signaling pathway has also been associated with lenalidomide resistance through its up-regulation of CD44, a hyaluron binding protein that enhances adhesion mediated drug resistance. Thus, multiple mechanisms contribute to drug resistance induced by the marrow microenvironment in MM and provide potential therapeutic targets to reverse or prevent development of drug resistance.

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Poster Abstracts

POSTER VIEWING (ODD ABSTRACT NUMBERS)

P001

Uptake of Nitrogen Containing Bisphosphonate by Breast and Prostate Cancer Cell Lines In Vitro

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Bisphosphonates are widely used to treat osteoporosis and problems related to cancer metastasis in bone. Bisphosphonates are classified in two main categories – nitrogen containing (N-BPs) and non-nitrogen containing ones. The mechanisms of BP uptake are incompletely characterized. In the current work we demonstrate that the main uptake pathway involved is dynamin dependent endocytosis. Further the dependence on clathrin varies between different cancer cell lines.
N-BPs inhibit farnesyl pyrophosphate synthase (FPPS) - enzyme of the mevalonate pathway. This leads to intracellular accumulation of the upstream metabolite isopentyl pyrophosphate (IPP) which can be further conjugated with adenosine-5'-triophosphate (ATP) by aminoacyl tRNA synthase.
MCF7, T47D and DU-145 cell lines were explored for Risedronate uptake. By using HPLC-MS we are able to detect the metabolites IPP and ApppI and to quantify them from *in vitro* samples. Cell viability as a percent of the control was used to demonstrate the N-BPs biological activity and estimate IC50 values. Inhibitors for different uptake mechanisms were used at effective concentrations with no or minimal cytotoxic effects to the cells.
Results: By using the various cellular uptake inhibitors we have established that the all the cells use the endocytotic, but not pinocytotic pathway for N-BP uptake. However, the exact endocytosis routes vary between the different cancer cell lines. N-BP internalization by T47D breast cancer cells is dynamin and clathrine dependent but MCF7 and DU-145 cells used clathrin independent but dynamin dependent endocytosis. Use of caveolin inhibitor methyl-β- cyclodextrin did not inhibit drug uptake, but surprisingly decrease cell viability probably due to the detectable increased production of toxic ApppI.
Discussion: MCF7, T47D and DU-145 are using different uptake pathways in internalizing N-BPs, but mainly dynamin-dependent. Interestingly MbCD increased the amount of IPP and ApppI and decreased the cell viability. Most probably this effect is counted to the intracellular cholesterol and MbCD complexion and subsequently mevalonate pathway activation. Activation of the pathway upstream from FPPS which is blocked by the N-BPs leads to higher accumulation of IPP and ApppI production.

P002

Randomised Phase II Placebo-Controlled Trial of Fulvestrant Plus Vandetanib in Postmenopausal Women with Bone Only or Bone Predominant, Hormone Receptor Positive Metastatic Breast Cancer (MBC). OCOG Zamboney Study

Mark Clemons[1], *Brandy Cochrane*[2], *Gregory Pond*[2], *Nadia Califaretti*[3], *Stephen Chia*[4], *Rebecca Dent*[5], *Xinni Song*[1], *Andre Robidoux*[6], *Sameer Parpia*[2], *David Warr*[7], *Kathleen Pritchard*[5,2], *Mark Levine*[2]
[1]Ottawa Hospital Cancer Centre, University of Ottawa, Ottawa, Ontario, Canada; [2]Ontario Clinical Oncology Group, McMaster University, Hamilton, Ontario, Canada; [3]Grand River Regional Cancer Centre, Kitchener, Ontario, Canada; [4]British Columbia Cancer Agency, Vancouver, British Columbia, Canada; [5]Sunnybrook Odette Cancer Centre, University of Toronto, Toronto, Ontario, Canada; [6]Centre Hospitalier de l'Université de Montréal, Montreal, Quebec, Canada; [7]The Princess Margaret Cancer Centre, Toronto, Ontario, Canada

Background: Biomarkers of bone turnover, including urine N-telopeptide (uNTx), reflect tumor-related bone breakdown and have been used as surrogate measures of response to systemic therapy in trials. Vascular endothelial growth factor (VEGF) levels correlate with extent of bone metastases. We assessed whether vandetanib, an inhibitor of VEGF, epidermal growth factor receptor and RET signalling, improved uNTx response when added to fulvestrant (F) in breast cancer patients with bone metastases.
Patients and Methods: Postmenopausal patients with bone only, or bone predominant, hormone receptor positive metastatic breast cancer (MBC) were randomised to F (500 mg IM day 1, 14, 28, then monthly) with either vandetanib (100 mg PO OD) (FV) or placebo (FP). The primary objective was uNTx response (>30% reduction from baseline). uNTx was collected at baseline, weekly to wk 4, at wk 12 and then every 12 wks. Secondary objectives included PFS, OS, RECIST response, pain and toxicity.
Results: 61 patients were allocated to FV and 68 to FP. Median age was 59, 18% had received 1 prior chemotherapy regimen and 74% prior endocrine therapy for MBC. uNTx response (n=127 pts) was 66% for FV vs. 54% for FP (p=0.21) (Figure 1). No difference was detected between groups for median PFS; 5.8 months for FV vs. 4.8 months for FP, HR=0.95 (95% CI: 0.65 to 1.38). 18 patients died in FV arm vs. 23 in the FP arm, HR=0.73 (95% CI: 0.39 to 1.37). For 62 patients with measurable disease, as per RECIST, clinical benefit rates were 41% and 43%, respectively, p=0.47. Serious adverse events were similar, 3.3% for FV vs. 5.9% for FP. Elevated baseline uNTx (>65 nM BCE/mmol Cr) was prognostic for PFS, HR=1.55 (95% CI: 1.04 to 2.30 and for mortality, HR= 2.48 (95% CI:

1.34 to 4.48). In an exploratory analysis, baseline uNTx was predictive of responsiveness to FV for PFS (interaction p-value=0.035) with HR=0.58 when uNTx >65 favouring FV but HR=1.31 when uNTx <65.
Conclusion: The addition of vandetanib to fulvestrant did not improve biomarker response, PFS or OS compared to fulvestrant alone in patients with bone metastases. Exploratory analyses confirmed that baseline bone turnover markers are prognostic for PFS and OS.

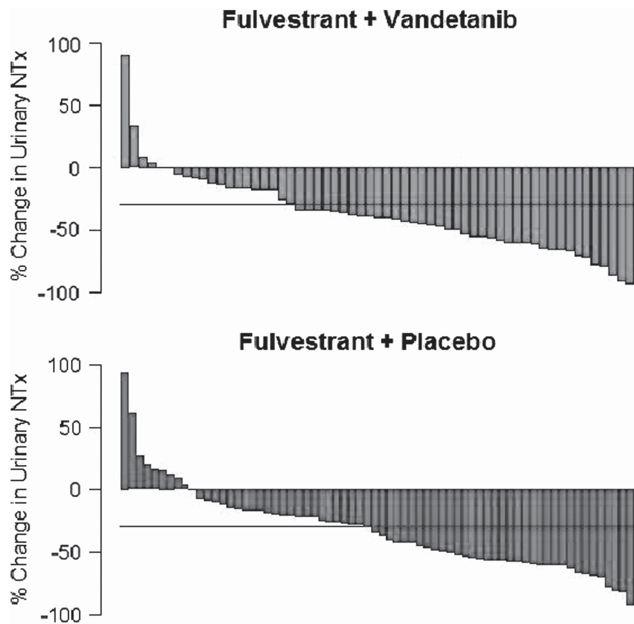

Figure 1. Waterfall Plot of % Change in Urine NTx from Baseline.

P003

Correlation of Baseline Biomarker Levels with Clinical Outcomes, Bone Turnover Markers and Response to Fulvestrant with and without Vandetanib in Patients with Bone Predominant Metastatic Breast Cancer. An OCOG Zamboney Sub-Study

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[1]Program for Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada; [2]University of Ottawa, Ottawa, Ontario, Canada; [3]McMaster University and Ontario Clinical Oncology Group, Hamilton, Ontario, Canada; [4]BC Cancer Agency, Vancouver, British Columbia, Canada

Background: Bone metastases (BM) are common in women with breast cancer (BCa) and often result in skeletal related events (SREs). The angiogenic factor VEGF regulates osteoclast activity and is associated with more extensive bone metastases and SRE risk in metastatic BCa. Blockade of VEGF signaling could thus be a therapeutic strategy for inhibiting BM, and possibly prolonging overall (OS) and progression-free survival (PFS). The Zamboney trial was a randomized placebo-controlled study designed to assess whether patients with bone predominant metastatic BCa benefited from addition of the VEGFR-targeting agent, vandetanib, to endocrine therapy with fulvestrant. As a companion study, evaluation of biomarkers and their potential association with response to vandetanib or SRE risk was performed.
Methods: Baseline overnight fasted serum from enrolled patients was analyzed for levels of various biomarkers including; VEGF-A, sVEGFR2, sVEGFR3, TGF-β1 and activinA by ELISA. Spearman correlation coefficients and Wilcoxon rank sum tests were used to investigate potential relationships between biomarker values and baseline clinical parameters. Prognostic and predictive ability of each marker was investigated using Cox proportional hazards regression with adjustments for treatment and baseline strata of serum CTx (<400 vs ≥400 ng/L).
Results: Of 129 enrolled patients, serum was available for analysis in 101; 51 in vandetanib and 50 in placebo arm. Mean age was 59.8 yrs. Median duration of prior bisphosphonate (BP) use was 12.9 m for 39 patients, 41 patients were on BP at time of enrollment and BP use was unknown for 2. Clinical characteristics were not significantly different between patients with or without serum biomarker data and serum markers were similar for patients by treatment arm. Baseline sVEGFR2 was prognostic for OS (HR=0.77, 95% CI=0.61 to 0.96, p=0.020), but was not significant for PFS (HR=0.90, 95% CI=0.80 to 1.01, p=0.085) nor time to first SRE (HR=0.82, 95% CI=0.66 to 1.02, p=0.079). Baseline CTx was not prognostic for time to first SRE (HR=1.36, 95% CI=0.94–1.96, p=0.099). No other marker showed any significant prognostic or predictive ability with any measured outcome.
Conclusions: In this clinical trial, sVEGFR2 appeared prognostic for OS, hence validation of sVEGFR2 should be conducted. The lack of correlation of baseline CTx with either OS or time to SREs is likely reflective of 41% of patients already receiving BP at the time of study entry.

P004

Pim Inhibition Preferentially Induces Myeloma Cell Death and Restores Drug Sensitivity in BCRP1-Expressing Myeloma Cells in Acidic Bone Lesions in Myeloma

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Bone marrow microenvironment in acidic bone lesions in myeloma (MM) confers drug resistance in MM cells. We recently demonstrated that Pim-2 kinase is up-regulated in both MM cells and bone marrow stromal cells (BMSCs) through their mutual interaction in MM bone lesions, and plays a pivotal role in tumor survival and bone loss in MM. The present study was undertaken to clarify the role of Pim inhibition in MM cell drug resistance in acidic bone lesions in MM. Acidic conditions as well as BMSCs enhanced Pim-2 expression and the phosphorylation of 4EBP1, a regulator of protein translation known as the substrate of Pim kinases, in MM cells. Although BMSCs enhance the survival of MM cells, acidic conditions rather enhanced the cytotoxic effects of the Pim inhibitor SMI-16a on MM cells even in cocultures with BMSCs. Furthermore, treatment with SMI-16a resumed and potentiated the cytotoxic activity of doxorubicin against MM cells blunted in acidic conditions. Cocultures with BMSCs or acidic conditions

significantly reduced the intracellular retention of auto-fluorescence emitting mitoxantrone and doxorubicin, substrates of the ABC transporter BCRP1, in BCRP1-expressing RPMI8226 cells as determined in flow cytometry. However, treatment with the Pim inhibitor SMI-16a substantially restored the intracellular levels of these drugs, which were more prominent in cocultures with BMSCs in acidic conditions. Consistently, the Pim inhibition minimized the size of a "side population", a highly drug-resistant fraction with enhanced BCRP1 activity, in RPMI8226 and KMS11 MM cells in acidic conditions. Interestingly, the Pim inhibition reduced BCRP1 expression at protein levels in MM cells in acidic conditions, which may be in part due to the suppression of protein translation by 4EBP1. Taken together, Pim-2 may become an important therapeutic target of MM cells which preferentially gain drug resistance in acidic bone lesions. Combinations of anti-cancer agents with Pim inhibitors warrant further study to overcome drug resistance in MM cells in bone lesions.

P005 Abstract presentation declined.
MicroRNA-27A Controls Osteoblast Growth

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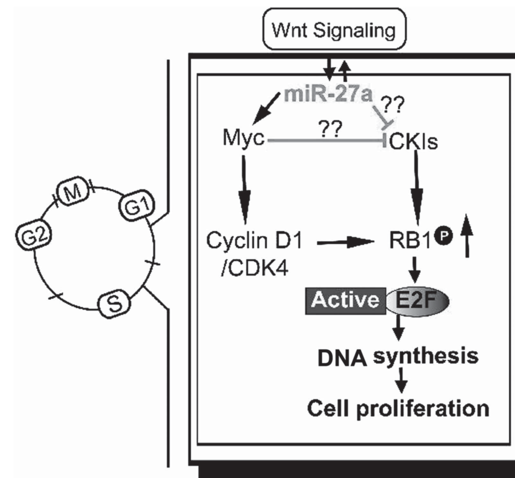
MicroRNA (miR) are evolutionarily conserved non-coding RNA of ~22 nucleotides that regulate gene expression by facilitating translational repression and mRNA degradation. Recently, several groups reported that miR-27a is a key miRNA in controlling cell proliferation and several forms of cancer pathogenesis. Wnt-β-catenin cellular signaling is also equally important in regulating numerous biological processes, including cellular growth and cancer. However, the mechanism(s) of miR-27a regulation for osteoblast growth is not clear. In this study we observed that Wnt signaling directly upregulates miR-27a expression and over expression of miR-27a in MC3T3-E1 also enhanced the expression of Wnt activators TCF and LEF but suppressive for GSK3β, DKK1 and APC expression, the potential antagonists of Wnt pathway. These findings reinforce a positive proliferative ambience involving both miR-27a and Wnt signaling. MiR-27a gain of function in pre osteoblast MC3T3-E1 cells increases RB1 phosphorylation that promotes G1-S cell cycle transition and activates cell growth while miR-27a loss of function decreases RB1 phosphorylation leading to cell cycle arrest. Immunoblot analysis of miR-27a stable MC3T3 cells showed enhanced expression for CyclinD1 and c-Myc. Increased association of histone H3K4 (activating) and decreased recruitment H3K27 (repressive) on c-Myc promoter, suggesting a significant enhancement of c-Myc gene transcription in miR-27 overexpressed cells. Cell cycle synchronization assay with nocodazole block and release in miR-27a stable cell lines revealed that c-Myc, Cyclin D1 expression peaked around 6hr after release. Western blot analysis with anti phospho RB1 antibody indicates a sharp increase at 6hrs after release. Therefore, our findings suggest that miR-27a gain of function induces the cell to progress from G1 to S phase by CyclinD1 induced Rb1 phosphorylation where c-Myc is maintaining increased level CyclinD1 expression. Taken together, our results establish a key role for Wnt signaling-miR-27a regulatory circuit to promote osteoblast cell cycle during proliferation by RB1 phosphorylation and c-Myc amplification. A feed forward and feedback loop between Wnt and activated miR-27a most probably inducing c-Myc and CyclinD1 expression to promote Rb1 phosphorylation and releasing E2F from Rb1 sequestration that constantly propels and maintains the cells in proliferation.



Regulation of osteoblast growth by miR-27a.

P006
Osteoblasts and Breast Cancer Cells Communicate via Exosomes and Gap Junctions to Create a Metastatic Niche in Bone Metastatic Breast Cancer

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Breast cancer preferentially metastasizes to the bone, where the five-year relative survival rate falls from 90% to <10%. While the precise mechanism underlying preferential metastasis is unknown, the bone likely provides a hospitable environment that both attracts breast cancer cells and allows them to colonize and grow. Besides affecting osteoblast and osteoclast properties, we have evidence that metastatic breast cancer cells create a unique bone niche by orchestrating extensive crosstalk with osteoblasts that may be involved in creating a niche permissive for breast cancer cell dormancy.
MC3T3-E1 murine osteoblasts (OBs) were grown to confluence, then MDA-MB-231 human breast cancer cells (BCCs) added at a ratio of 1 BCC to 10 OBs. Co-cultures were either fixed and stained for Cx43 expression, or examined for gap junction intercellular communication (GJIC). Next, OBs and BCCs were grown to confluence. Culture supernatants were removed and processed through a series of ultra-centrifugation and concentration steps to purify for exosomes. Then, OBs were differentiated for 10 days, and incubated with conditioned medium (CM) from BCCs. Twenty-one days later, OB-derived protein expression was assessed. Finally, OBs were grown to 4 (growth) and 10 days (early differentiation) and incubated with CM from BCCs. qPCR was used to assay for changes in OB-derived cytokine expression.

Connexin 43 expression was localized between BCCs and OBs; GJIC was also found. Both OBs and BCCs expressed exosomes, which were visualized using a transmission electron microscope. In the presence of BCC CM, OB-derived expression of alpha-smooth muscle actin was decreased, however FAP and MMP13 expression increased. OB-derived cytokines were increased in the presence of BCC CM, and included MCP-1 (CCL2), LIX (CXCL5), LIF, and NFATc2.
These data suggest that suggest there is extensive cross-talk between OBs and BCCs, and that OBs are an important source of cytokines in breast cancer bone metastasis. These findings implicate the bone microenvironment and cancer cell manipulation thereof in orchestrating metastatic tumor cell dormancy, colonization, and survival.
Supported by the NIH; 1RC1 CA146381, 1R01NS06994, P50 CA083639 for FCM; Ruth L. Kirschstein Institutional Research Service Award (NRSA) T32 CA079448 for KMB.

P007

PTH Promotes Development of Breast Cancer Metastasis In Vivo: evidence for a Role for Bone Cells in the Metastatic Niche

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Background: It has been proposed that tumour cell homing, and engraftment in bone requires the presence of ‘metastatic niches’ and osteoblast lineage cells have been proposed to be essential components of niches in bone. In this study we have used a mouse model of breast cancer bone metastasis to asses whether alterations in the differentiation and distribution of bone cells by pre-treatment with parathyroid hormone (PTH) affects bone metastases development.
Materials and Methods: Female 12-week-old balb/c nude mice were injected i.p. daily for 5 days with rhPTH 1–34 (80 µg/kg, Bachem) or PBS and sacrificed on day 5, 7, 10 and 15. Bone cell activity was analysed by ELISA (serum PINP and TRAP) and Ob and osteoclast (Oc) numbers on trabecular bone surfaces were measured on TRAP stained sections (Osteomeasure). Mice were injected with 7.5×10^4 MDA-MB-231-tdTomato-Luc2 tumour cells via the intra-cardiac route on day 5 of treatment with either PTH (n=7) or vehicle (PBS, n=6). Tumour growth was assessed *in vivo* by measuring luciferase activity.
Results: PTH treatment induced a significant increase in Ob numbers per mm trabecular bone surface at day 5 and 7 and significantly increased serum PINP levels on day 7 compared to mice treated with vehicle. In the PTH group Ob numbers decreased to control levels at day 10, which was associated with an increase in Oc numbers compared to PBS treated animals. Pre-treatment with PTH resulted in earlier tumour development and an increase in the number of tumours in the PTH treated mice (2.14 ± 0.74) compared to mice receiving PBS (0.50 ± 0.34).
Conclusion: The data demonstrate that PTH-induced changes in bone cell numbers are associated with increased number of individual breast tumour foci *in vivo*. This supports the notion that changing the bone microenvironment could alter tumour growth in the skeleton. We are currently investigating whether the detected increase in osteoclasts in addition to changes to osteoblastic cells post PTH treatment is essential for tumour development in the bone microenvironment.

P008

Dormancy in the Development of Prostate Cancer Bone Metastasis

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Rationale & Hypothesis: Skeletal metastases occur in >70% of patients with advanced prostate cancer. It has been proposed that metastasis-initiating cells in prostate cancer gain a foothold in bone by homing to a ‘metastasis niche’. We hypothesize that cells arriving in the skeleton are mitotically dormant and the bone metastasis niche maintains their dormancy.
Aim: To develop a novel methodology to identify mitotically dormant tumour cells and their roles in the initiation of growing lesions in bone.
Methodology: Cohorts of 6- and 16-week old male balb/c nude mice received intracardiac injections of a human prostate cancer cell line (PC3-NW1), stably transfected with a luciferase gene to facilitate monitoring of tumour growth *in vivo* and labelled with a fluorescent cell membrane dye (Vybrant DiD) to identify mitotically dormant cells. Animals were injected with either the whole population or with subpopulations shown to be either mitotically dormant or fast growing *in vitro* (isolated by FACS). The presence of growing tumours was monitored by an *In vivo* Imaging System (IVIS). Cohorts of animals were culled at different time points (1d and 1, 3, 6wks post injection) and the presence of mitotically dormant, DiD+ tumour cells in the tibiae were mapped by multiphoton microscopy.
Findings: PC3-NW1 cells formed growing tumours exclusively in the skeleton in ~70% of young mice from 2 weeks post injection. Individual DiD+ cells were found engrafted in the tibiae in close proximity (50 µm) to bone surfaces at all time points. Dormant (DiD+) tumour cells were observed in tibiae without growing lesions, within growing tumours in young animals and in tibiae of older mice in which tumours were not growing. Both mitotically dormant and proliferative tumour cells, generated from PC3-NW1 *in vitro* cultures, were able to form tumours following intracardiac injection. However, dormancy was re-established in the proliferating population when these cells became resident in bone.
Conclusions: Mitotic dormancy is an important feature of the PC3-NW1 prostate cancer cells that arrive in the skeleton in this xenograft model. Our studies suggest that dormancy exists in cells in this cell line grown *in vitro* and can be rapidly established in cells that home to bone. The dormancy is a state that cells can enter/exit rather than a specific subpopulation. The model developed provides the basis to study the control of dormancy in bone metastases and identify new targets for therapies.

P009

Human Myeloma Cells Express Adhesion Molecules, which are Mediators of Haemopoietic Stem Cell Dormancy

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Multiple myeloma is caused by the proliferation of plasma cells within the bone marrow microenvironment. Despite recent improvements in treatment, most patients eventually relapse and the reasons for this remain unclear. We hypothesise that some myeloma cells (MC) evade chemotherapeutic clearance because they occupy a protective niche within the bone marrow. In preliminary experiments, we have measured expression of cell adhesion molecules, previously implicated in haemopoietic stem cell dormancy, in a panel of human MC and bone marrow stromal cells.

Messenger RNA expression of cell adhesion molecules VLA-4, VCAM-1, LFA-1, ICAM-1, Notch-1 and Jagged-1 was measured, by qPCR, in the human MC lines JJN3, U266, OPM2, NCIH-929 and RPMI-8226, as well as in *ex vivo* MC lines JJN3*vv* and U266*vv* (collected from infiltrated marrow of NOD/SCID-GAMMA mice, following tail-vein administration). Data were normalised using GAPDH and relative expression was calculated using the ΔΔct method. Statistical analysis was performed using non-parametric One-Way Anova (Kruskal-Wallis) test, corrected for multiple comparisons using Dunn's test.

Selected human MC lines express the cell adhesion molecules LFA-1, ICAM-1, Notch-1 and Jagged-1 (cycle-threshold [ct] values 23.1–32.1). Interestingly, VLA-4 is expressed by all human MC tested, whereas expression of its complimentary ligand, VCAM-1, was not detected. LFA-1 was highly expressed by JJN3 and NCIH-929 cells (ct values 23.5, 23.1 respectively) and lower levels were detected in OPM2 and RPMI-8226 cells (ct values 32.9, 29 respectively). The LFA-4-ligand, ICAM-1, was expressed at similar levels by all MC tested (ct values 25.3–27.6), whereas Notch-1, and its complimentary ligand Jagged-1, were expressed on all MC lines except U266. Interestingly, expression of Notch-1 and Jagged-1 was observed on *ex vivo* U266*vv* cells suggesting that expression of these molecules may depend on one or more environmental cues within the bone marrow.

In conclusion, we have found that adhesion molecules, implicated in previous studies of haemopoietic stem cell dormancy, are differentially expressed in a panel of MC lines. Since there is complimentarity in expression of receptors and their ligands between the MC and osteoblast-like (MG63 and SAOS2) and endothelial (HUVEC) cells included in this study, it is tempting to speculate these molecules play may a role in tumour cell dormancy.

P010

Next Generation mRNA Sequencing and MicroRNA Profiling Highlight Pathways of Tumor - Stroma Crosstalk in Osteolytic Breast Cancer

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Tumor cells can affect and process their stromal microenvironment. The cellular and non-cellular stroma may facilitate bone colonization by providing niches for metastasis-initiating cells. Recent clinical studies show that primary breast tumors with a relatively high proportion of stroma (and few tumor cells) represent a group of patients with poor prognosis. The tumor-associated stroma is, therefore, not an innocent bystander and seems actively involved in formation and progression of malignant, osteotropic tumors.

In this study we generated gene expression profiles of the cancer-associated bone/bone marrow stroma in experimentally-induced metastases from human breast cancer. For this we inoculated 5000 MDA-MB-231 cancer cells into the proximal tibia of immune-deficient mice. Intra-bone tumor growth was monitored by bioluminescence imaging and affected bones were resected after 35 days. Sham-operated and intact tibias were used as controls. We utilized the chimeric mixture of human (tumor) and mouse (stroma) mRNA and control samples to perform Next Generation Sequencing (Illumina) on mouse and human transcriptomes. Moreover, microRNA (miR) profiling on PCR-based nanochips (Openarray) was performed on the same samples on both mouse and human platforms to identify tumor- and stroma-derived miRs.

This study identified 766 genes that are differentially expressed in the reactive bone stroma compared with sham-operated controls. Moreover, among the identified miRs we found 20.5 % being up- and 9 % down-regulated, representing a total number of 221. Mapping of selected leads into pathways using DAVID (v6.7) and DIANA-Mirpath (v2) tools identified significantly up-regulated pathways involved in the process of focal adhesion ($p<9.29E-21$), ECM-receptor interactions ($p<1.65E-20$), axon guidance ($p<0.008$) and TGF-β signalling ($p<0.04$), respectively. Due to sequence homology 62% of miRs cross-hybridize between both species and, as a result, the exact cellular origin could not always be determined.

In summary, our NGS data provide a stromal gene expression signature of osteolytic breast cancer highlighting the activation of multiple pathways in the supportive stroma. Furthermore, differential expression of miRs suggests that they may be involved in the regulation of differentially expressed genes identified by NGS. Clinical validation of selected leads is currently on-going.

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P011

SAMSN1 Underlies Genetic Susceptibility to Monoclonal Gammopahty of Undetermined Significance, the Requisite Precursor to Multiple Myeloma

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Multiple Myeloma (MM) is a cancer of plasma cells characterized by lytic bone disease. MM is preceded by monoclonal gammopathy of undetermined significance (MGUS), a preneoplastic plasma cell disorder that is not clinically treated. MGUS is associated with osteoporosis and increased fracture risk, emphasizing that the multi-step transformation to MM, both in B-cells and the bone marrow niche, is initiated prior to overt malignancy. Identifying MGUS/MM susceptibility loci will provide candidates for genetic screening to identify high-risk MGUS patients and will facilitate the development of preventative therapies to slow or halt progression to overt MM. The KaLwRij mouse, a spontaneous mutant arising from C57Bl/6 (B6), is a widely used model for MGUS and MM that parallels human pathology. Using a high-density SNP array, we found that KaLwRij, while closely related to B6, is a genetically distinct mouse strain. To identify candidate genes underlying MGUS and MM predisposition, we employed a novel integrative computational analysis, combining KaLwRij/B6 modified haplotype block analysis and low-power human MM/control GWAS. Our top candidate, Samsn1, is a negative regulator of B-cell activation. We found that KaLwRij mice had germline deletion of Samsn1, indicating that it may represent a susceptibility locus. Interestingly, Samsn1 was first identified as a gene differentially expressed in MM. Samsn1 is expressed within the hematopoetic compartment, including B-cells, macrophages, and osteoclasts (OC), key cell types that participate in MM pathology. Consistent with the reported function of SAMSN1, KaLwRij mice had enhanced naïve B-cell response and constitutive Lyn phosphrylation. Further, we found that KaLwRij macrophages and OC also had elevated pathway activation demonstrated by constitutive Lyn activation. Absence of Samsn1 leads to unregulated B-cell and macrophage/OC activation, priming both a pro-tumor bone microenvironment and B-cells for development of MGUS and predisposition to MM. Efforts are underway to sequence the Samsn1 gene from MM patients for evidence of germline inactivation. Our approach focuses on germline loci rather than malignant cell somatic mutations, advancing our understanding of host predisposition. This method will further the understanding of genetic susceptibility in plasma cell dyscrasias, providing novel targets for monitoring at-risk individuals and guiding the development of preventative therapeutics.

P012

Reliable Quantification of Low Numbers of Bone Metastatic Cells *In Vivo* in Xenograft Mouse Models

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Cancer relapse is due to micro-metastases and dormant cells that are able to escape radiation therapy and host immunity. The mechanisms that support the survival of small foci dormant cells are not known and difficult to study, particularly because sensitive techniques for detecting small numbers of metastatic cells in the bones are missing, even in pre-clinical models. We used an inexpensive extraction technique to simultaneously extract bone genomic DNA and RNA which allows for the detection of human metastatic cancer cells in mouse bones and expression studies within the same tissue. Via quantitative QPCR of Alu repeats, which number more than 10^6 copies per cell, we were able to achieve single cell sensitivity *in vivo*. Comparison with other commonly used detection techniques demonstrate that this technique is more sensitive, reliable and inexpensive than flow cytometric analysis of flushed marrow and orders of magnitude more sensitive than any imaging modality used for *in vivo* bone metastasis. Using the MDA-231 intracardiac bone metastasis model, we were able to accurately quantify the number of Alu-positive cells in long bones at multiple time points from 24 hours after injection up to 14 days by QPCR. Our data indicate that the vast majority of cells die within the first day after inoculation and only 10–30 cells are present within a single humerus, tibia, or femur at 24 hours, and that the threshold for *in vivo* imaging is greater than 10^4 cells per bone. This technique should facilitate the study of early determinants of metastatic cancer establishment in bone.

P013

A Randomized Placebo-Controlled Phase II Trial Evaluating the Effect of Hedgehog Inhibitor LDE225 on Bone Marrow Disseminated Tumor Cells in Women With Early Stage Estrogen Receptor Negative and Her2 Negative Breast Cancer

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Background: 20% of invasive breast cancers are triple negative (TN). Recurrence rates of 40–50% have been reported in those patients who do not achieve a complete pathological response to neoadjuvant therapy. Data suggest that micrometastases or disseminated tumor cells (DTCs) that persist despite chemotherapy are enriched with cells that have stem cell-like features and are associated with adverse prognosis. The Hedgehog (Hh) signaling pathway, which is involved in modulating epithelial-mesenchymal transition, maintenance of breast cancer stem cells, and osteoblast and osteoclast function, is an attractive therapeutic target for patients with TN tumors. In preclinical models, we have shown that Hh inhibition reduced breast cancer growth and metastasis.

Trial Design: This is a placebo-controlled, double blinded, randomized phase II trial in early stage TN breast cancer patients with detectable DTCs in their bone marrow (BM) at the completion of all therapy. LDE225 is administered at 400 mg orally daily for a 28 day cycle for 20 cycles. After 6 cycles, BM will be collected for analysis.
Specific Aims: The primary endpoint is the elimination of DTCs after 6 cycles of LDE225. Additional endpoints include: 2-year disease-free survival (DFS) and overall survival (OS), effect on BM Ptch1 gene expression, toxicity profile of LDE225, DFS and OS in patients with no DTCs at screening versus DTC-positive patients in the placebo arm.
Eligibility: Patients with ER-/Her2- stage I-III invasive breast cancers, who have completed all therapy and have detectable DTCs, as defined by a molecular based assay for DTCs performed in a CLIA-licensed facility, are eligible.
Statistical Methods: Based on preliminary data, 60% of patients in the LDE225 arm are expected to be Ptch1 positive, and 50% of these patients are expected to have eliminated DTCs after 6 cycles of treatment. The expected clearance rate in the remaining 40% of the LDE225 arm is 5% with an overall clearance rate of 32%. In the placebo arm, the expected clearance rate is 5% regardless of Ptch1 status. A sample of 68 patients, 34 in each treatment arm, will have power ~0.8 at a 0.05 significance level, after adjusting for one interim analysis, to detect a difference in DTC clearance rate of 32% in the LDE225 arm versus 5% in the placebo arm.
Accrual: Accrual is anticipated to begin August 2013. Target=34 per arm. NCT01757327

P014

Antibody-Based Therapy Targeting Integrin α5 is an Effective Strategy to Treat Experimental Breast Cancer Bone Metastasis

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Integrin α5β1 is a specific fibronectin receptor that is often upregulated in breast cancer cells undergoing epithelial-to-mesenchymal transition and in tumor-associated endothelial cells. Indeed, the disruption of α5β1 binding to fibronectin leads to tumor growth reduction through inhibition of angiogenesis. Additionally, α5β1-fibronectin interaction promotes *in vitro* the survival of growth-arrested breast cancer cells in the bone marrow microenvironment. This is in line with the observation that disseminated tumor cells (DTCs) in the bone marrow express α5β1 integrin. DTCs in the bone marrow represents the earliest sign of development of metastatic disease in patients. However, the involvement of tumor-derived α5β1 integrin in bone marrow metastasis formation remains poorly documented. Using a cohort of 427 radically resected breast cancer patients, we have found that a high α5 expression in primary tumors detected by qRT-PCR is a negative independent predictive factor of bone relapse. Moreover, compared with tumor-bearing animals treated with the vehicle, a preventive treatment of BALB/c immunodeficient mice bearing human breast MDA-MB-231/B02 tumors with a chimeric IgG4 monoclonal antibody that specifically binds to human integrin subunit α5 (M200;15 mg/kg three times per week starting from the day before intra-arterial tumor cell inoculation) significantly delayed the onset and reduced the extent of osteolytic skeletal lesions, as detected by bioluminescence and radiography.
Histomorphometric analysis of metastatic legs after 28 days of treatment confirmed that M200 substantially decreased skeletal tumor burden and increased the bone volume. When the bone marrow was flushed from the hind limbs of animals on day 7 after tumor cell inoculation, at which time there was no evidence of metastases, and placed in culture under puromycin selection, the growth of antibiotic-resistant DTCs colonies in the bone marrow from M200-treated mice was dramatically decreased compared with vehicle-treated animals. *In vitro*, M200 antibody did not affect MDA-MB-231/B02 cell survival. By contrast, it specifically inhibited MDA-MB-231/B02 cell adhesion to fibronectin and cell invasion.
Overall, our results suggest that α5β1 integrin expression in breast cancer cells facilitates bone marrow micrometastasis formation and the subsequent development of osteolytic lesions.

P015

Visualization of Tumor Cell Dormancy and Activation in the Skeleton by Two-Photon, Intra-Vital Imaging

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Cancer cells can exist in a dormant state in the skeleton and can be activated to form overt tumours. However, our understanding of these events is limited due to an inability to identify dormant cells, to follow their activation in bone and to purify and define their phenotype. Using multiple myeloma as a model, we developed novel labeling and intra-vital imaging techniques to visualize individual dormant cancer cells and their activation in live mice and performed transcriptome analysis to define their phenotype.
5TGM1eGFP murine myeloma cells were labeled with a membrane dye (Vibryant DiD), which is retained by dormant, non-dividing, cells (DiDHigh), but lost on sharing with daughter cells as they divide (DiDNeg). Cells were injected (i.v.) into C57BL-KalwRij mice and visualized after 1, 6, 14, 21, or 28 days, in the bone marrow microenvironment, of intact tibia of live mice by two-photon, intra-vital, microscopy. DiDHigh and DiDNeg cells were isolated for flow cytometric analysis and ST2.0 whole mouse genome array analysis, whereas CD138+ve cells myeloma cells and colonies were identified by immunohistochemistry.
Intra-vital microscopy identified limited numbers of individual, DiDHigh, cells directly opposed to endosteal bone surfaces at each time point, which was confirmed by flow cytometry

(171±31/10^6 total cells). Individual CD138+ve cells were seen by immunohistochemistry. DiDNeg/GFP+ve cells could be identified from day 14, which increased through to day 28. This was associated with formation of a limited number of overt DiDNeg/GFP+ve colonies and CD138+ve colonies at day 21 (14.8±1.1). Individual DiDHigh tumour cells remained evident at day 28. Microarray analysis identified a distinct transcriptome profile of DiDHigh cells when compared to DiDNeg cells. A panel of long non-coding RNAs were the most strongly up-regulated transcripts in DiDHigh dormant cells, consistent with the regulatory architecture of the genome playing a role in maintenance of dormancy.
These data demonstrate that two-photon, intra-vital microscopy can be used to visualize dormant cancer cells and their activation in the skeleton in live mice. These data also show that only a limited number of the dormant cells present in bone are activated to form tumour colonies. Furthermore, the DiDHigh cells have a unique transcriptome profile, which may be critical in retention of the dormant phenotype in bone.

P016

Myeloma Cell Dormancy is an Acquired State *In Vivo*

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Despite continually improving treatments in myeloma, patients eventually relapse. It has been suggested that some myeloma cells can evade chemotherapy by residing within protective niches in the bone marrow. We hypothesise myeloma cells reside within specialised niches close to bone and remain in a dormant state until activated. In this study we aimed to establish if myeloma cell dormancy is restricted to an intrinsic population of tumour cells or if it is an acquired state *in vivo*.
5TGM1-eGFP cells were labelled with a long-chain dialkylcarbocyanine membrane probe (Vybrant DiD) to monitor cell proliferation or dormancy and injected into C57BLkaLwRij mice. Tumour burden was measured by fluorescent activated cell sorting (FACS), immunohistochemistry and multiphoton microscopy; bone disease was measured by microCT and static histomorphometry. Sub-populations of proliferating (GFP/DID^{Neg}) or dormant 5TGM1 cells (GFP/DiD^{High}) were isolated from *in vitro* cultures or *ex vivo* from bone marrow flushes of 5TGM1 tumour-bearing mice by FACS. Cells were then characterised *in vitro* in osteoblast conditioned media assays or *in vivo* by injection into C57BLkaLwRij mice.
In a preclinical model of myeloma, we identified key stages in myeloma disease development. At the end stage of disease, both proliferating and dormant sub-populations of 5TGM1 cells were successfully isolated by FACS and characterised. *In vitro*, cells were cultured in osteoblast-conditioned media, and the proliferation of both populations was inhibited. Injection of these cells *in vivo* showed proportions of dormant cells could be activated to form tumour colonies; and proliferating cells could become dormant.
We have identified, isolated and characterised populations of dormant and proliferating myeloma cells, when cultured *in vitro* or *in vivo* both populations behaved in a similar manner. These findings suggest myeloma cell dormancy is an acquired state and targeting such cells in patients may prevent relapse.

P017

Identifying Haematopoietic Stem Cell (HSC) Niche Markers in Human Prostate Cancer Cells: Possible Mediators of Dormancy in Metastatic Cancer. Freyja Docherty, Ning Wang, Anne Fowles, Julia Hough, Clive Buckle, Ingunn Holen, Colby Eaton

Freyja Docherty[1], *Ning Wang*[1], *Anne Fowles*[1], *Julia Hough*[1], *Clive Buckle*[2], *Ingunn Holen*[1,2], *Colby Eaton*[1]
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Background/Rationale: Our recent studies with a human prostate cancer xenograft model show that tumour cells homing to bone are mitotically dormant. Others have suggested that tumour cells hijack HSC niches in this model[1]. In this study we have identified dormant cells in a prostate cancer cell line grown *in vitro* and tested the hypothesis that deployment of HSC niche components is a characteristic of dormant prostate cancer cells.
Methods: FACS was used to evaluate the following interacting pairs of HSC niche molecules in the human prostate cancer cell line PC3NW1 (PC3 expressing luciferase): CXCR4/CXCL12, Jagged/Notch, Tie 2/Ang1 and N-Cadherin. These cells were stained with Vybrant DiD cell membrane dye and dye retention over 14–21 days used to identify dormant cells. These were separated by FACS and HSC niche molecules expressed/present in dormant and non-dormant populations were compared by RT-PCR/immunofluorescence and for growth, in colony assays.
Results: FACS analysis of the entire PC3 population showed that N-Cadherin and Notch were present on >50% of cells. CXCR4 and Jagged were present on a minority population (<5%). We identified and isolated a slow growing, potentially mitotically dormant, cell sub-population from PC3-NW1 cells *in vitro* based on DiD retention for 14 days. Isolated dormant cells expressed significantly higher levels of CXCR4 and Jagged than the rapidly dividing population. Microscopically, DiD retaining cells were morphologically distinct being ~ twice the size of rapidly growing cells and immunofluorescence analysis confirmed the presence of high levels of CXCR4 in DiD+ve cells. Once separated both DiD+ve and –ve population formed growing colonies in monolayer.
Conclusions: These results suggest that the PC3 cell line contains a mitotically dormant phenotype with distinct characteristics and HSC niche factor profile. This may confer an increased ability to home to the skeleton in these cells. The study also shows that once isolated, this population is able to proliferate after long periods of dormancy. The factors that control entry/exit from a dormant state are potentially relevant to understanding homing to bone and the initiation of growing lesions *in vivo*.
Reference:
1. Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bonemarrow. Shiozawa Y. *et al.* J Clin Invest. 2011 Apr;121(4):1298–312.

P018
Altered Cortical Microarchitecture and Bone Metabolism in Patients with Monoclonal Gammopathy of Undetermined Significance

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Patients with monoclonal gammopathy of undetermined significance (MGUS) have an increased fracture risk, and we have previously shown that MGUS patients have altered trabecular bone microarchitecture compared with controls. However, there are no data on whether the porosity of cortical bone, which may play a greater role in bone strength and fracture occurrence, is increased in MGUS. We studied cortical porosity and bone strength (apparent modulus) using high-resolution peripheral quantitative computed tomography (HRpQCT) imaging of the distal radius in 50 MGUS patients and 100 matched controls. Compared to controls, MGUS patients had significantly higher cortical porosity [+16.8% (95% confidence interval [CI]: 14.5%, 18.7%); P<0.05] and lower apparent modulus [a measure of bone strength, −8.9% (95% CI: −11.7%, −6.5%); P<0.05]. Furthermore, MGUS patients with higher monoclonal protein concentrations (>2.0 g/dL) had significantly lower bone turnover (P=0.022) and tended to have higher circulating Dickkopf-related protein 1 levels. In conclusion, despite their larger radial bone size, MGUS patients have significantly increased cortical bone porosity and reduced bone strength relative to controls. In addition, MGUS patients with higher monoclonal protein concentrations have reduced bone turnover. This increased cortical porosity and suppression of bone formation may explain the increased fracture risk seen in MGUS patients.

P019
Selective Inhibition of Bet Bromodomains Epigenetic Signaling Interferes with the Bone-Associated Tumor Vicious Cycle

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Osteosarcoma is the most frequent primary bone tumor that develops mainly in young adults. The survival rate at 5 years is below 30% for patients with poor response to treatment or with metastasis. The vicious cycle established between bone associated tumors and bone resorption is the central problematic of the therapeutic strategies developed for primary bone tumors or bone metastasis. The bromodomain and extra-terminal domain (BET) protein family (including BRD4) is an important class of "histone reading protein" capable to recognize the N-acetylation of lysine residues on histone tails. Bromodomains act as a scaffold for molecular complexes at the recognized histones sites in order to regulate chromatin accessibility to transcription factors and RNA polymerase.

In this study we report for the first time the essential role of BRD4 in both osteoblastogenesis and osteoclastogenesis regulation. Indeed, a strong reduction of osteoblastic differentiation has been observed after BET protein inhibition associated to a transcriptional silencing of RUNX2 coincident with release of BRD4 from its locus. Moreover, we identified a BRD4-dependent RANKL activation of osteoclastogenesis on the NFATC1 promoter, leading to the inhibitory potential of JQ1 on osteoclast differentiation. Moreover the inhibition of BRD4 interferes with bone associated tumor progression through a transcriptional silencing of MYC and RUNX2 coincident with release of BRD4 from their respective locus. Indeed, in osteosarcoma tumor cell lines, BET inhibitor reduced cell growth in a dose-dependent manner and induced apoptosis with an increase of sub-G1 fraction and PARP cleavage. *In vivo*, BET inhibitor (IP; 50 mg/kg) significantly inhibits tumor growth by 70% and prolongs survival in both POS-1 syngenic and HOS xenograft models compared to control. Additionnally, these results were accompanied by a decrease of associated bone lesions.
Those new data support BET protein inhibition as a novel therapeutic strategy targeting simultaneously the three partners of the vicious cycle, using JQ1, a unique BET protein inhibitor. In this study, we uncover the therapeutic potential of BET bromodomain inhibition in bone associated tumors. We present for the first time a therapeutic strategy targeting an epigenetic recognition protein of acetyl lysines in the bone associated tumor vicious cycle.

P020
Lysophosphatidic Acid (LPA) Promotes Disseminated Tumor Cell (DTC) Formation through a ZEB1/MIR21-Dependent Activation Pathway

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Lysophosphatidic acid (LPA) is a natural bioactive lipid that promotes metastasis of many types of cancer cells. We have shown that blocking LPA receptor type 1 (LPA1) activity inhibits its early stage of bone metastasis by inhibiting motility and invasion of breast cancer cells. However, the signaling pathways and gene activation involved in this process have not been well characterized. Micro-RNAs (miR) are well known master regulators of gene expression. Based on the complete miR expression profile in human MDA-MB-231 breast cells stimulated by LPA, we found that miR-21 was one of the most up regulated miR. Using the Taqman RT-QPCR system, we found that LPA induced a rapid increase in miR-21 expression in MDA-MB-231 cells and in their highly osteotropic sub-clone MDA-B02 cells reaching a plateau by two to three folds ($EC50 = 0.1\,\mu M$) after 45 min of stimulation. MiR-21 is well known to act as an oncomiR promoting metastasis in multiple cancers. Also, it is known that miR-21 expression is controlled by several transcription factors. The full transcriptomic analysis of our breast cancer cell lines showed that among those transcription factors, ZEB1, STAT3 and cFos were up regulated by LPA. Interestingly, silencing ZEB1 expression in these cells using synthetic ZEB1-siRNAs abolished LPA-induced miR-21

expression whereas silencing STAT3 or cFos had no effect. Hence, silencing ZEB1 up-regulated the expression of miR-21 target genes PDCD4, PTEN and SPRY2 in MDA-MB-231 cells stimulated by LPA. RT-QPCR analyses also showed that LPA1 was the most abundant LPA receptor in MDA-MB-231 cells (LPA1>LPA2>>LPA6=LPA7>LPA5) whereas LPA3 and LPA4 were not detectable. We found that the treatment of our breast cancer cells with Ki16425, a LPA1/LPA3 antagonist, inhibited LPA-induced miR-21 expression. Silencing LPA1 expression in these cells using synthetic LPA1-siRNAs also abolished LPA-induced miR-21 expression. We showed in animal that Debio0719, another LPA1/LPA3 antagonist, inhibits implantation of disseminated tumor cells (DTC) into the bone marrow. Here, we found that the pretreatment of MDA-B02 cells with a specific blocker of miR-21 (mirVana miR-21 inhibitor) before injection of these cells in BALB/c nude mice decreased the incidence of DTC-positive animals and decreased the number DTCs at the metastatic sites. All together our results demonstrate that miR-21 controls the prometastatic activity of LPA involving LPA1-dependent activation of ZEB1 in breast cancer cells.

P021

The Role of Internal Fixation for Long Bone Metastasis Prior to Impending Fracture: an Experimental Model

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Background: Patients with long bone metastasis have many therapeutic options, including surgery. However, the appropriate time for surgical intervention and the use of internal fixation prior to impending fracture remains controversial. The purpose of this study was to establish a long bone metastatic model with internal fixation, and to determine whether prophylactic internal fixation for long bone metastasis prior to impending fracture would affect bone destruction, tumor progression, and mortality.
Materials and methods: We implanted VX2 tumor cells into the tibiae of 45 rabbits divided equally into three groups: internal fixation, control, and sham groups. Rabbits were monitored by X-ray and computed tomography, and blood serum levels were examined every 2 weeks.
Results: Computed tomography data revealed significantly higher bone destruction in rabbit tibiae in the sham and control groups compared with those in the fixation group; there were volumetric bone losses of 0.2, 0.4, and 2.3% in the fixation, sham, and control groups, respectively, at 3 weeks, which increased to 1.2, 2.5, and 6.1% at 5 weeks. Rabbits in the fixation group showed significantly prolonged survival (64.5±13.5 days) in comparison with rabbits in the sham group (50.3± 11.6 days) and control group (38.2±4.9 days).
Conclusion: Our results suggest that prophylactic internal fixation may hinder bone destruction and tumor progression, thus extending the survival period for patients with long bone metastasis.

P022

Non-Spine Bone Metastasis as an Initial Manifestation of Unknown Primary Cancer

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Introduction: In 3 to 15% of all patients with metastatic carcinomas, the type and site of the primary tumor is unknown. Approximately 5 to 20% of these patients, skeletal metastases are the first lesions to be detected. The identification of primary cancer provides valuable information in that orthopedic surgeons can choose operative options according to their primary cancer and life expectancy. There have been little published data focused on bone metastasis of pelvis and extremities as an initial manifestation of cancer for which orthopedic surgeons are more likely referred to. This study aims to describe the clinicopathologic characteristics of patients who presented with non-spine bone metastasis as an initial manifestation of unknown primary cancer (UPC).
Methods: We retrospectively reviewed 101 patients who were referred for bone metastases from UPC. There were 63 men and 38 women with a mean age of 58.2 years (range: 17.5–85.6 years). The average follow-up period was 21.9 months (range: 1–120 months).
Results: Pain was the most frequent initial symptom in 95.0%. The most frequent 3 locations of bone metastasis were the femur (n=43), pelvis (37), and humerus (12). Plain radiograph showed osteolytic metastasis in 79.2%. Primary cancer was identified in 96 cases (95.0%), in the order of lung cancer (38.6%), multiple myeloma (11.9%), renal cell carcinoma (10.9%), hepatocellular carcinoma (7.9%), thyroid carcinoma (6.9%), lymphoma (4.0%), and prostate cancer (4.0%). Multiple bone metastases were observed in 70.3% and distant organ metastasis in 46.5% in our study. Operative treatment was performed in 67 cases (66.3%) for a pathologic fracture (n= 23) or an impending fracture (44). Local recurrence rate after the operation was 11.9% and 5-year survival rate was 29.9%. Patients multiple metastasis had poorer prognosis than those who had solitary bone metastasis only (p= 0.010). In multivariate analysis, the overall mortality was found to be correlated with the presence of multiple bone metastases (HR 2.49; 95% CI 1.18–5.27, p=0.016).Survival rate of lung cancer was lower compared to those of thyroid carcinoma, and renal cell carcinoma, but the difference was not significant.
Coclusions: Bone pain was the most common initial symptom in patients with non-spine bone metastasis from UPC. Lung cancer was the most common primary, and patients with multiple bone metastasis had poor prognosis, possibly due to disseminated cancer and greater tumor burden.

P023

Preclinical Evidence of Potential Craniofacial Adverse Effect of Zoledronic Acid in Osteosarcoma Patients

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Background: Oncologic doses of zoledronic acid (ZOL) are currently evaluated in phase III clinical trials in Europe for the treatment of malignant primary bone tumors. The impact of such an intensive treatment on the craniofacial skeleton growth is a critical question in the context of patients with actively growing skeleton.
Methods: Two protocols adapted from pediatric treatments were developed for newborn mice (a total of 5 or 10 injections of ZOL 50 μg/kg every two days). Their impact on skull bones and teeth growth was analyzed by micro-CT and histology up to 3 months after the last injection. In parallel, panoramic radiographs of pediatric patients from the French OS2006 trial (chemotherapy +/− Zometa®) were analyzed for potential orofacial consequences.
Results: In mouse, ZOL administrations induced a transient delay of skull bone growth and an irreversible delay in incisor, first molar eruption and root elongation. Root histogenesis was severely impacted for all molars and massive odontogenic tumor-like structures were observed in lower incisors. Panoramic radiograph analysis of 23 pediatric patients treated by chemotherapy + Zometa® in the OS2006 protocol showed not significant increase of the percentage of tooth eruption delay comparatively to 21 patients treated by chemotherapy alone.
Conclusions: In mouse, oncologic doses of ZOL irreversibly disturbed teeth eruption and elongation, and delayed skull bone formation. In human, the same treatment may impact the permanent teeth eruption. These preclinical and clinical observations are crucial for the follow-up of pediatric patients treated with Zometa® in several European and American protocols.

P024

Bone Metastasis Inhibition in Prostate Cancer by Tasquinimod Involves Effects on Bone Microenvironment and Osteoblast Differentiation

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Introduction: Tasquinimod is a quinolone-3-carboxamide with antitumor activity via modulation of immune responses, inhibition of angiogenesis and inhibition of metastasis (George & Pili 2013). In an *in vivo* model of castration-resistant prostate cancer (CRPC), treatment with tasquinimod decreased the number of lymph node and lung metastases and inhibited tumor growth in bone (Jennbacken *et al* 2012). Also, in a randomized phase II trial tasquinimod showed proof of concept by demonstrating a significant clinical activity against bone metastasis in CRPC patients (Pili *et al* 2011). The drug is presently in phase III development (10TASQ10) for treatment of metastatic CRPC. We hypothesized that tasquinimod inhibits bone metastasis by affecting the bone microenvironment and bone homeostasis.
Methods: Under steroid deprived conditions, a cell culture model based on conditioned media (CCM) from human CRPC cells (LNCaP-19) on mouse osteoblasts (MC3T3-E1 clone 4) was used to simulate the interaction of the bone metastasis microenvironment. Influence of tasquinimod (10 μM) on tumor cell induced osteoblast differentiation was studied using Q-PCR.
In vivo effects of tasquinimod were studied in intact and castrated Balb/c nude mice after 2 weeks oral administration at 10 mg/kg/day (ad lib). mRNA was extracted from both bone and bone marrow compartments and analyzed using Q-PCR.
Results: Tasquinimod inhibited tumor cell-induced early osteoblast differentiation *in vitro*, indicated by lower expression of the transcription factors Runx2 and Osx, master regulators of osteoblast differentiation.
Tasquinimod decreased the expression of markers for bone remodeling (e.g. Alp1, Ocn, Opg) in castrated mouse femur but not in non-castrated mice. In the bone marrow, gene expression related to "homing to bone" and tumor establishment was influenced by castration and these effects were partly reversed by tasquinimod treatment. A normalization of androgen receptor mRNA expression was also observed in bone tissue and bone marrow cells after treatment with tasquinimod.
Conclusions: Tasquinimod affects osteoblast differentiation *in vitro* as well as bone remodeling in castrated mice. In addition, tasquinimod treatment *in vivo* normalizes the castration effects on properties involved in tumor homing. Our data show that tasquinimod targets the bone microenvironment, possibly explaining the reduced establishment and progression of bone metastasis in CRPC seen during tasquinimod treatment.

P025

Low-Intensity Continuous Ultrasound Promotes Antitumor Effects of a Clinical Dosing Regimen of Bisphosphonate in Animal Models of Breast Tumor Xenograft and Bone Metastasis

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Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption and have demonstrated clinical utility in the treatment of patients with bone metastases. There is also preclinical evidence suggesting that bisphosphonate zoledronic acid (ZOL) exhibits antitumor effects. However, high doses of ZOL used in most animal studies are incompatible with the dosing regimen that has been approved for the treatment of

bone metastases. Low-intensity continuous ultrasound (US) is known to enhance the local delivery of drugs in tissues. In the present study, we examined whether US could maximize the effects of a clinically relevant dose of ZOL in experimental breast cancer bone metastasis.

For tumor xenograft and bone metastasis protocols, human breast cancer cells were inoculated subcutaneously or into the tail artery of nude mice, respectively. Once tumor-bearing mice had palpable tumors or radiographically detectable bone metastases, these animals were randomly assigned to different treatment groups (vehicle, ZOL, US, ZOL+single US, ZOL+daily US; n=7 to 8 mice per group). For both protocols, a single clinically relevant dose of ZOL (0.1 mg/kg), single or daily US, or a treatment combining ZOL+US were administered to tumor-bearing mice. The size of tumor xenografts was measured using a Vernier caliper. Osteolytic lesions were detected by radiography. Tumor angiogenesis and tumor cell proliferation were assessed by immunohistochemistry. IPP accumulation within tumor cells, a surrogate marker of ZOL efficacy, was measured by mass spectrometry.

In vitro, a treatment of breast cancer cells with ZOL+US dramatically increased intracellular IPP accumulation, compared with ZOL alone. *In vivo*, US alone did not have any effect on bone metastasis and tumor outgrowth, compared with vehicle-treated animals. ZOL+daily US statistically significantly ($P < 0.01$) decreased bone destruction ($1.3 \pm 0.4\,mm^2$) compared with ZOL alone ($3 \pm 0.4\,mm^2$). This difference was accompanied with a sharp reduction in the tumor volume (TB/STV ratio: 11%) compared with ZOL-treated metastatic mice (TB/STV ratio: 46%), as determined by histomorphometry. ZOL+US treatment also inhibited growth of subcutaneous tumors in animals, when compared with ZOL alone. For both protocols, tumor angiogenesis and tumor cell proliferation were substantially reduced.

US facilitates the uptake of ZOL by tumor cells, thereby promoting its antitumor effects *in vivo*.

P026

Extra-Cellular Membrane Vesicles as Potential Mediators of Cancer Induced Bone Destruction in the Osteosarcoma Bone Microenvironment

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Osteosarcoma (OS) is an aggressive malignancy of bone affecting children, adolescents and young adults. The bone microenvironment (BME) is the main hub of all skeletal related pathological events in OS leading to tumor induced bone destruction, and decreasing overall bone quality and bone strength. In recent years, the significance of extracellular membrane vesicles (EMVs) as mediators of intercellular communication is emerging as a topic of great interest in bone biology. The role of OS-EMVs in modulating OS-bone microenvironment is unknown, and needs to be investigated. Our hypothesis is that the metabolic stress in the bone tumor microenvironment increases intracellular calcium levels in OS cells, which in turn induce cytoskeleton rearrangements, EMV biogenesis and release from cancer cells, and modulate expression of growth factors like transforming growth factor-β, matrix remodeling enzymes like MMPs, and proosteoclastic molecules such as RANKL in OS-derived EMVs. We had previously demonstrated the presence of EMVs in the preclinical bioluminescent osteosarcoma orthotopic mouse (BOOM) model (Garimella *et al*., 2013). In this study, we have successfully isolated and characterized EMVs from the conditioned media of 143B human OS cell cultures using differential ultracentrifugation, transmission electron microscopy (TEM), and western blotting. Size fractionation of 143B derived-EMVs by Nano-particle tracking analysis (NTA) indicated the size range as 50–200 nm in diameter. Transmission electron microscopy confirmed the ultrastructure of OS-EMVs and detected multivesicular bodies (MVBs). Biochemical characterization of 143B-EMVs revealed the presence of MMP-1, MMP-13 and RANKL. To our best knowledge, the expression of MMP1, MMP13, and RANKL in OS-EMVs is a novel finding. To test the role of calcium in EMV biogenesis, we monitored intracellular calcium concentration by Fura-2 loading, and observed that 143B cells actively mobilize calcium in the presence of ionomycin a calcium ionophore, and cause cytoskeleton arrangement. Pre-treatment with 10 M forskolin, an adenylate cyclase activator increased calcium mobilization of 143B OS cells and induced cytoskeleton rearrangements leading to vesicular biogenesis. In conclusion, these findings suggest a novel role for cAMP/calcium dependent signaling pathway as a candidate mechanism for EMV biogenesis; and EMVs as potential mediators of cancer induced bone destruction in the OS bone microenvironment.

P027

Denosumab for the Treatment of Persistent or Relapsed Hypercalcemia of Malignancy

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Background. Hypercalcemia of malignancy (HCM), often caused by tumor-induced bone resorption, is commonly treated with intravenous (IV) bisphosphonates. HCM may persist or relapse despite bisphosphonate therapy. Denosumab binds

to RANK ligand (RANKL) to inhibit osteoclast-mediated bone resorption.
Methods. In this single-arm, open-label study, patients with HCM (corrected serum calcium [CSC] >12.5 mg/dL) despite IV bisphosphonate treatment ≥7 and ≤30 days before screening received subcutaneous denosumab 120 mg on days 1, 8, 15, and 29, then every 4 weeks. The primary endpoint was the proportion of patients with CSC ≤11.5 mg/dL within 10 days of denosumab initiation.
Results. The study enrolled 33 patients; key baseline characteristics are shown in Table 1. By day 10, 21 patients (64%) reached CSC ≤11.5 mg/dL, including 7 of 13 patients (54%) with bone metastases and 14 of 20 (70%) without bone metastases. During the study, 23 patients (70%) reached CSC ≤11.5 mg/dL. A complete response (CSC ≤10.8 mg/dL) occurred in 12 patients (36%) by day 10 and in 21 patients (64%) during the study. In patients who reached CSC ≤11.5 mg/dL, the estimated median response duration was 104 days. By day 10, mean (SD) percentage reduction from baseline of urinary-n telopeptide (corrected for urinary creatinine) (uNTx/uCr) was 53% (36%). Of the 17 patients whose uNTx/uCr was ≥50 nmol/mmol at baseline, 14 (82%) reached CSC ≤11.5 mg/dL by day 10, including 8 of the 9 patients (89%) whose uNTx/uCr was reduced to <50 nmol/mmol. Day 10 reductions in uNTx/uCr and CSC were associated (Spearman rank correlation coefficient 0.6216, P<0.01). The most frequently reported serious adverse events were hypercalcemia (n=5, 15%) and dyspnea (n=3, 9%). Two patients had isolated episodes of CSC levels ≤8.0 mg/dL; none had CSC <7.0 mg/dL. No osteonecrosis of the jaw was reported.
Conclusions. In this study of patients with persistent or relapsed HCM despite recent IV bisphosphonate treatment, 64% of patients responded to denosumab within 10 days. The sustained duration of response observed in this study is a clinically favorable outcome for this population. Consistent with the known mechanism of action of denosumab, reduction of bone turnover was associated with reduction of CSC from baseline. No unexpected safety findings were identified. Denosumab may offer a new treatment option for HCM.

Baselie Patient Characteristics

Characteristic	Denosumab treatment group
Men, n (%)	21 (64)
Age, mean (SD)	60 (15)
Median (Q1, Q3) time from last bisphosphonate Treatment to enrollment, days	17 (13, 22)
ECOG performance status 2–4, n (%)	25 (76)
CSC, mg/dL, median (Q1, Q3)	13.7 (13.2, 14.2)
unTx/uCr, nmol/mmol, median (Q1, Q3)	76.9 (26.0, 148.2)
Proportion of patients with uNTx/uCr ≥ 50 nmol/mmol, n (%)	17 (52)

SD: standard deviation; Q1, Q3: interquartile range; CSC: corrected serum calcium, calculated as: {total serum calcium in mg/dL +[0.8 × (4–serum albumin in g/dL)]}; uNTx/uCR: urinary n-telopeptide corrected for urinary creatinine.

P028
Zingerone Protects Mouse Against the Radiation-Induced Lethality

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Radiation therapy is one of the most important paradigms used for checking and alleviating cancer in situations where surgical treatment is impracticable. A major problem associated with radiotherapy is the severe side effects resulting from the normal tissue toxicity on exposure to therapeutic doses of radiation. Although, a significant improvement in the cancer treatment with radiation has resulted in longer survival, at the same time there is growing evidence for the induction of radiotherapy induced secondary tumors in long time survivors. Therefore, the selective protection of normal tissues from the damaging effects of ionizing radiation is an unresolved problem.
Therefore, new approaches are needed to overcome these deleterious effects of ionizing radiation. Several chemical compounds and their analogues have been screened for their radioprotective ability. Therefore, now more importance is given to natural herbal compounds while screening radioprotective agents.
Zingerone (ZO), one of the active components, isolated from Z. officinale and its frequent use in India stimulated us to conceptualize and investigate the radioprotective activity of the ZO *in vivo*.
To evaluate acute toxicity and optimum dose selection of ZO, animals were treated with different concentrations (10–100 mg/kg.b.wt) of ZO and exposed to 10Gy gamma radiation. The radioprotective potential of ZO was evaluated by using the optimal dose of 20 mg/kg.b.wt. of ZO before exposure to different doses (7 –11 Gy) of gamma radiation. Radiation dose of 4.5 and 7.5 Gy was used in endogenous spleen colony forming unit assay and 3 Gy for mouse bone marrow micronucleus test. Radiation induced changes in endogenous antioxidants was also analyzed in the present study. Zingerone resulted in an increase in the LD50/30 by 1.8 Gy, giving a dose reduction factor (DRF) of 1.2 and also resulted in increased number of endogenous spleen colonies against 4.5 or 7.5 Gy irradiated mice. A significant reduction in micronucleated polychromatic, normochromatic erythrocytes and an increased PCE/NCE ratio was observed, and there was significant increase in the GSH and GST levels and a decreased LPx levels in ZO pretreated group. Our findings demonstrate the potential of ZO, in mitigating radiation induced animal mortality and antagonistic effect on the radiation induced cytogenetic damage.

P029

Long Term Use of Zoledronic Acid (ZOL) in Cancer Patients (PTS): Final Results of the Prospective Multicenter Lotuz Trial

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Background: Trial data documenting ZOL treatment is currently limited to approximately 2 years of therapy.
Methods: Pts with bone metastases from solid tumors (STM) or multiple myeloma (MM) and with at least 24 months of regular q3-4w ZOL therapy were followed prospectively for an additional 18 months. ZOL could be continued, interrupted or stopped at the discretion of the treating physician. End-points included ZOL exposure (% of expected per-label cumulative dose) and persistence (no treatment interruptions > 45 days), incidence of symptomatic skeletal related events (SRE), and safety.
Results: In all, 298 evaluable pts were enrolled (female n=201; median age 66y). The mean continuation rate of ZOL was 90.6%, even though only 28.0% of pts who completed follow-up (n=218) received uninterrupted per-label ZOL therapy. Exposure to ZOL decreased steadily with time in all pts, but was on average lower (50.0% vs 67.6%; p<0.001) and with higher discontinuation rates (IRR 1.95; p=0.002) in MM pts. ZOL infusions were extended beyond 15 minutes in 39%. ZOL continued to suppress the rate of SREs similarly during the 18 months study period (0.14 per person-year) as compared to the 18 months before inclusion (0.13 per person-year; p=0.9). At 18 months, 82.7% of pts were SRE free. In STM pts, persistent ZOL therapy reduced the SRE risk (HR 0.42; p=0.01) compared to interrupted treatment. This effect was more pronounced in breast cancer pts, where both higher ZOL exposure (HR 0.76 per 20% increase; p=0.009) and persistent ZOL therapy (HR 0.26; p=0.002) were associated with lower SRE risk. Renal deterioration occurred in 11 pts (3.7%), with a higher risk when ZOL dose was not adjusted for renal function (HR 3.96; p=0.03), as observed in 12.5% of pts. Symptomatic hypocalcemia was not reported, although adherence to supplemental calcium and vitamin D was only 18.5%. Acute phase reactions were infrequent (6.4%) and ONJ developed in 6.0% of pts. Invasive dental procedures or trauma were associated with increased ONJ risk (HR 4.67; p=0.002), with a 20% risk of ONJ after any of these events.
Conclusion: The continuation rate of ZOL beyond two years of therapy is high and the rate of clinically overt SREs remains low in this selected group. Nevertheless, ZOL treatment patterns were heterogeneous and deviating from per-label ZOL therapy resulted in a higher SRE risk. The long-term safety profile of ZOL was favorable, but adequate prevention strategies for ONJ remain important.

P030

Piceatannol Influences the Release of Active TGFb in Lysed Platelets and has a Context-Dependent Influence on Cell Proliferation

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Introduction: TGFb signaling has a profound importance for cell proliferation, differentiation and metastatic tumor growth. Latent TGFb is present in high concentrations in platelets. The latent form is inactive and bound to latent TGFb-binding proteins and peptides (LTBPs). Piceatannol has been recognized as a molecule with anti-inflammatory, anti-carcinogenic and anti-oxidative properties.
Aims: To investigate the effects of piceatannol on activation of latent TGFb and on fibroblast proliferation with and without lysed platelet preparations.
Hypotheses: Piceatannol will increase the release of active TGFb from the latent form.
Piceatannol will reduce fibroblast proliferation *in vitro*.
Methods: The TGFb concentrations were measured by ELISA in lysed platelet concentrates prepared at pH 3.5, 5.4 and 7.4. Fibroblasts were cultured with or without the addition of lysed platelets prepared at pH 5.4 and pH7.4. Fibroblast proliferation was assessed with the MTT kit.
Results: Piceatannol increased the release of active TGFb in lysed platelet preparations, particularly in the lower pH range. The addition of piceatannol resulted in a dose-dependent reduction of fibroblast proliferation. In contrast, piceatannol had a stimulatory effect on cell proliferation when preparations of lysed platelets were added to the cultures.
Conclusions: Lysed platelet preparations contain high concentrations of latent TGFb. Our findings indicate that piceatannol binds to LTBPs, which results in a higher release of active TGFb. Thus, piceatannol will not influence fibroblasts directly when it is bound to LTBPs and the released active TGFβ could improve fibroblast proliferation. When fibroblasts are cultured without lysed platelets, piceatannol could influence the fibroblasts directly and reduce proliferation. Inflammatory processes are reduced by active TGFb. The anti-inflammatory properties of piceatannol may act via the activation of TGFb.

P031

Microrna-Mediated Targeting of Runx2 Impairs Breast Cancer Metastasis and Progression of Osteolytic Disease

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Progression of breast cancer metastasis is associated with dysregulation of several bone-related transcription factors, such as Runx2. Several studies have shown that depletion

of Runx2 in bone metastatic cancer cell lines by RNA interference inhibits tumor cell activities and prevents metastatic bone disease, thus providing a basis for developing Runx2 as a therapeutic target for metastasis. In this study, we aim to evaluate the potential use of miRNAs that repress Runx2 as therapeutic targets for reducing tumor growth, and preventing metastasis to bone. Expression analysis of a panel of miRNAs established to target Runx2 revealed a reciprocal relationship between miRNA and Runx2 expression in breast epithelial cells (MCF-10a), non-metastatic MCF-7, and metastatic MDA-MB-231 breast cancer cell lines. Several miRNAs that target Runx2 are highly expressed in MCF-10a and MCF-7 cells that have nearly non-detectable levels of Runx2 protein while the expression is greatly dimished in MDA-MB-231 cells, suggesting that loss of specific miRNAs contributes to the elevation of Runx2 in metastasis. Reconstituting two of the miRNAs in MDA-MB-231-Luc cells reduced Runx2 expression, suppressed cell migration and invasion, and resulted in a modest decrease in tumor cell viability *in vitro*. Forced expression of these miRNAs also decreased mRNA expression of several Runx2 target genes including IL-11, MMP-13, and PTHrP that are all implicated in metastasis and vicious cycle of osteolytic disease. Consistently, ectopic expression of the miRNAs in MDA-MB-231-luc cells, followed by mammary gland implantation with additional intratumoral injection of the synthetic miRNA after 1 week, reduced tumor growth in the orthotopic site *in vivo* as well as spontaneous metastasis to bone. Furthermore, the miRNAs impaired tumor growth in the bone environment and bone resorption at the metastatic site. In summary, we have demonstrated that the abnormal expression of Runx2 in aggressive tumor cells is related to loss of specific miRNAs targeting Runx2, and that expressing miRNAs targeting Runx2 is effective in knocking-down Runx2 and the many downstream metastasis-related genes that Runx2 is known to regulate. We propose that synthetic miRNAs can be used as therapeutic targets, especially for master transcription factors, to prevent metastatic bone disease.

P032

TGF-β Inhibition Improves Bone Architecture and Reduces Tumor Burden when Combined with Velcade in Myeloma Bone Disease

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Multiple myeloma is often characterized by bone disease where the myeloma cells grow in bone and induce bone destruction. While many therapeutic strategies exist to treat the bone disease, none fully reverse bone loss or eliminate tumor burden. Since previous studies indicate that TGF-β inhibition improves bone volume and reduces tumor growth in several solid tumor models, we hypothesized that TGF-β inhibition would reduce tumor growth and improve bone architecture in myeloma-bearing mice. Our previous data have indicated that 1D11, a TGF-β inhibitory antibody (Genzyme) increases BV/TV but also tumor burden when the 5TGM1 myeloma cells were inoculated into the immune compromised, Rag 2–/– (Taconic), mice. Because we were concerned that the lack of B and T-cells may affect response to therapy, we repeated the study in the KalwRij model using combination therapy with the anti-myeloma drug, Velcade. Mice were treated with 10 mg/kg, 3x/week, of the anti-TGF-β antibody (1D11, Genzyme, n = ?) or the control antibody (13C4, n = ?) and with Velcade (.5 mg/kg, 3x/week) beginning at the time of tumor injection for 4 weeks. In this study, we found that Velcade + 1D11-treatment, but not 1D11-alone, reduced spleen weight (49%, p≤.001) and reduced IgG2bκ (40%, p≤.05) compared to the vehicle treated mice, indicating a reduction in tumor burden. Furthermore, both the 1D11 and the 1D11+Velcade groups displayed a reduction in μCT-derived cortical porosity (66%, p≤.05) and an increase in trabecular BV/TV (50%, p≤.001), whereas Velcade alone did not affect bone architecture. The increase in BV/TV was accompanied by a 1D11-related increase in vertebral body strength as assessed by biomechanical testing. Taken together these data demonstrate that 1D11 has significant positive effects on bone architectures, but does not reduce tumor burden in this myeloma model. However, when 1D11 was combine with Velcade a significant reduction in tumor burden was observed in addition to the effects on bone architecture, suggesting a potentially promising strategy for treating myeloma-induced bone disease.

P033

Osteocytes as a Cell of Origin for Osteoblastic Osteosarcoma

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Osteosarcoma (OSA) is the most common primary bone tumor and primarily impacts adolescents. Little is known about the etiology of OSA including the progenitor cells that give rise to the disease. It has been suggested OSA originates from osteoblasts; however, the potential of osteocytes (OCy) to be a source of origin for OSA has not yet been explored. OCy are terminally differentiated osteoblasts sequestered in mineralized bone. OCy maintain homeostasis through mechanotransduction. The goal of this study was to determine if the OCy's could serve as cells of origin for OSA.
To determine if OCy could grow as OSA, MLO-Y4 cells, a murine OCy cell line immortalized using SV-40 large T-antigen, were injected subcutaneously and orthotopically (into tibiae) into SCID mice. Radiographic and histologic measurements were taken from these mice to determine the growth rate and characteristics of the resulting tumors. To determine if OSA express OCy markers, murine, human, and canine OSA cell lines were subjected to quantitative PCR for expression of markers typically utilized in defining OCy, such as dentin matrix acidic phosphoprotein 1 (DMP1), podoplanin (PDPN), and fibroblast growth factor 23 (FGF23). Protein expression of OCy markers was validated in the murine and human OS cell lines. Clinical OSA were evaluated for expression of OCy markers using microarray data from ONCOMINE.

MLO-Y4 cells were tumorigenic when injected subcutaneously and orthotopically into SCID mice. Orthotopic tumors showed mixed osteoblastic/lytic lesions commonly associated with clinical OSA. Human and murine OSA cell lines were found to express OCy marker mRNA and protein. A significant over-expression of DMP1, a definitive OCy marker, was observed specifically in patients with osteoblastic osteosarcoma relative to other OS subtypes.

In summary, OSA cell lines express OCy-specific markers and the murine MLO-Y4 OCy cell line is tumorigenic in bone. These results suggest that we have identified OCy as a novel cell of origination for OSA. Investigation of OCy as mediators of OSA is critical for understanding the basic etiology of OSA and developing novel therapeutics to target a disease where little has been done to improve survival over recent decades.

P034

Serum Interleukin-8 and PTHRP(12–48) Mediate Distinct Breast Cancer Events

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Despite the improvements in population screening and health awareness, a significant number of patients present with advanced breast cancer and subsequent skeletal related events (SREs). Bone is a common site for cancer metastasis, and SREs connote a dramatic change in the prognosis for the patient and significantly increase the morbidity associated with disease. We recently identified PTHrP(12–48) as a biomarker in the circulation of women with breast cancer that correlates with and predicts bone metastasis. Similarly, we have also shown that the cytokine interleukin 8 (IL-8/CXCL8) is highly expressed in human metastatic breast cancer cells, and is able to directly stimulate osteoclastogenesis and bone resorption in an orthotopic mouse model of breast cancer bone metastasis. In this study, we investigated whether circulating levels of IL-8 and PTHrP(12–48) were associated with increased bone resorption and bone metastasis and investigated their biologic function. Using breast cancer patient serum, we measured IL-8 and PTHrP(12–48) levels and identified a significant correlation between both circulating biomarkers and increased bone resorption (NTx) in these patients. Interestingly, although IL-8 was positively correlated with bone resorption ($p<0.001$), it was not predictive of, or correlated with, the presence of bone metastasis (with or without NTx). In contrast, logistic regression models demonstrated that PTHrP(12–48) (in combination with serum NTx) predicted the presence of bone metastasis with high sensitivity and specificity. *In vitro*, IL-8 via its receptor CXCR1 was a potent inducer of osteoclast formation, resorption and motility whereas no positive effects of PTHrP(12–48) interaction with the PTH1 receptor were observed in any physiologic setting. The pro-osteoclastogenic activity of IL-8 was confirmed when transgenic mice expressing human IL-8 were examined and found to have a profound osteoporotic phenotype, with elevated bone resorption, inherently low bone mass and numerous fractures. Collectively, these data suggest that circulating PTHrP(12–48) and IL-8 may play important, but distinct, roles in breast cancer bone metastasis.

P035

Myeloablative Therapies Directly Cause Bone Loss: Implications for Preventing Bone Loss in Cancer Patients

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Reduced bone mass is one of the most common complication occurring in long-term survivors of cancer therapies. Aside from osteolytic lesions occurring in response to some cancers, the bone loss is most commonly attributed to the use of corticosteroids as part of the treatment regimes. Aims: In this study we sought to determine 1) the effects that common myeloablative therapies have on bone in the absence of corticosteroids or disease, and 2) whether the bisphosphonate, zoledronic acid (ZA) could protect against therapy-induced bone loss. Methods: Cohorts of eight-week old male mice were treated with different myeloablative therapies: lethal total body irradiation (IRR) with or without bone marrow transplantation (BMT) and single doses (150 mg/kg) of the chemotherapy agent, 5-fluorouracil or cyclophosphamide. We investigated the effects of a single dose of ZA (10 µg/kg) injected 3 days prior to BMT. We also undertook studies in allogeneic BMT patients to determine why they are more susceptible to bone loss, and to understand how rapidly the bone loss occurs in these patients. Results: All treatments caused rapid, irreversible loss of trabecular bone in mice compared to age-matched littermate controls, as assessed by micro-computed tomography. Bone loss was associated with higher bone turnover, as indicated by increases in the numbers of osteoblasts and osteoclasts, as measured using histomorphometry and serum protein. Inhibition of osteoclasts with ZA prevented the bone loss and was not detrimental to blood cell production. In each of the cancer therapy models, the greater bone resorption to bone formation was mediated, at least in part, by increased inflammation in response to therapy. In allogeneic BMT patients, DXA analysis showed significant bone loss was observed within 100 days post-BMT. Inflammatory cytokines were significantly increased in the serum taken from these patients between 8 and 15 days post-transplant. Conclusion: Bone loss occurs rapidly in response to a range of cytotoxic therapies independent of corticosteroid use or disease. This is likely due to inflammation occurring in response to myeloablation, leading to increased osteoclastogenesis. Furthermore, administration of a bisphosphonate prior to cancer therapy can prevent the bone loss. Collectively these studies suggest that blocking osteoclast activity prior to commencing cytotoxic therapies may reduce late fracture risks in cancer patients.

P036 Abstract presentation declined.
Osteocytes as Potential Targets of Proteasome Inhibitors in Multiple Myeloma Bone Disease

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Multiple myeloma (MM) is characterized by unbalanced and uncoupling bone remodeling. Recently we have shown that osteocytes are involved in MM bone disease (MBD) through an increased cell death and apoptosis. Accordingly MM patients are characterized by a reduced number of viable osteocytes related to the presence of osteolytic lesions. Proteasome inhibitors currently used in the treatment of MM are able to stimulate osteoblast formation but their potential effects on osteocytes are not known and have been investigated in this study.

The potential effect of Bortezomib (BOR) on osteocyte viability was checked in murine osteocytic cell line MLO-Y4 and in the human pre-osteocytic one HOB-01. BOR and the proteasome inhibitors MG262 and MG132 significantly blunted MM-induced osteocytic cell death. By a confocal microscopy analysis we show that proteasome inhibitors significantly reduced authophagy and at least in part apoptosis of osteocytes co-cultured with MM cells and blocked Dexamethasone (DEX)-induced autophagy. Parathyroid hormone short-term treatment potentiated the *in vitro* effects on osteocytes of the proteasome inhibitors.

To translate in a clinical perspective these *in vitro* observation we performed a histological evaluation on bone biopsies of a cohort of 37 newly diagnosis MM patients (31 symptomatic and 6 smoldering MM). Bone biopsies were obtained at the diagnosis and after an average time of 12 months of treatment or observation. Osteocyte viability was evaluated in a total of 500 lacunae per histological sections. A significant increase of the number of viable osteocytes was demonstrated in MM patients treated with BOR-based regimen as compared to those treated without BOR ($p=0.017$). By TUNEL assay and confocal microscopy we observed a significant reduction of both osteocyte apoptosis and authophagy in MM patients treated with BOR. On the other hand, any significant difference in osteocyte viability was not observed in patients treated with Thalidomide or Immunomodulatory drugs ($p=0.7$). A multiple regression non-parametric analysis showed that BOR had a significant positive impact on osteocyte viability ($p=0.042$) and counterbalanced the negative effect of DEX treatment ($p=0.035$).

Our data indicate that osteocytes are potential targets in MBD being affected by proteasome inhibitors that block osteocyte death supporting their use to preserve bone integrity in MM patients.

P037
Novel 99MTC(I)-Labeled Bone-Seeking Molecules for Bone Imaging

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Several malignant tumors, especially breast and prostate cancers, have high tendency to metastasize to bone. Moreover, the development and progression of bone metastases have a high impact in the quality of life of patients, causing drastic complications such as bone pain, pathologic fractures, hypercalcemia and spinal cord compression. Bisphosphonates (BPs) are a class of compounds with high affinity for the bone mineral matrix, binding strongly to the hydroxyapatite (HA) crystals and accumulate in areas of high bone metabolism such as metastases. Consequently, BPs are being extensively explored for the treatment of several bone diseases and as molecular imaging probes. In fact, complexes of 99mTc with BPs such as methylene diphosphonate (99mTc-MDP) and hydroxymethylene diphosphonate (99mTc-HMDP) are widely used as radiopharmaceuticals for the assessment of bone metastases. Despite their proven clinical success these radiopharmaceuticals present several limitations, namely low specificity, uncertainty in the radiopharmaceutical's molecular structure and long acquisition time after injection. Consequently, there is a need for rational design of novel 99mTc-bisphosphonates based radiopharmaceuticals with improved chemical and biological properties.

Aiming to find novel bone-seeking radiotracers, we have successfully synthesized and characterized a set of novel organometallic compounds of the type fac-[M(CO)3(k3-Pz-BP)] which contain a bisphosphonate unit (bone seeking agent), and the metal fragment fac-[M(CO)3] (M=99mTc, Re). The stable radioactive complexes were obtained with high yield and radiochemical purity (>95%) and have been characterized by comparing their chromatographic HPLC gamma-traces with the UV-vis traces of the respective Re surrogates. *In vitro* hydroxiapatite binding studies revealed that the complexes presented bone seeking potential. Herein, we will also present the biodistribution studies in Balb-c mice and compare their biological properties (e.g. bone-targeting properties) with 99mTc-MDP studied in the same conditions. The best complexes presented fast blood clearance and high bone uptake. Notably, the target to non-target ratios are considerably higher than the ones obtained for 99mTc-MDP, the gold standard for bone imaging in nuclear medicine.

P038

Pharmacologic Modification of the Osteoblastic Niche Affects Homing of Cancer Cells without Affecting Metastasis Development

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The number of cancer cells in the bone marrow correlates with decreased survival due to metastasis formation in patients with breast and prostate cancer. PTH injection has been used to increase the number of HSC niches and hence homing of cancer cells to the bone marrow suggesting a role for the osteoblastic niche in homing and bone metastasis formation. Our aim was to stimulate the osteoblasts without the osteoclasts and determine the effects of the osteoblasts on cancer cell homing and growth.

Administration of PTH and zoledronic acid (ZA) over 4 days resulted in a significant increase in BMD equivalent to treatment with ZA alone (CT vs. PTH/ZA: 44%, $p<0.05$). Osteoblast activity was increased 220% with PTH/ZA ($p<0.01$). Intracardiac injection of MDA-MB-231B+ cells in treated mice resulted in almost doubling of the number of cancer cells in the bone marrow after 24 hours (CT: 10.3 ± 3.7 vs. PTH/ZA: 18.4 ± 2.1 MDA cells/106 murine bone marrow cells, $p<0.05$). Neither PTH nor ZA alone affected the number of cancer cells, however. Using CD34+ human stem cells confirmed the increase in homing in the PTH/ZA group (by 65%, $p<0.05$).

Neither the percentage nor the number of HSC in the bone marrow was affected ($n=5$/group). Instead, mesenchymal stem cells (MSC) were diminished (by 32%, $p<0.05$). This decrease was confirmed *in vitro* (CFUs decreased by 21%, $n=3$ replicates/group). This suggests that a decrease in MSCs and not an increase in HSCs is responsible for the increase in homing in our model.

We then sought to determine whether increased homing of cancer cells to the bone marrow affected survival and cancer growth. Compared to CT, both ZA alone and PTH/ZA resulted in increased median survival from 7 weeks to 14 weeks (p<0.001). We were unable to find any decrease in survival in PTH/ZA compared to ZA alone, however, despite the increase in homing of cancer cells in the PTH/ZA group. Furthermore, the number of lesions at 3 weeks after intracardiac injection, total tumor burden and burden/lesion were not increased in PTH/ZA vs. ZA alone ($N=8$ mice/group).

Taken together, these data suggest that a change in mesenchymal stem cell numbers even in the absence of HSC changes affects the number of cancer cells that home to the bone marrow. In addition, the number of cancer cells that lodge in the bone marrow does not seem to be the key mediator for increased number of metastasis. Instead, changes in the cytokine environment in the bone marrow may be more important.

P039

Evidence for the Importance of the Osteoblastic Niche in the Response to Cancer

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The importance of the osteoblastic niche in hematopoiesis has been increasingly recognized. Fibronectin is a matrix protein that stimulates osteoblast (Ob) differentiation and can bind to immune cells. The aim of this work is to characterize how osteoblast fibronectin affects hematopoiesis and the implications of these effects.

To delete fibronectin in osteoblasts, collagen α1(I) attached to cre was introduced in fibronectin floxed mice. In the bone marrow (BM) CD11b+ cells were diminished (by 27%, p<0.0.05) in conditional knockout mice (cKO) compared to controls (CT). Culturing CT or cKO BM with CT Obs resulted in a comparable increase in the percentage of CD11b+. cKO Obs failed to induce an increase. This effect was further confirmed in transplantation experiments of cKO BM in CT mice and CT BM in cKO mice. Thus osteoblasts support CD11b+ differentiation.

Because CD11b+ cells define a population of cells (myeloid-derived suppressor cells: MDSCs) that inhibit the immune response to cancer we evaluated cancer growth. Induced skin B16 melanoma lesions were smaller in cKO mice (by 73%, $p<0.05$), and less CD11b+ cells could be detected in the tumor. Injection of MDA-MB-231B/luc+ in the tibia of T-cell deficient mice similarly resulted in hampered local tumor growth already during the first few days after injection (68%, $p<0.05$). This decrease remained up to the time of euthanasia but the difference to CT diminished (44%, $p<0.05$). This suggests that the effect of low CD11b+ cells is more pronounced during the early stages of cancer formation, and is not mediated by T-cells. Both, the cKO tumors and isolated CD11b+ cells from the cKO tumors showed a cytokine expression profile suggestive of a more immune active and less cancer supportive environment.

We then asked whether this immune effect could be enhanced to completely prevent cancer growth. We therefore used a fibronectin knockdown MDA cell line (Kd). Injecting these cells in CT results in decreased cancer growth and blood vessels (by 83 and 46%, $p<0.005$). Injecting Kd cells in cKO mice resulted in almost complete failure of cancer formation. Indeed, only 2 out of 11 Kd in cKO grew, while all control cells grew in CT mice ($p<0.05$). Thus, the absence of fibronectin in cancer cells combined with a decrease in CD11b+ cells results in the failure of cancer cells to induce tumor.

In summary, a decrease in functional CD11b+ cells together with diminished angiogenesis can combine to prevent cancer growth.

P040

Development of Short- and Long-Term Preclinical Models of Myeloma with Low Variability to Investigate the Effects of Therapeutics on Myeloma Bone Disease

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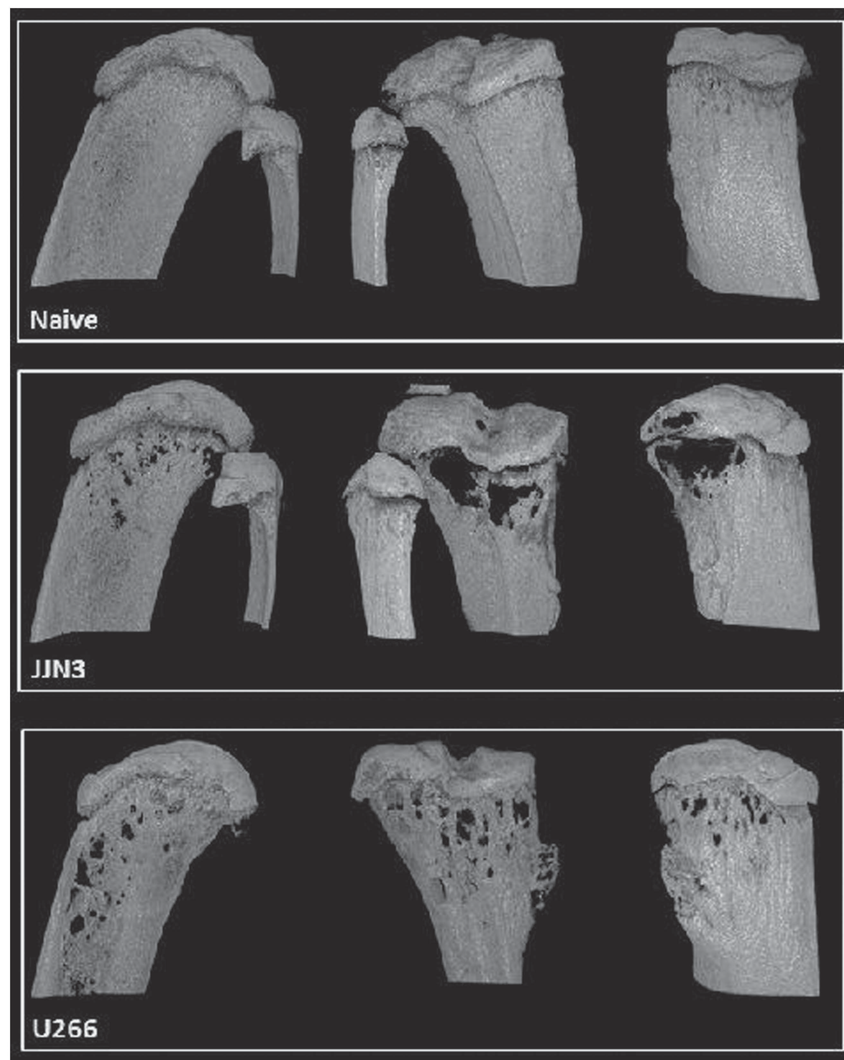
Preclinical animal models of multiple myeloma range from syngeneic mouse models to xenograft models of human myeloma in mice. Derivatives of these animal models are constantly being developed which has enhanced our understanding of myeloma. Here we describe two preclinical models of myeloma using the immune-suppressed NOD/SCID-GAMMA (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, NSG) strain of mice to specifically study the effects of therapeutic agents on myeloma osteolytic disease. NSG mice have recently been used successfully in developing human xenograft models of myeloma using OPM2, U266, RPMI-8226, HuNS1 and MM.1S cell lines to measure effects on tumour load. However, there is little or not data on the bone disease using this strain.

Groups of 7–8 week old female NSG mice (n=5/group) were injected intravenously via the tail vein with 100 μl PBS (naïve control), 1×10^6 JJN3 cells or 1×10^6 U266 cells. At the first signs of morbidity in each tumour group, all animals were sacrificed along with a control group. Micro-CT analysis was used to measure trabecular bone volume, trabecular number, trabecular thickness, number of cortical bone lesions and the total lesion area in the right tibiae. The numbers of osteoclasts and osteoblasts on tibial cortico-endosteal surfaces were also assessed using standard histomorphometric methods. Tumour burden was assessed in bone marrow flushes of the left femora at the end stage of disease by flow cytometry using an anti-human HLA-ABC-APC antibody. A therapeutic study was then performed injecting groups of female untreated NSG mice with PBS or JJN3 cells, and NSG mice treated with zoledronic acid (125 μg/kg 2x/wk) prior to injection of JJN3 cells. Statistical analysis was performed, where significance from the PBS controls was indicated by $p<0.05$.



Representative micro-CT images of the right tibiae from non-tumour (Naive) or tumour-bearing (JJN3 or U266) NSG mice.

Injection of JJN3 cells into NSG mice resulted in a short-term model of myeloma with animals becoming ill 3 weeks post-injection of tumour cells and injection of U266 cells resulted in a long-term model of myeloma with animals becoming ill 8 weeks post-injection of tumour cells. Both models showed low variability in tumour growth in bone, had osteolytic lesions as a result of increased in osteoclastic bone resorption and reduced osteoblastic bone formation. Treating NSG mice with zoledronic acid throughout the duration of the study prevented JJN3-induced bone disease.
Here we have shown, in addition to studying tumour load, NSG mice are an ideal strain to study myeloma bone disease.

P041

Osteolytica, Bespoke Osteolytic Lesion Analysis Software, Substantially Improves Accuracy of Cancer Induced Osteolytic Lesion Measurement Compared with Previous Sub-Optimal Methods

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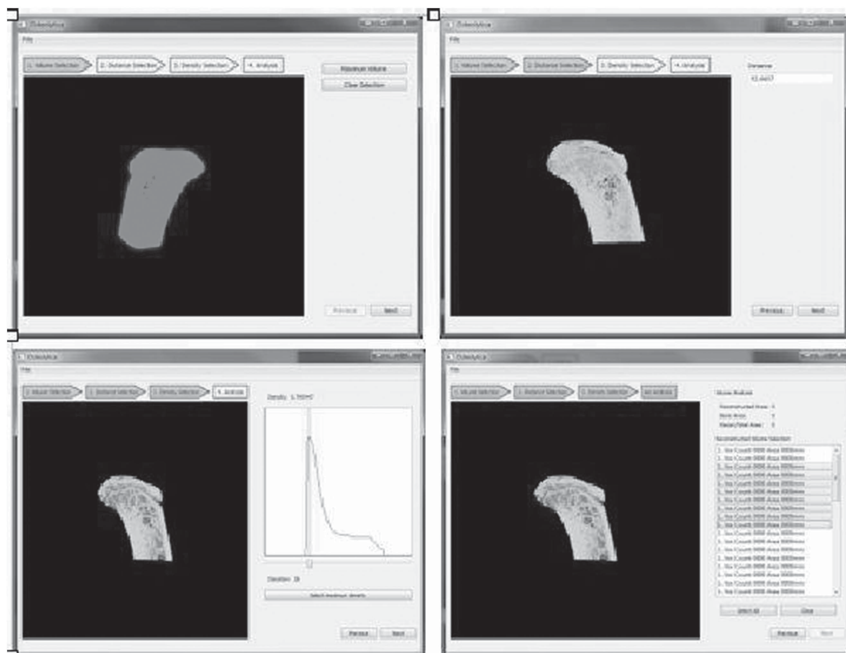
Bone is a complex 3d structure and measurement of the area and number of cancer induced osteolytic lesions is notoriously difficult. We have developed, in collaboration with computer modellers, a bespoke osteolytic lesion analysis software package which shows substantial improvement in the accuracy of osteolytic lesion measurement compared to previous sub-optimal methods of analysis which include manual counting and 2-d image analysis using ImageJ software. Data sets from microCT are analysed by Osteolytica software in four stages. Firstly, the region of bone to be analysed is selected; secondly, maximal lesion diameter is defined; thirdly, intact bone is reconstructed and lesions identified; finally, lesion area is analysed and subtracted from intact bone area. Results are expressed as both total lesion area and as a proportion of total bone area. We analysed five tibiae using ImageJ 2-d software, visualising static images of three tibial aspects. We then performed 3d analysis of the same five tibiae using Osteolytica software. Total proportion of lesion area was 45% higher using Osteolytica than ImageJ, p=0.012. Osteolytica software is a novel, accurate, rapid and robust method of analyzing osteolytic bone disease likely to supersede previous sub-optimal methods of analysis.



P042

Radium-223 Dichloride Monotherapy and Combination Therapy with Zoledronic Acid or Doxorubicin Improve Survival in a Mouse Model of Breast Cancer Bone Metastasis

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Bone metastases result in significant morbidity and poor prognosis. Radium-223 dichloride (Xofigo®) is an alpha-emitting calcium mimetic that binds to hydroxyapatite in bone and via efficient osteoaffinity provides targeted radiation therapy against bone metastases. Xofigo® received recently FDA-approval for the treatment of patients with castration-resistant prostate cancer with symptomatic bone metastases. We have previously reported that radium-223 decreases osteolysis and tumor burden in bone in a mouse model of breast cancer bone metastasis in preventive and micro-metastatic settings (Suominen *et al*. CIBD Meeting 2012), as well as in mice with

established bone metastases (Suominen *et al*. AACR Annual Meeting 2011).

Here, we investigated the effects of radium-223 dichloride monotherapy compared to and in combination with either doxorubicin (dox) or zoledronic acid (zol) on survival in a mouse model of established breast cancer bone metastasis. Human MDA-MB-231(SA)/GFP cells were inoculated intracardially into nude mice, and 15 days later, a single dose of vehicle, radium-223 dichloride (300 kBq/kg, iv) and/or zol (0.1 mg/kg, sc) was administered. Dox (5 mg/kg, ip) was administered once weekly. Radium-223 dichloride monotherapy extended time to sacrifice (P=0.039), unlike dox or zol monotherapy which did not improve survival as compared to the vehicle group. Radium-223 dichloride in combination with zol (P=0.004) or dox ($P < 0.001$) also extended time to sacrifice as compared to the vehicle but did not provide additional survival benefit as compared to the radium-223 dichloride monotherapy.

The effect of radium-223 dichloride in inducing double-strand breaks in cancer cells was evaluated by immunohistochemical staining of γ-H2AX molecules. A 3-fold increase in the number of tumor cells with double-strand breaks in the radium-223 dichloride-treated as compared to the vehicle control mice was observed ($P < 0.001$). This finding supports our previous observations that radium-223 dichloride has an effect on both tumor cells and osteoclasts.

In conclusion, radium-223 dichloride therapy alone or in combination with dox or zol increases survival in breast cancer bone metastasis mouse model via dual action by targeting tumor growth and osteolysis, both important players in the destructive vicious cycle of bone metastasis. Our findings strongly support the development of radium-223 dichloride for the treatment of patients with bone metastatic breast cancer.

P043

Combined Treatment with Halofuginone and Zoledronic Acid is More Effective to Inhibit Breast Cancer Bone Metastases Than Monotherapy

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Bone metastases cause significant morbidity and are incurable with current therapy. Halofuginone (Hfg), a plant alkaloid derivate, reduces osteolytic bone metastases through inhibition of TGFβ and BMP signaling, both of promote bone metastases and regulate bone remodeling. Here, we determine the effects of halofuginone combined with zoledronic acid (ZA), FDA-approved therapy for bone metastasis. We also tested the effect of Hfg and ZA on normal bone unaffected by cancer.

Hfg (5 μg/d) and ZA (5 μg/kg/3x per wk) alone or combined were administered in nude mice with MDA-MB-231 breast cancer bone metastases in a preventive treatment. Hfg and ZA alone significantly reduced osteolytic area compared to vehicle ($1.9\pm.9$ vs $3.5\pm2\,mm^2$, $p<0.001$ and $1.3\pm.4$ vs $3.5\pm.2\,mm^2$, $p<0.001$, respectively). Combined treatment with Hfg and ZA significantly reduced osteolysis area in mice better than either treatment alone (0.4 ± 0.1 vs $3.5\pm.2\,mm^2$, $p<0.001$). Histomorphometric analysis confirmed this.

Since TGF-β and BMP regulate bone remodeling, we treated female-nude mice unaffected by tumor with Hfg (5 μg/d) for 4 wks. Hfg significantly decreased whole body BMD (20 ± 3 vs $12\pm2\,g/cm^2$, $p<0.01$), as well as spine, tibia, and femur BMD. μCT analysis showed that Hfg (5 μg) significantly decreased trabecular bone volume. This was associated with increased osteoclast number as well as decreased osteoblast number, mineralizing surface and bone formation rate.

We then tested whether Hfg-induced bone loss can be prevented by the anti-resorptive ZA. Hfg (5 μg/d) and ZA (5 μg/kg/3x per wk) alone or combined were administered to female-nude mice over 4 wks. Mice treated with Hfg alone had lower BMD, as well as reduced bone stiffness measured by femoral 3-pt bending test. Concomitant treatment with ZA completely prevented the bone loss (40 ± 3 vs $10\pm2 g/cm^2$, $p<0.01$) and bone stiffness deficiencies induced by Hfg.

In conclusion, Hfg reduces breast cancer bone metastases in mice, and this effect is enhanced when Hfg is combined with ZA. Our data also show that Hfg induces bone loss in bone unaffected by tumor, which is rescued by the anti-resorptive ZA. Hfg completed Phase II trials in sarcoma patients and could rapidly be brought to the clinic for the treatment of bone metastases in breast cancer patients. However, due to its effects increasing osteoclast activity and reducing bone mass, Hfg should be combined with anti-resorptive therapy.

P044

Translocator Protein (TSPO) as a Marker for Early Tumor Establishment in Bone

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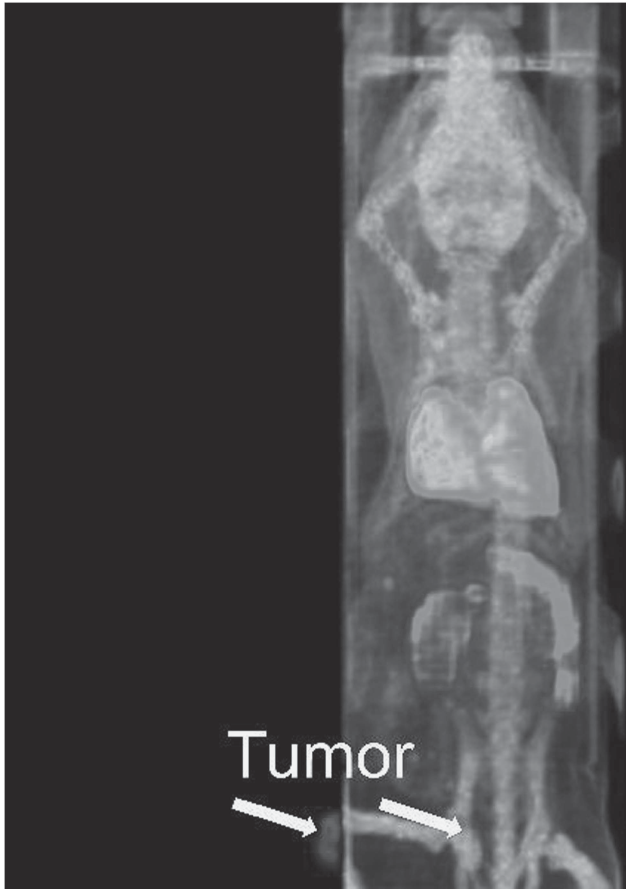
Current imaging modalities used for animal models of bone metastases primarily detect tumors at late stages of disease, which has led to a severe lack of knowledge of the mechanisms regulating early tumor establishment in bone *in vivo*. Therefore, it is critical that imaging techniques be developed that better detect tumor cells and bone destruction earlier in the progression of the disease. Specifically, we have examined the potential of an emerging positron emission tomography (PET) technique using the aryloxyanilide [18F]PBR06, a ligand specific for the translocator protein (TSPO), which we hypothesized would allow us to detect tumors in bone earlier and more precisely than the current imaging standards of fluorescence and radiography. In order to test the utility of TSPO PET with [18F]PBR06, 4-week old athymic nude mice were inoculated with 1×10^5 MDA-MB-231-GFP cells by intracardiac injection (n=13 mice). Imaging using Faxitron, FDG PET, TSPO PET, CT, and fluorescence began 7 days after tumor cell inoculation and continued through 4 weeks. After 3



weeks, obvious lesions were detectable throughout the skeleton by conventional imaging techniques (4 out of 13 mice). When compared to Faxitron and fluorescence imaging of the same mice, TSPO PET was able to detect lesions (10 out of 13 mice) 1 week prior to detection using other parameters. Immunohistochemistry confirmed TSPO expression in the tumor cells, verifying that the *in vivo* signal detected was due to tumor-specific uptake. Furthermore, we evaluated the expression of TSPO in the breast cancer cells at different stages of disease progression. To test this, 2.5×10^5 MDA-MB-231-GFP cells were seeded onto rigid (bone) and compliant (soft tissue) 2D polyurethane scaffolds and protein was harvested 48 hours later. Western blot analysis was performed with anti-TSPO antibody and detected with chemiluminescence. TSPO expression was 2-fold higher in MDA-MB-231-GFP cells grown on rigid scaffolds suggesting that TSPO is more highly expressed in tumors in a rigid bone environment rather than soft breast tissue. Our studies indicate that TSPO PEt allowed for earlier quantifiable information regarding tumor growth in bone. Therefore, we reason that TSPO imaging may be a promising clinical marker for the detection of small tumors in bone.

P045

Activation of the Wnt Pathway through use of AR79, a Glycogen Synthase Kinase 3β Inhibitor, Promotes Prostate Cancer Growth in Soft Tissue and Bone

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Due to its bone anabolic activity, multiple methods to increase Wnt activity, such as inhibitors of dickkopf-1 and sclerostin, are being clinically explored for bone-related diseases. Glycogen synthase kinase (GSK3β) inhibits Wnt signaling through inducing β-catenin degradation. Therefore, AR79, an inhibitor of GSK3β, is being evaluated as a bone anabolic agent. However, a concern of Wnt activation is its potential ability to promote tumor growth. Accordingly, the goal of this study was to determine if AR79 impacted progression of prostate cancer (PCa) tumors in soft tissue and bone. PCa tumors were established in subcutaneous and bone sites of mice followed by AR79 administration. Tumor growth, activation of β-catenin, cell proliferation (Ki67 expression) and apoptotic activity (caspase 3 activity) were measured. Additionally, PCa and osteoblast cells lines were treated with AR79 and β-catenin status, proliferation and proportion of the ALDH+CD133+ stem-like cells was determined. AR79 promoted PCa growth, decreased phospho-β-catenin expression and increased total and nuclear β-catenin expression in tumors and increased tumor-induced bone remodeling. Additionally, it decreased caspase 3 and increased Ki67 expression. In addition, AR79 increased bone formation in normal mouse tibiae. AR79 inhibited β-catenin phosphorylation, increased nuclear β-catenin accumulation in PCa and osteoblast cell lines and increased proliferation of PCa cells *in vitro*. Furthermore, AR79 increased the ALDH+CD133+ cancer stem cell-like proportion of the PCa cell lines. We conclude that AR79, while being bone anabolic, promotes PCa cell growth through Wnt pathway activation. This suggests caution should be used with the various Wnt pathway-activating agents, such as sclerostin antibody, being currently evaluated for therapy of bone disease.

P046

New Material Based on Chitosan Used for Bone Replacement

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The bones affected by cancer in two ways - the primary bone cancer and metastases. About the 50% of the tumors can spread or metastasize to the skeleton. The highest risk of bone metastases have cancer of lungs, breast, prostate, kidneys and thyroid. The treatment of bone cancer and metastatic bone disease includes non-operative (chemotherapy, radiation for bone, including radioisotope therapy) and operative procedure. The surgical treatment of bone cancer is becoming more common due to increased equipment surgery

and increased use of plastic materials for bone defects. Despite the use of a wide range of bone implants, a search for new materials which have a high degree of osteointegrative and osteostimilatory properties and to reduce the complications of the postoperative period and improve the quality of life of patients. Especially high need to develop new materials for countries with low levels of economy due to the high cost of commercial bone implants.
In our paper has been analyze a new biomaterial based on chitosan and calcium apatite for use as a bone implant. A series of chitosan/hydroxyapatite composites has been synthesized in aqueous medium from chitosan solution and soluble precursor salts by a one step coprecipitation method.
XRD patterns of the materials suggest the presence of nanocrystalline apatite with the average crystallite size of 20 nm. The results of IR spectroscopy studies suggest the presence of carbonate ions in the synthesized materials. Thus, this relatively simple synthesis procedure allows obtain composite materials with nanocrystalline carbonate-substituted hydroxyapatite similar to natural bone bioapatite.
The objective of the present study was to examine *in vivo* behavior of simple chitosan/hydroxyapatite scaffolds placed into a perforated tibial defect of model laboratory animals. In these experiments materials have shown good osteoconductive properties. Histomorphological studies have shown that the porous chitosan/hydroxyapatite materials undergo almost complete biodegradation. The complete replacement of porous chitosan/hydroxyapatite composite implant by newly formed bone tissue within bone defects in rats takes place on the 24th day of implantation.
The results of this study suggest the high potential of simple chitosan/hydroxyapatite composite scaffolds produced by the one-step co-precipitation method as a filling material for bone defect grafting including after the bone cancer disease operative treatment.

P047

Twist1 Expression in Breast Cancer Promotes Bone Metastasis Formation

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Background: The basic helix-loop-helix transcription factor Twist1 initially identified as a major regulator of tissue organization in early embryogenesis is aberrantly reactivated in human cancer. Twist1 expression in primary breast tumors is associated with disease aggressiveness and poor survival mainly by conferring growth advantage to tumor cells and by facilitating their intravasation in the circulation and their dissemination to the lungs. During these processes Twist1 promotes epithelial-to-mesenchymal transition of circulating and disseminating tumor cells (DTC). In this respect, Twist1 is expressed in breast cancer DTC that persist in the bone marrow after chemotherapy. However its involvement in breast cancer bone metastasis formation is unknown.
Results: To address this question, we chose the human osteotropic MDA-MB-231/B02 breast cancer cell line which has a mesenchymal phenotype, but does not express Twist1. Twist1 ectopic expression in B02 cells substantially increased the extent of osteolytic lesions in animals, being 50% larger than that of animals bearing mock-transfected tumors, as determined by radiography. This difference was accompanied with a sharp reduction of the bone volume (indicating a higher bone destruction) and a 2-fold increase in the tumor volume compared with mice bearing mock-transfected tumors, as determined by histomorphometry. Osteoclast activity was not alterated *in vivo*, neither *in vitro* by Twist1 expression pointing to its specific role in regulating skeletal tumor outgrowth. Additionally, the repression of Twist1 expression in B02 breast cancer cells in the presence of doxycycline (dox) abolished the stimulatory effect of tumor-derived Twist1 on bone metastasis formation *in vivo*. Importantly, examination of the bone marrow from untreated and dox-treated animals on day 7 after tumor cell inoculation, at which time there is no evidence of radiographic osteolytic lesions, revealed that the number of DTC colonies in the bone marrow from untreated mice was dramatically increased compared with that of dox-fed animals. *In vitro*, Twist1 expression promoted tumor cell invasion and enhanced microRNA-10b expression, a pro-invasive factor, but was dispensable for growth of breast cancer cells.
Conclusion: Overall, these results establish that Twist1 in breast cancer is important for the engraftment of tumor cells to the bone marrow, facilitating the subsequent formation of osteolytic bone metastases.

P048

Integrin Beta-3 and TGF-Beta Receptor Type II Cross-Talk Induces Osteolysis in Bone Metastatic Breast Cancer Cells

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The importance of the microenvironment in the regulation of tumor cell behavior and bone turnover is increasingly recognized. While many aspects of this environment have been explored, we have shown that the physical rigidity of bone can regulate gene expression of PTHrP and Gli2 in bone metastatic cells. Since studies in soft tissue tumors have demonstrated that $\alpha v\beta 3$ integrin mediates tumor response to rigidity, we hypothesized that the tumor response to bone rigidity was also integrin-dependent. It was found that this response was reliant on the cross-talk of integrins and TGF-β Receptor type II (RII). Limiting integrins $\alpha v\beta 3$, by utilizing a dominant negative Rho associated protein kinase (ROCK) construct, blocked the rigidity-mediated increase in PTHrP expression, while a dominant active construct increased PTHrP expression. Additionally, direct $\alpha v\beta 3$ inhibition by LM609, a $\alpha v\beta 3$ inhibitory antibody, reduced the expression of PTHrP and Gli2 by 2.8 and 10-fold respectively ($p \leq .01$), and reduced PTHrP expression in the ROCK dominant active cells by 9-fold ($p \leq .005$). To more precisely determine the importance of integrin $\beta 3$, we stably

transfected MDA-231 cells with β3 shRNA and measured the cells ability to respond to rigidity using 2D polyurethane (PUR) films. Integrin β3 knock-down reduced PTHrP expression by 54-fold in cells grown on rigid PUR films ($p \leq 001$), but not on compliant films. Since we have previously shown that RII is required for the mechanotransduction response, we investigated whether integrin and TGF-β pathways interacted, finding that β3 and RII co-localized by immunoprecipitation, suggesting a physical interaction between the receptors. Finally, since p38MAPK is known to play an important role in mechanotransduction and the MAPK inhibitor SB202190 reduced PTHrP expression on rigid films by 4-fold ($p \leq .01$), we investigated whether rigidity alone, in the absence of exogenous TGF-β, could upregulate p38 MAPK phosphorylation. It was found that p38 MAPK phosphorylation was increased on rigid films by Western blot, while no changes were observed in the expression of non-phosphorylated p38MAPK. No increase in p38 MAPK phosphorylation was observed in the ROCK dominant negative cells. Taken together these data indicate that integrin β3 is required for tumor cells to sense the rigidity of bone and cross-talks with the TGF-β pathway, suggesting that integrin inhibition may be a valuable target for inhibiting osteolytic tumor establishment in bone.

P049

Rank Expression on Circulating Tumor Cells (CTCS) in Metastatic Breast Cancer (MBC): Clinical Implications

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Background: It has recently been demonstrated that many solid tumors express RANK both in primary sites and in metastatic bone. Moreover RANK expression on cancer cells is associated with bone homing mechanism in early bone metastasis formation. The CTCs represent the “iquid” phase of metastasis and are considered an appealing biomarker for investigating the phenotype and the role of these migrating tumor cells. These evidence allow us to consider the RANK presence on the CTCs surface and its modulation during denosumab therapy as a potential predictor of treatment response in MBC or as a prognostic factor of skeletal outcome.
Experimental Design: An automated sample preparation and analysis system for enumerating CTCs (CellSearch) was integrated with a specific mAb for detecting RANK-positive CTCs. CTCs from blood were analyzed at baseline and at day2, 7, 14, 28 after the first denosumab administration. Time-to-first-SRE was estimated by Kaplan-Meier analysis and the p-value calculated by log-rank test according to CTC and RANK-positive CTC counts at each time point of the blood draw calendar. A companion algorithm (ΔAUC) was developed to express the difference between RANK-positive and RANK-negative CTC concentration-Time Area (AUC), as calculated according to the following formula:
ΔAUC= RANK-positiveCTC AUC - RANK-negativeCTC AUC

Results: 34 bone MBC patients (age 32–89) are currently included. At the first blood draw 5 MBC were CTC positive and RANK-negative and 16 out of 34 MBC were CTC positive and RANK-positive (RANK-positive CTCs from 3.6% to 100%). The detection of RANK-positive CTCs at day2 and day7 were associated with significant longer Time-to-first-SRE ($p=0.008$ and $p=0{,}019$ respectively). Categorizing the patients for positive ΔAUC value (that is expression of persistence of RANK-positive CTCs over the first 28 days of treatment) vs negative or 0 ΔAUC (that is expression of RANK-negative CTCs over the same period or balanced numbers of RANK-positive and RANK-negative CTCs) a median of 6 months of delay in terms of Time-to-first-SRE was observed in the positive ΔAUC group of MBC that retain RANK-positive CTCs.
Conclusion: For the first time we demonstrated that RANK expression is detectable by immunofluorescence on CTCs in MBC. The RANK-integrated test has potential for monitoring dynamic changes, in addition to CTC count, to evaluate SRE risk in patients under denosumab therapy. Accrual is ongoing, updated data will be presented at the meeting.

P050

Pharmacologic Inhibition of GSK-3β Activity Impairs Prostate Cancer Stem Cell Function and Suppresses Bone Metastasis

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Prostate cancer is the most common malignancy in males and preferentially metastasizes to the bone/bone marrow microenvironment. Clinical and experimental studies suggest that the subpopulation of cancer stem/progenitor cells (CSCs) play a pivotal role in prostate carcinogenesis, metastasis and therapy resistance. Selective depletion or inhibition of CSCs may thus provide a novel treatment approach for advanced, metastatic prostate cancer. The identification of key signaling pathways or factors in CSCs is, therefore, needed. In this study, we investigated the role of GSK-3β, a negative regulator of Wnt signaling, in prostate cancer stemness and migration *in vitro* and tumorigenicity and bone metastasis *in vivo*.
Selective inhibition of GSK-3β led to; 1. a selective reduction of the CSC subpopulation, 2. a strong decrease in clonogenic potential and 3. impaired migration of prostate cancer cell lines (PC-3, PC-3M-Pro4, C4-2B4, Du-145). These observed effects appear to be largely Wnt-independent since a canonical Wnt signaling suppressor (PNU-74654) could not reverse the anti-tumor effects of GSK-3β inhibition. In line with our *in vitro* data, pharmacologic inhibition of GSK-3β activity resulted in a near-complete loss of tumorigenicity and bone metastasis formation *in vivo*. Mechanistic studies revealed that suppression of GSK-3β activity disturbed the organization of the cytoskeleton, and in particular impaired F-actin polymerization. The latter may account for the observed strong anti-tumor effects of GKS-3β inhibition on CSC maintenance, CSC function, tumorigenicity and (bone) metastasis *in vivo*.
In conclusion, our study highlights a functional role of GSK-3β activity in prostate cancer stem cell biology, tumorigenicity and bone metastasis. Selective inhibition of this kinase may

provide a promising, novel treatment approach for metastatic prostate cancer.

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P051

Roles for Mesenchymal Stem Cells in Metastatic Prostate Cancer Progression and Osteogenesis in the Bone Microenvironment

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Bone is a common target for prostate metastases. In the bone microenvironment, prostate cancer cells induce extensive bone formation/osteogenesis by promoting osteoblast differentiation. This results in painful lesions that are difficult to treat. The precise mechanisms through which prostate cancer cells induce bone formation are not well understood. We posit that the recruitment of osteoblast precursors, known as mesenchymal stem cells (MSCs) to the bone microenvironment may contribute the formation of osteogenic lesions. Using in-vitro and in-vivo studies we aim to elucidate the effects of MSC's on prostate tumor progression in bone. To determine the effect of the MSCs on the growth of the prostate cancer cells, primary mouse MSCs were directly co-cultured at various ratios with luciferase expressing PAIII prostate cancer cells for 24 to 96 hours. Surprisingly, we observed that at a 1:1 ratio, MSCs consistently inhibited PAIII growth with a 55% reduction noted at 96 hours ($p<0.05$). To determine the impact *in vivo*, mice were intratibially inoculated with either PAIII, PAIII/MSCs (1:1), or MSCs alone (n=8/group, 104 total cells in 20 µl). Tumor growth was measured over 15 days using luminescence imaging. Subsequently the tibias were collected for x-ray, micro CT, and histological analysis. Consistent with our *in vitro* observations, we observed *in vivo* that at day 11, the average tumor size in the PaIII/MSC (1:1 ratio) group was 57.7% significantly smaller than the PAIII group alone ($p<0.05$). However, by day 15 the tumors were growing at comparable rates. Tumor bearing tibias were X-rayed and an overall decrease in tumor-induced osteolysis was observed between the PAIII/MSC and PAIII groups. µCT scans of tumor bearing bones show an increase in bone volume in bones injected with MSCs alone and PaIII/MSCs when compared to PAIIIs alone, a finding that was consistent with X-ray and hisotmorphometrical data. In conclusion we have found that in-vitro and in-vivo MSCs have an early inhibitory effect on the growth of prostate tumor cells but ultimately contribute to prostate cancer growth and osteogenesis. Currently, we are examining 1) the mechanisms through which prostate cancer cells drive MSC induced osteogenesis and 2) identifying the MSC derived factors that are inhibiting prostate cancer growth. Understanding the role of MSCs in the prostate tumor-bone microenvironment will lead to the identification of new therapeutic targets to treat the disease.

P052

Lung Cancer Stem-Like Cells (CSC) Display a Retarded Osseous Prometastatic Activity

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Current evidence indicates that a fraction of cells within tumors, called Cancer Stem Cells (CSC), display stem cell-like properties and overt chemoresistance. To enrich for CSC, *in vivo* limited dilution and spheres cultures have been widely used as assays for stem cell properties. These structures present a heterogeneous population of cells, containing a mixture of quiescent CSC together with highly proliferative cells. At present, the extent to which CSC also display prometastatic activity remains poorly understood.

Sphere cultures (SC) derived from a mouse lung adenocarcinoma (LACUN3) showed higher levels of CSC markers including a 5-fold increase in ALDH and a 4-fold increase in Sca-1 (Ly-6A/E), as compared to adherent cultures (non-SC). Consistent with their quiescent phenotype, SC displayed a marked cell cycle arrest in G0/G1 phase that was translated into a 15-fold reduction in *in vitro* growth kinetics as compared to the other conditions. More importantly, the evaluation of quiescence by fluorescent-dye retention was 80% higher in SC than in non-SC. As expected, SC presented a 6-fold higher colony formation activity in soft agar assay, as well as a 40% increase in resistance to paclitaxel. All these experiments were repeated with the human lung cancer cell line H460, where SC overexpressed ALDH and ABCG2 markers and were also endowed with quiescent properties.

To delineate the metastatic activity of SC, upon intracardiac inoculation into nude mice, bioluminescence imaging (BLI) showed a dramatic delay in the appearance of bone metastatic lesions as compared to non-SC. At day 21 postinoculation, BLI in SC-injected animals showed a striking decrease in osseous metastatic tumor burden as compared to non-SC mice. Analogous delay was observed when SC were disaggregated and cultured as adherent cells before inoculation. Similar results were obtained when *in vivo* experiments were repeated with other cell lines.

Interestingly, using subcutaneous injection, tumor growth was similar (SC vs non-SC). In contrast, assessment of osseous colonization activity by intratibial injection of SC cells showed a six-fold decrease in bone tumor burden as compared to non-SC injected mice.

Taken together, these data suggest that differentiation of CSC in the target organ might be required for the emergence of cell variants with high prometastatic activity. We postulate that microenvironmental factors imposed by each target metastatic organ might be crucial in this process.

P053

Nutlin-3 Treatment Spares Cisplatin-Induced Inhibition of Bone Healing While Maintaining Osteosarcoma Toxicity

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Osteosarcoma (OS) patients are treated by a closely timed combination of chemotherapy and surgery, yet remain at risk for postoperative complications associated with decreased bone repair. Cisplatin (CDP) is used to treat OS, yet the effects of CDP on bone repair are not well understood. CDP exposure can occur preoperatively, postoperatively, and even simultaneously with bone repair following OS surgery. Here we demonstrate that the inhibition of bone repair following CDP treatment can be prevented by the administration of a small molecule p53 inducer (nutlin-3). In Study 1, twenty 9-week-old C57BL/6 male mice received CDP + vehicle or CDP + nutlin-3. All mice received vehicle or nutlin-3 (20 mg/kg) IP on day 1, 3, and 4 prior to surgery. The day after the first nutlin injection, all mice received CDP (2 mg/kg/day) IP for two days (day 2 and 3 prior to surgery). On the fourth day after the second CDP injection, the mice underwent placement of an external fixator and osteotomy to the left tibia. Distraction osteogenesis (DO) began three days after surgery at a rate of 0.075 mm b.i.d for 11 days. Comparison of the distracted tibia (radiographs and MicroCT) demonstrated a significant increase in the mineralized area of bone healing in CDP/nutlin-3 treated (52.8% ± 3.7) versus CDP treated mice (30.9% ± 6.5) ($P<0.01$). Histological analysis confirmed the significant increase in bone healing in CDP/nutlin-3 (79.8% ± 2.1) versus CDP (51.3% ± 4.6) ($P<0.001$). Next, the combination of CDP and nutlin-3 treatment was tested in human osteosarcoma xenographs (SJSA-1 cells). Athymic 14 week old female nude mice were injected s.c. with 3×10^6 cells suspended in 0.2 ml Matrigel and PBS. Drug treatment began 7 days post implantation. CDP (9 mg/kg), nutlin-3 (20 mg/kg), and vehicle injections were performed per week as above. Primary tumors were dissected and measured two weeks post treatment. Both CDP and CDP/nutlin-3 treatment respectively resulted in a significant 67% ($P=0.002$) and 78% ($P<0.001$) inhibition of tumor growth relative to untreated vehicle controls. Collectively, these results demonstrate that an intervention involving the induction of p53, prior to surgery protects mesenchymal osteoprogenitors from the toxic effects of CDP, thereby facilitating bone healing, while maintaining the anti-tumor effect of CDP treatment. This scenario bodes well for the treatment of OS while allowing full potential for bone healing post-surgery.

P054

MIR25 as a Regulator of Cancer Stem Cells Maintenance and Bone Metastasis in Human Prostate Cancer via Modulation of Notch Signaling and Integrin Expression

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Experimental and clinical evidence highlight the importance of prostate cancer stem/progenitor cells in carcinogenesis, metastasis and therapy resistance. Previously, we reported that the ALDHhigh cellular subpopulation in human prostate cancer is phenotypically enriched for cancer stem/progenitor cells (CSCs). ALDHhigh cells display strong clonogenic and migratory potential when compared to the more differentiated ALDHlow subpopulation (transit amplifying and differentiated luminal cells). In this study, transcriptional profiling was performed on ALDHhigh vs ALDHlow cells revealing multiple, differentially expressed microRNAs (miRs). Here we investigated the involvement of miR-25 in the acquisition of an invasive phenotype and epithelial plasticity in osteotropic human prostate cancer cell lines (PC-3M-Pro4, C4-2B). We found that ALDHhigh subpopulation of human prostate cancer cells is enriched in stem/progenitor-like cells that show strong tumor- and metastasis-initiating properties in preclinical models of bone metastasis *in vivo*. Our miR profiling revealed that miR-25 was strongly downregulated in ALDHhigh CSCs compared to ALDHlow CSC-depleted prostate cancer cells. Similarly, in primary tumors from prostate cancer patients, the expression of miR25 strongly increased in the epithelial subpopulations during differentiation (stem cells -> transit amplifying cells -> luminal epithelial cells). When overexpressed, miR-25 induced dramatic changes in cell morphology and reduced the mesenchymal, invasive phenotype. Our target analysis reveals that miR25 directly targets multiple steps along the Notch pathway and blocks the acquisition of an invasive phenotype via suppression of integrin-αv (ITGAV) and integrin-α6 (ITGA6). We demonstrated, for the first time, that miR-25 directly targets the 3′UTR of Notch1 and validated this interaction at protein level. In conclusion, our data suggest that miR25 seems to be a master regulator of stem cell maintenance, invasiveness and epithelial plasticity in human prostate cancer through its interaction with Notch signaling and integrin expression. Our data are supported by published studies on the critical role for the Notch pathway and integrins in skeletal metastasis. The identification of miRs and functional validation of their target genes, as we have shown here for Notch, is crucial for the elucidation of the mechanisms involved in CSC maintenance, tumor progression and therapy resistance in human prostate cancer.

P055

How Bone Marrow Microenvironment Prepares the Bone Pre-Metastatic Niche for Breast Cancer Cells?

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Bone metastasis is an incurable complication of breast cancer (BC) affecting approximately 70% of advanced patients. Although novel findings demonstrate the bone marrow (BM)-microenvironment significance in BC progression, the majority of studies have focused on end-stage disease, but little is known about the how is the BM preparing the bone pre-metastatic niche. In this study, we demonstrated that BC induces substantial changes in peripheral blood (PB) and BM-microenvironments of untreated advanced patients without bone metastasis compared with healthy volunteers (HV). Data suggest that high RANKL, MIF and OPG levels in BC patient (BCP)-PB could play a role in the intravasation, angiogenesis, survival and epithelial-to-mesenchymal transition (EMT) phenotypes of circulating BC cells (BCCs). Interestingly, ICAM-1, VCAM-1 and PDGF-AB levels in BCP-BM plasma were significantly higher than HV-values, suggesting that they could be involved in the BCC escape from the blood vessels into the BM. We demonstrated that BCP-BM-mesenchymal stem cells could control the recruitment of the BCCs modifying the MCF-7 and MDA-MB231 cell migration. In addition to its angiogenic and EMT properties, PDGF-AB could be responsible for the higher proliferation of MDA-MB231 cells when we used BCP-BM plasma compared with HV-plasma. Finally, the high PDGF-AB, ICAM-1 and VCAM-1 levels in the BM plasma would increase bone resorption, leading to BCC invasion and proliferation. Taken together, the BM of untreated advanced BCP without bone metastasis provides an ideal environment for the development of the pre-metastatic niche.

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The Bone Marrow Niche Controls the "Stemness" of Disseminated Tumor Cells

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Background: Despite improvements in local treatments of prostate cancer (PCa), bone metastasis remains a major cause of death in PCa patients. The presence of disseminated prostate cancer cells in the bone marrow represents a major therapeutic challenge. The development of methods to identify and characterize disseminated tumor cells (DTCs) is therefore of critical importance to understanding the biology of DTCs and identifying therapeutic targets.
Methods & Results: Since it has been reported that the expression of the cytokeratin and EpCAM may vary considerably *in vivo* and *in vitro*, we explored the extent to which human specific HLA cell surface antigens could serve as alternative targets for the detection of human cells in murine tissues. We found that HLA-ABC antigen was highly identified on human PCa cell lines (PC3, DU145, LNCaP, and C4-2B). To determine if human PCa cells can be isolated from murine marrow with anti-HLA-ABC antibodies, intracardiac injections of PCa cells into SCID mice were performed. Intriguingly, disseminated PCa cells recovered from marrow were highly enriched in CD133+/CD44+ stem-like population. Compared to CD133+/CD44+ cells isolated from culture, the mRNA expression of the self-renewal genes Bmi1, KLF, and Nanog were dramatically increased in CD133+/CD44+ DTCs recovered from the marrow. Importantly, the CD133+/CD44+ DTCs recovered from the marrow of mice form sphere-like structures. Interestingly, the enrichment of CD133+/CD44+ population was not due to the effects of survival within the circulation, proliferation, or homing. The enrichment of CD133+/CD44+ population occurred only in the PCa cells spread to the bone marrow, but not lung or spleen. In addition, the enrichment of CD133+/CD44+ population was inhibited when mTOR signaling pathway was blocked prior to the injection. Moreover, when the CD133-/CD44- population was inoculated into the mice, the conversion into CD133+/CD44+ population was observed in PCa cells recovered from bone marrow.
Conclusions: In this study we developed an *in vivo* murine model in which the disseminated human PCa cells are recovered from murine marrow using flow cytometry. Our findings suggest that the bone marrow microenvironment, or niche, may play a potential role in the accumulation of tumor initiating PCa cells in the bone marrow. The pre-clinical model and strategy described will facilitate deeper insights into the nature and phenotype of DTCs.

P057

Tumor-Initiating Stem Cells are Regulated By α-Camkii-Induced Vegf in Human Osteosarcoma

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Osteosarcoma (OS) is among the most frequently occurring primary bone tumors, primarily affecting adolescents and young adults. Chemoresistance and disease recurrence are major challenges in the clinical management of OS and are thought to be caused by a small subpopulation of tumor-initiating stem cells (TISCs). Human OS TISCs are characterized by their expression of surface antigens CD117+ and Stro-1+, and the stem cell regulating transcription factors Sox2, Nanog, and Oct4. These OS TISCs are known to express high levels of receptors for vascular endothelial growth factor (VEGF). We have previously demonstrated that alpha-Ca2+/Calmodulin kinase two (α-CaMKII) regulates VEGF and its autocrine signaling functions in human OS. Here, we examine whether OS TISCs are regulated by α-CaMKII-induced VEGF. Using fluorescence-activated cell sorting, we discovered that

the pharmacologic inhibition of α-CaMKII or VEGF in 143B OS cells by tamoxifen (1 μM) or bevacizumab (1 μM), respectively, decreases the population of CD117+ and Stro-1+ TISCs, and the gene expression (60%) and protein levels (80%) of Sox2, NANOG, and Oct4. Additionally, we developed a novel preclinical xenograft mouse model to examine the recurrence and metastasis of human OS. 143B OS cells were intratibially injected into mice, and tumors were allowed to grow for 2 weeks. Hind limbs-containing tumors were then amputated, and mice were confirmed to be tumor free by bioluminescent imaging 7 days post-surgery. Mice were randomized into four treatment groups: saline, tamoxifen (500 μg/kg/day), and/or bevacizumab (5 μg/kg twice weekly) and monitored monthly by bioluminescent imaging for the development of metastasis. The incidence of pulmonary metastasis/recurrence in saline treated mice was 100% two months after amputation. However, the incidence decreased to 38% in bevacizumab-treated mice, 12% in tamoxifen-treated mice, and 0% when both drugs were used. The levels of the TISCs subpopulation in pulmonary metastasis were determined by immunohistochemistry for Sox2, NANOG, and Oct4. We show that the number of TISCs were significantly increased in the recurrent metastatic pulmonary tumors when compared to primary amputated tumors. Furthermore, we show that treatment with tamoxifen and/or bevacizumab significantly decreases the number of TISCs when compared with saline treated mice. Taken together, our results demonstrate that α-CaMKII-induced VEGF controls the levels of TISCs both *in vitro* and *in vivo*.

P058 Abstract presentation declined.

Primary Craniofacial Osteosarcomas Show Expression Patterns Distinct from Appendicular Osteosarcoma and Consistent with a Persistent Embryonic Neural Crest Cell Origin

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Osteosarcoma (OS) is the most common primary tumor of bone and the third most common malignancy in adolescents. OS accounts for approximately 40–60% of all primary malignant tumors of bone. Approximately 6–13% of OS tumors occur in the craniofacial skeleton. In patients with craniofacial osteosarcoma, the jaw is the primary site: 55% of tumors occur in the mandible and 31% occur in the maxilla. Chondroblastic osteosarcoma is the predominant histological phenotype of craniofacial osteosarcoma. Based on phenotypic differences including median age of onset, degree of cellular atypia, frequency of local versus distant recurrence and time until metastasis, there is strong evidence that primary osteosarcoma of the appendicular skeleton and primary osteosarcoma of the craniofacial skeleton represent separate and distinct diseases.

Primary craniofacial OS tumors arise in the parts of the skull that are derived from the neural crest. The neural crest is a multipotent population of cells that arises at the neural plate border in the vertebrate embryo. The neural crest contributes to numerous tissues throughout the body including progenitor tooth mesenchymal cells, osteoblasts and chondroblasts of the craniofacial skeleton. Neural crest stem cells have been found to persist into adulthood as a population of dormant multipotent stem cells with a high capacity for self-renewal and an extraordinary degree of plasticity. Indeed, their plasticity and capacity for self-renewal is surpassed only by pluripotent embryonic stem cells.

A comparison of the expression data from primary craniofacial OS with appendicular OS revealed that they had distinct expression patterns and that the craniofacial OS expression pattern had a high degree of similarity to expression patterns of neural crest stem cells as well as other tumors derived from neural crest cells suggesting that primary craniofacial OS tumors arise from the skeletogenic neural crest stem cells. Our data are thus consistent with a model in which primary craniofacial OS tumors represent a malignant transformation of the persistent neural crest-derived stem cells resident in the craniofacial skeleton and that these malignantly transformed stem cells may also function as cancer stem cells in the craniofacial OS tumors. Craniofacial OS tumors thus represent an opportunity to analyze a tumor that arises from embryonic rests of neural crest stem cells.

P059

Molecular Mechanisms of Bone Invasion by Oral Squamous Cell Carcinoma (OSCC)

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Aims Invasion of bone is a common complication of OSCC. This three-part study, aims to investigate signalling pathways involved in the crosstalk between OSCC cells, osteoblasts and osteoclasts. Results Firstly, conditioned medium (CM) was collected from OSCC cells (SCC15 and SCC25), and from osteoblasts of hFOB, then used for indirect co-culture: OSCC cells were treated with CM from osteoblasts and vice versa. Zymogenic activities of both MMP-2 and -9 were increased in OSCC cells following culture with CM from hFOB. The RANKL/OPG ratio, zymogen and protein expression of MMP-9 were increased in hFOB cultured with CM from OSCC cells. All targeted molecules were validated in clinical samples. Secondly, to determine which component of CM caused gene expression changes, OSCC cells of SCC25, HN5, and Tca8113 were artificially induced to display epithelial-mesenchymal transition (EMT) by adding 5 ng/mL of TGF-β1 to culture media for 1–3 days. Minimal immunochemical staining of VIM was found in SCC25 and HN5, while Tca8113 cells were strongly stained. In all cells assessed by real-time PCR, expressions of EMT markers Twist1 and N-cad were up-regulated; Snail1 and E-cad down-regulated. For osteoclast-related molecules, both MT1-MMP and RANKL were up-regulated while OPG down-regulated. CM of OSCC cells pre-treated with TGF-β1 could prolong the survival of mature osteoclasts up to 4 days. Thirdly, strong staining of MCP-1 protein was observed in OSCC cells and osteoclasts from clinical samples. SCC25 cells were found to have the highest expression of MCP-1 mRNA. A plasmid with the inhibitor of MCP-1 (7ND vector) was transfected into

SCC25 cells, and stabilized SCC25 cells with 0.6 μg of 7ND vector (SCC25-7ND) were generated. 10% CM of SCC25-7ND cells efficiently inhibited the formation of human osteoclasts, comparing with 10% CM of SCC25 cells. Additionally, OSCC cells were injected onto the surface of calvaria of nude mice. Well-differentiated SCC was formed in both groups of SCC25 and SCC25-7ND cell injections, with tumour cells invading bone, osteoclasts locating in typical resorption lacunae. TRAP staining showed significant differences between two groups for cell numbers of osteoclasts. Conclusions These studies prove that crosstalk between different types of cell appears to exist in invasion of bone by OSCC. Understanding, and ultimately interfering with these pathways, may provide therapeutic approaches.

P060

Effect of a Fully Human Monoclonal Antibody to Human RANKL (AMG 161) to Reduce Tumor-Induced Osteolysis and Inhibit Skeletal Tumor Burden in a Model of Breast Cancer Bone Metastasis Formation in Mice that Express Chimeric (MURINE/HUMAN) RANKL

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RANKL is an essential mediator of osteoclast function and survival. Tumor cells can induce RANKL expression in the bone stroma, causing activation of osteoclasts and ultimately bone breakdown and subsequent tumor growth. RANKL inhibition by OPG-Fc can prevent tumor-induced osteolysis, decrease skeletal tumor burden, and increase survival in preclinical models. Denosumab is an anti-huRANKL antibody approved for the prevention of skeletal-related events due to bone metastases from solid tumors. Selective anti-huRANKL antibodies do not bind murine RANKL, therefore efficacy cannot be tested in typical mouse models. We utilized a bone metastasis model in immunodeficient mice in which exon 5 from murine RANKL was replaced with its human ortholog (mu/huRANKL). AMG 161 is a fully human monoclonal antibody (IgG1) against human RANKL, which can bind chimeric mu/huRANKL. Human MDA-MB-231/B02-Luc breast carcinoma cells were injected into the tail artery of mu/huRANKL immunodeficient mice. This sub-population (B02) of the MDA-MB-231 human breast cancer cell line only metastasizes to bone. Mice were treated with AMG 161 (2.5 mg/kg, daily) or vehicle on day 0, coincidental with tumor cell inoculation, until study termination at day 37. Treatment with AMG 161 resulted in a substantial decrease in skeletal tumor burden as measured by bioluminescence imaging (BLI). Radiographic analysis on day 37 revealed a 90% decrease in the extent of osteolytic lesions in the hind limbs compared with that of vehicle-treated animals. Micro-CT analysis also confirmed that AMG161 protected against tumor-induced bone destruction. To examine the effect of AMG 161 on early tumor cell colonization in bone, the bone marrow from the hind limbs of vehicle- and AMG161-treated animals was collected on day 7, at which time there was no evidence of metastases, as judged by radiography, BLI, and histology. The bone marrow was then placed in culture under antibiotic selection, allowing the selective growth of antibiotic-resistant tumor cells. Compared with vehicle-treated mice, treatment with AMG 161 (0.3 or 3 mg/kg, 3X/week) resulted in a dose-dependent decrease in tumor colonies, reaching a nearly complete inhibition at the highest dose of antibody used. In conclusion, anti-RANKL antibody treatment blocked both the early engraftment and subsequent growth of established bone metastases, and inhibited tumor-induced bone destruction.

P061

RANK and RANK Ligand (RANKL) Expression in Primary Human Osteosarcoma (OS)

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RANKL is an essential mediator of osteoclast function and survival. RANK and RANKL expression in OS has been reported previously, but without documented validation of antibody specificity. This study assessed human RANK and RANKL expression in human OS and human OS cell lines using specific monoclonal antibodies, validated and optimized for immunohistochemistry (IHC) or flow cytometry.

RANK and RANKL expression were analyzed in a tissue microarray (TMA) of 79 human OS samples using IHC. RANK expression was analyzed in human OS cell lines using qPCR and flow cytometry. Antibodies against human RANK (N-1H8, N-2B10; Amgen) and RANKL (M366; Amgen) were used for IHC staining or flow cytometry. For human OS, IHC staining was scored using an H-score, which considers staining intensity and the percentage of positively stained tumor cells.

Analysis of primary human OS using IHC demonstrated that 44/79 (56%) expressed RANKL. However, the staining intensity was relatively low, and only 29/79 (37%) exhibited ≥10% RANKL-positive cells. A total of 4/70 (6%) OS samples exhibited RANK staining that was either within the spindeloid/anaplastic OS cells or a mononucleated myeloid cell. The extremely low intensity of RANK staining precluded a definitive differentiation of expression in the tumor vs. myeloid compartment in these cases. By contrast, RANK expression was clearly observed in myeloid osteoclast precursor compartment in 50/70 (71%) and in osteoclasts (35/70; 50%). The intensity and frequency of RANK and RANKL staining in OS samples was substantially less than that observed in primary giant cell tumor of bone (GCTB) samples. Analysis of human OS cell lines (MG63, SAOS, U20S) confirmed the absence of surface RANK expression.

In conclusion, a low intensity of RANKL expression was observed in tumor cells from human OS. However, RANK expression was not consistently observed in tumor cells from primary OS nor was RANK expression observed in human OS cell lines.

P062

Role of P63-Regulated Micrornas in the TGFβ-Induced Metastatic Dissemination

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The p53 family transcription factors p53, p63 and p73 make diverse contributions in development and cancer. P53 is functionally inactivated by mutation or other mechanisms in the majority of human cancers. P63 and p73 exhibit opposite functions: whereas p73 mediates apoptosis, p63 promotes proliferation and cell survival. When p53 is inactive in cancer cells, p63 and p73 keep their functionality, playing a very important role not only in the primary tumor development but in the metastatic dissemination. Despite being one of the crucial parameters in the patients' prognosis, the mechanisms of the metastatic process are still not fully elucidated.

The microRNAs (miRNAs) are a novel class of small non-coding RNAs that target mRNAs for inhibition, preventing their translation into proteins. miRNAs have been described recently to play an active role in cancer and in metastatic dissemination.

The aim of this study is to unveil the role of p63 and p73 in the metastatic dissemination through the regulation of a microRNAs network. We used either a model of osteosarcoma lung metastasis dissemination or a squamous cell carcinoma cell line (SCC) where p53 is mutated and p63 and p73 are highly expressed. In this context, the predominant isoform of p63 is known as ΔNp63a and it inhibits TAp73β, the predominant isoform of p73.

Expression profiling allowed us to identify p63-regulated miRs potentially targeting the TGFβ pathway. These miRs repress TGFβRII and Smad4 principally and thereby interfere with metastasis dissemination. Preliminary data demonstrate that these miRs are regulated by both p63 and p73, and that they are able to repress the activation of the TGFβ pathway *in vitro*, and to regulate tumor cells dissemination *in vivo*. Taken together, these findings support the hypothesis that a p63/p73-regulated miR program mediates metastasis dissemination, in different type of cancer, through the regulation of the TGFβ pathway.

These findings may provide new valuable tools for the clinicians such as predictive markers for metastasis development. Uncovering new therapeutic targets for the treatment of this critical step of the tumor development, that is metastasis dissemination or recurrence, is absolutely required and of extreme importance in the present context where few therapeutic alternatives are available.

P063

Involvement of Ubiquitin/Proteasome System in the Regulation of Epithelial-Mesenchymal Transition of Breast Cancer Cells in Bone

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Epithelial-mesenchymal transition (EMT) is a program through which epithelial cancer cells transdifferentiate to mesenchymal cancer cells with enhanced aggressiveness. Recent accumulating evidence showed EMT is regulated by the microenvironment in which cancer cells colonize. Since breast cancer preferentially spreads to bone, we examined whether bone microenvironment promoted EMT of breast cancer cells. MCF-7 human breast cancer cells expressing GFP (MCF-7/GFP) were inoculated into the mammary fad pad (MFP) and tibial bone marrow (TBM) of female athymic nude mice and examined for the expression of the mesenchymal marker Snail and epithelial marker E-cadherin. Real-Time PCR showed MCF-7/GFP tumors in the MFP expressed high E-cadherin and low Snail mRNA levels. In contrast, MCF-7/GFP tumors in TBM demonstrated increased Snail and decreased E-cadherin expression, suggesting that the bone microenvironment promoted EMT. Of note, administration of zoledronic acid (ZA) down-regulated Snail expression in tumors in TBM, thus ZA inhibited EMT in bone. ZA blocked the TGFβ-induced Snail increase and E-cadherin decrease and reversed TGFβ-induced mesenchymal cell shape into epithelial. These *in vitro* results suggest ZA inhibits EMT not only in osteoclast-dependent but also -independent mechanisms. Since Snail is a target of proteasomal degradation, we next examined whether ZA affected proteasomal degradation of Snail. The proteasome inhibitor MG132 restored ZA-decreased Snail expression, suggesting ZA decreases Snail via the ubiquitin/proteasome system. Differential microarray using mammary tumors in TBM isolated from untreated and ZA-treated mice identified the de-ubiquitinating enzyme, ubiquitin-specific enzyme 45 (USP45), as a candidate molecule involved in proteasomal degradation of Snail. ZA significantly inhibited USP-45 expression and activity, which in turn decreased Snail expression. Finally, to determine the biological significance of ZA inhibition of EMT of breast cancer in bone, the effects of ZA on mammary tumors in TBM were determined. ZA inhibited not only tumor progression in bone but also distant metastasis to lung and liver from bone. In conclusion, our results suggest the bone microenvironment promotes EMT in breast cancer. ZA inhibits EMT via stimulation of proteasomal degradation of Snail by suppressing the de-ubiquitinating enzyme USP-45. EMT inhibition by ZA then leads to suppression of aggressive behaviors of breast cancer in bone.

P064

Aging Osteoblasts Condition the Premetastatic Niche

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Bone metastases are responsible for tremendous morbidity and increased mortality in patients with breast cancer. Thus significant effort has focused on elucidating the mechanisms that alter the bone microenvironment and create a hospitable "premetastatic niche". Our recent work demonstrates that senescent or "aged" cells, which are known to accumulate in human tissue with age function analogously to cancer associated fibroblasts (CAFs) and stimulate tumor cell growth and progression. To determine how aging stromal cells impact the bone microenvironment and influence bone metastasis, we developed a novel conditional mouse model that allows us to temporally and spatially control the activation of senescent cells within the stromal compartment of young animals. Our model utilizes a stromal-specific Cre recombinase to conditionally induce senescence in a sub-population of osteoblasts in the bone. Our preliminary studies indicate that activation of senescence in osteoblasts increases breast cancer metastasis to the bone. Molecular analyses of senescent osteoblasts revealed that they stimulate significantly more osteoclast formation than their younger counterparts and fail to mineralize bone. Consistent with these observations, we find that *in vivo* the presence of senescent osteoblasts impairs bone formation and enhances bone absorption, suggesting that senescent osteoblasts alter the bone microenvironment and initiate the "vicious cycle" in the absence of tumor cells. Together these data suggest that senescent osteoblasts facilitate breast cancer bone metastasis by initiating the "vicious cycle" in the premetastatic niche. Our current work is focused on elucidating the mechanism(s) by which senescent osteoblasts alter the bone microenvironment. To this end, we have carried out RNA-sequencing (RNA-Seq) of mRNA from senescent and young osteoblasts isolated from our mice to identify senescence-specific factors that are currently under investigation. These factors will be discussed. Elucidation of the mechanism(s) by which age impacts the bone microenvironment and facilitates metastasis will yield promising therapeutic targets to treat or prevent breast cancer bone metastasis.

P065

Age Related Changes in the Bone Microenvironment Impact Breast Cancer Bone

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Senescence is a potent tumor suppressor mechanism that limits the growth of incipient cancer cells. However, when activated in the stromal compartment senescence leads to expression of a plethora of pro-tumorigenic factors referred to as the senescence associated secretory phenotype (SASP). Significantly, numerous SASP components are also expressed in cancer-associated fibroblasts (CAFs). Indeed, senescent cells recapitulate the tumor promoting capacity of CAFs. Here we uncover an important post-transcriptional regulatory mechanism that sustains SASP expression. We show that the mitogen-activated protein kinase p38 (p38MAPK) stabilizes SASP mRNA by reducing the occupancy of the RNA destabilizing protein AUF1 and Hsp27 on SASP mRNA UTRs. Inhibition of p38MAPK significantly reduced SASP mRNA stability by increasing AUF1's occupancy on SASP mRNAs, which abrogated SASP-mediated tumor growth. To examine the importance of the p38MAPK-driven protumorigenic factors, we developed an *in vivo* model capable of spatially and temporally controlling the activation of senescence in a sub-population of mesenchymal cells including osteoblasts in young animals. Using this model, we find that the activation of senescence significantly impacts osteoblast function. Indeed, senescent osteoblasts are unable to mineralize bone and increase osteoclast formation. These effects are mitigated upon p38MAPK inhibition, suggesting that p38MAPK is functionally critical to these activities. Finally, we find activation of senescence in this model increases bone metastasis in a breast cancer model. We are now investigating how senescent osteoblasts impact tumor seeding to the bone and growth within the bone. Elucidation of how an aged microenvironment impact bone homeostasis and breast cancer metastasis may lead to novel stromal-specific therapeutics.

P066

Interaction of Tumor Cells with the Hematopoietic Stem and Progenitor Cell Niche

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Background: The bone marrow microenvironment, with mesenchymal stromal cells (MSC) as the major cellular component supports self renewal and differentiation of hematopoietic stem and progenitor cells (HSPC). The microenvironment becomes the site of choice for HSPCs because of the chemo–attractants, immobilised growth factors and cell adhesion signaling network. The supportive functions of the niche could also be involved in its neoplastic transformation. Dissemination of breast cancer cells into the bone marrow has been described even in early stages of the disease. The study focuses on the influence of breast carcinomas on mesenchymal and hematopoietic progenitor cells of the bone marrow niche.
Results: The breast cancer cell lines -MCF-7 and MDA-MB231 caused a significant reduction in HSC adhesion to the MSCs (54% by MCF-7 cells; $p<0.001$ and 68% by MDA-MB231 cells; $p<0.0001$). Atomic -force microscopy based single-cell force spectroscopy studies also indicated a higher binding force between tumor cells and MSCs as compared to HSPCs. MCF-7 and MDA-MB231 cells express ICAM-1, which has been shown to promote breast cancer metastasis (Rosette *et al*, 2005; Schröder *et al*, 2011). Thus the competition between tumor cells and HSCs for binding to the niche might

be ICAM-1 dependent and might have implications on HSC homing to the niche. A cytokine array was performed to investigate if the breast cancer cell lines- MCF-7 and MDA-MB231 affect the cytokine profile of MSCs. The array showed altered secretion of growth factors and inflammatory molecules - bFGF, PDGF-BB (2.2 fold upregulation and 0.5 fold downregulation in tumor- MSC cocultures respectively), IL-17 and TNF-alpha (2 fold upregulation and 0.5 fold downregulation in tumor - MSC cocultures respectively) . Based on the cytokine array, a bFGF mediated synergistic increase in the proliferation of MSCs and breast cancer cells in coculture was observed. Interestingly the breast cancer cells caused a reduction in osteoblastic differentiation of MSCs by downregulation of PDGF-BB, indicating a reduced support for HSCs in the neoplastic niche.
Outlook and future directions: These findings indicate a perturbed bone marrow microenvironment upon tumor invasion. The possible role of altered cytokine secretion, downstream signaling in niche activation and bone turnover will be further studied using suitable *in vitro* and *in vivo* models to recapitulate tumor micrometastases to the HSC niche .

P067

Lysyl Oxidase Promotes Survival and Outgrowth of Colon Cancer Cells in the Bone Marrow, Enabling Bone Metastasis Formation

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Lysyl oxidase (LOX) catalyzes the cross-linking of collagens and elastin in the extracellular matrix, thereby regulating the tensile strength of many tissues, such as in the bone. In cancer, LOX plays a critical role on tumor growth and lung metastasis formation. Whether LOX also enables bone metastasis is unknown. In this study, we first showed that LOX is expressed in the stromal reaction of colorectal carcinoma and in the derived bone metastases. Furthermore, LOX expression in colon cancer cells enhanced the formation of osteolytic bone metastases in animals by promoting both skeletal tumor burden and osteoclast-mediated bone resorption. Conversely, the pretreatment of animals with the LOX inhibitor β-aminoproprionitrile or the silencing of LOX in colon cancer cells drastically reduced the formation of osteolytic lesions. Additionally, the treatment of animals with exogenous LOX enhanced the seeding of colon cancer cells in the bone marrow and their outgrowth as overt osteolytic bone metastases. Finally, LOX enhanced the attachment of colon cancer cells to collagen (but not fibronectin) and promoted cancer cell survival *in vitro*. In conclusion, our study provides novel evidence that LOX endows colon cancer cells with the ability to grow in the bone marrow and to stimulate the bone resorptive activity of osteoclasts.

P068

Novel Bone “Seeking” MMP-2 Inhibitors for the Treatment Of Multiple Myeloma

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Multiple Myeloma (MM) is a plasma cell malignancy that is hallmarked by areas of extensive bone destruction. Our group, and others have recently shown that individual host or tumor derived MMP-2 and MMP-9 play an important role in the progression of many cancers in the bone microenvironment, including MM. Our studies have shown MMP-2 to be localized to the tumor and host compartment of human and murine specimens of myeloma. These data support the design of selective inhibitors of individual MMPs for the treatment of multiple myeloma. Previous clinical trials with broad-spectrum matrix metalloproteinase (MMP) inhibitors failed, arguably because the precise roles of individual MMPs had not been fully appreciated. Therefore, in the current study, we have developed novel bone seeking MMP inhibitors (BMMPIs) that are highly selective to MMP-2 and structurally are based on the bone targeting bisphosphonates. *In vitro*, BMMPIs showed greater enzyme inhibition, and specificity for MMP-2 than previously synthesized bisphosphonates (e.g. Zoledronate, Etidronate and Tiludronate) with Ki values in the nanomolar range. Further treatment of the 5TGM1 mouse MM cell line with low micromolar concentrations of the BMMPI inhibitors significantly reduced cell viability through inhibition of cell growth ($p<0.05$). *In vivo*, 5TGMI tumor-bearing mice receiving BMMP inhibitors three times a week, showed significant increase in overall survival compared to vehicle and tiludronate treated mice over 80 days ($p<0.05$). This was associated with a reduction in tumor burden and protection from tumor-associated bone loss compared to vehicle treated mice as measured by x-ray and histomorphometry. These data suggest that given the roles for MMPs in tumor progression in bone, that our novel BMMPIs may be effective in the treatment of MM. We predict that MMP-2 specific BMMPIs may be more effective than current clinically used bisphosphonates for the treatment of MM, and may eliminate undesirable side effects of broad-spectrum MMP inhibitors due to their high specificity and bone seeking nature. We are currently continuing to characterize these BMMPI both as single agents, and in combination with existing MM treatments (eg. Bortezomib) for the treatment and eradication of MM.

P069

Rapamycin Alleviates Pain Associated with Bone Cancer in a Mouse Model of Breast Cancer Metastases to Bone

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In advanced breast cancer, bone metastases occur in 70% of patients. Managing the devastating pain associated with the

disease is difficult. Rapamycin is an immunomodulatory drug that targets the mammalian target of rapamycin (mTOR) pathway. Rapamycin was shown to decrease osteolysis associated with metastatic breast cancer in pre-clinical models and to reduce pain in inflammatory and neuropathic models. The aim of this study was to evaluate the effectiveness of rapamycin in reducing pain associated with experimental osteolytic metastases. Bone cancer was induced by intra-tibial injections of murine mammary carcinoma cells (4T1) in immunocompetent BALB/c mice and treated intraperitoneally for up to 5 weeks with vehicle, rapamycin or pamidronate (a bisphosphonate currently used to reduce bone loss in bone cancer patients). The control group received intra-tibial injection with saline (sham) and was treated with vehicle intraperitoneally. Cancer-induced osteolysis was observed histologically and radiographically 2–3 weeks following cancer inoculation and gradually increased with time. Alterations in nociceptive behaviors evoked by mechanical, thermal and cold stimuli and spontaneous nociceptive behaviors (limping and guarding) were evaluated. Significant hypersensitivity to sensory stimuli developed in cancer-bearing mice compared to sham 3 weeks following inoculation. Rapamycin decreased or delayed the development of cancer-induced mechanical, heat and cold hypersensitivity, while pamidronate reduced heat and cold hypersensitivity. Both rapamycin and pamidronate had a partial protective effect on cancer-induced limb guarding. Our data suggest that rapamycin may have efficacy in the management of pain associated with metastatic breast cancer.

P070
Stathmin Regulation of Bone Microenvironment in Prostate Cancer Bone Metastasis

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Prostate cancer (PCa) has a propensity to metastasize to the skeleton and causes osteosclerotic and osteolytic lesions. In the bone microenvironment, PCa cells which possess osteoblast and osteoclast properties interact with bone remodeling cells. However, the molecular mechanisms responsible for the role of bone microenvironment in development of PCa metastasis are yet to be elucidated. Recently, we have identified stathmin, a microtubule targeting protein, as a powerful regulator of bone remodeling. Null for stathmin in mice resulted in a skeletal phenotype attributed to dysfunction of both osteoblasts and osteoclasts. The stathmin gene (oncoprotein 18) is associated with a number of malignancies, including PCa. Therefore, we hypothesize that deficiency of stathmin will diminish PCa metastatic bone lesions by altering osteoblastic and osteoclastic bone remodeling in the bone microenvironment. To test the hypothesis, first, we generated immunodeficient stathmin knockout (KO) mice by crossbreeding the stathmin KO mice with immunodeficient Rag2 null mice in which both T and B cell functions are depleted. Then, we performed tumor inoculation studies on these mice. PCa C4-2B cells, which are capable of causing both osteolyitc and osteoslerotic metastasis, were inoculated by intracardiac injection into stathmin/Rag2 double knockout (dKO) mice (STMN–/–;Rag2–/–), control mice (STMN+/+;Rag2–/–), and non-immunodeficient wild-type mice (STMN+/+;Rag2+/+). X-ray and micro-computed tomography (µCT) scanning 6 weeks after tumor inoculation showed that while all wild-type STMN+/+;Rag2+/+ mice did not develop any bone metastasis, all STMN–/–;Rag2–/– and STMN+/+;Rag2–/– mice developed bone metastasis as evidenced by both osteolyitc lesions in metaphyseal regions of both the tibia and femur and osteoslerotic lesions on cortical bone of tibia. However, compared to STMN+/+;Rag2–/– control mice, stathmin deficiency in STMN–/–;Rag2–/– dKO mice substantially reduced both osteolyitc and osteoslerotic metastases. Histology provides further evidence for bone metastasis in these mice. These results demonstrate that stathmin plays a critical role in development and progression of PCa bone metastasis, suggesting that targeting the stathmin gene, which not only inhibits tumor growth but also alters the bone microenvironment, could be a promising therapeutic approach for PCa bone metastasis.

P071
Osteolysis Induced by Osteosarcoma Cells Implicates RANKL from Micro-Environmental Origin and is Independent of Rank Expression by Tumor Cells

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Background: Osteosarcoma, the most frequent malignant primary bone tumor, is characterized by an osteoid tumor tissue formation associated to a tumor-induced osteolysis. Such osteolysis corresponding to osteoclast differentiation and activation in the vicinity of the tumor is known to be under RANKL control. The question of the origin of RANKL implicated in the tumor-induced osteolysis is raised. Furthermore, expression of RANK in some osteosarcoma cells raises the second question of the potential direct effect of RANKL on tumor growth.
Methods: The mouse MOS-J osteosarcoma cell-line, expressing Rank at a very low level, was inoculated at para-osseous site in syngeneic C57BL/6 mice. The impact of a RANKL blocking antibody on the tumor-induced osteolysis and the tumor growth was analyzed. Injections were also performed on Rankl–/– and RankTg (overexpression in monocyte/macrophage lineage) mice. Finally, MOS-J cells modified to stably over-express RANK were injected and the impact on tumor growth and osteolysis compared with native MOS-J cells.
Results: The RANKL blocking antibody protected bone from tumor-induced osteolysis. No significant impact on the tumor growth was observed. Experiments with Rankl–/– mice evidenced that RANKL from the tumor cells was not sufficient by itself to induce bone resorption. Experiments with RankTg mice evidenced an increase of the osteolysis with no significant impact on the tumor growth. Finally, Rank overexpression

in the MOS-J cells has no effect on both the tumor growth and the tumor-induced osteolysis.
Conclusions: Reported data evidence that RANKL implicated in the tumor-induced osteolysis is not from tumor-cell origin but is rather synthetized by cells from the tumor microenvironment that need to be characterized. Regarding RANK expression by the tumor cells, the absence of effect suggests that RANKL has no direct effect on the tumor growth.

P072

Mechanical Loading Inhibits Metastasis-Mediated Osteolysis via Effects on Osteoclasts

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Increased mechanical stimulation following inoculation of metastatic breast cancer cells inhibited secondary tumor establishment and osteolysis in an *in vivo* loading model. To probe the cellular mechanisms mediating these results, we developed a 3D *in vitro* compressive loading model of bone metastasis to investigate whether loading altered tumor cell expression of the bone metastatic gene signature (MMP1, CXCR4, OPN) and genes that directly (IL-8, RANK, Dkk1) and indirectly (Runx2) regulate osteolysis. We also determined if tumor-secreted factors altered osteoclasts' (Oc) response to loading. We report that the inhibitory effects of loading on metastatic tumors are indirect due to altered Oc behavior.
Mineral-containing scaffolds were made from poly(lactide-co-glycolide) and hydroxyapatite, and seeded with 1.5e6 breast cancer cells (MDA-MB231) or pre-Ocs (RAW 246.7 murine monocytes). To test the effects of loading on MDAs, compression was applied to scaffolds (10% peak strain, 1 Hz) for 1 hr/day for 3 days. Gene expression was assessed via qRT-PCR, and cell number and viability were assessed via DNA content and live/dead staining. We also determined whether these effects were altered with increasing strain (peak 5–25%). To test whether tumor-secreted factors interfered with Oc behavior in response to loading, Oc-scaffolds were cultured with RANKL plus tumor-conditioned media (TCM), and gene expression of MMP-9 and cathepsin K was assessed.
Loading did not affect MDA cell proliferation and viability, as indicated by similar DNA content and live/dead staining in loaded and nonloaded groups. MDA expression of bone metastatic and osteolytic genes was unaffected by loading. However, Runx2 expression was significantly inhibited (20%) by loading (Fig. 1A). In pre-Ocs, tumor-secreted factors increased expression of MMP-9 and cathepsin K, and this effect was ameliorated by loading (Fig. 1B).
Mechanical loading did not directly affect the growth and viability of tumor cells; however, Runx2, a transcription factor that regulates secretion of osteolytic proteins such as PTHrP, was down-regulated in response to loading, suggesting that the inhibitory effects of loading on osteolysis are indirectly mediated by Ocs. Resulting changes in protein secretion and Oc function are currently being verified. Mechanical loading likely also modulates the effects of tumor-secreted factors on osteoblast function, which is being evaluated as well.

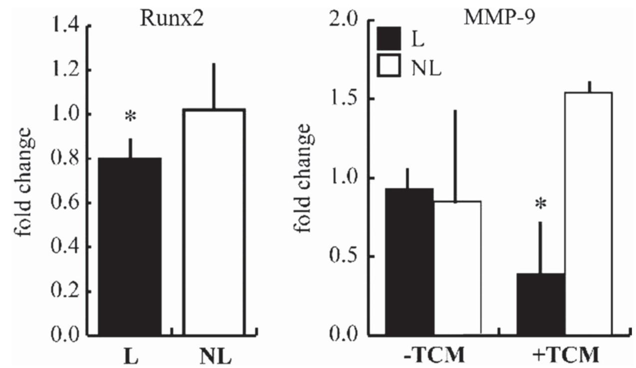

Fig 1: Mechanical loading decreased A) Runx2 expression in MDAs and B) MMP-9 expression in pre-Ocs cultured with tumor-secreted factors (+TCM). *vs. NL.

P073

Defining the Role of TGFβ in Osteogenic Prostate Cancer to Bone Metastases: an Integrated Biological/Mathematical Modeling Study

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Almost 90% of patients that succumb to prostate cancer will have bone metastases. The metastases are incurable with patients often experiencing intense pain. In bone, prostate cancer cells derive factors necessary for growth and survival by manipulating bone forming osteoblasts and bone resorbing osteoclasts, resulting in areas of excessive osteogenesis and osteolysis, respectively. Our group and others have defined transforming growth factor beta (TGFβ) as being a key factor in the progression of bone metastases. Using semi-quantitative immunohistochemical analysis, we have found in human specimens that TGFβ signaling is much higher in the cancer cells relative to the surrounding bone stroma. Therapeutic inhibition of TGFβ however, presents a dilemma since TGFβ can have differential effects on cell types in the tumor-bone microenvironment. Furthermore, current biological approaches are limited in their ability to assess multicellular parallel interactions over time but this can be overcome via the power of computational modeling. Thus, we have developed an integrated approach where biological results inform a predictive mathematical model, allowing for exploration of complex interactions mediated by TGFβ. The model was parameterized with empirical and published data. Simulations of the prostate cancer-bone microenvironment over a 208 day period generated a clinically relevant pathophysiological model with the following outputs: 1) that TGFβ is key for coordinating prostate cancer- induced phases of osteolysis and osteogenesis and 2) that mesenchymal stem cells (MSCs) are crucial for the osteogenic component of the disease. To test these hypotheses we performed biological studies using a TGFβ antibody-based inhibitor (1D11) that demonstrated: 1) TGFβ in prostate cancer cell conditioned media significantly contributes to MSC and osteoblast precursor recruitment and, 2) using an intratibial model of prostate cancer-induced osteogenesis (PAIII), we identified that TGFβ regulates prostate cancer induced osteolysis and osteogenesis (µCT, histomorphometry). Histological

analyses of *in vivo* specimens will further test the predicted outcomes of the mathematical model. Future studies are focused on evolving the model to incorporate tumor heterogeneity. We believe that integrated computational and biological approaches are key to the development of powerful models that can be used for the delivery of precision medicine to cure prostate to bone metastases.

P074

Markers of Breast Cancer Development Expressed by Epithelial Tumor and Stromal Cells: Future Perspectives

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Despite advance in the study of breast cancer (BC) progression mechanisms and novel therapeutic treatment development, it remains as the second leading cause of mortality among women. Classical diagnostic factors are not enough to prevent BC patients (BCP) from developing metastasis. The aim of this work was to study non-classical markers' expression [OPG, TRAIL, TRAIL receptors (R) (R1-4), RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6-R, MCSF and M-CSF-R] in tumor epithelial (TEpC) and stromal (SC) cells, evaluating the possible association between these parameters and the classical ones (age, ER, PR, HER2, tumor size and histological grade).
This was a prospective cohort study including 20 primary tumor biopsy (infiltrative ductal carcinoma, I-II stage BCP) and 10 non-neoplastic breast tissues (control). Evaluation of the % of positive cells was done by immunohistochemistry. Clinicopathological data was retrieved from pathology and medical records. Mann-Whitney test and Spearman's rank correlation coefficient were used ($p \leq 0.05$).
TRAIL-R2, -R3, -R4, RANK, RANKL, OPG, IL-6, IL-6R, CXCR4 showed significant higher expression in TEpC and BCP-SC than controls ($p<0.05$). Expression of SDF-1 and MCSF was significant higher in TEpC than control ($p=0.0286$; 0.0321). In BCP, the % of TRAIL, OPG and SDF-1 expression in TEpC was higher than BCP-SC ($p<0.0001$). In TEpC, TRAIL-R1 and -R4 expression was associated with TRAIL expression in them; IL-6R, CXCR4 and SDF-1 expression in TEpC was associated with IL-6, SDF-1 and CXCR4 in BCP-SC ($p=0.0236$; 0.0009; 0.0413). In BCP, the % of SC expressing TRAIL, IL-6 and SDF-1 was associated with TRAIL-R3, IL-6R and CXCR4 expression in them ($p=0.038$; 0.016; 0.0009). The % of TRAIL and RANKL expression in control-SC was associated with TRAIL-R3, -R4 and RANK expression in them ($p=0.0266$; 0.0152; 0.0039). In these TEpC TRAIL-R1, -R3 and OPG expression was associated with age ($p=0.0407$; 0.0147; 0.0361); RANKL and OPG expression was associated with HER2 ($p=0.0206$ inversely; 0.008); TRAIL-R2 and OPG expression was inversely associated with histological grade ($p=0.0088$; 0.0363). OPG and M-CSF-R expression was associated with desmoplasia ($p=0.0009$ inversely; 0.0008).
These data showed the importance of evaluating the non-classical parameters in TEpC and in BCP-SC. These results could be useful in identifying patients with more aggressive tumors at risk of bone metastasis, which may thus improve the available options for therapeutic intervention.

P075

The Impact of MMP-3 Generated PTHrP Peptides on Mesenchymal Stem Cell (MSC) Behavior: Implications for Prostate to Bone Metastases

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Prostate cancer commonly metastasizes to bone, generating mixed lesions that are both osteolytic and osteogenic. Matrix metalloproteinases (MMPs) have recently been implicated in regulating the bioavailability and activity of growth factors that control the "vicious cycle" of cellular interaction in the tumor-bone microenvironment. We have found that MMP-3, also known as stromelysin-1 and typically generated by host/stromal cells, is differentially expressed in the prostate tumor-bone microenvironment. Emerging data from our group indicates that host derived MMP-3 expression contributes to prostate tumor growth in bone.
Using an *in vivo* intratibial model of prostate to bone metastases (PaIII), we found that tumor growth, as measured by luminescence, and tumor-induced bone remodeling (µCT, histomorphometry) were significantly mitigated ($p<0.05$) in MMP-3 null mice compared to wild type controls. Using a candidate approach to examine potential mechanisms, we focused on parathyroid hormone-related protein (PTHrP), a powerful regulator of osteoblast behavior in the vicious cycle. We identified that MMP-3 processes mature PTHrP1-36 to yield unique PTHrP1-16, PTHrP17-26, and PTHrP27-36 fragments *in vitro*. To test the biological significance of the fragments we initially focused on testing their impact on osteoblast behavior. Using Boyden chamber migration assays, we observed a significant increase in migration of murine MSCs and MC3T3-E1 pre-osteoblasts in response to 10 nM PTHrP1-16 compared to that of PTHrP1-36. Further analysis via confocal microscopy revealed a reduction in actin stress fibers as well as variations in lamellipodia and vinculin organization of pre-osteoblasts treated with 10 nM PTHrP1-16. In addition, Western blots show a reduction of myosin light chain phosphorylation suggesting that PTHrP1-16 may mediate pre-osteoblast migration by modulating Rho-associated protein kinase (ROCK) activity.
Our data demonstrate that host MMP-3 contributes to prostate tumor growth and tumor induced changes in bone remodeling *in vivo*. Further, we have identified that mature PTHrP1-36 is subject to cleavage by MMP-3 resulting in PTHrP1-16, PTHrP17-26, and PTHrP27-36 fragments of which PTHrP1-16 significantly stimulates migration of murine MSCs and MC3T3-E1 pre-osteoblasts. Collectively, these data indicate that specific inhibition of MMP-3 and/or targeting MMP generated neo-epitopes would be efficacious for the treatment of prostate to bone metastases.

P076 Abstract presentation declined.

The Molecular Signature of the Stroma Response in Osteoblastic bone Metastasis Reveals Enrichment in Haematopoietic Stem Cell Niche Components

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In both primary and metastatic tumors cancer cell survival and growth strongly depend on stromal support. Prostate cancer (PCa) is highly osteotropic and preferentially induces osteoblastic lesions. The molecular cues responsible for survival/ growth of osteoinductive cancer cells by the bone stroma are not fully characterized.
We analyzed the stroma transcriptome in response to human osteoinductive PCa cells (VCaP and C4-2B) xenografted in immunocompromised mice. Species-specific cDNA-microarrays (Affymetrix) could successfully dissect the stroma (mouse) from the cancer cell (human) transcriptome. This consisted of 914 stromal genes differentially regulated in VCaP and/or C4-2B xenografts and referred as the osteoblastic bone metastasis-associated stroma transcriptome (ObBMST). Most likely this contains a universal, tissue- and cancer type-independent, response. Therefore, to define the bone-specific fraction of this signature, we subtracted publicly available gene sets representing stroma genes differentially regulated in inflammation/wound healing and in primary, non-osteotropic cancers (esophageal, gastric, pancreatic and colon cancer). The remaining list still shared genes differentially expressed in publicly available stroma signatures derived from primary breast and prostate cancer and these were also subtracted. The functional categorization of the top 86 genes in the remaining signature (“Core ObBMST”) revealed that 13 (15%) were encoding components of the haematopoietic stem cell- (HSC-) niche. RT-qPCR validation with mouse specific probes confirmed the differential expression of three representative HSC-niche genes (Ptn, Epha3 and Slit3) in experimental, osteoblastic bone metastasis.
This investigation reports for the first time stimulated gene expression of HSC-nice components as a relevant part of the stromal response in osteoblastic bone metastasis. The HSC-niche components revealed by this study are candidate to be also essential constituents of bone/bone marrow metastatic niche specifically supporting survival and growth of osteoinductive, PCa cells.

P077

A New Spectrum-Selective Cathepsin Inhibitor, VBY-825, Inhibits Bone Destruction in a Syngeneic 5TGM1 Multiple Myeloma Mouse Model

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Multiple myeloma (MM) is the second most common blood cancer after non Hodgkin lymphoma. It is a monoclonal B-cell neoplasia with clinical hallmarks of multiple osteolytic lesions causing bone pain, fractures and hypercalcemia. Chemo- or radiotherapy may induce remissions, but MM is generally thought to be incurable. Our aim was to observe the effects of a cathepsin inhibitor VBY-825 on bone lesions and tumor burden in the syngeneic 5TGM1 mouse MM model using immunocompetent C57BL/KaLwRij mice. VBY-825 is a potent inhibitor of cathepsins K, L, B, V, and S.
5TGM1 cells were inoculated via tail vein in 7 weeks old female C57BL/KaLwRij mice, which were divided to 4 groups: Control group received vehicle of VBY-825 (5% dextrose 10 ml/kg daily), Control group received bortezomib vehicle (3 ml/kg twice a week), Reference group received bortezomib (0.5 mg/kg twice a week) and Study group received VBY-825 (100 mg/kg daily). Administration of all compounds began one day before tumor cell inoculation and continued until day 34. Disease progression was followed by measuring the serum levels of paraprotein (IgG2b) and TRACP 5b, radiography, and body weight. The animals were sacrificed 5 weeks after inoculation, examined macroscopically, and their bones were collected for histomorphometric analysis.
The reference compound bortezomib had no effects on body weight but it delayed the disease progression based on IgG2b measurements. It also decreased the number and total area of osteolytic lesions, but not mean osteolytic lesion area (MOLA). VBY-825 had no effect on body weight or IgG2b level, frequency of soft tissue tumors or intraosseous tumor area. VBY-825 decreased total and MOLA, consistent with inhibited resorption. There was also a trend of increased relative trabecular bone area. Serum TRACP 5b activity in the VBY-825 treated group did not differ from the respective vehicle group, whereas the number of osteoclasts at tumor-bone interface was increased in VBY-825 treated animals. These findings suggest that VBY-825 decreased osteoclast function and resorption activity without decreasing the number of osteoclasts
In conclusion, VBY-825 had no effects on tumor growth but it inhibited bone destruction in this mouse model of MM, which is consistent with its potency on cathepsin S and K, which are known to be important in osteoclast-mediated bone resorption. VBY-825 is a promising candidate for the treatment of tumor-associated bone disease.

P078

Involvement of Roundabout Receptors in Breast Cancer Bone Metastasis Formation and Progression

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Roundabount (ROBO) receptors regulate axon guidance. Here, we compared the gene-expression profiles of human MDA-MB-231 breast cancer cells and its cell subpopulation that only metastasizes to bone (referred to as B02) and found that Robo1 and Robo4 receptors were overexpressed in B02 cells. Additionally, a high Robo4 expression in primary tumors from patients with breast cancer (n=254) correlated with poor prognosis and increased risk of bone metastasis relapse. The functions of Robo1 and Robo4 in tumor outgrowth and bone metastasis formation were therefore examined. The inoculation of Robo4-depleted cells in the mammary fat pad of mice led to a 50% reduction in tumor burden, whereas the outgrowth of Robo1-depleted tumors was substantially increased, compared with animals injected with mock-transfected B02 cells. For bone metastasis experiments, tumor cells were injected into the caudal artery of mice and these animals were analyzed by radiography on day 25 after tumor cell inoculation. The extent of osteolytic lesions was dramatically increased in animals bearing Robo1-depleted tumors, when compared with animals bearing Robo4-depleted tumors. This difference was accompanied with a sharp increase in skeletal tumor burden in mice bearing Robo1-depleted tumors, as determined by histomorphometric examination. *In vitro*, the treatment of parental B02 breast cancer cells with anti-Robo1 antibody promoted invasiveness, whereas anti-Robo4 antibody dose-dependently inhibited B02 cell invasion. Because Robo1 and Robo4 were regulating breast cancer cell invasion *in vitro* and bone metastasis formation *in vivo*, we hypothesized that these receptors might facilitate the engraftment of tumor cells in the bone marrow. To address this question, the bone marrow was flushed from the hind limbs of tumor-bearing animals on day 7 after tumor cell inoculation, at which time there was no evidence of metastases, and placed in culture under puromycin selection, enabling the growth of antibiotic-resistant tumor cells. Compared with mice inoculated with Robo1-depleted cells, the number of tumor cell colonies in the bone marrow from mice inoculated with Robo4-depleted cells was reduced by 75 to 80%. Overall, our results provide strong evidence that axon guidance receptors are involved in bone metastasis formation. They show for the first time that Robo1 and Robo4 in breast cancer cells have antagonistic functions, exhibiting anti-invasive and pro-invasive properties, respectively.

P079

Changes in the Transcriptome Signature of Bone-Forming Cells after Myeloma Cell Contact

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Multiple myeloma (MM) is a hemato-oncological malignancy caused by the expansion of one malignant plasma cell in the bone marrow. The proliferation of myeloma cells results in myeloma bone disease characterized by osteolytic lesions. This persisting skeletal destruction is caused by imbalanced bone remodeling as a result of the interaction between bone-forming cells and tumor that accelerates disease progression. To assess the transformation of bone-forming cells in the tumor microenvironment, global gene expression analyses for mesenchymal stem cells (MSC) and osteogenic precursor cells (OPC) were performed after myeloma cell contact.
For global gene expression analyses, we used Affymetrix GeneChip HG-U133 2.0 Plus Arrays. MSC and OPC, respectively, were co-cultured with the human plasmocytoma cell line INA-6 for 24 h followed by FACS separation. To allow for separation, INA-6 cells were stained with CellTracker® Green CMFDA prior to the co-culture set up. OPC were generated by 14 days of incubation of MSC with osteogenic differentiation media. Osteogenic differentiation was checked by alkaline phosphatase and alizarin red S staining. RNA samples of five different experiments were used for genome-wide expression analyses, the corresponding MSC and OPC served as controls. Data evaluation was carried out with the software R (normalization: RMA; filter $0.5 \leq$ log Fold change ≥ 0.5, AFFX), network analyses were performed by using the ClueGo plug-in for Cytoscape. The differential expression of target genes was evaluated by semiquantitative RT-PCR and densitometric analysis.
The co-culture with INA-6 cells yielded in changes of the transcriptome in bone-forming cells. MSC seem to be more susceptible for myeloma cell contact with 991 differentially expressed probe sets (718 genes) compared to OPC showing 552 differentially expressed probe sets (397 genes). The expression of genes involved in inflammation, angiogenesis, and hypoxia was increased, whereas the expression of genes linked to stem cell differentiation was decreased.
Selected candidates will be further investigated on the protein level *in vitro* by using primary myeloma cells and their potential role in myeloma will be assessed by examining tumor tissues. Contact-induced membrane proteins will be studied preferentially as they may be suitable targets for drug therapies and diagnostic markers for the early detection of MM.

P080

The Involvement of Microrna Alterations in Osteoclasts During Breast Cancer Bone Metastasis

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During osteolytic bone metastasis, found in a majority of late stage breast cancer patients, tumor cells activate bone resorbing osteoclast cells, leading to severe bone degradation, pain, and pathological fractures. Previous studies have shown the necessity of microRNAs (miRNAs) in osteoclast development; however, it is not currently known which miRNAs are essential for this process. We have revealed significant overlap of miRNA expression changes during osteoclast differentiation induced by the physiological osteoclastogenesis factor RANKL and by conditioned media from tumor cells. Furthermore, we revealed that ectopic expression of multiple candidate miRNAs, selected for their predicted targeting of known osteoclast genes, was capable of inhibiting osteoclast differentiation. Systemic treatment with a number of these pre-miRNAs revealed the capacity to influence osteoclast differentiation *in vivo*, enhancing bone volume and trabecular thickness. Importantly, treatment with multiple miRNAs significantly inhibited bone tumor burden and reduced bone lysis in a mouse metastasis model. Furthermore, our studies show that independently identified miRNAs with elevated expression during osteoclastogenesis are strong prognostic indicators of bone metastasis in patient serum samples. These results improve the functional understanding of miRNA regulation during osteoclast differentiation and have the potential to facilitate the development of novel bone metastasis therapeutics.

P081

Downregulation of PLCγ2/β-Catenin Pathway Promotes Activation and Expansion of Myeloid-Derived Suppressor Cells in Cancer

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Myeloid-derived Suppressor Cells (MDSC) promote tumor dissemination by mainly suppressing anti-tumor T-cell responses in many cancers. Although the mechanism of T-cell inhibition is well-established, the pathways leading to MDSC accumulation in bone marrow and secondary lymphoid organs remain unclear. We found that downregulation of PLCγ2 signaling in tumor-bearing mice induces aberrant expansion of MDSC in the bone marrow, spleen and tumor site. To analyze whether MDSC promote tumorigenesis in PLCγ2–/– mice, we adoptively transferred MDSC isolated from PLCγ2–/– or WT mice into subcutaneous tumor-injected WT mice. Animals receiving PLCγ2–/– MDSC displayed greater expansion of MDSC in the spleen and increased tumor burden compared to mice receiving WT MDSC. Furthermore, in addition to their increased proliferation as shown by higher BrdU incorporation, PLCγ2–/– MDSC were more efficient than WT cells to suppress CD8 T-cell proliferation via increased ROS and NO production. Mechanistically, PLCγ2–/– MDSC display reduced β-catenin levels and its rescued expression decreases MDSC expansion and tumor growth in PLCγ2–/– mice. In addition, the deletion of β-catenin in myeloid cells (LysM-Cre/β-cat.cKO), including MDSC, led to enhanced tumor growth and a 2-fold increase in MDSC compared to control mice. By contrast, constitutive β-catenin activation in myeloid cells (LysM-Cre/β-cat.CA) decreased tumor growth while MDSC percentage was reduced compared to control mice. Consistent with a role for β-catenin in MDSC during tumor progression, its expression was reduced in tumor-bearing WT mice compared to tumor-free animals. The relevance of the PLCγ2/β-catenin axis is further documented in MDSC from pancreatic cancer patients which show reduced phospho-PLCγ2 and β-catenin expression levels compared to healthy controls. Importantly, aberrant expansion of MDSC in tumor-bearing hosts is also likely to contribute to bone metastatic dissemination, even in the context of osteoclast (OC) blockade. Consistent with this hypothesis, PLCγ2–/– mice display increased bone tumor burden compared to WT animals despite reduced OC activity. The elevated rate of tumor growth in bone is due to impaired CD8 T-cell responses and correlates with greater MDSC accumulation in spleen of PLCγ2–/– mice. In conclusion, the downregulation of PLCγ2/β-catenin pathway occurs in mice and humans, leading to MDSC-mediated tumor expansion. Our data also implicates MDSC as new modulators of bone metastases.

P082

Two-Photon Imaging Reveals Tumour-Associated Myeloid Cells as the Cellular Targets Underlying the Anti-Tumour Activity of Bisphosphonates In Vivo

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Bisphosphonates (BPs) are the gold standard anti-resorptive treatment for metastatic bone disease. Nitrogen-containing BPs (N-BPs) also have anti-tumour effects in preclinical models and increased survival in several clinical trials of patients with breast cancer and myeloma. Although N-BPs can directly affect tumour cells and numerous other cell types *in vitro* by inhibiting protein prenylation, the exact mechanisms underlying these anti-tumour effects *in vivo* remain controversial, since BPs target rapidly to bone and there is no unequivocal evidence that N-BPs can directly affect tumour cells *in vivo*.

To finally answer this question, we determined the cell types capable of internalising BP *in vivo* in the 4T1 syngeneic murine breast cancer model by examining the distribution and cellular uptake of fluorescently-labelled N-BP (AF647-RIS) in mammary tumours in live BALB/c mice. Within minutes of tail vein injection, intravital 2-photon imaging revealed the flow of

N-BP into mammary tumour via the tumour vasculature, with slow diffusion into tumour tissue and binding to microcalcifications. Two hours after injection, intracellular fluorescent N-BP was associated almost entirely with tumour-associated macrophages (labelled *in vivo* by injecting mice with anti-F4/80-FITC). Flow cytometric analysis of the tumours 24 hours later confirmed that 66% of CD11b+F4/80+ tumour associated macrophages and 46% of the CD11b+Gr1+F4/80+ monocytic immature myeloid cells (often referred to as myeloid derived suppressor cells), but not tumour cells, had internalised the N-BP *in vivo*.
These studies provide the first conclusive evidence that N-BP can be rapidly internalised *in vivo* by myeloid cells in mammary tumours completely outside the skeleton. The abnormal vascular organisation of tumours may facilitate the local diffusion of N-BP and endocytic uptake by tumour-associated myeloid cells. Given that macrophages and immature CD11b+ myeloid cells suppress tumour immunosurveillance and promote tumour progression and metastasis, our studies indicate that the anti-tumour activity of N-BPs *in vivo* occurs via effects on these myeloid cell types and not via direct effects on tumour cells.

P083
CD105 Expression by Stromal Cells of Primary Tumors from Untreated Early Breast Cancer Patients Associates with Bone Metastatic Development

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Once breast cancer metastasizes to bone, mortality increases. Thus, there is an urgent need to identify patients with high risk to develop bone metastases at earlier-stage of disease. Several studies in breast cancer have confirmed that tumor-extracellular matrix and non-carcinoma cells, mainly cancer-associated fibroblasts, mesenchymal stem cells and inflammatory cells drive cancer progression and bone metastasis development. The aim of our research was to investigate their prognostic significance in untreated early-breast cancer patients. Therefore, we analyzed immunohistochemical expression of α-SMA and FSP (cancer-associated fibroblast-markers) and CD105 and CD146 (mesenchymal stem cell-markers) in stromal cells of 41 primary tumors of untreated-breast cancer patients with infiltrative ductal carcinoma (I/II stage) and 10 non-malignant breast tissues and investigated whether their expression and stromal features are associated with classical-prognostic markers, bone metastasis occurrence and skeletal disease-free survival of these patients. In this study, we demonstrated that high CD105 expression associates with bone metastases occurrence (p=0.0242) and shorter skeletal disease-free survival (p=0.0180). Moreover, we postulated it as an independent marker for early bone breast cancer-development (p=0.0440). Finally, we found a statistically association of high values of lymphocytic infiltrate with positive-HER2/neu (p=0.0138) and myxoid changes with high tumor size, poor differentiated tumors and positive-HER2/neu (p=0.0373, 0.0004 and 0.0310; respectively). In conclusion, this is the first demonstration of stromal-CD105 expression in primary tumors as an early marker of bone metastasis recurrence and shorter skeletal disease-free survival in breast cancer patients. This work also support an unfavourable prognostic significance of lymphocytic infiltration and myxoid changes in their tumors.

P084
The Osteoimmune Factor WNT5A Reduces Prostate Cancer Growth

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Patients with advanced metastatic prostate cancer develop osseous metastasis in 90% of the cases. However, the exact mechanism of the metastatic process is not fully understood. Wnt proteins are highly expressed in prostate cancer-induced metastases and have been implicated in tumor progression. Especially WNT5A was shown to be up-regulated in different cancer types, but its specific role in prostate cancer remains unclear. Here, we determined the WNT5A expression profile of different osteotropic prostate cancer cell lines and assessed the *in vitro* and *in vivo* effects of WNT5A on prostate cancer biology.
Three human prostate cancer cell lines were used, including PC3, C42B, and MDA PCa 2b cells, which generate osteolytic, mixed, and osteosclerotic lesions *in vivo*, respectively. To determine the impact of WNT5A on the biology of prostate cancer cells, we measured proliferation, apoptosis, and cytokine expression after WNT5A over-expression or WNT5A knock-down using siRNA. The impact of WNT5A on tumor growth in the bone microenvironment *in vivo* was determined using WNT5A over-expressing PC3-Luc cells that were injected into the tibia or the left ventricle into nude- or NSG mice.
WNT5A was differentially expressed in the different prostate cancer cell lines, being expressed highest in MDA-PCa-2b cells, followed by PC3 and C42B cells. Over-expression of WNT5A *in vitro* reduced proliferation by 39%, 65%, and 59% in PC3, C42B, and MDA-PCa-2b cells, respectively, and simultaneously induced apoptosis 2-8-fold. In contrast to WNT5A over-expression, knock-down of WNT5A stimulated cell proliferation and reduced apoptosis. Furthermore, we analyzed differences in cytokine expression and found that TNFα, IL-8, and IL-6 were expressed at low basal levels in C42B and MDA-PCa2b cells. In contrast, PC3 cells showed higher cytokine expression levels and after 48 h of WNT5A over-expression, TNFα, IL-8, and IL-6 levels were significantly increased. *In vivo* WNT5A over-expression in PC3-Luc cells nearly completely abolished intratibial (CO: 70% engraftment vs. WNT5A: 0% engraftment) as well as intracardic (CO: 80% engraftment vs. WNT5A: 20% engraftment) tumor engraftment compared to PC3-Luc cells with normal WNT5A levels.

These data indicate that WNT5A affects the lifespan of prostate cancer cells *in vitro* and *in vivo* and suggest that, WNT5A may emerge as a potential target for prostate cancer therapy.

P085

Optimisation of a Human-Specific Model of Breast Cancer Bone Metastasis

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Background: Bone is a preferential site for breast cancer metastasis, at which point the disease is considered incurable. The xenograft transplantations performed in immunocompromised mice most commonly used to study bone metastasis are limited by the lack of a true metastatic pathway and immune response. In addition, there are species differences involved in human cells growing in a murine environment. We have therefore developed a model where human breast cancer cells colonise human bone cores implanted in immunocompromised mice.
Materials & Methods: Trabecular bone cores ($0.5\,cm^3$) were prepared from the femoral heads of patients undergoing hip replacement surgery. Female NOD/SCID mice (n=20) underwent subcutaneous implantation of 2 bone cores per mouse. Mice were culled weekly (n=5 per group) for 4 weeks after implantation and bone cores and mouse spleen harvested. 2 mice per group were injected with calcein 24 hours prior to culling for labeling viable cells. Bone volume was assessed by µCT. Osteoclasts and blood vessels were identified following TRAP staining and immunohistochemical analysis (CD31). The human B cell content of the mouse spleen was detected by flow cytometry.
Results: There was a significant increase in the percentage of human B cells detected in mouse spleen by day 28 ($p<0.05$). Analysis of bone architecture showed no change at any time point in bone volume compared to day 0 controls. Osteocyte viability was reduced to 40% of day 0 controls by day 7 ($p<0.0001$) but were maintained at this level for all later timepoints. Viable cells were confirmed by multiphoton microscopy for calcein uptake, seen from day 21 onwards. The presence of osteoclasts on the bone surface appeared to increase with time. Revascularisation involved both human and mouse derived endothelial cells. IVIS imaging from a subsequent study with both human bone cores and human MDA-MB-231 mammary fat pad tumours (n=18) showed secondary tumour growth in the bone cores of 28% of mice.
Conclusions: Human bone cores remain viable and retain structure post-implantation providing the species-specific metastatic site. Revascularisation of the bone cores allows the transfer of human B cells to the mouse spleen and provides a route for circulating tumour cells to colonise human bone cores. Optimisation of human bone core viability as well as characterisastion of the early stages following bone implantation will conceivably improve the tumour take rate in this model.

P086

The MIR-218 -WNT Axis Promotes Osteomimicry of Osteolytic Breast Cancer Cells that Home to Bone

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Signaling pathways that are crucial in bone development, including Wnt and BMP, are also upregulated in breast cancer cells that grow aggressively in the bone microenvironment. Homing of breast cancer cells to bone is facilitated by their ability to express many osteoblast-related genes ('osteomimicry'). Here, we tested whether osteogenic miRNAs are aberrantly expressed in tumor cells metastatic to bone to support the osteomimetic properties. miR-218 is highly expressed in osteoblasts and promotes bone marrow stromal cell commitment and osteogenic differentiation, thus serving as a potential "osteomiR". We found that miR-218 expression is also significantly up-regulated in bone metastatic MDA-MB-231 breast cancer cells and is not detectable in normal MCF-10a mammary epithelial cells, suggesting a positive role of miR-218 in bone metastasis. Indeed, ectopic expression of miR-218 in MDA-MB-231-Luc cells promotes tumor growth in the bone microenvironment whereas inhibition of miR-218 results in impaired growth. Aggressive tumor growth is accompanied with increased bone resorption in the presence of miR-218 while antagonizing miR-218 protects from the development of osteolytic lesions. Wnt signaling is known to promote metastatic activity, and we demonstrate that the aberrant miR-218 expression positively correlates with high endogenous Wnt signaling activity as detected by the Wnt-responsive TopFlash reporter assay and expression of the Wnt transcriptional mediators. Mechanistically, miR-218 directly targets several inhibitors of Wnt and BMP signaling including Sclerostin, DKK2, sFRP-2, and TOB1, which were validated by several approaches. Ectopic expression of miR-218 further increases Wnt activity in MDA-MB-231 cells while inhibition of miR-218 decreases Wnt signaling. Importantly, bone sialoprotein (BSP) and osteopontin (OPN), both reported to be elevated in serum of breast cancer patients and CXCR-4, a chemokine receptor directly linked to bone metastasis are all upregulated in the presence of miR-218. Inhibition of Wnt signaling abolishes the miR-218-induced elevation, indicating a Wnt-dependent activation of these metastatic genes. We conclude that miR-218 activates Wnt and BMP signaling to enhance metastatic properties of breast cancer cells. Inhibition of miR-218 may serve as an attractive novel therapeutic approach for breast cancer-induced osteolytic disease.

P087

Blood Clots Do Not affect Metastasis Formation or Tumor Growth

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Cancer is associated with increased fracture risk, due either to metastasis or associated osteoporosis. Occasionally, a fracture site is later found to contain metastatic disease. Therefore, a fracture in patients with cancer often raises the question whether it is a pathologic fracture or whether the fracture itself might promote the formation of metastatic lesions. One of the first events in a fracture is the development of a hematoma, in which coagulation is activated. Blood clots contain proteins that directly affect tumor growth, such as thrombin, factor XIII, and fibrinogen. Furthermore, the platelets produce SDF-1, which is chemotactic for cancer cells. We therefore examined whether blood clot formation results in increased metastasis formation in a murine model of experimental breast cancer metastasis.

A clot was surgically induced in the bone marrow of the left tibia. Five Minutes later, MDA-MB-231B/luc+ breast cancer cells were introduced in the circulation by intracardiac injection. The number of cancer cells that homed to the intervention site was determined by quantitative RT-PCR. The presence of a blood clot was not associated with increased homing of cancer cells, as evidenced by comparable numbers of cells arriving at the site of intervention and the opposite side after 5 minutes, 1, 4, 24, and 48 hours of intracardiac cell injection. Thus, the presence of a blood clot does not offer a permissive environment for homing of cancer cells.

To evaluate metastasis formation and longitudinal growth, weekly bioluminescence measurements starting three weeks after intracardiac cancer cell injection were performed in a group that underwent clot induction and a control group. Survival was similar as was the number of macroscopic lesions. In the control group there were an average 4.3 tumors/mouse, and 4 localized to the left tibia. In the experimental group with clot formation there were an average of 4.6 tumors/mouse, and 2 localized to the left tibia (p=NS). Thus, the presence of a blood clot does not affect the development of a macroscopic lesion. In support of these findings, analysis of x-ray films revealed comparable sizes of lytic lesions between both groups.

In summary, the growth factors accumulating in a clot/hematoma are neither enough to promote cancer cell homing nor support growth. Accordingly, there is currently no evidence to support the possibility of metastasis formation in freshly injured areas in patients with cancer.

P088 Abstract presentation declined.

LYSYL Oxidase Drives Osteolytic Bone Lesions in a Breast Cancer Model via RANK Ligand Independent Effects On Osteoclasts: a New Player in the Vicious Cycle?

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Metastases to bone in breast cancer patients results in five year survival rates below 10%. The 'vicious cycle' of invading cancer cells uncouples bone remodelling resulting in increased osteoclast activity and a selective environment for tumour growth. The precise mechanisms behind how bone metastases initially establish in bone remain unclear. Lysyl oxidase (LOX) secreted from tumours increases metastasis and reduces patient survival. We have previously demonstrated that LOX plays a critical role in the formation of lytic lesions in bone, prior to cancer cell colonisation. In this study we provide exciting new evidence that LOX acts on osteoclasts independently of RANKL, increasing bone resorption and is an underlying mechanism of bone lesion formation in breast cancer. 4T1 cells were injected into the fat pads of syngeneic BALB/c mice and left for 3 weeks to develop primary tumours. TRAP stained tibiae from tumour bearing animals demonstrated a more than two-fold increase in osteoclast number compared to controls ($p=0.001$); genetic knockdown of LOX in the primary tumour abrogated these effects ($p=0.011$). The effect of LOX was studied further using human osteoclasts *in vitro*. Cultures treated with recombinant LOX in the absence of RANKL had a more than two-fold increase in cell number ($p<0.001$), a twelve-fold increase in total resorption ($p<0.001$) and more than five-fold increase in resorption per osteoclast ($p<0.001$) compared to RANKL treated controls. Immunohistochemical labelling for the master regulator of osteoclasts nuclear factor of activated T cells (NFAT) c1, demonstrated that recombinant LOX significantly increased NFATc1 translocation to the nucleus compared to RANKL ($p=0.017$). This affect was attenuated in the presence of a blocking LOX antibody ($p<0.001$). The addition of catalase to remove the by-product of LOX activity hydrogen peroxide (a potent reactive oxygen species) abrogated NFATc1 nuclear translocation ($p<0.0001$). These data demonstrate that LOX drives de novo osteoclastogenesis via a ROS mediated effect independently of RANKL. Identification of the mechanism behind disease mediated uncoupling of bone remodelling offers new therapeutic targets and inaugurates LOX as a novel player in the 'vicious cycle'.

P089

Modeling the Breast Cancer Metastatic Niche in Human Bone Tissues

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Bone is a preferential site of breast cancer metastasis and models are needed to study this process at the level of the

microenvironment. The presence of disseminated tumor cells (DTCs) in the marrow compartment during the early stages of breast cancer is associated with risk for later breast cancer recurrence. It is assumed that DTCs remain in a dormant state before transitioning into "the vicious cycle of metastasis", leading to the widespread destruction of ossified bone. To study this transition additional approaches are needed that will provide direct access to the microenvironment of both the marrow and ossified bone compartments. The co-culture of viable bone tissues with cancer cells offers a new model for characterizing these interactions directly within the 3-dimensional architecture of the bone microenvironment.

We have used bioluminescence imaging (BLI) and multiplex biomarker immunoassays to monitor dynamic breast cancer cell behaviors in co-culture with human bone tissue. Femur tissue fragments harvested from hip replacement surgeries were co-cultured with luciferase-positive MDA-MB-231-fLuc cells and BLI was performed to quantify breast cell proliferation, track migration relative to bone tissue, and monitor colonization of bone tissues. Breast cell colonization of bone tissues was confirmed with immunohistochemistry. Biomarkers in co-culture supernatants were profiled with MILLIPLEX® MAP immunoassays.

BLI demonstrated increased MDA-MB-231-fLuc proliferation ($p<0.001$) in the presence vs. absence of bones, and revealed breast cell migration toward bone. Immunohistochemistry illustrated MDA-MB-231-fLuc colonization of bone, and MILLIPLEX® MAP profiles of culture supernatants suggested breast/bone crosstalk.

We observed highly reproducible patterns of breast cell/bone tissue interactions within these cultures. Because of the accelerated time course of interactions, and the immediate quantitation afforded by BLI, this approach enables the direct, rapid study of dynamic processes within the metastatic niche. This approach circumvents the prolonged time course associated with *in vivo* metastasis models, offering the potential for high throughput perturbation of specific targets to identify and evaluate therapeutic interventions to prevent and treat bone metastasis.

P090

TMPRSS2:ERG Overexpression in a Prostate Cancer Cell Line Mimics Cell-To-Cell Contact with Osteoblasts

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The soil for systemic metastases in prostate cancer (PCa) is thought to be the bone. The invasion of the bone compartment by prostate tumor cells causes predominantly osteoblastic lesions with an increase in bone mineral density. The precise mechanisms underlying the homing and the invasion of prostate tumor cells in bone are poorly understood. The interaction between tumor cells and bone environment cells, such as osteoblasts, osteoclasts or bone marrow cells, is critical for the development of bone metastases.

Recurrent genomic rearrangements resulting in the fusion of the 5' untranslated region of a prostate-specific androgen-responsive gene to ETS family genes, coding for transcription factors, were found in prostate cancer tissues. The most frequent fusion gene identified, found in over 50% of prostate cancers and causing aberrant ERG over-expression in prostate cancer cells, is TMPRSS2:ERG (type II transmembrane serine protease, ETS-related gene). ERG is a transcription factor involved in skeletal formation and maintenance. We therefore hypothesized a link between Erg target genes expression and bone metastasis formation. Our recent work has shown that ERG over-expression in PCa cells upregulates target genes and more particularly Osteopontin (OPN) and Metalloproteinase-9 (MMP9), which are known to be involved in bone metastasis processes.

In order to identify the whole TMPRSS2:ERG target genes expression, we established TMPRSS2:ERG-expressing PC3 clones and investigated their transcriptomic expression profile using microarray analysis. Surprisingly, TMPRSS2:ERG involved in the regulation of a series of genes which have been described in native PC3 cells cocultured with osteoblasts under contact conditions. These genes have already been reported to participate in bone resorption and formation.

These results suggest that TMPRSS2:ERG may have osteomimetic properties and participate in the development and progression of prostate cancer bone metastases.

P091

PTHRP Promotes Osteoblastic Lesions Through C-Jun-Mediated Down-Regulation of DKK1 Expression in A Murine Model of Prostate Cancer Bone Metastasis

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Prostate cancer (PCa) bone metastases induce osteoblastic lesions through unknown mechanisms. The Wnt inhibitor dickkopf-1 (DKK1) has been shown to promote PCa growth in bone and inhibits Wnt-mediated osteoblast activity. As bone metastases progress, DKK1 expression decreases, which favors osteoblastic activity. Mechanisms that regulate DKK-1 expression in PCa are unknown. Parathyroid hormone-related protein (PTHrP) inhibits DKK1 expression through Jun-mediated inhibition of β-catenin activity on the DKK1 promoter *in vitro*. In this study, we examined if the ability of PTHrP to regulate DKK1 expression through c-Jun contributes to PCa-induced osteoblastic activity.

To determine if c-Jun modulation impacts PCa-mediated bone remodeling through downregulation of DKK1expression Jun and/or DKK1 were overexpressed in the osteolytic PC-3M PCa

cell line and cells were injected into mice tibiae. After 4 weeks, control PC-3M or PC-3M cells overexpressing DKK1 induced osteolytic lesions and decreased bone mineral content (BMC); overexpression of c-Jun reduced their osteolytic activity and restored BMC; whereas, overexpression of DKK1 reversed the ability of c-Jun to diminish the osteolytic phenotype. To determine if PTHrP mediates its effect on PCa bone metastasis and tumor growth in bone through inhibition of DKK1 *in vivo*, PTHrP and/or DKK1 expression was knocked down in PC-3M and cells were injected into mice tibiae. After 4 weeks, PC-3M cells induced osteolytic activity. DKK1 knockdown inhibited tumor growth and osteolysis. Knockdown of PTHrP alone increased osteolysis and tumor growth compared to control cells; however, the addition of knocking down DKK1 in the PTHrP knockdown cell lines reduced the osteolytic activity induced by PTHrP knockdown alone and decreased tumor volume.

In summary, these results, in combination with our previous *in vitro* studies, demonstrate that PTHrP inhibits osteolytic activity of PC-3M cells through Jun-mediated downregulation of DKK1 expression. Overall, this activity can contribute to the osteoblastic phenotype of PCa bone metastases.

P092

SOSTDC1: A Novel Soluble BMP and WNT Antagonist Involved in the Dysregulation of Bone Formation in Multiple Myeloma. Z. FARAAHI*1, C. EATON1 and P. CROUCHER2 AND C. BUCKLE3

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Rationale and Hypothesis: Multiple myeloma (MM) is characterised by destructive bone disease, mediated by an increase in osteoclastic bone resorption and impaired osteoblastic bone formation. The canonical Wingless-type (Wnt) and Bone Morphogenic Protein (BMP) signalling pathways have both been implicated in the regulation of bone formation. Our data show that SOSTDC1 is upregulated in the bone marrow (BM) of mice with osteolytic bone disease associated with myeloma. It is unclear whether SOSTDC1 regulates signalling in bone directly, and also which cells in the BM express SOSTDC1. We hypothesise that SOSTDC1 disrupts Wnt and BMP signalling in bone and is expressed by both myeloma cells (MC) and osteoblasts (OB) as a result of myeloma cell/osteoblast interaction. The objectives of this study were to (i) assess the effect of SOSTDC1 on mouse primary osteoblast (pOB) differentiation in the presence of activated BMP and Wnt signalling and (ii) to determine the conditions under which myeloma cells express SOSTDC1.

Materials and Methods: Mouse pOB were treated with recombinant SOSTDC1 protein, in the presence or absence of BMP2, BMP7 or Wnt3a. The effects of SOSTDC1 treatment on BMP and Wnt signalling were determined by measuring BMP-activated Smad phosphorylation and phosphorylated β-catenin respectively, using western blot. qPCR was used to assess the effects of SOSTDC1 on the regulation of genes involved in pOB differentiation. Flow-cytometry, immunofluorescence and western blot analysis were used to determine the distribution of SOSTDC1 in MC/OB cultures and co-cultures.

Results: SOSTDC1 suppressed BMP2- and BMP7-induced Smad phosphorylation and also Wnt3a-induced β-catenin phosphorylation in pOB ($P<0.05$). Both *Runx2a* and total *β-catenin* expression (qPCR) were reduced following treatment with SOSTDC1, suggesting that SOSTDC1 suppresses Wnt3a-induced pOB differentiation ($P<0.05$). Flow-cytometry, immunofluorescence and western blot data all suggest that SOSTDC1 is switched on in OB and increased in MC, when these two cells are cultured together.

Conclusions: Our findings suggest that increased SOSTDC1 levels may result from direct OB/MC contact and that this may result in suppression of OB differentiation. Taken together these results suggest that targeting SOSTDC1 may reduce osteolytic bone disease observed in MM.

P093

GFI1 and EPIGENETIC Regulation Of Runx2 In Multiple Myeloma Exposed Bone Marrow Stromal Cells

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Multiple myeloma (MM) is the most frequent cancer to impact the skeletal system by inducing osteolytic bone lesions that rarely heal even after treatments inducing remission. MM-induced alteration of the bone marrow microenvironment causes suppression of bone marrow stromal cell (BMSC) differentiation into functional bone-forming osteoblasts (OB). Evidence indicates that MM cells or TNFα plus IL-7 (TNFα/IL7) treatment causes suppression of the key OB differentiation factor Runx2, while up-regulating expression of IL6, which further supports MM cell growth. Previous studies in our laboratory have demonstrated that MM cell or TNFα/IL7 upregulate the transcription repressor Gfi1, which leads to Runx2 repression that endures long after removal of the MM cells and TNFα/IL7. Similarly, IL6 expression remained elevated. This led to the hypothesis that long term OB suppression results from Gfi1 binding to the Runx2 gene and recruitment of various chromatin modifiers. Therefore to determine the mechanisms of BMSC suppression, we investigated the MM-induced recruitment of co-repressors and epigenetic changes on the Runx2 and IL6 genes. We found that MM and TNFα/IL7 induced changes in the spatial distribution of various chromatin marks such as H3K4me3, H3K9ac, H3K36me3 H3K9me1, H3K27me3 and histone modifiers p300 and HDAC1 that were coordinate with binding of Gfi1 to the Runx2 promoter, decreased Pol II, and Runx2 repression, further arguing for Gfi1's direct role in Runx2 repression. The opposite histone changes occurred on the IL6 gene along with Pol II increases. Although a point mutation (N382S) in the Gfi1 DNA binding domain (DBD) known to block DNA binding in other systems does not do so on the Runx2 promoter Gfi1 binding site, Gfi1 with a 43 amino acid deletion of the DBD did not suppress

a Runx2 reporter and endogenous Runx2 mRNA expression. Novel sites of Gfi1 phosphorylation and acetylation affect Runx2 repression by Gfi1. Collectively our data suggest that MM cells induce various epigenetic changes along the Runx2 and IL6 genes, which are likely responsible for the prolonged alterations of BMSC physiology. Further, we provide additional evidence that Gfi1 mediates Runx2 repression directly. The understanding of the mechanisms associated with the suppressive effects of Gfi1 may lead to advancements of therapeutics for various inflammatory diseases causing homeostatic imbalance in the bone microenvironment such as MM and rheumatoid arthritis.

P094

Targeted Disruption of Beta3 Integrin in Myeloid Cells Promotes Tumor Growth through Enhanced M2 Macrophage Polarization

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Integrin beta 3 (B3) plays a critical role in bone in both healthy and pathologic states. B3–/– mice are protected from bone metastasis due to reduced platelet and OC activity. Increased B3 expression in breast cancer and melanoma is associated with more aggressive tumors, overexpression of $\alpha v\beta 3$ integrin in breast cancer increase bone metastasis. Despite these roles for B3 in both the tumor and bone compartments, inhibition of B3 in clinical trials have had mixed results. We hypothesize that this is due to differential effects on tumor cells and M1 (anti-tumor)—M2 (pro-tumor) macrophage polarization. Mice with targeted disruption of B3 in myeloid cells, knockout in myeloid (KOM) mice, have higher bone mass due to impaired OC function. Despite normal pathologic angiogenesis, KOM mice had enhanced tumor growth and increased M2 polarized macrophages in the tumor microenvironment. In murine tumor models (B16-F10 melanoma, MMTV-PyMT breast cancer), B3–/– and KOM mice had enhanced tumor burden compared to WT. By FACS analysis, we found that KOM and WT mice had equal numbers of tumor-associated macrophages (TAMs: CD11b+ Ly6c- F480+), but the M2 macrophage population (CD11b+ Ly6c- F480+ MHCII- CD206+) in the local tumor tissue was significantly increased in KOM mice. These data suggest that B3 regulates macrophage polarization, and the increased pro-tumor M2 macrophage population may contribute to the enhanced tumor growth in B3–/– and KOM mice. M2-polarizing cytokines IL-4 and IL-13 strongly induced B3 expression in bone marrow macrophages, while M1-polarizing cytokine IFN-g decreased B3 expression. *In vitro*, B3–/–macrophages showed enhanced proliferation and M2 macrophage marker expression (YM1, FIZZ1) compared to WT. M1 macrophage signaling acts on STAT1, while M2 polarization results in STAT6 activation. Under M2-polarizing conditions, B3–/– macrophages had decreased STAT1 (M1) phosphorylation but enhanced STAT6 (M2) phosphorylation. Overexpression of B3 in B3–/– macrophages partially rescued this shift in phosphorylation states. Together, these data suggest that integrin B3 signaling can promote anti-tumor M1 macrophage polarization and acts as a negative regulator of tumor progression. Therefore, the B3 integrin pathway remains a promising therapeutic target. Selective activation of the B3 pathway in TAMs may decrease tumor growth and metastasis.

P095

Targeting the Hedgehog Pathway to Inhibit Osteosarcoma Growth Through Dual Effects on Tumor and Microenvironment Cells

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Hedgehog (Hh) signaling contributes to the progression of many cancers through both cell-intrinsic and paracrine mechanisms. As Hh is a key regulator of bone development, this pathway is an attractive candidate for treatment of bone-localized cancers. We have shown that activated host Hh signaling generates a pro-tumorigenic microenvironment by inducing osteoclastic resorption and stromal production of tumor growth factors. The growth of osteosarcoma (OS), a primary bone cancer, is influenced by interactions with its microenvironment. Defining these coupling factors will identify novel targets for therapeutic intervention. We have shown that mice lacking the tumor suppressor ARF have enhanced rates of bone turnover, mimicking the adolescent growth period in which OS is prevalent. By crossing Arf–/– mice with those expressing Tax, an HTLV-1 oncogene that results in osteolytic tumors, we developed a model of high penetrant spontaneous OS that recapitulates many aspects of human disease. Pharmacological suppression of bone turnover prevented the development of OS, demonstrating that osteoclast (OC) activity stimulates OS growth. In agreement, Tax+Arf–/– OS cells directly stimulated osteoclastogenesis. Compared to normal osteoblasts (OB) and mesenchymal precursors, Tax+Arf–/– OS cells have increased expression of Hh pathway genes and are directly susceptible to Hh inhibitors (SMO antagonists). In particular, Tax+Arf–/– OS cells express high levels of the Hh ligands Sonic (SHH) and Indian (IHH), suggesting they may stimulate surrounding cells in a paracrine fashion. Conditioned media from Tax+Arf–/– OS cells enhanced bone marrow stromal cell production of pro-tumorigenic growth factors including IL-6 and IGF, generating a fertile niche. Furthermore, the increased OB and OC activity present in Tax+Arf–/– cells could be abrogated with SMO inhibitors. We have established an OS cell line from Tax+Arf–/– mice (TAN) with the ability to form mineralized tumors upon orthotopic intratibial injection. In this model, LDE225, a SMO antagonist currently in Phase I/II clinical trials, potently decreased tumor growth and bone pathology. This inhibition of OS was attributed to both direct tumor cytotoxic effects and modulation of pro-tumor microenvironment. As human OS have variable susceptibility to Hh inhibitors, we are testing a panel of cell lines to determine if microenvironment-directed activities alone exert clinically beneficial effects even in drug-resistant tumors.

P096

Blocking Syndecan-4 Inhibits Bone Metastasis Formation: Potential Involvement in the Prometastatic Activity of Autotaxin

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Autotaxin (ATX/NPP2) is a secreted glycoprotein that generates Lysophosphatidic acid (LPA) due to its lysophospholipase D activity. We have shown that ATX controls the progression of osteolytic bone metastases through the production of LPA in the tumor microenvironment. However, the molecular mechanisms involved in the local production of LPA at the bone metastatic site are still not well characterized. Binding of ATX to beta3 integrins has been proposed for LPA delivery to its receptors present at the surface of tumor cells. However, we found *in vitro* that the treatment of tumor cells with LM609 monoclonal antibody only partially inhibited ATX interaction with tumor cells indicating the involvement of other partners than alphavbeta3 integrins. Recent studies suggested that ATX could potentially interact with cell surface Heparan-sulfate proteoglycans (HSPs). Among the HSP family members, we found that syndecan-4 (SDC4) was commonly highly expressed in different cell lines. Silencing of SDC4 expression by synthetic siRNAs in MG-63 osteosarcoma cells and in 4T1 murine breast cancer cells, highly decreased cell adhesion on ATX. Additionally, pre-treatment of MG-63 cells with an anti-human SDC4 antibody (5G9) and pre-treatment of 4T1 cells with an anti-mouse SDC4 antibody (KY/8.2), but not with the isotypic control antibody, also decreased cell adhesion to ATX. We have already shown that 4T1 cells that induce the formation of osteolytic bone metastases in immunocompetent BALB/c mice, express active ATX and that stable silencing of ATX in these cells inhibited the extent of osteolytic lesions. Here, we showed that pre-treatment of 4T1 cells with the anti-mouse SDC4 antibody (KY/8.2) but not with the isotypic control antibody, before intravenous injection to BALB/c mice significantly decreased the number of medullar disseminated tumor cells. Moreover, pre-treatment of 4T1 cells with anti-mouse SDC4 antibody reduced the extent of osteolytic lesions after intraosseous injection. Altogether, these results demonstrated for the first time the role of SDC4 in bone metastasis formation and strongly suggested close interaction of SDC4/ATX at the cell surface during this process. These results may have important implications in the development of new therapies for patients with bone metastases.

P097

Role of the Tumor-Bone Microenvironment in Muscle Weakness and Cachexia

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Cancer cachexia is a devastating paraneoplastic syndrome that frequently occurs with breast cancer bone metastases and is characterized by weight loss and muscle weakness. Using a model of human breast cancer metastatic to bone (MDA-MB-231) we show that skeletal muscle dysfunction is independent of weight loss and not present in mice with primary breast cancer (no bone metastases).

Mice with bone metastases, compared to age-matched control mice, lost significant weight by 4wks (20.5 g $\pm$ 0.6 v. 23.2 g $\pm$ 0.4; $p<0.001$) due to loss of lean and fat mass. *Ex vivo* contractility of the extensor digitorum longus (EDL) muscle showed a significant reduction in specific force (corrected for muscle size) in tumor mice (213.2 kN/m^2 $\pm$ 17 v. 361.1 kN/m^2 $\pm$ 10; $p<0.001$) that correlated with greater osteolysis ($p<0.05$). To determine if muscle weakness was due to weight loss associated with reduced food consumption, we monitored food and water intake in mice with bone metastases. These mice had a 40% reduction in food intake during the last week before death. We restricted food of healthy mice by 40% for one week and despite a significant decrease in body weight, mice with caloric restriction exhibited no decrease in forelimb grip strength or EDL specific force, indicating that loss of muscle mass alone cannot account for muscle weakness associated with cancer. It is well established that cancer is associated with oxidative overload. We examined oxidation of muscle proteins and found that sarcomeric proteins (tropomyosin/myosin) and excitation-contraction coupling proteins (ryanodine receptor/Ca2+ release channel (RyR1) on the sarcoplasmic reticulum) were oxidized in mice with bone metastases. Oxidation of RyR1 causes depletion of the stabilizing subunit calstabin1 resulting in intracellular Ca2+ leak and muscle weakness. This was not observed in muscle from mice with primary breast cancer. Preventing oxidation-mediate loss of calstabin1 from RyR1 with a Rycal (S107) restored muscle function (431.0 kN/m^2 $\pm$ 19.4 v. 362.8 kN/m^2 $\pm$ 7.2; $p<0.0001$) with no affect on body weight. S107 treatment also increased intracellular Ca2+ release during tetanic stimulation.

Thus, there is a primary loss of muscle function in addition to weight loss in mice with bone metastases. These results demonstrate a mechanism for skeletal muscle dysfunction in cancer cachexia: leaky RyR1 channels. Moreover, these data show that the bone microenvironment plays a critical role in intracellular Ca2+ leak via remodeled RyR1.

P098

Multiple Myeloma Bone Marrow Derived Mesenchymal Stem Cells (MSCs) Show Decreased Osteogenesis in Part Due to Decreased Expression of Microrna HSA-MIR-199A-3P, MIR-15A-5P and MIR-16-5P

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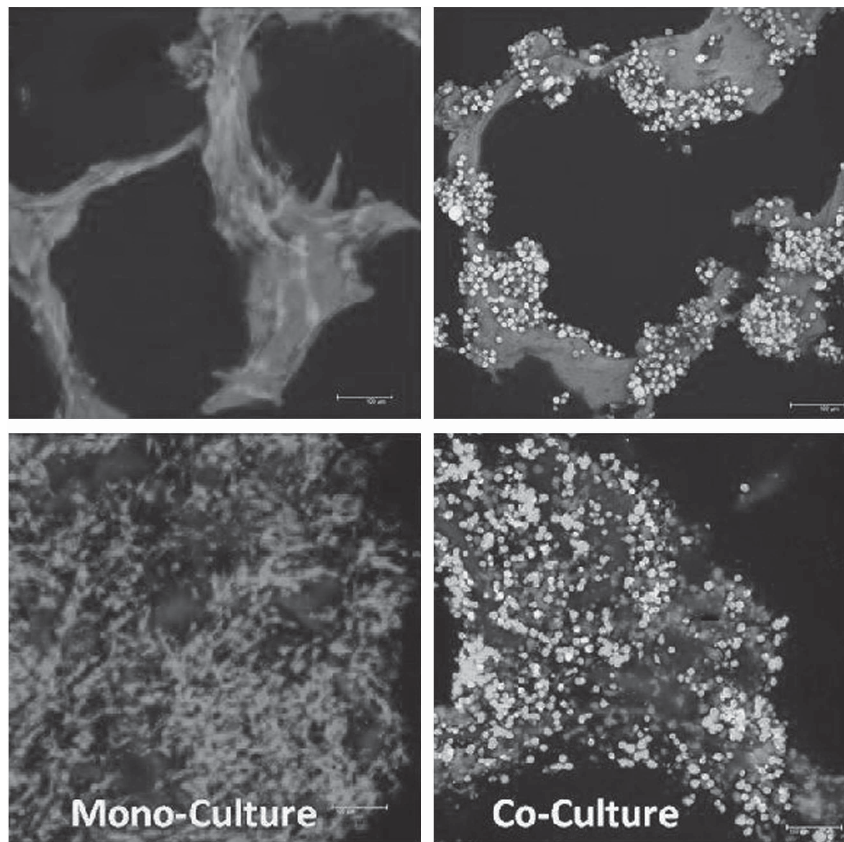
Multiple Myeloma (MM) involves clonal proliferation of malignant plasma cells within the bone marrow leading to a deterioration of bone structure and function. The disease creates a forward feedback system with local mesenchymal stem cells (MSCs) within the bone marrow that causes abnormalities in MSC function, but the exact mechanisms, cellular changes, roles of miRNAs, and downstream consequences within the stroma are largely unknown.

Proliferation and osteogenic differentiation of MSCs from myeloma patients (MM-MSCs) and normal donors (ND-MSCs) cultured with or without MM1S cells were characterized in 2D culture and in 3D porous silk scaffolds (Figure 1). MM-MSCs demonstrated decreased osteogenesis assessed by alizarin red staining (ARS) and qRT-PCR for osteopontin, osteocalcin, and RUNX2. Three-dimensional *in vitro* co-cultures assessed cell proliferation, cell-cell interactions and ECM production using confocal microscopy, IHC and histology, and demonstrated MM inhibition of MSC growth and osteogenesis. qRT-PCR and Nanostring were used to examine 800 miRNAs and 230 cancer-related mRNAs in clinical samples and *in vitro* co-cultures. Thirty-seven microRNAs were significantly different between MM- and ND-MSCs, including a down-regulation of miR-15a, miR-16, Let-f and miR-199a-3p, (>1.2 fold, $p<0.05$). Unbiased gene expression profiling showed discrete clustering of MM-MSCs distinct from ND-MSCs and 51 mRNAs significantly different between the groups (>1.2 fold, $p<0.05$). Higher expression of CDKN1A and CDKN2A was observed in MM-MSCs, suggesting a novel mechanism to explain their inhibited proliferation.

Of the miRs with decreased expressed in MM vs ND-MSCs and in the 3D model (MM.1S-co-cultured MSCs vs MSCs alone), 5 were investigated for potential osteoinductive effects using miRvana mimics. Overexpression of hsa-miR-199a-3p, miR-15a-5p and miR-16-5p increased MM-MSC expression of osteogenic markers (OPN, BGLAP, and RUNX2) and 199a-3p increased mineralization based on ARS.

Our 3D platform provides a simple, flexible, clinically relevant tool to model myeloma growth within bone. We utilized the 3D scaffold model system to investigate bone and cancer interactions during osteogenesis with non-destructive imaging techniques. The model recapitulated decreased bone formation as found in MM patients and proposed miR-199a-3p, miR-15a-5p and miR-16-5p as novel bone anabolic agents, which were confirmed to induce osteogenesis *in vitro* in clinical samples.



P099

Stem Cell Factor Enhances the Metastatic Potential of Prostate Cancer Cells In Vitro Jiménez-Sánchez Alejandro, Rocha-Zavaleta Leticia. Instituto de Investigaciones Biomédicas, Unam. México

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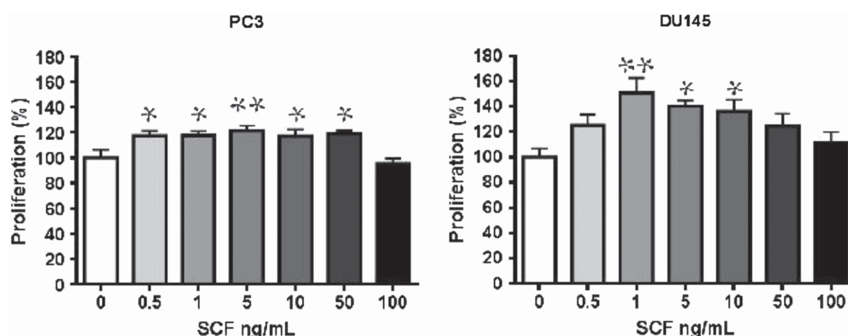
The bone marrow is commonly affected by metastasis. In fact, 90% of patients with advanced prostate cancer develop bone metastasis. The mechanism controlling this metastatic pattern is not fully understood. Stem Cell Factor (SCF) is one of the molecules orchestrating haematopoiesis within the bone marrow microenvironment. The SCF receptor (c-Kit) is considered a proto-oncogen, its activation prompts proliferation, survival, and migration of some cancer types. In prostate cancer the expression of c-kit has been documented. However, the participation of c-kit in the development of prostate cancer metastasis has not been fully explored. Here we assessed the effect of SCF in the metastatic potential of two human prostate cancer cell lines *in vitro*.

The expression of c-kit was demonstrated in two cell lines derived from prostate cancer, PC3 (bone marrow metastasis) and DU145 (brain metastasis), by RT-PCR and Western blot. Interestingly, flow cytometry analysis showed that only 1.32% of PC3 and 0.18% of DU145 cells express c-kit. To investigate the effect of SCF on proliferation, the cells were incubated with SCF (0–100 ng/mL), and evaluated using the MTT assay; we found that SCF induced a 20%-increase on PC3 proliferation at concentrations of 0.5–50 ng/mL, and a 50%-increase on DU145 at 1–10 ng/mL. Next, we evaluated the effect of SCF on survival in the absence of nutrients. We found that SCF was unable to protect PC3 and DU145 cells from death. Migration of cells was also assessed in the presence of SCF using Boyden chambers. 100 ng/mL SCF induced a 1.7-fold and a 3.3-fold increase of migrating cells in PC3 and DU145 cells, respectively. Finally, the effect of SCF on colony formation was evaluated. 10 ng/mL SCF was able to increase the number of cell colonies formed in both cell lines after 2 weeks incubation.

In conclusion, although c-Kit is expressed in a less than 2% of PC3 and DU145 cells, SCF stimulates proliferation, migration and cell colony formation, but it is unable to protect cells from lack of nutrient-mediated death. Our results suggest that SCF may be an important factor within the bone marrow microenvironment that fosters the formation of bone metastasis from prostate tumours.

Table 2 Effect of SCF on the metastatic potential of PC3 and DU145 cells *in vitro*

Assay	Proliferation			Survival			Migration			Colony formation		
SCF [ng/mL]	1	10	100	1	10	100	1	10	100	1	10	100
Fold increase												
PC3	1.18	1.21	0.95	0.86	0.73	0.88	0.73	0.67	1.76	0.64	1.44	0.76
DU145	1.51	1.36	1.11	0.97	0.94	1.14	0.37	2.05	3.39	0.66	1.49	0.56



Proliferation of PC3 and DU145 cells.

P100

Mutational Profiling of Synchronous Bone Metastases from Lung Adenocarcinoma: Feasibility and Results in a Prospective Cohort of 46 Patients (The Poumos Study)

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Background: Mutational profiling for targetable oncogenic drivers is recommended in the pretherapeutic workup of metastatic lung adenocarcinoma. Pathological and molecular diagnoses may be performed on specimens from metastatic lesions, when the primary pulmonary tumor is not accessible, either because of proximal location increasing the risk of transthoracic procedures, or when the material collected is insufficient in size. The feasibility of performing molecular diagnoses on small specimens from bone metastases has been questioned over time.
Methods: POUMOS was a prospective study, from our multidisciplinary group, aimed at evaluating the feasibility of routine percutaneous biopsy of synchronous bone metastases from lung adenocarcinoma, in order to perform pathological diagnosis and mutational profiling on the bone lesion. Results were correlated with that obtained on specimens from the primary tumor, if available. Technically, bone metastasis specimens were sent fresh for immediate formalin-fixation, and, if possible, snap-frozen. Decalcification of bone was performed using EDTA for a better preservation of cell morphology and DNA. Mutational profiling was conducted as recommended by the French National Cancer Institute (INCa). DNA extraction was performed after laser microdissection of bone metastases specimens, to concentrate tumoral DNA.
Results: Starting April 2011, 46 patients with lung adenocarcinoma and synchronous bone metastasis were enrolled. No grade 3–4 adverse effects were reported after the bone biopsy. Mutational profiling was obtained in 45 (98%) cases. EGFR mutation was observed in 6 (13%) patients, KRAS mutation in 14 (30%) patients, HER2, BRAF and PIK3CA mutations in 1 (2%) patient each. Updated results, especially correlations between the mutational profiles of primary lung tumors and bone metastases, will be reported at the meeting.
Conclusions: Our data demonstrate the feasibility of percutaneous biopsy of synchronous bone metastasis from lung adenocarcinoma to conduct mutational profiling for common oncogenic alterations. The establishment of multidisciplinary teams to ensure the coordination between clinicians, radiologists and pathologists, makes routine pathological and molecular diagnosis on bone metastasis specimens a fast, reliable and safe procedure. Moreover it allows if necessary a local antalgic treatment such as percutaneous cementoplasty after the biopsy was performed.

P101 Abstract presentation declined.

Bone Marrow Fat and Tumor Metabolism in Bone: New Insights into Growth and Survival of Metastatic Prostate Cancer in the Skeleton

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Bone is a predominant site of metastasis from prostate cancer (PCa). The incidence of skeletal lesions and death from this disease greatly increase with age and obesity, conditions, which increase bone marrow adiposity. Obese men are reported to have more than 3-fold higher risk of developing metastatic disease compared to normal weight men receiving the same treatment. Marrow fat is a metabolically active component of metastatic niche with detrimental effects on bone integrity and function: it accelerates bone resorption, induces oxidative/inflammatory stress, and alters functionality and metabolism of various cell types within the bone marrow microenvironment. It has been previously demonstrated that PCa cells are attracted to adipocytes within the metabolically active red bone marrow where metastases commonly occur; however, the mechanisms of marrow fat cell involvement in tumor cell adaptation and growth in the skeleton are not understood.
Herein, using models of diet-induced marrow adiposity and intratibial tumor growth we investigated how this adipocyte-rich bone marrow niche affects tumor cell growth and survival in bone. Our results demonstrate that bone marrow fat accelerates progression of skeletal tumors in mice. We show that tumor growth and invasiveness are fueled by lipid trafficking between bone marrow adipocytes and cancer cells and involves activation of FABP4-PPARγ axis in tumor cells. We reveal that lipid chaperone FABP4 is overexpressed in tumors from obese mice and in bone metastatic tissues from prostate cancer patients. We also provide evidence of FABP4 involvement in modulating growth and behavior of metastatic tumor cells by increasing the availability of energy-dense lipids. Our additional data, stemming from adipocyte-tumor cell co-cultures and lipidomics analyses indicate tumor cells promote lipolysis in adipocytes to drive fatty acid release into the microenvironment. Collectively, our data demonstrate that metastatic tumor cells have parasitic relationship with bone marrow adipocytes: they utilize marrow adipocyte-supplied lipids to thrive and progress in skeletal sites. Studies investigating molecular mechanisms behind marrow fat involvement in tumor metabolism are ongoing.

P102 Abstract presentation declined.
Investigating the Effects of Bone Marrow Adipocyte- and Macrophage-Derived CXCL1 and CXCL2 in Prostate Tumor Progression in Bone

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Prostate cancer (PCa) is the second leading cause of cancer-related deaths among men. Although PCa is a slowly progressing disease, eventually patients develop resistance to treatment leading to metastasis to distant sites including the bone. Recent evidence suggests that overweight and obesity are positively associated with biochemical recurrence and death from PCa. Obesity leads the accumulation of adipocytes within the bone marrow. This process stimulates infiltration and activation of inflammatory cells, particularly macrophages, to promote adiposity-induced inflammation in bone. CXCL1 and CXCL2 are potent pro-inflammatory chemokines secreted by macrophages and adipocytes that have been implicated in tumor growth and invasiveness in ovarian, prostate, and breast cancers. However, the roles of these cytokines in altering the bone marrow niche to promote tumor progression are not understood. Herein, we hypothesized that obesity-induced inflammation modulates both the host and tumor microenvironments and promotes PCa progression in bone via CXCL1 and CXCL2. Using Boyden chamber assays, we showed that PC3 cells are more invasive when treated with CXCL1 and CXCL2, and these effects are blocked with neutralizing antibodies. ELISA and PCR-analyses revealed that PC3 cells differentially express tumor-derived CXCL1 and CXCL2 when cultured in the presence of media conditioned by primary bone marrow derived adipocytes (Adipo CM). Interestingly, bone marrow macrophages (BMM) are more invasive toward PC3 cells and Adipo CM. BMM invasion was partially blocked by both neutralizing antibodies and a selective inhibitor to the receptor for these pro-inflammatory chemokines, CXCR2. Preliminary evidence also suggests that CXCL1 and CXCL2 signaling may be important in osteoclast differentiation that aids in tumor growth within the bone. Studies are ongoing to determine how adipocytes and tumor cells alter the function of BMMs and osteoclasts to favor tumor progression in the bone. To date, our studies suggest that PC3 cells interact with macrophages and adipocytes to drive tumor aggressiveness by CXCL1- and CXCL2-dependent mechanisms.

P103
CXCL14: A Critical Factor in Prostate Cancer Bone Metastasis

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Prostate cancer is the most diagnosed cancer and number one cancer killer among men. Prostate cancer metastases show a proclivity for the bone. Five year survival rates for localized disease are near 100%; however, presentation of metastasis worsens prognosis and reduces the survival rates to 31%. PCa bone metastasis occurs in approximately 90% of these advanced cases and is the principal source of morbidity and death in late stage disease. Chemokines play a pivotal role in PCa cell homing and dissemination to the bone.
Once established, PCa metastases alter the bone microenvironment and form osteoblastic lesions. CXCL14/BRAK is a well conserved chemokine with no known signaling receptor. CXCL14 is a primordial CXC type chemokine and shares high sequence homology with the well-established bone homing factor CXCL12. We have discovered CXCL14 to be up-regulated in prostate cancer bone metastasis (H-Score mean 172.5, n=113) as compared to soft tissue lesions (H-score mean 99.5, n=57) and primary disease. Expression of CXCL14 protein in the PCa xenograft line LuCaP 23.1 is similarly increased in mouse tibia versus subcutaneous tumor. CXCL14 mRNA expression is absent from ARCaPM, but CXCL14 can be induced through introduction into mouse bone and functional studies by our lab indicate an inhibitory effect of CXCL14 on CXCL12 induced PCa chemotaxis in the ARCaPM cell line. This data suggest that bone has intrinsic properties that increase prostate cancer CXCL14 expression.

P104
Differential Anti-Tumour Effects of Zoledronic acid in Breast Cancer According to ER Status and Levels of Female Hormones

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Background: Breast cancer clinical trials, pre- (neo-adjuvant) and post-surgery (adjuvant), have demonstrated enhanced anti-tumour efficacy of Zoledronic acid (Zol) in patients with oestrogen receptor (ER) negative disease (neo-adjuvant), and low levels of female hormones such as inhibin (adjuvant). The tumour suppressor activin is inhibited by both inhibin, and its binding protein follistatin. We hypothesize that Zol can differentially affect tumour activin/follistatin interactions according to ER status and presence of inhibin.
Objectives: 1. Determine effect of follistatin and activin on breast cancer cell proliferation.
2. Establish effect of Zol and inhibin on breast cancer cell secretion of follistatin.
3. Compare serum levels of follistatin in breast cancer patients treated +/– neo-adjuvant Zol.
Methods: *In vitro*: Effect of follistatin and activin on proliferation of ER-negative and ER-positive breast cancer cell lines was assessed using an MTS assay, following treatment with follistatin (240–2400 pg/ml) and/or activin (60–6000 pg/ml) for 1, 3 and 5 days. Effect of Zol on secretion of follistatin into supernatant, was evaluated in both cell lines with 25 µM Zol for 48 hours +/–10 ng/ml inhibin A.
Clinical samples: Serum follistatin (baseline, day 5 and day 21) was retrospectively analysed according to ER status (ER-positive n=28, ER-negative n=9) from patients with operable or locally advanced breast cancer who received neoadjuvant FE100C chemotherapy (CT) +/– Zol (4 mg i.v.) with first cycle.

Results: *In vitro*: Follistatin stimulated tumour cell proliferation, negating the tumour suppressive action of activin in both cell lines. Zol decreased secretion of follistatin from ER-negative cells (–57.2% of control) but not ER-positive cells. The presence of inhibin in the medium diminished the decline in follistatin secretion seen with Zol (–30.8% of control).
Clinical samples: Percentage change from baseline in serum follistatin was significantly different at day 5 in ER-negative patients only (median % change; CT+Zol –38.9%±10.62, CT +5.33%±7.34, p=0.0159 Mann Whitney).
Conclusions: The anti-tumour effect of Zol in ER-negative breast cancer cells may be linked to its ability to decrease follistatin secretion, and inhibin diminishes this effect of Zol. The ER dependent effect of Zol on follistatin secretion *in vitro* is mirrored in neo-adjuvant clinical serum samples, suggesting Zol's effect on follistatin is influenced by ER status.

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