**Functional Inhibition of Osteoblastic Cells in an in vivo Mouse
Model of Myeloid Leukemia**

by

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Curriculum Vitae

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List of Publications

1. Calvi LM, Bromberg O, Rhee Y, Weber JM, Smith JN, Basil M, **Frisch BJ**, Bellido T. Osteoblastic expansion induced by parathyroid hormone receptor signaling in murine osteocytes is not sufficient to increase hematopoietic stem cells. *Blood*. 2012.
2. **Frisch BJ**, Ashton JM, Xing L, Becker MW, Jordan CT, Calvi LM. Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. *Blood*. 2012;119:540-550.
3. **Frisch BJ**, Porter RL, Calvi LM. Hematopoietic niche and bone meet. *Curr Opin Support Palliat Care*. 2008;2:211-217. Review
4. **Frisch BJ**, Porter RL, Gigliotti BJ, Olm-Shipman AJ, Weber JM, O'Keefe RJ, Jordan CT, Calvi LM. In vivo prostaglandin E(2) treatment alters the bone marrow microenvironment and preferentially expands short-term hematopoietic stem cells. *Blood*. 2009;114:4054-4063.
5. Weber JM, Forsythe SR, Christianson CA, **Frisch BJ**, Gigliotti BJ, Jordan CT, Milner LA, Guzman ML, Calvi LM. Parathyroid hormone stimulates expression of the Notch ligand Jagged1 in osteoblastic cells. *Bone*. 2006;39:485-493.
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Abstract

Pancytopenia is a major cause of morbidity in acute myeloid leukemia (AML), yet its cause is unclear. Hematopoietic stem cells (HSCs) are necessary for replenishing the entire hematopoietic system throughout the lifetime of an organism. HSCs reside in the bone marrow and are reliant on a niche or microenvironment for regulatory cues. Normal osteoblastic cells have been shown to support hematopoiesis and hematopoietic stem cells within the marrow environment. Therefore understanding effects of leukemia on osteoblastic cells is an area of great interest.

To define effects of leukemia on osteoblastic cells, we employed a syngeneic, immunocompetent murine model of AML. Leukemic mice demonstrated inhibition of osteoblastic cells, with decreased serum levels of the bone formation marker osteocalcin. Osteoprogenitor cells and endosteal-lining osteopontin+ cells were reduced, and osteocalcin mRNA in CD45 negative marrow cells was diminished. This resulted in severe loss of mineralized bone. Osteoclasts were only transiently increased without significant increases in bone resorption, and their inhibition only partially rescued leukemia-induced bone loss. In vitro data suggested a leukemia-derived secreted factor inhibited osteoblastic cells. Since the chemokine CCL-3 was recently reported to inhibit osteoblastic function in myeloma, we tested its expression in our model, and in AML patients. Consistent with its potential novel role in leukemic-dependent bone loss, *CCL-3* mRNA was significantly

increased in malignant marrow cells from leukemic mice and from AML patient samples.

Based on these results, we propose that CCL3 production by leukemic cells in the marrow inhibits osteoblastic cells and uncouples their activity from the resorptive action of osteoclastic cells. This disruption of the normal marrow microenvironment is a potential factor in the observed loss of normal hematopoiesis in leukemia.

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Abbreviations

A-MEM	α -Minimum Essential Medium
AML	Acute Myelogenous Leukemia
BMP	Bone Morphogenic Protein
CBC	Complete Blood Count
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of Differentiation
CFU-C	Colony forming unit-culture
CFU-GEMM	Colony forming unit-granulocyte erythrocyte macrophage monocyte
CFU-GM	Colony forming unit-granulocyte monocyte
CFU-OB	Colony forming unit-osteoblast
CT	Computed tomography
CTX	C-terminal telopeptides
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	Chemokine (C-X-C motif) receptor 4
DKK-1	Dkkopf-1
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-related kinases
FACS	Flow assisted cell sorting
FBS	Fetal bovine serum
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GVL	Graft-versus-leukemia
GY	Gray
H&E	Hematoxylin and Eosin
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant

IGF	Insulin-like growth factor
IL	Interleukin
LRP	Low density lipoprotein receptor-related protein
LSC	Leukemia stem cell
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
OPG	Osteoprotegerin
OPN	Osteopontin
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
PSA	Prostate specific antigen
PTH	Parathyroid hormone
PTH-1R	Parathyroid hormone receptor 1
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B ligand
RBC	Red blood cell
RUNX2	Runt-related transcription factor 2
SDF-1	Stromal derived factor 1
SNS	Sympathetic nervous system
TGF- β	Transforming growth factor β
TNF	Tumor necrosis factor
YFP	Yellow fluorescent protein
ZA	Zoledronic acid

Foreword

Data from chapter 2, 3, and 4 of this thesis has been published in *Blood* in 2012 as a manuscript entitled: functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. The authors of the paper include Benjamin Frisch, John Ashton, Lianping Xing, Michael Becker, Craig Jordan, and Laura Calvi.

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Chapter 1:

Introduction

1.1 Hematopoietic stem cell niche

One of the critical and unique functions of the skeleton in terrestrial vertebrates is to provide the anatomical spaces for storing and facilitating differentiation of hematopoietic progenitors and precursors. The most immature cells capable of generating the entire hematopoietic system are hematopoietic stem cells (HSCs) found in bone marrow. True HSCs are defined as a single cell capable of generating the entire hematopoietic system throughout the lifetime of an adult (Baum, Weissman et al. 1992). To perform this task they must constantly balance self-renewal and differentiation. While cell autonomous processes play a role in HSC cell fate decisions, in vitro studies have continuously demonstrated a loss of long-term engraftment capacity when HSCs are grown in culture without a supportive stromal cell layer (Lawal and Calvi 2011). In addition, several studies have shown that disruptions in the microenvironment can lead to aberrant hematopoiesis and even hematopoietic malignancies in mice (Walkley, Olsen et al. 2007; Walkley, Shea et al. 2007; Raaijmakers, Mukherjee et al. 2010). This has led to the hypothesis that a specific microenvironment or niche is necessary for the maintenance and regulation of normal adult hematopoiesis and HSCs. Due to the singular homing of HSC to bone marrow spaces, it has long been suspected that non-hematopoietic cells contained in the skeleton, as well as their secreted products, including the matrix, may provide cellular and molecular components that are critical for the regulation of hematopoiesis and

HSCs. This chapter will review the principal cell types and skeletal signals that have been implicated as regulatory cellular components of the HSC niche. These components not only illustrate the extraordinary complexity of skeletal tissue, but also provide critical clues to novel therapeutic targets for HSC expansion in conditions of myeloablative injury.

1.1.1 Osteoblastic cells

Schofield first proposed the concept that HSCs are regulated by microenvironmental interactions in 1978 (Schofield 1978). It would take nearly 20 years before the first studies elucidating the cellular components of the HSC niche would be performed, and osteoblastic cells were identified early on as supportive of HSCs. In vitro, osteoblastic cells can maintain the stem cell activity of bone marrow (Taichman and Emerson 1994; Taichman, Reilly et al. 1996; Calvi, Adams et al. 2003). Additional murine studies have demonstrated that transplanted HSCs preferentially engraft at the endosteal surface, and in close contact with osteoblastic cells (Nilsson, Johnston et al. 2001; Zhang, Niu et al. 2003; Lo Celso, Fleming et al. 2009; Xie, Yin et al. 2009) moreover in vivo HSCs with high proliferative and long-term engrafting potential are found tightly adhered to the endosteal surface (Haylock, Williams et al. 2007). Increased osteoblastic numbers have been shown to correlate with increased HSCs (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003), and the converse experiments have shown that a loss of the osteoblastic lineage of cells results in disrupted hematopoiesis (Visnjic,

Kalajzic et al. 2001; Visnjic, Kalajzic et al. 2004). Treatment with parathyroid hormone (PTH) is capable of stimulating self-renewal of HSCs and increasing the HSC population despite the fact that HSCs do not express the PTH receptor, PTH1R, indicating a microenvironmentally mediated effect (Calvi, Adams et al. 2003). While osteoblasts produce many soluble factors that are known to be important for hematopoiesis (Marusic, Kalinowski et al. 1993; Taichman and Emerson 1994; Calvi, Adams et al. 2003), direct cell contact is necessary for osteoblastic cell support of HSC self-renewal in vitro (Calvi, Adams et al. 2003).

In addition to increasing HSCs, studies utilizing either PTH treatment or the constitutive activation of PTH1R on osteoblastic cells revealed that PTH signaling increases bone volume and the expression of the Notch ligand Jagged1 on osteoblastic cells (Calvi, Adams et al. 2003; Weber, Forsythe et al. 2006). Notch signaling is known to support stem cell self-renewal in other stem cell systems and has been implicated in the support of HSCs (Milner and Bigas 1999; Karanu, Murdoch et al. 2000; Karanu, Murdoch et al. 2001; Karanu, Yuefei et al. 2003). Further the inhibition of Notch signaling ablates the PTH dependent increase in HSC number (Calvi, Adams et al. 2003; Weber, Forsythe et al. 2006). Additional studies have further suggested that Notch signaling between osteoblasts and HSCs plays a role in maintaining repopulating potential (Chitteti, Cheng et al. 2010; Weber and Calvi 2010). A separate study questioned the importance of canonical Notch signaling in the

HSC niche and work remains to be done elucidating the precise role Notch signaling plays in the maintenance of HSCs (Maillard, Koch et al. 2008).

Annexin-2 is expressed by both osteoblasts and endothelial cells in the bone marrow, and treatment of mice with annexin-2 inhibitors impairs HSC homing and engraftment (Jung, Wang et al. 2007). Angiopoietin-1 is expressed by osteoblasts and promotes stronger adhesion and quiescence in HSCs through interactions with the receptor tyrosine kinase Tie2 (Arai, Hirao et al. 2004). Genetic deletion of both Tie1 and Tie2 in mice results in a loss of maintenance of HSCs in the marrow, again suggesting a role of angiopoietin-1 (Puri and Bernstein 2003). Osteoblastic cells also express Thrombopoietin, a regulator of HSC quiescence (Qian, Buza-Vidas et al. 2007; Yoshihara, Arai et al. 2007). Moreover, osteolineage cells produce local CXCL12, a critical regulator of HSC homing and maintenance (Ponomaryov, Peled et al. 2000).

Though direct cell contact is required for HSC maintenance in vitro short-lived secreted signals are also potential osteoblastic-dependent HSC regulators. Prostaglandin E₂ (PGE₂) is an arachidonic acid derivative that is produced by multiple cell types within the bone marrow including osteoblastic cells. Treatment of mice with PGE₂ increases short-term repopulating HSCs (Frisch, Porter et al. 2009) and ex vivo treatment of HSCs with dimethyl-PGE₂ increases HSC repopulating potential both in mouse bone marrow and in human cord blood samples (North, Goessling et al. 2007; Hoggatt, Singh et al. 2009; Goessling, Allen et al. 2011), one of the first illustrations of how

identification of HSC regulatory signals can be used pharmacologically for therapeutic purposes.

Osteoblastic cells are also critical regulators of osteoclastic activity. Osteoclastic cells were first implicated in HSC regulation when data demonstrated that lack of the Calcium sensor impaired the ability of HSCs to home to the skeleton (Adams, Chabner et al. 2006) increases in local calcium would be expected to co-localize with actively resorbing functional osteoclasts. Moreover, it was noted that treatment with strontium, which increases the osteoblastic pool but inhibits osteoclasts, did not expand HSCs (Lympieri, Horwood et al. 2008). In addition, increased osteoclastic bone resorption has been shown to reduce the levels of local CXCL12 and to stimulate mobilization of HSCs from the bone marrow (Kollet, Dar et al. 2006; Cho, Joo et al. 2010). Together these data have suggested a supportive role for osteoclasts in the HSC niche, and inhibition of osteoclasts has recently been associated with reduction of HSC numbers in vivo (Lympieri, Horwood et al. 2008). However, recent data using genetic murine models have suggested that osteoclasts are dispensable for HSC maintenance and mobilization (Miyamoto, Yoshida et al. 2011).

While numerous studies, using both in vivo and in vitro experimental models firmly implicate cells of the osteoblastic lineage in HSC maintenance and regulation, the differentiation stage of the osteolineage cell which supports HSCs has begun to emerge, and have supported the hypothesis

that immature cells in the osteoblastic lineage are critical for HSC regulation. Self-renewing osteoprogenitor cells in the marrow can form supportive HSC niches (Sacchetti, Funari et al. 2007). And most recently Nestin positive putative mesenchymal stem cells (MSCs) express high levels of genes implicated in HSC maintenance. Additionally these Nestin positive cells are spatially co-located with HSCs and depletion of the Nestin positive population results in HSC mobilization (Mendez-Ferrer, Michurina et al. 2010). Conversely, activation of the PTH1R in terminally mature osteocytes expands the osteoblastic population, but does not increase microenvironmental support for HSCs (Calvi, Bromberg et al. 2012). Data suggest that an immature osteoblastic cell is a critical factor of the HSC niche. The precise characteristics of the osteolineage cells responsible for HSC support remain of great interest since they could predict whether therapeutic strategies that stimulate osteoblastic cells could also achieve beneficial HSC effects.

1.1.2 Endothelial cells

The endothelium gives rise to the first definitive HSCs in the developing embryo (Chen and Zon 2009; Chen, Li et al. 2011) and there is mounting evidence that endothelial cells also play an important role in the maintenance of HSCs in the adult marrow. HSCs and hematopoietic progenitor cells localize to endothelial structures near the endosteal surface (Kiel, Yilmaz et al. 2005; Dahlberg, Delaney et al. 2011). Additionally angiocrine factors produced by endothelial cells support HSCs following

myeloablation (Butler, Nolan et al. 2010; Kobayashi, Butler et al. 2010) and expand immature hematopoietic cells ex vivo (Chute, Muramoto et al. 2006). Further, a study performed by Hooper et al. demonstrates that the recovery of sinusoidal endothelial cells is mediated by VEGFR2. Moreover hematopoietic reconstitution is severely delayed if VEGFR2 and thus sinusoidal endothelial cell recovery is lost (Hooper, Butler et al. 2009). These data strongly suggest a role of endothelial cells in the recovery of hematopoiesis following myeloablation. A more recent study by Ding et al. convincingly illustrates that endothelial cells express stem cell factor (SCF) at high levels. The authors show that conditional deletion of SCF from endothelial cells results in loss of marrow HSCs whereas deletion of SCF in osteoblastic lineage or directly from HSCs did not affect the HSC population (Ding, Saunders et al. 2012). Therefore endothelial cell derived SCF is an essential component in the normal maintenance of HSCs. Taken together these studies demonstrate the importance of endothelial cells, not just in the maintenance of HSCs during homeostatic conditions, but also in the recovery of the HSPCs following myeloablation.

1.1.4 CXCL12 abundant reticular cells

(C-X-C motif) ligand 12 (CXCL12) also known as stromal cell-derived factor 1 (SDF1) is a chemokine produced by osteoblasts and endothelial cells (Ponomaryov, Peled et al. 2000). CXCL12 signals through its receptor (C-X-C motif) receptor 4 (CXCR4) on HSCs to induce migration to and retention in

the bone marrow (Peled, Petit et al. 1999; Broxmeyer, Orschell et al. 2005; Kollet, Dar et al. 2006). HSCs were shown to be closely associated with CXCL12 abundant reticular (CAR) cells that are in turn closely associated with endothelial cells (Sugiyama, Kohara et al. 2006). The identity of these cells remains to be further characterized.

1.1.5 Sympathetic nervous system neuronal cells and glia

Treatment with G-CSF results in migration of HSCs from the marrow into the bloodstream through inhibition of osteoblastic cells and down-regulation of the CXCL12 (Petit, Szyper-Kravitz et al. 2002; Levesque, Hendy et al. 2003; Semerad, Christopher et al. 2005; Christopher, Liu et al. 2009). This process is regulated by sympathetic nervous system (SNS) neurons (Katayama, Battista et al. 2006). SNS neurons coordinate the circadian oscillation of HSC numbers in the marrow, and ablation of SNS neurons results in a loss of circadian controlled release of HSCs into the periphery. In addition sympathectomy of one tibia in a mouse results in altered expression of CXCL12 while the sham operated contralateral tibia is unaffected (Mendez-Ferrer, Lucas et al. 2008). Transforming Growth Factor β (TGF- β) also regulates HSC dormancy ex vivo (Yamazaki, Iwama et al. 2009). Glial cells are a major source of activated TGF- β in the bone marrow, are closely associated with HSCs, and produce numerous factors previously identified as playing a role in the HSC niche (Yamazaki, Ema et al. 2011). Ablation of this population in the bone marrow results in a loss of HSC dormancy and

ultimately of HSC numbers (Yamazaki, Ema et al. 2011). Thus, SNS neurons and glial cells are essential components of the G-CSF induced mobilization of HSCs, and are likely necessary for efficient retention of HSCs in the marrow.

1.1.6 Adipocytes

Adipocytic cells make up a large portion of the adult marrow. One adipocytic product, the adipokine adiponectin, has been demonstrated to increase proliferation of HSCs while retaining their repopulating potential (DiMascio, Voermans et al. 2007). Adiponectin, however, is not solely produced by adipocytes in the marrow, but is also expressed by osteoblastic cells (Berner, Lyngstadaas et al. 2004). In contrast to these pro-HSC effects of adipocytes, adipocyte numbers have more recently been described as inversely related to numbers of HSCs in the marrow by comparing anatomically distinct regions of the skeleton that display varying levels of adiposity (Naveiras, Nardi et al. 2009). Additional studies demonstrated that loss of adipocytes in the marrow, either genetically or pharmacologically, resulted in enhanced engraftment of HSCs and improved hematopoietic recovery following myeloablative injury (Naveiras, Nardi et al. 2009). More research into the role of adipocytes in the maintenance of HSCs is needed, however, they represent an intriguing area, as they are a very abundant cell type within the marrow space.

1.1.7 Macrophages and monocytes

Macrophages and monocytes are recent additions to the HSC niche's cellular milieu. They are involved in the G-CSF dependent mobilization of HSCs out of the marrow and depletion of the macrophage population results in loss of osteoblastic cells and increased HSC mobilization into the periphery (Winkler, Sims et al. 2010; Christopher, Rao et al. 2011). In addition, reduction of monocytic phagocytes from the marrow reduces the expression of genes associated with HSC retention in Nestin positive cells and specific ablation of macrophage populations resulted in the egress of HSCs from the marrow (Chow, Lucas et al. 2011). It is unclear whether macrophages and monocytes specifically interact with HSCs or function only in support of other niche components, however, it has been recently established that they play a pivotal role in the regulation of the HSC niche.

1.1.8 Emerging complexity of the HSC niche

The importance of the skeletal microenvironment in hematopoietic regulation is beginning to be elucidated and holds great promise for translation in hematopoietic recovery and in the treatment of hematopoietic malignancies as well as metastatic disease. While for simplicity there are as many HSC niches in the marrow as cell types that have been demonstrated as supportive of HSCs, the reality is almost certainly much more complex. In vivo imaging shows HSC localization to areas of close spatial relationship between vascular endothelial cells and endosteal osteoblasts suggesting that

both structures may be necessary components of a singular niche (Lo Celso, Fleming et al. 2009; Ellis, Grassinger et al. 2011). Therefore many cell types likely coordinate to provide one regulatory niche. An equally as likely scenario is that there are separate niches providing support for distinct and separate populations of HSCs, and or different potential HSC fates. Further study in this area has benefited greatly from interactions of the field of hematopoiesis with bone biology, and will likely continue to rapidly progress to increase our understanding and therapeutic use of the complex cellular relationships within the bone marrow.

1.2 Acute myelogenous leukemia

The word leukemia is derived from the Greek words *leukos* meaning white, and *haima* meaning blood. The German pathologist Rudolf Virchow first used the term leukemia in 1845 due to his observations that the blood of certain patients was composed largely of white blood cells (Virchow R 1860). We now know that leukemia is in fact a large and diverse family of diseases that involves the accumulation of abnormal cells derived from the lymphoid and myeloid arms of the hematopoietic system.

Acute myelogenous leukemia (AML) is a rapidly progressing de novo leukemia that involves the accumulation of immature cells of the myeloid lineage. AML is commonly classified using 2 main methods. The French-American-British (FAB) classification system categorizes AML into 8 different subtypes (Table 1) (Bennett, Catovsky et al. 1976). Though acute basophilic

leukemia has since been proposed as a ninth subtype (Duchayne, Demur et al. 1999). While the World Health Organization (WHO) categorizes AML into 4 main categories (Table 2) with each main category being divided into multiple sub-categories (Vardiman, Harris et al. 2002).

AML typically displays heterogeneity within the population of malignant cells. Within this heterogenic mixture of malignant populations it has long been postulated that there existed a stem cell compartment that was responsible for the production of the rest of the malignant populations. Lapidot et al. published the first solid evidence for AML being a stem cell based disease in 1994 (Lapidot, Sirard et al. 1994). In this study the authors demonstrated that human AML is capable of being replicated in immune deficient mice, and that only 1 in 250,000 peripheral blood cells is capable of recapitulating the disease. They further showed that the leukemia initiating cells were CD34+CD38-, a population that corresponds with immature normal hematopoietic cells. There remains some debate in the field over the validity of the leukemia stem cell (LSC) hypothesis. In syngeneic models of lymphoma it has been demonstrated that greater than 10% of the malignant population is capable of giving rise to the disease (Kelly, Dakic et al. 2007). Additionally a study in melanoma demonstrated that xenografts performed in more severely immune deficient mice led to greatly increased rates of tumor formation with approximately 25% of malignant cells capable of forming tumors (Quintana, Shackleton et al. 2008). In the case of AML however the

leukemia stem cell paradigm is well accepted (Bonnet and Dick 1997; Neering, Bushnell et al. 2007).

The majority of symptoms displayed by AML patients arise from the disruption of normal hematopoiesis (Hoffman 2005). Patients often present with anemia due to lack of red blood cells, or thrombocytopenia due to lack of platelet production. Additionally though the majority of patients present with a high white blood cell count often they are very prone to infection. This is due to the fact that the majority of the white blood cells present in the blood are in fact leukemic blasts that are incapable of performing normal immune functions (Hoffman 2005). The physical symptoms displayed by these patients often include fever, shortness of breath, bruising or bleeding, petechiae, weakness or tiredness, loss of appetite combined with weight loss, and bone and joint pain. Greater research efforts are needed, as the causes of bone and joint pain remain unknown, and the extent of bone involvement in AML remain largely unstudied.

1.2.1 Treatment and complications

SEER data from the United States indicates that the yearly rate of newly diagnosed AML ranges from: 0.4-1.5/100,000 in children up to age 15, 0.9-1.3/100 000 in adolescents and young adults, and 1.6-23.2/100 000 in adults over the age of 40, with a median age of diagnosis of 67 years. The rates from 2006-2008 translate into a lifetime overall risk of 0.39% for the development AML for men and women in the United States. Additionally, over

the time period of 2001-2007 the 5-year survival rate relative to the general population was 22.6% for men and women combined (Howlader 2011).

The standard therapy for virtually all subtypes of AML begins with induction therapy. This consists of the typical “3+7” regimen that includes 3 days of an anthracycline such as daunorubicin, at least 60 mg/m², idarubicin, 10-12 mg/m², or mitoxantrone, 10-12 mg/m², which is an anthracenedione. Starting at the same time as the anthracycline is a 7-day continuous iv infusion of cytarabine 100-200 mg/m² (Dohner, Estey et al. 2010). This treatment has been the standard of care for nearly 40 years (Yates, Wallace et al. 1973). Following induction therapy 70-80% of patients younger than 60 years of age will achieve complete remission. Despite this high rate of complete remission for this age group the overall 5-year survival rate is only 40-45% and relapse is common. The prognosis is worse for the 60 years and older population. Cure rates for this elderly population are less than 10% and the median survival time post-diagnosis is less than 1 year. The exception to these statistics is acute promyelocytic leukemia or M3 according to the FAB classification system. This subtype of AML accounts for approximately 10% of newly diagnosed AML and a 75% cure rate can be achieved with anthracycline, all-trans retinoic acid, and arsenic trioxide therapy (Roboz 2011).

The cause of relapse following complete remission is strongly suggested to be a remaining population of leukemic cells that are below the

detection limits of the standard diagnostic tests. This population has been termed minimal residual disease (MRD). For patients of low risk and often for those of intermediate risk, consolidation therapy is the standard course of treatment to eradicate MRD following induction therapy. Consolidation therapy generally consists of repeated cycles of cytarabine at a large dose of 3g/m^2 on days 1, 3 and 5. For the remaining intermediate risk and cytogenetically high-risk patients, an allogeneic hematopoietic stem cell transplant (HSCT) is the recommended course of treatment (Yanada, Matsuo et al. 2005; Cornelissen, van Putten et al. 2007; Basara, Schulze et al. 2009; Koreth, Schlenk et al. 2009). The benefits of allogeneic HSCT are great, though they are countered with a high rate of transplant-related mortality (TRM). The benefits of HSCT include very high-dose conditioning regimens as well as the graft-versus-leukemia (GVL) effect. These combine to provide the highest rates of relapse free survival, though the high rate of TRM, ranging from 15 to 50%, make it less beneficial than standard consolidation therapy in the low risk population. When considering allogeneic HSCT there are many factors that influence the rate of TRM including: patient age, disease stage, time interval from diagnosis to transplant, donor type, and donor-recipient sex combination (Gratwohl, Stern et al. 2009; Dohner, Estey et al. 2010). Therefore all of these factors are generally taken into account for each individual patient when deciding a course of treatment.

For patients that fail to achieve complete remission following induction therapy continued treatment using conventional chemotherapy provides almost no chance for disease-free survival (Wheatley, Burnett et al. 1999; Kern, Haferlach et al. 2003; Schlenk, Benner et al. 2003). For this group even allogeneic HSCT offers limited benefit with overall survival at 20-30% (Michallet, Thomas et al. 2000; Singhal, Powles et al. 2002; Fung, Stein et al. 2003). The treatment of this population remains an active area of clinical research.

Another population with poor prognosis is those whose age is greater than 60 years. Older age of diagnosis has been associated with secondary AML, and adverse cytogenetics. Additionally older age is associated with multiple comorbidities and more often result in treatment-related death (Appelbaum, Gundacker et al. 2006). Despite this, studies still suggest that standard inductive therapy provides a better quality of life and longer survival in these patients (Juliussen, Antunovic et al. 2009).

It has been suggested, and is gaining acceptance, that MRD is at least in part caused by the survival of LSCs following standard chemotherapeutic regimens. Since LSCs are a largely quiescent population of cells the typical chemotherapeutics used to treat AML may not effectively target them (Terpstra, Ploemacher et al. 1996; Guan, Gerhard et al. 2003; Guzman, Rossi et al. 2005). This is due to the fact that therapeutics such as cytarabine and anthracyclines primarily affect DNA replication and thus target actively

dividing cells (Guzman, Rossi et al. 2005). It has also been shown in xenograft assays that AML cells expressing markers of immaturity are more resistant to cytarabine treatment (Ishikawa, Yoshida et al. 2007). There is a growing body of literature demonstrating that LSCs can be specifically targeted while sparing normal HSCs (Guzman, Swiderski et al. 2002; Guzman, Rossi et al. 2005; Guzman, Li et al. 2007; Guzman, Rossi et al. 2007; Hassane, Guzman et al. 2008). This area of research still shows much promise in producing AML therapies with a greater chance of achieving long-term disease-free survival.

The remission rate of AML is relatively high at 70-80% for patients under the age of 60, however the 5-year survival rate for all age groups remains terribly low with reports ranging from 20-40%. These cure rates have remained relatively stable and the standard of treatment has not changed substantially for decades. New approaches to the treatment of AML are desperately needed and one area that remains underdeveloped is the interactions between leukemic cells and the environment in which they reside.

1.3 Leukemia's interactions with the microenvironment

Data in xenograft models have suggested that malignant leukemic cells disrupt normal interactions between HSCs and their microenvironment and induce changes in the marrow which decrease normal HSC niches and increase malignant niches (Colmone, Amorim et al. 2008). Additionally, it has been suggested that the marrow microenvironment can protect malignant

stem cells as immature acute myelogenous leukemia (AML) cells have been shown to reside near the endosteal surface of bone and these endosteal associated AML cells were resistant to chemotherapy treatment in xenograft models (Ishikawa, Yoshida et al. 2007; Lane, Wang et al. 2011). While xenograft models can be very useful tools, we must keep in mind that the environment in which the leukemia engrafts is not normal. The engraftment of human leukemia in mice necessitates that the mice be immune compromised. Moreover, engraftment of human cells in even the most immune compromised animals often requires prior myeloablative conditioning such as whole body irradiation, further altering the microenvironment. While these experiments can be informative, further work needs to be done such as validation in immune competent models.

The CXCL12/CXCR4 axis has been shown to be critical to the homing and retention of HSCs in the marrow. Leukemic cells often express CXCR4 and have a transendothelial migration in response to CXCL12 just as normal HSCs (Mohle, Bautz et al. 1998; Mohle, Schittenhelm et al. 2000). Expression of CXCR4 in leukemic cells can also predict an unfavorable outcome in AML (Rombouts, Pavic et al. 2004; Konoplev, Rassidakis et al. 2007). Targeting of the CXCL12/CXCR4 axis through CXCR4 inhibitory antibodies, CXCL12 inhibitory antibodies, or AMD3100 sensitizes malignant cells to chemotherapeutics (Tavor, Petit et al. 2004; Nervi, Ramirez et al. 2008; Zeng, Shi et al. 2009). These studies suggest that leukemic cells can hijack

mechanisms that are normally involved in the support of normal hematopoiesis, suggesting that the HSC niche may provide regulatory signals to leukemic cells as it does for HSCs.

Normal HSCs require close association to niche components if leukemic cells are hijacking the niche one would assume that they would also require colocalization. The attachment of AML cells to their environment by β integrins has been implicated in providing drug resistance. Integrin signaling increases the survival of leukemic cells through the phosphorylation of AKT (Tabe, Jin et al. 2007). Additionally MRD in AML was demonstrated to be dependent on the binding of very late antigen-4 on leukemic cells to the microenvironment (Matsunaga, Takemoto et al. 2003). Inhibition of β integrin-mediated adhesion can also overcome drug resistance and sensitize AML cells to Ara-C (Matsunaga, Fukai et al. 2008). CD44 is another cell-surface molecule implicated in the homing and lodgment of leukemic cells to their microenvironment (Avigdor, Goichberg et al. 2004; Krause, Lazarides et al. 2006). The inhibition of CD44-mediated adhesion can effectively eradicate human AML stem cells in a xenograft model (Jin, Hope et al. 2006).

The interactions between malignant hematopoietic cells and their microenvironment are continuing to be elucidated. These interactions are particularly exciting in that they could provide mechanisms by which MRD escapes traditional therapies. Additionally, it's appealing to consider that leukemia cells, and in particular LSCs, could be hijacking the mechanisms by

which normal HSCs maintain quiescence and self-renewal properties, namely the HSC niche. Hopefully further research into this exciting area of leukemia biology can provide therapeutically relevant targets for future therapies.

1.4 Osteoblastic and osteoclastic activities are coupled

Bone remodeling or bone turnover is a tightly coupled and highly regulated process that replaces approximately ten percent of the adult skeleton on a yearly basis. Bone remodeling maintains plasma calcium homeostasis and repairs micro-damage, maintaining bone integrity (Hadjidakis and Androulakis 2006). Bone turnover is accomplished by the activity of two main types of cells, osteoclasts that resorb old bone and osteoblasts that form new bone. These two processes are tightly coupled in order to maintain a constant bone volume and prevent either the overgrowth of bone by favoring bone formation, or the net destruction of bone by favoring bone resorption (Matsuo and Irie 2008).

The first descriptions of the coupling of bone formation and resorption was by Rasmussen and Bordier, who observed that the activities of osteoblasts and osteoclasts were coupled (Rasmussen and Bordier 1973). Though their observation of coupling has since been validated there were several specifics of their proposed model that have been shown to be false. They proposed that osteoclasts underwent fission in order to produce preosteoblasts thus ending the process of bone resorption and initiating the process of bone formation. We now know that osteoclasts and osteoblasts do

not share a common progenitor, with osteoblasts being derived from mesenchymal stem cells and osteoclasts from monocytes and macrophages of the hematopoietic system (Udagawa, Takahashi et al. 1990; Teitelbaum 2000; Wellik and Capecchi 2003).

The most widely accepted model of bone remodeling today involves 5 distinct phases: activation, resorption, reversal, formation, and resting (Baron 1996). The activation phase is initiated by the signaling of hormones and chemokines, including, PTH, IL-1 β , and TNF- α . These signals induce the release of signaling molecules such as MCP-1, CXCL12, and RANKL from bone-lining preosteoblastic cells. These locally produced signals activate osteoclast precursors that are derived from hematopoietic monocytes and macrophages. Activated osteoclast precursors then undergo cellular fusion to produce mature multinucleated osteoclasts (Hadjidakis and Androulakis 2006).

Following the emergence of mature osteoclasts the resorption phase begins. Mature osteoclasts attach to the bone surface through the binding of integrins to specific amino acid sequences present in bone matrix proteins present on the surface of bone (Hadjidakis and Androulakis 2006).

Osteoclasts resorb bone through the processes of acidification by the release of protons, as well as proteolysis by the secretion of a variety of enzymes such as cathepsins and collagenases. Through this process osteoclasts resorb a trench across the surface of trabecular bone at a rate of about 25

$\mu\text{m/day}$. The resorption process facilitates the release of growth factors that had been incorporated into the bone matrix during bone formation. These include TGF- β , BMP, IGF-I, and IGF-II. These growth factors help recruit preosteoblastic cells to the site of bone resorption (Mackie 2003).

The third phase in the process of bone turnover is the reversal phase. This phase marks the transition from resorption to formation. While the osteoclast is still actively resorbing bone, macrophage-like cells have been observed lining the freshly resorbed surface and have been implicated in the recruitment of osteoblastic cells to the site (Pettit, Chang et al. 2008). Additionally, inactive TGF- β liberated from the bone matrix has been shown to be activated by proteases and the acidified environment following bone resorption. This activated TGF- β can then locally regulate osteoblast function (Oreffo, Mundy et al. 1989). Subsequently, the formation phase begins as preosteoblasts fully differentiate and the newly formed mature osteoblastic cells start the deposition of new bone. New bone is formed in 2 successive processes; the first is the formation of osteoid matrix that consists largely of collagen. Following the formation of osteoid, mineralization takes place during which hydroxyapatite crystals cover the newly formed osteoid matrix to form fully mineralized bone. The amount of time it takes to completely replace the bone tissue lost during resorption is approximately 4 months (Hadjidakis and Androulakis 2006).

The final phase of bone remodeling is the termination phase. The termination of osteoclastic bone resorbing activity is the first to occur and is initiated by canonical Wnt signaling in osteoblastic cells approximately 2 weeks following the start of bone resorption. β -catenin activation in osteoblasts induces the expression of OPG that is a decoy receptor for RANKL. As the levels of OPG increase it starts to outcompete RANK on the surface of osteoclast precursors acting to halt the formation of mature osteoclasts (Sato, Nakashima et al. 2009). The termination of bone formation occurs as osteocytes secrete the protein sclerostin that binds to LRP5/6 on osteoblasts inhibiting Wnt signaling and repressing bone formation (van Bezooijen, Roelen et al. 2004; Li, Zhang et al. 2005). The termination of bone formation ends the process of bone remodeling at that site of the skeleton though globally in the skeletal system bone remodeling is a constant process.

Aside from the systemic factors mentioned earlier, there are local factors that have also been implicated in the initiation of bone remodeling. Osteocytes have been proposed to act as mechanosensors that signal to bone lining cells during times of mechanical stress. The signals from osteocytes in this process act as the activation phase of bone remodeling, and initiate signaling in bone lining preosteoblastic cells that attract and activate osteoclastic precursors (Huiskes, Ruimerman et al. 2000).

Another model for the mechanisms behind the tight spatial coupling of the bone remodeling processes involves osteoblast-like cells forming a

canopy over the remodeling site. These canopies are proposed to sequester the activities of osteoclastic and osteoblastic cells while permitting the penetration of blood vessels to supply necessary nutrients and potentially osteoblast and osteoclast precursors (Hauge, Qvesel et al. 2001). Further, disruption of the canopy was shown to result in an uncoupling of bone formation and bone resorption (Andersen, Sondergaard et al. 2009). In mice however it has been shown that the canopy cells express F4/80 and are likely macrophages rather than osteolineage cells (Pettit, Chang et al. 2008).

An important step in the spatial coupling of bone formation to bone resorption is the ability of osteoblasts to home to the freshly resorbed bone. An unbiased gene array approach that compared differentiated osteoclasts to undifferentiated precursors identified the proteins SP1, BMP6 and Wnt10b as factors released by osteoclasts that have been found to influence the differentiation of osteoblasts. SP1 and BMP6 were further implicated in the recruitment of osteoblasts as antagonistic antibodies against SP1 and BMP6 decreased the chemotaxis of osteoblast precursors. The addition of the Wnt10b antagonist DKK1 did not affect chemotaxis indicating that Wnt10b is involved in the differentiation of osteoblastic cells but not in their recruitment.

It has been proposed that osteoclasts may deposit factors in the resorptive site to regulate osteoblastic activity in addition to releasing factors that were previously incorporated in the bone matrix (Romano, Caton et al. 1997; Sheu, Schwarz et al. 2002). Tartrate resistant acid phosphatase

(TRAP 5b) is one factor that has been implicated (Sheu, Schwarz et al. 2002; Sheu, Schwarz et al. 2003). TRAP 5b is a secreted hydrolytic phosphatase that is highly expressed by osteoclasts, and as such acts as a marker for osteoclast lineage cells. During bone resorption TRAP is secreted by the osteoclast into the resorption lacuna and contributes to the degradation of bone matrix (Ek-Rylander, Flores et al. 1994). Following bone resorption TRAP has been identified as remaining tightly adhered to the freshly resorbed surface (Sheu, Schwarz et al. 2002). Further, TRAP null mice show no defects in the ability of osteoclasts to resorb bone, however the spatial coupling with osteoblastic bone formation is lost (Hayman, Jones et al. 1996). Another protein implicated in coupling is semaphorin 4D. Semaphorin 4D has been shown to be expressed at high levels by osteoclasts and to be involved in spatial coupling. Semaphorin 4D has been demonstrated to signal through its receptor Plexin-B1 on the surface of osteoblasts in order to repress osteoblastic function. Loss of this signaling pathway in osteoblastic cells results in osteoblasts localizing much closer to active osteoclasts than would be found in normal circumstances (Negishi-Koga, Shinohara et al. 2011).

The coupling of bone formation and bone resorption is absolutely critical to the healthy maintenance of the skeleton. There are many disease states that can result in the uncoupling of osteoblastic and osteoclastic activities such as rheumatoid arthritis and osteoarthritis. More relevant to this thesis is uncoupling that can occur in malignancy.

1.5 Disruption of bone homeostasis in malignancy

Though under normal circumstances bone formation and resorption are tightly coupled this coupling can be disrupted resulting in either overgrowth of bone by favoring bone formation, or the aberrant destruction of bone by favoring bone resorption. Hematologic malignancies that reside in the bone marrow and metastases of solid tumors to the bone are well known to result in disruptions in normal bone homeostasis.

1.5.1 Multiple myeloma

Multiple myeloma results from the transformation of hematopoietic cells and the accumulation of malignant plasma cells. One of the major morbidities of multiple myeloma is extensive and extremely destructive osteolytic lesions (Ludwig 2005). The involvement of aberrant bone resorption in multiple myeloma is associated with hypercalcaemia, pathologic fractures, compression of spinal vertebrae, and bone pain (Cocks, Cohen et al. 2007). Aberrant resorptive activity is also associated with a poorer prognosis (Jakob, Sterz et al. 2008).

Evidence has existed for nearly 30 years that bone destruction in multiple myeloma was mediated by a locally produced, secreted factor (Mundy, Raisz et al. 1974). Additionally the bone destruction in multiple myeloma is not seen throughout the skeleton, but rather in isolated pockets where malignant cells are present (Valentin-Opran, Charhon et al. 1982). The main regulator of osteoclast formation and activation is the RANKL-RANK-

OPG system that acts locally rather than systemically. The RANKL-OPG axis is disrupted by multiple myeloma in favor of greater RANKL availability (Giuliani, Bataille et al. 2001; Pearce, Sordillo et al. 2001; Sezer, Heider et al. 2003). Further RANKL signaling was shown to play a necessary role in the myeloma induced bone destruction as treatment with recombinant OPG inhibited osteoclast formation, prevented destruction of bone, and increased bone mineral density in mice with established multiple myeloma (Croucher, Shipman et al. 2001). There are 2 possible sources of RANKL in the marrow of multiple myeloma patients, one is cells of the microenvironment in which expression is being induced by multiple myeloma cells, and the other is direct expression by malignant myeloma cells. Direct expression of RANKL by multiple myeloma cells correlates with the presence of osteolytic lesions (Farrugia, Atkins et al. 2003; Heider, Langelotz et al. 2003). The Wnt inhibitor Dickkopf-1 (DKK-1), produced by myeloma cells, also disrupts the normally Wnt regulated expression of both RANKL and OPG in osteoblastic cells (Qiang, Chen et al. 2008). Therefore, it is likely that both changes in expression of RANKL and OPG in endogenous cells and production of RANKL by multiple myeloma cells contribute to the disrupted RANKL-OPG axis.

Though undoubtedly a central player in the osteolysis seen in multiple myeloma patients, the RANKL-RANK-OPG system is not the only mediator of skeletal disruption. CCL3 and CCL4, also known as macrophage

inflammatory protein 1 α (MIP-1 α) and MIP-1 β , respectively, also play a substantial role in the myeloma-induced osteolysis. CCL3 and CCL4 are inflammatory chemokines that signal through the chemokine receptors CCR1 and CCR5 (Sezer 2009). Myeloma cells secrete both CCL3 and CCL4. CCL3 induces chemotaxis of osteoclast progenitors and increases levels of osteoclast formation in vitro (Choi, Cruz et al. 2000; Choi, Oba et al. 2001; Abe, Hiura et al. 2002; Oyajobi, Franchin et al. 2003). Further, blockade of CCL3 and CCL4 signaling by antagonistic antibodies against CCL3, CCL4 or CCR5 blocks aberrant bone resorption (Abe, Hiura et al. 2002). Transfection of myeloma cells with an anti-sense construct against CCL3 is also capable of ameliorating the increase in bone resorption (Choi, Oba et al. 2001). The increase in bone resorption seen with CCL3 and CCL4 appears to still be dependent on RANKL as mice treated with CCL3 showed a dramatic increase in osteoclast formation, only when RANK was normally expressed.

Another cytokine implicated in increased osteoclast activity in multiple myeloma is CXCL12. Myeloma cells produce CXCL12, and its receptor CXCR4 is present on myeloma cells as well as osteoclasts and osteoclast precursors. Moreover increased CXCL12 levels in the serum of myeloma patients correlated with increased radiographic skeletal lesions. In vitro data showed an increase in osteoclast specific transcripts such as RANK, TRAP, MMP9 and Cathepsin K. Finally blockade of CXCR4 with a small molecule

inhibitor effectively blocked osteoclastogenesis stimulated by the myeloma cell line, RPMI-8226.

Members of the interleukin family of cytokines are also involved in the osteoclast formation and activation in multiple myeloma. IL-6 has long been recognized as a stimulator of osteoclast formation (Kurihara, Bertolini et al. 1990; de la Mata, Uy et al. 1995). IL-6 is produced both by myeloma cells as well as stromal cells within the bone marrow, and IL-6 signaling is thought to play a critical role in the pathophysiology of multiple myeloma (Hideshima, Bergsagel et al. 2004). IL-11 is also thought to act as a co-factor for the recruitment and differentiation of osteoclast precursors (Girasole, Passeri et al. 1994; Romas, Udagawa et al. 1996), and multiple myeloma cells can induce the release of IL-11 from osteoblastic cells potentially increasing osteoclastic bone resorption (Hjertner, Torgersen et al. 1999). The data demonstrating the activation of osteoclasts without a compensatory increase in osteoblastic activity is robust and convincing. Several pathways have been implicated and treatment with bisphosphonates is commonly used to control the osteolytic nature of multiple myeloma. The other side of bone turnover however, osteoblastic bone formation, is also a likely target to further the uncoupling of bone turnover.

Osteoblastic cells are found in decreased numbers in areas close to myeloma cells, and through cell to cell contact myeloma cells were shown to be able to suppress the activity of RUNX2, a critical mediator of osteoblasts

(Bataille, Chappard et al. 1991; Giuliani, Colla et al. 2005). Cell to cell contact of multiple myeloma cells and osteoblasts also resulted in a decrease in the expression of osteocalcin, a marker of mature osteoblastic cells (Barille, Collette et al. 1995). DKK-1 produced by myeloma cells has been shown to inhibit osteoprogenitor differentiation (Yaccoby, Ling et al. 2007; Qiang, Barlogie et al. 2008). Further, in a mouse model of multiple myeloma, treatment with an anti-DKK-1 antibody resulted in increased numbers of osteoblasts and an increase in bone density (Yaccoby, Ling et al. 2007). The cytokine IL-3 has also been shown to be upregulated in myeloma and is also a known inhibitor of osteoblastic differentiation (Ehrlich, Chung et al. 2005).

Most recently the chemokine CCL3 was shown to be a potent inhibitor of osteoblastic function. A study by Vallet et al. demonstrated that CCL3 inhibited osteoblastic cells, reducing both mineralization and osteocalcin expression. Further, inhibition of CCR1 signaling restored osterix and osteocalcin expression in spite of the presence of CCL3 (Vallet, Pozzi et al. 2011). While CCL3 has been well established as an initiator of aberrant osteoclastic bone resorption, this recent study demonstrates that it further uncouples bone remodeling by inhibiting osteoblastic bone formation.

1.5.2 Bone metastases of solid tumors

Bone metastases of solid tumors such as prostate cancer, breast cancer, lung cancer, thyroid cancer and renal cancer can also form osteolytic lesions using some of the same mechanisms as multiple myeloma (Roodman

2004). The cytokine IL-6 is well known to induce osteolysis in multiple myeloma and is highly expressed by renal, prostate and breast cancer cells (Tabibzadeh, Poubouridis et al. 1989; Blay, Schemann et al. 1994; Basolo, Fiore et al. 1996; Morrissey, Lai et al. 2010). Much like multiple myeloma, solid tumor metastases can also induce the expression of RANKL in bone marrow stromal cells. Breast cancer metastases express TNF, IL-1, prostaglandins, and PTHrP, all of which have been shown to increase the expression of RANKL in the stromal cell population (Pederson, Winding et al. 1999; Ono, Akatsu et al. 2002). PTHrP expressing breast carcinomas are more likely to develop bone metastases, and a much larger proportion of bone metastases express PTHrP than primary tumors, or metastases to sites other than bone (Powell, Southby et al. 1991). Thus, metastases of solid tumors uncouple bone turnover resulting in osteolysis utilizing many of the same mechanisms as multiple myeloma. However, solid tumor metastases can also uncouple bone turnover in the opposite direction resulting in large increases in bone volume.

Osteoblastic lesions are significantly less common than osteolytic lesions in bone metastases, but still occur relatively frequently in prostate and breast carcinomas. Though large amounts of bone are formed in osteoblastic lesions it is of poor quality and pathologic fractures are common. Endothelin-1 has been strongly implicated in the pathogenesis of osteoblastic lesions in both breast and prostate cancers. In prostate cancer endothelin-1 levels are

increased in the blood serum of patients with osteoblastic lesions (Nelson, Hedican et al. 1995). Moreover, in a xenograft model of breast cancer bone metastasis, treatment with an endothelin-1A receptor antagonist resulted in decreased osteoblastic metastases (Yin, Mohammad et al. 2003). Endothelin-1 may act to uncouple normal bone turnover by acting to decrease osteoclastic bone resorption (Alam, Gallagher et al. 1992). Several other factors have been implicated in osteoblastic lesions of metastatic bone disease such as uPA, PSA and PDGF (Rabbani, Desjardins et al. 1990; Killian, Corral et al. 1993; Achbarou, Kaiser et al. 1994; Yi, Williams et al. 2002). Tumors that have metastasized to bone are almost impossible to completely eradicate. The severity of resulting disease and lack of available treatments make this an important area of bone biology for further research.

1.6 Overview of the Thesis

Much of the morbidity and mortality in leukemia comes from cytopenias that develop in the normal hematopoietic system. Furthermore, regulation of the hematopoietic system is well established to be dependent on microenvironmental factors present in the bone marrow. Despite this there is very little known about the effects leukemia has on the marrow microenvironment. What is known about leukemia-microenvironmental interactions comes from studies performed using models lacking a normal microenvironment including xenografts and models requiring irradiation prior to engraftment of leukemia. These facts have led us to our central hypothesis

that **leukemia disrupts the marrow microenvironment by inhibiting osteoblastic cells and uncoupling bone formation from bone resorption.**

Disruption of the marrow microenvironment could lead to reduced support for normal hematopoiesis.

Osteoblastic cells are the best-described component of the hematopoietic niche in the bone marrow. Therefore, in Chapter 2 we test the hypothesis that a leukemic environment inhibits osteoblastic differentiation and function.

It is well established that osteoblastic and osteoclastic activities are tightly coupled. Therefore, in Chapter 3 we test the hypothesis that a leukemic environment uncouples the actions of osteoclastic and osteoblastic cells.

Based on evidence in other malignancies that uncouple bone turnover we expect that leukemic cells produce a factor that either directly or indirectly causes the observed uncoupling. Therefore, in Chapter 4 we test the hypothesis that leukemic cells produce a factor that results in the uncoupling of bone formation and bone resorption.

Finally in Chapter 5 we will discuss the significance of our findings and will address future problems to be addressed.

FAB Subtype	Name of Subtype
M0	Minimally differentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia without maturation
M2	Acute myeloblastic leukemia with granulocytic maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M4eo	Myelomonocytic together with bone marrow eosinophilia
M5a	Acute monoblastic leukemia
M5b	Acute monocytic leukemia
M6	Acute erythroid leukemias
M7	Acute megakaryoblastic leukemia

Table 1.1. French-American-British classification of AML

WHO Classification	Description
AML with characteristic genetic abnormalities	<ul style="list-style-type: none"> • AML with t(8;21) • AML with inv(16) • AML with t(15;17) <p>Higher rate of remission and often a better prognosis.</p>
AML with multilineage dysplasia	Includes patients with prior myelodysplasia or myeloproliferative disease. Often elderly patients with a poorer prognosis
AML and MDS, therapy-related	AML or MDS subsequent to prior Chemo or radiotherapy. Often have a poorer prognosis
AML not otherwise categorized	Subtypes of AML that do not fall into above categories.

Table 1.2. World Health Organization Classification of AML

Figure 1.1. Bone remodeling is a coupled process

Circulatory or locally produced factors induce bone lining stromal cells to produce factors that recruit, and initiate fusion of osteoclast precursors to produce terminally differentiated osteoclasts. During bone resorption osteoclasts produce, and liberate from the bone matrix, factors that recruit osteoblast precursors to the site of bone resorption. Osteoblast precursors then differentiate to fully mature osteoblasts and actively lay down new bone.

Chapter 2:

Mouse Model of Leukemia Inhibits Osteoblastic Cells

2.1 Introduction

Osteoblasts undergo a well-defined differentiation process that starts with the mesenchymal stem cell. The developmental process is initiated by signaling of bone morphogenic proteins (BMPs) through cell surface receptors. Subsequent signaling induces the production and activation of the critical osteoblastic transcription factor Runx2 that drives the development of osteoprogenitors (Ducy, Zhang et al. 1997; Lee, Kim et al. 2000). The osteoprogenitors then undergo a period of proliferation in which several markers are expressed including collagen-1, fibronectin, and TGF β (Lian and Stein 1992). In a transition from proliferation to fully mature mineralizing osteoblasts alkaline phosphatase is strongly upregulated. As osteoblasts enter the mineralization stage the markers osteopontin and osteocalcin will be produced (Lian and Stein 1992). The ultimate fate for an osteoblast following mineralization of new bone is to become a bone-lining cell, an osteocyte, or to undergo apoptosis. Approximately 85% of osteoblasts will become bone-lining cells or apoptose, with the remaining 15% becoming osteocytes embedded in the bone matrix (Hadjidakis and Androulakis 2006).

Cells of the mesenchymal/osteoblastic lineage play an essential role in the regulation of normal hematopoietic stem cells (HSCs) (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Visnjic, Kalajzic et al. 2004). In addition to data suggesting that activation of osteoblastic cells expands HSCs and that osteoblastic injury results in myeloablation (Calvi, Adams et al. 2003; Visnjic,

Kalajzic et al. 2004), specific disruptions of the osteoblastic compartment without genetic manipulation of the hematopoietic system results in a myeloproliferative disorder, demonstrating the important role osteoblasts play in hematopoietic stem and progenitor cell regulation (Walkley, Olsen et al. 2007).

In xenograft models, human acute myeloid leukemia (AML) cells reside at the endosteal surface of bone (Ishikawa, Yoshida et al. 2007; Ninomiya, Abe et al. 2007), where they are found in close proximity to osteoblastic cells, however the interactions between leukemia and these microenvironmental cells have not been clearly defined. Moreover, xenograft models, while beginning to elucidate in vivo intercellular relationships, juxtapose hematopoietic and non-hematopoietic cells from different species, and may not recapitulate normal leukemia-microenvironment regulatory interactions. Nonetheless, a number of xenograft studies have suggested that leukemia disrupts molecular mechanisms used by normal HSCs to home to the endosteal niche, including CD44 (Jin, Hope et al. 2006; Krause, Lazarides et al. 2006) and the well-established interaction between CXCR4 and its ligand CXCL12 (Rombouts, Pavic et al. 2004; Sipkins, Wei et al. 2005; Colmone, Amorim et al. 2008).

We hypothesized that a leukemic environment inhibits osteoblastic differentiation and function, which may impair support of normal hematopoiesis. To study interactions between leukemia and the marrow

microenvironment, we utilized a well-characterized murine model of myelogenous leukemia (Dash, Williams et al. 2002; Neering, Bushnell et al. 2007). In this model, leukemia is initiated by immature hematopoietic cells that have been engineered to co-express the BCR/ABL and Nup98/HoxA9 fusion products (Fig. 2.1 a-c), both of which have been documented in human leukemias, providing relevance of this model to human disease (Yamamoto, Nakamura et al. 2000). This model recapitulates blast-crisis Chronic Myelogenous Leukemia (bcCML) (Neering, Bushnell et al. 2007) with very rapid disease progression. Though, unlike bcCML, there is a lack of a preceding chronic disease. Therefore, it may closely represent the effects of AML on the normal marrow microenvironment. Further, this model utilizes mice that are immune competent and have not been pre-conditioned in any way in order to retain a normal microenvironment prior to induction of leukemia.

2.2 Materials and Methods

2.2.1 Model of AML

The MSCV-BCR/ABL-IRES-GFP, and MSCV-Nup98/HoxA9-YFP vectors (Fig. 2.1 a, b) were previously described¹⁶. Marrow cells from 6-8 week old male CD45.1 mice were enriched for hematopoietic stem and progenitor cells (HSPCs) by FACS sorting to purify the population of lineage negative, sca-1⁺, c-kit⁺ (LSK) cells. LSK cells were infected with both viral

vectors simultaneously as previously described (Neering, Bushnell et al. 2007). 6-8 week old male C57bl/6 primary recipients were sub-lethally irradiated (6Gy) using a ^{137}Cs source of radiation (GAMMACELL-40). Immediately following irradiation the mice were injected by tail vein with 2×10^4 leukemic cells in 0.1 mLs of phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum (FACS buffer). After 15 days the spleens of primary recipient mice were harvested and crushed using the plunger of a 3 mL syringe. The resulting cell suspension was strained using a 40- μm pore size cell strainer, resuspended in Cryostor CS10 (Biolife Solutions) at a concentration of 2×10^7 cells/mL and cryogenically stored in liquid nitrogen. To induce leukemia in animals used for all described experiments cells were thawed and 2×10^5 cells in 0.1 mLs FACS buffer were injected by tail vein into non-irradiated 6-8 week old male C57bl/6 mice. These are referred to as leukemic mice, and were sacrificed at the times after secondary transplantation as described for each experiment. Normal controls were always age and sex-matched to the leukemic mice in the same experiment.

2.2.2 Murine marrow, spleen, and peripheral blood cell collection

For hematopoietic analysis, marrow cells were flushed from the long bones of the hind limbs of mice using a 25-gauge needle. Spleen cells were collected by crushing the spleen inside of a 40- μm -pore cell strainer using the plunger from a 3ml syringe. Peripheral blood cells were collected by sub-mandibular bleeds and CBCs were obtained using a CBC-DIFF Veterinary

Hematology System (HESKA). Additionally blood was incubation for 20 minutes at room temperature in 2% 500,000 molecular weight dextran to precipitate the red blood cells in preparation for flow cytometric analysis.

2.2.3 Flow cytometric analysis and fluorescent activated sorting

Using cells collected as previously described red blood cells (RBCs) were lysed in 1 ml RBC lysis buffer (156 mM NH_4Cl , 127 μM EDTA and 12 mM NaHCO_3) for 5 minutes at room temperature, 1×10^7 cells were suspended in 100 μL FACS buffer and stained with appropriate antibodies. The cells were washed and data was collected on a LSR-II (Beckton Dickson). The data was analyzed using FlowJo software (Tree Star). For sorting, cells were prepared as described for flow cytometric analysis and GFP⁺/YFP⁺ cells were sorted using a FACS Aria cell sorter (Beckton Dickson) into FACS buffer.

2.2.4 Histology and Immunohistochemistry

Hind limbs were collected, cleaned of soft tissue, fixed in 10% neutral buffered formalin for 48 hours, and decalcified in 14% EDTA for 10 days. Tissues were then processed and embedded in paraffin, 5 μm thick sections were cut and used for hematoxylin and eosin staining (H&Es), immunohistochemistry. Immunohistochemical staining for GFP and OPN utilized the monoclonal JL-8 antibody (Clontech, 632380) and the AKm2A1 antibody (Santa Cruz biotechnology, inc. sc-21742) respectively. Both

immunohistochemical stains were performed using the M.O.M. kit (Vector Laboratories, inc. PK-2200) and were counterstained with hematoxylin.

2.2.5 Osteoblastic Cell Collection from long bones

Osteoblastic cells were collected from the long bones of the hind limbs according to a previously described protocol (Chitteti, Cheng et al. 2010). In brief, the long bones of the hindlimbs were cleaned of soft tissue, and the bone marrow was flushed with a 25-gauge needle and discarded. The resulting cleaned and flushed bones were cut into <1mm fragments and digested twice in collagenase (Stem Cell Technologies, 7902) for 30 and 60 minutes sequentially. Remaining fragments were removed from the collected digest by passing through a 40µm pore size cell strainer. The cells obtained were seeded in 6-well cell culture dishes at 1×10^6 cells per well for cell culture and bone nodule formation, or magnetically separated according to CD45 expression using the IMagnet system (BD biosciences, 552311) and a biotinylated CD45 antibody (eBioscience, 13-0451-82).

2.2.6 Bone Nodule Assay

Following 4 days in culture, media was changed to mineralizing media and 50 µg/ml L-Ascorbic acid 2-phosphate (Sigma), and 10mM Glycerol 2-phosphate disodium salt hydrate (Sigma) were added. At specified times following the addition of osteogenic media (see figure legends), cells were fixed with 10% neutral buffered formalin for 30 minutes followed by detection

of alkaline phosphatase activity using a staining buffer containing 100 mM Tris-HCL (Sigma), 0.005%w/v Naphthol AS MX-PO₄ (Sigma), and 0.03%w/v Red Violet LB salt (Sigma), with a final pH of 8.3 for 45 minutes. Following alkaline phosphatase staining, cells were von Kossa stained (2.5%w/v AgNO₃ (Sigma) for 30 minutes).

2.2.7 Osteoprogenitor Cultures from Marrow

Whole marrow flushed from the long bones of normal or leukemic mice was sorted as described for GFP⁻/YFP⁻ cells and seeded in 6-well tissue culture dishes at 4x10⁶ cells per well in α -MEM containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco) and then used for bone nodule assays.

2.2.8 ELISAs

All ELISAs were performed as indicated by manufacturer's instructions. CTX measurements were performed using the RatLaps ELISA (AC-06F1, Immunodiagnostic Systems Inc.). TRACP 5b protein measurement performed using Mouse TRAP ELISA (SB-TR103, Immunodiagnostic Systems Inc.). Osteocalcin measurement performed using the Mouse Osteocalcin EIA kit (BT-470, Biomedical Technologies). Bovine CTX from bone wafer culture media was measured using the Crosslaps® for Culture ELISA (AC-07F1, Immunodiagnostic Systems Inc.). Murine CCL3 protein measurement was performed using the Mouse CCL3/MIP-1 alpha Quantikine

ELISA Kit (MMA00, R&D systems). Human CCL3 protein measurement was performed using the Human CCL3/MIP-1 alpha Quantikine ELISA Kit (DMA00, R&D systems).

2.2.9 Analysis of relative gene expression by real-time RT-PCR

Total mRNA was extracted using the RNeasy kit (Qiagen) according to manufacturer's instructions. The Quantitect Reverse Transcription kit (Qiagen) was used to transcribe cDNA which was then diluted 1:50 in water, combined with SYBR Green PCR master mix (BioRad) and amplified using MyiQ Single Color PCR detection systems and software under the following conditions: 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 58°C (Osteocalcin, and Jagged1) or 60°C (CCL-3) for 30 seconds. Data were analyzed using the relative standard curve method, normalized to β -actin. All primers used for the analysis of murine gene expression throughout this thesis can be found in table 2.1.

Human gene expression was determined using Taqman technology (Applied Biosystems, Foster City, CA). Specific Taqman probes were obtained from Applied Biosystems: CCL3 (Hs00234142_m1) and GAPDH (Hs02758991_g1) cDNA amplification was performed according to manufacturers specifications using a LightCycler 480 II (Roche).

2.2.10 Light Microscopy

Histology slides were viewed at room temperature with a CKX41 upright microscope (Olympus, Tokyo, Japan). Cell culture dishes were viewed at room temperature with a BX41 inverted microscope (Olympus). All images were obtained with a DP70 digital microscope camera and DPController software (Olympus).

2.2.11 CFU-C analysis

Bone marrow cells flushed from the hindlimbs and sorted by FACS to purify GFP-/YFP- cells were resuspended in α -MEM (Mediatech) supplemented with 2% heat inactivated FBS at a concentration of 2×10^5 cells/mL; 0.4 mL cell-containing media was added to 4 mL methylcellulose containing M3434 media (Stem Cell Technologies). Cells were then plated in 35mm culture dishes and incubated at 37°. 12 days later colonies were counted.

2.2.12 MicroCT analysis

The right hindlimb of each mouse was fixed in 10% Formalin for 48 hours, and then stored in 70% ethanol at 4°C. The limbs were scanned on a Viva CT 40 (Scanco Medical) using a 55-kVp, 145- μ A current and a 300-ms integration time. A resolution of 12.5 μ m was used. A 2.0875-mm region that was 50 μ m above the growth plate in the femur and 50 μ m below the growth plate in the tibia was analyzed.

2.2.13 Statistical Analysis

For quantitative assays, treatment groups were reported as mean plus or minus SEM and compared using the Student *t* test in GraphPad Prism, Version 5.0b (GraphPad Software). Statistical significance was established at *P* less than or equal to 0.05.

2.3 Results

2.3.1 An immunocompetent in vivo model where progression of leukemia is identified first in marrow, then spleen and peripheral blood.

We utilized a previously characterized model of acute leukemia (fig. 2.1) (Dash, Williams et al. 2002; Neering, Bushnell et al. 2007). Leukemic cells can be identified and quantified based on their expression of Green Fluorescent Protein (GFP) and Yellow Fluorescent Protein (YFP) (fig. 2.2). In this model, secondary transplantation of the leukemia does not require irradiation of the recipient mice. Since our goal was to study the intact microenvironment, we chose to use as our experimental mice (heretofore designated as leukemic mice) the secondary transplantation recipients (fig. 2.2) and as controls sex and age-matched normal mice. Leukemic mice have rapid progression of disease that results in the accumulation of leukemic cells in the marrow identified by flow cytometric analysis (fig. 2.2) as well as morphology, and immunohistochemistry (fig. 2.3), though total cellularity of the marrow is unchanged (29.94 ± 2.48 vs 27.18 ± 2.69 , mono-nuclear

marrow cells/hindlimb, Normal vs Leukemic, n=13 from 3 separate experiments p=0.4595). Leukemic cells also accumulate rapidly in the spleen (fig. 2.2), followed by the blood (fig. 2.2). This sequence suggests initial engraftment of leukemic cells in the marrow and spleen followed by migration into the bloodstream.

2.3.2 Model of myeloid leukemia induces thrombocytopenia.

Peripheral blood analysis revealed that leukemic mice had elevated numbers of white blood cells (fig. 2.4). This is not a surprising finding as at late stages of disease more than 10% of nucleated peripheral blood cells are of leukemic origin. Hematocrit levels are not statistically different between normal and leukemic mice (fig. 2.4). In contrast platelet levels are dramatically reduced in leukemic animals (fig. 2.4). Indicating that thrombocytopenia, a common complication of AML, is a result of the leukemic environment in our model.

2.3.3 Leukemic environment alters levels of hematopoietic progenitors.

Due to the development of thrombocytopenia in our model and the common complication of cytopenias in AML, we analyzed the levels of hematopoietic progenitors in our model of myeloid leukemia. We performed colony forming unit assays in order to determine the levels of committed progenitors in the marrow of normal and leukemic mice. Marrow cells were sorted and the GFP-/YFP- fraction of marrow cells was used for the CFU-C

assays. In our model we saw a reduction in the numbers of total CFU-C, CFU-GEMM, or CFU-GM colonies formed (fig. 2.5). This indicates that in the leukemic environment the number of committed progenitors within the marrow is reduced.

We also used the sorted GFP-/YFP- cells to perform competitive transplantations. 5 mice were transplanted per experimental group. All of the recipients that received cells from a day 10 leukemic donor that had advanced disease and 2 of the recipients that received cells from a day 6 leukemic donor succumbed to leukemia. This indicates that the sorted population of cells retained cells with leukemogenic potential. There are two possibilities, either some GFP/YFP expression has been quenched in a population of leukemic cells, or the purity of the sort was inadequate and a sufficient number of GFP+/YFP+ cells were included in the supposed GFP-/YFP- population. Further investigation into this method will be needed to determine the cause of leukemia development in the recipient mice. Nevertheless we were able to analyze the recipients that were transplanted with sorted cells from normal mice, as well as the remaining recipients that received cells from day 6 leukemic mice. In these recipients there was no significant difference between engraftment rates at 4 weeks following transplantation (fig. 2.6). This is a measure of short-term engraftment and analysis of long-term engraftment may show different results. However this result does recapitulate what was seen with the CFU-C assays in regards to

committed progenitor cells. This indicates that in leukemic mice with a low level of disease there is no change in the numbers of cells capable of short-term repopulation of recipients.

2.3.4 Leukemia decreases osteoblastic cells.

Immunohistochemical examination of paraffin sections from the long bones demonstrated a severe decrease in osteopontin⁺ endosteal osteoblastic cells in leukemic compared to normal mice (fig. 2.7). Osteoblastic cells actively lay down bone; therefore a decrease in bone formation would be expected with osteoblastic inhibition. Global bone formation correlates well with serum osteocalcin levels, which were strongly suppressed in leukemic mice compared to sex and age-matched controls (fig. 2.8). Since osteocalcin is specifically expressed in mature osteoblastic cells, we measured osteocalcin expression in cells obtained from the long bones (femur and tibia) by collagenase digestion after bulk hematopoietic cells are removed by flushing. As expected, osteocalcin expression was present only in non-hematopoietic, CD45⁻ cells and in leukemic mice there was a nearly 400-fold decrease in osteocalcin expression (fig. 2.8), demonstrating loss of osteocalcin⁺ cells from the marrow microenvironment. Osteocalcin expression was already decreased at day 6, when only 10-15% of marrow mononuclear cells are leukemic (fig. 2.2). Additionally the expression of the notch ligand jagged1, a known HSC niche component is lost in CD45⁻ bone lining cells (fig. 2.9). In order to further qualify that osteocalcin expression was

lost in osteoblastic cells that line the endosteal bone surface we performed in situ hybridization for osteocalcin. This confirmed the loss of osteocalcin mRNA at the endosteal surface (fig. 2.10). Further we also performed in situ hybridization for collagen 1 that showed the same trend as osteocalcin, a loss of expression at the endosteal surface (fig. 2.10).

To quantify the presence of osteoprogenitors in this cell pool, we performed bone nodule analysis and found that cells from the leukemic mice had reduced capacity to form alkaline phosphatase and von kossa positive colonies (fig. 2.11). Notably, a small number of bone-attached CD45⁺ cells were still present, and in the cultures from leukemic mice, leukemic cells were detected by fluorescence, although they represented only a very small proportion of the total cells (data not shown). To eliminate leukemic cells from CFU-OB cultures, marrow was flushed and depleted of leukemic cells by FACS prior to plating. Cells from leukemic mice had reduced osteoprogenitor numbers compared to normal controls (fig. 2.11). These data suggest that, in addition to impaired function, osteoblastic and to a lesser degree, osteoprogenitor numbers are decreased in the long bones of leukemic mice.

2.3.5 Leukemia induces bone loss

Functional inhibition of osteoblastic cells would be expected to result in decrease in bone structures. Leukemic mice exhibited qualitative loss of trabecular structures, both in the metaphyseal region and the secondary ossification centers of the long bones. This loss of trabecular bone was

quantified by histomorphometric analysis (fig. 2.12). At day 10, quantification of mineralized bone volume (BV/TV) by micro-CT analysis demonstrated severe loss of trabecular and cortical bone in the femora of leukemic mice compared to age and sex-matched controls (fig. 2.13). Trabecular number and thickness were decreased and trabecular spacing was increased in leukemic mice, consistent with alterations in the bone microenvironment and loss of trabecular bone volume (fig. 2.13). The same trend of bone loss was also seen in the tibiae of leukemic mice compared to healthy controls (fig. 2.14). Therefore, in mice, development of AML results in net loss of mineralized bone.

2.4 Discussion.

This report establishes for the first time that a leukemic environment results in a severe loss of bone formation and bone-lining osteoblastic cells. Osteoblastic cells are a known component of the hematopoietic stem cell niche and as such represent an appealing target in the treatment of myeloid leukemias.

It is well known that leukemias cause cytopenias that result in significant morbidity and mortality. While these cytopenias can develop while the overall level of leukemic disease is relatively low and overcrowding in the marrow space is unlikely to be a cause we still know very little known about what causes these cytopenias to develop. The bone marrow microenvironment regulates normal hematopoiesis, and there is an extensive

body of evidence in support of osteoblastic cells importance in the regulation of HSCs (Taichman and Emerson 1994; Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Visnjic, Kalajzic et al. 2004; Adams, Martin et al. 2007; Xie, Yin et al. 2009). Due to a lack of knowledge about the effect AML has on the bone microenvironment we determined to investigate this interesting intersection of normal and malignant hematopoiesis. In our studies we utilized a syngeneic murine model of myeloid leukemia. We deemed it imperative to our studies that the mouse model utilized has a normal microenvironment under baseline conditions. Therefore, we chose to use a mouse model in which the recipient mice are immune competent and require no prior conditioning, such as irradiation, in order for leukemic cells to engraft. Moreover this model's relevance to human disease has been determined in prior studies and an identifiable leukemia stem cell compartment has been described (Dash, Williams et al. 2002; Neering, Bushnell et al. 2007).

Our results show that within the marrow microenvironment of this model osteoblastic cells are severely inhibited. The near complete loss of osteocalcin and collagen 1 expression within the marrow cavity, combined with the decreased numbers of osteoprogenitors suggests that the osteoblastic inhibition acts throughout the osteoblastic lineage. However, the fact that a significant number of osteoprogenitors remain suggests that it appears to be most severe on mature osteoblastic cells. Further, expression of the notch ligand jagged 1, a known component of the endosteal HSC

niche, is lost in bone lining CD45- cells. This result indicates that the osteoblastic cells lost are likely contributing to the endosteal niche. Moreover since osteoblasts typically persist for several months under normal conditions the rapidity of osteoblastic inhibition suggests that a factor in the leukemic marrow acts directly on mature osteoblastic cells rather than simply inhibiting osteoblastic differentiation.

Osteoblastic and osteoclastic functions are normally coupled to maintain a constant bone volume. Due to a rapid loss of osteoblastic function we determined to evaluate the bone volume in leukemic mice. Both histomorphometric and micro-CT analysis revealed a significant loss of bone in leukemic mice over the course of just 10 days. Though human AML patients rarely present with significant bone involvement, the median age of diagnosis is 67 years (Howlader N 2011). Bone turnover is well known to slow down significantly as people age. Moreover, there are numerous reports of bone events in pediatric cases of AML when osteoblastic activity is still very high (Sala and Barr 2007; Sinigaglia, Gigante et al. 2008). Further, due to the median age of diagnosis many patients may present with co-morbidities present in the bone that may obscure any skeletal complications arising from the myeloid leukemia. Finally, AML is a rapidly progressing disease that requires the administration of therapy as soon as the diagnosis is confirmed. Therefore, the analysis of possible skeletal complications is not a high priority prior to therapy.

In summary we have demonstrated a severe defect in osteoblastic activity in a mouse model of myeloid leukemia. This defect likely contributes to an observed loss of trabecular and cortical bone. Further, a defect in the osteoblastic population could contribute to the development of cytopenias in myeloid leukemias. This makes osteoblasts a potentially appealing target in leukemia and our data illustrate the need for further investigation into the role of osteoblasts in myeloid malignancies.

Gene Name	Sequence
β -actin 5'	GCCACTGCCGCATCCTCTT
β -actin 3'	GGAACCGCTCGTTGCCAATAG
Osteocalcin 5'	CCGCCTACAAACGCATCTACG
Osteocalcin 3'	GAGAGAGGACAGGGAGGATCAAG
CCL3 5'	AAGGATACAAGCAGCAGCGAGTA
CCL3 3'	TGCAGAGTGTCATGGTACAGAGAA
Jagged1 5'	CTTCAATCTCAAGGCCAGCC
Jagged1 3'	CAGGCGAAACTGAAAGGCAG

Table 2.1. Sequence of primers used to analyze murine gene expression by qRTPCR.

Figure 2.1. Syngeneic murine model of AML. A) Murine Stem Cell Virus (MSCV) construct containing BCR/ABL and GFP. B) MSCV construct containing Nup98/HoxA9 and YFP. C) Schematic representation of the transplant strategy used to produce the leukemic mice utilized.

Figure 2.3. Leukemic cells in the marrow can be visualized by immunohistochemical staining. Representative anti GFP immunohistochemistry of the femur's marrow space at the metaphysis. GFP is visualized by brown staining, with a hematoxylin counterstain.

Figure 2.4. Leukemic environment results in thrombocytopenia.

Complete CBCs were obtained from normal and day 10 leukemic mice. A) Total WBC count is reported as 10^3 cells/ μl . B) Hematocrit reported as % of blood volume. C) Platelet count is reported as $10^3/\text{ul}$. * $p \leq 0.05$, *** $p \leq 0.001$.

Figure 2.5. Leukemic environment reduces the number of hematopoietic progenitors in the marrow. Colony forming assays were performed using cells sorted for GFP/YFP negativity in methylcellulose containing media. A) Number of total colony forming units/2x10⁴ marrow cells. B) Number of CFU-GM colonies formed/2x10⁴ marrow cells. C) Number of CFU-GM colonies formed/2x10⁴ marrow cells. ** p≤0.01, *** p≤0.001.

Figure 2.6. Leukemic environment does not alter levels of non-leukemic short-term engrafting hematopoietic cells. Competitive transplantations were performed using sorted GFP/YFP negative cells from d6 leukemic and normal mice. A) Total blood cells arising from engrafted donor cells, % of total cells. B) CD11b⁺ peripheral blood cells from donor, % of CD11b⁺ cells. C) CD3e⁺ peripheral blood cells from donor, % of CD3e⁺ cells. D) B220⁺ blood cells from donor, % of B220⁺ cells.

Figure 2.7. Absence of bone lining osteopontin positive cells in leukemic femurs. Osteopontin immunohistochemistry was performed on paraffin embedded sections; representative images are shown of A) a naïve femur, and B) a leukemic femur. Osteopontin positive cells are stained brown and sections were counterstained with hematoxylin (blue). Arrowheads indicate osteopontin⁺ cells.

Figure 2.8. Osteocalcin protein and message levels are reduced in leukemic mice. A) Quantification of serum osteocalcin measured by ELISA. B) Real Time RT-PCR quantifying Osteocalcin RNA expression in osteoblast-like cells collected from the long bones of normal or leukemic mice at day 6 or 11 and magnetically separated based on CD45 expression, statistical significance was determined compared to Naïve mice (day 0) (n=5 samples per experimental group). *** $p \leq 0.001$.

Figure 2.9. Notch ligand Jagged 1 expression is lost in leukemic environment. Real Time RT-PCR quantifying Jagged 1 RNA expression in osteoblast-like cells collected from the long bones of normal or leukemic mice at day 6 or 11 and magnetically separated based on CD45 expression, statistical significance was determined compared to Naïve mice (day 0) (n=5 samples per experimental group. *** $p \leq 0.001$

Figure 2.10. Osteoblast specific mRNA is lost at the endosteal surface of bone in leukemic mice. A) in situ hybridization for osteocalcin mRNA in paraffin sections of the femur of WT, as well as day 6 and day 10 leukemic mice. The presence of osteocalcin mRNA is indicated by black staining. B) in situ hybridization for collagen-1 mRNA in paraffin sections of the femur of WT, as well as day 6 and day 10 leukemic mice. The presence of collagen-1 mRNA is indicated by red staining, with the areas of strongest collagen-1 expression stained black.

Figure 2.11. Leukemic environment reduces numbers of osteoblastic progenitors. CFU-OBs formed per 1×10^6 cells from A) cells collected by collagenase digestion of bone fragments after 15 days in culture and B) whole marrow after 28 days in culture. ** $p \leq 0.01$.

Figure 2.12. Leukemic environment induces trabecular bone loss as measured by histomorphometric analysis. A) Representative images of H&E stained Paraffin sections of the distal femur from a naïve and Leukemic mouse 10 days after transplant, 4x objective. B) % Trabecular bone at the metaphysis of the femur measured histomorphometrically. C) % Trabecular bone at the metaphysis of the tibia measured histomorphometrically.

** $p \leq 0.01$.

Figure 2.13. Leukemic environment induces bone loss in the femur as

measured by micro-CT. A) Representative micro-CT images from the metaphysis of the femur from naïve and leukemic mice. B) Micro-CT analysis of femur trabecular bone volume/total volume, C) femur cortical bone volume/total volume, D) Trabecular number, E) Trabecular thickness, and F) Trabecular spacing. * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 2.14. Leukemic environment induces bone loss in the tibia as measured by micro-ct. A) Representative micro-CT images from the metaphysis of the tibia from naïve and leukemic mice. B) Micro-CT analysis of tibia trabecular bone volume/total volume, C) tibia cortical bone volume/total volume, D) Trabecular number, E) Trabecular thickness, and F) Trabecular spacing. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Chapter 3:

**Mouse model of leukemia uncouples bone resorption
from bone formation.**

3.1 Introduction

Osteoclasts are responsible for the resorption of bone and are tightly coupled to bone formation. In normal physiology one of the main functions of osteoclasts is the resorption of bone in response to PTH in order to increase serum calcium levels.

Osteoclasts are identified as extremely large multinuclear cells that are attached to the surface of bone and stain positive for the activity of TRAP. Osteoclasts seal the area of resorption and acidify the area to dissolve the hydroxyapatite crystals, as well as release collagenases and other enzymes to degrade matrix proteins (Mackie 2003; Hadjidakis and Androulakis 2006). At the area of bone resorption the osteoclast's cell membrane is convoluted in order to increase the available surface area for secretion of protons and enzymes. This structure has been termed the ruffled border and is a unique feature of active osteoclastic cells (Blair, Teitelbaum et al. 1989; Vaananen, Karhukorpi et al. 1990).

Osteoclasts are formed by the fusion of osteoclast progenitors. These progenitors are of the hematopoietic lineage and have been identified as macrophages and monocytes (Boyle, Simonet et al. 2003; Hadjidakis and Androulakis 2006). The recruitment and proliferation of osteoclast precursors is mediated by macrophage-colony stimulating factor (M-CSF). Further differentiation down the osteoclast lineage is initiated by RANKL signaling through its receptor RANK (Boyce and Xing 2008). Several proteins that are

commonly associated and involved in cellular fusion processes have been identified to play a role in the fusion of osteoclast precursors including CD9, CD44, CD47 and SIRP α (Hayer, Steiner et al. 2005; Ishii, Iwai et al. 2006; Chen, Grote et al. 2007; Lundberg, Koskinen et al. 2007). Additionally the protein dendritic cell-specific transmembrane protein (DC-STAMP) plays a critical role in the development of multi-nucleated osteoclasts (Kukita, Wada et al. 2004; Yagi, Miyamoto et al. 2005; Mensah, Ritchlin et al. 2010).

Due to the disruption of bone formation, and the rapid loss of bone we hypothesized that a leukemic environment uncouples the processes of bone formation and bone resorption. We tested this hypothesis by analyzing the number and activity of osteoclasts in leukemic mice. Additionally osteoclasts have also been implicated as supportive of HSCs, and osteoclastic activity is known to support the proliferation of multiple myeloma cells, and cells of bone metastases from solid tumors (Matsumoto and Abe 2006; Buijs, Stayrook et al. 2011).

3.2 Materials and Methods

3.2.1 Zoledronic Acid Treatment

Mice were given a 0.25 mg/kg intraperitoneal injection of zoledronic acid bi-weekly for 2 weeks prior to induction of and throughout the course of disease.

3.2.2 Osteoclastogenic cultures

Spleen cells were collected and red blood cells were lysed as previously described. The remaining cells were cultured at a concentration of 8.75×10^5 cells/ml in alpha Minimum Essential Medium (α -MEM) containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco) (complete α -MEM), and 30 ng/ml M-CSF (R&D systems) for 2 days. Ten ng/ml RANKL (R&D systems) was added to the media and the cultures were continued for an additional 4 days and then TRAP stained. For osteoblastic co-cultures, osteoblastic cells were isolated as described. An osteoblast feeder layer was established by culturing 1.4×10^4 cells/well in 96 well plates in complete α -MEM supplemented with 1×10^{-9} M 1,25 dihydroxyvitamin D3 (Sigma, D1530). After 2 days 8.75×10^4 whole marrow cells from either normal or leukemic mice were added to each well. Half media changes were performed every 2 days. Cultures were ended and TRAP stained after 7 days.

For cultures utilizing bovine bone wafers, an IsoMet low speed saw with a diamond blade was used to cut 300 μ m thick wafers of bone from a 4mm square pillar of devitalized bovine cortical bone to produce 300 μ m x 4mm x 4mm bone wafers.

3.2.3 Scanning Electron Microscopy

Bovine bone wafers were removed from culture media and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 hours at 4°C. The next day the wafers were postfixed in cacodylate buffered 1.0% osmium

tetroxide, processed through a graded series of ethanol to 100% (x3), processed into a series of mixtures of 100% ethanol and hexamethyldisilazane (HMDS) before transitioning to 100% HMDS (x3). The last exchange of 100% HMDS covering the wafers was allowed to evaporate at room temperature overnight in an uncovered 12 well plate in a fume hood. The dried wafers were mounted onto aluminum stubs, sputter coated for 90 seconds with gold, and imaged using a Zeiss Supra 40VP Field Emission scanning electron microscope.

3.3 Results

3.3.1 Leukemic environment transiently increases osteoclast numbers in vivo

Bone formation and resorption are closely linked; therefore bone loss could result from increased bone resorption, in addition to decreased bone formation. C-terminal telopeptides (CTX) are released from the bone matrix when it is resorbed and are an established measure of global bone resorption (Risteli, Elomaa et al. 1993). When serum CTX levels were measured in the sera from leukemic and normal mice, there was no measured change in CTX levels from leukemic mice at any time analyzed during disease progression (fig.3.1).

To quantify osteoclasts, histological sections from normal and leukemic mice were stained for Tartrate Resistant Acid Phosphatase (TRAP) activity.

Multinucleated TRAP⁺ cells were scored as osteoclasts (fig.3.2). In leukemic mice, osteoclasts were mildly increased 6 days after induction of disease, but were decreased by 10 days in leukemic mice compared to controls (fig.3.2). Measurement of serum TRACP 5b has been validated as a measure of global osteoclast numbers (Aya, Alhawagri et al. 2005). In normal mice, the TRACP 5b serum levels were stable, contrasting with leukemic mice in which global TRACP 5b levels were initially mildly though not significantly increased and fell below normal by day 10 mirroring the histologic findings (fig.3.2). These data suggest that, in our model, there is an initial mild and transient increase in osteoclastic cells, but as the disease progresses to overt leukemia, osteoclastic cell numbers decline.

3.3.2 Leukemic cells do not differentiate into osteoclasts and do not resorb bone matrix

As osteoclasts are derived from the myeloid lineage, it is possible that leukemic cells may differentiate into osteoclasts. Therefore, we evaluated the ability of spleen-derived leukemic cells to differentiate into osteoclasts in vitro. Under osteoclastogenic conditions, cells from normal spleens produce abundant osteoclasts (fig. 3.3). In striking contrast, GFP⁺/YFP⁺ leukemic cells isolated from spleens produce no TRAP⁺ osteoclasts (fig. 3.3).

To determine if the initial increase in osteoclasts in vivo was the result of pro-osteoclastogenic signals from the leukemic clone, we cocultured marrow cells from either leukemic or normal mice with a normal osteoblastic

feeder layer in the presence of 1,25 dihydroxyvitamin D3. Under these culturing conditions, osteoclast formation was much more abundant in normal marrow cells as compared to leukemic (fig. 3.4). These data confirm the inability of leukemia cells to form mature osteoclasts *in vitro* and suggest that leukemic cells require a full leukemic microenvironment to stimulate the *in vivo* transient increase in osteoclasts. Moreover osteoclastic precursors from spleens of normal mice co-cultured with normal spleen cells or leukemic spleen cells under standard osteoclastogenic conditions form significantly fewer mature osteoclastic cells, further showing that the marrow microenvironment is likely required for the observed increase in osteoclasts *in vivo* (fig. 3.5).

Although leukemic cells do not differentiate into TRAP⁺ osteoclasts *in vitro*, they may be able to resorb bone matrix. To determine if leukemic cells resorb bone directly, we sorted GFP⁺/YFP⁺ cells from the spleens of leukemic mice and cultured them on bovine bone wafers. Spleen cells from naïve mice were also sorted for GFP⁻/YFP⁻ cells merely as a control for the effects of the cell sorter as these mice were non-leukemic and there was no GFP⁺/YFP⁺ cells present. Scanning electron microscopic images were obtained to visualize the surface of the bone wafers, where osteoclastic activity is identified by the presence of resorption pits with a rough appearance that were abundant in the normal controls (fig. 3.6). While viable leukemic cells were observed to adhere to the surface of the bone wafers, the bone matrix in

wells containing leukemic cells remained smooth and lacked resorption pits (fig. 3.6). The amount of bone resorption taking place was determined by the quantification of bovine CTX in the culture media. Normal osteoclast precursors under osteoclastogenic conditions readily resorb bone, as was demonstrated by the increase in bovine CTX. In contrast, leukemic cells under the same conditions did not increase bovine CTX above background levels (fig. 3.6). These data suggest that GFP⁺/YFP⁺ leukemic cells do not directly contribute to bone resorption.

3.3.3 Treatment with zoledronic acid blocks bone resorption but only partially rescues bone loss in leukemic mice

To determine whether loss of bone formation or increased numbers of osteoclasts was responsible for the loss of bone in leukemic animals we treated mice with Zoledronic acid (ZA), a highly potent osteoclastic inhibitor (Green, Muller et al. 1994) prior to transplantation with leukemic cells (fig. 3.7). This treatment was sufficient to inhibit the activity of osteoclasts as determined by serum CTX levels (fig. 3.7). The serum levels of osteocalcin, a well-established bone formation marker, were significantly decreased in normal ZA-treated mice (142 ± 28 vs 49 ± 1 ng/ml, vehicle vs. ZA-treated mice $p=0.0296$). This homeostatic compensatory decrease in bone formation in response to effective osteoclastic inhibition explains the lack of significant ZA-dependent increases in bone volumes in wildtype mice (fig. 3.8). In leukemic mice treated with ZA simultaneous to control mice treated with PBS,

serum CTX dropped to subnormal levels (fig. 3.7), confirming the inhibitory effect of ZA on osteoclasts in the setting of leukemia. Loss of trabecular bone volume was ameliorated by treatment with ZA (fig. 3.8). Trabecular number and spacing were also rescued, consistent with ZA-dependent protection of the trabecular bone volume loss induced by leukemia (fig. 3.8). In contrast, cortical bone volume loss remained unchanged by inhibition of resorption (fig. 3.8). When disease burden was quantified in leukemic mice treated with ZA, there was a mild decrease in disease burden in the marrow, but not in spleen or peripheral blood (fig. 3.9). In this model the phenotype of the leukemia stem cell (LSC) has been previously defined (Neering, Bushnell et al. 2007), however treatment with ZA did not result in measurable changes in the frequency of LSC in marrow or spleen (fig. 3.10). These data suggest that inhibition of bone resorption only partially reverses leukemic-induced bone loss without significantly changing disease progression.

3.4. Discussion

This report establishes an uncoupling of bone resorption from bone formation in a murine model of acute myeloid leukemia. Uncoupling of bone turnover is a significant problem in diseases like multiple myeloma and bone metastases of prostate and breast cancer, however it has not previously been reported in myeloid leukemias.

Despite a dramatic loss of bone formation and osteoblastic cells in this model of myeloid leukemia levels of bone resorption remain unchanged.

Further the number of osteoclastic cells is transiently, though significantly increased in leukemic mice as the disease progresses. As bone resorption and bone formation are tightly coupled under normal conditions these results indicate that leukemia cells produce, or induce the production of one or many uncoupling factors.

The severity of the bone loss observed is surprising due to the short time frame in which it occurs. We postulated that it could be possible for AML cells from this mouse model to directly differentiate into osteoclast-like cells capable of bone resorption. We deemed this a possibility, as this is a myeloid disease, and osteoclast precursors are myeloid derived. Further, it has been demonstrated in multiple myeloma as early as 1983 that malignant cells are capable of differentiating into osteoclast-like polykaryons and resorbing bone directly (McDonald, Schofield et al. 1983; Calvani, Cafforio et al. 2005; Andersen, Boissy et al. 2007). In order to test this hypothesis we first cultured leukemic cells under standard osteoclastogenic conditions and observed no TRAP⁺ polykaryons produced in the leukemic cultures. It is feasible that the presence of bone is required for the production of osteoclast-like cells. When cultured with wafers of bovine bone however we observed no bone resorption taking place in the leukemic cultures. These results indicate that, in vitro, direct stimulation of leukemic cells with osteoclastogenic conditions does not result in the production of cells capable of bone resorption. It is conceivable however that a more physiologic process could drive the production of bone

resorbing cells. Therefore a cell culture system utilizing co-culture of an osteoblastic feeder layer with marrow cells from either normal or leukemic animals was used. In this system osteoclastogenesis is driven more physiologically by factors produced by the osteoblasts when stimulated with dihydroxyvitamin d3. In these cultures there was a noted absence of osteoclastogenesis within the leukemic cultures indicating not only that under these conditions leukemic cells don't form bone resorbing cells, but also that the marrow of leukemic mice is deficient in its ability to form osteoclasts. This finding could partially explain the decrease in osteoclastic cell number observed in the late stages of disease. Further at late stages of disease leukemic mice are likely to be cytopenic and a lack of osteoclast precursors could also play a role in the lower numbers of osteoclasts.

Osteoclastic activity is known to release mitogenic cytokines from the bone matrix that act to fuel the growth of myeloma cells, and breast and prostate cancer metastases (Matsumoto and Abe 2006; Buijs, Stayrook et al. 2011). We hypothesized that in AML osteoclastic activity could provide the same pro-tumor effects as seen in other malignancies. To test this hypothesis we treated mice with the bisphosphonate zoledronic acid prior to induction of leukemia, and throughout the course of disease. Despite a dramatic reduction in the rate of bone resorption there was no difference in the number of leukemic cells in the spleen or blood. Though there was a small reduction in the number of leukemic cells in the marrow the number of immature leukemia

cells was not changed. Moreover, the use of zoledronic acid failed to prevent the loss of cortical bone indicating that the cortical bone defect is owed more to the loss of osteoblastic activity than the maintenance of osteoclastic activity.

In summary, these results demonstrate an uncoupling of bone formation and bone resorption. While this is commonly seen in multiple myeloma and bone metastases of solid tumors it has not previously been reported in myeloid leukemias. Further, through the use of the bisphosphonate zoledronic acid, we show that the maintenance of osteoclastic bone resorption is not essential or substantially beneficial to the progression of disease in this model.

Figure 3.1. Leukemic environment does not alter bone resorption levels.

Serum levels of carboxy-terminal collagen cross links (CTX) a marker of bone resorption as measured by ELISA.

Figure 3.2. Leukemic environment induces a mild and transient increase in the number osteoclasts. A) Representative images of paraffin sections. TRAP⁺ cells are pink and highlighted by arrowheads. B) Quantification of multi-nucleated TRAP positive cells in a 1mm square area just proximal of the distal growth plate in the femur from sections represented by the panels in B. C) Serum levels of TRACP 5b, the osteoclast specific TRAP enzyme, measured by ELISA (n=5 samples per experimental group. * p≤0.05.

Figure 3.3. Leukemic cells do not form osteoclasts under standard osteoclastogenic conditions. A) Representative light micrographs of normal and leukemic spleen cells under osteoclastogenic conditions in vitro. Pink cells are positive for TRAP activity. B) Quantification of TRAP+ cells in A.

Figure 3.4. Leukemic environment does not induce the formation of osteoclasts from bone marrow. A) Representative light micrographs of co-cultures containing normal osteoblasts with normal marrow cells or leukemic marrow cells. B) Quantification of TRAP+ cells formed per well from osteoblastic co-cultures with normal and leukemic marrows (n=5 samples per experimental group). *** $p \leq 0.001$.

Figure 3.5. Leukemic environment inhibits osteoclast formation in vitro.

Co-cultures were performed with osteoclast precursors obtained from the spleens of naïve mice, and either normal or leukemic spleen cells under standard osteoclastogenic conditions. A) Representative images of TRAP staining performed on day 6 of co-cultures with either normal or leukemic spleen cells. B) Quantification of the number of multinucleated TRAP+ osteoclasts formed per well. ** $p \leq 0.01$.

A **B**

Figure 3.6. Leukemic cells cannot resorb bone. A) Low and high power scanning electron micrographs of leukemia cells on bovine bone wafers. B) ELISA quantification of CTX released into culture media during culture of cells with bovine bone wafers (n=3 samples per experimental group). * $p \leq 0.05$.

Figure 3.7. Treatment of leukemic mice with zoledronic acid decreases levels of bone resorption. A) Schematic for the treatment schedule of leukemic and normal mice with zoledronic acid (ZA). Leukemia was initiated on day 0 following 2 weeks of ZA treatment. Injection of ZA denoted by arrows. B) Serum CTX levels in normal mice following 2 weeks of treatment with ZA. C) Serum CTX levels in normal and leukemic mice following the ZA treatment schedule. ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3.8. Zoledronic acid rescues trabecular but not cortical bone

loss. A) Trabecular bone volume/total volume B) cortical bone volume/total volume C) trabecular number and D) trabecular spacing in normal or leukemic mice as quantified by micro-CT analysis following treatment with zoledronic acid (n=5 samples per experimental group). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3.9. Treatment with zoledronic acid does not alter disease progression. Flow cytometric data quantifying bcCML cells as a percent of total mononuclear cells in A) the marrow, B) the spleen, and C, the peripheral blood from mice treated with either VEH or ZA.

* $p \leq 0.05$.

Figure 3.10. Treatment with zoledronic acid does not affect the number of immature leukemic cells. A) Flow cytometric gating strategy to isolate a population enriched for LSCs. Flow cytometric data quantifying a population of bcCML cells enriched for LSCs as a percent of total mononuclear cells in B) the marrow and C) the spleen. Each panel is a combination of 2 separate experiments.

Chapter 4:

Mechanism of action.

4.1 Introduction

The decrease in osteoblastic bone formation, unchanged levels of osteoclastic bone resorption and net bone loss in the leukemic environment highly suggest an uncoupling of osteoblastic and osteoclastic activity. Uncoupling of bone turnover has been observed in several malignancies, including multiple myeloma, and bone metastases of breast and prostate cancers, though to date has not been described in AML. Osteoblastic cells are known to play an important role in the hematopoietic stem cell niche, and in the regulation of normal hematopoietic development (Visnjic, Kalajzic et al. 2004; Shono, Ueha et al. 2010). Due to the observed, dramatic effect on osteoblastic cells the uncoupling event could have important clinical implications. In many patients cytopenias develop prior to a large tumor burden suggesting that leukemia cells are playing an active role in inhibiting normal hematopoietic development. If the inhibition of osteoblastic cells is playing a role in the development of cytopenias then the identification of a potentially targetable mechanism could be therapeutically relevant.

The uncoupling event could be mediated either by factors secreted by leukemic cells, or by factors that require direct cell-cell contact. Moreover leukemic factors could act directly on osteoblasts and osteoclasts, or they could relay through other microenvironmental cells that would then ultimately produce the uncoupling factor. There are many uncoupling signals that have been described in various systems including dkk1, CCL2, CCL3, CCL4,

SOST, noggin, RANKL, CXCL12, IL-6, IL-11, IL-3, and PTHrP most of which are secreted factors that do not require direct cell contact.

Due to the effect on osteoblastic and osteoclastic cells, the development of cytopenias in advance of a large tumor burden, and the factors that are involved in uncoupling events in other malignancies have led us to hypothesize that leukemic cells produce a secreted factor that inhibits osteoblastic cells and uncouples the process of bone turnover.

4.2 Materials and Methods

4.2.1 Patient Samples

Marrow aspirates were collected from the posterior iliac crest of both patients with AML and healthy volunteer donors. Marrow plasma was isolated by centrifugation and analyzed for protein levels by ELISA. Bone marrow mononuclear cells were isolated and CD34⁺CD38⁻ normal and leukemic cells were isolated as previously described (Majeti, Becker et al. 2009). Relative gene expression was determined by quantitative RT-PCR. All patients and volunteers provided written, informed consent in accordance with the Declaration of Helsinki on protocols approved by the Research Subjects Review Board of the University of Rochester.

4.2.2 Co-cultures and conditioned media

CFU-OB cultures were started as previously described. After 4 days in culture osteogenic media was added and 1×10^6 marrow cells from either normal or leukemic animals were added to the cultures. In addition separate cultures containing normal CFU-OB, and 1×10^6 bone marrow cells from leukemic mice were used to produce conditioned media that was added to normal CFU-OB cultures starting on the same day as the bone marrow cells from leukemic mice were added to the co-cultures.

4.3 Results

4.3.1 Expression of the chemokine CCL3 is increased in malignant marrow cells in leukemic mice

Osteoblastic inhibition by leukemia may be mediated by cell contact and/or by secreted molecules. CFU-OB co-cultures were performed with wildtype pre-osteoblasts as a feeder layer and leukemic cells from our murine model. Additionally normal CFU-OB cultures were treated with conditioned media from murine leukemic cell or normal CFU-OB cultures. Both the co-culture and conditioned media cultures showed a similar lack of alkaline phosphatase positive colonies (fig. 4.1) suggesting that a secreted factor(s) is (are) sufficient to suppress colony formation. CCL3 is a pro-inflammatory cytokine in the CC chemokine family that has been implicated in the pro-osteoclastic actions observed in multiple myeloma (Han, Choi et al. 2001;

Lentzsch, Gries et al. 2003). In addition, data have recently suggested that CCL3 inhibits osteoblastic cells in mice and in human in vitro studies (Vallet, Pozzi et al. 2011). The level of CCL3 in the media was elevated in both co-cultures and conditioned media (fig. 4.1). CCL3 protein levels were also increased in the marrow plasma and blood serum from leukemic mice compared to normal controls (fig. 4.2). To determine the cellular source of CCL3, mRNA was measured by quantitative RT-PCR of marrow mononuclear cells, cells digested from bone fragments of the hindlimbs that were separated based on surface CD45 positivity, and sorted GFP⁺/YFP⁺ leukemic cells. Bone-associated CD45⁺ cells from leukemic mice expressed higher levels of CCL3 mRNA compared to normal controls (fig. 4.3). In sorted cells, GFP⁺/YFP⁺ leukemic cells demonstrated the highest level of CCL3 expression (fig. 4.3). To assess whether CCL3 is also upregulated in patients with AML, CCL3 expression was quantified in sorted CD34⁺CD38⁻CD123⁺ human marrow cells, and found to be increased in marrow samples from AML patients compared to normal controls (fig. 4.4). In addition CCL3 protein levels were elevated in the marrow plasma from AML patients compared to normal controls (fig. 4.4). This data is in agreement with the analysis of microarray data previously published by Bullinger et al. in which 58 of 75 AML samples had elevated levels of CCL3 (fig. 4.5) (Bullinger, Dohner et al. 2004).

4.4 Discussion

CCL3 expression has previously been reported in AML though reports of its effect on AML cells have been somewhat contradictory (Eaves, Cashman et al. 1993; Ferrajoli, Talpaz et al. 1994). Further, *CCL3* is well known to cause bone destruction in multiple myeloma, and the role of *CCL3* in increasing bone resorption is well established. More recently a study by Vallet et al. showed that in multiple myeloma *CCL3* uncoupled bone turnover not only by increasing osteoclastic bone resorption, but also by decreasing osteoblastic bone formation. They also demonstrated that *CCL3*'s mechanism of action is mediated, at least in part, by signaling through its receptor *CCR1*. Following *CCL3* signaling through *CCR1* on the cell surface of osteoblasts *ERK* activation was observed followed by a decrease in osterix expression. Finally, osteocalcin expression is severely restricted in osteoblasts following treatment with *CCL3* (Vallet, Pozzi et al. 2011).

In our model we demonstrated that osteoblastic inhibition by leukemia cells is achieved by a secreted factor. We also showed that *CCL3* message is highly upregulated in cells of the leukemic mouse model, and that in leukemic mice *CCL3* protein is found at much higher concentrations in the marrow plasma and blood serum than in normal controls. Further we utilized samples from AML patients. In these samples we saw an upregulation of *CCL3* message. We also analyzed *CCL3* protein levels in the marrow plasma of

AML patients; CCL3 was detected at higher concentrations than normal controls in 4 of 6 samples analyzed. Finally we reanalyzed gene expression data that had previously been published by Bullinger et al. The reanalyzed data showed elevated expression levels of CCL3 in 58 of 75 samples.

We find it highly likely that CCL3 is playing a major role in the drastic reduction in osteoblastic cells in leukemic mice and the uncoupling of bone formation from bone resorption. It is well established that the receptors for CCL3 are expressed on osteoblastic cells, and the phenotype we see in our model of AML is in very strong agreement with what has been reported in multiple myeloma (Vallet, Pozzi et al. 2011). What our results fail to demonstrate is that CCL3 is the critical regulator of the uncoupling of bone formation and bone resorption. While we believe CCL3 to be at least partly responsible for the bone phenotype described further research is needed to determine if there are any other factors involved.

Figure 4.1. A soluble factor is sufficient to inhibit osteoblastic differentiation in a leukemic environment. A) Representative wells from CFU-OB cultures stained for alkaline phosphatase activity (pink). B) CCL3 levels in culture media from CFU-OB cultures (n=3 samples per experimental group). *** $p \leq 0.001$.

Figure 4.2. CCL3 protein levels are greatly increased in leukemic marrow plasma and blood serum. CCL3 protein levels in murine model of AML compared to normal controls at day 10 of disease. *** $p \leq 0.001$.

Figure 4.3. Leukemic cells express high levels of CCL-3. Relative expression of CCL3 in bone marrow mononuclear cells isolated from whole bone marrow of day 10 leukemic animals, cells sorted for GFP and YFP expression according to fig. 2.2, and cells liberated from bone fragments of day 10 leukemic animals by collagenase digestion and magnetically separated based on CD45 cell surface expression. * $p \leq 0.05$.

Figure 4.4. Human AML samples express high levels of CCL3. A) Relative expression of human CCL3 in primitive CD34+CD38-CD123+ AML cells compared to normal controls each bar represents a single AML sample normalized to 3 normal controls. B) CCL3 protein levels in human AML patient marrow plasma, each bar represents a single AML marrow sample compared to 7 normal controls. * $p \leq 0.05$.

GDS843 / 21614 / CCL3 : Adult acute myeloid leukemia: bone marrow and peripheral blood expression profiles (SHDJ) 1

Title: GDS843 / 21614 / CCL3 / Homo sapiens

Summary: Part of a study profiling 54 bone marrow and 65 peripheral blood samples from 116 adults with acute myeloid leukemia (AML). Results identify distinct gene expression signatures that correlate with clinical outcomes. Signatures used to construct a clinical outcome predictor using 133 genes.

Figure 4.5. CCL3 levels elevated in 58 of 75 AML samples. Re-analysis of micro-array data from 75 AML patients for expression levels of CCL3.

Chapter 5:

**Discussion, Clinical Significance and Future
Directions**

5.1 Discussion

It has long been known that osteoblastic cells support and expand HSCs in vitro (Taichman and Emerson 1994), and co-transplantation of osteoblastic cells with HSCs can increase engraftment rate (El-Badri, Wang et al. 1998). Work in our laboratory and others first identified osteoblastic cells as a regulatory component in the HSC niche through genetic means (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003). It has become evident that osteoblastic cells can both stimulate (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003) and limit HSC expansion (Nilsson, Johnston et al. 2005; Stier, Ko et al. 2005), promote quiescence (Arai, Hirao et al. 2004; Yoshihara, Arai et al. 2007; Fleming, Janzen et al. 2008), initiate HSC mobilization (Mayack and Wagers 2008), and mediate sympathetic nervous system regulation of HSCs (Katayama, Battista et al. 2006). In addition, bone progenitor dysfunction is sufficient to induce myelodysplasia and secondary leukemia (Raaijmakers, Mukherjee et al. 2010). Finally, recent data suggest that mesenchymal stem cells, which give rise to cells of the osteogenic lineage, regulate HSCs (Mendez-Ferrer, Michurina et al. 2010). In addition to increasing evidence that osteoblastic lineage cells act as orchestrators of HSC behavior, data strongly suggest that osteoblastic dysfunction results in pancytopenia (Visnjic, Kalajzic et al. 2004; Shono, Ueha et al.). We have shown that induction of myeloid leukemia in an in vivo immunocompetent non-irradiated murine microenvironment induces severe functional inhibition of osteoblastic cells

even when the burden of disease is relatively low and when leukemic cells are undetectable in blood. Therefore, osteoblastic damage by leukemia may be due to specific leukemic-initiated interactions rather than as a consequence of systemic disease. Osteoblastic inhibition in leukemia resulted in decreased bone formation and net bone loss, particularly in cortical bone, where osteoclastic inhibition could not compensate for the leukemic-dependent bone loss, despite significant reductions in osteoclastic activity.

The mechanisms by which AML inhibits osteoblastic cells may include both direct and indirect actions of leukemia cells on their microenvironment. However, review of microarray data comparing gene expression in total RNA obtained from sorted $\text{lin}^- \text{GFP}^+ \text{YFP}^+$ cells to $\text{lin}^- \text{GFP}^- \text{YFP}^-$ cells from this mouse model indicated no significant changes in the expression of genes encoding cytokines with potent inhibitory activity against osteoblastic cells, such as *dkk1*, *CCL2*, *CCL4*, *IL6*, *SOST* and *noggin* (Table 4.1), suggesting they are not likely direct mediators of the leukemia-induced osteoblastic defect. In contrast, we have shown significantly increased expression of the gene encoding the chemokine CCL3. Receptors for CCL3 (CCR1 and CCR5) are present on osteoblastic cells (Abbas, Zhang et al. 2003; Yano, Mentaverri et al. 2005), and recent data have demonstrated the inhibitory effects of CCL3 on both murine and human osteoblastic cells (Vallet, Pozzi et al. 2011). Our findings reveal that CCL3 is highly expressed both by our leukemic model as well as in human AML. Further the osteoblastic inhibition caused by CCL3

was reported to be most significant on mature osteoblastic cells. This correlates well with our data on osteoblast precursors that retained a significant population within the bone marrow. Additional experiments are needed to determine if CCL3 is indeed the key mediator of AML-dependent effects on the marrow microenvironment. CCL3 also has strong pro-osteoclastic effects (Han, Choi et al. 2001; Lentzsch, Gries et al. 2003) that may, in part, explain the transient increase in TRAP⁺ cells found in our model.

Bone turnover is a closely regulated event in which bone formation and bone resorption are tightly coupled. In the setting of dramatic loss of bone formation, a compensatory loss of osteoclastic bone resorption would be expected. In contrast, there was a mild and transient increase in osteoclastic cells in mice with leukemia. Moreover, leukemic-induced trabecular bone loss was completely blocked by osteoclast inhibition. Therefore, a leukemic environment results in uncoupling of bone formation and bone resorption. This effect is likely responsible for the trabecular loss, and may be important in the early stages of disease. We speculate that the decrease in osteoclastic number seen at later time points may be due, in part, to the leukemia-induced block in hematopoietic differentiation that could limit the pool of hematopoietic osteoclast precursors. Another alternative is that in addition to CCL3, leukemia may be producing a separate factor that acts to abrogate the osteoclastic activation normally seen with CCL3 upregulation. This other factor would have to still allow, or even act to worsen, the effect of CCL3 on

osteoblasts, as the osteoblastic inhibition only gets more severe as the disease progresses. We also know that in normal bone turnover osteolineage bone lining cells play major roles in the activation of osteoclastic bone resorption. We have demonstrated a progressive loss of bone lining osteolineage cells throughout the progression of leukemic disease. Hence the pro-osteoclastic effects of CCL3 may be maintaining a normal level of osteoclastic activity that would otherwise be lost due to coupling with bone formation. Each of these scenarios or a combination thereof is a possible cause of the somewhat surprisingly low levels of osteoclastic activity in light of the very high levels of CCL3 protein.

The progression of disease in this mouse model is extremely rapid. While acute leukemias also progress at a very rapid pace they develop more slowly than what occurs in this model. Therefore the use of additional models of myeloid leukemia such as a mouse model utilizing the translocation product MLL-AF9 will be important in verifying our findings. The use of this second model would allow a slower more physiologically relevant development of AML while still utilizing a syngeneic mouse model.

In summary, findings in this thesis support the concept that leukemia disrupts the normal marrow microenvironment, and particularly targets cells that have been demonstrated to support and regulate HSCs. We propose the following model: Leukemia cells produce CCL3 which either alone, or in conjunction with another factor uncouples bone turnover by stimulating

osteoclastic bone resorption, and reducing osteoblastic number and levels of bone formation. CCL3 achieves the osteoblastic inhibition by binding CCR1 on the surface of osteoblastic cells and through activation of ERK, reducing expression of osterix and osteocalcin, and resulting in the loss of osteoblastic function (fig. 5.1). Due to the known role of osteoblasts in the HSC niche and since the loss of normal hematopoietic function during human leukemia is a major determinant of morbidity and mortality (Lichtman 2000; Lane, Scadden et al. 2009), our data support investigation of a human osteoblastic defect as a result of AML.

5.2 Clinical Significance

Pancytopenia is the cause of significant morbidity in leukemia, and yet the mechanisms by which leukemia causes anemias remain poorly understood. Moreover anemias can develop when the overall tumor burden is still relatively low indicating an active role of leukemia cells in the inhibition of normal hematopoiesis. Despite this and the knowledge that the microenvironment plays a crucial role in the development of the normal hematopoietic system there is little known about the effects of myeloid leukemias on the bone marrow microenvironment.

To our knowledge this is the first report of a specific inhibition of osteoblastic cells in myeloid leukemias. While there is little data reporting the effects on bone in adult AML, these data are consistent with findings at diagnosis in pediatric acute leukemia, where decreased markers of bone

formation prior to corticosteroid treatment have been documented (Sala and Barr 2007; Sinigaglia, Gigante et al. 2008). The disruption in bone formation seen in pediatric leukemias may be reversible as, in spite of corticosteroid treatment in this disease, bone formation markers improve with reduction of disease burden after chemotherapy (Crofton, Ahmed et al. 1998). If the decreased markers of bone formation are indicative of a loss of osteoblastic cells the support of normal hematopoiesis could be adversely affected. Therapeutic intervention could potentially hasten the recovery of the normal hematopoietic system following treatment. Moreover as age increases the rates of bone turnover decrease. This fact could explain the lack of an observed significant bone effect in adult AML. The osteoblastic population is not as active even under healthy conditions so an inhibition of osteoblastic activity is unlikely to produce as dramatic of results on overall bone health as we have seen in our model.

Strategies for CCL3 inhibition have already been developed for the treatment of myeloma (Vallet, Raje et al. 2007), where CCL3 is an important mediator of osteolytic disease. Small molecule inhibitors of CCR1 have been shown to decrease myeloma tumor burden in a humanized mouse model. Therefore our findings, if verified in human AML, may suggest a therapeutic approach in which CCL3 inhibition may be used to ameliorate osteoblastic dysfunction, this could potentially lead to a faster recovery of the hematopoietic system following therapy.

5.3 Future Directions

The data presented in this thesis suggest many exciting questions for subsequent studies. Further investigation will lead to a greater understanding of the causes, extent, and significance of osteoblastic inhibition in leukemia. Further the therapeutic potential that CCL3 signaling suggests will need to be further investigated.

5.3.1 Is CCL3 the critical regulator of osteoblastic inhibition?

We have demonstrated that osteoblastic cells are strongly inhibited by leukemic cells both in vivo and in vitro. Moreover we have shown that the known osteoblastic inhibitor CCL3 is highly upregulated in our model of AML and that CCL3 protein accumulates at high levels in the marrow and blood serum. CCL3 is also highly expressed in human samples of AML and in several samples CCL3 protein accumulates at high levels in the marrow plasma. In this thesis however we have not shown that CCL3 is the critical regulator of osteoblastic inhibition. There are many tools available to further investigate CCL3 signaling in the setting of leukemia.

CCL3 knockout mice have been developed and represent a very good model in which to investigate the role of CCL3 (Chintalacharuvu, Wang et al. 2005). Using this model we propose to initiate leukemia in cells lacking CCL3. These cells could then be injected into wild-type mice in order to initiate a disease in which CCL3 signaling initiating from the leukemic cells has been eliminated.

Knockouts for CCR1 and CCR5 have also been developed (Hoshino, Iimura et al. 2010; Ferreira, Repeke et al. 2011). These models are also appealing, although there are some inherent complications. Global knockouts for the receptors of CCL3 are likely to have effects unrelated to the disease. CCR5 knockouts in particular have been shown to have baseline bone effects making this model unlikely to be very useful in pursuing the osteoblastic effect seen in leukemia (Ferreira, Repeke et al. 2011).

Therapeutic approaches are also an option. As previously discussed there is a small molecule inhibitor of CCR1 that has been previously used in models of multiple myeloma. There are also CCL3 inhibitory antibodies available that target either murine or human CCL3 (Vallet, Pozzi et al. 2011). Therefore, we propose to treat our mouse model of leukemia with a CCR1 inhibitor, and a murine CCL3 inhibitory antibody in vivo in separate experiments. Additionally, we propose to use these same treatments as well as a human CCL3 inhibitory antibody in vitro in co-culture assays. These experiments would further our understanding of the role CCL3 signaling plays in osteoblastic inhibition in myeloid leukemia.

5.3.2 Is the leukemic microenvironment less supportive of HSCs?

Leukemia is known to induce cytopenias and in our model of AML thrombocytopenia is very pronounced. Additionally, it is well established that osteoblasts play an essential role in the regulation of normal hematopoiesis, and in this thesis we have demonstrated a severe inhibition of osteoblastic

cells in a mouse model of myeloid leukemia. However, we have not directly shown that disruptions to the marrow microenvironment are a critical factor for the development of cytopenias.

We propose to utilize a co-culture system that allows us to use bone-lining cells from normal and leukemic mice to support immature hematopoietic cells *ex vivo*. Following co-cultures the number of immature hematopoietic cells can be quantified by CFU-C assays, flow cytometric analysis and competitive transplantation (Chitteti, Cheng et al. 2010).

Further, we have developed a new system in which we can isolate osteoblastic cells from bony spicules present in bone marrow aspirates from AML patients and normal volunteers. Using the same co-culture system as for the murine model of AML, but with bone-lining cells derived from human samples we can establish if a human leukemic microenvironment has impaired support for normal hematopoiesis.

5.3.3 Does leukemia affect the vascular niche?

In this thesis we have focused on the endosteal HSC niche and, in particular, osteoblastic cells. However there are other cell types involved in the support of normal hematopoiesis other than osteoblasts. Endothelial cells play a role in what has been termed the vascular or perivascular niche. The vascular niche plays a critical role in the recovery of normal hematopoiesis following myeloablative injury (Hooper, Butler et al. 2009), such as chemotherapeutic treatment for AML. In addition, myeloid leukemias have

been shown to increase angiogenesis (Schmidt and Carmeliet 2011). However, following myeloablative treatment for AML many patients are very slow to recover normal levels of hematopoietic cells. This suggests that although angiogenesis is increased in AML endothelial cells within a leukemic environment may be impaired in their ability to support normal hematopoiesis. We performed immunohistochemical staining for VEGF, a well-known angiogenic factor, in paraffin sections from the mouse model used in this thesis. A dramatic increase in the levels of VEGF protein was observed in the marrow of leukemic mice suggesting that aberrant levels of angiogenesis may be occurring.

Therefore, we propose to use this model of leukemia to further investigate the role of endothelial support for normal HSCs in AML. SCF has been described to be a critical factor of the endothelial, perivascular niche (Ding, Saunders et al. 2012). We propose to investigate the levels of SCF within the sinusoidal endothelial cell compartment in normal and leukemic mice.

5.3.4 Can osteoblastic dysfunction and bone loss be rescued in leukemic mice?

There are many disease states in which osteoblastic bone formation is either dysfunctional or uncoupled from osteoclastic bone resorption resulting in bone loss. These include but are not limited to rheumatoid arthritis, osteoarthritis, multiple myeloma, and bone metastases of solid tumors. In

these situations bone loss can be slowed either by inhibition of osteoclastic bone resorption, typically by treatment with bisphosphonates, or by stimulation of osteoblastic bone formation. To achieve stimulation of bone formation PTH is often given in therapeutic doses. In mice intermittent treatment with PTH is well known to increase bone mass (Calvi, Adams et al. 2003), and PTH has begun to be used in the treatment of human disorders as well.

Therefore we propose to treat leukemic mice with PTH in order to determine if osteoblastic activity can be either maintained or restored. This study would likely be combined with section 5.3.2 to determine if rescue of the osteoblastic compartment could also affect the support for normal hematopoiesis in leukemic mice.

Gene Name	Log ₂ Fold change	P-value
Dkk1	0.29	0.45
Ccl2	-0.67	0.01
Sost	0.46	0.14
Noggin	-0.09	0.72
Ccl4	0.27	0.48
IL6	0.80	0.15

Table 5.1. Genes associated with osteoblastic inhibition.

Figure 5.1. Model of AML induced uncoupling of bone turnover. AML cells produce CCL3 which alone or in conjunction with an unknown factor severely inhibits osteoblastic cells and maintains normal levels of osteoclastic activity resulting in a net loss of bone.

References

- Abbas, S., Y. H. Zhang, et al. (2003). "Tumor necrosis factor-alpha inhibits pre-osteoblast differentiation through its type-1 receptor." Cytokine 22(1-2): 33-41.
- Abe, M., K. Hiura, et al. (2002). "Role for macrophage inflammatory protein (MIP)-1alpha and MIP-1beta in the development of osteolytic lesions in multiple myeloma." Blood 100(6): 2195-202.
- Achbarou, A., S. Kaiser, et al. (1994). "Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo." Cancer Res 54(9): 2372-7.
- Adams, G. B., K. T. Chabner, et al. (2006). "Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor." Nature 439(7076): 599-603.
- Adams, G. B., R. P. Martin, et al. (2007). "Therapeutic targeting of a stem cell niche." Nat Biotechnol 25(2): 238-43.
- Alam, A. S., A. Gallagher, et al. (1992). "Endothelin inhibits osteoclastic bone resorption by a direct effect on cell motility: implications for the vascular control of bone resorption." Endocrinology 130(6): 3617-24.
- Andersen, T. L., P. Boissy, et al. (2007). "Osteoclast nuclei of myeloma patients show chromosome translocations specific for the myeloma cell clone: a new type of cancer-host partnership?" J Pathol 211(1): 10-7.
- Andersen, T. L., T. E. Sondergaard, et al. (2009). "A physical mechanism for coupling bone resorption and formation in adult human bone." Am J Pathol 174(1): 239-47.
- Appelbaum, F. R., H. Gundacker, et al. (2006). "Age and acute myeloid leukemia." Blood 107(9): 3481-5.
- Arai, F., A. Hirao, et al. (2004). "Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche." Cell 118(2): 149-61.
- Avigdor, A., P. Goichberg, et al. (2004). "CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow." Blood 103(8): 2981-9.

- Aya, K., M. Alhawagri, et al. (2005). "NF-(kappa)B-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis." J Clin Invest 115(7): 1848-54.
- Barille, S., M. Collette, et al. (1995). "Myeloma cells upregulate interleukin-6 secretion in osteoblastic cells through cell-to-cell contact but downregulate osteocalcin." Blood 86(8): 3151-9.
- Baron, R. (1996). Anatomy and Ultrastructure of Bone. Primer on the Metabolic Bone Diseases and Disorders of Mineral. M. J. Favus. Chicago, IL, Lippincott-Raven. 3.
- Basara, N., A. Schulze, et al. (2009). "Early related or unrelated haematopoietic cell transplantation results in higher overall survival and leukaemia-free survival compared with conventional chemotherapy in high-risk acute myeloid leukaemia patients in first complete remission." Leukemia 23(4): 635-40.
- Basolo, F., L. Fiore, et al. (1996). "Expression of and response to interleukin 6 (IL6) in human mammary tumors." Cancer Res 56(13): 3118-22.
- Bataille, R., D. Chappard, et al. (1991). "Recruitment of new osteoblasts and osteoclasts is the earliest critical event in the pathogenesis of human multiple myeloma." J Clin Invest 88(1): 62-6.
- Baum, C. M., I. L. Weissman, et al. (1992). "Isolation of a candidate human hematopoietic stem-cell population." Proc Natl Acad Sci U S A 89(7): 2804-8.
- Bennett, J. M., D. Catovsky, et al. (1976). "Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group." Br J Haematol 33(4): 451-8.
- Berner, H. S., S. P. Lyngstadaas, et al. (2004). "Adiponectin and its receptors are expressed in bone-forming cells." Bone 35(4): 842-9.
- Blair, H. C., S. L. Teitelbaum, et al. (1989). "Osteoclastic bone resorption by a polarized vacuolar proton pump." Science 245(4920): 855-7.
- Blay, J. Y., S. Schemann, et al. (1994). "Local production of interleukin 6 by renal adenocarcinoma in vivo." J Natl Cancer Inst 86(3): 238.
- Bonnet, D. and J. E. Dick (1997). "Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell." Nat Med 3(7): 730-7.
- Boyce, B. F. and L. Xing (2008). "Functions of RANKL/RANK/OPG in bone modeling and remodeling." Arch Biochem Biophys 473(2): 139-46.
- Boyle, W. J., W. S. Simonet, et al. (2003). "Osteoclast differentiation and activation." Nature 423(6937): 337-42.
- Broxmeyer, H. E., C. M. Orschell, et al. (2005). "Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist." J Exp Med 201(8): 1307-18.

- Buijs, J. T., K. R. Stayrook, et al. (2011). "TGF-beta in the Bone Microenvironment: Role in Breast Cancer Metastases." Cancer Microenviron 4(3): 261-81.
- Bullinger, L., K. Dohner, et al. (2004). "Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia." N Engl J Med 350(16): 1605-16.
- Butler, J. M., D. J. Nolan, et al. (2010). "Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells." Cell Stem Cell 6(3): 251-64.
- Calvani, N., P. Cafferio, et al. (2005). "Functional osteoclast-like transformation of cultured human myeloma cell lines." Br J Haematol 130(6): 926-38.
- Calvi, L. M., G. B. Adams, et al. (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature 425(6960): 841-6.
- Calvi, L. M., O. Bromberg, et al. (2012). "Osteoblastic expansion induced by parathyroid hormone receptor signaling in murine osteocytes is not sufficient to increase hematopoietic stem cells." Blood.
- Chen, A. T. and L. I. Zon (2009). "Zebrafish blood stem cells." J Cell Biochem 108(1): 35-42.
- Chen, E. H., E. Grote, et al. (2007). "Cell-cell fusion." FEBS Lett 581(11): 2181-93.
- Chen, M. J., Y. Li, et al. (2011). "Erythroid/Myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells." Cell Stem Cell 9(6): 541-52.
- Chintalacharuvu, S. R., J. X. Wang, et al. (2005). "An essential role for CCL3 in the development of collagen antibody-induced arthritis." Immunol Lett 100(2): 202-4.
- Chitteti, B. R., Y. H. Cheng, et al. (2010). "Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function." Blood 115(16): 3239-48.
- Cho, K. A., S. Y. Joo, et al. (2010). "Osteoclast activation by receptor activator of NF-kappaB ligand enhances the mobilization of hematopoietic progenitor cells from the bone marrow in acute injury." Int J Mol Med 26(4): 557-63.
- Choi, S. J., J. C. Cruz, et al. (2000). "Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma." Blood 96(2): 671-5.
- Choi, S. J., Y. Oba, et al. (2001). "Antisense inhibition of macrophage inflammatory protein 1-alpha blocks bone destruction in a model of myeloma bone disease." J Clin Invest 108(12): 1833-41.

- Chow, A., D. Lucas, et al. (2011). "Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche." J Exp Med 208(2): 261-71.
- Christopher, M. J., F. Liu, et al. (2009). "Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization." Blood 114(7): 1331-9.
- Christopher, M. J., M. Rao, et al. (2011). "Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice." J Exp Med 208(2): 251-60.
- Chute, J. P., G. G. Muramoto, et al. (2006). "Molecular profile and partial functional analysis of novel endothelial cell-derived growth factors that regulate hematopoiesis." Stem Cells 24(5): 1315-27.
- Cocks, K., D. Cohen, et al. (2007). "An international field study of the reliability and validity of a disease-specific questionnaire module (the QLQ-MY20) in assessing the quality of life of patients with multiple myeloma." Eur J Cancer 43(11): 1670-8.
- Colmone, A., M. Amorim, et al. (2008). "Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells." Science 322(5909): 1861-5.
- Cornelissen, J. J., W. L. van Putten, et al. (2007). "Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom?" Blood 109(9): 3658-66.
- Crofton, P. M., S. F. Ahmed, et al. (1998). "Effects of intensive chemotherapy on bone and collagen turnover and the growth hormone axis in children with acute lymphoblastic leukemia." J Clin Endocrinol Metab 83(9): 3121-9.
- Croucher, P. I., C. M. Shipman, et al. (2001). "Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma." Blood 98(13): 3534-40.
- Dahlberg, A., C. Delaney, et al. (2011). "Ex vivo expansion of human hematopoietic stem and progenitor cells." Blood 117(23): 6083-90.
- Dash, A. B., I. R. Williams, et al. (2002). "A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9." Proc Natl Acad Sci U S A 99(11): 7622-7.
- de la Mata, J., H. L. Uy, et al. (1995). "Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo." J Clin Invest 95(6): 2846-52.
- DiMascio, L., C. Voermans, et al. (2007). "Identification of adiponectin as a novel hemopoietic stem cell growth factor." J Immunol 178(6): 3511-20.

- Ding, L., T. L. Saunders, et al. (2012). "Endothelial and perivascular cells maintain haematopoietic stem cells." Nature 481(7382): 457-62.
- Dohner, H., E. H. Estey, et al. (2010). "Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet." Blood 115(3): 453-74.
- Duchayne, E., C. Demur, et al. (1999). "Diagnosis of acute basophilic leukemia." Leuk Lymphoma 32(3-4): 269-78.
- Ducy, P., R. Zhang, et al. (1997). "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation." Cell 89(5): 747-54.
- Eaves, C. J., J. D. Cashman, et al. (1993). "Unresponsiveness of primitive chronic myeloid leukemia cells to macrophage inflammatory protein 1 alpha, an inhibitor of primitive normal hematopoietic cells." Proc Natl Acad Sci U S A 90(24): 12015-9.
- Ehrlich, L. A., H. Y. Chung, et al. (2005). "IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma." Blood 106(4): 1407-14.
- Ek-Rylander, B., M. Flores, et al. (1994). "Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro." J Biol Chem 269(21): 14853-6.
- El-Badri, N. S., B. Y. Wang, et al. (1998). "Osteoblasts promote engraftment of allogeneic hematopoietic stem cells." Exp Hematol 26(2): 110-6.
- Ellis, S. L., J. Grassinger, et al. (2011). "The relationship between bone, hemopoietic stem cells, and vasculature." Blood 118(6): 1516-24.
- Farrugia, A. N., G. J. Atkins, et al. (2003). "Receptor activator of nuclear factor-kappaB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo." Cancer Res 63(17): 5438-45.
- Ferrajoli, A., M. Talpaz, et al. (1994). "Inhibition of acute myelogenous leukemia progenitor proliferation by macrophage inflammatory protein 1-alpha." Leukemia 8(5): 798-805.
- Ferreira, S. B., Jr., C. E. Repeke, et al. (2011). "CCR5 mediates pro-osteoclastic and osteoclastogenic leukocyte chemoattraction." J Dent Res 90(5): 632-7.
- Fleming, H. E., V. Janzen, et al. (2008). "Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo." Cell Stem Cell 2(3): 274-83.
- Frisch, B. J., R. L. Porter, et al. (2009). "In vivo prostaglandin E(2) treatment alters the bone marrow microenvironment and preferentially expands short-term hematopoietic stem cells." Blood 114(19): 4054-63.

- Fung, H. C., A. Stein, et al. (2003). "A long-term follow-up report on allogeneic stem cell transplantation for patients with primary refractory acute myelogenous leukemia: impact of cytogenetic characteristics on transplantation outcome." Biol Blood Marrow Transplant 9(12): 766-71.
- Girasole, G., G. Passeri, et al. (1994). "Interleukin-11: a new cytokine critical for osteoclast development." J Clin Invest 93(4): 1516-24.
- Giuliani, N., R. Bataille, et al. (2001). "Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment." Blood 98(13): 3527-33.
- Giuliani, N., S. Colla, et al. (2005). "Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation." Blood 106(7): 2472-83.
- Goessling, W., R. S. Allen, et al. (2011). "Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models." Cell Stem Cell 8(4): 445-58.
- Gratwohl, A., M. Stern, et al. (2009). "Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis." Cancer 115(20): 4715-26.
- Green, J. R., K. Muller, et al. (1994). "Preclinical pharmacology of CGP 42'446, a new, potent, heterocyclic bisphosphonate compound." J Bone Miner Res 9(5): 745-51.
- Guan, Y., B. Gerhard, et al. (2003). "Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML)." Blood 101(8): 3142-9.
- Guzman, M. L., X. Li, et al. (2007). "Rapid and selective death of leukemia stem and progenitor cells induced by the compound 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8)." Blood 110(13): 4436-44.
- Guzman, M. L., R. M. Rossi, et al. (2005). "The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells." Blood 105(11): 4163-9.
- Guzman, M. L., R. M. Rossi, et al. (2007). "An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells." Blood 110(13): 4427-35.
- Guzman, M. L., C. F. Swiderski, et al. (2002). "Preferential induction of apoptosis for primary human leukemic stem cells." Proc Natl Acad Sci U S A 99(25): 16220-5.
- Hadjidakis, D. J. and Androulakis, II (2006). "Bone remodeling." Ann N Y Acad Sci 1092: 385-96.
- Han, J. H., S. J. Choi, et al. (2001). "Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent

- of receptor activator of nuclear factor kappaB ligand." Blood 97(11): 3349-53.
- Hassane, D. C., M. L. Guzman, et al. (2008). "Discovery of agents that eradicate leukemia stem cells using an in silico screen of public gene expression data." Blood 111(12): 5654-62.
- Hauge, E. M., D. Qvesel, et al. (2001). "Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers." J Bone Miner Res 16(9): 1575-82.
- Hayer, S., G. Steiner, et al. (2005). "CD44 is a determinant of inflammatory bone loss." J Exp Med 201(6): 903-14.
- Haylock, D. N., B. Williams, et al. (2007). "Hemopoietic stem cells with higher hemopoietic potential reside at the bone marrow endosteum." Stem Cells 25(4): 1062-9.
- Hayman, A. R., S. J. Jones, et al. (1996). "Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis." Development 122(10): 3151-62.
- Heider, U., C. Langelotz, et al. (2003). "Expression of receptor activator of nuclear factor kappaB ligand on bone marrow plasma cells correlates with osteolytic bone disease in patients with multiple myeloma." Clin Cancer Res 9(4): 1436-40.
- Hideshima, T., P. L. Bergsagel, et al. (2004). "Advances in biology of multiple myeloma: clinical applications." Blood 104(3): 607-18.
- Hjertner, O., M. L. Torgersen, et al. (1999). "Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease." Blood 94(11): 3883-8.
- Hoffman, R. (2005). St. Louis, MO, Elsevier Churchill Livingstone.
- Hoggatt, J., P. Singh, et al. (2009). "Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation." Blood.
- Hooper, A. T., J. M. Butler, et al. (2009). "Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells." Cell Stem Cell 4(3): 263-74.
- Hoshino, A., T. Iimura, et al. (2010). "Deficiency of chemokine receptor CCR1 causes osteopenia due to impaired functions of osteoclasts and osteoblasts." J Biol Chem 285(37): 28826-37.
- Howlader N, N. A., Krapcho M, Neyman N, Aminou R, Waldron W, Alterkruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK. (2011). "SEER Cancer Statistics Review, 1975-2008." from http://seer.cancer.gov/csr/1975_2008/.
- Huiskes, R., R. Ruimerman, et al. (2000). "Effects of mechanical forces on maintenance and adaptation of form in trabecular bone." Nature 405(6787): 704-6.

- Ishii, M., K. Iwai, et al. (2006). "RANKL-induced expression of tetraspanin CD9 in lipid raft membrane microdomain is essential for cell fusion during osteoclastogenesis." *J Bone Miner Res* 21(6): 965-76.
- Ishikawa, F., S. Yoshida, et al. (2007). "Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region." *Nat Biotechnol* 25(11): 1315-21.
- Jakob, C., J. Sterz, et al. (2008). "Incorporation of the bone marker carboxy-terminal telopeptide of type-1 collagen improves prognostic information of the International Staging System in newly diagnosed symptomatic multiple myeloma." *Leukemia* 22(9): 1767-72.
- Jin, L., K. J. Hope, et al. (2006). "Targeting of CD44 eradicates human acute myeloid leukemic stem cells." *Nat Med* 12(10): 1167-74.
- Juliusson, G., P. Antunovic, et al. (2009). "Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry." *Blood* 113(18): 4179-87.
- Jung, Y., J. Wang, et al. (2007). "Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation." *Blood* 110(1): 82-90.
- Karanu, F. N., B. Murdoch, et al. (2000). "The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells." *J Exp Med* 192(9): 1365-72.
- Karanu, F. N., B. Murdoch, et al. (2001). "Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells." *Blood* 97(7): 1960-7.
- Karanu, F. N., L. Yuefei, et al. (2003). "Differential response of primitive human CD34- and CD34+ hematopoietic cells to the Notch ligand Jagged-1." *Leukemia* 17(7): 1366-74.
- Katayama, Y., M. Battista, et al. (2006). "Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow." *Cell* 124(2): 407-21.
- Kelly, P. N., A. Dakic, et al. (2007). "Tumor growth need not be driven by rare cancer stem cells." *Science* 317(5836): 337.
- Kern, W., T. Haferlach, et al. (2003). "Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial." *Blood* 101(1): 64-70.
- Kiel, M. J., O. H. Yilmaz, et al. (2005). "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." *Cell* 121(7): 1109-21.
- Killian, C. S., D. A. Corral, et al. (1993). "Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent

- TGF-beta and a proteolytic modulation of cell adhesion receptors." Biochem Biophys Res Commun 192(2): 940-7.
- Kobayashi, H., J. M. Butler, et al. (2010). "Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells." Nat Cell Biol 12(11): 1046-56.
- Kollet, O., A. Dar, et al. (2006). "Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells." Nat Med 12(6): 657-64.
- Konoplev, S., G. Z. Rassidakis, et al. (2007). "Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype." Cancer 109(6): 1152-6.
- Koreth, J., R. Schlenk, et al. (2009). "Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials." JAMA 301(22): 2349-61.
- Krause, D. S., K. Lazarides, et al. (2006). "Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells." Nat Med 12(10): 1175-80.
- Kukita, T., N. Wada, et al. (2004). "RANKL-induced DC-STAMP is essential for osteoclastogenesis." J Exp Med 200(7): 941-6.
- Kurihara, N., D. Bertolini, et al. (1990). "IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release." J Immunol 144(11): 4226-30.
- Lane, S. W., D. T. Scadden, et al. (2009). "The leukemic stem cell niche: current concepts and therapeutic opportunities." Blood 114(6): 1150-7.
- Lane, S. W., Y. J. Wang, et al. (2011). "Differential niche and Wnt requirements during acute myeloid leukemia progression." Blood 118(10): 2849-56.
- Lapidot, T., C. Sirard, et al. (1994). "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice." Nature 367(6464): 645-8.
- Lawal, R. A. and L. M. Calvi (2011). "The niche as a target for hematopoietic manipulation and regeneration." Tissue Eng Part B Rev 17(6): 415-22.
- Lee, K. S., H. J. Kim, et al. (2000). "Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12." Mol Cell Biol 20(23): 8783-92.
- Lentzsch, S., M. Gries, et al. (2003). "Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades

- mediating survival and proliferation in multiple myeloma (MM) cells." Blood 101(9): 3568-73.
- Levesque, J. P., J. Hendy, et al. (2003). "Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide." J Clin Invest 111(2): 187-96.
- Li, X., Y. Zhang, et al. (2005). "Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling." J Biol Chem 280(20): 19883-7.
- Lian, J. B. and G. S. Stein (1992). "Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation." Crit Rev Oral Biol Med 3(3): 269-305.
- Lichtman, M. A. (2000). "Interrupting the inhibition of normal hematopoiesis in myelogenous leukemia: a hypothetical approach to therapy." Stem Cells 18(5): 304-6.
- Lo Celso, C., H. E. Fleming, et al. (2009). "Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche." Nature 457(7225): 92-6.
- Ludwig, H. (2005). "Advances in biology and treatment of multiple myeloma." Ann Oncol 16 Suppl 2: ii106-12.
- Lundberg, P., C. Koskinen, et al. (2007). "Osteoclast formation is strongly reduced both in vivo and in vitro in the absence of CD47/SIRPalpha-interaction." Biochem Biophys Res Commun 352(2): 444-8.
- Lymperi, S., N. Horwood, et al. (2008). "Strontium can increase some osteoblasts without increasing hematopoietic stem cells." Blood 111(3): 1173-81.
- Mackie, E. J. (2003). "Osteoblasts: novel roles in orchestration of skeletal architecture." Int J Biochem Cell Biol 35(9): 1301-5.
- Maillard, I., U. Koch, et al. (2008). "Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells." Cell Stem Cell 2(4): 356-66.
- Majeti, R., M. W. Becker, et al. (2009). "Dysregulated gene expression networks in human acute myelogenous leukemia stem cells." Proc Natl Acad Sci U S A 106(9): 3396-401.
- Marusic, A., J. F. Kalinowski, et al. (1993). "Production of leukemia inhibitory factor mRNA and protein by malignant and immortalized bone cells." J Bone Miner Res 8(5): 617-24.
- Matsumoto, T. and M. Abe (2006). "Bone destruction in multiple myeloma." Ann N Y Acad Sci 1068: 319-26.
- Matsunaga, T., F. Fukai, et al. (2008). "Combination therapy of an anticancer drug with the FNIII14 peptide of fibronectin effectively overcomes cell adhesion-mediated drug resistance of acute myelogenous leukemia." Leukemia 22(2): 353-60.
- Matsunaga, T., N. Takemoto, et al. (2003). "Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal

- residual disease of acute myelogenous leukemia." Nat Med 9(9): 1158-65.
- Matsuo, K. and N. Irie (2008). "Osteoclast-osteoblast communication." Arch Biochem Biophys 473(2): 201-9.
- Mayack, S. R. and A. J. Wagers (2008). "Osteolineage niche cells initiate hematopoietic stem cell mobilization." Blood 112(3): 519-31.
- McDonald, D. F., B. H. Schofield, et al. (1983). "Direct bone resorbing activity of murine myeloma cells." Cancer Lett 19(2): 119-24.
- Mendez-Ferrer, S., D. Lucas, et al. (2008). "Haematopoietic stem cell release is regulated by circadian oscillations." Nature 452(7186): 442-7.
- Mendez-Ferrer, S., T. V. Michurina, et al. (2010). "Mesenchymal and haematopoietic stem cells form a unique bone marrow niche." Nature 466(7308): 829-34.
- Mensah, K. A., C. T. Ritchlin, et al. (2010). "RANKL induces heterogeneous DC-STAMP(lo) and DC-STAMP(hi) osteoclast precursors of which the DC-STAMP(lo) precursors are the master fusogens." J Cell Physiol 223(1): 76-83.
- Michallet, M., X. Thomas, et al. (2000). "Long-term outcome after allogeneic hematopoietic stem cell transplantation for advanced stage acute myeloblastic leukemia: a retrospective study of 379 patients reported to the Societe Francaise de Greffe de Moelle (SFGM)." Bone Marrow Transplant 26(11): 1157-63.
- Milner, L. A. and A. Bigas (1999). "Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation." Blood 93(8): 2431-48.
- Miyamoto, K., S. Yoshida, et al. (2011). "Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization." J Exp Med 208(11): 2175-81.
- Mohle, R., F. Bautz, et al. (1998). "The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1." Blood 91(12): 4523-30.
- Mohle, R., M. Schittenhelm, et al. (2000). "Functional response of leukaemic blasts to stromal cell-derived factor-1 correlates with preferential expression of the chemokine receptor CXCR4 in acute myelomonocytic and lymphoblastic leukaemia." Br J Haematol 110(3): 563-72.
- Morrissey, C., J. S. Lai, et al. (2010). "The expression of osteoclastogenesis-associated factors and osteoblast response to osteolytic prostate cancer cells." Prostate 70(4): 412-24.
- Mundy, G. R., L. G. Raisz, et al. (1974). "Evidence for the secretion of an osteoclast stimulating factor in myeloma." N Engl J Med 291(20): 1041-6.

- Naveiras, O., V. Nardi, et al. (2009). "Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment." Nature 460(7252): 259-63.
- Neering, S. J., T. Bushnell, et al. (2007). "Leukemia stem cells in a genetically defined murine model of blast-crisis CML." Blood 110(7): 2578-85.
- Negishi-Koga, T., M. Shinohara, et al. (2011). "Suppression of bone formation by osteoclastic expression of semaphorin 4D." Nat Med 17(11): 1473-80.
- Nelson, J. B., S. P. Hedican, et al. (1995). "Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate." Nat Med 1(9): 944-9.
- Nervi, B., P. Ramirez, et al. (2008). "Chemosensitization of AML following mobilization by the CXCR4 antagonist AMD3100." Blood.
- Nilsson, S. K., H. M. Johnston, et al. (2001). "Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches." Blood 97(8): 2293-9.
- Nilsson, S. K., H. M. Johnston, et al. (2005). "Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells." Blood 106(4): 1232-9.
- Ninomiya, M., A. Abe, et al. (2007). "Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice." Leukemia 21(1): 136-42.
- North, T. E., W. Goessling, et al. (2007). "Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis." Nature 447(7147): 1007-11.
- Ono, K., T. Akatsu, et al. (2002). "Involvement of cyclo-oxygenase-2 in osteoclast formation and bone destruction in bone metastasis of mammary carcinoma cell lines." J Bone Miner Res 17(5): 774-81.
- Oreffo, R. O., G. R. Mundy, et al. (1989). "Activation of the bone-derived latent TGF beta complex by isolated osteoclasts." Biochem Biophys Res Commun 158(3): 817-23.
- Oyajobi, B. O., G. Franchin, et al. (2003). "Dual effects of macrophage inflammatory protein-1alpha on osteolysis and tumor burden in the murine 5TGM1 model of myeloma bone disease." Blood 102(1): 311-9.
- Pearse, R. N., E. M. Sordillo, et al. (2001). "Multiple myeloma disrupts the TRANCE/ osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression." Proc Natl Acad Sci U S A 98(20): 11581-6.
- Pederson, L., B. Winding, et al. (1999). "Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity." Cancer Res 59(22): 5849-55.

- Peled, A., I. Petit, et al. (1999). "Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4." Science 283(5403): 845-8.
- Petit, I., M. Szyper-Kravitz, et al. (2002). "G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4." Nat Immunol 3(7): 687-94.
- Pettit, A. R., M. K. Chang, et al. (2008). "Osteal macrophages: a new twist on coupling during bone dynamics." Bone 43(6): 976-82.
- Ponomaryov, T., A. Peled, et al. (2000). "Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function." J Clin Invest 106(11): 1331-9.
- Powell, G. J., J. Southby, et al. (1991). "Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites." Cancer Res 51(11): 3059-61.
- Puri, M. C. and A. Bernstein (2003). "Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis." Proc Natl Acad Sci U S A 100(22): 12753-8.
- Qian, H., N. Buza-Vidas, et al. (2007). "Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells." Cell Stem Cell 1(6): 671-84.
- Qiang, Y. W., B. Barlogie, et al. (2008). "Dkk1-induced inhibition of Wnt signaling in osteoblast differentiation is an underlying mechanism of bone loss in multiple myeloma." Bone 42(4): 669-80.
- Qiang, Y. W., Y. Chen, et al. (2008). "Myeloma-derived Dickkopf-1 disrupts Wnt-regulated osteoprotegerin and RANKL production by osteoblasts: a potential mechanism underlying osteolytic bone lesions in multiple myeloma." Blood 112(1): 196-207.
- Quintana, E., M. Shackleton, et al. (2008). "Efficient tumour formation by single human melanoma cells." Nature 456(7222): 593-8.
- Raaijmakers, M. H., S. Mukherjee, et al. (2010). "Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia." Nature 464(7290): 852-7.
- Rabbani, S. A., J. Desjardins, et al. (1990). "An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells." Biochem Biophys Res Commun 173(3): 1058-64.
- Rasmussen, H. and P. Bordier (1973). "The cellular basis of metabolic bone disease." N Engl J Med 289(1): 25-32.
- Risteli, J., I. Elomaa, et al. (1993). "Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation." Clin Chem 39(4): 635-40.

- Roboz, G. J. (2011). "Novel approaches to the treatment of acute myeloid leukemia." Hematology Am Soc Hematol Educ Program 2011: 43-50.
- Romano, P. R., J. G. Caton, et al. (1997). "The reversal line may be a key modulator of osteoblast function: observations from an alveolar bone wound-healing model." J Periodontal Res 32(1 Pt 2): 143-7.
- Romas, E., N. Udagawa, et al. (1996). "The role of gp130-mediated signals in osteoclast development: regulation of interleukin 11 production by osteoblasts and distribution of its receptor in bone marrow cultures." J Exp Med 183(6): 2581-91.
- Rombouts, E. J., B. Pavic, et al. (2004). "Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia." Blood 104(2): 550-7.
- Roodman, G. D. (2004). "Mechanisms of bone metastasis." N Engl J Med 350(16): 1655-64.
- Sacchetti, B., A. Funari, et al. (2007). "Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment." Cell 131(2): 324-36.
- Sala, A. and R. D. Barr (2007). "Osteopenia and cancer in children and adolescents: the fragility of success." Cancer 109(7): 1420-31.
- Sato, M. M., A. Nakashima, et al. (2009). "Bone morphogenetic protein-2 enhances Wnt/beta-catenin signaling-induced osteoprotegerin expression." Genes Cells 14(2): 141-53.
- Schlenk, R. F., A. Benner, et al. (2003). "Risk-adapted postremission therapy in acute myeloid leukemia: results of the German multicenter AML HD93 treatment trial." Leukemia 17(8): 1521-8.
- Schmidt, T. and P. Carmeliet (2011). "Angiogenesis: a target in solid tumors, also in leukemia?" Hematology Am Soc Hematol Educ Program 2011: 1-8.
- Schofield, R. (1978). "The relationship between the spleen colony-forming cell and the haemopoietic stem cell." Blood Cells 4(1-2): 7-25.
- Semerad, C. L., M. J. Christopher, et al. (2005). "G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow." Blood 106(9): 3020-7.
- Sezer, O. (2009). "Myeloma bone disease: recent advances in biology, diagnosis, and treatment." Oncologist 14(3): 276-83.
- Sezer, O., U. Heider, et al. (2003). "RANK ligand and osteoprotegerin in myeloma bone disease." Blood 101(6): 2094-8.
- Sheu, T. J., E. M. Schwarz, et al. (2003). "A phage display technique identifies a novel regulator of cell differentiation." J Biol Chem 278(1): 438-43.
- Sheu, T. J., E. M. Schwarz, et al. (2002). "Use of a phage display technique to identify potential osteoblast binding sites within osteoclast lacunae." J Bone Miner Res 17(5): 915-22.

- Shono, Y., S. Ueha, et al. (2010). "Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation." Blood 115(26): 5401-11.
- Singhal, S., R. Powles, et al. (2002). "Allogeneic transplantation from HLA-matched sibling or partially HLA-mismatched related donors for primary refractory acute leukemia." Bone Marrow Transplant 29(4): 291-5.
- Sinigaglia, R., C. Gigante, et al. (2008). "Musculoskeletal manifestations in pediatric acute leukemia." J Pediatr Orthop 28(1): 20-8.
- Sipkins, D. A., X. Wei, et al. (2005). "In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment." Nature 435(7044): 969-73.
- Stier, S., Y. Ko, et al. (2005). "Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size." J Exp Med 201(11): 1781-91.
- Sugiyama, T., H. Kohara, et al. (2006). "Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches." Immunity 25(6): 977-88.
- Tabe, Y., L. Jin, et al. (2007). "Activation of integrin-linked kinase is a critical prosurvival pathway induced in leukemic cells by bone marrow-derived stromal cells." Cancer Res 67(2): 684-94.
- Tabibzadeh, S. S., D. Poubouridis, et al. (1989). "Interleukin-6 immunoreactivity in human tumors." Am J Pathol 135(3): 427-33.
- Taichman, R. S. and S. G. Emerson (1994). "Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor." J Exp Med 179(5): 1677-82.
- Taichman, R. S., M. J. Reilly, et al. (1996). "Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures." Blood 87(2): 518-24.
- Tavor, S., I. Petit, et al. (2004). "CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice." Cancer Res 64(8): 2817-24.
- Teitelbaum, S. L. (2000). "Bone resorption by osteoclasts." Science 289(5484): 1504-8.
- Terpstra, W., R. E. Ploemacher, et al. (1996). "Fluorouracil selectively spares acute myeloid leukemia cells with long-term growth abilities in immunodeficient mice and in culture." Blood 88(6): 1944-50.
- Udagawa, N., N. Takahashi, et al. (1990). "Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells." Proc Natl Acad Sci U S A 87(18): 7260-4.

- Vaananen, H. K., E. K. Karhukorpi, et al. (1990). "Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts." J Cell Biol 111(3): 1305-11.
- Valentin-Opran, A., S. A. Charhon, et al. (1982). "Quantitative histology of myeloma-induced bone changes." Br J Haematol 52(4): 601-10.
- Vallet, S., S. Pozzi, et al. (2011). "A novel role for CCL3 (MIP-1alpha) in myeloma-induced bone disease via osteocalcin downregulation and inhibition of osteoblast function." Leukemia 25(7): 1174-81.
- Vallet, S., S. Pozzi, et al. (2011). "A novel role for CCL3 (MIP-1alpha) in myeloma-induced bone disease via osteocalcin downregulation and inhibition of osteoblast function." Leukemia.
- Vallet, S., N. Raje, et al. (2007). "MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts." Blood 110(10): 3744-52.
- van Bezooijen, R. L., B. A. Roelen, et al. (2004). "Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist." J Exp Med 199(6): 805-14.
- Vardiman, J. W., N. L. Harris, et al. (2002). "The World Health Organization (WHO) classification of the myeloid neoplasms." Blood 100(7): 2292-302.
- Virchow R, C. F. (1860). Twenty lecutres delivered in the Pathological institute of Berlin during the months of February, March and April, 1858. New York, R. M. De Witt: 27-554.
- Visnjic, D., I. Kalajzic, et al. (2001). "Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice." J Bone Miner Res 16(12): 2222-31.
- Visnjic, D., Z. Kalajzic, et al. (2004). "Hematopoiesis is severely altered in mice with an induced osteoblast deficiency." Blood 103(9): 3258-64.
- Walkley, C. R., G. H. Olsen, et al. (2007). "A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency." Cell 129(6): 1097-110.
- Walkley, C. R., J. M. Shea, et al. (2007). "Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment." Cell 129(6): 1081-95.
- Weber, J. M. and L. M. Calvi (2010). "Notch signaling and the bone marrow hematopoietic stem cell niche." Bone 46(2): 281-5.
- Weber, J. M., S. R. Forsythe, et al. (2006). "Parathyroid hormone stimulates expression of the Notch ligand Jagged1 in osteoblastic cells." Bone 39(3): 485-93.
- Wellik, D. M. and M. R. Capecchi (2003). "Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton." Science 301(5631): 363-7.

- Wheatley, K., A. K. Burnett, et al. (1999). "A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties." Br J Haematol 107(1): 69-79.
- Winkler, I. G., N. A. Sims, et al. (2010). "Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs." Blood 116(23): 4815-28.
- Xie, Y., T. Yin, et al. (2009). "Detection of functional haematopoietic stem cell niche using real-time imaging." Nature 457(7225): 97-101.
- Yaccoby, S., W. Ling, et al. (2007). "Antibody-based inhibition of DKK1 suppresses tumor-induced bone resorption and multiple myeloma growth in vivo." Blood 109(5): 2106-11.
- Yagi, M., T. Miyamoto, et al. (2005). "DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells." J Exp Med 202(3): 345-51.
- Yamamoto, K., Y. Nakamura, et al. (2000). "Expression of the NUP98/HOXA9 fusion transcript in the blast crisis of Philadelphia chromosome-positive chronic myelogenous leukaemia with t(7;11)(p15;p15)." Br J Haematol 109(2): 423-6.
- Yamazaki, S., H. Ema, et al. (2011). "Nonmyelinating schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche." Cell 147(5): 1146-58.
- Yamazaki, S., A. Iwama, et al. (2009). "TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation." Blood 113(6): 1250-6.
- Yanada, M., K. Matsuo, et al. (2005). "Efficacy of allogeneic hematopoietic stem cell transplantation depends on cytogenetic risk for acute myeloid leukemia in first disease remission: a metaanalysis." Cancer 103(8): 1652-8.
- Yano, S., R. Mentaverri, et al. (2005). "Functional expression of beta-chemokine receptors in osteoblasts: role of regulated upon activation, normal T cell expressed and secreted (RANTES) in osteoblasts and regulation of its secretion by osteoblasts and osteoclasts." Endocrinology 146(5): 2324-35.
- Yates, J. W., H. J. Wallace, Jr., et al. (1973). "Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia." Cancer Chemother Rep 57(4): 485-8.
- Yi, B., P. J. Williams, et al. (2002). "Tumor-derived platelet-derived growth factor-BB plays a critical role in osteosclerotic bone metastasis in an animal model of human breast cancer." Cancer Res 62(3): 917-23.

- Yin, J. J., K. S. Mohammad, et al. (2003). "A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases." Proc Natl Acad Sci U S A 100(19): 10954-9.
- Yoshihara, H., F. Arai, et al. (2007). "Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche." Cell Stem Cell 1(6): 685-97.
- Zeng, Z., Y. X. Shi, et al. (2009). "Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML." Blood 113(24): 6215-24.
- Zhang, J., C. Niu, et al. (2003). "Identification of the haematopoietic stem cell niche and control of the niche size." Nature 425(6960): 836-41.