Implications and Regulation of Increasing Bone Marrow Fat in Age-related Bone Loss

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Dedication

This thesis is dedicated to my family-

My Parents- *Mr. Joseph Elbaz and*

Mrs. Irene Elbaz

My Brother-*Mr.Yannick Elbaz*

Abstract

Senile osteoporosis is associated with increasing levels of bone marrow fat. This accumulation of fat in the marrow cavity is a consequence of the predominant mesenchymal stem cell differentiation into the adipocyte fate at the expense of the osteoblast fate. Considering that these changes in stromal differentiation have a direct effect on bone health, we attempted to study bone marrow fat depots from a metabolic, lipotoxic and regulatory approach. In a fist attempt to study the potential metabolic role of bone marrow fat we observed the effect of calorie restriction (CR) on bone quality and marrow fat of aging rats subjected to a casein and soy protein diet. Bone quality and adipocyte quantification was obtained from rat tibia. Bone as well as adipogenic markers were quantified. CR was found to induce a significant decrease in bone quality. In contrast to CR rats, the *ad libitum* soy fed rats showed an overall better bone quality. Moreover, the results obtained showed that adipocytes were not mobilized during CR as no changes in leptin levels or adipocyte number were found. Finally we noticed that soy protein and not CR inhibited *PPARγ* expression, a transcription factor required for adipogenesis. In summary, results from this first approach showed that bone marrow fat does not participate in lipid metabolism during moderate stages of starvation and that the detrimental effect of CR on bone mass could be prevented using a soy protein regime. A second approach to this subject involved looking at the mechanism through bone and fat interact within the bone marrow. We hypothesized that bone marrow adipocytes' secretion of fatty acids (FA) induces changes in osteoblast differentiation, function and survival compatible with lipotoxicity. Using a co culture system of human pre-adipocytes and osteoblasts we showed that FAs negatively affect osteoblast differentiation and mineralization. Furthermore the effect could be prevented through the use of a FA syntase inhibitor, cerulenin. Gas chromatography/ mass spectrometry (GC\ MS) analysis of co-culture supernatants identified two predominantly present FA in the lipotoxic media: stearic and palmitic acid. These FA were shown to inhibit osteoblast differentiation and mineralization. Finally, osteoblasts survival was affected by the presence of adipocytes as per quantification of osteoblast survival using MTS-formazan. Osteoblast apoptosis, quantified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase 3/7 activation assays, was increased after exposure of osteoblasts to co-cultures and adipocyte media. In addition, direct exposure of osteoblasts to FA affected osteoblast survival through the induction of apoptosis. Results from this second set of experiments demonstrated that the paracrine release of FA within the bone marrow milieu could affect osteoblast differentiation, function and survival and therefore may contribute to age-related changes in bone mass. Finally the third approach elaborated on the fact that estrogens (E_2) regulate the differentiation of marrow precursors into osteoblasts. Using a model of young and old oophorectomized (OVX) mice, in this last approach we observed changes in marrow adipocytes, as well as changes in PPARγ and Sirt1 expression levels in bone marrow fat of young and old mice after OVX and treatment with either E_2 supplementation or vehicle alone. Results form this approach indicated that the absence of E_2 was associated with higher levels PPARγ and

lower levels of Sirt1, a known inhibitor of PPARγ and adipocyte differentiation. Moreover, old mice responded better to E_2 replacement in terms of reducing adipogenesis and PPARγ expression as well as increasing levels of Sirt1 expression. Our results suggest that the Estrogen regulation of age associated bone loss may be effected through a Sirt1 mediated mechanism.

In summary, in this thesis, we have provided evidence that demonstrates a predominant toxic role of marrow fat within the bone marrow milieu. In addition, we have found that marrow fat does not play a metabolic role in stages of moderate starvation. Finally, E_2 play an important role in the regulation of marrow adipogenesis, mainly in old bone. Taken together, our evidence has elucidated a new role of marrow fat in bone metabolism and could be the start point to therapeutic interventions that could target marrow fat as a potential anabolic treatment for osteoporosis in older persons.

Résumé

L'ostéoporose sénile est souvent associée à une augmentation de gras dans la moelle osseuse. Cette accumulation de gras est une conséquence d'une différentiation prédominante de cellules souches en adipocytes en lieu d'ostéoblastes. Prenant en considération ce changement de différentiation, nous avons décidé d'étudier le gras de la moelle osseuse de trois différentes approches : métabolique, lipo-toxique et régulatrice. Nous avons premièrement observé les effets d'une restriction calorique (RC) sur la qualité osseuse ainsi que sur le gras de la moelle osseuse de rats nourris de caséine ou de soja. Les marqueurs osseux et adipogéniques ont aussi été quantifiés. La RC fut identifiée comme une cause de réduction de qualité osseuse. Contrairement aux rats subjugués à une RC, les rats qui furent subjugués à une diète *« ad libitum »* de soja ont démontré une qualité osseuse supérieure. Aucuns changements n'ont été identifiés quant aux niveaux d'expression de leptin ou de cellules grasses. Ces derniers résultats indiquent que les cellules grasses de la moelle osseuse ne sont pas mobilisées pendant les périodes de RC. La diète de soja est responsable pour l'attenuation de l'expression de *PPARγ* .Les résultats de l'étude ont démontré en premier lieu que le gras de la moelle osseuse ne participe pas au métabolisme de lipides pendant les périodes de famines modérées ; puis en deuxième lieu que les effets de la RC sur les os peuvent être diminués par une diète de soja. En deuxième lieu, nous avons observé le mécanisme par lequel le gras de la moelle participe à la perte osseuse associé au vieillissement. Un modèle de co-cultures cellulaires de pré-adipocytes humains ainsi que d'ostéoblastes nous a permis de démontrer que les acides gras (AG) sécrétés par les adipocytes ont un effet inhibant sur la différentiation ainsi que sur la minéralisation des ostéoblastes. Aussi, nous avons observé que la présence d'adipocytes affectait négativement la survie d'ostéoblastes. Cette dernière information fut obtenue grâce à une quantification de MTS-formazan. Nous avons aussi mesuré l'apoptose d'ostéoblastes par la méthode TUNEL ainsi que par la quantification des caspases 3 et 7. Nous avons remarqué que les ostéoblastes exposés au milieu de co-cultures ainsi qu'à celui d'adipocytes étaient subjugués à plus de morts par l'apoptose. De plus, l'exposition directe des ostéoblastes aux AGs sélectionnés cause une apoptose générale d'ostéoblastes. Les résultats de cette deuxième vague d'expériences ont démontré que la sécrétion d'AGs dans la moelle osseuse peut affecter la différentiation d'ostéoblastes ainsi que leurs fonctionnalités et leurs survies. Ce mécanisme pourrait belle et bien participer aux changements osseux associés au vieillissement.

Nous avons aussi démontré que ces effets négatifs peuvent être prévenus à l'aide de la cerulenin, un inhibiteur de la synthéase d'acide gras. Les analyses chromatographique de gaz et spectrométrie de masse des super flottants de la co-culture ont démontrés que deux AG oxydés sont prédominants dans les milieux lipotoxiques : l'acide stéarique et l'acide palmitique. Ces derniers sont capables d'induire une augmentation dans les niveaux

d'activation de caspase 3\7 ainsi qu'une réduction dans les niveaux de différentiation et minéralisation d'ostéoblastes.

La troisième et dernière approche de notre étude se base sur la capacité qu'ont les estrogènes (E_2) à induire une différentiation ostéogéniques des cellules souches de la moelle osseuse. Utilisant un modèle de jeunes et vielles souris ovariectomisées (ovx), nous avons démontré que l'absence d'E_2 est associée à des niveaux élevés de PPARγ ainsi qu'à une baisse des niveaux d'expressions de Sirt1. De plus, les souris âgées répondent mieux à la supplémentassions d'E_2. Nos résultats démontrent que le contrôle de la perte osseuse associée au vieillissement est effectuée par un mécanisme où collaborent Sitr1 et E_2.

Dans cette Thèse, nous avons démontré des preuves supportant l'idée de la toxicité du gras de la moelle dans le milieu de la moelle osseuse. De plus nous avons démontré que lors des périodes de famines moyennes, le gras de la moelle ne participe pas aux réactions métaboliques. Finalement, nos résultats ont démontré qu'E_2 joue un rôle important dans le contrôle de la différentiation adipocytiques dans la moelle. Le gras de la moelle pourrait bel et bien permettre de nouvelles interventions thérapeutiques qui pourraient misées sur les aptitudes distinctes de ce gras afin de traiter l'ostéoporose chez les personnes âgées.

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Abbreviations

AC**:** *ad libitum*- and casein-fed

AS: *ad libitum*- and soy-fed

AIM: Adipogenic induction media

AMM: Adipogenic maintenance media

AKP: Alkaline phosphatise

AR: Alizarin Red

BMP: Bone Morphogenic Protein

BMPr: Bone Morphogenic Protein Receptors

Cbfa-1/RUNX2: Core binding factor alpha-1/ Runt-related transcription factor 2

CR: Caloric restriction

E2: Estrogens

FA: Fatty acids

IL: Interleukin

MAPK: Mitogen activated kinases

MSC: Mesenchymal stem cell

M-CSF: Macrophage Colony-Stimulating Factor

OB: Osteoblasts

OCN: Osteocalcin

OSX: Osterix

OPG: osteoprogerin

PPARγ2: Peroxisome proliferator activator receptor gamma 2

PUFA: Polyunsaturated Fatty acids

RANK-L: Receptor activator for nuclear factor κ B Ligand

RC: 40% CR and casein-fed

RS = 40% CR and soy-fed and compared with 2-month-old AC rats

Sirt1: Situin 1

TNF: Tumor necrosis factor

TUNEL: Terminal deoxynuclotidyl transferase d UTP nick end labelling

TZD: thiazolidinediones

Contribution of Authors

For the three manuscripts included in this thesis- Dr. Gustavo Duque was instrumental in supervising my research.

Manuscript-1: "*Effects of Long-Term Moderate Caloric Restriction and Dietary Protein Source on Bone and Marrow Fat of the Aging Rat"*

Animal colonies were developed and rats were treated at the Laboratory of Neuroendocrinology-University of Montreal (Drs. Pierrete Gaudreau and Guylaine Ferland). Dr. Duque and Mr. Daniel Rivas performed the bone phenotyping and metabolic studies. For the purposes of this project, I performed pathology stainings and cell biology experiments.

Manuscript-2: *"Inhibition of Fatty Acid Biosynthesis Prevents Adipocyte Lipotoxicity on Human Osteoblasts in Vitro*"

Published in the Journal of Cellular and Molecular Medicine

On this second project I performed all of the experiments except for the mass spectrometry which was performed at Metabolic Solutions in Nashua, New Hampshire. I also participated in the design of the project and data analysis.

Manuscript-3: "*Effect of Estrogens on Bone Marrow Adipogenesis and Sirt1 in Aging C57BL/6J Mice"*

Published in Biogerontology

The animal experiments were performed by Dr.Duque at the Royal Victoria Hospital (McGill University). I was provided the mice section and performed stainings, imaging and quantifications and participated on the design of the study.

Chapter 1. Introduction

The work presented connects the default adipocytic stem cell differentiation pathway to the biology of age-related bone loss focusing on the significance of age-related fat infiltration within the bone marrow milieu. The role of marrow fat in aging bone will be evaluated from its potential metabolic and toxic function and its role in the pathophysiology of osteoporosis, a disease characterized by weakened bone. A particular feature of osteoporosis in older subjects (senile osteoporosis) is the accumulation of fat in bone. We will look at this accumulation of fat as an inducer of changes in cell function and number within the bone marrow. To do so, we will look at its behavior, its function and its relevance in the aging process of bone. Overall, the publications and literature review of this Thesis will attempt to answer three specific questions regarding the relationship between fat and bone:

1. Does bone marrow fat participate in lipid metabolism?

2. Does bone marrow fat have a lipotoxic role?

3. What is the potential mechanistic link between aging and increasing bone marrow adipogenesis?

1.1. Osteoporosis as an Age Related Disease

Osteoporosis is a disease characterized by a low bone mass and a subsequent increase in the risk of fractures. Osteoporosis Canada states that the disease affects 1.4 million people and the number will only grow with the aging population. One in four women over the age of 50 has osteoporosis and one in 8 men over the age of 50 has the disease. It is estimated that the yearly

cost of treating patients with osteoporosis is around $1.3 billion in Canada alone (www.osteoporosis.ca). Therefore, further understanding the disease is crucial to the aging Canadian population as only more effective treatment of the disease will increase the quality of life of patients and alleviate the costs associated with the disease.

1.2. The Bone Marrow Milieu

The bone marrow is a spongy tissue located inside of long bones. It can further be divided into the myeloid tissue and bone marrow stroma. The myeloid tissue gives rise to the hematopoietic cell lineage whereas the stroma is composed of osteoblasts, fibroblasts, osteocytes, adipocytes and mesenchymal stem cells (MSCs). The hematopoietic stem cell of the myeloid tissue gives rise to blood cell types of the myeloid and lymphoid lineage. They give rise to osteoclasts which are derived from macrophages of the myeloid linage. The stroma is involved in the mesenchymal rather than hematopoietic differentiation (Alberts, B. 2002). Moreover, an imbalance in certain cellular levels in the marrow milieu may also be responsible for osteoporosis (Alberts, B. 2002). This later concept will be tested in this Thesis.

1.3. Mesenchymal Stem Cell (MSC)

The MSCs are pluripotent cells which can differentiate into a number of cell types and have the potential to replicate either in their differentiated or undifferentiated states (Valtieri and Sorrentino 2008) . Different culture conditions allow the in vitro differentiation of the MSC into the fate of choice (Pittenger, Mackay et al. 1999). A change in MSC replication and differentiation potentials seems to occur during the aging process (Zhou, Chen et al. 1999; Zhou, Greenberger et al. 2008). This change can be attributed to a number of factors of which include : changes in fate specific transcription and growth factors, cellular interactions, cytokines, adipokines and cell to cell signaling (Duque and Troen 2008)

1.4. Senile vs. Postmenopausal Osteoporosis

There are two types of primary osteoporosis: postmenopausal and senile osteoporosis. Postmenopausal osteoporosis, generally develops when estrogen levels decrease significantly. This process leads to an increase in bone resorption. Senile osteoporosis results when the processes of bone resorption and formation are no longer coordinated due to a decrease in osteoblast survival and differentiation (Chan and Duque, 2002); eventually bone breakdown prevails over bone building. In older women, both types often occur together (Cuccurullo, S. 2000). The projects performed by our research group deal mostly with senile osteoporosis which is not only marked by the deterioration of bone mass but is also marked by the accumulation of fat in bone .

1.5. Bone Marrow Fat: The Primary Motivator

Central to all of our studies is the age associated accumulation of fat in the bone marrow. Bone marrow fat content seems to increase with age. The work presented in this thesis pertains to this fat accumulation which does not seem to behave like other depots (Unger and Orci 2002). It appears to function in a way other than visceral and subcutaneous fat (Pei and Tontonoz 2004).

1.6. The Inverse Relationship

Multiple studies have demonstrated that there is in fact a direct interaction between fat and bone within the bone marrow milieu. Moreover, the fact that both the adipocyte and osteoblast cell lineages are derived from the MSC progenitor only brings more possibilities to that relationship. The dominant hypothesis in the field is that an inverse relationship exists between bone and fat in the marrow cavity such that adipocytes seen in the aging marrow exist at the expense of osteoblasts. (Gimble, Zvonic et al. 2006; Duque and Troen 2008)

1.7. Hypotheses

Considering that the mechanisms of increasing adipogenesis in aging bone as well as the mechanisms that explain this phenomenon remain partially elucidated, we hypothesized that:.

Hypothesis 1: In contrast to other types of fat (Unger and Orci 2002), (Picard and Guarente 2005), bone marrow fat does not participate in metabolism .

Hypothesis 2: Fatty acids (FA) secreted by marrow fat are toxic to osteoblast differentiation and function. These FA also affect osteoblast survival through the induction of osteoblast apoptosis.

Hypothesis 3: E_2 deprivation and accumulation of oxidative stress are responsible for the increasing levels of marrow adipogenesis in aging bone.

1.8. Thesis Outline

This thesis is organized as follows: The first and second chapter will introduce the research topic and the necessary concepts to understand the rationale behind our work as well as a review of the related literature. We will review concepts of bone turnover and the differentiation of the MSC, while considering and elaborating on the two hypotheses in the field regarding to the relationship of bone and fat. We will also review the major growth and transcription factors involved in MSC differentiation. Additionally we will look at the concepts relating to the apoptosis of bone cells, bone marrow fat and the regulation of hormones in the context of aging. We will finish chapter 2 by defining the rationale behind the projects performed.

The core of this thesis will be presented in chapters 3, 4 and 5 where three manuscripts will be presented. Finally, in chapter 6, we will summarize the results obtained in the three studies and answer the questions asked earlier in the introduction. Using the work we performed, we will further attempt to fill some of the gaps that still exist in the literature regarding the role and regulation of marrow fat.

1.9. Thesis contributions

This thesis is an assessment of the role and regulation of bone marrow fat. We have tested our hypotheses using both in vitro and in vivo models. Briefly, our results demonstrated that bone marrow fat does not participate in metabolism during moderate stages of starvation, that certain FA secreted by adipocytes are toxic to normal osteoblast function and further induce apoptosis, that this lipotoxic effect could be prevented by inhibiting FA synthesis and finally, that the regulatory effect of estrogen on bone marrow fat could be partially executed through a Sirt1-mediated mechanism.

The first study (Chapter 3), "*Calorie Restriction induces Changes in Bone and Fat of Aging Rats: Comparison of two Different Protein Regimes",* was performed to observe the effect of CR and soy based diets on bone quality and bone marrow fat quantity of aging rats. Using the results obtained from the differential diet and CR model, our study not only confirmed that the detrimental effects on bone quality that CR induces could be reverted on a soy-based diet, but also demonstrated that bone marrow fat is not affected by moderate stages of starvation.

The second study (chapter 4),"*Inhibition of Fatty Acid Biosynthesis Reverts Adipocyte Lipotoxicity on Human Osteoblasts In Vitro",* was performed to observe the effect of adipocyte secretion of FA on osteoblast differentiation, mineralization and survival. More importantly, we assessed whether this lipotoxic effect was reverted by adding an inhibitor of FA synthase into the media. Using a coculture model of human osteoblasts and adipocytes, we attempted to mimic the relationship between these two cell types in marrow milieu. Our results demonstrated the lipotoxic effect of adipocyte secretions on osteoblasts. Furthermore, the study showed that stereate and palmitate not only negatively affect the differentiation and mineralization of osteoblast but also induce osteoblast apoptosis. This toxic effect was reverted by cerulenin.

The third and final study (chapter 5),"*Effect of Estrogens on Bone Marrow Adipogenesis and Sirt1 in Aging C57BL/6J Mice",* was performed to determine the process through which the regulatory effect of E_2 on bone marrow adipogenesis in a young and old ovarectomized (OVX) mice is effected. Our results showed that E_2 replacement has a greater effect on the marrow adiposity of older mice than younger OVX mice. Furthermore, we found evidence that the regulatory effect of E_2 on marrow adiposity may be induced through a Sirt1 mediated mechanism.

Chapter 2. Background, Literature Review

2.1. Bone related processes: The process of bone turnover

Bone is a dense connective tissue, primarily composed of osseous tissue. The amount and quality of bone tissue is regulated by three different cell types namely osteoblasts, osteocytes, and osteoclasts. Osteoblasts are mononucleated bone-forming cells whereas osteocytes are formed from osteoblasts which have migrated to the bone matrix. Finally, the osteoclasts are the cells responsible for bone resorption (Alberts, B. 2002). Although rigid, bone is far from static. The dynamic nature of the bone is dictated by the remodeling process whereby bone resorption and formation occurs at the same site and allows for cortical and trabecular bones to be replaced as they are resorbed (Alberts, B. 2002). A balance between resorption and formation allows for the normal bone remodeling process, any deviation from this balance results in bone disorders

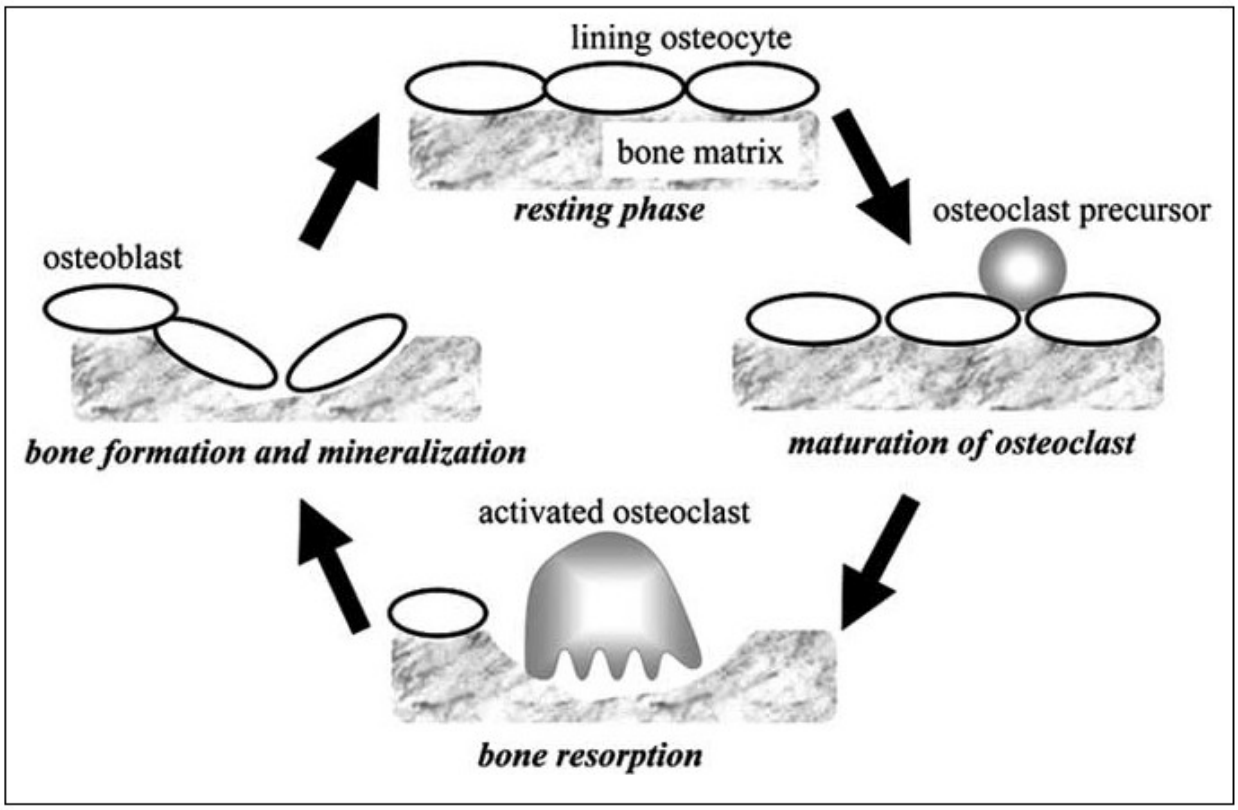


FIGURE 2.1 Bone Remodeling: Bone turnover, also known as bone remodeling involves both the osteoblasts and the osteoclasts. Osteoclasts eat away at the old bone at the same site were bone is formed by osteoblasts. The turnover process involves the resorption of the bone by the osteoclasts followed by reversal stage and finally the laying of the osteoid by osteoblasts and mineralization at the same site where it was originally destroyed.

(Tanaka, Nakayamada et al. 2005)

The bone forming activity is performed by the MSC-derived osteoblasts whereas the bone resorptive activity is performed by monocyte/macrophage derived cells, osteoclasts (Pei et al., 2008).The declining function of osteoblasts or the enhanced activity of osteoclasts causes osteoporosis (Kim, Ahn et al. 2008).The balance between both processes is lost after the 3rd decade of life due to an increased bone resorption and a lagging bone formation (Allan, Hausler et al. 2008). The remodeling process starts with the osteoclasts destroying the old bone. To do so, they work in a small group digging a tunnel through the old bone. At the same site where the old bone is destroyed; the osteoblasts secrete an organic matrix. This last process is known as the

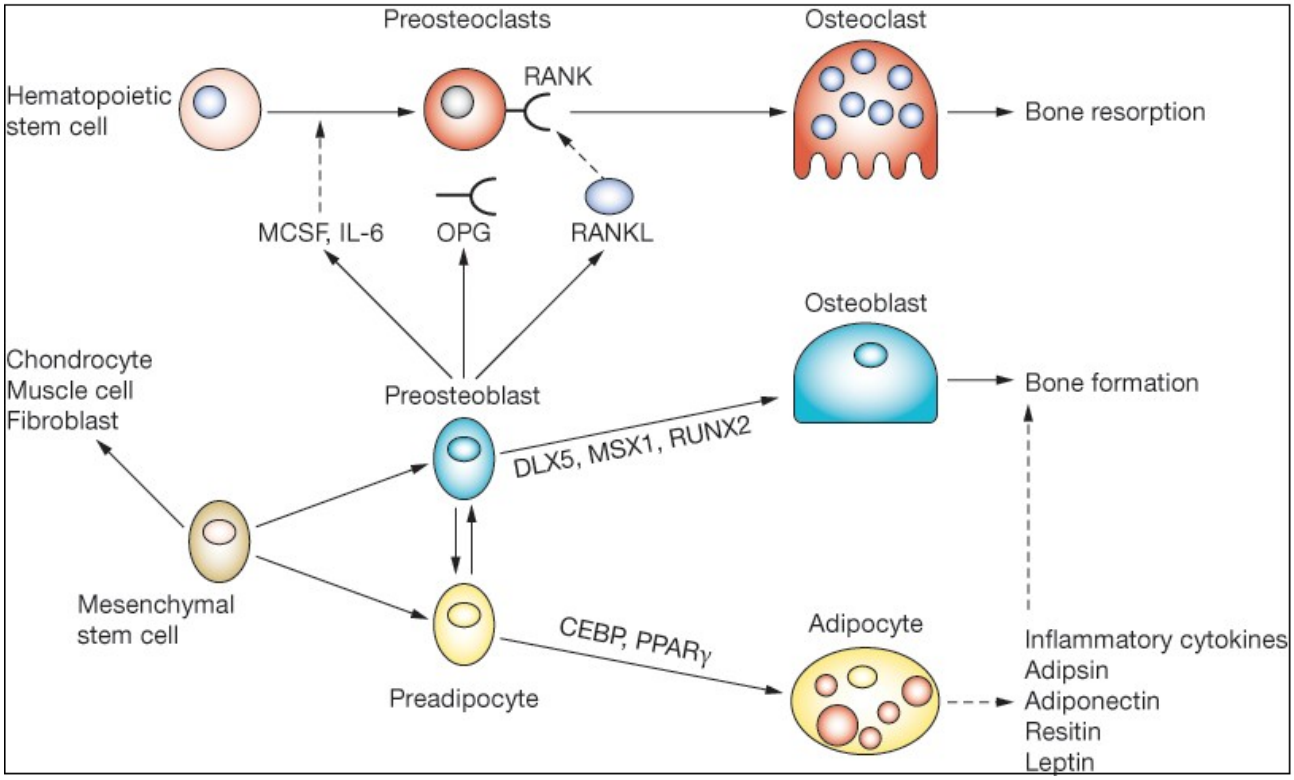
laying of the osteoid (un-mineralized bone). This matrix is eventually mineralized and new bone is formed at the site of resorption (Pei and Tontonoz 2004).

Moreover, the coordination of the bone turnover process requires two cytokines produced by the stromal cells and osteoblast; namely: macrophage colony-stimulating factor (M-CSF), and receptor activator of nuclear factor-kappa B ligand (RANK-L). Osteoclast precursors express the RANK receptor, which when bound to the osteoblast secreted RANK-L, stimulates the differentiation and activation of osteoclasts and inhibit osteoclast apoptosis. Finally, osteoprogerin (OPG), a decoy generated by osteoblasts, binds to RANKL and regulates osteoclastogenesis through regulation of the RANK-RANKL interaction. The process of bone turnover requires a balance in these regulatory cytokines to attain homeostasis. Any alterations in the number or function of MSCs or osteoblasts, as occurs in the aging bone, would affect the secretion of these cytokines and therefore the bone turnover process as a whole.

2.2 MSC: Osteoblastogenesis vs. Adipogenesis in the Aging Bone

Bone remodeling relies on the differentiation of the MSC into osteoblasts, an appropriate number of mature osteoblasts and an adequate osteoblast and osteocytes survival. As in other organs, bone marrow MSCs have the potential to either replicate in their undifferentiated state or into adipocytes, chondrocytes, myocytes, osteoblasts, and as described lately, beta pancreatic islets (Mao 2008). In vitro control of the mesenchymal fate is possible. Researchers have shown that the MSCs can be differentiated into either adipocytes or osteoblasts with the appropriate culture conditions (Pittenger, Mackay et al. 1999). Of special interest, when considering the accumulation of fat in the bone marrow are the osteogenic and adipogenic fates. The fulfillment of theses fates is dependant on the recruitment of MSC, the release growth factors, and the expression of fate specific transcription factors.

The transcription factor necessary for osteoblastogenesis is runt-related transcription factor 2 (Runx2). The most important adipogenic transcription factor is the peroxisome proliferator activated-receptor γ2 family of proteins PPARγ2. It is often coined the master regulator of adipogenesis as no factor has been found to induce normal adipogenesis in its absence (Muruganandan, Roman et al. 2008). Other relevant regulators of adipogenesis are the CCAAT-enhancer-binding proteins (C/EBPs), three of which (α, β, δ) have been found to be adipogenic promoters (Muruganandan, Roman et al. 2008).The most important growth factors concerning the adipogenic and osteogenic fates are the bone morphogenic proteins (BMP), the largest protein subfamily of the transforming growth factors beta superfamily, and the Leucine Zipper WNT proteins (Krishnan, Bryant et al. 2006), (Duque 2008).



(Rosen and Bouxsein 2006)

Figure 2.2 Osteoclastogenesis vs.Osteoblastogenesis vs Adipogenesis: Osteoclast are derived from the hematopoietic stem cell and require a number of factors secreted by preosteoblasts to mature.The differentiation of MSC into the osteoblast fate requires the activation of RunX2, TGFB and osterix. The activation of the adipocytic fate requires the expression of the of the PPARγ and CEBPα factors.

2.2.1. Age Associated Changes in MSCs

Senile osteoporosis is not only associated with a decline in osteoblast number but also with an increase in the number of adipocytes within the bone marrow. In the marrow cavity, the MSC seems to preferentially differentiate into adipocytes with increasing age (Duque, 2008). Basically, an age associated shift in commitment of the stromal cell seems to occur in the osteoporotic bone marrow. This shift could be due to an altered balance of transcription factors (Moerman, Teng et al. 2004).

Moerman et al. showed that factors necessary for the osteoblast fate were decreased in older mice marrows while those necessary for the adipogenic fate were increased. Furthermore, they examined the differentiation potential of the mouse MSCs and noticed that MSCs of older mice developed far fewer osteoblast colonies and far more adipocyte colonies than those of younger mice (Moerman, Teng et al. 2004). Not only is there a shift in differentiation but there also is a shift in replication. Zhou et al. demonstrated that the MSC's replication potential decreases with aging. Their work indicated that MSCs from older patients (mean 67+/- 8) had a doubling time 1.7 times greater than younger patients (mean 41+/- 10) (Zhou, Greenberger et al. 2008).

Additionally, stem cells, which usually combat the aging process by replenishing damaged cells, lose their ability to do so as they acquire age associated defects. It was originally thought that those bone marrow stem cell were protected from aging, however increasing evidence is suggesting otherwise. (Gross L 2007), (Fehrer and Lepperdinger 2005). With age MSCs in culture show not only replicative senescence but also appear quiescent. Not only do the aging MSCs loose multipotentiality, but they also express changes in telomere lengths and replication potential, all factors which render them less productive with age.

2.2.2.Cbfa1/Runx2:The Osteogenic Transcription Factor

The Cbfa1/Runx2 gene was originally cloned from fibroblasts and shown to be preferentially expressed in osteoblasts. It is part of the Runx family of proteins of which three have been identified in mammals: Runx1 (alternative nomenclature Cbfa2), required for hematopoiesis, Runx3 (Cbfa3) which controls neurogenesis in the dorsal root ganglia and cell proliferation in the gastric epithelium, and finally the one of interest,Runx2 (Coffman 2003). All of the Cbfa or Runx genes share the same alpha DNA binding domain. It is a conserved 128 amino acid domain, called the runt domain because of its homology to the Drosophila pair-rule gene, runt. The Cbfa or Runx genes function through that Runt DNA binding domain and can bind DNA as a either a monomer or with more stability a heterodimer (Otto, Thornell et al. 1997), (Ito 1996).

The Cbfa1/Runx2 transcription factor is expressed early on in the stem cell lineage and is essential for osteogenic maturation (Komori, Yagi et al. 1997). Cbfa1/RunX2 is essential for the differentiation of the MSC into the osteogenic fate. Moreover, mutations in the Cbfa1 gene were shown to have a detrimental effect on the skeletal development and bone formation in both animals and humans (Komori, Yagi et al. 1997). Komori et al. showed that Cbfa1/RunX2 -/- mice died just after birth with a complete lack of bone formation. Further studies were performed by the same group observing the effect of heterozygous mutations in Cbfa1, Cbfa/RunX2 +/- mice; the group reported incomplete skeletogenisis and severe impairement of osteogenesis, very similar to what is seen in human disorder, cleidocranial dysplasia which is due to a haploinsufficiency of the Cbfa1/RunX2 gene(Komori, Yagi et al. 1997) (Lee, Thirunavukkarasu et al. 1997).

2.2.3. PPARγ: The Aadipogenic Transcription Factor

The PPARγ nuclear transcription factor is part of the peroxisome proliferator activated receptor family of protein. PPARs function through heterodimerization with retinoid X receptor and regulate the expression of genes through the binding of the peroxisome proliferator hormone response elements (PPREs, response elements). Of interest, is the PPARγ family since the protein encoded by the PPARγ gene is a regulator of adipocyte differentiation. The γ subfamily can be further divided in 2 subgroups: γ1 & γ2. The γ2 subtype is specific to adipose tissue and is the one against which antibodies are used when trying to detect adipogenesis. The γ2 subtype is specific to adipose tissue whereas the γ1 subtype is expressed to a lesser extent in adipose tissue as well as in many other tissues. PPARγ2 has an additional 30 N-terminal amino acids relative to PPARγ1 (Napimoga, Vieira et al. 2008). Some debate exists regarding the role of each isoform in adipogenic differentiation, however it is established that both are expressed during the induction of adipogenesis (Muruganandan, Roman et al. 2008).

With age, MSCs which would normally differentiate into osteoblast in the bone marrow seem to preferentially express PPARy2 and differentiate into adipocytes (Duque, 2008). Moreover PPARγ2 has been proposed to play a major contributing role in osteoporosis; its ability to induce adipogenesis while inhibiting osteogenesis, may be responsible for the infiltration of fat in the bone marrow. The idea is based on findings that the expression of PPARγ2 has been found to regulate osteogenic development negatively while positively inducing adipogenic differentiation (Duque 2003). Lecka-Ceznick et al. previously showed that the activation of the PPARy2 transcription factor in pluripotent cells induced the adipocyte fate and blocked Cbfa1/Runx2 activation, inhibiting the osteoblast fate (Lecka-Czernik, Moerman et al. 2002). A similar effect was observed in osteoblastic cell lines where the ectopic expression of PPARγ2 suppressed the

osteogenic transcription factor, Cbfa1/RunX2 (Akune, Ohba et al. 2004), (Moerman, Teng et al. 2004). On the other side of the spectrum, PPARγ2 happloinsufficient mice were shown to have a lower number of marrow adipocytes as well as an increased bone mass (Akune, Ohba et al. 2004). Subsequent invitro studies of PPARγ-/- embryonic stem cells demonstrated their spontaneous differentiation into the osteogenic fate (Akune, Ohba et al. 2004). Moreover, rosiglitazone, a PPARγ activator, was also shown to suppress osteoblast differentiation in part through the down regulation of Cbfa1/RunX2. Ali, Weinstein et al reported that osteoblastic cells in the later stages of differentiation were resistant to the negative effect of rosiglitazone suggesting that the detrimental effect of PPARγ happens early on in osteoblast differentiation. They suggested that the suppression of differentiation of osteoblast progenitors could be involved in the relationship between PPARγ and osteoblasts (Ali, Weinstein et al. 2005) .

The involvement of PPARγ in osteoclastogenesis is becoming increasingly evident. It allows for a regulation of bone turnover not only through the inhibition of bone specific markers but also through the activation of osteoclastogenesis. Recent data in mice where PPARγ was deleted in osteoclasts but not in osteoblasts showed that PPARγ mutants developed osteopetrosis, a disease characterized by increased bone mass. These defects were attributed to an underactive osteoclast differentiation and an impaired receptor activator nuclear factor Kβ ligand signaling (Wan, Chong et al. 2007). Moreover thiazolidinediones (TZDs), a PPARγ agonists used in the treatment of type 2 diabetes, have been shown to cause bone loss in both mice and rats (Ali, Weinstein et al. 2005),(Li, Pan et al. 2006), (Wan, Chong et al. 2007). This effect was attributed in part to an overactive bone resorption.

Taken together, these findings suggests that PPARγ2 regulates the adipogenic, osteogenic and osteoclastogenic differentiation and should be considered an important drug target (Duque, 2003).

2.2.4. CEB/Ps

CEB/Ps are Leucine zipper transcription factor involved in promoting adipogenic differentiation. Six CEB/P isoforms exist; four of them (α,β,γ,δ) are expressed in adipocytes. The alpha isoform is considered a major adipogenic transcription factor. Following its induction, C/EBPα activates PPARγ and promotes adipogenesis. Both transcription factors have a synergic relationship and promote each other. Forced expression of C/EBPα in NIH 3T3 cells resulted in adipose conversion whereas the prevention of C/EBPα induction inhibited adipocyte differentiation. Moreover, the ectopic expression of C/EBPα promotes adipogenesis. Fibroblast lacking C/EBPα showed a decreased level of adipogenesis along with a decrese in PPARγ (Gimble, Zvonic et al. 2006).

2.2.5. The role of Growth Factors

The MSC differentiation into its desired fate is not only regulated by transcription factors but is also regulated in response to secreted growth factors. Maximal achievement of the differentiation fate is dependant on the release of fate specific growth factors. As mentioned earlier the two most important ones regarding the osteogenic/adipogenic debate are the bone morphogenic proteins (BMP) and the WNT proteins (Zhou, Greenberger et al. 2008) .

2.2.5.1 Bone Morphogenic Proteins (BMPs)

BMPs are growth factors secreted by osteoblasts that participate in bone and cartilage formation. Specific members of the BMP family are involved in osteoblast differentiation, fracture repair, skeleton formation, basal organ development, brain development and overall prenatal development. BMP-2, BMP-4, BMP-6, and BMP-7 are the most interesting to our topic as they are well known to participate in osteoblast differentiation (Muruganandan, Roman et al.

2008).The BMP mode of action starts with its to a to a type II Bone Morphogenic Protein receptor (BMPr), which recruits and phosphorylates the type I receptor. A sequence of events follows leading to the formation of a Smad complex and its subsequent translocation to the nucleus where it acts in conjunction with transcription factors (See Figure 2.3) (Muruganandan, Roman et al. 2008).

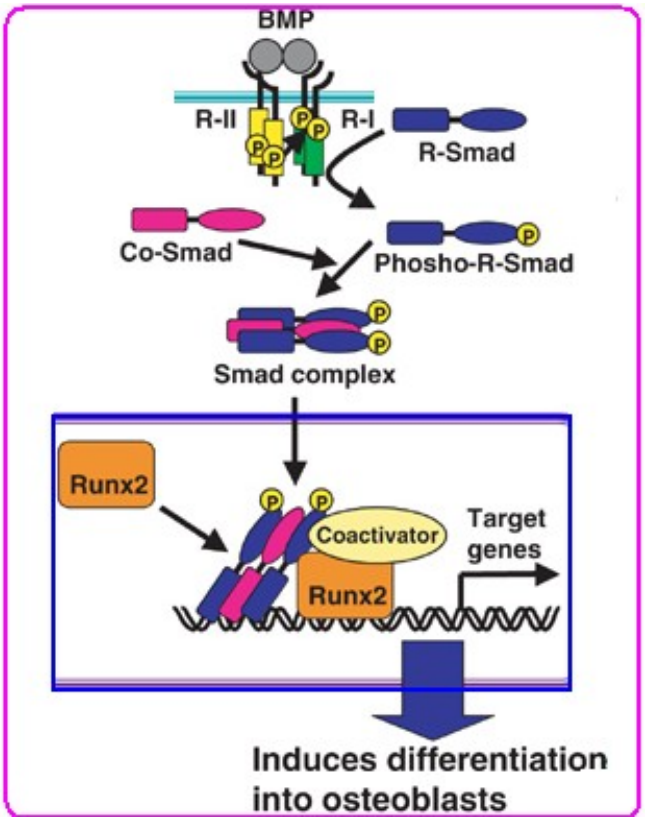


Figure 2.3: BMP/Smad/RUNX2 mode of action: The BMP/BMPR interaction leads to the recruitment of Smad proteins, their phosphorylation and complex formation. The complex translocates to the nucleus and acts in conjuction with a transcription factor on a target gene; in this case Runx2 and osteoblastogenesis. Adapted from (Miyazono, Maeda et al. 2004)

Another pathway which could occur after the BMP/BMPR interaction may be one which employs the p38 mitogen activated protein kinase (MAPK). Figure 2.4A below shows the two pathways mentioned with regard to the adipogenic differentiation. However, both the Smad mediated interaction and p38-MAPK could also lead to the transcriptional activation of Runx2 and subsequent osteogenic differentiation (Muruganandan, Roman et al. 2008) .

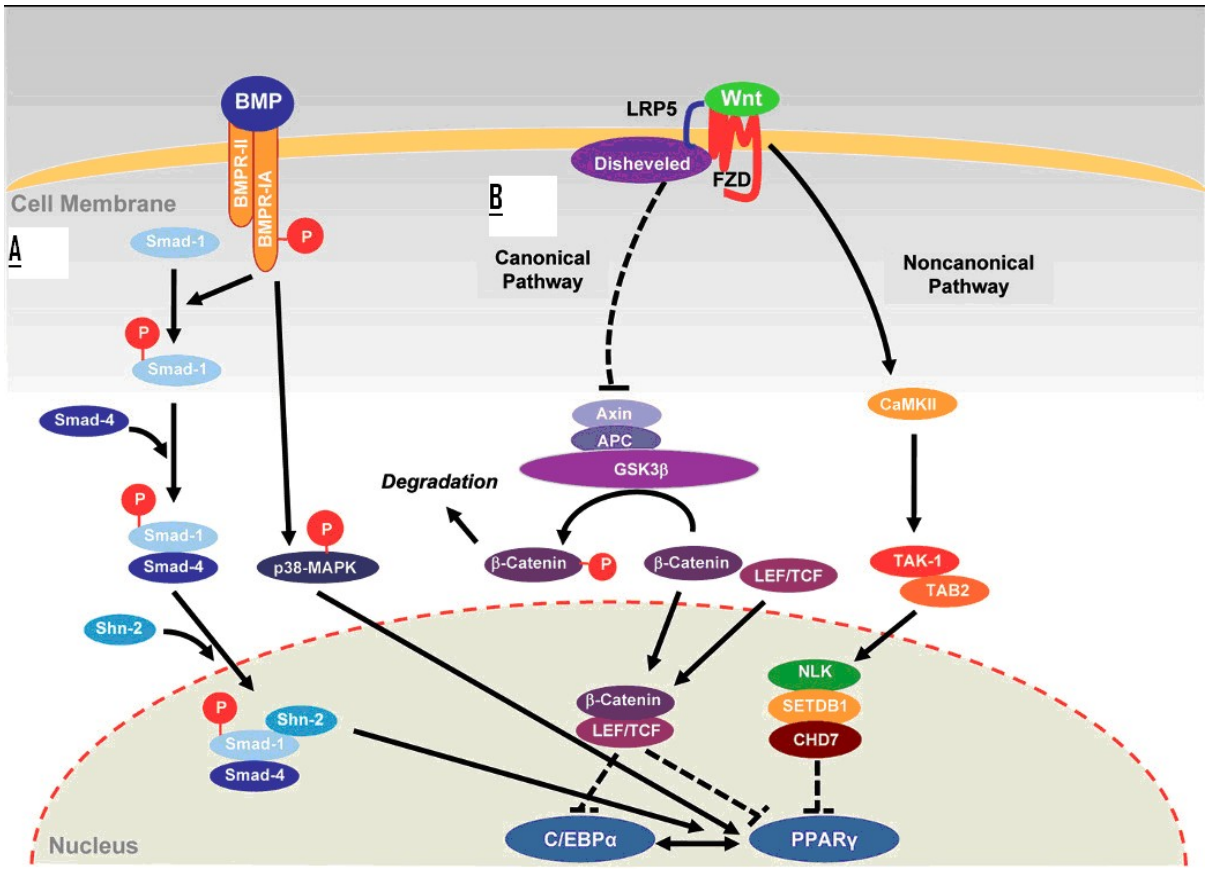


Figure 2.4: Wnt\BMP mode of action. (A)-Regulation of adipogenic differentiation by BMP mediated signaling Upon BMP/BMPRs interactions two pathway of regulation for the adipogenic differentiation are possible. The first involves the smad complexes; smad1 gets phosphorylated and forms a complex with smad4 which can act with Shn-2 and C/EBPα on the PPARγ receptor resulting on its concurrent activation, thereby promoting adipogenic differentiation. A similar transcriptional activation could be reached through the p38-MAPK mechanism. (B)- Inhibition of adipogenic differentiation by Wnt mediated signaling Regulation of adipogenic transcriptional factors (C/EBPa and PPARy) by Wnt signaling molecules occurs upon binding to FZDs which directs intracellular canonical and non-canonical signals. The canonical pathway enhan-ces the stability of B-catenin and represses PPARy ransactivation via the LEF/TCF pathway while the non-canonical Wnt signal activates CaMKII and MAPK kinase kinases such as TAK-1 and TAB2 cause a complex formation which represses PPARγ activation further maintaining preadipocytes in an undifferentiated state. (Muruganandan, Roman et al. 2008)

The concentration of BMPs present also appears to play a role in the determination of the fate expressed. Work by Wang, Israel et al. suggests a dose dependent relationship between BMP-2 and fate differentiation in C3H10T1/2 mouse embryonic stem cells; such that a high dose of BMP-2 is conducive to osteoblast and chondrocyte differentiation whereas lower doses of the growth factor are conducive to an adipogenic differentiation of the stem cell (Wang, Israel et al.

1993). .Also interesting, BMP signaling could target osteogenic differentiation through a Cbfa1 independent process. Recent work by Wang et al. suggests an osterix (OSX) mediated pathway which works independently of Cbfa1/RunX2 and is also initiated by BMP activated p38-MAPK (Wang, Goh et al. 2007), (Muruganandan, Roman et al.2008).

2.2.5.2 Closing the loop with the Wnt Signaling Pathway

Other relevant growth factors in osteogenic differentiation are the Wnt glycoproteins which act through two types of pathways: The canonical and non canonical pathways. The canonical pathway starts with the Wnt proteins binding to frizzled receptors and further directing a sequence of events to ultimately change the expression of genes relevant for cell growth and differentiation. Ultimately the activated canonical pathway leads to a build up of β-catenin in the cytosol which translocates to the nucleus and acts on cell growth. The non canonical pathway functions independently of β-catenin, through mediators illustrated in figure 2.4B. (Muruganandan, Roman et al. 2008).

Understanding that senile osteoporosis requires a balance between bone and fat in the marrow cavity makes the concept of blocking adipogenesis very relevant. Taking the concept of balancing adipogenesis and osteogenesis along with the idea that the disruption of β-catenin signaling causes spontaneous adipocyte conversion of cell types both in vivo and in vitro (Ross, Hemati et al. 2000; Bennett, Ross et al. 2002; Castro, Shin et al. 2004), further strengthens the idea that canonical Wnt signaling may indeed be a determinant factor of osteoblast versus adipocyte differentiation in bone marrow-derived MSCs (Muruganandan, Roman et al. 2008). Wnt molecules could also directly promote osteogenesis through a direct induction of Runx2 via a canonical pathway; this has been shown in both in vitro and in vivo models (Gaur, Lengner et al. 2005). Taken together, the presented literature suggests that β-catenin signaling not only inhibits adipogenesis (Figure 2.) but also promotes osteogenic differentiation (Gaur, Lengner et

al. 2005),(Muruganandan, Roman et al. 2008). Moreover, canonical signaling seems to promote osteogenic proliferation and differentiation as well as the renewal of stem cells (Reya and Clevers 2005),(Muruganandan, Roman et al. 2008),(Kato, Patel et al. 2002). Taking all the concepts presented regarding Wnt siganling, it is increasingly evident that the ability of Wnt signaling to both promote osteoblastogenesis and actively repress adipogenesis makes Wnt an important player in the maintenance of a balance between cell fates in the marrow milieu.

Transcription and growth factors work together such that a balanced MSC osteoblast and adipocyte differentiation can be reached as it is critical to the maintenance of healthy bone (Zhang, Yang et al. 2008). Better understanding of the mechanisms by which this balance is modulated could shine a new light on the treatment of senile osteoporosis.

2.3. Aging, Adiposity and Marrow Fat

Aging is associated with a redistribution of fat in the human body. Past middle age, fat depots which have reached their peaks decline and establish in other locations. Due to a loss of lean body mass which occurs with age, the percentage of body fat does not significantly change with age; however fat infiltrates new organs and tissues. Of these: bone marrow, muscle, and liver are commonly infiltrated by fat and are associated with age related disorders such as type 2 diabetes, hypertension, and atherosclerosis(Kirkland, Tchkonia et al. 2002; Cartwright, Tchkonia et al. 2007),(Barbagallo et al., 2001,and Okosun et al., 2001).

Fat infiltration of the liver observed in familial partial lipodystrophy is associated with the onset of diabetes. Fat infiltration of both the bone marrow and muscles could be attributed to the dysdiferentiation of MSCs into adipocyte like cells in non adipose tissue (Cartwright, Tchkonia et al. 2007). Moreover the age associated infiltration of muscle by fat is associated with sacropenia (the loss of muscle mass and strength with aging) as well as insulin resistance (Visser,

Goodpaster et al. 2005), whereas the accumulation of fat in bone is associated with lowered bone mass and osteoporosis. The diseases and conditions derived from the infiltration of organs and tissues by fat suggest the toxicity of its accumulation.

2.3.1: Depot Specificity and Changes in Adipogenesis

The pattern through which fat is lost is somewhat specific. Fat from subcutaneous stores is the first to be lost with age. It appears to be redistributed to visceral stores. Moreover, as fat cells are lost from one depot and established in new depots, they appear to behave differently (Cartwright, Tchkonia et al. 2007). Kirkland reported a similar effect as his group found that fat cells extracted from one depot and cultured behaved differently than those from other depots (Kirkland, Tchkonia et al. 2002). Even after numerous rounds of ex vivo population doubling, fat cells from particular depots appeared to express depots specific properties (Cartwright, Tchkonia et al. 2007). Furthermore, Duque et al. (unpublished data) in a proteomic profile comparing subcutaneous fat to bone marrow fat, reported that bone marrow adipocytes express higher levels of factors that have the potential to promote adipogenesis, decrease osteoblastogenesis and enhance the number of osteoclasts.than their subcutaneous counterparts. Taken together the results from both the Duque and the Kirkland studies suggest that fat from different locations express different toxicity levels.

Moreover, the shift from subcutaneous fat towards increased visceral fat results in increased ratios of central (intra-abdominal) to peripheral fat which is associated with insulin resistance, astherosclerosis and diabetes (Cnop et al., 2003; Kahn et al 2001) ; thereby predisposing the aging subject to metabolic dysfuntions (Despres, 2006; Lakka et al.,2002; Slawik and Vidal-Puig, 2006; Unger, 2005). This effect only strengthens the idea that fat depots at different locations have specific roles and exert different levels of toxicity.

The adipogenic differentiation also appears to change with aging. The expression of PPARγ and C/EBPα appears to be lower in preadipocytes from older than younger rats whereas the expression of anti adipogenic transcription factors seems to increase with age (Karagiannides et al., 2001, 2006). Fat depots which decline with aging would probably experience decreases in adipocyte specific transcription factors. Another important consideration involves the transdifferentiated mesenchymal cells earlier mentioned. Preadipocytes isolated from old rats do not appear to differentiate into mature fat cells; they express a partial adipocyte phenotype (Karagiannides et al.2001, 2006). The dysdifferentiated MSC progenitor cells which become preadipocyte appear to express a similar condition as they are not full adipocytes but rather mesenchymal adipocyte-like default cells (Kirkland et al., 2002).

Also, the ability of white adipose tissue to store lipids is altered with age. Usually white adipose tissue neutralizes free FA and converts them into triglycerides. The ability of white adipose tissue to do so declines with age and results in a lipotoxic environment for non adipose tissue leading to a metabolic syndrome (cluster of diseases including: diabetes, hypertension, dyslipidemia, atherosclerosis,visceral obesity) (Cartwright, Tchkonia et al. 2007).

Finally, new evidence looking at the behaviour of fat depots has suggested that adipocytes can function as paracrine and endocrine organs secreting adipocytokines such as leptin, adiponectine and tumor necrosis factors (Duque et al. unpublished data) with a potential deleterious effect on the cells in their vicinity.

In summary, studies regarding the redistribution of fat that occurs with age all point out the loss of fat from where it should be and the establishment of fat in locations where it should not. Depending on its location fat behaves differently. Moreover, Bone marrow fat appears to be especially toxic as it expresses elevated levels of toxic cytokines(namely the basic fibroblast growth factor, Resistin and Troy) and decreased levels of anti-adipogenic proteins (matrix

metalloproteinase 3) when compared to subcutaneous fat (Duque et al. unpublished data). Bone marrow fat is thus a specific fat depot and should be considered more toxic to non adipose systems than other depots.

2.4. Bone vs Fat: Interdependent Vs. Inverse

Two important hypotheses exist in the field regarding the relationship between adipogenesis and osteogenic in the marrow cavity: The inverse relationship between bone and fat and the interdependent relationship between bone and fat.

The first hypothesis concerning the relationship between the adipogenic and osteogenic fates was based on the fact that adipocytes and osteoblasts share a common mesenchymal origin (Beresford, Bennett et al. 1992), (Ducy, Zhang et al. 1997). Beresford, Bennett et al. proposed that the regulation of the adipocyte and osteoblast fate occurred at an early stage in the precursor cell (Beresford, Bennett et al. 1992). Moreover it was proposed that an inverse relationship exists between bone and fat in the marrow cavity such that MSCs are differentiated into adipocytes at the expense of osteoblast (Beresford, Bennett et al. 1992), (Ducy, Zhang et al. 1997) (Gimble, 2006). The concept later evolved into the idea that adipocytes may be the default state of the stromal differentiation (Kirkland 2002), (Chan and Duque 2002).The inverse hypothesis was an obvious suggestion as PPARy was shown to inhibit osteogenesis through the stimulation of the MSC differentiation into the adipogenic fate (Beresford, Bennett et al. 1992), (Ducy, Zhang et al. 1997). Other work in the field completed the inverse loop and showed that the opposite was also possible when looking at osteogenic induction and its concurrent inhibition of adipogenic differentiation (Gimble, Hua et al. 1995),(Gimble, Zvonic et al. 2006). Most of the evidence mentioned in earlier and later sections supports the inverse relationship.

The second hypothesis points towards an interdependent relationship between bone and fat in the marrow cavity (Gimble, Zvonic et al. 2006). This idea is based on the MSC plasticity, the ability

of these cells to transdifferentiate (Gimble, Zvonic et al. 2006). This concept challenges much of what is accepted in the inverse relationship hypothesis, moreover, it questions the use of cell specific markers as relevant evidence in functionality (Gimble, Zvonic et al. 2006). Groups supporting this hypothesis suggest that the relationship between the fates is not based on the MSC precursor but rather on transdifferentiation of differentiated cells. The idea of cell plasticity in the two cell types was shown earlier in Nutal et al.'s work where differentiated osteoblasts from the human trabecular bone underwent adipogenic differentiation under specific culture condition (Nuttall, Patton et al. 1998). The concept that the communication between adipocytes and osteoblasts occurs at differentiated or semi-differentiated state does not come without controversy.

Work performed by Akune et. al disproved the interdependent relationship of fates in the aging marrow. They showed that PPARγ deficiency induced embryonic stem cell (ES) differentiation into osteoblasts and that the reintroduction of PPARγ would lead to the adipogenic differentiation of ES (Akune, Ohba et al. 2004). Furthermore, the group also showed that $PPAR\gamma^{+/-}$ bone marrow cells developed more osteoblast colonies than the WT cultures, indicating an increase of osteoblastogenesis from bone marrow progenitors by PPARγ haploinsufficiency. These results indicated that the relationship between osteogenesis and adipogenesis occurred at the stromal level and was indeed inverse.

Along the lines of the interdependent argument; why would certain PPARγ ligands of thiazolidinedione family block osteoblast differentiation without inducing adipogenesis in murine cell lines (Lecka-Czernik, Moerman et al. 2002),(Lazarenko, Rzonca et al. 2006),(Gimble, Zvonic et al. 2006) ? This finding would suggest that relationship could not always be inverse. Finally, further complicating the story and negating the inverse relationship, recent work in diabetic mice showed that blocking PPARγ was not successful in increasing bone mass (Botolin and McCabe 2006).

Finding data supporting both arguments is not difficult. However, determining which is ultimately correct can not be done. Most of the literature points towards the inverse relationship but the interdependent relationship does fill some of the gaps left in the literature by the inverse hypothesis therefore both hypothesis are complementary.

2.5. Apoptosis in the aging bone

The shift from osteogenic differentiation to adipogenic differentiation in the marrow cavity has been presented here as being rooted in changes of both transcription and growth factors.

However, there is intriguing evidence suggesting that marrow fat may be responsible for the premature death of osteoblasts.

Apoptosis is a gene regulated organized cell death. It is perpetrated by activated **c**aspases, cysteine-aspartic acid proteases, which cleave their substrate at the post-aspartic-acid residue bonds. Cleavage of the proenzyme is required for the pro-cascapase to be active, become a caspase and further activate a cascade of caspases which eventually lead to a non inflammatory and organized cell death.

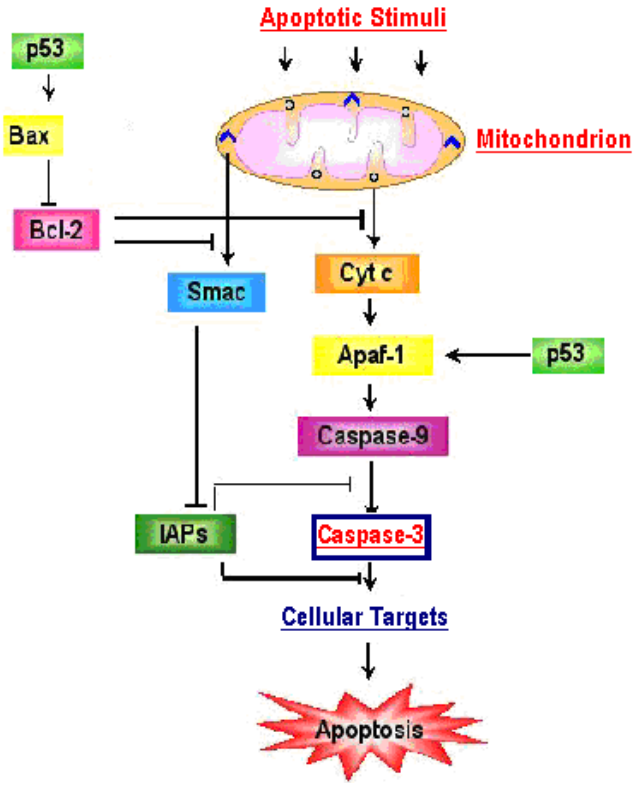


Figure 2.5. Apoptosis: Simplified version of the Apoptotic signaling pathway. Modified from: weizmann.ac.il/home/ligivol/apoptosis_project/apoptosis.jpg .

Apoptosis itself is not a product of aging; it occurs at every stage of life and is involved in the proper development of the embryo **(**Carrington 2005**).** It is for the most part a protective mechanism that allows our body to clear malignant cells. However, in the context of aging and disease, loss of cells in vital structures or organs leads to a decrease in function (Joaquin and Gollapudi 2001).In the context of bone turnover, increased loss of osteoblasts disrupts the balance of the turnover process, gives the opportunity for adipogenic differentiation of the stromal cell in the marrow cavity, and can ultimately lead to osteoporosis.

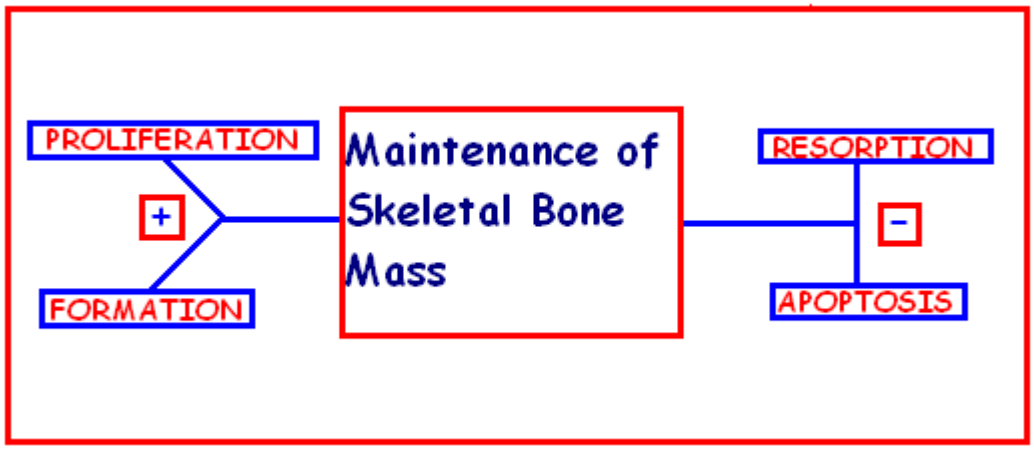


Figure 2.6: The Maintenance of Skeletal Bone Mass: The skeletal mass is maintained through a balance between proliferation, differentiation and apoptosis, resorption.

(Adapted from Weinstein and Manolagas 2000)

2.5.1 Oxidative stress, aging and apoptosis

Increased levels of oxidative stress have been shown to be associated with a lower bone density and reduced cell viability(Shouhed, Kha et al. 2005),(Sohal, Mockett et al. 2002). Additionally, it has been reported that low oxygen tension (pO2), which is related to elevated levels of oxidative stress, negatively affects osteogenic differentiation (D'Ippolito, Diabira et al. 2006). Regarding apoptosis, it has been proposed that free radicals cause damage to the mitochondria which

releases its usually internalized cytochrome C; when released it could activate the apoptotic pathway(Joaquin and Gollapudi 2001). This theory, however, has not been effectively proven; as apoptosis' regulation occurs through a balance of pro and anti apoptotic signals,free radical induced cytochrome C release could be negligible in the balance (Joaquin and Gollapudi 2001).

2.5.2 The Implications of Vitamin D: Fighting off apoptosis

The link between vitamin D and osteoporosis has been discussed at both a scientific and public level. Public agencies such as Osteoporosis Canada have promoted vitamin D as being a key factor in calcium absorption which is essential to the maintenance of healthy bones. In vivo work has shown the ability of vitamin D to stimulate bone formation as well as to increase the number of osteoblast precursor cells in intact rats (Ono, Watanabe et al. 1997). Vitamin D has been shown to be an inducer of osteogenic differentiation and of MSCs through the upregulation of Cbfa1/Runx2 and BMPs (Griffith, Yeung et al. 2006; Maehata, Takamizawa et al. 2006), (Sammons, Ahmed et al. 2004), (Duque, El Abdaimi et al. 2004).

Vitmain D was also shown to inhibit adipogenesis through inhibition of the Fas pathway and PPARγ2 (Mulholland, Dedhar et al. 2005),(Duque, Macoritto et al. 2004) . Moreover, its role in protecting osteoblast from an apoptotic death has been shown in a variety of cell lines and signaling pathways. Welsh J. showed that 1,25(OH)2D3, the active form of vitamin D is able to protect human MG-63 osteosarcoma cells from TNFα and ceramide induced apoptosis (Welsh J., 1997). Additional work by Duque et al. has demonstrated that 1,25 $(OH)_2D_3$ could function as a protector of Fas ligand mediated apoptosis through both mitochondrial and Fas related pathways (Duque, El Abdaimi et al. 2004). Duque et al. suggested that upon Fas ligand binding to the Fas receptor on the osteoblast, 1,25$(OH)_2D_3$ could mediate the inhibition of osteoblast apoptosis either somewhere in the cascade of caspases or at mitochondrial level (Duque, El Abdaimi et al. 2004)

2.5.3 Osteoblast Apoptosis in the "Fatty" Bone

An important consideration regarding apoptosis and the accumulation of fat in bone is the interdependency of cell types within the bone and the marrow cavity such that the increased apoptosis of a cell type will signal changes in other cell types (Carrington 2005). In practice this means that osteoblast apoptosis gives adipocytes a chance to proliferate where they normally would not. Taking the evidence that apoptosis becomes dysregulated with age (Joaquin and Gollapudi 2001); irregular osteoblast apoptosis may slow down the process not only directly but also indirectly by promoting the accumulation of fat in the marrow cavity. Weinstein and Manolagas have looked at the effect of osteoblast apoptosis on bone turnover and have proposed a causal relationship between the osteoblast apoptosis and the dysregulated turnover (Weinstein and Manolagas 2000). Better understanding the effect of adipocyte proliferation and differentiation on osteoblast survival could suggest new therapeutic approaches to the treatment of ostroporosis.

Duque et al demonstrated an increased osteoblast apoptosis in response to adipocyte secretions (Ch.4, Duque et al, unpublished data). If one were to consider apoptosis as an important and determinant factor in the aging bone, any insight regarding that process becomes relevant in our attempts to reduce bone loss associated with aging. The toxicity of the cytokines and fatty acids released by adipocytes, thereby remain at the center of our studies and will be discussed in the section pertaining to the lipotoxicity of bone marrow fat.

2.6. Bone Marrow Fat

2.6.1. Bone Marrow Fat: The Inducer of a Lipotoxic Disease

The consequences of the age-related fat infiltration in the bone marrow do not appear to be completely understood; however, similar fat infiltrations in other organs have been reported to have a detrimental effect. Newly arrived adipocytes in the pancreas affect beta cells' function and induce their death through lipoapoptosis (Unger and Orci 2002), (Duque 2008). As will be presented here, the lipotoxicity of bone marrow fat elaborates not only on the inhibitory effect of marrow fat on osteoblasts, but also on the idea of fat-induced apoptosis in the bone.

The increased bone marrow adipogenesis that comes with age leads to the secretion of factors which affect osteoblast differentiation and mineralization (Maurin, Chavassieux et al. 2002). Furthermore, in chapter 4, the concept of osteoblast apoptosis in the context of fatty acid release from adipocytes and their concurrent toxicity on osteoblasts will be discussed. The idea was extended from prior work in the field where the presence of adipocytes was shown to affect osteoblast proliferation in a model of co culture (Maurin, Chavassieux et al. 2000; Maurin, Chavassieux et al. 2002). The co culture model aims to mimic the relationship between adipocytes and osteoblast in the marrow cavity.

The lipotoxicity of marrow fat relies on the idea that adipokines and fatty acids secreted from adipocytes have a toxic effect on osteoblast in the marrow milieu (Maurin, Chavassieux et al. 2002). Adipocytes synthesize and secrete a number of factors and adipokines including leptin, tumor necrosis factor-α and interleukin-6. Much of the work performed regarding these adipokines suggest that these compounds are not responsible for the toxic effect of marrow fat on osteoblast (Maurin, Chavassieux et al. 2002). Taking a look at the non fatty acid adipocyte secretions: Leptin has been reported to activate osteogenic differentiation, TNF-α and IL-6 are known to stimulate osteoclast activity. More specifically, TNF-α has already been reported to

promote osteoblastic differentiation (Centrella, McCarthy et al. 1988); and, although IL-6 enhances the effects of other cytokines and hormones on bone resorption, it does not appear to have a direct effect on osteoblast proliferation and activity (Fried, Bunkin et al. 1998). On the other hand, groups have shown that the release of adipocytokines such as TNF-α could be responsible for the toxicity of the bone marrow fat (Unger and Orci 2002).

Not much is known about the toxicity of adipocyte-released factors on osteoblast in the marrow cavity, however the available data seems to also suggest a toxic effect mediated by fatty acids (Maurin, Chavassieux et al. 2000).

The nature and behaviour of fatty acids accumulated in the marrow cavity is however quite debatable. Lecka-Czernik *et al.* have reported increasing levels of oxidized FA in the aging bone marrow (Canalis, McCarthy et al. 1991; Lecka-Czernik, Moerman et al. 2002), whereas others have identified increasing levels of polyunsaturated fatty acids in the aging marrow (Maurin, Chavassieux et al. 2002).

However, not all FA have a negative effect on osteoblast proliferation. Some have been reported to promote bone health whereas others are known to negatively affect bone health (Coetzee, Haag et al. 2007), (Cornish, MacGibbon et al. 2008). A number of studies in the field of nutrition have demonstrated the beneficial effect of certain dietary fatty acids on bone health(Griel, Kris-Etherton et al. 2007),(Watkins, Li et al. 2000)

Much of the available literature suggests PUFAs (polyunsaturated FA) are specifically toxic to osteoblasts in the aging marrow. Maurin et al showed that two specific cis-n-3 PUFAs (arachidonic acid (AHA), docosahexanoic acid (DHA)) dose-dependently inhibited hOB cell and MG-63 cell proliferation (Maurin, Chavassieux et al. 2002). Additionally, eicosapentaenoic acid (EPA) has also been described as an inhibitor of cell proliferation(Coetzee, Haag et al. 2007). All three fatty acids were reported to not have an apoptotic effect on osteoblast, they seem

to stop cell cycle at G1 instead (Diascro, Vogel et al. 1998), (Coetzee, Haag et al. 2007), (Maurin, Chavassieux et al. 2002). Duque et al., in support to Lecka-Czernik et al's identification of oxidized fat in the aging marrow, have identified two saturated, oxidized FA, namely stearic and palmitic acid as negatively affecting osteoblast differentiation, proliferation and survival. Those saturated FA were identified as inducers of osteoblast apoptosis (Elbaz et al. unpublished data). Ideas surrounding the lipotoxicty of bone marrow fat are still at early stages; further work needs to be done in the field before the mechanism of action of fatty acid on osteoblast proliferation, differentiation and survival is properly identified.

2.6.2 Targeting Bone Marrow Fat

Targeting bone marrow fat in both the diagnosis and treatment of osteoporosis is not so far away. With the wealth of data that has been collected in the field, we are approaching the development of new treatments in the field of senile osteoporosis. Additionally, recent studies have shown an inverse relationship between marrow fat and bone marrow density (Verma, Rajaratnam et al. 2002). Those studies are suggesting the identification and quantification of bone marrow fat through non invasive magnetic resonance imaging in the diagnosis of osteoporosis (Duque 2008). In addition, evidence provided in this thesis suggest that lipotoxicity could be pharmacologically prevented which may constitute a new therapeutic approach to senile osteoporosis in the future.

2.7. Aging, Hormones and Osteoporosis

In terms of Postmenopausal osteoporosis, the protective function of estrogens (E_2) against bone resorption relies strongly on its regulation of osteoclasts (Mano, Hakeda et al. 2001) . Osteoclast progenitors respond to E_2 through an estrogen receptor and block the overactivity of osteoclasts. However, the age-related decline in estrogens deregulates the normal osteoclastic activity, allowing for osteoclastic overactivity, thereby promoting bone resorption (Shevde, Bendixen et al. 2000). E_2 are also known as regulators and promoters of osteoclast apoptosis. Once again, the declining levels of the hormone deregulate the process and allow for longer osteoclast survival rates, thereby increasing bone resorption. The regulation of bone resorption by estrogens is also done at an osteoblast level. E_2 decrease osteoblastic death by decreasing the production of cytokines such as interleukin (IL-1 and IL-6) and TNF-α from stromal cells. Finally, E_2 were also shown to increase stromal cell expression of osteogenic markers (Dang, van Bezooijen et al. 2002), (Nasu, Sugimoto et al. 2000).

The role of hormones on the regulation of geriatric osteoporosis is less documented. Prior work has shown that E2 treatment of stromal cells not only increased markers of osteogenic differentiation such as Cbfa1 and alkaline phosphatase but also decreased expression levels of PPARγ (Dang, van Bezooijen et al. 2002). Moreover, recent work has shown that E_2 deprivation enhances marrow fat infiltration. It was also demonstrated that the regulatory role of E_2 in the marrow allows for a reduction in adipocyte size and a prevention in increases of adipocyte numbers (Syed, Oursler et al. 2008).

Sirt 1, an NAD-dependent deacetylase, encodes for a member of the sirtuin family of proteins. It has recently been reported to be a suppressor of PPARy (Picard and Guarente 2005). Picard et al proposed that Sirt1 protein activates a critical component of calorie restriction in mammals. Upon food withdrawal, Sirt1 protein represses the genes that are controlled by PPARγ (Picard,

Kurtev et al. 2004). Sirt1's ability to suppress PPARγ was identified in both subcutaneous and visceral fat. In bone marrow stem cells, it was found to reduce adipocyte formation and promote osteoblast differentiation (Backesjo, Li et al. 2006).

Our group suggests that within the bone marrow, E_2 inhibition of adipogenesis could be done in a Sirt1 dependent manner. The novel protein just described may be the missing modulator in the regulation of adipogenesis.

2.7.1 Aging, Hormones and Oxidative Stress

E_2 deprivation involves not only an increased adipogenesis but also involves increases in oxidative stress. Fat is a factor of oxidative stress such that increased levels of fat lead to elevated levels of oxidative stress (Zhang, Dong et al. 2005). The association between adiposity and oxidative stress relies on the fact that fat produces elevated levels of reactive oxygen species (ROS) due to a overactive NADPH oxidase. The production of ROS combined with the reduction in antioxidant defense ecountered in fat direct increases in oxidative stress. Moreover, many parameters of oxidative stress are elevated during lipid oxidation (Zhang, Dong et al. 2005).

The involvement of fat in oxidative stress brings a new player in the regualtion of oxidative stress parameters: Sirt1. It has the ability to regulate adipogenesis and thus the synthesis of ROS. Moreover it was reported in mesangial cells to prevent oxidative stress induced apoptosis through P53 deacetylation (Kume, Haneda et al. 2006).

Estrogen could thus potentially signal the activation Sirt1 and prevent oxidative stress. In periods of estrogen deprivation Sirt1 would not be activated and adipogenesis as well as the synthesis of ROS would be increased. Finally, a concurrent reduction of the antioxidant defense would follow, ultimately leading to increases in oxidative stress

2.8. Rationale

Knowledge surrounding the infiltration of bone by fat is pointing towards a lipotoxic effect of adipogenesity on bone. The process of how this lipotoxicity is delivered is still in hypothetical stages. Moreover, the regulation of this fat is still unknown and although the behavior of bone marrow fat was identified as being different from that of visceral and subcutuaneous fat, the parameters that makes it different have not all been clearly defined.

In an attempt to fill some of the gaps in the literature, we propose that bone marrow fat does not participate in metabolism during moderate periods of stress but does secrete oxidized fat which have a toxic effect on normal osteoblast activity. Furthermore we suggest that E2 could mediate the accumulation of fat in the marrow milieu through a Sirt1 mediated mechanism.

Finally, although concepts regarding bone marrow fat have not all been clearly defined, our work along with the available data are suggesting that targeting marrow fat is the next step to take in the treatment of senile osteoporosis.

Chapter 3

Effects of Long-Term Moderate Caloric Restriction and Dietary Protein Source on Bone and Marrow Fat of the Aging Rat

This chapter is a reproduction of: ***"Effects of Long-Term Moderate Caloric Restriction and Dietary ProteinSource on Bone and Marrow Fat of the Aging Rat".***

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Summary

Contrary to its beneficial effect on other organs, caloric restriction (CR) has shown divergent effects on bone, which could be due to the protein regime utilized. Additionally, the effect of

CR on bone marrow fat, a common feature of age-related bone loss, remains unknown. In this study, the effect of moderate CR on bone quality and marrow fat of aging rats was assessed comparing two different protein regimes namely casein or soy protein. Male Sprague Dawley rats (8 month-old) were subjected for 12 months to 40% CR diet and compared to rats fed *ad libitum*. Tibiae were obtained for bone quality and adipocyte quantification. Additionally, serum levels of bone markers, calciotropic hormones and leptin were quantified. Finally, immunohistochemistry/immunofluorescence for adipogenesis transcription factors (leptin and PPARγ) as well as quantification of Sirt1, a marker of adipocyte function, were performed.There was a significant decrease in bone quality in the CR groups. In contrast, the group fed *ad libitum* and soy showed better bone quality and higher levels of bone formation. Furthermore, adipocytes were not mobilized upon CR as demonstrated by no changes in adipocyte number and local expression of leptin. In addition, soy protein but not CR had an inhibitory effect on PPARγ expression. Finally, CR induced higher levels of Sirt1 protein expression while decreasing Sirt1

mRNA. In summary, this study demonstrates that, independently of changes found in adipogenesis transcription factors, bone marrow adipocytes are not mobilized upon CR. Additionally; the negative effect of CR on bone mass was prevented using a soy protein regime.

3.1 Introduction

In addition to a loss of bone mass, senile osteoporosis is characterized by the accumulation of fat within the bone marrow at the expense of bone tissue (Gimble *et al*., 2006). Although changes in adipogenic factors that may explain this shift between fat and bone have been widely described (Bennett *et al*., 2005; Zhou *et al*., 2007), the role of marrow fat accumulation remains unclear (Gimble, 1990; Gimble and Nuttall, 2004). Caloric restriction (CR) has been shown to delay the development and the severity of age-related diseases in multiple organs and systems (Roth *et al*., 1999). Considering that senile osteoporosis is the consequence of the accumulation of fat at the expense of bone (Duque, 2007) and that CR reduces white fat mass in several tissues (Iwasaki *et al*., 1988; Linford *et al*., 2007), it might be expected to exert a preventive effect from the appearance of senile osteoporosis.

In fact, CR has been shown to decrease bone volume and induce structural changes of the trabecular bone of proximal tibiae (Tatsumi *et al*., 2007) and to reduce distal femoral bone mineral contents in rodents (Sanderson *et al*., 1997). Similar data were reported in humans (Radak, 2004; Villareal *et al*., 2006). This effect has recently been associated with the suppression of bone formation, in rats and mice tibiae (Tatsumi *et al*., 2007). However, differences in dietary protein sources, an important variable to consider when assessing bone cells function, may also play a role but has not yet been considered in this type of studies. In addition, the effect of CR and dietary protein source on bone marrow adipocytes remains unknown. Marrow fat has been proposed to differ from visceral and subcutaneous fact in several

aspects. It has been postulated that bone marrow adipocytes remain in an undifferentiated state known as mesenchymal adipocytic-default cells (Kirkland *et al*., 2002), which could be toxic to the cells in their vicinity (Maurin *et al*., 2000). In the particular case of CR, the identification of changes in bone marrow adiposity may help assessing whether or not bone marrow fat has a metabolic role, induces toxicity or simply occupies the space created by the age-related bone loss.

Previous studies on CR models have demonstrated that the potentially negative effects of CR could be prevented using different dietary protein sources. For instance, one study has shown that, in contrast to casein-fed animals, soy-fed animals showed longer survival and lower incidence of nephropathy (Iwasaki *et al*., 1988). Since soy protein has demonstrated a beneficial effect on bone by increasing bone mass due to its phytoestrogenic effect (Figard *et*

al., 2006), it is tempting to hypothesize that using soy protein as the main protein source could not only prevent the bone loss seen in CR animals but also have an effect on the potential changes in bone marrow fat induced by CR. The objective of the present study was therefore to investigate the effect of long-term moderate CR in combination with casein or soy dietary protein, on bone quality and marrow fat of aging male Sprague Dawley rats.We have found that, 1) the negative effect of CR on bone could be prevented using soy as the source of protein, and 2) unlike those of other adipose tissues and regardless of the protein source, bone marrow adipocytes are not mobilized upon CR.

3.2Experimental Methods

Animals and experimental procedures: Two-month-old male Sprague-Dawley rats (Charles Rivers Canada, St-Constant, QC) were individually housed in wire-bottom cages, in a room under controlled temperature (22°C), humidity and lighting (cycles of 12 h: lights on at 07:00 h, ambient light intensity of 45 Lux). The animals were fed chow diet containing 20% casein or soy, 20% sucrose, 29.5% corn starch, 14% maltodextrin, 5.5% corn oil, 5% cellulose, 3.5% AIN-93G vitamin mix, 0.3% methionine, 0.3% choline bitartrate and 0.001% tertiary butylhydroquinone (Harland, Teklad, Madison, WI). At 8 months, each group (casein- and soy-fed) was randomized in 2 groups: *ad libitum*-fed and 40% CR. Implementation at this age allows to avoid interference with the period of rapid growth. Rats were submitted to a 20% restriction for two weeks and to 40% thereafter. The regimen provided comparable amounts of mineral and vitamins when consumed at 60% of *ad libitum* feed diet. All animals had free access to water. Body weight (BW) and food intake were recorded every two weeks. Four groups (n=10 per group) of aged rats were studied: **AC =** *ad libitum*- and casein-fed, **AS** = *ad libitum*- and soy-fed, **RC** = 40% CR and casein-fed, **RS** = 40% CR and soy-fed. Aging rats were weighted regularly until sacrifice. They were sacrificed at 20 months of age along with 2-month-old *ad libitum* casein fed rats (n=10), between 08:30 and 10:30 h, by complete blood withdrawal *via* the abdominal aorta under pentobarbital anesthesia (5 mg/100g BW). Macroscopic evaluation indicated the absence of major pathology or tumor. The animal protocol was approved by the animal Care Committee of the University of Montreal in compliance with the guidelines of the Canadian Council on Animal Care. The aging rats were supplied by the Aging Rat Colonies platform from the Quebec Network for studies on aging (www.rqrv.com).

Tissue handling: All analyses were performed essentially as described previously (Richard et al., 2005). Briefly, 2 and 20-month old rats were sacrificed with CO2. Tibiae were fixed intact for 36 h in 4% paraformaldehyde (PFA), rinsed thoroughly in PBS, and processed for paraffin

embedding. Serial 4-µm sections were cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Richmond Hill, Ontario, Canada). Blood was also collected and sera were kept at -80°C for determination of serum concentrations of bone markers and hormone levels.

Von Kossa staining: After overnight fixation in 4% PFA and rinsing in PBS, the left tibia was embedded in Poly-methylmethacrylate (MMA) or a mixture of 50% MMA and 50% glycolmethacrylate (GMA). Serial 4- to 6-µm sections of MMA-embedded tissues were stained with von Kossa as previously described (Richard *et al*., 2005). Images were captured using a Leica DMR microscope (Leica Microsystems, Nussloch, Germany), equipped with a Retiga 1300 camera (Q imaging, Burnaby, British Columbia, Canada) and the primary histomorphometric data obtained using Bioquant Nova Prime image analysis software (Bioquant Image Analysis Corp, Nashville, TN, USA). Nomenclature and abbreviations conform to those recommended by the American Society for Bone and Mineral Research (Parfitt *et al*., 1987).

Serum biochemistry:Serum biochemistry was determined at the Rodent Diagnostics Lab (McGill University, Montréal, Quebec, Canada) using routine automated techniques as previously described (Duque *et al*., 2005). Commercial immunoassays were used to determine serum levels of osteocalcin (Immutopics, San Clemente, CA, USA), C-telopeptides (RatLaps ELISA, Nordic Bioscience, Herlev, Denmark), 17- estradiol (IBL Immuno-Biological, Hamburg, Germany), Parathyroid Hormone (PTH) (Immutopics, San Clemente, CA, USA), 25-hydroxyvitamin D (25OH-vitamin D) (Immunodiagnostics Systems, Fountain Hills, AZ, USA) and leptin (R & D Systems, Minneapolis, MI, USA).

Immunohistochemistry: Sections (4-µm) were mounted on silane-coated glass slides (Fischer Scientific, Springfield, NJ, USA). Paraffin was removed with three washes of xylene and rehydrated with washes of graded ethanol (80%–50%–30%) and PBS. Non-specific binding was blocked by addition of goat normal serum for 1 hour. Sections were then incubated with

antibodies against PPARγ Santa Cruz Biotechnology, Santa Cruz, CA, USA) for either 4 h at room temperature or 8–24 hours at 4°C. After washing with PBS, hydrogen peroxide complexed rabbit anti-mouse IgG were added to the sections at room temperature for 30 min, followed by a 30 min incubation with 0.6% hydrogen peroxide ± chromogen. Immunohistochemical staining was performed using the human ABC staining system (Santa Cruz Biotechnology). Positive cells showed a brown nucleus with punctate brown staining from the peroxidase-labelled antibody and blue ounterstaining from the haematoxylin.

Immnunofluorescence: Sections were treated as described above, omitting the final treatment with hydrogen peroxide. After fixation in 4% paraformaldehyde, sections were washed with PBS and then incubated in PBS with 10% blocking serum for 20 min to suppress non-specific binding of IgG. Sections were incubated with antibodies against PPAR_2, Sirt-1 and leptin (Santa Cruz Biotechnology) with 1.5% blocking serum overnight at 4oC and then incubated with fluorescein-conjugated secondary antibody (FITC-Santa Cruz, Santa Cruz, CA, USA) diluted to 2 □g/ml in PBS with 1.5% blocking serum for 45 min. Control slides were incubated with normal rabbit IgG according to manufacturer's instructions, and triplicate tests and control slides were included in immunodetection. Fluorescence was quantified using Image software (Image Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA).

Oil red O staining of bone marrow fat: Sections were deparafinized in different washes of xylene and ethanol as previously described (Duque *et al*., 2004). Oil red O (Sigma-Aldrich Canada LTD, Oackville, ON) was dissolved in propylene glycol, slowly, while stirring and heat to 100°C stirring constantly. The solution was filtered and stored in a 60°C oven. Sections were deparafinized and immersed in propylene glycol, two changes, 5 min each. Sections were then immersed in the oil red solution and kept at 60°C for 72 hours. Sections were then washed in 85% Propylene glycol for 3 min and counterstained with haematoxylin for 3 min. After washing with PBS, sections were mounted and images captured using a Leica DMR microscope (Leica

Microsystems) equipped with a Retiga 1300 camera (Qimaging, Burnaby, British Columbia, Canada). The number of adipocytes and fat volume quantification was performed by three different observers as previously described (Duque *et al*., 2004).

In Situ hybridization histochemistry: The protocol used was adapted from the technique described by Simmons *et al*. (Simmons *et al*., 1998). Briefly, bone sections were deparaffined, then fixed for 20 minutes in PFA (4%), digested for 25 min at 37°C with proteinase K (10 µg/ml), acetylated with acetic anhydride (0,25% in 0,1 M triethanolamine, pH8) and dehydrated through alcohol gradient (50, 70, 95 and 100%). Slides were then dried for the night, and then the antisense 35S labeled Sirt1 Crna probe diluted at 10 million cpm/ml in hybridization solution was spotted on the slides. The slides were sealed under a coverslip and incubated overnight at 60°C on a slide warmer. The next day, coverslips were removed and slides were rinsed four times in SSC 4X. The slides were digested for 30 min at 37°C with RNAse A (20µg/ml), washed through gradient of SSC (2X, 1X, 0,5X and 0,1X) and dehydrated through alcohol gradient. After drying, the slides were apposed to an autoradiography film for 72h. The slides were then defatted in toluene and dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA), exposed for 3 weeks and developed in D19 developer (Eastman Kodak) for 3,5 min at 14°C and fixed in Rapid Fixer (Eastman Kodak) for 5 minutes. The slides were rinsed in distilled running water for 1h, counterstained with eosin 0,05%, dehydrated through alcohol gradient, cleared in toluene and cover slipped with PDX.

Antisense 35S-labeled Sirt1 cRNA probe: The Sirt1 cRNA probe was generated by cloning the partial rat Sirt1 cDNA, obtained after PCR (upper primer: 5'-CCA AGG CCA CGG ATA GGT C- 3', lower primer: 5'-CAC AGC AAG GCG AGC ATA AAT- 3') in pGEM-T Easy vector (PROMEGA, Madison, WI), and linearized with *NotI* and *NCoI* (New England Biolabs, Ipswich, MA, USA) for antisense and sense probes, respectively. Radioactive riboprobes were synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, 10 mM dithiothreitol, 6 mM MgCl,

40 mM Tris (pH 7,9), 0,2 mM ATP/GTP/CTP, 35S-UTP, 40 U RNasin (Promega, Madison, WI), and 20 U of T7 and SP6 RNA polymerase for, respectively, antisense and sense probes of Sirt1 cRNA for 60 min at 37°C. The DNA templates were treated with 100 µl of DNAse solution (1 µl DNAse, 5 µl of 5 mg/ml tRNA, 94 µl of 10 mM Tris/10 mM MgCl2). The preparation of the riboprobes was accomplished the phenol-chloroform extraction and ammonium acetate precipitation. The specificity of the probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

Quantitative analysis of the hybridization signals: The hybridization signals revealed on NTB2 dipped nuclear emulsion-dipped slides were analyzed using a light microscope (Olympus, BX60) equipped with a black and white video camera (Diagnostic Instruments, RT slider model 2.3.0, Sterling Heights, MI, USA) coupled to a computer by using Image software (Image Pro Plus 6.0, MediaCybernetics). The optical density (OD) of the hybridization signal was measured under darkfield illumination at a magnification of 20X. The OD determination was performed on six different fields on both sections for each 4 animals in each group. The OD was corrected for the background signal. The OD values obtained were then averaged. For each given analyzed area of the bone section, the densitometric analyses were performed in all the groups without changing the light source of the microscope and the exposure time of the camera.

Statistical methods: Data are presented as mean ± SEM. Comparisons of bodyweight curves were done with one way ANOVA for repeated measures. Comparisons among the young and aged groups were made by ANOVAfollowed by Neuman-Keul's test to determine statistical differences between specific groups. Comparisons of the *ad libitum*-fed and CR rats with different protein sources were made by 2 x 2 ANOVA. In some cases comparisons between specific groups were made by Student's *t* test. In all experiments, a value of $P < 0.05$ was considered significant

3.3 Results

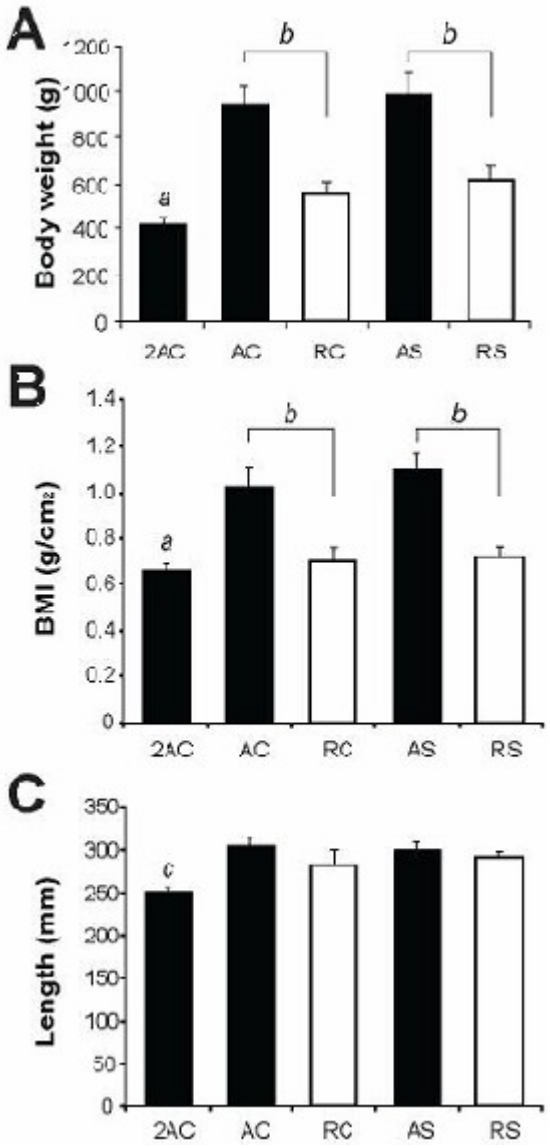

FIGURE 3.1: Effect of Long-term Moderate CR and Dietary Protein Source on Body Weight (A), Body Mass Index (BMI) (B) and total length (C) of aged rats.

Eight-month-old rats were exposed to 12 months of CR (40%) or fed *ad libitum* and received either casein or soy as protein source. All aged groups showed a significant increase in body weight (A) as compared with their 2-month-old controls (*a*, $p<0.001$). When compared with aged rats fed *ad libitum*, CR rats showed a significant reduction in body weight (*b,* $p < 0.001$). In the case of BMI (B), all aged groups showed a significant increase in BMI as compared with their 2-month-old controls (*a*, $p<0.01$). Additionally, aged CR rats showed a significant reduction in BMI as compared with their *ad libitum*-fed counterpart ($p <0.01$). Finally, rats' length (C) significantly increased in both CR and *ad libitum* fed groups as compared to 2-month-old controls (*c*, $p <0.01$) with non-significant difference in length seen between the CR and *ad libitum* aged rats.

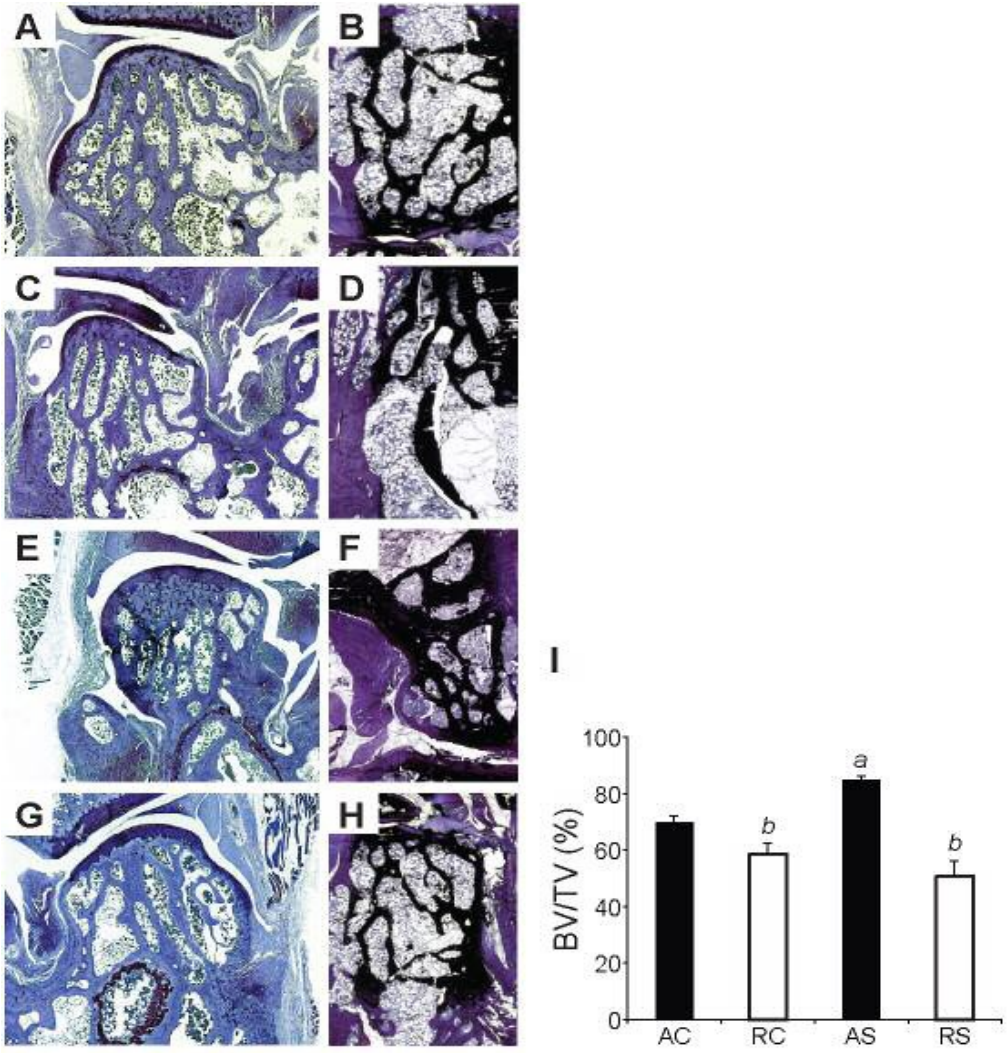

FIGURE 3.2: Effect of Long-term Moderate CR and Dietary Protein Source on Histologic Parameters of Undercalcified Tibiae of Aged Rats.

Sections of undecalcified tibiae were stained with toluidine blue to evaluate matrix (A,C,E and G, blue staining) and von Kossa to determine calcification (B,D,F and H, black staining). Four groups aged groups are presented: AC= *ad libitum* and casein fed (A and B), RC= 40% CR and casein-fed and (C and D), AS= *ad libitum* and soy-fed, (E and F) and 40% CR and soy-fed (G and H). Bone quality was assessed as the ratio between bone volume (BV) and trabecular volume (TV) and expressed in percentage (Fig. 2L). All 20-month-old rats showed a significant reduction in BV/TV as compared to the 2-month old *ad libitum* controls (considered as 100% BV/TV). Rats fed *ad libitum* receiving a soy protein regime (AS) showed a significantly lower decrease in BV/TV as compared to the other three groups (Fig. 2E, F and I) (*a*, $p<0.05$). Additionally, CR rats fed either with casein (C and D) or soy protein (G and H) showed a significant decrease in BV/TVin the proximal tibial epiphysis as compared to aged rats fed *ad libitum* (I) (*b*, $p<0.05$).Five sections per rat were analyzed. Three regions of interest (ROI) per section were quantified. Magnification at source was ×40. Micrographs are representative of four to six screened in each group of animals.

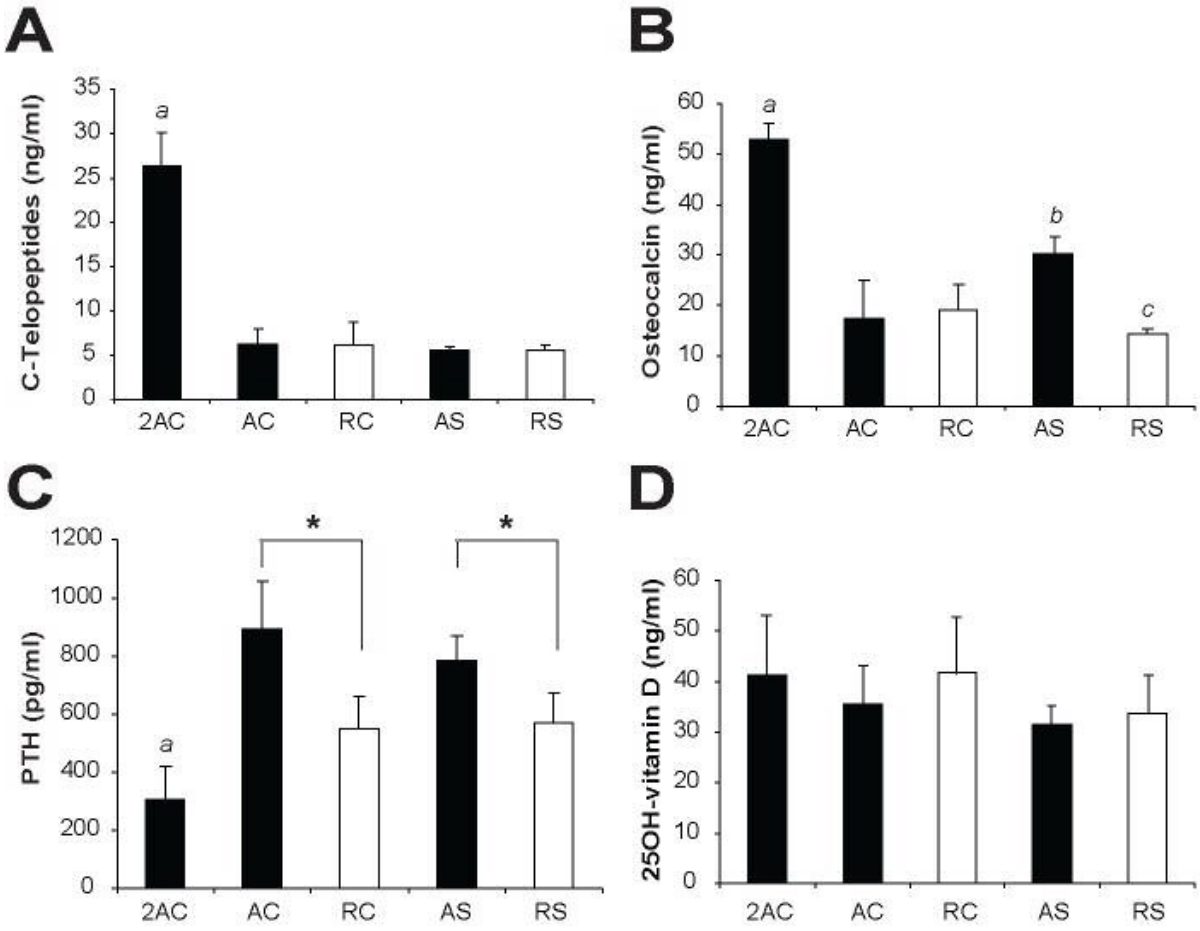

FIGURE 3.3: Changes in Serum Bone Biomarkers and Calciotropic Hormones in Aged Rats Fed Casein or Soy and Submitted or not to Long-term Moderate CR.

Serum immunoreactive C-telopeptides (A), osteocalcin (B), PTH(C), and 25-OH vitamin D (D).Values are means ± SEM. In panel A: 2-month-old controls had significantly higher levels of bone resorption (C-telopeptides) than all the other groups, (*a*, $p < 0.001$). In panel B: 2-monthold controls had significantly higher levels of bone formation (osteocalcin) than the aged rats (*a*, $p< 0.001$). The decrease in osteocalcin was significantly lower in the AS group as compared with the other aged groups (*b*, $p< 0.01$). Finally, the RS group showed the lower levels of bone formation as compared with all the other groups (*c*, $p<0.01$). In panel C: 2-month-old controls showed significantly lower PTH levels than all aged groups (*a*, $p<0.01$) whereas aged rats fed *ad libitum* showed higher levels of PTH than the CR groups (*$p<0.01$). In panel D: Nosignificant differences in serum levels of 25(OH) vitamin D were found.

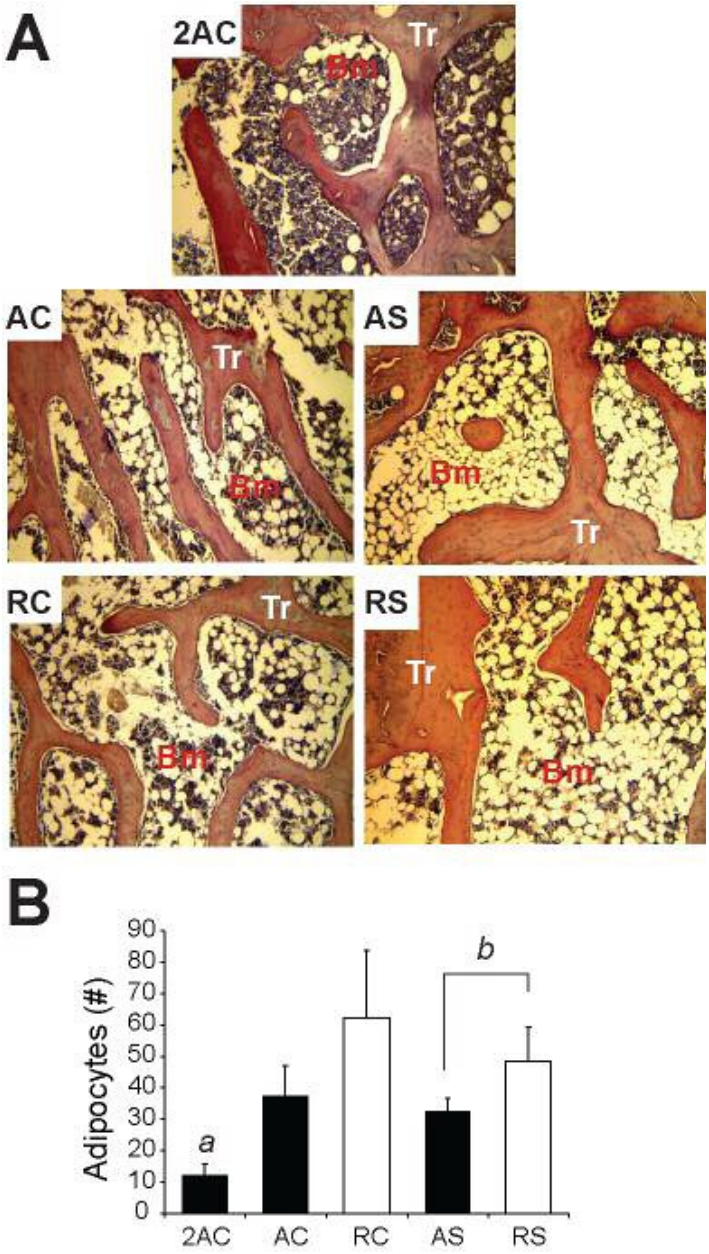

FIGURE 3.4: Quantification of Tibial Bone Marrow Adipocytes in CR Rats receiving either Casein or Soy and Submitted or not to Long-term Moderate CR.

(A) Longitudinal sections of proximal tibiae from 2-month-old rats fed *ad libitum* (2AC) and 20-month-old rats fed *ad libitum* or CR receiving either casein or soy as protein sources. Sections were stained with oil red O for adipose tissue. Haematoxylin was used as counterstaining. Adipocytes stained strong red with blue nuclei while all other bone marrow cells were stained light blue by haematoxylin. Adipocyte number (B) was quantified by three different observers looking at ten different fields per section. A marked increase in adipocyte number was seen in all aged groups as compared to their 2-month-old controls (*a*, $p< 0.01$). Additionally, the AS rats showed a significantly lower amount of adipocytes per field (*b*, $p<0.05$) as compared with all the other aged groups. Note that most of the bone marrow (Bm) space is occupied by fat (empty spaces) in all aged groups. Tr=bone trabeculae. Magnification at source was ×40. Micrographs are representative of four to six screened in each group of animals.

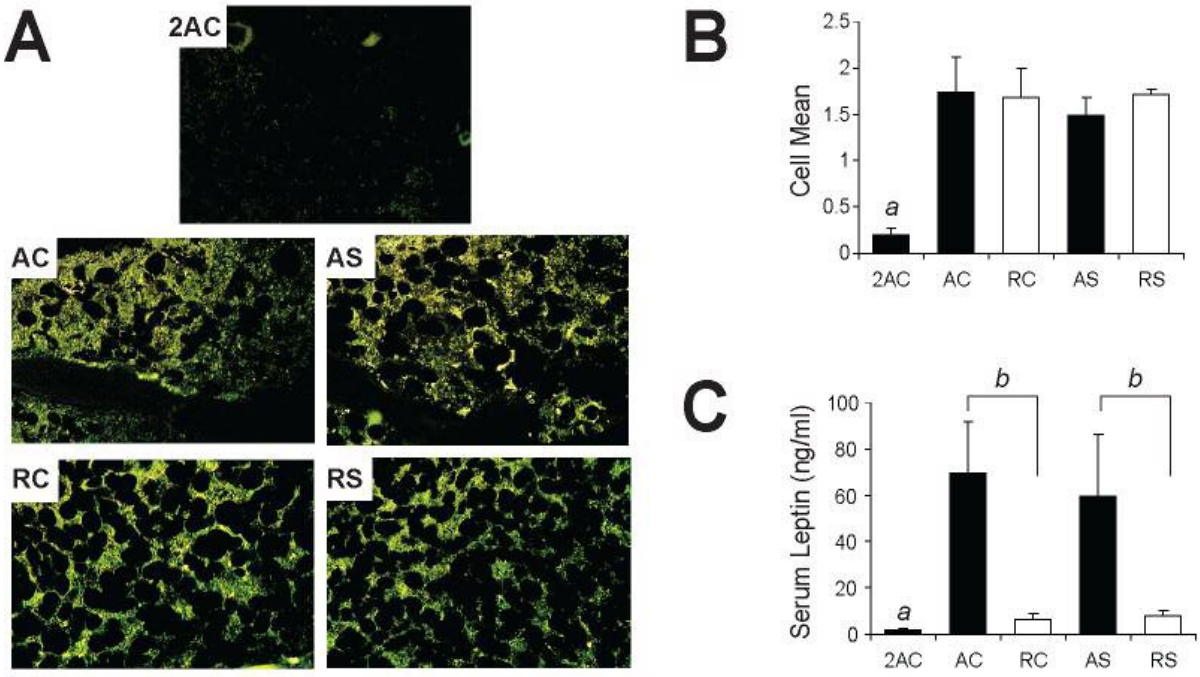

FIGURE 3.5: Comparison between Leptin Expression within the Tibial Bone Marrow and Serum Levels of Leptin CR in Aged Rats Fed Casein or Soy and Submitted or not to Long-term Moderate CR.

(A) Immunofluorescence analysis of leptin mRNA levels withinthe bone marrow in 2-month-old (2AC) and 20-month-old rats fed ad libitum or CR, receiving either casein or soy protein as protein sources. A marked age-related increase in leptin expression/marrow area was observed in all aged groups. Furthermore, quantification of leptin expression/marrow area did not show any difference between the aged groups (B).In contrast, (C)serum were significantly higher in all the aged groups, both *ad libitum*-fed and CR, when compared to the 2-month old controls (*a*, $p< 0.05$). Additionally, CR significantly decreased serum levels of leptin when compared to aged rats fed *ad libitum* independently of the protein source (*b*, $p< 0.001$). Magnification at source was ×40. Micrographs are representative of four to six screened in each group of animals.

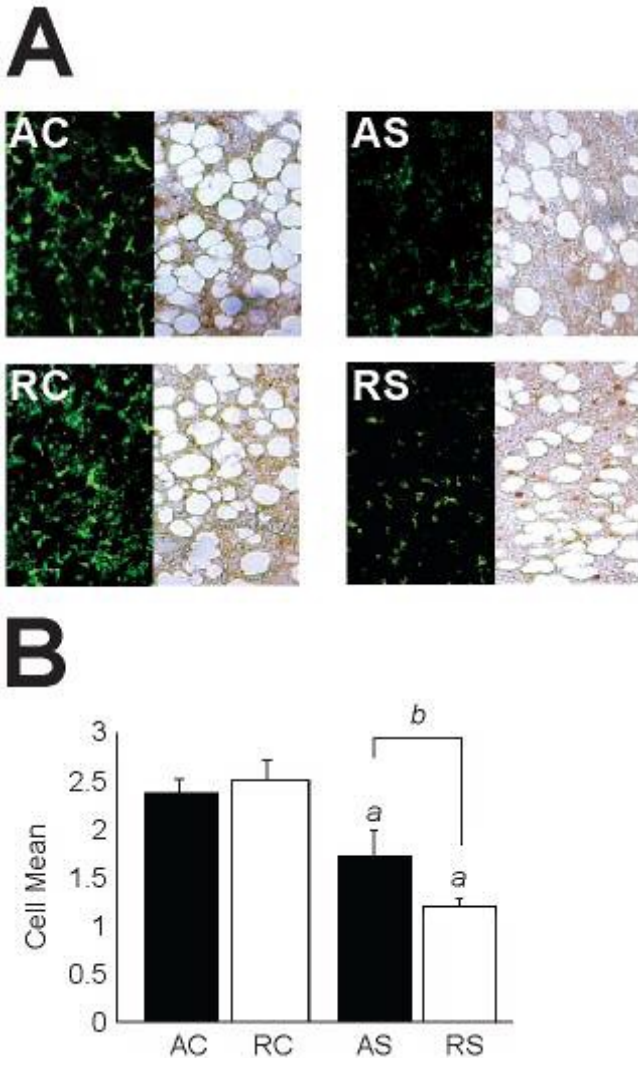

FIGURE 3.6: Changes in PPARγ immunoreactive levels of Tibiae of Aged Rats Fed Casein or Soy and Submitted or not to Long-term Moderate CR.

(A) Immunofluorescence (left panels) and immunohistochemistry (right panels) analysis of PPARγ expression in aged rats either fed *ad libitum* or under CR and receiving either casein or soy protein. (B) Quantification of PPARγ expression in the proximal tibiae shows a significant reduction in the soy-fed as compared to the casein-fed aged rats (*a*, $p < 0.05$). Additionally, a significant difference between AS and RS groups was found (*b*, $p < 0.05$). Magnification at source was ×40. Fluorescence was quantified using image analysis software (Image Pro Plus 6.0, Media Cybernetics). Micrographs are representative of four to six screened in each group of animals.

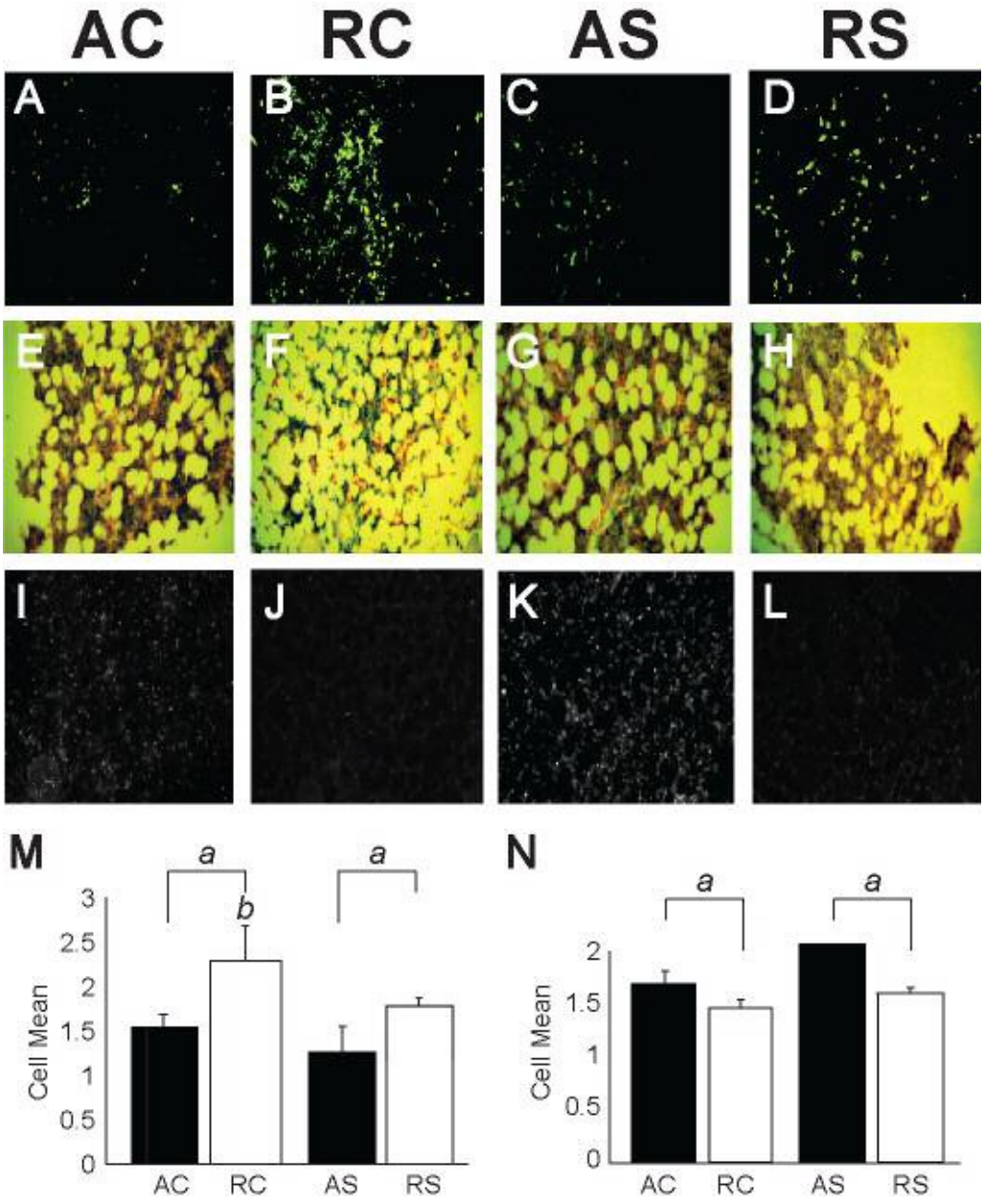

FIGURE 3.7: Immunofluorescence and *in situ* Hybridization Analysis of Sirt1 in Tibiae of Aged Rats fed Casein or Soy and Submitted or not to Long-term Moderate CR.

A-D**:** Immunofluorescence localization of Sirt1 protein showed significantly upregulated expression in the tibiae of CR aged rats fed either casein (RC, panel B) or soy (RS, panel D)compared to aged rats fed *ad libitum* (AC, panel A and RS, panel D). Levels of expression were quantified using image software and are shown in panel M (*a*, $p<0.01$). E-L**:** CR reduces Sirt1 mRNA expression in aged rats fed with either casein (F and J) or soy(H and L) as the source of dietary protein when compared with aged rats fed ad libitum (E,I,Gand K). Panel N shows the quantification of hybridization signals under a light microscope and using image analysis software (Image Pro Plus 6.0, Media Cybernetics) (*a*, $p<0.01$).Magnification at source was ×40. Panels E to H show light microscopy whereas panels I to L show dark field images. Micrographs are representative of four to six screened in each group of animals.

Effect of CR on Body Weight, Body Mass Index (BMI) and Length:

The effect of age and dietary intervention was examined in four groups of aged rats: **AC** = *ad libitum*- and casein-fed, **AS** = *ad libitum*- and soy-fed, **RC** = 40% CR and casein-fed, **RS** = 40% CR and soy-fed and compared with 2-month-old **AC** rats. As shown in Figure 3.1, aging rats (20-month-old) had a significant increase in body weight (Figure 3.1A) and body mass index (BMI) (Figure 3.1B) as compared to the young (2-month-old) controls. In addition, 20-monthold rats exposed to long-term moderate CR had a body weight 1.4 times lower than 20-month old rats fed *ad libitum* ($p < 0.001$), and 1.5 times higher than 2-month-old controls fed *ad libitum* (Figure 3.1A, $p < 0.01$). Additionally, BMI was 30% lower in the CR rats as compared with their *ad libitum* counterparts (Figure 3.1B, $p <0.01$). There was not difference in either body weight or BMI changes based on the protein source. Finally, rat length (Figure 3.1C) was increased by 1.3-fold in all 20-month-old rats compared with 2-month-old controls ($p <0.01$) regardless of the diet.

Effects of Aging and Diet on Tibial Bone Quality:

We assessed bone quality by measuring changes in bone volume/total tissue volume (BV/TV) using von Kossa and Toluidine blue stained sections of plastic embedded tissue. All 20-month old rats showed a significant reduction in BV/TV as compared to the 2-month old *ad libitum* controls (considered as 100% BV/TV). As shown in Figure 2, CR rats (Figure 3.2, C, D, G, and H) fed either with casein or soy protein showed a significant decrease in bone volume per tissue volume (BV/TV) in the proximal tibial epiphysis as compared to aged rats fed *ad libitum* ($p<0.05$). In contrast, rats fed *ad libitum* receiving a soy protein regime (AS) showed a lower decline in BV/TV as compared to the other three aged groups (Figure 3.2, E, F and I).

Effects of Aging and Diet on Serum Biomarkers and Calciotropic Hormones:

To assess if the changes found in bone morphometry correlated with bone serum biomarkers of bone metabolism we analyzed C-telopeptide, as an index of bone resorption, and osteocalcin, as an index of bone formation (Figure 3.3, A and B). A significant and age-related decrease in both markers was found in all 20-month-old groups compared with 2-month old controls ($p<0.001$). The decrease in osteocalcin levels was significantly smaller in the AS group ($p<0.01$), compared to the other aged groups, suggesting that *ad libitum* soy could stimulate bone formation. In contrast, the RS group showed the lowest levels of osteocalcin ($p< 0.01$). Finally, no diet effect was seen for serum C-telopeptide. Serum levels of the calciotropic hormones, parathyroid hormone (PTH) and vitamin D (25hydroxy vitamin D) were also measured. All groups showed a significant increase in PTH levels as compared to the 2-month old controls (Figure 3.3C; $p<0.01$). Additionally, an important increase in serum PTH levels was present in 20-month-old *ad libitum*-fed rats (Figure 3C;$p<0.001$), as previously reported using other conditions of CR in aging rats (Kalu *et al*., 1988), which was reverted by CR. Finally, changes in serum PTH were not associated with any significant difference in serum levels of vitamin D (25OH) (Figure 3D).

Effects of Aging and Diet on Bone Marrow Adiposity:

A common feature associated with aging in multiple species is an increase in bone marrow adipogenesis (Rosen and Bouxsein, 2006). The role of bone marrow fat remains undefined but has been proposed to be metabolic, toxic or passive occupation of the space left by a reduction in bone mass (Gimble, 1990). Since CR has been shown to induce a mobilization of adipocytes as a source of energy in other fat tissues (Das *et al*., 2004), it was of interest to determine if CR influenced adipocyte mobilization from the bone marrow. As shown in Figure 3.4, there was an age-related increase in bone marrow fat and adipocyte number in all 20-month-old groups, CR and fed *ad libitum*, when compared to the 2-month-old controls. This increase was significantly

lower in the AS group ($p<0.01$). Interestingly, this is the same group that showed improved bone quality (Figure 3.2E and F) and a higher level of osteoblastic activity as evidenced by serum osteocalcin (Figure 3.3B). This finding suggests that contrarily to other tissues, bone marrow adipocytes are not mobilized under CR, but occupy the space left during bone loss. Furthermore, given the proposed role of leptin in fat metabolism and bone growth, serum levels of leptin were quantified (Figure 3.5, A and B) and compared to *in situ* expression of leptin within the bone marrow (Figure 3.5C). As previously reported (Anderlova *et al*., 2006) serum levels of leptin were significantly increased with aging and reduced by CR ($p <0.001$) (Figure 3.5, A and B) likely due to the decrease of adipose tissue mass outside the bones. However, quantification of leptin expression/marrow area, although increased by aging (Figure 3.5, A and B) did not show differences between the aged groups suggesting that local expression of leptin within the bone marrow was not affected by CR (Figure 3.5A) and correlating with thefact that these adipocytes did not seem to be mobilized upon CR.

Effects of Aging and Diet on PPARγ and Sirt1 Expression and Activity:

The sirtuin family of nicotinamide adenine dinucleotide-dependant deacetylases plays an important role in aging and metabolic regulation (Imai, 2007). Among the sirtuin family, CR upregulates Sirt1 in white adipose tissue while depleting adipocytes of fatty acids due to repression of PPARγ (Michan and Sinclair, 2007). To assess if CR has a similar effect on PPARγ and Sirt1 in bone marrow fat, changes in PPARγ and Sirt1 immunoreactive levels and Sirt1 mRNA levels were examined (Figures 3.6 and 3.7). In agreement with previous reports (Duque *et al.*, 2003) aged rats showed significantly higher expression of PPARγ within the bone marrow as compared to the 2-month-old controls (data no shown). Furthermore, a reduction in PPARγ was noted in SA rats ($p <0.01$) compared to AC, with RS rats showing the lowest level of PPARγ expression ($p<0.001$) (Figure 6, A-C). Furthermore, in agreement with previous reports (Cohen *et al*., 2007), immunofluorescence localization of Sirt1 was significantly increased in both CR

groups (Figure 7, B and D) compared with the age-matched *ad libitum* groups (Figure 7, A and C) with a significantly higher expression in the casein fed group ($p<0.01$) (Figure 7B). *In-situ* hybridization of Sirt1-mRNA shows a reduction in Sirt1 mRNA levels in both CR groups ($p<0.01$) (Figure 7J and L).

Taken together, this data suggests that contrary to the situation seen in subcutaneous and visceral white fat, the lack of adipocytes mobilization during CR is independent of changes in PPARγ expression. For example, despite the significantly lower levels of PPARγ expression in CR rats fed soy, the number of adipocytes within the bone marrow remains the same. Additionally, the discrepancy in Sirt1 protein and Sirt1 mRNA levels suggest that CR may promote Sirt1 translation and/or protein stability in the bone marrow, which have no impact on adipocyte number.

3.4 Discussion

In this study, we examined the impact of CR on the two major features of age-related bone loss, namely a decrease in bone mass and an increase in marrow fat (Rosen and Bouxsein, 2006). CR has been shown to extend the lifespan of laboratory animals, while significantly reducing the incidence of numerous age-related diseases regardless of species or gender (Roth *et al*., 1999).

The first study that serendipitously associated CR with changes in bone architecture was conducted by McCay *et al*. while examining the effect of CR on longevity (McCay *et al*., 1939). As an unexpected finding, the bones of CR rats were noted to be fragile compared with non- CR controls. Subsequent studies have suggested that this effect was due to extreme dietary deprivation and calcium deficiency (Harrison and Fraser, 1960). Another study (LaMothe *et al*., 2003) investigated the effect of age and CR on bone geometry and mechanics in the axial and appendicular skeleton of F-344 Brown-Norway rats, showed that CR adversely affected bone geometry and mechanics independently of a reduction in body mass. Finally, a recent study proposed that the detrimental effect of CR on bone is due to low bone turnover that could be prevented through leptin inhibition by beta blockers (Tatsumi *et al*., 2007). A common feature in studies looking at the effect of CR is the use of casein as a source of dietary protein. In fact, casein has not been shown to have any effect on bone mass. In a model of male rats fed *ad libitum* in which casein was compared with soy as the source of protein, it was shown that casein had no apparent effects on bone microarchitecture whereas the soy fed rats showed a significant improvement in all the parameters in an animal model fed *ad libitum* (Soung *et al*., 2006). We extended these observations to a comparison between two protein regimens in a model of CR that had previously been assessed and validated for the evaluation of age-related neuroendocrine changes (Girard *et al*., 1998). In this model, a long-term moderate caloric intake (60% of *ad libitum*) induced a significant reduction in body weight and BMI as compared with the *ad libitum*-fed groups.

Changes were induced by aging in both bone microarchitecture and serum biomarkers of bonemetabolism in aged rats exposed to CR or fed with two different protein sources. Male rats were used in order to avoid the effect of estrogenic deprivation on bone during menopause. All aged rats, restricted and *ad libitum*, showed a significant reduction in bone quality. However, in agreement with previous studies showing that soy protein, more specifically soy isoflavones, could protect against age-related bone loss (Khalil *et al*., 2005) the reduction in bone mass was less in the AS group. This positive effect of soy protein in rats fed *ad libitum* correlated with the changes in biochemical markers of bone formation (osteocalcin) found in the same group with no effect on markers of bone resorption (C-telopeptide).

This effect was also independent of either PTH or vitamin D levels. In agreement with previous studies (Kalu *et al*., 1984), food restriction reduced the high serum levels of PTH seen in aged rats without affecting bone resorption as suggested by serum levels of C-telopeptide. Finally, vitamin D levels were similar in all groups with no evidence of deficiency in the CR animals.

Additionally to testing the effect of two different proteins on bone structure, we were also interested on testing the effect of CR on the second component of age-related bone loss, which is the increasing adipogenesis. Typically, both visceral and subcutaneous fat respond to CR by mobilizing adipocytes, a phenomenon that occurs earlier in visceral fat (Das *et al*., 2004). Additionally, the responsiveness of lipid metabolism-related genes to fasting is more sensitive in visceral fat than in subcutaneous fat in CR rats (Wang *et al*., 2007). However, the response of bone marrow fat to CR remains undefined.

As expected, young rats showed significantly lower marrow fat and adipocyte numbers when compared to the aged groups. It was also shown that, contrary to other types of fat, bone marrow fat did not respond to CR by mobilizing its adipocytes' reservoir. The observation that the AS

group had less marrow fat in the presence of a higher bone mass supports the “occupying space” hypothesis for the bone marrow fat.

In addition to adipocyte number, we also examined leptin expression within the bone marrow space. Leptin, which is highly expressed by adipocytes, has been associated with either stimulation or inhibition of bone formation depending on the cell origin of its secretion (Coen,2004; Hamrick and Ferrari, 2007). Rats exposed to CR have been shown to have a dramatic reduction in their serum leptin (Gallardo *et al*., 2005) as a consequence of a significant reduction in subcutaneous and visceral fat. In addition, CR reduces the local expression of leptin in these two types of fat (Das *et al*., 2004). However, this does not appear to be the case in bone marrow fat where none of the groups, either restricted or *ad libitum*, showed a reduction in levels of leptin expression within the bone marrow independently of the reduction in serum levels, again suggesting a lack of fat mobilization.

From a mechanistic point of view, we assessed if CR aging rats receiving either casein or soy in their diet showed differences in the expression of two major genes that are affected by both aging and CR: PPARγ and Sirt1. PPARγ levels increase with aging in bone marrow leading to an increase in the differentiation of MSC into adipocytes (Duque *et al*., 2004; Lecka-Czernick and Suva, 2006). Although it has been suggested that PPARs mediate the effects of CR (Linford *et al*., 2007), information about the impact of CR on PPARγ remains limited. Indeed, recent studies on Sirt1 have demonstrated that it promotes fat mobilization in adipocytes by repressing PPARγ (Picard et al., 2004). Sirt1 is a member of the sirtuins, a group of regulators of cell response to stress and senescence (Imai, 2007) which is not only an important determinant of adipocyte differentiation from mesenchymal stem cells (MSC) but alsoincreases in white fat during CR (Picard and Guarente, 2005).

In this study, we found that in agreement with previous studies looking at changes in PPARγ expression induced by soy protein in white fat (Ricketts *et al*., 2005), both groups receiving soy protein showed significantly less PPARγ within the bone marrow compared with rats fed casein diet. With respect to Sirt1 expression, CR induced higher levels protein expression while reduced Sirt1-mRNA in both restricted groups. Taking together these findings suggest that contrary to the effect of CR on Sirt1 and PPARγ in white fat where induction of high levels of Sirt1 expression promotes signals of fat mobilization, this is not the case in bone marrow fat. Further studies looking at the specific response of bone marrow adipocytes to Sirt1 are required. In summary, using a model of aged Sprague Dawley male rats we have tested the impact of CR and two different protein sources on the cellular features of age-related bone loss. We have found that: 1- The decrease in bone quality associated with aging and CR could be reduced by the positive effect of soy protein on bone formation; 2- Bone marrow adipocytes are not mobilized upon CR; and 3- Bone marrow fat responds differently to CR than subcutaneous and visceral fat in terms of expression of adipocyte-regulating genes.

In conclusion, the detrimental effect of aging and CR on bone quality could be partially reverted by dietary soy. Additionally, the fact that CR did not induce the mobilization of bone marrow fat suggests that bone marrow adipocytes are not involved in the metabolic response during periods of CR.

3.5 Acknowledgements

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Transition to Chapter 4

In the ***"Effects of Long-Term Moderate Caloric Restriction and Dietary ProteinSource on Bone and Marrow Fat of the Aging Rat"*****,** our group showed that bone marrow fat depots do not participate in metabolism during moderate stages of starvation. Contrary to visceral and subcutaneous fat depots which are usually mobilized during CR as a source of energy (Das *et al*., 2004); bone marrow fat depots do not appear to be mobilized.

A central hypothesis regarding the relationship of bone and fat in the marrow cavity is that adipocytes occupy space left empty by bone loss. Furthermore, Soy isoflavones were previously shown to have antiresorptive and anabolic effects on bone. Taken together, our results confirmed these concepts as *ad libitum* soy fed rats appeared to have less marrow fat in the presence of a higher bone mass. These results further reinforced the idea that the fat infiltration of the marrow milieu is a consequence of bone loss and that bone marrow fat may just occupy space.

Subsequently, we hypothesized that the infiltration of fat could be toxic to osteoblast differentiation, mineralization and survival. We believed in a lipotoxic effect of fat in the marrow milieu. The ideas presented in chapter 4, "Inhibition of Fatty Acid Biosynthesis Prevents

Adipocyte Lipotoxicity on Human Osteoblasts *In Vitro"*, elaborate on the toxicity of adipocytes, a concept earlier presented by Maurin AC, Chavassieux et al. Using a co culture model of adipocytes and osteoblasts, similar to the one established by Maurin AC et al, our group demonstrated the toxic effect of fatty acid synthesis on osteoblast behavior and survival. From a global perspective, we propose in the following manuscript, that the age associated accumulation of fat in the marrow milieu is not only a consequence of bone loss but also promotes further bone loss through an induction of osteoblast apoptosis and a reduction of osteogenic mineralization and differentiation.

The manuscript presented in chapter 4 will not only test the concepts earlier introduced but will also show that the toxic effect of adipocytes on osteoblasts could be prevented through an inhibition of FA synthesis.

Chapter 4

Fatty Acid Biosynthesis and Lipotoxicity in Bone

This chapter is a reproduction of the following Manuscript: ***"Inhibition of Fatty Acid Biosynthesis Prevents Adipocyte Lipotoxicity on Human Osteoblasts In Vitro"***

Alexandre Elbaz, Xiying Wu, Daniel Rivas, Jeffrey M. Gimble , Gustavo Duque

ABSTRACT

While increased bone marrow fat in age-related bone loss has been associated with lower trabecular mass, the underlying mechanism responsible remains unknown. We hypothesized that marrow adipocytes exert a lipotoxic effect on osteoblast function and survival through the reversible secretion of fatty acids (FA) into the bone marrow microenvironment. We have used a two-chamber system to co-culture normal human osteoblasts (NHOst) with differentiating adipocytes in the absence or presence of an inhibitor of FA synthase (cerulenin) and separated by an insert that allowed unidirectional trafficking of soluble factors only and prevented direct cell-cell contact. Supernatants were assayed for the presence of FA using mass spectophotometry. After 3 weeks in co-culture, NHOst showed significantly lower levels of differentiation and function based on lower mineralization and expression of alkaline phosphatase, osteocalcin and Runx2. In addition, NHOst survival was affected by the presence of adipocytes as determined by MTS-Formazan and TUNEL assays as well as higher activation of caspases 3/7. These toxic effects were inhibited by addition of cerulenin. Furthermore, culture of NHOst with either adipocyte-conditioned media alone in the absence of adipocytes themselves or with the addition of the most predominant FA (stearate or palmitate) produced similar toxic results. Finally, Runx2 nuclear binding was affected by addition of either adipocyte conditioned media or FA into the osteogenic media. We conclude that the paracrine release of FA within the marrow milieu can

contribute to the age-related changes in bone mass and can be prevented by the inhibition of FA synthase.

4.1. Introduction

The redistribution of body fat is a prevalent feature of the aging process (Gimble, Robinson et al. 1996; Cartwright, Tchkonia et al. 2007). With aging, body fat appears to accumulate in depots where it should not, infiltrating normal tissues such as muscle and pancreas (Unger and Orci 2002). Bone marrow is no exception since fat content increases within this hematopoietic and osteogenic tissue, making it an ectopic fat depot in older aged individuals (Gimble, Robinson et al. 1996),(Duque 2008), (Gimble, Zvonic et al. 2006).

Various theories have been proposed for the role and the cause of the increased bone marrow fat (Duque 2008), (Gimble, Zvonic et al. 2006), (Rosen and Bouxsein 2006) . It has been proposed that the conversion of osteoblast progenitors into adipocyte-like cells accounts for the increased bone marrow fat at the expense of bone forming cells(Duque 2008), (Gimble, Zvonic et al. 2006).It has also been hypothesized that an inverse relationship exists in the marrow cavity between bone and fat where fat would occupy the space left empty by age-related bone loss or altered hematopoietic capacity (Beresford, Bennett et al. 1992), (Pei and Tontonoz 2004).

The accumulation of reactive lipids in metabolically active tissues such as pancreatic islets, liver, heart and skeletal muscle leads to lipotoxicity (Unger and Orci 2002), (Slawik and Vidal-Puig 2006). Lipotoxicity has shown to contribute to the pathophysiology of insulin resistance, type 2 diabetes, steatotic liver disease, and heart failure (Unger and Orci 2002), (Slawik and Vidal-Puig 2006). In the case of aging bone marrow, similar lipotoxity could impact mature osteoblasts negatively due to their close proximity to marrow adipocytes (Diascro, Vogel et al. 1998).

In fact, there is evidence suggesting that bone marrow fat plays a toxic role similar to that seen in other organs, resulting in the further loss of mature osteoblasts. *In vitro* studies have found that co-culture of osteoblasts with adipocytes results in a decrease of osteoblasts proliferation (Maurin, Chavassieux et al. 2000). A further study has associated this negative effect on osteoblast proliferation with the presence of polyunsaturated fatty acids (FA) in the media (Maurin, Chavassieux et al. 2002). In agreement with this *in vitro* data, studies looking at age-related changes in bone marrow fat *in vivo* have reported that increasing levels of FA oxidation by bone marrow adipocytes may inhibit osteoblast differentiation (Lecka-Czernik, Moerman et al. 2002). However, the mechanisms and the potential reversibility of this lipotoxic effect have not been assessed.

In this study, we have looked at the potential mechanisms that explain the lipotoxic effect of adipocyte infiltration within the bone marrow. Using a system of co-cultures (Maurin, Chavassieux et al. 2000) we have exposed human osteoblasts to either human differentiating pre-adipocytes or their conditioned media. We have identified the predominant FA released by the adipocytes in our model and documented their effect on osteoblast differentiation, function and survival. Additionally, this lipotoxic effect was reversed by cerulenin, an inhibitor of FA synthase. Finally, we found that adipocytes affect not only osteoblast proliferation but also their function and survival through the inhibition of Runx2-nuclear binding and the activation of caspases. In conclusion, this evidence could provide a new understanding of the interaction between fat and bone within the marrow microenvironment and the potential regulation and prevention of lipotoxicity on bone metabolism.

4.2. Experimental Methods

Normal Human Osteoblasts (NHOst): NHOst as well as media were purchased from Lonza (Walkersville, MD, USA). Cells were obtained from healthy 24-year-old male donors (n=3).

Cells at passages three and four from time of marrow harvest were used in these experiments. NHOst were plated in growth media at 37°C in a humidified atmosphere of 5% CO_2. Growth media was composed of osteoblast basal medium media (C-3208, Lonza) containing 10% of FBS, 0.1% ascorbic acid, and 0.1% gentamicin. The cells were allowed to grow for 2 days or until confluence at which point they were differentiated (day 0). Cells were differentiated into mature osteoblasts using a combination of Osteoblast Basal Medium, the Growth Medium kit (CC-4193, Lonza) and finally the differentiation kit, which included 0.5ml hydrocortisone (200μM) and 5 ml β-glycerophosphate (1 M).

Normal Human Pre-adipocytes: Human Pre-adipocytes were obtained at Pennington Biomedical Research Center (Baton Rouge, LA, USA) from liposuction aspirates as previously described (DeLany, Floyd et al. 2005) (Dubois, Floyd et al. 2008). Briefly, aspirates from subcutaneous adipose tissue sites were obtained from male and female subjects *(n=6)* undergoing elective procedures in plastic surgical offices of the Baton Rouge region. Ethical approval in accordance with the Human Tissue Act 65, 1983, was obtained from the Ethics Committee at Pennington Biomedical Research Center. The mean age and BMI (+/- S.D.) of the subjects were 38 +/-14 years and 30.4 +/-7.1, respectively. Tissues were washed three to four times with phosphate buffered saline and suspended in an equal volume of PBS/0.5 mM calcium chloride supplemented with 1% bovine serum and 0.1% collagenase type I pre-warmed to 37 °C. The tissue was placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged for 5 min at 300 x *g* at room temperature. The supernatant was removed, and the pelleted stromal vascular fraction (SVF) was resuspended in Stromal Medium (Dulbecco's modified Eagle's medium/F-12, from Invitrogen Grand Island, NY, USA, 10% fetal bovine serum, 1% antibiotic/antimycotic) and plated at a density of 0.156 ml of tissue digest/cm2 of surface area in T225 flasks using Stromal Medium for expansion and culture. This initial passage of the primary cell culture is referred to as "Passage 0." Following the first 48 h of incubation at

37 °C at 5% CO2, the cultures were washed with PBS and maintained in Stromal Medium until they achieved 80-90% confluence. Cells were harvested by trypsin digestion, suspended in 10% dimethylsulfoxide/10%DMEM/F12 Ham's/80% bovine serum albumin at 0.5 $X10^6$ cells/ml, and cryopreserved in liquid nitrogen until required for experimental use.

Normal Human Pre-adipocytes –Differentiation: The cryopreserved cells were thawed and plated in Stromal Medium until they reached confluence. At this time, differentiation was induced using the Adipogenic Differentiation media composed of Dulbecco's modified Eagle's medium/F-12 (pH 8.4) with 3% fetal bovine serum, 33μM biotin, 17 μM pantothenate, 1 μM bovine insulin, 1 μM dexamethasone, 0.25mM isobutylmethylxanthine, 5 μM rosiglitazone, and 100 units of penicillin, 100 μg of streptomycin, 0.25μg of Fungizone. After 3 days, Adipogenic Differentiation Medium was changed to Adipocyte Maintenance Medium, which was identical to the induction medium except for the removal of both isobutylmethylxanthine and rosiglitazone(Dubois, Floyd et al. 2008).

Oil Red O staining and its quantification:To demonstrate adipogenesis, Oil Red O staining was used as previously described (Duque and Rivas 2007) . Differentiated adipocytes were considered those polygonal in shape, with eccentrically located nuclei, considerable cytoplasm and lipid droplets scattered throughout. Mean adipocyte number was accounted in 10 fields of cells for each condition. For Oil red O quantification, cultures were fixed in Baker's formal calcium, washed in 60% isopropanol and stained with double-filtered Oil Red O solution to show for lipid accumulation. Oil Red O was extracted from cells using 100% ethanol and measured at 540 nm, using ELx800 Universal Microplate Reader (Bio-tek instruments Inc, USA). Oil Red O concentration was determined against known standards of Oil Red O (0.02 mg/ml to 10.00 mg/ml).

Co-cultures of adipocytes and osteoblasts: Differentiating pre-adipocytes, were cultured in

apical compartments of cell culture inserts (Pore size: 0.45 μm, growth area: 4.2 cm^2, Falcon-BD Bioscience, Franklin Lakes, NJ, USA) with NHOst grown in the basal compartment of 6-well plates (growth area: 9.6 cm^2). These inserts prevent cell-to-cell contact and allow unidirectional flow of media from the apical to basal compartments when contact between both media is avoided(Maurin, Chavassieux et al. 2000). Pre-adipocytes were seeded on inserts (50,000/cm^2) in the absence of underlying NHOst for 48 h to reach confluence. Inserts containing pre-adipocytes were transferred to plates with confluent NHOst monolayers (40,000/cm^2) in the basal compartment. Both compartments received fresh culture medium and the co-culture was maintained for 3 weeks. Three types of co-culture were performed as previously described (Maurin, Chavassieux et al. 2000): (1) differentiating pre-adipocytes were plated in the apical side of the insert with osteoblasts plated on the basal compartment (AD/OB), with unidirectional media flow going from the adipocyte to the osteoblast side; (2) pre-adipocytes were plated on the basal compartment and osteoblasts plated on apical side (OB/AD), with unidirectional media flow going from the osteoblast to the adipocyte side; or (3) Adipocytes differentiating media alone was placed in the apical side with NHOst plated in the basal compartment (-/OB). During all culture manipulations, contact between the medium in the bottom and the membrane was carefully avoided. Culture media from the both sides of the membrane was changed every three days. Supernatants were collected and placed at -20 ^oC for further experiments and future analysis. After 21 days of co-culture, cells were stained for alkaline phosphatase (ALP) and alizarin red or trypsinized for protein extraction.

***Cerulenin treatment*:** Human differentiating preadipocytes were plated in co-culture as previously described (DeLany, Floyd et al. 2005), (Dubois, Floyd et al. 2008). Treatment with either cerulenin (10 nM) or vehicle alone was started at day one of co-cultures. To analyze the effect of cerulenin on the co-cultures, six different conditions were analyzed: AD/OB treated and untreated, OB/AD treated and untreated and -/OB treated and untreated. Cells were treated for 21

days with media being replaced every three days. Additionally, to assess the potential direct effect of cerulenin on NHOst, cells were plated in six well plates and treated for 21 days with either cerulenin (10 nM) or vehicle alone. Media was replaced every three days.

Determination of Osteoblast Function*:* As previously reported (Karsenty 2001), osteoblasts placed in osteogenic media express ALP and fully mineralize at week three of differentiation. To identify changes in osteoblast function induced by the presence of adipocytes in co-culture ALP staining was performed at week 3 using 0.25% naphthol AS-MX ALP solution with fast blue RR salt (Sigma, St. Louis, MO, USA). After washing with PBS, cells were incubated with naphthol solution mixture for 1 hour at room temperature. The resulting purple, insoluble, granular dye deposit indicated sites of ALP activity. Additionally, calcium deposition was also quantified using 1% Alizarin red S (Lab Chem Inc, Pittsburgh, PA, USA). Briefly, after Alizarin red staining, matrix mineralization was quantified by extracting the Alizarin red staining with 100mM cetypyridinium chloride (Sigma, St. Louis, MO, USA) at room temperature for 3 h. The absorbance of the extracted Alizarin red S stain was measured at 570 nm. Data represented as units of Alizarin red S per mg of protein in each culture after correction for cell number. Six wells were analyzed per experimental condition. Experiments were performed in triplicate.

Determination of cell viability: To test whether adipocyte-secreted factors have any effect on cell survival, NHOst were seeded in 96 well plates. Upon reaching confluence, the cells were treated with 100□l/well of supernatant obtained from the osteoblast side of the membrane under each of the six different co-culture plus treatment conditions. At timed intervals (24, 48,72 and 96h), MTS-Formazan cell viability assays (Promega, Madison, WI, USA) were performed and corrected for cell number as previously described (Duque, El Abdaimi et al. 2004). Briefly, a stock solution of MTS was dissolved in PBS at a concentration of 5mg/ml and was added in a 1:10 ratio (MTS/DMEM) to each well, incubated at 37^{o}C for 2h, and the optical density determined at a wavelength of 490 nm on a microplate reader model 3550 (Biorad, Hercules,

CA, USA). In preliminary experiments the absorbance was found to be directly proportional to the number of cells over a wide range (2 x 10^2 to 5 x 10^4 cells/well). The percent survival was defined as [(experimental absorbance - blank absorbance)/control absorbance - blank absorbance)] x 100, where the control absorbance is the optical density obtained for 1 x 10^4 cells/ well (number of cells plated at the start of the experiment), and blank absorbance is the optical density determined in wells containing medium and MTS alone.

Western Blot Analysis: Cells were plated in co-culture as previously described. At day 21, osteoblasts were lysed in ice-cold buffer [20 mM Tris, pH 7,9, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 400 mM NaCl, and 0.5 ml glycerol containing protease inhibitor tablets (Roche Diagnostics Canada, Laval, QC, Canada)], freeze-thawed 3 times in a dry ice-ethanol bath and centrifuged at 11500 rpm for 15 minutes to remove insoluble material. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, California, USA). Samples were then aliquoted and stored at -80^{o}C. Protein lysates (10 µg per lane) were separated on 15% SDS-polyacrylamide gels and blotted on to nitrocellulose membrane. Blots were blocked with 5% (w/ v) skim milk in 1% Tween 20 in PBS and incubated overnight at 4^{o}C with primary antibodies against Runx2 (PC287L, Calbiochem, San Diego, CA, USA), an essential transcription factor for osteoblast differentiation and function (Karsenty 2001) and osteocalcin (OCN) (sc-80902, Santacruz biotechnology, Santacruz, CA), an osteoblast specific protein downstream of Runx2 activation (Karsenty 2001). After three washes with the Tween solution, the membrane was incubated with rabbit anti-goat antibody conjugated with horseradish peroxidase. The secondary antibody was detected with chemiluminescence reagent (Perkin-Elmer Life Sciences, Inc, Boston, MA, USA) and exposed to X-ray film (Eastman Kodak Bio-max, Rochester, NY, USA).

Apoptosis Detection: Two techniques were used to quantify apoptotic changes namely TUNEL and caspase 3/7 assays. For quantification of DNA cleavage TUNEL reaction was performed using the Apoptag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon, Temecula,

CA, USA) as previously described (Duque, El Abdaimi et al. 2002). Briefly, 6 x 10^5 NHOst cells were seeded in 2-well glass chamber slides (Nalge Nunc, Rochester, NY, USA) and left in culture in osteoblast growth medium for 48 hours at which point they were treated with supernatants from the osteoblast side of all co-culture systems. After 72 h of treatment, cells were fixed in 4% paraformaldehyde for 10 min, washed in 10 mM Tris–HCl, pH 8.0, and preincubated for 10 min at room temperature in the reaction buffer for terminal deoxynucleotidyl transferase reaction (200 mM potassium cacodylate, 0.22 mg/ml BSA, and 25-mM Tris–HCl, pH 6.6). The preincubation buffer was then removed, and a reaction mixture containing 500 U/ml terminal deoxynucleotidyl transferase, 25 mM $CoCl_2$, and 40 μM biotinylated dUTP was added for 60 min at 37°C. The reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate for 25 min at room temperature and for 60 min at room temperature in the dark. Propidium Iodide (PI) (SIGMA) was added to cell suspensions at a concentration of 5 μg/ml. Slides were mounted and observed through fluorescence microscopy. The proportion of apoptotic cells was quantified in ten fields per well by three different observers. This experiment was repeated three times.

Furthermore, occurrence and mechanism of apoptosis were assessed by analysis of caspase-3/7 activity. NHOst (6 x 10^5) were seeded in 6-well plates and left in culture in osteoblast growth medium for 48 hours at which point they were treated with supernatants from the osteoblast side of all co-culture systems. At timed intervals (24-72h), osteoblasts were lysed in ice-cold buffer [20 mM Tris, pH 7,9, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 400 mM NaCl, and 0.5 ml glycerol containing protease inhibitor tablets (Roche Diagnostics Canada, Laval, QC, Canada)], freeze-thawed 3 times in a dry ice-ethanol bath and centrifuged at 11500 rpm for 15 minutes to remove insoluble material. Protein concentrations were determined as previously described. Caspase-3/7 activity was measured by using Caspase Glo-3/7 assay systems (Promega). Samples

(100 µl) were gently mixed with Caspase-Glo substrate (100 µl) and the luminescence of each sample was measured by using Luciferase assay system (Promega).

Gas chromatography/Mass spectrometry (GC/MS): Supernatants (2.5 ml) obtained from both sides of the cerulenin-treated and untreated co-culture conditions were collected at week 3 of adipocyte differentiation. Samples were analyzed for FA by GC-MS at Metabolic Solutions, Inc. (Nashua, NH, USA). To be considered as relevant as a potential lipotoxic factor in our model, any FA found by GC/MS of the analyzed media should fulfill the following criteria: be significantly detected only when adipocytes are present in the co-culture system, have the capacity of being detected in the media obtained from the osteoblast side of the co-cultures, and finally, be significantly affected by the presence of cerulenin. In all experiments, supernatants obtained from normal adipocytes were analyzed for FA by GC-MS as controls.

Treatment of osteoblasts *with FA* – After identification of the predominant FA in our model, NHOst were plated and treated with either stearate or palmitate at normal physiological concentrations employed in other published cell models (Kim, Kim et al. 2008),(Eitel, Staiger et al. 2002). A FA known to stimulate osteoblast function (linolate) was used as control at a dose previously used in other study(Platt, Rao et al. 2007). Briefly, NHOst were plated at a density of 4 x 10^5 cells in six-well plates containing osteoblast growth media at 37ºC in a humidified atmosphere of 5% CO_2. After 80% confluence media was replaced with osteoblast growth media alone or containing stearate (0.5-1 mM), palmitate (100-250 □M), or linolate (25-50□M). At week 3 of treatment, cells were fixed and ALP and alizarin red staining and quantification were performed as previously described.

Runx2 activity measurement: Active Runx2 binding to DNA was determined using the ELISA-based Runx2 activation TransAM™ kit (Active Motif, Rixensart, Belgium) as previously described (Akter, Rivas et al. 2008). The Trans-AM Runx2 Kit contains a 96-well plate on which

an oligonucleotide containing a Runx2 consensus-binding site (5'-AACCACA-3') has been immobilized. The active form of Runx2 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in the Trans-AM Runx2 Kit recognizes an accessible epitope on Runx2 protein upon DNA binding. Addition of a secondary horseradish peroxidase (HRP)-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectophotometry (450 nm). To quantify active Runx2 binding, 15-20 mg of nuclear extract obtained from NHOst, cultured in osteoblast growth medium for 48 hours and treated with supernatants from the osteoblast side of all co-culture systems, was measured using the Trans-AM Runx2 Kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA).

Statistical analysis: All data are expressed as mean ± SD of three replicate determinations. Unless otherwise stated, all experiments were repeated three times. Statistical analysis was performed by two-way ANOVA for time course analysis and Student’s t-test for comparison between groups. A probability value of $p < 0.05$ was considered statistically significant.

4.3 Results

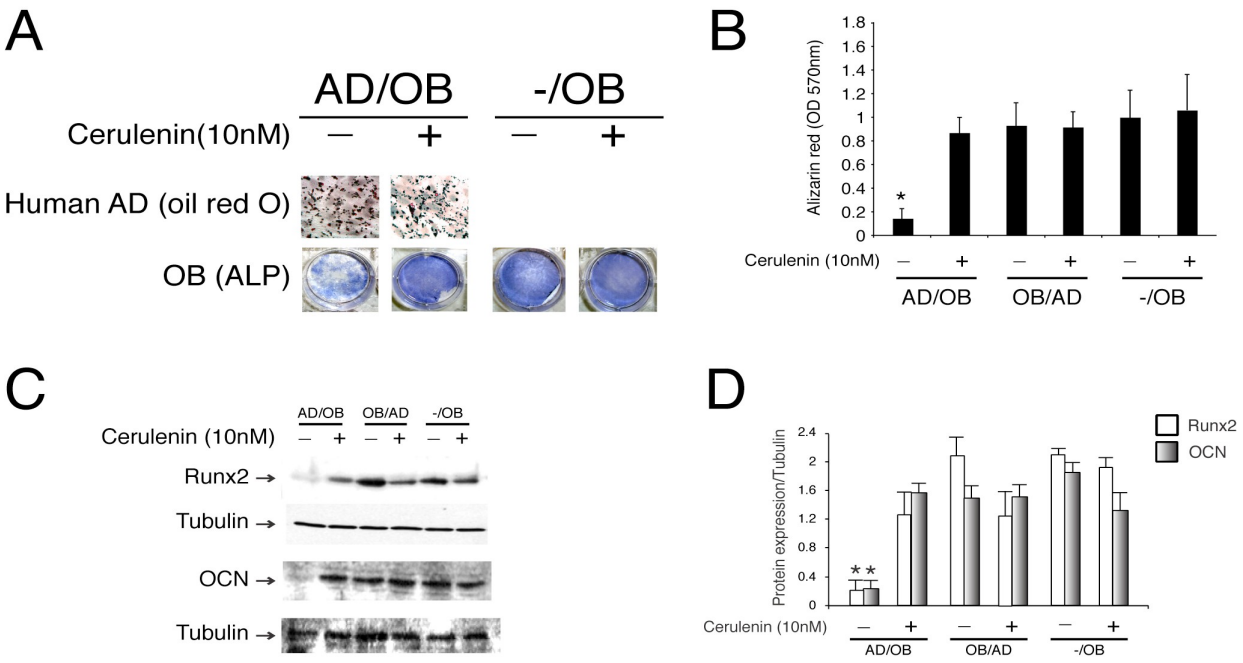

FIGURE 4.1: **The Effect of Adipogenesis on Osteogenic Differentiation and Mineralization in a Co Culture System**

(A-B) Mature human osteoblasts were plated in co-culture with differentiating human adipocytes (AD) treated with either cerulenin (10nM) or vehicle alone. After three weeks in co-culture, adipocytes were stained with oil red O (A, upper panels) to identify fat droplets and osteoblasts were stained with alkaline phosphatase (ALP) (A, lower panels) and alizarin red (B) to identify function and mineralization respectively. Addition of cerulenin to the adipocytes media did not affect adipocytes' capacity to produce fat droplets. In contrast, both osteoblast function and mineralization were significantly affected by the presence of adipocytes on top of the membrane. This inhibitory effect was prevented by addition of cerulenin (10nM) to the media ($p < 0.001$). (C,D) Expression levels of Runx2 and osteocalcin (OCN) quantified by western blot analysis were significantly reduced by the presence of adipocytes (*$p < 0.001$) and recovered by addition of cerulenin to the media. The data are representative of three different experiments

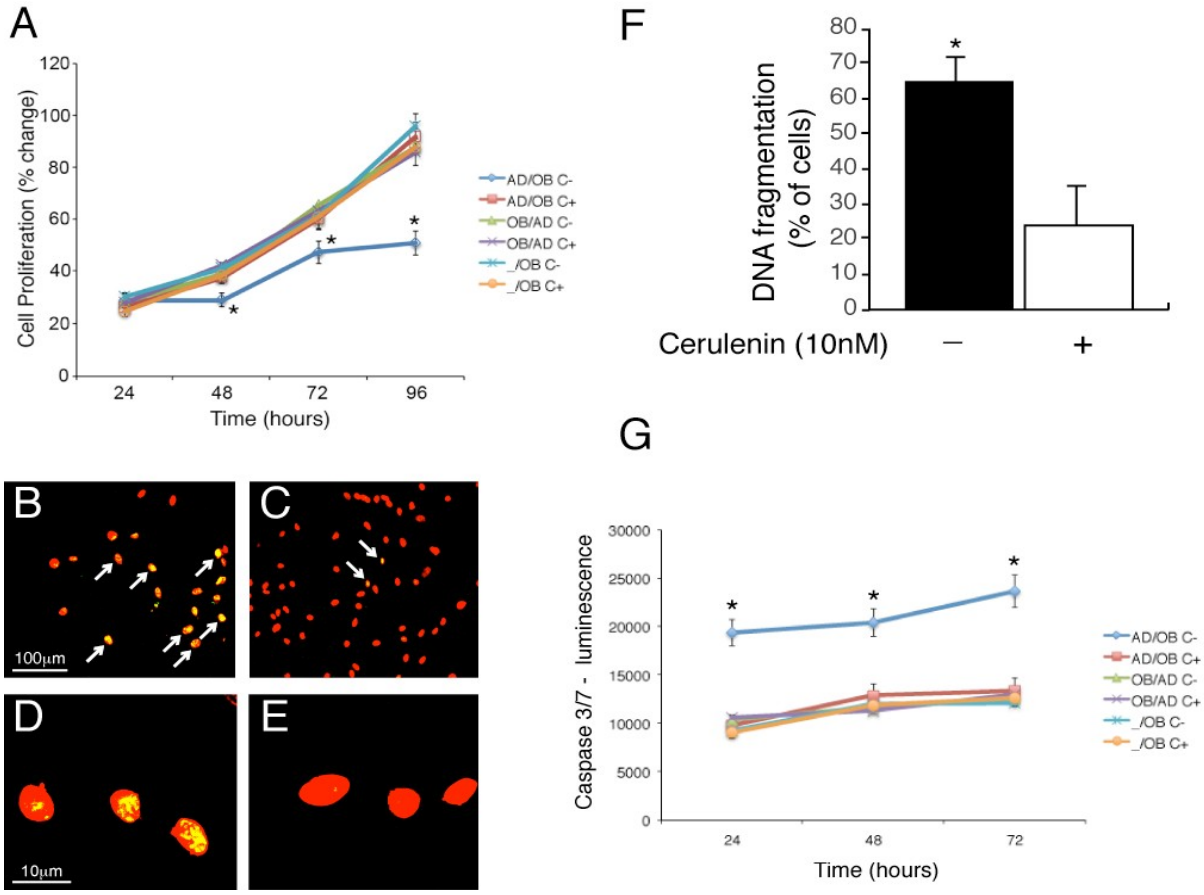

FIGURE 4.2: (A) Cerulenin prevents the inhibitory effect of adipocyte on osteoblast survival. Supernatants were collected from the osteoblasts side of the membrane in the six different conditions (AD/OB, OB/AD, -/OB with and without cerulenin). Human osteoblasts were cultured for 24 h in 96-well plates. At 24h, media was replaced with the supernatants and cell proliferation was measured at timed intervals (24-96h). Cell proliferation was significantly decreased in osteoblasts cultured with the supernatant from the AD/OB untreated cells after 48,72 and 96 hours in culture (*p< 0.001). In contrast, osteoblasts exposed to supernatants from all other conditions either treated or untreated with cerulenin showed a progressive increase in cell proliferation at all timed intervals.

(B-F) Inhibition of FA synthase prevents osteoblast apoptosis induced by adipocyte-secreted factors. Human osteoblasts were cultured in 2 well slides for 24 hours. After 24 hours, media was replaced with media obtained from normal confluent human adipocytes treated with either cerulenin (10nM) or vehicle alone. After 72 hours in culture, media was removed and cells showing apoptotic cells were identified (B-E) and quantified (F) using TUNEL assay. The TUNEL assay was able to detect a higher percentage of cells with DNA fragmentation (arrows) in the NHOst treated with supernatants obtained from untreated (B,D and F) as compared with cerulenin-treated (C,E and F) adipocytes (F)(*p< 0.01). (B and C: scale bar 100 µm), (D and E: 10 µm).

(G) Caspase activity

Caspase-3 and -7 activity was assayed using the Caspase-Glo luminescence assay and data represent the mean values (SD) of triplicate cultures. NHOst treated with supernatants obtained from untreated adipocytes (AD/OB-) showed higher caspase 3/7 activity as compared with cerulenin-treated adipocytes (*p< 0.001).

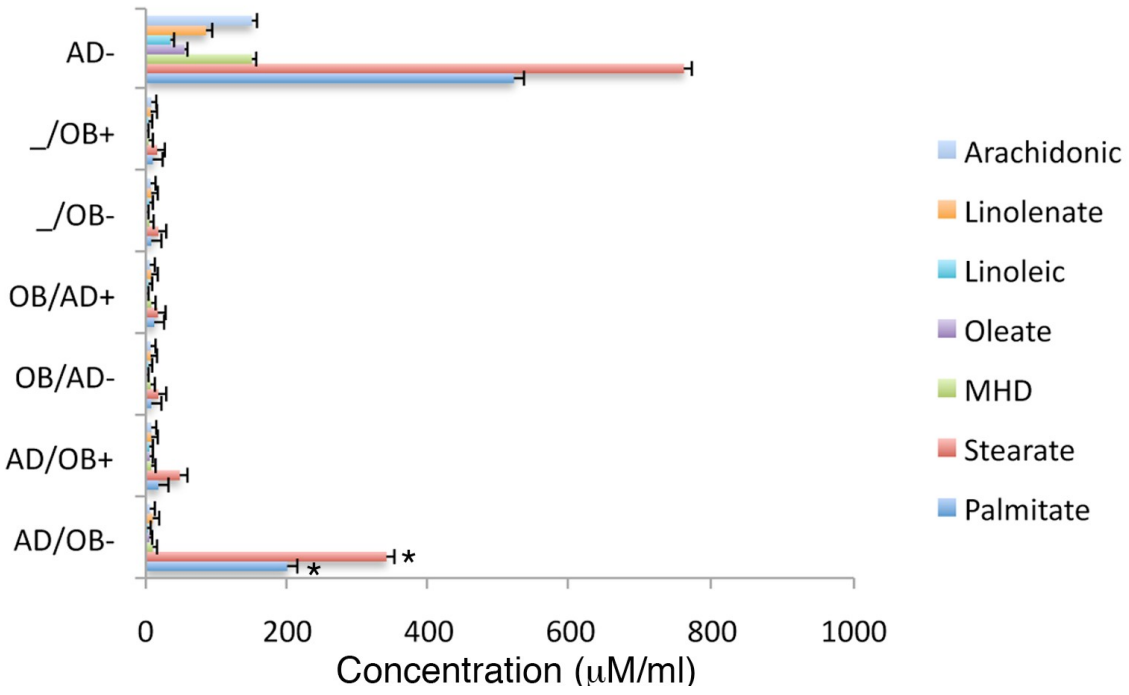

FIGURE 4.3: Fatty acid composition

Fatty acid composition of supernatants collected from the osteoblast side in co-culture with human adipocytes treated with either cerulenin or vehicle alone and analyzed by GC/MS showed the presence of seven fatty acids. In addition, a significantly higher amount of two FA, palmitate and stearate, was found in the supernatants obtained from the AD/OB condition treated with vehicle alone (*p< 0.01; ** p<0.001 for untreated AD/OB vs. all other conditions). The levels of these two FA were significantly reduced after treatment with cerulenin (□p<0.001). MHD: Methylhexadecanoic acid

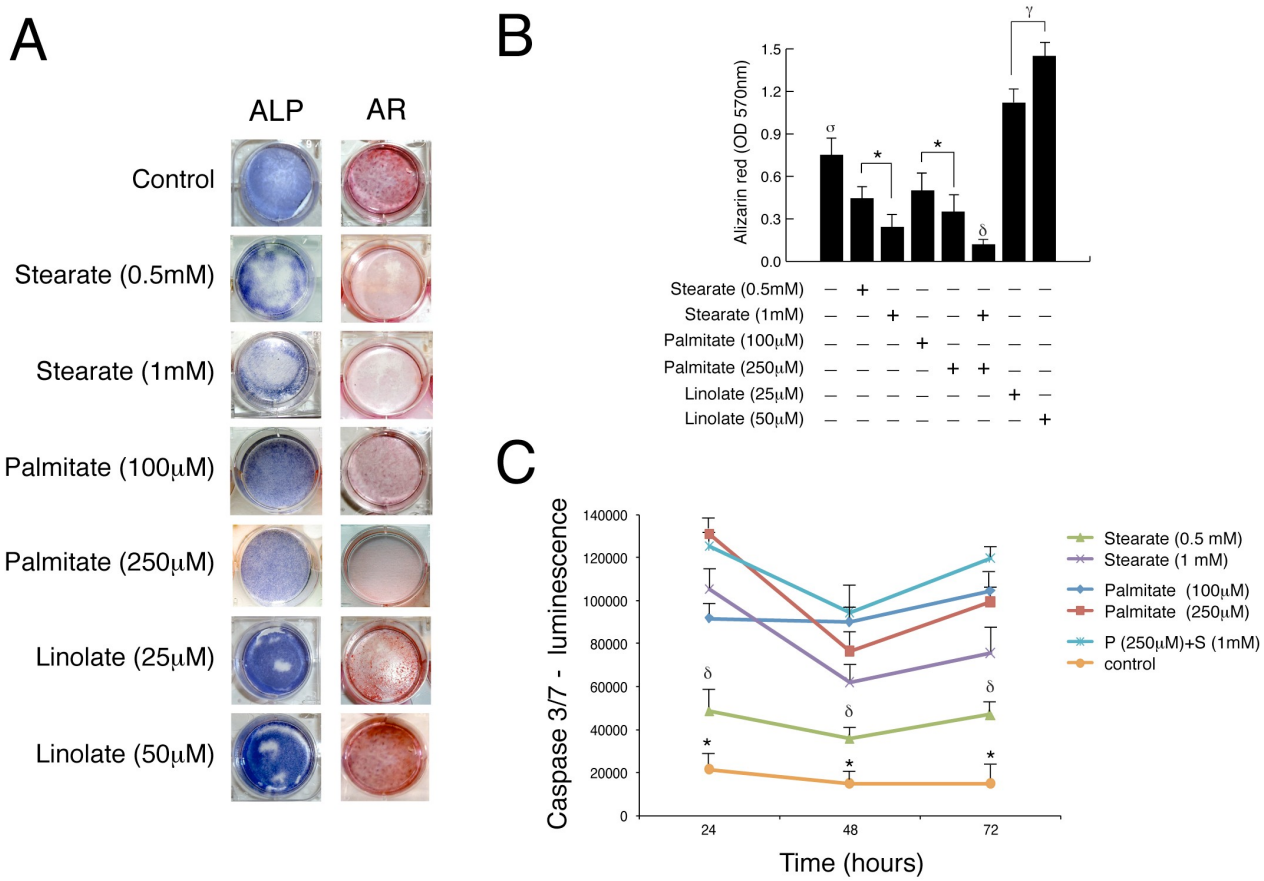

FIGURE 4.4: Stearate and palmitate affect osteoblasts function and mineralization.

Osteoblasts treated with either stearate or palmitate showed a significant reduction in alkaline phosphatase (ALP) (A, left panels) and alizarin red (AR) (A, right panels and B) as compared with both untreated and linolate -treated cells. Additionally, linolate-treated osteoblasts showed higher function and mineralization than untreated osteoblasts. Finally, combination of both FA was found to potentiate their negative effect on mineralization (B, δ $p<0.001$ vs. all other conditions). * $p<0.001$ for dose dependent effect**;** **$p<0.01$ untreated cells vs. FA treated cells; γ $p<0.01$ for linolate dose-dependent effect; σ $p<0.01$ for control vs. all other conditions.

(C) Caspase-3/7 activity was assayed using the Caspase-Glo luminescence assay and data represent the mean values (SD) of triplicate cultures. NHOst treated with either stearate or palmitate showed higher caspase 3/7 activity as compared with untreated cells (*$p< 0.001$). δ $p<0.01$ Stearate (0.5mM)vs. all other conditions

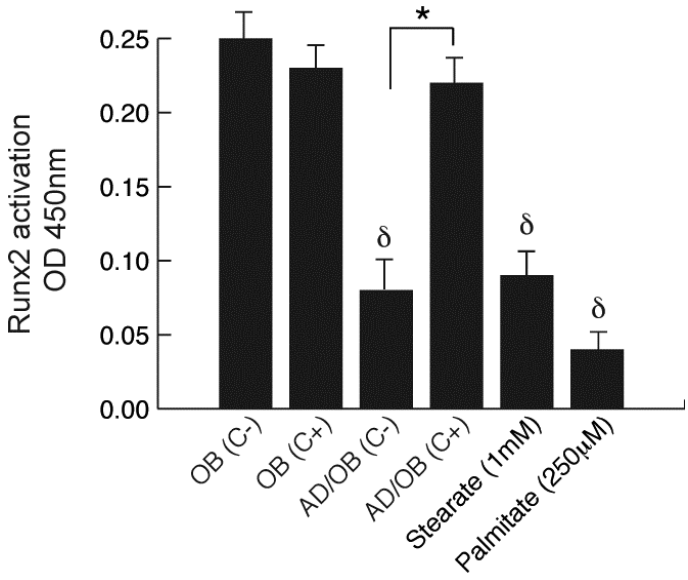

FIGURE 4.5: Effect of adipocyte-secreted factors on Runx2 nuclear-binding activity in human osteoblasts. Runx2 DNA-binding activity was determined using ELISA-based Runx2 activation kit and quantified by colorimetry. Osteoblasts exposed to supernatants obtained from adipocytes as well as treated with stearate and palmitate showed a significantly lower activity of the Runx2 nuclear complex in the nuclei as compared with controls. This effect was reverted by addition of cerulenin into the adipogenic media. Finally, addition of cerulenin into osteogenic media did not show a direct effect on the complex in normal osteoblasts. Values are mean ± SEM of 6 wells per group in three independent experiments; $^{*}p < .001$ cerulenin treated vs. matched untreated cells. δ $p<0.01$ for cells exposed to adipogenic factors vs., control (-/OB).

Effect of Inhibition of FA Synthase on Adipocytes Differentiation in Co-cultures:

The initial studies evaluated the effect of the fatty acid synthase inhibitor cerulenin on NHOst adipogenesis. As shown in figure 1A (upper panels), addition of cerulenin to the adipogenic medium did not affect the preadipocytes' capacity to differentiate and produce fat droplets. Quantification of oil red O showed no differences between cerulenin treated vs. untreated differentiating adipocytes (data not shown).

Inhibition of FA Synthase Prevents the Inhibitory Effect of Adipocytes on Osteoblast Function: NHOst were plated in co-culture with human pre-adipocytes induced to undergo adipogenesis in the absence or presence of the FA synthase inhibitor cerulenin (10nM) or vehicle alone. After three weeks in co-culture, osteoblasts were stained with ALP and alizarin red to identify function and mineralization respectively. As shown in Figure 4.1A and B, both osteoblast function and mineralization were significantly inhibited by the presence of adipocytes in the co-culture. This inhibitory effect was prevented by addition of cerulenin (10nM) to the media ($p< 0.001$). Furthermore, expression of Runx2 and OCN was significantly reduced by the presence of adipocytes in the apical side of the co-cultures (Fig 4.1 C and D) ($p< 0.001$). Finally, this reduction in Runx2 and OCN expression was prevented by addition of cerulenin to the media in the AD/OB condition ($p<0.001$).

Inhibition of FA Synthase Prevents the Inhibitory Effect of Adipocytes on Osteoblast Proliferation*:* Supernatants were collected from the osteoblast side of the membrane in the six different conditions (AD/OB, OB/AD, -/OB with and without cerulenin). NHOst were cultured for 24 h in 96-well plates. At 24h, media was replaced with the respective conditioned media and cell proliferation was measured at timed intervals (24-96h) as previously described (Duque, El Abdaimi et al. 2004) . Cell proliferation was significantly decreased in osteoblasts cultured with the supernatant from the AD/OB condition in the absence of cerulenin after 48, 72 and 96 hours in culture ($p< 0.001$)(Fig. 4.2A). In contrast, osteoblasts exposed to supernatants from all other conditions either treated or untreated with cerulenin showed a progressive increase in cell proliferation at all timed intervals (Fig. 4.2A).

Inhibition of FA Synthase Prevents Osteoblast Apoptosis Induced by Adipocyte-secreted Factors: NHOst were cultured in 2 well slides for 24 hours. After 24 hours, media was replaced with conditioned media obtained from normal confluent differentiated human adipocytes treated with either cerulenin (10nM) or vehicle alone. After 72 hours in culture, media was removed and

the number of apoptotic cells was quantified using TUNEL assay. As shown in figure 4.2B-F, osteoblasts cultured in media obtained from untreated adipocytes showed significantly higher percentage of DNA fragmentation (Fig. 4.2 B,D and F) than osteoblasts grown in media obtained from cerulenin-treated cells (Fig. 4.2 C,E and F)($p< 0.01$). Finally, no difference in the percentage of DNA fragmentation (± 8%) was found in NHOst maintained in routine culture medium and treated with either cerulenin or vehicle alone in the absence of adipocyte media.

Furthermore, we determined caspase 3/7 activity as these are important indicators of the caspase-activated apoptosis. As shown in figure 2G, osteoblasts grown in media obtained from the osteoblast side of the untreated AD/OB condition showed higher concentrations of caspase 3/7 activity than all the other conditions ($p<0.001$). This effect was reverted by addition of cerulenin to the media ($p<0.001$).

Identification of Changes in FA After Treatment with Cerulenin*:* Supernatants were collected from the both sides of the membrane in the six different conditions (AD/OB, OB/AD, -/OB with and without cerulenin). GC/MS analysis of all supernatants identified the presence of seven FA (Figure 4.3). However, palmitate and stearate represented 98% of all FA identified ($p<0.001$) in the supernatants. In addition, supernatants obtained from the basal chamber of the AD/OB condition showed significantly higher levels of palmitate and stearate as compared with the osteoblastic side of all the other conditions (Fig. 4.3, $p<0.001$). Furthermore, the levels of these two FA were significantly reduced to basal levels after treatment with cerulenin in the AD/OB condition (Figure 4.3, $p<0.001$).

Stearate and Palmitate Affect Osteoblasts Function and Survival: As shown in Figure 4.4, osteoblasts treated with stearate and palmitate, the predominant FA found in the supernatants, showed a significant and dose-dependent reduction in function (ALP) (Fig. 4.4A) and mineralization (AR) (Fig. 4A and B) as compared with both untreated and linolate-treated cells

($p<0.01$). Lower concentrations of palmitate and stearate showed minimal toxicity (data no shown). Additionally, linolate-treated osteoblast showed higher function and mineralization than untreated osteoblasts (Fig. 4.4A and B, $p<0.01$). Furthermore, the inhibitory effect on mineralization was potentiated by combination of both palmitate and stearate FA in the media (Fig. 4.4B). Finally, higher levels of caspase 3/7 activity were found after addition of either palmitate or stearate into the media (Fig. 4C, $p<0.01$).

Runx2 Transcriptional Activity in Osteoblasts is Affected by the Presence of Adipocyte-secreted Factors: We tested the effect of all lipotoxic conditions on Runx2 nuclear complex binding. As shown in Figure 5, ELISA analysis of Runx2 complex binding showed a significant reduction in DNA binding activity in nuclear extracts obtained from osteoblasts exposed to supernatants from the basal chamber in the AD/OB condition as well as osteoblasts treated with either stearate and palmitate as compared with control (-/OB) ($p<0.001$). The additional presence of cerulenin under the AD/OB condition reverted this inhibitory effect ($p<0.001$). Finally, treatment of NHOst with cerulenin did not affect Runx2 nuclear binding as compared with untreated cells.

4.4. Discussion

In this study, we have demonstrated that the toxic effect of adipocyte-derived factors on osteoblasts *in vitro* not only includes a reduction in proliferation and function but also increasing levels of apoptosis. Additionally, we have found that this lipotoxic effect could be reverted by inhibition of FA biosynthesis. Finally, we have identified stearate and palmitate as the predominant FA secreted by human adipocytes that can account for the toxic effect of adipocytes in our model.

In our experiments, we exposed NHOst to human pre-adipocytes obtained from human subcutaneous fat (DeLany, Floyd et al. 2005). Based on previous evidence indicating that FA

could be responsible of the lipotoxic effect of fat cells on osteoblasts *in vitro* (Maurin, Chavassieux et al. 2002), and considering that differentiating pre-adipocytes release high levels of FA, we tested whether inhibition of FA biosynthesis by cerulenin could prevent this lipotoxic effect of differentiating adipocytes. First, we looked at the direct effect of cerulenin on NHOst *in vitro*. No direct effect of cerulenin was found on either the markers of osteoblast function or survival. Subsequently we tested whether, as in previous reports (Schmid, Rippmann et al. 2005), the action that we observed after addition of cerulenin into the media was simply a reduction of the adipogenic process due to the inhibition of adipogenesis. We found that adipogenesis and production of fat droplets were not affected by the presence of cerulenin whereas it was able to inhibit the secretion of FA by differentiating adipocytes. This could be explained by the dose used in our co-cultures, which was lower than the dose previously reported (Schmid, Rippmann et al. 2005).

Furthermore, we found that in agreement with previous studies(Maurin, Chavassieux et al. 2000), the presence of adipocytes in co-cultures with osteoblasts affects the capacity of the latter to proliferate and function. Furthermore, we extended these observations by assessing the potential mechanism and by attempting to reverse this lipotoxic effect. Our results indicate that the presence of fatty acid synthase inhibitor, cerulenin, reverted this negative effect as suggested by a recovery in the levels of osteoblasts proliferation, expression of Runx2, OCN, and ALP expression, and mineralization capacity.

The toxicity of lipids, or lipotoxicity, and specifically lipid-induced apoptosis or lipoapoptosis, is a potential mechanism of several diseases such as non-alcoholic steatohepatitis (Malhi, Bronk et al. 2006) and type II Diabetes Mellitus (El-Assaad, Buteau et al. 2003). The common apoptotic pathways activated by the presence of ectopic fat include the activation of caspase 3/7 and ceramide (Malhi, Bronk et al. 2006), (Unger and Orci 2002). In the case of bone, osteoblast apoptosis plays an important role in the pathophysiology of age-related bone loss (Weinstein and

Manolagas 2000). However, the mechanisms that trigger osteoblast apoptosis remain unclear. Therefore, we hypothesized that fat infiltration could play a role in this process, thus constituting a new type of lipoapoptosis. To test this hypothesis, we determined the apoptotic changes (DNA fragmentation) as well as the activation of caspase 3/7 in osteoblasts grown in adipocyte-conditioned medium. We found that osteoblasts exposed to adipocyte-conditioned medium showed a significantly higher prevalence of apoptotic changes and higher levels of caspase 3/7 activation. As in our previous experiments, cerulenin reversed these effects suggesting that the secretion of FA could be the mechanism accounting for lipoapoptosis in bone.

Furthermore, we attempted to identify the FA that could play a pivotal role in this lipotoxic effect. We found that palmitate and stearate are not only the predominant FA present in the lipotoxic media but also that these two FA significantly affect osteoblast function and survival. Interestingly, these two FA have been shown not only to have a toxic effect on several cell types including hepatocytes (Malhi, Bronk et al. 2006) , beta cells (El-Assaad, Buteau et al. 2003), and osteoblasts (Salari, Rezaie et al. 2008), but also in the particular case of palmitate, it has been reported as one of the most prevalent FA secreted into the bone marrow (Deshimaru, Ishitani et al. 2005) as well as a potent inducer of apoptosis in osteoblasts (Kim, Ahn et al. 2008). In agreement with previous studies looking at lipotoxicity in other cell models (Malhi, Bronk et al. 2006; Kim, Ahn et al. 2008) both, palmitate and stearate showed a lipotoxic effect in our model through the induction of apoptotic changes and the activation of caspases 3/7.

Recently, Cornish et al (Cornish, MacGibbon et al. 2008) have reported that FA could have a stimulatory effect on thymidine incorporation by mature osteoblasts, therefore having a positive effect on osteoblast function. However, their conclusions are hard to compare with our results not only because their cell model is considerably different but also because higher concentrations of FA were less consistent in their effect.

The significance of our findings goes beyond the concept of lipotoxicity. In contrast to pancreas where fat infiltration affects the function of normal beta cells without involving changes in cell differentiation (Unger and Orci 2002), bone adipocytes and osteoblasts share the same precursor, indicating that increasing bone marrow adipogenesis not only happens at expense of osteoblast differentiation but also could have a direct inhibitory effect on osteoblast differentiation through the inhibition of critical transcription factors for osteoblastogenesis (Diascro, Vogel et al. 1998). To assess this potential mechanism, we looked at the effect that the presence of adipocyte-secreted factors may have on Runx2-nuclear binding. Our results indicate that the presence of either differentiating adipocytes or FA in the same milieu than mature osteoblasts affects Runx2 nuclear binding therefore affecting the expression of osteogenic proteins downstream the Runx2-activated complex, such as OCN, constituting an additional mechanism of age-related bone loss.

In summary, in this study we have successfully reversed the lipotoxic effect of adipocytes on osteoblasts through the inhibition of FA synthase. In addition, we have not only identified the predominant FA that could explain the toxic effect of fat on bone cells in our particular model but also we have proposed a mechanism of lipotoxicity in bone. Although this toxic effect could also be exerted through either the induction of peroxisome proliferator activator receptor gamma (PPARγ)-related pathways (Cornish, MacGibbon et al. 2008) or the activation of other pro-apoptotic factors in the osteoblasts, further studies looking at the potential activated pathways should be pursued. Likewise, the use of FA synthase inhibitors as a potential therapeutic approach for senile osteoporosis merits further investigation.

4.5. Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research, the Nepean Medical Research Foundation, and the Pennington Biomedical Research Foundation. Dr. Duque holds a Fellowship from the University of Sydney-Medical Research Foundation.

Transition to Chapter 5

In the "Inhibition of Fatty Acid Biosynthesis Prevents Adipocyte Lipotoxicity on Human Osteoblasts *In Vitro",* we showed that the in vitro toxic effect of adipocytes not only included a reduction in osteoblast mineralization and differentiation but also increased levels of osteoblast apoptosis. The later effect was showed by an increase in DNA fragmentation, observed in TUNEL assays as well as through increased levels of caspase 3/7 activation. Furthermore, our group identified two saturated FA as inducers of this lipotoxic effect. Available data already showed the increased presence of oxidized fats in the aging marrow (Lecka-Czernik, Moerman et al. 2002); as well as the ability of saturated fats to negatively affect BMD in men (Corwin et al , 2006). Further corroborating our results

The in vitro data collected in chapter 4 suggests inhibiting FA biosynthesis for the treatment of osteoporosis. Moreover, the accumulated data in both chapters 3 and 4 demonstrate that bone marrow fat could be a mediator of osteoporosis as it has become increasingly evident that bone marrow fat participates in the age associated bone weakening process. However, the link between the wholesome aging process and marrow fat infiltration remains unknown.

Since there is a clear correlation between marrow fat and low levels of E2, we were interested in identifying a potential link between aging, estrogen deprivation and MSC differentiation within the bone marrow. In fact, the regulation of bone marrow fat by E2 is the topic of chapter 5. In the "*Effect of Estrogens on Bone Marrow Adipogenesis and Sirt1 in Aging C57BL/6J Mice"* our group shows that estrogen mediate their regulation of adipogenesis through a Sirt1 mediated pathway.

Chapter 5

Effect of Estrogens on Bone Marrow Adipogenesis and Sirt1 in Aging C57BL/6J Mice

This chapter is a reproduction of the following Manuscript:**"*Effect of Estrogens on Bone Marrow Adipogenesis and Sirt1 in Aging C57BL/6J Mice*",**

Alexandre Elbaz, Daniel Rivas , Gustavo Duque

Abstract

Age-related bone loss has been associated with high levels of marrow adipogenesis. Estrogens (E_2) are known to regulate the differentiation of marrow precursors into osteoblasts. In addition, E_2 interact with other nuclear receptors (i.e. PPARγ) and their regulatory factors (Sirt1) thus regulating the differentiation of subcutaneous and visceral adipocytes. Since most of the *in vivo* studies looking at all the potential associations between nuclear receptors, E_2 and sirtuins has been performed in subcutaneous and visceral fat *in vitro*, in this study, we compared two groups of animals, young and old, and analysed the changes in marrow adipocytes and in the expression of PPARγ and Sirt1 in bone marrow fat after either oophorectomy (OVX) or oophorectomy plus E_2 (OVX+E_2) replacement. We found that absence of E_2 was associated with higher levels of PPARγ and lower levels of Sirt1 most significantly in the old group. In addition, old mice responded better to E_2 replacement in terms of reducing adipogenesis and PPARγ expression as well as increasing levels of Sirt1 expression. Our findings represent a new understanding of the role of E_2 in age-related bone loss, which could be mediated through the regulation of Sirt1 expression within the bone marrow. In addition, this evidence suggests that old individuals may show a better response to E_2 administration in terms of reverting the most important cellular changes in age-related bone loss.

5.1 Introduction

Estrogens (E_2) are known to increase bone mass through the regulation of osteoclastic activity (i.e. resorption) (Chaiamnuay and Saag, 2006). Osteoclast progenitors usually respond to E_2 through their conventional E_2 receptors thus decreasing osteoclastic activity (Zallone 2006). This process is affected during the menopause where E_2 decrease with a subsequent increase of osteoclast activity and bone loss representing the main pathophysiological mechanism of post-menopausal osteoporosis (Lund 2008).

In contrast to post-menopausal osteoporosis, age-related bone loss is due not only to high levels of resorption but also to increasing levels of bone marrow adipogenesis at expense of osteoblastogenesis thus decreasing bone formation (Duque and Troen 2008).

With aging, the progressive decline in serum levels of E_2 could explain the high levels of bone resorption seen in age-related bone loss (Raisz and Seeman 2001). Furthermore, *in vivo* studies, both in rats (Sottile et al. 2004) and humans (Syed et al. 2008), have suggested that E_2 deprivation enhances marrow fat infiltration by increasing bone marrow adipocyte number and size which could be reverted by E_2 replacement. The mechanisms explaining these findings have not been elucidated.

Among the mechanisms that have been proposed to explain increasing adipogenesis in age-related bone loss, high levels of expression of the nuclear receptor peroxisome proliferator activator gamma (PPARγ) is the most widely accepted and studied (Duque and Troen 2008; Shockley et al. 2007). Pharmacological induction of PPARγ inhibits osteoblasts differentiation while favours adipogenic conversion of precursor cells (Moerman et al. 2004). In contrast, downregulation of PPARγ *in vivo* is associated with higher bone mass (Akune et al. 2004).

Considering that both, PPARγ and E_2 receptors, belong to the nuclear binding receptors that

share similar coactivators (Kumar and Thompson 1999), the effect of E_2 on adipogenesis could be associated with their interaction with PPARγ in precursor cells. In fact, several studies have suggested an inhibitory effect of E_2 on PPARγ (Foryst-Ludwig et al. 2008) therefore reducing adipogenesis. This effect has been tested using several types of E_2 and E_2-like compounds (Foryst-Ludwig et al. 2008; Liao et al. 2008).

Among these compounds resveratrol is a phytoestrogen with both a potent inhibitory effect on adipogenesis (Rayalam et al. 2008) and a stimulatory effect on osteoblastogenesis (Backesjo et al. 2006). The effect of resveratrol on mesenchymal stem cells differentiation is exerted through the activation of the nuclear NAD-dependent protein deacetylase Sirt1 (Backesjo et al. 2006). Interestingly, Sirt1 activation inhibits the action of PPARγ and adipocyte differentiation in subcutaneous and visceral fat (Picard et al. 2004). However, the role of Sirt1 in bone marrow adipogenesis *in vivo* remains unknown.

Since most of the *in vivo* studies looking at the potential association between nuclear receptors, E_2 and sirtuins has been performed in subcutaneous and visceral fat *in vitro*, in this study, we compared two groups of animals, young and old, and analysed the changes in the expression of PPARγ and Sirt1 in bone marrow fat after either oophorectomy (OVX) or oophorectomy plus E_2 (OVX+E_2) replacement. We found that absence of E_2 was associated with higher levels of PPARγ and lower levels of Sirt1 most significantly in the old group. In addition, old mice responded better to E_2 replacement in terms of reducing adipogenesis and PPARγ expression as well as increasing levels of Sirt1 expression.

Our findings represent a new understanding of the role of E_2 in age-related bone loss, which could be mediated through the regulation of Sirt1 expression within the bone marrow. In addition, this evidence suggests that old individuals may show a better response to E_2 administration in terms of reverting the most important cellular changes in age-related bone loss.

5.2 Experimental Methods

Animal tissue preparation: All protocols were approved by the McGill University Animal Care Utilization Committee and carried out in accordance with the requirements of the Canadian Council on Animal Care. Young (5 months) and old (22–24 months) female C57BL/6J mice (Jackson Labs, Bar Harbor, Maine) were either gonadally-intact, OVX or OVX and E_2 (estratien-3,17-β-diol Sterloids Inc., Witten, NH, USA) treated (OVX+E_2). Mice were maintained in an isolated, pathogen-free colony with a 12-h light/dark photoperiod and food and water ad libitum. In normal animals, reproductive cyclicity patterns were established by daily vaginal smears. Young intact mice showed all stages of the reproductive cycle and old intact mice were in persistent diestrus. Surgery was performed during diestrus and tissue collected as previously described (Duque et al. 2004). Under anesthesia with ketamine (8.5 mg/100 *g*) and xylazine (0.3 mg/100 *g*) *i.m,* bilateral OVX surgery was performed using a posterior surgical approach and 5 mm silastic capsules containing E_2 crystals mixed in silastic adhesive were then implanted subcutaneously (s.c.) in the nape of the neck for OVX+E_2 animals. These capsules produce mean circulating levels of E_2 of 12 to 18 ng/ml, i.e. typical of diestrus animals, whereas levels of estradiol for OVX animals were undetectable. The completeness of gonadectomy and efficiency of E_2 treatment was determined by the appearance of vaginal cytological smears exhibiting the absence or presence of mature epithelial cells, respectively. Post-mortem uterus weight was taken as an index of the effect of E_2 deprivation (OVX) or replacement (E_2 treatment) and was significantly reduced ($p < 0.05$) in OVX group (44 ± 1 mg, mean ± s.e.m) compared to intact (76 ± 2 mg) or OVX+E_2 mice (64 ± 3 mg).

Six treatment groups were evaluated. a) Normally cycling (young – 5 months, n=6; old – 24 months, n=6) (intact): b) OVX (young – 5 months, n=6; old – 24 months, n=6): c) OVX treated

with 17 β Estradiol (young – 5 months, n=6; old – 24 months, n=6). Animals were sacrificed by decapitation. Limbs were placed in 4% paraformaldehyde for further histology analysis.

Decalcification of tissues: EDTA-glycerol (EDTA-G) solution was prepared as previous described [27]. 14.5 g EDTA, 1.25 g NaOH, and 15 ml glycerol were dissolved in distilled water and the pH was adjusted to 7.3. The solution was then made up to 100 ml and stored at 5ºC.

After fixation, specimens were serially washed for 12 hours at 5ºC in each of the following solutions: 0.01M PBS containing 5% glycerol, 0.01 M PBS containing 10% glycerol, and 0.01 M PBS containing 15% glycerol. The specimens were then decalcified in EDTA-G solution for 10-14 days at 5ºC. This EDTA-G solution was replaced every 5 days. Progression of decalcification was checked by X-ray. To remove EDTA and glycerol from the decalcified tissues, they were washed at 5C for 12h successively in 15% sucrose and 15% glycerol in PBS; 20% sucrose and 10% glycerol in PBS; 20% sucrose and 5% glycerol in PBS; 20% sucrose in PBS; 10% sucrose in PBS; 5% sucrose in PBS and 100% PBS as previously described (Duque et al. 2004a).

Adipocyte quantification: For adipocyte quantification, the right femur was cleaned of soft tissue, fixed for 36 h in 4% paraformaldehyde, rinsed thoroughly in PBS, decalcified, and processed for paraffin embedding. Serial 4-μm sections cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Richmond Hill, Ontario, Canada) were deparaffinized in xylene and immersed in 2 changes of propylene glycol before staining with haematoxylin/eosin to quantify adipocyte number as described previously (Duque et al. 2004a). Adipocyte number was measured by tracing out individual adipocytes in all the fields analyzed; adipocytes appear as distinct, translucent, yellow ellipsoids in the marrow cavity. All measurements were done at a 40X magnification using the Bioquant image analysis software (Bioquant Corp. Nashville, TN, USA. Photomicrographs were captured at a 40X magnification.

Detection of PPARγ and Sirt1 by Immunofluorescence: After decalcification, bone samples were embedded in low-melting-point paraffin in a Shandon Citadel 2000 automatic tissue

processor (Shandon Scientific Limited, Runcorn, UK). Coronary and transverse sections (4μ) were made for the epiphyseal parts and the shaft, respectively. Sections were mounted on silane-coated glass slides (Fischer Scientific, Springfield, NJ, USA) and paraffin was removed with three washes of xylene and rehydrated with ethanol washes (80-50-30%) and PBS. Non-specific binding was blocked by addition of goat serum for 1 hour. Sections were then incubated with either goat polyclonal IgG PPARγ or mouse monoclonal Sirt1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 1.5% normal blocking serum overnight at 4°C and then incubated for 45 minutes with either phytoerythrin-conjugated mouse for PPARγ or FITC-conjugated donkey anti mouse for Sirt1 (Santa Cruz, Santa Cruz, CA, USA) diluted to 2 μg/ml in PBS with 1.5% normal blocking serum.

Quantification of fluorescence staining: Bioquant image analysis was used to analyze fluorescent images. Threshold intensities for each picture were determined and from this the number of pixels within the picture that were above the threshold intensity were calculated and considered positively stained. The positively staining pixels were expressed as a percentage of the total pixels in the picture.

Controls

In all immunofluorescence detection methods for PPARγ and Sirt1, non-immune mouse serum and mouse IgG, in place of the primary antibody, were included as controls. Three tests and three control slides were included for each variable, and runs were performed on at least three different blocks from the same bone on separate occasions.

Statistical analysis: All results are expressed as mean ± SEM of 3 replicate determinations, and statistical comparisons are based on one-way analysis of variance (ANOVA) or Student's T-test. A probability value of $p<0.05$ was considered significant.

5.3 Results

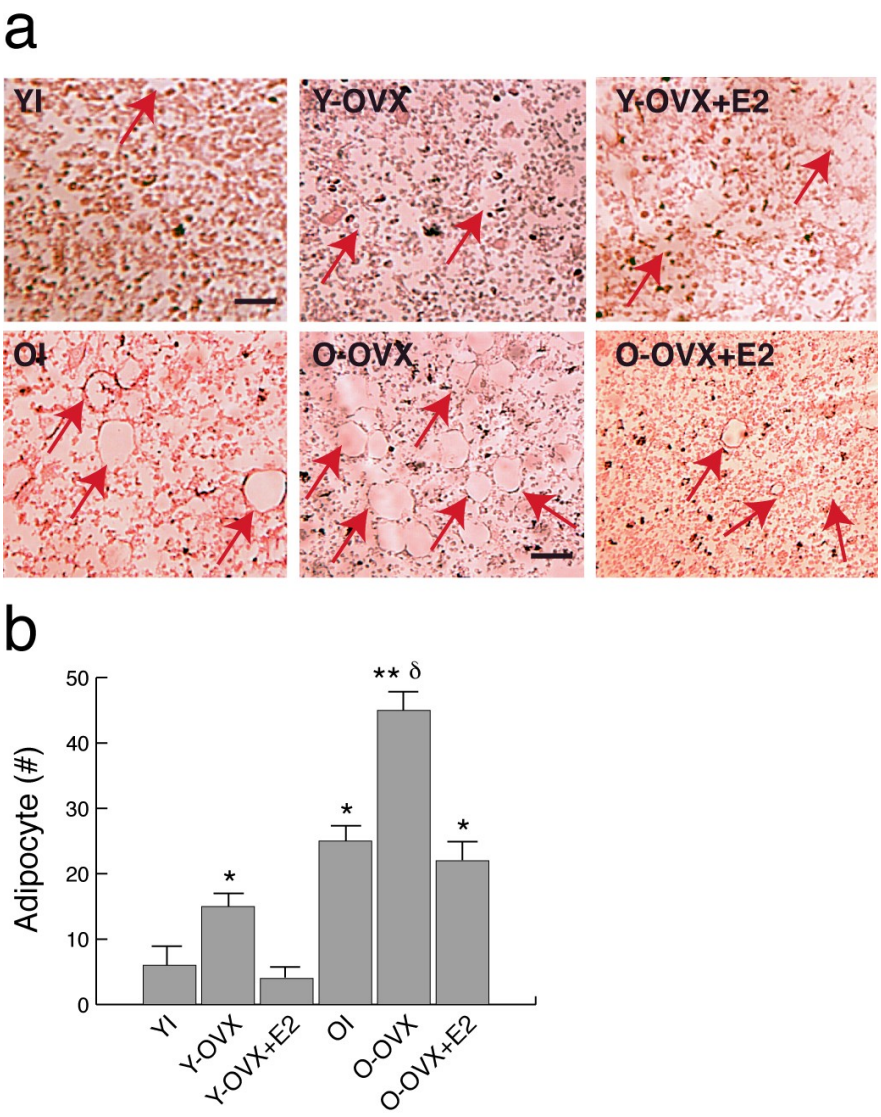

FIGURE 5. 1: Changes in Bone Marrow Fat and Adipocyte number in young and old OVX C57BL/6J mice. Sections of undecalcified bone were stained with H/E and images captured at original magnifications of 40X to evaluate fat infiltration (a, white areas, red arrows) and adipocyte number per field (b). Both, young and old intact mice, showed an age-related infiltration of bone marrow by fat with more significant infiltration happening in the old group. In addition, OVX significantly increased marrow fat infiltration in both young and old mice. This increase in marrow fat was normalized by E_2 replacement. Magnification at source was 40X. Micrographs are representative of four to six screened in each group of animals. Average of adipocyte number was quantified in 10 fields per section. Bars=50μm. *$p<0.01$ compared with young intact mice; **$p< 0.001$ compared with young intact mice, δ $p< 0.001$ compared with old

intact mice

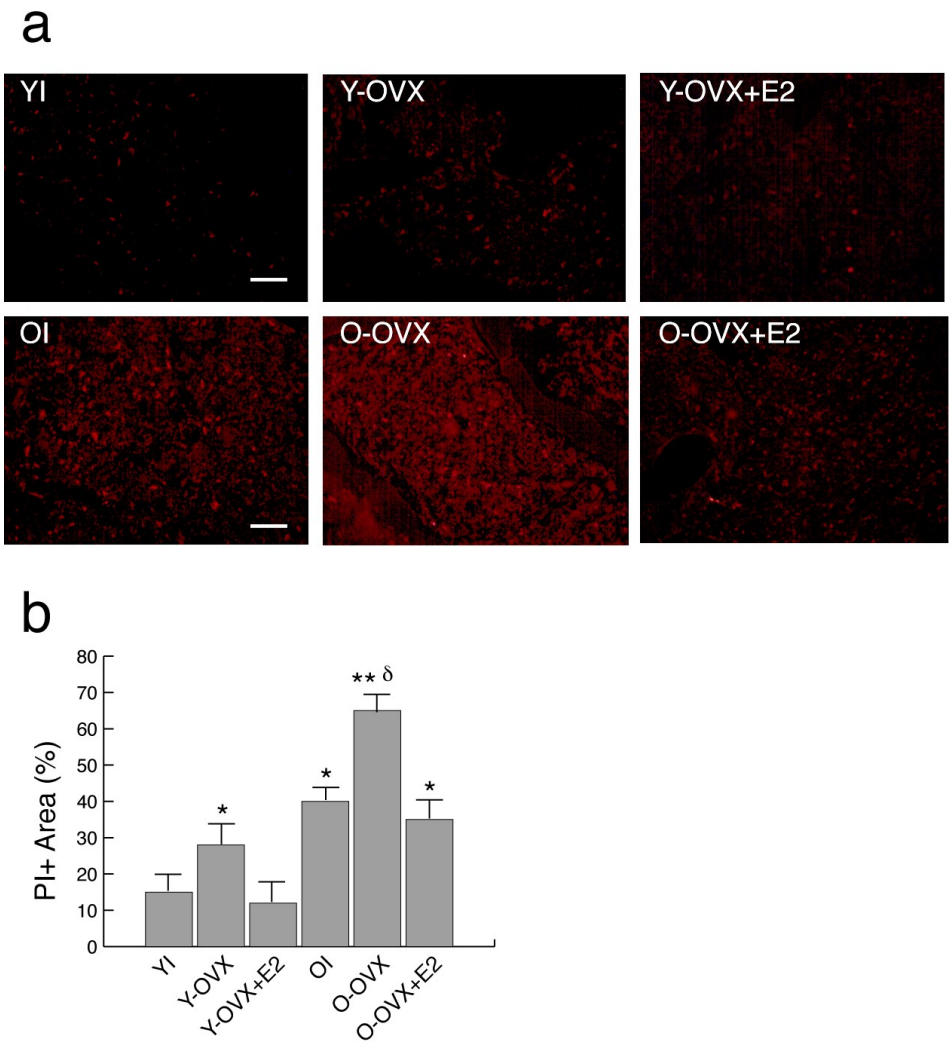

FIGURE 5.2: Changes in PPARγ expression in the bone marrow of young and old OVX C57BL/6J mice. After decalcification, bone samples were embedded in low-melting paraffin, coronary sections were made for the epiphyseal parts and the shaft respectively. Sections were treated as described in materials and methods. In both cases sections were incubated with PPARγ2 antibody (green fluorescence). Each longitudinal section was analyzed using Bioquant image analysis. The positively staining pixels were expressed as a percentage of the total pixels in the picture (b). PPARγ expression was lower in all young mice groups as compared with the old mice groups (a). Quantification of PPARγ expression by immunofluorescence showed a significant increase in bone marrow expression after OVX in both young and old mice returning to normal levels after E2 replacement. In addition, old OVX showed a significantly higher increase in PPARγ expression as compared with the young OVX mice. Bars=50μm. *$p<0.01$ compared with young intact mice; **$p< 0.001$ compared with young intact mice, δ $p< 0.001$ compared with old intact mice

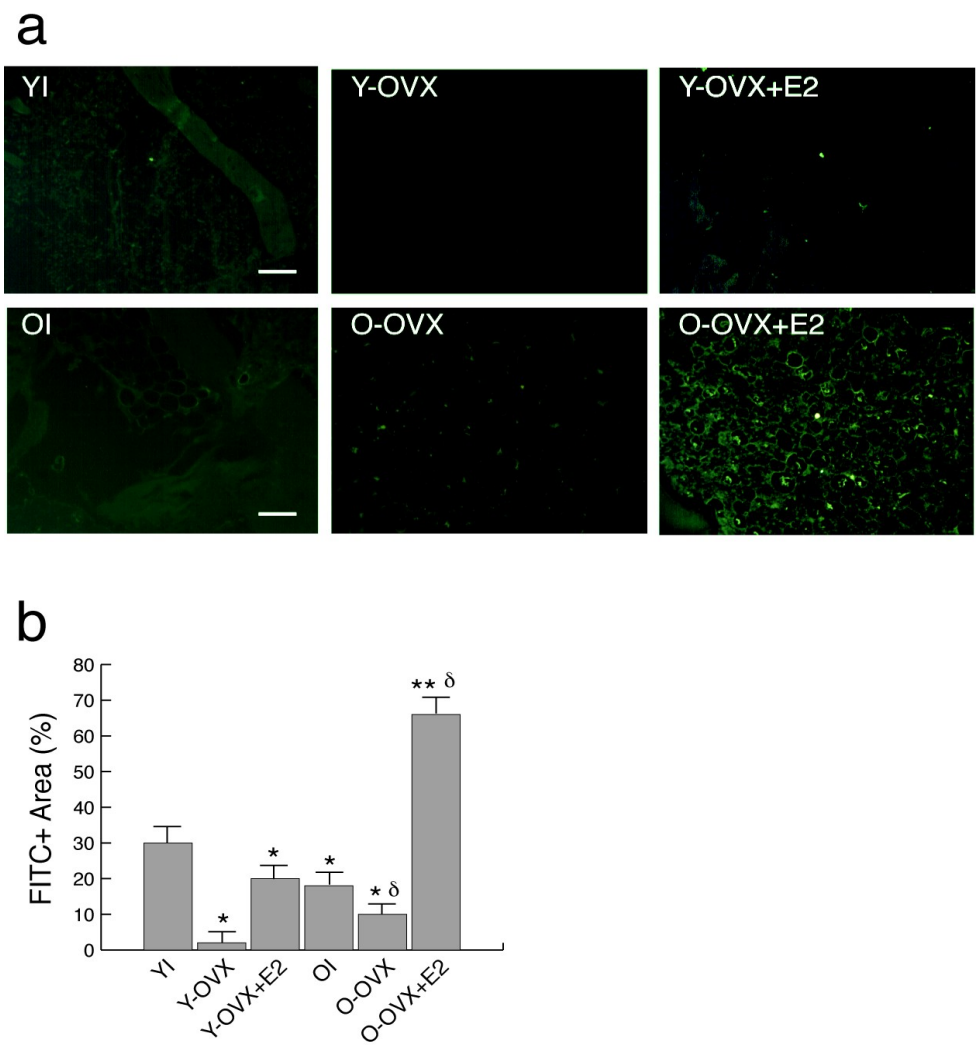

FIGURE 5.3: Changes in Sirt1 expression in the bone marrow of young and old OVX C57BL/6J mice. After decalcification, bone samples were embedded in low-melting paraffin, coronary sections were made for the epiphyseal parts and the shaft respectively. Sections were treated as described in materials and methods. In both cases sections were incubated with Sirt1 antibody (red fluorescence). Each longitudinal section was analyzed using Bioquant image analysis. The positively staining pixels were expressed as a percentage of the total pixels in the picture (b). Sirt1 expression was higher in young intact mice groups as compared with the old intact group (a). Quantification of Sirt1 expression by immunofluorescence showed a significant decrease in bone marrow expression after OVX in both young and old mice returning to normal levels after E2 replacement. In addition, old OVX showed a significantly higher increase in Sirt1 expression after E_2 replacement as compared with the young mice. Bars=50μm. *$p<0.01$ compared with young intact mice; **$p< 0.001$ compared with young intact mice, δ $p< 0.001$ compared with old intact mice**.**

Intact aging mice showed a significantly higher adipocyte number as compared with the young mice.In both young and old mice, OVX induced higher levels of adipocytes number as compared

with their intact controls. In both young and old OVX mice, E_2 replacement induced a significant reduction in adipocyte number ($p<0.01$). This reduction was significantly higher in the old OVX+E_2 group ($p<0.001$).PPARγ expression was lower in all young mice groups as compared with the old mice groups. Quantification of PPARγ expression by immunofluorescence showed a significant increase in bone marrow expression after OVX in both young and old mice (Fig. 2, $p<0.01$) returning to normal levels after E_2 replacement. Furthermore, in contrast with PPARγ expression, OVX induced a significant reduction in Sirt1 expression in both young and old mice (Figure 3). In addition, young intact mice showed higher levels of Sirt1 than old intact mice (Figure 3). Expression levels of Sirt1 in young and old OVX were normalized by E_2 replacement. Interestingly, E_2 replacement in old mice significantly induced a higher increase in Sirt1 expression in bone marrow of old OVX mice as compared with E_2-replaced young OVX mice.

5.4 Discussion

In this study, changes in PPARγ and Sirt1 expression were compared after either OVX or OVX+E_2 replacement in young vs. old C57BL/6J mice. Our data indicate that OVX induced a significantly higher increase in bone marrow fat in old mice as compared with young mice. In addition, the increase in marrow fat in OVX old mice correlated with higher levels of PPARγ expression within the bone marrow, which was normalized after E_2 replacement. Furthermore, Sirt1 levels within the bone marrow were significantly reduced after OVX in both young and old mice. Finally, old OVX mice showed a significantly higher increase in Sirt1 expression after E_2 replacement as compared with OVX+E_2 young mice.

The increasing levels of bone marrow adipogenesis associated wit age-related bone loss have been correlated with higher levels of expression of PPARγ by marrow cells (Duque et al. 2004b). The mechanisms that explain this age-related increase in PPARγ are a subject of intense research. Levels of ligands for PPARγ such as polyunsaturated fatty acids and eicosanoids are known to

increase with aging (Moerman et al. 2004). In the case of E_2, it is known that E_2 act as modulators of the marrow stromal precursor differentiation and therefore constitutes an important determinant factor in balancing osteoblastogenesis over adipogenesis (Hong et al. 2006).

Previous studies in young OVX rats (6 and 9 month-old) have demonstrated an increase in bone marrow adipogenesis (Sottile et al. 2004). However, the effect of OVX and E_2 on marrow adipogenesis has not been assessed in an accepted mouse model of age-related bone loss, therefore in this study we compared young (5 month) vs. very old (24 month) C57BL/6J mice. In agreement with previous studies in young rats (Sottile et al, 2004), our findings in young and old mice suggest that E_2 replacement reversed adipogenesis to baseline.

In an attempt to elucidate a potential mechanism that could partially explain the role of E_2 deficiency in increasing bone marrow adipogenesis with age, we looked at changes in Sirt1 expression within the bone marrow after both OVX and OVX+E_2. Considering that Sirt1 is a known inhibitor of PPARγ in adipocytes obtained from subcutaneous and visceral fat (Picard et al. 2004), and that Sirt1 expression is increased by the presence of E2 in *in vitro* models (Rasbach and Schnellmann 2008), it is tempting to hypothesize that high levels of PPARγ after OVX would correlate with low levels of Sirt2, which would be corrected by E_2 replacement.

In our model, OVX induced both high levels of marrow adipogenesis and PPARγ expression in bone marrow of both young and old mice. However, this increase was significantly higher in the old mice. A particularly interesting finding of our study is that E_2 replacement had a more significant effect in old than young animals suggesting that old individuals who receive E_2 would benefit more than young individuals in terms of inhibition of marrow adipogenesis. In fact, a recent study by Syed et al (2008) in female subjects (mean age 63 year-old) treated with E_2 reported a significant decrease in marrow adipogenesis. According with our findings, older

subjects would benefit more of E_2 replacement. Further studies looking at this effect in older populations should be pursued.

Although our data is unable to establish a direct causal relationship between high levels of PPARγ and low levels of Sirt1 after OVX, we provide evidence correlating the changes seen in Sirt1 with those seen in PPARγ expression after OVX and E_2 replacement. In addition, the dramatic recovery in Sirt1 expression after E_2 replacement in old mice suggests that E_2 replacement has a positive stimulatory effect on Sirt1 expression in bone. The significance of this finding should be studied in the future.

In summary, using an aging mouse model of OVX and E_2 replacement we have found that increasing levels of adipogenesis within the bone marrow after OVX could be associated with high levels of PPARγ and low levels of Sirt1 expression. High levels of marrow adipogenesis were normalized by E_2 replacement with a significantly higher response in the old mice.

In conclusion, E_2 play an important role in the pathogenesis and treatment of age-related bone loss. The understanding of the mechanisms of action of E_2 on bone marrow cellularity would provide us with new potential therapeutic approach to promote osteoblastogenesis at expense of adipogenesis and thus increase bone mass and prevent osteoporosis in our aging population.

5.5 Acknowledgements

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Chapter 6: General Discussion

6.1 Summary of Contributions

In this thesis we assessed the role and regulation of bone marrow fat. We demonstrated that bone marrow fat does not participate in metabolism during periods of CR ; furthermore, we showed that adipocytes and their secreted FA inhibit osteoblast differentiation and function and induce osteoblast apoptosis *in vitro*. Finally, we provided evidence that E_2could regulate fat accumulation in the marrow cavity through a Sirt1 mediated pathway. Although our work was discussed in prior sections, key ideas and results will be reiterated and further analyzed in the context of our global project.

6.1.1 Calorie Restriction: Its Effects on Bone Quality and Adiposity

In chapter 3 our group observed the effect of CR and differential protein source on bone mass and fat infiltration of the marrow cavity. Prior work in the field had shown that although CR delayed the effects of diseases associated with aging and increased lifespan of certain test animals, it caused increased bone fragility (McCay *et al*., 1939). The later detrimental effect was proposed to be due to the dietary deprivation that comes with CR (Harrison and Fraser, 1960) and reduced bone turnover (Tatsumi *et al*., 2007). The work presented in chapter 3 confirmed theses effects and extended the available literature by describing the effect of CR on marrow adiposity.

In agreement with the established literature (McCay *et al*., 1939), all aged rats experienced a decrease in bone quality, however, *ad libitum* soy fed rats showed a less severe decrease in bone quality. The later further confirms earlier work which reported the beneficial effects of soy isoflavones as promoters of bone quality (Khalil *et al*. 2005). Moreover, our results indicated that marrow fat infiltration occurred independently of CR when bone formation was decreased. In comparison to the two month old control, all aged rats regardless of calorie restriction showed

increased bone marrow fat infiltration. However, *ad libitum* soy fed rats expressed reduced marrow adiposity in the presence of higher bone formation (as indicated by the BV/TV in figure 3.2 I). Together, these effects support the inverse relationship between bone and fat in the marrow cavity such that fat infiltration increases when bone formation is decreased.

In summary, our results indicated that the detrimental effect of CR on bone mass could be diminished by a dietary soy protein intake. Moreover, our work shows that contrary to other fat depots, bone marrow adipocytes are not mobilized during moderate stages of starvation. Bone marrow fat does not appear to be used as an energy source. Finally our results suggest that adipocyte mobilization is independent of PPARy expression as the reduction in PPARy expression in CR soy fed rats (figure 3.7) does not correlate with a decreased number of adipocytes in the bone marrow cavity.

6.1.2. Lipotoxicity of Bone Marrow Fat

Although our work in chapter 3 supports the idea that bone marrow fat "occupies space" left empty by bone loss, it does not provide great insight on the functional role of this fat depot.

Fat infiltration of a number of organs is known to be toxic and alter cell functionalities; moreover prior work has suggested that adipocyte secretions of FA negatively alter osteogenic proliferation and differentiation (Maurin, Chavassieux et al. 2002). Our group hypothesized that within the bone marrow, adipocytes, through their release of FA, are mediators of toxicity on normal osteoblast behaviour.

To test this lipotoxic effect, we elaborated a co culture model and observed the relationship between pre-adipocytes and osteoblasts. More specifically, we looked at the effect of FA secretion on osteogenic differentiation, mineralization and survival.

Results in chapter 4 indicated that adipocyte secretions of FA have the ability to reduce osteogenic differentiation (as seen by lowered levels of alkaline phosphatase in 4.1A and decreased expression of osteogenic transcription factor, Cbfa1/RunX2, in fig.4.1C&D when FA were present in the osteoblast media) and mineralization (as seen by the decreased levels of alizarin red expression in fig.4.1B). These detrimental effects could be prevented by the introduction of cerulenin, a FA synthase inhibitor,

Earlier work had already suggested that FA could be responsible for the toxic effects of fat cells on osteogenic proliferation and maturation (Maurin, Chavassieux et al. 2002), however, the ability of FA to induce apoptotic osteoblast death is a newer concept.

Our work goes on to prove the later effect, as osteoblasts exposed to adipocyte-conditioned medium expressed a significantly higher prevalence of DNA fragmentation as well as higher levels of caspase 3/7 activation, both markers of apoptotic cell death (fig.4.3).

We extended the concept of toxicity and identified two predominant saturated FA in the lipotoxic adipocyte-conditioned media. We showed in figure 4.4 that both palmitate and stereate reduce osteogenic differentiation and mineralization (fig.4.4 A and B) and increase caspase3/7 activation, hence inducing apoptosis. Finally our work also demonstrated that the lipotoxic effect of fatty acids is exerted through Cbfa1/RunX2 nuclear binding factor thereby affecting osteoblast and bone specific markers. Although our results can be understood in the context of a co culture, they can be translated to the marrow cavity where adipocytes and osteoblasts are in close interaction.

Prior groups have reported an increased presence of oxidized fats in the aging bone marrow (Lecka-Czernik, Moerman et al. 2002). Both stereate and palmitate, oxidized fats, were shown in our studies to induce osteoblast apoptosis as well as to decrease osteogenic proliferation and maturation. Bone marrow fat could thus mediate the introduction of more fat in the marrow milieu

by disrupting osteoblasts, further allowing for fat infiltration, as fat cells would occupy space left empty by osteoblast death and lowered osteogenic proliferation.

Adding to this idea, both adipocytes and osteoblasts originate from the same mesenchymal precursor. Although we have not tested for it, bone marrow fat could potentially inhibit the expression of osteoblast promoting transcription factors through the activation of PPARy pathways, hence promoting the differentiation of stromal cells into fat at the expense of bone cells (Cornish, MacGibbon et al. 2008). Further investigations are required to determine if this toxic effect is exerted through such a pathway or through an alternative pathway that directly induces the activation of a cascade of caspases.

6.1.3 Hormone Regulation of Bone Marrow Fat

Although our work in chapter 4 suggests a lipotoxic relationship between bone and fat, the regulation of bone marrow fat remains unclear. E_2 have been reported to regulate the differentiation of marrow precursors into osteoblast (Hong et al. 2006). Moreover, estrogen deprivation increased fat infiltration of the marrow cavity (Sottile et al. 2004).We attempted in chapter 5 to identify the players involved in the estrogen regulation of bone marrow fat.

E_2 regulation of both visceral and subcutaneous fat seems to involve the interaction of E_2 with nuclear receptors and regulatory factors (Hong et al. 2006).We hypothesized that a similar interaction may dictate the E_2 regulation of bone marrow fat. We therefore attempted to identify the mechanism through which this regulation occurs by observing changes in marrow adipocytes, PPARγ and Sirt1 expression after ovariectomy with and without estrogen replacement in young vs. old C57BL/6J mice. In accordance with prior findings, our results showed that OVX mice both young and old had a significantly higher marrow fat infiltration than their normal counterparts. The effect was reverted by E2 replacement. The recovery was more significant in older mice suggesting that older patients would benefit more from E_2 replacement for the treatment of osteoporosis.

Our group also noted estrogen as a potential regulator of SIRT1. We observed that estrogen supplementation in mice correlated with lower marrow adiposity as well as lower levels of Sirt1.

The activation of Sirt1 by E_2 may be responsible for this decreased adiposity, however our results fail to conclusively prove this effect. Literature surrounding the NAD-dependent deacetylase, Sirt1, supports a mechanism through which activated Sirt1 inhibits PPARγ activity during periods of lowered caloric intake (Picard, Kurtev et al. 2004). No reports are available proving or disproving such a regulation in the marrow fat depots. Our results suggest that the regulation of bone marrow adiposity by estrogen involves Sirt1 and PPARy. Although our data does not provide sufficient proof for a causal relationship between high levels of PPARy and low levels of Sirt1 after OVX, a correlation can be suggested. From our results, one can hypothesize that estrogen replacement signals for increased Sirt1 expression which in turn would cause a decrease PPARy expression levels.

Our results provide new evidence far beyond the usual understanding of the role of E_2 supplementation in the treatment of osteoporosis. However the mechanism through which E_2 mediates its regulation of stromal differentiation still needs to be understood as it could provide a new therapeutic approach to promote osteoblastogenesis at expense of adipogenesis within the marrow milieu.

6.2. Future Work

From a clinical perspective our work suggests that the future treatment of osteoporosis should target bone marrow fat. Whether it be through the inhibition of fatty acid biosynthesis in the bone marrow or through the regulation of adipogenesis in marrow cavity, proper treatment of osteoporosis should take into consideration not only the age associated decrease in bone formation but also the increased fat accumulation. Regulating the apparent inverse relationship between the

stromal fates appears to be the missing link in the proper treatment of the geriatric disease. Our work provides two new therapeutic approaches for the treatment of osteoporosis:

1- The treatment of senile osteoporosis should capitalize on bone marrow fat as a promoter of osteoporosis rather than a marker of the disease. Inhibiting FA secretion from bone marrow adipocytes could allow the promotion of healthy bone through the normalization of osteoblastogenesis and osteoblast survival.

2- The treatment of osteoporosis could also involve E_2, regulating the stromal differentiation into the osteogenic fate through the inhibition of PPARγ activity by Sirt1.

Generally speaking knowledge on bone marrow fat is scarce, however, our work reports interesting findings which could be complemented by the experiments suggested below.

We reported in chapter 4 that the adipocyte secretions of FA cause osteoblast apoptosis, however, the effects of these findings on the bone turnover could be more complex than just allowing for the infiltration of fat in the marrow cavity. FA secreted not only induce osteoblast apoptosis but could also activate PPARγ and thus signal for more fat infiltration. In that sense fat begets fat and the infiltration of the bone marrow by fat could be promoted by two pathways; a PPARγ mediated one and one induced by the activation of a cascade of caspases signaling osteoblast death. Understanding which of the pathways is responsible for the infiltration or if a combination of the two is responsible is one of many missing links in the literature.

Another concern that arises when extending the results obtained from the co culture to the marrow cavity lies in the nature of the cells used. In the co culture system presented in chapter 4, normal human osteoblasts as well as human pre adipocytes from subcutaneous source were employed, however using bone marrow derived cells could allow the elaboration of more accurate suggestions on the behavior of cell types in the aging marrow. To further confirm the results obtained, bone

marrow mesenchymal cells could be differentiated in co culture in an attempt to develop a similar yet more complete model.

Furthermore, cerulenin was employed in the lipotoxicity study presented in chapter 4, however, clinically speaking, using C75 (FA synthase inhibitor which works through the same mechanism as cerulenin but is not toxic to human) could involve a number of nutritional implications. Thus, inhibiting the toxic release of lipids through the use of antilipolytic molecules from bone marrow adipocytes could not only be relevant, but could further simplify the lipotoxic relationship proposed. A similar co culture system using nicotinic acid (niacin, vitamin B_3) instead of cerulenin is one of the next steps necessary in the study of lipotoxicity.

Another future step in the study should include looking at the relationship between osteoclasts and adipocytes in a co culture system. Repeating the same experimental design but replacing osteoblasts by osteoclasts could provide additional information on the way lipotoxicity affects bone turnover. The available literature leads us to suspect that FA would activate PPARγ which in turn could activate osteoclastogenesis through the c-fos gene (Wan, Chong et al. 2007). However experiments need to be performed to prove such an effect.

Our results in chapter 5 suggest that a possible causal relationship exists between high levels of PPARy and low levels of Sirt1 after OVX , however much more work needs to be done before conclusively reporting such a relationship in bone marrow. A more complete study needs to be designed to determine the effect of $E_{2,}$ on Sirt1 and its subsequent effect on PPARγ expression in the bone marrow.

Finally, as more knowledge is assembled surrounding transdifferentiation of cells, a future arena for the treatment of senile osteoporosis could involve turning the existing fat in the bone marrow into osteoblasts. Studies have reported the ability of adipocytes to transdifferentiate into osteoblasts (Kuehnle and Goodell 2002), (Duque 2008). Another important consideration is that the majority

of fat cells in the bone marrow do not seem to be mature adipocytes, they appear to be ‘’Mesechymal Adipocytic Default’’ (MAD) cells. These cells are in a permanent stage of de-differentiation acting mostly as pre-adipocytes. At such a stage, the MAD cells are more susceptible to trans differentiation into osteoblasts. Moreover, from a clinical perspective, this would lower the risk of trans-differentiating unwanted fat in other organs (Kirkland et al., 2002), (Duque, 2008). More work is needed for this concept to be considered clinically relevant.

As the mechanisms through which bone marrow fat not only is regulated but exerts its lipotoxic function are unraveled, proper therapeutic approaches that will capitalize on targeting bone marrow fat should be made available for the treatment of senile osteoporosis.

Bibliography

Akter, R., D. Rivas, et al. (2008)."Effect of Lamin A/C Knockdown on Osteoblast Differentiation and Function." J Bone Miner Res.

Akune T, Ohba S, Kamekura S, et al (2004) PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 113:846-855

Ali, A. A., R. S. Weinstein, et al. (2005). "Rosiglitazone causes bone loss in mice by suppressing osteoblast differentiation and bone formation." Endocrinology 146(3): 1226-35.

Allan, E. H., K. D. Hausler, et al. (2008). "EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts." J Bone Miner Res 23(8): 1170-81.

Anderlová K, Kremen J, Dolezalová R, Housová J, Haluzíková D, Kunesová M, Haluzík (2006) The influence of very-low-calorie-diet on serum leptin, soluble leptin receptor, adiponectin and resistin levels in obese women. *Physiol Res*. 55, 277-283.

Arjmandi BH (2005) Soy isoflavones may protect against orchidectomy-induced bone loss in aged male rats. *Calcif Tissue Int.* 76, 56-62.

Bäckesjö CM, Li Y, Lindgren U, et al (2006) Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells. J Bone Miner Res 21:993-1002

Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, MacDougald OA (2005) Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci USA*. 102, 3324-3329.

Beresford, J. N., J. H. Bennett, et al. (1992). "Evidence for an inverse relationship between the ifferentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures." J Cell Sci 102 (Pt 2): 341-51.

Botolin, S. and L. R. McCabe (2006). "Inhibition of PPARgamma prevents type I diabetic bone marrow adiposity but not bone loss." J Cell Physiol 209(3): 967-76.

Canalis, E., T. L. McCarthy, et al. (1991). "Growth factors and cytokines in bone cell metabolism." Annu Rev Med 42: 17-24.

Cartwright, M. J., T. Tchkonia, et al. (2007). "Aging in adipocytes: potential impact of inherent, depot-specific mechanisms." Exp Gerontol 42(6): 463-71.

Carrington, J. L. (2005). "Aging bone and cartilage: cross-cutting issues." Biochem Biophys Res Commun 328(3): 700-8.

Castro, C. H., C. S. Shin, et al. (2004). "Targeted expression of a dominant-negative N-cadherin in vivo delays peak bone mass and increases adipogenesis." J Cell Sci 117(Pt 13): 2853-64.

Centrella, M., T. L. McCarthy, et al. (1988). "Tumor necrosis factor-alpha inhibits collagen synthesis and alkaline phosphatase activity independently of its effect on deoxyribonucleic acid synthesis in osteob last-enriched bone cell cultures." Endocrinology 123(3): 1442-8.

Chaiamnuay S, Saag KG (2006) Postmenopausal osteoporosis. What have we learned since the introduction of bisphosphonates? Rev Endocr Metab Disord 7:101-112

Chan, G. K. and G. Duque (2002). "Age-related bone loss: old bone, new facts." Gerontology 48(2): 62-71.

Coetzee, M., M. Haag, et al. (2007). "Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E(2) and parathyroid hormone on osteoprotegerin and RANKL secretion by MC3T3-E1 osteoblast-like cells." J Nutr Biochem 18(1): 54-63.

Coffman, J. A. (2003). "Runx transcription factors and the developmental balance between cell proliferation and differentiation." Cell Biol Int 27(4): 315-24.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M,

de Cabo R, Sinclair DA (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science*. 305, 390-392.

Cornish, J., A. MacGibbon, et al. (2008). "Modulation of osteoclastogenesis by fatty acids." Endocrinology 149(11): 5688-95.

Corwin R.L, Hartman J.T, Maczuga A.S, Graubard A. B, (2006), Dietary Saturated Fat Intake Is Inversely Associated with Bone Density in Humans: Analysis of NHANES III, Nutritional Epidemiology, 136, 159-165.

D'Ippolito, G., S. Diabira, et al. (2006). "Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells." Bone 39(3): 513-22.

Dang, Z. C., R. L. van Bezooijen, et al. (2002). "Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis." J Bone Miner Res 17(3): 394-405.

Das M, Gabriely I, Barzilai N (2004) Caloric restriction, body fat and ageing in experimental models. *Obes Rev*. 5,13-19.

Day, T. F., X. Guo, et al. (2005). "Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis." Dev Cell 8(5): 739-50.

DeLany, J. P., Z. E. Floyd, et al. (2005). "Proteomic analysis of primary cultures of human adipose-derived stem cells: modulation by Adipogenesis." Mol Cell Proteomics 4(6): 731-40.

Deshimaru, R., K. Ishitani, et al. (2005)."Analysis of fatty acid composition in human bone marrow aspirates." Keio J Med 54(3): 150-5.

Diascro, D.D.,Jr., R.L. Vogel, et al. (1998). "High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells." J Bone Miner Res 13(1): 96-106.

Dubois, S. G., E. Z. Floyd, et al. (2008)."Isolation of human adipose-derived stem cells from biopsies and liposuction specimens." Methods Mol Biol 449: 69-79.

Ducy, P., R. Zhang, et al. (1997). "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation." Cell 89(5): 747-54.

Duque, G. (2008). "Bone and fat connection in aging bone." Curr Opin Rheumatol 20(4): 429-34.

Duque G, Troen BR (2008) Understanding the mechanisms of senile osteoporosis: new facts for a major geriatric syndrome. J Am Geriatr Soc 56:935-941

Duque, G., K. El Abdaimi, et al. (2004). "Vitamin D inhibits Fas ligand-induced apoptosis in human osteoblasts by regulating components of both the mitochondrial and Fas-related pathways." Bone 35(1): 57-64.

Duque, G., K. El Abdaimi, et al. (2002). "Estrogens (E2) regulate expression and response of 1,25-dihydroxyvitamin D3 receptors in bone cells: changes with aging and hormone deprivation." Biochem Biophys Res Commun 299(3): 446-54

Duque G, Macoritto M, Kremer R (2004a) Vitamin D treatment of senescence accelerated mice (SAM-P/6) induces several regulators of stromal cell plasticity. *Biogerontology.* 5, 421-429.

Duque G, Macoritto M, Kremer R (2004b) 1,25(OH)2D3 inhibits bone marrow adipogenesis in senescence accelerated mice (SAM-P/6) by decreasing the expression of peroxisome proliferator-activated receptor gamma 2 (PPARgamma2). Exp Gerontol 39:333-338.

Duque G, Macoritto M, Dion N, Ste-Marie LG, Kremer R. 1,25(OH)2D3 acts as a bone forming agent in the hormone-independent senescence-accelerated mouse (SAM-P/6). Am J Physiol Endocrinol Metab. 2005 Apr;288(4):E723-30.

Duque G (2007) As a Matter of Fat: New Perspectives on the Understanding of Age-Related Bone Loss, *BoneKEy-Osteovision*, 4, 129-140.

Duque, G. and D. Rivas (2007). "Alendronate has an anabolic effect on bone through the differentiation of mesenchymal stem cells." J Bone Miner Res 22(10): 1603-11.

Eitel, K., H. Staiger, et al. (2002). "Different role of saturated and unsaturated fatty acids in beta-cell apoptosis." Biochem Biophys Res Commun 299(5): 853-6.

El-Assaad, W., J. Buteau, et al. (2003). "Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death." Endocrinology 144(9): 4154-63.

Fehrer, C. and G. Lepperdinger (2005). "Mesenchymal stem cell aging." Exp Gerontol 40(12): 926-30.

Figard H, Mougin F, Gaume V, Berthelot A (2006) Combined intervention of dietary soybean proteins and swim training: effects on bone metabolism in ovariectomized rats. *J Bone Mineral Metab*. 24, 206-212.

Forget-Richard A, Komarova S, Tremblay ML, Li W, Li A, Gao YJ, Henderson JE (2005) Ablation of the Sam68 RNA binding protein protects mice from age-related bone loss. *PLoS Genet.* 1:e74

Foryst-Ludwig A, Clemenz M, Hohmann S, et al (2008) Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. PLoS Genet

4:e1000108

Fried, S. K., D. A. Bunkin, et al. (1998). "Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid." J Clin Endocrinol Metab 83(3): 847-50.

Gallardo N, Arribas C, Villar M, Ros M, Carrascosa JM, Martínez C, Andrés A (2005) ObRa and ObRe are differentially expressed in adipose tissue in aged food-restricted rats: effects on circulating soluble leptin receptor levels. *Endocrinology.* 146, 4934-4942.

Gimble JM (1990) The function of adipocytes in the bone marrow stroma. *New Biol.* 2:304-312.

Gimble, J. M., C. E. Robinson, et al. (1996). "The function of adipocytes in the bone marrow stroma: an update." Bone 19(5): 421-8.

Gimble JM, Nuttall ME (2004) Bone and fat: old questions, new insights. *Endocrine.* 23, 183-188.

Gimble JM, Zvonic S, Floyd ZE, Kassem M, Nuttall ME (2006) Playing with bone and fat. *J Cell Biochem.* 98, 251-266.

Girard N, Ferland G, Boulanger L, Gaudreau P (1998) Long-term calorie restriction protects rat pituitary growth hormone-releasing hormone binding sites from age-related alterations. Neuroendocrinology. 68, 21-29.

Griel, A. E., P. M. Kris-Etherton, et al. (2007). "An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans." Nutr J 6: 2.

Griffith, J. F., D. K. Yeung, et al. (2006). "Vertebral marrow fat content and diffusion and perfusion indexes in women with varying bone density: MR evaluation." Radiology 241(3): 831-8.

Gross L., Gross L (2007) Mechanisms of Aging in Bone Marrow Stem Cells. PLoS Biol 5(8): e215 doi:10.1371/journal.pbio.0050215

Harrison M, Fraser R (1960) Bone structure and metabolism in calcium deficient rats. *J*

Endocrinol. 21, 197.

Holloszy JO (2006) Bone mineral density response to caloric restriction-induced weight loss or exercise-induced weight loss: a randomized controlled trial. *Arch Intern Med.* 166, 2502-2510.

Hong L, Colpan A, Peptan IA (2006) Modulations of 17-beta estradiol on osteogenic and adipogenic differentiations of human mesenchymal stem cells. Tissue Eng 12:2747-2753Imai S (2007) Is Sirt1 a miracle bullet for longevity. *Aging Cell.* 6, 735-737.

Isales, C. M., L. Min, et al. (1999). "Acetoacetate and beta-hydroxybutyrate differentially regulate endothelin-1 and vascular endothelial growth factor in mouse brain microvascular endothelial cells." J Diabetes Complications 13(2): 91-7.

Ito, Y. (1996). "Structural alterations in the transcription factor PEBP2/CBF linked to four different types of leukemia." J Cancer Res Clin Oncol 122(5): 266-74.

Iwasaki K, Gleiser CA, Masoro EJ, McMahan CA, Seo EJ, Yu BP (1988) The influence of dietary protein source on longevity and age-related disease processes of Fischer rats. *J Gerontol.* 43, B5-12.

Joaquin, A. M. and S. Gollapudi (2001). "Functional decline in aging and disease: a role for apoptosis." J Am Geriatr Soc 49(9): 1234-40.

Kalu DN, Hardin RR, Cockerham R, Yu BP, Norling BK, Egan JW. (1984) Lifelong food restriction prevents senile osteoporosis and hyperparathyroidism in F344 rats. *Mech Ageing Dev.* 26, 103-112.

Karsenty, G. (2001). "Minireview: transcriptional control of osteoblast differentiation." Endocrinology 142(7): 2731-3.

Kato, M., M. S. Patel, et al. (2002). "Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor." J Cell Biol 157(2): 303-14.

Khalil DA, Lucas EA, Smith BJ, Soung DY, Devareddy L, Juma S, Akhter MP, Recker R,

Kim, J. E., M. W. Ahn, et al. (2008). "AMPK activator, AICAR, inhibits palmitate-induced apoptosis in osteoblast." Bone 43(2): 394-404.

Kim, J. E., Y. W. Kim, et al. (2008). "AMP-activated protein kinase activation by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) inhibits palmitate-induced endothelial cell apoptosis through reactive oxygen species suppression." J Pharmacol Sci 106(3): 394-403.

Kirkland JL, Tchkonia T, Pirtskhalava T, Han J, Karagiannides I (2002) Adipogenesis and aging: does aging make fat go MAD? *Exp Gerontol.* 37, 757-767.

Komori, T., H. Yagi, et al. (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell 89(5): 755-64.

Krishnan, V., H. U. Bryant, et al. (2006). "Regulation of bone mass by Wnt signaling." J Clin Invest 116(5): 1202-9.

Kuehnle, I. and M. A. Goodell (2002). "The therapeutic potential of stem cells from adults." BMJ 325(7360): 372-6.

Kumar R, Thompson EB (1999) The structure of the nuclear hormone receptors. Steroids 64:310-319

Kume, S., M. Haneda, et al. (2006). "Silent information regulator 2 (SIRT1) attenuates oxidative stress-induced mesangial cell apoptosis via p53 deacetylation." Free Radic Biol Med 40(12): 2175-82.

LaMothe JM, Hepple RT, Zernicke RF (2003) Selected contribution: Bone adaptation with aging and long-term caloric restriction in Fischer 344 x Brown-Norway F1-hybrid rats. *J Appl Physiol.* 95, 1739-1745.

Lazarenko, O. P., S. O. Rzonca, et al. (2006). "Netoglitazone is a PPAR-gamma ligand with selective effects on bone and fat." Bone 38(1): 74-84.

Lecka-Czernik, B., E. J. Moerman, et al. (2002). "Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation." Endocrinology 143(6): 2376-84.

Lecka-Czernik B, Suva LJ (2006) Resolving the Two "Bony" Faces of PPAR-gamma PPAR Res. Sep 19, Article ID 27489.

Lee, B., K. Thirunavukkarasu, et al. (1997). "Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia." Nat Genet 16(3): 307-10.

Li, M., L. C. Pan, et al. (2006). "Surface-specific effects of a PPARgamma agonist, darglitazone, on bone in mice." Bone 39(4): 796-806.

Liao QC, Li YL, Qin YF, et al (2008) Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. J Cell Biochem 104:1853-1864

Linford NJ, Beyer RP, Gollahon K, Krajcik RA, Malloy VL, Demas V, Burmer GC,

Lund KJ (2008) Menopause and the menopausal transition. Med Clin North Am 92:1253-1271.

Maehata, Y., S. Takamizawa, et al. (2006). "Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: roles of osterix, an osteoblast-related transcription factor." Matrix Biol 25(1): 47-58.

Mao, J. J. (2008). "Stem cells and the future of dental care." N Y State Dent J 74(2): 20-4.

Malhi, H., S. F. Bronk, et al. (2006). "Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis." J Biol Chem 281(17): 12093-101.

Mano, H., Y. Hakeda, et al. (2001). "Estrogen directly down-regulates the bone-resorbing activity of mature osteoclasts through nuclear estrogen receptor alpha." Cytotechnology 35(1): 17-23.

Maurin AC, Chavassieux PM, Frappart L, Delmas PD, Serre CM, Meunier PJ (2000) Influence of mature adipocytes on osteoblast proliferation in human primary cocultures. *Bone.* 26, 485-489.

Maurin, A. C., P. M. Chavassieux, et al. (2002). "Role of polyunsaturated fatty acids in the inhibitory effect of human adipocytes on osteoblastic proliferation." Bone 31(1): 260-6.

McCay CM, Maynard LA, Sperling C and Barnes LL (1939) Retarded growth, life span,

ultimate body size and age changes in the albino rat after feeding diets restricted in calories. *J Nutr.* 18: 1-13.

Michan S, Sinclair D (2007) Sirtuins in mammals: insights into their biological function. *Biochem J.* 404,1-13.

Miyazono, K., S. Maeda, et al. (2004)."Coordinate regulation of cell growth and differentiation by TGF-beta superfamily and Runx proteins." Oncogene 23(24): 4232-7.

Moerman EJ, Teng K, Lipschitz DA, et al (2004) Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. Aging Cell 3:379-389

Mulholland, D. J., S. Dedhar, et al. (2005). "Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know?" Endocr Rev 26(7): 898-915.

Muruganandan, S., A. A. Roman, et al. (2008). "Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: Cross talk with the osteoblastogenic program." Cell Mol Life Sci.

Napimoga, M. H., S. M. Vieira, et al. (2008). "Peroxisome proliferator-activated receptor-gamma ligand, 15-deoxy-Delta12,14-prostaglandin J2, reduces neutrophil migration via a nitric oxide pathway." J Immunol 180(1): 609-17.

Nasu, M., T. Sugimoto, et al. (2000). "Estrogen modulates osteoblast proliferation and function regulated by parathyroid hormone in osteoblastic SaOS-2 cells: role of insulin-like growth factor (IGF)-I and IGF-binding protein-5." J Endocrinol 167(2): 305-13.

Nishimura, R., K. Hata, et al. (2002). "Core-binding factor alpha 1 (Cbfa1) induces osteoblastic differentiation of C2C12 cells without interactions with Smad1 and Smad5." Bone 31(2): 303-12.

Nuttall, M. E., A. J. Patton, et al. (1998). "Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders." J Bone Miner Res 13(3): 371-82.

Ono, Y., H. Watanabe, et al. (1997). "Synthetic studies of vitamin D analogs. XXIV. Synthesis of active vitamin D3 analogs substituted at the 2 beta-position and their preventive effects on bone mineral loss in ovariectomized rats." Chem Pharm Bull (Tokyo) 45(10): 1626-30.

Otto, F., A. P. Thornell, et al. (1997). "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell 89(5): 765-71.

Parfitt AM, Drezner MR, Glorieux FH, Kanis JA, Malluche H, et al. (1987) Bone histomorphometry: Standardization of nomenclature, symbols and units. *J Bone Miner Res.* 2, 595–610.

Pei, L. and P. Tontonoz (2004). "Fat's loss is bone's gain." J Clin Invest 113(6): 805-6.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature.* 429, 771-776.

Picard F, Guarente L (2005) Molecular links between aging and adipose tissue. *Int J Obes (Lond).* 29 Suppl 1:S36-39.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science 284(5411): 143-7.

Platt, I., L. G. Rao, et al. (2007). "Isomer-specific effects of conjugated linoleic acid on mineralized bone nodule formation from human osteoblast-like cells." Exp Biol Med (Maywood) 232(2): 246-52.

Rabinovitch PS (2007)Transcriptional response to aging and caloric restriction in heart and adipose tissue. *Aging Cell*. 6, 673-688

Radak TL (2004) Caloric restriction and calcium's effect on bone metabolism and body composition in overweight and obese premenopausal women. *Nutr Rev.* 62, 468-481.

Raisz LG, Seeman E (2001) Causes of age-related bone loss and bone fragility: an alternative view. J Bone Miner Res 16:1948-1952

Rasbach KA, Schnellmann RG (2008) Isoflavones promote mitochondrial biogenesis. J Pharmacol Exp Ther 325:536-543

Rayalam S, Yang JY, Ambati S, et al (2008) Resveratrol induces apoptosis and inhibits

adipogenesis in 3T3-L1 adipocytes. Phytother Res 22:1367-1371

Reya, T. and H. Clevers (2005). "Wnt signalling in stem cells and cancer." Nature 434(7035): 843-50.

Richard S, Torabi N, Franco GV, Tremblay GA, Chen T, Vogel G, Morel M, Cléroux P,

Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF(2005) Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. *J Nutr Biochem.* 16, 321-330.

Roth GS, Ingram DK, Lane MA (1999) Calorie restriction in primates: will it work and how

will we know? *J Am Geriatr Soc.* 47:896-903.

Rosen, C. J. and M. L. Bouxsein (2006). "Mechanisms of disease: is osteoporosis the obesity of bone?" Nat Clin Pract Rheumatol 2(1): 35-43.

Ross, S. E., N. Hemati, et al. (2000). "Inhibition of adipogenesis by Wnt signaling." Science 289(5481): 950-3.

Salari, P., A. Rezaie, et al. (2008). "A systematic review of the impact of n-3 fatty acids in bone health and osteoporosis." Med Sci Monit 14(3): RA37-44.

Sanderson JP, Binkley N, Roecker EB, Champ JE, Pugh TD, Aspnes L, Weindruch R (1997)

Influence of fat intake and caloric restriction on bone in aging male rats. *J Gerontol A Biol Sci Med Sci.* 52, B20-25.

Sammons, J., N. Ahmed, et al. (2004). "The role of BMP-6, IL-6, and BMP-4 in mesenchymal stem cell-dependent bone development: effects on osteoblastic differentiation induced by parathyroid hormone and vitamin D(3)." Stem Cells Dev 13(3): 273-80.

Schmid, B., J. F. Rippmann, et al. (2005). "Inhibition of fatty acid synthase prevents preadipocyte differentiation." Biochem Biophys Res Commun 328(4): 1073-82.

Schwarz, E. J., M. J. Reginato, et al. (1997). "Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription." Mol Cell Biol 17(3): 1552-61.

Shevde, N. K., A. C. Bendixen, et al. (2000). "Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression." Proc Natl Acad Sci U S A 97(14): 7829-34.

Shockley KR, Rosen CJ, Churchill GA, et al (2007) PPARgamma2 Regulates a Molecular Signature of Marrow Mesenchymal Stem Cells. PPAR Res 2007:81219

Shouhed, D., H. T. Kha, et al. (2005). "Osteogenic oxysterols inhibit the adverse effects of oxidative stress on osteogenic differentiation of marrow stromal cells." J Cell Biochem 95(6): 1276-83.

Simmons DM, Arriza JL, Swanson LW (1989) A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-probe. *J. Histotechnol*.12, 169–181.

Slawik, M. and A. J. Vidal-Puig (2006). "Lipotoxicity, overnutrition and energy metabolism in aging." Ageing Res Rev 5(2): 144-64.

Sottile V, Seuwen K, Kneissel M (2004) Enhanced marrow adipogenesis and bone resorption in estrogen-deprived rats treated with the PPARgamma agonist BRL49653 (rosiglitazone). Calcif Tissue Int 75:329-337

Sohal, R. S., R. J. Mockett, et al. (2002). "Mechanisms of aging: an appraisal of the oxidative stress hypothesis." Free Radic Biol Med 33(5): 575-86.

Soung DY, Devareddy L, Khalil DA, Hooshmand S, Patade A, Lucas EA, Arjmandi BH

(2006) Soy affects trabecular microarchitecture and favorably alters select bone-specific gene expressions in a male rat model of osteoporosis. *Calcif Tissue Int.* 78:385-389.

Syed FA, Oursler MJ, Hefferanm TE, et al (2008) Effects of estrogen therapy on bone marrow adipocytes in postmenopausal osteoporotic women. Osteoporos Int 19:1323-1330

Tanaka, Y., S. Nakayamada, et al. (2005). "Osteoblasts and osteoclasts in bone remodeling and inflammation." Curr Drug Targets Inflamm Allergy 4(3): 325-8.

Tatsumi S, Ito M, Asaba Y, Tsutsumi K, Ikeda K (2007) Life-long caloric restriction reveals

biphasic and dimorphic effects on bone metabolism in rodents. *Endocrinology.* Nov 8;

Unger, R. H. and L. Orci (2002). "Lipoapoptosis: its mechanism and its diseases." Biochim Biophys Acta 1585(2-3): 202-12.

Valtieri, M. and A. Sorrentino (2008). "The mesenchymal stromal cell contribution to homeostasis." J Cell Physiol 217(2): 296-300.

Verma, S., J. H. Rajaratnam, et al. (2002). "Adipocytic proportion of bone marrow is inversely related to bone formation in osteoporosis." J Clin Pathol 55(9): 693-8.

Wang, E. A., D. I. Israel, et al. (1993). "Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells." Growth Factors 9(1): 57-71.

Wan, Y., L. W. Chong, et al. (2007). "PPAR-gamma regulates osteoclastogenesis in mice." Nat Med 13(12): 1496-503.

Wang Z, Masternak MM, Al-Regaiey KA, Bartke A (2007) Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice. *Endocrinology.* 148, 2845-2853.

Watkins, B. A., Y. Li, et al. (2000). "Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats." J Nutr 130(9): 2274-84.

Weinstein, R.S.and S.C. Manolagas (2000). "Apoptosis and osteoporosis." Am J Med 108(2): 153-64.

Zallone A (2006) Direct and indirect estrogen actions on osteoblasts and osteoclasts. Ann N Y Acad Sci 1068:173-179

Zhang, W., N. Yang, et al. (2008). "Regulation of mesenchymal stem cell osteogenic differentiation by glucocorticoid-induced leucine zipper (GILZ)." J Biol Chem 283(8): 4723-9.

Zhou, G., Y. Chen, et al. (1999). "CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia." Hum Mol Genet 8(12): 2311-6.

Zhou, S., J. S. Greenberger, et al. (2008). "Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts." Aging Cell 7(3): 335-43.

Appendix