TYPE I DIABETIC OSTEOPOROSIS AND OSTEOBLAST APOPTOSIS

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ABSTRACT

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Type I diabetes is a metabolic disorder that affects roughly 1 million people in the United States. Medical advances are lengthening patient lifespan resulting in longer exposure to hyperglycemia causing secondary complications including bone loss. Bone formation and density are decreased in T1-diabetic mice. During diabetes onset, levels of blood glucose and pro-inflammatory cytokines (including tumor necrosis factor α (TNFα)) are increased. I hypothesized that early diabetes onset can promote osteoblast death resulting in diabetic bone loss. Indeed, examination of type I diabetic mouse bones demonstrates a greater than 2-fold increase in osteoblast TUNEL staining and increased expression of pro-apoptotic factors. Osteoblast death was amplified in both pharmacologic and spontaneous diabetic mouse models. Co-culture studies demonstrate that diabetic bone marrow cells increase osteoblast caspase 3 activity and Bax:Bcl-2 RNA ratio. Administration of TNF-neutralizing antibody prevented diabetic marrow-induced osteoblast death. Several apoptotic mechanisms could account for this increase in osteoblast death. Two known mediators of osteoblast death, TNFα and ROS, are increased in T1-diabetic bone. TNFα and oxidative stress are known to activate caspase-2, a factor involved in the extrinsic apoptotic pathway. This dissertation determines that caspase-2 is not required for diabetes-induced osteoblast death or bone loss indicating another apoptotic factor is sufficient. Bax, a pro-apoptotic factor that mediates the intrinsic death pathway is also elevated in T1-diabetic bone. Deficiency of Bax results in protection against diabetes-induced osteoblast death and decreased bone density. Targeting osteoblast apoptosis is a possible

therapeutic strategy for preventing diabetes-induced osteoporosis. One commonly used treatment against osteoporosis is bisphosphonates. Here, I demonstrate that bisphosphonate therapy prevents diabetes-induced osteoblast death and bone loss. Furthermore, diabetes induced bone marrow inflammation can also contribute to osteoblast death and is another potential therapeutic target for inhibiting osteoporosis. Administration of the non-steroidal anti-inflammatory drug, aspirin, resulted in enhanced bone formation markers and decreased diabetic hyperglycemia, but did not protect against diabetic bone loss. This dissertation focuses on the relevance of osteoblast apoptosis and bone marrow inflammation as two potential mediators of diabetes-induced bone loss and addresses two commonly used therapies as preventive treatments for T1-diabetes-induced osteoporosis.

.....I dedicate this dissertation to my husband and best friend Cory, who is my strength and comfort through good times and bad. To my parents, Bob and Pati, my never veering pillars of support and to my brother, Joff, my role-model and constant comedic relief. I cannot thank you each of you enough for your support and love you have given me throughout my graduate school studies and my entire life. I love you all eternally.

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α-MEM	alpha-minimal essential media
AGE	advanced glycation end produce
AIF	apoptosis inducing factor
aP2	adipocyte fatty acid binding protein
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BADGE	bisphenol-A-diglycidyl ether
BAK	Bcl-2 homologous antagonist/killer
BAX	bcl-2 associated protein-X
BCL-2	Bcl-2-like protein 2
BCL-W	B-cell lymphoma 2
BID	BH3 interacting domain death agonist
BIM	BCL2L11-Bcl-2-like protein 11
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BMU	basic metabolic unit
BVF	bone volume fraction
C/EBP	CCAAT enhancer binding protein
COX	cyclooxygenase
DNA	deoxyribonucleic acid

dpi	days post first injection
DR	death receptor
FADD	fas-associated death domain
FoxO1	forkhead transcription factor family
GLUT	glucose transporter
HbA_{1C}	glycated hemoglobin
IFN-γ	interferon gamma
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist
Ins2	insulin 2
KO	knockout
LEF	lymphoid enhancer factor
MC3T3-E1	pre-osteoblast cell line
M-CSF	macrophage colony stimulating factor
MOI	moment of inertia
MOM	mitochondrial outer membrane
MSC	mesenchymal stem cell
NSAID	non-steroidal anti-inflammatory drug
NOD	non-obese diabetes
OC	osteocalcin
OPG	osteoprotegrin
OVX	ovarectomy
PCD	programmed cell death

PPARγ	peroxisome proliferator-activated receptor gamma
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
ROS	reactive oxygen species
RunX2	runt related transcription factor 2
STZ	streptozotocin
T1-diabetes	type 1 diabetes mellitus
T2-diabetes	type 2 diabetes mellitus
TNFα	tumor necrosis factor alpha
TRAP	tartrate-resistant acid phosphatase
WT	wild type

CHAPTER 1. LITERATURE REVIEW

1.1. BONE

The skeleton is the framework for the body. It is responsible for protecting the internal organs, maintaining mineral homeostasis and acid-base balance, housing hematopoietic cells within the marrow space, and serving as a reservoir for various growth factors (1). The skeleton is highly dynamic and adaptive, and reflects the genetic and environmental impacts placed on it. It is one of the strongest biological materials, and can support more weight than some forms of steel.

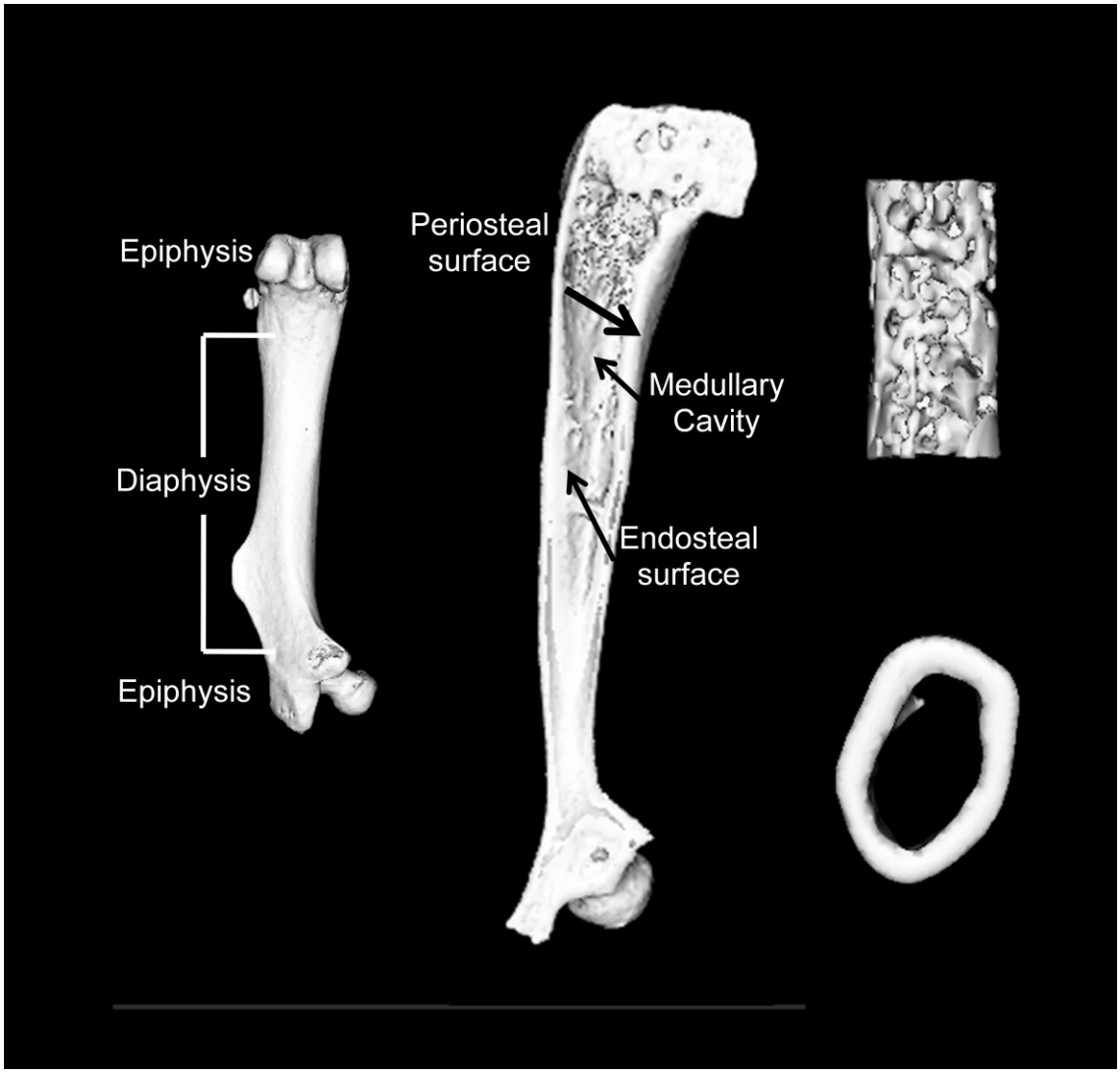
1.1.1. Molecular Composition of Bone

Bone is a highly active metabolic structure that is composed of various inorganic compounds deposited on an organic framework. The mineral composition of bone consists mainly of calcium phosphate in the form of hydroxyapatite crystals (2-4). This compound takes up roughly 25% of the total bone volume, but accounts for nearly 50% of the total weight of bone due to its high density (5) . The skeleton is responsible for storing most of the body's content of carbonate, magnesium and sodium, in addition to calcium and phosphate. Nearly 99% of the body's total calcium content is provided or stored in bone.

1.1.2. Bone Anatomy and Development

At the anatomical level, bone is made up of two types of bone: trabecular (spongy) and cortical (compact). The adult human skeleton is composed of 80% cortical bone and 20% trabecular bone (1). Cortical bone is the dense solid structure that surrounds the hollow bone marrow shaft. It is composed of a network of cylindrical osteons called Haversian systems that run longitudinally down the bone shaft. Cortical bone contains an outer periosteal surface that is important for appositional growth and fracture repair (figure 1). This surface generally increases in older adults. The inner endosteal surface of bone has a higher remodeling activity than the periosteal surface; this is thought result from its direct exposure to marrow contents (figure 1). Both layers of cortical bone undergo minimal bone remodeling in comparison to trabecular bone. Trabecular bone is the site of high remodeling and bone growth. It is composed of a honeycomb-like network of osteons called packets (figure 1). In long bones, the shaft is referred to as the diaphysis while the ends are the epiphysis (figure 1).

Figure 1. **Mouse femur structure and anatomy.** Micro-computed tomography three-dimensional isosurface images demonstrating the anatomical structures of the mouse femur. The femurs exterior surface (left image) illustrates the shaft (diaphysis) and end regions (epiphysis) while displaying the proximal (hip ball) and distal (knee joint) regions of the femur. The middle image represents the internal structures of the mouse femur, medullary cavity and endosteal surface. The right images illustrate trabecular and cortical bone.



Skeletal development occurs through two distinct pathways: intramembranous and endochondral ossification. Intramembranous ossification is responsible for development of flat bones such as the skull, mandible and clavicle. It occurs when the mesenchymal stem cells (MSC) condense and directly differentiate into osteoblasts that synthesize and mineralize new bone matrices (4, 6-8). Endochondral ossification is responsible for bone formation for the rest of the skeleton. This form of bone formation occurs when MSC's condense into chondrocytes laying down a stiff layer of cartilage (6, 8). Eventually, the cartilage calcifies, and then osteoclasts and blood vessels invade the template and attract osteoblasts to replace the cartilage template. This repetitive pattern of cartilage differentiation, calcification of the cartilage, new bone formation, and resorption is characteristic of endochondral ossification (6, 8). Longitudinal bone growth involves osteoclasts, osteoblasts and chondrocytes at the growth plate. The growth plate cartilage layer produces cartilaginous projections termed spicules that project between the chondrocytes and osteoblasts junction (6). Osteoblasts adhere to these cartilage extensions and replace them with a bony matrix. This lengthens the spicules causing the growth plate to move centrifugally (6). A study examining osteogenesis imperfecta, a model deficient in osteoblast function, revealed that without osteoblasts longitudinal bone growth would not occur (9). Similarly without osteoclasts to remodel this area, longitudinal growth would not occur (10).

1.1.1.1. Osteoblasts

Osteoblasts are responsible for synthesis and secretion of the organic matrix that makes up new bone. Osteoblasts are derived from multipotent mesenchymal stem cells (MSC), which can differentiate into various cell lineages, including osteoblasts, adipocytes, chondrocytes, neurons, and myoblasts, within the bone marrow stroma (11-15). Bone formation occurs through a series of events: MSC differentiating into osteoblast precursor cells (preosteoblasts), osteoblast maturation, mineralization of new bone, and finally mineralization of the matrix. Each phase in an osteoblast's life is highly regulated (figure 2). Osteoblast differentiation from MSCs is highly regulated by the transcription factor Runx2 (cbfa 1). Reports indicate that mice deficient in Runx2 die at birth due to the failure of osteoblast formation (16). Runx2 is also important for replenishing the number of osteoblasts after bone loss. Many other factors (e.g. osterix, bone morphogenic protein, and Wnt/β-catenin pathway) can promote osteogenesis, possibly through the regulation of RunX2 expression.

Pre-osteoblasts cannot form new bone, therefore osteoblasts must mature to synthesize new bone by the production and deposition of type I collagen. Type I collagen is the most important and abundant extracellular matrix protein in bone. It provides the structure, strength and flexibility of bone, as well as facilitating bone growth through aiding hydroxyapatite deposition (4). As the osteoblasts mature they secrete extracellular matrix proteins, including osteocalcin, which can bind to bone mineral, but is also found in the serum. Because of its limited expression, osteocalcin serves as a useful biochemical marker for osteoblast maturation and activity. Recent studies also suggest

that a modified form of osteocalcin regulates insulin secretion from the pancreas (17). Once the bone extracellular matrix is mineralized, the osteoblasts lose their synthetic abilities. The fully mature osteoblast has three potential final fates: it can become a bone-lining cell, get incorporated into the mineralized bone as an osteocyte, or undergo apoptosis.

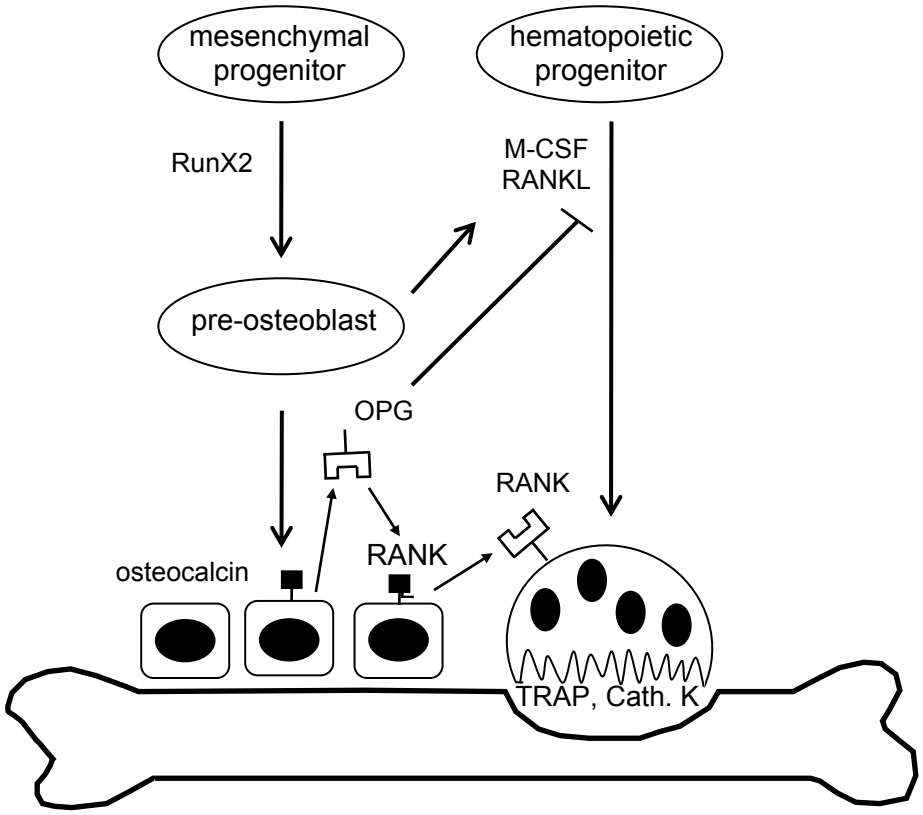
The osteocyte is the most abundant cell type found in bone. They are 10 times more plentiful then osteoblasts in an adult human bone (18). Mature osteocytes neither form nor destroy bone; instead, they maintain the adaptive properties of bone (structure and flexibility) to accommodate alterations in mechanical or chemical stimuli (4). Physically, ostocytes are dendritic-like cells located within the lacunocanalicular network of bone. They communicate with each other, bone-lining cells and osteoblasts through long cell bodies contained in the lacunae that radiate in high densities perpendicular to the bone surface.

1.1.1.2. Osteoclasts

Osteoclasts are multinucleated cells derived from hematopoetic stem cells through the monocyte/macrophage lineage. Monocyte precursors differentiate into osteoclasts when they interact with osteoblasts. Osteoclasts are responsible for bone resorption and are commonly located mainly in the trabecular bone region (19). Bone resorption occurs when osteoclasts bind the bone surface through the $\alpha_v\beta_3$ integrin, creating a leak-proof seal important for a low pH microenvironment. The characteristic "ruffled border" of the osteoclast is a complex structure of interfolded finger-like

projections that penetrate the bone matrix forming the resorption lacunae (figure 2) (12, 20, 21). Osteoclasts then secrete acids (e.g., hydrochloric acid) and proteolytic enzymes (e.g., matrix metalloptroteinases or cathepsin K) that digest the mineralized bone leaving erosion pits to recruit osteoblasts. Measurements of osteoclast activity occur through various markers and receptors that are unique to osteoclasts including tartrate resistance acid phosphatase (TRAP), cathepsin K secretion, and the expression of vacuolar proton ATPase (figure 2) (12, 22, 23). Osteoclast/osteoblast intercellular communication is vital for osteoclast mediated bone resorption and bone remodeling (12). First, osteoblasts secrete a paracrine factor macrophage colony-stimulating factor (M-CSF) that binds to its receptor on osteoclast precursors promoting osteoclastogensis. Osteoblasts also secrete an anti-osteoclastogenesis molecule osteoprotegerin (OPG), a decoy receptor that binds receptor activator of NFκB ligand (RANKL) expressed on the surface of osteoblasts (figure 2) (12, 24-26). Unlike OPG, RANKL is a potent activator of osteoclastogenesis by binding its receptor RANK on osteoclasts (6, 12, 26). Together, osteoblasts and osteoclasts are responsible for bone remodeling to maintain healthy bones.

Figure 2. **Osteoblast/Osteoclast intercellular interaction.** Osteoblasts derive from mesenchymal precursors, while osteoclasts are derived from hematopoietic progenitors. Pre-osteoblasts(expresssing RunX2) aid in osteoclast differentiation through expression of M-CSF and RANKL. Once mature, osteoblasts, which secrete ostocalcin, express RANKL which binds its receptor RANK on osteoclasts to promote bone remodeling. Osteoblasts also produce a decoy receptor OPG, which binds RANKL, thus inhibiting the RANKL/RANK binding, decreasing bone resorption and remodeling. Osteoclasts secrete several acids and proteases (TRAP and Cathepsin K) to promote bone resorption.



1.1.1.3. Bone Remodeling

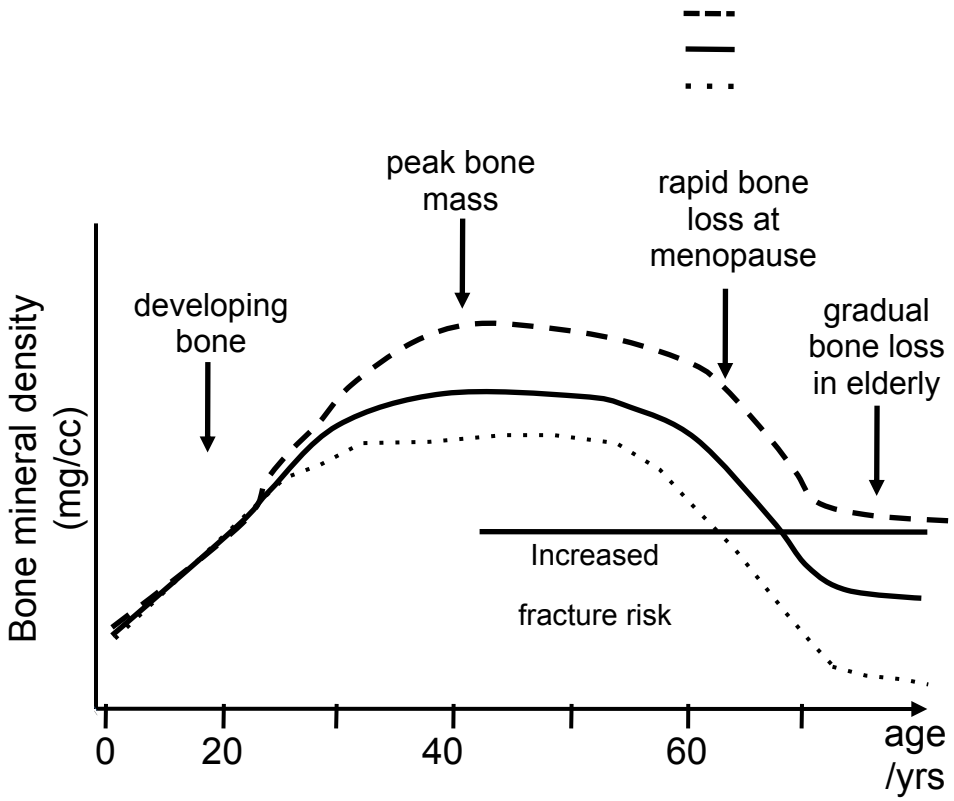
Bone is a highly dynamitic and adaptive tissue that is continuingly remodeling. Roughly 10% of bone is replaced each year and the entire skeleton is renewed every 10 years (27). Bone remodeling during growth and development optimizes strength, while maintaining lightness for mobility. First, bone is resorbed by osteoclasts then deposited by osteoblasts on the same location. These cells form the basic metabolic unit (BMU) and align themselves parallel to the longitudinal Haversian canals (28, 29). In order for the BMU to function properly, osteoclasts and osteoblasts must be replenished over time. BMU's continual advancement through cortical bone can take as much as 6-9 months during which the size and spacing of the BMU is maintained for optimal bone remodeling (29). As the BMU advances, osteoclasts resorb bone, while osteoblasts mineralize new bone. BMUs are also seen in trabecular bone, where regions of bone are removed (pits) and a population of osteoblasts are aligned over the pit to fill it with bone. This balance between bone resorption and formation is key to proper bone health and fracture repair. An imbalance in either resorption or formation can result in serious consequences, including osteoporosis.

1.2. OSTEOPOROSIS

Osteoporosis is commonly referred to as a "silent disease" because patients are often unaware their bone heath is deteriorating until they have significant enough bone loss to cause a fracture. It is characterized by a decrease in bone density of more than 2.5 standard deviations below average (figure 3). A decrease in bone density by 1 standard

deviation (termed osteopenia) results in increased risk of fracture by roughly 2.4 times. The most common fractures are generally observed in the hip, spine, and forearm (30-45). Osteoporosis is quickly becoming a public health problem due to roughly 20 million Americans suffering from decreased bone density (45). Of those roughly 80% are women. Research suggests that 1 out of 3 American women have osteoporosis and roughly 75% of women over the age of 65 suffer from a bone fracture (46, 47). Although osteoporosis is thought to be a women's disorder, men are susceptible to developing decreased bone density. Studies indicate that over 2 million men in the United States develop osteoporosis and another 12 million are at increased risk (48). In addition, over 30% of hip fractures occur in men and the mortality rate related to fractures is higher in men then women (49-52). It is estimated that by the year 2050, hip fractures will increase by 310% in men and 240% in women (53). Osteoporosis is responsible for approximately 1.5 million fractures annually and costs nearly 15 billion dollars in hospitalizations and health care (54). Not only do financial burdens cause stressful situations but also nearly 20% of hip fracture patients die within a year (55). Osteoporosis can also be a secondary complication associated with various diseases including diabetes. Therefore, understanding the mechanisms accounting for osteoporosis is critical to reducing its occurrence.

Figure 3. **Increased fracture risk in T1-diabetic patients.** Normal individuals (solid Line) will develop bone during early adolescence and reach peak bone mass in their 30's. Menopause for women rapidly reduces bone density, while males have a more gradual loss. Diabetic patients (dotted line) do not develop enough bone early on leaving them at greater risks in their later years.



1.3. DIABETES

1.3.1. Glucose homeostasis

Glucose is the most commonly used fuel for generation of energy rich ATP in the tissues. Blood glucose levels are regulated by the hormones insulin and glucagon and are dependent on the balance between output or the breakdown of energy and input from dietary absorption or release from storage. High blood glucose levels (such as after a meal) stimulate insulin secretion from the pancreas into the blood. This decreases plasma glucose concentrations by promoting glucose uptake, intracellular glucose metabolism and glycogen synthesis in various tissues including muscle, fat and liver. Glucose transportation regulated by insulin occurs in these tissues through the glucose transporter GLUT-4. In the presence of elevated insulin concentrations, GLUT-4 containing vesicles fuse with the plasma membrane resulting in greater glucose uptake into these tissues.

1.3.2. Epidemiology and classifications

Diabetes mellitus is a metabolic disorder where either a lack of insulin production or tissue insulin resistance results in impaired tissue glucose uptake. Nearly 23.6 million people, roughly 7.8% of the total population in the United States, are affected by one of the three major forms of diabetes: Type I, Type II or gestational (56). Type I (T1) diabetes is an autoimmune mediated disease that results from chronic destruction of the pancreatic islet beta cells resulting in hyperglycemia (fasting serum glucose levels greater than 125 mg/dl) and little or no insulin production. Therefore, insulin replacement

therapy is essential. T1-diabetes is predominantly diagnosed in children or young adolescents and affects approximately 4 million people in the United States, Canada and Europe (57). Recently however, an increasing number of adults are developing late-onset T1-diabetes. An estimated 1 in every 300 individuals are at risk for developing T1-diabetes in North America (57) and costs the United States alone nearly $160 billion dollars in 2002 to treat and counteract this disease (58).

Type II diabetes or adult onset diabetes, is the most prevalent form of diabetes, and is associated with insulin resistance in specific tissues (e.g., pancreas, brain, and muscle) and increased insulin production by the pancreas. Constant demand placed on the pancreas for insulin production eventually results in pancreatic β-cell death leading to insulin deficiency. A third form of diabetes, gestational diabetes, occurs during some pregnancies creating tissue specific insulin resistance.

1.3.3. Secondary complications

Secondary complications resulting from diabetes include neuropathy, nephropathy, retinopathy, myopathy, and bone loss (13, 59-63). Reports have shown that greater than 50% of all patients diagnosed with T1-diabetes suffer from bone loss and are at increased risk for fractures (43, 64-66). Diabetic osteoporosis is well documented, making it a useful model to study mechanisms of bone loss associated with metabolic pathology (67-70). However, the underlying mechanism(s) of T1-diabetic induced bone loss remain unknown.

1.4. TYPE I DIABETIC OSTEOPOROSIS

1.4.1. Clinical Research

Osteoporosis is widely accepted as a secondary complication of T1-diabetes and results in increased fracture risk in various bones examined (30-41, 43). Bone loss and increased fracture risk are reported in the hip (36, 39, 40, 71-77), spine (36, 39, 77, 78), and feet (73, 79) of T1-diabetic patients. Diabetes-induced osteoporosis is independent of gender and age with several studies demonstrating that both male and female adult and adolescent diabetic patients have decreased lumbar spine bone mineral density and increased fracture risk (31, 36, 43, 80-82). Suppressed bone formation is a major mechanism accounting for decreased bone mass in diabetic patients (45). Patients with T1-diabetes should be considered as high-risk individuals for osteoporotic fractures, as well as family history, fracture history, nutritional content to properly determine the correct therapeutic regime (76).

1.4.2. Rodent models of T1-diabetes

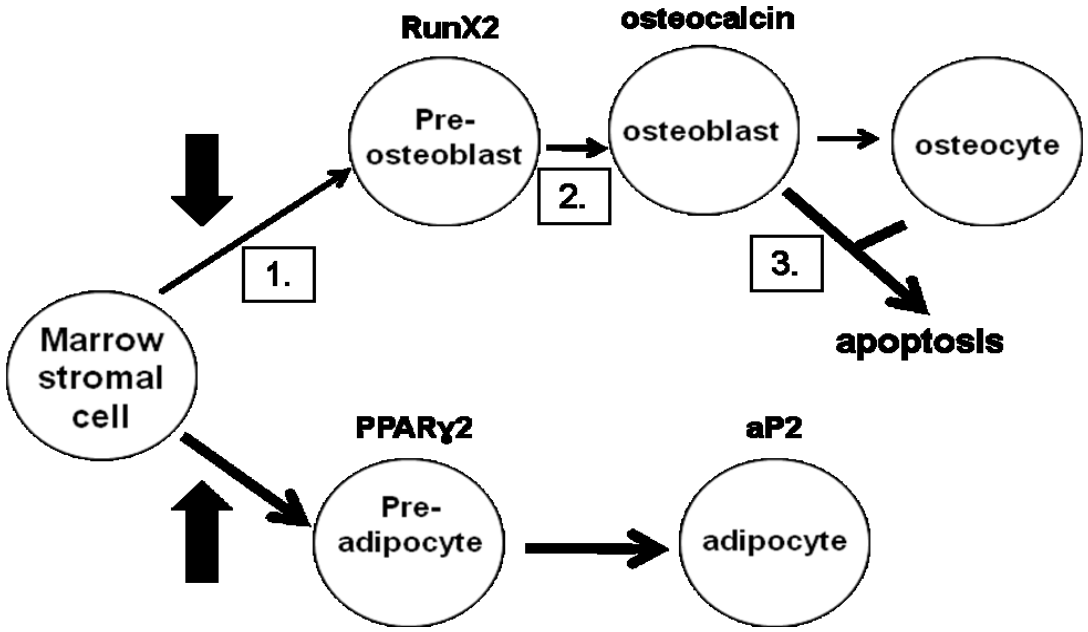
Several T1-diabetic rodent models exist. There are genetically predisposed models that spontaneously develop diabetes, such as non-obese diabetic (NOD) mouse and the $Ins2^{+/-}$ (Akita) mice, and there are pharmacologic models such as the multiple low-dose streptozotocin (STZ) injection mouse models. The latter involves STZ entering

the pancreatic β-cells and causing toxicity and cell death, which therefore halts insulin production. Our lab and others have demonstrated that all rodent models display decreased bone mineral density due to suppressed osteoblast function and not by increased osteoclast activity (13, 83). While diabetes onset is difficult to predict in the NOD and $Ins2^{+/-}$ models, it is easily induced in a reproducible manner at any mouse age or genetic background using STZ (13, 84). Thus, STZ allows for easier investigation within transgenic mouse models and the ability to examine early time points during disease onset.

1.4.3. Mechanisms of T1-diabetic bone loss

Bone formation is regulated at three key stages: lineage selection, osteoblast differentiation and osteoblast death (figure 4). Mesenchymal stem cells (MSC) can differentiate into various cell types including, osteoblasts, adipocytes, fibroblasts, myoblasts, and chondrocytes. Changes in MSC lineage selection could increase one cell lineage (i.e., adipocytes) at the cost of another (i.e., osteoblasts) and lead to reduced bone formation. Similarly, increased osteoblast death could quickly result in fewer bone forming cells and less bone formed. Often, it is a combination of regulatory effects (increased death and altered lineage selection) that leads to bone loss. Whether they contribute synergistically or independently in T1-diabetes remains unknown, but both topics are discussed later in this dissertation.

Figure 4. **Potential mechanisms regulating bone formation in T1-diabetes-induced bone loss.** Osteoblasts are derived from mesenchymal stem cells that can also give rise to adipocytes. As the osteoblast lineage is selected (1. First regulation point) the transcription factor Runx2 is expressed. Further maturation of osteoblasts (2. Second point of regulation) leads to osteocalcin expression, commonly used as a late stage marker for osteoblast development. Osteoblasts have three final fates: become a bone-lining cell, an osteocyte or under apoptosis (3.Third point of osteoblast regulation). In diabetes, mesenchymal stem cells select adipogenesis over osteogenesis. At this time, PPARγ2 is expressed from pre-adipocyte-like cells followed by aP2 expression from mature adipocytes. Elevated osteoblast apoptosis is a key mediator in regulating the T1-diabaetic bone phenotype and in the topic of this dissertation.



1.4.3.1. Marrow Adiposity

A reciprocal relationship often (but not always (85, 86)) exists between bone marrow adiposity and bone density (13, 87, 88). It is suggested that mesenchymal stem cells, capable of differentiating into both adipocytes and osteoblasts, favor adipogenesis at the expense of osteogenesis (13). This mechanism has been implicated in contributing to bone loss in models of aging, unloading and T1-diabetes (13, 84, 89-91). However, several T1-diabetic studies have observed no relationship. Inhibition of adipocyte linage factor, PPARγ2 in T1-diabetes, alleviated diabetes induced marrow adiposity but did not prevent bone loss (84). T1-diabetes induced vertebral bone loss also did not correlate with elevated marrow adiposity (88). Furthermore, leptin-treated diabetic mice displayed reduced marrow adiposity in long bones, still showed decreased trabecular bone loss (86). Taken together, these findings suggest that the increase in marrow adiposity, alone, cannot account for diabetic bone loss. Additional factors, possibly increased osteoblast apoptosis could account for the decrease in bone density and osteoblast activity under diabetic conditions.

1.4.3.2. Osteoblast apoptosis

Roughly 60-80% of all osteoblasts and 100% of osteoclasts undergo apoptosis, implying an important physiological role for cell death in regular maintenance, remodeling, and fracture repair (92). Osteoblast cell death can occur through activation of the two main apoptotic pathways: extrinsic and intrinsic. The extrinsic pathway is activated through membrane bound receptors, where extracellular ligands (e.g. TNFα,

Fas, TRAIL) bind specific death receptors (DR), such as DR5 or Fas-associated death domain (FADD). Both DR5 and FADD contain an effector death domain (DED) that recruits DED-containing proteins, like procaspase 8. Caspases are a group of proteases that contain a large and small subunit proceeded by an N-terminal prodomain (93, 94). The two subunits of each caspase associate together producing the active site of each enzyme. Caspases can be generally classed into two categories; initiators (caspase-1, -2, -8, -9) or effector caspases (caspase-3) (94). The initiator caspases are autocatalytic, whereas activation of effector caspases relies on the initiator caspases for cleavage at an aspartic acid cleavage site.

Intrinsic apoptosis pathway refers to mitochondrial mediated cell death and is initiated by an imbalance between pro-apoptotic (Bax, Bak, Bim, Bid) components and anti-apoptotic (Bcl-2, Bcl-xL) factors. Bcl-2-associated X protein (Bax) is an important pro-apoptotic protein that, unlike other Bcl-2 pro-apoptotic family members that are frequently found in the mitochondrial membrane, resides mainly in the cytosol. During periods of cell stress, Bax expression is elevated in the cytosol promoting translocation to the mitochondrial outer membrane (MOM) (95). At the MOM, Bax can dimerize with another Bax protein and insert itself into the extracellular membrane creating pores that allow free diffusion of an intermembrane protein, cytochrome c, across the membrane (93, 96-100). Cytochrome c binds Apaf-1 forming an apoptosome that activates caspase-9. Simultaneously, AIF (apoptosis-inducing factor) is released from the mitochondria and migrates to the nucleus resulting in DNA fragmentation and subsequent activation of caspase-3 (101, 102). Inhibition of mitochondrial pore formation can occur when Bax dimerizes with anti-apoptotic factors, such as Bcl-2 or Bcl-xL (103, 104). The ratio

between Bax and anti-apoptotic Bcl-2 family members determines the fate of the cell. Increased levels of Bax and/or decreased levels of Bcl-2 have been reported to influence multiple disorders including osteoporosis. Reports indicate elevated levels of Bax expression and decreased Bcl-2 expression during osteoblast apoptosis (105, 106). Over-expressing Bcl-2 in osteoblasts (*in vitro*) leads to increased osteocalcin (mature osteoblast marker) expression and osteoblast differentiation, while decreasing osteoblast death (107) and preventing age-induced bone loss (108).

Osteoblast death has been demonstrated to play an important role in models of bone loss associated with unloading (109), aging (92), periodontal disease in T2-diabetic rodents (110) and T1-diabetes (111). The underlying mechanism responsible for diabetes-induced osteoblast death remains unclear; however, several studies have investigated specific factors such as advanced glycation end-products (AGE), reactive oxygen species (ROS), and pro-inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α□) □(112-114), which are known to induce osteoblast apoptosis (114, 115). T1-diabetes associated hyperglycemia can lead to non-enzymatic glycation of both intracellular and extracellular proteins, resulting in the accumulation of AGE. In diabetic bone, AGE promotes cell death of osteoblasts and can delay fracture healing [58,104] [105]. *In vitro* studies demonstrate that AGE can induce osteoblast apoptosis through activation of caspase-3 and caspase-8 (105). Additionally, AGE elevates pro-inflammatory cytokines and activates production of ROS. Studies have shown that inhibition of AGE in diabetic mice resulted in decreased expression of pro-inflammatory cytokines, increasing wound healing (58).

In addition to AGE, ROS levels are also elevated in diabetic mice, perhaps as a result of hyperglycemia, AGE accumulation and superoxide production (116). *In vitro* studies have reported elevated oxidative stress inhibits osteoblast differentiation and induces apoptosis (117-119). Rodent studies demonstrate that markers of oxidative DNA damage, such as urinary output of 8-hydroxydeoxyguanosine, are increased in diabetic rats and mice compared to controls and are suppressed by insulin treatments (112, 120). Furthermore, diabetic mice overexpressing an intracellular antioxidant, thiroredoxin-1, are moderately protected from diabetes-induced bone loss (112, 116). Both AGE and ROS are two possible mediators of osteoblast death and likely play a key role in the diabetic bone phenotype.

Since osteoblast apoptosis plays a significant role in regulating diabetic bone loss, the identification of factors that inhibit osteoblast death is a key area of research. Interestingly, a member of the Forkhead transcription factor family, FoxO1, inhibits free radicals thus protecting against osteoblast apoptosis and oxidative stress. One study demonstrated that FoxO1 coordinates an antioxidant response in osteoblasts by inhibiting the p53-mediated signaling pathway known to induce cell death (121). Taken together, apoptosis, induced through various synergistic or independent mechanisms, plays a vital role in contributing to the ultimate diabetic bone pathology.

1.4.3.3. Bone marrow inflammation

T1-diabetes is strongly associated with inflammation. Hyperglycemia contributes to increased pancreatic inflammation through the recruitment of cytokine releasing, Th1 and Th2 cells. During an immune response, either in genetic or environmentally induced diabetes, Th1 cells release pro-inflammatory cytokines that activate circulating lymphocytes, killer T-cells and macrophages that destroy the pancreatic β-cells decreasing insulin production (122, 123). In humans, pro-inflammatory cytokines have been found elevated in diabetic serum compared to controls. One recent study found an increase in intracellular levels of TNFα in $CD8^+$ T cells at the time of T1-diabetes diagnosis (124). Similar results are reported in rodent models of T1-diabetes. Pro-inflammatory cytokines, such as TNFα and IL-1, are elevated in the serum of diabetic mice compared to controls, but also (and more importantly) TNFα and IL-1 messenger RNA expression is increased directly in diabetic bone (83). Furthermore, treatment of diabetic mice with anti-TNFα specific inhibitors such as pegsunercept and entanercept reduced osteoblast and chondrocyte apoptosis during fracture healing (125-127).

To address if the bone marrow is involved in producing the toxic cytokines within the bone, we co-cultured bone marrow cells isolated from control and T1diabetic mice with osteoblasts, either primary or MC3T3-E1 pre-osteoblast lines. Our findings demonstrated that T1-diabetic bone marrow induced osteoblast death (discussed in Chapter 2) (111). Furthermore, the addition of anti-TNFα antibodies to the cultured osteoblasts reduced the diabetic marrow death response (111). These results suggest a role for bone marrow inflammation and pro-inflammatory cytokines, specifically TNFα,

in contributing to osteoblast death and diabetes-induced bone loss. Thus, decreasing bone inflammation with either a non-steroidal anti-inflammatory drug (NSAID) (discussed below and in Chapter 6) or a specific cytokine antagonist (TNFα-neutralizing antibodies) may serve as a potential therapy for diabetic osteoporosis.

1.4.4. Treatment of T1-diabetes bone loss

1.4.4.1. General Treatment Options

To date no perfect therapy for diabetic-induced bone loss is available; however, maintaining a well-balanced diet (calcium and vitamin D intake levels) and regular exercise can provide a first line of defense. Studies show that diabetic patients can have lower levels of serum vitamin D and calcium compared to healthy non-diabetic patients (32, 127-132), indicating that administration of vitamin D or calcium could normalize the deficiency and be beneficial to diabetic patients. Previous reports suggest that vitamin D therapy promotes diabetic bone health (133), however rodent studies demonstrate conflicting results on bone loss associated with type I diabetes. Some reports suggest that vitamin D reduces diabetic bone loss (134-137), while others report no benefit to bone health (134). In addition to adequate calcium and vitamin D intake, regular exercise can promote bone remodeling in young non-diabetic males and females (138, 139), as well as in ovariectomized (140) and orchidectomized rats (141). Little is known about exercise and its beneficial effects on T1-diabetic induced-bone loss. A study on spontaneous diabetic female BB rats demonstrated no beneficial results occurred in bone from

treadmill therapy (142). T2-diabetes patients, however, greatly benefit from regular exercise through weight loss, as well as through increasing load on the bones to improve bone density (143).

1.4.4.2. Bisphosphonate treatment

Bisphosphonates are considered the first-line pharmacological defense for osteoporosis in aging patients (144-147). These drugs selectively integrate into bone mineral surfaces and increase bone mineral density (148-154) by inhibiting osteoclast-mediated resorption, recruitment, differentiation, and apoptosis. They are reported to prevent both vertebral and non-vertebral fractures (155-158). Previous studies suggest that bisphosphonates not only inhibit osteoclast-mediated resorption, but they also inhibit osteoblast death (159, 160). Because osteoblast death is elevated in T1-diabetes (111), bisphosphonates could potentially benefit bone health in diabetic patients. *In vitro* studies report that bisphosphonates protected rodent-derived osteoblasts from AGE-induced death (161). Few studies have addressed the impact of bisphosphonate therapy on type 2 diabetes and none have addressed the effects on type 1-diabetes patients. One clinical study involving patients on long-term, 4 years, bisphosphonate treatment did not prevent bone loss in postmenopausal women with type 2 diabetes (162). In contrast, Keegan et al. found that both type 2 diabetic and non-diabetic women benefitted in overall bone health from bisphosphonate therapy (163). Interestingly, in this study more diabetic women were reported taking estrogen supplements compared to the non-diabetic women; this could have altered any positive bone results due to estrogen's positive

impact on bone formation (163). It is important to note that patients with diabetes taking bisphosphonate therapy can show increased (albeit rare) incidence of osteonecrosis of the jaw at high doses (commonly used to treat cancer patients) compared to non-diabetic patients (164). A recent discovery is a potential link between bisphosphonate therapy and increases in rare femur fractures (165, 166). At present time, the benefits of bisphosphonate treatment in reducing fracture risk still outweigh both potential rare complications in the general population. With the right dosing regimen and frequency, bisphosphonate treatment could decrease both osteoclast activity and osteoblast apoptosis, thus improving type 1 diabetes associated osteoporosis. More research is needed to fully understand and confirm this hypothesis.

1.4.4.3. Anti-inflammatory therapy

Aspirin is a commonly used broad-spectrum non-steroidal anti-inflammatory drug (NSAID). It has been reported as a beneficial additive for treating T2-diabetes by ameliorating diabetic-related inflammation (167, 168) and decreasing blood glucose levels in T1-diabetic rats (169). Aspirin binds and inhibits cyclooxygenase (COX) enzyme function resulting in decreased prostaglandin, specifically PGE_2, expression. COX exists as two isoforms: COX-1 and COX-2. COX-1 is the constitutively active form and highly expressed in various tissues throughout the body. It functions to maintain normal prostaglandin levels. COX-2, however, is an inducible enzyme correlated to inflammatory insults and is more highly expressed in osteoblasts than COX-1 (170, 171). In a pre-osteoblastic cell line, MC3T3-E1, COX-2 was highly induced after

serum TNFα treatment (172). Reports have indicated that primary calvaria cells cultured from COX-2 deficient mice displayed increased osteoblast growth and proliferation (173). Aspirin treatments on bone health are controversial. Aspirin treatments have been shown advantageous to bone complications. Reports showed that aspirin treatment attenuated Fas-induced bone marrow stromal cell apoptosis (174) and improved bone mineral density in both trabecular and cortical bone compared to nonusers in aged populations (175, 176). Examination of aspirin's effects on bone density illustrated aspirin improved bone density measurements (trabecular and cortical) in ovariectomized mice by increasing RunX2 (osteoblast differentiation) expression. Additionally, aspirin treatment increased Wnt signaling *ex vivo*, a key element in promoting osteoblast lineage selection (174). In contrast, NSAIDs are reported to suppress bone repair and bone remodeling while only mildly inhibiting bone formation (177). This is thought to occur through suppression of prostaglandins, specifically PGE_2 a known stimulator of bone formation, increased bone strength and bone volume (177, 178).

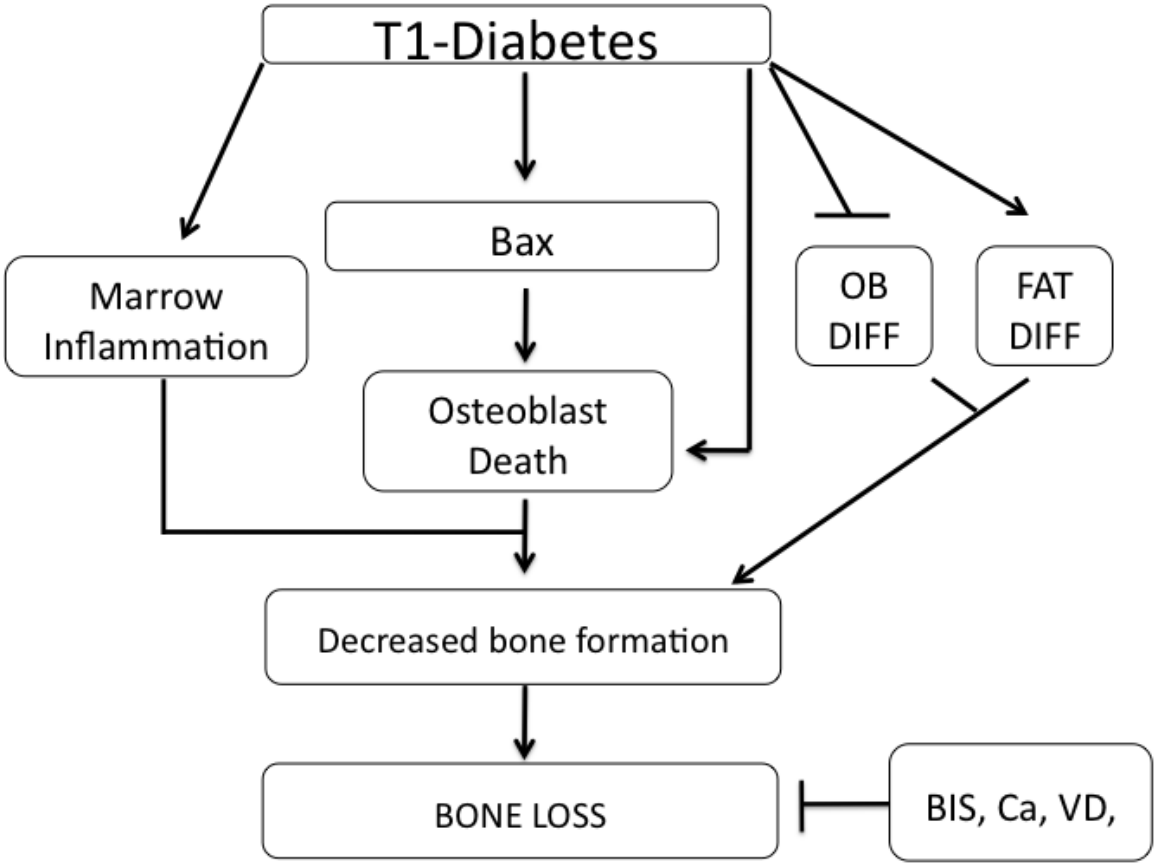
Prostaglandins are a group of lipid mediators that are derived from arachidonic acid in various tissues and help maintain local homeostasis (179). Among them, PGE_2 plays an important role in bone metabolism (180). *In vitro* studies demonstrate that addition of PGE_2 to bone cultures results in elevated bone resorption and possibly bone formation (178, 180). *In vivo* rodent models suggest that multiple injections of PGE_2 result in increased bone formation through activation of the EP4 (4 subtypes of the PGE receptor) receptor (180). Additionally, clinical studies indicate that PGE_2 therapy induces osteoblastogensis and osteoclastogenesis, however using this as a treatment

option has been hindered by secondary complications (178). Since aspirin is a widely accepted treatment for decreasing inflammation and has been shown beneficial in age and OVX-related bone loss, treatment with aspirin in our model of T1-diabetes may attenuate diabetic-induced bone inflammation and bone loss.

1.5. SUMMARY

In summary, T1-diabetes is associated with increased fracture risk and decreased bone density throughout the body in adults and children. The mechanism accounting for diabetic bone loss is complex and involves multiple mediators and pathways (figure 5). Medical advances are lengthening patient life spans resulting in increased exposure to diabetic conditions and compounding those problems associated with natural aging. Future clinical and laboratory research is needed to continue to improve our understanding of diabetic bone loss so we can attempt to counteract its effects with new and improved therapeutic tools.

Figure 5. **Mechanism of T1-diabetic bone loss**. Diabetes increases marrow inflammation, bone Bax expression, osteoblast death and adipocyte differentiation, while decreasing osteoblast differentiation. Taken together, these factors mediate decrease bone formation resulting in bone loss. Several therapeutic approaches are used today as bone loss prevention, including bisphosphonate therapy, calcium and vitamin D supplements.



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CHAPTER 2. THE BONE MARROW MICROENVIRONMENT CONTRIBUTES TO TYPE I DIABETES INDUCED OSTEOBLAST DEATH

2.1. ABSTRACT

Type I diabetes increases an individual's risk for bone loss and fracture, predominantly through suppression of osteoblast activity (bone formation). During diabetes onset, levels of blood glucose and pro-inflammatory cytokines (including tumor necrosis factor α (TNFα)) increased. At the same time, levels of osteoblast markers are rapidly decreased and stay decreased chronically (i.e., 40 days later) at which point bone loss is clearly evident. We hypothesized that early bone marrow inflammation can promote osteoblast death and hence reduced osteoblast markers. Indeed, examination of type I diabetic mouse bones demonstrates a greater than 2-fold increase in osteoblast TUNEL staining and increased expression of pro-apoptotic factors. Osteoblast death was amplified in both pharmacologic and spontaneous diabetic mouse models. Given the known signaling and inter-relationships between marrow cells and osteoblasts, we examined the role of diabetic marrow in causing the osteoblast death. Co-culture studies demonstrate that compared to control marrow cells, diabetic bone marrow cells increase osteoblast (MC3T3 and bone marrow derived) caspase 3 activity and the ratio of Bax:Bcl-2 expression. Mouse blood glucose levels positively correlated with bone marrow induced osteoblast death and negatively correlated with osteocalcin expression in bone, suggesting a relationship between type I diabetes, bone marrow and osteoblast death. TNF expression was elevated in diabetic marrow (but not co-cultured osteoblasts); therefore, we treated co-cultures with TNFα neutralizing antibodies. The antibody

protected osteoblasts from bone marrow induced death. Taken together, our findings implicate the bone marrow microenvironment and TNFα in mediating osteoblast death and contributing to type I diabetic bone loss.

2.2. INTRODUCTION

Type I diabetes is an autoimmune disease characterized by the loss of insulin producing pancreatic beta islet cells and by hypoinsulinemia and hyperglycemia. Potential complications resulting from type I diabetes include retinopathy, neuropathy, and nephropathy. Human and animal studies demonstrate that bone loss is another complication (1-7). As a result, type I diabetes is a risk factor for bone fractures (5, 8-10) that are often difficult to heal (11-14). Nearly 20% of type I diabetic patients age 20-56 have enough bone loss to meet the criteria for being osteoporotic (7, 15) and both males and females are vulnerable (16-20). Bone loss can result from decreased osteoblast activity (bone formation) and/or increased osteoclast activity (bone resorption). Previous studies demonstrate that type I diabetes decreases osteoblast activity, while effects on osteoclast activity are variable (21-31).

Thus, suppressed osteoblast activity is a key mediator of bone loss in type I diabetes, although its cause is unknown. Osteoblast activity can be regulated at several levels. Increased bone marrow stromal cell lineage progression to adipocytes at the expense of osteoblasts (32) is suggested by the increased bone marrow adiposity in diabetic models (21, 23, 33). Another mechanism is decreased osteoblast maturation as is indicated by the suppression of late stage osteoblast maturation markers in chronic diabetic animals and humans (21, 34, 35). Enhanced osteoblast death is yet another mechanism that can suppress osteoblast activity and cause bone loss, but has not been examined in type I diabetic bone under basal (non-stimulated) conditions.

Cell death is critical for the development, maturation and adaptation of the body's organs, including bone. Increased osteoblast and osteocyte death has been demonstrated under conditions where rapid bone remodeling takes place such as with limb disuse/unloading (36, 37). A role for osteoblast death in type I diabetic bone loss is suggested by the increase in pro-apoptotic, pro-inflammatory cytokines in the bone environment (24). One of the identified pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα), has previously been shown to suppress osteoblast maturation as well as promote osteoblast death *in vitro* (38-49). We also previously demonstrated that this pro-inflammatory cytokine is elevated during early stages of type I diabetes onset (24).

Knowing that type I diabetes onset is marked by the rapid suppression of osteoblast markers as well as concomitant hyperglycemia and increased bone inflammation (24), a role for bone marrow changes (inflammatory cells, inflammatory mediators) in promoting osteoblast death and the type I diabetic bone pathology is implicated. Therefore, we set out to identify the type I diabetic bone changes and their impact on osteoblast viability. We utilized two mouse models. The first involves pharmacologic-induction of type I diabetes by streptozotocin injection, which causes pancreatic beta-islet inflammation in rodents similar to what is observed in humans (50-52). This approach provides a precise time course of disease onset and we have previously demonstrated that this model causes a decrease in bone density similar to humans (7, 14-20, 53) and increases marrow adiposity (21, 54). Bone loss has been demonstrated in both male and female mice and in femur, tibia, vertebrae and calvaria (55). To exclude model specific effects, we also examined spontaneous diabetic mice

(Ins2$^{+/-}$). This mouse model exhibits bone loss and increased marrow adiposity similar to the pharmacologic streptozotocin model and the spontaneous NOD model (54)(Coe et al., manuscript submitted). Our findings are the first to demonstrate that the onset of diabetes does indeed cause an increase in osteoblast death. Bone marrow-osteoblast co-cultures indicate that diabetic marrow is one mediator of osteoblast death. Addition of TNFα neutralizing antibody to marrow-osteoblast co-cultures implicates a role for marrow TNFα and inflammation in osteoblast death.

2.3. MATERIALS AND METHODS

2.3.1. Diabetic mouse models

Pharmacologic induction of type I diabetes in adult (15-16 weeks) male mice (C57BL/6 and Balb/c (in previous studies); Harlan Laboratories, Houston, TX) was performed by 5 daily intraperitoneal injections with streptozotocin (40 μg/g body weight in 0.1 M citrate buffer). Controls were injected with buffer alone. In some experiments, male $Ins2^{+/-}$ mice (C57BL/6 background; Jackson Laboratory, Bar Harbor, Maine) were used. These mice become spontaneously diabetic at approximately 4-5 weeks of age. Diabetes was monitored by blood glucose measurements (>300 mg/dl) made immediately using a glucometer or later by using a Glucose Assay Kit (Sigma, Saint Louis, MO). All mice were kept on a light/dark (12h/12h) cycle at 23^{o}C, and received food (standard lab chow) and water ad libitum. Animal studies were conducted in accordance with the Michigan State University All-University Committee on Animal Use and Care.

2.3.2. RNA Analysis

Whole tibiae were isolated, crushed and RNA extracted using TriReagent according to manufacture protocol (Molecular Research Center Inc., Cincinnati, OH). RNA integrity was verified by formaldehyde-agarose gel electrophoresis. Synthesis of cDNA was performed using the Superscript II kit with as described by the manufacturer (Invitrogen, Carlsbad, CA). cDNA was amplified by PCR using the iQ SYBR Green

Supermix (Bio-Rad, Hercules, CA). Osteocalcin, TNFα and hypoxanthine-guanine phosphoribosyl transferase (HPRT; not modulated under diabetic conditions) were amplified using primers previously described (24, 56). Bax and Bcl-2 were amplified using the following primers: Bax 5'-GCT GAC ATG TTT GCT GGC AAC-3' and 5'-TGT CCA GCC CAT GAT GGT TCT GAT-3', Bcl-2 5'- GTG GAT GAC TGA GTA CCT GAA CC-3' and 5'-AGC CAG GAG AAA TCA AAC AGA G-3'. Real time PCR was carried out for 40 cycles using the iCycler (Bio-Rad, Hercules, CA). Each cycle consisted of 95^{o}C for 15 seconds, 60^{o}C for 30 seconds (except for osteocalcin, which had an annealing temperature of 65^{o}C) and 72^{o}C for 30 seconds. RNA-free samples, a negative control, did not produce amplicons. Melting curve and gel analyses (sizing and sequencing) verified single products of the appropriate base pair size.

2.3.3. Bone histology and TUNEL assays

Isolated bones were fixed in formalin, decalcified, embedded and sectioned at 5 micron thickness. To detect cell death *in vivo*, the TACS•XL® Basic In Situ Apoptosis Detection Kit was used according to manufacturer protocol (Trevigen Inc., Gaithersburg, MD). This method is a variation of terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL, that uses a brominated nucleotide (BrdU) to label DNA fragment ends rather than dUTP. Positive controls included slides incubated with nuclease. Five trabecular regions were examined for each mouse. Total osteoblasts counted ranged between 17 and 240 per bone. In most cases, over 1000 total osteoblasts were counted per condition.

2.3.4. Osteoblast and marrow co-culture

Osteoblasts (MC3T3-E1 cell line (57)) were plated at 10,000 cells per well of a 24 well plate. Upon reaching confluency, the media was supplemented with 2mM inorganic phosphate and 25μg/ml ascorbic acid. Co-culture experiments began when osteoblasts were cultured for a total of 14 days.

Bone marrow was harvested/flushed from femurs using a syringe containing α-MEM. Cells were centrifuged and resuspended with 10 milliliters complete media. A cell suspension aliquot was mixed with Turks solution for counting and cells were plated at 2 million cells per transwell permeable support (5 μm membrane; Corning, Fisher Scientific). Transwells were placed in wells containing differentiating osteoblasts. After 24 hours, osteoblasts were harvested for caspase 3 activity or RNA (Bax, Bcl-2) analyses. For neutralizing antibody studies, anti-TNFα antibody (R&D Systems) was added to the media at a concentration of 2 μg/ml at the time of marrow transwell addition. Osteoblasts were harvested 24 hours later to assess caspase 3 activities.

In some experiments (Figure 9B), bone marrow-derived osteoblasts were used (rather than MC3T3 cells). These cells were obtained by seeding flushed marrow cells (noted above) into each well of a 24 well plate at a concentration of 100,000 cells per well. Upon reaching confluency the media was supplemented with 2 mM inorganic phosphate and 25 μg/ml ascorbic acid and cells were cultured for an additional 14 days prior to the start of co-culture experiments.

2.3.5. Caspase 3 activity measurement

Caspase 3 activity was determined using the Caspase-Glo 3/7 Assay Kit according to manufacturer protocol (Promega, Madison, WI). Caspase 3 activity was then measured using a luminometer. Data was collected, the value for background subtracted, and values reported as luminescence (relative light units, RLU).

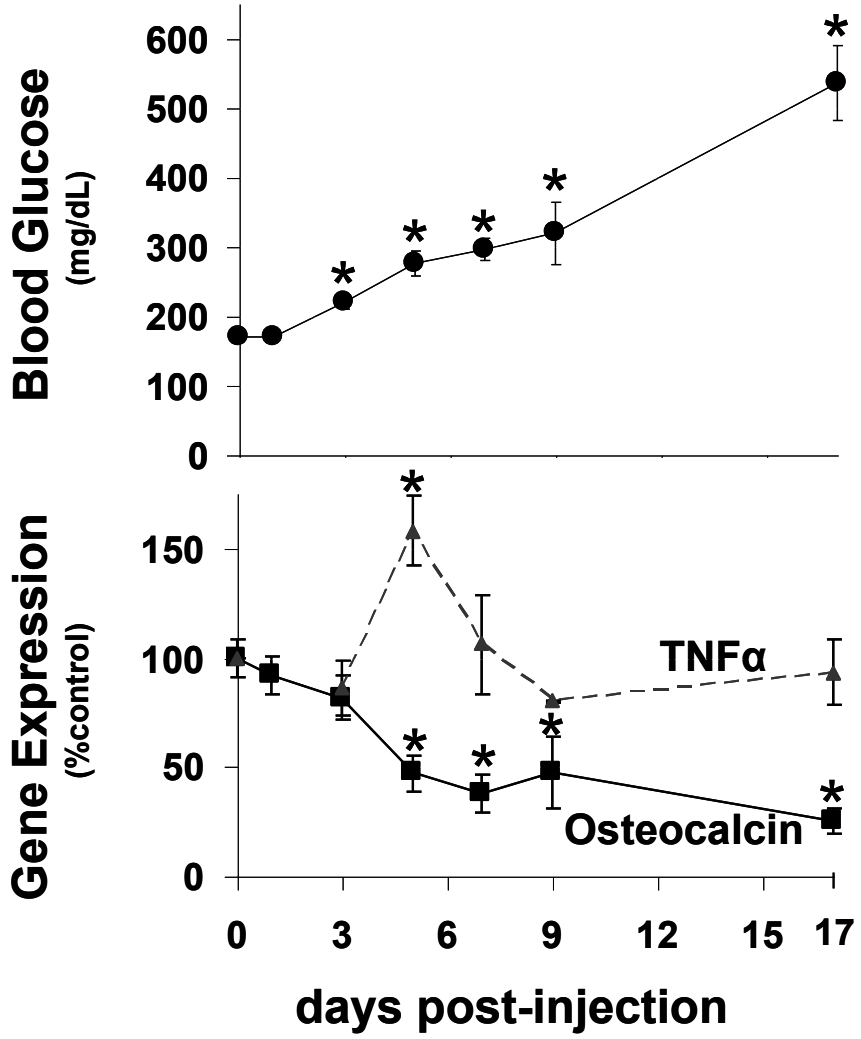
2.3.6. Statistical analysis

Statistical analyses were performed using Microsoft Excel data analysis program for student's t-test analysis and Pearson's correlation coefficient values. A p-value of less than 0.05 was considered statistically significant. Values are expressed as averages ± SE.

2.4. RESULTS

To identify mechanisms accounting for changes in bone during the onset of type I diabetes, diabetes was induced in adult male mice (15-16 weeks of age) by injection of streptozotocin a pancreatic beta-islet cell cytotoxin. As we previously reported, these mice display a significant decrease (35-50%) in tibia trabecular bone volume at 40 days post-injection (dpi, table I)(58). Three days after the first streptozotocin injection (3 dpi), non-fasting blood glucose levels significantly increased to 221 mg/dl (figure 6), a level that would be prognostic of diabetes in humans. With extended time, blood glucose levels continued to rise above 400 mg/dl while control levels stayed below 200 mg/dl (figure 6; table I). Previously we reported that serum TNFα levels transiently increased at 3 dpi (from 168±40 to 463±125 pg/ml in diabetic mice) and serum osteocalcin levels decreased at 5 dpi (from 436±42 to 261±30 ng/ml in diabetic mice) and remained suppressed (24). Similarly, bone TNFα mRNA expression transiently increased, while osteocalcin mRNA expression decreased in bone RNA extracts of diabetic mice at 5 dpi, (figure 6)(21, 54) and remains suppressed at extended time points, including 40 dpi (table I).

Figure 6. **Type I diabetes onset, marked by increasing blood glucose levels, is associated with increased TNFα expression and, at the same time, suppressed osteocalcin expression.** Diabetic mouse blood glucose (mg/dl) and whole bone RNA levels of TNFα (triangles) and osteocalcin (squares) at 1 - 17 days after the first streptozotocin injection (dpi). RNA levels are calculated relative to HPRT, a transcript which is not modulated by diabetes, and expressed as a percentage of control levels. Values are averages ± SE ($n \geq 5$ per point); * $p<0.05$ compared to control mice (set at 100%).



Thus, part of the mechanism of diabetic bone changes, in particular suppression of bone anabolic processes, lies in the early and rapid suppression of osteocalcin. Decreased maturation, altered osteoblast lineage selection (choosing the adipocyte over osteoblast lineage) and increased osteoblast death can contribute to the suppression of osteocalcin expression. Here, we tested the hypothesis that type I diabetes promotes osteoblast death. We focused on the 5 dpi time point because this is the time point where the key early changes occur (figure 6).

Table I. **Type I diabetic mouse parameters at 40 days**

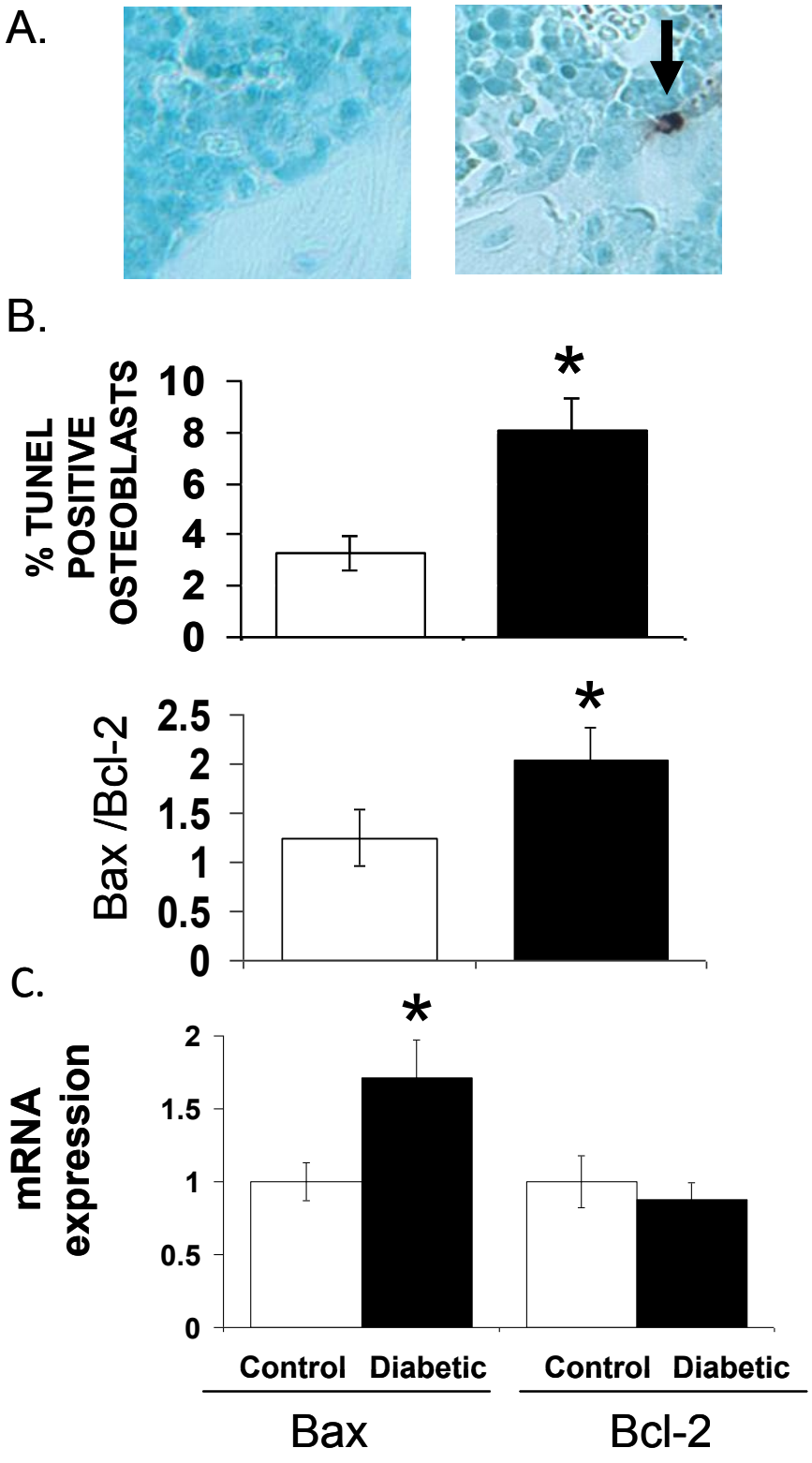
	Control	**Diabetic**
Body Mass (g)	29.4 ± 0.38	26.5 ± 0.65*
Blood Glucose (mg/dl)	182 ± 8	517 ± 17*
Bone volume fraction(%)	15.4 ± 1.3	9.9 ± 1.1*
Tibia Osteocalcin/ HPRT RNA	1 ± 0.2	0.3 ± 0.1*

n≥10 mice per group, * p<0.05

Consistent with our hypothesis, we observed a greater than 2-fold increase in TUNEL positive osteoblasts on diabetic compared to control bone surfaces at 5 dpi (figure 7B). This data was obtained from more than 30 mice per condition and over 1500 total osteoblasts were counted per condition. Consistent with this finding, the ratio of BAX (pro-apoptotic) to Bcl-2 (anti-apoptotic) expression levels, commonly used to obtain a general picture of cell viability, increased type I diabetic compared to control mouse bones (figure 7B). Analysis of theexpression of the individual genes demonstrates that an increase in Bax expression is responsible for elevating the ratio in diabetic mice, while Bcl-2 expression remained unchanged (figure 7C).

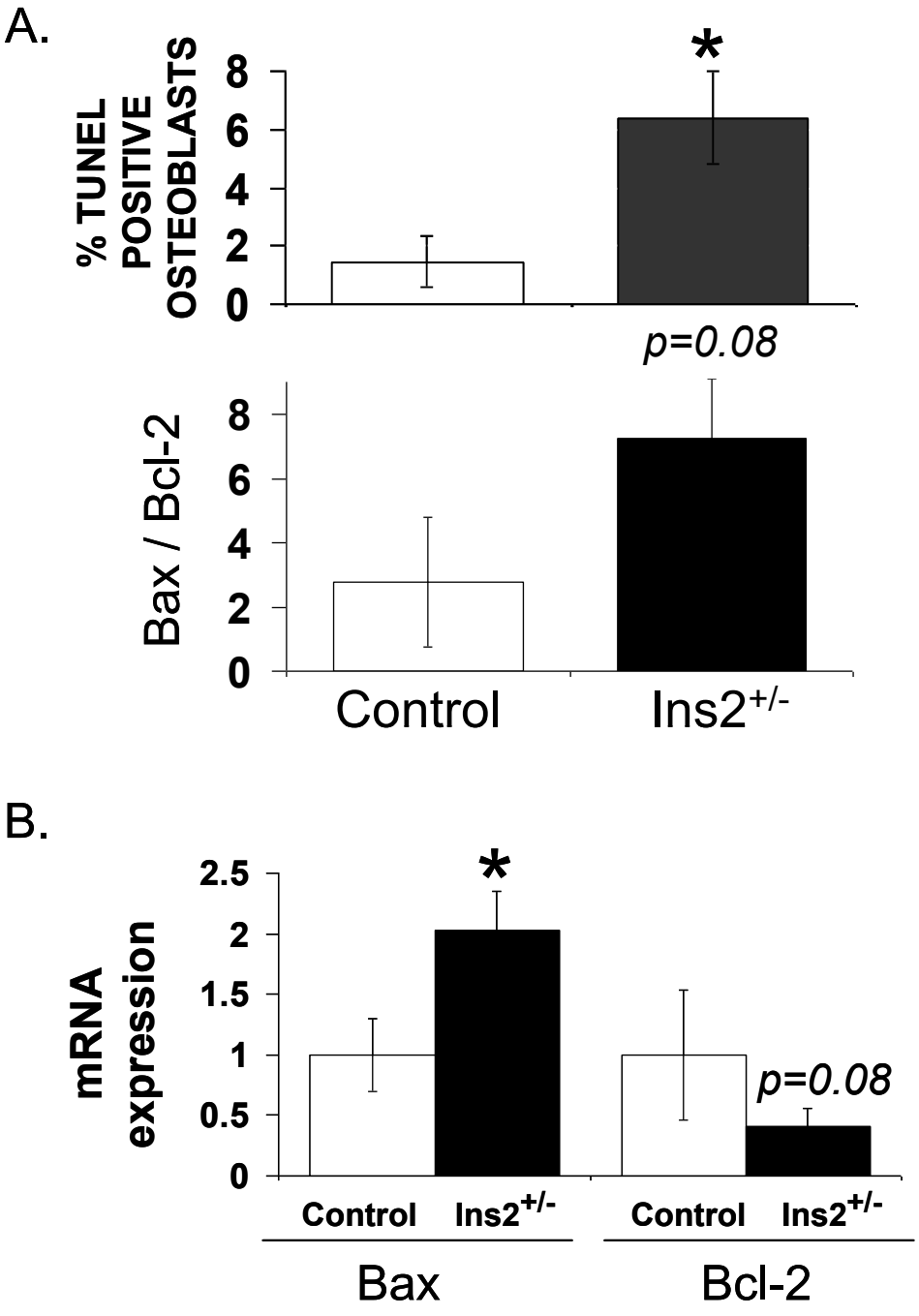
Figure 7. **Type I diabetes onset increased the percentage of TUNEL positive osteoblasts in vivo and increased BAX expression in tibias.** A. Representative images of control (left) and diabetic (right) bone sections. The arrow indicates a TUNEL positive osteoblast. B. The percentage of TUNEL positive osteoblasts and tibial BAX/Bcl-2 RNA expression ratios in control (white bars) and streptozotocin-induced diabetic (dark bars) mice at 5 days post injection (dpi). C. Individual levels of Bax and Bcl-2 RNAs in control and diabetic bones at 5 dpi. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$ per group); * denotes $p<0.05$. For TUNEL assay $n>32$ per condition. For Interpretation to the references of color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure7(cont'd)



To confirm that the effects we observe are not due to the direct action of streptozotocin, we examined TUNEL positive osteoblasts in spontaneously diabetic $Ins2^{+/-}$ mice. This non-obese type I diabetic mouse model has a point mutation in the A chain of insulin that ultimately triggers pancreatic beta cell death and causes type I diabetes (59). Hyperglycemia is first detectable at approximately 4 weeks of age in male mice and by 10 weeks of age bone loss is apparent. In this experiment, we harvested $Ins2^{+/-}$ and control $Ins2^{+/+}$ mice at 6 weeks of age to ensure induction of diabetes. Non-fasting blood glucose levels of $Ins2^{+/-}$ mice were highly elevated (462 ± 61 mg/dl) compared to littermate wild type mice (190 ± 17 mg/dl). Examination of the percentage of TUNEL positive osteoblasts in the $Ins2^{+/-}$ mice indicated a significant increase (greater than 3-fold) in diabetic ($Ins2^{+/-}$) compared to the control ($Ins2^{+/+}$) mouse bones (figure 8A), confirming our findings in the streptozotocin-diabetic mouse model. The ratio of tibia Bax/Bcl-2 expression was increased, but did not reach statistical significance ($p=0.08$; figure 8A); this appeared to result from the variability seen in Bcl-2 expression in control bones (figure 8B). However, Bax expression itself was statistically increased in diabetic $Ins2^{+/-}$ compared to control mouse bones (figure 8B) consistent with the STZ model and in line with the observed increase in TUNEL positive osteoblasts.

Figure 8. **Osteoblast death is increased in spontaneous type I diabetic Ins2 mice.** A. Similar to studies in the streptozotocin pharmacologic diabetic mouse model, the percentage of TUNEL positive osteoblasts, and the Bax:Bcl-2 ratio were elevated in spontaneously diabetic ($Ins2^{+/-}$ strain; black bars) and littermate control (wild type; white bars) mouse bones. B. RNA expression of Bax and Bcl-2 individually expressed relative to HPRT in control (white bars) and $Ins2^{+/-}$ (black bars). Control and diabetic mice were 6 weeks of age. Bars represent the average value ± SE ($n \geq 5$); * denotes $p<0.05$.



We next asked the question: what is occurring during the onset of diabetes that could cause an increase in osteoblast death? Based on our past studies, we considered the potential of type I diabetes to alter the bone marrow which could serve as a potential mediator of the altered osteoblast activity. To test this hypothesis, we isolated bone marrow from control and diabetic (5 dpi) mice and cultured the marrow in transwells inserted into wells containing an osteoblast cell line, MC3T3-E1 (figure 9A). After 24 hours, caspase 3 activity (a marker of cell death) increased by nearly 2-fold in osteoblasts co-cultured with diabetic compared to control bone marrow (figure 9B). Accordingly, the Bax to Bcl-2 ratio was also elevated (figure 9C). To confirm that this effect was not specific to the streptozotocin model, we isolated bone marrow from $Ins2^{+/-}$ mice, co-cultured the marrow with osteoblasts, and examined osteoblast death. Consistent with the pharmacologic streptozotocin model, marrow from the spontaneous diabetic model also increased osteoblast caspase 3 activity by more than 1.7-fold compared to control bone marrow (figure 9B). To verify that the response was not specific to the MC3T3-E1 osteoblast cell line, we co-cultured mature bone marrow-derived osteoblasts with normal and diabetic marrow. Consistent with the MC3T3 osteoblast response, bone marrow-derived osteoblasts also exhibited a significant increase in caspase 3 activity when co-cultured with marrow isolated from type 1 diabetic mice. This increase was smaller than what was seen in the MC3T3-E1 cultures and may be the result of cell heterogeneity with regard to cell type as well as cell stage. Taken together, these findings indicate that diabetic bone marrow, alone, is capable of impacting osteoblasts.

Figure 9. **Diabetic mouse bone marrow is capable of increasing osteoblast death *in vitro***. A. Representation of the co-culture system utilized in the study. Bone marrow cells are cultured in transwells containing a 10 μm thick membrane with 0.4 μm pores at the bottom of the well to allow diffusion of factors between this cell compartment and the osteoblasts (MC3T3-E1 or bone marrow stromal (BMS)-derived osteoblasts) plated at the bottom of the well. B. After co-culturing with marrow cells for 24 hours, osteoblast caspase 3 activity and Bax/Bcl-2 RNA levels were determined. Bone marrow for these studies was obtained either from 15-week old control (white bar) and streptozotocin-injected (black bar) mice at 5 dpi (top and bottom graphs) or from control (white bar) and diabetic $Ins2^{+/-}$ (black bar) mice at 6 weeks of age (middle graph). The bottom graph examined marrow effects on bone marrow stromal (BMS) osteoblast cells. C. Osteoblast Bax/Bcl-2 expression relative to HPRT, from co-cultures with control (white bar) and diabetic (black bar) marrow. Values represent averages ± SE (n>5 different mouse marrow co-cultures per condition); * denotes $p<0.05$.

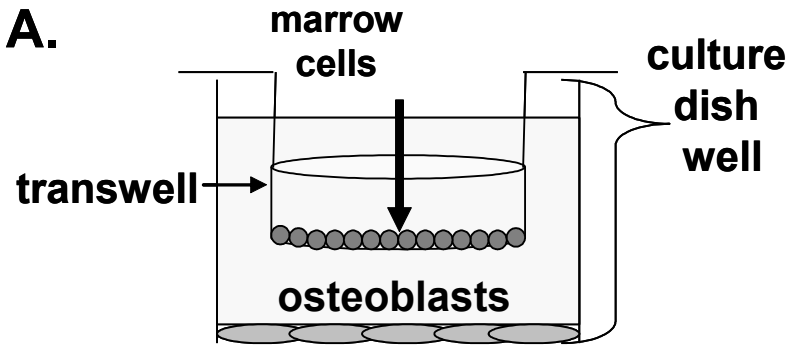
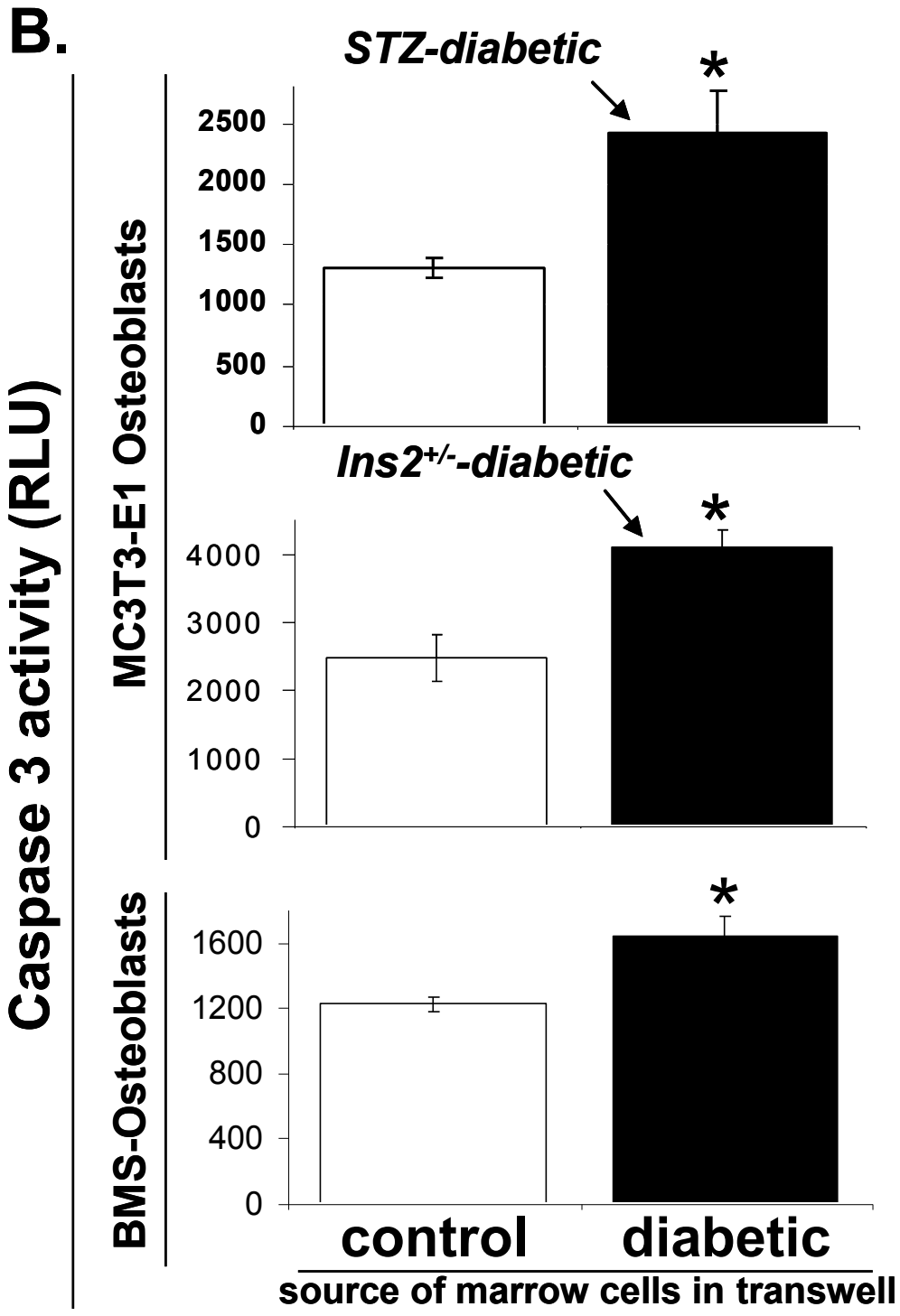


Figure 9 (cont'd)



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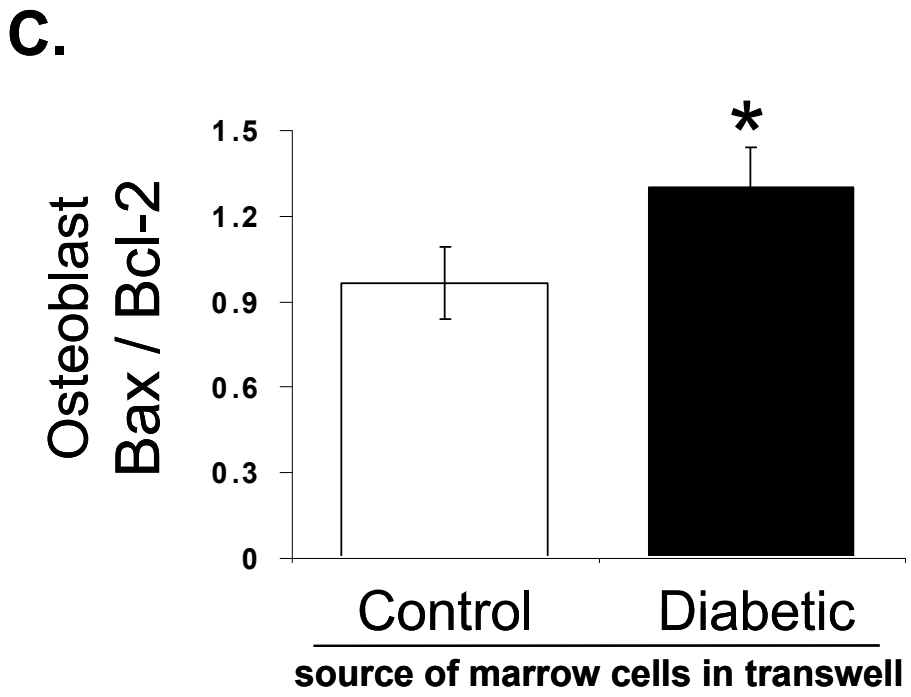
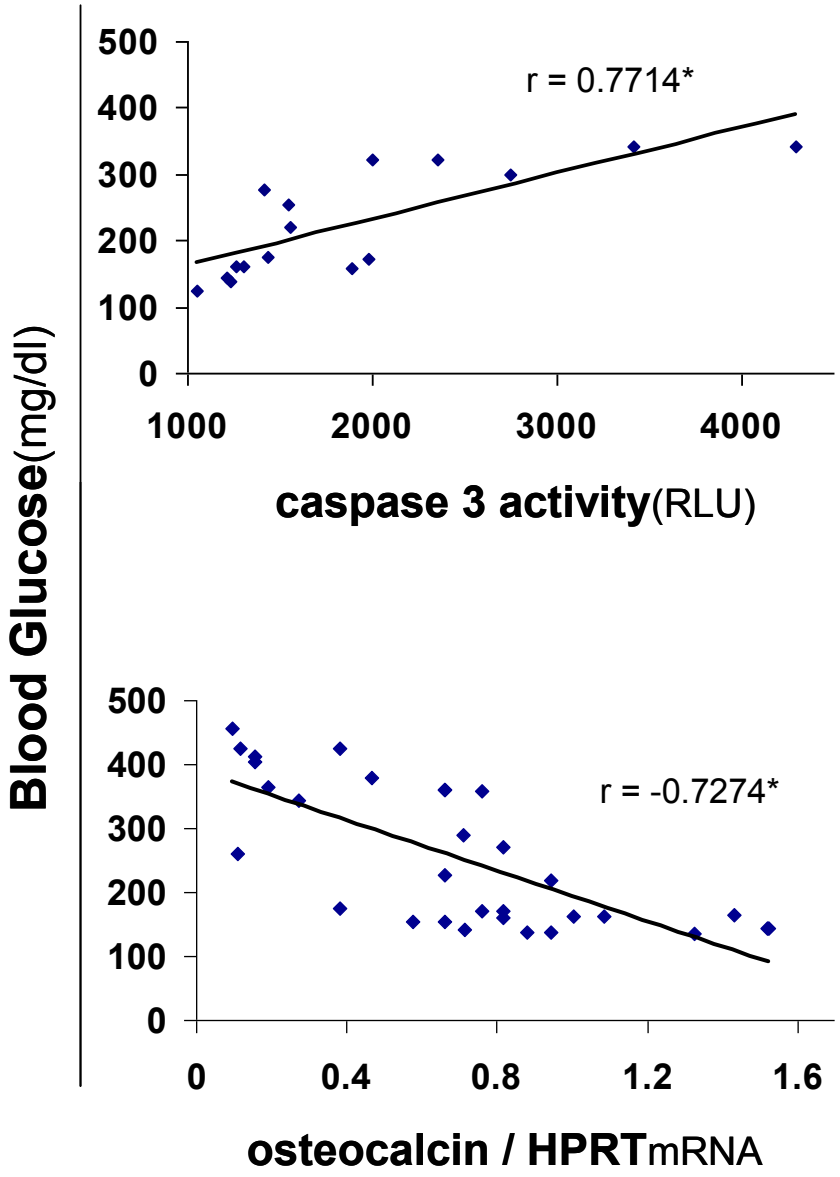


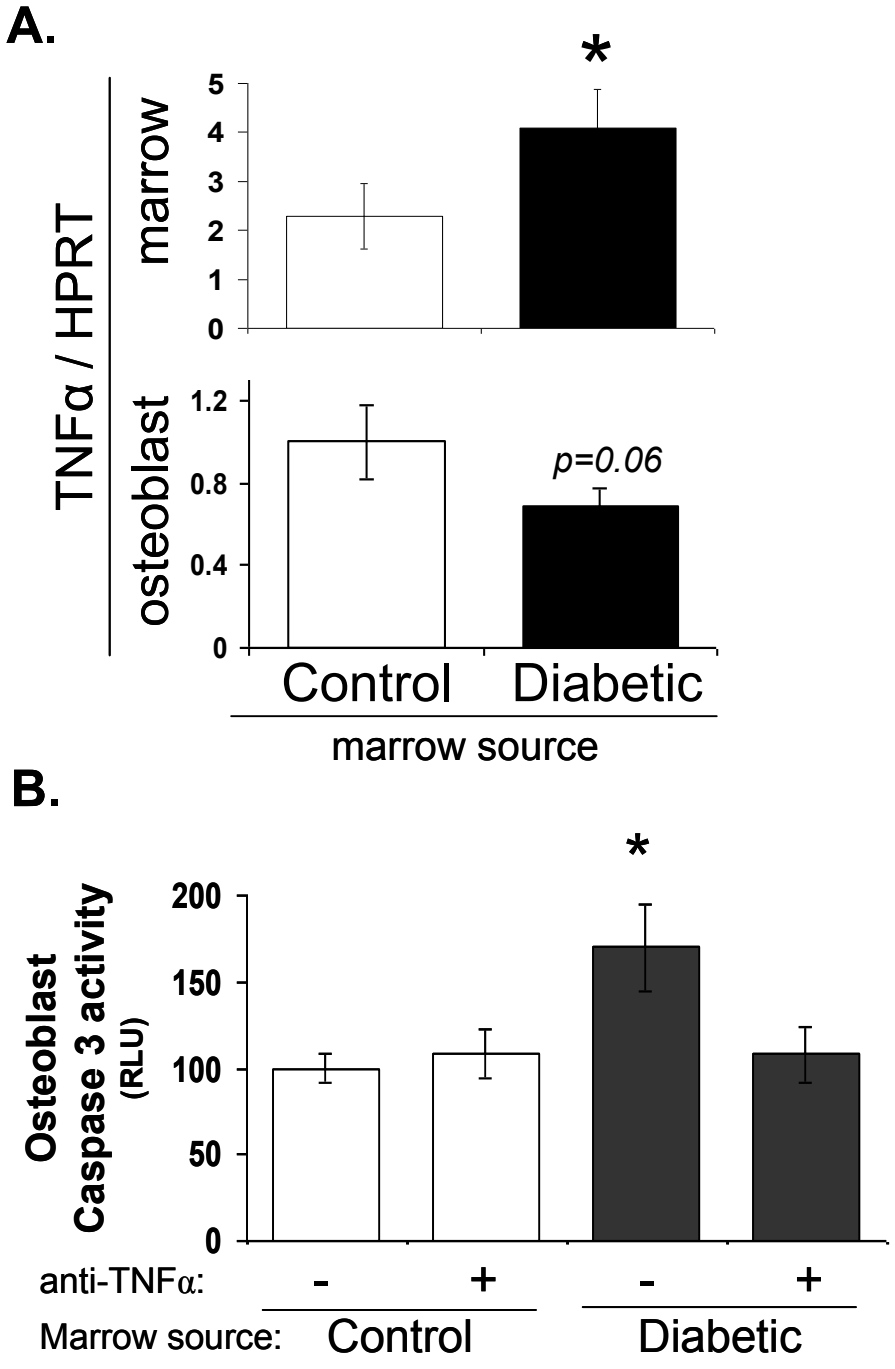
Interestingly, it was noted that some marrow isolates were especially effective at inducing caspase 3 activity. Therefore, we examined the correlation between mouse blood glucose levels and the ability of the isolated bone marrow cells to stimulate osteoblast caspase 3 activity (figure 10). We obtained a Pearson's correlation coefficient value of 0.7714 (two tailed $p<0.0005$; r confidence interval between 0.447 and 0.917) indicating that mouse blood glucose levels are positively associated with the ability of bone marrow cells to induce osteoblast death. Consistent with suppressed osteoblast activity in vivo, blood glucose levels were negatively correlated with expression of the osteoblast marker, osteocalcin (figure 10). The Pearson's correlation coefficient was -0.7274 (two tailed $p<0.0001$; r confidence interval between -0.860 and -0.502). Thus, disease severity, characterized by blood glucose levels, is linked to marrow-osteoblast toxicity and osteoblast death.

Figure 10. **Blood glucose correlates with osteoblast caspase 3 activity *in vitro* and osteocalcin expression *in vivo*.** The top scatter plot illustrates the positive correlation between elevated mouse blood glucose levels (mg/dl) and the ability of mouse marrow cells to induce co-cultured osteoblast caspase 3 activity (n=16). The bottom scatter plot illustrates the negative correlation between mouse blood glucose levels and mouse tibial osteocalcin gene expression (n=31). Comparisons were computed using a Pearson's correlation coefficient value (r); * $p<0.0005$.



Diabetes onset is characterized by an inflammatory response marked by increased expression of cytokines, including TNFα in serum as well as in whole bone RNA (24)(figure 6). Therefore, we hypothesized that TNFα may be one of the marrow factors contributing to diabetes induced osteoblast death. Examination of TNFα mRNA levels in bone marrow isolated at 5 dpi demonstrates, as expected, that TNFα expression is increased in diabetic compared to control mouse marrow (figure 11A). Whereas, no increase was seen in osteoblasts co-cultured with control or diabetic marrow; although, we did observe a trend to decreased expression in osteoblasts co-cultured with diabetic bone marrow. The latter may be the result of some type of negative feed back response. To test if TNFα is involved in mediating increased osteoblast death in our diabetic marrow-osteoblast co-cultures, we treated co-cultures with neutralizing TNFα antibody. After 24 hours we measured caspase 3 activity in the osteoblast cultures. Our results indicate that treatment with neutralizing TNFα antibody prevented the increase in diabetic marrow induced osteoblast death (figure 11B), indicating a role for TNFα in promoting osteoblast death in this system.

Figure 11. **TNFα is involved in mediating diabetic marrow induced osteoblast death**. A. Bone marrow was harvest from 15 week old control (white bars) and STZ-diabetic (black bars) mice at 5 dpi and analyzed for TNFα expression (top graph). Osteoblasts plated at the bottom of the co-culture were also analyzed for TNFα expression (bottom graph). B. Marrow was cultured for 24 hours in transwells above osteoblasts (cultured for 14 days). Cells were treated with serum (vehicle) or TNFα neutralizing antibody. After 24 hours, osteoblasts were harvested for caspase 3 activity. Values represent averages ± SE (n≥5 different mouse marrow co-cultures per condition); * denotes $p<0.05$.



2.5. DISCUSSION

Type I diabetes is associated with bone loss marked by decreased osteoblast activity that is typically coupled with unchanged or decreased osteoclast activity. Here we demonstrate that type I diabetes onset induces a bone microenvironment that is detrimental to osteoblasts. Specifically, increased osteoblast death is evident at 5 dpi in streptozotocin-diabetic mouse bones consistent with a reduction in expression of a mature osteoblast marker, osteocalcin. Increased osteoblast death is evident in both spontaneous and pharmacologically induced diabetic mouse models consistent with the described bone loss in both models (54). This bone adaptation to type I diabetes may represent a switch in energy from anabolic processes (building bone) to fighting disease.

Cytokines such as TNFα, IL-1 and IFN-γ are known to decrease osteoblast viability, growth and maturation (38-49, 60). Because of the increased cytokine expression during early bone phenotype changes, we hypothesized that inflammation and pro-inflammatory cytokine production contributed to osteoblast death during the early onset of diabetes. Previous reports indicate that mononuclear/macrophage cells isolated from diabetic mice and humans display greater IFN-γ, TNFα, IL-1β, and IL-6 secretion and/or expression compared to non-diabetics (61-63) and could be involved in the increase in cytokine expression in bone as well as changes in the bone marrow itself. Other conditions marked by increased pro-inflammatory cytokine production, such as rheumatoid arthritis (without corticosteroid treatment), also demonstrate decreased bone formation similar to what is observed in diabetic bones (21, 35, 54, 64). Osteoclast activity, which one would expect to be increased in an elevated pro-inflammatory cytokine environment, is decreased or unaltered in both type I diabetes and rheumatoid arthritis

(21, 35, 54, 64). These inflammatory diseases have interesting similarities, suggesting some areas for potential overlapping mechanisms. More closely related to our studies are those examining periodontal disease in type II diabetic rodent models. These studies indicate that in response to a challenge of *P. gingivalis* db/db diabetic mouse bones exhibit greater expression of MCP-1, MIP, and TNFα mRNA and increased osteoblast death compared to control mice (65-68). Similar to our findings, treatment with a pan-caspase inhibitor or an inhibitor of TNFα was successful in reducing gingivalis induced bone loss and cell death (38, 69).

Our studies revealed that bone marrow isolated from control and type I diabetic mice is capable of inducing osteoblast death *in vitro*; similar to what is observed *in vivo*. Thus, the early induction of osteoblast death seen in diabetes is likely not solely the result of the metabolic effects of diabetes directly on osteoblasts. Rather, diabetes onset affects the bone marrow cells which then contribute to altered osteoblast function and death. Treatment of osteoblasts *in vitro* with TNFα neutralizing antibody prevented diabetic marrow induced death, implicating a key role for TNFα in this process. However, it should be noted that other cytokines were also observed to be increased during type I diabetes onset and could also contribute to bone pathology *in vivo* during early or later stages of diabetes progression (24). In particular, granulocyte-colony stimulating factor (G-CSF) has been recently demonstrated to indirectly increase osteoblast apoptosis through its modulation of the bone marrow (70). We tested G-CSF inhibitory antibodies in our co-culture system (data not shown) but were unable to suppress osteoblast apoptosis. While this suggests that G-CSF does not mediate osteoblast death *in vitro*, it does not exclude G-CSF from playing an important role in mediating compositional changes in the bone marrow seen in diabetes and warrants future investigation.

Could the increase in osteoblast death in diabetic mice be serving some physiologic function? It could be important for suppressing anabolic bone formation activity, but may serve additional or alternate roles. In particular, apoptotic cells can attenuate inflammation (71). Therefore, the death of osteoblasts on the bone surface adjacent to the marrow may be involved in a feedback loop to suppress chronic diabetic marrow inflammation. Communication between bone marrow and osteoblasts is critical for the regulation of bone formation, hematopoesis, and perhaps tissue healing (72, 73). A recent study inducing osteoblast deficiency in mice demonstrates profound alterations in the bone marrow, marked by decreased cell number and decreased lymphoid, erythroid and myeloid cell populations (74). Therefore, while we show that the marrow is promoting osteoblast death, the response is most likely a two-way street and the loss of osteoblasts may also contribute to altering bone marrow cells in diabetic mice.

In summary, we demonstrate type I diabetes increases osteoblast death through local bone marrow changes. We propose that TNFα is a key mediator of the type I diabetic bone marrow effects on osteoblasts during diabetes onset, however with extended time additional factors such as reactive oxygen species and hyperglycemia may also be involved in increasing osteoblast death and thereby causing bone loss. Future studies will focus on the mechanisms accounting for these responses and identifying potential therapeutic targets.

2.6. ACKNOWLEDGEMENTS

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CHPATER 3. CASPASE 2 DEFICIENCY PROTECTS MICE FROM DIABETES-INDUCED MARROW ADIPOSITY

3.1. ABSTRACT

Type I (T1) diabetes is an autoimmune and metabolic disease associated with bone loss. Bone formation and density are decreased in T1-diabetic mice. Correspondingly, the number of TUNEL positive, dying osteoblasts increases in T1-diabetic mouse bones. Two known mediators of osteoblast death, TNFα and ROS, are increased in T1-diabetic bone. TNFα and oxidative stress are known to activate caspase-2, a factor involved in the extrinsic apoptotic pathway. Therefore, we investigated the requirement of caspase-2 for diabetes-induced osteoblast death and bone loss. Diabetes was induced in 16-week old C57BL/6 caspase-2 deficient mice and their wild type littermates and markers of osteoblast death, bone formation and resorption, and marrow adiposity were examined. Despite its involvement in extrinsic cell death, deficiency of caspase-2 did not prevent or reduce diabetes-induced osteoblast death as evidenced by a 2-fold increase in TUNEL positive osteoblasts in both mouse genotypes. Similarly, deficiency of caspase-2 did not prevent T1-diabetes induced bone loss in trabecular bone (BVF decreased by 30% and 50%, respectively) and cortical bone (decreased cortical thickness and area with increased marrow area). Interestingly, at this age, differences in bone parameters were not seen between genotypes. However, caspase-2 deficiency attenuated diabetes-induced bone marrow adiposity and adipocyte gene expression. Taken together, our data suggests that caspase-2 deficiency may play a role in promoting marrow adiposity under stress or disease conditions, but it is not required for T1-diabetes

induced bone loss.

3.2. INTRODUCTION

Type I (T1) diabetes affects over 800,000 men and women in the United States and is commonly characterized by elevated blood glucose levels (greater than 200 mg/dl) and little or no insulin secretion. Many known secondary complications arise from T1-diabetes including osteoporosis. Both male and female patients are vulnerable to decreased bone density and increased fracture risk (1-12). T1-diabetic rodent models (pharmacological and spontaneous) exhibit decreased bone density comparable to the human bone phenotype (*13*). To date, bone loss been detected in all bones examined (e.g. femur, tibia, calvaria, vertebrae) in mouse models of T1-diabetes and is independent of gender (*13-15*), similar to humans. Although exact mechanisms of T1-diabetic bone loss remain unknown, recent studies have demonstrated a role for decreased osteoblast number and activity in diabetic bone (*13-16*). Decreased bone formation can occur through multiple mechanisms including altered mesenchymal stem cell linage selection (adipocytes over osteoblasts), reduced osteoblast differentiation, and increased osteoblast apoptosis (*15, 17*). Past studies in our lab have demonstrated that bone loss occurs irrespective of marrow adiposity/altered stem cell lineage selection (*13, 18, 19*). This has led us to focus on the role of osteoblast apoptosis in diabetic bone loss.

All osteoclasts and roughly 60-80% of osteoblasts (*20*) undergo programmed cell death (PCD) as an integral physiological component of bone remodeling, fracture repair and rejuvenation. PCD has been implicated in bone adaptation with unloading (*21, 22*), aging (*23*) and bone-destructive diseases such as rheumatoid arthritis, periodontal diseases, and T1-diabetes (*17, 24-26*). Recently, our lab demonstrated an increase in osteoblast TUNEL staining during diabetes onset (*17*) which is consistent with the first

detectable suppression of osteocalcin gene expression in bone (*27*). Therefore, increased osteoblast apoptosis may play a critical role in mediating T1-diabetic bone loss.

TNFα and reactive oxygen species (ROS) are two known mediators of osteoblast death (*28-34*). Several studies have identified increased TNFα and ROS in diabetic bone (*27, 35-37*) suggesting a possible role for each in mediating diabetes induced osteoblast death. TNFα levels have been shown to be elevated in diabetic serum, but more importantly, TNFα mRNA levels/expression are also elevated in the bone microenvironment during diabetes onset (*17, 27, 38*); this is the time when increased osteoblast death and reduced osteocalcin expression are first observed (*27*). Furthermore, the increased osteoblast death induced by isolated diabetic marrow cells is suppressed by treatment with TNFα neutralizing antibodies (*17*). Correspondingly, entanercept or pegsunercept, TNFα inhibitors used clinically, suppress diabetes-enhanced death of fibroblasts, bone-lining cells and osteoblasts arising from periodontal disease *in vivo* (*24, 39, 40*). Similarly, ROS can contribute to diabetic complications and cell death. Past studies demonstrate that ROS levels are elevated in bones of T1-diabetic mice (*37*) as well as in spontaneously developing T2-diabetic Torii rats (*41*). Hyperglycemia, AGE accumulation, superoxide production and/or pro-inflammatory cytokines (TNFα and INFγ) are thought to contribute to the elevated ROS levels (*32, 35*). When oxidative stress is reduced, a notable reduction in osteoblast apoptosis is observed (*33, 34*). Reduction of oxidative stress in diabetic mice, by overexpressing an intracellular antioxidant, thioredoxin-1 (TRX), attenuates bone loss (*35, 37*). Taken together, both TNFα and ROS likely contribute, to some extent, to the diabetic bone phenotype.

Cells can die by two main apoptotic regulatory pathways: intrinsic and extrinsic. Intrinsic apoptosis, also known as mitochondria-mediated cell death, is regulated through the balance of pro-apoptotic factors (Bax, Bak, Bim, Bid) and anti-apoptotic factors (Bcl-2 and Bcl-xl). The extrinsic pathway can be activated by extracellular ligands, including TNFα and Fas, which bind membrane bound receptors (i.e. TNFR) and trigger a cascade of caspase activations (*31*). ROS enhances intracellular oxidative stress and thereby triggers a cascade of caspase activations (*42*). Studies show that both ROS and TNFα activate multiple caspases including, caspase-2, -3, -8 and -9 (*42, 43*). Caspases are a family of proteins that contain a cysteine residue capable of cleaving an aspartic acid-containing motif on the next downstream caspase. Two different caspase groups are involved in PCD: initiators and effectors. Initiator caspases (i.e. caspase 2, -8, -9) are responsible for activation of the effector caspases (i.e. caspase-3, -7) that cleave an array of cellular substrates (*44*).

Little is known about which caspase contribute to cell death in diabetic bone. Here we focused on the role of caspase-2, which is the most conserved caspase, sharing sequence homology with most initiator caspases but functioning more closely to the effector caspases, which makes it an intriguing caspase to study (*45*). Caspase-2 can be activated in response to cell stress, oxidative stress and TNFα treatment (*46-48*), all conditions that are elevated in diabetic mouse bone. Additionally, caspase-2 deficient mouse embryonic fibroblasts, derived from mesenchymal stem cells (which can differentiate into osteoblasts), showed protection from drug induced cell death, indicating a role for caspase-2 in mesenchymal-derived cell death (*49*). Here we demonstrate that caspase-2 is not required for type I diabetes induced osteoblast death or bone loss, but

surprisingly, caspase-2 deficiency attenuates diabetes-induced marrow adiposity.

3.3. MATERIALS AND METHODS

3.3.1. Animals

Diabetes was induced in C57BL/6 adult (15-16 week old) caspase 2 deficient (*50*) and wild type littermate male mice by 5 consecutive daily intraperitoneal injections of streptozotocin (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Corresponding genotype controls were given citrate buffer alone. Mice were maintained on a 12-hour light, 12-hour dark cycle at 23°C, given standard lab chow and water *ad libitum*. Diabetes was confirmed by blood glucose measurements (>300 mg/dl) obtained with an Accu-Check compact glucometer (Roche Diagnostics Corporation, Indianapolis, IN). Mice were euthanized at 5 or 40 days after diabetes induction and total body, tibialis anterior, and subcutaneous femoral fat pad recorded. Animal procedures were approved by the Institutional Animal Care and Use Committee

3.3.2. Genotyping

DNA was obtained from tail snips of wild type and caspase-2 deficient mice. Caspase-2 deficient mice have a mutation that deletes the exon encoding the QACRG active site of the enzyme and a portion of the subsequent exon that encodes for the caspase-2 short isoform. This deletion inactivates both the long and short form of caspase-2. Genotyping assessed the presence or absence of this region as previously

described (*51*). PCR products were separated on a 1.5% agarose gel to ensure the presence or absence of an amplicon band.

3.3.3. RNA Analysis

Immediately after euthanasia, tibias were cleaned of soft tissue and one was snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted from the bone using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA integrity was determined by formaldehyde-agrose gel electrophoresis. cDNA was synthesized using Superscript II Reverse Transcriptase Kit and oligo dT (12-18) primers (Invitrogen, Carlsbad, CA). cDNA was amplified by quantitative real time PCR with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers. Primers for fatty acid binding protein (aP2), Bcl-2 associated protein-X (Bax), B-cell lymphoma-2 (Bcl-2), hypoxanthine-guanine phosphoribosyl transferase (HPRT), osteocalcin, tumor necrosis factor-α (TNFα), tartrate resistant acid phosphatase 5 (TRAP5) are previously described (*16, 17*). HPRT served as a housekeeping gene; its levels were not altered in bone by diabetes. Each amplification cycle consisted of 95°C for 15 seconds, 60°C for 30 seconds (except osteocalcin which had an annealing temperature of 65°C) and 72°C for 30 seconds. RNA-free samples were used as a negative control and did not produce amplicons. PCR products were separated on 1.5% agarose gel electrophoresis and sequenced to verify that the desired gene is being amplified.

3.3.4. Micro-computed Tomography (μCT) analysis

Fixed femurs (stored in 70% alcohol) were scanned using the GE Explore μCT system at a voxel resolution of 20um from 720 views with a beam strength of 80kvp and 450uA and an integration time of 2000ms. Scans included bones from each condition and a phantom bone to standardize the grayscale values and maintain consistency between runs. Using the system's auto-threshold (1000) and an isosurface analysis, trabecular bone densities (bone volume fraction (BV/TV), bone mineral density (BMD) and content (BMC), trabecular number (Tb. N) and trabecular thickness (Tb. Th) were measured in the trabecular region defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis, and excluding the outer cortical shell. Cortical bone measurements were determined with a $2mm^3$ region of interest (ROI) in the mid-diaphysis, with the exception of cortical bone mineral content and bone mineral density, which were measured using a smaller 0.1 mm^3 ROI. Cortical BMC, BMD, moment of inertia, thickness, perimeters and areas and trabecular densities were computed by the GE Microview Software for visualization and analysis of volumetric image data.

3.3.5. Bone Histology and Histomorphometry

Bones fixed in formalin underwent dehydration, clearing and infiltration on a routine overnight processing schedule. Samples are then paraffin embedded and sectioned at 5μm using a rotary microtome. Osteoclasts were identified, by staining for

TRAP (Sigma). Osteoclasts, osteoblasts and adipocytes were identified in the trabecular region of the femur, defined at 0.17mm under the growth plate and extending 2mm toward to diaphysis. Osteoclast and osteoblast surfaces were measured in three trabecular regions for each mouse and expressed as a percent of total trabecular surface. Visible adipocytes, greater than 30μm, were counted in the same trabecular region.

Cell death was determined using a TACS•XL® Basic In Situ Apoptosis Detection Kit (TUNEL, Trevigen Inc., Gaithersburg, MD) on femur sections; osteoblasts with positive nuclei were counted and expressed as a percentage of total osteoblasts counted per bone. Positive controls included slides incubated with nuclease. Five trabecular regions were examined for each mouse. Total osteoblasts counted ranged between 83 and 190 per bone. Over 500 total osteoblasts were counted per condition.

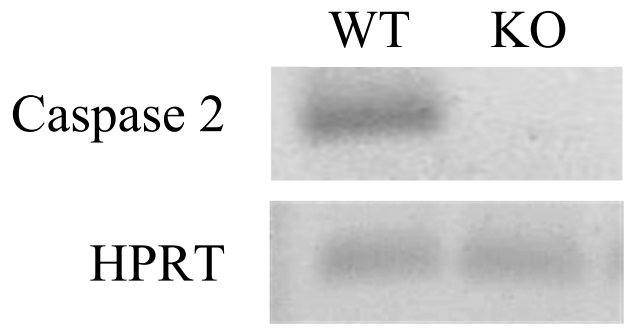
3.3.6. Statistical Analysis

All data is presented as mean ± standard error. Statistical significance (p-value < 0.05) of main effects (genotype difference or diabetes) as well as genotype x diabetes interaction (which determines if diabetes alters the genotype effect or vise versa) was determined using factorial analysis of variance (ANOVA) with SPSS statistical software (Chicago, IL). Student's t-test was also used to determine significance where necessary.

3.4. RESULTS

Cellular caspase-2 can be activated by oxidative stress and TNFα signaling, all of which are present in T1-diabetes. To test if capsase-2 is required for the induction of diabetic bone pathology, and in turn could be a therapeutic target, we induced diabetes in caspase-2 deficient and littermate wild type mice. The absence of caspase-2 was determined by genotyping and confirmed by the lack of detectable caspase-2 mRNA in bone RNA isolates from deficient mice (figure 12).

Figure 12. **The absence of detectable caspase-2 mRNA in tibias RNA isolates from deficient mice.** Caspase-2 mRNA expression was examined in wild type (left) and caspase-2 deficient (right) bone. PCR products were subsequently run on a 1.5% agarose gel for the absence of caspase-2. RNA levels are expressed relative to the housekeeping gene HPRT.



At 15-16 weeks of age wild type and caspase 2 deficient mice were injected with streptozotocin (to induce diabetes) or vehicle (controls). Blood glucose levels illustrated that caspase-2 deficiency did not impact diabetes induction or severity. Specifically,

diabetic wild type and diabetic caspase-2 deficient mice displayed similar elevations in blood glucose levels, averaging 459 mg/dl and 474 mg/dl, respectively (table II). Consistent with previous studies, T1-diabetes caused decreases in total body mass (12% and 17%, respectively) and femoral fat pad mass (64% and 60%, respectively) in both wild type and caspase-2 deficient mice. Although there was a trend toward decreased lean muscle mass in diabetic mice, it did not reach statistical significance (unlike previous studies). Taken together, caspase-2 deficiency did not modify general physiologic responses to T1-diabetes.

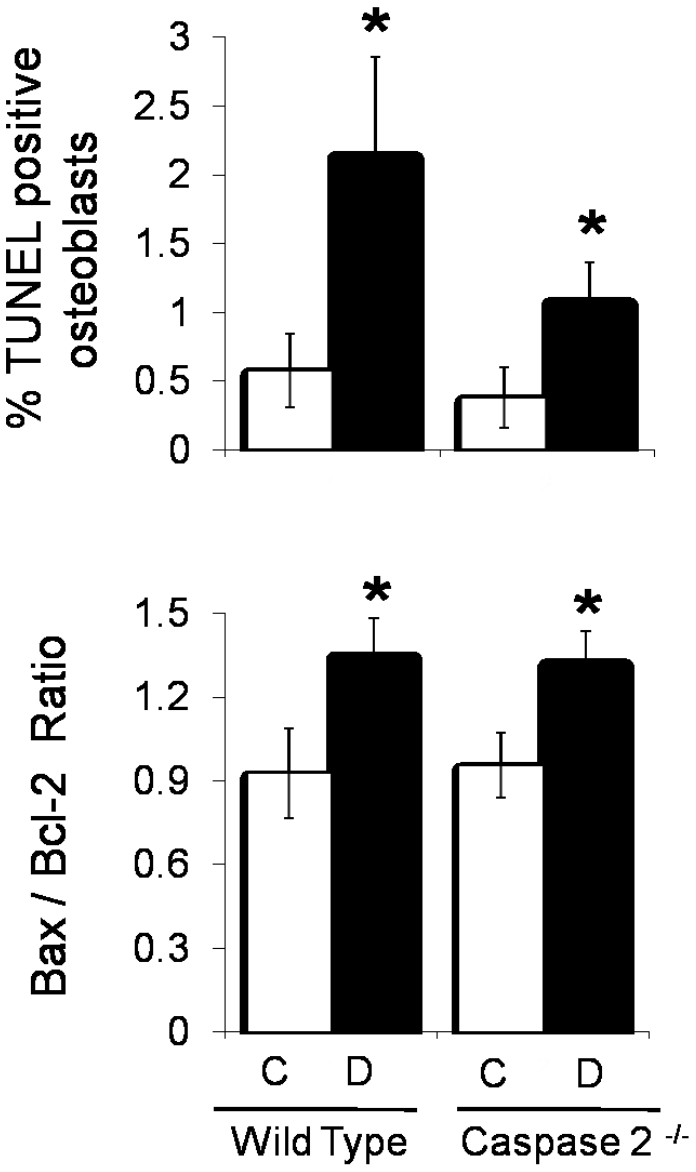
Table II. **Type I diabetic mouse parameters**

	Wild Type		Caspase 2 $^{-/-}$	
	Control	Diabetic	Control	Diabetic
Total Body Mass (g)	31.0 ± 0.8	27.3 ± 1*	29.2 ± 1	24.3 ± 1.6*
Blood Glucose (mg/dL)	163 ± 6	459 ± 18*	158 ± 8	474 ± 31*
Tibialis Anterior Mass (mg)	80 ± 4	75 ± 4	76 ± 5	67 ± 5
Femoral Fat Pad Mass (mg)	360 ± 37	128 ± 18*	322 ± 31	128 ± 31*

$n \geq 10$ mice per group, * $p<0.05$

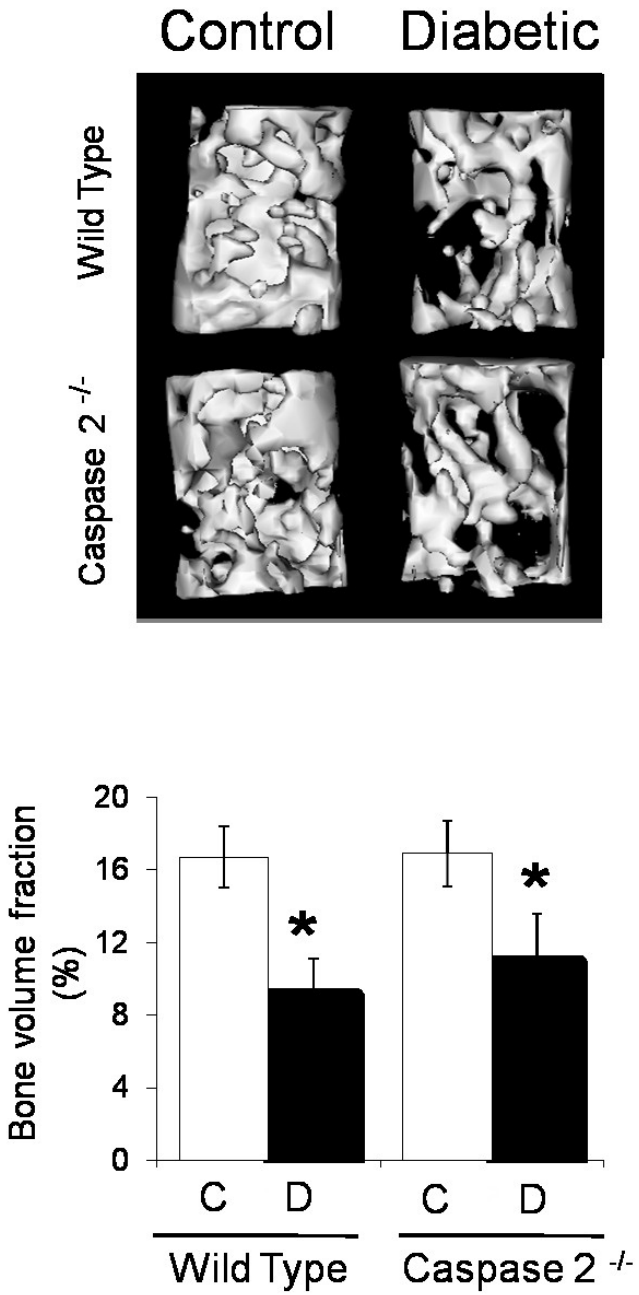
The onset of T1-diabetes increases osteoblast death (*17*). Therefore, we examined if the absence of caspase-2 altered T1-diabetes induced osteoblast death at 5 days post injection. As expected, the percentage of TUNEL positive osteoblasts in femur sections increased significantly in diabetic wild type mice by nearly 4-fold (figure 13). Diabetic caspase-2 deficient mice also displayed an increase in TUNEL positive osteoblasts compared to genotype controls; however, it was somewhat less than in wild type mice. Statistical analyses, however, found no significant difference in the percentage of TUNEL positive osteoblasts between genotypes. We also examined the ratio of Bax/Bcl-2 mRNA levels, which positively correlates with increased cell death. The ratio was elevated in both diabetic wild type and diabetic caspase-2 deficient mice compared to corresponding genotype control mice, but did not differ between wild type and deficient control mice, consistent with TUNEL analysis.

Figure 13. **Caspase-2 deficiency does not prevent T1-diabetes-induced osteoblast death.** Percentage TUNEL positive osteoblasts and BAX/Bcl-2 RNA ratio from bone of control (white bars) and streptozotocin-induced diabetes (black bars) in caspase-2 deficient and wild type littermate mice at 5 days post injection (dpi) is shown. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$ per group); * denotes $p<0.05$. For TUNEL assay n >500 osteoblasts per condition were counted.



Next, we examined the impact of caspase-2 deficiency on diabetic bone loss at 40 days post-diabetes induction, a time point when mice lose a significant amount of bone (*14, 15*). Femurs were imaged by micro-computed tomography and representative 3-dimensional isosurface images are shown (figure 14).

Figure 14. **Decreased bone volume fraction in wild type and caspase-2 deficient diabetic mice.** Representative μCT isosurface images of trabecular bone volume in distal femurs were obtained from control and diabetic caspase-2 deficient and wild type mice. Graphical representation of bone volume fraction is displayed from control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $p<0.05$.



As expected, wild type diabetic mice lost an average of 50% BVF. Caspase-2 deficient diabetic mice also lost BVF although it was somewhat less (30%), but not significantly different from wild type diabetic mice. Cortical bone parameters did not differ between genotypes (figure 15, table III). However, T1-diabetes did have a significant effect on cortical bone parameters of both wild type and caspase-2 deficient mice. Specifically, cortical thickness was decreased and the inner (marrow/endosteal) cortical perimeter was increased (table III). These changes resulted in a larger bone marrow area; this response was apparent in both diabetic genotypes.

Figure 15. **Decreased cortical bone parameters in wild type and caspase-2 deficient diabetic mice.** Representative μCT isosurface slices of the cortical bone (mid-diaphysis) in femurs obtained from control and diabetic caspase-2 deficient and wild type mice. Graphical representation of cortical bone thickness at 40 dpi from control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $p<0.05$.

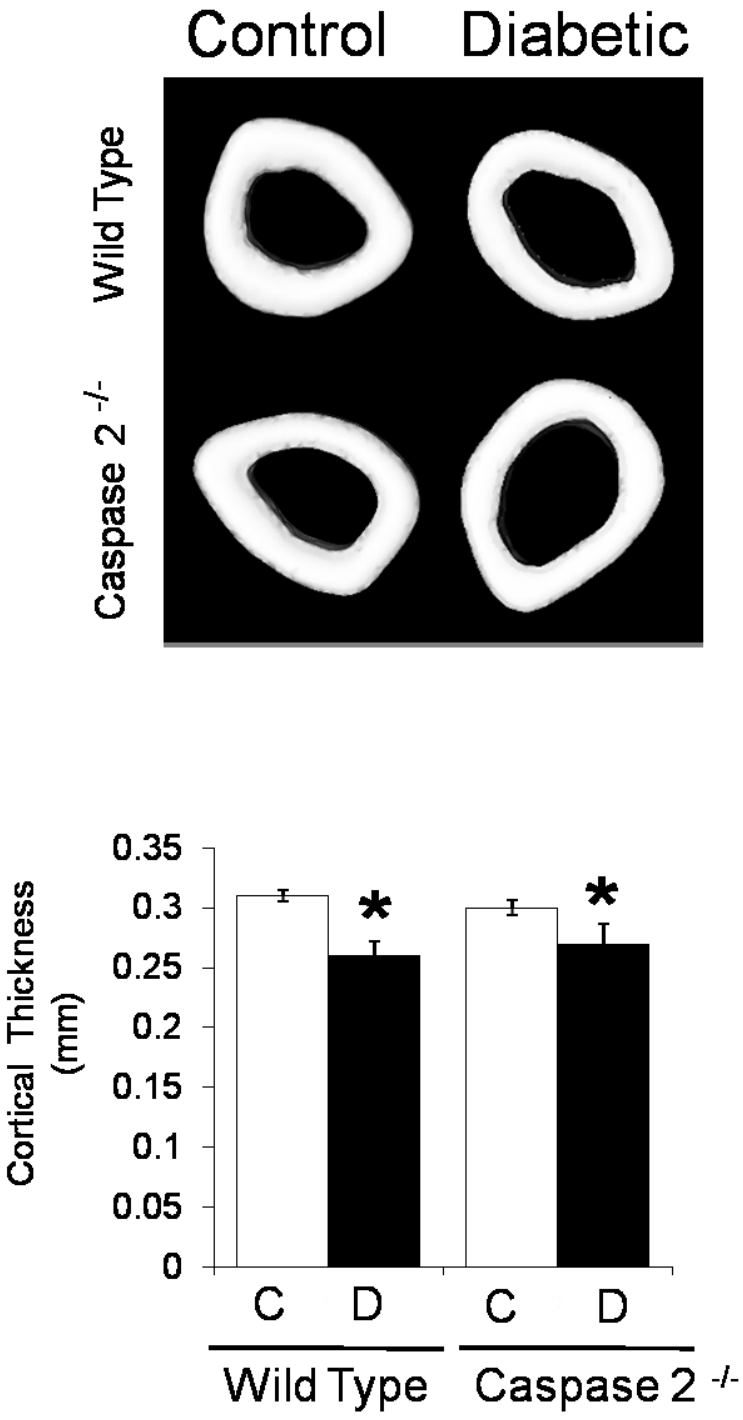


Table III. Trabecular and cortical μCT bone parameters.

	Wild Type		Caspase 2 -/-	
Trabecular	Control (n=14)	Diabetic (n=11)	Control (n=12)	Diabetic (n=7)
BMC (mg)	0.63 ± 0.03	0.51 ± 0.04*	0.66 ± 0.03	0.54 ± 0.05*
BMD (mg/cm^2)	210 ± 10	164 ± 12*	217 ± 10	178 ± 17*
BVF (%)	16.7 ± 1.7	9.4 ± 1.7*	16.9 ± 0.2	11.2 ± 2.4*
Tb Th (μm)	39.8 ± 1.2	31.3 ± 1.8*	40.3 ± 1.19	34.7 ± 2.38*
Tb N (mm^{-1})	4.06 ± 0.30	2.79 ± 0.37*	4.13 ± 0.41	3.35 ± 0.50*
Cortical				
Th (mm)	0.31 ± 0.005	0.26 ± 0.012*	0.3 ± 0.006	0.27 ± 0.017*
Ixx (mm^4)	0.26 ± 0.02	0.25 ± 0.02	0.26 ± 0.02	0.28 ± 0.03
Inner P (mm)	3.23 ± 0.06	3.48 ± 0.08*	3.25 ± 0.06	3.54 ± 0.09*
Outer P (mm)	5.26 ± 0.07	5.21 ± 0.09	5.24 ± 0.06	5.32 ± 0.09
Marrow Area (mm^2)	0.75 ± 0.03	0.87 ± 0.04*	0.77 ± 0.03	0.90 ± 0.04*
Cortical Area (mm^2)	1.25 ± 0.03	1.08 ± 0.06*	1.24 ± 0.03	1.15 ± 0.09
BMC (μg)	0.25 ± 0.02	0.26 ± 0.02	0.29 ± 0.02	0.24 ± 0.02
BMD (mg/cm^2)	1093 ± 28	1041 ± 40	1159 ± 23^	1047 ± 42*

Significance: * $p<0.05$ compared to genotype control; ^ $p<0.05$ compared to wild type control. *Abbreviations:* BMC, bone mineral content, BMD, bone mineral density, BVF, bone volume fraction, Tb Th, trabecular thickness, Tb N, trabecular number, Th, thickness, P, perimeter.

Histomorphometry and molecular phenotyping of osteoblasts and osteoclasts indicated that T1-diabetes suppressed osteoblast parameters. Tibia osteocalcin mRNA levels and the percentage of osteoblasts per total trabecular surface area were reduced in wild type and caspase-2 deficient mice (figure 16), consistent with an increase in TUNEL positive osteoblasts early in disease progression (figure 12). Osteoblast parameters did not differ between control wild type and caspase-2 deficient mice, as previously reported (*52*), although, osteoblast surface displayed a trend to decrease in caspase-2 deficient ($p<0.06$). Markers of resorption indicated that osteoclast surface area was not significantly altered by diabetes (figure 17). However, caspase-2 deficiency alone decreased TRAP5 mRNA levels (expressed by matures osteoclasts) in diabetic mice.

Figure 16. **T1-diabetes decreased bone formation markers in caspase-2 deficient and wild type mice.** Control (white bars) and diabetic (black bars) caspase-2 knockout and wild type littermates were examined at 40 dpi for the percent osteoblast surface per total trabecular surface and tibia osteocalcin mRNA expression. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $p<0.05$.

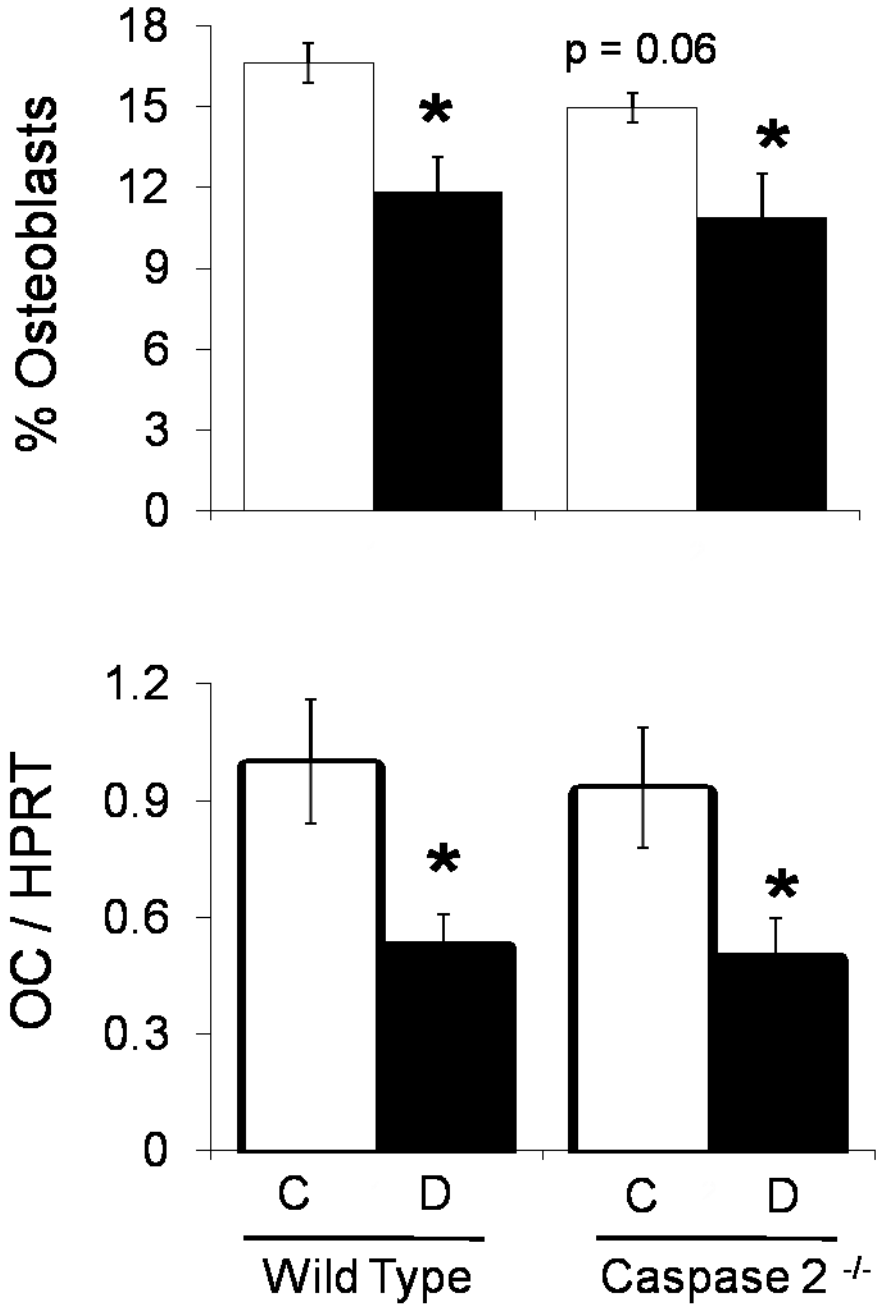
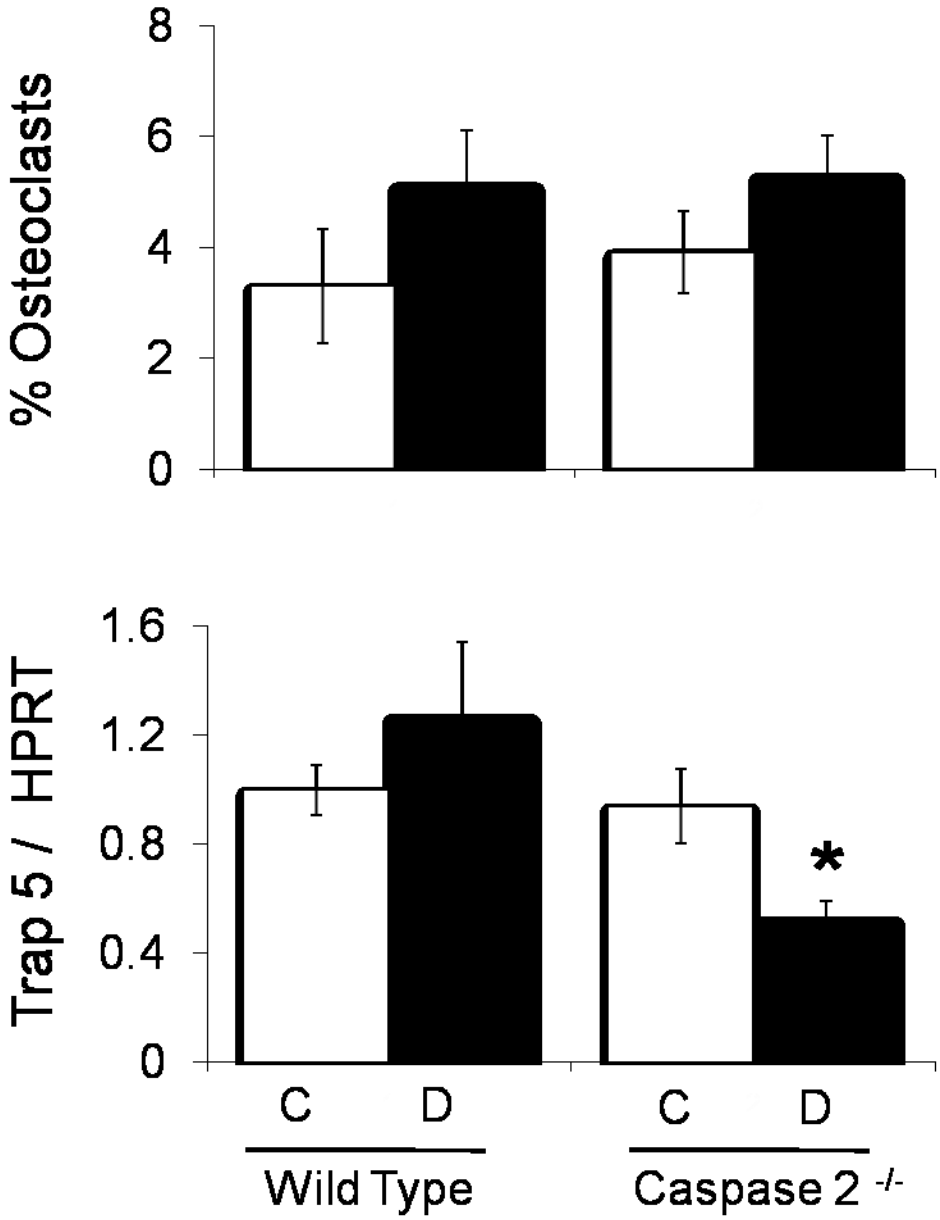


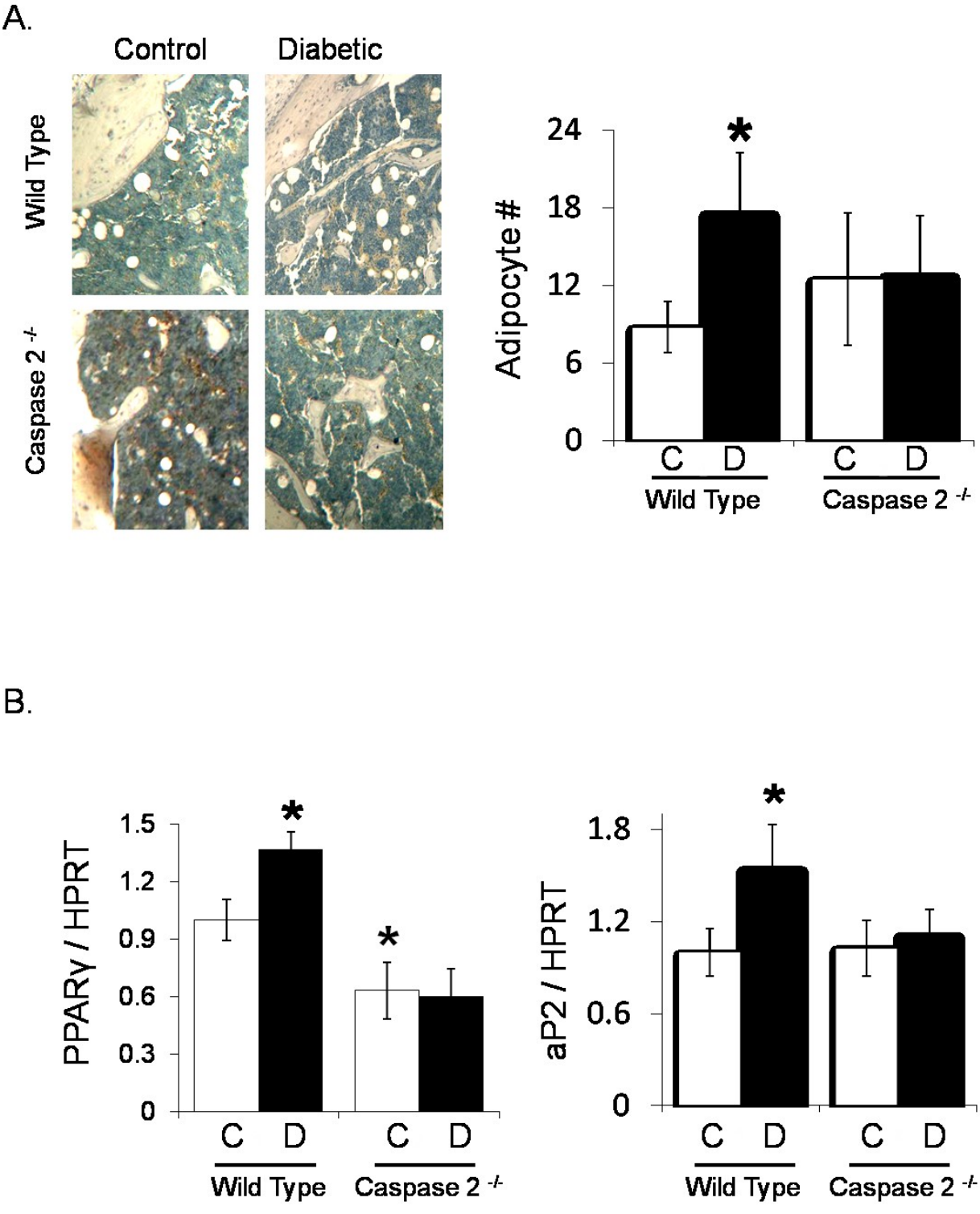
Figure 17. **Bone resorption parameters unchanged in caspase-2 deficient and wild type mice in response to T1-diabetes.** Control (white bars) and diabetic (black bars) caspase-2 knockout and wild type littermates were examined at 40 dpi for percent osteoclast surface per total trabecular surface and bone Trap5 mRNA expression. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $p<0.05$.



Previous reports demonstrated elevated marrow adiposity in T1-diabetic animal models (*13-15*); therefore we also investigated markers of adiposity. As expected, wild type T1-diabetic mice displayed a 2-fold increase in marrow adipocyte number and elevated tibia PPARγ (early adipocyte marker) and aP2 mRNA levels (mature adipocyte marker), by 40% and 67%, respectively (figure 18). Interestingly, caspase-2 deficiency prevented diabetic-induced marrow adipocyte number and gene expression (figure 18). We also observed that caspase 2 deficiency effectively reduced bone PPARγ levels in control mice (figure 18B).

Figure 18. **Caspase-2 deficiency prevents T1-diabetes induced marrow adiposity.** A) Representative adipocyte images were obtained from femurs of control and diabetic caspase-2 deficient and wild type mice. Graphical representation of adipocyte numbers in control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. B) Expression of early and late adipocyte mRNA markers, PPARγ (early marker measured at 5 dpi to capture changes in differentiation) and aP2 (expressed in mature adipocytes measured at 40 dpi), were examined and calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$-8 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of genotype, diabetes and genotype x diabetes on adipocyte parameters, * denotes $p<0.05$ by student t-test.

Figure 18 (cont'd)



3.5. DISCUSSION

Enhanced cellular apoptosis is associated with diabetic complications including retinopathy (*27, 53-55*), nephropathy (*56*) and bone loss (*17*) and it's repair (*24, 53*). The association of T1-diabetes with increased pro-inflammatory cytokines, (i.e. TNFα and IL-1β) (*27*) and cell and oxidative stress (*35*) led us to test the requirement of caspase-2 for the development of T1-diabetic bone pathology. Here we demonstrate that caspase-2 deficiency does not change trabecular or cortical bone parameters under control or diabetic conditions, compared to wild type mouse responses. Of surprise is that caspase-2 deficiency reduced diabetes induced marrow adiposity, suggesting a role for caspase-2 in promoting marrow fat under stress/disease conditions.

Little is known about the molecular mechanisms leading to increased osteoblast death in T1-diabetes. Our studies are the first to investigate the requirement of caspase-2 in this pathology. Although important for cell death, caspase-2 appears not to be a main contributor to T1-diabetes-induced osteoblast death. Redundancy and/or compensation from another caspase, e.g. caspase-8 or -12, as previously reported in neurons (*57*), and germ cells (*58*) could address why deletion of caspase-2 resulted in osteoblast death. However, we did not observe a change in caspase-8 mRNA levels between caspase-2 deficient and wild type control and diabetic tibias (data not shown). It is also possible that disrupting caspase-2 may not prevent activation of an intrinsic apoptotic mediator like Bax or Bid, both of which are suggested to be linked to activation of downstream caspases (e.g. caspase-3 and -9) in AGE and ROS induced osteoblast death (*36*).

The lack of a genotype difference in bone density is consistent with previous reports demonstrating that older 14-month-old caspase-2 deficient mice display similar trabecular BMD

to wild type mice (*52*). Interestingly, previous reports suggest that caspase-2 deficiency may compromise removal of oxidatively damaged cells (*52*), which could contribute to an overall elevation in oxidative stress components (e.g. ROS) and have a negative impact on organ health. Consistent with this idea, capsase-2 deficient mice at 24-26 months of age exhibit lower BMD than wild type mice as a result of increased bone resorption (*52*).

Surprisingly, caspase-2 deficiency did not significantly alter diabetes-induced changes in trabecular and cortical bone density parameters. While the percentage of trabecular bone loss was slightly decreased in caspase-2 deficient mice, the actual values were not significantly different between diabetic wild type and caspase-2 deficient mice and are consistent with previous reports in diabetic mice (*13, 15*). Cortical bone parameters were also altered as a result of diabetes, consistent with previous studies in rodent models (*59*) and humans (*60, 61*), and was not genotype dependent. If caspase-2 were required for diabetes-induced osteoblast cell death, deficient mice would have exhibited altered osteoblast markers and reduced bone loss. Interestingly, caspase-2 may have positive effects on bone, as seen in old mice (*52*), can its loss could have compounded diabetic bone loss through increased bone resorption. We may not have observed this because our mice are young and diabetes can suppress osteoclast activity and bone turnover in general.

What is the role of caspase-2 in osteoblasts? Our studies suggest that it is not critical for mediating osteoblast apoptosis and may exhibit redundancy. Capsase-2 may also act in other cellular functions. Similarities between cell death and cell cycle progression suggest that some apoptosis mediators may participate in cell cycle regulation (*62*). Caspase-2 is demonstrated to participate in cell cycle regulation, DNA repair and tumor suppression, to even a greater extent than PCD (*45, 49, 63, 64*). Cells deficient in caspase-2 had partial loss of the G2/M checkpoint

modulator resulting in cells continuing to cycle within this timeframe (*63, 64*). Thus, it may be possible that adipocyte precursors have reduced differentiation potential.

Interestingly, caspase-2 deficient diabetic mice were protected from increases in marrow adiposity. Although elevated marrow adiposity is commonly observed in both pharmacological and spontaneously diabetic mice (*13, 14*), several studies have reported that inhibition of marrow adiposity did not prevent diabetes-induced bone loss. These findings suggest that the inverse relationship between marrow adiposity and bone loss does not always occur (*18, 19*). Since early markers of adipocyte differentiation (PPARγ) are decreased in caspase-2 deficient mice, one hypothesis is that caspase-2 expression is involved in adipocyte differentiation. Sterol regulatory element binding protein (formally named adipocyte determination and differentiation factor 1) is required for adipocyte differentiation (*65, 66*) and is thought to mediate increased caspase-2 activity in these cells. Disrupting caspase-2 by siRNA impairs lipid levels in human adipocytes (*65, 66*), suggesting a role for caspase-2 in fatty acid and triglyceride synthesis. A second hypothesis is that caspase-2 is involved in modifying (cleaving) PPARγ and affecting its ability to regulate adipogenesis and adipocyte differentiation (*67, 68*). Several studies demonstrate that TNFα can activate many caspases, including 1 through 9, in adipocytes and lead to PPARγ cleavage (*67, 68*). Consistent with these findings, broad-spectrum caspase inhibitors decrease TNFα-mediated PPARγ cleavage and fragmentation (*67, 68*). Taken together, eliminating caspase-2 could result in altered transcriptional effects of PPARγ that in turn decrease adipogenesis and adipocyte differentiation, thus preventing T1-diabetes-induced marrow adiposity.

In summary, inhibition of caspase-2 prevented T1-diabetes induced marrow adiposity, but did not attenuate diabetes induced osteoblast death or bone loss. Our results suggest that

caspase-2 is not involved in diabetes-mediated osteoblast death and may play a more vital role in other cell functions. Further investigation is needed to understand the mechanisms mediating T1-diabetes associated osteoblast death.

3.6. ACKNOWLEDGEMENTS

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CHAPTER 4. BAX DEFICIENCY PROTECTS AGAINST TYPE I DIABETES-INDUCED OSTEOBLAST DEATH AND BONE LOSS.

4.1. ABSTRACT

T1-diabetes is a metabolic disorder associated with decreased bone formation and bone loss. Previously, our lab reported an elevation in osteoblast death and Bax mRNA expression in T1-diabetic mouse bones. Bax is an important pro-apoptotic factor of the mitochondrial-mediated (intrinsic) death pathway, which determines cell viability based upon the balance of pro-apoptotic and anti-apoptotic factors. Intrinsic apoptosis is associated with bone loss and osteoblast death in models of unloading, aging, and T1-diabetes. Here we hypothesize that elevated Bax expression is involved in mediating osteoblast death and bone loss in T1-diabetes. To test this, we induced T1-diabetes in 16-week old male C57BL/6 Bax deficient and wild type littermate mice. TUNEL analysis demonstrated that Bax deficiency protects osteoblasts from diabetes-induced death. Furthermore, Bax deficiency attenuated losses in trabecular bone volume and bone formation markers observed in diabetic mice. Markers of bone resorption remained unchanged. Marrow-osteoblast co-culture studies demonstrate that Bax deficient osteoblasts are protected from diabetic marrow induced increases in osteoblast caspase 3 activity. Furthermore, we identified that Bax deficiency prevents diabetic suppression of Wnt10b, a key factor that promotes osteoblast viability and maturation. Taken together, our results suggest a role for the intrinsic cell death pathway, specifically the pro-apoptotic factor Bax, in T1-diabetes induced osteoblast death and bone loss.

4.2. INTRODUCTION

Type I (T1) diabetes is an auto-immune disorder characterized by chronic destruction of the insulin producing beta cells in the pancreas resulting in little to no insulin production and hyperglycemia. Medical advances and therapies are increasing diabetic patient lifespan, however proper control over insulin remains difficult, leading to long-term consequences such as bone loss. Greater than 50% of all patients diagnosed with T1-diabetes suffer from bone loss (1, 2). Patients, men and women of all ages, with T1-diabets have an increased fracture risk at multiple bone sites including the spine, femur, radius and feet (3-18). Animal models of T1-diabetes display comparable bone loss and fracture risks to patients and provide a way to extensively investigate mechanisms contributing to diabetes-induced bone loss (19-21).

T1-diabetes targets osteoblasts and their subsequent bone formation (19-22). Exact mechanisms responsible for suppressed osteoblast function remain unknown, but likely involve a combination of suppressed lineage selection, viability and maturation. Our lab became interested in the role of osteoblast death since roughly 65% of all osteoblasts undergo apoptosis and die (23), making apoptosis an important mechanism regulating osteoblast number. Furthermore, the number of TUNEL positive osteoblasts increases during diabetes onset (24) and coincides with the first detectable suppression of the mature osteoblast marker, osteocalcin (25). Therefore, osteoblast apoptosis may play a critical role in mediating T1-diabetic bone loss. This would be consistent with observed increases in osteoblast death in other rodent models of bone loss including unloading (26) aging (23) and periodontal disease (27).

Osteoblast death can occur through two main pathways, extrinsic and intrinsic apoptosis. Extrinsic apoptosis is triggered by extracellular ligands binding membrane bound death receptors and activating the caspase cascade (28). Caspases are a family of proteins that contain a cysteine residue capable of cleaving an aspartic acid-containing motif on the next downstream caspase. Two different caspase groups are involved in PCD: initiators and effectors. Initiator caspases (i.e. caspase 2, -8, -9) are responsible for activation of the effector caspases (i.e. caspase-3, -7) that cleave an array of cellular substrates (28). Recently, our lab demonstrated that mice deficient in caspase 2, an initiator caspase involved in TNFα and reactive oxygen species (ROS) induced apoptosis (both of which are elevated in T1-diabetes bone (25, 29-31)), did not prevent T1-diabetes induced osteoblast death or bone loss (Coe et al.). This data suggests another pathway is responsible for mediating osteoblast death in diabetic bone.

Intrinsic (mitochondrial-mediated) apoptosis is regulated by the Bcl-2 (B-cell lymphoma-2) family members that consists of nearly 20 pro- (Bax, Bak, Bim, Bad, Bid) and anti-apoptotic (Bcl-2, Bcl-xL) proteins, all of which contain Bcl-2 homology (BH) domains (32). There are four distinct BH domains: BH1, BH2, BH3, and BH4. Most of the anti-apoptotic factors, such as Bcl-2, Bcl-xL contain all four BH subtypes. Pro-apoptotic factors however, are divided by the composition of their BH domains. Some pro-apoptotic factors (Bax and Bak) contain BH1, BH2, and BH3, while others (Bim, Bid, Bad) only have the BH3 domain, indicating they share the short BH3 domain that is vital for dimerization with other Bcl-2 family members and is crucial for their killing effect (32). The pro-apoptotic proteins that contain only the BH3 domain activate the multi-domain factors, Bax and Bak, to carry out cell death. Intrinsic death occurs

through the balance of these pro-apoptotic and anti-apoptotic factors. High expression of Bax promotes apoptosis, while a high expression of Bcl-2 inhibits apoptosis (28, 33-36). After receiving an apoptotic stimulus (e.g. cellular stress or cytotoxic stimuli), Bax undergoes a conformational change and translocates from the cytosol to the mitochondrial membrane. At the extracellular membrane Bax binds and inserts itself creating a transitional pore capable of governing the membranes permeability (28, 33, 35-39). Cytochrome c is then released from the mitochondria into the cytosol where it binds and activates Apaf-1 and procaspase-9 forming an "apoptosome" (28, 33, 36, 40). Simultaneously, AIF (apoptosis-inducing factor) is released from the mitochondria and migrates to the nucleus resulting in DNA fragmentation and subsequent activation of caspase-3 (28, 33). Together, the apoptosome and DNA fragmentation result in cell morphology changes and engulfment by surrounding cells.

The intrinsic apoptosis pathway is demonstrated to be involved in the regulation of bone health. Mice deficient in Bax are reported to be protected from age-induced bone loss (41) and enhanced fracture healing (42). *In vitro* studies report that inhibition of Bax expression through siRNA in murine osteoblasts and human primary stromal cells were protected from 18-hour serum starvation-induced apoptosis by 85-90% (52). Furthermore, *in vitro* over-expression of anti-apoptotic factor Bcl-2, which heterodimerizes and counteracts Bax, results in increased osteoblast differentiation and decreased osteoblast apoptosis (43). Correspondingly, mice over-expressing Bcl-2 in osteoblasts were protected from glucocorticoid-induced osteoblast death (53).

In the current study we tested the requirement for Bax in mediating T1-diabetes induced osteoblast death and bone loss. Our findings demonstrate that mice deficient in

Bax were protected from diabetes-induced osteoblast death as well as from reduced bone density and bone formation markers. Bax deficient osteoblasts were also protected from diabetic-marrow induced osteoblast death.

4.3. MATERIALS AND METHODS

4.3.1. Diabetic Mouse Models

Diabetes was induced in adult (15-16 week old) male C57BL/6 Bax deficient and wild type littermates by 5 daily intraperitoneal injections of streptozotocin (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Controls were given citrate buffer alone. Mice were maintained on a 12-hour light, 12-hour dark cycle at 23°C, given standard lab chow and water *ad libitum*. Diabetes was confirmed 12 days after initial injection using an Accu-Check compact glucometer (Roche Diagnostics Corporation, Indianapolis, IN) with a drop of blood from the saphenous vein. Total body, tibialis anterior, and subcutaneous femoral fat pad mass were recorded. Animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

4.3.2. Genotyping

Bax deficiency was determined from ear snips from wild type, heterozygote and knockout mice and extracted using a DNeasy Blood & Tissue Kit (QIAGEN Group) according to manufacture's instructions. The absence of Bax was determined by PCR with three primers specific for Bax (41, 44). Wild type mice displayed a single 304 base pair band that corresponded to the endogenous, undisrupted Bax gene, while Bax-deficient mice showed a single band of 507 base pair amplified from the sense and neo primers within the Bax construct (41, 44, 45). Heterozygote mice, used for breeding,

displayed both the 304 base pair and 507 base pair bands. PCR products were then separated on a 1.5% agrose gel to ensure presence or absence of bands.

4.3.3. RNA Analysis

Immediately after euthanasia, one tibia and femur were cleared of soft tissue and snap frozen in liquid nitrogen and stored at -80°C. Frozen tibias were crushed under liquid nitrogen conditions, homogenized, and placed in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA integrity was determined through formaldehyde-agrose gel electrophoresis. cDNA was synthesized through a reverse transcriptase reaction utilizing Superscript II Reverse Transcriptase Kit and oligo dT (12-18) primers (Invitrogen, CarlsBad, CA) and then amplified by quantitative real time PCR with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers. Forward and reverse primers used as previously stated: aP2, Bad 5'-GAG GAA GTC CGA TCC CGG AA-3' and 5'-CGG CGG TTT GTC GCA TCT GT-3', Bak 5'-ACA GCA GGT TGC CCA GGA CA-3' and 5'-CCA TTT CTG GGG CTC CAG GA-3' Bax, Bcl-2, Bcl-w 5'-CGG GCT CTA GTG GCT GAC TT-3' and 5'-GCA CTG TCC TCA CTG ATG CC-3', Bid 5'-CCT GCT GGT GTT CGG CTT TC-3' and 5'-CGT GTG GAA GTC ATC ACG GA-3', Bim (EL) 5'-GGT AAT CCC GAC GGC GAA GGG AC-3' and 5'-AAG AGA AAT ACC CAC TGG AGG ACC-3' HPRT, osteocalcin, and TRAP5 (22, 24, 46). Expression of HPRT was not altered by diabetes and was used as a housekeeping gene. Real time PCR was carried out for 40 cycles, each cycle consisting of 95°C for 15 seconds, 60°C for 30 seconds (except osteocalcin which had an annealing

temperature of 65°C) and 72°C for 30 seconds. RNA-free samples were used as negative controls and did not produce amplicons. PCR products were separated on 1.5% agrose gel electrophoresis and sequenced to verify the desired gene is being amplified.

4.3.4. Micro-computed Tomography (μCT) analysis

Fixed femurs in 70% ethanol were scanned using the GE Explore μCT system at a voxel resolution of 20um from 720 views with a beam strength of 80kvp and 450uA. Integration time for each scan was 2000ms. Scans included bones from each condition and a phantom bone to standardize the grayscale values and maintain consistency between runs. Using the systems auto-threshold (1000) and isosurface analysis confirmation, trabecular bone densities (bone volume fraction (BV/TV), bone mineral density (BMD) and content (BMC), and trabecular thickness (tb. th), number (tb. n) and spacing (tb. sp)) were measured in the trabecular region defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis, and excluding the outer cortical shell. Cortical bone measurements are determined with a 2-mm^3 region of interest (ROI) in the mid-diaphysis. Cortical thickness, moment of inertia, cortical area, marrow area, total area, inner perimeter and outer perimeter as well as all trabecular parameters were computed using the GE Microview Software for visualization and analysis of volumetric image data.

4.3.5. Bone Histology and Histomorphometry:

Bones were fixed in 10% buffered formalin, transferred to 70% EtOH after 24 hours, and processed for dehydration, clearing and infiltration on a routine overnight processing schedule. Samples were then paraffin embedded on a Sakura Tissue Tek II embedding center and 5μm sections prepared. Osteoclasts were identified, by staining for tartrate resistant acid phosphatase (TRAP) (Sigma). The number or surface area of osteoclasts, osteoblasts and adipocytes was determined in the trabecular region of the femur (defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis). Osteoclast and osteoblast surface was measured in three trabecular regions for each mouse and expressed as a percent of total trabecular surface. Visible adipocytes, greater than 30 μm, were counted in the same trabecular region.

Cell death

Cell death was determined using a TACS•XL® Basic In Situ Apoptosis Detection Kit (TUNEL, Trevigen Inc., Gaithersburg, MD) on the L1-3 vertebrae or femur sections. Osteoblasts with positive nuclei were counted and expressed as a percentage of total osteoblasts counted per bone. Positive controls included slides incubated with nuclease. Five trabecular regions were examined for each mouse. Total osteoblasts counted ranged between 20 and 100 per bone.

Mineral apposition rate

Mice were injected intraperitoneally with 200µl of 10mg/ml calcein (Sigma, St. Louis, MO, USA) dissolved in saline at 7 and 2 days prior to harvest. L3-L4 vertebrae were fixed in formalin at time of harvest then transferred to 70% ethanol 24 hours later. Vertebrae were then embedded, sectioned and examined under UV light. Five images were taken and the distance between the calcein lines and their length along the bone surface was measured and used to calculate MAR.

4.3.6. Serum Measurements

Blood was collected at the time of harvest, allowed to clot at room temperature for 5 minutes, then centrifuged at 4,000 rpm for 10 minutes. Serum was removed and stored at -80°C. Serum went through no more than one freeze/thaw cycle. Serum TRAP5b was measured using a MouseTRAP assay kit (SB-TR103, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) according to the manufacturer's protocol.

4.3.7. Osteoblast and marrow co-culture

Bone marrow-derived osteoblasts were obtained by seeding flushed marrow cells into wells of a 24 well plate at a concentration of 100,000 cells per well. Cells were cultured for 14 days prior to the start of co-culture experiments. Upon reaching confluency the media was supplemented with 2mM inorganic phosphate and 25µg/ml ascorbic acid.

Bone marrow was harvested/flushed from femurs using a syringe containing serum free α-MEM. Cells were centrifuged and resuspended with 10 milliliters complete media. A cell suspension aliquot was mixed with Turks solution for counting and cells were plated at 2 million cells per transwell permeable support (5μm membrane; Corning, Fisher Scientific). Transwells were placed in wells containing differentiating osteoblasts. After 24 hours, osteoblasts were harvested for caspase 3.

4.3.8. Caspase 3 activity measurement

Caspase 3 activity was determined using the Caspase-Glo 3/7 Assay Kit according to manufacturer protocol (Promega, Madison, WI). Data was collected, the value for background (media over plated osteoblats with no transwell) subtracted and values reported as luminescence (relative light units, RLU).

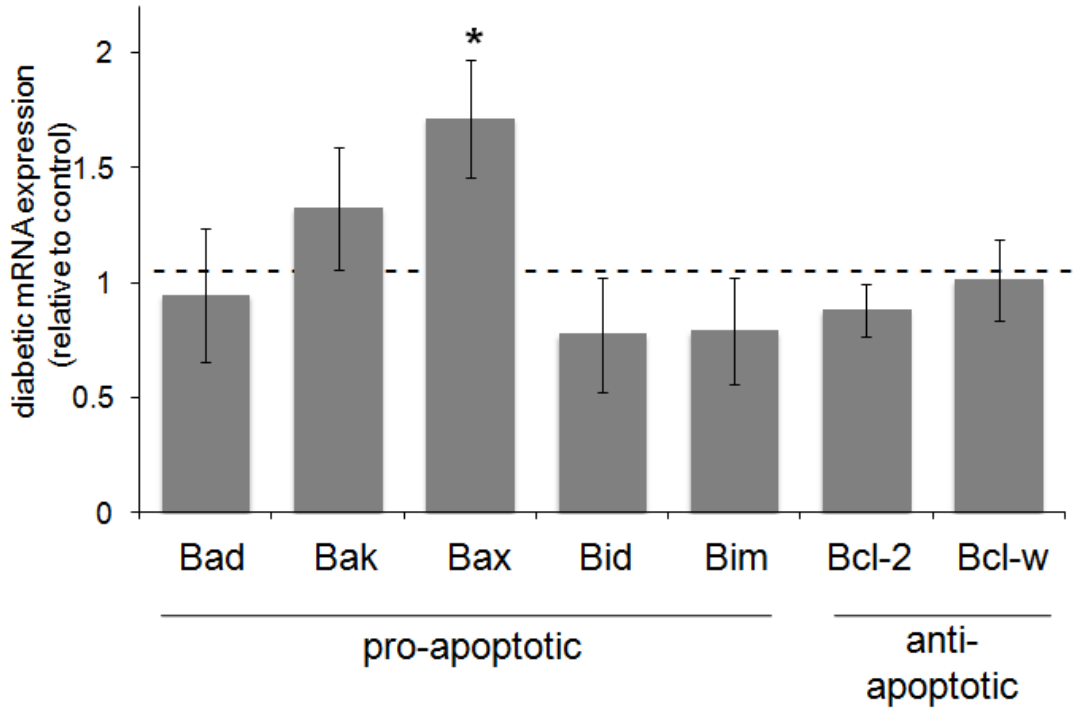
4.3.9. Statistical Analysis

All data is presented as mean ± standard error. Statistical significance (p-value < 0.05) of main effects (genotype difference or diabetes) as well as genotype x diabetes interaction (which determines if diabetes alters the genotype effect or vise versa) was determined using factorial analysis of variance (ANOVA) with SPSS statistical software (Chicago, IL). Student's t-test were used where needed.

4.4. RESULTS

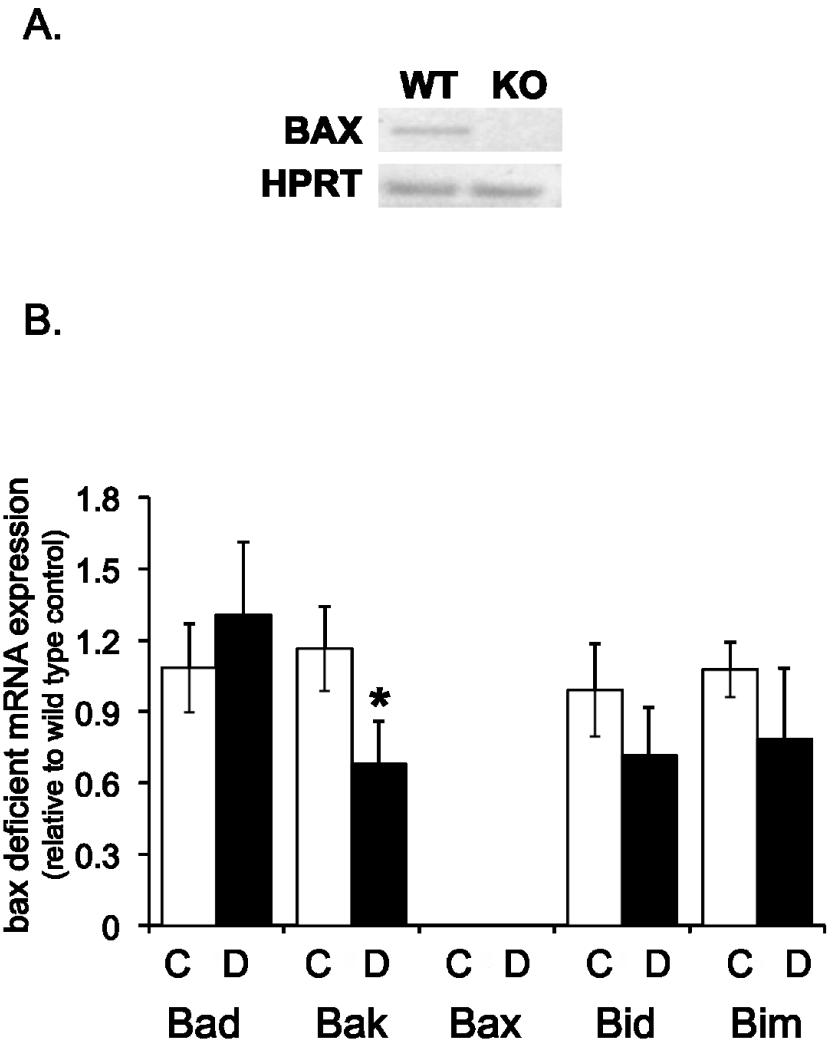
Elevated osteoblast death has previously been reported in T1-diabetic mouse bone (47). To establish the impact of T1-diabetes on the level of expression of factors in the intrinsic apoptosis pathway, mRNA levels of multiple Bcl-2 family members (both pro- and anti-apoptotic) were examined at 5 days post injection (dpi), when osteoblast death is observed. Of the factors tested, only Bax gene expression was significantly elevated in diabetic bone compared to vehicle treated controls (figure 19). RNA levels of Bak, the pro-apoptotic factor that displays some redundant properties to Bax, trended to increase in diabetic bone, but did not reach significance. Expression of other pro-apoptotic factors, Bad, Bid and Bim, were either unchanged or trended to decrease in diabetic bone compared to controls (figure 19). Additionally, anti-apoptotic factors, Bcl-2 and Bcl-w, did not change in diabetic compared to control bone (figure 19). These results suggest that, rather than a decrease in anti-apoptotic factors, the elevation of pro-apoptotic Bax could be involved in T1-diabetes induced osteoblast death.

Figure 19. **Bax mRNA expression is elevated in diabetic bone.** Various pro-apoptotic (Bad, Bak, Bax, Bid and Bim) and anti-apoptotic (Bcl-2 and Bcl-2) factors were examined at 5 dpi (time point were osteocalcin reduction and increased osteoblast death is first detected) in diabetic mice and expressed relative to control mice (set to 1). RNA levels are calculated relative to the housekeeping gene HPRT. Values are expressed as MEAN ± SE ($n \geq 10$ per group); * denotes $p<0.05$.



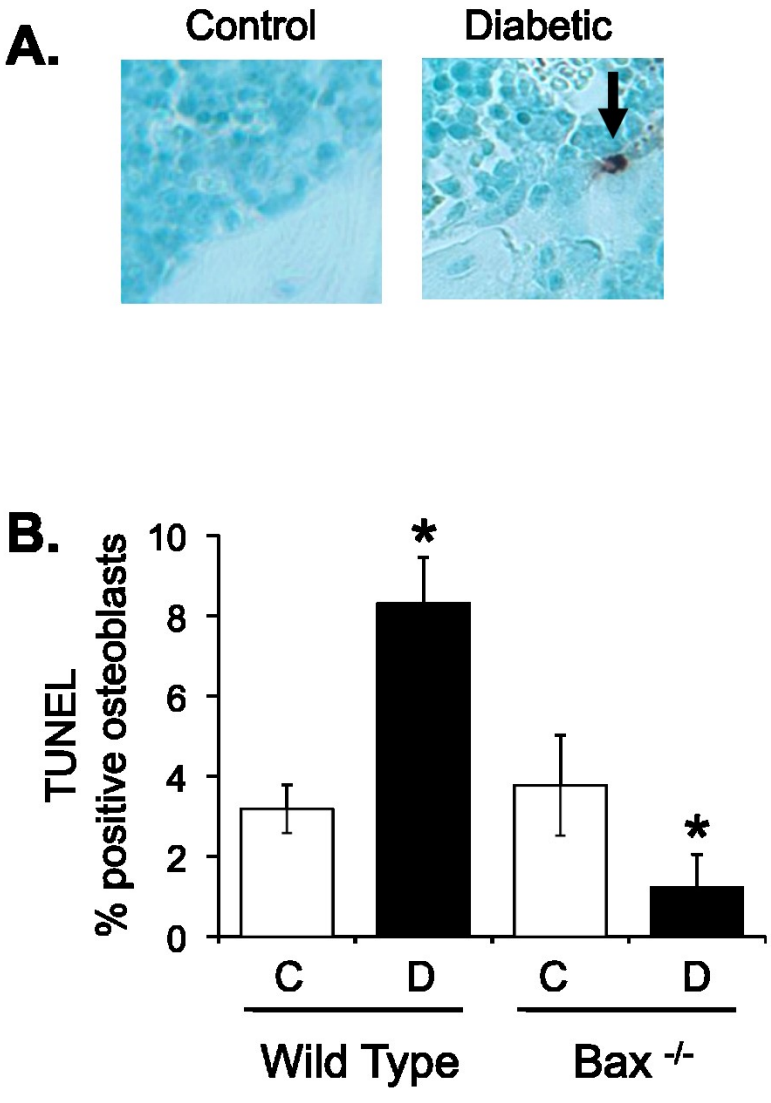
To test if Bax is required for the diabetic bone pathology, we induced diabetes in Bax deficient and littermate wild type mice. The absence of Bax was determined by genotyping and confirmed by the lack of detectable Bax mRNA expression in bone RNA isolates from deficient mice (figure 20). At 15-16 weeks of age male wild type and Bax deficient mice were injected with streptozotocin (to induce diabetes) or vehicle (controls). At 5 dpi, RNA from control and diabetic Bax deficient mice was extracted and analyzed for potential compensation by other pro-apoptotic factors, specifically Bad, Bak, Bid, Bim. Interestingly, none of the factors were elevated in Bax-deficient mice. Bak however, was significantly decreased in the diabetic mice compared to genotype controls (figure 20).

Figure 20. **No pro-apoptotic compensation for the absence of detectable Bax in tibia RNA.** A) Bax RNA expression was examined in wild type (left) and Bax deficient (right) bone. PCR products were then run on a 1.5% agrose gel for the absence of Bax expression. RNA levels are expressed relative to the housekeeping gene HPRT. B) Various pro-apoptotic (Bad, Bak, Bax, Bid and Bim) factors were examined at 5 dpi in tibias from control and diabetic Bax-deficient mice and expressed relative to wild type control littermates (set to 1). RNA levels are calculated relative to the housekeeping gene HPRT. Values are expressed as MEAN ± SE (n≥10 per group); * denotes $p<0.05$.



To determine if Bax deficiency alters T1-diabetes-induced osteoblast death, we examined osteoblast death at this time point. As expected (based on our past studies), the percentage of TUNEL positive osteoblasts in trabecular bone increased 4-fold in wild type diabetic mice compared to genotype controls (figure 21). Bax deficiency, however, protected mice from diabetes-induced osteoblast death and actually reduced osteoblast death lower than control levels. Bax deficient control mice did not display altered osteoblast death compared to wild type controls (figure 21). This may result from the inability to detect changes at the low level of osteoblast death in control mice or alternatively suggests that Bax is important for mediating disease/stress induced osteoblast death and does not play a significant role in day-to-day regulation of osteoblast death.

Figure 21. **Bax deficiency prevents diabetes-induced osteoblast death.** A) Representative images of TUNEL staining in control (left) and diabetic (right) trabecular bone at 5 dpi. The arrow demonstrates a positive TUNEL stained osteoblast. B) Graphical representation of TUNEL staining in control (white bars) and diabetic (black bars) wild type and Bax-deficient mouse bones. Bars represent the average value ± SE ($n \geq 5$ per group); * denotes $p<0.05$. For interpretation to the references of color in this and all other figures, the reader is referred to the electronic version of this dissertation.



Increased osteoblast death could contribute to T1-diabetes induced bone loss. Because Bax deficient mice do not display enhanced osteoblast death during diabetes onset, we examined if mice were also protected from bone loss. Mice were examined at 40 dpi, a time where significant bone loss is evident (19, 21). First we examined the effect of Bax deficiency on general mouse parameters. Bax deficiency alone decreased blood glucose levels and femoral fat pad mass (similar to previous reports (41)) and trended to decrease lean muscle mass (although this did not reach statistical significance) when compared to wild type mice (table IV). However, Bax deficiency did not impact T1-diabetes induction or physiologic responses to T1-diabetes. Specifically, diabetic wild type and diabetic Bax deficient mice displayed similar elevations in blood glucose levels, averaging 503 mg/dl and 517 mg/dl, respectively (table IV). Both diabetic wild type and Bax deficient mice displayed decreases in total body mass (9% and 8%, respectively), femoral fat pad mass (56% and 41%, respectively) and tibialis anterior mass (49% and 36%, respectively) compared to their respective genotype controls. Thus, Bax deficiency led to leaner mice (consistent with previous reports (41)) but did not modify general physiologic responses to T1-diabetes.

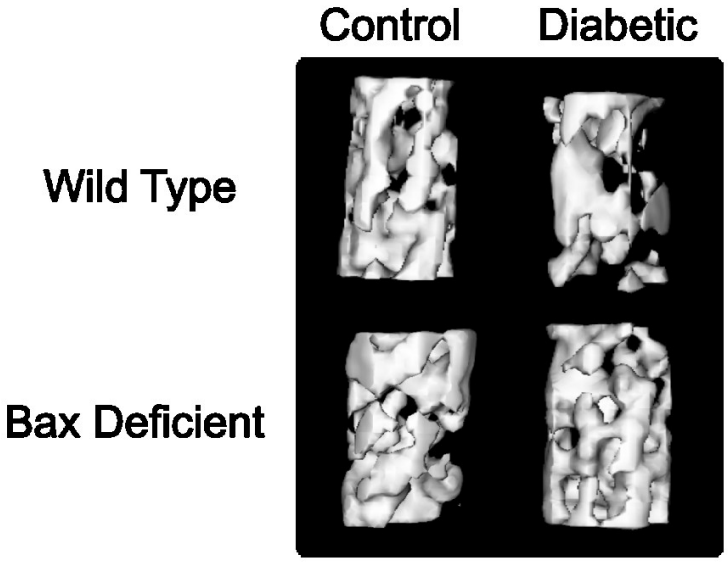
Table IV. Body parameters in control and diabetic Bax deficient and wild type mice.

	Wild Type		Bax Knockout	
	Control (n=13)	Diabetic (n=12)	Control (n=8)	Diabetic (n=11)
Body Mass (g)	29.8 ± 0.51	27.1 ± 0.76*	28.9 ± 0.56	26.6 ± 0.65*
Glucose (mg/dl)	197 ± 9	503 ± 24*	172 ± 11^	517 ± 25*
Fem. Fat Mass (mg)	34.1 ± 2.7	14.9 ± 1.7*	22.0 ± 1.2^	13.0 ± 1.1*
Tibialis Mass (mg)	17.1 ± 6.8	8.7 ± 0.6*	10.3 ± 0.7	6.7 ± 0.7*

*$p \leq 0.05$ compared to respective control.
^$p \leq 0.05$ compared to wild type control.

To examine the role of Bax in diabetic bone loss, femurs were imaged by micro-computed tomography and representative 3-dimensional isosurface images are shown (figure 22). As expected, wild type diabetic mice lost a significant amount of bone volume fraction (BV/TV), bone mineral density (BMD), bone mineral content (BMC) and trabecular thickness (tb. th). Bax deficient mice, on the other hand, were protected from diabetes-induced bone loss and altered trabecular bone parameters (figure 22, table V). While Bax deficiency in control mice did not affect trabecular bone measurements compared to wild type mice, cortical bone parameters displayed differences between genotypes. Specifically, Bax deficiency increased cortical bone thickness while decreasing inner and outer parameters resulting in a decreased marrow area (table V). Diabetes resulted in no cortical bone changes in either wild type or Bax deficient mice compared to respective genotype controls.

Figure 22. **Bax deficiency protects against T1-diabetes-induced bone loss.** Representative μCT isosurface images of trabecular bone volume fraction in distal femurs were obtained from control and diabetic Bax deficient and wild type mice at 40 dpi. Graphical representation of bone volume fraction is displayed from control (white bars) and diabetic (black bars) Bax knockout and wild type mice. Bars represent the average value ± SE (n≥10 per group); * denotes p<0.05.



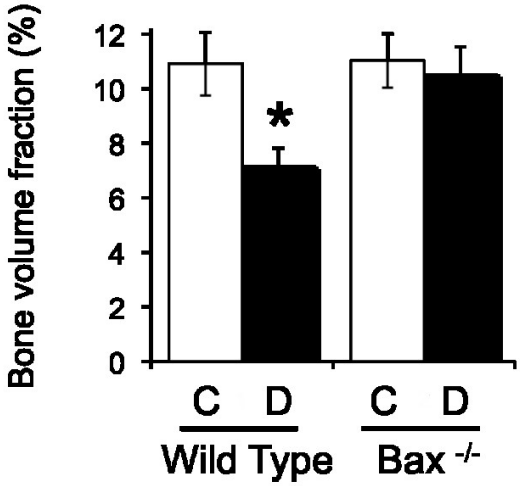


Table V. μCT femur parameters in control and diabetic Bax deficient and wild type littermates.

	Wild Type		Bax Deficient	
Trabecular	Control	Diabetic	Control	Diabetic
BMC (mg)	0.72 ± 0.04	0.54 ± 0.06*	0.72 ± 0.03	0.68 ± 0.03
BMD (mg/cc)	235 ± 13	204 ± 9*	242 ± 9	235 ± 8
BVF (%)	22.0 ± 3.0	15.2 ± 4.4*	21.8 ± 2.4	24.4 ± 2.1
Tb. Th (μm)	45.2 ± 3.5	37.8 ± 1.7*	43.5 ± 1.7	45.4 ± 2.2
Tb. Sp (mm)	0.18 ± 0.02	0.23 ± 0.03	0.18 ± 0.03	0.15 ± 0.01
Tb. N (1/mm)	4.7 ± 0.4	3.9 ± 0.3	4.9 ± 0.5	5.3 ± 0.3
Cortical				
Thickness (mm)	0.249 ± 0.010	0.244 ± 0.008	0.302 ± 0.009^	0.297 ± 0.015
MOI (mm^4)	0.121 ± 0.009	0.114 ± 0.010	0.103 ± 0.010	0.093 ± 0.004
I. Perimeter (mm)	2.38 ± 0.10	2.27 ± 0.10	1.79 ± 0.08^	1.90 ± 0.06
O. Perimeter (mm)	4.00 ± 0.03	3.83 ± 0.10	3.71 ± 0.06^	3.79 ± 0.06
Marrow Area (mm^2)	0.40 ± 0.04	0.37 ± 0.03	0.19 ± 0.05^	0.26 ± 0.02
Cortical Area (mm^2)	0.74 ± 0.03	0.69 ± 0.04	0.79 ± 0.03	0.80 ± 0.04
Total Area (mm^2)	1.14 ± 0.03	1.06 ± 0.05	1.02 ± 0.04^	1.05 ± 0.03
Ct. Area/ Tt. Area (%)	65.6 ± 2.5	65.6 ± 2.2	77.9 ± 1.6^	75.4 ± 2.1

Abbreviations: BMC, bone mineral content, BMD, bone mineral density, BVF, bone volume fraction, Tb. Th, trabecular thickness, Tb. Sp, trabecular spacing, Tb. N, trabecular number, MOI, moment of inertia, I. Perimeter, inner perimeter, O. Perimeter, outer perimeter, Ct. Area/Tt. Area cortical/total area. $n \geq 8$ per condition. *Statistics:* *$p \leq 0.05$ compared to respective control. ^$p \leq 0.05$ compared to wild type control.

Type I diabetes is associated with decreased osteoblast number and activity and either decreased or no change in osteoclast activity, therefore we examined several markers of bone formation and resorption to assess the impact of Bax deficiency on these parameters. Interestingly, Bax deficiency alone did not affect either bone formation or bone resorption parameters, which is consistent with Bax deficiency not affecting osteoblast viability under control conditions. However, under T1-diabetic conditions Bax deficiency completely inhibited the diabetes-induced suppression of bone formation parameters. In contrast, similar to previous studies, diabetic wild type mice displayed suppressed dynamic and static osteoblast parameters such as mineral apposition rate, tibia osteocalcin mRNA levels and the percentage of osteoblast surface per total trabecular surface (figure 23). We also examined markers of resorption: serum TRAP5b, TRAP5 mRNA levels and osteoclast surface per total trabecular surface (figure 24). Serum, histomorphometric analyses and RNA expression indicates that osteoclast parameters were not significantly altered by diabetes (consistent with previous reports) in either wild type or Bax deficient mice.

Figure 23. **Diabetes suppression of bone formation is attenuated in Bax-deficient mice.** Dynamic (mineral apposition rate, top graph), and static (osteocalcin mRNA expression, middle graph and osteoblast surface, bottom graph) markers of bone formation were examined in control (white bars) and diabetic (black bars) from Bax knockout and wild type mice. Osteocalcin mRNA levels are calculated relative to the housekeeping gene HPRT. Osteoblast surface is expressed as a percent surface per total trabecular surface. Bars represent the average value ± SE (n≥10 per group); * denotes $p<0.05$.

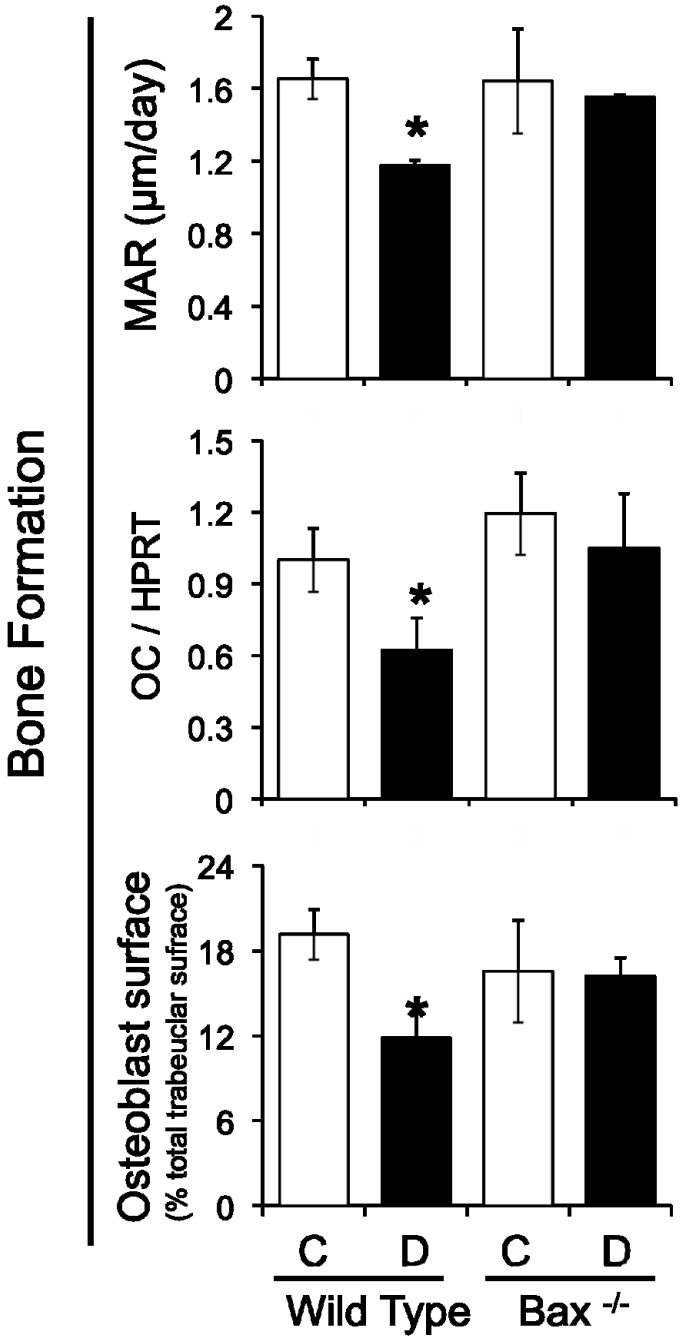
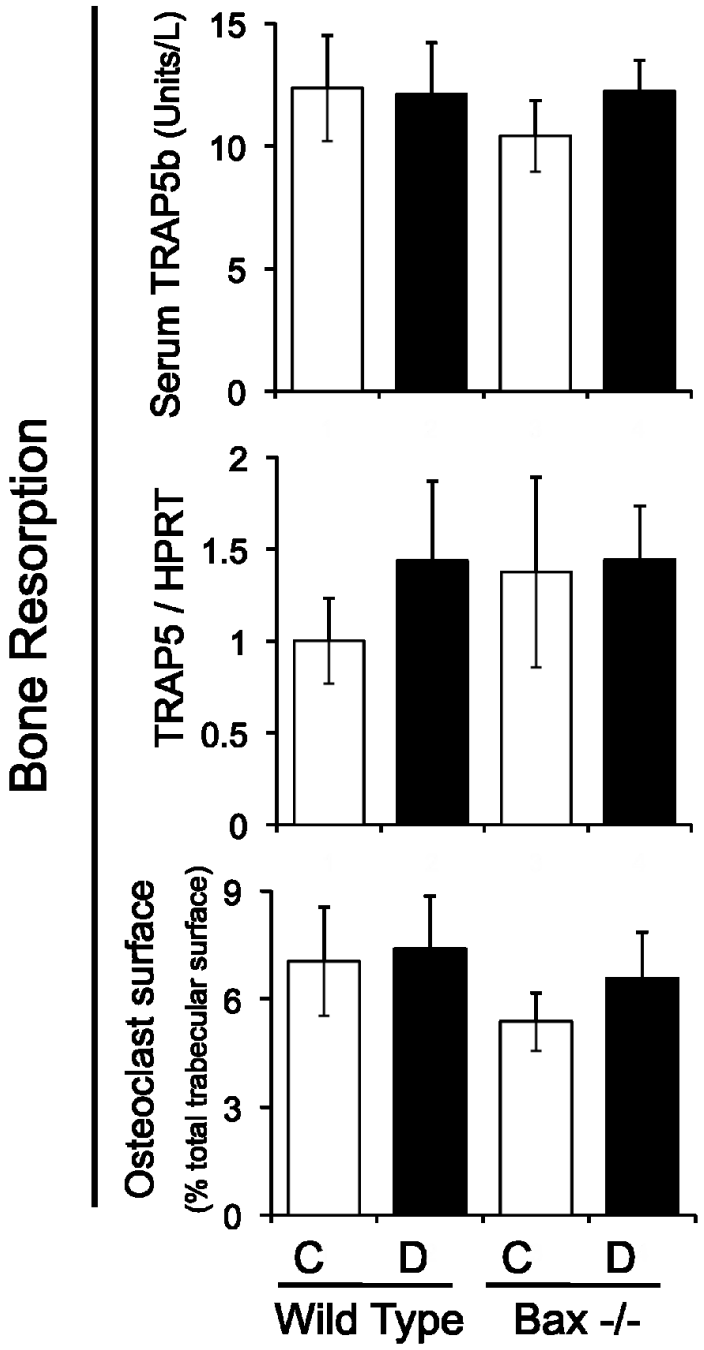
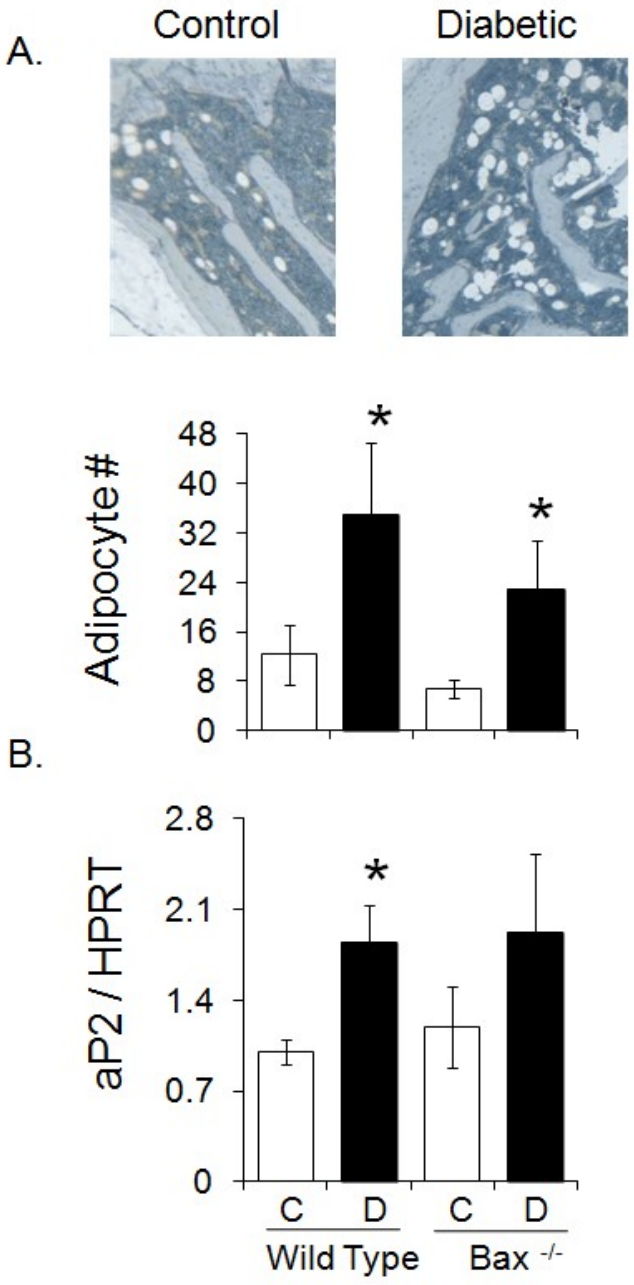


Figure 24. **Bone resorption remains unchanged by diabetes in wild type and Bax-deficient mice.** Serum TRAP5b (top), TRAP5 mRNA expression (middle) and osteoclast surface were not changed in diabetic (black bars) compared to control (white bars) in Bax-deficient and wild type littermates at 40 days post injection. TRAP5 mRNA levels are calculated relative to the housekeeping gene HPRT. Osteoclast surface is expressed as a percent surface per total trabecular surface. Bars represent the average value ± SE ($n \geq 10$ per group); * denotes $p<0.05$.



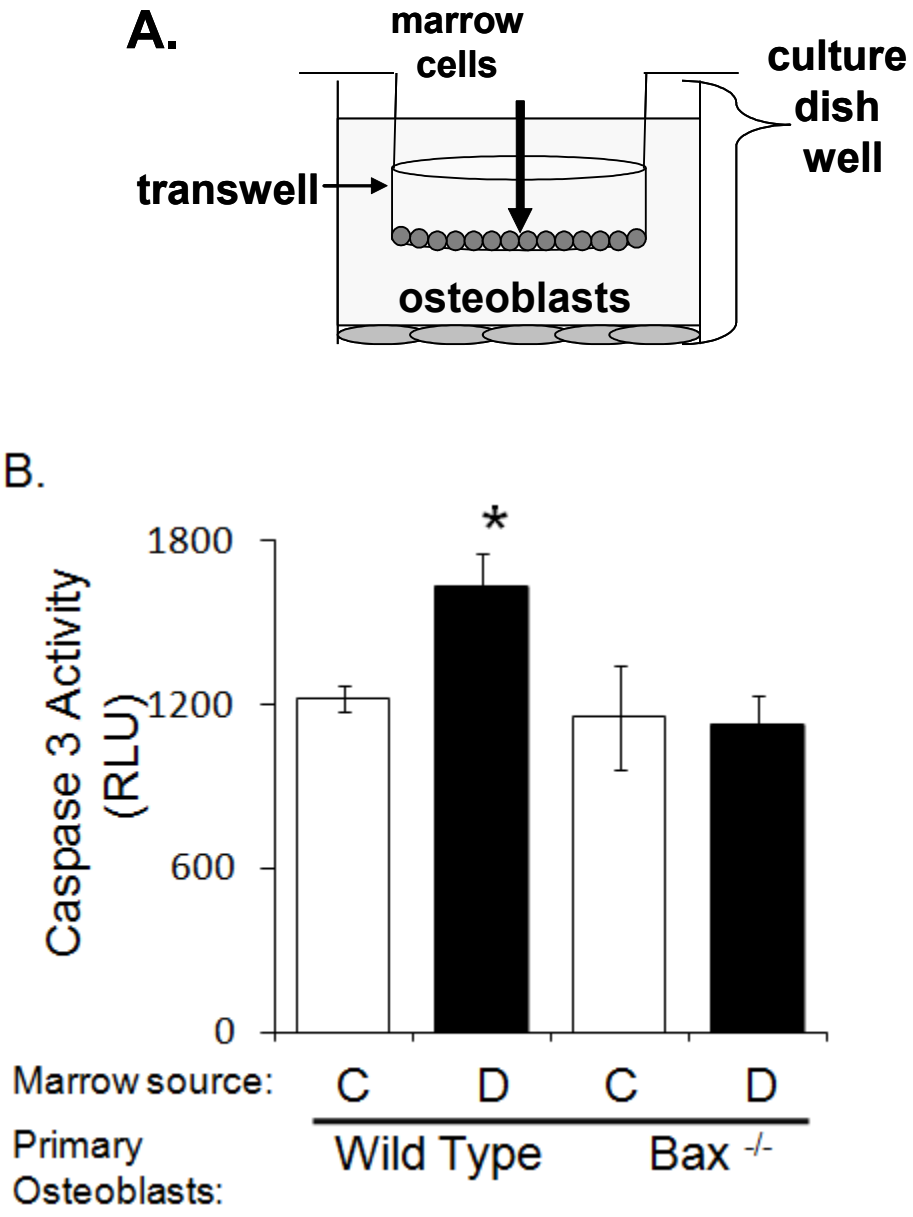
Diabetes increases marrow adiposity in mice (19-21, 48); therefore, we measured marrow adipocyte levels and aP2 (marker for mature adipocytes) mRNA expression in wild type and Bax deficient control and diabetic mice. As expected, T1-diabetes elevated marrow adipocyte number and aP2 expression in wild type mice. Bax deficiency did not prevent diabetes-induction of marrow adiposity, marked by increased adipocyte numbers and aP2 expression (figure 25). This finding suggests that Bax expression does not play a major role in disease induced adipocyte lineage selection or differentiation.

Figure 25. **Diabetes-induced marrow adiposity occurs in Bax knockout and wild type mice.** Adipocyte images represent adipocyte numbers from the marrow of control and diabetic Bax deficient and wild type mice. Graphical representation of adipocyte number and aP2 mRNA expression (a marker of mature adipocytes) displayed from control (white bars) and diabetic (black bars) Bax knockout and wild type mice. Mature adipocyte marker aP2 mRNA levels are calculated relative to the housekeeping gene HPRT. Values are expressed as MEAN ± SE (n≥10 per group); * denotes p<0.05.



Previously, we reported that diabetic marrow induces osteoblast death when co-cultured in transwells over osteoblasts (MC3T3-E1 or primary osteoblasts) (24). Based on our mouse data, we hypothesize that Bax deficient osteoblasts are protected from diabetic marrow-induced osteoblast apoptosis. To test this, we isolated bone marrow cells from control and diabetic (5 dpi) wild type mice and cultured the marrow in transwells over primary osteoblasts isolated from either wild type or Bax deficient mice (figure 26). After 24 hours, caspase 3 activity (a marker of cell death) increased significantly in wild type derived osteoblasts co-cultured with diabetic marrow. In contrast, Bax deficient osteoblasts did not display increased caspase 3 activity when co-cultured with diabetic marrow (figure 26). This implicates elevated osteoblast Bax expression in the signaling pathway mediating T1-diabetes induced osteoblast death.

Figure 26. **Bax-deficient osteoblasts are protected from diabetic marrow induced osteoblast death.** A) Representation of the co-culture system utilized in the study. Bone marrow cells are cultured in transwells containing a 10 µm thick membrane with 0.4 µm pores at the bottom of the well to allow diffusion of factors between this cell compartment and the osteoblasts (bone marrow stromal (BMS)-derived osteoblasts from Bax-deficient or wild type mice) plated at the bottom of the well. B) After co-culturing with marrow cells for 24 hours, osteoblast caspase 3 activity was determined. Bone marrow for these studies was obtained from 5 dpi control (white bar) and diabetic (black bar) mice. Values are expressed as MEAN ± SE (n≥3 per group); * denotes $p<0.05$.



4.5. DISCUSSION

Apoptosis is a programmed cell suicide that is physiologically important in development of many organ systems including bone. The maintenance of skeletal mass is based on balancing bone cell proliferation and apoptosis as well as bone formation and resorption (23). Disrupting one of these four factors could lead to complications resulting in increased fracture risk and decreased overall bone health. Suppressed bone formation is an accepted secondary complication of T1-diabetes (20-22). Previously, we demonstrated that T1-diabetes results in elevated osteoblast apoptosis and increased Bax expression in bone (24). Here, we investigated the role of Bax in mediating diabetes-induced osteoblast death and its significance in regulating the diabetic bone phenotype. Our findings demonstrate that Bax is required for diabetes-induced osteoblast death and bone loss.

Previous studies show that murine osteoblasts and primary human bone marrow stromal cells require Bax-mediated osteoblast death during serum starvation (52). Inhibition of Bax expression through siRNA in these cells protected osteoblasts from 18-hour serum starvation-induced apoptosis by 85-90% and displayed significantly reduced levels of caspase 3 activity (52). Interestingly, this study also reported that Bax deficiency in the presence of normal serum levels did not decrease control apoptosis levels (52), consistent with our data. Correspondingly, Bax deficiency alone did not increase apoptosis in cartilage cells or bone marrow cells (42). Osteoblasts over-expressing Bcl-2, the anti-apoptotic counterpart to Bax, decreased osteoblast death *in vivo* (53) and *in vitro* (43) in response to glucocorticoid-induced apoptosis. Additionally,

the only anabolic therapy used to treat osteoporosis, intermittent parathyroid hormone (PTH), is suggested to promote bone formation through the up-regulation of Bcl-2 expression in osteoblasts in a Runx2-dependent manner (54, 55) thus inhibiting osteoblast death. This data suggests that regulation of bone cell apoptosis has remarkable therapeutic value for the treatment of osteoporosis.

In addition to preventing osteoblast death, Bax deficiency eliminated diabetes-induced bone loss. Similarly, Bax-deficient female mice are protected from age-induce bone loss (41). These mice displayed elevated whole body, trabecular, and cortical BMD at 7 months old and even higher bone density levels in older (20-22 months) mice (41). They also displayed enhanced bone strength in both femur and vertebral sites (41). Additionally, Bax-knockout mice displayed increased mineralized fracture callus and had a 25% greater BMC then wild type mice 14 days post-fracture (42). Male mice overexpressing Bcl-2 exclusively in osteoblasts resulted in increased bone volume fraction compared to wild type littermates. In contrast to these beneficial results, female Bcl-2 transgenic mice displayed reduced bone volume at 2-months old (53). This could be due to pre-puberty estrogen levels. Correspondingly, Bax-knockout mice that underwent bilateral ovariectomys were not protected from bone loss in either cortical or trabecular bone (41). These results indicate the importance of ovarian function and estrogen levels on bone.

In summary, our studies indicate that the pro-apoptotic factor, Bax, mediates T1-diabetes-induced osteoblast death and plays a vital role in regulating diabetes-induced bone loss. We propose that Bax deficiency inhibits bone loss and osteoblast apoptosis through eliminating diabetes-induced suppression of Wnt10b. This data could provide a

potential biomarker for regulating osteoblast death and bone loss and/or be used as a target for therapeutic treatments of osteoporosis in the diabetic patient population.

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CHAPTER 5. ALENDRONATE PREVENTS DIABETES-INDUCED BONE LOSS THROUGH INHIBITION OF OSTEOBLAST DEATH.

5.1. ABSTRACT

Type I (T1) diabetes is an autoimmune disease associated with bone loss. Previous studies demonstrate that T1-diabetes decreases osteoblast activity, while increasing osteoblast death. Therefore, therapies targeting osteoblast death may prevent diabetes-induced bone loss. One commonly used therapy for osteoporosis is bisphosphonates. These drugs inhibit osteoclast and osteoblast activity and promote apoptosis. Taken together, we hypothesized that administration of alendronate (commonly proscribed bisphosphonate) could prevent the diabetes-induced bone loss through the inhibition of osteoblast apoptosis. To test this, we induced diabetes in 16-week-old male C57BL/6 mice. Both control and diabetic mice were given once a week injections of alendronate and compared to non-treated control and diabetic mice. Bone measurements of osteoblast death, bone formation, bone resorption and marrow adiposity were tested. Examination of trabecular BV/TV indicated alendronate therapy fully attenuated the diabetes-induced osteoporosis. Additionally, markers of bone formation and bone resorption were both decreased in response to alendronate therapy. The diabetes-induced marrow adiposity was observed in both alendronate treatment and non-alendronate treated diabetic mice compared to respective controls. Alendronate therapy alone did decrease marrow adiposity beyond normal control levels. How does bisphosphonate therapy protect against diabetic bone loss? Examination of osteoblast apoptosis illustrated that alendronate therapy protects osteoblasts from diabetes-induced osteoblast death. Furthermore, alendronate therapy increased the ultimate stress needed

to fracture femurs. Taken together, our results suggest that alendronate therapy prevents diabetes-induced bone loss by inhibiting diabetes associated osteoblast death.

5.2. INTRODUCTION

Type I (T1) diabetes is an autoimmune disease characterized by little to no insulin production and hyperglycemia. Medical advances to maintain euglycemia are increasing patient lifespan, however even today, proper maintenance of glucose levels remains difficult. Prolonged exposure to hyperglycemia results in increased occurrence of secondary complications such as neuropathy, nephropathy, retinopathy and osteoporosis [1-6]. Osteoporosis is defined as 2.5 standard deviations below average bone density in humans. A decrease in bone density by even 1 standard deviation results in increased fracture risk by roughly 2.4 times. Bone loss and increased fracture risk are reported evident in the hip [7-16], spine [8, 15-17], and feet [11, 18] of T1-diabetic patients and is independent of gender and age. Rodent models of T1-diabetes display comparable decreases in bone density and increased fracture risk to T1-diabetic patients, making it a useful model to study. Diabetic bone loss is well documented in both the pharmacological and spontaneous mouse models as well as diabetic patients to result from the suppression of bone formation [6, 19, 20].

Bone formation is regulated at multiple stages including (but not limited to) stromal cell lineage selection and osteoblast death. Mesenchymal stem cells that differentiate into osteoblasts also differentiate into adipocytes. Elevated marrow adiposity is associated with diabetic bone loss [6, 21, 22] however, inhibition of marrow adiposity does not prevent diabetes-induced bone loss [23, 24] implementing another mechanism may be sufficient for mediating bone loss. Recently, we reported elevated osteoblast apoptosis in early diabetes onset [25]. A time point which coincides with the first detectable decrease in osteocalcin expression [25]. Furthermore, inhibition one pro-

apoptotic factor, specifically Bax, resulted in protection against T1-diabetic bone loss and osteoblast death (Coe, et al). Therefore, a therapy that targets osteoblast death could prevent diabetes-induced bone loss.

To date no perfect therapy exists for treating diabetes-induced bone loss. The most commonly used preventive therapy for decreased bone density is bisphosphonates [26-29]. These drugs selectivity integrate into bone mineral surfaces and increase bone mineral density [30-36] by inhibiting osteoclast-mediated resorption, recruitment, differentiation, and apoptosis. Furthermore, they are reported to prevent both vertebral and non-vertebral fractures [37-40]. Previous studies suggest that bisphosphonates not only inhibit osteoclast-mediated resorption but they also inhibit osteoblast death [41, 42], thus making them a possible beneficial therapy for treating diabetic patients. *In vitro* studies report that bisphosphonates protect rodent-derived osteoblasts from AGE-induced death (previously shown elevated in diabetic bone) [43]. Clinically, type 2 diabetic and non-diabetic women taking bisphosphonates benefitted in overall bone health [44]. Taken together, the goal of this study was to determine if diabetic mice treated with weekly alendronate therapy, a commonly used bisphosphonate, would prevent diabetes-associated osteoporosis by inhibiting osteoblast death. Here, we demonstrate that alendronate therapy prevented diabetes-induced bone loss through inhibition of osteoblast death. Additionally, bisphosphonate therapy decreased the bone stiffness but also decreased total bone remodeling.

5.3. METHODS

5.3.1. Diabetic Mouse Models

Diabetes was induced in adult (15-16 week old) male C57BL/6 mice by 5 consecutive intraperitoneal injections of streptozotocin (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Controls were given citrate buffer alone. Alendronate was delivered by subcutaneously injections in control and diabetic mice at a concentration of 2mg/kg/week, a dose consistent with the literature [45]. Mice were maintained on a 12-hour light, 12-hour dark cycle at 23°C, given standard lab chow and water *ad libitum*. Diabetes was confirmed 12 days after initial injection using an Accu-Check compact glucometer (Roche Diagnostics Corporation, Indianapolis, IN) with a drop of blood from the saphenous vein. Total body, tibialis anterior, and subcutaneous femoral fat pad mass were recorded. Animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

5.3.2 RNA Analysis

Immediately after euthanasia, one tibia and femur were cleared of soft tissue and snap frozen in liquid nitrogen and stored at -80°C. Frozen tibias were crushed under liquid nitrogen conditions, homogenized, and placed in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA integrity was determined through formaldehyde-agrose gel electrophoresis. cDNA was synthesized through a reverse transcriptase reaction utilizing Superscript II Reverse Transcriptase Kit and oligo dT (12-18) primers (Invitrogen, Carlsbad, CA), amplified by quantitative real time PCR with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and gene-specific primers. Forward and

reverse primers used as previously stated: aP2, Bax, Bcl-2, HPRT, osteocalcin, TRAP5, [22, 25, 46]. Expression of HPRT does not alter in diabetes and therefore used as a housekeeping gene. Real Time PCR was carried out for 40 cycles, each cycle consisting of 95°C for 15 seconds, 60°C for 30 seconds (except osteocalcin which has an annealing temperature of 65°C) and 72°C for 30 seconds. RNA-free samples were used as a negative control and did not produce amplicons. PCR products were separated on 1.5% agrose gel electrophoresis and sequenced to verify the desired gene is being amplified.

5.3.3. Micro-computed Tomography (μCT) analysis

Fixed femurs in 70% ethanol were scanned using the GE Explore μCT system at a voxel resolution of 20um from 720 views with a beam strength of 80kvp and 450uA. Integration time for each scan was 2000ms. Scans included bones from each condition and a phantom bone to standardize the grayscale values and maintain consistency between runs. Using the systems auto-threshold (800) and isosurface analysis confirmation, trabecular bone densities (bone volume fraction (BV/TV), bone mineral density (BMD) and content (BMC), and trabecular thickness (tb. th), number (tb. n) and spacing (tb. sp)) were measured in the trabecular region defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis, and excluding the outer cortical shell. Cortical bone measurements are determined with a 2-mm^3 region of interest (ROI) in the mid-diaphysis. Cortical thickness, moment of inertia, cortical area, marrow area, total area, inner perimeter and outer perimeter as well as all trabecular parameters were computed using the GE Microview Software for visualization and

analysis of volumetric image data.

5.3.4. Bone Histology and Histomorphometry

Bones were fixed in 10% buffered formalin, transferred to 70% EtOH after 24 hours, and processed for dehydration, clearing and infiltration on a routine overnight processing schedule. Samples were then paraffin embedded on a Sakura Tissue Tek II embedding center and 5μm sections prepared. Osteoclasts were identified, by staining for tartrate resistant acid phosphatase (TRAP) (Sigma). The number or surface area of osteoclasts, osteoblasts and adipocytes was determined in the trabecular region of the femur (defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis). Osteoclast and osteoblast surface was measured in three trabecular regions for each mouse and expressed as a percent of total trabecular surface. Visible adipocytes, greater than 30 μm, were counted in the same trabecular region.

Cell death

Cell death was determined using a TACS•XL® Basic In Situ Apoptosis Detection Kit (TUNEL, Trevigen Inc., Gaithersburg, MD) on the L1-3 vertebrae or femur sections. Osteoblasts with positive nuclei were counted and expressed as a percentage of total osteoblasts counted per bone. Positive controls included slides incubated with nuclease. Five trabecular regions were examined for each mouse. Total osteoblasts counted ranged between 20 and 100 per bone.

Dynamic bone formation

Mice were injected intraperitoneally with 200µl of 10mg/ml calcein (Sigma, St. Louis, MO, USA) dissolved in saline at 7 and 2 days prior to harvest. L3-L4 vertebrae were fixed in formalin at time of harvest then transferred to 70% ethanol 24 hours later. Vertebrae were then embedded, sectioned and examined under UV light. Five images were taken and the distance between the calcein lines and their length along the bone surface was measured and used to calculate MAR. Mineralizing surface was calculated by measuring total calcein labeling per total trabecular surface. Bone formation rate was calculated by the equation BFR=mineralizing surface X mineral apposition rate as previously stated [47, 48].

5.3.7. Serum Measurements

Blood was collected at the time of harvest, allowed to clot at room temperature for 5 minutes, then centrifuged at 4,000 rpm for 10 minutes. Serum was removed and stored at -80°C. Serum went through no more than two freeze/thaw cycles. Serum TRAP5b and Osteocalcin were measured using a Mouse TRAP and OC assay kits (SB-TR103, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA and BT-470, Biomedical Technologies Inc., Stoughton, MA, USA respectively) according to the manufacturer's protocol.

5.3.8. Statistical Analysis

All data is presented as mean ± standard error. Statistical significance (p-value < 0.05) of main effects (treatment or diabetes) as well as treatment x diabetes interaction

(which determines if diabetes alters the treatment effect or vise versa) was determined using factorial analysis of variance (ANOVA) with SPSS statistical software (Chicago, IL). Student's t-test were used where needed.

5.4. RESULTS

To test if alendronate therapy prevented diabetes-induced bone loss, we induced diabetes in 16-week-old male C57BL/6 mice (controls given vehicle alone) and divided both control and diabetic mice into two groups; alendronate treated and non-alendronate treated. First, we examined the effect of alendronate on general mouse parameters. Alendronate decreased both femoral and visceral fat pad mass by 12.5% and 42%, respectively, compared to non-alendronate treated controls (table VI). It did not, however, prevent any of the diabetes induced physiological changes. Specifically, diabetic mice with or without alendronate treatment displayed comparable elevations in blood glucose levels when compared to their respective controls (averaging 511 mg/dl and 494 mg/dl, respectively) (table VI). Both diabetic mouse groups, alendronate treated and non-alendronate treated, had decreased total body mass (14% and 13%, respectively), femoral fat pad mass (62% and 61%, respectively), tibialis anterior mass (24% and 25%, respectively), and visceral fat mass (94% and 96%, respectively) when compared to their respective treatment controls. Two-way ANOVA analyses indicated that diabetes alone was responsible for the observed physiological changes (table VI).

Table VI. Body parameters in control and diabetic with or without alendronate.

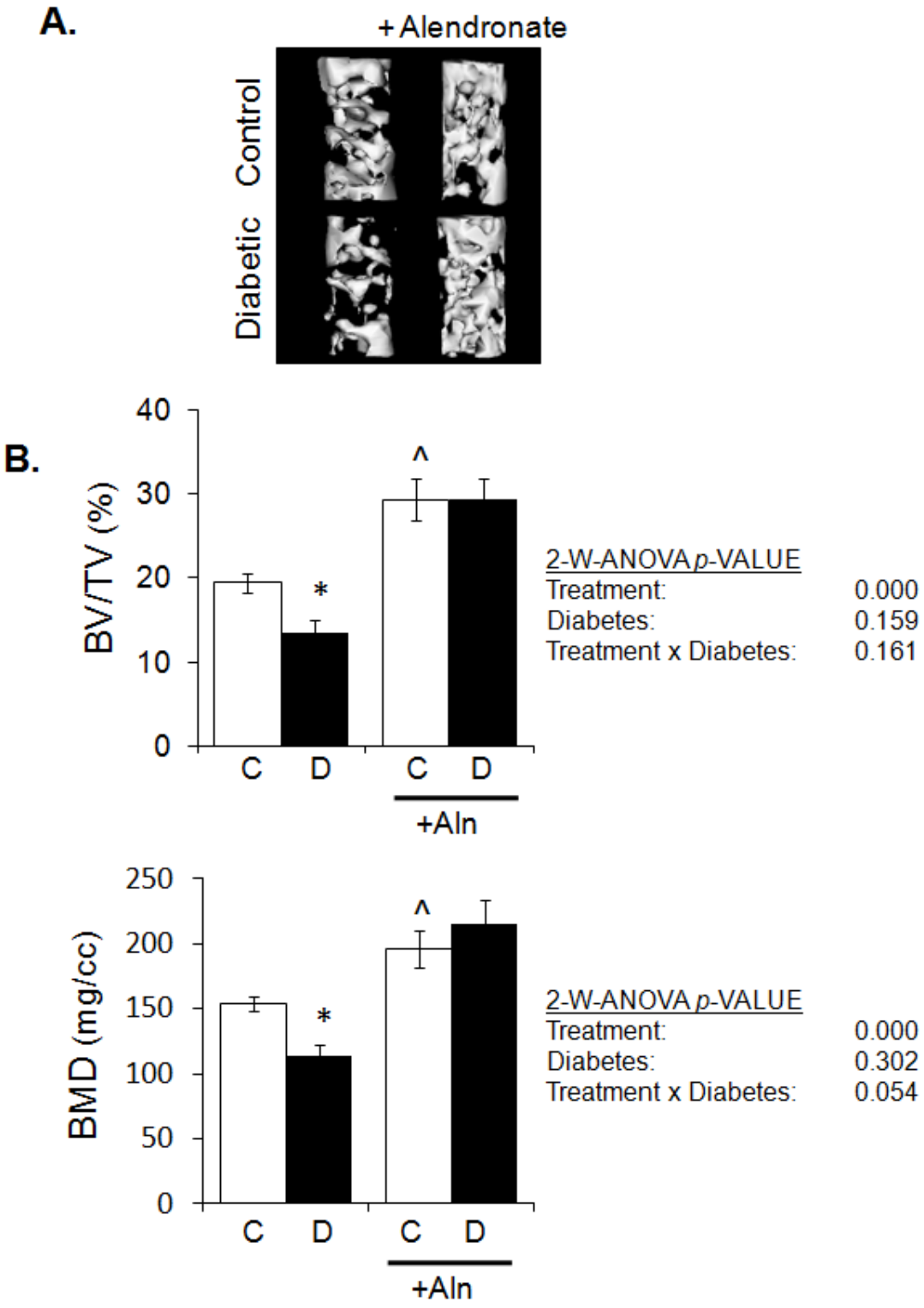
			Alendronate		2-W-ANOVA *p*-VALUE		
	Control (n=7)	Diabetic (n=7)	Control (n=8)	Diabetic (n=8)	Treatment	Diabetes	Treatment X Diabetes
Body Mass (g)	28.4 ± 0.9	24.6 ± 1.0* (-13%)	27.4 ± 0.6	23.7 ± 0.5* (-14%)	0.242	0.000	0.902
Blood Glucose (mg/dl)	156 ± 7	494 ± 29* (217%)	166 ± 7	511 ± 34* (208%)	0.557	0.000	0.862
Fem Fat Mass (mg)	15.8 ± 1.6	6.1 ± 0.7* (-61%)	12.5 ± 1.1^ (-12.5%)	4.8 ± 0.7* (-62%)	0.046	0.000	0.377
Tibialis Mass (mg)	3.2 ± 0.2	2.4 ± 0.3* (-25%)	3.3 ± 0.8	2.5 ± 0.3* (-24%)	0.731	0.006	0.994
Vis Fat Mass (mg)	6.88 ± 1.67	0.17 ± 0.01* (-96%)	4.01 ± 0.61^ (-42%)	0.26 ± 0.17* (-94%)	0.115	0.000	0.095

Abbreviations: Fem. Fat, femoral fat pad mass, Vis. Fat, visceral fat pad mass. *Statistics*: * $p<0.05$ compared to respective control. ^ $p<0.05$ compared to untreated controls. (%) change indicated compared to control littermates and vehicle treated control.

Next, we determined if alendronate therapy prevented diabetes-induced bone loss. We examined bone density parameters using μCT three dimensional isosurface imaging (Figure 27A). As expected, diabetes resulted in a 31% reduction in bone volume fraction (BV/TV) and 27% reduction in bone mineral density (BMD) (Figure 27B) as well as decreases in trabecular spacing, thickness and number (table VII). In contrast, alendronate therapy increased both BV/TV and BMD by 54% and 27%, respectively, beyond non-treated control levels. Furthermore, alendronate treatment increased trabecular number by 25% compared to non-treated controls. Alendronate therapy prevented the diabetes-induced bone loss is all trabecular parameters. When analyzed by two-way ANOVA, bisphosphonate treatment impacted BV/TV and BMD levels ($p<0.000$ and $p<0.000$, respectively) while treatment X diabetes only affected BMD ($p<0.054$).

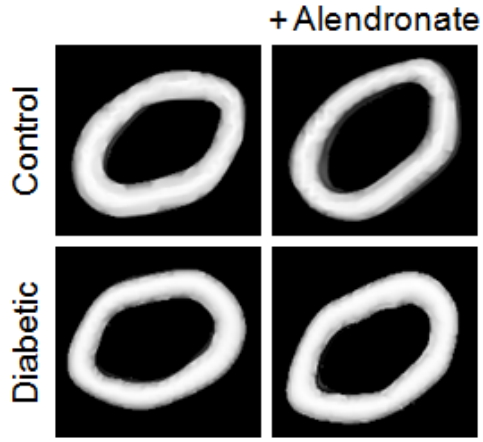
Figure 27. **Alendronate prevents diabetes-induced bone loss.** A) Representative μCT isosurface images of trabecular bone volume/total volume from distal femurs were obtained from control and diabetic mice treated with or without alendronate at 40 dpi. B) Graphical representation of bone volume/total volume and bone mineral density (BMD) is displayed from control (white bars) and diabetic (black bars), non-alendronate treated and alendronate treated mice. Bars represent the average value ± SE (n=7-8 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of bisphosphonate treatment, diabetes and bisphosphonate treatment x diabetes on bone density measurements. * denotes $p<0.05$ from respective control. ^ denotes $p<0.05$ relative to non-treated control.

figure 27 (cont'd)



To determine if alendronate therapy affected cortical bone similar to trabecular bone we measured several cortical bone parameters. Consistent with previous reports, diabetes decreased cortical thickness, cortical area and the percent cortical area/total area compared to control bones (Figure 28, table VII). Alendronate treatment prevented any diabetes-induced decreases in all cortical bone parameters measured. Interestingly, control mice given alendronate had a 5% decrease in inner perimeter (table VII), which could result in increased bone strength.

Figure 28. **Decreased cortical thickness in both alendronate and non-alendronate diabetic mice.** Representative μCT isosurface images of cortical thickness from the mid-diaphysis region of femurs were obtained from control and diabetic mice treated with or without alendronate. Graphical representation of cortical thickness in millimeters is displayed from control (white bars) and diabetic (black bars), non-alendronate treated and alendronate treated mice. Bars represent the average value ± SE (n=7-8 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of bisphosphonate treatment, diabetes and bisphosphonate treatment x diabetes on cortical bone thickness * denotes $p<0.05$ from respective control.



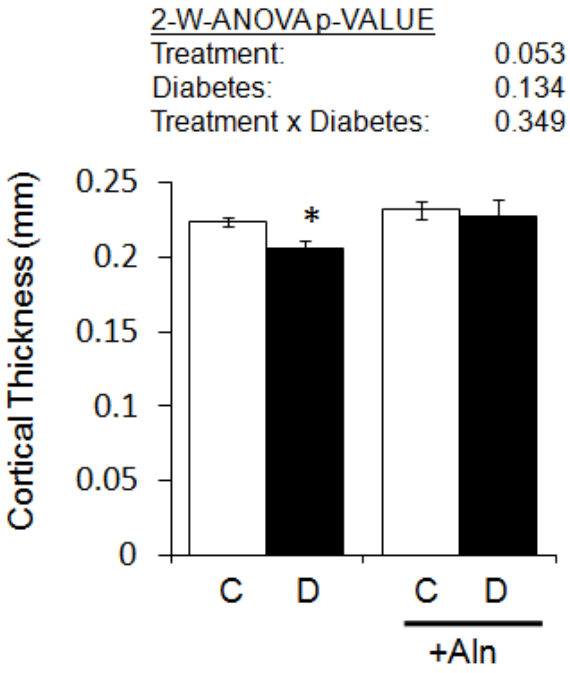


Table VII. μCT femur parameters in control and diabetic vehicle and alendronate treatment littermates.

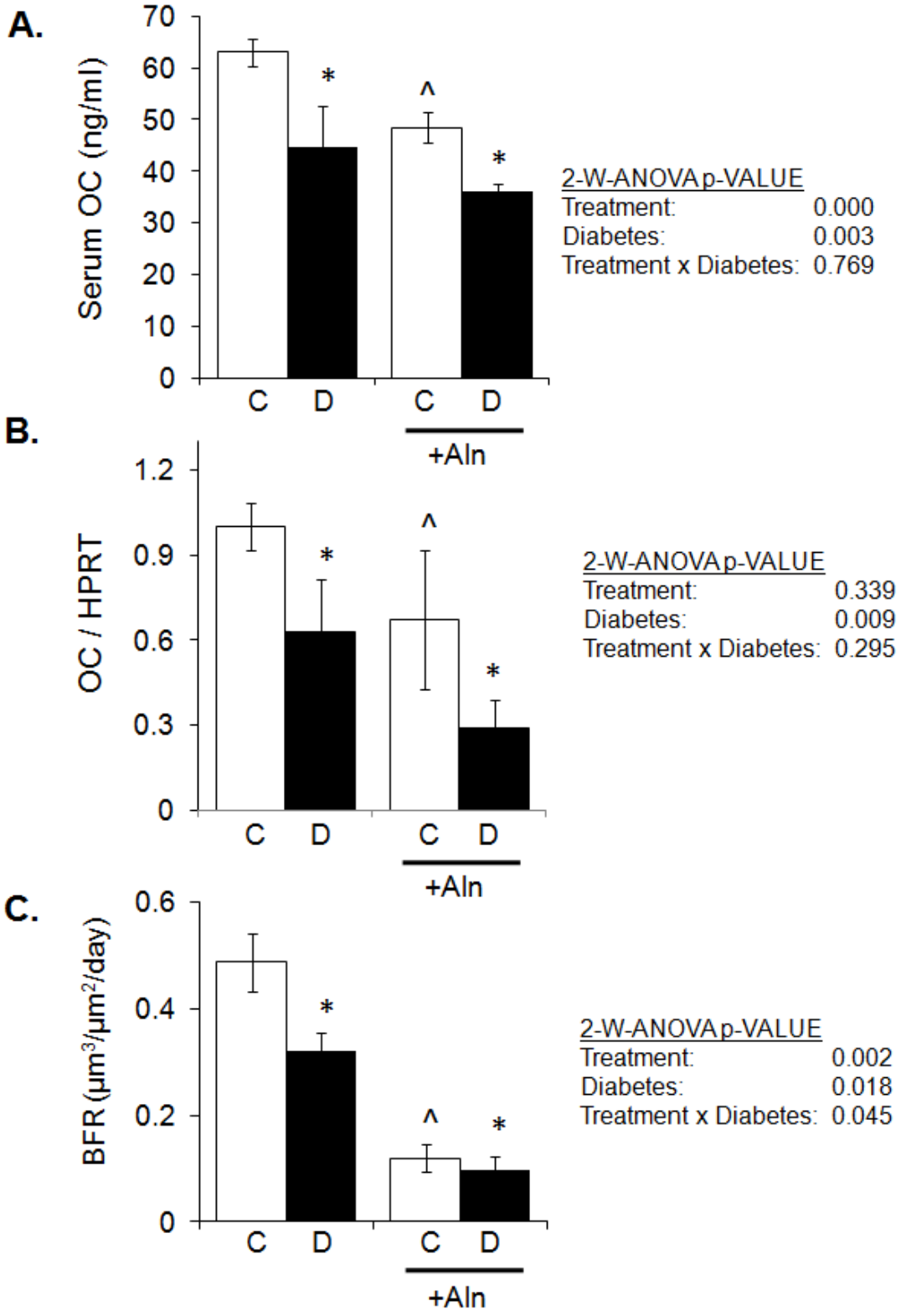
			Alendronate	
Trabecular	Control	Diabetic	Control	Diabetic
BMC (mg)	0.48 ± 0.02	0.34 ± 0.02*	0.60 ± 0.05*	0.65 ± 0.05*^
BMD (mg/cc)	154 ± 5	113 ± 9*	195 ± 14*	216 ± 18*^
BV/TV (%)	19.4 ± 1.2	13.4 ± 1.6*	29.8 ± 4.1*	28.5 ± 2.1*^
Tb. Th (μm)	40.0 ± 1.2	36.4 ± 2.0*	48.7 ± 5.1$	54.9 ± 7.1*^
Tb. N (1/mm)	4.79 ± 0.26	3.56 ± 0.27*	5.97 ± 0.22*	5.96 ± 0.19*^
Tb. Sp (mm)	0.17 ± 0.01	0.26 ± 0.03*	0.12 ± 0.01*	0.11 ± 0.01*^
Cortical				
Ct. Th (mm)	0.220 ± 0.003	0.206 ± 0.005*	0.230 ± 0.006	0.230 ± 0.011^
I_{max} (mm^4)	0.20 ± 0.03	0.16 ± 0.01	0.19 ± 0.02	0.18 ± 0.02
Ec. Pm (mm)	3.68 ± 0.07	3.79 ± 0.07	3.53 ± 0.06*	3.57 ± 0.06^
Ps. Pm (mm)	5.09 ± 0.08	5.01 ± 0.07	5.00 ± 0.07	5.01 ± 0.06
Ma. Ar (mm^2)	0.93 ± 0.03	0.96 ± 0.04	0.86 ± 0.03	0.89 ± 0.03
Ct. Ar (mm^2)	0.92 ± 0.02	0.78 ± 0.03*	0.93 ± 0.03	0.92 ± 0.04^
Tt.Ar (mm^2)	1.85 ± 0.06	1.76 ± 0.05	1.79 ± 0.04	1.81 ± 0.04
Ct. Ar/ Tt. Ar (%)	49.9 ± 0.44	44.6 ± 1.56*	52.0 ± 1.09	50.8 ± 1.58^
BMD (mg/cc)	919 ± 27	961 ± 26	914 ± 17	973 ± 24*

Significance: * $p<0.05$ compared to non-treated control; ^ $p<0.05$ compared to non-treated diabetic $ $p<0.07$ compared to non-treated control. *Abbreviations*: BMC, bone mineral content, BMD, bone mineral density, BV/TV, bone volume fraction, Tb. Th, trabecular thickness, Tb. Sp, trabecular spacing, Tb. N, trabecular number, I_{max}, maximum moment of inertia, Ec. Pm, Endocortical perimeter, Ps. Pm, Periosteal Perimeter, outer perimeter, Ma. Ar, Marrow area, Ct. Ar, Cortical area, Tt. Area, total area.

Type I diabetes is associated with decreased osteoblast number and activity and generally no change in osteoclast activity (although controversial); therefore we examined several markers of bone formation and resorption to assess the impact of alendronate therapy on these parameters. Consistent with previous reports, diabetes induction resulted in decreased serum and tibia osteocalcin levels by 30% and 37%, respectively. Diabetic mice administered with alendronate therapy also decreased serum and tibia osteocalcin levels by 25% and 80%, respectively (Figure 29). Two-way ANOVA analysis indicates that these changes in osteocalcin expression are due to both bisphosphonate treatment and diabetes induction ($p<0.000$ and $p<0.003$, respectively), however, treatment X diabetes had no affect. Alendronate treatment alone significantly decreased serum and gene osteocalcin expression (Figure 29). Consistent with decreased osteocalcin levels that are static bone formation makers, diabetes and alendronate therapy (administered separately or together) decreased the dynamic measurement bone formation rate. Specifically, bisphosphonate treatment resulted in an 80% reduction in bone formation rate as indicated by two-way ANOVA ($p<0.002$), while diabetes decreased BFR by 40% ($p<0.018$).

Figure 29. **Bone formation decreased as a result of both diabetes and bisphosphonate therapy.** A & B) Serum and tibia mRNA osteocalcin expression was analyzed in non-treated and alendronate treated in control (white bars) and diabetic (black bars) mice. Bars represent the average value ± SE (n=7-8 per group). RNA levels are calculated relative to the housekeeping gene HPRT. Statistical analyses by factorial analysis of variance (ANOVA) C) Mice were injected with calcein dissolved in saline 7 and 2 days prior to harvest. L3-L4 vertebrae sections were photographed under UV light and the distance between the calcein lines were measured. Bone formation rate is graphed and bars represent the average value ± SE (n=7-8 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of bisphosphonate treatment, diabetes and bisphosphonate treatment x diabetes on markers of dynamic bone formation. * denotes $p<0.05$ from respective control. ^ denotes $p<0.05$ relative to non-treated control.

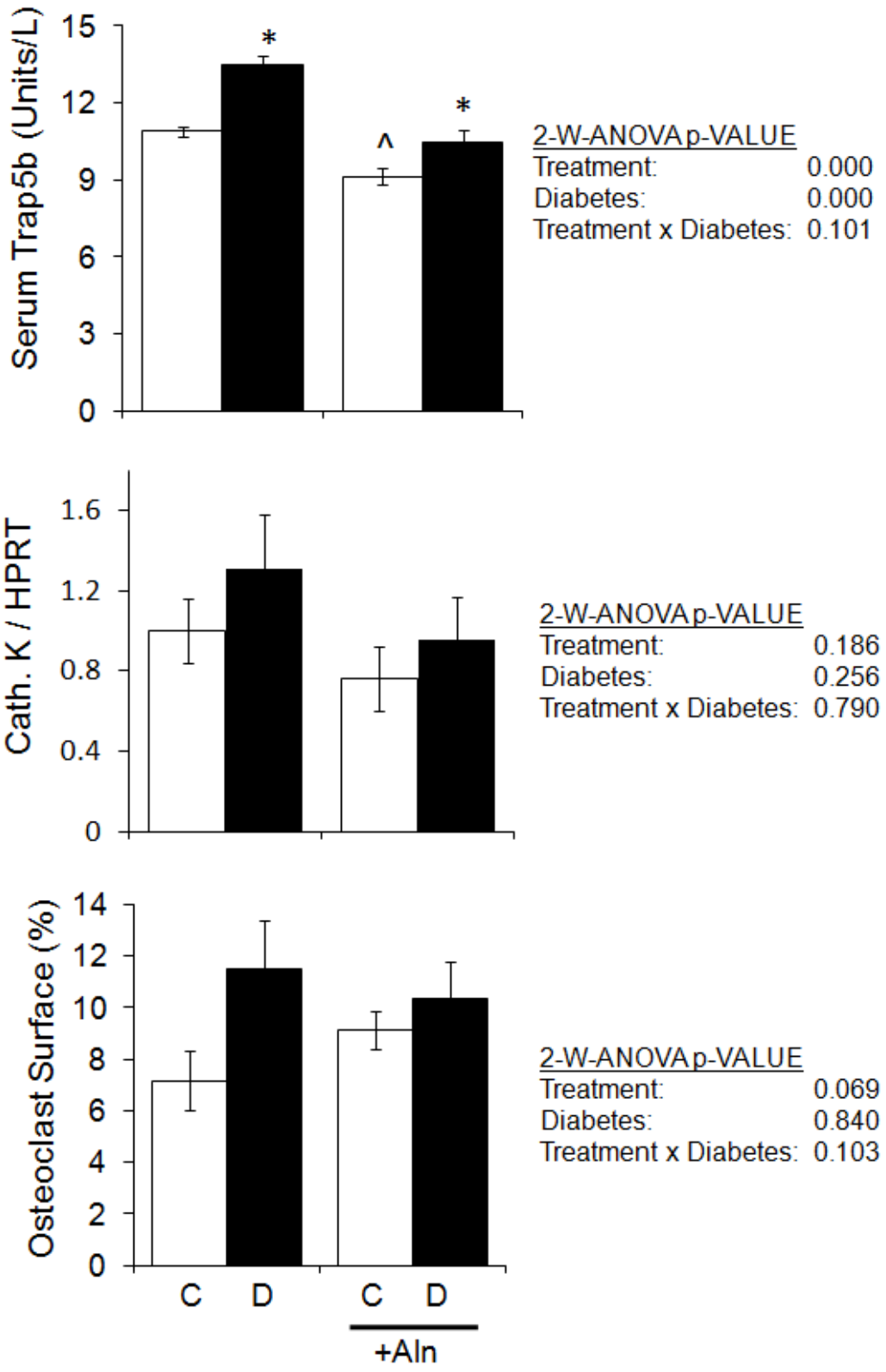
Figure 29 (cont'd)



Since bisphosphonates are known suppressors of bone resorption we examined serum Trap5b levels, cathepsin K mRNA expression and percent osteoclast surface per total trabecular surface. As expected, alendronate therapy decreased serum Trap5b levels by 18% compared to non-treated controls, while cathepsin K expression and percent osteoclast surface were not changed (Figure 30). In contrast, diabetes increased serum Trap5b levels compared to control levels by 11% in non-alendronate treated diabetic mice and 20% in alendronate treated diabetic mice. Two-way ANOVA analysis indicated that bisphosphonate treatment and diabetes are both responsible for these changes ($p<0.000$ and $p<0.000$, respectively).

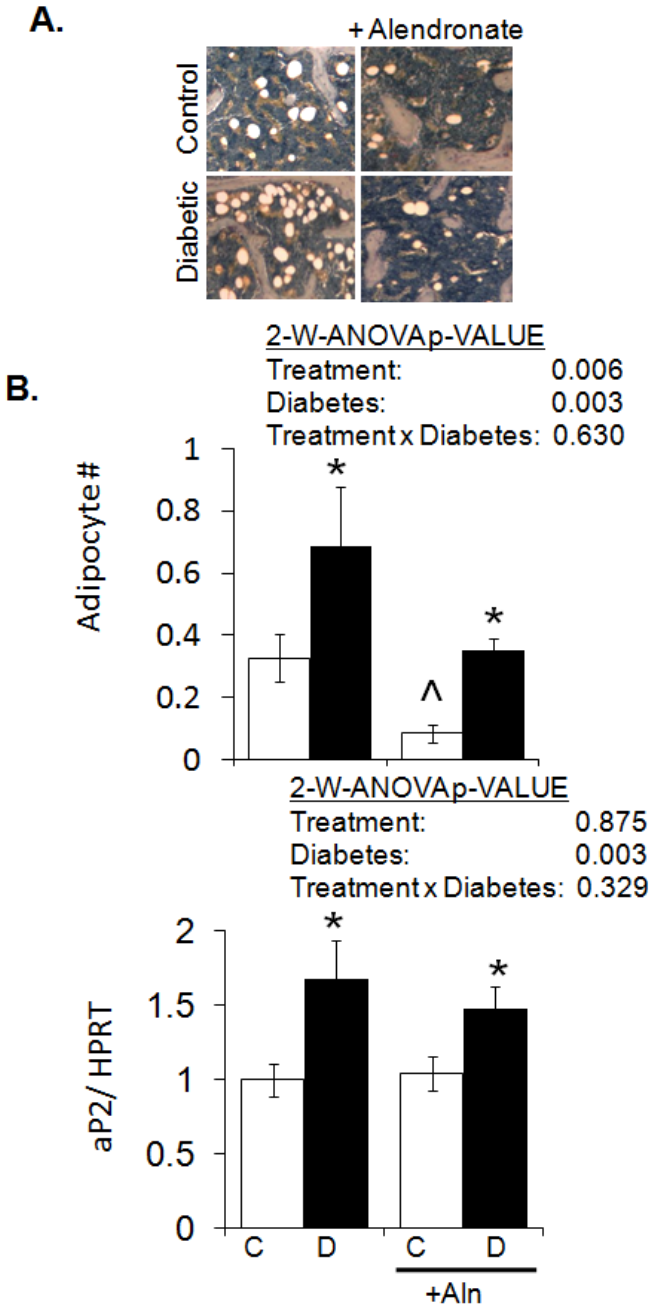
Figure 30. **Alendronate therapy suppresses bone resorption.** Serum TRAP5 expression, cathepsin K mRNA, and osteoclast surface were analyzed in non-treated and alendronate treated in control (white bars) and diabetic (black bars) mice. Bars represent the average value ± SE (n=7-8 per group). RNA levels were calculated relative to the housekeeping gene HPRT. Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of bisphosphonate treatment, diabetes and bisphosphonate treatment x diabetes on bone resorption markers. * denotes $p<0.05$ from respective control. ^ denotes $p<0.05$ relative to non-treated control.

Figure 30 (cont'd)



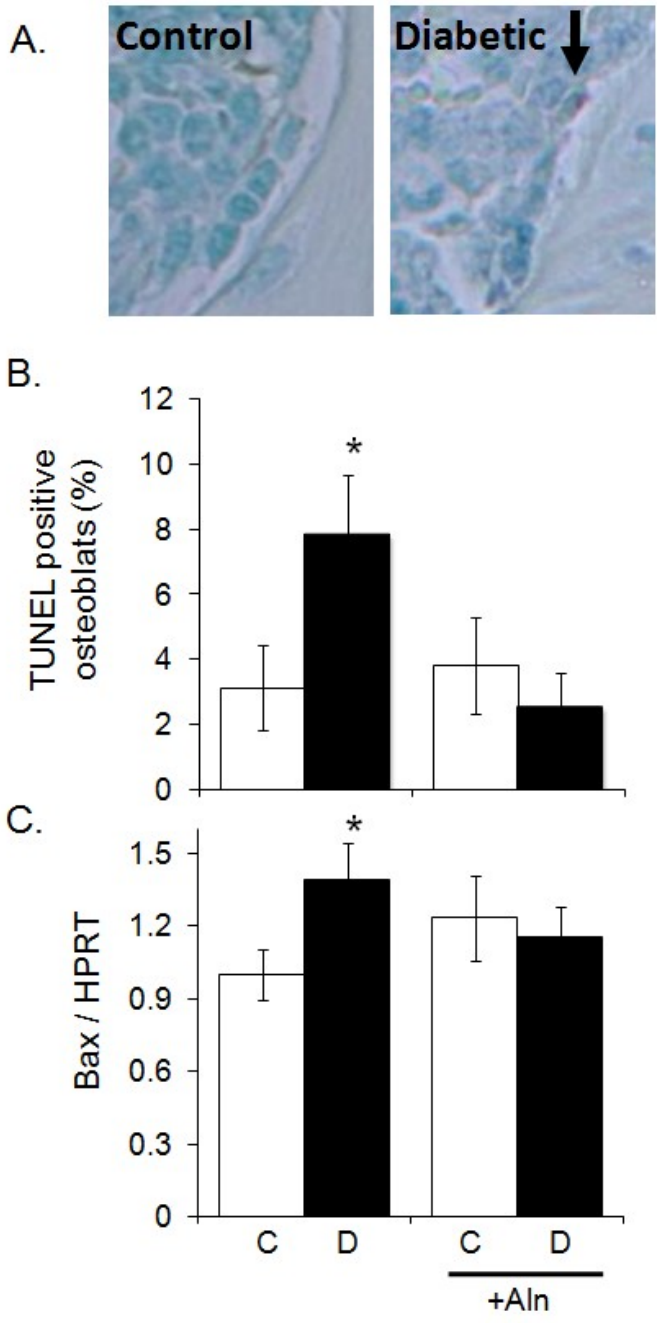
Diabetes increases marrow adiposity in mice [6, 21, 24, 49]; therefore, we measured marrow adipocyte levels and aP2 (marker for mature adipocytes) mRNA expression in control and diabetic mice with or without alendronate. Consistent with previous reports, diabetes induced a significant 2-fold increase in adipocyte number and a 60% increase in tibia aP2 expression (Figure 31) as indicated by 2-way ANOVA analysis ($p<0.003$, and $p<0.003$, respectively). Interestingly, alendronate therapy did not prevent diabetes-induced marrow adiposity, but did reduce the severity of adipocyte number. Two-way AVOVA analysis indicated that bisphosphonate treatment alone decreased adipocyte number compared to non-treated controls ($p<0.004$).

Figure 31. **Diabetes induces marrow adiposity while alendronate therapy alone decreases control levels.** Representative images of bone marrow adiposity from distal femurs were obtained from control and diabetic mice treated with or without alendronate. Graphical representations of adipocyte number and aP2 mRNA expression are displayed from control (white bars) and diabetic (black bars), non-alendronate treated and alendronate treated mice. Bars represent the average value ± SE (n=7-8 per group). RNA levels are calculated relative to the housekeeping gene HPRT. Statistical analyses by factorial analysis of variance (ANOVA). * denotes $p<0.05$ from respective control. ^ denotes $p<0.05$ relative to non-treated control.



Since alendronate therapy did not prevent the diabetes-induced decreases in bone formation or increases in bone resorption and marrow adiposity, what is responsible for the bone protection in the diabetic alendronate treated mice? One possible mechanism is decreased osteoblast death (previously demonstrated elevated in T1-diabetic bone). Therefore, we examined osteoblast death parameters in alendronate treated and non-treated control and diabetic mice at the time when osteoblast death occurs, 5 dpi. As expected, the percentage of TUNEL positive osteoblasts in femur sections increased nearly 4-fold in diabetic mice compared to control mice (Figure 32A). Correspondingly, pro-apoptotic factor Bax was elevated in diabetic bone by 40% compared to controls (Figure 32B). Diabetic mice treated with alendronate prevented this diabetes-induced increase in TUNEL positive osteoblasts and inhibited the elevation of Bax expression. Alendronate therapy alone displayed no effect on osteoblast death compared to non-treated controls. The inhibition of osteoblast death could account for the observed bone protection in alendronate treated diabetic mice.

Figure 32. **Alendronate inhibits diabetes-induced osteoblast death.** A) Representative images of TUNEL staining in control (left) and diabetic (right) trabecular bone at 5 dpi, the time when osteoblast death is first detectible. The arrow demonstrates a positive TUNEL stained osteoblast. B) Graphical representation of TUNEL staining and Bax mRNA expression from control (white bars) and diabetic (black bars) non-treated and alendronate treated mice. Bax expression is calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$ per group); * denotes $p<0.05$ relative to respective controls. For interpretation to the references of color in this and all other figures, the reader is referred to the electronic version of this dissertation.



5.5. DISCUSSION

Bisphosphonate therapy is the first line of pharmacological defense against osteoporosis. Previous studies have demonstrated that bisphosphonates selectively integrate into bone mineral surfaces and increase bone mineral density [30-36] by inhibiting osteoclast-mediated resorption, recruitment, differentiation, and apoptosis. Previous studies suggest that bisphosphonates not only inhibit osteoclast-mediated resorption but they also inhibit osteoblast death [41, 42]. Previously, we demonstrated that T1-diabetes results in elevated osteoblast apoptosis and increased Bax expression in bone [25]. Here, we demonstrate that alendronate prevents osteoblast death and elevated Bax expression and prevents diabetes-induced bone loss.

Few studies have addressed the impact of bisphosphonate therapy on type 2 diabetes and none have addressed the effects on type 1-diabetes patients. One clinical study involving patients on long-term, 4 years, of bisphosphonate treatment did not prevent bone loss in postmenopausal women with type 2 diabetes [50]. Additionally, a study performed on 13 post-menopausal Japanese women with type 2 diabetes taking bisphosphonates for 6 or 12 months demonstrated elevated bone density in the lumbar spine at both time points [51]. In contrast, Keegan et al. found that both type 2 diabetic and non-diabetic women benefitted in overall bone health from bisphosphonate therapy [44]. Interestingly, in this study more diabetic women were reported taking estrogen supplements compared to the non-diabetic women. This is important to note since estrogen could have altered any beneficial bone results due to estrogen's positive impact on bone formation [44].

Although bisphosphonates are thought to function as anti-resorptive drugs there are several lines of evidence that bisphosphonates suppress bone formation, differentiation [52, 53], and osteoblast apoptosis [42]. Gangoiti, et al. reported that mouse and rat osteoblasts treated with advanced glycation end products to induce cell death (elevated in T1-diabetes) in addition to various bisphosphonates were protected from death at low concentrations; however osteoblasts treated with high concentrations of bisphosphonates resulted in apoptosis [43]. Similar to our findings, mice treated with alendronate prevented vertrebral osteoblast and osteocyte apoptosis associated with prednisolone administration [42]. Although inhibition of osteoblast apoptosis is beneficial for T1-diabetic bone, the anti-resorptive properties remain problematic. Recently, a study demonstrated a new bisphosphonate that does not affect osteoclast activity or bone turnover but still inhibits osteoblast and osteocyte apoptosis in response to glucocorticoid insults [54]. Specifically, this bisphosphonate analog did not affect circulating or mRNA expression of bone resorption or formation markers (e.g. C-telopeptide and osteocalcin, respectively) [54]. This variation of bisphosphonate might be a better option for the T1-diabetic patient population.

Bisphosphonates are known to suppress bone resorption markers [35, 55-57] and induce osteoclast apoptosis *in vitro* and *in vivo* [41, 42, 54]. Our finding is consistent with a previous study that reported that alendronate decreased serum osteocalcin and urinary NTX, while preventing glucocorticoid-induced bone loss in both the lumbar spine and femur [54, 58]. Interestingly, diabetes alone resulted in elevated bone resorption in T1-diabetic mice. The role of bone resorption in diabetic bone loss is variable. Some studies have indicated elevated osteoclast number at 16 days post-fracture in diabetic rats.

Additionally, increasing the dose of streptozotocin (drug used to induce diabetes in rodents) from 40 mg/kg to 65 mg/kg significantly elevated bone resorption markers [22]. The majority of clinical studies, however, have indicated decreased or no changes in several markers of bone resorption [60-62]. It is possible that our 50 mg/kg was a high enough dose to induce the changes in bone resorption, but still does not solely account for the bone loss, as the decrease in bone formation must be included.

Elevated marrow adiposity is often (but not always [23, 49]) inversely related to diabetes-induced bone loss [21, 24]. Here we show that alendronate treatment decreased adipocyte number, but did not affect diabetes-induced marrow adiposity. Consistent with our data, a recent clinical study indicated that bisphosphonate therapy suppressed marrow adipocyte number by as much as 20% in post-menopausal women [63]. Additionally, a clinical study that examined over 2400 postmenopausal women treated with risedronate (another bisphosphoante) demonstrated significant decreases in adiposity while increasing BMD levels and reducing the incidence of vertebral and non-vertebral fractures [64]. The lack of a decrease observed in aP2 expression in the control mice treated with alendronate could be due to other cells present in the bone marrow microenvironment that expression aP2. One study reported that hematopoietic cells, specifically monocytes, express both early and late adipocyte markers, PPARγ and aP2, respectively. Elevated levels of monocytes in the bone marrow could accompany the previously reported increase in inflammation in T1-diabetic bone [19]. This could account for the similar levels in aP2 expression between the non-treated compared to the bisphosphonate-treated control mice [65].

It is important also to note that studies investigating diabetic patients taking bisphosphonate therapy can have increased (albeit rare) incidence of osteonecrosis of the jaw at high doses commonly used to treat cancer patients compared to non-diabetic patients [66]. Additionally, a recent discovery examines the possible link between bisphosphonate therapy and increases in rare femur fractures [67, 68]. Nonetheless, to date the benefits of bisphosphonate therapy in combating osteoporosis still outweigh the potential rare complications in the majority of patients. New and improving bisphosphonate therapy remains an important area of research.

5.6. ACKNOWLEDGEMENTS

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CHAPTER 6: ASPIRIN TREATMENT: GOOD OR BAD FOR T1-DIABETIC BONE?

6.1. ABSTRACT

Diabetes is strongly associated with increased fracture risk. During T1-diabetes onset, levels of blood glucose and pro-inflammatory cytokines (including TNFα) are increased. At the same time, levels of osteoblast markers are rapidly decreased and stay decreased 40 days later at which point bone loss is clearly evident. Administration of TNF-neutralizing antibodies to osteoblasts prevented diabetic marrow-induced osteoblast death, indicating a role for pro-inflammatory cytokines in mediating osteoblast apoptosis. Here we investigate a commonly used non-steroidal anti-inflammatory drug, aspirin, to prevent bone loss. To test this, we induced diabetes in 16-week-old male C57BL/6 mice and administered aspirin in the drinking water. Our results demonstrate that aspirin therapy decreases diabetic mouse blood glucose levels, but did not affect bone density in either control or diabetic mice (diabetic mice displayed significantly less bone density regardless of treatment),. Bone formation markers increased in control but not diabetic mice, but did not increase bone volume in either control or diabetic mice. Aspirin treatment did not affect markers of bone resorption or prevent the previously reported diabetes-induced marrow adiposity. Taken together, our results suggest that low dose aspirin therapy does not negatively impact control mice, but could amplify bone loss in T1-diabetic mice.

6.2. INTRODUCTION

Type I (T1) diabetes is a metabolic disorder that affects nearly 1 million Americans. Intensive treatments for diabetic patients to maintain euglycemia are critical but remain difficult. Prolonged exposure to hyperglycemia results in increased incidence of secondary complications such as retinopathy, neuropathy, neuropathy, heart disease and osteoporosis [1-6]. Osteoporosis increases fracture risk. Both male and female T1-diabetic adults and adolescents are vulnerable to decreased bone density and increased fracture risk [7-18]. Animal models of T1-diabetes display comparable bone loss to patients thus allowing for extensive investigation of bone pathologies and mechanisms contributing to diabetes-induced bone loss [6, 19, 20]. To date, bone loss in T1-diabetic mice appears to occur at a number of skeletal sites and is independent of gender [6, 19, 20], similar to what is seen in human patients. While the exact mechanisms accounting for T1-diabetes-induced bone loss remain unknown, recent studies demonstrate a role for bone inflammation [6, 19-22].

T1-diabetes is strongly associated with inflammation. Hyperglycemia contributes to increased systemic inflammation, as well as local (bone) inflammation [23]. Recent reports demonstrate an elevation of several pro-inflammatory cytokines, including serum and bone TNFα, IFNγ, and IL-1, during diabetes onset [23, 24]. At the same time osteoblast death is increased and osteocalcin expression is reduced [22, 23]. IFNγ, TNFα, and IL-1 are known mediators of osteoblast death [25-33]. Furthermore, treatment with TNFα neutralizing antibodies reduces osteoblast death caused by diabetic marrow cells [22]. Correspondingly, two clinically used TNFα inhibitors, entanercept or pegsunercept, suppress diabetes-enhanced death

of fibroblasts, bone-lining cells, and osteoblasts arising from periodontal disease *in vivo* [34-36]. Thus, decreasing bone inflammation could prevent diabetes-induced bone loss.

A commonly used anti-inflammatory drug, aspirin, is known to reduce diabetes related inflammation [37, 38] and decrease blood glucose levels in T1-diabetic rats [39]. Aspirin binds and inhibits cyclooxygenase (COX) enzyme and decreases prostaglandin (PG) production [40]. COX exists as two isoforms: COX-1 and COX-2. COX-1 is constitutively active and highly expressed in various tissues throughout the body and functions to maintain normal prostaglandin levels. COX-2 is an inducible enzyme correlated with inflammation and more highly expressed in osteoblasts compared to COX-1 [41, 42]. Some studies (although controversial) have advocated that aspirin treatment is advantageous to bone health by improving bone mineral density in trabecular and cortical bone parameters in aged populations [43, 44]. Similar results indicate that ovariectomized mice treated with aspirin had higher bone density than non-aspirin treated mice. Furthermore, aspirin has been reported to decrease bone marrow stromal cell apoptosis [45].

Based on the above studies, we hypothesized that bone inflammation, illustrated by increased pro-inflammatory cytokine levels, contributes to the diabetic bone pathology. Here, we demonstrate that regular, low dose aspirin treatment decreases blood glucose levels in diabetic mice, but does not prevent diabetes-induced bone loss. Additionally, aspirin treatment increased diabetic marrow adiposity and bone loss beyond the normal diabetic phenotype.

6.3. METHODS

6.3.1. Diabetic Mouse Models

Diabetes was induced in adult (15-16 week old) C57BL/6 male mice (Harlan Laboratories, Indianapolis, Indiana) by 5 daily intraperitoneal (IP) injections of streptozotocin (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Controls were given citrate buffer alone. Aspirin was delivered in the water of control and diabetic mice on 1-day post first injection (dpi) at a concentration of 200μg/kg (comparable to a human dose of 70mg/kg) for the entire experiment (40 dpi). The water was changed every third day to maintain dosage. Mice were maintained on a 12-hour light, 12-hour dark cycle at 23°C, and given standard lab chow and water *ad libitum* (if not treated with aspirin). Body weight and food intake were monitored during diabetes induction and throughout the experiment. Diabetes was confirmed 12 days after initial STZ injection using an Accu-Check compact glucometer (Roche Diagnostics Corporation, Indianapolis, IN) with a drop of blood from the saphenous vein. Total body, tibialis anterior, and subcutaneous femoral fat pad mass were recorded. Animal procedures have been completed with the approval of Michigan State University Institutional Animal Care and Use Committee.

6.3.2. RNA Analysis

Immediately after euthanasia, one tibia and femur were cleared of soft tissue and snap frozen in liquid nitrogen and stored at -80°C. Frozen tibias were crushed under liquid nitrogen conditions, homogenized, and placed in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA integrity was determined through formaldehyde-agarose gel electrophoresis. cDNA was synthesized through a reverse transcriptase reaction utilizing Superscript II Reverse Transcriptase Kit and oligo dT (12-18) primers (Invitrogen, Carlsbad,

CA), amplified by quantitative real time PCR with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and gene-specific primers. Forward and reverse primers used as previously stated: aP2, HPRT, OPG, and RANKL [21, 22, 46]. Expression of HPRT does not alter in diabetes and therefore used as a housekeeping gene. Real Time PCR was carried out for 40 cycles, each cycle consisting of 95°C for 15 seconds, 60°C for 30 seconds (except osteocalcin which has an annealing temperature of 65°C) and 72°C for 30 seconds. RNA-free samples were used as a negative control and did not produce amplicons. PCR products were separated on 1.5% agarose gel electrophoresis and sequenced to verify the desired gene is being amplified.

6.3.3. Micro-computed Tomography (μCT) analysis

Femurs and vertebrae were fixed in 70% EtOH and scanned using the GE Explore μCT system at a voxel resolution of 20um from 720 views with a beam strength of 80kvp and 450uA. Scans included bones from each condition and a phantom bone to standardize the grayscale values and maintain consistency between runs. Using the systems auto-threshold (800) and an isosurface analysis, trabecular bone densities: bone volume fraction (BV/TV), bone mineral density (BMD) and content (BMC), and trabecular thickness (Tb. th), number (Tb. n) and spacing (Tb. sp) are measured in the trabecular region defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis, and excluding the outer cortical shell. Cortical bone measurements are determined with a 2-mm^3 region of interest (ROI) in the mid-diaphysis. Cortical BMC, BMD, moment of inertia, cortical thickness, perimeter and area and trabecular parameters were computed by the GE Microview Software for visualization and analysis of volumetric image data.

6.3.4. Bone Histology and Histomorphometry:

Bones are fixed in 10% buffered formalin and transferred to 70% EtOH after 24 hours where they underwent dehydration, clearing and infiltration of fixed femurs on a routine overnight processing schedule. Samples are then paraffin embedded on a Sakura Tissue Tek II embedding center and sectioned at 5μm on a Reichert Jung 2030 rotary microtome. Osteoclasts were identified, by staining for tartrate resistant acid phosphatase (TRAP) (Sigma). Osteoclasts and adipocytes were identified in the trabecular region of the femur are measured in the trabecular region defined at 0.17mm under the growth plate of the femur extending 2mm toward to diaphysis. Osteoclast surface was measured in three trabecular regions for each mouse and expressed as a percent of total trabecular surface. Visible adipocytes, greater than 30μm, were counted in the same trabecular region.

Mineral apposition rate: Mice were injected intraperitoneally with 200μl of 10mg/ml calcein (Sigma, St. Louis, MO, USA) dissolved in saline 7 and 2 days prior to harvest. L3-L4 vertebrae were fixed in formalin at time of harvest then transferred to 70% ethanol 24 hours later. Vertebrae were then embedded and sectioned at 5μm on a on a Reichert Jung 2030 rotary microtome. Sections were photographed (5 images per bone) under UV light and the distance between the calcein lines were measured.

6.3.5. Serum Measurements

Blood was collected at the time of harvest, allowed to clot at room temperature for 5 minutes, then centrifuged at 4,000 rpm for 10 minutes. Serum was removed and stored at -80°C. Serum went through no more than two freeze/thaw cycles. Serum TRAP5b and Osteocalcin were measured using a Mouse TRAP and OC assay kits (SB-TR103, Immunodiagnostic Systems

Inc., Fountain Hills, AZ, USA and BT-470, Biomedical Technologies Inc., Stoughton, MA, USA respectively) according to the manufacturer's protocol.

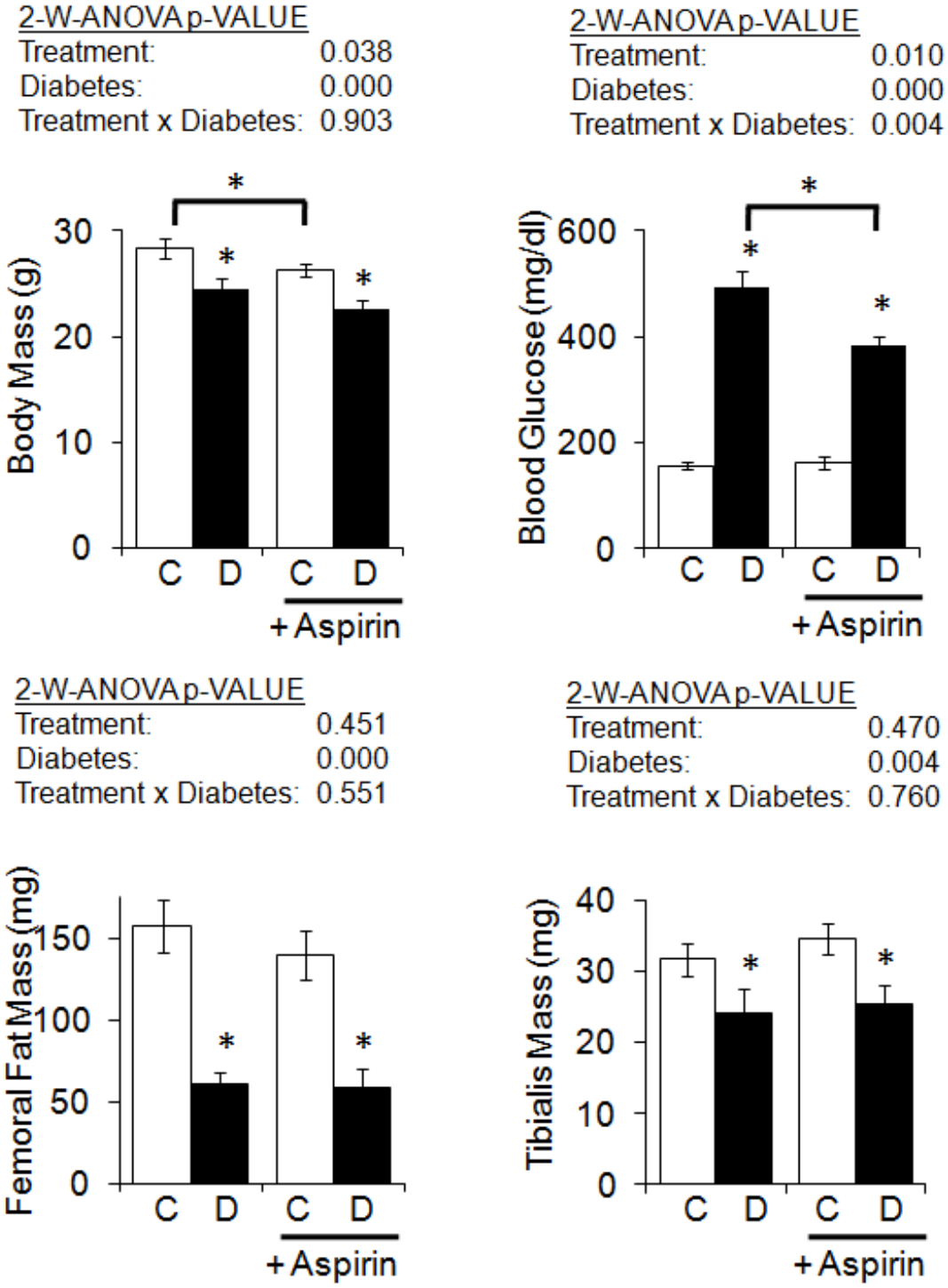
6.3.6. Statistical Analysis

All data is presented as mean ± standard error. Statistical significance (p-value < 0.05) of main effects (aspirin treatment or diabetes) as well as aspirin treatment x diabetes interaction (which determines if diabetes alters the treatment effect or vise versa) was determined using factorial analysis of variance (ANOVA) with SPSS statistical software (Chicago, IL). Student's t-test was used where needed.

6.4. RESULTS

Previous reports indicate that type 1 diabetes causes bone inflammation. To test if a general anti-inflammatory therapy could be used to treat T1-diabetic bone loss, we induced diabetes in aspirin and non-aspirin treated mice. After 40 days, mice were examined. Consistent with previous reports, blood glucose levels were decreased in treated compared to non-treated diabetic mice (383mg/dl and 494mg/dl, respectively). Analysis by 2-way ANOVA indicates that aspirin treatment, diabetes and treatment X diabetes all significantly affected blood glucose levels ($p<0.010$, $p<0.000$, and $p<0.004$, respectively). In contrast, aspirin treatment did not significantly modify any other general physiologic responses to T1-diabetes. Specifically, diabetes induction with or without aspirin treatment relative to corresponding controls decreased total body mass (14% and 14%, respectively), tibialis anterior mass (33% and 27%, respectively) and femoral fat pad mass (61% and 58%, respectively) (figure 33). Interestingly, aspirin therapy alone decreased the body weight of control mice (by 7%; $p<0.038$).

Figure 33. **Aspirin decreases blood glucose levels in diabetic mice compared to non-treated diabetic mice, but did not prevent other diabetes-induced physiological changes.** Body mass, blood glucose, peripheral fat mass and muscle (tibialis) mass were collected at time of harvest for control (white bars) and diabetic (black bars) mice treated with aspirin or without aspirin. Bars represent the average value ± SE (n=7 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of aspirin treatment, diabetes and treatment x diabetes on body parameters. * indicates $p<0.05$.



To determine if aspirin treatment prevented T1-diabetes-induced bone loss, we imaged femurs by micro-computed tomography. Representative 3-dimensional isosurface images are shown in figure 34. Consistent with previous studies, diabetic mice lost an average of 30% bone volume fraction (BV/TV) and displayed decreased bone mineral content, bone mineral density, and trabecular number. While aspirin treatment did not alter control mouse bone parameters, it did increase the severity of the diabetic bone phenotype, with mice losing nearly 53% BV/TV compared to aspirin treated control mice. Cortical bone parameters decreased in both non-aspirin and aspirin treated diabetic mice compared to respective controls by 8% and 13%, respectively, resulting in decreased cortical area and decreased percent cortical area per total area (figure 35, TABLE VIII).

Figure 34. **Aspirin treatment does not prevent diabetes-induced trabecular bone loss.** Representative μCT isosurface images of trabecular bone volume fraction (BV/TV) in distal femurs were obtained from control and diabetic mice with or without aspirin treatment. Graphical representation of BV/TV is displayed from control (white bars) and diabetic (black bars) in the presence or absence of aspirin. Bars represent the average value ± SE (n=7 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of treatment, diabetes and treatment x diabetes on bone volume fraction. * indicates $p<0.05$.

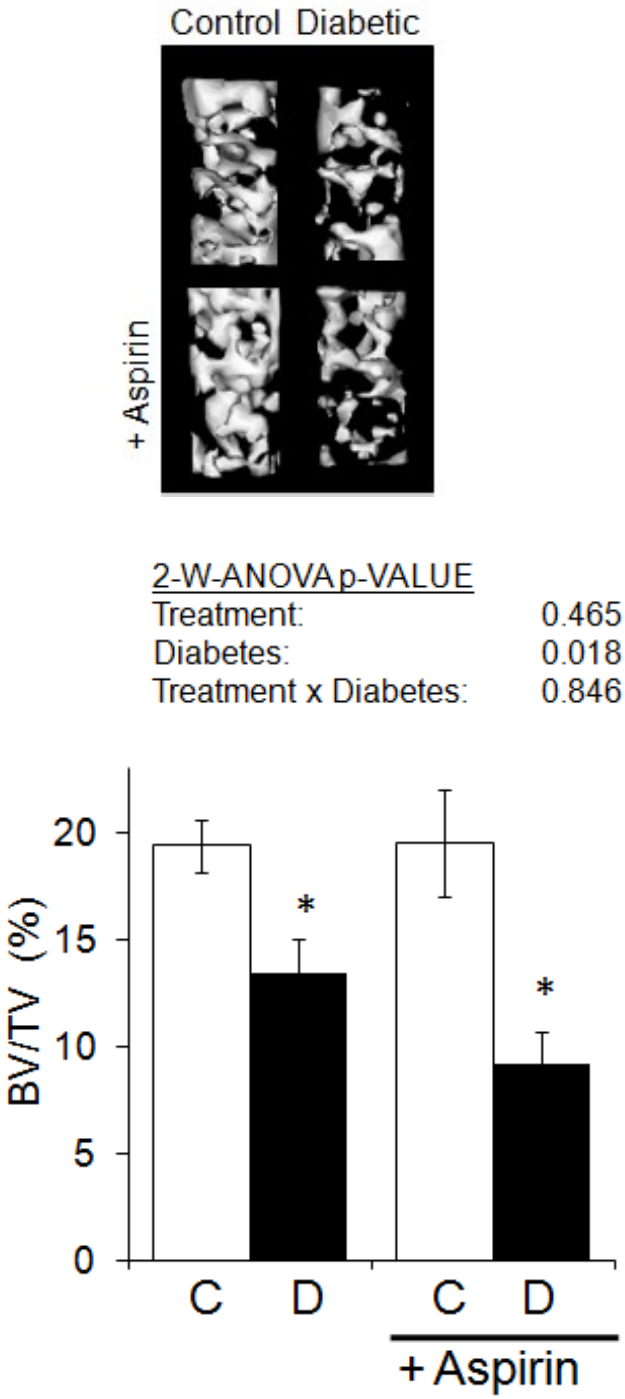
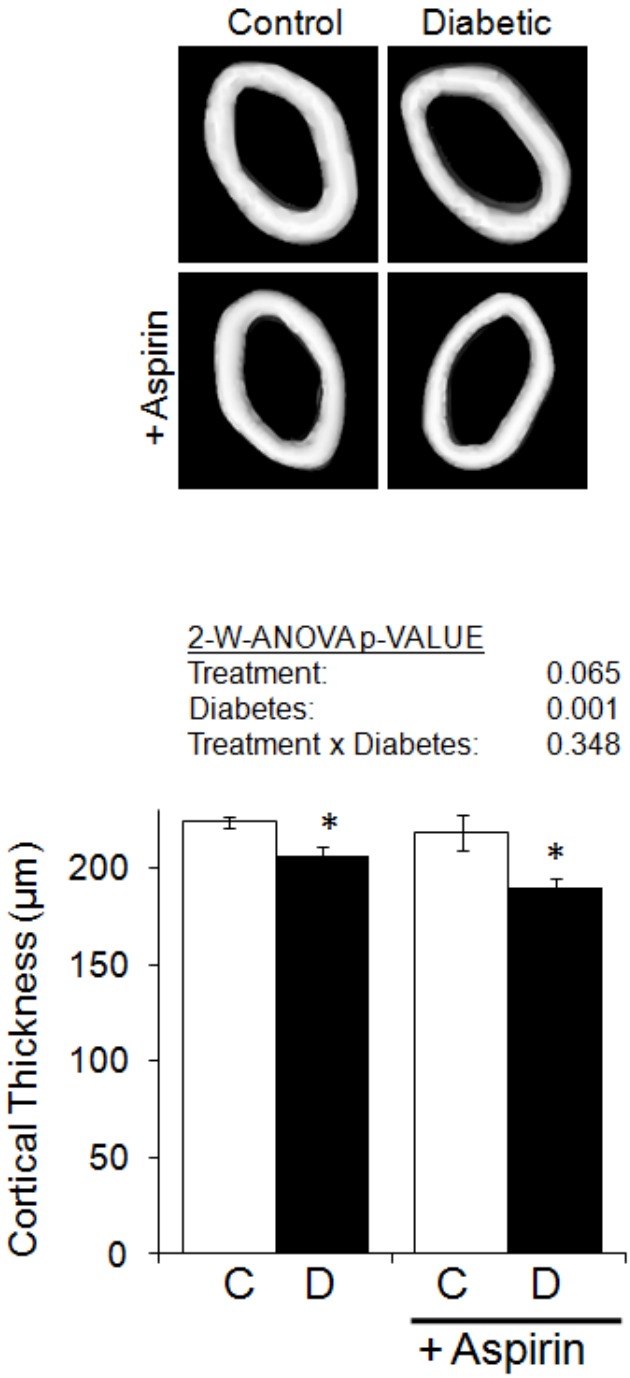


Table VIII. µCT femur parameters in control and diabetic mice.

			+Aspirin	
Trabecular	Control (n=7)	Diabetic (n=7)	Control (n=7)	Diabetic (n=7)
BMC (mg)	0.48 ± 0.02	0.34 ± 0.02*	0.43 ± 0.04	0.33 ± 0.03*
BMD (mg/cc)	154 ± 5	113 ± 9*	143 ± 13	108 ± 10*
BV/TV (%)	19.4 ± 1.2	13.4 ± 1.6*	19.1 ± 3.2	9.2 ± 1.5*
Tb. Th (µm)	40.0 ± 1.2	36.4 ± 2.0	40.0 ± 3.9	33.2 ± 2.8
Tb. N (1/mm)	4.79 ± 0.26	3.56 ± 0.27*	4.56 ± 0.42	3.27 ± 0.53*
Tb. Sp. (mm)	0.17 ± 0.01	0.26 ± 0.03*	0.19 ± 0.02	0.32 ± 0.05*
Cortical				
Ct. Th (mm)	224.2 ± 2.8	206.4 ± 4.7*	218.4 ± 9.0	189.5 ± 5.0*
I_{max} (mm^4)	0.2 ± 0.03	0.16 ± 0.01	0.18 ± 0.01	0.12 ± 0.01*
Ec. Pm (mm)	3.68 ± 0.07	3.79 ± 0.07	3.76 ± 0.06	3.97 ± 0.11
Ps. Pm (mm)	5.09 ± 0.08	5.01 ± 0.07	5.07 ± 0.06	5.07 ± 0.09
Ma. Ar (mm^2)	0.93 ± 0.03	0.96 ± 0.04	0.96 ± 0.03	1.05 ± 0.04
Ct. Ar (mm^2)	0.92 ± 0.02	0.78 ± 0.03*	0.88 ± 0.05	0.70 ± 0.03*
Tt.Ar (mm^2)	1.85 ± 0.06	1.76 ± 0.05	1.83 ± 0.05	1.75 ± 0.05
Ct. Ar/ Tt. Ar (%)	49.9 ± 0.4	44.6 ± 1.6*	47.8 ± 1.7	40.1 ± 1.5*

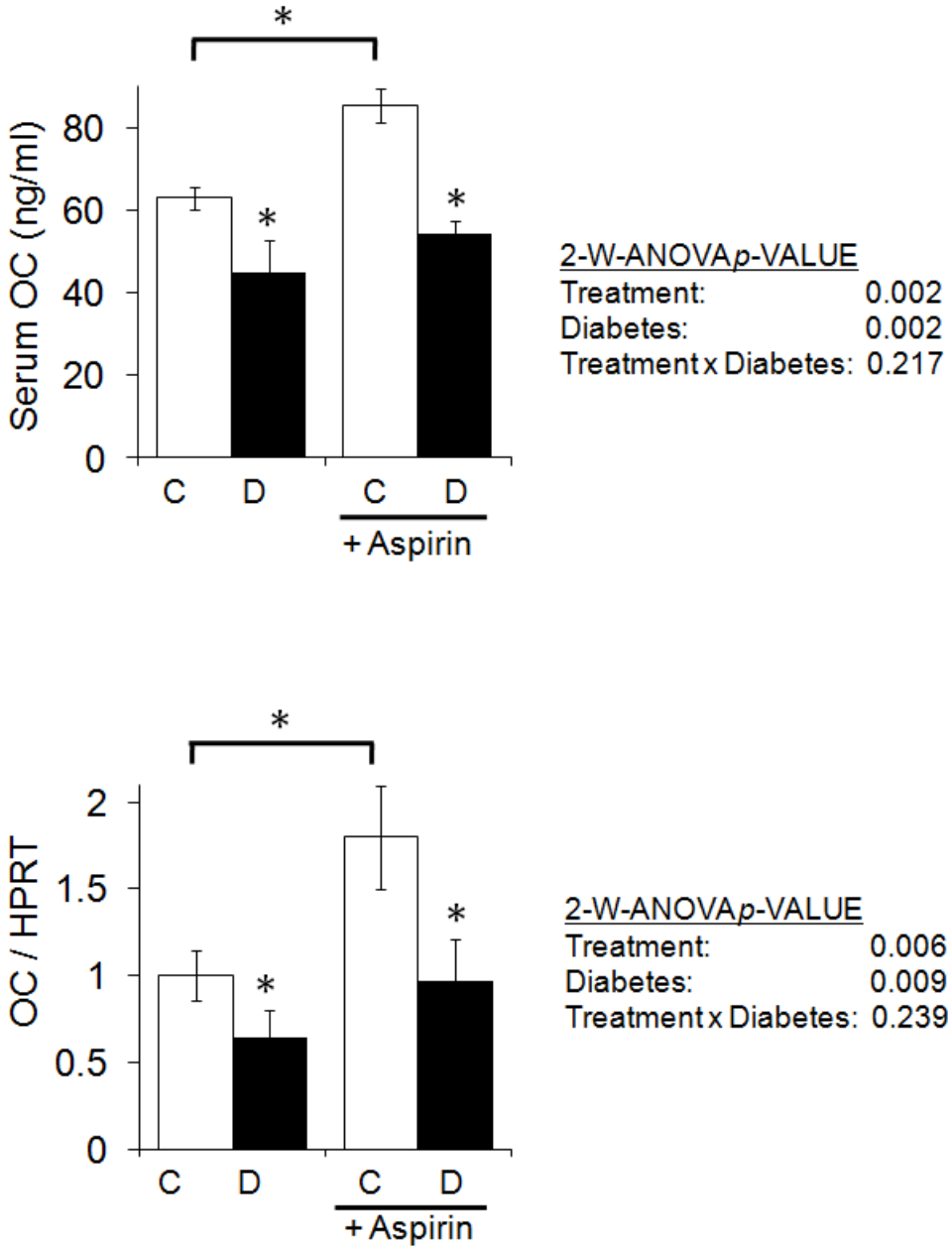
Significance: * $p<0.05$ compared to non-treated control; ^ $p<0.05$ compared to non-treated diabetic. *Abbreviations*: BMC, bone mineral content, BMD, bone mineral density, BV/TV, bone volume fraction, Tb. Th, trabecular thickness, Tb. Sp, trabecular spacing, Tb. N, trabecular number, I_{max}, maximum moment of inertia, Ec. Pm, Endocortical perimeter, Ps. Pm, Periosteal Perimeter, outer perimeter, Ma. Ar, Marrow area, Ct. Ar, Cortical area, Tt. Area, total area.

Figure 35. **Cortical bone thickness is decreased in diabetic mice.** Representative μCT isosurface images of cortical bone thickness from the mid-diaphysis of femurs were obtained from control and diabetic mice with or without aspirin treatment. Graphical representation of coritical bone thickness is displayed from control (white bars) and diabetic (black bars) in the presence or absence of aspirin. Bars represent the average value ± SE (n=7 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of treatment, diabetes and treatment x diabetes on cortical thickness. * indicates $p<0.05$.



T1-diabetes is a known suppressor of bone formation; therefore, we investigated the role of aspirin therapy on several markers of bone formation. Consistent with previous studies, diabetes decreased serum osteocalcin levels (-29%) and tibia osteocalcin mRNA levels (-40%) compared to control mice. Interestingly, aspirin treatment alone elevated serum and tibia osteocalcin levels (35% and 60%, respectively) in control mice. Aspirin therapy did not, however, prevent diabetic suppression of bone formation markers (figure 36). Analysis by 2-way ANOVA indicated that aspirin treatment as well as diabetes induction affected serum and tibia osteocalcin levels ($p<0.002$ and $p<0.002$, respectively, and $p<0.009$ and $p<0.006$, respectively). However, there was no interaction between treatment and disease.

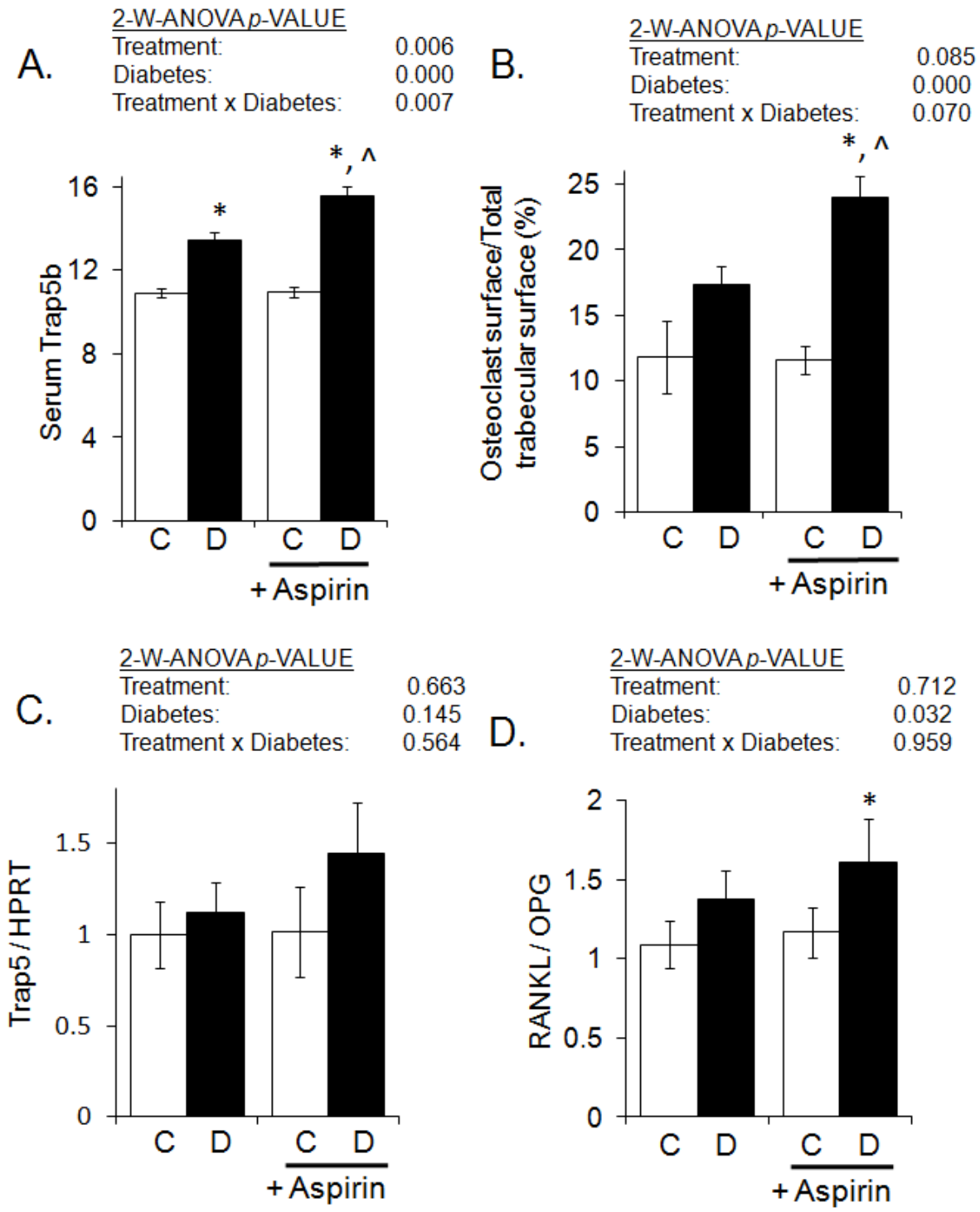
Figure 36. **Aspirin treatment increases bone formation markers**. Serum and tibia mRNA osteocalcin expression was analyzed in control and diabetic mice and treated with or without aspirin water. Bars represent the average value ± SE (n=7 per group) in control (white bars) and diabetic (black bars) in the presence or absence of aspirin. RNA levels are calculated relative to the housekeeping gene HPRT. Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of aspirin, diabetes and aspirin x diabetes on bone formation markers. * indicates $p<0.05$.



We also examined markers of bone resorption: serum TRAP5b, osteoclast surface per total trabecular surface, and Trap5 and RANKL/OPG mRNA ratio. Serum TRAP5b and the percent osteoclast surface/total trabecular surface were elevated in diabetic mice by 30% and 47%, respectively in non-aspirin treated mice; the former reached statistical significance. In aspirin treated mice, both serum Trap5b and osteoclast surface were significantly increased by 42% and 107%, respectively (figure 37). Two-way ANOVA analysis indicated that aspirin therapy, diabetes treatment, and aspirin X diabetes all account for the increase in serum TRAP5b levels ($p<0.006$, $p<0.000$, and $p<0.007$, respectively). Enhanced levels of resorption in aspirin treated diabetic mice could lead to the trend toward greater bone loss in this condition (figure 34). Furthermore, we measured the mRNA ratio RANKL/OPG expression and observed elevated ratios in aspirin treated diabetic mice compared to respective control, while non-treated diabetic mice displayed trended to have an elevated RANK/OPG ratio (figure 37). Two-way ANOVA analysis determined that diabetes alone is responsible for these changes ($p<0.032$).

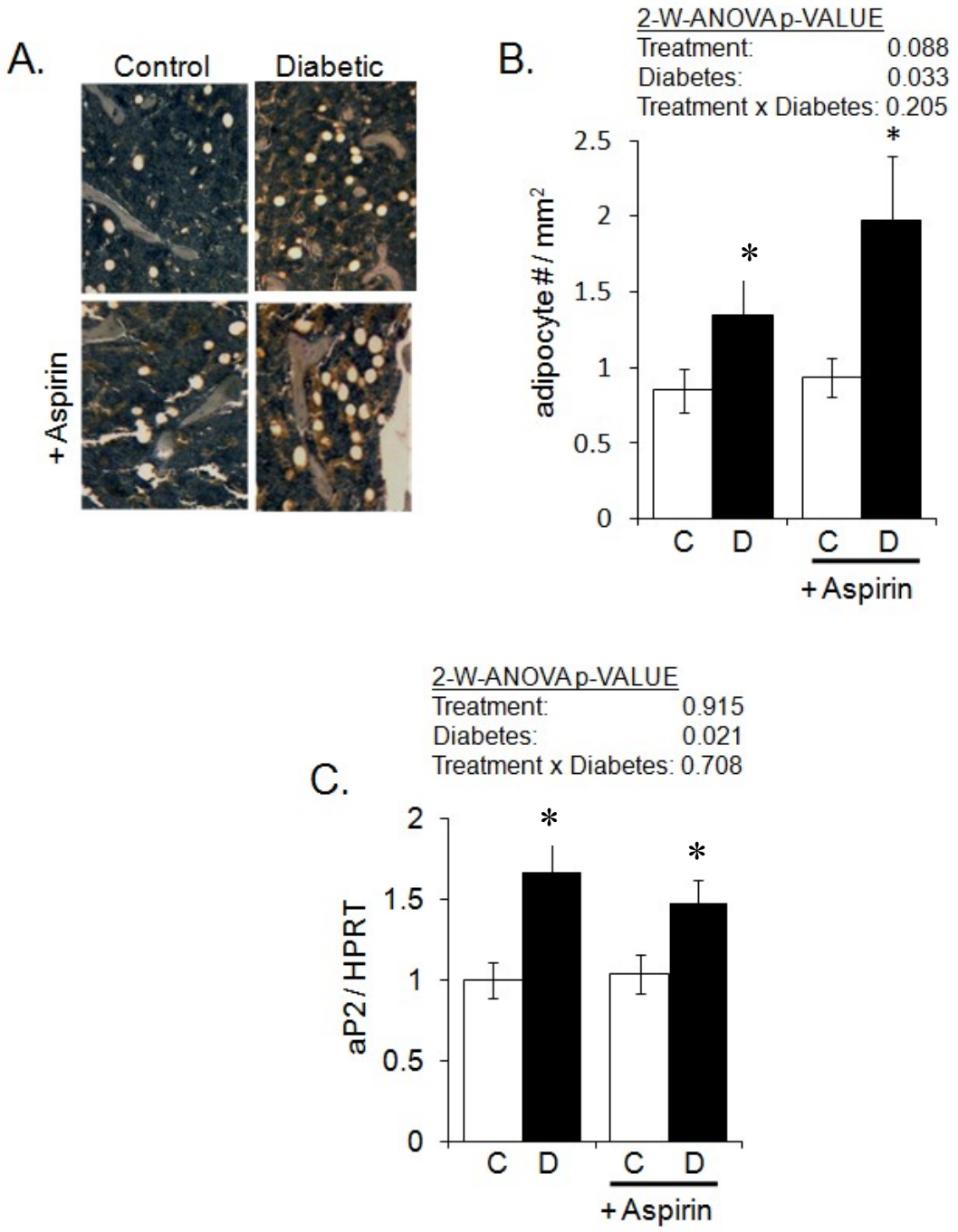
Figure 37. **Increased bone resorption results from diabetes induction.** Serum TRAP5 expression, percent osteoclast surface per total trabecular surface, and RANKL/OPG ratio and Trap5 mRNA were analyzed in control (white bars) and diabetic (black bars) mice in the presence or absence of aspirin treatment. Bars represent the average value ± SE (n=7 per group). RNA levels were calculated relative to the housekeeping gene HPRT. Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of aspirin treatment, diabetes and aspirin treatment x diabetes on bone resorption and remodeling markers. * indicates $p<0.05$ compared to respective control. ^ indicates 0.05 compared to non-treated diabetic mice.

Figure 37 (cont'd)



Previous reports indicate a significant increase in bone marrow adiposity in response to diabetes; therefore, we measured tibia aP2 (marker for mature adipocytes) mRNA expression and total adipocyte number per total marrow area in response to aspirin therapy. As expected, diabetes induction resulted in a 70% increase in aP2 expression and a 92% increase in adipocyte number/marrow area compared to control mice (figure 38). Aspirin therapy did not prevent the diabetes-induced marrow adiposity. Specifically, aP2 expression increased 50% in aspirin treated diabetic mice compared to controls while adipocyte number/marrow area increased 171% (figure 38). Statistical analysis by 2-way ANOVA indicated that diabetes affected adipocyte number and aP2 expression ($p<0.001$ and $p<0.023$, respectively), while aspirin treatment alone did not alter marrow adiposity (figure 38).

Figure 38. **Diabetes increased marrow adiposity in aspirin treated and non-treated mice.** Representative adipocyte images were obtained from femurs of control and diabetic mice given aspirin water or regular water. Graphical representation of adipocyte numbers in control (white bars) and diabetic (black bars) mice with or without aspirin treatment. Expression late adipocyte mRNA marker, aP2 was also examined and calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE (n=7 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of aspirin, diabetes and aspirin x diabetes on adipocyte parameters. * indicates $p<0.05$ compared to respective control.



6.5. DISCUSSION

T1-diabetes and its associated hyperglycemia contribute to increased systemic inflammation as well as local bone inflammation [23]. For example, studies demonstrate elevated levels of pro-inflammatory cytokines, such as TNFα, in diabetic serum as well as in the bone during diabetes onset [22-24]. Inflammation in the bone microenvironment is known to contribute to bone loss. Therefore, decreasing bone marrow inflammation could serve as a potential therapeutic strategy for inhibiting bone loss. Here, we investigated the influence of aspirin therapy on control and diabetic mouse bone parameters. Despite aspirin treatment reducing diabetic blood glucose levels, it did not prevent the diabetes-induced bone pathology.

Research investigating aspirin's effects on serum blood glucose levels dates back to 1877 when researchers found that high doses of sodium salicylate (aspirin) reduced glucosuria in diabetic patients, however conflicting results have been reported [38, 47-53]. More recent clinical studies report that T2-diabetic patients showed a 40 mg/dl decrease in fasting plasma glucose levels and a 21% reduction in blood glucose levels during a mixed-meal tolerance test after only 2 weeks of aspirin treatment [38]. Additionally, diabetic rodents treated with aspirin for a short term (7 days) or long-term (45 days to 5 months) regimes display significantly decreased blood glucose and HbA1c levels, similar to our findings [39, 49, 50]. In contrast, a small clinical study showed that neither aspirin treatment nor ibuprofen reduced fasting blood glucose levels in T2-diabetic patients, however this study had a small number of subjects compared to other studies, making their results difficult to draw conclusions from [54]. How does aspirin treatment reduce hyperglycemia? It is hypothesized that aspirin contributes to

decreased basal rates of hepatic glucose production, enhanced tissue insulin sensitivity, decreased insulin clearance, and decreases oxidative stress in diabetic patients [38] [50].

Aspirin treatment and its effects on bone remain controversial. Some studies indicate significant beneficial effects on both trabecular and cortical bone density parameters. One clinical report indicates that aspirin users had elevated whole body BMD (4.2%) and total hip (4.6%) BMD compared to non-users [55][43]. Additionally, aspirin treatment improved bone density and remodeling in OVX mice as well as in aged human population by inhibiting bone resorption [43-45]. Similar to the increased osteogenesis seen in aspirin treated OVX mice, our studies revealed that aspirin increased serum OC levels in control mice, although BV/TV did not change. The former study did not include aspirin treated controls, so we cannot make a direct comparison for this condition. In our study we also observed a decrease in body weight in aspirin treated controls, which could contribute to the lack of a notable change in BV/TV.

NSAIDs can also suppress bone remodeling, especially at high doses. This is thought to result from a reduction in prostaglandins specifically PGE_2 [55, 56]. *In vivo* rodent models demonstrate that injections of PGE_2 increase bone formation [58] and induce bone remodeling [56]. NSAIDs could also reduce bone formation through general suppression of basal inflammation. NSAID suppression of inflammation has been proposed to be detrimental to certain stages of fracture repair, since some cytokines may promote osteogenesis and angiogenesis which are required for healing [57]. On the other hand, too much inflammation, can suppress fracture healing as indicated in a study in T1-diabetic mice where treatment with a TNFα improved fracture healing [36].

Although we observed a decrease in serum glucose levels and elevated osteocalcin the question remains why doesn't aspirin treatment prevent T1-diabetes-induced bone loss.

Previously, we have demonstrated elevated pro-inflammatory cytokines, IL-1, TNFα, and IL-6 mRNA expression in bone [23]. These same cytokines are known to mediate osteoblast PGE_2 production and promote bone resorption through enhanced expression of COX-2 [59]. Osteoblasts treated with neutralizing antibodies against TNFα protected them from diabetic marrow-induced osteoblast death, indicating a strong role for these pro-inflammatory cytokines in diabetic bone. It is possible that aspirin is too broad of an anti-inflammatory to prevent bone specific inflammation. Therefore, more specific inhibitors may be required to prevent the diabetic bone pathology.

In summary, low dose aspirin treatment decreased blood glucose levels but did not prevent T1-diabetes-induced bone loss or marrow adiposity. It is possible that a more specific inhibitor of pro-inflammatory cytokines, such as TNF-neutralizing antibodies could provide more profound anti-inflammatory effect and target diabetes-induced inflammation and osteoblast apoptosis. Further research is needed to accurately dissect which pro-inflammatory cytokines are key to mediating diabetes-induced inflammation and bone loss.

6.6. ACKNOWLEDGEMENTS

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