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FOXO1 Deletion Reverses the Effect of Diabetic-Induced Impaired Fracture Healing

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Diabetes impairs fracture healing. Our laboratory previously suggested that a dominant mechanism was the premature loss of cartilage during endochondral bone formation. Based on these results we tested the hypothesis that chondrocytes regulate osteoclast formation in diabetes-impaired fracture healing and that it is controlled by the transcription factor FOXO1.

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Closed fracture of the femur was induced in mice with lineage-specific FOXO1 deletion in chondrocytes Col2α1Cre+/FOXO1L/L and Col2α1Cre-/ FOXO1L/L as control mice that had FOXO1 gene present. Mice were rendered diabetic by multiple streptozotocin injections. The normoglycemic group received vehicle alone. Specimens were collected at 10 days (cartilage formed), 16 days (transition from cartilage to bone formation) and 22 days (primary bone formed). The cartilage area was measured using safranin-O/fast green staining. Osteoclasts were counted as TRAP positive cells. The expression of cathepsin K and RANKL were determined by quantitative immunostaining with specific antibodies compared to control IgG. Micro CT was used to measure the callus volume and the mineralized bone. Statistical analysis was done using a one-way ANOVA test. Results with p

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Our data indicates that FOXO1 drives RANKL expression in chondrocytes and resorption of cartilage. FOXO1 deletion reverses diabetes-enhanced osteoclast formation and prevents the premature loss of cartilage, which results in a better bone healing.

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FOXO1 DELETION REVERSES THE EFFECT OF DIABETIC-INDUCED

IMPAIRED FRACTURE HEALING

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ACKNOWLEDGMENT

Table of Contents

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Closed fracture of the femur was induced in mice with lineage-specific FOXO1 deletion in chondrocytes Col2α1Cre^{+}/FOXO1$^{L/L}$ and Col2α1Cre^{-}/ FOXO1$^{L/L}$ as control mice that had FOXO1 gene present. Mice were rendered diabetic by multiple streptozotocin injections. The normoglycemic group received vehicle alone. Specimens were collected at 10 days (cartilage formed), 16 days (transition from cartilage to bone formation) and 22 days (primary bone formed). The cartilage area was measured using safranin-O/fast green staining. Osteoclasts were counted as TRAP positive cells. The expression of cathepsin K and RANKL were determined by quantitative immunostaining with specific antibodies compared to control IgG. Micro CT was used to measure the callus volume and the mineralized bone. Statistical analysis was done using a one-way ANOVA test. Results with $p < 0.05$ were considered significant.

Results:

All the groups showed similar cartilage areas at day 10. At day16 the diabetic group had 77% less cartilage area than the normal (P <0.05). The accelerated cartilage loss in the diabetic group was completely reversed when FOXO1in chondrocytes was deleted (P<0.05). Diabetic mice had 4.4 times more cathepsin K positive cells in comparison to normal littermates. This change could be accounted for by changes in RANKL expression, which was 2-fold greater in the diabetic mice, compared to the normal mice (P <0.05). However, when FOXO1 in chondrocytes was deleted in the diabetic animals, osteoclasts numbers were reduced to normal levels (P <0.05). Bone volume in the diabetic group was 29% and 34% less than the normoglycemic animals on day 16 and day 22 respectively (P <0.05). However, the effect of diabetes was rescued to normal levels upon FOXO1 deletion in chondrocytes (P <0.05).

Conclusion:

Our data indicates that FOXO1 drives RANKL expression in chondrocytes and resorption of cartilage. FOXO1 deletion reverses diabetes-enhanced osteoclast formation and prevents the premature loss of cartilage, which results in a better bone healing.

LIST OF ABBREVIATIONS

ADAMTS   Disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin-type 1 motifs

AGE   Advanced Glycation End-products

BMD   Bone Mineral Density

BMP   Bone Morphogenetic Protein

ECM   Extracellular matrix

EDTA   Ethylenediaminetetraacetic acid

FOXO1   Forkhead box protein O1

GSEA   Gene Set Enrichment Analysis

ICTP   Type 1 Collagen Cross-linked Carboxy-terminal Telopeptide

IF   Immunofluorescence

IFN-γ   Interferon gamma

IGF   Insulin-like Growth Factor

IHC   Immunohistochemistry

IL-1α   Interlukine-1 alpha

IL-1β   Interlukine-1 beta

IL-6   Interlukine-6

MCSF   Macrophage Colony Stimulating Factor

MMP   Matrix Metalloproteinase

MFI	Mean fluorescence intensity
NO	Nitric Oxide
OPG	Osteoprotegerin
PBS	Phosphate-buffered saline
PDGF	Platelet-derived Growth Factor
PKC	Protein Kinase C
RAGE	Receptor for AGE
RANK	Receptor Activator of NFκB
RANKL	Receptor Activator of NFκB Ligand
ROS	Reactive Oxygen Species
SEM	Standard error of the mean
STZ	Streptozotocin
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor alpha
TRAIL	TNF related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor

Introduction

<u>Normal Fracture Healing:</u>

Long bone fracture healing is a dynamic process that involves complex and coordinated activities of stem cells, inflammatory cells, chondrocytes, osteoblasts, osteoclasts, and other cell types. (L. Gerstenfeld, Cullininane, Barnes, Graves, & Einhorn, 2003). This occurs via two dynamic interactive responses. The first is the intramembranous bone formation, which is characterized by direct bone formation without using cartilage as a template. The periosteum is the primary source of cells involved in this phase. Those cells are predominantly osteoprogenitor and undifferentiated mesenchymal cells (Einhorn, 1998). The second is the endochondral bone formation. Endochondral bone formation is a process that involves recruitment, proliferation, and differentiation of undifferentiated mesenchymal stem cells into cartilage producing chondrocytes (Einhorn, 1998). Cartilage is eventually calcified, resorbed and replaced by bone.
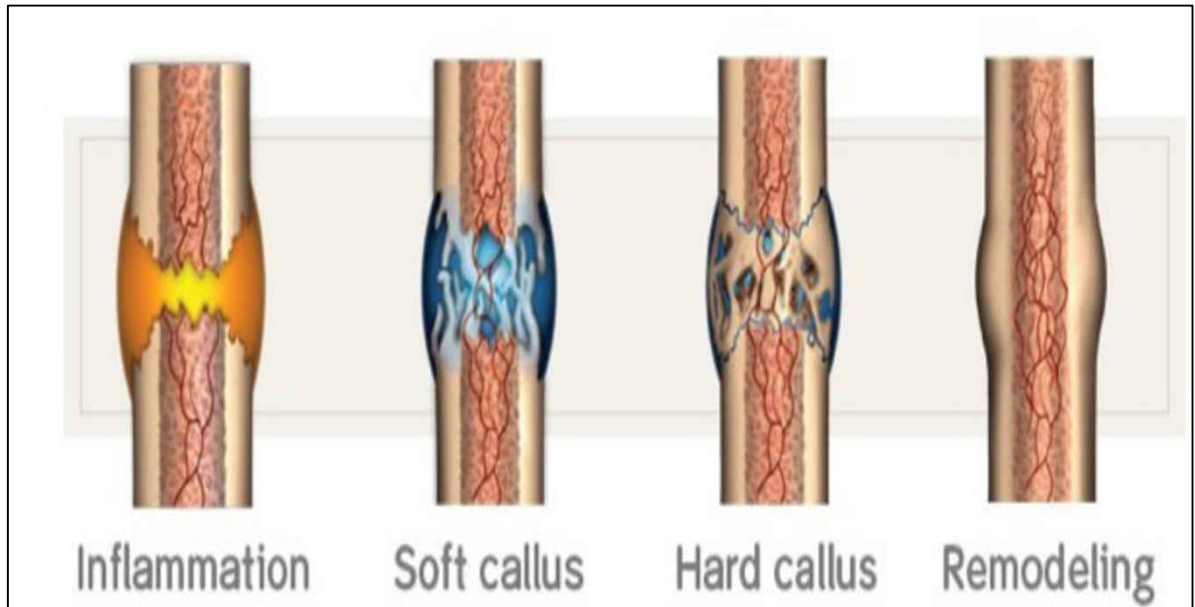
Figure1: Long bone fracture healing stages. The callus primarily consists of hematoma and immune cells at the inflammatory phase, un-calcified cartilaginous soft callus at the second phase, calcified intermixed hard callus, and finally the remodeling phase at which the callus will remodel to restore the original morphology.

The fracture healing process can be divided into three interconnected phases, initial inflammatory phase, regenerative phase, and finally a remodeling phase. Like most other injuries, at the initial phase an inflammatory process will start and a hematoma will form from blood cells extravasating the damaged blood vessels. Those cells will produce cytokines, which will increase the blood capillary permeability (Einhorn, 1998). In addition to the blood cells, this phase involves the infiltration of different inflammatory cells including macrophages. Macrophages release various cytokines and chemokines that help to recruit other inflammatory cells, enhance the angiogenesis, and finally promote matrix formation. (Einhorn, Majeska, Rush,

Levine, & Horowitz, 1995; L. C. Gerstenfeld et al., 2003). Furthermore, at this early stage platelets play a primary role in the initial healing phase. They aggregate and are considered a major source of platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) that are important for both the recruitment and the differentiation of mesenchymal stem cells (Bolander, 1992). Bone-marrow-derived MSCs differentiate to either cartilage forming chondrocytes or bone forming osteoblasts; both of which are critical for endochondral ossification of fractured calluses. MSCs mediate and enhance angiogenesis in addition to their role in reducing inflammation and apoptosis. (Caplan & Dennis, 2006; Cho, Gerstenfeld, & Einhorn, 2002; Shea, Edgar, Einhorn, & Gerstenfeld, 2003) .

The initial stability of the fractured segments is provided by the cartilage. Chondrocytes will populate the fracture area and will deposit soft matrix that constitutes the soft callus. Those chondrocytes express type II collagen in addition to aggrecan which is a significant feature of mature chondrocytes (Sandberg, Aro, Multimaki, Aho, & Vuorio, 1989). Chondrocytes will then release growth factors and cytokines that induce osteogenesis (L. C. Gerstenfeld et al., 2002). Chondrocytes will then differentiate into a hypertrophic phase characterized by expressing type X collagen (Barnes, Kostenuik, Gerstenfeld, & Einhorn, 1999). Cartilage matrix undergoes degradation by collagenases and proteases, predominantly MMP and disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin-

type 1 motifs (ADAMTs). Cartilage degradation and breakdown facilitate the

formation and the invasion of new blood vessels via VEGF and angiopoietin

dependent pathways that facilitate the recruitment of osteoclasts and

osteoblasts (Peng et al., 2005). RANKL also induces the production of

chemokines, suggesting an amplification loop during recruitment of precursors

and differentiation of osteoclasts [12]. Little attention was given to

chondrocytes as a source of RANKL in the literature. Martínez-Calatrava,

Maria J., et al. looked at the expression of RANKL in chronic arthritis rabbit

model. They have found that chondrocytes had very high RANKL expression

in the osteoarthritis group and the expression of RANKL was proportional to

the articular bone loss observed in that group.(Martinez-Calatrava et al., 2012)

The apoptosis phase of the hypertrophic chondrocytes is a critical

phase during fracture repair (F. Y. Lee, Choi, Behrens, DeFouw, & Einhorn,

1998; Li, White, Connolly, & Marsh, 2002). There is a direct relationship

between the vasculature and the rate of apoptosis and resorption of cartilage.

This process is mediated in part by the tumor necrosis factor alpha (TNF-α). It

has been reported that TNF-α enhances the apoptosis of chondrocytes and

upregulates the levels of pro-resorptive cytokines that regulate the remodeling

phase by osteoclasts (L. C. Gerstenfeld et al., 2003).

The main source of the bone forming cells (osteoblasts) that repair the

fractured bone during endochondral ossification remains controversial. There

are two different theories; the first one is that the fully differentiated

hypertrophic chondrocytes undergo programmed apoptosis and the progenitor cells from the blood vessels penetrate the cartilage callus which is undergoing degradation to replace the cartilage with bone. The second hypothesis is that some of the fully differentiated hypertrophic chondrocytes do not undergo apoptosis; however, they transdifferentiate into bone forming osteoblasts. Recent investigations using contemporary genetic lineage-specific tracing methods concluded the both pathways work parallel to each other. However, chondrocyte transdifferentiation into osteoblasts was the predominant pathway during the endochondral process (Bahney et al., 2014; X. Zhou et al., 2014).
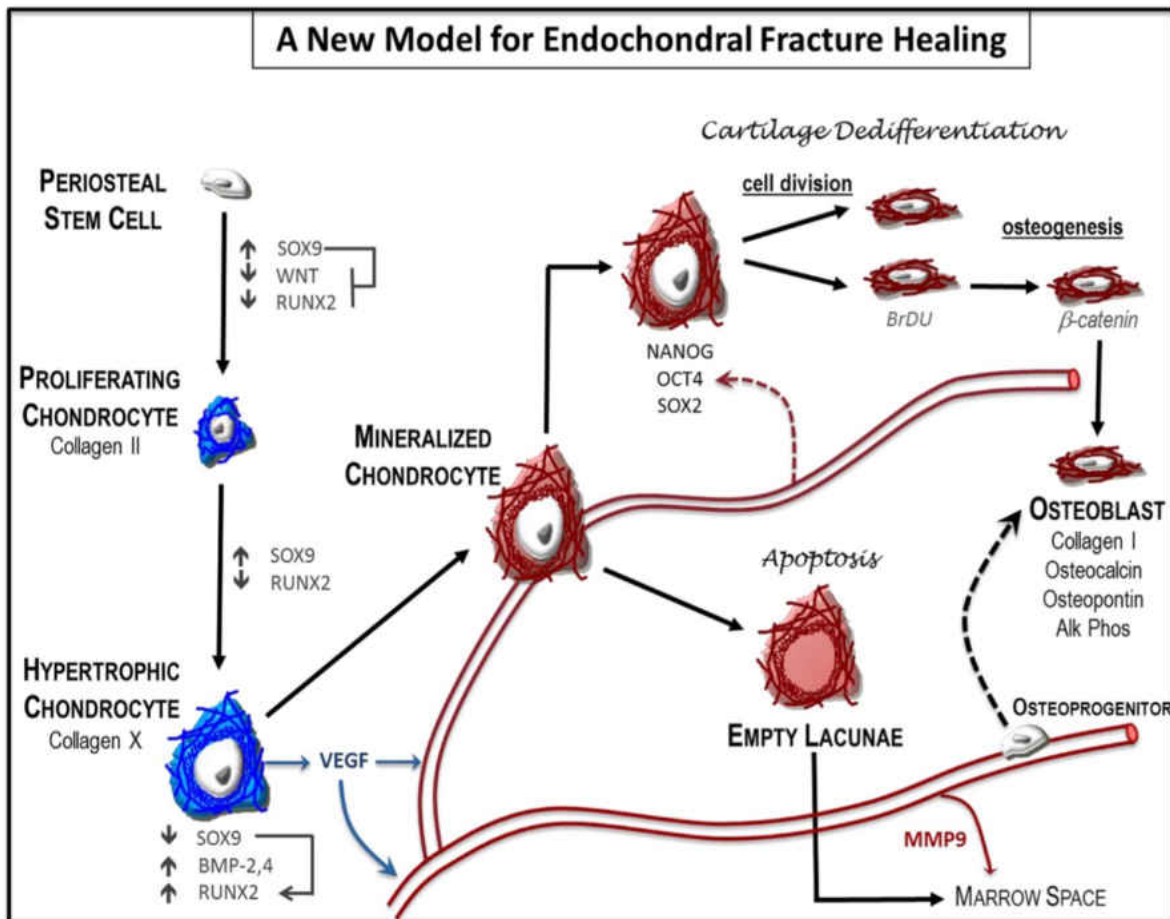
Figure 2: A new endochondral ossification model for fracture healing. Stem cells from the periosteum and the endosteum will differentiate to form bone and cartilage forming cells. The early soft callus will be formed by the osteochondral progenitors differentiating into chondrocytes. Those chondrocytes undergo hypertrophic differentiation and will secrete potent angiogenic factors that result in the vascular invasion at the transitional zone which will facilitate the mineralization of the cartilage. Some hypertrophic chondrocytes will undergo apoptosis to create marrow space within the trabecular bone. Other hypertrophic chondrocytes regain some stem cell-like properties by expressing the pluripotent transcription factors OCT4, SOX2 and NANOG. These large cells re-enter the cell cycle, divide and then transform into osteoblasts. (Picture copied from Bahney et al., 2014)

Osteoclasts are the cells that normally line bones and are responsible for its resorption. Osteoclasts are large multinucleated cells that range between 150-200 µm in diameter. During fracture healing, osteoclasts resorb the cartilage within the healing callus. Receptor activator of NFκB Ligand (RANKL), Macrophage colony stimulating factor (MCSF), and Osteoprotegerin (OPG) are considered the key regulators of osteoclasts activation (Kon et al., 2001). Cathepsin K is a member of peptidase C1 protein family which is expressed predominantly by osteoclasts and it is secreted into the resorptive pit. Cathepsin K is a protease that is capable of catabolizing type I collagen, elastin, and gelatin that enables the osteoclasts to resorb calcified tissue such as bone and cartilage. Once the osteoclast reaches the resorption site, cathepsin K is secreted into the resorptive pit from the ruffled border. It transmigrates by the intercellular vesicles with reactive oxygen species formed by TRAP to breakdown the ECM. A cathepsin K knock out mouse model showed an osteopetrotic phenotype. Osteopetrosis is defined as "stone bone" and it is also known as marble bone disease. It is a genetic disorder that affects the bone making it extremely dense and harder. On the other hand, Osteoporosis is another more common condition in which the bone becomes very brittle and has a very low density. In osteopetrosis, the number of osteoclasts may be reduced, normal, or increased. Most importantly, osteoclast dysfunction mediates the pathogenesis of this disease.(Aker et al., 2012)

While the cartilage is undergoing resorption, osteoblasts secrete bone matrix which undergoes calcification to form the woven bone which represents the bony callus (Dimitriou, Tsiridis, & Giannoudis, 2005). Bone morphogenic proteins are upregulated during this phase and they regulate osteoblasts recruitment, differentiation, and maturation (Cho et al., 2002). At the final stage of the fracture healing, this bony callus will remodel to the primary structure and morphology of the unfractured bone. Another set of cytokines are elevated during the remodeling phase including, but not limited to, TNF-α, IL-1 and IL-6 (Kon et al., 2001).

Diabetes Mellitus:

Diabetes mellitus (DM) is a serious concern in the United States and worldwide as the incidence of this chronic disease has been rapidly increasing in the past 20 years. Today it is estimated that 415 million people worldwide are diagnosed with diabetes (IDF DIABETES ATLAS 6th edition 2015). DM is a disease characterized by high blood glucose level (hyperglycemia) due to insulin deficiency or due to the inability of the body cells to respond to the insulin secretion. There are two major types of diabetes mellitus, type 1(TIDM) and type 2 (T2DM).

Type 1 is characterized by the destruction of Beta cells in the pancreas. The cause of type 1 diabetes is not known, although a number of possible hypotheses have been proposed. These predominant hypotheses are genetic

susceptibility, a diabetogenic trigger, and the exposure to an antigen. Type 1 diabetes usually occurs following autoimmune response initiated by pancreatic inflammation and predominant infiltration of macrophages into the pancreatic islets (O'Reilly et al., 1991). Macrophages will stimulate the cytotoxic T-lymphocytes which will secrete antibodies against beta cells (Jun, Santamaria, Lim, Zhang, & Yoon, 1999). They will also stimulate the production of a number of proinflammatory cytokines such as inertleukin-1β (IL-1β), interferon-γ (IFN-γ), TNF-α. The combination of inflammatory cells infiltration and the production of the proinflammatory cytokines will lead to further destruction of beta cells promoting their apoptosis (Christen et al., 2001; Green, Eynon, & Flavell, 1998; Suarez-Pinzon et al., 1999; von Herrath & Oldstone, 1997).

Type 2 diabetes is more prevalent and it is associated with very high insulin resistance. It has been reported that the incidence of type 2 diabetes is directly related to increased visceral adipogenesis (Colditz et al., 1990; Kahn & Flier, 2000; Mokdad et al., 2003). Adipocytes can release molecules that alter the insulin response by the body, which will enhance the insulin resistance. (Greenberg & Obin, 2006). During the early onsets of type 2 diabetes, the pancreatic beta cells will overproduce insulin to compete with the body's resistance. This hyperinsulinemia usually leads to an increase in pancreatic cell mass (Dickson & Rhodes, 2004). Thus, these individuals do not develop diabetes. However, in many cases, the beta cell mass will undergo apoptosis. This results in the inability of the body to compensate for the insulin resistant

status and subsequently will result in sustained hyperglycemia leading to a diagnosis of type 2 diabetes. Thus, type 2 diabetes is characterized by both insulin resistance coupled with insufficient production of insulin to overcome the insulin resistance (Donath, Storling, Maedler, & Mandrup-Poulsen, 2003).

Furthermore, a less common form of diabetes is gestational diabetes. Some pregnant women may undergo temporary changes in their metabolism including lowering their insulin sensitivity. The reduced insulin sensitivity will result in glucose intolerance. This condition is called gestational diabetes mellitus and it is defined as glucose intolerance first recognized in pregnancy (Dornhorst & Beard, 1993). The epidemiological studies of gestational diabetes show that it is widely variable and ethnicity-dependent. A family history of diabetes, as well as the maternal weight were all considered as predisposing factors to gestational diabetes (H. King, 1998; Xiong, Saunders, Wang, & Demianczuk, 2001).

Diabetic complications:

Mechanisms of Diabetic Complications

Diabetes is associated with a number of reported factors that can lead to diabetic complications such as enhanced oxidative stresses, enhanced formation of advanced glycation end products (AGEs), increased the levels of protein kinase C activation (PKC), and shunting through the polyol pathway (Figure 3).

Diabetes is associated with downregulation of anti-oxidants as well as upregulation of superoxides (Giugliano, Ceriello, & Paolisso, 1996; Hunt & Wolff, 1991; Wolff & Dean, 1987). One of the negative impacts of hyperglycemia can happen through overloading the mitochondria. Once the mitochondria becomes overloaded its electron transport chains becomes dysregulated. This dysregulation leads to the release of electrons which react with oxygen and results in overproduction of superoxides, which will increase the oxidative stress inside the cell (Brownlee, 2005). It has been reported that the overexpression of reactive oxygen species (ROS) can harm cell membranes and can denature proteins. (Baynes & Thorpe, 1999). Furthermore, ROS can enhance the immune response through the activation and stimulation of cytokine secretions (Millar, Phan, & Tibbles, 2007; Mitra & Abraham, 2006). In addition, ROS was reported to be associated with enhancing inflammation by activating nuclear translocation of nuclear factor of kappa light chain gene enhancer in B-cells NF-κB (Gloire, Legrand-Poels, & Piette, 2006).

Many reports showed that aging, obesity, and hyperglycemia are associated with enhanced formation of AGE's (He, Koschinsky, Buenting, & Vlassara, 2001; He et al., 2000; G. L. King & Brownlee, 1996; Stitt & Curtis, 2005; Vlassara & Bucala, 1996; Wada & Yagihashi, 2005; Yan, Ramasamy, Naka, & Schmidt, 2003). AGE's can cause cross-linking of the cell membrane as well as denaturing proteins, which will result in altering many cellular and

molecular activities. (G. L. King & Brownlee, 1996; Stitt, He, & Vlassara, 1999; Vlassara & Bucala, 1996). AGE's can also enhance the recruitment of different inflammatory cells including mononuclear phagocytes. Furthermore, AGE's can also increase osteoclastic activities and facilitate bone resorption in cultured mouse bone cells (Miyata et al., 1997). Finally, it has been reported that AGE's- RAGE combination blocked MSC differentiation to chondrocytes or osteoblasts and it enhanced the programed cell death (Kume et al., 2005).

Hyperglycemia is a sustained state of excessive glucose levels inside the tissues. The excess glucose leads to the activation of the polyol pathway and subsequently elevates the levels of both sorbitol and fructose (Gabbay, 1973). Shunting through the polyol pathway increases the production of AGE's, ROS, and nitric oxide (NO) (Cameron & Cotter, 1992; Hamada, Araki, Horiuchi, & Hotta, 1996; G. L. King & Brownlee, 1996; Stevens et al., 1994). High glucose is associated with activating PKC, nuclear translocation of (NF-κB), and dephosphorylating of the inhibitor of NF-κB kinase (IKK). Blocking aldose reductase in diabetic conditions blocks these effects (Ramana, Bhatnagar, & Srivastava, 2004a, 2004b; Ramana, Friedrich, Srivastava, Bhatnagar, & Srivastava, 2004; Ramana et al., 2005; Srivastava, Ramana, & Bhatnagar, 2005).

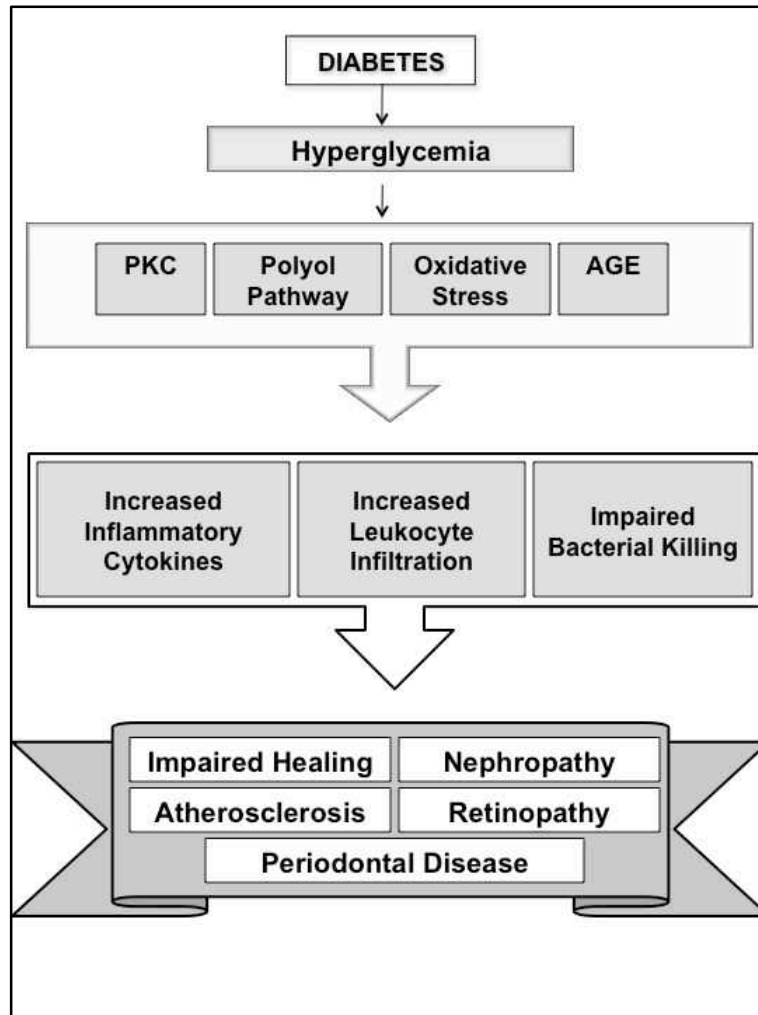**Figure 3: Mechanisms of diabetic complications**. Hyperglycemia caused by diabetes leads to an increased oxidative stress, activation of the polyol pathway, the formation of AGE's, and increased PKC activity. These factors increase proinflammatory cytokine expression, enhance PMN and monocyte recruitment, and impair bacterial killing. These alterations may contribute to complications seen in diabetes.

Diabetes and bone fractures:

It has been reported that there is an increased risk of fractures of the long bones in patients with TIDM (odds ratio = 2.2) and T2DM (odds ratio = 2.8) (Hernandez, Do, Critchlow, Dent, & Jick, 2012). Furthermore, in T1DM the relative risk of hip fracture was 6.9 after adjusting for age, body mass index and smoking (Forsen, Meyer, Midthjell, & Edna, 1999; Vestergaard, Rejnmark, & Mosekilde, 2005). One of the possible mechanisms is through decreased bone mineral density and osteopenia. (Hamada et al., 2007; Jehle, Jehle, Mohan, & Bohm, 1998; Kemink, Hermus, Swinkels, Lutterman, & Smals, 2000; Piepkorn et al., 1997; Tuominen, Impivaara, Puukka, & Ronnemaa, 1999). Kemink et al. reported that 67% of diabetic men and 57% of diabetic women had osteopenia of either the femoral neck and/or the lumbar spine. They have also reported that the diabetic patients had a lower level of plasma insulin-like growth factor (IGF) in comparison to the normal control. Furthermore, they have indicated that within the diabetic group of patients, those who had femoral neck osteopenia had significantly lower plasma IGF-1 levels compared to those without osteopenia (Kemink et al., 2000). The mechanisms of osteopenia caused by diabetes have been thoroughly investigated. In one investigation, they found that individual with type 1 diabetes had lower BMD and they found a drastic reduction in the serum levels of osteocalcin. Osteocalcin is a noncollagenous hormone secreted primarily by osteoblasts and odontoblasts in a vitamin K-dependent

process. Osteocalcin plays a major role in the mineralization of hard tissues in addition to its role in the homeostasis of calcium. Osteocalcin also behaves as a hormone that stimulates pancreatic cells to release more insulin into the system.
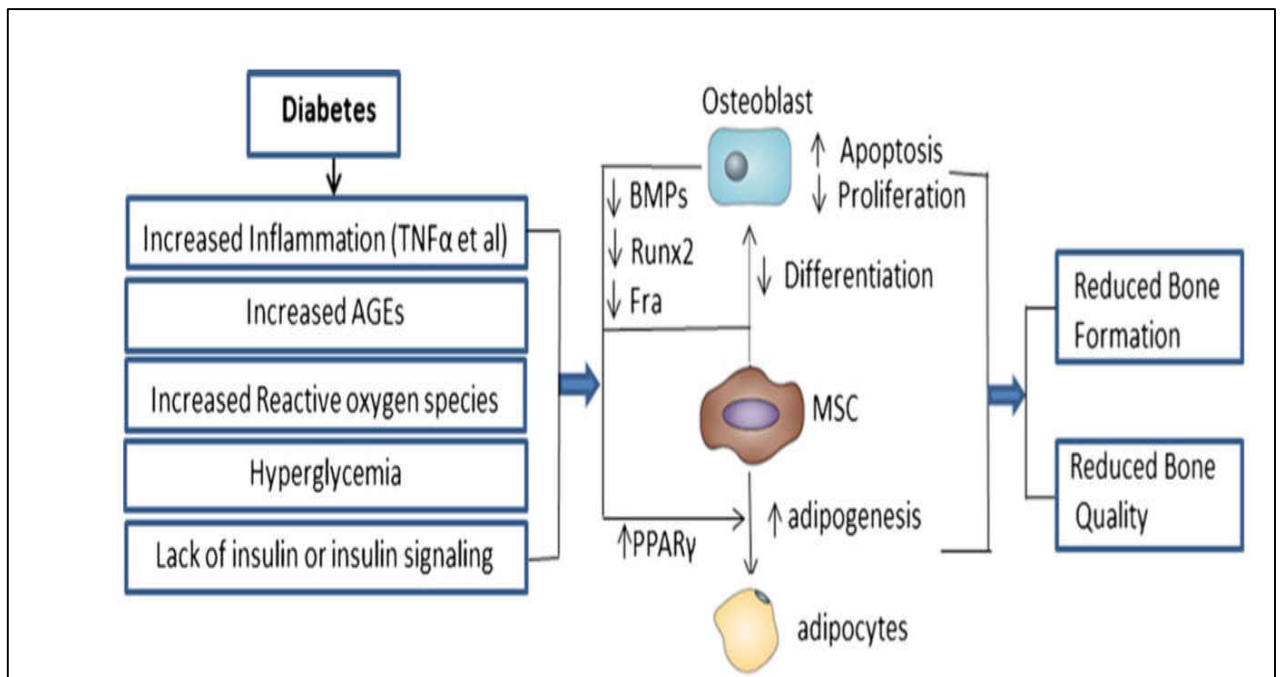

Figure 4: Mechanisms of how diabetes reduces bone formation. The hyperglycemia caused by diabetes will enhance and prolong the inflammation status of the body which will enhance the formation of AGEs, ROS and reduction in insulin signaling. Those events will affect osteoblasts and will reduce bone formation via dysregulating BMPs, Runx2, or Fra1. Copied from (Jiao, Xiao, & Graves, 2015)

Another group of investigators suggested that the osteopenia caused by diabetes is not only due to decreased bone formation but, also as a result of increased bone resorption associated with IGF deficiency. They have measured urinary deoxy pyridinium crosslinks, which is a marker of bone resorption, in diabetic patients and they have reported that it was significantly upregulated compared to matched controls (Bjorgaas, Haug, & Johnsen, 1999). Since type 1 diabetics are typically deficient in insulin, it has been proposed that the hypoinsulinemic status rather than the hyperglycemic effects of diabetes is the causative factor for bone dysregulation (Thrailkill, Lumpkin, Bunn, Kemp, & Fowlkes, 2005). However, a study on euglycemic mice, mice with tissue-specific insulin receptor deficiency, showed that those mice had normal bone mass. Besides, those mice had normal mRNA levels of both osteocalcin and runt-related transcription factor 2 (Runx2). All those findings indicate that osteopenia caused by diabetes is due to hyperglycemia rather than hypoinsulinemia (Irwin, Lin, Motyl, & McCabe, 2006).
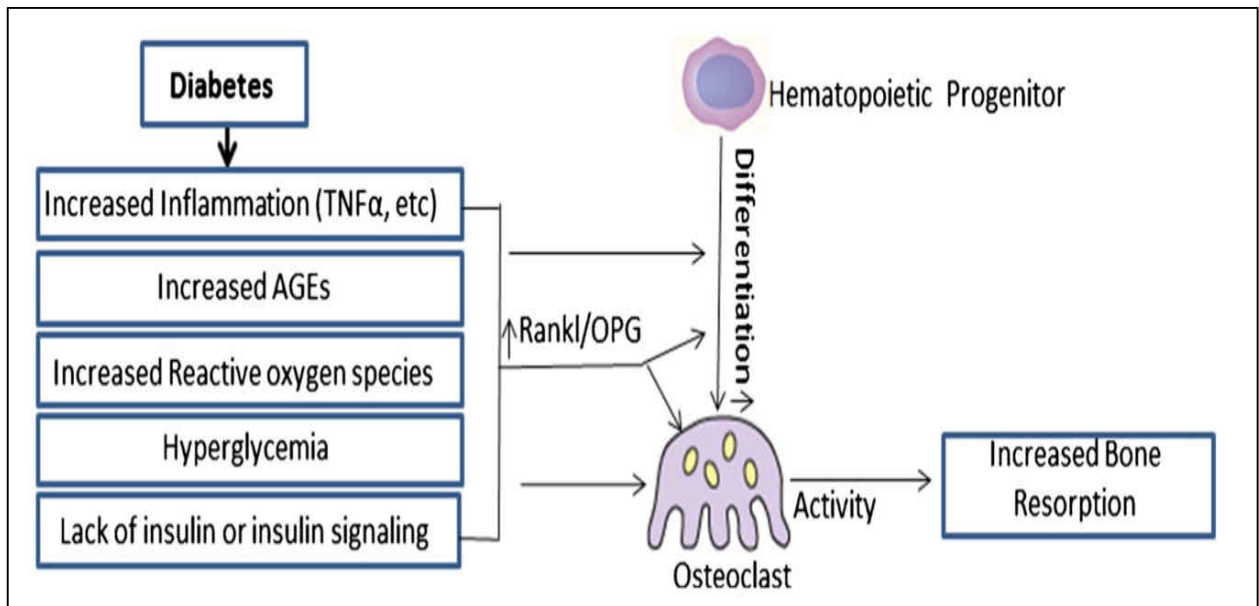
Figure 5: Mechanisms of how diabetes increases osteoclastogenesis.

Hyperglycemia caused by diabetes enhances inflammation and the production of AGEs, ROS, and will decrease insulin signaling. This will lead to alteration in the RANKL/OPG ratio which will increase osteoclast formation (Copied from (Jiao et al., 2015).

Streptozotocin-induced animal model:

Streptozotocin (STZ) induced diabetes is a commonly used animal model because it is straightforward and reproducible (Lenzen, 2008). STZ (2-deoxy-2-(3-methyl-3- nitrosourea)-1-D-glucopyranose) is a broad-spectrum antibiotic synthesized by Streptomyces achromogenes. It has a structure that is similar to glucose and N-acetyl glucosamine (GlcNAc) which facilitate its transportation to the pancreatic β cells. This transport is exclusive by glucose transport protein GLUT2 but not by other transporters (figure 6). It has been reported that β cells have very high levels of GLUT2 which would explain the high toxicity of STZ on β cells (Schnedl, Ferber, Johnson, & Newgard, 1994; Z. Wang & Gleichmann, 1998). Once STZ is transported into the cells, it induces cell death through different suggested pathways. First, the formation of carbonium ions (CH3+) will result in activating poly ADP-ribose synthetase resulting in DNA methylation. The second pathway works via increased production of hydrogen peroxide and other free radicals that will promote the cell's death. Finally, STZ might increase nitric oxide levels within the cells which will accelerate the cell death process. (Szkudelski, 2001; Tesch & Allen, 2007)
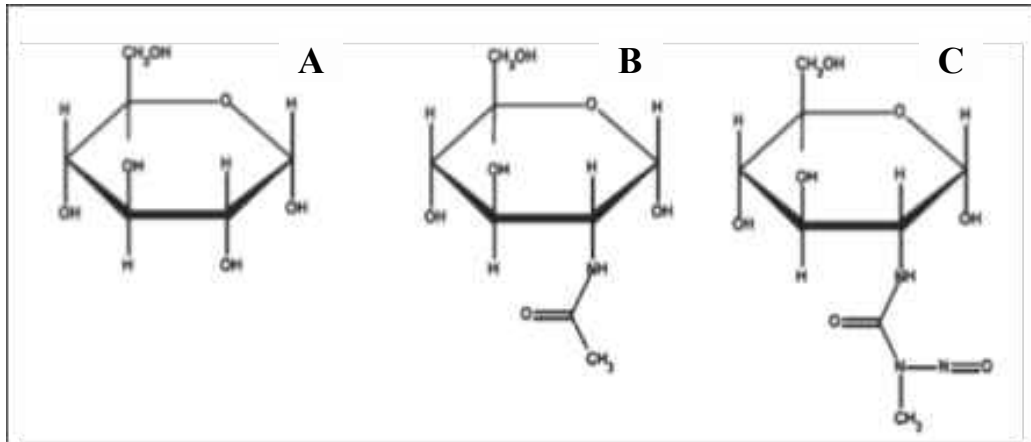
Figure 6: Chemical structures of (A) glucose, (B) N-acetyl glucosamine and (C) streptozotocin

Several studies using STZ-induced diabetes showed the negative impact of the disease on bone. There was a significant reduction in bone mineral density as well as bone mineral content in STZ induced diabetic animals. Moreover, the levels of osteocalcin in the serum were also significantly reduced compared to non-diabetic animals. (Botolin et al., 2005; Hou, Zernicke, & Barnard, 1991). Also, STZ induced diabetic animals had around 20-30% decrease in the biomechanical strength of both femurs and tibias (Hou et al., 1991; Reddy, Stehno-Bittel, Hamade, & Enwemeka, 2001). As previously mentioned, diabetes is associated with enhanced AGE's production, and the role of upregulated AGE's in diabetic complications is already well established. Santana and colleagues showed the association between higher levels of RAGE in diabetic animals and the reduction in bone forming capability of STZ-induced diabetic mice compared to the matched controls. Besides, there was a significant reduction in the bone forming ability

upon treating normal animals with AGE's to levels matching the diabetic animals (Santana et al., 2003). Alikhani et al. showed both *in vitro* and *in vivo* the upregulation in osteoblast apoptosis associated with AGE's treatment which might explain the significant reduction in the bone forming capacity associated with AGE's upregulation. In addition to increasing osteoblasts apoptosis, it has been reported that AGE's were associated with increased osteoclastic activities through upregulating the production of the proinflammatory cytokine IL-6 (Miyata et al., 1997)

Our lab has previously shown that diabetes causes premature cartilage resorption. Although the mechanism of how diabetes negatively affects the fracture healing is unknown, our lab has proposed a dominant mechanism which is the premature cartilage resorption via upregulation in osteoclastic activates. Our lab has shown that diabetes resulted in smaller cartilage areas at the transitional time. (Kayal et al., 2007). During fracture healing, there was double the number of osteoclasts in the calluses of diabetic animals compared comparison to normal animals. The striking change in osteoclast numbers was associated with significantly higher mRNA levels of RANKL in the diabetic animals (Kayal et al., 2007). Tsentidis et al. suggested a reduction in IGF1 as a mechanism of diabetes enhanced total s-RANKL (Tsentidis et al., 2016). Our lab has also shown that the diabetic-induced upregulated osteoclastogenesis was TNF-α dependent, and when the diabetic mice were treated with a TNF-α inhibitor, they exhibited normal osteoclastic activities. TNF-α upregulation in

diabetes may reduce the capacity to downregulate other inflammatory and apoptotic genes which have been linked to the reduction in bone coupling in diabetic animals. Prolonged overexpression of TNF-α leads to upregulation in nuclear factor-kappa-B activity and will diminish the levels of fra-1 and runx2 which are potent stimulators for osteoblast migration, proliferation, and differentiation. Furthermore, TNF-α overexpression has been linked to the reduction in osteoblast numbers through increasing programed cell death via activating caspase 3 and altering Bax/Bcl-2 ratio (Coe, Irwin, Lippner, & McCabe, 2011). In another investigation, our lab has shown that diabetes causes upregulation of inflammatory cytokines and chemokines expressed by chondrocytes. The overexpression of those cytokines and chemokines might play an important role in the diabetic-induced impaired fracture healing. Furthermore, those cytokines and chemokines were mediated in part by the transcription factor forkhead box O1 (FOXO1) (Alblowi et al., 2013).

FOXO1:

Forkhead box (FOX) transcription factor was first found in Drosophila. It was then cloned and characterized in humans by homology. FOXO is a family of transcription factors involved in many cellular and molecular events. FOXO transcription factors are a member of a big family of forkhead proteins characterized by the presence of a winged-helix DNA binding domain called Forkhead box (Almeida, 2011). They are currently the largest family of transcription factors in humans, and they have been grouped according to

their structural characteristics into four subclasses; FOXO1 (or FKHR), FOXO3 (also known as FKHRL1), FOXO4 (which is also called AFX) and FOXO6 (Greer & Brunet, 2005). All the members of this subfamily are highly expressed in bone and cartilage forming cells, except FOXO6, which is expressed exclusively in the brain (Jacobs et al., 2003). It has been reported that these family members are key regulators in processing various stimuli such as inflammation, hormonal changes, increased oxidative stresses and free radicals into different physiological and pathological events. Furthermore, FOXO1 activities regulate several genes that control glycogenesis and gluconeogenesis.
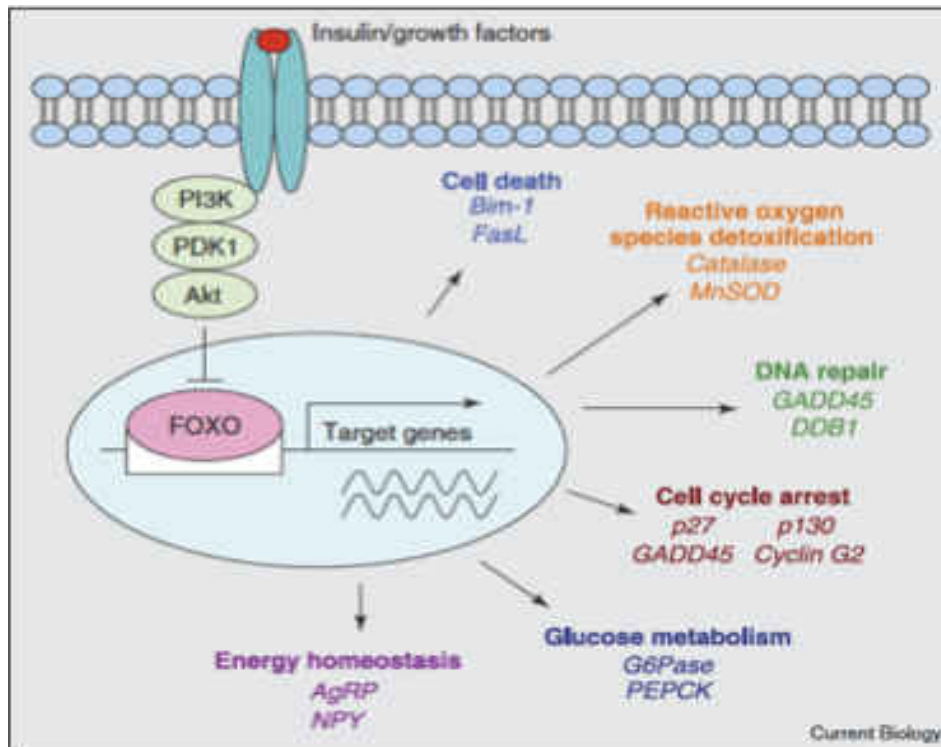
Figure 7: Mechanisms of FOXO1 activation. FOXO1 translocate into the nucleus in the absence of insulin which will lead to activation of all the FOXO1 dependent cascades. (picture copied from Current Biology)

Insulin plays a major role in regulating FOXO1 activities since the deactivation of this transcription factor is mediated by the insulin/PI3K/AKT signaling pathway. Several pathways are activated through insulin receptor signaling. One of those pathways is Ras which will activate MAP kinase. Furthermore, insulin also induces the phosphorylation of insulin receptor substrate 1 (IRS-1) and IRS-2 and the activation of phosphatidylinositide-3-kinase (PI3K), which will subsequently activate AKT. Insulin resistance can lead to the reduction in the phosphorylation of IRS-1/IRS-2. The reduction in IRS-1 and IRS-2 activities will reduce PI3K activities and will increases MAP

kinase activation. AKT signaling regulates the activation of FOXO1 and is critical in controlling homeostasis. FOXO1 is deactivated by phosphorylation via AKT and SGK1 at three different sites. The phosphorylation process of FOXO1 reduces the DNA binding activities of this transcription factor and will facilitate its transport outside the nucleus to the cytoplasm, which will result in the inhibition of FOXO-dependent transcriptional activities. Acetylation of FOXO1 has been associated with the reduction in its activities (Lalmansingh, Karmakar, Jin, & Nagaich, 2012; Tikhanovich, Cox, & Weinman, 2013). On the contrary, deacetylation of FOXO1 increases its activities. It has been reported that members of the sirtuin family of deacetylases, silent information regulator -1 (Sirt-1) and Sirt-2, increase the transcriptional and DNA binding activities of FOXO1 via deacetylation. (Kobayashi et al., 2005)

Our lab has shown that there was a significant elevation in FOXO1 DNA binding activities in diabetic fracture calluses. When TNF-α was inhibited by pegsunercept treatment, FOXO1 nuclear translocation diminished (Alblowi et al., 2009). Furthermore, high glucose inhibits FOXO1 binding to certain promoters and causes FOXO1 to elevate the expression of cytokines that interfere with healing (Zhang et al., 2015).

Specific Aims:

Diabetes mellitus is a common chronic disease that affects more than 230 million people worldwide. A significant complication of diabetes is impaired fracture healing (Cozen, 1972; Herskind, Christensen, Norgaard-Andersen, & Andersen, 1992; Loder, 1988). Long bone fractures heal primarily by endochondral bone formation. Chondrocytes deposit cartilage to form a soft callus, which also acts as an anlagen for later bone formation (L. C. Gerstenfeld et al., 2002). We have proposed that diabetes interferes with fracture healing by causing premature loss of cartilage through upregulation of osteoclast activity. This concept was supported by findings that the number of osteoclasts was doubled in the calluses of diabetic animals in comparison to the normal animals and coincided with the upregulation of pro-inflammatory and pro-apoptotic factors including TNF-α (Alblowi et al., 2013; Kayal et al., 2007).

Based on findings that TNF-α induces FOXO1, we tested the **hypothesis that Foxo1 deletion in chondrocytes will improve the bone healing in diabetic mice** by regulating the expression of a critical pro-osteoclastogenic factor, RANKL. To investigate whether FOXO1 activation in chondrocytes is a critical factor in diabetes-impaired fracture healing we examined mice with lineage-specific deletion of FOXO1 in chondrocytes and determined the impact on fracture repair in diabetic animals.

Materials and methods:

Animal model and Induction of diabetes:

Cre recombinase is an enzyme from the integrase superfamily that uses a mechanism similar to topoisomerase to produce a lineage-specific recombination. Specifically, it catalyzes the site-specific recombination between the LoxP sites on both ends. The result of this recombination at the specific sites depend on the sequence and the location of the LoxP sites. The DNA that is located between the two floxed sites will be excised which will drive tissue specific or site specific gene deletion.

All Animal experiments were conducted in conformity with the University of Pennsylvania Institutional Animal Care and Use Committee. In the current study lineage-specific FOXO1 deletion in chondrocytes was obtained by crossing mice with floxed FOXO1 alleles, provided by Ronald A.DePhino (MD Anderson Cancer Center) as previously described in the literature (Paik et al., 2007), with mice expressing Cre recombinase under the control of col2α1 (obtained from the Jackson Laboratory). This breeding scheme produced the experimental Col2α1Cre$^+$/FOXO1$^{L/L}$ and Col2α1Cre$^-$/FOXO1$^{L/L}$ which were used as controls. Between two to five mice were housed per cage under standard conditions with a 14-hours light/ 10-hours dark cycles. All procedures were done on adult mice between the age of 10-16 weeks. Mice were rendered diabetic by intraperitoneal (I.P) injections of

streptozotocin (50mg/kg) (Sigma, St. Louis, MO) in 10mM citrate buffer once every day for 5 consecutive days (Graves et al., 2005). Control mice were injected with 10mM citrate buffer alone. Blood glucose was measured weekly from blood obtained from a small laceration in the tail. The weight of the mice was monitored as well as the mice hydration status. Mice were considered diabetic when blood glucose levels exceeded 250 mg/dl for two consecutive measurements. Mice typically become diabetic after 10 days from starting the injections. If animals did not develop hyperglycemia after the first round of injections, they were given a second round of STZ and if they still did not respond they were euthanized. Experiments were carried out when mice had been diabetics for at least 3 weeks.
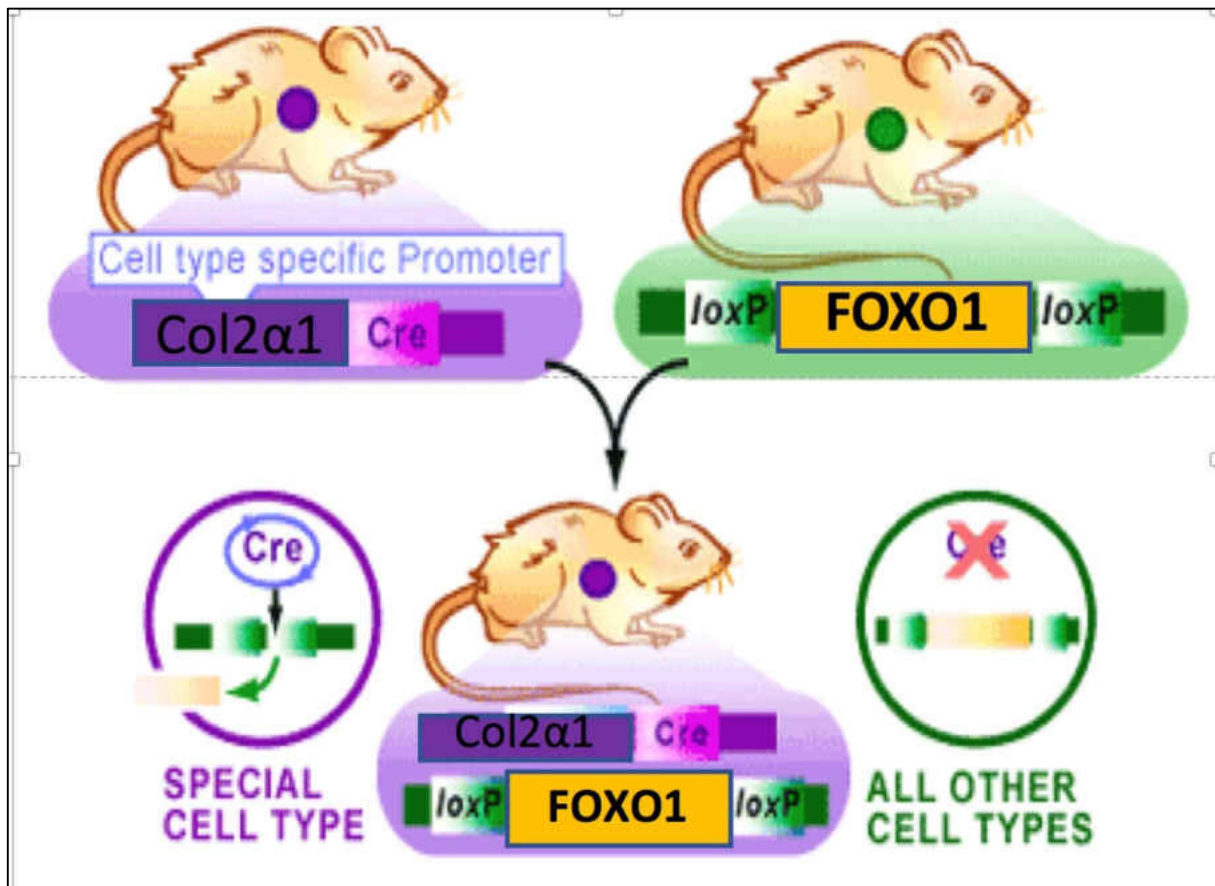
Figure 8: The mouse model and the breeding scheme. An Illustration of the mouse model and the breeding scheme used in the study utilizing Cre lox system. Col2α1 promoter was used to drive FOXO1 deletion.

Femoral fracture:

An incision was made lateral to the knee and the tendon for the quadriceps femoris muscle was pushed medially to expose the articular surface of the femur. A 27-gauge needle was used to gain access to the medullary canal and a 30-gauge spinal needle was inserted for fixation. After suturing the incision, a controlled closed simple transverse fracture was created by blunt trauma using a guillotine device. The animals were

euthanized and femurs were harvested at 3 different time points: day 10, day 16, and day 22 post fracture. The fracture sites were evaluated physically and radiographically at euthanasia time. All the fractures that weren't mid-diaphyseal or the grossly comminuted fractures were excluded from the study. An illustration of the study time line is presented in figure 9.
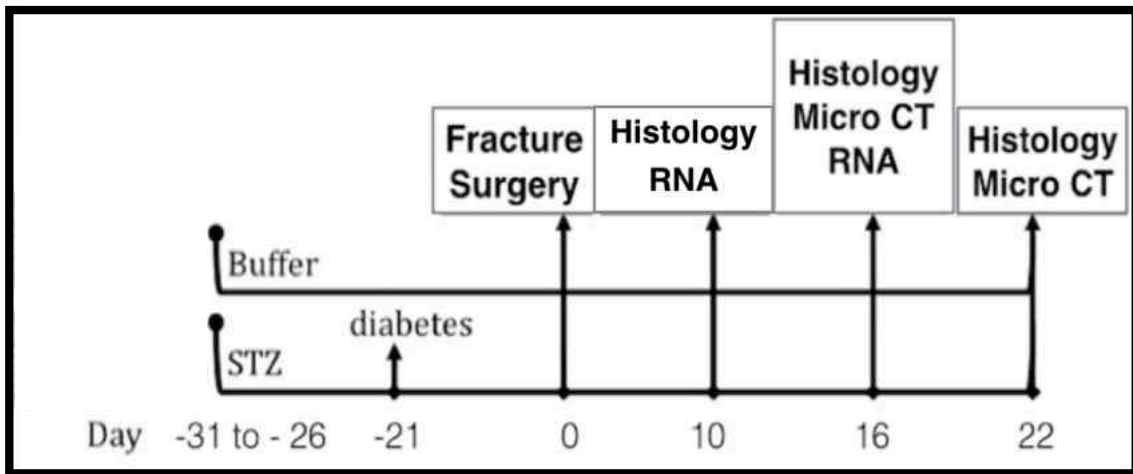

Figure 9: Study time line illustration showing the animal distribution and the different study groups.

Micro-Computed Tomography and Image Analysis:

To evaluate the total callus and the bone volume, the fractured femurs were scanned individually using a desktop micro-computed tomography system (Scanco μCT40, Scanco Medical, Basserdorf, Switzerland). The scan axis coincided nominally with the diaphysial axis, 70kVp and 114mA were used to scan the samples. The integration time was 200ms with 19μm voxel size. Constrained, 3-D Gaussian filter (sigma=0.8, support=1) was used for noise reduction. A fixed, global threshold was chosen that represented the

29

transition in X-ray attenuation between the mineralized and the unmineralized tissue. The 3D reconstruction and the image data were analyzed using software provided by the system manufacturer to quantify the bone volume of the fracture callus using the same threshold.

Histology and Histomorphometric Analysis:

The harvested femurs with a small amount of surrounding muscle and soft tissues were fixed for 24 hours in cold 10% paraformaldehyde followed by decalcification in 10% EDTA (Boston Bioprodicts, Worcester, MA) for 5 weeks. The internal fixation pin was removed and the samples were then processed in ascending ethanol concentrations. After processing, the pieces were embedded in paraffin, sectioned at 4 microns and prepared for staining. Sectioning and histomorphometric measurements were performed according to methodologies described in (L. C. Gerstenfeld, Wronski, Hollinger, & Einhorn, 2005). Cross-sectional transverse sections were sampled along the long axis of the bone including the fracture site itself. The callus area, cartilage area, and new bone area were measured in sections stained with safranin-O/fast green. The area of each tissue was assessed by computer assisted image analysis using NIS elements AR software (Nikon). Measurements were made by one examiner under blinded conditions, with the results confirmed by a second blinded examiner.

Safranin-O/Fast Green Stain:

Slides were baked, deparaffinaized in xylene and hydrated in graded ethanol series from 100 to 80% for 2-3 minutes each. To stain the nuclei, slides were placed in Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 30 seconds then rinsed in slow running water for at least 5 minutes. Fast green (Fisher Scientific, Fair Lawn, NJ) was used as a background stain. The slides were placed in the stain for 7 minutes and then rinsed in 1% acetic acid for 1 second. After that, slides were placed in safranin-O (Sigma-Aldrich, St. Louis, MO) for 10 minutes to stain the cartilage. This was followed by dehydration in which slides go through graded series of ethanol from 80 to 100% and then placed in xylene until cover slipping.

Mechanical testing:

A separate set of mice were utilized for mechanical testing. The mice were euthanized 5 weeks post-fracture to evaluate the mechanical properties of the broken femur at the remodeling phase. Both the experimental and the contralateral femurs were harvested. The femurs ends were cemented and torsional testing was carried out on a servo hydraulic testing machine (MTS Corp., Minneapolis, MN) using a 20 Nm reaction torque load cell (Interface, Scottsdale, AZ). Femurs were tested to failure at an actuator head displacement rate of 1°/second. Max torque, stiffness, toughness, and modulus of rigidity were tested according to the method described by Manigrasso and O'Connor 2004. For each parameter, the means and

standard deviations were calculated.

TRAP staining:

Osteoclast formation was determined by counting the number of multinucleated, tartrate-resistant acid phosphatase (TRAP) positive cells lining cartilage. After deparaffinization and hydration, as previously mentioned, slides were placed in 0.2M acetate buffer for 30 minutes. The buffer was prepared from 16mg/ml of 0.2M sodium acetate (Sigma-Aldrich, St. Louis, MO) and 11.6mg/ml of 50mM tartaric acid (Sigma-Aldrich, St. Louis, MO) with a pH of 5.0. After 30 minutes, 0.5mg/ml naphthol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO) and 1.1 mg/ml fast red TR salt (Sigma-Aldrich, St. Louis, MO) were added to the solution and the slides were incubated at 37°C for 10 to 15 minutes. The slides were then placed in Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 30 seconds to stain the nuclei and rinsed in slow running water for 5 minutes. Slides were left over night to dry and cover slipped the next day.

Immunohistochemistry in histological sections:

To confirm the TRAP staining results, sections from formalin-fixed, paraffin-embedded samples were used for IHC staining using cathepsin K antibody. Deparaffinization and hydration were performed as mentioned above and antigen retrieval was performed using pressure cooker (2100-Retriever Aptum, Southampton, UK) at 120°C with 10mM of citric acid with a pH of 6.0. Samples were then blocked with avidin-biotin (Vector Laboratories,

Burlingame, CA) and nonimmune serum matching the secondary antibody.

Slides were then incubated overnight with anti-cathepsin K antibody (Abcam:

AB19027) at 4°C as well as a matched control IgG. The slides were then

rinsed the next day and quenched with 3% hydrogen peroxide and incubated

with biotin labeled secondary antibody (Santa Cruz Biotechnology, Santa

Cruz, CA). Hematoxylin was used as a counter stain followed by slide

hydration and cover slipping. Analysis of the cartilage was performed by

comparison with safranin-O/fast green stained serial sections. Analysis was

done blindly by one examiner with the results confirmed by a second blinded

examiner.

Immunofluorescence in histological sections:

Paraffin embedded slides were used for *in vivo* immunofluorescence assays. After deparaffinization and hydration, pressure cooker (2100-Retriever Aptum, Southampton, UK) was used at 120°C with 10mM of citric acid with a pH of 6.0 for antigen retrieval followed by non-specific binding blocking for 55 minutes. Finally, an overnight incubation with anti-RANKL antibody (Santa Cruz sc9073) or anti-cleaved caspase 3 antibody (Cell Signaling Technology 96615) at 4°C as well as the appropriate isotype-matched negative control IgG was done. Primary antibody was localised by Biotinylated secondary antibody (Vector Laboratories). Avidin–biotin peroxidase enzyme complex (ABC) and tyramide signal amplification (TSA) were used to amplify the signals. Alexa Fluor 546–conjugated streptavidin (Invitrogen S-11225) was used to visualize the staining. Finally, the slides were mounted with DAPI (Sigma-Aldrich). Images were captured at different magnifications (4x, 20x, and 40x magnification) using a fluorescence microscope (ECLIPSE 90i; Nikon). The exposure time was set to the experimental group based on the IgG control signals. The quantification was performed with the aid of NIS Elements AR image analysis software. The percentage of positive cells was obtained by dividing the immunopostive cells over the total cell number measured by DAPI.

Primary Cell culture:

Primary costal chondrocytes were isolated from (2-4 days old) experimental Col2α1Cre$^+$/FOXO1$^{L/L}$ and control Col2α1Cre$^-$/FOXO1$^{L/L}$ mice. Briefly the mice skin was dissected, the ribs were isolated, incubated in Pronase for 45 minutes to dissolve the soft tissue, followed by 60 minutes in Collagenase D to dissociate the hard tissue from the remaining soft tissues. Finally, the rib cage was incubated in Collagenase D for 3-5 hours to release the cells. Chondrocytes were cultured in DMEM media supplemented with 10% FBS and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific). All cell cultures were maintained in a 5% CO2 humidified incubator at 37°C. Cells were put into starvation for 2 days (low serum media) followed by either serum stimulation for 14 hours or hypertrophic differentiation induction for 4 days using DMEM with 10% FBS supplemented with 10mM β-glycerophosphate and 50µg/ml ascorbic acid, RNA extraction was done using Quick-RNA Microprep kit (Zymo). Real-time quantitative RT-PCR of RANKL gene was performed.

Reverse Transcription and Real Time PCR:

For a given experiment RNA was isolated from cells and SYBR® Green reverse transcription reagents (Applied Biosystems, Foster City, CA) was used for first-strand cDNA synthesis and amplification. Using a thermocycler, samples were incubated at 37°C for 60 minutes, followed by 95°C for 5 minutes and then held at 4°C until transferred to -20°C for storage.

Real time PCR was done on StepOnePlus Real-Time PCR System using 10μl

of master mix SYBR® Green gene expression assays (Applied Biosystems,

Foster City, CA). Each experiment was performed three times and the results

from the three separate experiments were combined in order to derive mean

values and standard of error.

In vitro Immunofluorescence:

Primary chondrocytes isolated from Col2α1Cre[+]/FOXO1[L/L] and control

Col2α1Cre[-]/ FOXO1[L/L] mice were seeded in 96-well plates. They were

incubated in low glucose (5mM d-glucose), high glucose (25mM d-glucose) for

5 days. In another set, cells were incubated with CML-BSA (200μg/ml) for 3

days, which was prepared by chemical modification of BSA (Sigma-Aldrich) as

previously described (Alikhani et al., 2010). The control cells were incubated

with unmodified BSA. The cells were fixed for 10 minutes with 10%

formaldehyde, permeabilized in 0.5% Triton X-100 for 5 min. Non-specific

blocking was done with 2% nonimmune serum matching the secondary

antibody followed by primary anti-RANKL antibody (Santa Cruz sc9073).

Primary antibody was localised with biotinylated secondary antibody and Alexa

Fluor 546–conjugated streptavidin and finally DAPI was used to stain the

nuclei. Images were captured by a fluorescence microscope (Nikon Eclipse Ti)

with the same exposure time for both the control and the experimental group.

The mean fluorescence intensity (MFI) was measured using NIS Elements AR

image analysis software.

TUNEL Assay:

Apoptotic cells were detected by DeadEnd™ Colorimetric TUNEL System (Promega, WI, USA) which detects apoptotic cells by labeling and detecting DNA strand breaks by the TUNEL method. To distinguish apoptotic chondrocytes from other cells the images were combined with bright-field channel to confirm the cell morphology. In addition, stain was compared with a saffranin-o/fast green stain to verify the location of the chondrocytes. Slides were first depraffinaized and hydrated in the same manner mentioned. Slides were then incubated at room temperature for 15 minutes in diluted proteinase K solution and then rinsed with PBS. This was followed by 5 minute incubation in endogenous oxidation blocking solution, which is 3% hydrogen peroxide, at room temperature and then rinsed in PBS. After that, slides were incubated at room temperature for 30 seconds in equilibration buffer, then in working strength TdT enzyme for an hour at 37°C. Slides were then incubated in working strength stop/wash buffer for 10 minutes at room temperature, they were then rinsed in PBS and incubated in anti-digoxignenin conjugate for 30 minutes at 37°C. Slides were rinsed again in PBS and mounted with DAPI to stain the nuclei.

Chromatin Immunoprecipitation Assay:

Chromatin immunoprecipitation assays (ChIP) were carried out with ChIP It Kit (Active Motif) using approximately 1.5 x10^7 ATDC5 murine chondrogenic cells. Hypertrophic differentiation was induced for 6 days using DMEM/F12 media supplemented with 50µg/ml ascorbic acid and 10mM β-glycerophosphate. Different groups of cells at 70 to 80% confluency were separately treated with TNF-α (20ng/ml) for 30 minutes, CML-BSA (200µg/ml) or unmodified BSA (200µg/ml) as a control for 3 days, and high glucose (25mM) for 5 days. ChIP was done following the manufacturer's instructions. Formaldehyde was used to fix the cells, followed by a PBS wash and glycine to stop the fixation process. The cells were collected by scraping and pelleted. A Dounce homogenizer was used to lyse the cells and to release the nuclei. An enzyme was used to shred the chromatin followed by Anti-FOXO1 antibody (Santa Cruz Biotechnology, Inc.) or control polyclonal nonspecific IgG (Cell Signaling, Danvers, MA) incubation. Protein G-coupled beads were used to precipitate the antibodies. Quantitative real-time PCR for RANKL and caspase-3 promoter which has a consensus FOXO1 element, was performed in triplicates.

Luciferase reporter assay:

Transient transfection with luciferase reporter constructs was performed by senior lab members, Dr Guangyu Dong and Citong Zhang, using Lipofectamine™ 3000 (Thermo Fisher Scientific) in 48-well plates as

described before (Zhang et al., 2015). Briefly, ATDC5 cells were incubated in low serum culture media for 5 days with 5 or 25mM d-glucose, or with CML-BSA (200µg/ml) for 3 days, or TNF-α (10ng/ml) for 12 hours before running the assay. Cells were cotransfected with RANKL luciferase reporter (provided by Dr. Charles A. O'Brien, University of Arkansas for Medical Sciences, Little Rock, AR) (O'Brien, Kern, Gubrij, Karsenty, & Manolagas, 2002) or Caspase-3 reporter together with pGL3 luciferase control vector, FOXO1ADA or (Addgene) plasmid (referred to FOXO1) that is constitutively transported to the nucleus, pcFOXO1 which is a wild type FOXO1, or pCMV control plasmid. Two days after transfection, cells were lysed, Firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. Firefly luciferase activities were divided by Renilla activities to normalize for transfection efficiency. Experiments were performed two to three times with similar results.

The role of FOXO1 in chondrocytes proliferation and differentiation:

The number of proliferative and hypertrophic chondrocytes was counted in the healing callus in Saf/O stained slides to measure the cell density. Furthermore, Primary chondrocytes were extracted from experimental FOXO1 deleted and normal WT neonates. Cells were incubated in serum free media, stimulated with serum for 14 hours and the expression of cell cycle genes was determined by quantitative qPCR. Another set of cells were used to measure the proliferation curve. Cells were grown for 5 days, live images were taken

every day and finally DAPI was used to stain the nuclei. The number of cells was quantified at each time point.

To study the effect of FOXO1 on chondrocytes differentiation, primary cells were treated with differentiation media for 4 and 10 days, quantitative qPCR were performed to detect the expression of known differentiation markers. Another set of cells were cultured and Alician blue staining was performed at 0,4,7, and 14 days to evaluate the matrix formation abilities of the cells. The absorbance of the dye was detected using TECAN odometer.

FOXO1 binding activities and nuclear translocation: (ongoing experiment)

To better understand how high glucose regulates FOXO1 activations. ATDC5 murine chondrogenic cells will be seeded in 6-well and 96-well plates. Hypertrophic differentiation will be induced using DMEM/F12 media supplemented with 50μg/ml ascorbic acid and 10mM β-glycerophosphate. Cells will be incubated in high glucose containing media with the use of the following; insulin, AKT inhibitor, TLR4 inhibitor, Sirt-1 inhibitor, HDAC inhibitor, N-Acetyl-L-cysteine, and L-NAME hydrochloride. To explore the effect of insulin, phosphorylation, acetylation and ROS on FOXO1 activation under high glucose conditions.

Nuclear protein will be extracted from the 6-well plates, and will be used to

evaluate FOXO1 binding activities using FOXO1 Transcription Factor Assay

Kit (Abcam).

The 96-well plates will be used for immunostaining with FOXO1 antibody

compared to matched IgG control. FOXO1 nuclear localization will be

quantified.

Statistics:

Statistical analysis between the groups were determined by analysis of variance, one-way ANOVA, Tukey's post-hoc test to denote significance. $P < 0.05$ was considered statistically significant. All the results are expressed as the mean ± SEM. (*) indicates a significant difference between normal and diabetic ($P<0.05$). (+) indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group ($P<0.05$).

For the *in vitro* experiments, Student's t test was used for the comparison between the study groups. $P < 0.05$ was considered statistically significant.

RESULTS

<u>Diabetes validation:</u>

To establish that the animals were diabetic, blood was obtained from the tail of the animal and blood glucose was measured. The experimental group, which was treated with streptozotocin, had a mean blood glucose level of 322 mg/dl (STD ±58), while the control group, which had the vehicle alone, had a mean blood glucose level of 85 mg/dl (STD ±10). This indicates that the experimental group was rendered diabetic by streptozotocin and the control group remained normoglycemic. In addition, the blood glucose level was also measured at the euthanasia time to confirm the established findings.

<u>Validating the animal model:</u>

To confirm the presence or absence of Cre recombinase in the animals, genotyping was performed via PCR using both Cre and FOXO1 primers. Genomic DNA was obtained from the mice's ears/tails before starting the experiment and the results were validated at the time of euthanasia.

A

FOXO1

Cre

B   Col2 staining

C   FOXO1 staining WT

D   FOXO1 staining KO

Col2

FOXO1

Col2a1 Cre-.FOXO1$^{L/L}$

FOXO1   cartilage   bone   10 X

DAPI / FOXO1   cartilage   bone

Col2a1 Cre+.FOXO1$^{L/L}$

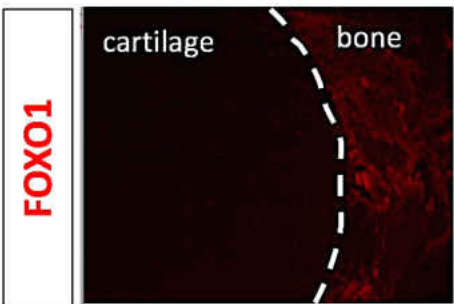FOXO1   cartilage   bone

DAPI / FOXO1
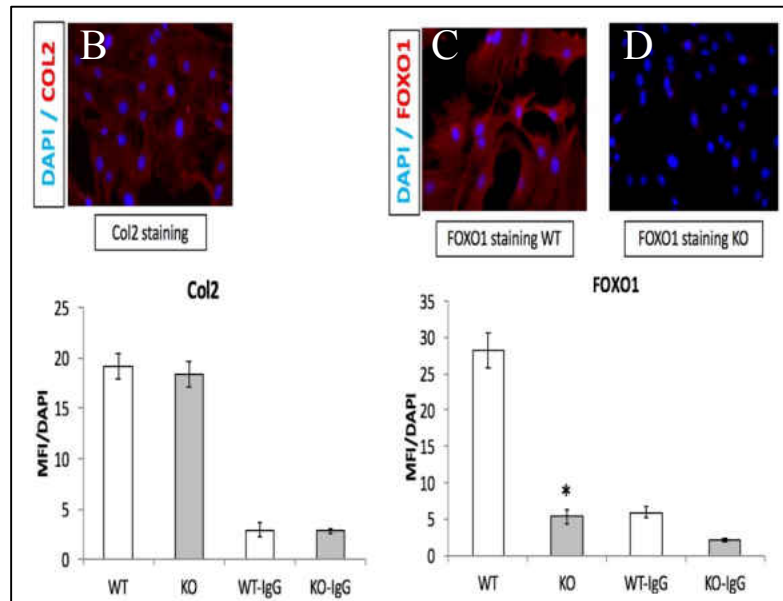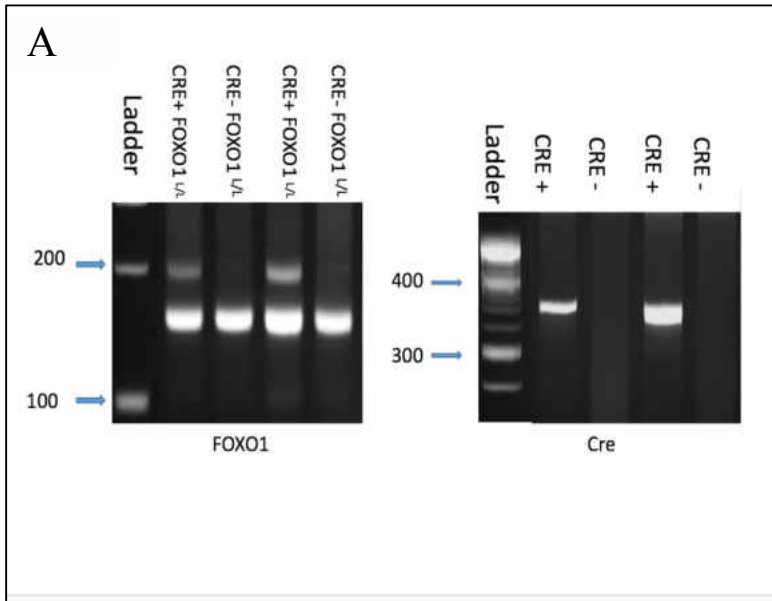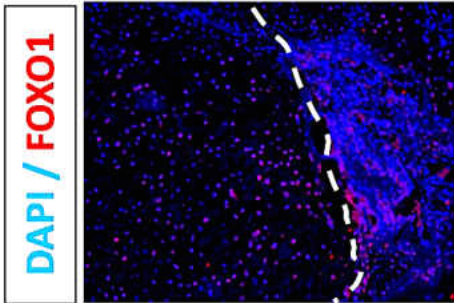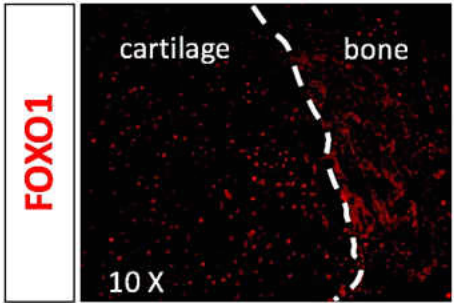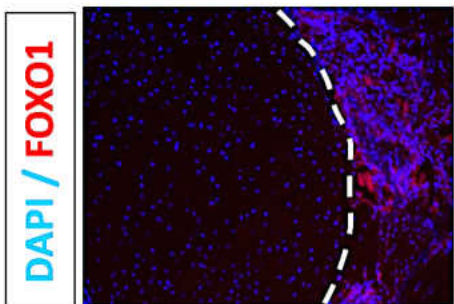
Figure 10: Col2a1Cre deletes FOXO1 in Chondrocytes only.

(A) Electrophoresis of PCR amplified FOXO1 and Cre recombinase primers to establish genotyping for each animal. **(B)** Immunofluorescence of primary chondrocytes using Col2 specific antibody to confirm the cells' phenotype. **(C)** Immunofluorescence staining of primary chondrocytes from WT and **(D)** FOXO1 deleted mice using FOXO1 specific antibody to confirm that the experimental chondrocytes didn't' express FOXO1. (**E)** *In vivo* Immunofluorescence staining of fractured callus from both WT and FOXO1 deleted mice using FOXO1 specific antibody to show the lineage-specific deletion. * indicates a significant difference between WT and FOXO1 deleted group using Students t-test. P<(0.05).

Histomorphometric Analysis:

Experiments were carried out focusing on the stage of cartilage removal and bone formation, days 10, 16, and 22 during fracture repair. Callus size and cartilage area were measured on transverse sections of fracture midline stained with safranin-O/fast green stain. The histomorphometric analysis of fracture callus showed that there was no significant difference between the cartilage areas of the normoglycemic, diabetic, and diabetic mice with FOXO1 deletion in chondrocytes 10 days post fracture (P>0.05). On day 16, the diabetic group had 77% less cartilage than the normoglycemic group (P<0.05), and FOXO1 deletion in chondrocytes of the diabetic animals increased the cartilage area by 369% (4.7 fold) (P<0.05) to match the values of the normoglycemic group (P>0.05; Figure 11).

To evaluate the significance of premature cartilage resorption on the callus, the callus size and new bone formed area were measured. Fracture calluses of the diabetic control group were 43% smaller on day 16 and 37% smaller on day 22 compared to the normoglycemic control group(P<0.05). FOXO1 deletion in chondrocytes in diabetic animals significantly increased the callus size on day 16 by 64% and on day 22 by 52% when compared to diabetic control groups (P<0.05). This parameter was comparable to that of normoglycemic animals (P>0.05; Figure12). Newly formed bone area was also quantified histologically. Diabetes decreased new bone formation by 47% on day 16 and by 51% on day 22(P<0.05). FOXO1 deletion from the diabetic

animals increased the new formed bone areas at day 16 by 77% and at day

22 by 96% (P<0.05) and this was comparable to that of normoglycemic groups
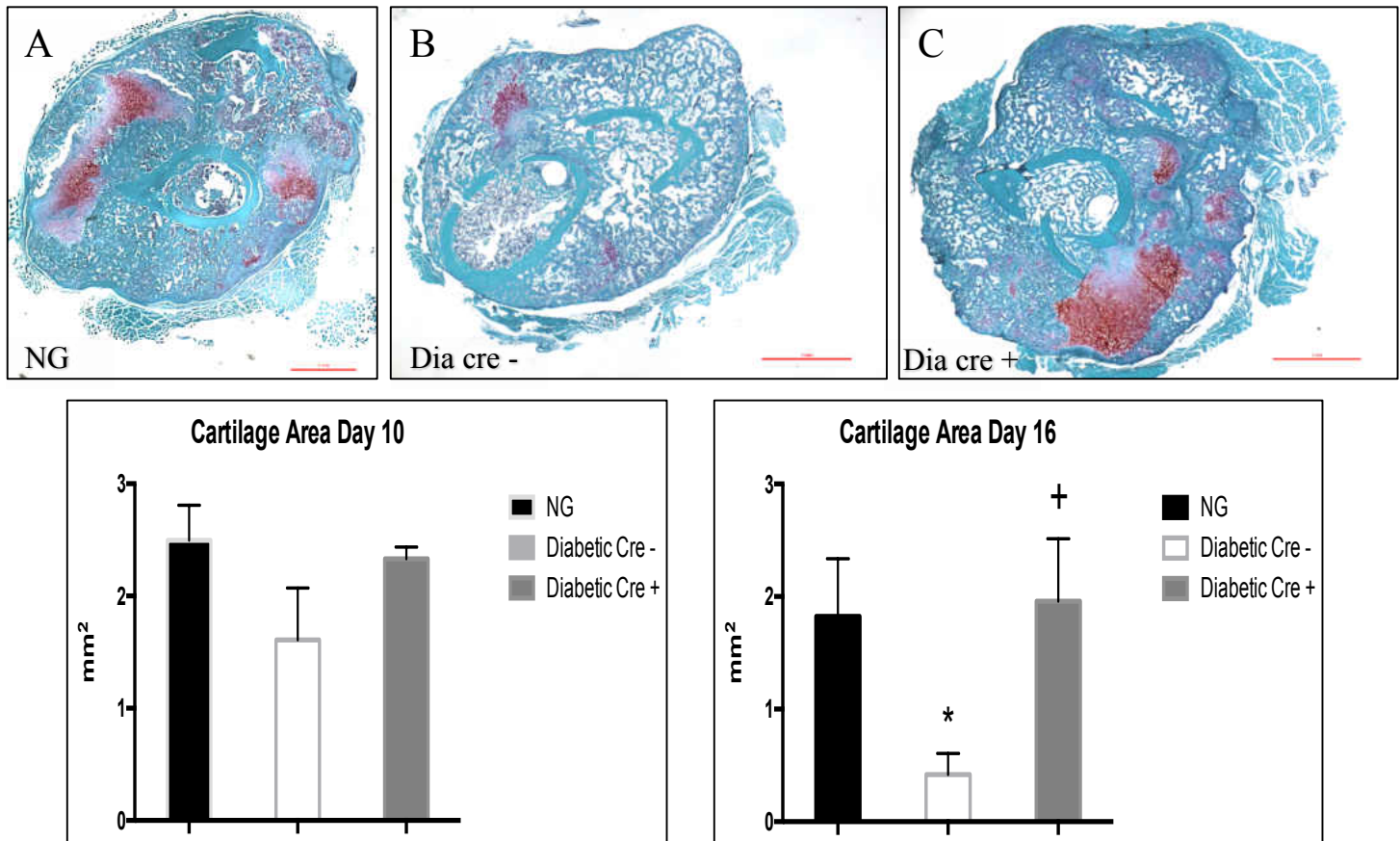
(P>0.05; Figure 12).

Figure 11: Diabetes significantly reduces cartilage area prematurely, which is rescued by FOXO1 deletion in chondrocytes. Comparison of cartilage area between **(A)** normoglycemic**, (B)** diabetic control, and **(C)** diabetic with FOXO1 deletion using safranin-O/fast green stained sections to measure the area of cartilage within each callus. Data are expressed as mean ± SEM. * indicates a significant difference between normal and diabetic ($P<0.05$). + indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group ($P<0.05$)

Micro CT:

The differences in callus size and bone formation in on day 16 and 22 were confirmed by high-resolution μCT. On day 16 post fracture, the total callus volume of the diabetic group was 39% smaller and the bone volume was 29% smaller compared to the normoglycemic group (P<0.05). On the other hand, FOXO1 deletion from chondrocytes in diabetic animals increased the total volume by 59% and the bone volume by 33% (P<0.05), to volumes similar to that of the normoglycemic group (P>0.05). The same trend was observed on day 22. When compared to the normal group, the diabetic group had 41% smaller total volume and 34% smaller bone volume (P<0.05). Upon FOXO1 deletion in the diabetic group, the total volume increased by 65% and the bone volume increased by 57%(P<0.05) to match the normoglycemic group (P>0.05; figure 12)
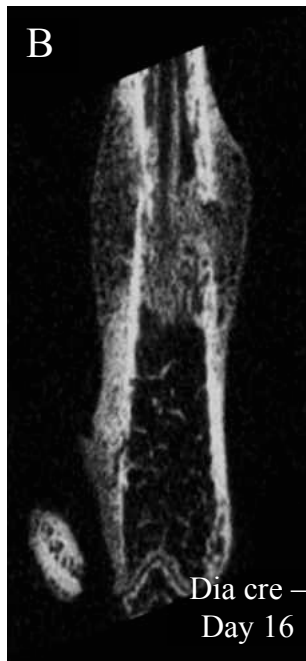
A — NG Day 16
B — Dia cre – Day 16
C — Dia cre + Day 16

μCT

Day 16 Bone Volume
NG
Diabetic Cre -
Diabetic Cre +

Day 22 Bone Volume
NG
Diabetic Cre -
Diabetic Cre +

Day 16 Total Volume
NG
Diabetic Cre -
Diabetic Cre +

Day 22 Total Volume
NG
Diabetic Cre -
Diabetic Cre +

Histology

New Formed Bone Day 16
NG
Diabetic Cre -
Diabetic Cre +

New Formed Bone Day 22
NG
Diabetic Cre -
Diabetic Cre +

Callus Size Day 16
NG
Diabetic Cre -
Diabetic Cre +

Callus Size Day 22
NG
Diabetic Cre -
Diabetic Cre +

50

Figure 12: Premature cartilage resorption caused by diabetes resulted in significantly smaller callus and bone volume. FOXO1 deletion rescued the callus and the bone volume to normal levels. Comparison of callus size and new bone formation in in **(A)** normoglycemic, **(B)** diabetic control, and **(C)** diabetic with FOXO1 deletion using μCT scans and histology. The callus was defined as the area within the peripheral fibrous capsule, excluding the original cortical bone. Data are expressed as mean ± SEM. * indicates a significant difference between normal and diabetic (P<0.05). + indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group (P<0.05)

Mechanical testing:

To evaluate if the quality of the healed femurs is different among the study group, torsional mechanical testing was performed on a separate set of mice. The contralateral (non-fractured) legs were tested first, the results show that the base line values of both diabetic groups were identical. However, the mechanical testing of the experimental side showed that the diabetic control mice had significantly lower maximum torque to failure strength, stiffness, toughness, and modulus of rigidity compared to normal control mice. FOXO1 deletion restored the max torque, the stiffness, and the modulus of rigidity to levels comparable to the normal control.
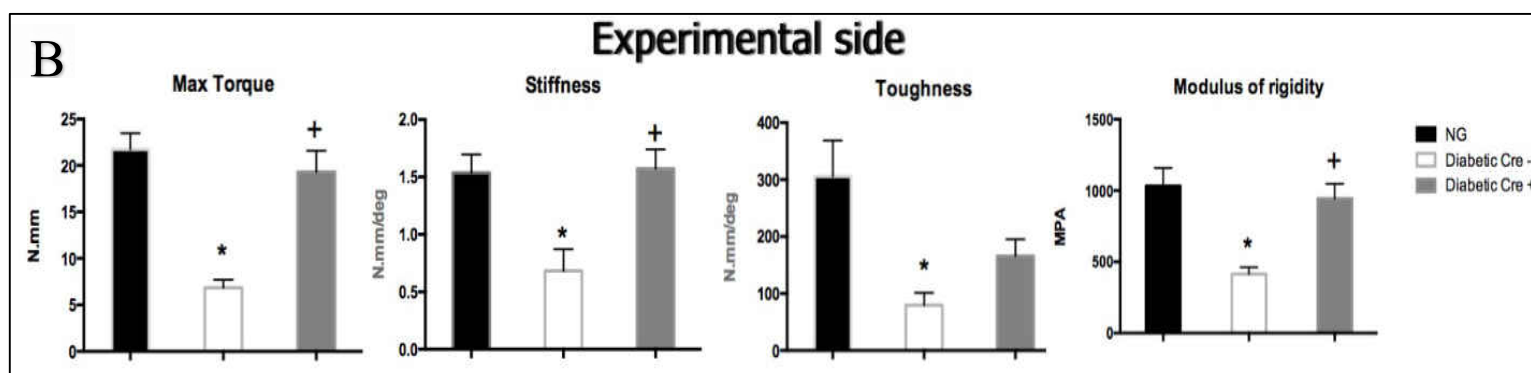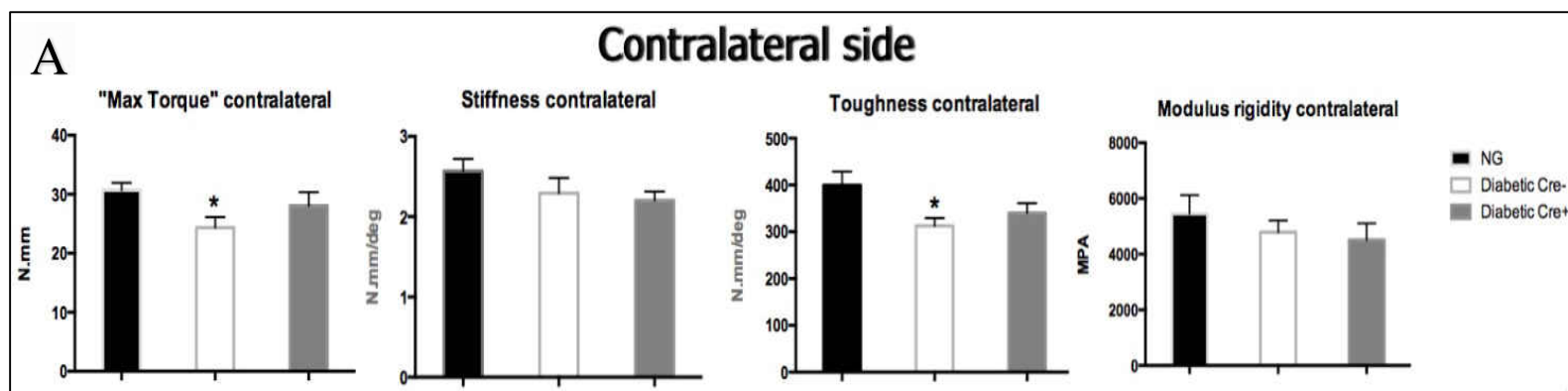
Figure 13: Diabetes significantly alters the mechanical strength of the healing callus. FOXO1 deletion restored the strength to normal levels.
(A) Comparison of callus strength using torsional mechanical testing revealed that both diabetic groups had similar base line values. **(B)** Diabetic control mice had significantly lower maximum torque to failure strength, stiffness, toughness, and modulus of rigidity compared to normal control mice. FOXO1 deletion restored the max torque, the stiffness, and the modulus of rigidity to levels comparable to the normal control. Data are expressed as mean ± SEM. * indicates a significant difference between normal and diabetic (P<0.05). + indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group (P<0.05)

Osteoclasts:

In order to further investigate the process of cartilage resorption, the number of osteoclasts lining cartilage was measured on transverse callus sections stained with TRAP. The diabetic group had 2.8 times more TRAP positive cells lining cartilage than the normoglycemic group on day 10 (P<0.05). The Diabetic group with FOXO1 deletion in chondrocytes had 4.5 times less TRAP positive cells than the diabetic control group (P<0.05; Figure 14). Furthermore, immunohistochemical staining using cathepsin K specific antibody was used to validate the osteoclast numbers obtained from TRAP stain. Ten days post fracture, the diabetic group had 4.4 times more cathepsin K positive cells lining cartilage than the normoglycemic group. Diabetic group with FOXO1 deletion in chondrocytes had 6.5 times less cathepsin K positive cells than the diabetic control group (P<0.05; Figure 14).
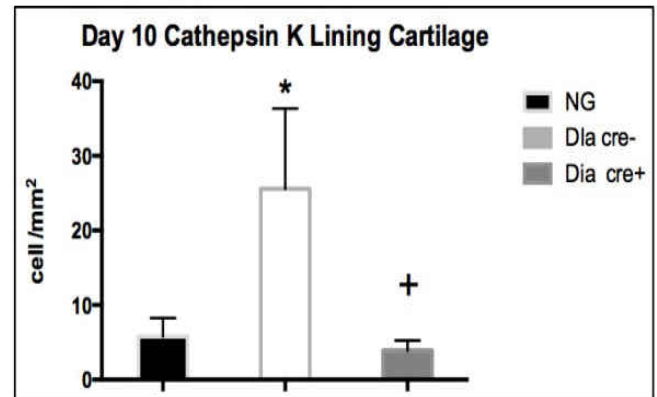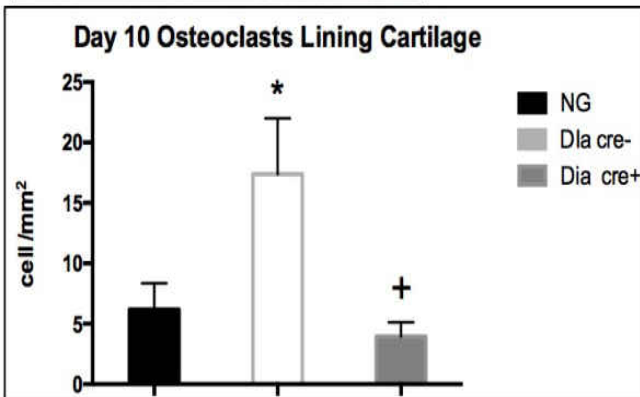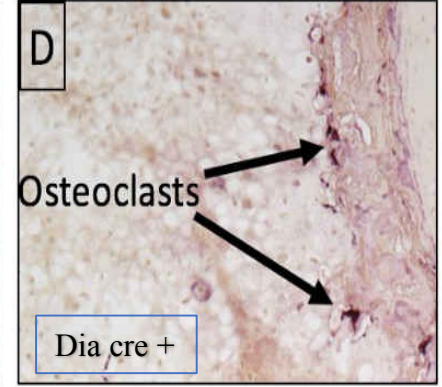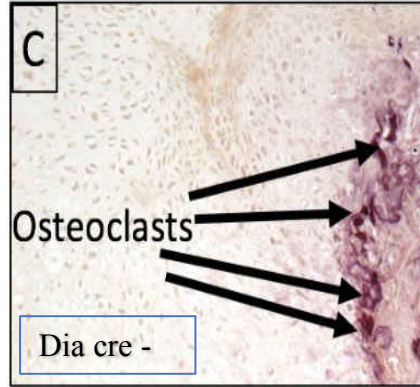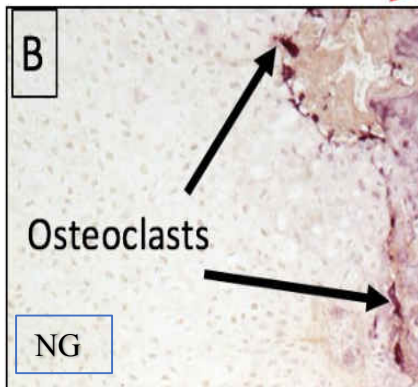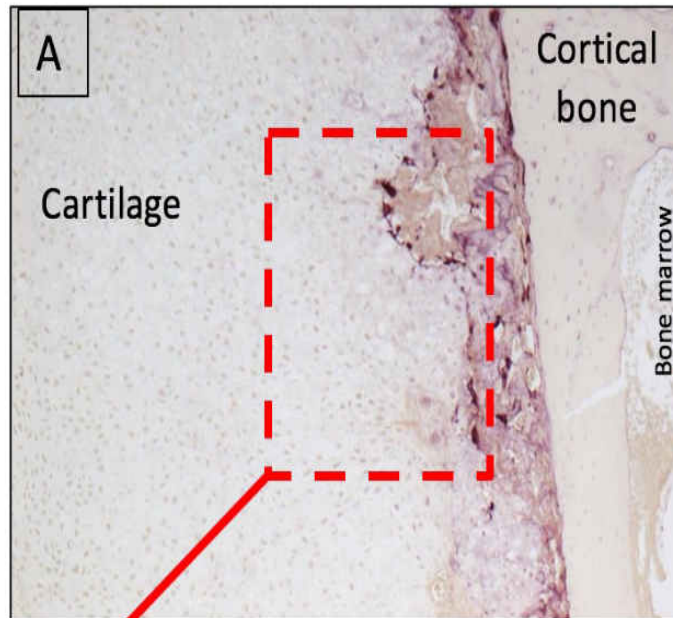
Figure 14: Diabetes significantly increases osteoclasts formation, FOXO1 deletion reduced the osteoclasts numbers to normal level.
Comparison of osteoclast numbers in **(A,B)** normoglycemic, **(C)**diabetic control, and **(D)** diabetic with FOXO1 deletion using TRAP stained sections. The obtained results were confirmed with cathepsin K IHC staining. Data are expressed as mean ± SEM. * indicates a significant difference between normal nd diabetic (P<0.05). + indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group (P<0.05).

In vivo RANKL:

To provide an insight into how FOXO1 expression by chondrocytes may regulates osteoclasts activation, quantitative analysis was performed to evaluate RANKL expression in chondrocytes by immunofluorescence using RANKL specific antibody compared to control IgG antibody. Diabetes led to 1.9-folds increased in the percentage of RANKL positive chondrocytes on day 10 and 1.7-fold increase on day 16 post fracture compared to the normoglycemic control group. On day 10 post fracture FOXO1 deleted group had 9 times less RANKL positive chondrocytes and on day 16 they had 23 times less RANKL positive chondrocytes compared to the diabetic group ($P<0.05$; Figure 15). Those results indicate that diabetes enhances chondrocytes RANKL expression in the fracture calluses and the expression was mediated by FOXO1.
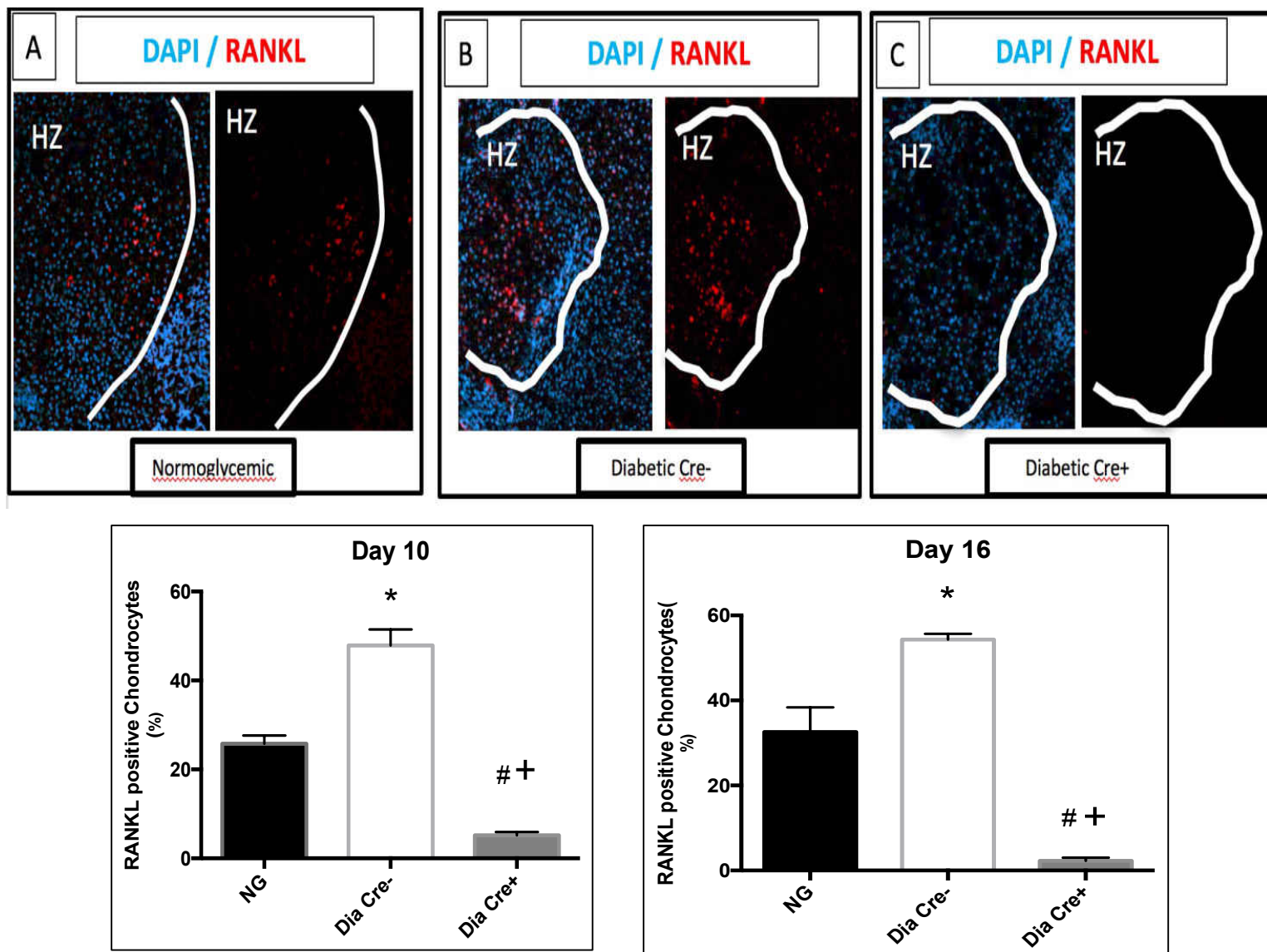
Figure 15: Diabetes increases chondrocytes' RANKL expression which is FOXO1 dependent. Immunofluorescence images from **(A)** normoglycemic, **(B)**diabetic control, and **(C)** diabetic with FOXO1 deletion using RANKL specific antibody to evaluate the Chondrocytes' RANKL expression in the fracture callus. **(D,E)** Quantitative data from day 10 and 16 post fractures. **(HZ) Hypertrophic zone.** Data are expressed as mean ± SEM. * indicates a significant difference between normal and diabetic (P<0.05). + indicates a significant difference between the diabetic animals with FOXO1 deletion and the diabetic control group (P<0.05). # indicates a significant difference

between the diabetic animals with FOXO1 deletion and the normal group. **
indicates a significant difference between the WT and FOXO1 KO.

In vitro RANKL expression :

To investigate whether FOXO1 regulates RANKL expression in chondrocytes under diabetic conditions, primary chondrocytes were isolated from experimental Col2α1Cre^{+}/FOXO1$^{L/L}$ and from control Col2α1Cre^{-}/ FOXO1$^{L/L}$ newborn mice. The cells were cultured and quantitative qPCR analysis of RANKL mRNA levels was carried out after serum stimulation and differentiation induction. Chondrocytes with FOXO1 deletion had 46% less RANKL mRNA level at base line, 37% less RANKL expression after 14 hours of serum stimulation, and 54% less RANKL expression after 4 days of hypertrophic differentiation induction.

Another set of isolated cells were incubated in high glucose and advanced glycation end product followed by immunofluorescence staining using RANKL specific antibody. Under high glucose condition, control group showed 62% increase in RANKL mean fluorescence intensity, FOXO1 deleted group showed 85% reduction in RANKL expression under low glucose and 69% reduction under high glucose treatment. Similar to the high glucose conditions, incubation with AGE led to 61% increase in RANKL expression in the control group compared to serum control incubation, FOXO1 deletion reduced RANKL expression by 82% under AGE incubation (Figure 15).
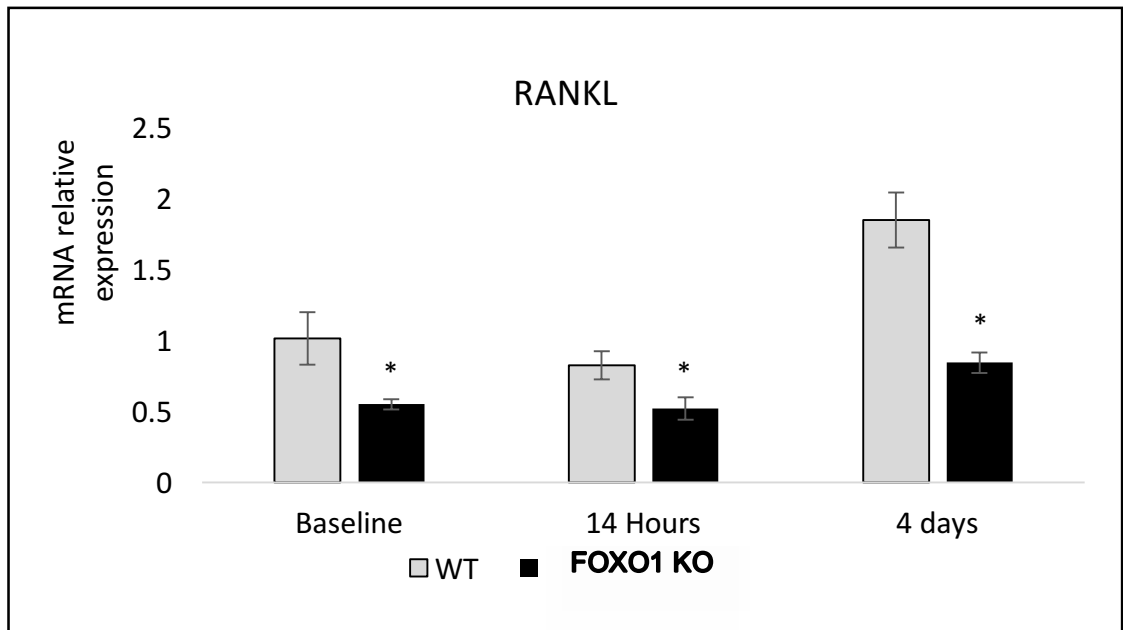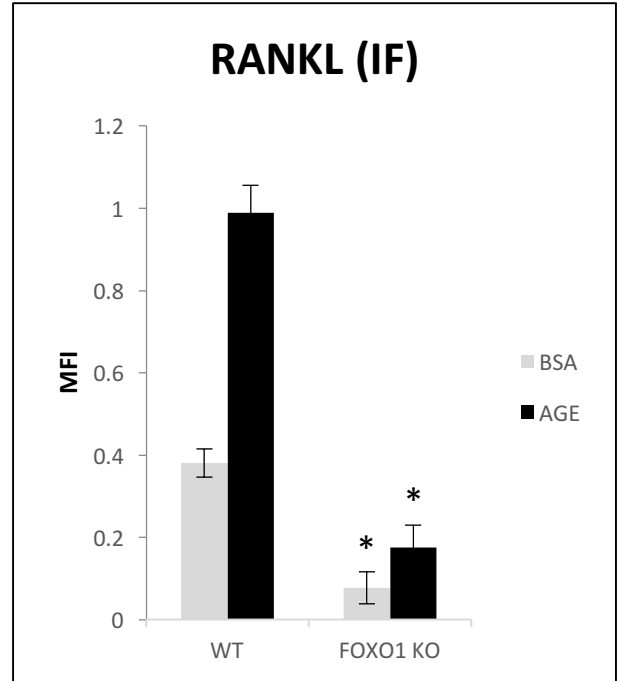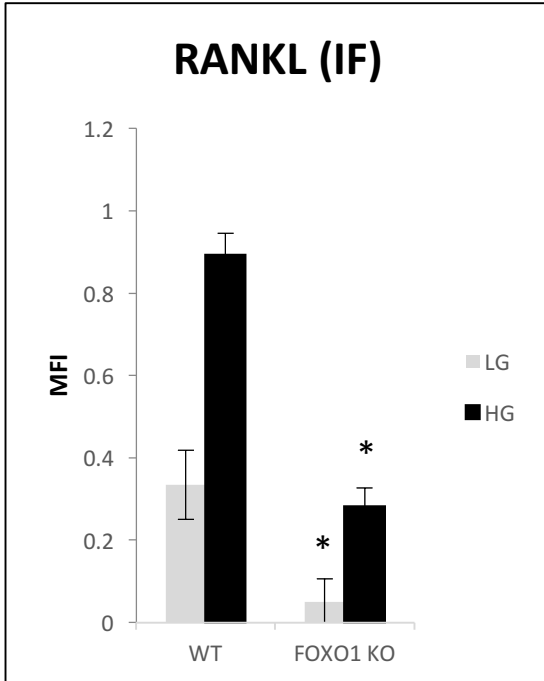
RANKL (IF)

RANKL (IF)

RANKL

Figure 16: *In vitro* **diabetic conditions increase chondrocytes' RANKL expression which is FOXO1 dependent** *In vitro* RANKL expression was also evaluated using primary chondrocytes, isolated from control mice (WT) as well as experimental FOXO1 deleted mice (FOXO1 KO), treated with low glucose (LG) and high glucose (HG) containing culture media. Another set of cells were treated with advanced glycation end product (AGE) or unmodified bovine serum albumin. To better understand how FOXO1 modulate osteoclast formation by regulating chondrocytes' RANKL expression. Primary chondrocytes precursor were isolated from *Col2a1Cre$^+$/FOXO1$^{L/L}$* (FOXO1 KO) and from the wild type (WT) *Col2a1Cre $^-$/FOXO1$^{L/L}$* stimulated with serum for 14 hours or cultured in chondrogenic media to induce chondrogenic differentiation for 4 days as described in the materials and methods sections. **(A)** Quantitative RT-PCR analysis of RANKL mRNA levels at different time points. Data are expressed as mean ± SEM. * indicates a significant difference between WT and FOXO1 deleted group using Student T test (P<0.05).

Luciferase reporter, and ChIP assays:

To verify the association and the possible regulation of RANKL by FOXO1, a luciferase reporter and ChIP assays were carried out. For the Luciferase reporter, ATDC5 cells were cotransfected with either FOXO1 plasmid (FOXO1ADA) or vector. TNF-α, AGE, and high glucose doubled the RANKL luciferase relative expression with FOXO1 plasmid compared to the vector control. (Figure 17).

In addition, Chromatin immunoprecipitation (ChIP) assays showed that FOXO1 interacted with the RANKL promoter under diabetic conditions. High glucose resulted in 10-fold enrichment of FOXO1 association with the RANKL promoter compared with control IgG precipitates. In addition, AGE resulted in 13-fold, and finally TNF-α resulted in 33-fold enrichment. (Figure 17).
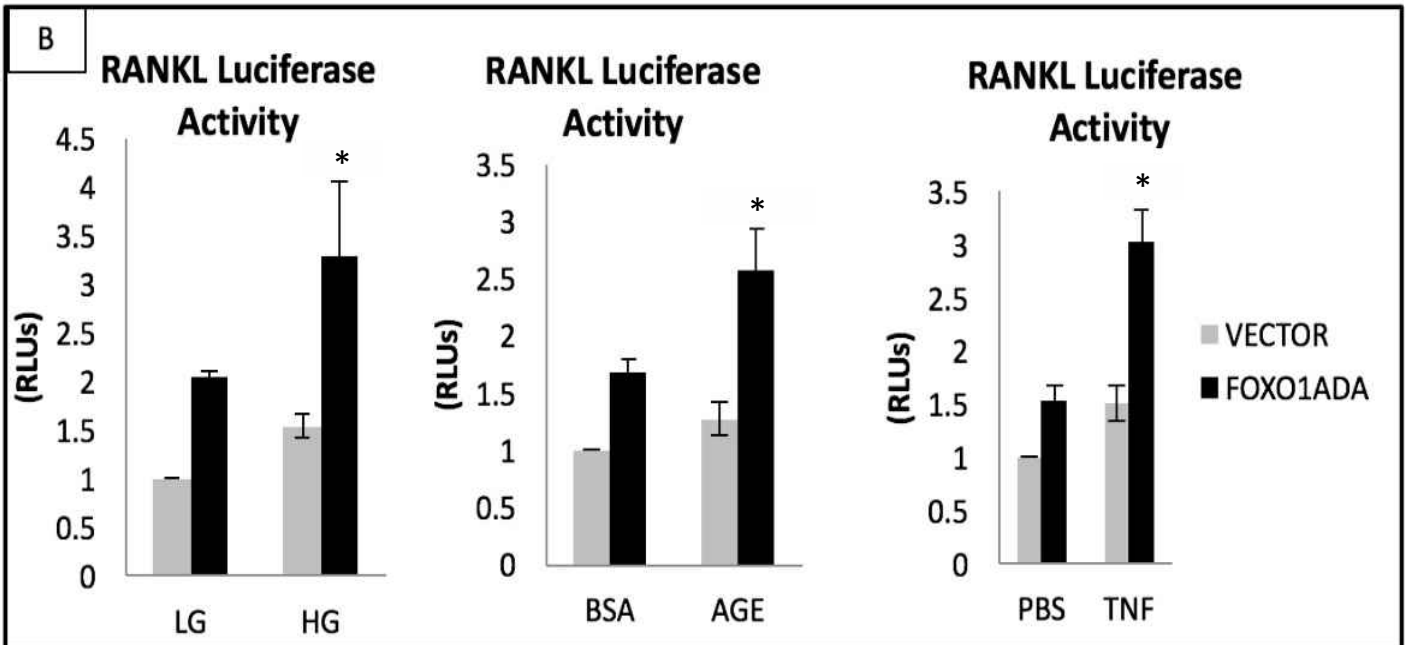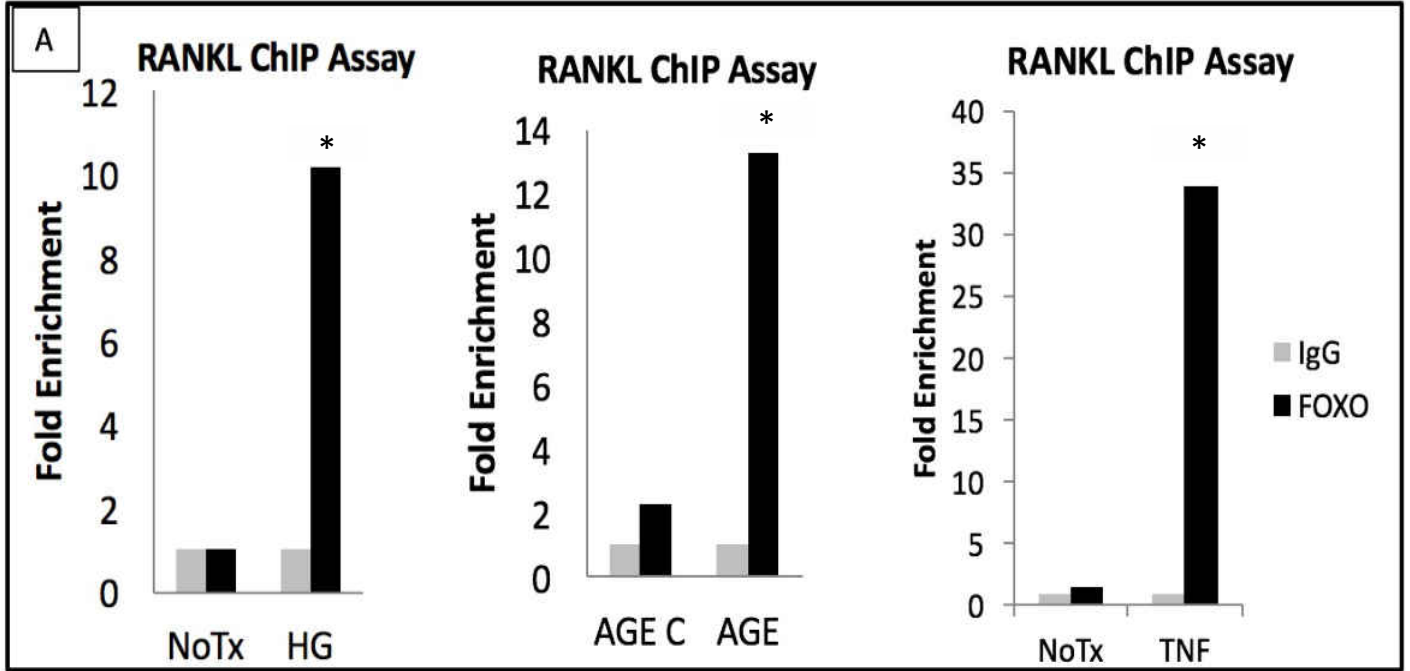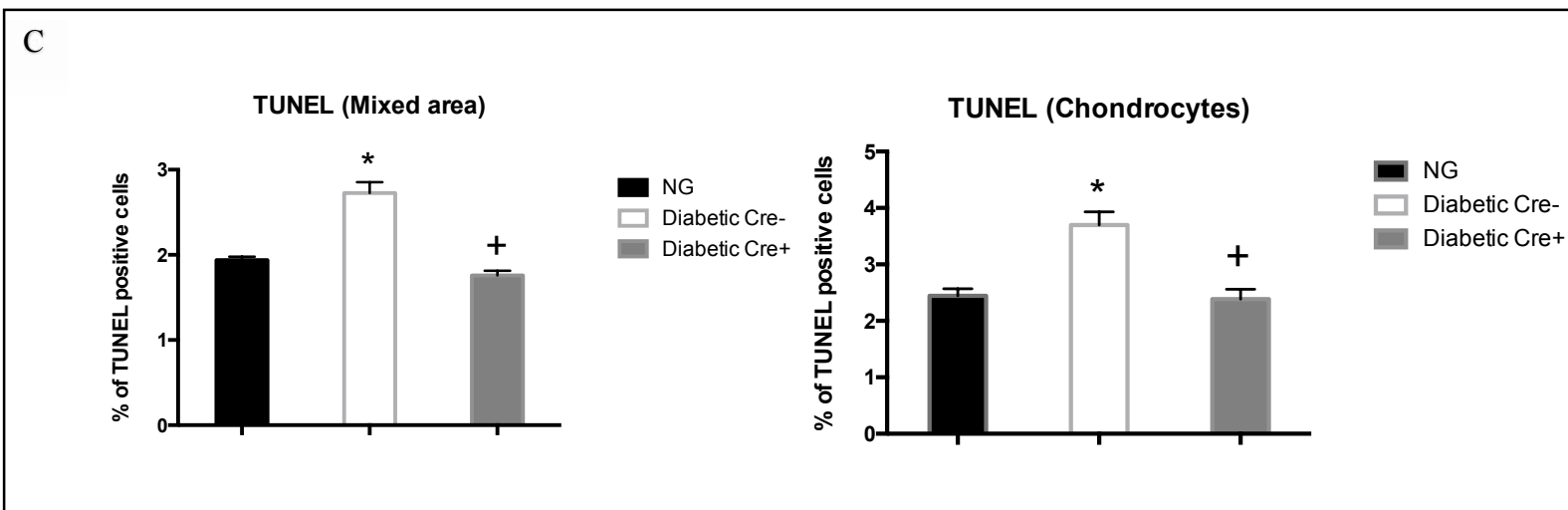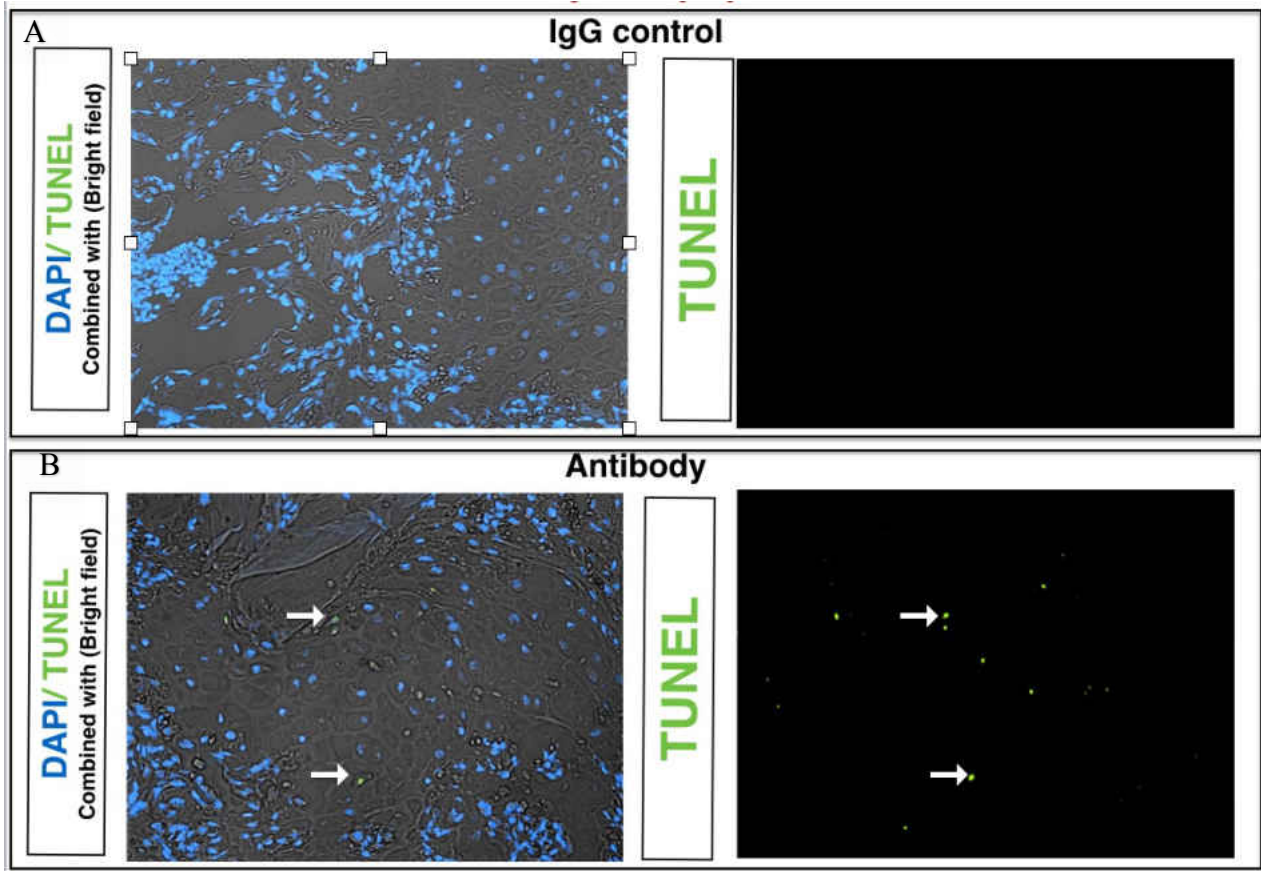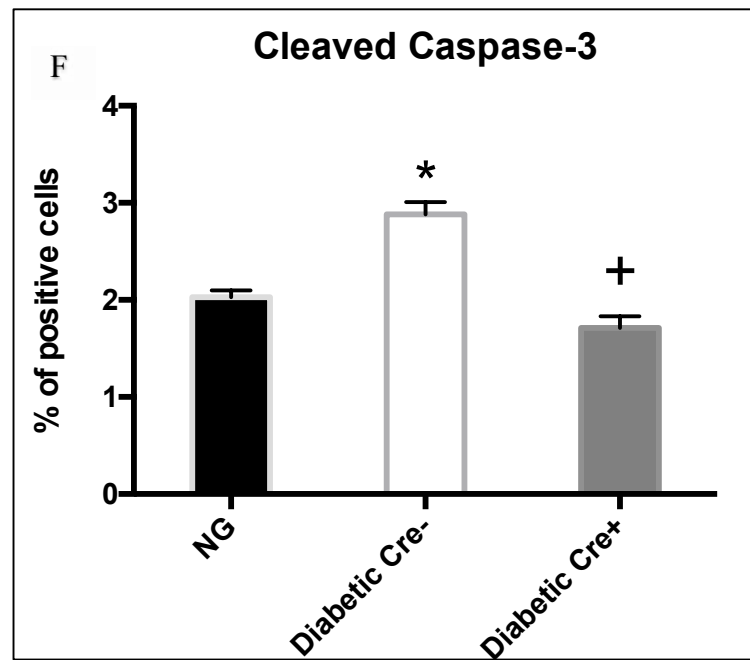
Figure 17: Chondrocytes' FOXO1 modulates diabetic fracture healing by regulating RANKL expression (A) The interaction of the RANKL promoter and FOXO1 was determined by quantitative real-time PCR of the pulled down chromatin using anti-FOXO1 IgG compared to matched control IgG. (B) RANKL luciferase reporter assay after cotransfection with control (vector) or FOXO1 plasmid (FOXO1ADA). Data are expressed as mean ± SEM. * indicates a significant difference between (IgG and FOXO1antibody), and between (control vector and the FOXO1ADA) using Student's T test (P<0.05).

Chondrocytes Apoptosis & cartilage degradation:

To test wither premature cartilage resorption was the only mechanism of early cartilage loss in diabetic animals, chondrocytes apoptosis was measured using TUNEL assay was conducted at day 16 post fracture. In addition, cleaved caspase-3 expression by chondrocytes was detected using immunostaining. Finally, ChIP and luciferase reporter assays were performed using ATDC5 cells under different *in vitro* diabetic conditions to study the association between FOXO1 and caspase-3 promoter. We found that the number of apoptotic chondrocytes was significantly higher in the diabetic group compared to the normoglycemic. FOXO1 deletion reduced the number of apoptotic chondrocytes to normal levels. Furthermore, diabetic animals has 50% more chondrocytes expressing caspase-3 compared to normal animals. FOXO1 deletion completely blocked the up-regulated expression of caspase-3 caused by diabetes. *In vitro* experiment shows that there is a significant increase in FOXO1 binding activities to caspase-3 promoter under high glucose treatment and AGE treatment compared to IgG control. Finally, the luciferase reporter assay shows that FOXO1 regulates caspase-3 expression under *in vitro* diabetic model.

IgG control

A

DAPI/ TUNEL
Combined with (Bright field)

TUNEL

Antibody

B

DAPI/ TUNEL
Combined with (Bright field)

TUNEL

C

TUNEL (Mixed area)

% of TUNEL positive cells

*

+

■ NG
□ Diabetic Cre-
■ Diabetic Cre+

TUNEL (Chondrocytes)

% of TUNEL positive cells

*

+

■ NG
□ Diabetic Cre-
■ Diabetic Cre+

IgG control
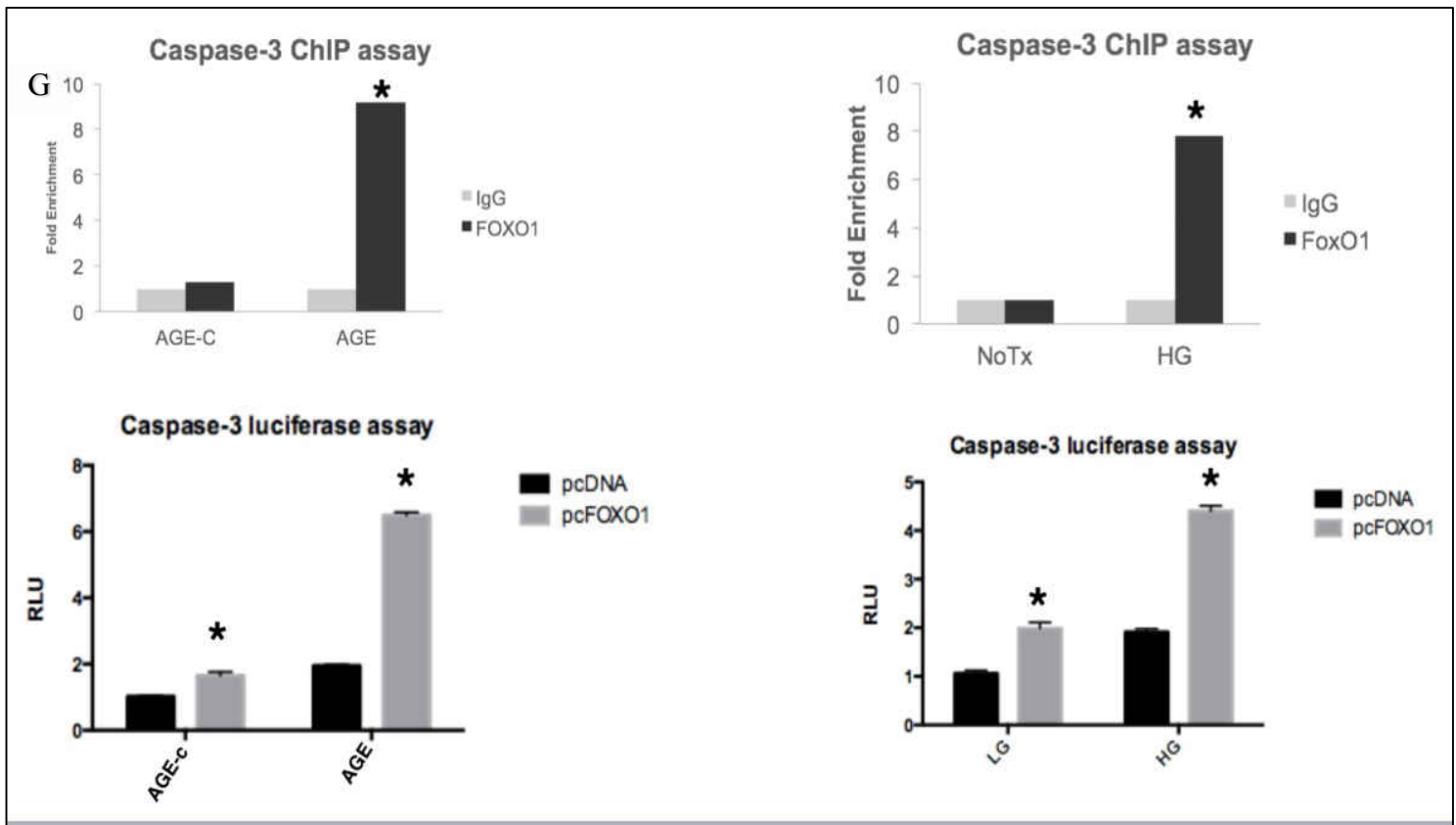
Antibody

Cleaved Caspase-3
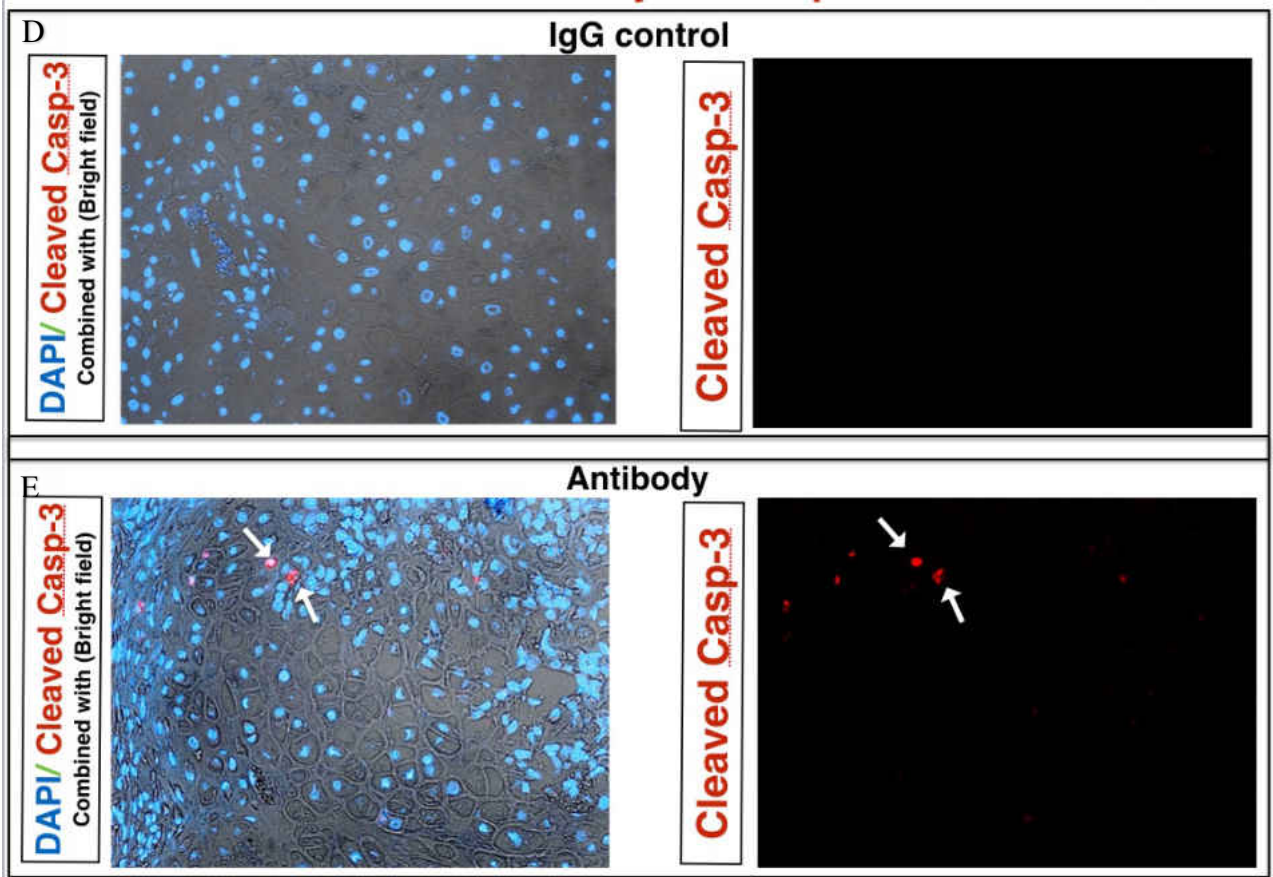
Figure 18: Diabetes significantly increases chondrocytes apoptosis and caspase-3 expression during fracture healing. FOXO1 deletion reduces both parameter to normal levels. (A) An immunofluorescence images of the IgG control. **(B)** An immunofluorescence images of the experimental group using TUNEL assay. **(C)** Quantified data showing that diabetic control mice has around 50% increase in apoptotic cells compared to normal group. FOXO1 deletion reduced it to normal levels. **(D)** An immunofluorescence pictures of the IgG control. **(E)** An immunofluorescence pictures of the experimental group using cleaved caspase-3 antibody. (F) Quantified data showing that diabetic control group has around 50% increase in caspase-3 expressing chondrocytes compared to the normal group. FOXO1 deletion rescued caspase-3 expressing cells to levels similar to the normal group. **(G)** ChIP and Luciferase reporter assay shows that both high glucose containing media (HG) and AGE increases FOXO1 binding activities to Caspas-3 as well as regulating its expression at the transcriptional level.

The role of FOXO1 in chondrocytes proliferation and differentiation:

The number of hypertrophic and proliferative chondrocytes was determined in the healing callus by counting the cells in Safranin-O stained sections. The number of cells was normalized by the measured area. FOXO1 deletion significantly increased the number of the proliferative, but not the hypertrophic, chondrocytes in the healing callus.
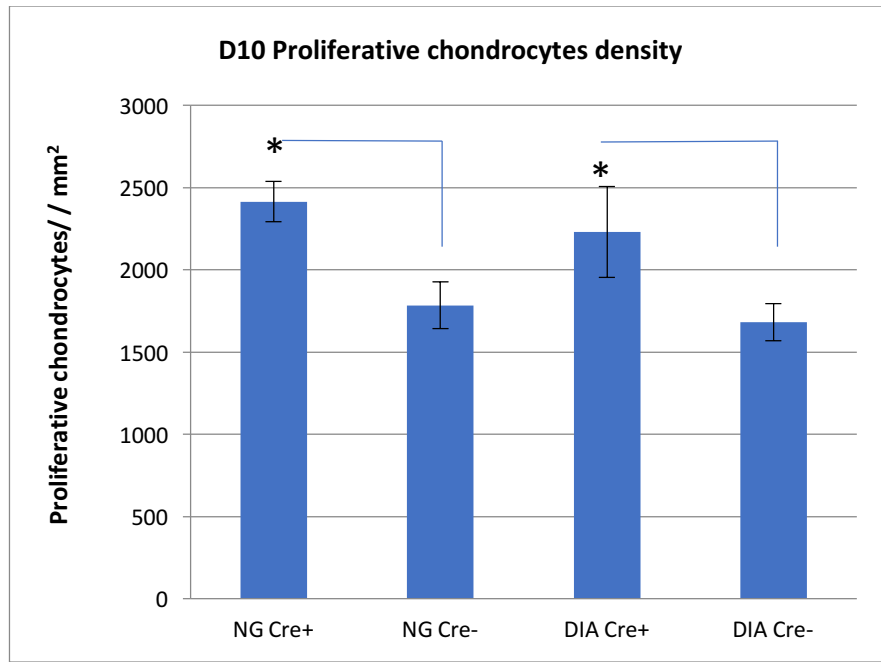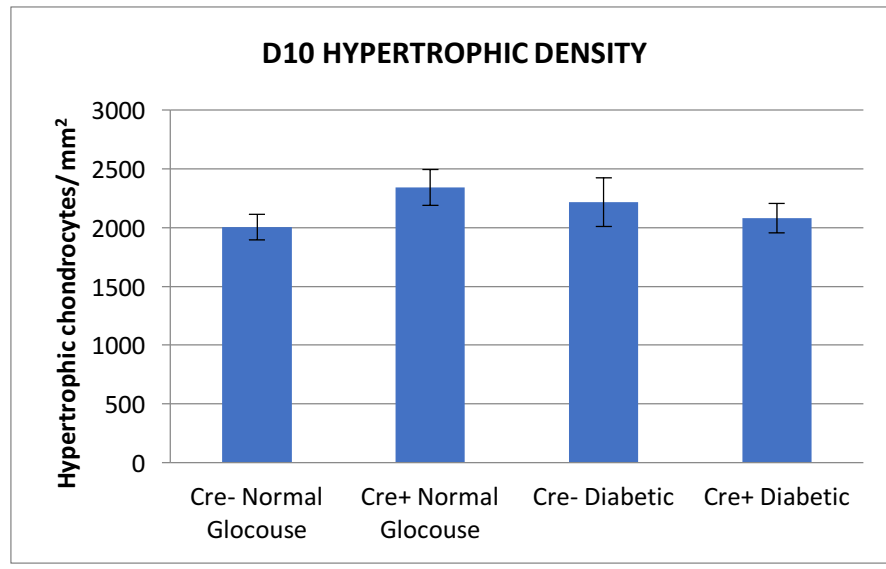
Figure 19: FOXO1 deletion increases the number of proliferative chondrocytes in the healing callus. (A) The quantified data shows that FOXO1 deletion increases the number of proliferative chondrocytes compared to the normal control, in addition, FOXO1 deletion in diabetic animals increased the proliferative count over the wild type diabetic control. (**B)** The number of hypertrophic chondrocytes was no statistically different between the study groups.

Primary chondrocytes proliferation curve:

WT and cKO cells were grown in DMEM with 5% FBS in 96-well plate pictures were taken for live cells during 0-4day and then fixed on the 5th day and stained with DAPI.
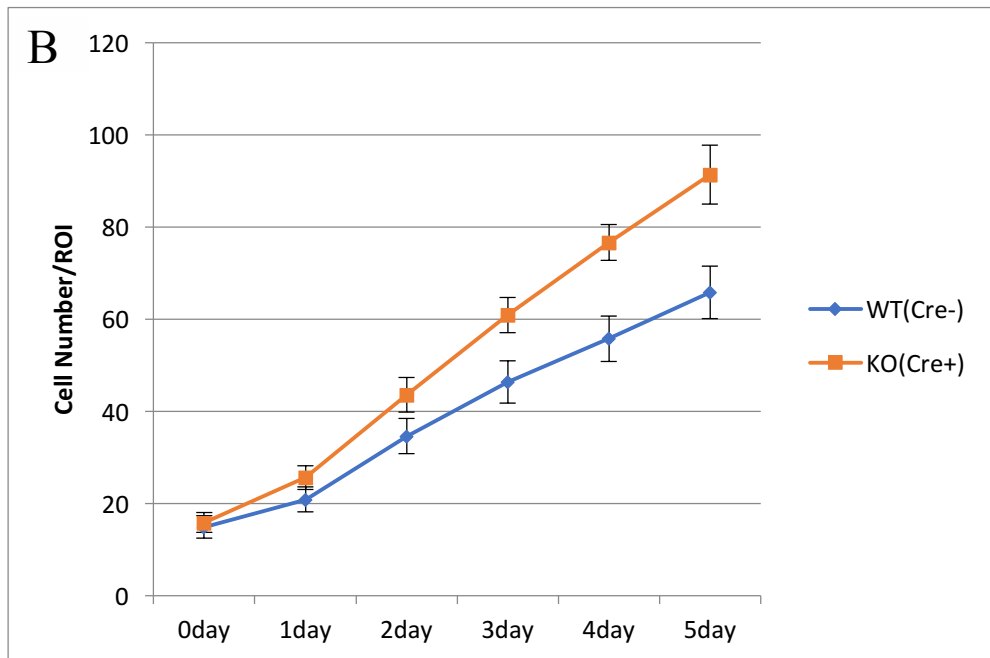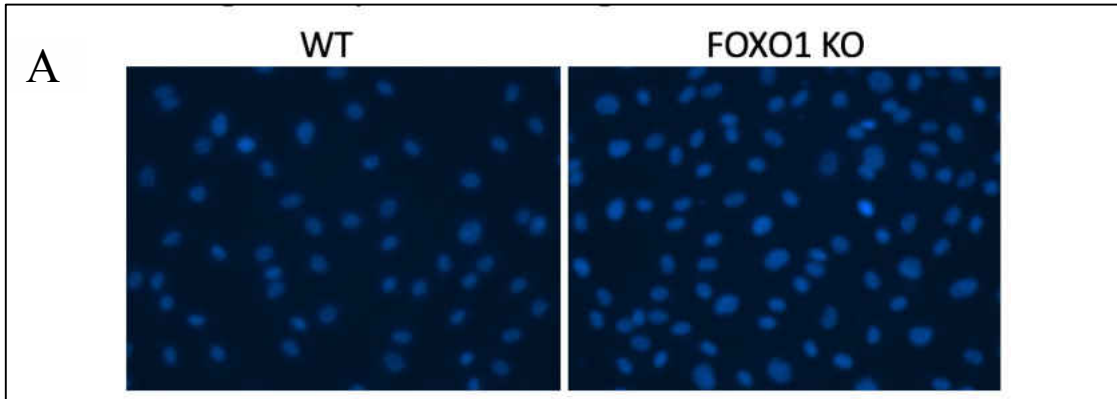
Figure 20: FOXO1 deletion enhances chondrocytes proliferation (A) An image of DAPI staining of primary chondrocytes isolated from both experimental (FOXO1 KO) and wild type control (WT) after 5 days in culture. **(B)** The proliferation curve of the primary cells shows a significant increase in cell proliferation upon FOXO1 deletion.

The expression of cell cycle genes in primary chondrocytes:

To better understand how FOXO1 deletion increases chondrocytes proliferation. Primary costal chondrocytes were isolated from both the experimental mice and wild type control. The expression of different cell cycle genes after 48 hours of cell starvation followed by 14 hours stimulation with 10%FBS was evaluated using quantitative real time PCR.
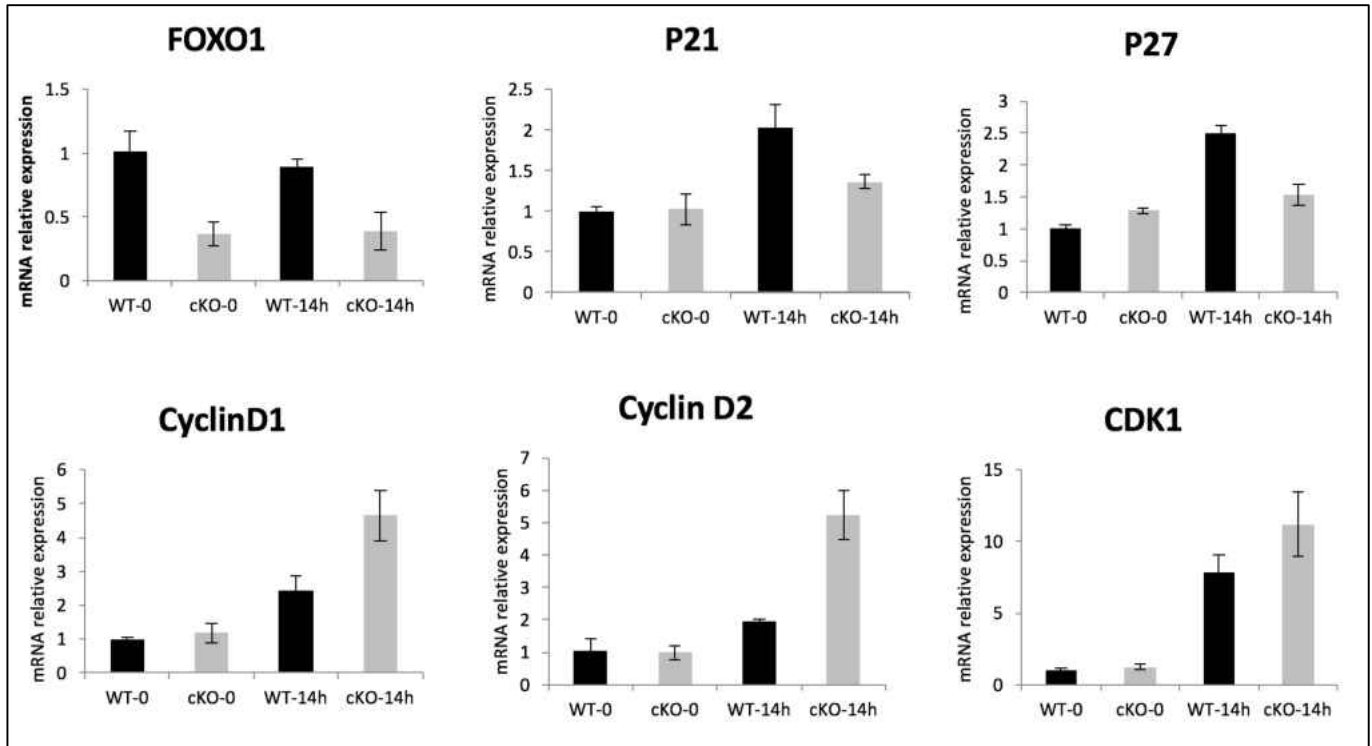
Figure 21: FOXO1 deletion enhances chondrocytes proliferation via regulating cell cycle genes expression. qPCR results show the effect of FOXO1 on cell cycle gene expression. FOXO1 deletion decreases the expression of p21, p27 which are a potent down-regulator for cells proliferation. In addition, FOXO1 deletion significantly up-regulated CD1, CD2, and CDK1, which are critical phosphorylation of variety of proteins, leads to cell cycle progression.

The role of FOXO1 in chondrocytes differentiation:

Primary costal chondrocytes from experimental mice and wild type control were isolated, cultured, and differentiated with differentiation medium (growth medium supplemented with 50 μg/ml ascorbic acid and 0.5 mM NaH2PO4). mRNA levels of differentiation genes were detected using qPCR. Alcain Blue staining was used for another set of cell to detect the matrix formation at different time points 0day, 4days, 7days, and 14days. The results show that FOXO1 deletion has no effect on chondrocyte differentiation or matrix formation ability.
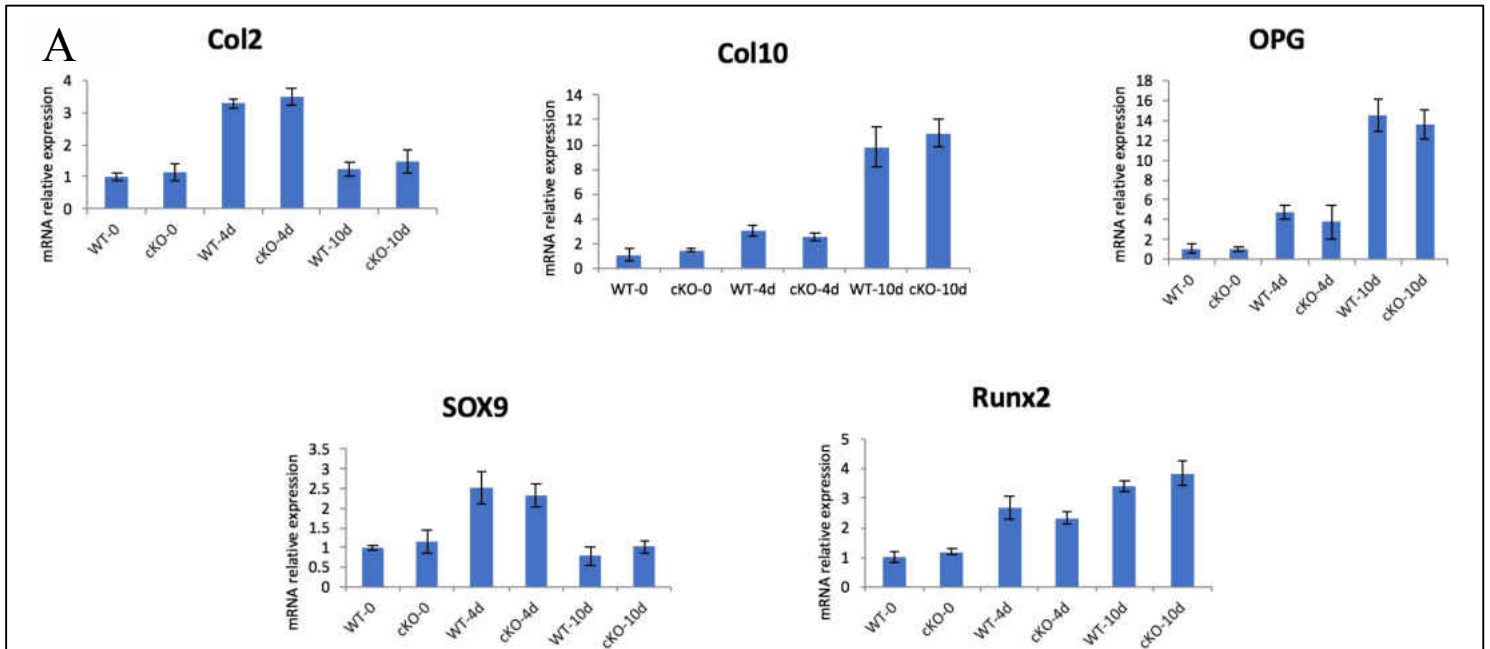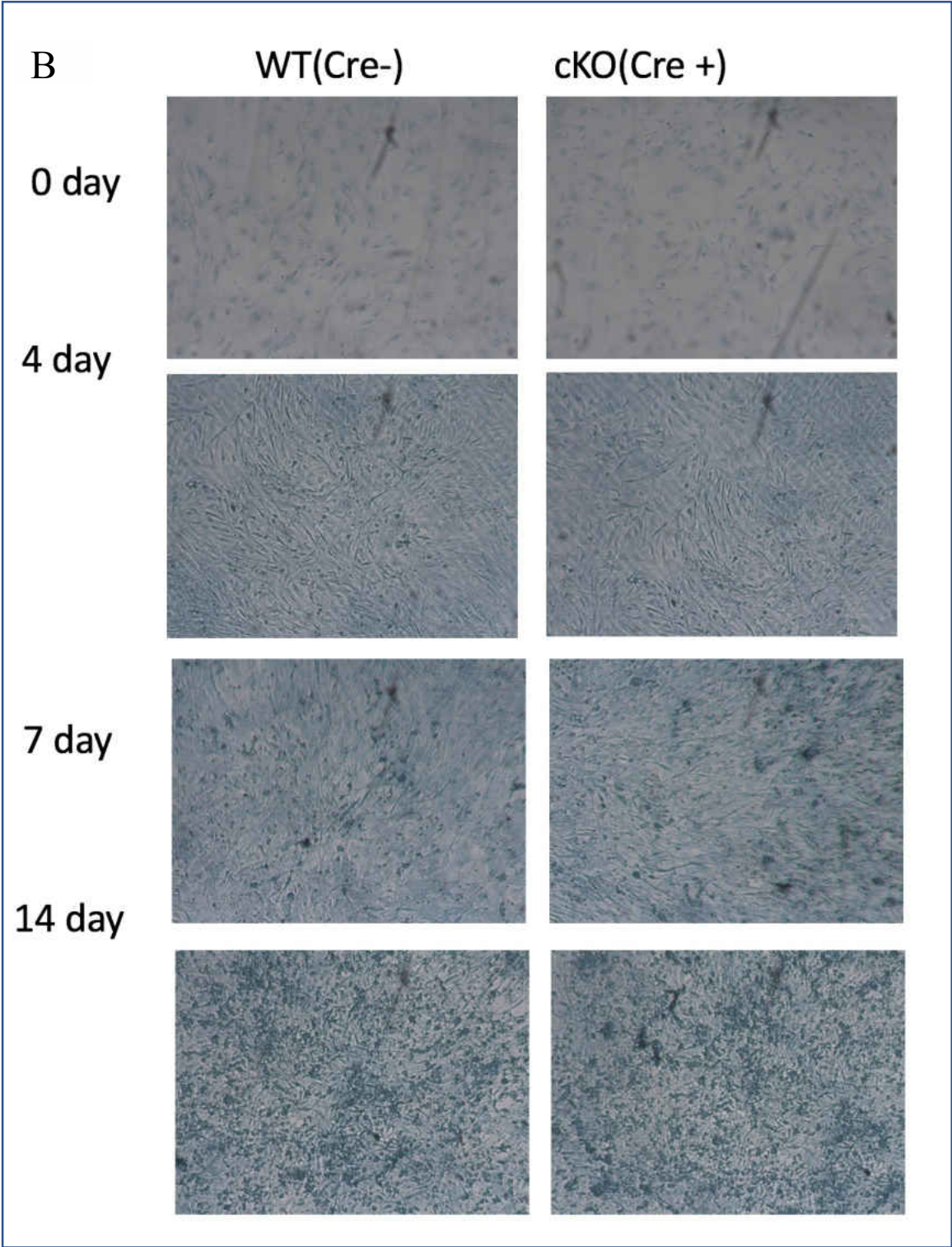
Figure 22: (A) qPCR results show that FOXO1 deletion doesn't alter the expression of different known differentiation markers. (B) Alician blue staining at different time point for both the experimental and the control group that shows similar matrix forming abilities. (C) Quantified data from the odometer shows comparable absorbance at all the studied time points.

Discussion

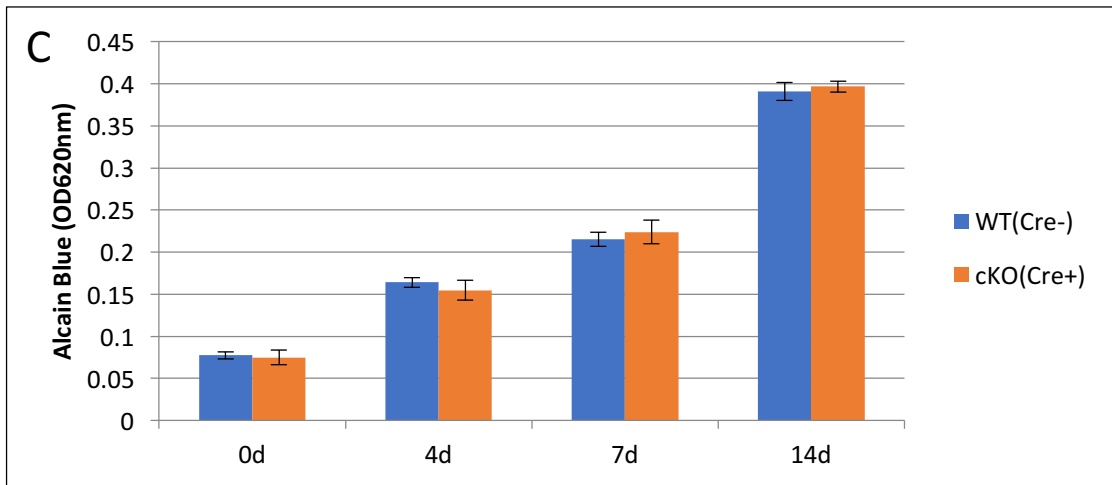Diabetes is a significant disease with many complications, particularly if left uncontrolled. It has been reported that diabetes negatively affects the skeleton integrity and it impairs fracture healing process (Clarke, 2010; Saller, Maggi, Romanato, Tonin, & Crepaldi, 2008). STZ induced diabetes significantly affected the calluses in the current investigation. There was a significant reduction in the cartilage area at the transitional time point, which resulted in a significantly smaller callus volume and smaller bone volume within the healing callus. Several parameters were compared by Botolin and McCabe between STZ induced diabetic mice and non-obese diabetic (NOD) mouse model which develops type 1 diabetes mellitus spontaneously. Both animal models showed comparable bone character, and they both showed a reduction in bone trabeculation with increased marrow adipocytes. They also showed a similar reduction in osteocalcin mRNA in the tibia. (Botolin & McCabe, 2006). Furthermore, BB rats, which develop type 1 diabetes spontaneously, exhibited a comparable decrease in serum osteocalcin levels, bone mineral density, and mechanical properties to STZ induced diabetic mice (J Verhaeghe et al., 1989; J. Verhaeghe et al., 1990). Those findings validate the effect of multiple low dose STZ diabetic model on bone.

The mechanism of how diabetes impairs the fracture healing is not entirely understood. It has been previously reported that the diabetic animals

had significantly smaller calluses than those in normal animals. Those investigations attributed the reduction in size to decreased cartilage and bone formation (Gooch, Hale, Fujioka, Balian, & Hurwitz, 2000; Topping, Bolander, & Balian, 1994; Tyndall, Beam, Zarro, O'Connor, & Lin, 2003). In our investigation, there was no significant difference in the cartilage areas of the study groups 10 days post fracture, which suggests that diabetes doesn't significantly alter the cartilage formation phase. This finding was consistent with what was reported by Kayal et al, in regards to the expression of collagen I, II, or X which wasn't significantly different between normal mice and diabetic controls which suggests that the overall anabolic rate of chondrocytes or osteoblasts is similar at that stage.

Cartilage is critical for fracture healing because it acts as a stabilizer for the fractured bony segments maintaining their close proximity. It also acts as a template for bone deposition as well as providing essential cytokines, chemokines, and growth factors. In the current study, diabetic animals exhibited significantly smaller cartilage areas at day 16, which is a critical time point for the transition of the healing callus from cartilage to bone, compared to normal animals. The cartilage areas were restored to normal levels upon FOXO1 deletion in the diabetic animals. The histology and micro CT of both day 16 and 22 in this study showed the impact of premature cartilage resorption caused by diabetes on the fracture healing. Diabetic animals showed significantly smaller calluses and bone volumes compared to normal

animals, which was consistent with the finding of Kayal et al. and Alblowi et al. FOXO1 deletion in diabetic animals rescued both the callus and the bone volumes to normal levels. We can attribute those changes to the significantly smaller cartilage area in the diabetic mice at the transitional time point. Naik et al. showed that there was delayed fracture healing in old mice and it was associated with smaller cartilage areas in comparison to the young mice (Naik et al., 2009). Besides, insulin resistant mice with impaired fracture healing exhibited premature chondrocyte differentiation which resulted in smaller cartilage areas and subsequently smaller calluses (Shimoaka et al., 2004). On the other hand, FOXO1 deletion from chondrocytes in diabetic animals in our study resulted in the maintenance of the cartilage at the transitional time and led to a better overall healing assessed both radiographically and histologically.

Inflammation is essential for normal long bone fracture healing but it is very important to be critically regulated temporally and spatially. The first stage of the healing process involves an inflammatory response which is critical for attracting cells and releasing certain molecules essential for the progression of the healing process. Furthermore, inflammation is also critical at the late bone remodeling phase which is highly regulated by the expression of IL-1 and IL-6 (Kon et al., 2001). Kayal et al. reported that these cytokines were elevated at the transitional time point (day 16) which is the period of cartilage resorption, not the bone remodeling. This confirms the uncontrolled inflammatory status of

the diabetic calluses compared to normoglycemic and FOXO1 deleted mice. It is well established that inflammation is associated with increased osteoclast recruitment and bone resorption (Kindle, Rothe, Kriss, Osdoby, & Collin-Osdoby, 2006; Yu, Huang, Collin-Osdoby, & Osdoby, 2004). Many studies have reported that diabetes was associated with increased osteoclastogenesis. Suzuki et al. reported that patients diagnosed with DM had higher TRAP serum level in comparison to healthy individuals. They have also indicated that those diabetic patients had higher concentrations of Type 1 Collagen Cross-linked Carboxy-terminal Telopeptide in their urine which confirms the increased resorption rate. (Suzuki et al., 2005). Maes and their group have shown that the cartilage was not effectively removed when the number of osteoclasts decreased during endochondral ossification (Maes et al., 2006). The opposite was observed in the current investigation, the diabetic animals had higher osteoclasts and cathepsin K positive cells lining cartilage consistent with the accelerated cartilage resorption observed in the diabetic animals. Our lab has also previously reported that there was an increase in TNF-α, MCSF, and RANKL in diabetic fracture healing (Kayal et al., 2007). These cytokines have been shown to induce the differentiation of bone marrow hematopoietic precursor cells into bone-resorbing osteoclasts (Cappellen et al., 2002). It has been shown that diabetes upregulates the TNF-α related protein (TRAIL). TRAIL enhances chondrocyte apoptosis and it blocks osteoclast inhibition effect of OPG (Emery et al., 1998). Furthermore,

TNF-α stimulates the osteoblast expression of both MSCF and RANKL which will indirectly activate the osteoclastogenesis (Cenci et al., 2000; Kaplan, Eielson, Horowitz, Insogna, & Weir, 1996). Our lab previously examined the mechanism of how diabetes accelerates cartilage resorption through mRNA profiling and GSEA during the cartilage resorption stage of fracture healing. We have found that diabetes upregulated couple of TNF-α dependent inflammatory pathways which were associated with activation and maturation of osteoclasts (Alblowi et al., 2009). Those inflammatory pathways were mediated in part by FOXO1. FOXO1 deletion from chondrocytes in the current investigation resulted in the reduction of the number of osteoclasts lining cartilage as well as cathepsin K positive cells. This reduction of osteoclastiogenesis resulted in preserving the cartilage for a longer time to set the stage for bone deposition.

MMP13 is one of the matrix metalloproteinases superfamilies. Its active enzyme is collagenase 3 which is secreted in its inactive form. It gets activated when its pro-domain cleaved. It has been reported that MMPs are involved in the breakdown of the extracellular matrix of cartilage and, to a lesser extent, bone, which can be explained by the superior ability of this gene to cleave type II collagen more efficiently than types I and III. Kayal et al. have previously reported that there was no difference between normoglycemic and diabetic animals in mRNA levels of MMP13 at the transitional time point (day 16), which is highly expressed during fracture healing and plays a major role in

cartilage remodeling (E. R. Lee, 2006; Yamagiwa et al., 1999) This finding might indicate that the quality of the cartilage was not affected by diabetes and the increased osteoclastic activities were the dominant mechanism explaining the accelerated cartilage lost observed in diabetic animals.

It has been also reported that different inflammatory cytokines are involved in osteoclast activation. However, RANKL/OPG ratio was suggested to have the most and the final influential effect. (Hofbauer & Heufelder, 2001). The accelerated cartilage resorptions in the current investigation may be explained mechanistically by the increased number of osteoclasts in the healing callus which was directly proportional to the expression of RANKL by chondrocytes. Diabetic animals had almost 2 times more chondrocytes expressing RANKL in the healing callus compared to the normoglycemic control group. There was almost complete shutdown of RANKL expressions by chondrocytes upon FOXO1 deletion at all time points which indicate that RANKL expression was mediated by FOXO1 in the fractured callus. Gerstenfeld et al. also showed an increase of the residual cartilage in the healing callus when inflammation was inhibited by non-selective anti-inflammatory drugs (L. C. Gerstenfeld et al., 2007). Thus, the retention of the cartilage by FOXO1 deletion in diabetic animals can be mechanistically explained by the reduction in the up-regulated inflammatory signals caused by diabetes, the reduced inflammatory status rescued the osteoclasts numbers to normal levels

The mechanism of diabetes and the lack of insulin during fracture healing has been controversially discussed in the literature. Our lab has previously shown that treating diabetic animals with insulin restored normal fracture healing on both tissue and molecular levels. Since insulin treatment resulted in significantly larger calluses and bone areas compared to non-insulin treated diabetic animals. In addition, the osteoclastic activities, as well as tissue degradation markers were all significantly reduced to normal level upon insulin treatment. This was suggestive that the defect in healing process noticed in diabetes was a direct result of hyperglycemia. The anabolic effect on both bone and cartilage has been proposed by many groups. (Thrailkill et al., 2005). The negative impact of diabetes on the healing calluses were completely blocked by Gandhi and colleagues upon injecting insulin locally to the fractured bone (Gandhi, Beam, O'Connor, Parsons, & Lin, 2005). Nevertheless, local insulin delivery might be mediating its effect via upregulating IGF-1 activities, which is a potent stimulator for both bone and cartilage formation and does not necessarily represent the physiologic conditions (Irwin et al., 2006). Our results are also in agreement with this finding since both the diabetic control animals and the diabetic mice with FOXO1 deletion both lacked insulin, however the reduction in the inflammatory status mediated through FOXO1 deletion restored the impaired healing to a normal level.

The direct relationship between BMD and the mechanical loading on the skeleton is well established. The focus of the current investigation was directed toward better understanding and studying the effect of T1DM on bone. Individuals with T1DM are usually associated with nutritional deficits, low body weight, and lower BMD. In contrary to TIDM, women and men diagnosed with T2DM usually have normal to high BMD in comparison to matched non-diabetic individuals. (Rakic et al., 2006; van Daele et al., 1995). People with lower Body Mass Index (BMI) have a higher risk of developing osteoporosis in addition to their increased fracture risk compared to matched peers. The increased BMD in patients with T2DM could be explained as a biomechanical adaptation of their body to the higher load they are subjected to since patients withT2DM have higher lean mass and higher weight compared to matched control. This biomechanical strain can induce bone formation in patients with T2DM.(Travison, Araujo, Esche, Beck, & McKinlay, 2008). As mentioned earlier, insulin is considered a potent anabolic factor for cartilage and bone formation, the hyperinsulinemic states observed in individuals with T2DM can also enhance the bone formation and will lead to increased BMD (Barrett-Connor & Kritz-Silverstein, 1996; Thrailkill et al., 2005). Adults diagnosed with T2DM are at higher risk of fracture despite their higher BMD. The mechanism is yet not fully understood. Diabetes is associated with a state of constant and chronic multisystem disruption. The changes in homeostatic

regulation might have a detrimental effect on bone and skeleton. The upregulation in the AGE's could disturp the collagen cross-linking and alter the bone structure (Saito & Marumo, 2010; Viguet-Carrin et al., 2006; X. Wang, Shen, Li, & Agrawal, 2002). Furthermore, bone turnover cycles might also be affected which will alter the microstructure of the bone marrow. Altered marrow might negatively impact the proliferation of MSC and their capability to differentiate into bone forming osteoblasts rather than adipocytes. It is well known that multiple factors regulate differentiation and maturation of MSC into its committed lineage(Sethe, Scutt, & Stolzing, 2006; S. Zhou et al., 2008). The swings in anabolic, catabolic, hormonal, and inflammatory status associated with diabetes could negatively impact MSC's integrity which will increase the fragility of bones.(Cramer et al., 2010; Kassem, 1997; Kume et al., 2005) Thus, the bone mineral density should not be used solely to assess the fracture risk associated with diabetic patients.
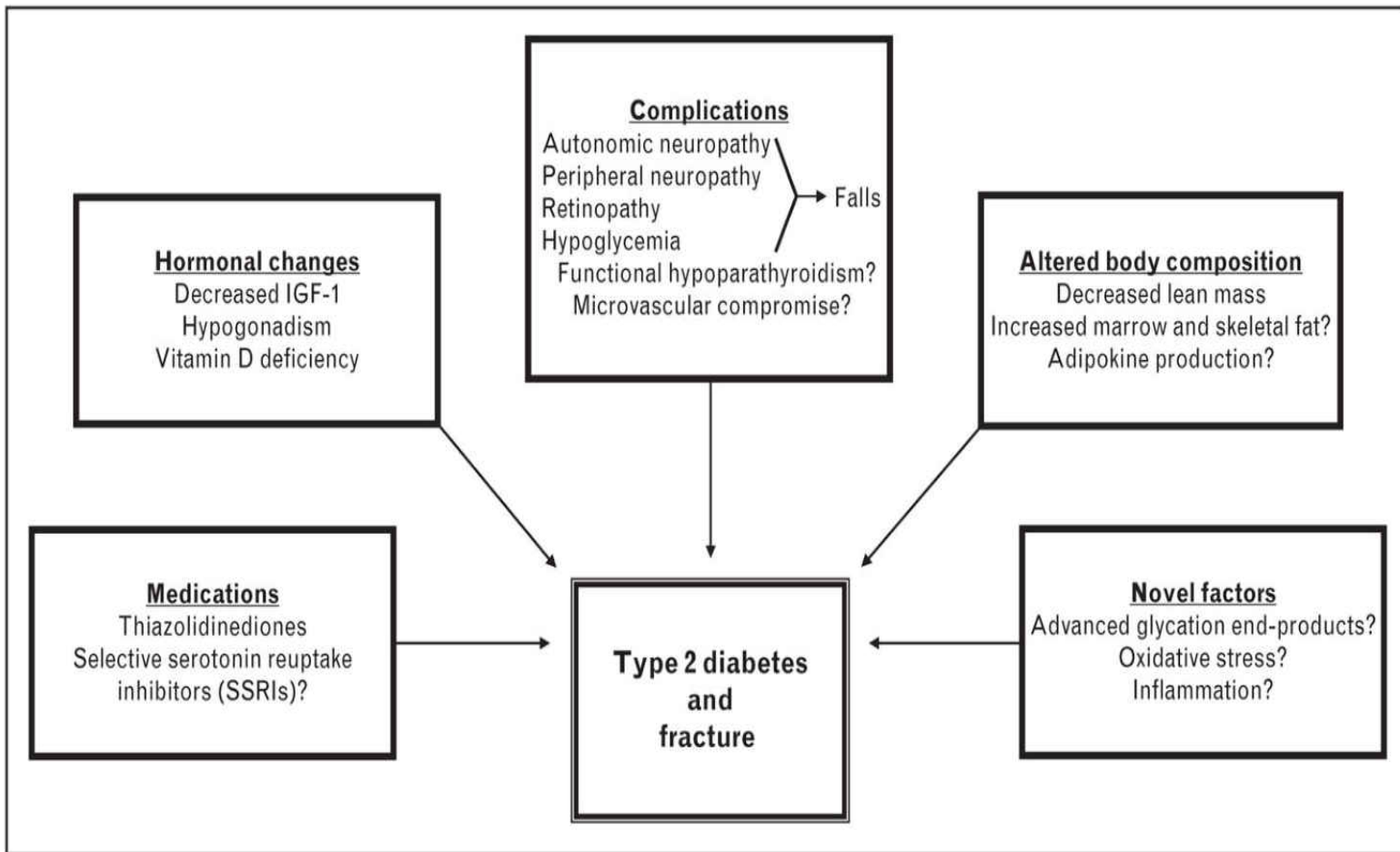
Figure 23: Possible mechanisms of how type 2 diabetes might increases bone fragility and fracture risk.

Long bones are not the only bones associated with increased

osteoclastogenesis, but also the alveolar bone, which supports the teeth in the

mouth, had a higher resorption rate associated with diabetic animals

compared to normal animals (Kohsaka, Kumazawa, Yamasaki, & Nakamura,

1996; Villarino, Sanchez, Bozal, & Ubios, 2006). There was an upregulation in

TNF-α, IL-1β, and the chemokine monocyte chemotactic protein 1 (MCP-1) in

the serum and gingival crevicular fluid of patients with diabetes and they were

associated with more severe periodontitis. Ligature-induced periodontitis is a

well-established model to study periodontal disease. In this model, diabetic

mice had a stronger and prolonged inflammation associated with increased

osteoclastic activities, larger eroded bone area, reduced bone formation (Liu et

al., 2006). Upregulated osteoclastogenesis was shown on a molecular level in

a diabetic periodontal animal model. (Mahamed et al., 2005).

Apical periodontitis (AP) is an inflammatory lesion which surrounds the roots of

the teeth. It is caused by microbial invasion to the root canal system. The

primary goal of any Endodontic therapy is the prevention or the treatment of

AP. The prevalence of individuals with AP associated with at least one tooth

was reported be around 40-50%.(Saunders & Saunders, 1998). It has been

reported that diabetic animals develop larger AP than non-diabetic controls.(A.

Fouad, Barry, Russo, Radolf, & Zhu, 2002). In addition, human studies

indicated a delayed healing of the AP lesions in diabetic individuals compared

to normal individuals.(A. F. Fouad & Burleson, 2003). The mechanism of how

diabetes facilitates the development of AP or how it retards the healing has not been thoroughly investigated. This study might open the door to study possible different pathways and mechanisms using an STZ-induced diabetic animal model. The role of FOXO1, RANKL, and TNF-α in the development and healing of AP in diabetes will be my primary research interest in the near future.

CONCLUSIONS:

❖ Diabetes has a significant effect on fracture healing by enhancing premature cartilage removal via up-regulating the osteoclastic activities.

❖ Premature cartilage resorption resulted in smaller calluses and bone volume at later time points.

❖ Chondrocytes are a major source of RANKL which was FOXO1 dependent.

❖ Lineage specific FOXO1 deletion down-regulated the osteoclastogenesis in diabetic animals to normal levels and the premature loss of cartilage was completely reversed.

❖ We demonstrated, for the first time, that all the negative impacts from diabetes on callus size and bone formation were completely blocked when the cartilage was maintained to the right time. Furthermore, this is the first time it has been shown that FOXO1 modulates RANKL levels, which led to alteration in the up-regulated osteoclastogenesis.

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Supplemental Experiments

The role of FOXO1 deletion in the development and the maturation of the growth plates:

To study if the cartilage phenotype observed during fracture healing in the diabetic is specific to the cartilage forms during the healing process and not other types of cartilage, the growth plates of 7 days post-natal mice as well as adult mice were studied using Safranin/O fast green stain. The adult joint spaces were also scanned using high resolution micro CT to evaluate the bone volume and the total growth plate volume.

Cre -   Cre +
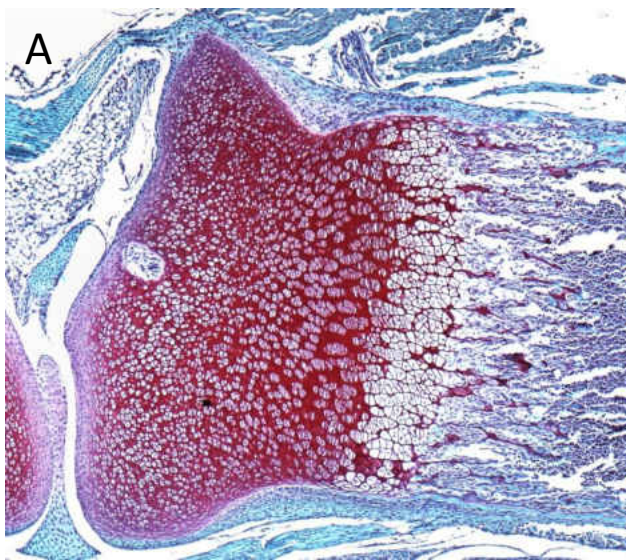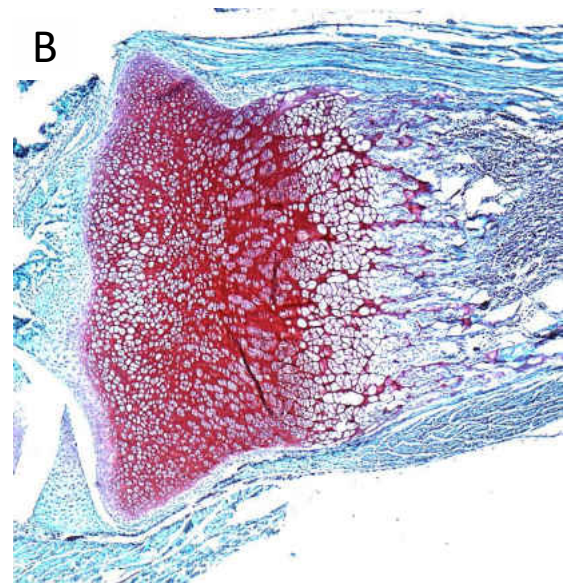
C 7days chondrocytes' zones of the tibial growth plate
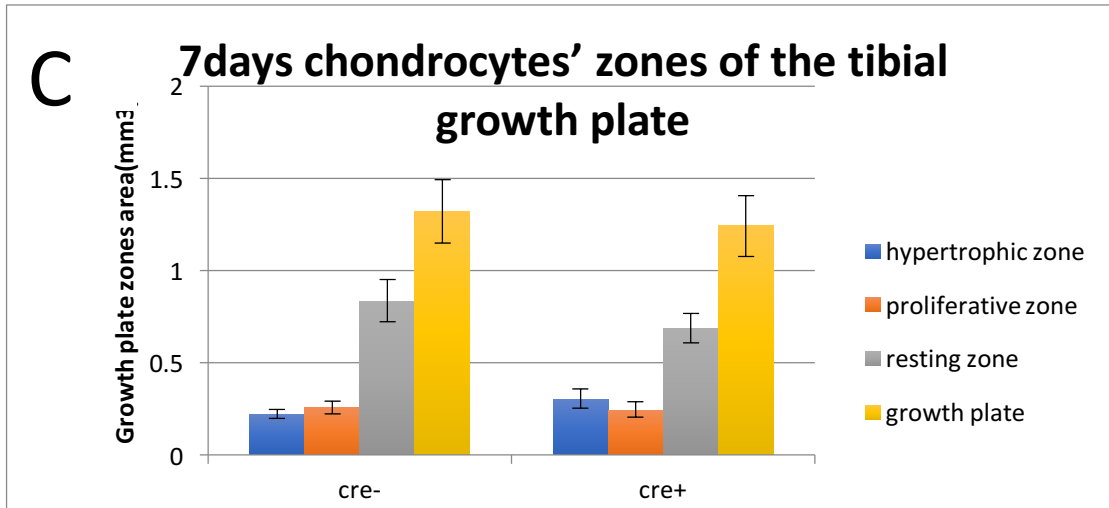
Supplemental Figure 1: (A,B) Safranin/O staining of a representative samples from both normal and FOXO1 deleted neonate. **(C)** FOXO1 deletion didn't change the growth plates zones.

A

Low magnification
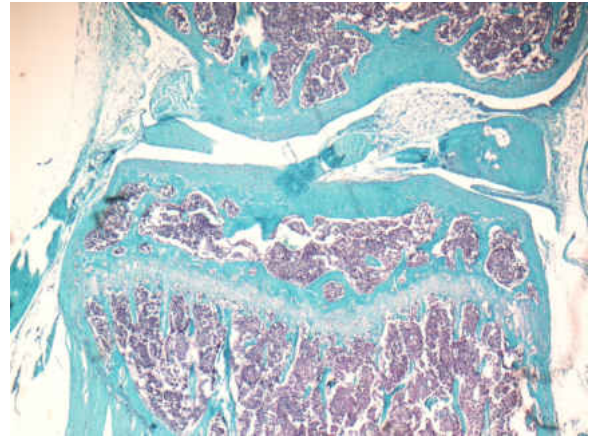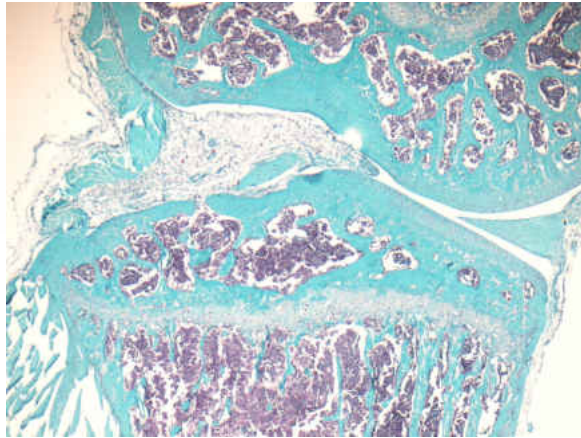
High magnification

Cre -   Cre +

B

Cre -   Cre +

C

TV

BV

BV/TV

BMD

D

TV

BV/TV

BMD
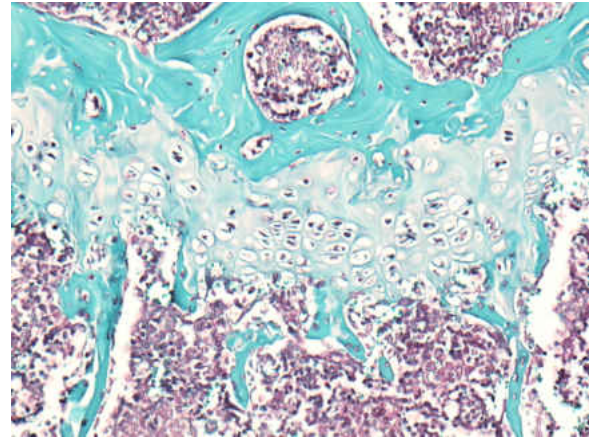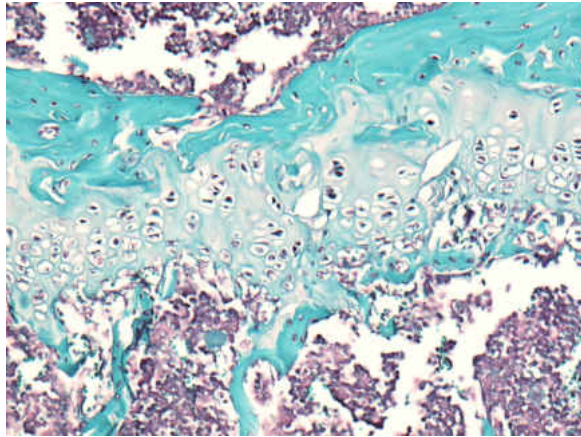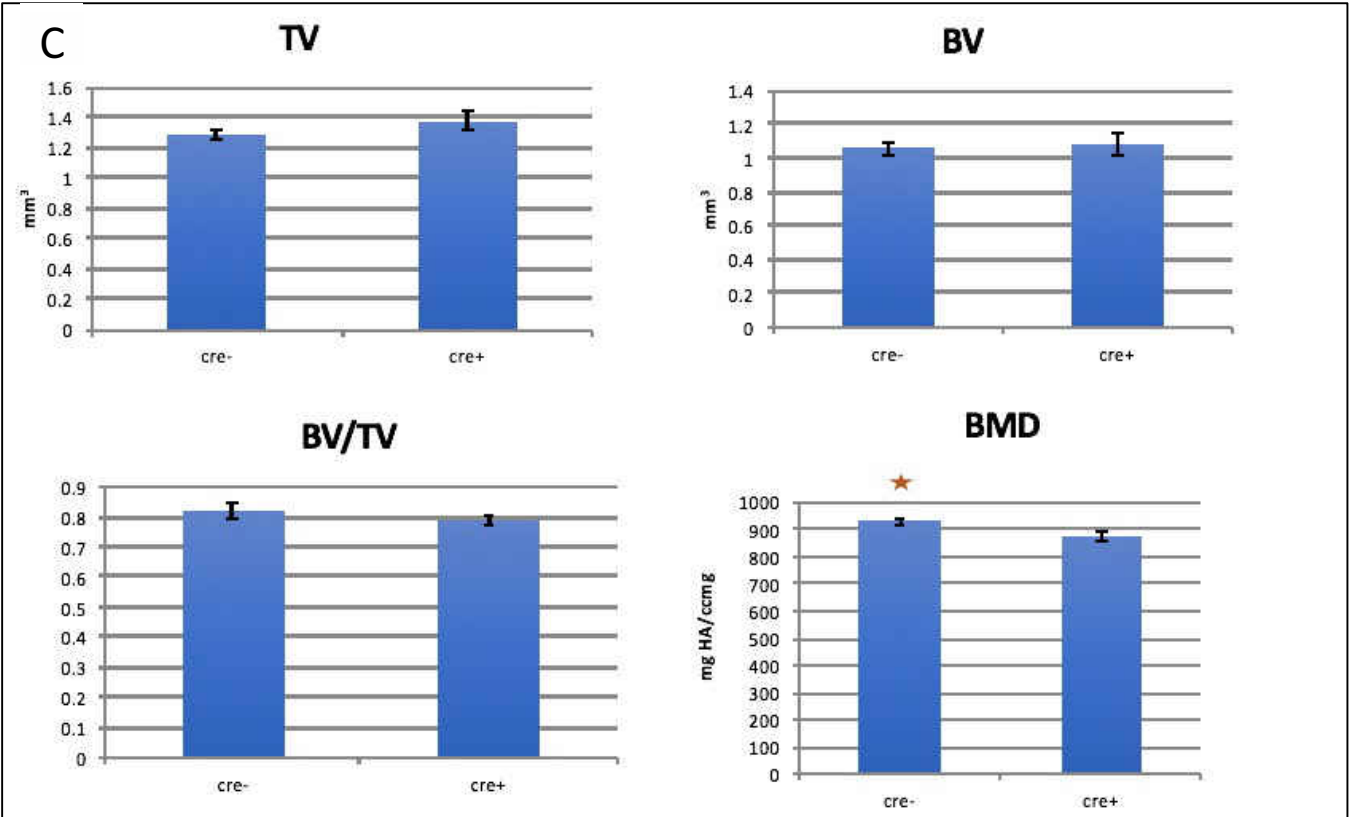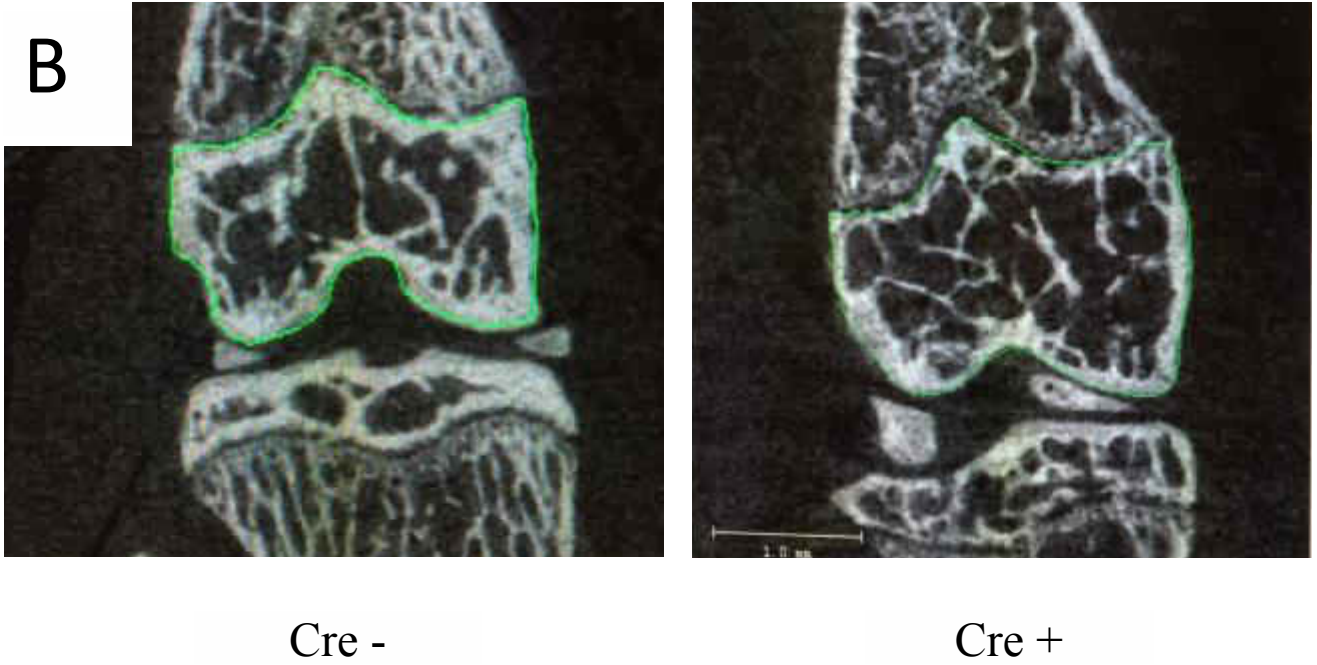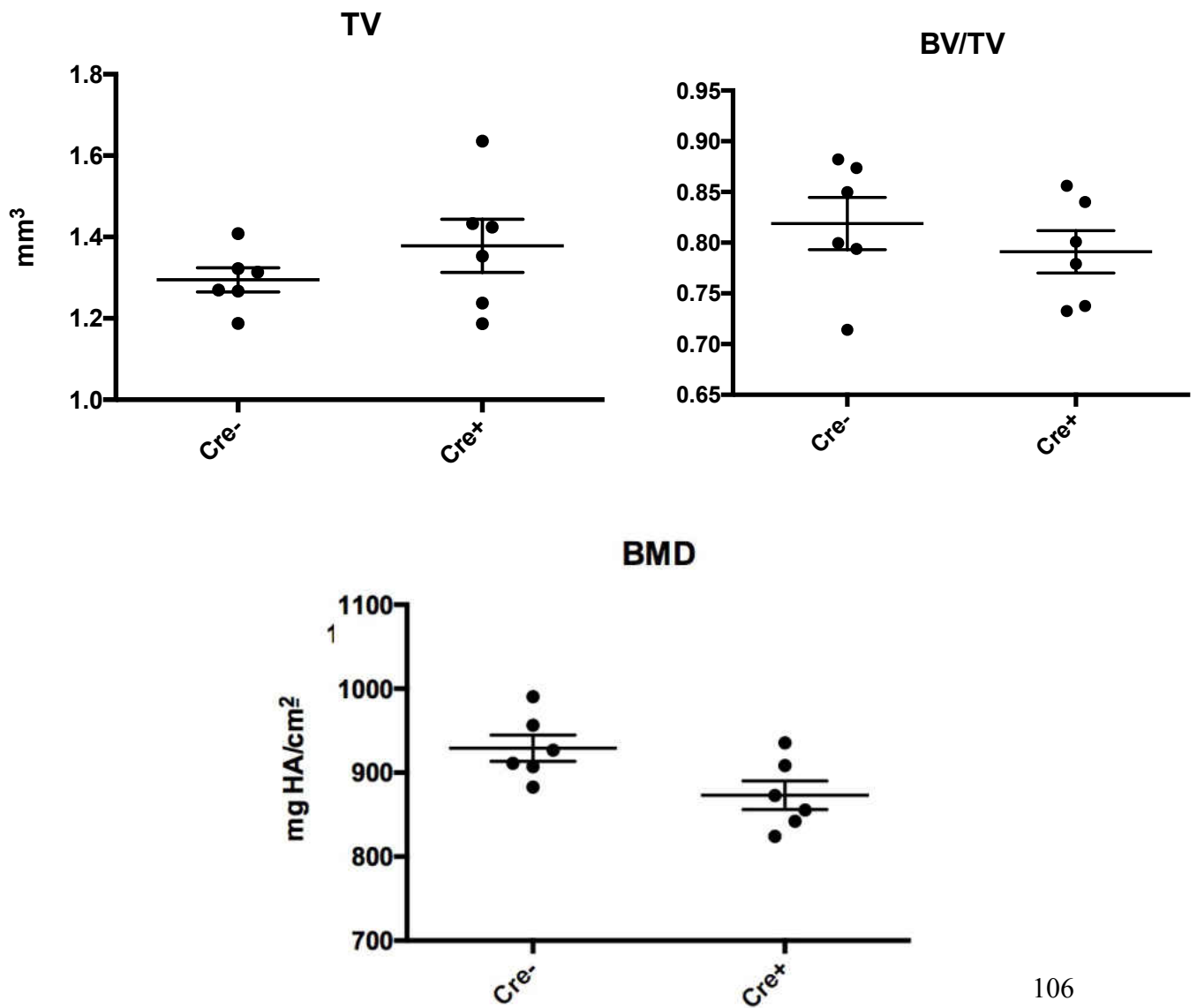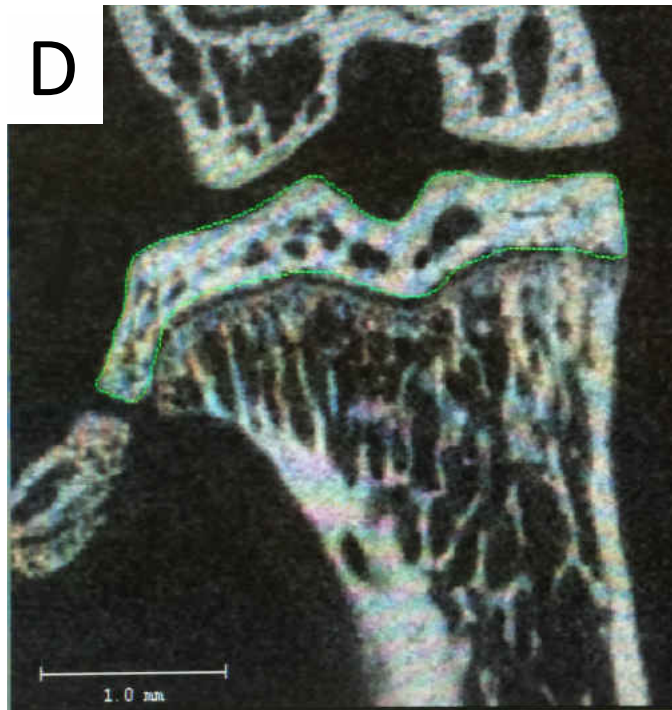
Supplemental Figure 2: (A) Safranin/O staining of a representative samples from both normal and FOXO1 deleted adult growth plates. The images shows no difference in the orientation of the chondrocytes and the size of the growth plates between both groups **(B)** Micro CT images for femoral heads that shows comparable sizes between the WT and FOXO1 deleted mice. **(C)** Quantified data showing no differences in the total volume (TV) and bone volume (BV) **(D)** Tibial plateau µ CT image and quantified data shows no significant difference between the study groups.

Ongoing experiments

Collaborative work with Dr. Dodge:

  To study the effect of FOXO1 deletion on the mineralization of cartilage, paraffin imbedded sections from day 10 post fracture were stained with Masson's Trichrome, Alician blue, and Picrosirius Red. Quantification of the stained sectioned will be carried on to study the differences between the groups.
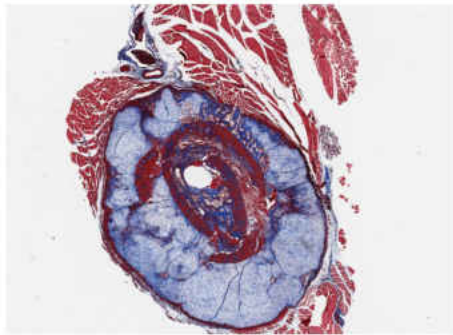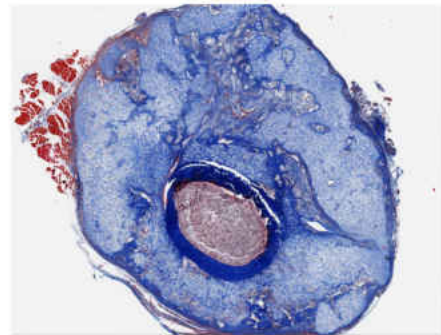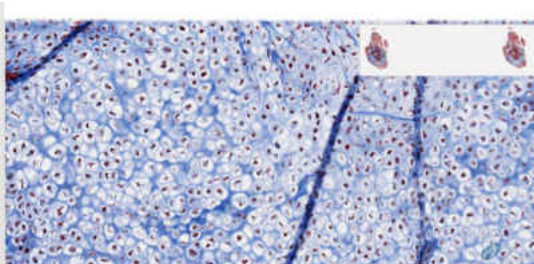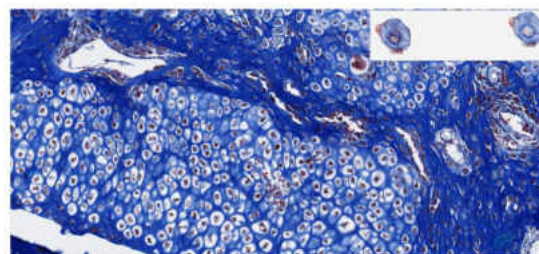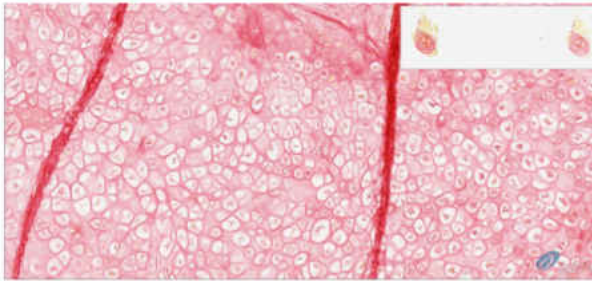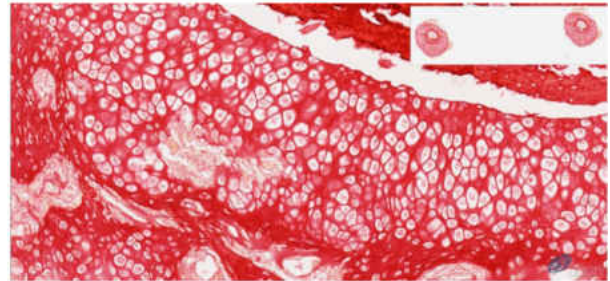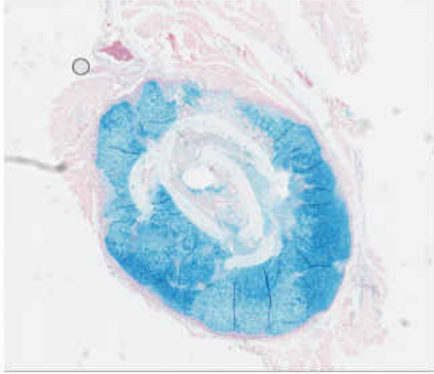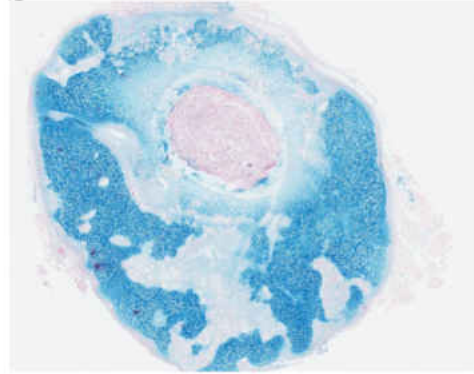
Picrosirius Red
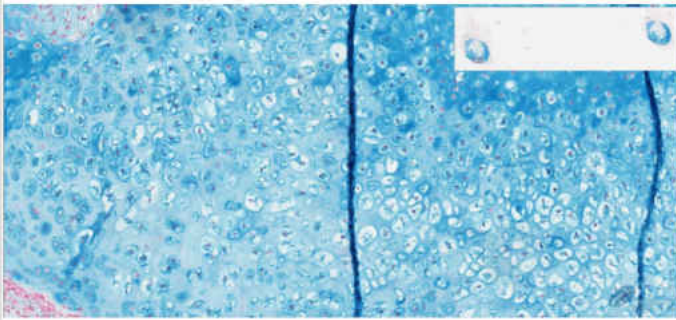

WT control

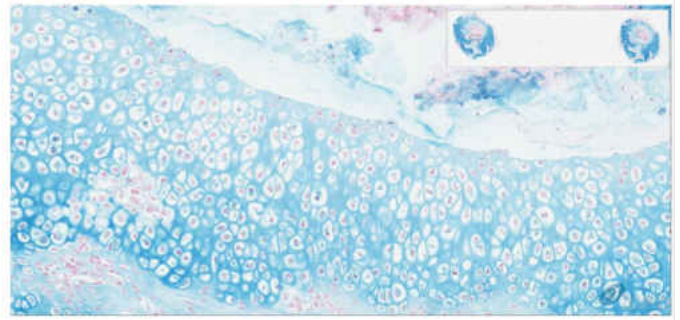FOXO1 deleted

20x WT control

FOXO1 deleted

Alcian blue

WT control

FOXO1 deleted

20x   WT control

FOXO1 deleted

Supplemental Figure 3: Fracture slides from day 10 stained with **(A)** Masson's Trichrom **(B)** Picrosirius Red **(C)** Alcian blue.

RNA sequencing:

RNA was isolated from fracture calluses from 8 different study groups. NG WT and FOXO1 deleted, Diabetic WT and FOXO1 deleted on both day 10 and day 16 post fracture the RNA was sent for RNA-Seq using next generation sequencing. In addition, RNA was isolated from both day 7 and day 13 from NG WT and FOXO1 deleted mice to look at the temporal effect of FOXO1 deletion on cartilage maturation and degradation.

Furthermore, primary chondrocytes isolated from WT control or FOXO1 deleted mice were cultured and treated under different condition to study the role of FOXO1 in chondrocytes proliferation, differentiation, apoptosis, and angiogenesis under both normal condition and high glucose cultured media. RNA was isolated from those cells and sent for RNA-Seq using next generation sequencing.

Chondrocytes trans differentiation:

An in Vivo study is taking place to evaluate the chobdrocytes – osteoblast trans differentiation and wither; 1- diabetes alter this process, 2- FOXO1 mediate the trans differentiation process involved in the process. Linage specific labeling was achieved using ROSA tomato reporter mouse under the effect of Col2 promoter.

FOXO1 activation under high glucose and AGE conditions:

To better understand how high glucose regulates FOXO1 activations. ATDC5 murine chondrogenic were seeded and hypertrophic differentiation was induced using DMEM/F12 media supplemented with 50µg/ml ascorbic acid and 10mM β-glycerophosphate. Cells were incubated in high glucose containing media with the use of the following; insulin, AKT inhibitor, TLR4 inhibitor, Sirt-1 inhibitor, HDAC inhibitor, N-Acetyl-L-cysteine, and L-NAME hydrochloride. Another set of cells were incubated with AGE or unmodified BSA control and were treated with the same inhibitors. To explore the effect of insulin, phosphorylation, acetylation and ROS on FOXO1 activation under high glucose conditions. Nuclear protein was extracted, western blot will be done. The 96-well plates will be used for immunostaining with FOXO1 antibody compared to matched IgG control. FOXO1 nuclear localization will be quantified.