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Deletion of FOXO1 in Chondrocytes Rescues the Effect of Diabetes on Mechanical Strength in Fracture Healing

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Abstract

Diabetes increases the risk of fracture, impairs fracture healing and causes rapid loss of the fracture callus cartilage, which was linked to increased FOXO1 expression in chondrocytes. We recently demonstrated that deletion of FOXO1 in chondrocytes blocked the premature removal of cartilage associated with endochondral bone formation during fracture healing. However, the ultimate impact of this deletion on mechanical strength was not investigated and remains unknown. Closed fractures were induced in Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice with lineage specific deletion of FOXO1 in chondrocytes compared to littermate controls. Type 1 diabetes was induced by multiple low dose streptozotocin treatment. Thirty-five days after fracture micro CT analysis showed that diabetes significantly reduced callus volume and bone volume ($P<0.05$), both which were reversed by FOXO1 deletion in chondrocytes. Diabetes significantly reduced mechanical strength measured by maximum torque, stiffness, modulus of rigidity and toughness and FOXO1 deletion in diabetic mice rescued each parameter ($P<0.05$). Diabetes also reduced both bone volume and mechanical strength in non-fractured femurs. However, FOXO1 deletion did not affect bone volume or strength in non-fractured bone. These results point to the important effect that diabetes has on chondrocytes and show for the first time that the premature removal of cartilage induced by FOXO1 in chondrocytes has a significant impact on the mechanical strength of the healing bone.

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Conflict of interest statement

All authors declare no conflict of interest.

Keywords

Bone; chondrocyte; forkhead; FOXO; hyperglycemia; diabetic; long bone; mechanical testing; transcription factor

INTRODUCTION

Insulin synthesis is substantially reduced in Type 1 diabetes mellitus (T1DM) resulting in hyperglycemia and impacts bone by increased risk of fracture and impaired fracture healing[1–3]. Reduced growth factor expression associated with T1DM decreases osteoblast activity and limits callus formation[4–6]. The eexpression of multiple inflammatory cytokines is increased in the fracture calluses of T1DM mice during the transition from cartilage to bone[7, 8] and is linked to an increase in osteoclasts that leads to premature loss of cartilage[5, 8]. However, the ultimate impact on the strength of the healing bone is unknown.

FOXO1 is a member of the mammalian forkhead box O family of transcription factors (FOXO1, FOXO3, FOXO4 and FOXO6) that have a characteristic winged-helix DNA binding domain[9]. FOXO1 regulates gene expression important for controlling apoptosis, oxidative stress, and proliferation[10]. FOXO1 is active in mesenchymal stem cells, chondrocytes, and osteoblasts and is the most studied and abundant FOXO member in bone[11, 12]. In aging, FOXO1 expression in chondrocytes protects the cells from apoptosis induced by oxidative stress[13] and in normal fracture healing FOXO1 induces chondrocytes to express VEGFA and enhance angiogenesis[14] However, in conditions where inflammation is clearly elevated FOXO1 can have the opposite effect. For example, diabetes can lead to increased FOXO1 activation and increased apoptosis in callus chondrocytes that is mediated by high levels of TNF[8] while in normal conditions it can have an important protective effect on chondrocytes and enhance resistance to osteoarthritis[15]Interestingly, diabetes leads to increased resorption of callus cartilage, which has been proposed to impair fracture healing in diabetics[16]. FOXO1 can promote expression of inflammatory mediators and RANKL expression in cultured chondrocytes[17]. In vivo, diabetes enhances FOXO1 activation.[18] Lineage specific deletion of FOXO1 in chondrocytes reduces FOXO1 expression and rescues the premature resorption of cartilage in diabetic fracture healing.[18]

These prior studies suggest that FOXO1 expression in callus chondrocytes may be a focal point for the negative effects of diabetes on fracture healing. Here we measured the effects of lineage-specific deletion of FOXO1 on the mechanical properties of the healing bone in diabetic mice. The results show for the first time that lineage-specific deletion of FOXO1 in chondrocytes rescues the negative effect of diabetes on mechanical strength of the healing fracture site. The results indicate that enhanced activity of FOXO1 in chondrocytes has a negative regulatory effect on the subsequent endochondral bone that is formed.

Method:

Animal Model—Animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. To investigate the role of FOXO1 in fracture healing, a modified allele of FOXO1 was used in which exon 2 is flanked by loxP sites

(FOXO1^{L}) to produce a null allele following Cre mediated recombination[19]. To produce chondrocytes with null alleles of FOXO1, a transgene expressing Cre recombinase from the collagen2α1 promoter (Col2α1.Cre^{+}) was used[20]. The Col2α1.Cre transgene has previously been shown to restrict Cre recombination to chondrocytes[6, 21]. Standard breeding schemes were used to produce Col2α1.Cre$^{+/-}$.FOXO1$^{L/L}$ (Col2α1.Cre^{+}.FOXO1$^{L/L}$) and Col2α1.Cre$^{-/-}$.FOXO1$^{L/L}$ (Col2α1.Cre$^{-/-}$.FOXO1$^{L/L}$). Mouse genotypes were confirmed by PCR analysis of DNA from a tail biopsy.

T1DM was induced in experimental mice beginning at 8 weeks of age by intraperitoneal injections of streptozotocin (STZ, 50 mg/kg, Sigma-Aldrich, St. Louis, MO) administered once per day for 5 days. Mice were considered to be diabetic when blood glucose levels exceeded 220 mg/dl. Mice were diabetic for at least 3 weeks prior to fracture. Control mice were treated identically but with vehicle (sodium citrate buffer).

Experimental mice were diabetic Cre^{+}.FOXO1$^{L/L}$ (DB Cre^{+}) and diabetic control littermates Cre^{-}.FOXO1$^{L/L}$ (DB Cre^{-}), which were compared to normal glycemic mice without FOXO1 deletion. Each group had 8 to 11 mice.

Immunofluorescence of Histological Sections—Fracture calluses were harvested on day16. Samples were fixed for 24 hours in cold 4% paraformaldehyde then decalcified for five weeks by incubation at room temperature in 10% EDTA solution before embedding in paraffin blocks. Transverse sections were prepared, de-waxed and antigen retrieval was performed at 120°C in 10mM citric acid, pH 6.0 (2100-Retriever, Aptum, Southampton, UK) for 20 minutes followed by non-specific blocking with nonimmune serum matching the secondary antibody for 55 minutes. Sections were incubated overnight at 4°C with either anti-FOXO1 antibody or matched IgG control antibody (1:200; Santa Cruz sc-11350, Dallas, TX, US). Sections were then incubated with a biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, US) followed by avidin-biotin-peroxidase enzyme complex (ABC) (Vector, PK-4000), which was localized with Alexa Fluor 546–conjugated streptavidin (1:400; Invitrogen S-11225, Carlsbad, CA, US) followed by mounting with DAPI containing mounting media (Sigma-Aldrich, St. Louis, Missouri, US). The final signal was enhanced by tyramide signal amplification (1:50; Adipogen, CDX-B0270-M100). Images were captured with a Nikon Eclipse 90i microscope equipped for epifluorescence (Tokyo, Japan) and the exposure time was set so that no signal was detected in IgG controls.

Femur fracture production—Mice were anesthetized with ketamine and xylazine prior to production of a closed, transverse, mid-diaphyseal fracture in the right femur as described previously[22]. The leg was scrubbed with a 10% povidone-iodine and a 5 mm medial, para-patellar incision was made to expose the femoral condyle. A hole was drilled into the femoral intramedullary canal at the intracondylar notch using a 30-gauge needle. A 0.01” stainless steel pin was inserted retrograde into the femoral canal to stabilize the impending fracture and secured in place with the tip of 30 g needle. The incision was closed with 5–0 resorbable suture and the fracture was induced with a 3-point impact-bending device. The animals were allowed free, unrestricted weight bearing after recovery from anesthesia.

MicroCT analysis—Mice were euthanized 35 days after fracture. Femurs were excised, cleaned of soft tissue and stored at −20°C in saline-saturated gauze. All femurs were scanned in saline at room temperature using a VivaCT40 (SCANCO USA, Inc. Wayne, PA) with 10 μm cubic voxel size. Two density calibration phantoms (0.25 g/cm^3 and 0.75 g/cm^3) were included in each scan to calibrate density determinations. For each slice, we draw the ROI of callus exclude the old cortical bone. bone volume (BV), tissue volume(TV) and total mineral density (TMD)were calculated from the sum of the areas by the slice thickness of each bone. BV/TV. BV/TV was calculated according to the BV and TV. Each group consisted of eight to eleven mice.

Mechanical testing—Following CT evaluation, all specimens were tested by the Biomechanics Core of the Penn Center for Musculoskeletal Disorders. Specimens were equilibrated at room temperature in PBS for 30 minutes prior to mechanical testing. Each end of the femur was embedded in poly-methylmethacrylate (PMMA) within an acrylic tube ensuring proper orientation and affixed to the actuating mechanism. Torsional mechanical integrity was evaluated with an electromechanical materials testing system (Instron 5542, Instron, Norwood, MA) with a custom torsion device consisting of a stepper motor (AM1524; MicroMo Electronics, Inc., Clearwater, FL) and a 0.035 Nm torsional load cell (TFF400; Futek, Irvine, CA). The resultant torque-angular displacement data was used to calculate torsional stiffness (Nm/deg), maximum torque (Nm), and maximum rotation (deg). The area under the torque-angular displacement curve was used to calculate toughness (Nmdeg). Finally, the maximum torsional rigidity was calculated by the following formula: $\frac{TL}{\varphi}$, where T=maximum torque, L=free length of specimen, and φ=angular displacement (degrees)at maximum torque. Each group consisted of eight to eleven mice.

PCR assay:

Fractured calluses were collected immediately after euthanasia, trimmed and soft tissue removed so that specimens primarily consisted of the fracture callus and immediately frozen in liquid nitrogen. RNAqueous TM (Ambion®, Waltham, MA USA) was used following the manufacturer's instructions to extract the total RNA. Reverse transcription was performed using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster City, CA, USA). mRNA levels of TNF-α were measured in triplicate using StepOnePlus™ Real-Time PCR System (Thermo- Fisher Scientific, Waltham, MA, US). Results were normalized with the housekeeping gene RPL32, a ribosomal protein.

Histologic stain and chondrocyte counts:

Safranin O/fast green staining was used to identify the cartilage area and nuclei were stained with Harris's modified hematoxylin (Fisher Scientific, Fair Lawn, NJ). Images were obtained from day 22 specimens to assess bone formed and on day 10 specimens to assess chondrocyte numbers. For the latter five fields consisting of cartilage were randomly chosen in each specimen and the number of chondrocytes were counted per mm of cartilage per field. For statistical purposes the animal was the unit of measurement.

Statistics—Differences between groups were determined by ANOVA with Tukey's post-hoc tests. $P < 0.05$ was considered to be statistically significant. Results are expressed as the mean ± SEM.

RESULTS

FOXO1 deletion

The current animal model was previously verified by Alharbi et al [16]. Briefly, Using Cre-Lox methods, FOXO1 was targeted for lineage-specific deletion in chondrocytes. Mouse genotypes were tested by PCR analysis of tail biopsy DNA before starting the experiment and the results were validated at the euthanasia time. (Fig 1a). Immunofluorescent staining was utilized using FOXO1 specific antibody to confirm targeted deletion of FOXO1 in chondrocytes but not other cell types in the experimental group. FOXO1 expression was evident in areas of cartilage and bone in mice lacking the Col2α1-Cre transgene (Col2α1-Cre^{-}.$FOXO1^{L/L}$) (Fig 1d). No FOXO1 expression was detected in chondrocytes in the cartilage area of Col2α1−Cre^{+}.$FOXO1^{L/L}$ mice but it was detected in bone areas (Fig 1e).

Diabetes significantly affects fracture healing that is reversed by FOXO1 deletion in chondrocytes

Mice were euthanized at 35 days after fracture to examine the callus by μCT. Cross sectional images were obtained in the area of the fracture site in healing femurs from all cohorts tested (Fig 2). The callus size of normoglycemic group at day 35 is shown in Fig 2a along with cross-sectional images of the callus in diabetic and diabetic group with FOXO1 deletion. The callus size for the diabetic group is noticeably smaller than the normoglycemic (Fig 2b). Deletion of FOXO1 in the diabetic group restored the callus size to one that approximates the normoglycemic (Fig 2c). Histologic sections taken on day 22 after fracture exhibited similar changes (Fig 2e–g). The hard callus was smaller in the diabetic group and the negative impact of diabetes on the hard callus was reversed by FOXO1 deletion in chondrocytes.

Quantitative measurements of the microCT was undertaken. In the diabetic mice the total volume (TV) of the fracture callus was 39% smaller and bone volume (BV) was 34% ($P<0.05$) smaller compared to normoglycemic control mice (Fig 3a and b; $P<0.05$). However, BV/TV and total mineral density (TMD) values were not significantly different between the normoglycemic and diabetic mice (Fig 3c and d; $P>0.05$). Lineage-specific deletion of FOXO1 in chondrocytes restored the callus TV and BV in the diabetic group. Deletion of FOXO1 increase callus TV and BV by 34% and 28%, respectively, relative to the diabetic Col2α1.Cre^{-}.$FOXO1^{L/L}$ mice (Fig 3a and b; $P<0.05$). Deletion of FOXO1 in chondrocytes had no effect on BV/TV or TMD. Furthermore, in normoglycemic group, TV and BV of Col2α1.Cre^{+}.$FOXO1^{L/L}$ mice were smaller respectively 21% and 20.5% than Col2α1.Cre^{-}.$FOXO1^{L/L}$ mice($P<0.05$), no difference in other measurements

Diabetes reduces fracture callus mechanical strength that is restored by deletion of FOXO1 in chondrocytes

Torsion testing was used to test the mechanical property of the healing mouse femurs 35 days after fracture (Fig 4). Diabetes reduced maximum torque by 69%, stiffness by 56%, toughness by 74%, and shear modulus by 60% 35 days after fracture (Fig 4a–d; $P<0.05$). The loss of mechanical strength caused by diabetes was reversed by deletion of FOXO1 in chondrocytes. The healing fractures of diabetic Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice were significantly stronger compared to diabetic Col2α1.Cre^{-}.FOXO1$^{L/L}$ mice (Fig 4). Maximum torque, stiffness, toughness, and shear modulus were 182%, 130%, 108%, and 128% greater, respectively in the diabetic group with lineage specific FOXO1 deletion (Fig 4a–d; $P<0.05$). No significant difference in mechanical strength was detected between the calluses of normal control and diabetic Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice (Fig 4a–d; $P>0.05$). There was a tendency for the mean callus toughness of the diabetic Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice to be less than that of normal control mice, but this difference was not significant (Fig 4c; $P>0.05$).

Effect of diabetes and lineage specific FOXO1 deletion on intact femur morphometry and strength

The effects of diabetes and chondrocyte-specific deletion of FOXO1 on bone homeostasis was measured in the contra-lateral, intact femurs of the normal control mice, the diabetic Col2α1.Cre^{-}.FOXO1$^{L/L}$ mice, and the diabetic Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice (Fig 5). Diabetes caused a 17% reduction in TV and BV of the intact femurs compared to normoglycemic control mice (Fig 5a and c; $P<0.05$). FOXO1 deletion in chondrocytes had no effect on TV or BV of the non-fractured diabetic femurs (Fig 5a and c $P>0.05$).

Torsional mechanical testing of the intact femurs found that maximum torque was 21% less and toughness was 22% less in diabetic mice compared to normoglycemic control mice (Fig 6a and c; $P<0.05$). However, no significant difference in stiffness or shear modulus were found between the intact femurs of diabetic compared to normoglycemic mice (Fig 6b and d; $P>0.05$). In contrast to the effect on the mechanical strength of fractured femurs, deletion of FOXO1 in chondrocytes had no effect on the mechanical strength of the non-fractured intact femurs of the diabetic mice (Fig 6 $P>0.05$).

Diabetes and FOXO1 deletion in diabetic animals does not affect the number of chondrocytes per cartilage area but does modulate TNF-α mRNA levels.

In the callus 10 days after fracture there was no significant difference in the number of chondrocytes per cartilage area in the three groups examined (Fig 7a; $P>0.05$). However, in diabetic mice the TNF- α mRNA levels in the fracture callus were 250% higher than normoglycemic fractures (Fig 7b $P<0.05$). Deletion of FOXO1 in chondrocytes rescued the dysregulation of TNF-α caused by diabetes (Fig 7b, $P<0.05$) so that there was no significant difference in TNF- α mRNA in the normal control and diabetic Col2α1.Cre^{+}.FOXO1$^{L/L}$ mice (Fig 7b; $P>0.05$).

DISCUSSION

To investigate the role of FOXO1 we examined diabetic mice with lineage specific deletion of FOXO1 (Col2α1. Cre^{+}.$FOXO1^{L/L}$) and compared the results to diabetic mice with wild-type FOXO1 (Col2α1. Cre^{-}.$FOXO1^{L/L}$). The experimental mice express Cre recombinase under the regulation of a response element of the collagen-2α1 promoter[6, 21] effectively deleted FOXO1 as shown by genotyping that was restricted to chondrocytes as shown by immunofluorescence. The T1DM model we used, multiple low dose streptozotocin injection has many similarities with T1DM in humans including decreased bone mineral density, reduced bone mineral content and diminished bone strength[23–25]. Fractures in streptozotocin induced diabetic mice healed with smaller calluses with less bone and reduced strength as was demonstrated in previous studies[26–28]. However, the impact of diabetes was rescued by deletion of FOXO1 in chondrocytes with significant improvements in several parameters of mechanical strength. To our knowledge these are the first results to demonstrate that the effect of diabetes on chondrocytes plays a central role in the impact of diabetes on the strength of the healing bone.

Diabetes significantly reduced mechanical strength tested by maximum torque, stiffness, modulus of rigidity and toughness. FOXO1 deletion in chondrocytes blocked the negative effect of diabetes on maximum torque, stiffness and rigidity and partially reversed the effect on toughness. The near complete rescue of mechanical strength by lineage-specific deletion of FOXO1 in chondrocytes was unexpected and may be due to the effect on bone volume rather than an effect on mineral density based on micro CT results. For example, BV/TV was not affected by FOXO1 deletion.

There are different potential mechanisms through which FOXO1 could affect chondrocytes and the fracture healing process. In normal conditions FOXO1 may contribute to a robust fracture repair as shown by evidence that FOXO1 activation in chondrocytes leads to greater VEGFA expression and angiogenesis[15]. It has also been shown to protect chondrocytes in articular cartilage[14]. Moreover, reduced expression of FoxO transcription factors in chondrocytes increases susceptibility to cell death, which is associated with reduced expression of antioxidants[12]. However, in diabetes Foxo1 appears to have a different function and induces expression of pro-osteoclastogenic factors such as TNF-α and RANKL[17]. We have previously proposed that elevated FOXO1 induced by high levels of TNF in diabetic fractures would lead to greater resorption of cartilage thus diminishing the anlagen for bone formation, which would result in smaller bone volumes and mechanical strength[8]. In support of this diabetes increases RANKL expression in chondrocytes during fracture healing[18] and lineage specific deletion of FOXO1 in chondrocytes reduces diabetes-enhanced RANKL expression and reverses diabetes-increased osteoclast numbers to normal levels[18]. Here we found that TNF-α mRNA levels were increased in diabetic fractures and identified FOXO1 as a driving force, since the increase in TNF in day 10 diabetic fractures was rescued by FOXO1 deletion in chondrocytes.

An alternative mechanism through which FOXO1 activation in diabetic fractures could affect fracture healing is by promoting chondrocyte cell death. Diabetes-enhanced TNF stimulates chondrocyte apoptosis in vivo[17] and in vitro, this has been shown to occur

through a mechanism involving FOXO1 mediated pro-apoptotic gene expression[17]. Previous studies have suggested that diabetes affects chondrocytes through its effect on collagen production[28]. The impact of diabetes has been linked to reduced proliferation of chondroprogenitor cells, diminished chondrocyte proliferation and reduced expression of matrix proteins[29]. We have previously shown that FOXO1 over-expression reduces proliferation of osteoblasts[29]. Thus, it is also possible that high levels of FOXO1 activity observed in chondrocytes in diabetic calluses also impairs chondrocyte proliferation reducing the amount of cartilage. However, at the day 10 time point we did not see a difference in chondrocyte density in diabetic fractures with or without FOXO1 deletion. Thus, additional experiments will need to be carried out to investigate whether FOXO1 plays an important role in chondrocyte apoptosis, proliferation of matrix production in healing fractures of diabetic animals.

In summary, we found that diabetes affected bone volume and the mechanical strength of bone, which was rescued by FOXO1 deletion in chondrocytes. In non-fractured femurs in mice that were hyperglycemic for up to 3 months there was reduced total callus and volume and reduced mechanical strength as measured by maximum torque and toughness. However, deletion of FOXO1 in chondrocytes had no effect on the non-fractured bone. This result is not surprising since the non-fractured site would not be expected to be significantly affected by lineage specific deletion of FOXO1 in chondrocytes.

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Highlights

1. Diabetes impairs fracture healing and reduces mechanical strength of the facture callus.
2. Deletion of FOXO1 in chondrocytes can rescue the effect of the diabetes on reduced bone formation establishing that FOXO1 has a detrimental effect on the hard callus by affecting chondrocytes.
3. Deletion of FOXO1 in chondrocytes can rescue the negative effect of the diabetes on the reduced mechanical strength of the hard callus.

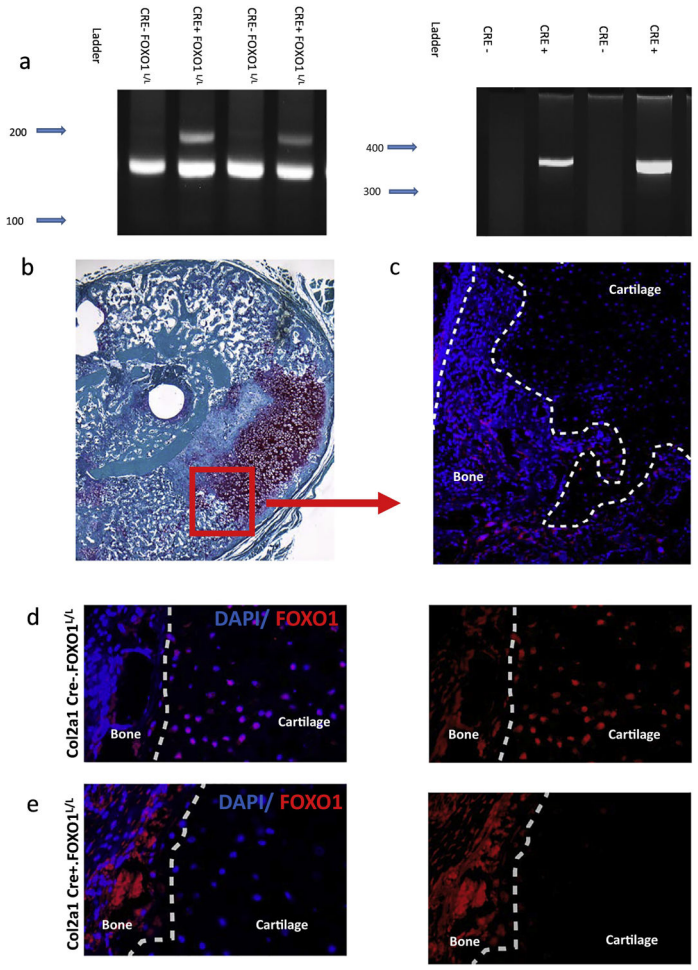

Fig1.

Cre recombinase deletes FOXO1 in chondrocytes of experimental animals but not marched Cre- control mice. (a) Genotyping of experimental Col2α1Cre^{+}.FOXO1L/L and littermate control Col2α1Cre^{-}.FOXO1L/L mice. FOXO1 with flanking loxP sites (149bp) is detected in all groups, while FOXO1 with exon deletion (198bp) is found only in mice with Cre recombinase (352bp). (b) Photo micrographs of a histologic section from the fracture site of an experimental (Cre+) mouse was stained with safranin O/fast green. (c) A photo micrograph of a serial section from the same specimen in (b) was examined by immunofluroesence for antibody specific for FOXO1 (red) and nuclei were identified by DAPI counterstain (blue). (d & e) ections from a representative experimental Cre+ and control Cre- mouse were examined by immunofluorescence with antibody specific for FOXO1 and nuclei identified by DAPI counterstain. No signal was detected in matched control antibody (data not shown). Original magnification 200X. (d & e) Photo micrographs of the fracture site examined by immunofluorescence from control Col2α1Cre-.FOXO1L/L and experimental Col2α1Cre-.FOXO1L/L, respectively. Magnification 400X.

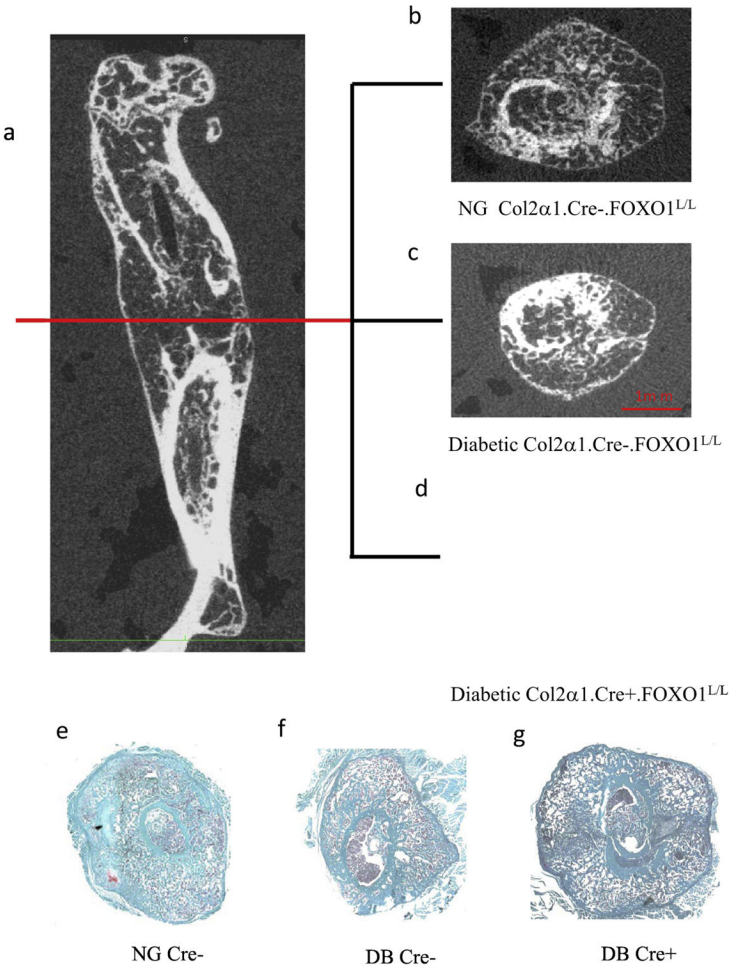

Fig 2.

Changes of callus size of the fracture femurs at 35 days after fracture Section images of fractured femurs. (a) longitudinal section images of fractured femur. (b, c, d) Micro CT cross sectional images of normoglycemic Col2α1.Cre- FOXO1 $^{L/L}$, matched diabetic Col2α1.Cre- FOXO1 $^{L/L}$ or diabetic Col2α1.Cre^{+} FOXO1 $^{L/L}$ mice. (e, f, g) Histologic cross sectional images of safraninO/fast green stained sections 22 days after fracture.

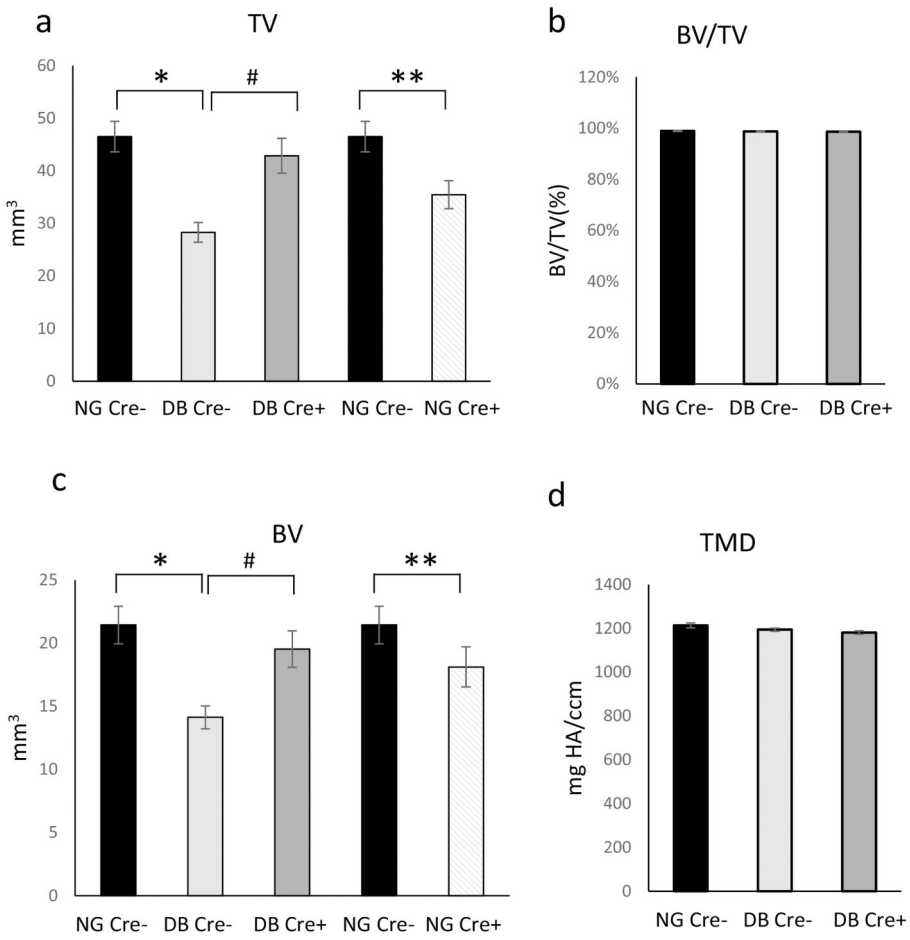

Fig 3.

Changes in bone parameters of fractured femurs 35 days after fracture. Micro-CT analysis was performed for fractured femurs in normoglycemic or diabetic mice with Col2α1.Cre^{-}.FOXO1$^{L/L}$ or Col2α1.Cre^{+} FOXO1$^{L/L}$ genotypes. Each value is mean ± SEM for n=8–11 per group. *P<0.05 for NG vs matched Diab group; # P<0.05 for matched Cre+ vs Cre- group.

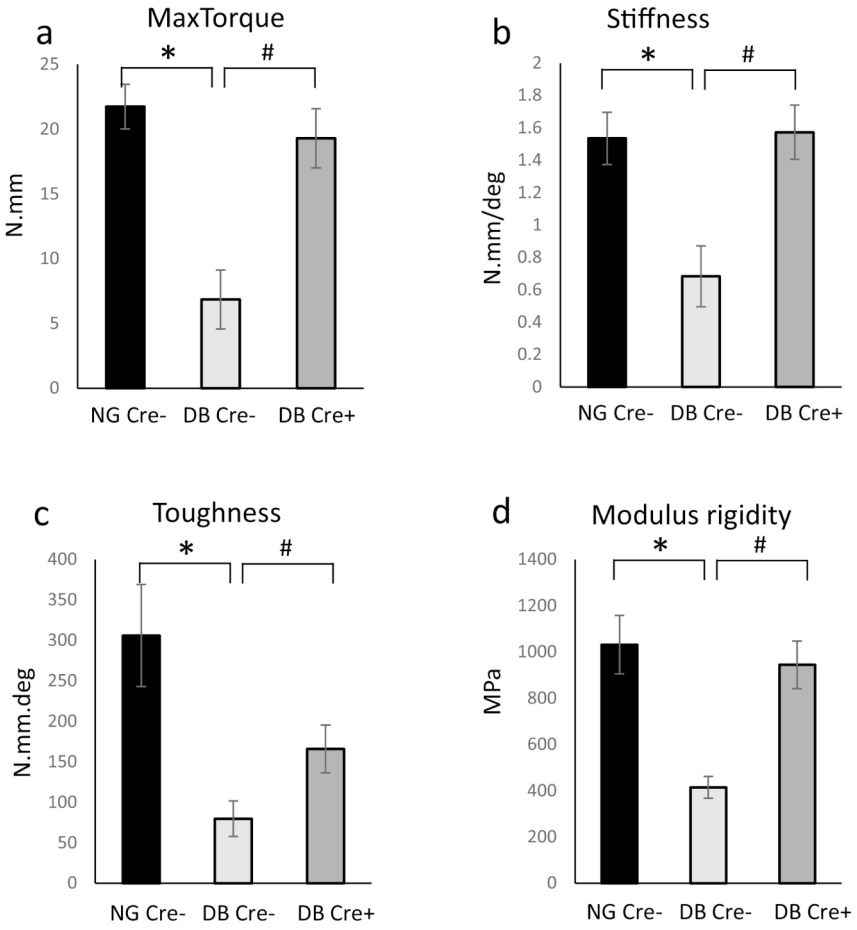

Fig 4.

Changes in mechanical properties of fractured femurs 35 days after fracture. Mechanical tests were performed for fractured femurs in normoglycemic Cre-, diabetic Cre- or diabetic Cre+ mice as indicated 35 days after fracture. Each value is mean ± SEM for n=8–11 per group. *P<0.05 for NG vs matched Diab group; # P<0.05 for matched Cre+ vs Cre- group.

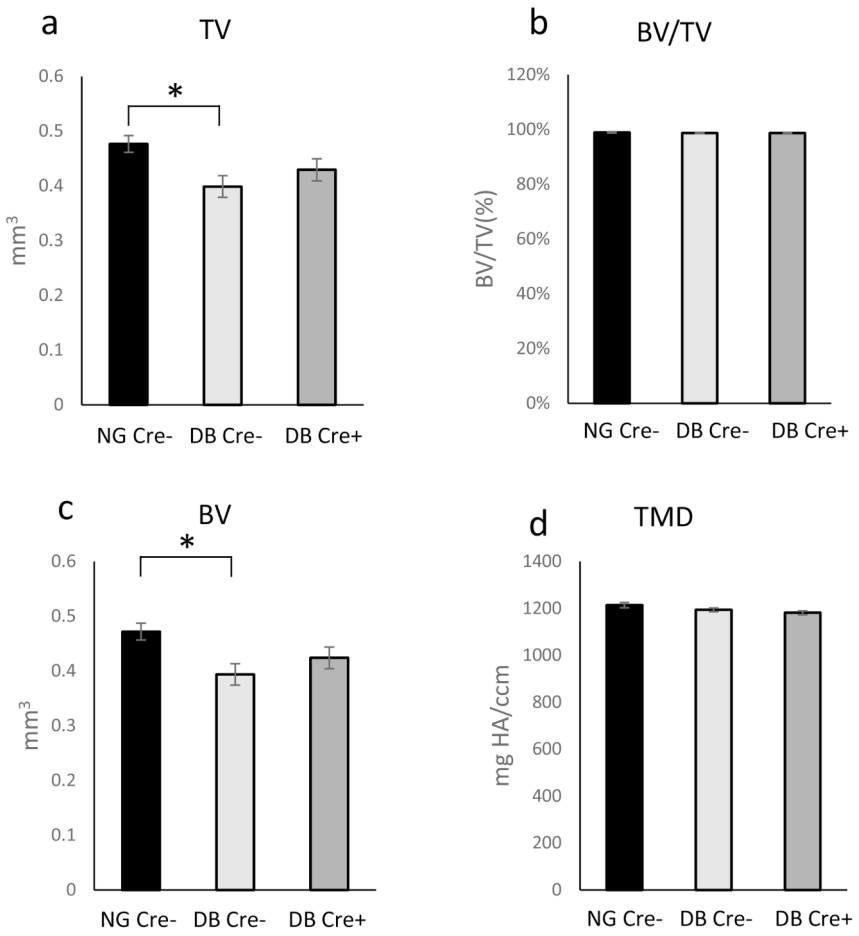

Fig 5.

Changes in bone parameters of contralateral non-fractured femurs 35 days after fracture. Micro-CT analysis was performed on the contralateral non-fractured femurs in normoglycemic Cre-, diabetic Cre- or diabetic Cre+ mice as indicated 35 days after fracture. Each value is mean ± SEM for n=8–11 per group. *P<0.05 for NG vs matched Diab group; # P<0.05 for matched Cre+ vs Cre- group.

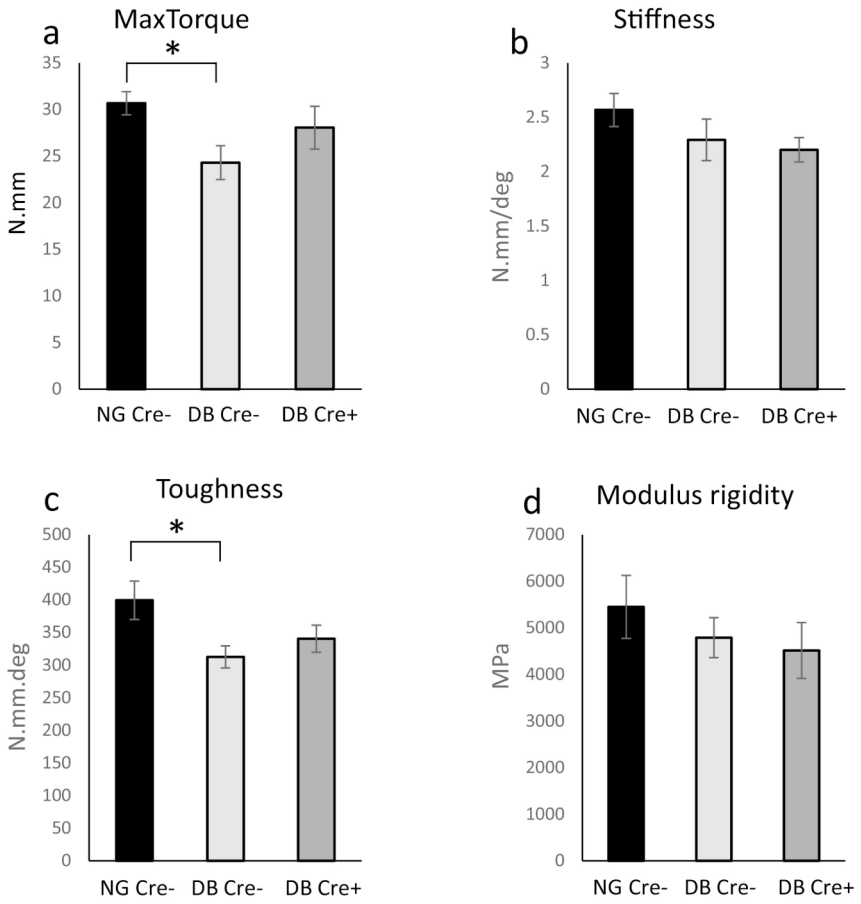

Fig 6.

Changes in mechanical properties of non-fractured femurs 35 days after fracture.

Mechanical tests were performed in contralateral non-fractured femurs in normoglycemic Cre-, diabetic Cre- or diabetic Cre+ mice as indicated 35 days after fracture. Each value is mean ± SEM for n=8–11 per group. *P<0.05 for NG vs matched Diab group; # P<0.05 for matched Cre+ vs Cre- group.

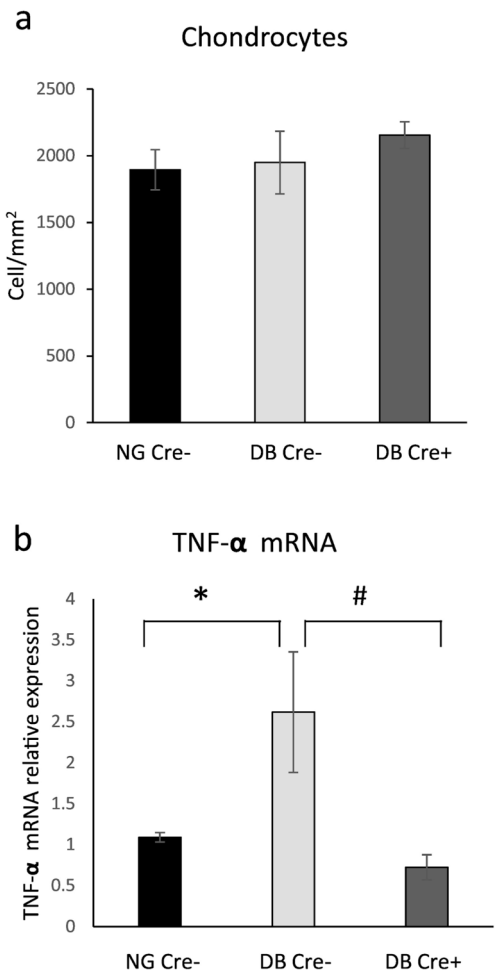

Fig 7.

Changes in chondrocyte numbers and TNF-α mRNA levels. (a) Histologic sections were prepared from day 10 fractures and stained with safranin/fast green. The number of chondrocytes per cartilage area was counted in the indicated groups. (b) Total RNA was extracted from 10 day fractures and measured TNF-α mRNA level by real-time PCR. *P<0.05 for NG vs matched Diab group; # P<0.05 for matched Cre+ vs Cre- group.