Pathophysiological mechanism of bone loss in type 2 diabetes involves inverse regulation of osteoblast function by PPARγ coactivator-1α and skeletal muscle atrogenes: adiponectin receptor 1 as a potential target for reversing diabetes-induced osteopenia

Running title: Adiponectin receptor-1 and PGC-1α protect against type-2 diabetes-induced osteopenia

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Abstract

Type 2 diabetes is associated with increased fracture risk and delayed fracture healing; the underlying mechanism however remains poorly understood. Here we made a systematic investigation of skeletal pathology in leptin receptor-deficient diabetic mouse in C57/BLKS background (db). Compared with wild-type (wt), db mice displayed reduced peak bone mass and age-related trabecular and cortical bone loss. Poor skeletal outcome in db was contributed by high glucose and non-esterified fatty acid (NEFA)-induced osteoblast apoptosis that was associated with PPARγ coactivator 1-α (PGC-1α) downregulation and upregulation of skeletal muscle atrogenes in osteoblasts. Osteoblast depletion of the atrogene, muscle ring finger protein-1 (MuRF1) protected against gluco- and lipotoxicity-induced apoptosis. Osteoblast-specific PGC-1α upregulation by 6-C-β-d-glucopyranosyl-(2S,3S)-(+-)5,7,3’,4’-tetrahydroxydihydroflavonol (GTDF), an adiponectin receptor 1 (AdipoR1) agonist as well as metformin in db mice that lacked AdipoR1 expression in muscle but not bone, restored osteopenia to wt levels without improving diabetes. Both GTDF and metformin protected against gluco- and lipotoxicity-induced osteoblast apoptosis and depletion of PGC-1α abolished this protection. While AdipoR1 but not AdipoR2-depletion abolished protection by GTDF, metformin action was not blocked by AdipoR-depletion. We conclude that PGC-1α upregulation in osteoblasts could reverse type 2 diabetes-associated deterioration in skeletal health.
Introduction

Type 2 diabetes typically is a mid age-onset (>40 years) metabolic disease that affects multiple organ systems. Increasing evidence indicates that type 2 diabetes is associated with increased fracture risk; especially vertebral and hip fractures in older patients (1-4). Since chronic inflammation participates in diabetes pathogenesis and is also a prerequisite for osteoclast activation; increased bone resorption is considered as the likely cause of increased fracture risk in this disease. Intriguingly however, available studies suggest that in type 2 diabetes, there is an increased risk of hip fractures at a higher bone mineral density (BMD) value than non-diabetics (5) and increased incidence of vertebral fractures at BMD values comparable to non-diabetics(6). Thus, the reasons for increased skeletal fragility in type 2 diabetes patients remain largely unexplained.

Type 2 diabetes patients also display delayed fracture healing resulting in poorer outcomes following hip fracture (7). Reduced fracture healing in type 2 diabetes is attributed to decreased collagen content, defective cross-linking, alterations in collagen sub-type ratios and collagen defects due to accumulation of advanced glycation end-products (8-10), which could impair osteoblast function. These could also lead to compromised bone material strength and increased cortical porosity affecting bone quality as shown in postmenopausal women suffering from type 2 diabetes (11; 12).

Several mouse models of type 2 diabetes are available however; they poorly represent human skeletal fragility observed in typical type 2 diabetes patients(13). Unlike typical mid-age type 2 diabetes onset in humans, when skeletal maturity has already been attained, in genetically engineered diabetic mice, the onset of diabetes occurs at 4-8 weeks, an age comparable to adolescence in humans at which skeletal maturity has not been attained (14). However, the average onset age is falling in humans and is becoming increasingly common
among those aged less than 30 years including children and adolescents in different ethnic
groups (15-18). This early onset diabetes is characterized by increased disease severity and
pancreatic β cell failure than typical type 2 diabetes (19-21). Data on the impact of early-
onset type 2 diabetes on human skeletal health are limited, although a recent report indicates
that prediabetic children with impaired glucose tolerance have low mineral content and low
bone mass (22). We thus believe that the distinction in skeletal phenotype between early and
mid-age-onset type 2 diabetes might be important for the following reason. While, skeleton
of children/adolescents predominantly undergoes modeling-directed growth (resulting in net
increase in bone mass due to enhanced osteoblastic activity), adult skeletons predominantly
experience remodeling (no net bone gain), and thus type 2 diabetes in these two cases may
affect skeletal health in different manner.

Although monogenic obese and diabetic mice models such as leptin receptor-deficient
diabetic mice essentially differ from the polygenic disease origin in human type 2 diabetes, it
however, may serve as a suitable model for deciphering the skeletal outcomes in this disease.

Here we investigated the skeletal phenotype in leptin receptor-deficient genetically
obese diabetic mice (in C57BLKS background; db) that manifest severe diabetes including
pancreatic β cell failure. Our study also involved identification of factors crucial for type 2
diabetes-induced skeletal effects. Furthermore, modulation of such factors by therapeutic
intervention on diabetic skeleton was also assessed.
Research Design and Methods

**Reagents and kits:** Cell culture reagents were from Life Technologies. Fine reagents were from Sigma-Aldrich unless indicated otherwise. Globular adiponectin (gAd) was from ATGen Global. $^{125}$I (20 MBq) was from BARC (Mumbai, India). Kits for plasma biochemical parameters were: glucose (Pointe Scientific), lipids and creatinine (Randox Laboratories LTD, Mumbai, India), insulin (Millipore), adiponectin (B-Bridge International Inc), osteocalcin (USCN life Science Inc). ELISA kits: PAI-1, MCP-1, leptin and resistin (Millipore). TUNEL assay kit: Roche Applied Science. GTDF (purity >98%) was purified as reported (23), metformin (Met; purity 97%) and pioglitazone (Pio; purity ≥98%) were from Sigma-Aldrich.

**Animal Experiments:** Wild type (wt; C57BLKS/J) or diabetic (db; BKS.Cg-Dock7^m^+/+, Lepr^{db}/J and B6.db; B6.BKS(D)-Lepr^{db}/J ) ice were housed at 22±3°C temperature and a 12h light/dark cycle. All animals had access to standard chow diet and water *ad libitum*. Time course studies were conducted at AALAC accredited animal facility of Zydus Research Centre (ZRC), Ahmedabad following approval from Institutional Animal Ethics Committee (IAEC) of ZRC. The db or wt mice used were originally from Jackson laboratories and the colonies were maintained at ZRC. Drug treatment studies using 10 wk old db mice from Harlan laboratories, Netherlands (BKS.Cg- + Lepr^{db}+/+Lepr^{db}/OlaHsd) were conducted at Syngene International Ltd (Bangalore, India) in their AALAC accredited animal facility, following ethical approval from IAEC. In both studies, all the animals were randomized into...
groups based on blood glucose levels and body weight. Vehicle groups received 0.5%
Carboxymethylcellulose and treatment groups received GTDF (10mg/kg), Met (350mg/kg)
and Pio (10mg/kg) once a day, by oral gavage for 30 days. The doses of Met in adult human
range from 850-2550mg/day which comes to 14.6-42.5mg/kg (considering the average
human weight to be 60kg). The adult human dose of Pio is 15-45mg/day which corresponds
to 0.25-0.75mg/kg. Formula for dose translation from human to mouse was based on body
surface area: human equivalent dose (mg/kg) = animal dose (mg/kg) × (animal $K_m$ / human
$K_m$) (24). Feed intake and body weight were measured every day and on day 31 the animals
were sacrificed. Plasma and tissues were collected and stored at -80°C until further analysis.

**Micro-computed tomography (µCT):** µCT of excised bones was carried out using Sky
Scan 1076 CT scanner (Aartselaar) as described earlier for mice bone (25; 26) and following
the general guidelines for the assessment of bone microarchitecture in rodents using µCT
(27). For scanning, source voltage was set to 50 kV and current, 200 µA. X-ray source
rotation step size was 0.84° over a trajectory of 180°. Reconstructions were made using the
nRecon V1.6.9.4 software (SkyScan) to create 2D 2000 × 2000 pixel images with a beam
hardening correction set to 10% with dynamic range −1,000 to 11,000 Hounsfield units. By
drawing ellipsoid contours, trabecular bone was extracted using the CT analyzer software. In
the femur epiphysis region, 200 slices were selected leaving 50 slices from the start of the
growth plate as a reference point. Cortical parameters were determined at femur mid-
diaphysis by 2D analysis. From the start of the growth plate as a reference point, 200 slices
were selected in the cortical region, leaving 500 slices as offset (to exclude the trabecular
region). For BMD calibration, the hydroxyapatite phantom rods of 2mm diameter with
known BMD (0.25g/cm³ and 0.75 g/cm³) were employed. For each analysis, the estimated
mineral density of the bone tissue was determined based on the linear correlation between
µCT attenuation coefficient and BMD (28).
Bone biomechanical strength: Three-point bending test on femur was performed using a Bone Strength Tester (Muromachi, TK-252C) as previously reported (29).

Determination of the bone lining cells: Deparaffinized and hydrated femoral epiphysis sections (5µm) of different groups were stained with H&E and bone lining cells were visualized by light microscopy. 10 sections/mouse (n=6) were used for counting by two independent researchers blinded to experimental design.

Cell culture and induction of differentiation: Mouse calvarial osteoblasts (MCO) were obtained from 1-2 day old mouse pups as described earlier (26; 30). Bone marrow cells (BMC) from 10 to 12 wk old male wt and db mice were isolated and were cultured and differentiated into osteoblasts as described earlier (31).

Radio iodination of gAd and radioligand binding experiment: 10µg gAd was radioiodinated by iodogen method using precoated iodination tubes (Pierce) according to manufacturer’s instructions. Excess $^{125}$I was removed by using PD 10 desalting column (GE Healthcare). For binding assays, osteoblast and myocytes in 24-well plates were incubated with increasing concentrations of $^{125}$I-gAD in PBS supplemented with 0.1% BSA for 2h (at which binding equilibrium was achieved), following which the cells were washed and lysed. Nonspecific binding for each concentration was determined using 200-fold excess of cold gAD. Specific binding was calculated by subtracting nonspecific binding from total binding.

RNAi experiments: siRNAs were from Dharmacon. MCO were transfected with 0.1µM of each siRNA per well using DharmaFECT 1 transfection reagent (Dharmacon) in 6-well plates. 72h after transfection, cells were treated as indicated and were analyzed as required.

QPCR, immunoblotting and immunohistochemistry: These studies were performed using standard procedures as previously described (32; 33). Primer sequences for QPCR are listed in supplementary Table 3. Antibodies and dilutions for immunoblotting: anti-PGC-1α
(ST1202; Calbiochem; 1:2000). Anti-AMPK, phospho-AMPK, (Thr 172) and cleaved
caspase-3 (Cell Signaling Technology; 1:1000). AdipoR1, AdipoR2, MuRF1 and β-actin
antibodies were from Santacruz Biotechnology (1:1000 except β-actin; 1:3000). **TUNEL**
**assay** was performed as described earlier (33). For immunohistochemistry, femur epiphysis
transverse sections (5µm) were deparaffinized, hydrated and after antigen retrieval, incubated
with mouse anti-Runx-2 (Abcam, 1:1600) along with anti-PGC-1α (1:2000), pAMPK (1:500)
or MuRF1(1:500) at 4°C overnight. The sections were then washed and incubated with
fluorescent Alexa Fluor-goat anti-mouse and goat anti-rabbit IgG (H + L) (1:1500) (Life
Technologies) for 1h at RT. Sections were also stained with DAPI and visualized by
fluorescent microscopy. Image-Pro plus 6.1 software (MediaCybernetics) was used for
quantification of microscopic data, where five randomly selected fields from six bone
sections per group were analyzed.

**Osteoblast differentiation assay:** MCOs at 70-80% confluence were trypsinized and $2 \times 10^3$
cells/well were seeded in 96-well plates. After 24h, cells were given various treatments for 48
h in osteoblast differentiation medium. Alkaline phosphatase (ALP) activity was measured
using a fluorometric kit (Biovision) according to the manufacturer’s protocol.

**Cell viability assay:** MCO in 96-well plates ($2 \times 10^3$ cells/well) were treated with increasing
concentrations of glucose, palmitic acid and dexamethasone (dex) with or without GTDF
and metformin for 48h. Osteoblast viability was assessed by MTT assay as reported (33).

**Flow cytometry-based determination of apoptosis:** Annexin V-FITC Apoptosis Detection
kit (Sigma) was used to determine apoptosis. Briefly, the treated cells were trypsinized and
washed with PBS. $1 \times 10^6$ cells/mL were resuspended in binding buffer and labeled with 5µL
annexinV-FITC and 10µL propidium iodide for 10 minutes in the dark. Cells fluorescence
was measured on a FACSCalibur flow cytometer (Becton Dickenson) and analyzed using
CellQuest Pro software.
Data analysis and Statistics: Results are expressed as mean ± SE. All data were analyzed using GraphPad Prism 5.0. Statistical analyses were performed using one or two-way ANOVA as appropriate followed by Bonferroni’s post test.

Results

Lack of peak bone mass achievement and age-related osteopenia in BKS.Cg-Dock7+/+
Leprdb/db/J (db) mice

3D µCT-based evaluation of trabecular microarchitecture in 8, 12 and 16 wk old C57BLKS/J (wt) and db revealed that db femur epiphysis at all ages displayed loosely connected trabecular network than wt mice (Fig 1A). wt mice displayed bone gain at 12 wk, characterized by significantly higher BMD, trabecular bone volume (BV/TV), trabecular number (Tb.N) and connectivity density (Conn.D) followed by trabecular loss at 16 wks manifested by the decreases in these parameters (Fig. 1A). db mice did not gain bone at any age and were osteopenic throughout, characterized by significantly lower BMD, BV/TV, Tb.N, trabecular thickness (Tb.Th), Conn.D, and higher trabecular separation (Tb.Sp) than wt (Fig. 1A). Age-based comparison between db mice also revealed progressive osteopenia characterized by significantly higher Tb.sp at 16 wk, and other parameters when compared to 8wk old db showed a decreasing trend with age.

Femur mid-diaphysis of db mice representing cortical bone showed a thinner cortex than wt mice at all ages (Fig 1B). At 8 wk, BMD, average cortical thickness (Ct.Th), and cortical area (Ct.Ar) were comparable between wt and db, the latter group displayed
significantly lower periosteal perimeter (Ps.Pm) and endocortical perimeter (Ec.Pm). At 12 and 16 wk, all cortical parameters in db were significantly lower than corresponding wt groups (Fig. 1B). Like trabecular parameters, cortical parameters including BMD, Ct.Th and Ct.Ar in 12 wk old wt were significantly higher than 8 wk old wt mice, and at 16 wk, these parameters displayed a decreasing trend compared to 8 wk old wt (Fig.1B). db mice showed no cortical gain at any age (Fig. 1B).

Osteoblast apoptosis is associated with both primary and secondary osteoporosis (34) and compared to wt femur epiphysis in db mice across all age groups displayed remarkably increased osteoblast apoptosis (Fig. 1C, Supplementary Figure 1A).

Periosteal or bone lining cells serve as a source of osteogenic precursors (35). At 12 wk, wt but not db bones displayed significantly higher periosteal cell number compared to the 8wk groups (Fig. 1D, Supplementary Figure 1B) and db mice at all ages had significantly lower periosteal cell number compared to wt. (Fig. 1D). The osteogenic surrogate, serum osteocalcin (OCN) level significantly dropped with age in both mice; however as reported earlier (36)db mice had significantly lower OCN level than wt (Fig 1J).

Compared to wt non-fasting and fasting glucose was significantly higher in 8wk db mice (>280 and >150 mg/dL respectively) which further increased to >450 and > 200mg/dL at 12 wk and remained steady thereafter (Fig. 1E-F). db mice also displayed significant non-fasting and fasting hyperinsulinemia (Fig. 1H-I), however, insulin levels in 16wk db was significantly less than the 8wk old db mice, probably due to β-cell apoptosis which is typical of the db strain. Intriguingly, despite the decrease in insulin, 16wk old db mice had comparable blood glucose to 12wk old db, although we can’t explain it, presumably it was due to elimination of glucose through urine, which again is a trait in db mice. NEFA level in db mice was significantly higher than wt at all ages and no age-dependent change was
observed (Fig. 1G). Consistent with earlier reports (37; 38), db mice at 8 wk displayed a significantly lower adiponectin level than wt which decreased further with age (Fig. 1K).

**Glucose and palmitate directly induce osteoblast apoptosis**

We next assessed if NEFA and glucose, the two major mediators of diabetic pathology could directly affect osteoblast viability. Both palmitate and glucose induced loss of mouse calvarial osteoblast (MCO) viability and apoptosis *in vitro* (Fig 2A-2B). Apoptosis-related cysteine protease, caspase-3 activation assay revealed that palmitate and dexamethasone (Dex) but not glucose enhanced cleaved (active) caspase-3 levels (Fig 2C).

**db mice display suppression of PGC-1α and increase in skeletal muscle atrogene expression in bone**

Consistent with enhanced osteoblast apoptosis, db femur epiphysis displayed significantly higher p53 expression than wt at all ages (Fig. 3A). Among the db group, 16 wk old had significantly higher p53 than the 8wk old (Fig 3A). Consistent with peak bone gain (Fig. 1A-B), wt mice displayed significantly higher Runx2 (key osteogenic factor) expression at 12 wk followed by a decline at 16 wk while Runx2 mRNA in db femur epiphysis was significantly lower than wt at all ages (Fig. 3A). Further, among the db group, Runx2 expression significantly declined with age (Fig 3A).

Muscular PGC-1α expression is suppressed in diabetes (39; 40). Since in osteoblasts PGC-1α is PTH-responsive (41) and its expression increases during osteoblast differentiation (42), we assessed its skeletal expression. Like Runx2, PGC-1α expression in wt but not db, peaked at 12 wk and then declined and compared to wt, PGC-1α expression in db bones was significantly lower and showed significant decline with age (Fig. 3A).
Diabetes and obesity negatively influence muscular health by increasing atrogenes \((43)\) that are involved in protein catabolism. Increasing PGC-1\(\alpha\) expression and activity downregulates these atrogenes and prevents muscle atrophy under diverse stresses \((44)\). Since some of these atrogenes are reported in bone, and genetic ablations of the E3 ubiquitin ligase MuRF1 and lysosomal protease (cathepsin-L) prevent unloading \((45)\) and ovariectomy-induced bone loss \((46)\), we assessed their skeletal expression. Compared to wt, db femur had significantly higher MuRF1, atrogin-1 and cathepsin-L transcripts and their levels in db but not wt mice increased significantly with age \((\text{Fig. } 3\text{A})\). Consistent with mRNA expression, PGC-1\(\alpha\) protein level was drastically lower in db than wt mice of corresponding age groups \((\text{Fig. } 3\text{B})\). MuRF1 protein level conversely, was modestly higher in 8 wk old db mice than in wt, but dramatically increased at 12 and 16 wk \((\text{Fig. } 3\text{B})\).

Consistent with their ability to directly induce osteoblast apoptosis, palmitate or glucose alone robustly enhanced MuRF1 and atrogin-1, and decreased PGC-1\(\alpha\) mRNA and protein in MCO \((\text{Fig.3C-D; } \text{Dex was used as positive control})\).

**Bones of insulin resistant mice express functional AdipoR1**

We next asked if modulating PGC-1\(\alpha\) expression and activity could ameliorate diabetes-induced osteopenia. Adiponectin signaling via AdipoR1 in particular modulates PGC-1\(\alpha\) \((47; 48)\) and therefore we first assessed its expression in wt and diabetic skeleton at different ages.

We recently reported that compared to B6.db, db mice at 12 wk displayed severely depleted muscular AdipoR1 protein and impaired response to gAd \((32)\). Interestingly, AdipoR1 was readily detectable in the wt, B6.db and db bones while consistent with our previous report \((32)\) muscular AdipoR1 was markedly reduced in db but not B6.db mice \((\text{Fig})\).
4A). In agreement with differential AdipoR1 expression, acute gAd exposure caused AMPK phosphorylation in muscle and bones of wt, but only in bones of db mice (Fig. 4B).

To decipher intact AdipoR1 expression in bones while its depletion in db skeletal muscle, we assessed microRNA-221 (miR-221) and RNA binding protein polypyrimidine tract binding protein (PTB) levels as they negatively regulate AdipoR1 expression (49). miR-221 level in bone and muscle across all ages was significantly higher in both diabetic strains than wt, although compared to bone, differences in muscle was higher (2-3 fold in bone vs. 3-6 fold in muscle; Fig. 4C). PTB expression pattern however was different from miR-221. While 8 wk old bones had similar PTB expression across the groups, 12 and 16 wk old db bones displayed modest but significantly higher PTB expression than both wt and B6.db (Fig. 4C). The skeletal muscle however showed a dramatically higher PTB expression in 12 and 16 wk old db mice (5-20 fold) than both wt and B6.db. Taken together, PTB and miR-221 expression pattern appeared to correlate with the loss of AdipoR1 in skeletal muscle of db mice.

**GTDF, an AdipoR1 agonist and Met reverses osteopenia in diabetic mice**

Recently, we showed that the osteoanabolic agent GTDF (23) acting as an AdipoR1 agonist ameliorated diabetes in B6.db but not in db mice (32). Since AdipoR1 action in bone induces osteogenic effect (50) and db bones were AdipoR1-intact (Fig. 4A), we reasoned that despite its inability in ameliorating diabetes GTDF may still show osteoanabolic effect in db mice.

Testing the effect of GTDF along with standard anti-diabetic drugs necessitated sufficient number of age and sex-matched db mice that was not available in our colony and thus we procured fresh db mice in identical genetic background. This also allowed us to
confirm that the skeletal phenotype observed in db mice (Fig. 1) were not due to breeding and
maintenance-associated local factors. Trabecular and cortical parameters of both db mice
were comparable (Supplementary Figure 2A and 2B) and both displayed AdipoR1
expression in bone but not skeletal muscle (Supplementary Figure 2C). These newly
acquired db mice were then used for further studies.

We treated db mice with GTDF for 4 wk at a dose (10mg/kg) that failed to rescue
diabetes in them (32), so that any osteogenic outcome would not be a consequence of
improved diabetic phenotype. We compared the skeletal effects of GTDF with clinically used
antidiabetic drugs, Met (AMPK/PGC-1α activator) and Pio (PPARγ agonist).

Assessment of metabolic parameters revealed while final body weight significantly
increased in Pio-treated mice as expected, GTDF or Met did not alter it (Supplementary
Table 1). ECHO-MRI data showed that Pio but not GTDF or Met significantly increased fat
mass, while none of the treatments altered lean mass or water content (Supplementary Table
1). Pio, but not GTDF or Met significantly decreased fasting and non-fasting blood glucose
and Pio alone significantly reduced plasma TG and VLDL levels (Supplementary Table 2).

In gross observation by μ-CT, deterioration of femoral and tibial trabecular
architecture was readily observed in vehicle treated db, while both GTDF and Met caused
substantial improvement (Fig 5A). Compared with vehicle-treated db, GTDF-treated db mice
femur had significantly higher BV/TV, Tb.N, Tb.Th and Conn.D, and lower Tb.Sp, and all
the parameters were comparable to wt (Fig. 5A), suggesting complete trabecular restoration.
Although BV/TV, Tb.N and Tb.Th in Met-treated group were restored to the wt level, these
mice still had significantly lower Conn.Dn and higher Tb.Sp than wt (Fig. 5A), indicating a
partial restoration. Pio modestly but significantly improved BV/TV, Tb.N, Tb.Th and Conn.D
in db femur but failed to reduce Tb.Sp, and except Tb.Th and Tb.N, could not restore other
parameters to wt level (Fig. 5A). Tibial trabecular data show that except Tb.Sp, all
parameters in db were significantly lower than wt (Fig. 5A). GTDF-treated db mice displayed
significant improvement in all tibial parameters and except BV/TV and Tb.Sp, rest were
restored to the wt level (Fig. 5A), suggesting substantial tibial restoration. Met caused partial
tibial restoration as only Tb.Th and Conn.D were comparable to wt (Fig. 5A). Pio was
ineffective in restoring tibial cancellous bone as all parameters were lower than wt (Fig. 5A).

Biomechanical strength of femur diaphysis assessed by three-point bending
showed db mice had significantly lower strength parameters than wt (data not shown) and
GTDF and Met but not Pio-treated db mice displayed significantly higher resistance to
bending than vehicle-treated db as evidenced from higher energy, failure load and
stiffness (Fig 5B).

Further, GTDF and Met but not Pio–treated db had higher periosteal cell number
(Fig. 5C and Supplementary Figure 3). Osteogenic effect of GTDF and Met was further
evident from significantly higher Runx2 and OCN mRNAs in femur epiphysis of db mice
treated with them whilst Pio had no effect (Fig 5D). Further, GTDF and Met but not Pio
significantly lowered expression of adipogenic markers PPARγ and C/EBPα in db (Fig
5D).

In support of direct effects of GTDF and Met on osteoblasts, the cytotoxic and
apoptosis-inducing effects of glucose and palmitate on osteoblasts were mitigated by GTDF
and Met but not Pio (Fig 5E and F).

AMPK is a key downstream mediator of AdipoR1 signaling, which phosphorylates
and thereby activates PGC-1α (47). Osteoblasts in vehicle-treated db femur epiphysis had
severely depleted pAMPK compared to wt (Fig. 6A). Consistent with AdipoR1-intact status
of diabetic bone; GTDF-treated db epiphysis had significantly higher pAMPK than vehicle-
treated db (Fig. 6A). Met activates AMPK and thereby stimulates osteoblast differentiation
(51; 52) and consistent with this finding Met-treated db mice displayed significantly higher
pAMPK than vehicle control (Fig. 6A). Pio-treated db also had modest but significantly
higher pAMPK than vehicle-treated db (Fig. 6A). Similar to AMPK, PGC-1α expression in
osteoblasts was severely lower in vehicle-treated db mice and GTDF and Met but not Pio
restored it (Fig. 6A and Supplementary Fig. 4). MuRF1 displays an inverse correlation with
PGC-1α [(Fig. 3 and (44)] and here too MuRF1 was strongly expressed in vehicle-treated db
osteoblasts and GTDF and Met but not Pio–treated db mice its expression was significantly
lowered (Fig. 6A and Supplementary Fig. 4). These results were confirmed by
immunoblotting using whole marrow-free femur and tibia, where, while PGC-1α was
undetectable in vehicle and Pio -treated db, it was strongly expressed in GTDF or Met-treated
db mice (Fig 6B). Conversely, MuRF-1 level was high in vehicle and pio -treated db bones
while in GTDF and Met –treated bones it was drastically lower (Fig 6B).

**AdipoR1 and PGC-1α mediate the effects of GTDF in osteoblasts**

We next determined the roles of AdipoR1, AdipoR2, PGC-1α and MuRF1 in
osteoblasts by individually silencing them. Fig 7A shows confirmation of silencing of these
proteins in osteoblasts.

MuRF1 depletion reduced both palmitate and glucose-induced apoptosis in MCOs by
>50% (Fig 7B), suggesting the mediatory role of this atrogene in osteoblast apoptosis.

We asked if the protection conferred by GTDF against palmitate- and glucose-
mediated MCO apoptosis (Figure 5G) was AdipoR1-dependent. Indeed siAdipoR1 but not
siAdipoR2 or siC (non-silencing control siRNA) abolished anti-apoptotic effects of GTDF
Fig. 7C and Supplementary Fig. 5). siPGC-1α also abrogated GTDF effect (Fig 7C). As expected, the anti-apoptotic effect of Met was attenuated by siPGC-1α but not siAdipoR1 (Fig 7C).

Since both GTDF and Met restored trabecular and cortical parameters in db mice, which essentially indicated osteoanabolic actions of these compounds, we assessed GTDF and Met-mediated osteoblast differentiation. In control MCO (siC transfected) ALP activity was significantly stimulated by GTDF and Met (Fig 7D, gAd and BMP-2 was used as positive controls for AdipoR1 and differentiation respectively). Silencing PGC-1α abolished the induction of ALP activity stimulated by all agents (Fig 7D). Upon AdipoR1 silencing, the ALP stimulatory effect of gAd or GTDF but not BMP-2 was abolished (Fig 7D). Silencing AdipoR2 failed to impact the stimulatory effect of gAd or GTDF on ALP activity (Fig 7D). These data suggest the specific role of AdipoR1 and PGC-1α in GTDF-mediated osteoblast differentiation. Interestingly, basal ALP activity in MCOs was also significantly depleted in presence of siPGC-1α and siAdipoR1 but not siAdipoR2 (Fig. 7D) indicating that the autonomous activities of two proteins might also be required for osteoblast differentiation.

Discussion

Although there are numerous rodent models of type 2 diabetes, few have undergone thorough assessment of bone structure in relation to peak bone mass achievement and age-related bone loss. Amongst various mouse models that represent human obesity-associated with type 2 diabetes, leptin receptor-deficient mice in a C57-BLKS background represent severe phenotypes (53). In our hands, wt mice in C57-BLKS background displayed peak bone mass achievement at both trabecular and cortical sites at 12 wk whilst db mice did not. Several reports have shown osteopenia in leptin receptor-deficient mice (6; 13) with
the exception of one (54) which showed high bone mass compared to wt littermates. Our
data corroborates the finding of Williams et al., showing trabecular osteopenia in
approximately 11 week old male db (36). In addition, db mice showed severe age-related
bone loss. From these observations, it appears that db mice had an early termination of
peak bone gain and/or early onset of age-related bone loss. Bone accrual is critically
dependent on osteoblast number and function and since db mice displayed robust apoptosis
in osteoblasts at all ages, it appears that lack of viable osteoblasts and resultant deficiency
in osteoblast function in the db bones was responsible for their failure in peak bone mass
achievement and accelerated the development of age-related osteopenia. Bone formation is
consistently lower in type 2 diabetes patients compared to non-diabetics, as evidenced by
lower serum osteocalcin (55) and as reported before(36), we have observed that db mice
too had markedly lower osteocalcin levels at all ages than wt.

Glucose and palmitate induced mouse primary osteoblast apoptosis at pathologically
relevant concentrations. The extent of cytotoxicity induced by palmitate and glucose was
comparable to dex, a potent inducer of osteopenia (33). It thus appears that the combined
effect of glucotoxicity and lipotoxicity causes osteopenia that is equivalent in severity with
Dex and diabetic bones indeed shared common features of Dex-induced osteoporosis as bone
was lost at both trabecular and cortical sites.

Tumor suppressor p53 is a negative regulator of osteoblast differentiation as it
suppresses Runx2 expression and hypermorphic p53 mutation in mice causes osteopenia (56).
In db bones, p53 was increased and Runx2 suppressed, suggesting reduced osteoblast number
and differentiation. Skeletal muscle atrogenes were also elevated in the db bones and showed
age-related increase, which could also negatively impact osteoblast survival and
differentiation. In fact silencing MuRF1 did confer robust protection against palmitate and
glucose-mediated MCO apoptosis. It is possible that other atrogenes (atrogin-1 and cathepsin-L) may too play key roles in osteopenia induced by various stresses, as these factors were also induced in db bones and glucose and palmitate-treated MCO. Recent reports support such notion as mice lacking these atrogenes are protected from osteopenia under diverse stresses (45; 46). By contrast, the muscle anabolic factor, PGC-1α exhibited an age-related decline in db mice. Therefore it appears that like skeletal muscle, a reciprocal relationship between MuRF1 and PGC-1α exists in osteoblasts. Thus loss of osteoblast population and function in db bones may occur due to suppression of PGC-1α and induction of atrogenes. The regulation and possible interaction of p53 and Runx2 with MuRF1 and PGC-1α would be interesting future topic of investigation.

AdipoR1 increases insulin sensitivity and promotes cellular energy expenditure by activating AMPK and PGC-1α and AdipoR1 activation represents an attractive therapeutic approach for the treatments of obesity and type 2 diabetes (48). Previously, we showed that db skeletal muscle is deficient in AdipoR1 expression (32). By contrast, here we showed that diabetic bones are AdipoR1-intact. The explanation behind this appears to involve two negative AdipoR1 regulators, PTB and miR-221 (49), as both, especially PTB, were expressed at much higher levels in skeletal muscle than bones of diabetic mice.

Adiponectin and its receptors are expressed in bone marrow stromal cells that suggest their potential role in bone metabolism (57). C57BL6/J mice treated with adenoviral-derived adiponectin had increased trabecular bone mass and enhanced mineralization activity of osteoblasts (58). Mice harboring porcine AdipoR1 transgene had higher bone volume and trabecular number than the age- and sex matched controls (50). Cultures of bone marrow stromal cells from adiponectin knockout mice show significantly less osteogenesis than cultures from adiponectin-intact mice (57). Together, these reports suggest that adiponectin
may regulate bone formation in autocrine/paracrine manner in addition to endocrine mode. Although AdipoR1 expression in db bones was comparable to wt, osteopenia in db could be attributed to their observed hypo-adiponectinemia.

To investigate whether AdipoR1 in bone and more specifically osteoblasts of diabetic mice could be pharmacologically targeted to mitigate osteopenia through an osteoanabolic mechanism, we made use of GTDF. We have shown that GTDF improved diabetic phenotypes in B6.db mice with intact AdipoR1 in skeletal muscle and liver but not db that lacked functional AdipoR1 in these tissues (32). Thus db mice with skeletal (but not muscular) expression of functional AdipoR1 allowed us to selectively activate AdipoR1 in bone without correcting the diabetic phenotype. The most common clinically used drugs Met and Pio were used at their pharmacologically relevant doses for comparison of skeletal effects between the drugs.

Neither GTDF nor Met improved diabetic phenotypes in db but Pio was modestly effective, where although it failed to reduce NEFA yet it reduced fasting and non-fasting serum glucose, which however were still significantly higher than in wt. GTDF and Met improved trabecular microarchitecture, increased bone strength and bone lining cells. Pio was mostly ineffective as some improvement in femur epiphysis was seen but tibia metaphysis, where bone is actually accrued less than femur, showed no improvement. Also, strength parameters were not improved by Pio and neither did it increase periosteal cell number. The modest skeletal improvement by Pio could be due to modest mitigation of diabetic phenotypes as Pio failed to confer protection against gluco- and lipotoxicity on isolated osteoblasts. The skeletal improvement by GTDF and Met without altering diabetic phenotype as well as the protection imparted by them on isolated osteoblasts against glucose and palmitate assault clearly indicates that GTDF and Met had direct osteoblastic effect. Our data
also gains support from previous reports where Met has been shown to directly induce osteoblast differentiation (51; 59)) and protect against high glucose-mediated inhibition of osteoblast growth (60).

Although, both GTDF and Met restored bones of db mice by likely preservation of osteoblasts against gluco- and lipotoxicity, the molecular mechanism of action of the two were different. Silencing AdipoR1 but not AdipoR2 abrogated the pro-survival and differentiation-promoting effects of GTDF thus suggesting its AdipoR1-specific effect in osteoblasts as observed earlier in myocytes (32). Skeletal effects of Met however was blocked by silencing PGC-1α but not AdipoR1, suggesting that the osteoanabolic effect of the drug was mediated by PGC-1α and not AdipoR1, which was supported by the fact that Met failed to replace ¹²⁵I-gAd in a radioligand competition assay (data not shown). Salient findings of our study are summarized in Fig 8.

In conclusion, this study showed that diabetic mice in BLKS background have poor achievement of peak bone mass and age-related development of severe osteopenia and, inducing and activating PGC-1α in osteoblasts by GTDF or Met completely reverses osteopenia in these mice without correcting diabetic phenotypes.

**Author Contributions:** SS conceived the study, MPK, AKS, AAJ, MY, SoS, HK, AG, JSM, MCT, SK, GKN and MMG performed experiments, MPK, AKS, JRG, NC and SS designed experiments, AKT, RR, MMG, MRJ, RM, JRG, NC and SS supervised experiments, all authors analyzed data and contributed to discussion. NC and SS wrote the paper. NC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Acknowledgements: SS and NC acknowledge funding from CSIR network project ASTHI. Authors acknowledge sophisticated analytical instrument facility of CDRI for helps with confocal microscopy and flowcytometry. AKS and AG were supported by fellowships from CSIR. MPK and MY were supported by ICMR. HK, JSM, SoS and SK were supported by UGC.

Conflict of interest statement: AAJ and MRJ are employees of Zydus Research Center, the research and development arm of Cadila Healthcare Ltd. All authors have no conflict of interest.

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Figure Legends

Figure 1. Evaluation of bone phenotypes in 8, 12 and 16 wk old wt and db mice. A. Age and genotype-dependent changes in trabecular microarchitecture. µCT analysis was carried out using Sky Scan 1076 CT scanner (Aartselaar). Left panel shows representative images from 3D-µCT analysis of femur epiphyses (trabecular bone) from 8, 12 and 16 wk old wt and db mice. Bar graphs on the right show quantification. B. Age and genotype-dependent changes in cortical parameters. Femur mid-diaphysis representing cortical bone was analyzed by µCT. Left: 2D-µCT; representative images. Right: Quantification. A and B; n=6 bones/group. C. db mice display enhanced osteoblast apoptosis. Quantification of apoptotic osteoblasts was performed by dual TUNEL (DNA fragmentation marker) and Runx2 (osteoblast marker) staining of femur epiphysis sections (6 bones/group; 5 fields/bone) followed by confocal microscopy (Carl Zeiss LSM 510 Meta). Runx2+ve, TUNEL+ve cells were plotted as percent of total Runx2+ve cells, representative images in Supplementary Figure 1A. D. db mice display reduced bone lining cells. H&E stained femur epiphyses
sections (6 bones/group; 5 fields/bone) were used for counting by light microscopy by two
independent researchers blinded to experimental design; Representative microscopic images
in Supplementary Figure 1B. E-J. Serum biochemical parameters in wt and db mice, N=6/group. All graphs in the figure are mean ± SE. *8 wk vs. 12 or 16 wk age groups, # wt vs.
db (corresponding age groups). *,#p<0.05, **,##p<0.01, ###p<0.001 as determined by two
way ANOVA followed by Bonferroni’s post test. Tb.N; average trabecular number per unit
length, Conn.Dn; trabecular connectivity normalized by tissue volume, Tb.Sp; mean distance
between trabeculae.

Figure 2. Glucose and free fatty acid (palmitate) are sufficient for inducing osteoblast
apoptosis. A. Glucose and palmitate induce loss of osteoblast viability. MCO were treated
for 24h as indicated and cell viability was determined by an MTT assay and plotted as %
viable cells compared to vehicle treated controls. Dexamethasone (Dex) was used as positive
control. The glucose and long chain free fatty acid (palmitate) concentrations used here were
based on available references (12; 24; 54; 61). Data are mean ± SE of 3 independent
experiments performed in triplicates. *P<0.05, ***P<0.001 compared to vehicle treated
control as determined by one way ANOVA followed by Bonferroni’s post test. B. Glucose
and palmitate induce osteoblast apoptosis in a concentration-dependent manner. Mouse
calvarial osteoblasts were treated for 24h as indicated and apoptosis was analyzed by
Annexin-V-FITC and PI staining followed by analysis in a FACScalibur flowcytometer
(Beckton and Dickinson). Representative dot plots are shown. Right panel shows
quantification from 2 independent experiments. C. Palmitate but not glucose induces caspase-
3 activation. MCOs were treated for 24h as indicated and cleaved caspase-3 levels was
determined by immunoblotting. Data are representative of 3 independent experiments. V;
Vehicle; V for Dex treatment was 0.1% DMSO. V for glucose treated group was medium containing 5.5mM glucose and V for palmitate group was medium supplemented with 4% BSA.

**Figure 3. Diabetic bones exhibit enhanced skeletal muscle atrogenes and suppressed PGC-1α expression.**

A. Quantitative real time PCR (QPCR)-based gene expression analysis. Total RNA from femur epiphyses of indicated mice (N= 3/group) were isolated, reverse transcribed and were assessed for expression of indicated genes in triplicates. β-actin was used for normalization. Data are mean ± SE. *8 wk vs. 12 or 16 wk age groups, #wt vs. db *.*p<0.05, **p<0.01, ***p<0.001 as determined by two way ANOVA followed by Bonferroni’s post test.

B. Immunoblot-based evaluation of PGC-1α and MuRF1 protein levels in femur epiphyses. β-actin was used as loading control. N=3.

C. QPCR-based gene expression analyses in MCO. MCO were treated as indicated for 24h and mRNA expressions were analyzed. β-actin was used for normalization. Data are mean ± SE of 3 independent experiments performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test.

D. Evaluation of PGC-1α and MuRF1 expression by immunoblotting. β-actin was used as loading control. Data are representative of 3 independent experiments with similar results.

**Figure 4. db bone but not skeletal muscle express AdipoR1, bind to gAd and induce AMPK phosphorylation.**

A. Evaluation of AdipoR1 protein level in wt or diabetic mice. Total protein was isolated from femur epiphysis or extensor digitorum longus (EDL) muscles from wt, B6.db or db mice and AdipoR1 expression was determined by immunoblotting. N=3.

B. Radioligand saturation assays in isolated osteoblasts or myocytes. Osteoblasts derived from bone marrow cultures or myoblasts isolated from 12 wk old wt or db mice were differentiated in their respective differentiation mediums. Cells were incubated with 125I-gAd
(specific activity 407.43 Ci/mmole) for 2h (at this time point binding equilibrium was reached) and following washes the cells were lysed and radioactivity was determined in a gamma counter. Data are mean ± SE of 3 independent experiments performed in duplicates. Photomicrographs above the line graphs show representative phase-contrast images of the cells before they were used for $^{125}$IgAd binding assay (objective 10×, magnification 100×). C. db bones but not skeletal muscle display gAd-sensitivity. wt or db mice were intraperitoneally injected with V (PBS) or gAd. 30 min following injection, mice were sacrificed and total protein from EDL muscles and marrow free femurs were used for determination of pAMPK and AMPK levels by immunoblotting. N=3/group. D. Determination of age and strain-dependent bone and skeletal muscle expression of PTB and miR-221. For miR-221, total miRNA from bone and gastrocnemius muscle were isolated using a micro RNA isolation kit. miRNA-221 values were normalized with U6 expression and plotted. For PTB, total RNA isolated from the same tissues was used. Data are mean ± SEM of 3 independent experiments performed in triplicates. *8 wk vs. 12 or 16 wk age groups, #wt vs. B6.db or db, @@B6.db vs. db. *,#,@p<0.05, **,##,@@p<0.01, ###,@@@p<0.001 as determined by two way ANOVA followed by Bonferroni’s post test.

Figure 5. AdipoR1 agonist GTDF and AMPK/PGC-1α activator metformin (Met) but not PPARγ agonist pioglitazone (Pio) improved bone phenotype in diabetic mice. 12 wk old db mice were treated with GTDF or Pio (10mg/kg b.w.) or Met (350mg/kg b.w.) for 4 wk (comparison of skeletal parameters with db and AdipoR1 expression are in Supplementary Figure 2ACC. Metabolic parameters and the plasma biochemistry are in Supplementary Tables 1 and 2). All graphs are mean ± SE. *wt vs db (all treatments), #vehicle-treated db vs other treatment groups. A. Evaluation of trabecular restoration by GTDF, Met and Pio. Femur and tibia epiphyses (white and black bars respectively) from indicated animals (n=10/group) were evaluated by μCT. Representative images are shown along with quantification data
B. Evaluation of bone strength. Bone strength parameters of the same animals were determined by 3-point bending test. N=10/group. C. GTDF and Met but not Pio increase the number of trabecular lining cells. H&E stained femur epiphyses sections (10 bones/group) were used for counting by two independent researchers blinded to experimental design. Representative images are in Supplementary Figure 3. D. GTDF and Met enhanced osteoblast formation markers and suppressed adipogenic marker expressions in femur epiphysis. Femur epiphysis from db mice (n=3; performed in triplicates) treated as indicated were assessed for the expression of indicated mRNAs by QPCR. E. GTDF and Met but not Pio ameliorate palmitate and glucose-induced loss of osteoblast viability. MCOs were pretreated with GTDF (0.1µM), Met (100µM) or Pio (1µM) for 24h followed by treatment with indicated concentrations of glucose or palmitate for a further 24h. Cell viability then was assessed by MTT assay. Data are mean ± SEM of three independent experiments performed in triplicates. F. GTDF and Met protect against palmitate and glucose-induced apoptosis. MCO were pretreated with GTDF (0.1µM) or Met (100µM) for 24h followed by 24h incubation in medium containing indicated concentration of glucose or palmitate. Apoptosis was assessed by Annexin-V-FITC and PI staining followed by flowcytometry. Representative dot plots from 2 independent experiments with similar results are shown. *V vs. treatment groups. *p<0.05, **p<0.01, ***p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test.

Figure 6. GTDF and Met but not Pio induce pAMPK, PGC-1α and suppress MuRF1 expression in db mice. A. Evaluation of osteoblast expressions of pAMPK, PGC-1α and MuRF1. Femur epiphysis sections obtained from 6 mice/group were probed with indicated antibodies and visualized by fluorescent microscopy (Carl zeiss, AX10-Imager. M2). Representative photomicrographs are shown (objective 20×, magnification 200×).
Microscopic quantifications are given as percent cell count. B. PGC-1α and MuRF1 expression in femur or tibia epiphyses from mice treated as indicated. N=3. *Comparison of db with wt mice. #Comparison between vehicle-treated db mice with GTDF, Met or Pio-treated groups. *:# p<0.05, **:## p<0.01, ***:#### p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test.

Figure 7. MuRF1 is necessary for palmitate or glucose-induced osteoblast apoptosis and AdipoR1 and PGC-1α are required for GTDF-induced protection against palmitate and glucose-mediated osteoblast apoptosis. A. siRNA-mediated silencing of PGC-1α, MuRF1, AdipoR1 and AdipoR2. MCO were transfected with 0.1µM of each siRNA and expression of indicated proteins was assessed by immunoblotting. Data are representative of 3 independent experiments with similar results. B. MuRF1 depletion protects against glucose and palmiate-induced osteoblast apoptosis. 48h after transfection with indicated siRNAs, MCO were incubated in medium containing indicated concentrations of palmitate or glucose for further 24h. Apoptosis was then assessed by flowcytometry. One representative set of dot plots from 2 independent experiments is shown. Bar graph is quantification from two independent experiments (mean±SE). C. GTDF protection against glucose and palmitate-induced osteoblast apoptosis was compromised by AdipoR1 and PGC-1α silencing. 48h after transfection with indicated siRNAs, MCO were incubated in medium containing glucose (22mM) or palmitate (500µM) supplemented with vehicle, GTDF (0.1µM) or Met (100µM) for further 24h. Apoptosis was then assessed by flowcytometry. One representative set of dot plots from 2 independent experiments is shown. Bar graph is quantification from two independent experiments. AdipoR2 silencing failed to influence GTDF or Met effects; supplementary Figure 5. D. GTDF and gAd-mediated osteoblast differentiation is dependent on PGC-1α and AdipoR1 but not AdipoR2. 48h after transfection with indicated siRNAs, MCO were incubated in differentiation medium supplemented with vehicle, GTDF.
(0.1µM), gAd (1µg/ml), BMP2 (0.1µg/ml) or Met (100µM). 24h after treatment, osteoblast differentiation was determined by fluorometric quantification of the differentiation marker alkaline phosphatase (ALP). Data are mean ± SE of 3 independent experiments performed in duplicates. *Vehicle vs. treatment groups, #siC vs. other siRNA transfected groups. ***p<0.001, #p<0.05, ###p<0.001 as determined by two way ANOVA followed by Bonferroni’s post test.

Figure 8. Schematic diagram summarizing the pathophysiological mechanisms of bone loss in db mice and a potential osteoanabolic role of AdipoR1. In osteoblasts gluco-/lipotoxicity induces downregulation (-) of pAMPK and PGC-1α (a key regulator of cellular energy metabolism and whose expression and activity are modulated by AdipoR1) and upregulation (+) of several skeletal muscle atrogenes (atrophy related genes that are involved in protein catabolism) resulting in impaired survival and differentiation of these cells. Osteopenia in db mice is associated with reduced expression of osteogenic genes likely due to decreased PGC-1α and increased atrogenes. Atrogenes could in turn activate p53 leading to inhibition of osteoblast survival and function. Osteopenia in db mice could be reversed by GTDF (an AdipoR1 agonist) or Met (acting independent of AdipoR1 activation) but both converge to PGC-1α to promote osteogenic genes and suppress atrogenes that ultimately improve skeletal health by a likely osteoanabolic mechanism. (Stimulation) and (inhibition).

Supplementary information includes Supplementary Figures 1-5 and and Supplementary tables 1-3.
Figure 3
Femur epiphysis

A

B

C

D

Relative mRNA

PGC-1α
MuRF1
β-actin

8 weeks
12 weeks
16 weeks

wt
db
wt
db
wt
db
wt
db

1
2
3
4
5
6
7
8
9
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11
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Diabetes

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Figure 5

A

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B

Bone strength parameters
- Energy (mJ)
- Ultimate load (N)
- Stiffness (N/mm)

C

Periodontal lining cells
- V
- GTDF
- Met
- Pio

D

Relative mRNA
- RUNX1
- OPG
- RANKL
- CEBPα

E

Palmitate (µM)
- 250
- 500
- 750

Glucose (mM)
- 11
- 22
- 44

F

Glc (22mM)
- V
- Glc+GTDF
- Glc+Met

Palm (500µM)
- V
- Palm+GTDF
- Palm+Met

Annexin V-FITC

270x406mm (600 x 600 DPI)
Diabetes

Figure 6

A

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B

Femur

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Tibia

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<td>Met/GDF</td>
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Cell count (%)

- pAMPK/Runx2
- PGC-1α/Runx2
- MuRF1/Runx2

319x568mm (600 x 600 DPI)
Diabetes
Figure 8

Glucose/NEFA

Metformin (?)

GTDF

AdipoR1

pAMPK

PGC-1α

Atrogene

OCN, Runx2

P53

Osteoblast survival/Differentiation
Supplementary Figure 1. Age and diabetes-dependent changes in osteoblast viability and periosteal cell number. 

A. Bones from db mice display apoptotic osteoblasts. 5µm thick femur sections from indicated mice were analyzed by TUNEL and Runx2 staining followed by confocal microscopy (bars 50µm). DAPI was used as a nuclear stain. Representative images are shown. N=6 bones/group; 5 fields/bone section. 

B. db bones display severe depletion of periosteal cells. Femur epiphysis sections were stained with H&E and visualized by light microscopy. Arrows indicate periosteal cells (flattened nuclei). Representative images are shown (objective 40X, magnification 400X). N=6 bones/group; 5 fields/bone section.
Supplementary Figure 2. db mice from ZRC and Harlan (New db) display comparative skeletal features. A. µCT derived parameters for trabecular microarchitecture between 14 wk old wt, db and new db (n=6 bones/group), were compared. B. µCT derived cortical parameters between 14 wk old wt, db and new db (n=6 bones/group), were compared. *p<0.05, **p<0.01, ***p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test. C. Both db and New db retain skeletal but not muscle AdipoR1 expression. AdipoR1 expression was determined in femur epiphyses or EDL muscles by immunoblotting. N=3.
Supplementary Figure 3. GTDF and Met but not Pio enhance surface lining cells in diabetic mice. Femur epiphysis sections were stained with H&E and visualized by light microscopy (objective 40X, magnification 400X). Arrows indicate perisoetal cells (flattened nuclei). Representative images are shown. N=6 bones/group; 5 fields/bone section.
Supplementary Figure 4. GTDF and Met but not Pio enhance PGC-1α and suppresses MuRF1 mRNA expression. Total RNA from femur epiphyses of vehicle (V) or GTDF, Met or Pio–treated db mice were analyzed for PGC-1α and MuRF1 expression. N=3. **p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test.
Supplementary Figure 5. Silencing AdipoR2 fails to affect GTDF action of glucose and palmitate-induced osteoblast apoptosis. 48h after transfection with indicated siRNAs, MCO were incubated in medium containing glucose (22mM) or palmitate (500µM) supplemented with vehicle, GTDF (0.1µM) or Met (100µM) for further 24h. Apoptosis was then assessed by flowcytometry. One representative set of dot plots from 2 independent experiments is shown.
Supplementary Table 1. Pioglitazone but not GTDF and Met alter metabolic parameters in diabetic mice. Mice were subjected to Echo MRI (Model no. 700) on indicated days. Fed glucose was measured as described in methods section. *p<0.05, ***p<0.001 as determined by one way ANOVA between different treatment groups (corresponding ages) followed by Bonferroni’s post test.

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<td>26</td>
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**Other Parameters**

| Body weight | 46.8±0.9 | 49.5±1.2 | 47.5±0.4 | 49.9±0.9 | 46.7±0.4 | 50.8±2.2 | 46.9±0.8 | 57±0.8*** |
| Fed Glucose (mg/dl) | 337±11 | 531±22 | 334±11 | 611±34 | 357±26 | 529±50 | 355±24 | 368±41*** |
| Number of animals | 16 | 16 | 16 | 16 | 6 | 6 | 6 | 6 |
Supplementary Table 2. Comparison of fasting serum biochemical parameters in db mice treated as indicated for 28 days. ND; not done. *p<0.05, ***p<0.001 as determined by one way ANOVA followed by Bonferroni’s post test.

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<tr>
<td>Glucose (mg/dL)</td>
<td>590±48</td>
<td>516±44</td>
<td>479±55</td>
<td>340±56***</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.59±0.34</td>
<td>1.81±0.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.8±0.09</td>
<td>0.8±0.06</td>
<td>1.3±0.2</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>140±10</td>
<td>159±26</td>
<td>154±18</td>
<td>156±7</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>83±6</td>
<td>96±16</td>
<td>87±8</td>
<td>52±5*</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>81±6</td>
<td>92±15</td>
<td>79±10</td>
<td>80±4</td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>17±1</td>
<td>19±3</td>
<td>17±2</td>
<td>10±1*</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>74±6</td>
<td>79±11</td>
<td>73±6</td>
<td>66±4</td>
</tr>
<tr>
<td>PAI-1 (pg/mL)</td>
<td>20117±3544</td>
<td>10933±4491</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>13±4</td>
<td>25±9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td>108316±23410</td>
<td>95392±33863</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Resistin (pg/ml)</td>
<td>1904±442</td>
<td>2168±486</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.6±0.12</td>
<td>0.7±0.06</td>
<td>0.5±0.1</td>
<td>0.8±0.02</td>
</tr>
</tbody>
</table>
**Supplementary Table 3. Oligonucleotide sequences used for qPCR. Orientation (5’→3’)**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>p53</td>
<td>CCGTGTGGTTCATCCCTGTA</td>
<td>TTTGGATTTTTAAGACAGAGTCTTTGTA</td>
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<tr>
<td>Runx2</td>
<td>AAGTGCCTGAACCAACTTTCT</td>
<td>TCTCGGGCTGCTAGTGA</td>
</tr>
<tr>
<td>Cathapsin-L</td>
<td>CAAAATAAGAATAATAATGGCTGGTTGCA</td>
<td>TGTAGCTTTCCATACCCCATT</td>
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<tr>
<td>Atrogin-1</td>
<td>AGTGAGGACCCTACTGTG</td>
<td>GATCAACGCTTGCAATCT</td>
</tr>
<tr>
<td>MuRF1</td>
<td>CCTGCAGAGACCAACAGGA</td>
<td>GGCCTAGAGGCTGCAAAACT</td>
</tr>
<tr>
<td>GLU-L</td>
<td>AGTCTGAAGGCTTCCAACAGC</td>
<td>AAGGGGTCTCAAAACATGG</td>
</tr>
<tr>
<td>PTB</td>
<td>TCTACCCAGTGACCCTTGAC</td>
<td>GAGCCTGGAGAAAGTCGATGC</td>
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<tr>
<td>PGC-1α</td>
<td>AGCCGTGACCCTGACAACAGAG</td>
<td>CTGACATTGGTTCTAGTGCTAAG</td>
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<tr>
<td>PPARγ</td>
<td>CAGGCCGAGAGGGAGAAGCT</td>
<td>GGCTCGAGATCAGACGAGACT</td>
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<td>C/EBPα</td>
<td>AAACACGGCAAGTGGAGAC</td>
<td>TGTCCAGTTTACGGCTCAG</td>
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<tr>
<td>OCN</td>
<td>CTGACAAAGGCTTCTAGTCCAA</td>
<td>GCGGGCGAGTGCTTCCACTA</td>
</tr>
<tr>
<td>β-actin</td>
<td>CCTCACCTCCCCAAAAGC</td>
<td>GTGGACTCAGGGCATGGA</td>
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