Published in final edited form as:

Calcif Tissue Int. 2012 August ; 91(2): 139–148. doi:10.1007/s00223-012-9623-4.

Rosiglitazone Inhibits Bone Regeneration and Causes Significant Accumulation of Fat at Sites of New Bone Formation

Lichu Liu,

Arkansas Children's Hospital Research Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA

James Aronson,

Arkansas Children's Hospital Research Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Department of Orthopaedic Surgery, Center for Orthopaedic Research, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Shilong Huang,

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Yalin Lu,

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Piotr Czernik,

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Sima Rahman,

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Center for Diabetes and Endocrine Research, University of Toledo Health Sciences Campus, Toledo, OH, USA

Vipula Kolli,

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Larry J. Suva, and

Department of Orthopaedic Surgery, Center for Orthopaedic Research, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Beata Lecka-Czernik

Department of Orthopaedic Surgery, University of Toledo Health Sciences Campus, Toledo, OH, USA

Center for Diabetes and Endocrine Research, University of Toledo Health Sciences Campus, Toledo, OH, USA

© Springer Science+Business Media, LLC 2012

Correspondence to: Beata Lecka-Czernik, beata.leckaczernik@utoledo.edu.

S. Huang, Department of Orthopaedic Surgery, Tongji Hospital and Medical College, Huazhong University of Science & Technology, 1095 Jiefang Avenue, Wuhan, People's Republic of China

The authors have stated that they have no conflict of interest.

Departments of Orthopaedic Surgery, Physiology and Pharmacology, University of Toledo Health Sciences Campus, 3000 Arlington Avenue, Toledo, OH 34614, USA

Beata Lecka-Czernik: beata.leckaczernik@utoledo.edu

Abstract

Thiazolidinediones (TZDs), peroxisome proliferator-activated receptor gamma activators, and insulin sensitizers represent drugs used to treat hyperglycemia in diabetic patients. Type 2 diabetes mellitus (T2DM) is associated with a twofold increase in fracture risk, and TZDs use increases this risk by an additional twofold. In this study, we analyzed the effect of systemic administration of the TZD rosiglitazone on new bone formation in two in vivo models of bone repair, a model of drilled bone defect regeneration (BDR) and distraction osteogenesis (DO) and a model of extended bone formation. Rosiglitazone significantly inhibited new endosteal bone formation in both models. This effect was correlated with a significant accumulation of fat cells, specifically at sites of bone regeneration. The diminished bone regeneration in the DO model in rosiglitazone-treated animals was associated with a significant decrease in cell proliferation measured by the number of cells expressing proliferating cell nuclear antigen and neovascularization measured by both the number of vascular sinusoids and the number of cells producing proangiogenic vascular endothelial growth factor at the DO site. In summary, rosiglitazone decreased new bone formation in both BDR and DO models of bone repair by mechanisms which include both intrinsic changes in mesenchymal stem cell proliferation and differentiation and changes in the local environment supporting angiogenesis and new bone formation. These studies suggest that bone regeneration may be significantly compromised in T2DM patients on TZD therapy.

Keywords

Rosiglitazone; Diabetes; Bone healing; Osteoblast; Angiogenesis; Adipocyte

Introduction

Clinically, bone homeostasis and bone repair are affected in patients with type 2 diabetes mellitus (T2DM) [reviewed in 1]. Regardless of normal or even high bone mineral density, T2DM patients are at increased risk of fractures [2, 3]. The therapeutic use of antidiabetic the thiazolidinediones (TZDs) rosiglitazone and pioglitazone correlates with bone loss and further increases fracture risk, placing TZDs in a category of drugs associated with secondary osteoporosis [1]. Additional risk factors for the development of TZD-induced secondary osteoporosis are gender (women), age (elderly), and duration of treatment [4-7].

TZDs exert their antidiabetic effects by activating the adipocyte-specific transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ), which controls glucose and fatty acid metabolism [8]. In bone, PPAR γ controls the differentiation of cells from the mesenchymal and hematopoietic lineages toward bone-forming osteoblasts and bone-resorbing osteoclasts, respectively [9-14]. Numerous mechanistic studies in animals and in in vitro cell models suggest that the TZDs uncouple normal bone remodeling by decreasing bone formation and/or increasing bone resorption; however, it is still unclear which of these processes are responsible for the bone loss and increased fractures observed in humans [10, 11, 13, 15-18]. PPAR γ , specifically its PPAR γ 2 isoform, commits marrow-derived mesenchymal stem cells (MSCs) toward the adipocyte lineage and suppresses their differentiation toward the osteoblast lineage. These effects translate in vivo to significantly decreased osteoblast numbers and bone formation rate with a simultaneous increase in the number of adipocytes in the bone marrow [10, 19].

Although the detrimental effects of TZDs on bone mass and increased fracture rate are well documented, surprisingly there is a paucity of evidence describing the effect of TZDs on bone healing. Bone healing is a complex cascade of cellular events that requires the differentiation of osteoblasts from the pool of MSCs, neovascularization, and primary bone formation that precedes the remodeling phase consisting of bone resorption and secondary bone formation [20]. In the studies described here, the effect of systemic administration of rosiglitazone on new bone formation was studied in two in vivo bone repair models, drilled bone defect regeneration (BDR) and tibial distraction osteogenesis (DO) [21-25]. Both models allowed a detailed analysis of the effect of TZD-activated PPAR γ on the process of new bone formation, fat accumulation, and neovascularization that have been shown to be critical for normal bone healing [20]. These studies provide compelling evidence that bone regeneration and healing are likely to be affected in individuals who are on TZD therapy.

Materials and Methods

Animals

Sixteen-week-old male mice of either C57BL/6 strain (Jackson Laboratories, Bar Harbor, ME) or an unrelated a/a strain (agouti control VY/WffC3Hf/Nctr-a, colony maintained at the University of Arkansas for Medical Sciences) [26, 27] were used in these studies. C57BL/6 mice were used for the BDR experiments, whereas a/a mice were used for the DO studies. The design of both experiments followed the same scheme. To mimic the clinical situation in which patients experience fracture and undergo subsequent healing while on TZD therapy, treatment with rosiglitazone was initiated 2 weeks prior to the initial bone injury for either BDR or osteotomy for DO studies. Rosiglitazone treatment was continued for the next 2 weeks after either introduction of the drilled defect or DO osteotomy. For BDR studies, the experimental groups consisted of four mice/group and for DO studies six mice/group were used. Mice were fed a controlled amount of chow either nonsupplemented or supplemented with rosiglitazone at a concentration of 0.14 mg/g chow. The average intake of rosiglitazone calculated at the end of the experiment was 16 μ g/g body weight/day. After surgery, mice were housed in individual cages in constant temperature—(22 °C) and humidity—(50 %) controlled rooms on a 12-h light/12-h dark cycle. All research protocols were approved by the Institutional Animal Care and Use Committee at the University of Toledo Health Sciences Campus (for BDR) and at the University of Arkansas for Medical Sciences (for DO).

Drilled BDR Regeneration Model

The drilled BDR model consists of a defect created in the lateral surface at the tibial midshaft by drilling a 0.9-mm-diameter hole through a single cortex. The regeneration process comprises intramembranous bone formation [21-23]. This model allows for the creation of a reproducible bone defect, which regenerates in a timely, ordered fashion. The bone-healing sequence consists of an initial inflammatory response (first week), followed by hard callus formation with poorly organized woven bone (second week) and a phase of primary bone remodeling, which leads to final bone healing (third and fourth weeks) (Fig. 1). In the present experiments bone regeneration was continued for a period of up to 2 weeks until mice were killed. Operated tibiae and intact contralateral control tibiae were harvested for microCT analysis of both the mineralized bone component and the marrow fat component, as described below.

DO Model

DO represents a model of bone regeneration through purely intramembranous bone formation induced in response to low-energy distraction [28]. Mouse tibiae underwent placement of a two-ring external fixator spanning a mid-shaft osteotomy, as previously

described [28]. Distraction began 1 day after surgery (24-h latency) at a rate of 0.075 mm twice a day (total 0.15 mm/day) and was continued for 14 days. After the distraction period, mice were killed and both tibiae (distracted and contralateral) were harvested for μ CT and histological analyses.

μ CT Visualization and Volumetric Analysis of Bone in BDR and DO Models

Images of bone specimens from the BDR model were acquired at 70 kV X-ray energy and 114 μ A intensity using the μ CT-35 system (Scanco Medical, Bassersdorf, Switzerland) at the University of Toledo Health Science Campus and reconstructed using 6 μ m isometric voxel at 2,048 samples and 1,000 projections. Image analysis was conducted on 220 consecutive cross-sectional slices encompassing the defect and including 0.2 mm of intact bone at each end of the specimen. Image segmentation was done at global threshold conditions by applying an optimized gray-scale threshold of 220–1,000 with a 3-dimensional (3-D) noise filter set to sigma 1.2 and support 2.0. Bone volumes were calculated directly from the sum of the individual voxel volumes in 3-D reconstructions. Images of bone specimens resulting from DO experiments were acquired at 55 kV X-ray energy and 70 μ A intensity using the μ CT-40 system (Scanco Medical) at the University of Arkansas for Medical Sciences and reconstructed using 12.4 μ m isometric voxel and analogous sample and projection settings, as previously described [29]. Image analysis was conducted on 200 cross-sectional slices encompassing the defect and including 0.4 mm of intact bone at each end of the specimen. Image segmentation was done by applying an optimized gray-scale threshold of 245–1,000 with a 3-D filter set to sigma 0.8 and support 1.0. Minor differences between measurements obtained with the two different μ CT systems regarding X-ray energies, image resolutions, and 3-D filtering were appropriately corrected.

μ CT Visualization and Volumetric Analysis of Lipid Content in Bone Marrow Surrounding the BDR Site

Following completion of bone imaging, specimens containing BDR and contralateral counterparts were separately decalcified in 10 mL of 2 % HCl and 1 % EDTA for 3.5 h at room temperature with slow agitation. Next, specimens were washed for 5 min in PBS buffer (pH 7.0) and stained for 1 h in solution containing 2 % osmium tetroxide prepared in 0.1 M sodium cacodylate buffer (pH 7.4). Staining was carried out in an exhaust hood and away from light due to osmium tetroxide toxicity and light sensitivity. Images of lipid depositions were acquired at 70 kV energy and 114 μ A intensity using the μ CT-35 system and reconstructed using 12 μ m isometric voxel at 1,024 samples and 500 projections. Image segmentation was done under global threshold conditions by applying a gray-scale threshold of 480–1,000 using a permille scale with a 3-D noise filter set to sigma 1.2 and support 2.0. Lipid volumes were calculated directly from individual voxel volumes in 3-D reconstructions.

Histological and Immunohistochemical Analyses of the DO Gap

Distracted and contralateral tibiae were decalcified in 5 % formic acid, dehydrated, and embedded in paraffin [30, 31]. Longitudinal (coronal) sections of 5–7 μ m thickness were cut on a microtome (Leitz 1512, Wetzlar, Germany) and stained with hematoxylin and eosin. Sections were selected to represent a central gap location from the mid-sagittal plane including all four cortices and the medullary canal proximally and distally. Adipocyte number was analyzed in a quadrilateral region of interest (ROI) which included both the proximal and distal endosteal new bone measured from the inside corners of the cortices adjacent to the medullary canals. Adipocytes were identified as empty spaces with the characteristic oval shape. Osteoblasts expressing osteocalcin were detected with antiosteocalcin polyclonal antibody (ALX-210-333; Alexis Biochemical, San Diego, CA). Cells expressing proliferating cell nuclear antigen (PCNA), vascular endothelial growth

factor (VEGF), and CD34, a marker of endothelial and hematopoietic cells, were detected by immunohistochemistry using polyclonal antibodies against each: sc-7907, sc-507, and sc-18917, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Nonimmune serum, nonimmune IgGs (rabbit, goat, rat), and biotinylated secondary antibody were purchased from Vector Laboratories (Burlingame, CA).

Statistical Methods

Statistical analyses of differences between the control and experimental groups for all μ CT and immunohistochemical analyses were performed using Student's *t*-test. All data are reported as the mean $\pm$ standard deviation (SD), and differences are considered statistically significant when $p < 0.05$.

Results

Rosiglitazone Decreased New Bone Formation and Caused Significant Fat Accumulation within the Bone Defect in the BDR Model

The BDR model comprises a bone defect introduced by drilling a hole across a single cortex of the tibial diaphysis and its regeneration for a period of 4 weeks. The effect of rosiglitazone on bone regeneration was assessed in animals which were preconditioned to rosiglitazone for 2 weeks before a defect was generated and 2 weeks after a defect was created. Tibial bones were harvested 2 weeks after surgery, during the phase of most active bone formation. This phase comprises high osteoblast activity involved in the formation of woven bone and an effective process of neovascularization that is supported by the local bone marrow microenvironment (Fig. 1) [21-23]. Harvested bones were analyzed with μ CT for a volume of newly formed bone and a volume of fat accumulated at the defect site. As shown in Fig. 2a, c, the total volume of newly formed bone within the BDR defect in mice treated with rosiglitazone was 35 % lower than in control mice. More detailed analysis demonstrated that rosiglitazone treatment specifically decreased endosteal bone formation (by 60 %), whereas formation of new bone in the periosteal area was unaffected (Fig. 2c).

The decrease in new bone formation due to rosiglitazone treatment was associated with substantial fat infiltration into the same region (Fig. 2a, b, d). The fraction of fat occupying the region of the drilled defect undergoing regeneration was significantly increased in mice receiving rosiglitazone (33.3 % rosiglitazone vs. 0.4 % control, $p < 0.05$) (Fig. 2d, total fat). This increase was primarily due to an increase in fat content in the endosteal region (27.3 % rosiglitazone vs. 0.3 % control, $p < 0.05$) (Fig. 2d, endosteal fat) rather than the periosteal region (9.2 % rosiglitazone vs. 0.1 % control) (Fig. 2d, periosteal fat). The significant increase in fat volume following rosiglitazone treatment was specific to the area where bone regeneration occurred since rosiglitazone administration increased fat content in the corresponding region of uninjured contralateral tibia only to approximately 1.5 % (Fig. 2e). These data suggest that the mesenchymal cells involved in the process of new bone formation express PPAR γ and are prone to proadipocytic differentiation in response to rosiglitazone-activated PPAR γ .

Rosiglitazone Decreased New Bone Formation and Caused Accumulation of Fat in the DO Gap

To study the effects of rosiglitazone treatment further, we employed another model of bone regeneration, namely, tibial DO. The new bone formation in this model is induced by a gradual, low-energy mechanical distraction to the murine tibia [20, 24, 25]. Bone is formed in the distraction gap by intramembranous ossification and follows a well-described pattern of uniform microcolumns parallel to the distraction force [20, 24]. DO produces a large number of bone-forming zones that are completely lined by active osteoblasts and initially

uncoupled from the presence or activity of osteoclasts. Thus, DO represents a model uniquely suited for in vivo study of osteoblast function during direct trabecular bone formation.

As in the BDR studies, mice undergoing DO were preconditioned with rosiglitazone before surgery, as described in “Materials and Methods” section; and treatment continued for the following 2 weeks after surgery, which covered the entire DO process. At the end of the experiment, operated and contralateral tibiae were harvested and new bone formation in the operated tibia was analyzed with μ CT. As shown in Fig. 3a, new bone formation in the DO gap was significantly compromised in mice treated with rosiglitazone. Compared to untreated control mice, the fraction of total new bone formed in the DO gap was decreased by 22 % in mice receiving rosiglitazone (Fig. 3b, total new bone). Detailed analysis showed that the fraction of new bone that was formed in the endosteal area was decreased by 82 % (Fig. 3b, endosteal new bone), whereas the fraction of bone that was formed in the periosteal area was decreased by 33 % compared to nontreated control (Fig. 3b, periosteal new bone). As in the BDR model, rosiglitazone induced fat accumulation within the DO gap (Fig. 3c, d). In untreated animals adipocytes were virtually absent within zones of new bone formation, whereas their number increased by approximately 1,000-fold in the same zones in animals treated with rosiglitazone (Fig. 3c, d). In contrast, the increase in adipocyte number in the corresponding area of the contralateral tibia was relatively modest, with an average of approximately tenfold (Fig. 3c).

An analysis of osteoblast numbers on the surface of newly formed bone at the DO site showed no difference in the number of osteocalcin-positive cells present in control and rosiglitazone-treated animals (data not shown). This indicates that rosiglitazone did not affect more mature osteoblasts once new bone had formed but, rather, their precursors at the stage of lineage commitment to adipocytes or osteoblasts.

Rosiglitazone Decreased a Number of PCNA-Positive Cells at the DO Site

Intramembranous bone formation in the DO gap requires the differentiation of mesenchymal progenitor cells to osteoblasts. The prerequisite step for osteoblast differentiation is cell proliferation [32]. The PCNA protein is specifically expressed in cells undergoing mitotic division and serves as a marker of cell proliferation. As shown in Fig. 4a, the number of PCNA-positive cells in the DO gap was significantly decreased in rosiglitazone-treated compared to control animals. Interestingly, rosiglitazone treatment affected not only the number but also the distribution of PCNA-positive cells in the zone of new bone formation. In nontreated mice, a dense and highly organized line of proliferating cells was present along the leading edge of the developing bone microcolumns (Fig. 4a, left microphotograph). In contrast, in animals treated with rosiglitazone this arrangement was disorganized and led to a relative decrease in the number of PCNA-positive cells along the microcolumn leading edges (Fig. 4a, right microphotograph).

Rosiglitazone Decreased a Number of Cells Expressing VEGF and a Number of Vascular Sinusoids at the DO Site

Angiogenesis is essential to new bone formation by supplying necessary nutrients, oxygen, and cells for this process [20, 33]. New vessel formation is supported by mesenchymal cells producing proangiogenic factors including VEGFs and angiopoietins in response to FGF and PDGF cytokines supplied locally by other bone marrow components. At the DO site, the formation of new bone columns is coupled to a parallel formation of vascular sinusoids [24, 25]. Compared to controls, the number of cells present at the DO site and producing VEGF was decreased by 33 % in rosiglitazone-treated animals (Fig. 4b). The decrease in the number of VEGF-positive cells was paralleled by a 75 % decrease in the number of vascular

sinusoids in the DO gap of animals receiving rosiglitazone compared to control. These data imply that the negative effect of rosiglitazone on new bone formation includes inhibition of new vessel formation, which can be attributed to changes in a local environment supporting angiogenesis.

To determine the extent to which rosiglitazone treatment may affect the angiogenic environment provided by cells of mesenchymal origin, we reanalyzed our existing microarray database representing the rosiglitazone-activated PPAR γ 2 transcriptome in U-33/ γ 2 cells, which serve as a model of bipotential marrow MSCs in which osteoblast and adipocyte differentiation is under the control of PPAR γ 2 [9, 12]. As shown in Table 1, the microarray data suggest that rosiglitazone may have a negative effect on mesenchymal cell support for angiogenesis. Indeed, in the model of U-33/ γ 2 cells rosiglitazone decreased the expression of factors implicated in the recruitment and differentiation of endothelial cells, including angiopoietins 1 and 4 and VEGFc. It also decreased the expression of two FGF receptors, FGFR2 and -3, and PDGF receptor β , suggesting decreased responsiveness to FGF and PDGF signals, which are recognized as positive regulators of VEGF and angiopoietin production in marrow mesenchymal cells (Table 1) [25, 34].

Discussion

The studies described herein were initiated to address the important question of whether bone regeneration and repair are affected by the antidiabetic TZDs. Both bone repair models, BDR and DO, confirmed that new bone formation is significantly compromised in animals treated with rosiglitazone and that this effect is accompanied by a significant accumulation of fat within the zone of new bone formation. The diminished bone formation is also associated with decreased proliferation of nonadipocytic and likely preosteoblastic mesenchymal cells, which are located in close proximity to the surface of newly formed bone. Rosiglitazone treatment also elicited significant changes in the local bone marrow microenvironment that support angiogenesis, which is essential for the bone regeneration process [20, 25, 33].

The rosiglitazone inhibitory effect on bone formation and stimulatory effect on fat accumulation were entirely specific to the endosteal compartment associated with the presence of local contiguous bone marrow osteogenic precursors and osteoinductive properties. Interestingly, the effect on periosteal new bone formation and periosteal fat accumulation was marginal, suggesting a differential sensitivity to rosiglitazone of the endosteal and periosteal pools of osteoblastic precursors.

The significant accumulation of fat within the bone regeneration zone is of particular interest. It has been shown previously that TZDs change MSC lineage allocation toward adipocytes and that this occurs at the expense of osteoblasts in both in vitro and in vivo models of bone remodeling [9, 10, 35]. However, the results presented here suggest that the mesenchymal cells which are present at the injury site are especially sensitive to the effects of TZD treatment. The compromised bone regeneration in the presence of systemic rosiglitazone administration correlates with the significant increase in the fat cell number at the regenerating site, an effect that exceeds by several orders of magnitude the fat accumulation in nonregenerating bone. This may suggest that either mesenchymal cells which accumulate at the site of injury are more responsive to the PPAR γ proadipocytic activation or the appearance of significant amounts of fat results from the large number of multipotential MSCs which accumulate at the site of bone regeneration.

Besides committing MSCs toward the adipocyte lineage, rosiglitazone also inhibits their proliferation and alters their support for angiogenesis, which are prerequisite conditions for

bone regeneration in respect to osteoblast differentiation and the formation of a supporting microenvironment [20, 25, 36, 37]. The exuberant occurrence of new blood vessels at the regenerating site is determined by the osteoblast-like cells' ability to produce proangiogenic factors including VEGFs and angiopoietins, which support endothelial cell migration and differentiation [20, 37, 38]. The production of these factors is regulated by cytokines such as FGFs and PDGFs, which signal through the specific receptors present on the surface of mesenchymal cells. Our data indicate that rosiglitazone decreases the responsiveness of mesenchymal cells to FGF and PDGF signaling, which decreases the production of VEGF and angiopoietins [33, 34]. These data indicate that PPAR γ plays an essential role in the regulation of support for angiogenesis at the site of bone repair and regeneration. Indeed, it has been previously shown that PPAR γ plays a pivotal role in organizing the formation of new vasculature during placental development [39]. Although not studied here, there is also the possibility that rosiglitazone directly affects endothelial cell progenitors involved in vessel formation. Recent studies have demonstrated that TZDs inhibit the migration of endothelial cells by targeting their response to VEGF [40]. Thus, TZDs' profound antiangiogenic effect during bone regeneration may comprise both a decreased support of MSCs and a decreased responsiveness of endogenous endothelial cells to proangiogenic factors. In addition, the large quantities of fat may exert a local endocrine effect that is negative to the new vessel formation and bone regeneration.

In summary, rosiglitazone affects the bone regeneration process by at least two mechanisms. The first is intrinsic to mesenchymal progenitor cells and affects their ability to proliferate and differentiate into bone-forming osteoblasts while inducing adipocyte differentiation. The second affects the local microenvironment supporting angiogenesis. The present studies provide the first indication that bone healing in patients on antidiabetic TZD therapy may be significantly impaired and may indicate a need for caution during the orthopedic treatment of such patients.

Acknowledgments

This work was supported by the following funds (to B. L.-C.): NIH/NIA AG 028935 and the American Diabetes Association's Amaranth Diabetes Fund 1-09-RA-95.

References

1. Lecka-Czernik B. Bone loss in diabetes: use of antidiabetic thiazolidinediones and secondary osteoporosis. *Curr Osteoporosis Rep.* 2010; 8:178–184. [PubMed: 20809203]
2. Vestergaard P, Rejnmark L, Mosekilde L. Diabetes and its complications and their relationship with risk of fractures in type 1 and 2 diabetes. *Calcif Tissue Int.* 2009; 84:45–55. [PubMed: 19067021]
3. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. *Am J Epidemiol.* 2007; 166:495–505. [PubMed: 17575306]
4. Kahn SE, Zinman B, Lachin JM, Haffner SM, Herman WH, Holman RR, Kravitz BG, Yu D, Heise MA, Aftring RP, Viberti G. Rosiglitazone-associated fractures in type 2 diabetes: an analysis from a diabetes outcome progression trial (ADOPT). *Diabetes Care.* 2008; 31:845–851. [PubMed: 18223031]
5. Loke YK, Singh S, Furberg CD. Long-term use of thiazolidinediones and fractures in type 2 diabetes: a meta-analysis. *CMAJ.* 2009; 180:32–39. [PubMed: 19073651]
6. Meier C, Kraenzlin ME, Bodmer M, Jick SS, Jick H, Meier CR. Use of thiazolidinediones and fracture risk. *Arch Intern Med.* 2008; 168:820–825. [PubMed: 18443256]
7. Habib ZA, Havstad SL, Wells K, Divine G, Pladevall M, Williams LK. Thiazolidinedione use and the longitudinal risk of fractures in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab.* 2010; 95:592–600. [PubMed: 20061432]
8. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem.* 2008; 77:289–312. [PubMed: 18518822]

9. Lecka-Czernik B, Gubrij I, Moerman EA, Kajkenova O, Lipschitz DA, Manolagas SC, Jilka RL. Inhibition of *Osf2/Cbfa1* expression and terminal osteoblast differentiation by PPAR- γ 2. *J Cell Biochem.* 1999; 74:357–371. [PubMed: 10412038]
10. Lazarenko OP, Rzonca SO, Hogue WR, Swain FL, Suva LJ, Lecka-Czernik B. Rosiglitazone induces decreases in bone mass and strength that are reminiscent of aged bone. *Endocrinology.* 2007; 148:2669–2680. [PubMed: 17332064]
11. Lecka-Czernik B. PPARs in bone: the role in bone cell differentiation and regulation of energy metabolism. *Curr Osteoporos Rep.* 2010; 8:84–90. [PubMed: 20425615]
12. Shockley KR, Lazarenko OP, Czernik PJ, Rosen CJ, Churchill GA, Lecka-Czernik B. PPAR γ 2 nuclear receptor controls multiple regulatory pathways of osteoblast differentiation from marrow mesenchymal stem cells. *J Cell Biochem.* 2009; 106:232–246. [PubMed: 19115254]
13. Wan Y, Chong LW, Evans RM. PPAR- γ regulates osteoclastogenesis in mice. *Nat Med.* 2007; 13:1496–1503. [PubMed: 18059282]
14. Wei W, Wang X, Yang M, Smith LC, Dechow PC, Wan Y. PGC1 β mediates PPAR γ activation of osteoclastogenesis and rosiglitazone-induced bone loss. *Cell Metab.* 2010; 11:503–516. [PubMed: 20519122]
15. Sottile V, Seuwen K, Kneissel M. Enhanced marrow adipogenesis and bone resorption in estrogen-deprived rats treated with the PPAR γ agonist BRL49653 (rosiglitazone). *Calcif Tissue Int.* 2004; 75:329–337. [PubMed: 15549648]
16. Zinman B, Haffner SM, Herman WH, Holman RR, Lachin JM, Kravitz BG, Paul G, Jones NP, Aftring RP, Viberti G, Kahn SE. Effect of rosiglitazone, metformin, and glyburide on bone biomarkers in patients with type 2 diabetes. *J Clin Endocrinol Metab.* 2010; 95:134–142. [PubMed: 19875477]
17. Grey A, Bolland M, Gamble G, Wattie D, Horne A, Davidson J, Reid IR. The peroxisome-proliferator-activated receptor- γ agonist rosiglitazone decreases bone formation and bone mineral density in healthy postmenopausal women: a randomized, controlled trial. *J Clin Endocrinol Metab.* 2007; 92:1305–1310. [PubMed: 17264176]
18. Gruntmanis U, Fordan S, Ghayee HK, Abdullah SM, See R, Ayers CR, McGuire DK. The peroxisome proliferator-activated receptor- γ agonist rosiglitazone increases bone resorption in women with type 2 diabetes: a randomized, controlled trial. *Calcif Tissue Int.* 2010; 86:343–349. [PubMed: 20354684]
19. Wren TA, Chung SA, Dorey FJ, Bluml S, Adams GB, Gilsanz V. Bone marrow fat is inversely related to cortical bone in young and old subjects. *J Clin Endocrinol Metab.* 2011; 96:782–786. [PubMed: 21177790]
20. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *J Dent Res.* 2008; 87:107–118. [PubMed: 18218835]
21. Kim JB, Leucht P, Lam K, Luppen C, Ten Berge D, Nüsse R, Helms JA. Bone regeneration is regulated by wnt signaling. *J Bone Miner Res.* 2007; 22:1913–1923. [PubMed: 17696762]
22. Campbell TM, Wong WT, Mackie EJ. Establishment of a model of cortical bone repair in mice. *Calcif Tissue Int.* 2003; 73:49–55. [PubMed: 14506954]
23. Monfoulet L, Rabier B, Chassande O, Fricain JC. Drilled hole defects in mouse femur as models of intramembranous cortical and cancellous bone regeneration. *Calcif Tissue Int.* 2010; 86:72–81. [PubMed: 19953233]
24. Aronson J. Experimental and clinical experience with distraction osteogenesis. *Cleft Palate Craniofac J.* 1994; 31:473–481. discussion 481–472. [PubMed: 7833340]
25. Aronson J. Modulation of distraction osteogenesis in the aged rat by fibroblast growth factor. *Clin Orthop Relat Res.* 2004; 425:264–283. [PubMed: 15292818]
26. Wolff GL, Roberts DW, Mountjoy KG. Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. *Physiol Genomics.* 1999; 1:151–163. [PubMed: 11015573]
27. Duhl DM, Vrieling H, Miller KA, Wolff GL, Barsh GS. Neomorphic agouti mutations in obese yellow mice. *Nat Genet.* 1994; 8:59–65. [PubMed: 7987393]

28. Aronson J, Liu L, Liu Z, Gao GG, Perrien DS, Brown EC, Skinner RA, Thomas JR, Morris KD, Suva LJ, Badger TM, Lumpkin CK Jr. Decreased endosteal intramembranous bone formation accompanies aging in a mouse model of distraction osteogenesis. *J Regen Med.* 2002; 3:7–16.
29. Perrien DS, Akel NS, Edwards PK, Carver AA, Bendre MS, Swain FL, Skinner RA, Hogue WR, Nicks KM, Pierson TM, Suva LJ, Gaddy D. Inhibin A is an endocrine stimulator of bone mass and strength. *Endocrinology.* 2007; 148:1654–1665. [PubMed: 17194739]
30. Aronson J, Shen XC, Gao GG, Miller F, Quattlebaum T, Skinner RA, Badger TM, Lumpkin CK Jr. Sustained proliferation accompanies distraction osteogenesis in the rat. *J Orthop Res.* 1997; 15:563–569. [PubMed: 9379266]
31. Perrien DS, Brown EC, Aronson J, Skinner RA, Montague DC, Badger TM, Lumpkin CK Jr. Immunohistochemical study of osteopontin expression during distraction osteogenesis in the rat. *J Histochem Cytochem.* 2002; 50:567–574. [PubMed: 11897810]
32. Aubin JE. Regulation of osteoblast formation and function. *Rev Endocr Metab Disord.* 2001; 2:81–94. [PubMed: 11704982]
33. Caplan AI, Correa D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. *J Orthop Res.* 2011; 29:1795–1803. [PubMed: 21618276]
34. Lieu C, Heymach J, Overman M, Tran H, Kopetz S. Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. *Clin Cancer Res.* 2011; 17:6130–6139. [PubMed: 21953501]
35. Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. *Endocrinology.* 2002; 143:2376–2384. [PubMed: 12021203]
36. Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop Relat Res.* 1994; 301:124–131. [PubMed: 8156663]
37. Kumar S, Wan C, Ramaswamy G, Clemens TL, Ponnazhagan S. Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. *Mol Ther.* 2010; 18:1026–1034. [PubMed: 20068549]
38. Lewinson D, Maor G, Rozen N, Rabinovich I, Stahl S, Rachmiel A. Expression of vascular antigens by bone cells during bone regeneration in a membranous bone distraction system. *Histochem Cell Biol.* 2001; 116:381–388. [PubMed: 11735002]
39. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, Chrast R, Desvergne B. PPARgamma in placental angiogenesis. *Endocrinology.* 2010; 151:4969–4981. [PubMed: 20810566]
40. Goetze S, Eilers F, Bungenstock A, Kintscher U, Stawowy P, Blaschke F, Graf K, Law RE, Fleck E, Grafe M. PPAR activators inhibit endothelial cell migration by targeting Akt. *Biochem Biophys Res Commun.* 2002; 293:1431–1437. [PubMed: 12054675]

Fig. 1.

Sagittal μ CT renderings of murine tibia model of drilled bone defect regeneration (BDR). The skeletal injury was generated in the tibia of a C57BL/6 mouse as described in “Materials and Methods” section. Mice were killed at different time points after surgery, representing the inflammatory (1 week), hard callus (2 weeks), and remodeling (3 and 4 weeks) phases of healing [21]. New bone at the injury site with a poorly organized trabecular structure forms approximately 2 weeks postsurgery. Three weeks postsurgery the injury site is covered by a layer of bone on the periosteal side and the process of remodeling of newly formed woven bone in the endosteum is initiated. Almost complete cortical bone restoration and woven bone remodeling are observed at 4 weeks postsurgery.

Fig. 2.

The effect of rosiglitazone on bone regeneration and fat accumulation in the BDR model. Two weeks after surgery both tibiae, with a defect and contralateral, were harvested for μ CT assessment of mineralized tissue mass and fat mass at the site of the defect or at the corresponding site in the contralateral tibia. **a** Representative μ CT renderings of horizontal cross sections of the tibia with a drilled cortical defect harvested from control and rosiglitazone-treated animals. *MT* renderings of mineralized tissues, *FT* renderings of fat stained with osmium tetroxide after bone demineralization, as described in “Materials and Methods” section. **b** μ CT renderings of the whole tibia harvested 2 weeks postsurgery, demineralized, and stained for fat with osmium tetroxide. *White brackets* mark the area of the drilled bone defect. **c** μ CT measurements of a new bone formed either at the entire

defect site (total new bone) or at the endosteal (endosteal new bone) or periosteal (periosteal new bone) location of the bone defect. **d** μ CT measurements of fat content at the entire defect site (total fat) or at the endosteal or periosteal location of the defect site. **e** Measurement of fat content in the contralateral nonoperated tibia in the region corresponding to the drilled defect in the operated tibia. *Gray bars*, control; *black bars*, rosiglitazone-treated animals. * $p < 0.05$ vs. control (Color figure online)

Fig. 3.

The effect of rosiglitazone on bone regeneration and fat accumulation in the DO model. **a** μ CT renderings of newly formed bone in the DO gap after 2 weeks of distraction. **b** μ CT measurements of new bone occupying either the entire DO gap (total new bone [TNB]) or the endosteal (endosteal new bone [ENB]) or periosteal (periosteal new bone [PNB]) location of the DO gap. **c** Histological visualization of adipocytes at the DO site and in the corresponding region of contralateral, nonoperated tibia (CO) from mice treated or not with rosiglitazone. Specimens were stained with hematoxylin and eosin. Note decreased new bone formation at the DO site and large quantities of fat (white oval spaces) accumulated in the DO zone of microcolumn formation and in the marrow adjacent to the DO site in rosiglitazone-treated animals (magnification $\times 10$). **d** Quantification of adipocytes at the DO

site of nontreated and rosiglitazone-treated mice. *Gray bars*, control; *black bars*, rosiglitazone. * $p < 0.05$ vs. control (Color figure online)

Fig. 4.

Immunohistochemical analyses of PCNA-positive and hematopoietic VEGF-producing cells and the number of hematopoietic sinusoids at the DO gap after 2 weeks of distraction. **a** Visualization and quantification of cells stained for PCNA (*brown*) on the surface and in close proximity to the newly formed bone at the DO site. Magnification $\times 40$. **b** Quantification of VEGF-positive cells in the DO gap of control and rosiglitazone-treated animals. Microphotograph illustrates a typical location of VEGF-positive cells on the surface of new bone formed in the DO gap (*brown*). **c** Number of hematopoietic sinusoids in the DO gap area. Sinusoids were identified by the presence of CD34-positive cells in the surrounding peripheral area (*brown*). *Red arrows* indicate cells stained positively for the tested antigen. Magnification $\times 40$. *Gray bars*, control; *black bars*, rosiglitazone. * $p < 0.05$ vs. control (Color figure online)

Table 1Rosiglitazone effect on mRNA expression of proangiogenic factors in U-33/ γ 2 cells analyzed by microarray

Gene symbol	Gene name	Fold change vs. control	<i>p</i> Value
<i>Angpt1</i>	Angiopoietin 1	-4.1	2.99E-06
<i>Angpt4</i>	Angiopoietin 4	-6.0	<1.00E-06
<i>VEGFC</i>	Vascular endothelial growth factor c	-6.9	<1.00E-06
<i>FGFR2</i>	Fibroblast growth factor receptor 2	-3.7	<1.00E-06
<i>FGFR3</i>	Fibroblast growth factor receptor 3	-2.7	5.01E-06
<i>PDGFR-β</i>	Platelet-derived growth factor receptor β	-1.8	0.008

U-33/ γ 2 cells were treated for 72 h with 1 μ M rosiglitazone, and gene expression was analyzed using GeneChip[®] Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), as described [12]. All microarray data are available at the NCBI Gene Expression Omnibus (GEO) database with accession number GSE10192