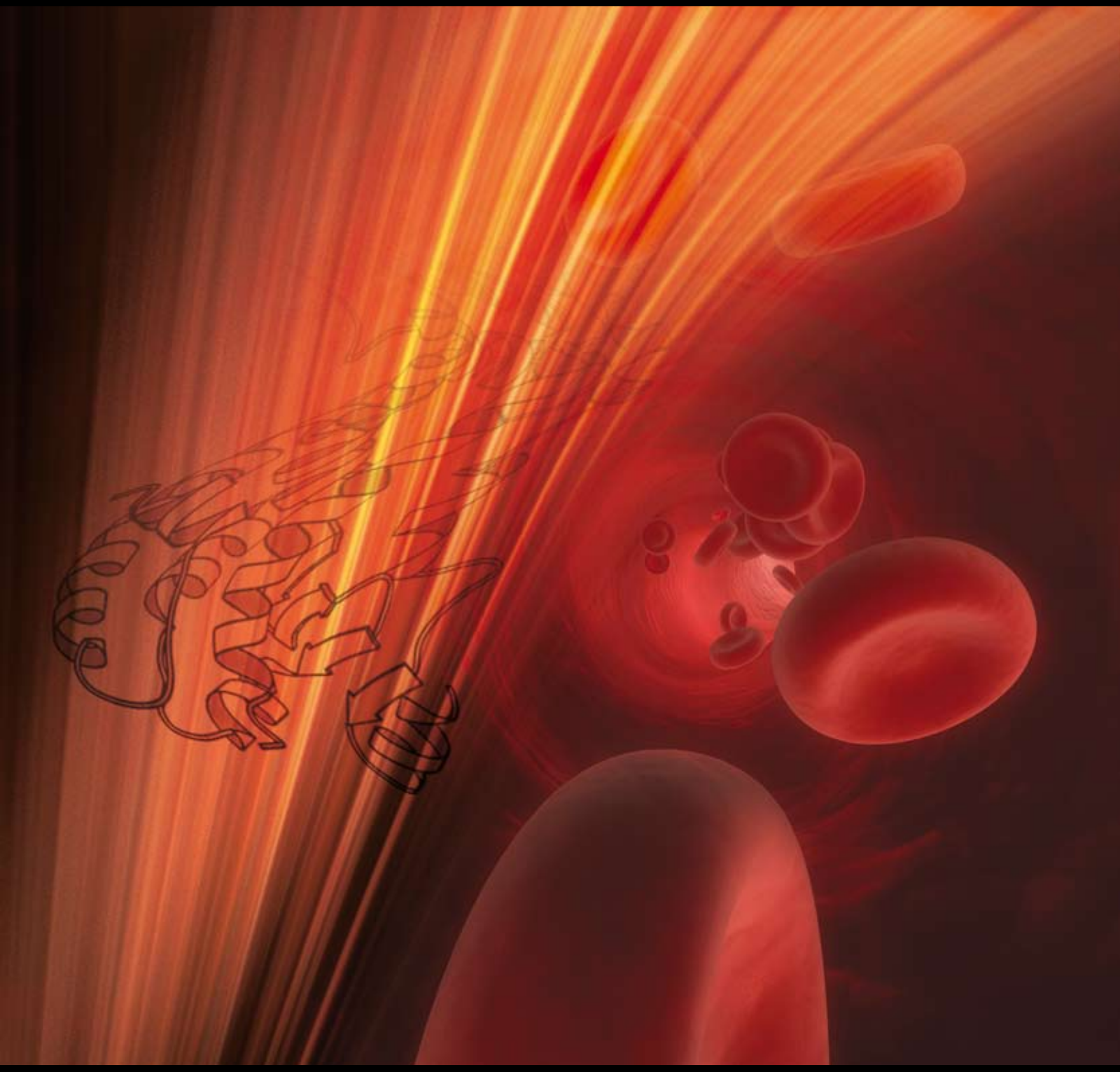
PPAR Research

PPARs and Bone Metabolism

Guest Editor: Beata Lecka-Czernik



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Editorial
PPARs and Bone Metabolism

Beata Lecka-Czernik

Department of Geriatrics, Reynolds Institute on Aging, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

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Welcome to the inaugural *special* issue of *PPAR Research*: *PPARs and Bone Metabolism*. In addition to the key roles PPARs play in numerous processes including glucose and fat metabolism, inflammation, cancer, and central nervous system maintenance, a new role for PPAR-γ has recently emerged: the maintenance of bone homeostasis during aging and disease. In this premier issue we have assembled what is close to a comprehensive overview of the role of PPAR-γ in the control of bone maintenance. This takes into account PPAR-γ's role in mesenchymal stem cell lineage allocation, possible cross-talk with relevant nuclear receptors, examination of PPAR-γ gene polymorphisms and bone mineral density in humans, a role of PPAR-γ in bone loss due to skeletal disuse, evidence that human bone is vulnerable to antidiabetic therapies with PPAR-γ agonists, the thiazolidinediones, and evidence that the antiosteoblastic activity of PPAR-γ can be separated from its proadipocytic and antidiabetic activities by using selective modulators. We also present a novel hypothesis that PPAR-γ acts as a regulator of chondrocyte development and cartilage homeostasis. We realize that we have not covered all aspects of PPARs involvement in the control of bone maintenance; however, this introduction should serve as a competent first attempt to present these new aspects of bone biology to the broader audience of our readers.

Beata Lecka-Czernik

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Review Article

Fine-Tuning Reception in the Bone: PPARγ and Company

Z. Elizabeth Floyd,[1] Sanjin Zvonic,[1] Mark E. Nuttall,[2] and Jeffrey M. Gimble[1]

[1] *Stem Cell Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA*
[2] *Centocor, Johnson and Johnson, Horsham, PA 19044, USA*

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PPARγ plays a central role in the formation of fat. Regulation of PPARγ activity depends on numerous factors ranging from dietary ligands to nuclear hormone coactivators and corepressors to oxygen-sensing mechanisms. In addition, the interplay of PPARγ with other nuclear hormone receptors has implications for the balance between adipogenesis and osteogenesis in mesenchymal stem cells of the bone marrow stroma. This review will explore a range of factors influencing PPARγ activity and how these interactions may affect osteogenesis.

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INTRODUCTION

This special issue focuses on the latest findings relating to the role of PPARs in bone metabolism. This review uses the broader scope of the nuclear hormone receptor superfamily to assess the relationship between adipogenesis and osteogenesis, both in vitro and in vivo, and their underlying regulatory mechanisms. While PPARγ takes center stage, the vitamin D_3, estrogen, LXR (liver X receptor), and related receptors are used as examples to explore the potential impact of coactivators and corepressors on bone marrow-derived mesenchymal stem cell (MSC) differentiation. The role of dietary and endogenous ligands, such as genistein, long chain fatty acids, and resveratrol, are evaluated in the context of nuclear receptor regulation of bone physiology and pathology.

Bone marrow stroma MSCs give rise to a number of cell types, including osteoblasts and adipocytes [1, 2]. Bone formation is regulated by Runx2/Cbfa 1, a member of the runt homology domain transcription factor family [3–6] while fat formation depends on the peroxisome proliferator-activated receptor gamma (PPARγ) [7–9]. A number of studies suggest that bone formation is related inversely to adipocyte formation in the marrow cavity [2, 10]. In vitro studies using bone marrow-derived MSCs find that induction of adipocyte differentiation inhibits osteoblastic bone formation [2, 10]. Likewise, agents inducing osteoblast differentiation inhibit adipogenesis [11]. These findings are consistent with the results of Akune et al [12] demonstrating that haploinsufficiency of PPARγ promotes bone formation.

The reciprocal relationship between PPARγ levels and osteogenesis is particularly evident with increased age [12, 13], supporting a role for PPARγ in bone development and osteoporosis associated with aging. The increasing age of the population and osteoporosis associated with aging indicates a need to further explore the regulation of PPARγ with respect to bone formation. The interplay of PPARγ with other nuclear receptors and the regulation of PPARγ by a range of cofactors in other tissue types may offer insights into potential therapeutic targets for regulating bone formation.

PPARγ: CROSSTALK WITH THE CLASSICAL NUCLEAR RECEPTORS

Originally described as an "orphan" nuclear receptor [14–17] having no known ligand, the peroxisome proliferator-activated receptor-γ (PPARγ) has since been identified as the target of the widely-used thiazolidinedione (TZD) class of antidiabetic drugs. Although the thiazolidinediones are well described as synthetic ligands of PPARγ, the endogenous PPARγ ligand has remained elusive. Long chain fatty acid derivatives are known to activate PPARγ [18–20], but the affinity of these natural ligands for PPARγ is well below the affinity of *bona fide* classical nuclear receptor ligands. However, there is now an evidence that nitric oxide derivatives of linoleic acid are potent adipogenic agonists at levels of 133 nM, well within the physiological range [21].

In vitro analyses demonstrate that various PPARγ ligands (rosiglitazone, 9,10 dihydroxyoctadecenoic acid,

15-deoxy12,14-PGJ2) not only induce murine bone marrow stromal cell adipogenesis but also inhibit osteogenesis [22]. However, in vivo models suggest that not all PPARγ ligands exhibit the same effects [23–25]. For example, long term treatment of mice with the thiazolidinedione troglitazone increased bone marrow adipocyte content without reducing bone mass and trabecular volume [23]. In contrast, treatment of mice with rosiglitazaone, a thiazolidinedione with higher affinity for PPARγ, decreased bone mineral content, bone formation rates, and trabecular bone volume while increasing adipogenesis [24, 25].

In addition to PPARγ, other nuclear hormone receptors control critical adipogenic and osteogenic steps. Among these are the estrogen and vitamin D receptors and the interplay between PPARγ and these receptors has implications regarding the regulation of bone and fat formation in the bone marrow.

The effects of estrogen on bone and adipose tissue formation have long been recognized in rodent and canine ovariectomy models. In vitro studies using murine bone marrow MSCs have found that estrogen reciprocally promotes osteogenesis while inhibiting adipogenesis [26, 27]. In vitro studies using murine bone marrow MSCs have found that the soy phytoestrogen diadzein exhibits a dose dependent biphasic response: low concentrations of diadzein increase osteogenesis and decrease adipogenesis while higher doses have the opposite effect [28]. The reciprocal relationship between osteogenesis and adipogenesis is attributed to a balance between diadzein-induced activation of ER (estrogen receptor) and PPARγ [28]. The importance of a balance between ER and PPARγ activities is further illustrated by studies indicating that activation of PPARγ with the thiazolidinedione rosiglitazone in ovariectomized rats is associated with increased bone resorption [29]. Indeed, recent studies show that a point mutation in the ligand binding domain (exon 6, C161T) of PPARγ is associated with decreased levels of osteoprotegerin in postmenopausal women [30]. However, future studies are needed to determine the role of estrogen receptor and PPARγ "cross-talk" in adipogenesis and osteogenesis. Estrogen can exert stimulatory effects on bone formation in the absence of the estrogen receptor alpha (ERα) [31]. Although estrogen-mediated changes in bone marrow adipogenesis were not determined in the absence of ERα, the results suggest that any reciprocal relationship between bone and fat formation may not require activation of the estrogen receptor.

Crosstalk between PPARγ and vitamin D receptor (VDR) activated pathways also plays a role in the balance between bone and fat formation. The inbred SAM-P/6 (senescence accelerated mice-P/6) murine strain provides a model of accelerated senescence characterized by osteopenia and increased bone marrow fat mass [32]. Recent studies found that 1.25 $(OH)_2$ vitamin D_3 treatment inhibited adipogenesis in the SAM-P/6 mice [33]. This correlated with a 50% reduction in PPARγ mRNA and protein levels as well as a decrease in Oil Red O positively stained cell numbers [33]. Additional studies indicate that 1.25 $(OH)_2$ vitamin D_3 bound VDR blocks adipogenesis by downregulating

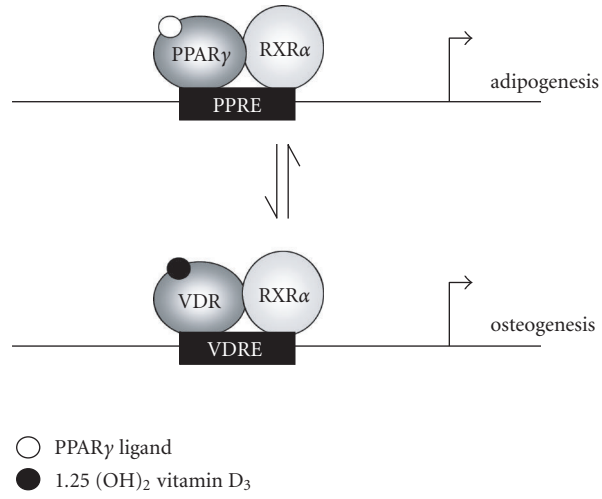

Figure 1: PPARγ and vitamin D receptor interactions with RXRα may function as a switch between adipogenesis and osteogenesis.

C/EBPβ (CAAT/enhancer binding protein), a critical inducer of PPARγ transcription early in adipogenesis [34]. However, ligand-free VDR appears to be necessary for adipogenesis as "knockdown" of VDR using siRNA prevents the formation of fat cells [34].

It is tempting to speculate that the inverse relationship between adipogenic and osteogenic differentiations in the bone marrow stroma may involve competition between PPARγ and other nuclear receptors such as the vitamin D receptor for their common obligate heterodimeric partner, RXRα (retinoid X receptor) [35] (see Figure 1). In this role, RXRα is well positioned to regulate the transcriptional activity of its binding partners. PPARγ activity is regulated by PPARγ ligands as well as the RXRα ligand, 9-cis-retinoic acid, even in the absence of PPARγ ligand binding [36]. Indeed, adipogenesis is inhibited in the presence of 9-cis-retinoic acid in the murine TMS-14 stromal cell line [37]. Inhibition of adipogenesis is accompanied by a decrease in PPARγ protein levels and suggests a decrease in PPARγ transcriptional activity [37]. Conversely, VDR activity is not affected by 9-cis-retinoic acid binding to RXRα alone [38]. However, 1.25 $(OH)_2$ D_3-bound VDR enhances heterodimerization with RXRα, resulting in increased VDR activity [38]. The variable response of PPARγ and VDR to RXRα ligand binding is consistent with the idea that RXRα heterodimerization may serve as a dynamic switch in the "decision" to undergo adipogenesis or osteogenesis.

PPARγ AND LXR: A CONNECTION BETWEEN LIPID METABOLISM AND BONE FORMATION

The liver X receptor subfamily of nuclear receptors, LXRα and LXRβ, are pivotal in the conversion of cholesterol to bile acids. While the LXR gene was originally identified as an "orphan receptor" based on its heterodimerization with the 9-*cis* retinoic acid receptor RXR, subsequent studies identified cholesterol metabolites as endogenous LXR ligands [39].

LXR proteins are abundant in adipocytes and recent studies suggest cross-talk between PPARγ and the LXRs during adipogenesis [40–43]. Although the effect of LXR agonists on adipogenesis is unclear [41, 44], several studies in murine 3T3-L1 cells link LXR to adipogenesis [41–44]. Homozygous LXR$\alpha/\beta^{-/-}$ mice have smaller adipose tissue depots compared to their wild type littermates, suggesting that LXR regulates lipid storage [42, 43]. This effect is attributed to LXRβ since adipose tissue is decreased in LXR$\beta^{-/-}$ but not LXR$\alpha^{-/-}$ mice [43]. There is evidence that LXR activates the PPARγ promoter and enhances adipogenesis in 3T3-L1 cells [44] while other studies indicate that the LXR promoter in adipocytes is regulated by PPARγ [42]. These findings suggest that PPARγ and the LXR proteins positively interact in the formation of adipocytes. However, LXR ligands, such as the oxysterols 20S and 22R hydroxycholesterol, inhibit adipogenesis induced by the PPARγ ligand troglitazone [45]. These studies did not determine if the effects of the oxysterols in adipogenesis were LXR-mediated, leaving open the possibility that the effects are LXR-independent. It would be interesting to examine the effects of the LXR ligands on adipose tissue and PPARγ activity in the LXR$\alpha^{-/-}\beta^{-/-}$ mouse model.

The interplay of LXR and PPARγ in bone formation is relatively unexplored. While inhibiting adipogenesis, the oxysterols 20S and 22R hydroxycholesterol enhance osteogenesis [45, 46]. However, inhibition of cholesterol synthesis and presumably 20S and 22R hydroxycholesterol by the statin compounds also enhances bone formation [47], and suggests decreases in LXR ligands that are associated with osteogenesis. At present, these contradictions are difficult to reconcile and future studies examining the relationship between LXR (liganded or unliganded) and PPARγ in adipogenesis and osteogenesis should provide important insights into these complex interactions.

PPARγ AND THE NUCLEAR RECEPTOR COREGULATORS: POTENTIAL ROLES IN BONE FORMATION

The transcriptional activity of the nuclear receptors is also mediated by interactions of the receptors with a large group of proteins classified as coactivators and corepressors of nuclear receptor activity. A major category of the coactivators is the p160 family of proteins that includes the cAMP response element binding protein (CBP)/p300 and steroid receptor coactivators (SRC)-1,-2,-3, which recruit histone modifiers to the chromatin structure (reviewed in [48]). A second category of coactivators includes subunits of the mediator complex such as the PPAR-binding protein (PBP)/thyroid hormone receptor-associated protein (TRAP) 220/vitamin D receptor-associated protein (DRIP) 205 [49–51]. These coactivators interact with the general transcriptional machinery to control assembly of the transcription preinitiator complex [49]. TRAP220/DRIP205, originally cloned as a coactivator of the vitamin D receptor [50], interacts directly with PPARγ [51]. TRAP 220 $(-/-)$ fibroblasts fail to undergo adipogenesis, indicating that TRAP 220 acts as a PPARγ-selective coactivator [51]. An additional coactivator, peroxisome proliferator-activated receptor gamma interacting protein (PRIP), serves to link TRAP220/DRIP205 bound PPARγ to the CBP/p300 coactivator [52]. PRIP $(-/-)$ mouse fibroblasts are also refractory to PPARγ-stimulated adipogenesis [53]. Although these coactivators are relatively unexplored in the regulation of osteogenesis, the essential role of PPARγ in regulating the balance between fat and bone formation strongly implies a role for PPARγ-coactivator interactions in osteogenesis. This possibility is supported by studies examining the effects of loss of SRC-1 [54–56]. In brown adipocytes, PPARγ activity is regulated by interaction with SRC-1 and the PPARγ cofactor 1(PGC-1) [57]. PPARγ target genes involved in adipogenesis are decreased in SRC-1 and p/CIP (p/300 cointegrator-associated protein) knockout mice [54]. This is associated with increased metabolic rates and activity levels, indicating a role for SRC-1/PPARγ interactions in energy balance [54]. Other studies using SRC-1 $(-/-)$ mice have demonstrated that SRC-1 plays a role in bone responses to estrogen following ovariectomy, particularly in the metabolically active trabecular bone [55, 56]. Further studies will be needed to determine if SRC-1 interactions with PPARγ influence responses to estrogen in metabolically active bone. However, the effects on bone formation associated with the loss of SRC-1 are expected to be complex given the general interaction of SRC-1 with nuclear receptors, including the estrogen and vitamin D receptors.

A second group of coregulators of PPARγ activity are the nuclear corepressors, nuclear hormone receptor-corepressor (N-CoR) [58], and silencing mediator of retinoid and thyroid hormone receptor (SMRT) [59]. Repression of nuclear receptor activity by N-CoR/SMRT involves recruitment of histone deacetylases to the transcriptional machinery (reviewed in [60]). PPARγ and VDR belong to a group of nuclear receptors that interact with N-CoR and SMRT in the absence of ligand [61, 62]. Ligand binding results in disengagement with the corepressors and recruitment of coactivators (reviewed in [60]). Studies using siRNA "knockdown" of N-CoR and SMRT in murine 3T3-L1 adipocytes show that these corepressors regulate PPARγ activity during adipogenesis [63]. These results are consistent with other studies indicating that the loss of fat mass associated with calorie restriction is due to increased interaction of PPARγ with N-CoR and SMRT [64]. Calorie restriction activates the histone deacetylase Sirt1, which recruits the N-CoR/SMRT corepressor to PPARγ leading to inhibition of PPARγ activity in adipocytes [64]. Very little is known about the effects of calorie restriction on bone formation. However, studies using resveratrol, a plant polyphenol that, like calorie restriction, activates Sirt1, may offer some insight. Recent studies in ovariectomized rats show that resveratrol treatment increases bone mineral density [65]. In addition, resveratrol increases the expression of osteocalcin and osteopontin in human bone marrow MSCs [66]. This upregulation of osteoblast markers is associated with increased responses to 1, 25 $(OH)_2$ vitamin D_3 that are accompanied by increases in expression of the vitamin D receptor [66]. These results hint at a relationship between repression of PPARγ activity in adipocytes via interaction with N-CoR/SMRT and activation of vitamin D receptor responses in osteoblasts.

Unraveling a potential relationship between repression of PPARγ activity via interaction with N-CoR/SMRT and enhancement of bone formation may provide new therapeutic targets in treating osteoporosis in the aging population. An important area for exploration involves regulation of PPARγ transcriptional activity via ubiquitin-proteasome-dependent degradation. The ubiquitin-proteasome system is responsible for the degradation of short-lived proteins in eukaryotes, including the nuclear receptors (reviewed in [67]). PPARγ is targeted for degradation under basal [68] and ligand-activated conditions [69]. Recent studies show that components of the ubiquitin-proteasome system responsible for targeting substrates for degradation also function as nuclear receptor coactivators and corepressors [70–72]. Indeed, subunits of the N-CoR/SMRT complex are ubiquitin ligases that target substrates for degradation by the 26S proteasome [72]. These components, TBL1/TBLR1 (transducin β-like 1/transducin β-like 1 related protein), are required for exchange of corepressors for coactivators upon ligand binding for a number of nuclear receptors, including PPARγ [72]. TBL1/TBLR1 act as adaptors for recruiting components of the ubiquitin-proteasome system to the liganded receptor [72]. In addition, deletion of TBL1 from mouse embryonic stem cells precludes the ability of these cells to undergo adipogenesis as judged by staining for neutral lipids and decreased gene expression of PPARγ and PPARγ targets such as adipsin [72]. Given the reciprocal relationship between adipogenesis and osteogenesis, these results suggest a role for interactions of components of the ubiquitin-proteasome system with PPARγ (and other nuclear receptors) in determining the balance between bone and fat formation.

OTHER COREGULATORS OF PPARγ

Additional components of the transcriptional complex also influence PPARγ activity and the differentiation of mesenchymal stem cells into either adipocytes or osteoblasts. New findings have identified a coactivator protein, known as the transcriptional coactivator with PDZ binding motif (TAZ), that is shared between Runx2 and PPARγ [73, 74]. In murine cell models, the TAZ protein localized to the osteocalcin promoter in the presence of bone morphogenic protein-2 (BMP-2) and coactivated Runx2 and osteogenesis while directly suppressing PPARγ and adipogenesis [73]. Although not structurally related to β-catenin, TAZ is proposed to be functionally similar to β-catenin as a regulatory switch in determining the balance between osteoblast and adipocyte development [74]. Wnt signaling stimulates osteogenesis by induction of osteogenic factors such as Runx2 [75] while suppressing adipogenesis in mesenchymal stem cells [76, 77]. Activation of the Wnt signaling pathway leads to activation of β-catenin, which interferes with PPARγ transcriptional activity [78]. Conversely, suppression of Wnt signaling [77] and activation of PPARγ [78] destabilize β-catenin, resulting in adipogenesis. Future studies will be needed to determine if β-catenin functions as a direct corepressor of PPARγ activity in a manner analogous to the TAZ protein. Finally, ligand-activated PPARγ itself suppresses both the expression and activity of Runx2 [79], adding another regulatory layer to the balance between bone and fat formation.

Any exploration of PPARγ's influence over bone formation must take into account the effect of oxygen tension on the development of fat and bone. It is here that the reciprocal relationship between bone and fat formation seems to disappear. The bone marrow mesenchymal stem cells (bone marrow MSC) are normally exposed to oxygen tensions lower than the atmospheric oxygen tension of 21%. In vitro studies indicate that low oxygen levels block induction of adipogenesis from human and murine MSCs [80]. Human MSCs accumulate lipid inclusions at low oxygen tensions, but the appearance of lipids is unaccompanied by expression of PPARγ or the downstream PPARγ target genes required for adipogenesis [81]. Adipogenesis is similarly inhibited under low oxygen conditions in human adipose-derived mesenchymal stem cells (ASC) [82]. However, reduced oxygen tension is also associated with decreased osteogenesis in the human ASCs [82, 83], suggesting parallel regulation of bone and fat development under these conditions. While hypoxic conditions (2% oxygen) do not inhibit Runx2 transcriptional activity [84], PPARγ transcriptional activity is inhibited under the same conditions [85]. PPARγ inhibition is mediated by HIF-1α, a hypoxia inducible transcription factor governing a range of cellular responses to low oxygen levels [85]. HIF-1α mediated repression of PPARγ activity depends on an HIF-1α regulated transcriptional repressor, DEC1/Stra13 [85]. Interestingly, HIF-1α/DEC1 inhibition of PPARγ under hypoxic conditions does not involve histone deacetylation, raising the possibility that the classical nuclear receptor coactivators and corepressors are not required in this process.

CONCLUSIONS AND FUTURE QUESTIONS

These observations suggest that regulation of PPARγ activity may lie at the heart of determining if bone and fat development proceed along parallel or reciprocal directions. Efforts to understand the regulation of PPARγ transcriptional activity have uncovered interplay of PPARγ and other nuclear hormone receptors that is intricately regulated by a range of coregulators. The coregulators extend beyond the classical coactivators and corepressors to include enzymes of the ubiquitin-proteasome system, components of the Wnt and BMP-2 signaling pathways, β-catenin and TAZ, and oxygen-sensing factors such as DEC1/Stra13. As research progresses in defining the role of PPARγ and other nuclear hormone receptors in osteogenesis, some of the questions to be answered will include the following

(1) Will new insights into MSC adipogenesis and osteogenesis be gained as the ligands for "orphan" nuclear hormone receptors are identified?
(2) How do additional components of the transcriptional apparatus, such as histone acetylases and histone deacetylases, contribute to the effects of PPARγ and related nuclear hormone receptors?
(3) How does ubiquitin-proteasomal targeting of PPARγ and related nuclear hormone receptors coordinately regulate MSC adipogenesis and osteogenesis?

(4) Will these avenues of investigation have the potential to yield novel therapeutic targets or identify small molecules for osteoporosis, osteopenia, and related bone disorders?
(5) Do adipokines exert either an anabolic or catabolic effect on osteogenesis?

This field of research has advanced rapidly since the discovery of PPARγ over a decade ago. As new investigators are recruited to this intriguing and clinically relevant field, we anticipate that the pace of scientific progress will continue to accelerate.

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Research Article

The Genetics of *PPARG* and the Skeleton

Cheryl Ackert-Bicknell[1] and Clifford Rosen[1,2]

[1] *The Jackson Laboratory, Bar Harbor ME 04609, USA*
[2] *St. Joseph's Hospital, The Maine Center for Osteoporosis Research and Education, Bangor ME 04401, USA*

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Osteoporosis is a complex metabolic bone disorder. Recently it has been appreciated that the "obesity in bone" phenomenon occurs at the expense of bone formation, and that is a key component of the pathology of this disease. Mouse models with altered bone expression levels of peroxisome proliferator-activated receptor gamma (PPARG) impact bone formation, but genetic studies connecting *PPARG* polymorphisms to skeletal phenotypes in humans have proven to be less than satisfactory. One missense polymorphism in exon one has been linked to low bone mineral density (BMD), but the most studied polymorphism, Pro12Ala, has not yet been examined in the context of skeletal phenotype. The studies to date are a promising start in leading to our understanding of the genetic contribution of *PPARG* to the phenotypes of BMD and fracture risk.

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INTRODUCTION

Osteoporosis currently affects 10 million Americans and an additional 34 million Americans are considered at risk for osteoporosis and fracture (http://www.nof.org/ accessed June, 2006). The World Health Organization (WHO) defines osteoporosis as having a BMD with a T-score of less than −2.5 [1], yet in the Rotterdam prospective study of 7806 men and women over the age of 55, only 44% of women and 21% of the men with a nonvertebral fracture had a T-score of −2.5 or lower [2] suggesting a need for additional means for predicting fracture risk. A variety of studies have been done to examine other risk factors for osteoporosis, both for the purpose of determining who should undergo further screening and more importantly, who is at risk for fracture. Osteoporosis and the clinically measurable phenotypes such as BMD and fracture incidence have proven to be very complicated genetic traits with quantitative trait loci (QTLs) for various bone phenotypes found on almost every chromosome in both humans and mice (reviewed in [3, 4]). Yet BMD is not an independent phenotype, rather it is associated with many other phenotypes and pathologies such as diabetes mellitus [5] and coronary artery disease [6]. Body weight is positively correlated to bone mass and in load-bearing skeletal sites, increased adiposity is associated with higher BMD, yet adiposity still influences BMD at non-load-bearing sites such as the forearm [7]. PPARG's role in insulin sensitivity and obesity, as well as work done with mesenchymal stem cells have made PPARG an attractive candidate gene in studies examining the genetic basis of bone density.

Meunier et al [8], were the first to show that women with osteoporosis had an increased accumulation of marrow adipocytes as determined from iliac crest biopsies [8]. More recent studies have not only confirmed this observation, but have also shown that volume fraction of the marrow cavity occupied by adipocytes increased with age in both men and women and that this is coincident with a decrease in trabecular bone volume. This increase in adipocyte volume is exacerbated in osteoporotic patients [9, 10]. More importantly, the increased adipocyte volume seen in osteoporotic patients is negatively correlated with bone formation rate (BFR) [10].

Osteoblasts, the cells responsible for the formation of bone, are derived from marrow mesenchymal stem cells. This multipotential stem cell is also able to give rise to chondrocytes, muscle cells, marrow stromal cells, and adipocytes [11]. Lineage allocation is determined by the activation of lineage-specific transcription factors such as RUNX2 (CBFA1), an osteoblast-specific transcription factor or PPARG, a nuclear receptor shown to be key for the maturation of adipocytes [12, 13]. In preosteoblast cell lines, it has been shown that expression of PPARG2 can force a commitment to the adipogenic pathway [14], an occurrence that can be mimicked by the addition of the pharmacological PPARG ligand BRL4965 [15]. In studies of aging mice, it has been shown that the increase in adipocyte volume in the bone

marrow seen with aging is coincident with an increase in expression of PPARG2 [16].

PPARG GENE STRUCTURE, FUNCTION, AND GENETIC LOCATION

PPARG is one of three PPAR nuclear receptors and while widely expressed, it is primarily found in white adipose tissue. Like all nuclear receptors, PPARG is composed of three domains: the N-terminal domain A/B domain, a two-zinc finger containing DNA-binding domain, and a C-terminal ligand-binding domain (17–19). PPARG forms a heterodimer with the retinoic X receptor-alpha and this complex binds to the PPRE (PPAR response element), a direct repeat of the sequence AGGTCA separated by a single nucleotide spacer, in the target gene [17]. Several classes of compounds, both endogenous and exogenous, have been found to act, at least in part, as ligands for PPARG and included polyunsaturated fatty acids such as arachidonic acid, prostaglandins-like compounds, oxidized lipids such as 9-HODE, and the widely used pharmacological thiazolidinedione (TZD) compounds (20).

PPARG is located in humans on 3p25.3 at Mb position 12.3 to 12.45 and in mouse on chromosome 6 at 115.8 to 115.93 Mb (http://www.ensembl.org v.37, release date: February, 2006). The gene is composed of nine exons, four promoters and yields four transcripts via alternate promoter use and splicing [18–20]. All transcripts contain the exons numbered one through six. It is the alternate promoters and leader exons that yield the four distinct transcripts. As shown in Figure 1, *PPARG*1 is transcribed from the g1 promoter and consists of exons A1, A2 and the ubiquitous exons one through six [18, 19] and is considered to be universally expressed [20]. *PPARG*2, which is only found in adipose tissue [21], is transcribed from the third promoter, which is referred to as g2, and consists of exon B and exons one through six [18, 19]. *PPARG*3, also ubiquitously expressed [20], is transcribed from the second promoter g3 and consists of exons A2 and one through six [19]. The last isoform characterized in humans *PPARG*4 does not contain any of the three leader exons, and rather is expressed directly from the g4 promoter found immediately in front of exon one [20]. Little is known about the g4 transcript, although a recently characterized mutation in humans suggests a key role for this transcript in adipocyte biology [22]. All of the transcripts of *PPARG*, with the exception of the transcript generated from the g2 promoter, yield the same protein product. The protein product yielded by the g2 promoter's transcript PPARG2 contains 30 extra amino acids on the N-terminus. These extra 30 amino acids have been shown to increase the transcriptional activity of PPARG2 by 5–10-fold over that of PPARG1 (26).

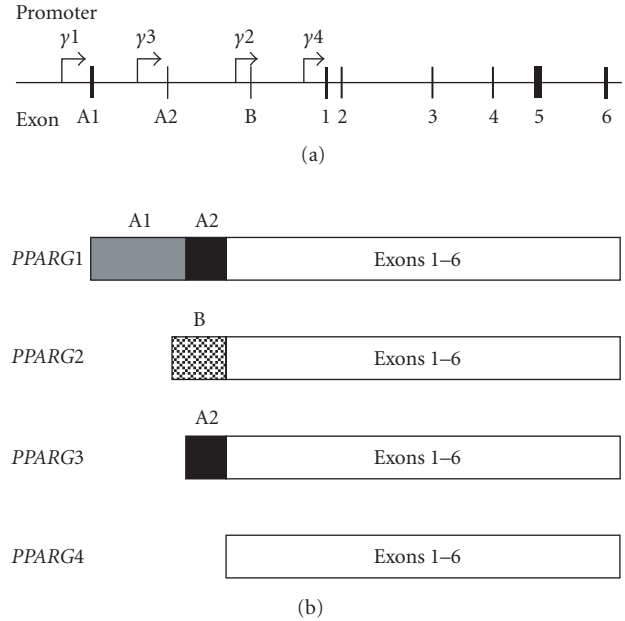

Figure 1: A schematic representation of the *PPARG* gene. (a) The *PPARG* gene is composed of nine exons, named A1, A2, B, 1, 2, 3, 4, 5, and 6, respectively, and four promoters. (b) There are four major *PPARG* transcripts, all of which contain exons 1 through 6. Expression of each transcript is controlled by one of the four promoters. All of the transcripts yield the same protein, except for the γ2 transcript, which codes for 30 additional amino acids on the N-terminus.

GENETIC MAPPING STUDIES IN HUMANS

Of all of the many genome wide scans published to date, only Deng et al [23] report a QTL for BMD in the vicinity of the *PPARG* gene. They showed a forearm-specific BMD QTL with a peak at D3S1297 (3p26) with a modest LOD score of 1.82 [23]. A recent meta analysis was done by Lee et al using data from 11 separate genome-wide scan studies comprised of 3097 families with 12 685 individuals of a variety of ethnic backgrounds [24]. These investigators found suggestive evidence for a QTL for BMD in human on 3p25.3 to 3p22.2, the exact region of the *PPARG* gene. The study by Deng et al was not one of the studies used in this analysis [24]. While studies have examined the heritability of fracture risk [4], no study to date has mapped a QTL for fracture risk to 3p25.

Several mutations have been discovered in *PPARG* in human and have been investigated for their role in obesity, diabetes, and metabolic syndrome and as such are reviewed elsewhere [25]. Four studies published to date have investigated the genetic association of *PPARG* polymorphisms and bone in humans, as summarized in Figure 2 and Table 1.

A silent His477His (C → T, rs3856806) mutation has been identified in humans in the 161st base pair (bp) of the sixth exon of *PPARG* and is referred to in the literature as C161T (as numbered from the beginning of exon 6) or C1431T (as numbered from the ATG start site). While this single nucleotide polymorphism (SNP) may actually be in linkage disequilibrium (LD) with another more causative mutation, the T allele has been associated with increased plasma leptin and adipose tissue mass [30] as well as improved lipid profiles in type II diabetes [31, 32]. Two studies have examined this polymorphism in the context of bone.

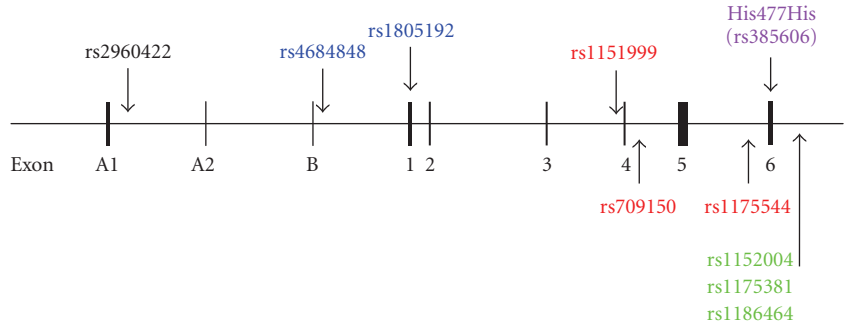

Figure 2: Physical location of the studied human *PPARG* polymorphisms. Several of these SNPs have been shown to be in high LD. All SNPs within an LD block are shown as the same color.

Table 1: A summary of the SNP alleles and associated bone phenotypes as studied to date in humans. The alleles are given in parenthesis after the SNP number with the major allele in the study population listed first. For SNP rs2960422 (∗) no allele frequency in this population was reported by the authors. SNPs rs11512999, rs709150, and rs1175544 (∗∗) showed no association with either BMD or BUA when analyzed separately, but an association with BMD was found for the haplotype of rs11512999 (A), rs709150 (C), and rs1175544 (C) in women.

SNP	Allele	Study population	Phenotype	Reference
His477His (rs3856806, C > T)	C/T or T/T	Postmenopausal Japanese women	Increased total body BMD	[26]
His477His (rs3856806, C > T)	any	Pre-and Postmenopausal Korean women	No association with BMD	[27]
rs2960422∗	A/G	Men and women in mainland China	Increased risk for low BMD in premenopausal women only	[28]
rs1805192 (C > G)	C/C	Caucasian men and women	Site-specific higher BMD in females and lower in males	[29]
rs4684848 (G > A)	any	Caucasian men and women	No association with BMD	[29]
rs1151999 (A > C), rs709150 (C > G) and rs1175544 (C > T) ∗∗	A, C, and C alleles inherited as a block only	Caucasian men and women	Site-specific lower BMD in women	[29]
rs1152004 (T > C)	any	Caucasian men and women	No association with BMD	[29]
rs1175381 (T > C)	T/C or C/C	Caucasian men and women	Site-specific lower BMD in women	[29]
rs1186464 (A > G)	any	Caucasian men and women	No association with BMD	[29]

In the first study of 394 postmenopausal Japanese women, an association between carriers of at least one T allele and increased total body BMD was observed [26]. A more recent study of 138 premenopausal and 125 postmenopausal Korean women showed no association with this SNP and any marker of bone formation, bone resorption, or BMD at the spine or hip, with the exception of serum osteoprotegerin (OPG) [27]. In this study, the authors showed a relationship between low OPG levels and the T allele [27]. While these two studies contradict one another, it must be remembered that first, the cohort size in these studies were very small and second, this is a silent polymorphism and is likely in LD with a more causative mutation. Studies with larger sample sizes and studies involving different ethnic groups must be done in order to get a more comprehensive picture regarding any association of this SNP with bone biology.

Two studies have looked at associations between SNPs in the *PPARG* gene and bone in larger human cohorts. A study of 6743 Chinese men and women examined a single SNP upstream of the first promoter of *PPARG* (rs2960422) and showed a modest increase in the risk of low BMD with the heterozygous state of this allele, but only in premenopausal women. No association was found in either men or postmenopausal women [28]. It must be noted that to date, this SNP has only been examined in this one ethnic group.

A more comprehensive study of SNPs in *PPARG* and their association with aspects of bone density has been done in the Framingham Offspring cohort [29]. The population of study consisted of 740 men and 776 women, with an average age of 61 years old, who were primarily Caucasians. Eight SNPs constituting three LD blocks were investigated for association with femoral neck, greater trochanter or spine BMD

as well as with broadband ultrasound attenuation (BUA) of the calcaneus. The location of these SNPs and the LD blocks is summarized in Figure 2. Only one coding SNP was assessed in this study, rs1805192. This SNP, located in the universal exon one, codes for the substitution of an alanine (Ala) for the wild-type proline (Pro) but is not to be confused for the much-studied Pro12Ala polymorphism found in exon B [29]. Homozygosity for the more common Pro allele was associated with increased BMD at both the femoral neck and lumbar spine as well as increased BUA in women, when the data was adjusted for age and estrogen status. Conversely, men with this same allele had lower femoral neck and trochanter BMD [29]. A full examination of this amino acid substitution has not been undertaken to date but computer modeling programs designed to predict the implications of amino acid change suggest that this substitution could have structural consequences [33, 34]. The C allele of the SNP rs1175381 located distal to the polyadenylation signal was associated with lower BMD at all sites measured in women. No association with men was reported [29]. Lastly, a haplotype block of three SNPs with the associated alleles shown in brackets, rs1151999 (A), rs709150 (C) and rs1175544 (C), was in women, also associated with lower BMD of the femoral neck, trochanter, and lumbar spine, but no association was found in men. Interestingly all of these allele-BMD associations were found to be independent of BMI or type II diabetes (36).

All of the findings presented in these four studies need to be confirmed in other cohorts. Both the Chinese cohort study and the Framingham study are ongoing studies and it is hoped that future publications from these two groups will include an examination of such well-studied SNPs such as the Pro12Ala and the His477His SNP. While these studies did correct for factors such as menopausal status, there may well be other confounding and/or interacting factors that have not been taken into account in these studies, thus masking important results. Previous studies have shown *PPARG* allele by environment interactions for a variety of non bone phenotypes, warranting more comprehensive studies of this gene and bone [35–37].

BONE BIOLOGY OF THE *Pparg* KNOCKOUT ANIMAL

Homozygous knock out $Pparg^{tm1Tka}$ mice die at embryonic day 10.5 to 11 pc due to placental insufficiency and cardiac defects, making any meaningful examination of skeletal biology impossible [38]. In contrast, the *Pparg* heterozygous knockout mouse ($Pparg^{+/-}$) is viable and appears to have normal development of all major organs. Akune et al have thoroughly examined the bone biology of this haploinsufficient *Pparg* mouse [39]. The $Pparg^{+/-}$ male mice show marked increase in trabecular bone volume at 8 weeks of age as compared to wild-type, and while the volume fraction of trabecular bone (BV/TV) of the distal femur did decrease with age in both genotypes, the $Pparg^{+/-}$ mouse maintained a higher BV/TV than the wild-type controls through 52 weeks of age. Histological analysis showed a more than 50% increase in the number of osteoblasts and a doubling in the total bone formation rate (BFR) of the haploinsufficient mice, leading to the conclusion that the function of individual osteoblasts was not affected. This increase in osteoblast number was coincident with a trend for a decrease in adipocyte number. The number of adipocytes in the marrow increased in the wild-type controls with age, but no change in adipocyte number was observed in the $Pparg^{+/-}$ mice by 52 weeks of age. The effects of estrogen loss in females on bone, in the context of low PPARG were also examined. The loss of one *Pparg* allele was not protective to bone, as the $Pparg^{+/-}$ ovariectomized (OVX) mice lost the same proportion of bone after OVX, as the wild-type OVX mice lost when compared to the appropriate genotypic sham operated mice [39]. Although Rieusset et al, in a separate study, report slight total body growth retardation in the $Pparg^{+/-}$ male but not female mice [40], Akune et alfound no such growth retardation.

SENESCENCE-ACCELERATED MOUSE P6

The senescence-accelerated series of mice (SAM) were created in the 1970s as model for the study of physiological decline with aging. Two series of mouse lines were created: the SAMR series served as control lines and the SAMP lines were selected for signs of advanced aging. The SAMP6 line was created from the SAMR3 line, from a pedigree that showed spontaneous leg fractures with advanced age [41]. While indistinguishable from the SAMR1 control strain at one month of age, bones from the SAMP6 mice showed decreased trabecular bone volume, decreased cortical thickness, lower areal BMD, and lower BFR as early as three months of age. The SAMP6 mice also showed a decreased bending strength and increased brittleness, and are considered an excellent model of the senile osteoporosis observed in humans [42, 43]. The SAMP6 mice show an increase in marrow adiposity with aging [44] and a coincident decrease in osteoblast precursor cells evident as early as three months of age [42]. More recently, it has been shown that *Pparg2* mRNA levels increase in the marrow with aging in these mice, yet this could be blocked by a yet-to-be-determined mechanism upon the administration of $1,25(OH)_2D_3$ (49).

MAPPING STUDIES IN MICE

Two separate mouse mapping crosses in mice have identified a QTL for an aspect of bone density or geometry on the distal 6th chromosome (Chr) in the vicinity of the *Pparg* gene. Klein et al have identified QTL for femoral cross-sectional area, with a broad peak that includes the genetic location of *Pparg* in a C57BL/6J (B6) by DBA/2J cross [45]. Drake et al have shown a QTL for bone density that colocalized with adipose tissue mass and bone torsional strength QTLs in the same genetic location as Klein et al in a cross of the same two strains, but only after the mice were fed a high fat diet [46].

Our laboratory has conducted intensive studies of a Chr 6 QTL found in a cross of B6 by C3H/HeJ(C3H), *Bmd*8 [47]. A congenic mouse was made for the purpose of studying this QTL in isolation from the large number of other BMD

affecting QTLs found on other chromosomes. The ensuing strain B6.C3H-6T (6T) was made by introgressing the region of 6th Chr encompassed by the markers *D6Mit*93 and *D6Mit*150 from C3H onto a B6 background by 9 generations of selective backcrossing, followed by several generations of intercrossing. The resulting mouse is homozygous for B6 alleles for the entire genome except for the region between *D6Mit*93 and *D6Mit*125, which is homozygous for the C3H alleles [48]. The biology of the 6T mouse has been well studied. This strain has lower BMD than either the B6 background strain, or the C3H donor strain. 6T mice have a smaller periosteal circumference, slightly shorter femurs, and a lower BFR as compared to the B6 background strain [48]. There are several candidate genes in the congenic region of the 6T mouse for the various phenotypes seen in the 6T mouse, including, but not limited to *Pparg*, arachidonate 5-lipoxygenase (*Alox5*), adiponectin receptor 2 (*AdipoR2*), and *Wnt5b*. While not all of the phenotypes seen in the 6T mouse can be explained by a single gene alteration, the 6T mouse does have a strikingly opposite phenotype than that seen in the $Pparg^{+/-}$ mouse for several key phenotypes. For example, the 6T mouse has increased numbers of marrow adipocytes and significantly lower trabecular bone volume at all sites measured when compared to the background strain [48, 49]. Marrow stromal cell cultures show that there are less alkaline phosphatase staining colonies as compared to B6 control cultures as soon as 7 days after culture, suggesting a decrease in osteoblastogenesis [49].

Yet the biology of the 6T mouse is not clear cut. Increased fat feeding (increase in % kcal from fat), which provides more exogenous ligand for *Pparg*, does not increase total body fat in the female 6T mouse, as it does in the B6 control strain, nor does it affect the number of marrow adipocytes. However, decreased fat feeding does improve the BV/TV in 6T to levels seen in control fed B6 mice [50]. Differences in *Pparg* transcript levels have been found in both the liver and in the bone when comparing 6T back to the background B6 strain [49]. In addition, several polymorphisms in both coding and noncoding regions of *Pparg* have been found when comparing B6 to C3H. While no nonsynonymous SNPs have been found, several intriguing promoter polymorphisms have been found as well as 12 SNPs in the 3′ UTR (Ackert-Bicknell, unpublished data). Both the biology of the 6T mouse as well as the number of polymorphisms in *Pparg* suggest a key role for this gene in the bone phenotype of the 6T mouse.

Our original F2 genetic mapping-cross suggested that this Chr 6 QTL interacted with a locus on the 11th Chr (56). The *Alox15* gene, which codes for an enzyme key in the formation of 15S-HETE, an endogenous ligand for PPARG (57), is located on Chr 11 at 70069811–70077674 Mb (http://www.ensembl.org v.37, release date: February, 2006) and knockout mice for this gene show higher femoral BMD and femoral stiffness [51]. Associations with BMD have been found in human with SNPs in *ALOX12*, the gene that codes for the human functional homologue to the mouse *Alox15* [52]. Another member of the ALOX gene family, *Alox5*, is located approximately 1 Mb distal to *Pparg* on mouse Chr 6 and also likely produces a ligand for PPARG. While expression of *Alox15* is much more widespread, the expression of *Alox5* appears to be more limited with the greatest expression levels seen in bone and white blood cells (http://symatlas.gnf.org/SymAtlas/).

It is interesting to speculate about the causative gene or genes in the 6T mouse. In some ways, the phenotypes of the 6T mice mimic phenotypes of the $Pparg^{+/-}$ mouse, such as the resistance fat feeding induced obesity [50, 53], yet in other respects, the 6T mouse is the exact opposite of the $Pparg^{+/-}$ mouse. Are alterations in the *Pparg* gene the cause of this, or is PPARG the mediator of this action under the control of another gene, such as a member of the *Alox* gene family? Cellular differentiation in bone cell lineages, as driven by PPARG, has been shown to be dependant on the type of PPARG ligand present [54], further suggesting the alterations in ligand processing and/or the ability of PPARG to respond appropriately, may contribute to the interesting physiology of the 6T mouse. Additional experiments are in progress to elucidate the genetic mechanisms responsible for the phenotypes seen in the 6T mouse.

PPARG, DIABETES, AND OBESITY

The Pro12Ala polymorphism has been found in a variety of ethnic populations [25] and has been shown to decrease both the binding of PPARG/RXR heterodimers to the PPRE and their ability to activate gene transcription [55]. This polymorphism has not been studied with regard to an association with bone density, but it has been examined in the context of several other physiological and pathological states that are known to impact bone health. While a clear association between this polymorphism and BMI or obesity is lacking, a vast number of studies performed to date have linked the Ala allele with decreased risk for type II diabetes (reviewed in [25, 56]). The few patients described with dominant negative *PPARG* mutations present with early onset and severe insulin resistance [57] and a few studies have suggested that the His477His mutation may actually be a better predictor of type II diabetes in certain ethnic populations than the Pro12Ala mutations [32, 58, 59]. Increased fracture rates are seen in patients with type II diabetes despite an overall increase in BMD [5, 60].

In contrast, patients with type I diabetics often have osteopenia even after long periods of good metabolic control. These patients frequently have a decrease in markers of bone formation, such as serum alkaline phosphatase and osteocalcin, as this is thought to be indicative of insufficient bone accrual beginning at a very young age [60]. These observations of low bone formation are confirmed in an inducible mouse model of type I diabetes. Type I diabetic male mice have been shown to have lower BFR, and the maturation of osteoblasts from these mice is inhibited [61]. PPARG expression is shown to be increased in concert with an increase in marrow adiposity in these same mice, as well as other markers of adipocyte maturation, suggesting a mechanism for the low bone mass seen in type I diabetes [61].

Leptin (gene symbol *Lep*), a hormone secreted by adipose tissue, is thought to inhibit bone formation, as evidenced by the fact that both the *ob/ob* (leptin-deficient) and *db/db* (leptin-receptor-deficient) mice have increased bone mass and increased bone formation rate [62]. It is thought that leptin mediates its actions on bone via the sympathetic nervous system [63]. It has been proposed that PPARG suppresses *Lep* gene expression, as expression of *Lep* is increased in the $Pparg^{+/-}$ mice [64], providing yet another mechanism by which PPARG may influence the biology of bone. In humans, the His477His polymorphism has been shown to be associated with plasma leptin levels in obese subjects, yet it may be argued that this is more a reflection of the effects of PPARG on adipose tissue mass [30].

SUMMARY

PPARG is indisputably important for bone acquisition as is clearly demonstrated by the phenotype of the $Pparg^{+/-}$ mouse. While a promising start has been made with regard to the usefulness of genetic typing for *PPARG* as predictor of BMD and fracture risk, too few studies have been completed for any conclusive statements to be made. The associations between *PPARG* and three major influences on BMD, leptin, obesity, and diabetes, are encouraging. Genetic mouse models of low BMD, such as SAMP6 and 6T, are invaluable tools for the further study of PPARG in bone.

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Review Article

PPAR Gamma Activity and Control of Bone Mass in Skeletal Unloading

P. J. Marie[1,2] and K. Kaabeche[1,2]

[1] *Laboratory of Osteoblast Biology and Pathology, INSERM U606, 75475 Paris Cedex 10, France*
[2] *Faculty of Medicine, University of Paris 7, 75251 Paris Cedex 05, France*

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Bone loss occuring with unloading is associated with decreased osteoblastogenesis and increased bone marrow adipogenesis, resulting in bone loss and decreased bone formation. Here, we review the present knowledge on the role of PPARγ in the control of osteoblastogenesis and bone mass in skeletal unloading. We showed that PPARγ positively promotes adipogenesis and negatively regulates osteoblast differentiation of bone marrow stromal cells in unloading, resulting in bone loss. Manipulation of PPARγ2 expression by exogenous TGF-β2 inhibits the exaggerated adipogenesis and corrects the balance between osteoblastogenesis and adipogenesis induced by unloading, leading to prevention of bone loss. This shows that PPARγ plays an important role in the control of bone mass in unloaded bone. Moreover, this opens the possibility that manipulation of PPARγ may correct the balance between osteoblastogenesis and adipogenesis and prevent bone loss, which may have potential implications in the treatment of bone loss in clinical conditions.

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1. INTRODUCTION

The maintainance of both bone mass and bone microarchitecture is controlled by the balance between bone resorption and formation. At the cellular level, this balance is largely dependent on the number and activity of bone forming and resorbing cells. Any alteration in the number or activity of bone cells will result in an imbalance between resorption and formation, resulting in microarchitecture deterioration and altered bone mass and strength.

The control of bone forming cells is largely influenced by weight bearing and exercise that induce mechanical forces on the skeleton. Mechanical forces induce anabolic effects by promoting bone formation at multiple levels [1–3]. Bone formation is a complex process that is dependent on the recruitment, differentiation, and function of osteoblasts. The osteogenic process starts by the commitment of osteoprogenitor cells into osteoblasts under the control of transcription factors, followed by their progressive differentiation into mature osteoblasts [4, 5]. In the recent years, the development of cellular, molecular, and genetic studies has led to the identification of a number of important transcription factors that are essential in the control of bone formation. Specifically, several studies have provided evidence for a role of PPARγ in the control of bone formation and bone mass through modulation of bone marrow stromal cell differentiation. In this brief review, we summarize the present knowledge on the role of PPARγ in the control of osteoblastogenesis and bone mass, with a particular reference to skeletal unloading.

Reciprocal relationship between osteoblastogenesis and adipogenesis in the bone marrow

Several conditions associated with bone loss such as aging [6], glucocorticoid treatment [7], estrogen deficiency [8], or immobilization [9] are characterized by decreased osteoblastogenesis associated with increased adipogenesis in the bone marrow. This supports the concept that there is a reciprocal relationship between adipocyte and osteoblast differentiation [10]. Early studies showed that bone marrow stromal cells can be differentiated into several lineages in vitro [11–13], and that differentiation towards one lineage is dependent on local or hormonal factors [14]. Further studies showed that clonal marrow stromal cells can be differentiated into adipocytes, osteoblasts, or chondrocytes in different species including humans [15–17]. Notably, a single marrow stromal cell may have multipotential competence in vitro and differentiation towards one pathway restricts expression of other lineage-specific genes [18]. This provides evidence that adipocytes and osteoblasts are derived from a

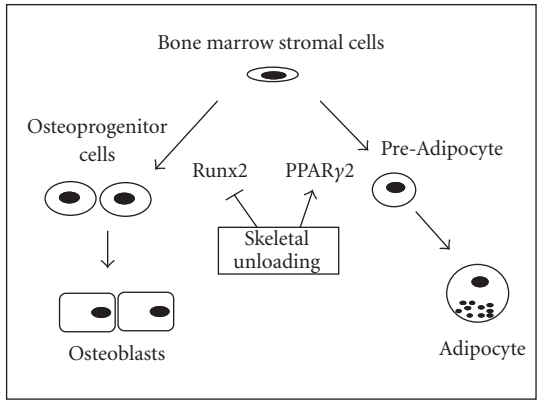

Figure 1: The in vivo differentiation of bone marrow stromal cells towards adipocytes and osteoblasts is governed by the balance between PPARγ2 and Runx2 expression. In unloaded bone, decreased Runx2 and increased PPARγ2 expression result in decreased osteoblastogenesis, increased adipogenesis, and bone loss.

common mesenchymal stromal cell and that a reciprocal relationship exists between osteoblastogenesis and adipogenesis in the bone marrow [10].

PPARγ2 is a positive promoter of adipogenesis and a negative regulator of osteoblastogenesis

The mechanisms involved in adipogenesis have been studied extensively in adipose tissue. The differentiation of preadipocytes into mature adipocytes is primarily controlled by peroxisome proliferator-activated receptor γ (PPARγ) which is a key transcription factor involved in adipocyte differentiation [19]. PPARγ exists in two isoforms PPARγ1 and PPARγ2 as a result of alternative splicing. PPARγ2 is expressed at high levels in fat tissue and is essential for adipogenesis in vitro and in vivo. CCAAT/enhancer binding proteins (C/EBP) are other important transcription factors that control the expression of adipocyte genes by acting synergistically with PPARγ to activate adipocyte gene expression [20]. In vitro, C/EBPs activate the expression of PPARγ and C/EBPα and promote PPARγ2 activity in preadipocyte cultures, which contributes to the expression of genes that characterize the adipocyte phenotype [21].

In bone, recent advances have been made in the role of PPARγ in the interconversion of marrow stromal cells into osteoblasts or adipocytes in vitro (Figure 1). In cultured murine and human cells, PPARγ agonists and overexpression of PPARγ2 induce the differentiation of bone marrow stromal cells into the adipocyte lineage and negatively regulate osteoblast differentiation by repressing the osteoblast specific transcription factor Runx2 [22–24]. There is also evidence that PPARγ negatively regulates osteoblast differentiation. For example, activation of PPARγ with a thiazolidinediones with high affinity for PPARγ increases adipogenesis and decreases osteoblastogenesis in vitro [25–27]. Additionally, activation of PPARγ with rosiglitazone in mice or ovariectomized rats decreases Runx2 expression and bone formation, and increases adipogenesis in the bone marrow, resulting in decreased bone mass [28, 29]. Consistently, PPARγ haploinsufficiency in mice was shown to decrease adipogenesis and to increase Runx2 expression and bone formation, resulting in increased bone mass [30]. These findings indicate that PPARγ positively promotes adipogenesis and negatively regulates osteoblast differentiation of bone marrow stromal cells in vivo, suggesting that PPARγ is a negative regulator of bone mass.

Skeletal unloading decreases osteoblast differentiation and induces bone loss

A representative model of bone loss resulting from alterations in osteoblasts is skeletal unloading [31]. Skeletal unloading induced by hind limb suspension rapidly causes a marked trabecular bone loss in the long bone metaphysis, resulting mainly from reduced trabecular thickness and number associated with inhibition of endosteal bone formation [32]. Although both the number and activity of osteoblasts are decreased in the unloaded metaphyseal bone [32, 33], the number of osteoblasts is more affected than their activity [34]. Although the mechanisms underlying bone loss induced by unloading in rats are not fully understood, bone loss does not appear to result from changes in serum corticosteroid, 25-hydroxyvitamin D or PTH levels [31]. However, there is some evidence that skeletal unloading may result in part from to decreased expression [34] or response [35] to local growth factors.

The cellular mechanisms underlying the alterations of bone formation induced by skeletal unloading in rats have been partly identified [36]. We initially showed that the decreased bone formation in unloaded rat bone results from an impaired recruitment of osteoblast precursor cells in the bone marrow stroma and in the metaphysis [33]. In addition to affect osteoblast recruitment, skeletal unloading in this model alters the function of differentiated osteoblasts. This is reflected by the decreased expression of bone matrix type-1 collagen and osteocalcin and osteopontin mRNA levels [37–40], which correlates well with the decreased bone matrix synthesis measured at the tissue level [32, 33]. These findings indicate that removal of mechanical forces on the skeleton rapidly alters both the recruitment of osteoblast progenitor cell and the function of differentiated osteoblasts, resulting in a marked reduction of bone formation. Such alterations are consistent with the effects of unloading in other rat models in which there is a reduction of the osteogenic capacity of bone marrow osteoblast precursor cells and a decreased expression of bone matrix proteins in rat long bones [41, 42].

PPARγ controls the osteoblast/adipocyte relationship in unloaded bone

The altered bone metabolism induced by skeletal unloading is asociated with alterations in transcription factor expression. Specifically, the decreased osteoblastogenesis and bone formation induced by skeletal unloading in rats are

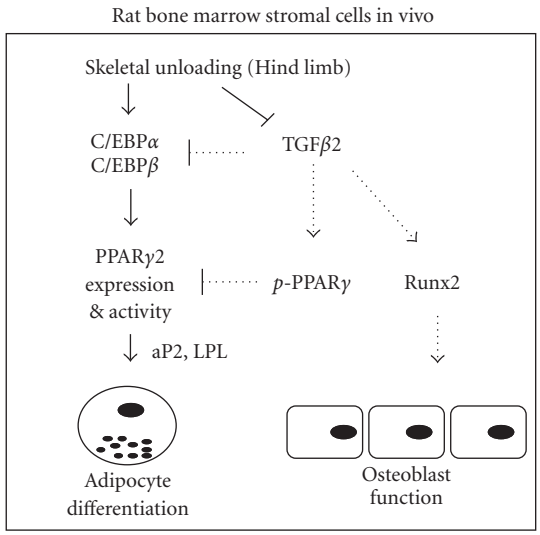

Figure 2: Skeletal unloading decreases TGF-β expression and activates the expression of C/EBPα, C/EBPβ, and PPARγ2, resulting in activation of adipocyte gene expression such as adipocytic differentiation-related genes adipocyte binding protein (aP2) and lipoprotein lipase (LPL) in bone marrow stromal cells. Exogenous TGF-β2 (dotted lines) reduces C/EBPα, C/EBPβ, and PPARγ expressions, induces PPARγ phosphorylation (p-PPARγ), and increases Runx2 expression, resulting in decreased adipogenesis, increased osteoblast function, and prevention of bone loss.

associated with reduced Runx2 expression [34]. Additionally, we showed that skeletal unloading is associated with increased adipocyte differentiation in the bone marrow stroma [43], suggesting that unloading not only impairs osteoprogenitor cell differentiation into osteoblasts but also promotes adipocyte differentiation. The exagerated reciprocal relationship between osteoblastogenesis and adipogenesis may account for the decreased bone formation associated with the increased bone marrow adipogenesis in unloaded rats (Figure 1).

Interestingly, the adipogenic differentiation of bone marrow stromal cells in unloaded bone is consistent with the temporal gene expression observed during adipocyte differentiation in vitro. Specifically, skeletal unloading in rats increases C/EBPα and C/EBPβ expression followed by increased expression of PPARγ, resulting in activation of adipocyte gene expression such as adipocytic differentiation-related genes adipocyte binding protein (aP2) and lipoprotein lipase (LPL) in bone marrow stromal cells [44] (Figure 2). Thus, PPARγ with other transcription factors are involved in adipogenic conversion of bone marrow stromal cells in vivo, indicating that PPARγ is a negative regulator of bone mass in unloaded rats.

The mechanisms underlying the expression of Runx2 and PPARγ in unloaded bone may involve decreased signaling pathways that are normally transmitted by loading. Mechanical forces are believed to transduce signals through cell-matrix interactions [45–48]. Part of the communication between the matrix and cells is ensured by integrins which interact with bone matrix proteins [49]. In bone, integrin-matrix interactions are important modulators of osteoblast differentiation in vitro [50, 51]. It is thus possible that the lack of mechanical strain is induced by unloading results in decreased integrin-matrix interactions and signaling, and consequently decreased osteoblast differentiation. This is supported by the finding that mechanical forces increase Runx2 expression in cultured preosteoblastic cells [52]. One recent study indicates that stretching induces downregulation of PPARγ2 and adipocyte differentiation in mouse preadipocytes [53], suggesting that mechanical forces may play a dual role in the control of Runx2 and PPARγ expression in preosteoblasts.

How mechanical signals may modulate PPARγ expression or activity and thereby induce adipogenesis rather than osteoblastogenesis in bone marrow stromal cells is not fully understood. One interesting hypothesis is that specific pathways controlling osteoblastogenesis/adipogenesis may be sensitive to biomechanical forces. For example, changes in cell shape or modulation of the cytoskeletal-related GTPase RhoA were recently found to induce stem cell adipogenic or osteoblast differentiation [54]. Additionally, multiple signal pathways, including ERK and Wnt signaling, may control the balance between adipogenesis and osteoblastogenesis in vitro [53, 55]. It remains however to determine which pathway may be involved in the altered balance between osteoblastogenesis and adipogenesis in vivo.

TGF beta is a negative regulator of PPARγ and adipogenesis in unloaded rats

Transforming growth factor beta (TGF-β) is an important regulator of bone formation by modulating osteoblastic cell proliferation and differentiation [56]. Additionally, TGF-β is also an important modulator of adipocyte differentiation. TGF-β inhibits adipogenesis in preadipocyte cell lines and reduces adipocyte differentiation in vitro [57, 58]. In vivo, we found that skeletal unloading results in a rapid reduction in TGF-β1 and TGF-β receptor II mRNA expression in bone marrow stromal cells [34]. Others found reduced TGF-β2 mRNA levels in bone marrow stromal cells in this model [37], suggesting that TGF-β signaling may mediate part of the altered bone formation induced by unloading. Although diminished, TGF-β receptors can still be activated by TGF-β since we showed that exogenous TGF-β2 in unloaded rats increased Runx2 expression and osteoblastogenesis, resulting in prevention of trabecular bone loss [59]. Beside this positive effect on osteoblastogenesis, TGF-β2 administration downregulated the expression of C/EBPα, C/EBPβ, and PPARγ in bone marrow stromal cells, and reduced the expression of adipocyte genes such as aP2 and LPL in bone marrow stromal cells, thus preventing the adipocyte conversion of bone marrow stromal cells induced by unloading [43, 44]. This indicates that TGF-β is a negative regulator of PPARγ and adipogenesis in unloaded rats (Figure 2).

One mechanism by which TGF-β may negatively regulate adipogenesis in unloaded rats is through MAPK activation.

TGF-β is known to induce phosphorylation of PPARγ in adipocyte cells, and MAPK-dependent PPARγ phosphorylation results in the reduction of PPARγ transcriptional activity and repression of adipocyte differentiation [60–62]. In vitro, ERK activation was found to induce osteogenic differentiation of human mesenchymal stem cells, whereas its inhibition induces adipogenic differentiation [63]. In unloaded bone, we showed that TGF-β2 increased PPARγ phosphorylation and inhibited adipocyte differentiation of bone marrow stromal cells through MAPK phosphorylation [44]. Thus, exogenous TGF-β can inhibit the excessive adipogenic differentiation induced by skeletal unloading by reducing PPARγ2 expression, resulting in the inhibition of adipogenesis. This effect, combined with the upregulation of Runx2 expression and osteoblast differentiation induced by exogenous TGF-β on bone marrow stromal cells, leads to correcting the imbalance between osteoblastogenesis and adipogenesis and results in a positive effect on bone mass (Figure 2). This demonstrates that appropriate manipulation of PPARγ2 expression in vivo can lead to prevent bone loss in unloaded bone.

CONCLUSION

There is now clear evidence that PPARγ plays an important role in the control of marrow stromal cell differentiation to osteoblasts or adipocytes in unloaded bone. In this model, PPARγ positively promotes adipogenesis and negatively regulates osteoblast differentiation of bone marrow stromal cells, indicating that PPARγ is a negative regulator of bone mass. This concept provides a possible target for therapeutic intervention in osteopenic disorders characterized by altered osteoblast and adipocyte differentiation of bone marrow stromal cells [64]. As an example, we showed that exogenous manipulation of PPARγ expression by TGF-β can inhibit adipogenesis induced by skeletal unloading and correct the balance between osteoblastogenesis and adipogenesis, resulting in prevention of bone loss. This opens the possibility that manipulation of PPARγ may have potential implications in the treatment of bone loss associated with immobilization [65].

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Review Article

Diabetes, TZDs, and Bone: A Review of the Clinical Evidence

Ann V. Schwartz

Department of Epidemiology and Biostatistics, University of California, San Francisco, 185 Berry Street, Suite 5700, San Francisco, CA 94107, USA

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Evidence from rodent and in vitro models suggests that activation of PPAR-γ by thiazolidinediones (TZDs) causes increased bone marrow adiposity and decreased osteoblastogenesis, resulting in bone loss. TZDs are prescribed for the treatment of diabetes, providing an opportunity to determine whether PPAR-γ activation also impacts bone in humans. In addition, since type 2 diabetes is associated with higher fracture risk, an understanding of the clinical impact of TZDs on bone is needed to guide fracture prevention efforts in this population. This review summarizes current findings regarding type 2 diabetes and increased fracture risk and then considers the available evidence regarding TZD use and bone metabolism in humans.

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INTRODUCTION

Thiazolidinediones (TZDs) are an effective treatment for diabetes that increase insulin sensitivity through activation of peroxisome proliferator-activated receptor (PPAR)-γ. Activation of PPAR-γ by TZDs may also cause an increase in bone marrow adiposity and a decrease in osteoblastogenesis, resulting in reduced bone formation [1]. TZDs are reported to cause bone loss in some [1–4], but not all [5], rodent models. However, little information is available on the effects of TZDs on bone in humans.

The increased use of TZD treatment is taking place in the context of growing evidence that type 2 diabetes (T2DM) is associated with a higher risk of fracture. If TZDs cause bone loss in humans, use of TZD treatments could add to this increased fracture risk. Reports on TZD use and fracture risk are not currently available, in part because widespread use of TZDs is relatively recent. Some limited clinical and observational studies have addressed the impact of TZD use on bone turnover and bone density. This review presents current evidence that type 2 diabetes is associated with higher fracture risk and then considers the available evidence regarding the impact of TZD use on bone in humans.

TYPE 2 DIABETES IS ASSOCIATED WITH AN INCREASED RISK OF FRACTURE

Nonvertebral fractures

Until relatively recently, type 2 diabetes was not considered a risk factor for fracture. Type 2 diabetes is associated with increased weight which provides protection from most fractures. In 1980, a large retrospective study using Mayo Clinic records reported that diabetes was not associated with increased risk of fracture except at the ankle [6]. However, recent studies have reported that those with type 2 diabetes are at higher risk for hip (Table 1) [7–14], proximal humerus [9, 10], foot [9, 15], and all nonvertebral fractures combined (Table 2) [9, 10, 13, 16].

As shown in Table 1, the age-adjusted effect estimates for the relative risk of hip fracture associated with type 2 diabetes range from 1.1 to 5.8 in older women and 1.0 to 7.7 in older men. Diabetes is generally associated with being overweight and with higher bone mineral density (BMD). Thus, with adjustment for body size and/or BMD in these studies, the relative risks are somewhat higher.

Impaired glucose metabolism

Two studies have also considered increased fracture risk in those with impaired glucose metabolism. In both studies, those with impaired glucose metabolism as well as those with diabetes had higher BMD than those with normal glucose homeostasis. In the Rotterdam Study, impaired glucose tolerance, compared with normal glucose tolerance, was associated with a reduced risk of nonvertebral fracture, adjusted for BMD and body size (HR = 0.80; 95% CI: 0.63–1.00) [13]. Results from the Health, Aging, and Body Composition Study (Health ABC) showed a modest increase in nonvertebral fracture risk in those with impaired fasting glucose but

Table 1: Age-adjusted relative risk of hip fracture for older adults with type 2 diabetes.

Study	Gender	Age	RR	95% CI
Cardiovascular Disease in Norwegian Countries (1993) [7]	Women	35–49	5.8	2.2–15.7
	Men	35–49	7.7	2.4–24.5
Nord-Trondelag Health Survey (1999) [8]	Women	50–74	1.7	1.1–2.7
	Men	50–74	1.0	0.4–2.6
Study of Osteoporotic Fractures (2001) [9]	Women	≥ 65	1.5	1.1–2.0
Iowa Women's Health Study (2001) [10]	Women	55–69	1.8	1.2–2.4
Hispanic EPESE (2002) [11]	Men and Women	≥ 65	1.6*	1.0–2.4
Tromso Study (2005) [12]	Women	25–98	1.7	1.0–3.0
	Men	25–98	1.4	0.5–4.0
Rotterdam Study (2005) [13]	Women	≥ 55	1.1	0.7–1.6
	Men	≥ 55	1.4	0.7–2.8
Malmo Preventive Project (2005) [14]	Women	28–58	4.1	1.8–9.3
	Men	27–61	7.7	4.4–13.7

*Adjusted for age, gender, current smoking, BMI, history of stroke.

Table 2: Adjusted relative risk of nonvertebral fracture with type 2 diabetes.

Study	Gender	Age	RR	95% CI
Study of Osteoporotic Fractures (2001) [9]	Women	≥ 65	1.3	1.1–1.5
Iowa Women's Health Study (2001) [10]	Women	55–69		
Insulin treated	—	—	1.5	1.1–1.9
Not insulin treated	—	—	1.1	1.0–1.3
Tromso Study (2005) [12]	Women	25–98	1.1	0.7–1.7
	Men	25–98	1.2	0.6–2.5
Health ABC Study (2005) [16]	Men and Women	70–79	1.6	1.1–2.5
Rotterdam Study (2005) [13]	Men and Women	≥ 55	1.3	1.0–1.8

confidence intervals were wide (adjusted HR = 1.34; 95% CI: 0.67–2.67) [16].

Vertebral fractures

Based on findings from three studies that identified vertebral fractures from spine radiographs, it appears that the risk of vertebral fractures may not be increased with type 2 diabetes. Diabetic women aged 50 years and older in a Canadian study had no increase in prevalent vertebral fractures (OR = 0.92; 95% CI: 0.67–1.25) [17]. Additionally, there were no differences in prevalent vertebral fracture by diabetes status among older women with low bone density enrolled in the Fracture Intervention Trial [18]. For incident vertebral fractures, the study of osteoporotic fractures (SOF) reported no increased risk in women with type 2 diabetes over an average of 3.7 years (OR = 1.1; 95% CI: 0.69–1.83) [9].

Reasons for increased fracture risk: falls and bone strength

The reasons for increased fracture risk with type 2 diabetes are not well understood. T2DM have average or higher BMD even after adjustment for body size [19–21]. However, diabetic bone may be more fragile for a given BMD [22]. We have also found evidence in the Health ABC Study that older white women with diabetes were losing bone at the hip more rapidly than those without diabetes, even though the diabetic women had higher BMD at baseline [23]. The increased bone loss was partly accounted for by greater weight loss in the diabetic, compared with nondiabetic, women. Weight loss correlates with bone loss and increased bone turnover in older adults [24, 25]. The reasons for increased weight loss with diabetes in this cohort are not known. However, a study in the Pima Indians reported that weight loss after the onset of diabetes was found in those who were not treated with hypoglycemic medications [26].

T2DM is also associated with an increased risk of falls [27–30]. More frequent falls are known to increase fracture risk, and this probably accounts for at least some of the higher fracture risk with diabetes. However, adjustment for frequency of falls has not fully explained the association between diabetes and fracture in previous studies [9, 16]. It is likely that other factors such as decreased bone strength and bone loss also contribute to increased fracture risk [31].

Similar to well-known findings in the broader population, lower BMD predicts fracture in older adults with diabetes. Among the older diabetic adults in the Health ABC Study, those who experienced a fracture had an average total hip BMD at baseline that was 15% lower than those without fracture [16]. Thus, although type 2 diabetes is associated with higher BMD, loss of BMD would still be expected to increase fracture risk.

It has been suggested that TZD use may explain some of the increased fracture risk observed in older adults with type 2 diabetes [32]. However, the data for studies reporting increased fracture risk with diabetes were generally acquired before use of TZDs for diabetes treatment. Troglitazone was available in the USA from 1997 to 2000 when it was removed from the market because of rare cases of fatal liver disease. Pioglitazone and rosiglitazone were first available for prescription in the USA in 1999. In 2001, TZDs accounted for 17% of market share for oral hypoglycemic medications [33]. It is unlikely that TZD use accounts for the currently published reports of an increased fracture risk with type 2 diabetes.

TZDS AND BONE LOSS

Evidence from rodent and in vitro models

Several lines of evidence from rodent and in vitro models point to the possibility that treatment with TZDs causes bone loss. The results of these investigations are reviewed in accompanying articles in this special issue and are only mentioned briefly here. In rodent models, Rzonca et al [1] and others [2] have reported bone loss with rosiglitazone treatment in mouse models, and Sottile et al [3] found bone loss in ovariectomized rats treated with rosiglitazone although no effect was seen in intact animals. Jennermann et al reported decreased BMD with pioglitazone treatment in rats [4]. However, Tornvig et al reported that troglitazone treatment did not cause bone loss in mice [5].

In vitro studies have shown that PPAR-γ activation with TZDs promotes the differentiation of precursor cells into adipocytes and inhibits their differentiation into osteoblasts [34, 35]. These effects of PPAR-γ activation are not necessarily bound together. Use of ligands other than rosiglitazone to activate PPAR-γ has been shown to promote only the proadipogenic or only the antiosteoblastogenic pathways [36, 37]. These results suggest that it may be possible to identify PPAR-γ activators that promote insulin sensitivity without inhibiting osteoblastogenesis [38].

Evidence from clinical studies

The current clinical studies of TZD use and bone are limited in size and study design and have not produced consistent results.

Bone turnover

A study in 33 type 2 diabetic patients found that troglitazone treatment (400 mg per day) for four weeks reduced markers of bone turnover, including formation and resorption markers, by a modest amount (7–13%) [39]. This may reflect a direct effect of troglitazone on bone metabolism, or it may be an indirect result of improved glycemic control on bone. The impact of hyperglycemia on bone is not well studied but some reports indicate that improved glycemic control is associated with a reduction in bone turnover [40]. In this study of troglitazone, baseline mean A1C was 8.4%, and was essentially unchanged after 4 weeks of treatment. Mean FPG was reduced, although the change was not statistically significant with 4 weeks of treatment, and this may at least partly account for the reduction in bone turnover with troglitazone use.

In another study of troglitazone use, Watanabe et al treated 25 patients (14 women) with type 2 diabetes for 12 months with 400 mg per day. Similar to the previous report, this study found that levels of urine type 1 collagen *N*-telopeptide and serum bone alkaline phosphatase were modestly reduced after the first month of treatment by 14% and 9%, respectively. However, both markers had returned to baseline levels after 12 months of troglitazone treatment [41].

Bone density

Watanabe et al also reported that, for the patient group as a whole, lumbar spine BMD *Z*-scores were not changed after 12 months of troglitazone. When patients were divided into those who did (responders; $N = 17$) or did not (nonresponders; $N = 8$) experience a reduction in leptin levels during treatment, the leptin responders had less bone loss compared with the nonresponders. Bone loss in the nonresponders was similar to a group of nondiabetic controls with hypercholesterolemia. The responders also had greater reductions in A1C compared with the nonresponders. The BMD results in the subgroups defined by leptin changes could be due to direct troglitazone effects, improved glycemic control, or chance.

Using data from the Health, Aging, and Body Composition Study (Health ABC), an observational cohort study, we assessed TZD use and bone loss over four years among participants with type 2 diabetes [42]. Participants were white and black, physically able, men and women, aged 70–79 years at baseline [23]. We analyzed changes in whole body, lumbar spine (derived from whole body), and hip BMD.

There were 666 diabetic participants in Health ABC, and 69 of them reported TZD use at an annual visit, including troglitazone ($N = 22$), pioglitazone ($N = 30$), and/or rosiglitazone ($N = 31$). In repeated measures models adjusted for potential confounders associated with TZD use and BMD, each year of TZD use was associated with greater bone loss at the whole body (additional loss of −0.61% per year; 95% CI: −1.02, −0.21% per year), lumbar spine (−1.23% per year; 95% CI: −2.06, −0.40% per year), and trochanter (−0.65% per year; 95% CI: −1.18, −0.12% per year) in women, but not men, with diabetes.

The average whole body bone loss among diabetic women who were not using a TZD in Health ABC was 0.4% per year. TZD use appears to increase whole body bone loss

by a factor of 2.5. Bone loss is a potent predictor of fracture risk, suggesting that TZD use may be associated with a measurable effect on skeletal health.

For women who used TZDs continuously, these results predict an additional whole body bone loss of 3% over five years. A cross-sectional difference of 1 SD in whole body BMD, or a difference of about 10% in BMD, corresponds to an increased hip fracture risk of 60% [43]. Thus, long-term use of TZDs by diabetic women may add substantially to their fracture risk. This burden is in addition to any increased risk of fracture associated with diabetes.

In contrast to these observational findings in Health ABC, the study of one year of troglitazone administration found that bone loss at the lumbar spine was not increased beyond changes expected with age [41]. However, the results of these two studies may not be inconsistent. The Health ABC study found increased bone loss with TZD use only in women. The troglitazone study included only 14 women, and this group may have been too small to detect increased bone loss confined to women. It is also possible that troglitazone has a different effect than the other TZDs on bone and that the results from the Health ABC Study are driven by effects of rosiglitazone and/or pioglitazone rather than troglitazone. In rodent models the one reported study with troglitazone did not find bone loss, although troglitazone did induce adipogenesis in bone marrow [5]. In contrast, rosiglitazone and pioglitazone have produced bone loss as well as adipogenesis in rodent models [1–4]. Different effects of these medications on bone metabolism would be consistent with reports that the results of PPAR-γ activation depend on the particular ligand [36].

Limitations of DXA scans

The standard approach for measuring changes in BMD is dual X-ray absorptiometry (DXA), employed in both of the clinical studies discussed here. However, there are inherent limitations of DXA scans for studying changes in bone density associated with TZD use. Increases in body weight may cause artifactual changes in BMD while increases in bone marrow fat may cause artifactual decreases in BMD as measured by DXA.

To derive BMD values, DXA must assume values for soft tissue mass over- and underlying bone. These soft-tissue values are derived from the surrounding soft tissue, and a higher proportion of fat in the surrounding soft tissue results in an over-estimation of the true value of BMD [44]. With weight gain, the fat composition in the different areas of soft tissue may change at different rates, introducing artifactual increases or decreases in measured BMD changes [45]. The effect of weight change on DXA measurements may depend on the particular scanner and software version used [46]. Thus, the increased weight and body fat associated with TZD use could tend to artificially increase or decrease any observed bone loss.

Bone marrow is included in the DXA scan of bone tissue and an increase in bone marrow fat may artificially lower the DXA measurement, the opposite effect of weight gain described above [47]. The effect of TZD on bone marrow composition has not been addressed in human studies. In rodent models, TZD use has been reported to increase [1], and to have no effect on [2], bone marrow fat. If TZD use causes an increase in bone marrow fat in humans, this could artificially lower bone density as measured by DXA.

FUTURE DIRECTIONS

Given the evidence of bone loss in rodent models, it would be prudent to understand the impact of TZD treatment on bone in humans, especially as these medications are being considered for prevention as well as treatment of diabetes. A carefully designed clinical trial is needed at this juncture to test whether TZD use causes bone loss. New research will particularly need to address the limitations of DXA scans in the context of a treatment that changes body composition and may alter bone marrow composition. Now that TZD use has become more prevalent, data from larger observational studies with fracture outcomes could be used to address the association between TZD use and fracture risk.

CONCLUSION

Clinical studies to date on TZD and bone have been limited by small size and relatively short duration of TZD use. In addition, studies to date have either been observational or have included only a treatment group. Separate clinical data are not available on the two TZDs currently in use, rosiglitazone and pioglitazone. Thus, we do not know if the bone loss observed with TZD administration in some rodent models is also occurring in type 2 diabetic patients treated with TZDs. A well-designed clinical trial, planned specifically to examine the impact of TZD use on bone density, would clarify this issue. Because older adults with type 2 diabetes are at increased risk of fracture, further study of TZDs is needed to assess the possible risk of bone loss. At the same time, clinical studies of the effect of TZDs on bone could provide valuable insights into the role of PPAR-γ in bone metabolism.

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Review Article

Resolving the Two "Bony" Faces of PPAR-γ

Beata Lecka-Czernik[1] and Larry J. Suva[2]

[1] *Department of Geriatrics, Reynolds Institute on Aging, University of Arkansas for Medical Sciences, 629 Jack Stephens Drive, Little Rock, AR 72205, USA*
[2] *Department of Orthopaedic Surgery, Center for Orthopaedic Research, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*

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Bone loss with aging results from attenuated and unbalanced bone turnover that has been associated with a decreased number of bone forming osteoblasts, an increased number of bone resorbing osteoclasts, and an increased number of adipocytes (fat cells) in the bone marrow. Osteoblasts and adipocytes are derived from marrow mesenchymal stroma/stem cells (MSC). The milieu of intracellular and extracellular signals that controls MSC lineage allocation is diverse. The adipocyte-specific transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ) acts as a critical positive regulator of marrow adipocyte formation and as a negative regulator of osteoblast development. *In vivo*, increased PPAR-γ activity leads to bone loss, similar to the bone loss observed with aging, whereas decreased PPAR-γ activity results in increased bone mass. Emerging evidence suggests that the pro-adipocytic and the anti-osteoblastic properties of PPAR-γ are ligand-selective, suggesting the existence of multiple mechanisms by which PPAR-γ controls bone mass and fat mass in bone.

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INTRODUCTION

The two-faced ancient Roman god Janus, represents the inseparable relationship between opposites. The nuclear receptor and transcription factor PPAR-γ has many "faces" in regard to its activities, but its proadipocytic and antiosteoblastic activities in bone closely resemble the two inseparable faces of Janus.

The decreased rate of bone formation and the number of osteoblasts that occurs with aging correlate inversely with an increase in the fat content and a number of adipocytes in the bone marrow [1]. The apparent inverse relationship between osteoblast and adipocyte differentiations and their shared mesenchymal progenitor origin led to the formulation of the hypothesis that binds these two phenotypes and makes them inseparable [2, 3]. According to the shared precursor hypothesis, an increase in adipocyte differentiation occurs at the expense of osteoblast differentiation, and vice versa. However, in some circumstances, adipocytic and osteoblastic differentiation may occur independently [4, 5], suggesting either an existence in adult marrow of separate pools of progenitor cells responding to proosteoblastic and proadipocytic stimuli differently and/or separate regulatory mechanisms of both osteoblast and adipocyte differentiations. This review summarizes the existing evidence supporting either the "simultaneous" scenario or the "independent" scenario. We cite examples, in which the proadipocytic and antiosteoblastic activities of PPAR-γ can be modulated either simultaneously or independently using ligands of different chemical structures. We also summarize the evidence indicating that PPAR-γ is an important regulator of bone homeostasis and marrow mesenchymal stem cell (MSC) differentiation.

Osteoblasts, bone-forming cells, and adipocytes, fat cells, are derived from a common marrow MSC compartment, which also serves as a source of progenitors for fibroblasts, muscle, and cartilage cells, and functions as hematopoiesis-supporting stroma [6, 7]. The commitment of MSCs towards either the adipocyte or osteoblast lineage occurs by a stochastic mechanism [8], in which lineage-specific transcription factors, such as Runx2, Dlx5, and Osterix for osteoblasts and PPAR-γ2 and C/EBPs for adipocytes, are activated (Figure 1) [9].

Aging is associated with changes in the status of MSCs and in the milieu of intrinsic and extrinsic signals that determine the differentiation of MSCs towards osteoblasts and/or adipocytes [1, 10–12]. These changes modulate the continuing dialog between phenotype-specific transcription factors and signals from the microenvironment that collectively determines MSC lineage allocation. With aging, the status of MSCs changes with respect to their differentiation

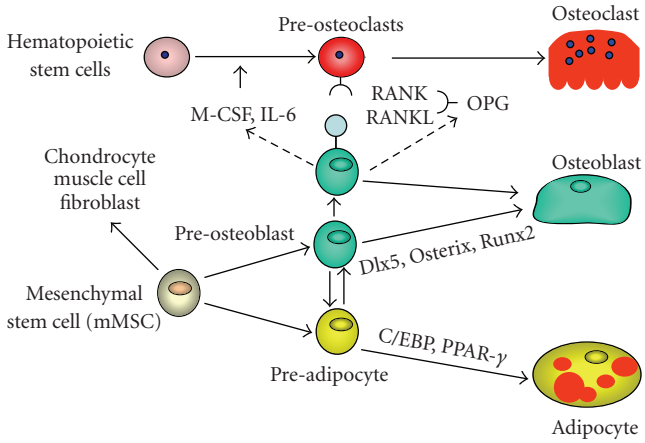

Figure 1: Schematic representation of bone cell development.

potential, such that commitment to the osteoblast lineage decreases, whereas commitment to the adipocyte lineage increases [1, 10]. These changes in cellular differentiation are reflected in the expression profile of phenotype-specific gene markers in undifferentiated MSCs. The expression of the osteoblast-specific transcription factors, Runx2 and Dlx5, and osteoblast markers, collagen and osteocalcin, is decreased, whereas expression of the adipocyte-specific transcription factor PPAR-γ2 and a gene marker of adipocyte phenotype, fatty acid-binding protein 4 (FABP4), is increased [10]. Aging also results in alterations in the bone marrow microenvironment. MSC support for osteoclastogenesis is enhanced due to the increased production in the marrow of macrophage colony-stimulating factor (M-CSF) and RANKL, two proosteoclastic cytokines required for physiological bone resorption [13–16]. Moreover, bone marrow derived from old mice produces unknown PPAR-γ activator(s) that stimulates adipocyte differentiation and suppresses osteoblast differentiation [10]. Interestingly, in humans osteoblast differentiation can be affected by either a presence of mature marrow adipocytes [17], polyunsaturated fatty acids, which are natural ligands for PPAR-γ [18], or serum derived from older women [12].

PPAR-γ REGULATES BONE MASS

PPAR-γ nuclear receptor is an essential regulator of lipid, glucose, and insulin metabolism [19]. The receptor is expressed in mice and humans as two different isoforms, PPAR-γ1 and PPAR-γ2, due to alternative promoter usage and alternative splicing [20–22]. PPAR-γ2 differs from PPAR-γ1 by 30 additional amino acids on its N-terminus. PPAR-γ1 is expressed in a variety of cell types, including osteoblasts, whereas PPAR-γ2 expression is restricted to adipocytes, including marrow adipocytes, and is essential for differentiation and maintenance of their phenotype and function [9, 23]. PPAR-γ belongs to the family of nuclear receptor transcription factors, and its activation requires heterodimer formation with another nuclear receptor, retinoid *X* receptor (RXR), and binding of a specific ligand. Natural ligands for PPAR-γ comprise polyunsaturated fatty acids and metabolites of prostaglandin J_2, whereas synthetic ligands include the antidiabetic thiazolidinediones (TZDs) [24].

An important role of PPAR-γ in the maintenance of bone homeostasis has been demonstrated in several animal models of bone accrual [25, 26] or bone loss [27–30], regulated by the status of PPAR-γ activity. Decreased PPAR-γ activity in PPAR-γ-haploinsufficient mice or in mice carrying a hypomorphic mutation in the PPAR-γ gene locus led to increased bone mass, due to increased osteoblastogenesis from bone marrow progenitors, but not due to effects on mature osteoblast activity or cells of the osteoclast lineage [25, 26]. Moreover, age-related osteopenia did not develop in PPAR-γ-haploinsufficient mice [25]. In contrast, activation of PPAR-γ via the administration of rosiglitazone, an antidiabetic TZD, to rodents resulted in significant decreases in bone mineral density (BMD), bone volume, and changes in bone microarchitecture [27–30]. The bone loss observed was associated with the expected reciprocal changes in the structure and function of bone marrow; a decreased number of osteoblasts and an increased number of adipocytes [27, 30]. Indeed, we had previously demonstrated in U-33/γ2 cells, a model of murine marrow mesenchymal cell differentiation, that activation of the PPAR-γ2 isoform by rosiglitazone converted cells of the osteoblast lineage to terminally differentiated adipocytes irreversibly suppressing the osteoblast phenotype via the inhibition of osteoblast-specific gene expression [9].

While the antiosteoblastic effect of PPAR-γ2 on osteoblast differentiation is well established, its effect on osteoclast development is less clear. In vitro, PPAR-γ activation in osteoclast precursor cells inhibits their differentiation [31, 32], whereas activation of PPAR-γ in cells of mesenchymal lineage increases their support to osteoclastogenesis [33]. In vivo, and in contrast to other animal models, bone loss due to rosiglitazone administration to ovariectomized rats resulted from increased bone resorption, but not decreased bone formation [28]. These results indicate that at least in some circumstances, bone loss due to PPAR-γ activation may involve increased bone resorption.

Since TZDs have only been approved for clinical use in the treatment of type II diabetes since 1999, their effects on human bone are just emerging. Early observations indicated that the 4-week administration of troglitazone to patients with poorly controlled type II diabetes who exhibited high bone turnover resulted in a significant decrease in metabolic bone markers, such as urinary deoxypyridinoline, urinary type I collagen C-terminal telopeptide, and serum bone-type alkaline phosphatase [34]. Recent analysis of data from the Health, Aging, and Body Composition cohort indicate that TZD use for more than 3 years results in the acceleration of bone loss, at approximately 1% per year in older postmenopausal women [35].

Emerging evidence from studies of PPAR-γ gene polymorphism in humans strongly suggests a role for this transcription factor in the regulation of bone mass. A silent $C \rightarrow T$ transition in exon 6, which is common to both PPAR-γ

isoforms, results in a lower bone density and a predisposition to osteoporosis in postmenopausal Japanese women [36]. The same polymorphism in a population of healthy middle-age Korean women was associated with lower levels of circulating osteoprotegerin, a negative regulator of osteoclast development, but no changes in bone density [37]. Another polymorphism in the STAT5B regulatory element in the alternative promoter of the human PPAR-γ1 protein was associated with increased height and plasma low-density lipoprotein cholesterol concentrations in a French population [38]. Similarly, analysis of a population from the Framingham Offspring study revealed several novel polymorphic changes in the coding region of PPAR-γ that correlated independently with bone mineral density (BMD) at different skeletal sites [39]. A more detailed review of the associations between PPAR-γ genomic polymorphism and bone status can be found in this issue [40].

As mentioned above, natural ligands of PPAR-γ include polyunsaturated fatty acids and their oxidized derivatives, the levels of which increase in the circulation with aging. We showed previously that oxidized forms of linoleic acid serve as ligands for PPAR-γ2 in marrow MSC and activate either its proadipocytic and/or antiosteoblastic properties [4]. Oxidized fatty acids are generated in the enzymatic reactions controlled by lipoxygenases. It was demonstrated that three of them, 5-, 12-, and 15-lipoxygenases, are involved in the regulation of bone mass in mice and human. The disruption of either 5- or 15-lipoxygenase in mice led to increased bone mass [41, 42], whereas in humans polymorphic changes in the locus for 12- or 15-lipoxygenases correlated with changes in BMD in normal subjects or in postmenopausal women, respectively [43, 44].

Age-related osteoporosis is typified by a low serum IGF-1 level and a particular pattern of fat redistribution [45–47]. IGF-1 serves an important regulatory role in bone acquisition and maintenance of the adult skeleton, although its role in mesenchymal stem cell allocation towards the osteoblastic and adipocytic lineages remains unclear [46, 48]. Recent advances in genetic techniques to manipulate the mouse genome have resulted in several murine models that provide insights into the skeletal actions of IGF-1 and its potential interaction with other bone regulatory mechanisms.

One such animal model reflecting the relationship of IGF-1 with bone and fat consists of the congenic B6.C3H-6T (6T) mouse, which is a C57BL/6J (B6) mouse that carries a region of the C3H/HeJ (C3H) sixth chromosome [49]. Compared to B6, the 6T strain is characterized by low BMD, increased marrow fat, a reduced serum IGF-1 concentration, and reduced mRNA levels of IGF-1. Interestingly, the PPARγ gene is within the carried-over C3H-like region. Moreover, our recent results suggest that IGF-1 production in bone is under the control of the PPARγ gene [50].

ROLE OF MARROW FAT AND ITS SIGNIFICANCE FOR THE MARROW MICROENVIRONMENT

As mentioned above, the PPAR-γ transcription factor is essential for both extramedullary and bone marrow fat development [19, 25], yet bone marrow adipocyte biology and function are not well understood. The marrow adipocyte phenotype is similar to that of adipocytes present in white and brown fat tissues, but the unique location of these cells in bone directs their more specialized functions [3]. For years, marrow fat was merely considered as a cellular component of bone that served a passive role by occupying a space no longer needed for hematopoiesis. However, recent developments suggesting that marrow fat plays an essential role as an endocrine organ involved in lipid and glucose metabolism place marrow fat under a new research spotlight. With advancing age, fat infiltrates bone marrow cavities, especially in the long bones [51]. From the perspective of adipokine production and glucose utilization, which is similar to white and brown fat, it is likely that marrow fat serves a variety of endocrine functions.

A relatively well-characterized role of marrow adipocytes is to support hematopoiesis by producing the necessary cytokines and providing heat for hematopoietic cell development. In addition, marrow fat may participate in lipid metabolism by clearing and storing circulating triglycerides and may provide a localized energy reservoir for emergency situations affecting, for example, osteogenesis (eg, bone fracture healing) [3]. Marrow adipocytes also produce several cytokines, but two adipokines, whose expression is under the PPAR-γ control, leptin and adiponectin, are currently the focus of increased attention as possible regulators of bone mass.

Leptin is produced by fat cells, and its primary role is the regulation of satiety through the effects on central nervous system [52]. Leptin expression increases during a starvation period resulting in decreases in growth, fertility, and bone mass; its expression decreases when energy intake increases. Leptin is thought to regulate bone mass through two alternative pathways: one involving a direct stimulatory effect on bone growth, when acting on bone cells through its receptors; and another, which is indirect, involving a hypothalamic relay that suppresses bone formation, when acting on central nervous system [52]. Thus, when acting locally on bone, leptin increases BMD, bone mineral content (BMC), and bone-formation rate, while it decreases the number and the size of bone marrow adipocytes [52]. In contrast, when injected into a hypothalamic ventricle, leptin decreases bone mass in the spine [53]. This activity is presumably mediated via β2-adrenergic receptors signaling, which regulates the expression of RANKL in osteoblasts [54].

Another adipokine, adiponectin, was recently discovered to be an insulin-sensitizing hormone produced by fat tissue [55]. Clinical studies implicate adiponectin as an independent predictor of bone mass; circulating levels of adiponectin correlate inversely with bone mass in humans [56]. Adiponectin and its receptors, similar to leptin and its receptors, are expressed by cells of the osteoblast lineage [57–60]. In vitro, adiponectin inhibits adipocyte formation and stimulates osteoblast proliferation and differentiation via the MAPK signaling pathways [59], however adiponectin-deficient or transgenic for its expression mice did not show bone abnormalities [60]. Since adiponectin can act on bone through either an autocrine/paracrine pathway and/or an

endocrine pathway as a hormone secreted from fat tissue, Shinoda et al. concluded that adiponectin may have three distinct actions on bone: a positive action of locally produced adiponectin through an autocrine/paracrine pathway, a direct negative effect of circulating adiponectin, and a positive indirect action of circulating adiponectin via the enhancement of insulin signaling [60].

EVIDENCE FOR THE RECIPROCAL RELATIONSHIP BETWEEN BONE LOSS AND OSTEOBLAST AND ADIPOCYTE DEVELOPMENT

Accumulating in vivo and in vitro evidences support the hypothesis that increased adipocyte formation occurs at the expense of osteoblast development. In humans, the association between bone loss and increased marrow adiposity is visible not only during aging, but also during conditions of skeletal disuse, such as microgravity or paraplegia [51, 61, 62]. In animals, skeletal unloading results in bone loss, which is also associated with an increase in the marrow fat compartment [63–66].

In contrast, the lack of adipose tissue has been associated with increased bone formation. In patients with congenital generalized lipodystrophy, a lack of body fat is accompanied by skeletal abnormalities, such as increased bone density, a thickened calvarium, and scoliosis [67, 68]. An animal model of lipodystrophy due to a hypomorphic mutation in the PPAR-γ gene exhibits both decreased marrow fat content and increased bone mass [26]. On the other hand, embryonic fibroblasts carrying a null mutation in the PPAR-γ gene spontaneously differentiate towards osteoblasts and do not possess the capability to differentiate towards adipocytes [25]. Strong evidence for a reciprocal relationship between adipocyte formation and bone loss is provided by studies that have examined the effect of TZDs, highly specific PPAR-γ agonists, on bone and bone marrow cell differentiation, as described above [27–30]. In support of this evidence, we have previously demonstrated in an in vitro model of marrow mesenchymal cell differentiation (U-33/γ2 cells) that activation of the PPAR-γ2 isoform by rosiglitazone converted cells of the osteoblast lineage to terminally differentiated adipocytes and irreversibly suppressed both the osteoblast phenotype and osteoblast-specific gene expression [9].

In the SAMP6 mouse model of involutional osteopenia associated with early senescence, low bone mass results from a diminished ability of MSCs to differentiate towards osteoblasts [69, 70]. Simultaneously, MSCs of SAMP6 mice exhibit an increased commitment towards the adipocyte lineage [71]. The impaired marrow osteogenesis is associated with a reduction in endochondral, but not periosteal, new bone formation, which suggests a defective differentiation of osteogenic progenitors present in the bone marrow [72]. Importantly, this defect is completely corrected when bone marrow derived from normal nonosteopenic mice is transplanted into irradiated SAMP6 mice [73]. Allogeneic bone marrow transplantation resulted in histologically normal trabecular bone and bone density and restored circulating levels of interleukin (IL)-11, RANKL, and IL-6, all cytokines involved in the regulation of bone remodeling.

The terminal differentiation of MSC towards osteoblasts and adipocytes results from the selective activation of specific programs of gene expression, which are controlled by phenotype-specific transcription factors, such as Runx2 and PPAR-γ, respectively. However, the control of expression and the activity of these factors, and their precise role in MSC lineage allocation, remain poorly understood. The recent identification of TAZ (transcriptional coactivator with PDZ-binding motif) provides some insight into how the activity of transcriptional regulators may be controlled and suggests that TAZ may act as a molecular switch in the differentiation of MSC to osteoblasts and adipocytes [74, 75]. TAZ protein functions in the convergance of extracellular signals from the cytoplasm to the nucleus [74], where it binds to the large number of transcription factors including Runx2 and PPAR-γ [76]. Binding of TAZ to Runx2 strongly coactivates Runx2-dependent gene transcription, while binding to PPAR-γ suppresses PPAR-γ-dependent gene transcription. Interestingly, closely related to TAZ protein, Yes-associated protein, YAP, acts as a strong repressor of Runx2 transcriptional activity and osteoblast differentiation in a manner that requires Src/Yes kinases activity [77]. However, its effect on adipocyte differentiation and PPAR-γ activity remains to be determined. Nevertheless, TAZ and YAP transcriptional modulators are suggested to be functionally related to β-catenin with respect to their role in integration of extracellular, membrane, and cytoskeletal-derived signals to influence mesenchymal stem cell fate [74].

Recent discoveries identifying an important role for the Wnt signaling pathway in postnatal bone accrual, by regulating osteoblast and osteoclast development, have provided major advances in our understanding of skeletal biology [78, 79]. Wnts are soluble glycoproteins that engage receptor complexes composed of Lrp5/6 and frizzled proteins, which induce a cascade of intracellular events that stabilize β-catenin, facilitating its transport to nuclei where it binds Lef1/Tcf transcription factors, and alters gene expression to promote osteoblast expansion and function. The first indication that Wnt signaling plays a critical role in bone formation came from human studies where inactivating mutations in the Wnt coreceptor LRP5 were shown to cause osteoporosis [80]. In contrast, gain of function mutations in LRP5 that increase Wnt signaling results in higher bone density in humans and mice [81, 82]. The Wnt pathway has also been implicated in the regulation of lineage allocation of MSC. Animals that express Wnt10b under the control of FABP4 in marrow are characterized by high bone mass, which is maintained during aging [83, 84]. Interestingly, Wnt 10b suppresses PPAR-γ expression and adipocyte development [83] and vice versa, PPAR-γ2 suppresses Wnt10b expression in U-33/γ2 cells [4]. Recent findings indicate that Wnt pathway not only regulates osteoblast development towards bone-forming cells, but it also controls osteoblast support of osteoclastogenesis [85, 86].

EVIDENCE FOR NONRECIPROCAL BONE LOSS AND OSTEOBLAST AND ADIPOCYTE DIFFERENTIATIONS

In some circumstances, osteoblast and adipocyte differentiations may have a nonreciprocal nature. Recently, we demonstrated that administration of the selective TZD netoglitazone to animals resulted in extensive accumulation of marrow fat, but did not affect bone mass [5]. Similar findings were reported previously by Tornvig et al [87], who demonstrated that the administration of another TZD, troglitazone, to apolipoprotein E-deficient mice for 10 months did not affect bone mass, although it increased the number of marrow adipocytes and appeared to affect the marrow hematopoietic compartment. These data suggest that in vivo antiosteoblastic and proadipocytic activities of PPAR-γ can be independently activated by selective PPAR-γ modulators.

The nonreciprocal character of osteoblast and adipocyte differentiations is also supported by several animal models of bone mass regulation that are not directly related to PPAR-γ activity in MSCs. Mice deficient in 11β-hydroxysteroid dehydrogenase type 1 ($HSD1^{-/-}$), an enzyme that converts inactive cortisone into active cortisol, exhibit normal bone formation and bone loss with aging in the absence of marrow adipocytes [88]. Conversely, overexpression of the transcriptional regulator δFosB in cells of the osteoblast lineage resulted in an increased number of osteoblasts and increased bone formation, with no effect on the number of marrow adipocytes [89]. In another murine model, deletion of the early B-cell factor gene, *EBF1*, results in a significant increase in osteoblast number and bone formation, in the face of the marrow cavity being filled with fat [90]. In total, these data suggest that the Janus-like osteoblast-adipocyte relationship is more complex than first thought and likely subject to selective regulation.

DIVERGENT EFFECT OF PPAR-γ ACTIVATORS ON THE PROADIPOCYTIC AND ANTIOSTEOBLASTIC ACIVITIES

The ligand-binding pocket of PPAR-γ is promiscuous and binds a variety of molecules with different affinities [24]. We showed that PPAR-γ2 activation in osteoblast cells using natural and artificial ligands with distinct pharmacophores and binding affinities resulted in a divergent activation of the proadipocytic and antiosteoblastic activity of PPAR-γ2 [4]. For example, using a variety of oxidized linoleic acid derivatives (eg, its epoxy-, hydroxy- and dihydroxy-derivatives) we were able to demonstrate that the proadipocytic and antiosteoblastic activities of PPAR-γ2 can be separated. These results suggested that PPAR-γ2 effects on osteoblast and adipocyte phenotypes are mediated by distinct regulatory pathways that are differentially modulated depending on the nature of the ligand. Moreover, they suggested that there may be selective PPAR-γ2 modulators that have beneficial activities as insulin sensitizers, without adverse effects on bone. Therefore, we have tested whether any of the available FDA-approved antidiabetic TZDs also modulate PPAR-γ2 activities differently.

Using U-33/γ2 cells, in which osteoblast and adipocyte differentiation is under the control of constitutively expressed PPAR-γ2 [4, 9], we compared the antiosteoblastic and proadipocytic activities of troglitazone, pioglitazone, and rosiglitazone. The proadipocytic activity was measured as number of U-33/γ2 cells accumulating fat, and antiosteoblastic activity was measured as the suppression of alkaline phosphatase enzyme activity, in response to treatment with different doses of tested TZD. As showed in Figure 2, U-33/γ2 cells responded to this treatment in a dose-dependent manner and the antiosteoblastic and proadipocytic activities of tested TZDs correlated with their ligand binding affinity for PPAR-γ (rosiglitazone (EC_{50} = 0.04 μM) > pioglitazone (EC_{50} = 0.5 μM) > troglitazone (EC_{50} = 0.8 μM)) [24], with the exception to troglitazone, which appeared to have higher proadipocytic activity than pioglitazone.

Next, we measured the effect of TZDs on the expression of adipocyte and osteoblast signature genes using quantitative real-time PCR. We tested their effect on gene expression in U-33/γ2 cells and primary bone marrow cultures in concentrations that induced fat accumulation in 50% of U-33/γ2 cells. As shown in Table 1, the effects of tested TZDs, at doses which were equally effective for fat accumulation in U-33/γ2 cells, were similar. Although primary bone marrow cells responded to these treatments with a different magnitude than U-33/γ2 cells, all tested TZDs equally induced both proadipocytic and antiosteoblastic properties of PPAR-γ in both U-33/γ2 and primary bone marrow cells.

These effects are in contrast to the effects of another TZD, netoglitazone [5]. Netoglitazone appears to be a synthetic PPAR-γ ligand that separates the proadipocytic and antidiabetic activities from the antibone activity in vivo. Netoglitazone administered at a dose equally effective as rosiglitazone in lowering blood glucose in a murine model of type 2 diabetes did not induce bone loss, affect changes in bone microarchitecture, or alter bone-specific gene expression. Interestingly, netoglitazone, which possesses weak proadipocytic activities in vitro effectively induced marrow adipocyte formation in vivo. Regardless of the discrepancies between the in vitro and in vivo proadipocytic effects of netoglitazone, these results indicate that it is possible to separate the proadipocytic and antiosteoblastic activities of PPAR-γ in vivo. They also suggest that in vivo, at least some of the marrow cells are responsive to netoglitazone and thereby mediating the proadipocytic activity. Interestingly, it appears that this population of cells is not involved in production of bone-forming osteoblasts. Collectively, these data suggest that these effects are modulated by the cellular environment and/or the availability of specific cofactors required for PPAR-γ activity [91].

CONCLUSIONS

Osteoporosis, obesity, and diabetes are the most common pathologies seen in highly industrialized countries and the cost impact to treat these diseases is enormous and still growing. Since PPAR-γ is positioned at the cross-roads of the control of bone mass, energy expenditure, and glucose

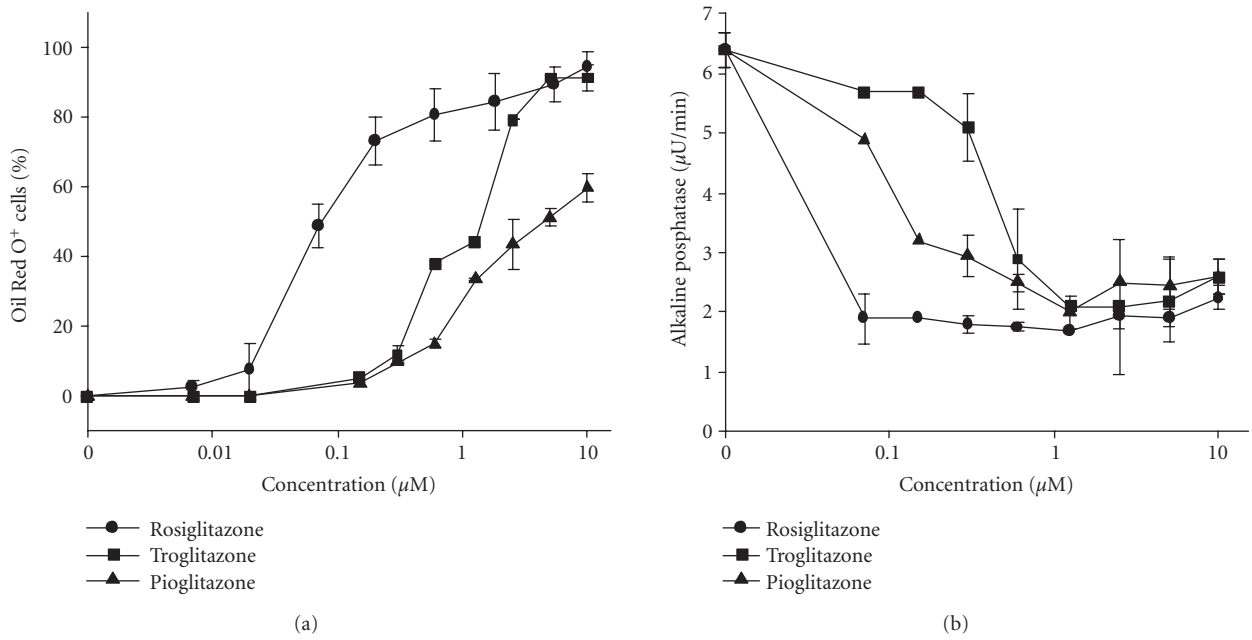

Figure 2: The effect of tested glitazones on adipocyte (a) and osteoblast (b) phenotypes of U-33/γ2 cells. U-33/γ2 cells represent marrow mesenchymal bipotential progenitor cells, which differentiation towards osteoblast and adipocyte is under the control of PPAR-γ2 transcription factor. Cells were treated for 3 days with different doses of tested PPAR-γ agonists and cultures were either stained for fat with Oil Red-O or subjected to alkaline phosphatase enzyme activity assay as previously described [4].

Table 1: The effects of TZDs on osteoblast and adipocyte gene markers.

Treatment	Cell type	PPAR-γ2	FABP4	Dlx5	Runx2	OC	Coll
Rosiglitazone[(a)]	U-33/γ2	4.0[(d)]	2,558.0	0.18	0.23	0.01	0.26
	Bone marrow	74.8	94.4	0.27	0.14	0.13	0.18
Pioglitazone[(b)]	U-33/γ2	2.4	1,857.0	0.15	0.21	0.01	0.19
	Bone marrow	367.8	84.0	0.40	0.39	0.16	0.14
Troglitazone[(c)]	U-33/γ2	2.9	2,234.0	0.14	0.19	0.01	0.18
	Bone marrow	160.8	108.0	0.39	0.32	0.07	0.17

TZDs concentrations: [(a)] 1 μM; [(b)] 6 μM; [(c)] 10 μM; [(d)] values represent fold of gene expression in cells treated with TZDs versus untreated control.

metabolism, changes in its activity, which occur either naturally during aging or during antidiabetic therapy using TZDs, may result in unwanted effects on the skeleton. The attractive possibility to separate specific PPAR-γ activities may allow for the development of selective antidiabetic modulators that will also be safe for the skeleton. Such a possibility ensures that there will be a continued discovery effort to identify pharmacophores that will be of benefit for both bone and glucose metabolism.

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Research Article

Peroxisome Proliferator-Activated Receptor-γ Promotes Adipogenic Changes in Growth Plate Chondrocytes In Vitro

Lai Wang, Yvonne Y. Shao, and R. Tracy Ballock

Orthopaedic Research Center, Department of Orthopaedic Surgery and Biomedical Engineering, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA

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Chondrocytes and adipocytes are two differentiated cell types which are both derived from mesenchymal cells. The purpose of this study was to investigate whether peroxisome proliferator-activated receptor-γ (PPARγ), a transcription factor involved in lineage determination during adipogenesis, is able to induce adipogenic differentiation in growth plate chondrocytes. Isolated epiphyseal chondrocytes were infected with a PPARγ adenovirus or treated with the PPARγ agonist ciglitazone. Both of these treatments resulted in lipid droplet accumulation and expression of the adipogenic markers aP2, lipoprotein lipase, and adipsin in chondrocytes. Proteoglycan matrix synthesis was decreased in the PPARγ-infected cells, as was the expression of the chondrogenic genes Col2a1 and aggrecan. Growth plate cells transfected with a PPARγ expression plasmid under the control of the collagen α1(II) promoter also demonstrated a similar adipogenic changes. Terminal differentiation of growth plate chondrocytes induced by thyroid hormone was also inhibited by overexpression of PPARγ and ciglitazone treatment, with decreased expression of alkaline phosphatase and Runx2/Cbfa1 genes. These in vitro data suggest that PPARγ is able to promote adipogenic differentiation in growth plate chondrocytes, while negatively regulating chondrogenic differentiation and terminal differentiation.

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INTRODUCTION

Longitudinal growth of the skeleton is a result of endochondral ossification that occurs at the growth plate [1]. Endochondral ossification is a multistep process that includes differentiation of mesenchymal cells into chondrocytes, cell proliferation, hypertrophic differentiation, matrix mineralization, apoptosis, vascular invasion, and eventually the replacement of the cartilage by bone.

The first step of growth plate development is the commitment of mesenchymal stem cells to the chondrogenic lineage. Mesenchymal stem cells exhibit a high differentiation plasticity. They are capable of differentiating into chondrocytes, osteoblasts, adipocytes, and other tissues of mesenchymal origin [2]. Interconversion between mesenchymal phenotypes is thought to be under control of specific transcription factors, including the Sox family in chondrogenesis [3], Runx2/Cbfa1 in osteogenesis [4], and PPARγ (peroxisome proliferator-activated receptor-γ), and C/EBP (CCAAT/enhancer-binding protein) in adipogenesis [5].

PPARγ is a key transcriptional regulator of adipogenesis [5]. PPARγ is also expressed in preosteoblastic cells and is thought to play a role in regulation of bone metabolism. PPARγ and PPARγ activators inhibit the maturation of preosteoblastic cells to osteoblasts [6–8]. Free fatty acids activate PPARs and induce adipocyte-like differentiation of osteosarcoma cell lines [6]. Lecka-Czernik et al observed that PPARγ2 negatively regulates stromal cell plasticity by suppressing expression Osf2/Cbfa1 and osteoblast-like biosynthetic activity, while promoting differentiation to adipocytes [7]. Conversely, PPARγ insufficiency enhances osteogenesis through increased osteoblast formation from bone marrow progenitors. Homozygous PPARγ-deficient ES cells fail to differentiate into adipocytes, but increase bone mass by stimulating osteoblastogenesis from bone marrow progenitors [8].

Transdifferentiation of chondrocytes to adipocytes has been previously reported by Heermeier et al, who observed that chondrocytes of the mouse xiphoid process undergo transdifferentiation into adipocytes in the presence of 10% fetal calf serum [9].

Based on the finding that PPARγ is expressed in growth plate chondrocytes [10], as well as the evidence that PPARγ is able to compete with the thyroid hormone receptor (TR) for binding to retinoic acid receptor X to inhibit growth plate cell hypertrophy [11], the purpose of this study was to

investigate whether PPARγ and its ligands are able to promote adipogenic differentiation and suppress chondrogenic differentiation in growth plate chondrocytes.

MATERIALS AND METHODS

Cell culture

Chondrocytes were isolated from the resting zone of the distal femoral growth plate of 2-day old neonatal Sprague-Dawley rats by sequential digestion in trypsin/EDTA (Invitrogen, Carlsbad, Calif) for 1 hour at 37°C, followed by 0.3% collagenase type I (Worthington, Lakewood, NJ) for 4 hours at 37°C [12]. Cells were resuspended in DMEM/F12 medium (Invitrogen) supplemented with a defined media supplement (ITS+1, Sigma, St Louis, Mo) and plated in monolayer at a density of 5×10^5 cells/cm^2, or in a pellet culture of 1×10^5 cells/mL as indicated [12]. Tri-iodothyronine (T3, Sigma) at a concentration of 100 ng/mL and ciglitazone (BioMol, Plymouth Meeting, Pa) at a concentration of 5 μM were added to the medium as indicated.

Immunoblotting

Total cellular protein was extracted from chondrocytes treated with 5 μM of ciglitazone using RIPA buffer [9]. An equal amount of protein was subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. The blots were incubated with anti-PPARγ and anti-actin (Santa Cruz Biotechnology, Santa Cruz, Calif) followed by a HRP-conjugated secondary antibody. Immunoreactive proteins were visualized by Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). Immunoblot bands were quantitated with Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY).

Adenovirus infection

Recombinant adenovirus carrying PPARγ1 (Ad-PPARγ) was kindly provided by Dr J. L. Jameson (Northwestern University Medical School, Chicago, Ill). Ad-PPARγ contains mouse PPARγ1 cDNA driven by the CMV promoter/enhancer with an SV40 polyadenylation sequence [13]. Ad-Gal, which contains β-galactosidase driven by CMV promoter, was used to evaluate the efficiency of gene transduction. Eighteen hours after plating in monolayer, growth plate chondrocytes were infected with adenoviral vectors at a multiplicity of infection (MOI) of 100. Fresh media were added 24 hours after infection and incubated for 72 hours to collect the cell protein extracts. β-galactosidase expression was detected in 80% of cells after 24 hours of infection with Ad-Gal. Expression of introduced PPARγ genes was confirmed by immunoblot.

Plasmid construction and transient transfection

The full-length cDNA of mouse PPARγ was excised by *Asp718/NheI* digestion from pCMX-PPARγ (kindly provided by Dr R. Evans, Salk Institute, La Jolla, Calif). The ends of this fragment were blunted with Klenow polymerase and ligated to a blunt-ended BamHI site in the p1757 plasmid containing the rat α1(II) collagen promoter (kindly provided by Dr Y. Yamada, NIDR, Bethesda, Md) [14]. The cDNA encoding the mouse PPARγ was thus located downstream of the rat α1 (II) collagen promoter element (−977 to +110). Nucleotide sequence analysis confirmed the correct orientation of the PPARγ cDNA.

Growth plate cells were transfected with 10 μg of p1757-PPARγ or p1757 as a negative control by lipofection (Fugene 6, Roche, Indianapolis, Ind) in the presence of 4 units/mL of hyaluronidase. Sixteen hours later, the cells were trypsinized and centrifuged to pellets cultured in DMEM/F12 plus ITS+ supplements [11].

Histochemical staining

For the analysis of adipogenic differentiation, adipogenesis and lipid accumulation in the growth plate cells were examined by staining with Oil Red-O. After 10 days of culture, cells were washed gently with PBS followed by staining with a filtered solution of 0.5% Oil Red-O (Sigma) in 60% isopropanol for 20 minutes. After washing cells with PBS three times, cells were kept in 75% glycerol solution and observed under a phase-contrast microscope.

Alcian blue staining was used to detect chondrocyte-specific proteoglycans at 10 days of culture. Cells were stained with a 4 : 1 ratio of 0.1 M HCl/0.5% Alcian blue stock (0.5% Alcian blue in 95% ethanol) overnight at 37°C in a humidified atmosphere. Cells were then washed twice with PBS to stop reaction and once with 70% ethanol to reduce background.

For alkaline phosphatase (ALP) staining, cultured plates were rinsed with PBS at 10 days of culture, fixed in 3.7% formaldehyde at room temperature for 10 minutes, and stained in the dark for 30 minutes in a 0.1 M Tris-HCl solution (pH 8.5) containing 0.2 mg/mL of Napthol AS-MX phosphate and 0.6 mg/mL of Fast Blue BB salt (Sigma).

Quantitative real-time RT-PCR

The expression of chondrocyte or adipocyte-specific RNA markers was analyzed using quantitative real-time RT-PCR. Total RNA was isolated from cultured growth plate chondrocytes using the RNeasy Kit (Qiagen, Valencia, Calif) 4 days after adenovirus infection or plasmid transfection. Reverse transcription was performed using random primers and Superscript III (Invitrogen). Real-time PCR reactions were conducted in an ABI Prism 7700 Sequence Detection System using SYBR Green PCR core reagents (Applied Biosystems, Foster City, Calif). The comparative C_T method ($\Delta\Delta C_T$ method) was utilized for relative quantitation of gene levels of expression. 18S rRNA was used as an internal control for normalization of target gene expression. The forward and reverse primers for the amplifications are listed in Table 1.

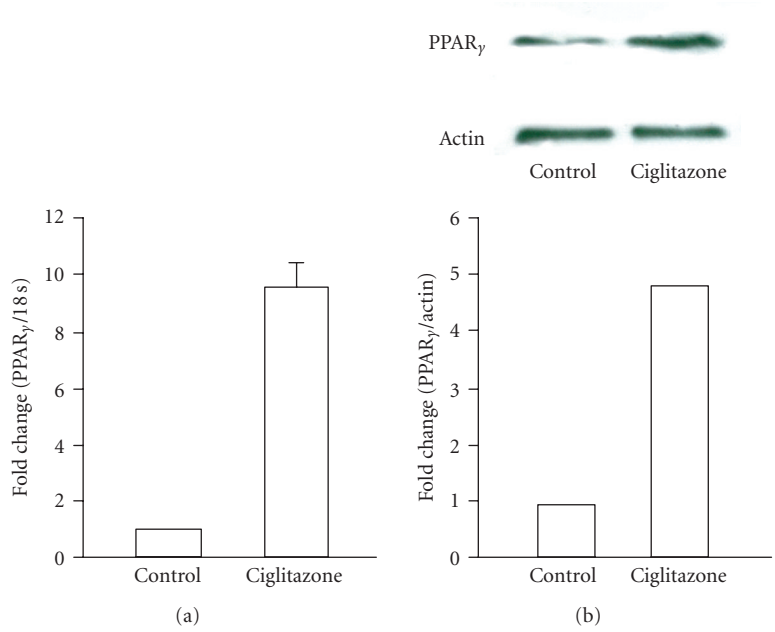

Figure 1: Ciglitazone promotes PPARγ expression in the growth plate chondrocytes. Growth plate chondrocytes in pellet cultures were incubated in the presence or absence of 5 μM of ciglitazone for 4 days. (a) Total RNA was collected and real-time PCR was performed to quantitate PPARγ mRNA levels, which were normalized with respect to endogenous 18S rRNA levels. (b) Proteins were extracted for immunoblotting to detect PPARγ expression and the immunoblots quantitated using Kodak 1D image analysis software. Actin was used as an internal control.

Statistical analysis

The data for real-time PCR are represented as mean ± standard deviation. Values are assessed by one-way ANOVA with the Bonferroni post-hoc test and Student t test at a significance level of $P < .05$.

RESULTS

Ciglitazone upregulates PPARγ expression in growth plate chondrocytes

Treatment of growth plate cells with ciglitazone resulted in increases of both PPARγ mRNA and protein. PPARγ mRNA was increased 9-fold after addition of ciglitazone (5 μM) for 4 days (Figure 1(a)), while PPARγ protein levels were increased approximately 5-fold (Figure 1(b)).

PPARγ induces adipogenic differentiation in growth plate chondrocytes

Phase-contrast microscopy demonstrated that the Ad-PPARγ-infected growth plate chondrocytes acquired the morphology characteristic of adipocytes after culture in monolayer for 10 days. Approximately 50% of the cells had accumulated vacuoles, which were positive for Oil Red-O staining of lipid accumulation (Figure 2(a)). The control cells infected with Ad-Gal demonstrated few Oil Red-O positive vacuoles. Cells treated with 5 μM of ciglitazone alone for 10 days also showed enhanced Oil Red-O staining (20% of the total cells), while the combination of ciglitazone treatment and Ad-PPARγ infection further enhanced lipid accumulation (over 70% of cells staining positively with Oil Red-O).

Table 1: Primer sequences used for real-time PCR.

Genes	Primers	
aP2	Forward	5′-GGCTTCGCCACCAGGAA-3′
	Reserve	5′-CCCTTCTACGCTGATGATCAAGT-3′
LPL	Forward	5′-GGGTCGCCTGGTCGAAGT-3′
	Reserve	5′-AAAGTGCCTCCATTGGGATAAA-3′′
Adipsin	Forward	5′-CCGATGTCCTGCAGCAACT-3′
	Reserve	5′-CATGGTACGTGCGCAGATTG-3′
COL2A1	Forward	5′-GGTGGAGCAGCAAGAGCAA-3′
	Reserve	5′-CGTCGCCGTAGCTGAAGTG-3′
Aggrecan	Forward	5′-CTAGCTGCTTAGCAGGGATAACG-3′
	Reserve	5′-CCGCAGAGTCACAAAGACCAA-3′
ALP	Forward	5′-GCCGGCAGGACACAGACT-3′
	Reserve	5′-GGTTGCAGGGTCTGGAGAGTATA-3′
Runx2/	Forward	5′-TTTAGGGCGCATTCCTCATC-3′
Cbfa1	Reserve	5′-GGAGGGCCGTGGGTTCT-3′
18S	Forward	5′-AGTCCCTGCCCTTTGTACACA-3′
	Reserve	5′-GATCCGAGGGCCTCACTAAAC-3′

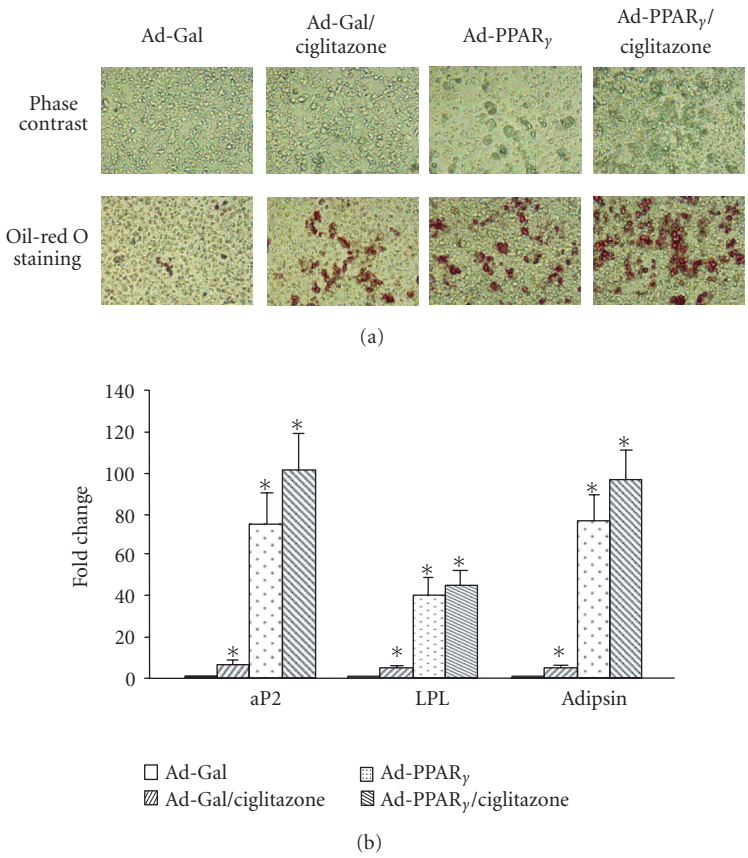

Figure 2: Adipogenic changes in the growth plate cells in which PPARγ was overexpressed or cells treated with ciglitazone. (a) Growth plate cells were infected with Ad-PPARγ or Ad-Gal followed by a 10-day incubation in the presence or absence of ciglitazone (5 μM). Cells were observed under the phase contrast microscope with 10-fold magnification. Oil Red-O staining shows lipid accumulation within the cells. (b) Quantitative real-time PCR analysis of the adipogenic marker genes aP2, LPL, and adipsin, in growth plate cells at day 4 of the culture. Ad-Gal- infected cells were used as controls. Gene expression levels were normalized with respect to endogenous 18S rRNA. $^*P < .05$ versus the expression in control cells.

To characterize the phenotype of the transformed cells in more detail, the cells were cultured in three-dimensional cell pellets and the expression of adipocyte differentiation marker genes examined by real-time RT-PCR at day 4 of the culture period. Compared with the control samples, the levels of expression of the adipogenic marker genes aP2, LPL,and adipsin increased 6.6-, 4.4- and 4.6-folds, respectively, on day 4 in the 5 μM ciglitazone-treated cells (Figure 2(b)). Expression of aP2, LPL, and adipsin genes increased in the Ad-PPARγ-infected cells by 75.1-, 40.2-, and 76.6-folds, respectively in the absence of ciglitazone, and 101.1-, 44.9-, and 96.3-folds, respectively, in the presence of ciglitazone (5 μM).

In order to address the possibility that PPARγ was acting on an undifferentiated mesenchymal stem cell as opposed to a differentiated chondrocyte, p1757-PPARγ expression plasmid was generated in which a PPARγ cDNA was placed under the transcriptional control of the rat COL2A1 gene promoter and enhancer sequences. Oil Red-O staining of the p1757-PPARγ-transfected cells maintained in three-dimensional pellet culture for 10 days showed markedly increased lipid accumulation (Figure 3(a)). Real-time RT-PCR demonstrated that aP2, LPL, and adipsin mRNA expressions were upregulated 21.1-, 12.9-, and 17.9-folds, respectively, compared with the cells transfected with the empty p1757 plasmid at day 4 after transfection (Figure 3(b)).

PPARγ induces loss of chondrocytic phenotype in growth plate cells

Alcian blue staining was used to detect the accumulation of cartilage-specific proteoglycan. At day 10, the control cultures of growth plate cells still accumulated abundant proteoglycan (Figure 4(a)). No significant difference in proteoglycan accumulation was observed in the growth plate chondrocytes that were treated with 5 μM of ciglitazone alone. Compared to the Ad-Gal-infected cells, the Ad-PPARγ-infected cultures were stained less intensely with Alcian blue, especially the surrounding of the cells that contained vacuoles. Addition of 5 μM of ciglitazone to the Ad-PPARγ-infected cultures resulted in a further decrease in proteoglycan matrix.

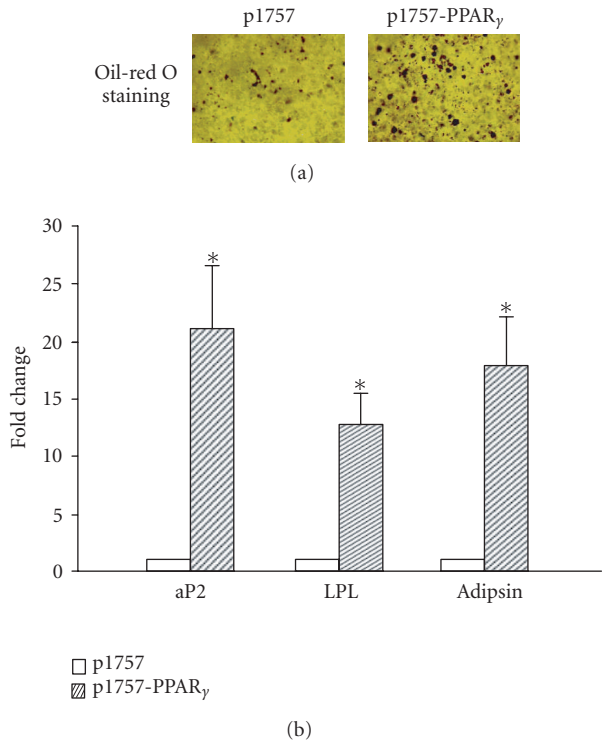

FIGURE 3: Adipogenic changes in the growth plate chondrocytes transfected with a PPARγ plasmid under the control of a collagen α1(II) promoter. (a) Growth plate cells were transfected with the p1757-PPARγ plasmid. Transfected cells were maintained in pellet cultures for 10 days, followed by Oil Red-O staining. (b) Quantitative PCR analysis of adipogenic markers of the p1757-PPARγ transfected growth plate cells at day 4 after transfection. Cells transfected with the empty p1757 plasmid were used as controls. Gene expression levels were normalized with respect to endogenous 18S rRNA. $^*P < .05$ versus the expression in control cells.

Quantitative RT-PCR demonstrated that the chondrocyte-specific genes COL2A1 and aggrecan were downregulated by both PPARγ and ciglitazone (Figure 4(b)). Treatment with 5 μM of ciglitazone for 4 days resulted in a 33% decrease of COL2A1 mRNA and a 17% decrease in aggrecan mRNA expression. Combination of both PPARγ adenovirus and 5 μM of ciglitazone resulted in a 50% decrease of COL2A1 mRNA and a 22% decrease in aggrecan mRNA expression.

PPARγ inhibits T3-induced hypertrophy and mineralization in growth plate chondrocytes

Thyroid hormone is a crucial regulator in growth plate chondrocyte hypertrophic differentiation and matrix mineralization [15–17]. Growth plate cells treated with thyroid hormone and 5 μM of ciglitazone demonstrated decreased alkaline phosphatase histochemical staining compared to cells treated with T3 alone (Figure 5(a)). Quantitative RT-PCR analysis of growth plate cells in pellet cultures treated with T3 and 5 μM of ciglitazone for 4 days confirmed a 64% decrease in ALP mRNA compared to cells treated with T3 alone (Figure 5(b)). Infection with PPARγ adenovirus in cells treated with T3 also decreased expression of ALP mRNA approximately 71% in the absence of ciglitazone, and 76% in the presence of 5 μM of ciglitazone. Runx2/Cbfa1 is expressed in chondrocytes as they initiate chondrocyte hypertrophy and maturation. Ciglitazone at a concentration of 5 μM decreased the T3-induced expression of Runx2 by 36%. Ad-PPARγ infection decreased the expression of Runx2 mRNA by 54% in the absence of ciglitazone and by 66% in the presence of 5 μM of ciglitazone.

DISCUSSION

Growth plate chondrocytes originate from multipotential mesenchymal stem cells that can differentiate into other cell types including adipocytes. We present evidence in this study that growth plate cells continue to display differentiation plasticity and are able to undergo adipogenic changes and a reciprocal decrease of chondrocytic markers when PPARγ is overexpressed.

It has been previously reported that chondrocytes are able to transdifferentiate into adipocytes in vitro [9]. The fatty acid content of the serum added to the culture media has been implicated as a potential cause of this transdifferentiation process [6]. We used a serum-free culture system in these experiments to avoid the possibility that fatty acids in the serum might induce the adipogenic changes observed.

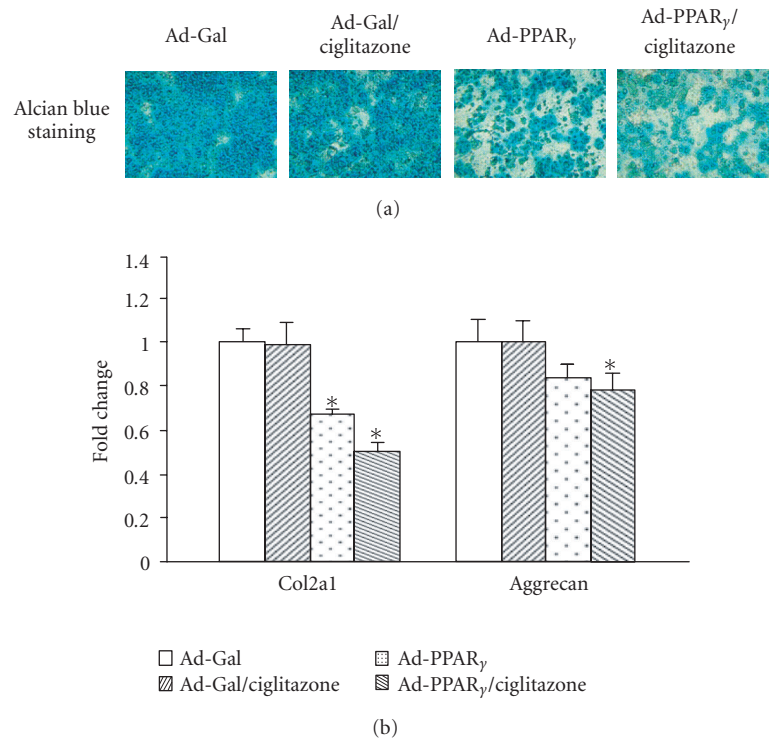

Figure 4: Loss of the chondrogenic phenotype in growth plate cells in which PPARγ was overexpressed or cells treated with ciglitazone. (a) Growth plate cells were infected with Ad-PPARγ or Ad-Gal followed by a 10-day incubation in the presence or absence of ciglitazone (5 μM). Alcian blue staining shows the accumulation of chondrocyte-specific matrix. (b) Quantitative PCR analysis of chondrogenic genes, COL2A1, and aggrecan, in growth plate cells infected with Ad-PPARγ or treated with ciglitazone at day 4 of treatment. Ad-Gal-infected cells were used as controls. Gene expression levels were normalized with respect to endogenous 18S rRNA. $^*P < .05$ versus the expression in control cells.

Ciglitazone is one of the thiazolidinedione classes of antidiabetic compounds which can activate PPARγ [18]. Ciglitazone not only increases endogenous PPARγ transcriptional activity [11], but also upregulates PPARγ mRNA and protein expression in growth plate chondrocytes, as observed in this study.

Activation of endogenous PPARγ by ciglitazone or adenoviral overexpression of PPARγ in growth plate chondrocytes resulted in acquisition of adipogenic features in both high-density monolayer cultures and three-dimensional pellet cultures of growth plate chondrocytes, as evidenced by cell morphology, lipid accumulation, and expression of adipocyte marker genes aP2, LPL, and adipsin. Growth plate cells maintained in monolayer cultures seemed to acquire features of the adipocytic phenotype and lose features of the chondrocytic phenotype more readily than those in the pellet cultures (data not shown).

To confirm that the adipocyte-like cells were differentiated directly from chondrocytes and not from other cell types such as undifferentiated mesenchymal stem cells, growth plate cells were transfected with a PPARγ plasmid under the control of a collagen α1(II) promoter. Acquisition of the adipogenic phenotype in these transfected cells was similar to the cells infected with an adenovirus encoding PPARγ and driven by the CMV promoter/enhancer.

While PPARγ and ciglitazone converted cells of the chondrocyte lineage to an adipocytic phenotype, features of the chondrocyte phenotype were simultaneously suppressed. PPARγ inhibited the ability of chondrocytes to terminally differentiate into hypertrophic cells, and suppressed the expression of genes encoding chondrocyte-specific extracellular matrix proteins.

Slipped capital femoral epiphysis (SCFE) is an obesity-related hip disease in children characterized by weakness in the growth plate of the proximal femur, delayed skeletal maturation, and eventual mechanical failure of the physis [19–21]. We speculate that obesity may induce the expression of PPARγ isoforms in growth plate chondrocytes, resulting in phenotypic changes that interrupt normal skeletal maturation at the growth plate through interference with thyroid hormone signaling. This interference with thyroid hormone-mediated terminal differentiation of growth plate cells and resulting decreased mineralization of the cartilage matrix would be expected to reduce the resistance of the growth plate to shear stresses. Therefore this delay in maturation at the growth plate, combined with both the increased mechanical stress resulting from increased body weight and the decreased shear stress resulting from delayed maturation, may combine to cause the proximal femoral epiphysis to slip.

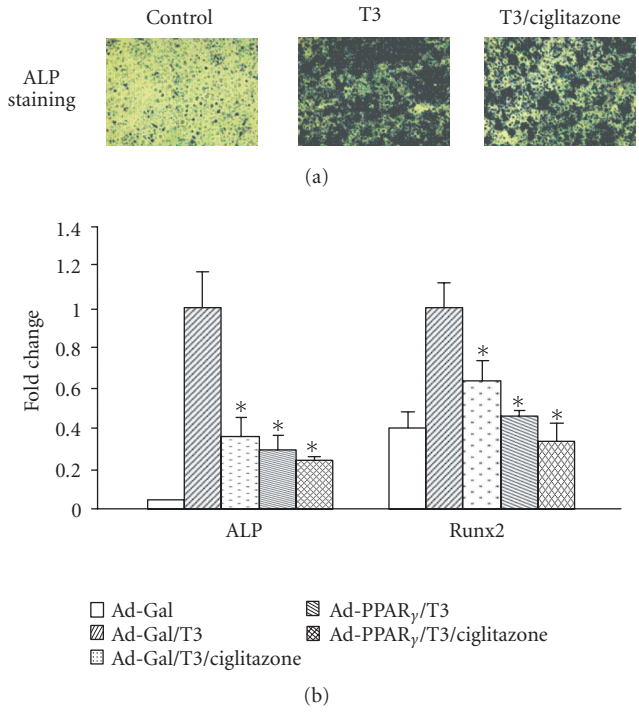

Figure 5: Inhibition of T3-induced hypertrophy and mineralization in the growth plate cells by PPARγ overexpression or ciglitazone treatment. (a) Growth plate cells were treated with T3 (100 ng/mL) in the presence or absence of ciglitazone (5 μM) for 10 days. Alkaline phosphatase (ALP) staining was used as a marker of terminal differentiation of growth plate chondrocytes. Positive stainings were colored in dark blue. Negative-stained background was colored in light green. (b) Quantitative PCR analysis of ALP and Runx2 genes 4 days after growth plate cells were infected with Ad-PPARγ or treated with ciglitazone. Ad-Gal-infected cells without T3 or ciglitazone treatment were used as controls. Gene expression levels were normalized with respect to endogenous 18S rRNA. $^*P < .05$ versus the expression in the Ad-Gal-infected cells with T3-treatment.

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