A New Concept Underlying Stem Cell Lineage Skewing That Explains the Detrimental Effects of Thiazolidinediones on Bone

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ABSTRACT
Bone-marrow adipogenesis is an aging-related phenomenon and is correlated with osteoporosis. The latter is a prevalent bone disease in the elderly leading to increased fracture risk and mortality. It is widely hypothesized that the underlying molecular mechanism includes a shift in the commitment of mesenchymal stem cells (MSCs) from the osteogenic lineage to the adipogenic lineage. Lineage skewing is at least partially a result of transcriptional changes. The nuclear transcription factor peroxisome proliferator-activated receptor γ (PPAR-γ) has been proposed as a major decision factor in MSC lineage commitment, promoting adipogenesis at the expense of osteogenesis. Here we found that PPAR-γ acted unexpectedly to stimulate osteoblast differentiation from human bone marrow-derived MSCs. Both rosiglitazone-mediated activation and overexpression of PPAR-γ caused acceleration of osteoblastic and adipocytic lineages upon regulation through elements of the skeleton. They can enter, among others, the bone marrow adipogenesis that is caused by a switch of mesenchymal stem cells (MSCs) into the adipogenic lineage [4, 5]. Pluripotent MSCs in the bone marrow surround trabecular elements of the skeleton. They can enter, among others, the osteoblastic and adipocytic lineages upon regulation through specific endocrine, paracrine, or autocrine signals. It is currently hypothesized that there is a competitive balance between osteoblasts and adipocytes that underlies a cross-talk between complex signaling mechanisms inducing one’s cell fate and ultimately suppressing the alternative lineage (recently reviewed in [6]). At least partially, MSC lineage commitment is regulated at the transcriptional level [7]. Herein, runx2 and osterix are considered as important osteoblast-specific transcription factors. Its main function is to regulate fatty acid uptake and storage (reviewed in [15–17]). Activation of PPAR-γ occurs upon binding to a variety of ligands that can be nutritionally derived endogenous polyunsaturated fatty acids and eicosanoids [18–20] or synthetic compounds including TZDs (reviewed in [21]). The latter are pharmaceutical compounds with insulin-sensitizing function that are frequently prescribed to type II diabetic patients. The single PPARγ gene undergoes alternative splicing and promoter...

INTRODUCTION

Osteoporosis is a prevalent skeletal disorder in the elderly that is characterized by impaired bone strength and increased fracture risk. It is associated with reduced life quality and increased mortality [1–3]. Bone strength is negatively correlated with bone marrow adipogenesis in aging and patients with osteoporosis. Furthermore, the recently observed reduced bone mass and increased risk of peripheral fractures in thiazolidinedione (TZD)-treated patients are suggested to result from bone marrow adipogenesis that is caused by a switch of mesenchymal stem cells (MSCs) into the adipogenic lineage [4, 5].
usage, giving rise to two proteins: PPAR-γ1 and PPAR-γ2. The latter contains an additional N-terminal exon that results in a higher ligand-independent transactivation capacity when compared to PPAR-γ1 [22]. Expression of PPAR-γ2 is mainly limited to adipocytes, whereas PPAR-γ1 is found to be ubiquitous abundantly [23, 24]. We have recently demonstrated dynamic expression patterns of PPAR-γ1 protein variant in osteoblasts and osteogenic hMSCs. Expression levels increase during the differentiation process and reach a plateau during the late phase that is defined by the onset of extracellular matrix mineralization [25]. This has suggested a role of PPAR-γ in osteogenesis besides the reported role in adipogenic MSC commitment.

In the current study, we investigated the impact and identity of PPAR-γ-regulated processes in osteoblast differentiation. We used both direct and indirect approaches to perturb PPAR-γ signaling in various human MSC and osteoblast models and analyzed the long-term osteoblastic phenotype outcome. In a condition of increased PPAR-γ activity, a first phase of accelerated differentiation was followed by a second phase of increased accumulation of reactive oxygen species (ROS) and apoptosis. Cells from the adipogenic lineage were protected from rosiglitazone-induced ROS accumulation and apoptosis and revealed a stronger expressed oxidative stress response network when compared to the osteogenic lineage. The results of the current study lead to a new concept of PPAR-γ action that challenges the role of PPAR-γ as MSC lineage decision maker and direct suppressor of osteoblast differentiation. This new concept is based on (1) evidence for a stimulatory role of PPAR-γ in both osteoblast and adipocyte differentiation from MSCs and (2) the revealed differential susceptibilities of osteogenic and adipogenic lineages to PPAR-γ ligand-induced ROS stress and apoptosis. Resistance of adipocytes but not osteoblasts to TZD-induced ROS and apoptosis builds the molecular basis for the new concept that adds to the explanation of clinically observed bone marrow adipogenesis, diminished bone formation, and increased fracture rate in TZD-treated patients.

### Materials and Methods

#### Cell Culture

SV-HFO preosteoblasts [26], normal human osteoblasts (NHOst: Lonza, Basel, Switzerland, http://www.lonza.com), and human bone marrow-derived mesenchymal stem cells (hMSC; Lonza) were cultured as described previously [27]. MSCs were derived from two different donors, and passages 6 and 7 were used for the experiments. Adipogenic differentiation was induced by culturing confluent hMSCs in differentiation medium that was supplemented with 100 nM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX), and 60 µM indomethacin.

#### Human Tissues

The tissues used in this publication were provided by Erasmus MC Tissue Bank (Dr. Riegman, Erasmus MC, Rotterdam, The Netherlands, http://www.tubafrost.org). Total RNA was isolated as described below.

#### Constructs

PPAR-γ1 expression construct containing PPAR-γ1 open reading frame in pCMV6-XL4 vector was purchased from Origene (Rockville, MD, http://www.origene.com), and PPAR-γ2 expression construct was kindly provided by Prof. J. Auwerx (Insitut Clinique de l’Hôpital, Strasbourg, France, http://www.ics-mci.fr). Sh-RNAi constructs targeting PPAR-γ were obtained from TRC library TRC-Hs1.0 (human) via OpenBiosystems (http://www.openbiosystems.com), and sequences are listed in supporting information Table S1.1.

#### Transfection Studies

SV-HFO and hMSC were cultured as described above until 60% confluence was reached. Transfection was achieved by electroporation using the appropriate Amaxa nucleofector kit according to the manufacturer’s recommendations (Lonza).

#### Quantification of mRNA Expression

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (Q-RT-PCR) were carried out as described recently [25]. Primer and probe sequences as well as their concentrations are listed in supporting information Table S1.2. Q-RT-PCR efficiencies were similar and about 100% for all reactions. Results for PPARA, total PPARG, PPARG2, and PPARD are displayed in supporting information Figure S1.

#### DNA, Protein, Alkaline Phosphatase Activity, and Mineralization Assays

Alkaline phosphatase (ALPL), DNA, protein, and calcium measurements were performed as described previously [27].

#### Quantification of Lipid Vesicle Formation

Cells were fixed in 10% formaline/phosphate-buffered saline (PBS), washed with 60% (v/v) isopropanol, and subsequently incubated with Oil red O working solution (Sigma, St. Louis, http://www.sigmaaldrich.com; 60% (v/v) Oil red O in deionized water). Staining solution was removed by washing with deionized water. Then culture plates were dried and remaining stain was dissolved in isopropyl alcohol solution (Sigma; 4% (w/v) igepal in isopropanol). Absorbance was measured photometrically at 490 nm.

#### Measurement of Reactive Oxygen Species

Total reactive oxygen species were measured using the cell-permeable non-fluorescent probe 2’,7’-dichlorofluorescin diacetate (DCF-DA; Sigma), which is de-esterified intracellularly and turns into highly fluorescent 2’,7’-dichlorofluorescin upon oxidation. Culture medium was replaced with 10 µM DCF-DA in modified ringer buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 25 mM Hepes, 6 mM glucose, 1.2 mM MgSO4, and 1 mM CaCl2; pH 7.4) and incubated for 1 hour at 37°C in the dark. Cultures were washed with PBS and supplemented with differentiation medium, and the fluorescence signal was subsequently quantified.

Superoxide radicals were measured using MitoSOX red mitochondrial superoxide indicator (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) according to the manufacturer’s instructions. Microscopic images were processed using Cell profiler software (http://www.cellprofiler.org).

#### Apoptosis Measurement

Apoptotic cells were counted by fluorescent-activating cell sorting using FacsCanto II apparatus (BD Biosciences, San Diego, http://www.bdbiosciences.com) after staining with 1 µg/ml FITC-labeled annexin-V (IQ-Products, Groningen, The Netherlands, http://www.iqproducts.nl) and 0.25 µg/ml propidium iodide (Nexins Research, Netherlands, http://www.nexins.com) according to the manufacturer’s instructions.

#### Statistics

Data presented are the results of at least two independent experiments performed in at least triplicate. Values are the means ± s.e.m. Significance was calculated using the Student’s t-test.

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**RESULTS**

**PPAR-γ1 Is the Predominant Expressed Isoform in Osteoblasts**

The three PPAR isoforms, α, β/δ, and γ, share high structural and physiological homology. We investigated expression levels of PPAR-α, −β/δ, and −γ in osteoblasts and adipocytes. Primer pairs were designed to detect all mRNA molecules that encode the ligand-binding domain of each PPAR isoform. Because the PPAR gene gives rise to PPAR-γ1 and PPAR-γ2 splice variants that differ only in their N-terminal region but contain identical ligand-binding domains, an additional primer pair was designed to detect the PPAR2-specific exon. Total PPAR-γ was between 2- and 500-fold higher expressed than PPAR-α or −β/δ in human osteoblasts, osteogenic hMSCs, and human bone tissue samples (Fig. 1A and 1B). PPAR-γ was the predominant expressed isoform in human adipocytes and human fat tissue as well (Fig. 1C). The splice variant PPAR-γ1 accounted for more than 99% of total expressed PPAR-γ in bone (Fig. 1A and 1B). In fat, both PPAR-γ variants were expressed, which is consistent with the data reported by others (Fig. 1C) [28, 29]. We had shown earlier that PPAR-γ1 expression level was dynamically regulated during osteogenic differentiation [25]. Functionality of endogenous PPAR-γ signaling in osteoblasts and osteogenic hMSCs

**Figure 1.** PPAR isoform expression in human bone and fat. Expression levels of PPAR isoforms PPAR-α, −β/δ, and −γ were determined by quantitative real-time polymerase chain reaction (Q-RT-PCR). To discriminate between both PPAR-γ splice variants, a PPAR2-specific primer pair was used (red bars). (A): Human pre-osteoblast model SV-HFO and osteogenic hMSCs, (B) human bone tissue from sternum and crista supraepicondylaris, and (C) adipogenic hMSCs and human fat tissue. (D): Functionality of PPAR-γ in SV-HFO and osteogenic hMSC. Expression levels of the primary PPAR target gene ANGPTL4 were analyzed by Q-RT-PCR in SV-HFO and osteogenic hMSC at day 3 of differentiation that were incubated for 3 hours with control, 1, or 10 μM rosiglitazone. SV-HFO and hMSC experiments were independently repeated. Data points represent means of three biological replicates ± s.e.m. Asterisks (*) denote values that were determined to be significantly different (p < 0.05) from those of controls. Q-RT-PCR data from bone and fat tissue are the mean of two technical replicates. To provide an idea about absolute expression levels of the three PPAR isoforms in cells, Ct values of osteogenic hMSCs at day 3 are provided: Ct (PPARA) = 26.18 ± 0.38; Ct (total PPARG) = 23.69 ± 0.04; Ct (PPARG2) = not detected; Ct (PPARD) = 26.63 ± 0.25. The following Ct values were detected in adipogenic hMSC at day 3 of differentiation: Ct (PPARA) = 27.81 ± 0.18; Ct (total PPARG) = 22.06 ± 0.22; Ct (PPARG2) = 28.23 ± 0.26; Ct (PPARD) = 28.19 ± 0.23. Abbreviations: hMSC, human mesenchymal stem cell; PPAR, peroxisome proliferator-activated receptor.
was shown by dose-dependent induction of expression of the primary PPAR target gene ANGPTL4 within 3 hours after treatment with the specific PPAR-γ agonist rosiglitazone in SV-HFO and hMSCs at day 3 of differentiation (Fig. 1D).

**Activated PPAR-γ Stimulates Both Adipocyte and Osteoblast Differentiation from Human Mesenchymal Stem Cells**

To test whether activation of PPAR-γ results in a switch from osteoblastic towards adipocytic lineage commitment, hMSC cultures were differentiated with dexamethasone and β-glycerophosphate into the osteogenic or with dexamethasone, indomethacin, and 3-isobutyl-1-methylxanthine into the adipogenic lineage and continuously supplemented with control, 0.1, 1, or 10 μM rosiglitazone and analyzed at day 15 for (A) extracellular matrix mineralization by Alizarin red staining and (B) lipid vesicle formation by Oil red O staining. In panel A in the Alizarin red staining of the adipo + control and adipo + 10 μM rosi condition, light pink aspecific staining can be observed. This is not mineralization but adipocytes, as can be clearly seen from the accumulation of lipid droplets. The experiment was repeated with a second sample of MSCs from the same donor, and data were confirmed in a second MSC donor as well. Data points from staining semiquantification plots below represent the mean of four biological replicates ± s.e.m. Asterisks (*) denote values that were determined to be significantly different (p < .05) when compared to respective controls. Abbreviations: adipo, adipogenic; osteo, osteogenic.

**Rosiglitzone Stimulates Osteoblast Differentiation in a PPAR-γ-Dependent Manner**

To further elucidate the role of PPAR-γ in osteoblast differentiation, the effects of continuous rosiglitazone supplementation on expression levels of osteoblast differentiation markers RUNX2, collagen type IA1 (COL1A1), osteopontin (SPP1), osteocalcin (BGLAP), activating transcription factor 4 (ATF4), and FOS-like antigen 1 (FOSL1 or FRA1) and alkaline phosphatase (ALP) activity were analyzed in osteogenic hMSC cultures. Expression levels of all osteoblast differentiation markers were elevated in rosiglitazone-supplemented cultures in a differentiation stage-dependent manner. RUNX2 and COL1A1 expression levels were significantly higher in rosiglitazone-treated hMSCs when compared to respective controls during the early phase of differentiation (day 3; Fig. 3A). Expression levels of ATF4, FOSL1, BGLAP, and SPP1 were significantly elevated by rosiglitazone in hMSCs at day 15 (Fig. 3A). ALP activity was dynamically regulated in control condition and reached its peak at day 15 of culture. Treatment with rosiglitazone caused an accelerated ALP activity pattern (Fig. 3B) and resulted in increased mineralization (Fig. 3C). Rosiglitazone had similar effects on the differentiation of the human pre-osteoblast cell line SV-HFO and NHOst (data not shown). Rosiglitazone treatment resulted in a significant upregulation of the primary PPAR target gene ANGPTL4 (Fig. 3A) and a significant increase in PPARG1 expression levels, whereas PPARG2 expression remained absent (data not shown).
Figure 3. Role of rosiglitazone-activated PPAR-γ in osteoblast differentiation. (A): Expression levels of osteogenic differentiation marker genes RUNX2, COL1A1, ATF4, FOSL1, BGLAP, and SPP1 and primary PPAR target gene ANGPTL4 were quantified in continuously 10 μM rosiglitazone- or control-treated hMSC cultures at multiple time points during osteoblast differentiation (induced by dexamethasone and β-glycerophosphate) by quantitative real-time polymerase chain reaction (Q-RT-PCR). (B): ALP activity and (C) extracellular matrix mineralization was quantified in hMSC cultures that were continuously supplemented with 10 μM rosiglitazone- or control-treated hMSC cultures at multiple time points during culture. (D): hMSC cultures were transfected with shRNAi constructs targeting PPARα ligand-binding domain or scrambled control. Total PPAR, PPAR2, PPARα, PPARδ, ANGPTL4, and ALPL expression levels were quantified 5 days after transfection by Q-RT-PCR. (E): hMSC cultures were transfected with PPARG1 of PPARG2 expression constructs or empty vector control. Total PPARG, PPARG2, and ALPL expression levels were quantified 3 days after transfection by Q-RT-PCR. Data from panels (A)–(C) were reproduced in an independent experiment with a second sample of cells from the same MSC donor. The experiment of panels (D) and (E) was repeated independently in a second MSC donor. Data points represent means of four biological replicates ± s.e.m. Asterisks (*) denote values that were determined to be significantly different (p < .05) when compared to respective controls. Abbreviations: ALP, alkaline phosphatase; ATF4, activating transcription factor 4; BGLAP, osteocalcin; COL1A1, collagen type IA1; FOSL1, FOS-like antigen 1; hMSC, human mesenchymal stem cell; PPAR, peroxisome proliferator-activated receptor; SPP1, osteopontin.

Figure 4. Role of rosiglitazone-activated peroxisome proliferator-activated receptor γ (PPAR-γ) in osteoblast apoptosis and mitochondrial dysfunction. (A): DNA content and (B) apoptosis measured as a percentage of annexinV-FITC-positive cells in hMSC cultures that were continuously supplemented with 10 μM rosiglitazone or control. Apoptosis was quantified in a similar manner in (C) hMSC cultures transfected with PPARG1 or PPARG2 expression constructs at day 15 of culture and in (D) hMSC cultures transfected with shRNAi constructs targeting PPARG ligand-binding domain at day 5 of culture. (E): Expression levels of CASP3 and CASP6 were determined by quantitative real-time polymerase chain reaction (Q-RT-PCR) at multiple time points in hMSC cultures that were supplemented continuously with 10 μM rosiglitazone or control. (F) Levels of superoxide radicals were determined by Mitosox staining (white spots in top panels), and data displayed are from mineralization onset at day 15 of culture. Total reactive oxygen species were semiquantified at culture days 1, 7, and 14. (G) Expression levels of markers of mitochondrial dysfunction BAX, CYCS, APAF1, VDAC1, and SLC25A4 were measured by Q-RT-PCR in hMSC cultures supplemented with 10 μM rosiglitazone or controls at multiple time points. Q-RT-PCR based analysis of BAX and CYCS expression in hMSC cultures that were transfected with PPARG1 or PPARG2 expression constructs (H) or shRNAi constructs targeting PPARG ligand-binding domain (I). Data from panels (A), (B), (G), (E), and (F) were reproduced in an independent experiment with cells from the same MSC donor. The experiments of panel (C), (D), (H), and (I) were repeated independently in MSCs from a second donor. Data points represent means of at least three biological replicates ± s.e.m. Asterisks (*) denote values that were determined to be significantly different (p < .05) different when compared to respective controls. Abbreviations: APAF1, apoptotic peptidase activating factor 1; BAX, Bcl2-associated X protein; CASP, caspase; CYCS, cytochrome C; DCF-DA, 2′,7′-dichlorofluorescin diacetate; hMSC, human mesenchymal stem cell; rosi, rosiglitazone; SLC25A4, mitochondrial solute carrier family 25 member 4; VDAC1, voltage-dependent anion channel 1.
In order to assess the involvement of PPAR-γ, hMSCs were transfected with shRNAi constructs targeting mRNA sequences that encode the ligand-binding domain of PPAR-γ. Expression of PPARG was significantly downregulated and ALPL expression was significantly reduced in PPAR-γ-shRNA cultures when compared to controls three days after transfection (Fig. 3D). Interestingly, PPARA expression levels were higher in both PPAR-γ-shRNA cultures when compared to controls (Fig. 3D). A similar observation in regard to the inversed relationship between PPARA and PPARG...
expression was described earlier by Patel et al. [30]. However, it is unlikely that this PPARα expression will compensate for the knockdown of PPARγ because the expression of the primary PPAR target gene ANGPTL4 was diminished in PPARγ-shRNA cultures (Fig. 3D). To investigate the impact of increased PPARγ expression, hMSC cultures were transfected with PPARγ expression constructs under the control of CMV promoter. ALP activity levels were significantly higher in PPARγ overexpressing cells (Fig. 3E). Interestingly, both PPARγ-1 and PPARγ-2 overexpression resulted in higher ALP activity when compared to empty vector controls (Fig. 3E).

The Prodifferentiative Effect of Rosiglitazone is Followed by Enhanced PPARγ-Dependent Apoptosis and ROS Accumulation

We investigated the effect of rosiglitazone on osteoblast apoptosis, ROS accumulation, and expression of genes involved in mitochondrial dysfunction. The chosen concentration of 10 μM rosiglitazone is well within the range measured in TZD-treated patients [31–34]. DNA content was significantly lower in rosiglitazone-treated osteogenic hMSC cultures when compared to control during late differentiation phase (Fig. 4A). The reduction in DNA content was paralleled by increased apoptosis (Fig. 4B). Similar effects of rosiglitazone treatment on DNA and apoptosis were confirmed in SV-HFO and NHOst (data not shown). Overexpression of PPARγ-1 or PPARγ-2 resulted in increased apoptosis that was further enhanced by supplementation with rosiglitazone (Fig. 4C). Conversely, the pro-apoptotic effect of rosiglitazone was reduced in hMSCs that were transfected with PPARγ-shRNAi constructs (Fig. 4D). To assess whether expression of genes involved in apoptosis is affected by rosiglitazone, mRNA levels of apoptosis-related cysteine peptidases caspase 3 (CASP3) and caspase 6 (CASP6) were quantified. Expression levels of both genes showed a tendency to increase or were stable during osteogenic differentiation of hMSCs, and continuous rosiglitazone treatment significantly increased expression levels in a differentiation-dependent manner (Fig. 4E). Superoxide radical levels were significantly elevated in rosiglitazone-treated cultures during late differentiation phase (Fig. 4F). We further investigated the expression of genes involved in mitochondrial dysfunction. Bcl2-associated X protein (BAX), cytochrome C (CYCS), apoptotic peptidase activating factor 1 (APAF1), voltage-dependent anion channel 1 (VDAC1), and mitochondrial solute carrier family 25 member 4 (SLC25A4) were quantified. Expression levels of all genes were elevated in rosiglitazone-supplemented hMSC cultures at the onset of mineralization when compared to respective controls (Fig. 4G). We investigated the role of PPARγ in mitochondrial-dependant apoptosis by overexpression and knockdown studies and subsequent analysis of CYCS and BAX expression. PPARγ overexpression revealed modest increases in CYCS and BAX expression levels (Fig. 4H), whereas downregulation of PPARγ resulted in significantly reduced CYCS and BAX transcript levels (Fig. 4I).

Differential Robustness of Adipocytes and Osteoblasts to Rosiglitazone-Induced Oxidative Stress and Apoptosis

Taken together so far, the data above provide evidence for an involvement of PPAR-γ in osteogenic differentiation from hMSCs. The lack of evidence for a direct lineage switch towards the adipogenic lineage away from the osteogenic lineage gives rise to the question of how the commonly reported reduced number of osteoblasts and increased number of adipocytes is caused in models of stimulated PPAR-γ activity. Alternatively to a direct suppressive action of PPAR-γ on osteoblast differentiation, age-related shifts in stem cell commitment may be secondary to lineage-specific differences in susceptibility to oxidative stress and apoptosis, as has been hypothesized by Warren and Rossi for the hematopoietic system [35]. In order to test this hypothesis, we measured the percentage of apoptotic cells in osteogenic and adipogenic cultures that were continuously supplemented with increasing concentrations of rosiglitazone during several time points of differentiation. Interestingly, only the osteogenic but not the adipogenic lineage revealed dose-dependent increases in apoptosis upon stimulation with rosiglitazone (Fig. 5A). Also, the amount of superoxide radicals was increased only in the osteogenic lineage upon rosiglitazone treatment, whereas the adipogenic lineage was protected from this (Fig. 5B). To further investigate whether the observed difference in rosiglitazone-induced apoptosis between both lineages is a result of transcriptional differences in oxidative stress response genes, we compared mRNA levels of well characterized genes involved in oxidative stress response, in particular, genes encoding proteins with antioxidant capacity, phase I and II metabolizing enzymes, and components of the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2)-mediated oxidative stress response signaling pathway, in osteogenic and adipogenic cultures at several time points during differentiation. Interestingly, genes encoding the antioxidant proteins catalase (CAT), heme oxygenase 1 (HMOX1), and mitochondrial superoxide dismutase 2 (SOD2) were significantly higher expressed in adipocytes (Fig. 5C). Also, transcripts encoding the phase I and II metabolizing enzymes epoxide hydrolase 1 (EPHX1), microsomal glutathione S-transferase 1 (MGST1), and glutamate cysteine ligase, modifier subunit (GCLM) were more strongly expressed in adipocytes than in osteoblasts throughout the differentiation process (Fig. 5C). On the other hand, glutathione S-transferase alpha 4 (GSTA4), glutathione S-transferase kappa 1 (GSTM1), and NAD(P)H dehydrogenase, quinone 2 (NQO2) transcript levels were only significantly higher expressed in adipocytes at later time points during culture but did not reveal any differences during early differentiation (Fig. 5C). Smooth muscle gamma 2 subunit of actin (ACTG2) and protein kinase C, alpha (PKCα) are components of the nuclear factor NFE2L2-mediated oxidative stress response signaling pathway and were significantly higher expressed in the adipogenic compared to the osteogenic lineage (Fig. 5C). Interestingly, mitogen-activated protein kinase kinase kinase 1 (MAP3K5) was more strongly expressed in the osteogenic compared to the adipogenic lineage. This observation is in line with the findings above, because activation of MAP3K5 has been shown to mediate activation of mitogen-activated protein kinase 14, also known as p38 MAPK (MAPK14), which would lead to inhibition of phosphorylated NFE2L2 [36]. In summary, the NFE2L2-mediated oxidative stress response signaling network was more strongly expressed in the adipogenic compared to the osteogenic lineage, suggesting that the osteogenic lineage is more susceptible to oxidative stress and apoptosis. These data support a role for DNA damage and genomic maintenance in lineage skewing of hMSCs in aging and aging-related diseases that are associated with suppressed osteogenesis, in particular in TZD-induced bone loss.

DISCUSSION

The current study has revealed three major findings. First, we have shown that activation of PPAR-γ unexpectedly accelerates human osteoblast differentiation by using a variety of direct and indirect perturbation studies in several osteoblast...
Figure 5. Differential analysis of apoptosis, superoxide levels, and oxidative stress response gene expression in osteogenic and adipogenic lineages. (A): Apoptosis measured as a percentage of annexin V-FITC-positive cells in hMSC cultures that were differentiated towards osteogenic or adipogenic lineage and continuously supplemented with control, 0.1, 1, or 10 μM rosiglitazone. (B): Superoxide levels were quantified by Mito-sox staining and subsequent Cell profiler image analysis software in hMSC cultures that were differentiated towards osteogenic or adipogenic lineage and continuously supplemented with control, 0.1, 1, or 10 μM rosiglitazone. (C): Expression levels of oxidative stress response genes were determined in hMSC cultures that were differentiated towards osteogenic or adipogenic lineage and continuously supplemented with control, 0.1, 1, or 10 μM rosiglitazone by quantitative real-time polymerase chain reaction analysis at multiple time points during culture. Data from panels (A) and (B) were independently repeated in an experiment with a second sample of cells from the same MSC donor. The experiment from panel (C) was repeated with MSCs from a different donor. Data points are means of at least three biological replicates ± s.e.m. Asterisks (*) denote values that were determined to be significantly different (p < 0.05) when compared to respective controls. Abbreviations: ACTG2, smooth muscle gamma 2 subunit of actin; adipo, adipogenic; CAT, catalase; EPHX1, epoxide hydrolase 1; GCLM, glutamate cysteine ligase, modifier subunit; GSTA4, glutathione S-transferase alpha 4; GSTK1, glutathione S-transferase kappa 1; HMOX1, heme oxygenase 1; MAP3K5, apoptosis signal regulating kinase 1; MGST1, microsomal glutathione S-transferase 1; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; NQO2, NAD(P)H dehydrogenase, quinone 2; osteo, osteogenic; PRKCA, protein kinase C, alpha; SOD2, superoxide dismutase 2.
insulin \[41, 42\], insulin-like growth factor I \[43\], or estrogen that are important for bone metabolism, such as, leptin \[40\], bone marrow \[5, 9, 38, 39\], (b) modulation of hormone levels

MSCs into adipocytes on the expense of osteoblasts in the first sight. But interestingly, we have found that RUNX2 expression is time-dependently affected by rosiglitazone. During early osteogenesis, expression levels are elevated in rosiglitazone-supplemented hMSCs and SV-HFO when compared to respective controls. However, during later differentiation stage, RUNX2 expression is diminished by rosiglitazone and PPAR-\(\gamma\) expression is increased in parallel. At first glance, this observation may be interpreted as transdifferentiation from the osteogenic towards the adipogenic lineage. However, it is well established that RUNX2 possesses a dual role in osteogenic differentiation with a positive role in osteogenic lineage commitment and a suppressive action on osteoblast maturation \[46, 47\]. The fact that expression levels of two well known osteoblast maturation factors FOSL1 and ATF4 are upregulated at the same time point while RUNX2 levels are decreased suggests that rosiglitazone treatment causes stimulation of osteoblast maturation but not necessarily a transdifferentiation from an osteoblastic towards an adipocytic cell phenotype.

The involvement of PPAR-\(\gamma\) in adipogenesis is well established \[29, 48, 49\]. We have confirmed a dose-dependent pro-adipogenic effect of rosiglitazone in human MSCs that have been supplemented with dexamethasone, IBMX, and indomethacin to differentiate into the adipogenic lineage. However, supplementation of osteogenic medium (dexamethasone and b-glycerophosphate) with rosiglitazone is not sufficient to induce adipogenesis in hMSC cultures, as we have shown in two different donors.

We have described earlier that endogenous PPAR-\(\gamma\)-1 expression is dynamically regulated during the differentiation process of human osteoblasts and osteogenic hMSCs whereas PPAR-\(\gamma\)-2 expression is limited to adipocytes \[25\]. Rosiglitazone treatment results in homologous upregulation of PPAR-\(\gamma\)-1 expression levels, but PPAR-\(\gamma\)-2 expression remains virtually absent in the human osteoblast models SV-HFO \[25\], NHOst, and osteogenic hMSCs (data not shown). Therefore, the supplementation of osteoblast and osteogenic hMSC cultures with rosiglitazone is likely to reflect a condition of increased PPAR-\(\gamma\)-1 activity. However, we have not observed any differences between PPAR-\(\gamma\)-1- and PPAR-\(\gamma\)-2-overexpressing cells in regard to their osteoblastic phenotype. Therefore, these data do not provide unambiguous evidence for a PPAR-\(\gamma\) variant-specific effect. Future detailed analysis of PPAR-\(\gamma\)-variant-specific interactions with the transcription initiation machinery as well as primary PPAR-\(\gamma\)-variant-specific target genes in osteoblasts will be necessary to definitely answer this question.

Our finding that rosiglitazone supplementation stimulates osteogenic differentiation from hMSCs, osteoblast maturation, and extracellular matrix mineralization reveals an unexpected role of PPAR-\(\gamma\) in osteogenic differentiation. Detailed analysis of the osteoblastic phenotype at multiple time points shows an acceleration of osteoblast development by activated PPAR-\(\gamma\) as shown by the early stimulation of RUNX2 and COL1A1 expression, ALP activity, and mineralization. We speculate here about a role of PPAR-\(\gamma\) as energy sensor and signaling component for the osteoblastic developmental process. This view is supported by the differentiation phase-dependent up-regulation of PPAR-\(\gamma\) reaching the highest expression levels during mineralization onset \[25\]. The osteoblast differentiation process consists of several highly energy-demanding phases including matrix production, maturation, and

Figure 6. Schematic representation of the new concept derived from this study that adds to the explanation of thiazolidinedione (TZD)-induced bone loss. TZD-mediated activation of peroxisome proliferator-activated receptor \(\gamma\) (PPAR-\(\gamma\)) accelerates osteoblast differentiation as indicated by the line graph illustrating the shift in time. Concomitantly there is a stimulation of adipogenesis from MSCs. The phase of accelerated osteoblast differentiation, energy expenditure, and reactive oxygen species production is ultimately followed by increased osteoblast apoptosis, whereas the survival of adipocytes is not affected by TZDs. Therefore, osteogenic and adipogenic lineages reveal differential susceptibilities to TZD-induced apoptosis. This concept builds the molecular basis for the clinically observed bone marrow adipogenesis, diminished bone formation, and increased fracture rate in TZD-treated patients. Abbreviations: MSC, mesenchymal stem cell.

A vast number of mechanistic studies have provided insight into TZD action on bone. Putative mechanisms include suppression of bone formation and stimulation of bone resorption that are caused by (a) preferential differentiation of MSCs into adipocytes on the expense of osteoblasts in the bone marrow \[5, 9, 38, 39\], (b) modulation of hormone levels that are important for bone metabolism, such as, leptin \[40\], insulin \[41, 42\], insulin-like growth factor I \[43\], or estrogen

\[44\], and (c) stimulation of osteoclastogenesis by regulation of c-fos expression \[45\].

The evidence for a preferential differentiation of MSCs into adipocytes at the expense of osteoblasts is, to date, based on mechanistic studies in murine osteoblastic cells that report a suppression of runx2 expression and promoter activity \[5, 9, 38, 39\]. The observed stimulation of RUNX2 expression by activated PPAR-\(\gamma\) in human MSCs and osteoblasts in the current study is, therefore, in contrast to the reported data in murine osteoblastic cells at first sight. But interestingly, we have found that RUNX2 expression is time-dependently affected by rosiglitazone. During early osteogenesis, expression levels are elevated in rosiglitazone-supplemented hMSCs and SV-HFO when compared to respective controls. However, during later differentiation stage, RUNX2 expression is diminished by rosiglitazone and PPAR-\(\gamma\) expression is increased in parallel. At first glance, this observation may be interpreted as transdifferentiation from the osteogenic towards the adipogenic lineage. However, it is well established that RUNX2 possesses a dual role in osteogenic differentiation with a positive role in osteogenic lineage commitment and a suppressive action on osteoblast maturation \[46, 47\].

The fact that expression levels of two well known osteoblast maturation factors FOSL1 and ATF4 are upregulated at the same time point while RUNX2 levels are decreased suggests that rosiglitazone treatment causes stimulation of osteoblast maturation but not necessarily a transdifferentiation from an osteoblastic towards an adipocytic cell phenotype.

The involvement of PPAR-\(\gamma\) in adipogenesis is well established \[29, 48, 49\]. We have confirmed a dose-dependent pro-adipogenic effect of rosiglitazone in human MSCs that have been supplemented with dexamethasone, IBMX, and indomethacin to differentiate into the adipogenic lineage. However, supplementation of osteogenic medium (dexamethasone and b-glycerophosphate) with rosiglitazone is not sufficient to induce adipogenesis in hMSC cultures, as we have shown in two different donors.

We have described earlier that endogenous PPAR-\(\gamma\)-1 expression is dynamically regulated during the differentiation process of human osteoblasts and osteogenic hMSCs whereas PPAR-\(\gamma\)-2 expression is limited to adipocytes \[25\]. Rosiglitazone treatment results in homologous upregulation of PPAR-\(\gamma\)-1 expression levels, but PPAR-\(\gamma\)-2 expression remains virtually absent in the human osteoblast models SV-HFO \[25\], NHOst, and osteogenic hMSCs (data not shown). Therefore, the supplementation of osteoblast and osteogenic hMSC cultures with rosiglitazone is likely to reflect a condition of increased PPAR-\(\gamma\)-1 activity. However, we have not observed any differences between PPAR-\(\gamma\)-1- and PPAR-\(\gamma\)-2-overexpressing cells in regard to their osteoblastic phenotype. Therefore, these data do not provide unambiguous evidence for a PPAR-\(\gamma\) variant-specific effect. Future detailed analysis of PPAR-\(\gamma\)-variant-specific interactions with the transcription initiation machinery as well as primary PPAR-\(\gamma\)-variant-specific target genes in osteoblasts will be necessary to definitely answer this question.

Our finding that rosiglitazone supplementation stimulates osteogenic differentiation from hMSCs, osteoblast maturation, and extracellular matrix mineralization reveals an unexpected role of PPAR-\(\gamma\) in osteogenic differentiation. Detailed analysis of the osteoblastic phenotype at multiple time points shows an acceleration of osteoblast development by activated PPAR-\(\gamma\) as shown by the early stimulation of RUNX2 and COL1A1 expression, ALP activity, and mineralization. We speculate here about a role of PPAR-\(\gamma\) as energy sensor and signaling component for the osteoblastic developmental process. This view is supported by the differentiation phase-dependent up-regulation of PPAR-\(\gamma\) reaching the highest expression levels during mineralization onset \[25\]. The osteoblast differentiation process consists of several highly energy-demanding phases including matrix production, maturation, and

Schematic representation of the new concept derived from this study that adds to the explanation of thiazolidinedione (TZD)-induced bone loss. TZD-mediated activation of peroxisome proliferator-activated receptor \(\gamma\) (PPAR-\(\gamma\)) accelerates osteoblast differentiation as indicated by the line graph illustrating the shift in time. Concomitantly there is a stimulation of adipogenesis from MSCs. The phase of accelerated osteoblast differentiation, energy expenditure, and reactive oxygen species production is ultimately followed by increased osteoblast apoptosis, whereas the survival of adipocytes is not affected by TZDs. Therefore, osteogenic and adipogenic lineages reveal differential susceptibilities to TZD-induced apoptosis. This concept builds the molecular basis for the clinically observed bone marrow adipogenesis, diminished bone formation, and increased fracture rate in TZD-treated patients. Abbreviations: MSC, mesenchymal stem cell.
mineralization [50], which is coordinated by a switch from glycolysis to respiration that involves increased mitochondrial biogenesis [51]. A role of PPARs in mitochondrial energetics has been demonstrated recently in a mouse model of mitochondrial myopathy [52] and T-cells [53]. Mitochondrial function of T-cells is improved when the concentrations of PPAR-γ agonists used are just in the sufficient range to induce transcriptional activity of PPAR-γ, whereas higher concentrations result in T-cell death [53]. In addition, high concentrations of PPAR-γ ligands induce mitochondrial dysfunction in human hepatocarcinoma cells [54] and astrocytes [55]. Mitochondrial dysfunction leads to partial reduction of oxygen that causes the formation of reactive oxygen species [56]. The latter activate signaling cascades, resulting in apoptosis [57].

Alternatively to a direct suppression of transcriptional networks involved in osteoblast differentiation, age-related shifts in mesenchymal stem cell commitment may be secondary to lineage-specific differences in susceptibility to oxidative stress and apoptosis, as has been hypothesized by Warren and Rossi for the hematopoietic system [35]. Our findings that rosiglitazone-stimulated PPAR-γ results in increased expression levels of genes involved in mitochondrial dysfunction, the generation of reactive oxygen species, and apoptosis of the osteogenic lineage but not the adipogenic lineage may explain the reduced number of fully differentiated osteoblasts in vivo. This reduction of mature osteoblasts due to premature death is likely to cause an immature or non-optimal bone matrix with lower quality. Further evidence for an apoptotic mechanism in PPAR-γ-mediated bone loss has been provided recently by Elbaz and co-workers. The authors have shown that fatty acids, which are natural PPAR-γ agonists, are released by adipocytes and cause osteoblast lipotoxicity by an apoptotic mechanism in a co-culture system consisting of normal human osteoblasts and pre-adipocytes [58]. In addition, Jung et al. have reported a caspase-dependent mechanism of troglitazone-induced apoptosis that is associated with downregulation of ERK and upregulation of p38 in the murine osteoblastic cell model MC3T3-E1 [59]. Kim and co-workers have published earlier the involvement of a MAPK signaling pathway upstream of mitochondria-dependent mechanism in cigitazone-induced MC3T3 apoptosis [60]. Our data are also supported by the in vivo work of Sorocanu and co-workers, who have found decreased osteoblast number and activity due to increased apoptotic death of osteoblasts and osteocytes in five-week-old male non-diabetic C57BL/6 mice that have been treated an additional 90 days with rosiglitazone [61]. Furthermore, bone of these mice is histologically characterized by decreased trabecular bone volume and increased marrow space with no significant change in bone marrow adiposity [61]. In contrast to this, Ali et al. have described bone loss associated with an increase in marrow adipocytes with no significant change in osteoblast lifespan in five-month-old Swiss Webster mice that have been treated with rosiglitazone for 28 days [11]. Divergent skeletal responses to rosiglitazone treatment have been observed more recently in a study with adult female mice from four different inbred strains (C3H/HeJ, DBA/2J, C57BL/6J, and A/J) [62]. It is, therefore, likely that multiple mechanisms contribute to TZD-induced bone loss and that, in mice studies, those are strongly dependent on the genetic background.

The new concept that derives from this study and explains TZD-induced bone loss stresses the importance of mitochondrial dysfunction, oxidative stress, and apoptosis in mesenchymal stem cell lineage skewing and bone loss. The involvement of oxidative stress in osteoblast apoptosis and bone loss has been shown in C57BL/6 mice with advancing age [63]. In these mice, an increase in the prevalence of apoptotic osteoblasts and a corresponding decrease in osteoblast number and bone-formation rate are accompanied by increased oxidative stress and diminished Wnt signaling, a critical regulator of bone formation [63]. A β-catenin-dependent mechanism of oxidative stress-induced PPAR-γ expression and suppressed Wnt3a signaling has been described recently by the same group [64]. Interestingly, despite increased PPAR-γ expression, oxidative stress, diminished Wnt signaling, and reduced osteoblast number in vertebrae of aged C57BL/6 mice, marrow adipocytes are not increased at this site [64]. Therefore, the diversion of MSCs to adipocytes may not be an obligatory part of the oxidative stress-induced anti-osteogenic cascade.

Human osteoblast cell models require glucocorticoids for proper initiation of differentiation and extracellular matrix (ECM) mineralization [27]. However, glucocorticoid-treated male Swiss Webster mice exhibit an increase in osteoblast apoptosis in vertebrae and show apoptosis in 28% of the osteocytes in metaphyseal cortical bone [65]. Increased osteoblast and osteocyte apoptosis is also found in patients with glucocorticoid-induced osteoporosis [65]. Taken together, the actions of glucocorticoids and TZDs appear to have similarities, and it is conceivable that both nuclear receptors (glucocorticoid receptor and PPAR-γ) interact on common transcriptional networks that play a role in osteoblast apoptosis. In support of this, we have shown recently that PPAR-γ expression is directly induced by the synthetic glucocorticoid dexamethasone in human osteoblasts [25]. This suggests that PPAR-γ is a component of the glucocorticoid-mediated apoptosis pathway in bone.

CONCLUSION

In conclusion, our findings provide a new concept for TZD-induced bone loss and bone marrow adipogenesis. This concept is based on differences between osteogenic and adipogenic lineages in the susceptibility to oxidative stress, mitochondrial dysfunction, and apoptosis that is triggered by activated PPAR-γ. Therefore, improving mitochondrial function may be a successful strategy to prevent aging-related and TZD-induced bone loss.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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