Thiazolidinedione-induced lipid droplet formation during osteogenic differentiation

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Abstract

Chronic administration of the insulin-sensitising drugs, thiazolidinediones (TZDs), results in low bone mineral density and 'fatty bones'. This is thought to be due, at least in part, to aberrant differentiation of progenitor mesenchymal stem cells (MSCs) away from osteogenesis towards adipogenesis. This study directly compared the effects of rosiglitazone, pioglitazone, and netoglitazone treatment on osteogenesis and adipogenesis in MSCs derived from subcutaneous (SC) or visceral (PV) white adipose tissue. MSCs were isolated from adipose tissue depots of male Wistar rats and characterised using flow cytometry. The effects of TZD treatment on osteogenic and adipogenic differentiation were assessed histologically (day 14) and by quantitative PCR analysis (*Ppar γ 2* (*Pparg2*), *Ap2* (*Fabp4*), *Adipsin* (*Adps*), *Mx2*, *Collagen I* (*Col1a1*), and *Alp*) on days 0, 7, and 10. Uniquely, lipid droplet formation and mineralisation were found to occur concurrently in response to TZD treatment during osteogenesis. Compared with SC MSCs, PV MSCs were more prone to lipid accumulation under controlled osteogenic and adipogenic differentiation conditions. This study demonstrated that the extent of lipid accumulation is dependent on the nature of the *Ppar* ligand and that SC and PV MSCs respond differently to *in vitro* TZD treatment, suggesting that metabolic status can contribute to the adverse effects associated with TZD treatment.

Key Words

- ▶ rosiglitazone
- ▶ netoglitazone
- ▶ pioglitazone
- ▶ mesenchymal stem cells

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Introduction

Thiazolidinediones (TZDs; also known as glitazones) have a unique insulin-sensitising action (Bergeron *et al.* 2006); however, the clinical use of TZDs for the treatment of type 2 diabetes mellitus is currently challenged by reports of an inadequate safety profile (Cariou *et al.* 2012). In addition to body weight gain (van Dieren *et al.* 2012), fluid retention (Karalliedde *et al.* 2006), congestive heart failure (Gitt *et al.* 2012), and cancer (Mamtani *et al.* 2012), the chronic administration of TZDs may result in low bone mineral density and increased fracture rates (Loke *et al.* 2009, Solomon *et al.* 2009). Understanding the

molecular mechanisms underlying TZD-induced side effects is central to the further development and successful therapeutic use of these drugs.

In rodent models, TZD-induced low bone mineral density and reduced bone formation have been attributed to the promotion of adipogenesis at the expense of osteogenesis (Johnson *et al.* 1999, Lecka-Czernik *et al.* 1999, Sottile & Seuwen 2000, Shockley *et al.* 2007, Nishii *et al.* 2009, Cho *et al.* 2012). This is thought to be due to a shift in progenitor cell lineage allocation, as osteoblasts and adipocytes originate from the same

mesenchymal stem cell (MSC) population (Owen 1988). The commitment of MSCs during differentiation towards either adipogenesis or osteogenesis involves a stochastic mechanism under the control of lineage-specific transcription factors. Runt domain-containing protein, RUNX2 (also known as CBFA1 or OSF2), Osterix and msh homoeobox 2 (*Msx2*) play an essential role in osteoblast differentiation, and expression of these transcription factors is restricted to cells with an osteoblastic lineage (Ducy *et al.* 1997, Komori *et al.* 1997, Satokata *et al.* 2000, Nakashima *et al.* 2002, Nakashima & de Crombrughe *et al.* 2003). However, osteoblastic commitment may be radically usurped through activation of peroxisome proliferator-activated receptor gamma (*Pparγ* (*Pparg*)), which promotes adipogenesis (Nishii *et al.* 2008). Nishii *et al.* (2008) demonstrated that treatment of immortalised bone marrow stromal cells (TBR31-2) with the *Pparγ* agonist troglitazone resulted in a marked decrease in osteogenic-specific genes (*Runx2*, alkaline phosphatase (*Alp*), bone Gla protein (*Bgp* (*Bglap*)), and type I collagen (*Col1a1*)) and induced the expression of the adipocytic markers adipsin (*Adps*) and lipoprotein lipase (*Lpl*). Furthermore, Cho *et al.* (2012) indicated that rosiglitazone-induced activation of *Pparγ* reduced collagen and osteocalcin (BGLAP) levels in mouse primary bone marrow-derived MSCs and prevented the mRNA expression of *Runx2*. These inhibitory effects of *Pparγ* activation on osteoblastogenesis are further exemplified in *Pparγ* null mutant embryonic stem cells, which spontaneously differentiate into osteoblasts, and in heterozygous *Pparγ*-deficient mice, which exhibit high bone mass and increased osteoblastogenesis (Akune *et al.* 2004).

There are two distinct isoforms of *Pparγ* (Zhu *et al.* 1995) in humans and mice. *Pparγ1* (*Pparg1*) is predominantly expressed on osteoblasts, whereas *Pparγ2* (*Pparg2*) is specific to the adipocyte lineage (Lecka-Czernik *et al.* 1999, Ren *et al.* 2002). Naïve cells express lower levels of both *Pparγ1* and *Pparγ2* isoforms compared with lineage-committed cells (Sewter *et al.* 2002). Shockley *et al.* (2007) investigated the expression of gene markers associated with stem cell phenotype using DNA microarray analysis of U-33 stromal cells transfected with *Pparγ2* (U-33/ γ 2) or vector control cells treated with TZDs. The authors demonstrated that rosiglitazone-induced activation of *Pparγ2* down-regulates the expression of multiple stemness (*Abcg2*, *Egfr*, and *Cd44*) genes. Taken together, these results suggest that *Pparγ* negatively regulates stromal cell plasticity and promotes adipogenesis at the expense of osteogenesis.

These findings have recently been contradicted by Beck *et al.* (2013), who demonstrated that *ex vivo* TZD exposure

stimulates adipogenesis, but does not directly alter osteoblast differentiation or lineage commitment in non-diabetic human bone marrow-derived MSCs. In the same study, the *in vivo* treatment of type 2 diabetic patients with rosiglitazone and pioglitazone did, however, decrease *ex vivo* osteogenic differentiation of bone marrow-derived MSCs, indicating an indirect negative effect on osteoblasts. This suggests that metabolic status influences the effects of TZDs on bone quality. The objective of this study was thus to investigate the influence of TZDs on both adipogenic and osteogenic differentiation of primary MSCs with distinct metabolic profiles. This study therefore utilised primary MSCs derived from either visceral (PV) or subcutaneous (SC) adipose tissue, as depot-specific metabolic differences exist between PV and SC adipose tissue (Dusserre *et al.* 2000, Lafontan & Berlan 2003).

In addition to the metabolic influence as a possible explanation as to why studies utilising various *Pparγ* agonists have yielded diverse results, another possible explanation for the diverse outcome of studies is that different types of TZDs are known to have distinct yet subtle difference in the affinity for *Pparγ* activation. Generally, high-affinity *Pparγ* ligands such as rosiglitazone, troglitazone, and pioglitazone decrease osteoblast proliferation and promote differentiation into an adipocytic phenotype (Johnson *et al.* 1999, Lecka-Czernik *et al.* 1999, Sottile & Seuwen 2000), whereas low-affinity *Ppar* pan agonists, such as netoglitazone (Chang *et al.* 2007), have been reported to have a little negative effect on the skeleton in animal models. Lazarenko *et al.* (2006) investigated the pro-adipocytic and anti-osteoblastic activities of netoglitazone compared with those of rosiglitazone in immortalised U-33/ γ 2 stromal cells, primary bone marrow MSCs, and *in vivo* in C57BL/6 mice. The authors demonstrated that the expression of osteoblast-specific gene markers (*Runx2*, *Dlx5*, *Bglap*, and *collagen*) remained unchanged and that *in vivo* netoglitazone treatment did not result in trabecular bone loss.

As differences between various types of TZDs and metabolism can potentially influence the outcome of studies investigating the effect of TZD treatment on osteogenesis, this study aimed to directly compare the influence of rosiglitazone, pioglitazone (high affinity for *Pparγ*), and netoglitazone (low affinity for *Pparγ*) on the differentiation potential of primary MSCs with distinct metabolic profiles.

Materials and methods

This research study was approved by the Stellenbosch University Animal Ethics committee and complies with

the South African Animal Protection Act (Act no 71, 1962). All experiments were conducted in accordance with the South African Medical Research Council Guidelines on Ethics for Medical Research.

Isolation of rat adipose-derived MSCs

Male Wistar rats ($n=3$) that were 10 weeks old with an average weight of 250 ± 10 (mean $\pm$ s.d.) g were used in all experiments. Animals were killed by injecting 12 mg/kg sodium pentobarbitone (Eutha-naze Ref83/91, #22079248DJ, Bayer), after which mesenchymal stromal cells were isolated from inguinal SC and peri-renal PV adipose tissues. The isolation procedure was adapted from Huang *et al.* (2002) as described previously (Sadie-Van Gijzen *et al.* 2010). Briefly, the excised tissue was rinsed with 75% (v/v) ethanol (EtOH) and placed in high-glucose (4.5 g/l glucose; L-glutamine) DMEM (BioWhittaker, #BE12-604F, Lonza, Basel, Switzerland). Tissue samples were diced and digested at 37 °C for 30 min in 0.075% (w/v) collagenase I (CLS1, #XOM12195J, Worthington, Lakewood, NJ, USA) dissolved in Hanks' balanced salt solution (BioWhittaker, #10-508F, Lonza) containing 1.5% BSA. Digested samples were subjected to centrifugation for 5 min at 250 g and the resulting pellets were washed with PBS. The pellets containing MSCs were re-suspended in DMEM containing 20% foetal bovine serum (FBS; Biochrom, S0615, 0459B, Berlin, Germany) and seeded into two 100 $\times$ 20 mm culture dishes (#430167, Corning Life Sciences, Tewksbury, MA, USA). MSCs were maintained at 37 °C, in 90% humidified air with 5% CO₂. Growth media containing 20% FBS were replaced after 24 h with standard growth media (SGM) containing high-glucose (4.5 g/l) DMEM with 1% penicillin/streptomycin (BioWhittaker, #DE17-6026, Lonza) supplemented with 10% FBS (Biochrom, S0615, 0459B). For culture expansion, cells were treated with 0.5% trypsin (200 mg/l Versene EDTA, #BE171616, Lonza) and sub-cultured at a dilution of 1:4. All subsequent experimental procedures were carried out on cells in passage two and were repeated in triplicate wells for MSCs derived from three animals.

MSC characterisation

MSCs reaching 80% confluence in passage two under standard culturing conditions were harvested through trypsinisation and re-suspended in PBS containing 1% BSA (#A4503-100G, Sigma-Aldrich). Cell suspensions of 1×10^6 cells per 100 μ l were stained with mouse anti-rat FITC-conjugated CD90 (Thy-1; 5 μ l, #MR5001, Invitrogen)

or FITC-conjugated CD45 (2.5 μ l, #MR6901, Invitrogen) fluorescent antibodies. Flow cytometry was performed on the FACSCalibur using the CellQuest software. A total of 15 000 events were recorded and data analysis was performed using the WinMDI 2.9 (J.Trotter) and Flow Jo Vx (Treestar) software.

Medium conditions for differentiation

MSCs that were 2 days post-confluence in passage two were used for all differentiation experiments. Standard adipogenic media (AM) contained SGM (refer to section 'Isolation of rat adipose-derived MSCs') supplemented with 10 μ M insulin (19278-5ML, SLBD5980, Sigma Life Sciences), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, STBC7632V, Sigma Life Sciences), 1 μ M dexamethasone (D4902-100MG, BCBK1265V, Sigma Life Sciences), and 56 μ M indomethacin (17378-5G, 064K1207, Sigma Life Sciences). Standard osteogenic differentiation media (OM) contained SGM supplemented with 10 nM dexamethasone (D4902-100MG, BCBK1265V, Sigma Life Sciences), 50 μ M ascorbic acid (A4544-25G, SLBB4446V, Sigma Life Sciences), and 10 mM β -glycerophosphate (G5422-100G, 029K54241V, Sigma Life Sciences). Stock solutions of dexamethasone and indomethacin were prepared with ethanol, whereas IBMX and GW9662 (#70785, Cayman Chemicals, Ann Arbor, MI, USA) stock solutions were prepared with dimethyl sulphoxide (DMSO, 1029873, Merck). Corresponding volumes of ethanol and DMSO were added to all cell culture conditions as vehicle controls.

TZD treatment

MSCs undergoing either adipogenesis or osteogenesis under differentiation conditions were treated with 1 μ M rosiglitazone (rosi), pioglitazone (pio), netoglitazone (neto) (Table 1) or indomethacin (indo; affinity for PPAR γ : $K_d \sim 10^{-7}$ M, Lee *et al.* 2012). Stock solutions of TZDs were prepared with DMSO (1029873, Merck). A corresponding volume of DMSO was added to SGM for culturing untreated cells to serve as a vehicle control. MSCs were treated for 21 days during osteogenic differentiation with the media being changed twice weekly. For adipogenic differentiation, MSCs were treated for 14 days with the media being changed three times per week.

Histology

Lipid accumulation during both adipogenesis and osteogenic differentiation was assessed by Oil red O staining.

Table 1 Properties of thiazolidinediones used in this study

	PPAR γ affinity (K_d)	PPAR γ activation (EC_{50} , nM)	Antihyperglycaemic potency ^a (ED25, mg/kg)
Rosiglitazone	43 nM ^b	30–200 ^c	7.1
Pioglitazone	NA	400–500 ^d	13.9
Netoglitazone	Ten- to 50-fold lower affinity than rosiglitazone ^e	8000 ^e	2.7

NA, not available.

^aDetermined in KK-A γ mice.^bMouse PPAR γ 1 ligand-binding domain, Lehmann *et al.* (1995).^cLehmann *et al.* (1995), Reginato *et al.* (1998), and Sakamoto *et al.* (2000).^dLehmann *et al.* (1995) and Sakamoto *et al.* (2000).^eReginato *et al.* (1998).

Briefly, the media were discarded and cells were stained with 0.7% (w/v) Oil red O prepared in 70% (v/v) isopropanol. After 30 min at room temperature, the stain was removed and cells were washed three times with distilled water before being photographed for image analysis. Mineralisation during osteogenic differentiation was assessed by Alizarin red S staining. Briefly, cells were washed twice with PBS before being fixed in 70% EtOH (v/v) for 5 min. Fixed cells were washed with distilled water and stained with 100 mM Alizarin red S (pH 4.0–4.1; 9436, Amresco, Solon, OH, USA) overnight at room temperature. Unbound Alizarin red S was removed by washing three times with distilled water and once with PBS before being photographed for image analysis. Image acquisition was carried out using an Olympus light microscope (CKX41, CachN 10 $\times$ /0.25 PhP objective, EOS600D Canon digital camera). All experiments were carried out in triplicate for MSCs derived from three rats

and four images (one image from each quartile) were taken per well. For each treatment condition, a total of 48 images were thus analysed using the ImageJ software (version 1.46, nih.gov). The extent of lipid accumulation or mineralisation was expressed as the percentage of positively stained surface area per well.

RNA isolation and quantitative real-time PCR

Total RNA was isolated on days 7 and 10 of differentiation using the RNeasy RNA isolation kit (Qiagen, #74106) as per the manufacturer's instructions. DNase-treated RNA samples were used as templates for cDNA synthesis, using 20-(dT) as a primer with Promega ImProm-2 reverse transcriptase. Real-time semi-quantitative PCR was carried out on a Rotor-Gene (Qiagen) using the Quantace Sensimix kit. Relative gene expression (Table 2) was calculated according to the ΔC_t method (Pfaffl 2001) by comparison

Table 2 Primer sequences and conditions used in semi-quantitative real-time PCR

Transcripts (reference)	Primer sequences (5'–3')	Product size (bp)
<i>Pparγ2</i> (<i>Pparg2</i>) (Tanabe <i>et al.</i> 2004)	F: ACT GCC TAT GAG CAC TTC AC R: CAA TCG GAT GGT TCT TCG GA	448
<i>Ap2</i> (<i>Fabp4</i>) (Fukuen <i>et al.</i> 2005)	F: TGA AAT CAC CCC AGA TGA CAG R: CTC ATG CCC TTT CAT AAA CT	194
<i>Adipsin</i> (<i>Adps</i>) ^a	F: CAC GTG TGC GGT GGC ACC CTG R: CCC CTG CAA GTG TCC CTG CGG T	476
<i>Msx2</i> (Kato <i>et al.</i> 2009)	F: TCA CCA CGT CCC AGC TTC TAG R: AGC TTT TCC AGT TCC GCC TCC	178
Alkaline phosphatase (<i>Alp</i>) (Kuroda <i>et al.</i> 2005)	F: GAG CAG GAA CAG AAG TTT GC R: GTT GCA GGG TCT GGA GAG TA	202
Collagen 1 α 1 (An <i>et al.</i> 2006)	F: GAC GTC CTG GTG AAG TTG G R: AGC CAC GAT GAC CCT TTA TGR	588
<i>Arbp</i> (<i>Rplp0</i>) (van Wijngaarden <i>et al.</i> 2007)	F: AAA GGG TCC TGG CTT TGT CT R: GCA AAT GCA GAT GGA TCG	91

^aPrimers designed based on the rat *Adipsin* gene sequence in GenBank: NCBI Reference Sequence NM_001077642.1.

with the housekeeping gene acidic ribosomal phosphoprotein (*Arbp* (*Rplp0*)) (van Wijngaarden *et al.* 2007).

Statistical analysis

Values are expressed as mean $\pm$ s.d. Statistical analysis was performed using the Statistica software (StatSoft, version 10). Factorial ANOVA with Tukey's *post hoc* test was used to determine the group effect, treatment effect or group $\times$ treatment effect. Groups were classified as either SC MSCs or PV MSCs. Treatment effect was classified as the effect of a specific TZD on MSC differentiation in specific culture medium conditions. The level of significance was set at $P < 0.05$.

Results

MSC characterisation

Primary MSCs isolated from SC and PV rat adipose tissue depots were characterised using cell surface markers to determine the purity of the expanded cell populations used for subsequent experiments. The progenitor cell-associated surface marker CD90 (Thy-1) was highly expressed on both SC ($99.4 \pm 0.3\%$) and PV ($97.3 \pm 1.1\%$) MSCs, whereas very few cells expressed the haematopoietic lineage-specific marker CD45 (SC $1.2 \pm 1.0\%$; PV $0.6 \pm 0.2\%$) (Fig. 1). The isolated MSCs furthermore did not spontaneously differentiate and could thus be characterised as naïve.

Adipogenesis

No lipid accumulation was evident under SGM conditions (Fig. 2A and B). MSCs were cultured in standard adipogenic media (AM) in order to compare the rate of adipogenic differentiation between SC and PV MSCs. A higher percentage of lipid accumulation was evident in PV MSCs ($5.97 \pm 0.98\%$) compared with SC MSCs ($3.03 \pm 0.45\%$) ($P < 0.01$; Fig. 2A and B). The importance of *Ppar γ* activation during adipogenesis was assessed by either the addition of a *Ppar γ* antagonist (GW9662) or by the removal of the *Ppar γ* agonist (indomethacin) from the adipogenic media. Lipid accumulation was significantly ($P < 0.001$) reduced in both SC and PV MSCs in the presence of GW9662 and in the absence of indomethacin (AM^{Indo-}) (Fig. 2A and B). The capacity of each of the TZDs to rescue adipogenesis in cells grown either in the presence of GW9662 or in the absence of indomethacin in adipogenic media (AM) was assessed. Rosiglitazone and

Figure 1

MSC surface marker expression. (A) SC MSCs: CD90 + CD45. (B) PV MSCs: CD90 + CD45. A total of 15 000 events were recorded, of which 9948 ± 401 and 8977 ± 745 were gated as SC and PV respectively.

pioglitazone were able to partially rescue lipid accumulation (SC > PV MSCs) in the presence of GW9662 and in the absence of indomethacin from the differentiation media (Fig. 2C and D). Netoglitazone was unable to rescue lipid accumulation in the presence of GW9662 in either cell type, i.e. SC or PV MSCs (Fig. 2C). In the absence of indomethacin from the differentiation media, netoglitazone was, however, able to partially rescue lipid accumulation (PV > SC MSCs; Fig. 2D). Despite the lack of indomethacin and the addition of GW9662, rosiglitazone, and pioglitazone, but not netoglitazone, were still able to partially rescue adipogenesis (Fig. 2E). TZD treatment

Figure 2

Adipogenesis: lipid accumulation. (A) Oil red O staining (day 14). (B) Lipid accumulation under different medium conditions without TZD treatment. (C) TZD treatment in AM with PPAR γ antagonist (GW9662). (D) TZD treatment in AM without indomethacin. (E) TZD treatment in AM without indomethacin and with added GW9662. Statistical analysis: * $P < 0.01$, *** $P < 0.001$ indicate significant difference from AM (B) $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$ \$\$}P < 0.001$ indicate significant difference between cell types within the same media and TZD treatment conditions (B and D).

Factorial ANOVA with Tukey's *post hoc* test. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ indicate significant difference from control media within the same cell type. + $P < 0.05$ and +++ $P < 0.001$ indicate significant difference between TZD treatments in the same cell type. $^{\$}P < 0.05$ and $^{\$ \$\$}P < 0.001$ indicate significant difference between cell types within the same media and TZD treatment conditions. A full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-14-0425>.

under standard adipogenic conditions (AM) did not enhance lipid accumulation compared with AM without TZD treatment (data not shown).

Osteogenesis

No mineralisation or lipid accumulation was evident in either SC or PV MSCs maintained in SGM (Fig. 3A and B). MSCs were cultured in standard OM in order to compare osteogenic differentiation between SC and PV MSCs. Mineralisation was evident in both SC ($10.3 \pm 2.0\%$) and PV MSCs ($8.6 \pm 1.1\%$) under standard OM conditions (Fig. 3B and C). The addition of the PPAR γ antagonist GW9662, however, resulted in the highest levels of mineralisation (% surface area stained positive for Alizarin red) in both cell types (SC MSCs $25.5 \pm 3.0\%$ and PV MSCs $27.7 \pm 4.6\%$) (Fig. 3B and C). To determine whether TZD

treatment has a negative influence on osteogenesis, MSCs subjected to standard osteogenic conditions (OM) were treated with either rosiglitazone, pioglitazone or netoglitazone. TZD treatment did not negatively affect mineralisation in either SC or PV MSCs compared with OM without TZD treatment (Fig. 3C and E). In the presence of GW9662, TZD treatment did, however, reduce mineralisation compared with OM+GW9662 without TZD treatment ($P < 0.01$) (Fig. 3E), suggesting that TZD treatment counters the positive effect of GW9662 on mineralisation. Pronounced lipid accumulation was evident in PV MSCs during osteogenesis by treatment with indomethacin ($5.4 \pm 0.8\%$), rosiglitazone ($13.9 \pm 1.7\%$), or pioglitazone ($10.6 \pm 1.3\%$) compared with OM without TZD treatment ($0.4 \pm 0.1\%$) (Fig. 3F and G). However, netoglitazone treatment did not result in lipid accumulation during osteogenesis (Fig. 3F). During osteogenesis, PV MSCs were

Figure 3

Osteogenesis: mineralisation. (A) No evidence of mineralisation in SGM with or without PPAR γ antagonist (GW9662). (B) Mineralisation in different medium conditions without TZD treatment. (C) Alizarin red S staining (day 21) demonstrating mineralisation with lipid droplets evident with TZD treatment. (D) Mineralisation with TZD treatment in OM. (E) Mineralisation with TZD treatment in OM + GW9662. (F) Lipid accumulation with TZD treatment in OM. (G) Lipid accumulation with TZD treatment in OM + GW9662. Statistical analysis: factorial ANOVA with

Tukey's *post hoc* test. $^{##}P < 0.01$ and $^{###}P < 0.001$ indicate significant difference between SGM and other medium conditions (B). $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant difference between OM and OM + GW9662 (B) and significant TZD treatment effect (D, E, F and G) in the same cell type. $^{SSS}P < 0.001$ indicate significant difference between SC and PV MSCs in the same treatment conditions. A full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-14-0425>.

more prone to lipid accumulation than SC MSCs in response to TZD treatment (Fig. 3F and G). Lipid droplet accumulation was furthermore evident in areas of mineralisation (Fig. 3C) in both SC and PV MSCs.

Relative mRNA expression of adipogenic genes

The relative gene expression levels of the adipocytic genes *Pparγ2*, *Ap2* (*Fabp4*), and *Adipsin* were determined to assess whether TZD treatment skews osteogenesis towards adipogenesis. Consistent with histological observations, PV MSCs were more adipogenic than SC MSCs. In PV MSCs, on day 10 of adipogenic differentiation (AM), the expression of adipogenic genes (*Pparγ2*, *Ap2*, and *Adipsin*) was significantly elevated ($P < 0.01$) compared with control conditions (SGM). Despite significant lipid accumulation observed with TZD treatment during osteogenesis, *Pparγ2* expression during rosiglitazone, pioglitazone, and netoglitazone treatment was less compared with AM and not significantly different from control media (SGM; Fig. 4A and B). Rosiglitazone and pioglitazone treatment induced modest expression of *Ap2* (relative expression, AM=1; Rosi 0.53 ± 0.2 ; Pio 0.47 ± 0.2) on day 10 in PV MSCs, whereas netoglitazone did not (relative expression, AM=1; 0.03 ± 0.01) (Fig. 4C and D). TZD treatment during osteogenesis was, however, unable to induce the expression of *adipsin* (Fig. 4E and F).

Relative mRNA expression of osteogenic genes

The relative gene expression levels of the osteogenic genes, *Msx2*, *Collagen I*, and *Alp*, were assessed to determine whether TZD treatment has a negative influence on osteogenesis. TZD treatment did not downregulate the expression of the pro-osteogenic genes (*Msx2*, *Collagen I*, and *Alp*) during osteogenesis (Fig. 5A, B, C, D, E, and F). In PV MSCs, pioglitazone treatment increased *Msx2* expression after 10 days of osteogenic differentiation, whereas no effect was observed in SC MSCs (Fig. 5A and B).

Discussion

This is the first investigation to directly compare the effect of three different TZDs on the differentiation of MSCs towards the adipogenic or osteogenic lineage. Uniquely, it was found that although TZD treatment did not negatively affect mineralisation, *Pparγ* ligands (indomethacin, rosiglitazone, and pioglitazone) promoted the accumulation of lipid droplets during osteogenesis. In

addition, the partial inhibition of basal *Pparγ* activity under standard osteogenic conditions markedly increased mineralisation. The absence of a *Pparγ* agonist from the adipogenic media or the addition of a *Pparγ* antagonist reduced lipid accumulation and indicated the known importance of *Pparγ* activation during adipogenesis (Lecka-Czernik *et al.* 2002). The direct treatment comparison between three different TZDs demonstrated that unlike rosiglitazone and pioglitazone, the *Ppar* pan agonist, netoglitazone, was not able to rescue adipogenesis in the presence of the *Pparγ* antagonist GW9662 and induced the least lipid accumulation during osteogenesis. Despite indirect evidence pointing towards the skewing of MSC differentiation towards adipogenesis with TZD treatment, the expression of the adipogenic genes *Pparγ* and *Ap2* was only modestly increased compared with SGM, whereas the expression of the adipocyte-specific marker *Adipsin* was not induced by TZD treatment. Taken together, the data therefore suggest that aberrant lipid droplet formation occurred concurrently with mineralisation and that differentiation was not overtly skewed towards adipogenesis.

The negative influence of TZDs on bone mineral density has been as attributed to the promotion of adipogenesis at the expense of osteogenesis, i.e. reduced bone formation due to a shift in MSC lineage allocation. Challenging this generally accepted view, Bruedigam *et al.* (2010) proposed an alternative concept explaining the detrimental effects of TZDs on bone. The authors utilised human preosteoblasts and osteoblasts that were already committed to the osteogenic lineage and demonstrated that the addition of rosiglitazone to osteogenic differentiation medium accelerated osteoblast differentiation and was followed by an accumulation of reactive oxygen species that lead to osteoblast apoptosis, whereas cells from the adipogenic lineage (adipocytes) were not apoptotic following rosiglitazone treatment. In the same investigation, MSCs pre-treated with rosiglitazone did not preferentially differentiate into adipocytes. Taken together, these findings suggest that cells of the osteogenic lineage are more susceptible to TZD-induced apoptosis than cells of the adipogenic lineage (Bruedigam *et al.* 2010). The authors concluded that compared with adipocytes, oxidative stress and apoptosis are preferentially triggered in osteoblasts by activated *Pparγ*, explaining the suppressive action of TZD treatment on bone (Bruedigam *et al.* 2010). In the current investigation, TZD treatment during osteogenesis (without GW9662) did not negatively influence mineralisation. The addition of a *Pparγ* antagonist (GW9662) to

Figure 4

Pro-adipogenic gene expression. The relative mRNA expression of *Pparγ2* (A and B), *Ap2* (C and D), and *Adipsin* (E and F) in SC MSCs and PV MSCs. Statistical analysis: factorial ANOVA with Tukey's *post hoc* test. * $P < 0.05$

and ** $P < 0.01$ indicate significant treatment effect compared with AM at the same time point. Note: values are expressed as the relative expression of *Pparγ2*, *Ap2*, or *Adipsin* compared with the housekeeping gene, *Arbp*.

osteogenic media without TZD treatment did, however, significantly increased mineralisation. In an investigation similar to that by Bruedigam *et al.* (2010), Wang *et al.* (2012) investigated the effect of TZD treatment on

the trans-differentiation of preosteoblasts (bone marrow-derived MSCs differentiated towards the osteogenic lineage) into adipocytes. Following either 5 or 14 days of osteogenic differentiation of MSCs, osteogenic media

Figure 5

Pro-osteogenic gene expression. The relative mRNA expression of *Msx2* (A and B), *Collagen I* (C and D), and *alkaline phosphatase* (E and F) in SC MSCs and PV MSCs. Statistical analysis: factorial ANOVA with Tukey's *post hoc* test.

* $P < 0.05$ indicate significant treatment effect compared with OM at the same time point. Note: values are expressed as the relative expression of *Msx2*, *Collagen I*, or *Alp* compared with the housekeeping gene, *Arbp*.

were changed to adipogenic media with or without the addition of pioglitazone (21 days). The authors demonstrated that MSCs subjected to osteogenic conditions for shorter durations retained a stronger ability to differentiate into adipocytes. Trans-differentiation was furthermore enhanced by pioglitazone treatment and was associated with decreased mRNA expression of osteogenic genes (*Runx2* and *Alp*) and increased *Ppar γ* expression.

In the current investigation, simultaneous mineralisation and lipid droplet formation were evident with indomethacin, rosiglitazone, and pioglitazone treatment during osteogenesis. Despite modest increases in the expression of *Ap2* with rosi- and pio-treatment, the change in *Ppar γ* levels was insufficient to induce the downstream expression of the adipocyte-specific marker, *Adipsin* (Duan *et al.* 2014). Novel data from this study therefore suggest that lipid droplet formation during mineralising conditions may not necessarily be the result of the skewing of differentiation towards adipogenesis. Netoglitazone treatment during osteogenesis did, however, result in less lipid accumulation compared with rosiglitazone and pioglitazone treatment, TZDs that have a high affinity for *Ppar γ* , which may be due to the limited ability of netoglitazone to activate *Ppar γ* . This is consistent with *in vitro* studies utilising U-33 stromal cells transfected with *Ppar γ 2* (U-33/y2), vector control cells (U-33/c) and primary bone marrow MSC cultures, which showed that netoglitazone is less effective than rosiglitazone in inducing lipid accumulation (Lazarenko *et al.* 2006). Contradicting the *in vitro* observations, analysis of the trabecular bone marrow composition in the proximal tibia of CD7BC/6 mice following 7 weeks of TZD treatment demonstrated similar increases in adiposity with netoglitazone and rosiglitazone treatment (Lazarenko *et al.* 2006). The authors did, however, demonstrate that despite the accumulation of adipocytes, chronic netoglitazone administration had little to no negative effect on the skeleton *in vivo* (Lazarenko *et al.* 2006). Taken together with the above findings, data from the current investigation suggest that the extent of TZD-induced lipid accumulation is highly dependent on the nature of these *Ppar* ligands.

Lecka-Czernik *et al.* (2002) have previously investigated the influence of diverse *Ppar γ* ligands on MSC (Swiss-Webster mice bone marrow-derived MSCs and U-33/y2) differentiation. The authors demonstrated that, although some naturally occurring *Ppar γ* ligands (9,10-dihydroxyoctadecanoic acid and 15-deoxy- $\Delta^{12,14}$ -PGJ₂) stimulate adipogenesis and inhibit osteoblast

differentiation, other ligands (9,10-epoxyoctadecanoic acid and thiazolidine acetamide ligand GW0072) prevent osteogenesis but do not stimulate adipogenesis. The authors suggested that the pro-adipogenic and anti-osteoblastogenic effects of *Ppar γ* are regulated by diverse downstream regulatory pathways that can be differentially modulated depending on the nature of the ligand. *Ppar* pan-agonist and dual-agonist drugs such as netoglitazone were thus developed to combine the triglyceride-lowering and HDL cholesterol-elevating effects of the *Ppar α* (*Ppara*) agonists with the insulin sensitisation elicited by the *Ppar γ* agonists (Liu *et al.* 1998, Upton *et al.* 1998, Pickavance *et al.* 1998, 2003, Chang *et al.* 2007), while limiting negative effects on the skeleton (Lazarenko *et al.* 2006). Unfortunately, to date, *Ppar γ /Ppar α* dual agonist treatment is associated with unacceptable adverse effects that have delayed clinical application of these drugs (Chang *et al.* 2007). In addition to the diverse nature of *Ppar* ligands, the current study also demonstrated that the metabolic status of MSCs influences the ability of TZDs to induce lipid droplet accumulation in both adipogenic and osteogenic medium conditions.

SC adipose tissue has a higher propensity for lipogenesis and fatty acid uptake than its PV counterpart (for review, see Lafontan *et al.* (2003)). Nonetheless, this study demonstrated that, compared with SC MSCs, PV MSCs are more prone to lipid accumulation under controlled differentiation conditions *in vitro*. The difference in the extent of lipid accumulation between PV and SC MSCs can in part be explained by their divergent lipogenic responses to insulin. Sadie-Van Gijzen *et al.* (2010) demonstrated that insulin contributes to lipid accumulation in PV MSCs but is not essential for adipogenesis in SC MSCs. The authors furthermore demonstrated that the presence of insulin in adipogenic media increased SC MSC proliferation and suggested that, in hyper-insulinaemic states, SC MSCs increase in number while maintaining intracellular lipid content in a non-pathological range, whereas PV MSCs are more prone to hypertrophy during lipid accumulation. In this study, the substitution of indomethacin with various TZDs in adipogenic differentiation media demonstrated that MSCs derived from adipose tissue with diverse lipogenic responses to insulin also have a diverse response to specific TZDs. PV MSCs were more responsive to pioglitazone and netoglitazone, whereas SC MSCs had a higher percentage of lipid accumulation when treated with rosiglitazone. Furthermore, during osteogenesis, the addition of indomethacin, rosiglitazone, and pioglitazone induced lipid droplet formation only in PV MSCs. This study is thus the first to demonstrate that metabolically

diverse mesenchymal stromal cells respond differently to *in vitro* TZD treatment.

Conclusion

It is important to identify whether factors such as obesity and altered metabolism in addition to drug treatment can affect stem cell behaviour specifically because of the fact that, in the context of type 2 diabetes, patients often have altered metabolic profiles. Owing to the severity of the 'off-target' effects of TZDs, clinical administration of these drugs has been under the spotlight in recent years (Yau *et al.* 2013). Pioglitazone is, however, still available for clinical use despite the potential long-term adverse effects, such as 'fatty bones', which may progress towards osteoporosis. The novel evidence in this study demonstrates that lipid droplet formation and mineralisation can occur concurrently in response to TZD treatment during osteogenesis and that lipid accumulation was more pronounced in MSCs derived from peri-renal PV adipose tissue than in MSCs derived from its SC counterpart. This investigation furthermore demonstrates that the extent of lipid accumulation is dependent on the nature of the *Ppar* ligand and that metabolic status can contribute to the adverse effects associated with TZD treatment. Unfortunately, owing to the limitations associated with the *in vitro* nature of this study, the observations cannot be extrapolated to the *in vivo* situation. Future studies will therefore focus on *in vivo* TZD treatment models, comparing the *ex vivo* differentiation of bone marrow-derived MSCs from healthy/normal and obese animals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All the authors contributed to the conceptual design of the study and interpretation of data. Drs M v, E A, and I L C were involved in the experimental procedures, sample collection and sample analysis. Dr M v was responsible for data analysis and writing of the manuscript. Prof. W F F contributed to the interpretation of data and editing of the manuscript.

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