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Enhanced bone formation in lipodystrophic PPARγ$^{hyp/hyp}$ mice relocates haematopoiesis to the spleen

Terrie-Anne Cock[1], Jonathan Back[1]*, Florent Elefteriou[2], Gérard Karsenty[2], Philippe Kastner[1], Susan Chan[1] & Johan Auwerx[1,3+]*

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, Illkirch, France, [2]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA, and [3]Institut Clinique de la Souris, Illkirch, France

The peroxisome proliferator-activated receptor gamma (PPARγ) controls adipogenesis and metabolism. We demonstrate here that the absence of PPARγ in fat has potent osteogenic activities, which affect haematopoiesis. The congenital absence of PPARγ in fat of lipodystrophic PPARγ$^{hyp/hyp}$ mice, strongly enhanced bone mass and consequentially reduced the bone-marrow cavity. Consistent with this, PPARγ$^{hyp/hyp}$ mice had a significant decrease in bone marrow cellularity and resorted to extramedullary haematopoiesis in the spleen to maintain haematopoiesis. Our data indicate that antagonizing PPARγ activity in fat could be an effective way to combat osteoporosis and suggest that haematopoietic function should be scrutinized in lipodystrophic subjects.

Keywords: osteoporosis; nuclear receptors; lipodystrophy; extramedullary haematopoiesis

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INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that controls not only adipogenesis but also the endocrine function of adipose tissue (Cock *et al*, 2004). PPARγ activity is governed by binding of fatty acids or synthetic compounds with antidiabetic activity, including thiazolidinediones, such as rosiglitazone and pioglitazone (Picard & Auwerx, 2002). These compounds represent one of the most effective therapies for type 2 diabetes. PPARγ activation, however, induces multiple effects that go beyond the control of metabolism and range from effects on cell proliferation and immune function, to the most recent implication in bone homeostasis.

The influence of PPARγ on bone homeostasis was first indicated by a silent single-nucleotide polymorphism of PPARγ that was associated with lower bone mineral density (BMD) (Ogawa *et al*, 1999) and higher leptin levels (Meirhaeghe *et al*, 1998). Genetic, genomic and pharmacological studies suggested that lipoxygenase 12/15, an enzyme that produces endogenous PPARγ ligands (Klein *et al*, 2004), and synthetic PPARγ agonists (Rzonca *et al*, 2004) adversely affect bone formation in mice. The interpretation of such studies, using natural and synthetic PPARγ agonists, is complicated because they reflect PPARγ-dependent and PPARγ-independent effects (Picard & Auwerx, 2002). Genetic PPARγ-deficient animal models are not fraught with such problems and allow careful dissection of receptor-mediated effects. The recent observation that heterozygous PPARγ deficiency in mice enhanced bone mass due to increased osteoblastogenesis is consistent with a role of PPARγ in determining bone mass (Akune *et al*, 2004), but it is still not defined in which tissue PPARγ contributed to enhanced bone formation.

We independently ascertained the contribution of PPARγ to bone homeostasis, in the severely lipodystrophic PPARγ$^{hyp/hyp}$ mice, which have a hypomorphic mutation at the PPARγ2 locus that not only results in an absence of PPARγ2 expression but also drastically reduces PPARγ1 levels to almost undetectable levels in white adipose tissue (WAT; Koutnikova *et al*, 2003). This congenital and selective deficit in PPARγ expression in WAT of the PPARγ$^{hyp/hyp}$ mice results in lipodystrophy, which severely impedes bone marrow haematopoiesis, favouring extramedullary haematopoiesis in the spleen, highlighting the importance of PPARγ in WAT in whole-body homeostasis.

RESULTS

The absence of PPARγ changes bone architecture

Previously, we demonstrated that the PPARγ$^{hyp/hyp}$ mice, which congenitally lack PPARγ in WAT, were severely lipodystrophic (Koutnikova *et al*, 2003). Dual-energy X-ray absorptiometry (DEXA) analysis in PPARγ$^{hyp/hyp}$ mice showed that both sexes not only had a lower fat mass (Fig 1A) but also an increased BMD and bone area (Fig 1B,C). Micro-computed tomography (micro-CT)

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, 67404 Illkirch, France
[2]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA
[3]Institut Clinique de la Souris, 67404 Illkirch, France
*These authors contributed equally to this work
+Corresponding author. Tel: +33 388 653425; Fax: +33 388 653201;
E-mail: auwerx@igbmc.u-strasbg.fr

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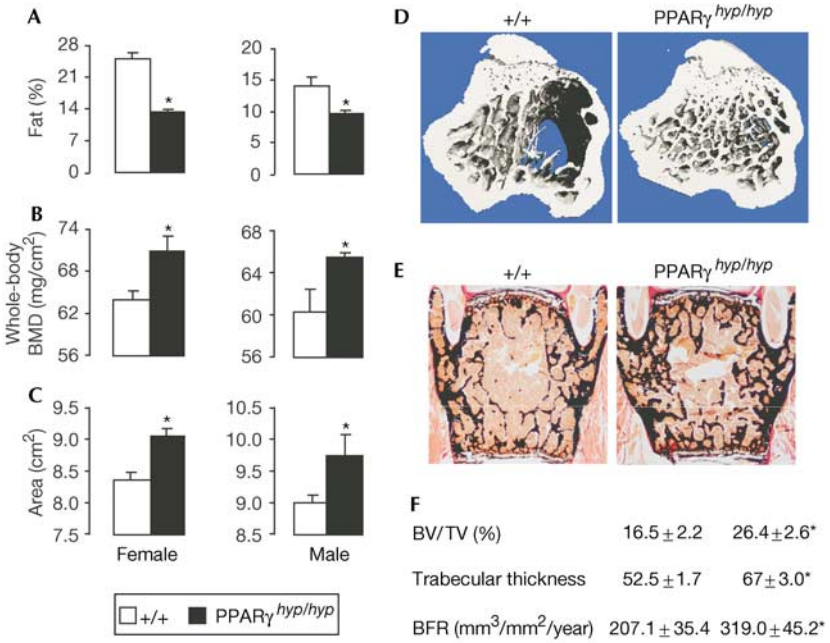

Fig 1 | Increased bone mass in lipodystrophic PPARγ$^{hyp/hyp}$ mice. (**A–C**) DEXA analysis of adult wild-type (white bars) or PPARγ$^{hyp/hyp}$ mice (black bars; $n>10$) in both sexes quantifying (**A**) whole-body fat content, (**B**) whole-body BMD and (**C**) bone area. (**D**) Micro-CT image and (**E**) histological analysis of vertebrae of 3-month-old wild-type and PPARγ$^{hyp/hyp}$ mice. Mineralized bone matrix is stained in black by von Kossa. (**F**) Histomorphometric parameters, bone volume over tissue volume (BV/TV), trabecular thickness and BFR ($n=8$; $^*P<0.05$).

and histomorphometric analyses showed an increase in bone content (ratio of bone volume/trabecular volume (BV/TV), 38%) and trabecular thickness (22%) in lumbar vertebrae of PPARγ$^{hyp/hyp}$ mice (Fig 1D–F). Similar increases in bone content and trabecular thickness were observed in femurs (not shown). These increases are secondary to increased bone formation rate (BFR, 35%; Fig 1F), demonstrating that the decrease in PPARγ activity in fat enhances bone formation.

The absence of PPARγ in PPARγ$^{hyp/hyp}$ mice affected both osteoblast and osteoclast activities. The increased osteoblast activity in bone of PPARγ$^{hyp/hyp}$ mice was signified by increased mRNA levels of the osteoblast-specific transcription factors Dlx5 and Runx2/Cbfa1 and markers such as 1(I)collagen and osteocalcin (Fig 2A). Bone formation and resorption are coupled (Harada & Rodan, 2003); hence with the increase in BFR in PPARγ$^{hyp/hyp}$ mice, it was not surprising that genes that typify the osteoclastic lineage, tartrate-resistant acid phosphatase (Trap) and cathepsin K (CathK) were increased (Fig 2A). Decreases in PPARγ activity in this lipodystrophic mouse model also robustly reduced leptin levels (Fig 2B), which was shown to enhance bone formation in the mouse (Ducy *et al*, 2000; Takeda *et al*, 2002). Also in humans, low leptin levels in patients with lipodystrophy have been correlated with enhanced bone formation (Elefteriou *et al*, 2004). Because of the increased bone content, the PPARγ$^{hyp/hyp}$ mice had 40% less total bone marrow cells (Fig 2C). Bone marrow is the principal site of adult haematopoiesis; peripheral blood and leukocyte counts were, however, not different between the genotypes (Fig 2D and data not shown).

Extramedullary erythromyelopoiesis in enlarged spleens

We also investigated alternative sites for haematopoiesis in the spleen and liver. Whereas spleens of neonatal PPARγ$^{hyp/hyp}$ mice were normal in size, adult PPARγ$^{hyp/hyp}$ mice showed a twofold increase in spleen size and cell number (Fig 3A–C). Adult PPARγ$^{hyp/hyp}$ livers were normal in size and function (Koutnikova *et al*, 2003), and showed no clinical indications of portal hypertension or haematopoiesis (not shown). The PPARγ$^{hyp/hyp}$ spleens showed no signs of lipid accumulation or infiltrative diseases, but only a pronounced accumulation of megakaryocytes, which is indicative of extramedullary haematopoiesis (Fig 3B). To evaluate the existence of extramedullary haematopoiesis, different haematopoietic lineages were examined in PPARγ$^{hyp/hyp}$ animals. In bone marrow, absolute numbers of myeloid (Gr1$^+$:granulocyte specific and CD11b/Mac1$^+$:myeloid specific in rodents and humans) and erythroid (TER119$^+$:erythroid specific) cells were reduced in PPARγ$^{hyp/hyp}$ mice, but the proportions remained unchanged between the genotypes (Fig 3D). In contrast, the proportion and absolute numbers of myeloid and erythroid cells were consistently increased in the PPARγ$^{hyp/hyp}$ spleen. The relative and absolute size of the population of immature haematopoietic cells (negative for differentiation markers and positive for the cytokine receptor c-kit) was decreased in bone marrow and increased in spleen (Fig 3D). The absolute numbers of all types of erythroid and myeloid progenitors declined in PPARγ$^{hyp/hyp}$ bone marrow (Fig 3E). Conversely, all progenitors were markedly increased in the PPARγ$^{hyp/hyp}$ spleen (Fig 3E). Although cell numbers were reduced, the proportion of erythroid and myeloid cells in the PPARγ$^{hyp/hyp}$ bone marrow did not differ

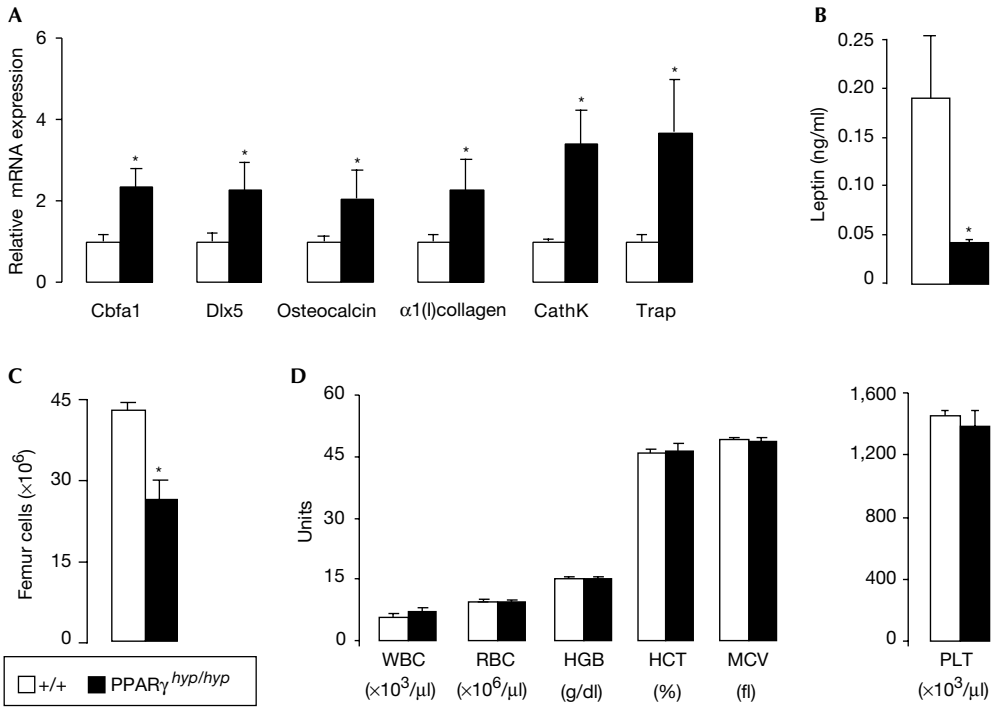

Fig 2 | Increased bone formation and consequential deceased bone cavity of PPARγ$^{hyp/hyp}$ mice. (**A**) Relative expression levels of mRNA of osteoblast- and osteoclast-specific markers in bone and bone marrow of wild-type and PPARγ$^{hyp/hyp}$ mice, determined by RT–PCR ($n=4$); CathK, cathepsin K; Trap, tartrate-resistant acid phosphatase. (**B**) Serum leptin levels ($n>10$). (**C**) Bone marrow cell number within femurs ($n=4$). (**D**) Complete blood count analysing white (WBC) and red blood cell (RBC) counts, haematocrit (HCT), haemoglobin (HGB), erythrocyte indexes (mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume (MCV)) and platelets (PLT) in PPARγ$^{hyp/hyp}$ and wild-type male mice ($n=8$–10; $^*P<0.05$).

from wild type (not shown), suggesting that erythroid and myeloid cell differentiation is not modified by the changed bone microenvironment, but just limited. Overall, these observations suggest that bone marrow haematopoiesis is lost because of increased bone area and decreased bone marrow space in PPARγ$^{hyp/hyp}$ animals. The haematopoietic defect is offset by extramedullary haematopoiesis in the PPARγ$^{hyp/hyp}$ spleen. Extramedullary haematopoiesis is also reported in humans, in which bone marrow space is reduced due to osteopetrosis (Freedman & Saunders 1981; Caluser *et al*, 1995).

Haematopoietic defects are indirect

The changes observed in bone physiology and haematopoiesis between the two genotypes were not related to differences in PPARγ expression in spleen, bone, bone marrow or haematopoietic precursors (Fig 4A). The only difference in gene expression was the virtual absence of expression of both PPARγ1 and 2 within adipose tissue of PPARγ$^{hyp/hyp}$ mice (Fig 4A). Unfortunately, PPARγ isoform-specific knockout animals will have to be generated to determine the relative contribution of each specific PPARγ isoform to the bone phenotype and to exclude that the loss of PPARγ2 may lead to specific alterations of the bone phenotype, which are independent of the overall levels of PPARγ1 in WAT.

To determine whether the haematopoietic phenotype was due to an intrinsic defect of PPARγ$^{hyp/hyp}$ haematopoietic cells, we performed bone marrow reconstitution experiments. Bone marrow cells from PPARγ$^{hyp/hyp}$ or wild-type mice, both expressing the Ly-5.2 marker, were transferred into lethally irradiated wild-type C57Bl/6 mice expressing the Ly-5.1 marker (C57Bl/6$^{Ly\text{-}5.1}$) and vice versa. Successful reconstitution (85–95%) by haematopoietic cells of donor origin was confirmed by analysis of surface markers seven weeks later (not shown). When C57Bl/6$^{Ly\text{-}5.1}$ mice were reconstituted with either PPARγ$^{hyp/hyp}$ or wild-type cells, no differences were detected in the number of erythroid (CFU-E), myeloid (CFC-M) or mixed (CFC-EM) progenitor cells in bone marrow or spleen (Fig 4B and data not shown). Conversely, when PPARγ$^{hyp/hyp}$ or wild-type littermate mice were reconstituted with donor cells from C57Bl/6$^{Ly\text{-}5.1}$ mice, fewer haematopoietic progenitors were detected in bone marrow (CFU-E were reduced) and significantly more erythroid (BFU-E and CFU-E), myeloid (CFC-M) and mixed (CFC-EM) progenitors were present in spleens of PPARγ$^{hyp/hyp}$ relative to wild-type recipients (Fig 4B and data not shown). These data demonstrate that the bone marrow deficiency in PPARγ$^{hyp/hyp}$ mice is not due to an intrinsic defect of the haematopoietic cells but rather to changes in bone cavity. These conclusions are further supported by our observations that fetal liver haematopoiesis is normal in PPARγ$^{hyp/hyp}$ mice (Fig 4C).

DISCUSSION

Adipocytes and osteoblasts both originate from common mesenchymal stromal precursor (MSP) cells. *In vitro* models suggested that PPARγ activation stimulates adipocyte differentiation of MSP cells at the expense of osteoblastogenesis (Lecka-Czernik *et al*, 2002). This was recently substantiated *in vivo* in

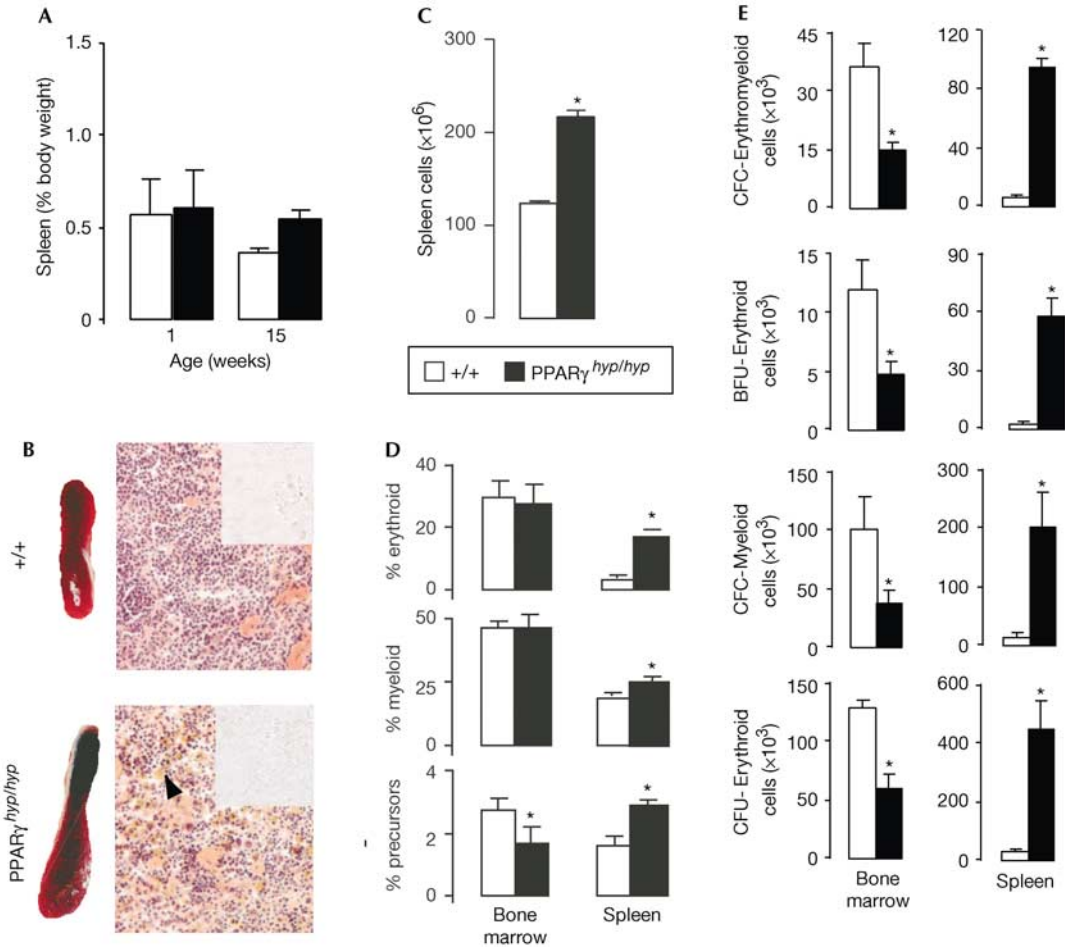

Fig 3 | Haematopoietic rearrangement in PPARγ$^{hyp/hyp}$ mice due to decreased bone marrow cellularity. (**A**) The spleen/body weight in neonatal and adult wild-type and PPARγ$^{hyp/hyp}$ mice. (**B**) Gross morphology, histology (× 6,000) and Oil-Red O staining of the spleen of adult PPARγ$^{+/+}$ and PPARγ$^{hyp/hyp}$ mice; megakeratocytes are indicated by the arrowhead. (**C**) Spleen cell number of adult mice. (**D**) Proportion of erythroid, myeloid and precursor populations determined using flow cytometry in bone marrow and spleen ($n = 4$). (**E**) The methylcellulose colony assay measured the absolute number of each type of progenitor per organ (spleen and two femurs for bone marrow) for PPARγ$^{hyp/hyp}$ and wild-type animals ($n = 4$). Erythromyeloid progenitor quantification was performed. CFC-Erythromyeloid cells are immature progenitors, which give rise to mixed erythromyeloid colonies; BFU-Erythroid and CFC-Myeloid cells are immature progenitors with respective erythroid or myeloid potential; CFU-Erythroid cells are mature erythroid progenitors (*$P < 0.05$).

heterozygous PPARγ-deficient mice (Akune *et al*, 2004) and confirmed in this study in PPARγ$^{hyp/hyp}$ mice, which like PPARγ +/− mice showed enhanced bone formation. Moreover, low leptin levels in PPARγ$^{hyp/hyp}$ mice suggest that PPARγ not only drives competition between adipogenic and osteoblastic differentiation of MSP cells but also could have an important antiosteogenic endocrine role. Adipocytes secrete adipokines, such as leptin, which have antiosteogenic activity in both mice and humans (Ducy *et al*, 2000; Takeda *et al*, 2002; Elefteriou *et al*, 2004). The contribution of other adipokines, such as adiponectin and interleukins, to bone remodelling cannot be excluded (Manolagas & Jilka, 1995; Yokota *et al*, 2002). As age-related bone loss is associated with bone marrow lipid accumulation (Nuttall & Gimble, 2000), it has also been suggested that PPARγ could drive the age-related increase in adipocytes and decline of osteoblasts in bone, due to enhanced ligand availability (fatty acids; Lecka-Czernik *et al*, 1999, 2002). The importance of ligand availability for the antiosteogenic actions of PPARγ was recently illustrated by the improved bone density and strength following genetic and pharmacological inhibition of 12/15-lipoxygenase, an enzyme that generates endogenous PPAR ligands (Klein *et al*, 2004). Conversely, PPARγ activation by thiazolidinediones reduces bone integrity, in as little as seven weeks in mice (Rzonca *et al*, 2004). So far, there are no clinical data that correlate changes in BMD with treatment with PPARγ agonists. It would be of considerable concern if such effects were to occur in the many patients with type 2 diabetes who are receiving thiazolidinediones. Given the species differences in bone homeostasis, it is imperative that eventual effects of PPARγ agonists on bone integrity are ascertained in clinical studies.

Recently, evidence has been accumulating that osteoblasts support haematopoietic stem cell development (Calvi *et al*, 2003; Zhang *et al*, 2003). The enhanced bone formation in PPARγ$^{hyp/hyp}$ animals hence leads us to expect more haematopoietic precursor

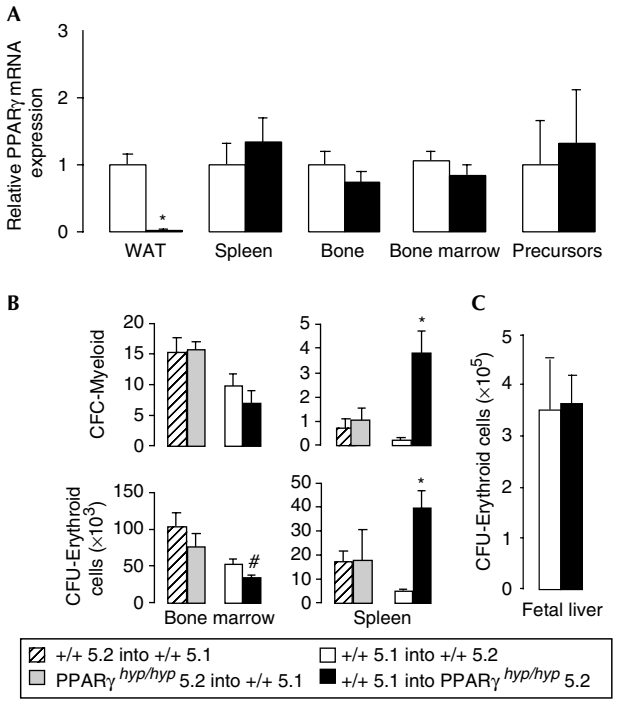

Fig 4 | PPARγ$^{hyp/hyp}$ mice do not have intrinsic haematopoietic abnormalities. (**A**) Relative level of PPARγ mRNA in white adipose tissue, bone, spleen, bone marrow and haematopoietic precursor cells from bone marrow of wild-type and PPARγ$^{hyp/hyp}$ mice ($n = 4$; $^*P < 0.05$). (**B**) Haematopoietic reconstitution 7 weeks after transplantation of bone marrow cells into lethally irradiated recipients. Absolute number of erythroid (CFU-Erythroid) and myeloid (CFC-Myeloid) progenitor cells per organ ($n = 4$). Bone marrow cells from PPARγ$^{hyp/hyp}$ (grey bars) or wild-type littermates (Ly-5.2; striped bars) were transplanted into C57Bl/6$^{Ly\text{-}5.1}$ recipient mice or donor cells from C57Bl/6$^{Ly\text{-}5.1}$ mice were transplanted into wild-type (Ly-5.2; white bars) and PPARγ$^{hyp/hyp}$ (Ly-5.2; black bars) mice. (**C**) Erythroid progenitors (CFU-Erythroid) in E18.5 embryonic livers for each genotype ($n = 4$; $^*P < 0.05$, $\#P < 0.06$).

cells. Even though we did not analyse the haematopoietic stem cell compartment in PPARγ$^{hyp/hyp}$ bone marrow, the large decrease in both erythro/myeloid colony-forming precursors and the Lin$^-$c-kit$^+$ population argues against an increase in the haematopoietic stem cell population. In fact, positive effects of osteoblasts on haematopoiesis requires an increase in contact area between osteoblastic and haematopoietic cells (Calvi *et al*, 2003; Zhang *et al*, 2003). A massive increase in bone formation will lead to a decrease of this contact area, which adversely affects haematopoiesis, as seen in PPARγ$^{hyp/hyp}$ mice. The result would force migration of the haematopoietic precursor cells to the spleen, where we demonstrated a robust increase in haematopoietic precursors. Bone marrow reconstitution experiments in lethally irradiated mice demonstrated that the bone marrow deficiency in PPARγ$^{hyp/hyp}$ mice was not due to an intrinsic defect of haematopoietic cells but rather to changes in bone cavity. The increased number of spleen cells, on repopulating sublethally irradiated PPARγ$^{hyp/hyp}$ recipient mice with wild-type donor cells, further proved that the restricted bone marrow space was directly causing the increase in spleen cell numbers.

Extramedullary haematopoiesis has previously been described in osteopetrotic mice (Tagaya *et al*, 2000) and humans (Freedman & Saunders 1981; Caluser *et al*, 1995). Our study suggests that an enlarged spleen observed in other lipodystrophic mouse models and patients with lipodystrophy (Moitra *et al*, 1998; Shimomura *et al*, 1998; Misra & Garg, 2003) cannot be attributed to infiltrative processes or steatotic liver dysfunction and portal hypertension alone, but also to bone abnormalities. Increased bone volume not only decreases bone total cellularity but it may also change cell–cell interactions within bone microenvironment, which are important for haematopoietic differentiation (Okubo *et al*, 2002). In view of this, the proportion of haematopoietic cells present in the bone marrow did not differ between genotypes, indicating that the possible alterations in cell–cell interactions do not lead to a major disruption of haematopoiesis. Hence, reduction in erythro-myelopoiesis within bone marrow is most likely a consequence of space limitations. These bone abnormalities, together with the decreased expression of adipokines that support haematopoietic differentiation (Bennett *et al*, 1996; Mikhail *et al*, 1997), cause the extramedullary haematopoiesis in PPARγ$^{hyp/hyp}$ mice. No haematopoietic abnormalities have so far been reported in human lipodystrophic or lipoatrophic patients. This could be due to the apparently normal peripheral blood analysis in these patients, which does not incite physicians to analyse this further. Splenomegaly is, however, a common clinical abnormality in human lipodystrophy (Rajab *et al*, 2002; Misra & Garg, 2003). Furthermore, enhanced bone formation was reported in human lipodystrophy patients (Elefteriou *et al*, 2004). These observations hence warrant systematic investigation of bone homeostasis and haematopoiesis in response to severe changes in adiposity in humans.

In summary, the specific absence of PPARγ in fat robustly increases bone mass by favouring osteogenic over adipogenic differentiation of MSP cells. In addition, absence of PPARγ in adipocytes limits their capacity to secrete antiosteogenic signalling factors, such as leptin, further enhancing the bone phenotype. Previous studies on the role of PPARγ in bone homeostasis did not allow us to make this conclusion or enable to unequivocally pinpoint PPARγ specifically in WAT as causative factor for the changes in bone architecture, as they involved changes in PPARγ activity in all tissues (agonists (Rzonca *et al*, 2004) and the heterozygous PPARγ +/− mice model (Akune *et al*, 2004)). If our data and other recently published mouse studies can be extrapolated to humans, inhibition of PPARγ activity could be an interesting strategy to combat osteoporosis. Furthermore, the splenomegaly in lipodystrophic patients also needs to be revisited, as it might reflect reactivation of haematopoiesis that occurs in the wake of the reduction in bone marrow cavity.

METHODS

Animal experiments. Generation of PPARγ$^{hyp/hyp}$ mice on a hybrid C57BL/6J/129 SV background has been described previously (Koutnikova *et al*, 2003). Unless otherwise stated, female PPARγ$^{hyp/hyp}$ and wild-type littermates fed regular rodent chow were used at 4 months of age. Blood and tissue were collected as described previously (Koutnikova *et al*, 2003).

Bone analysis. Bone histology on lumbar vertebrae and BFR by calcein double labelling were performed as described previously (Takeda *et al*, 2002). Micro-CT analysis of L5 vertebrae was

performed at 12 μm resolution (Scanco Medical, Bassersdorf, Switzerland). Lumbar vertebrae were embedded in methylmethacrylate, sectioned and stained with von Kossa, or left unstained for calcein labelling. Histomorphometric measurements were performed using the OsteoMeasure system (OsteoMetrics, Atlanta, GA). BMD and body fat mass were evaluated in anaesthetized mice by DEXA (PIXIMUS^{TM}, GE Medical Systems, Buc, France).

Haematological assays. Bone marrow and spleen cells were obtained from adult animals. Liver cells were taken from 17.5-day embryos. Methylcellulose colony assays and quantification of erythroid precursors have been described previously (Back *et al*, 2004). The following primary antibodies were used: phycoerythrin (PE)-anti-TER119, biotin-anti-CD71, biotin-anti-CD117 (c-kit), biotin-anti-CD11b (Mac1), PE-anti-Gr1 (all from Pharmingen, San Diego, CA, USA), purified anti-B220 (clone RA36B2), anti-Ly5.2 and PE-anti-IgM. Lineage staining was performed using antibodies against TER-119, CD3, CD4, CD8, B220, NK1.1, GR1 and F4/80. Secondary antibodies include cyanin (Cy) 5-anti-rat IgG and PE-anti-rat IgG (Jackson Immunoresearch, West Grove, PA, USA), Cy5 or fluorescein (FITC)-conjugated streptavidin.

Bone marrow reconstitutions. Recipient mice were irradiated at 9 Gy, and reconstituted 1 day later with 10×10^6 donor bone marrow cells. Mice were analysed 7 weeks after reconstitution. Donor cells or host cells were identified by the appropriate Ly-5.1 or Ly-5.2 expression by flow cytometry.

RNA analysis. RNA preparation and quantitative RT–PCR were described previously (Koutnikova *et al*, 2003). Sequences of primers used are available at http://www-igbmc.u-strasbg.fr/Auwerx or in Rzonca *et al* (2004). Glyceraldehyde-3-phosphate dehydrogenase mRNA or 18S rRNA was used as a control.

Data analysis. Data are presented as mean ± s.e.m. and statistical differences analysed using Student's *t*-test.

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