



Microparticles Carrying Peroxisome Proliferator-Activated Receptor Alpha Restore the Reduced Differentiation and Functionality of Bone Marrow-Derived Cells Induced by High-Fat Diet

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ABSTRACT

Metabolic pathologies such as diabetes and obesity are associated with decreased level of circulating and bone marrow (BM)-derived endothelial progenitor cells (EPCs). It is known that activation of peroxisome proliferator-activated receptor alpha (PPAR α) may stimulate cell differentiation. In addition, microparticles (MPs), small membrane vesicles produced by activated and apoptotic cells, are able to reprogram EPCs. Here, we evaluated the role of MPs carrying PPAR α on both phenotype and function of progenitor cells from mice fed with a high-fat diet (HFD). HFD reduced circulating EPCs and, after 7 days of culture, BM-derived EPCs and monocytic progenitor cells from HFD-fed mice displayed impaired differentiation. At the same time, we show that MPs bearing PPAR α , MP^{PPAR α +/+}, increased the differentiation of EPCs and monocytic progenitors from HFD-fed mice, whereas MPs taken from PPAR α knockout mice (MP^{PPAR α -/-}) had no effect on the differentiation of all types of progenitor cells. Furthermore, MP^{PPAR α +/+} increased the ability of progenitor cells to promote in vivo angiogenesis in mice fed with HFD. The in vitro and in vivo effects of MP^{PPAR α +/+} were abolished in presence of MK886, a specific inhibitor of PPAR α . Collectively, these data highlight the ability of MPs carrying PPAR α to restore the failed differentiation and functionality of BM-derived cells induced by HFD. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:135–145

SIGNIFICANCE STATEMENT

This article reports that microparticles (MPs) carrying peroxisome proliferator-activated receptor alpha (PPAR α) are able to reprogram ex vivo expansion of bone marrow-derived cells and to improve their angiogenic activity in high-fat-fed mice. Accordingly, MPs bearing PPAR α could favor the formation of new vessels in order to improve supply of oxygen and nutrients of target tissues.

INTRODUCTION

Metabolic syndrome is a complex disorder consisting of central obesity, hyperglycemia, hypertension, and hyperlipidemia [1]. In this pathology, increased blood glucose levels, insulin resistance, oxidative stress, dyslipidemia, and hypertension, compromise the vasculogenic ability to restore the endothelium, originating the vascular endothelium damage [2]. This is thought to be due, at least partially, to the dysfunction of bone marrow (BM)-derived endothelial progenitor cells (EPCs) biological activities and to the presence of pro-inflammatory state [3]. Interestingly, previous studies have demonstrated a link between reduced numbers and functionality of circulating EPCs and increased risk of cardiovascular diseases [4], such

as diabetes and metabolic syndrome [2], but the mechanisms leading to the failed angiogenic function are not completely elucidated. It has been recognized that EPCs are a heterogeneous population and their cellular identity is classified according to their morphology, function, and growth potential. Stem cell antigen 1 (Sca1) and vascular endothelial growth factor receptor type 2 (Flk1) double positive cells have been considered as EPCs in the past. Increased levels of Sca1/Flk1 positive EPCs have been associated with improved reendothelialization in animal and clinical studies [5, 6]. Thus, one can advance the hypothesis that ex vivo expansion of EPCs might be a promising strategy to overcome the clinical problem of limited cell numbers in cardiovascular diseases.

Microparticles (MPs) released from the plasma membrane blebs upon activation and/or apoptosis, can act as signaling elements in physiological or pathological conditions. Importantly, MPs can transfer receptors and organelles between cells and deliver mRNA and proteins into cells creating cell-cell interaction [7]. It is also demonstrated that MPs are able to program progenitor cells to repair tissue injury [8].

Emerging evidence indicates that members of the peroxisome proliferator-activated receptor (PPAR) superfamily play a central role in the regulation of metabolic homeostasis and in cell differentiation. Among them, PPAR α , implicated in lipid homeostasis and inflammation, represents a drug target for the treatment of some risk factors of metabolic syndrome [9]. Furthermore, PPAR α regulates cardiac progenitor differentiation via NADPH oxidase [10]. We have shown that PPAR α regulates EPC maturation and myeloid lineage differentiation through a NADPH oxidase-dependent mechanism in mice [11]. In addition, we reported that PPAR α carried by MPs stimulate cell differentiation through the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway in “healthy” mice [12]. However, it is not known whether MPs carrying PPAR α (MPs^{PPAR α +/+}) affect BM-cell differentiation and the failed angiogenesis in the context of obesity. Thus, the aim of this study is to investigate the capacity of MPs^{PPAR α +/+} to induce differentiation and neo-vascularization in an experimental model of obesity such mice fed with high-fat diet (HFD).

MATERIALS AND METHODS

Animal and Experimental Design

Male PPAR α wild type (C57BL/6N, 8 weeks-old) mice were used in this study. They were maintained at 23°C, conditioned to a 12 hours light/dark cycle. The animals were allowed free access to water and standard diet (SD) or HFD (37% Kcal from fat, 47.5% from carbohydrates, SAFE, Augy, France) for 12 weeks. Blood was obtained after 18 hours fasting period by cardiac exsanguinations under isoflurane-induced anesthesia, and plasma was collected and stored at -80°C until analysis. Tissues, including liver, adipose tissues and heart were removed, washed with phosphate-buffered saline (PBS), and weighed.

We also used homozygous PPAR α -null mice on pure C57BL/6 background and corresponding wild-type for circulating MP isolation. PPAR α knockout mice were generated by Pr. Frank Gonzalez (NIH, Bethesda, PPAR α knockout mice MD) [13].

All animal studies were carried out using approved institutional protocols (no. CEEA.2012.125) in accordance with Guide for the Care and Use of Laboratory Animals published by U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Circulating MP Isolation

Circulating MPs were isolated from venous blood obtained by cardiac puncture from wild type and corresponding PPAR α knockout mice. Whole blood was centrifuged two times at 1,500g for 5 minutes at 20°C to obtain platelet-free plasma (PFP). After circulating MPs were concentrated from PFP by two series of centrifugations at 16,000g for 45 minutes and resuspended in 0.9% saline salt solution and stored at 4°C until subsequent use. MP quantification was performed using calibrated 10 μ m-sized beads of known concentration on FC500 cytometer (Beckman Coulter, Villepinte, France). The physiological circulating concentration of MPs was used in all experiments as previously reported by Benameur et al. [12].

Dot Blot Assay

Circulating MPs isolated from wild type or PPAR α -deficient mice fed with SD were collected. Five micrograms of MPs were spotted onto nitrocellulose membrane. Membranes were then saturated at room temperature for 10 minutes in TBS-T (20 mM Tris base, 61.5 mM NaCl pH 7.8 and 0.1% Tween 20) buffer containing 5% bovine serum albumin (BSA). After washing, the membrane was incubated with the primary antibodies anti-PPAR α (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at room temperature for 1 hour. A secondary anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) was then added to the membrane. The protein-antibody complexes were detected by ImmunoCruz kit (Santa Cruz Biotechnology) according to the protocol of manufacturer. Blots were quantified by densitometry using Image J software.

Characterization of MP Phenotype

Membrane MP subpopulations of HFD- and normal-fed mice were discriminated in PFP according the expression of membrane-specific antigens by flow cytometer. Phenotype of endothelial MPs was performed using anti-CD54 labeling, characterization of platelet, leukocyte, erythrocyte MPs, and MPs from progenitor cells was performed using anti-CD61, anti-CD45, anti-Ter-119/erythroid cell, and anti-CD133 labeling, respectively, (BioLegend, San Diego, CA). Also, Annexin V (BioVision Inc., Mountain View, CA) binding was used to numerate phosphatidylserine-expressing circulating MPs. Irrelevant mouse IgG was used as an isotype-matched negative control for each sample.

Biochemical Analyses

Levels of glucose, triglycerides, total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol in plasma were determined by spectrophotometry UV/visible Roche/Hitachi Modular P (Roche Diagnostics, Mannheim, Germany) using enzyme-linked immunosorbent assay and Kone-lab/T Series) according to the protocol of the manufacturer. Serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) kit (Mouse insulin Elisa kit, Millipore, Billerica, MA; ref: EZRMI-13K) following the manufacturer's instructions.

Isolation, Culture, and Characterization of BM-Derived Cells

Femurs and tibiae were dissected and carefully cleaned from adherent tissues. Then, the tips of each bone were removed and BM was collected by flushing out the content with DMEM (Lonza, Walkersville, MD). BM-derived cells were obtained by isolating mononuclear cells from BM of mice fed with normal chow and HFD using Histopaque H1083 (Sigma-Aldrich, St Louis, MO) density-gradient centrifugation, as previously described [11–16]. Immediately after isolation, total mononuclear cells (10⁶ cells/cm²) were plated on culture dishes coated with fibronectin (BD Biosciences, San Jose, CA, 10 μ g/ml) and maintained in EGM-2 endothelial medium Bullet Kit system (Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum. After 24 hours, nonadherent cells were removed by washing with PBS, and adherent cells were cultured in EGM-2 endothelial medium in the presence of circulating MPs from WT or PPAR α -null mice and GW7647, specific PPAR α agonist, (Santa Cruz Biotechnology, 1 μ M), for the whole culture period of 7 days. In another set of experiments, PPAR α inhibitor, MK886 (Santa Cruz Biotechnology, 20 μ M) was

preincubated with circulating MPs^{PPAR α +/+} and MPs^{PPAR α -/-} for 1 hour, and then centrifuged and washed to eliminate excess of MK886, before to be added to BM-derived cells. Cell characterization was performed at day 0, immediately after isolation and after 7 days of culture. Three BM subpopulations were identified by flow cytometry using the following antibodies: EPCs (Sca1-PE/Cy7-Flk1-PE), monocytes (Sca1-PE/Cy7-CD14-FITC), leukocytes (Sca1-PE/Cy7-CD45-PE/Cy5) (BioLegend).

After 7 days of treatment with MPs, adherent EPCs were analyzed by confocal microscopy and by flow cytometry. Adherent cells were incubated with 2.5 μ g/ml 1, 19-dioctadecyl-3,3,39,39-tetramethyl-indocarbocyanine-labeled acetylated-LDL (Dil-Ac-LDL) (Harbor Bioproducts, Norwood, MA) for 2 hours and then, fixed with 2% paraformaldehyde and counterstained with 10 μ g/ml FITC-labeled lectin from *Bandeiraea simplicifolia* (Sigma-Aldrich) for 1 hour at 37°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology) at room temperature for 5 minutes. Double positive cells (Dil-ac-LDL⁺/FITC-lectin⁺), identified as EPCs by confocal microscopy and by three independent investigators, were counted in five randomly selected high-magnification fields of each culture slide by using a computer-based program.

White Blood Cell Count

After 12 weeks of diet, peripheral blood specimens were obtained from tail of mice. A peripheral blood smear was made by placing a drop of blood on one end of the slides, and using a spreader slide to disperse the blood over the slides's length. Then, the slides were stained with *May-Grünwald-Giemsa* (Sigma-Aldrich) to distinguish the various blood cells to be examined microscopically.

In Vivo Matrigel Plug Assay

After treatment, cells were detached and were mixed with 500 μ l of Matrigel (ECMgel Sigma-Aldrich) with recombinant basic fibroblast growth factor (bFGF) (300 ng/ml, Peprotech, Rocky Hill, NJ). This mixture was injected subcutaneously on the back of male mice fed either with normal chow or HFD. One week after the initial injection, the Matrigel plug was removed, photographed, weighed, and homogenized in lysis buffer and incubated for 24 hours at 4°C and then, disrupted with a Polytron (PRO250, PRO Scientific, Monroe, CT). Hemoglobin levels were determined using the Drakin method (Sigma-Aldrich) according to manufacturer's instructions.

PPAR α Reporter Assays

The transcriptional activity of mouse PPAR α was determined by measuring luciferase activity in PPAR α reporter assays (Indigo Biosciences, State College, PA) according to the manufacturer's instructions. The reporter cells, derived from Chinese hamster ovary cells, constitutively express mouse hybrid PPAR α , of which the DNA-binding domain (DBD) has been substituted with the DBD of GAL4, a transcription activator protein in yeast. These cells also incorporate a luciferase reporter gene driven by the GAL4 upstream activation sequence, which is the binding site for the DBD of GAL4. Thus, the effects of circulating MPs from WT or PPAR α -null mice on the activation of PPAR α can be determined by measuring the luciferase activity. Briefly, mouse PPAR α reporter cells were dispensed into the wells of a 96-well assay plate and immediately treated for 24 hours with circulating MPs from WT or PPAR α -null mice and with two selective PPAR α agonists, GW7647 (1 μ M), and serial dilution of GW590735. In

Table 1. Metabolic parameters of mice fed normal chow or high fat-diet (HFD) for 12 weeks

	Normal chow	HFD
Triglyceride (mg/dl)	85.2 \pm 14.2	131.3 \pm 26.7
Cholesterol (mg/dl)	87.5 \pm 3.64	145 \pm 7.23***
HDL (mg/dl)	84.4 \pm 6.74	157.7 \pm 11.3***
LDL (mg/dl)	7.75 \pm 1.11	17.69 \pm 2.5**
HDL/LDL	13.61 \pm 1.97	6.26 \pm 0.87**
Glucose (mg/dl)	137.8 \pm 27.38	236.9 \pm 8.29*
Insulin (mU/l)	3.2 \pm 1.05	9.9 \pm 1.28*

Values are represented as means \pm SEM; n = 6–12 animals/genotype. *, p < .05; **, p < .01; ***, p < .001 versus normal chow. Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

another set of experiments, PPAR α inhibitor, MK886 (20 μ M) was preincubated with circulating MPs^{PPAR α +/+} and MPs^{PPAR α -/-} for 1 hour, before to be added to reporter cells. At the end of the treatment, the medium was discarded and the luciferase detection reagent was added. Luminescence was measured using a Perkin Elmer Wallac 1420 Victor2 Multilabel Counter (Marshall Scientific, Hampton, NH).

Statistical Analysis

All data are represented as mean \pm SEM; n represents the number of mice. Significant differences between values in experimental and control group were calculated by Mann–Whitney U tests (nonparametric) using Prism software package 5.00 (GraphPAD Software, San Diego, CA). Differences were considered significant when p < .05.

RESULTS

High-Fat Diet Caused Metabolic Changes in Mice

Anatomical changes induced by HFD are shown in Supporting Information Table S1. After 12 weeks, no significant difference in body weight was observed between mice fed either with normal chow or HFD, but consumption of HFD increased epididymal and visceral fat weight. In addition, HFD feeding significantly led to increased levels of total, HDL- and LDL-cholesterol, glucose and insulin when compared with mice fed with normal chow (Table 1).

HFD Reduces Circulating MPs from Progenitor Cells

Flow cytometry analysis of circulating MPs (Supporting Information Fig. S1) showed that the total number of circulating MPs was not significantly different between HFD-and normal chow-fed mice indicating that HFD did not affect the circulating levels of MPs. Phenotypical characterization of cellular origin of MPs showed an increase of \sim 2.6-fold and \sim 2.8-fold of the circulating levels of platelet (CD41⁺) and procoagulant (annexin V⁺), respectively, as well as a reduction of MPs from progenitor cells (3.2-fold) in HFD-fed mice compared with normal chow-fed mice. Thus, these results demonstrate that HFD affect the cellular origins of MPs, in particular alters MP release from progenitor cells.

Circulating EPCs and In Vitro Cultured BM-Derived EPC Decline in HFD-Fed Mice

As expected, the number of Sca1/Flk1 positive EPCs in peripheral blood decreased in HFD-fed mice compared with those receiving

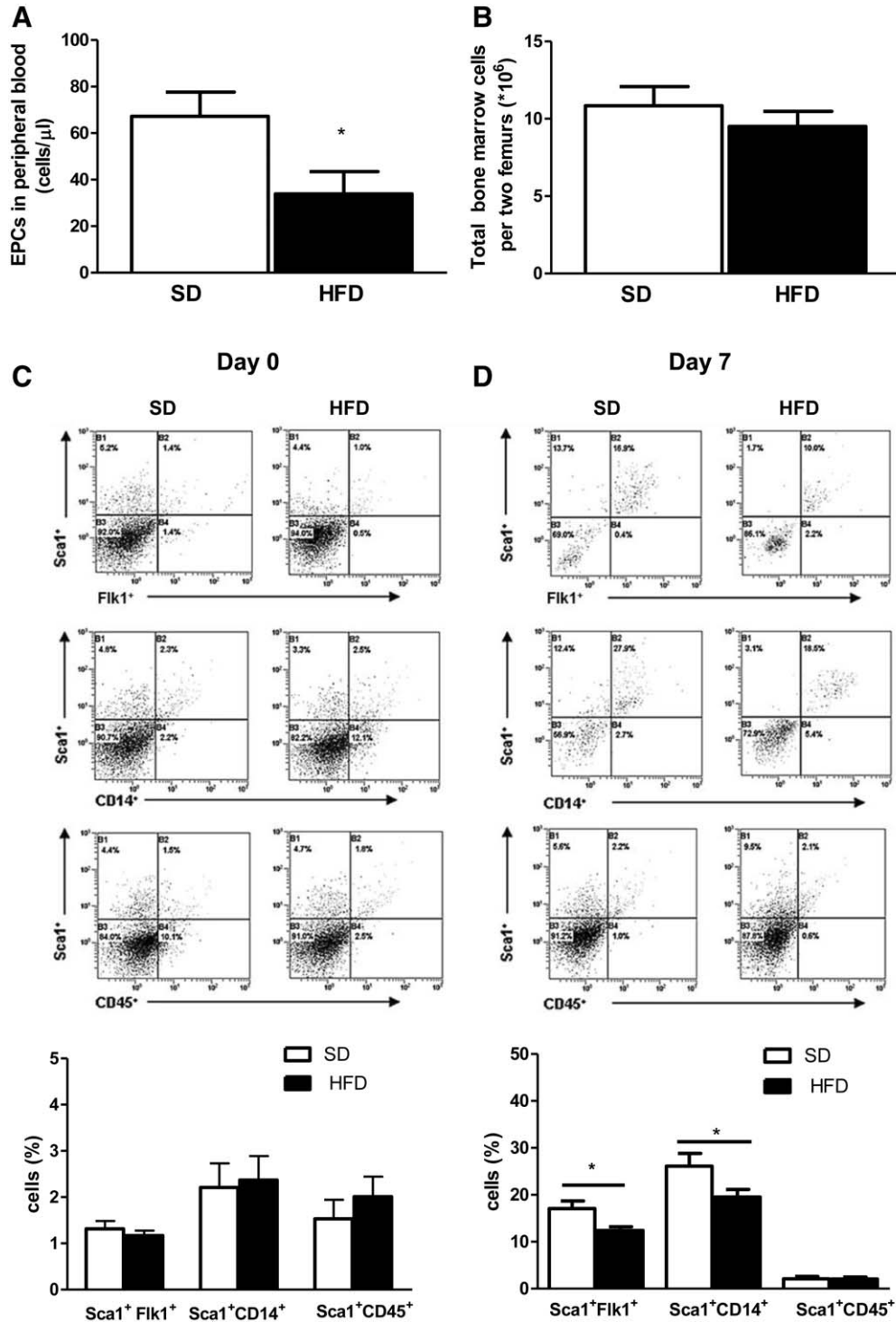


Figure 1. HFD impairs mobilization of bone marrow (BM)-derived cells. At sacrifice day, circulating EPCs in peripheral blood (**A**) and total numbers of progenitors from BM (**B**) of mice was performed by flow cytometry. Count of cells was determined using double-positive staining for Sca1⁺/Flk1⁺ (EPCs). (**C, D**): At day 0 and after 7 days of culture, flow cytometer characterization of BM-derived cells was performed. Cells with double-positive staining for Sca1⁺/Flk1⁺ were identified as differentiating EPCs, Sca1⁺/CD14⁺ were identified as monocytes, Sca1⁺/CD45⁺ were identified as leukocytes. Data are shown as mean values \pm SEM ($n = 8-16$) *, $p < .05$. Abbreviations: EPCs, endothelial progenitor cells; HFD, high-fat diet; Sca1, stem cell antigen 1; SD, standard diet.

normal chow, whereas BM levels of progenitor cells were not different between the two groups (Fig. 1A, 1B). These findings implicate that EPC mobilization from BM to circulation was markedly impaired in HFD-fed mouse model.

In previous studies, “early” EPCs were quantified by flow cytometry, and the most popular surface marker combinations comprise a heterogeneous composition of CD14⁺/CD45⁺ positive cells, with hematopoietic and endothelial potential [14]. For this

Table 2. Effects of high-fat diet (HFD) on white blood count of the mice

	LYM	MONO	NEU	EOS	BASO
Normal chow	85.5 ± 1.83	0.59 ± 0.05	11.77 ± 1.98	1.85 ± 0.64	0.36 ± 0.17
HFD	83.3 ± 1.87	1.62 ± 0.18*	14.2 ± 1.782	1.11 ± 0.26	0.28 ± 0.09

All values are means ± SEM ($n = 6$). *, $p < .05$ versus normal chow.

Abbreviations: BASO, basophils; EOS, eosinophils; LYM, lymphocytes; MONO, monocytes; NEU, neutrophils.

reason, also, monocytic (Sca1⁺/CD14⁺) and leukocytic (Sca1⁺/CD45⁺) progenitors were identified by flow cytometry.

At day 0, immediately after isolation, HFD had no effect on the all types of progenitor cells in both groups (Fig. 1C). However, after 7 days of culture expansion, HFD reduced the subpopulation of EPCs and monocytic progenitor cells (Fig. 1D). Since leukocytic progenitor population was not affected by HFD, we next decide to investigate whether HFD modules the function of EPCs and monocytic progenitors.

Changes in White Blood Cells Induced by HFD

To determine whether HFD can affect peripheral sites, blood was collected from two groups of mice. Analysis of complete blood counts revealed an increase in monocytes in mice fed with HFD (Table 2). Overall, these results indicate that HFD-related changes occurring in the BM are also manifest in blood.

Effects of Circulating MP^{PPAR α +/+} and MP^{PPAR α -/-} on BM-Derived EPC Differentiation from HFD-Fed Mice

As shown above, HFD feeding induced a reduction on expansion of EPCs from HFD-fed mice (see Fig. 1D). Once verified that only MPs isolated from wild type (MP^{PPAR α +/+}) expressed PPAR α (Supporting Information Fig. S2), we assessed whether MPs carrying PPAR α can favor in vitro EPC expansion. Circulating MPs isolated from wild type or PPAR α -deficient mice were added to adherent BM-derived cells from normal- and HFD-fed mice for the whole culture period (Fig. 2A, 2B). After 7 days of culture, BM-derived cells were characterized by the presence of endothelial-specific lectin BS-I binding [15] as well as double expression of Sca1⁺/Flk1⁺ for differentiation analysis. By confocal microscopy, we found that cells from HFD-fed mice displayed a reduced Dil-Ac-LDL/lectin labeling when compared with mice fed with normal chow (Fig. 2A, 2B). Furthermore, both MP^{PPAR α +/+} and MP^{PPAR α -/-} induced an increase Dil-Ac-LDL⁺/lectin⁺ uptake typical for endothelial lineage in BM-derived cells from animal fed with normal chow. However, in BM-derived cells from HFD-fed mice, only MP^{PPAR α +/+} were able to increase the number of Dil-Ac-LDL⁺/lectin⁺ cells (Fig. 2B). These results were confirmed by flow cytometry (Fig. 2C, 2D). In addition, the specific PPAR α agonist GW7647 induced similar effects of MP^{PPAR α +/+}.

To test whether the MPs effects are primarily mediated by PPAR α carried by MPs, we preincubated MPs isolated from WT or PPAR α -null mice with PPAR α antagonist (MK886, 20 μ M) (Fig. 2C, 2D). Interestingly, the preincubation of MP^{PPAR α +/+} with MK886 abolished the effects of MP^{PPAR α +/+} on EPC differentiation in both groups of mice (Fig. 2D). In contrast, MK886 treatment appeared to reduce Sca1⁺/Flk1⁺ expansion induced by MP^{PPAR α -/-} of BM-derived cells from normal-fed mice, but not from HFD-fed mice. These results suggest that PPAR α is essential for the effects of MPs on EPC maturation from HFD-fed mice.

Effects of MPs on Differentiation of Monocytic Progenitor Cells from HFD-Fed Mice

Since HFD affects the differentiation of hematopoietic lineage toward monocytic cells [16], we investigate whether circulating MPs isolated from WT or PPAR α -deficient mice are able to restore, in vitro, this impairment. Unfractionated BM-derived cells were cultured in the absence or in the presence of MP^{PPAR α +/+} or MP^{PPAR α -/-} for 7 days, and then, analyzed by flow cytometry.

We found that, after 7 days of culture, GW7647 and MP^{PPAR α +/+} were able to increase the differentiation of monocytic progenitors from either normal- or HFD-fed mice, respectively, (Fig. 3A, 3B). In contrast, MP^{PPAR α -/-} had no effect on the differentiation of monocytic progenitor cells under the same experimental conditions (Fig. 3B), suggesting that the presence of PPAR α is necessary for MPs to develop monocytic differentiation. In addition, no significant effect was observed on leukocyte progenitor cells regardless of diet (data not shown).

Next, to assess whether MPs carrying PPAR α are implicated in hematopoietic progenitor cell differentiation, we cultured for 7 days BM-derived cells with MPs pre-treated with MK886. As shown in Figure 3A and 3B, MK886 treatment prevented the effects of MP^{PPAR α +/+} on BM-derived cells from the two groups of mice. Moreover, BM-derived cells either from normal- or HFD-fed mice treated with MP^{PPAR α -/-} alone or in the presence of MK886 did not modify monocytic differentiation.

Together these data suggest that in mice with early metabolic alterations monocytic differentiation is rescued by MPs carrying PPAR α .

In Vivo Modulation of Angiogenic Properties of BM-Derived EPCs by MPs

We further characterized the angiogenic ability of MPs by Matrigel plugs subcutaneously injected into normal- and HFD-fed mice. After 7 days, mice were sacrificed, and blood vessel growth into the gels was quantified by measuring hemoglobin content of the plugs, as shown in Figure 4A.

Prior, we addressed whether the HFD induced functional alterations in BM-derived cells. As expected, hemoglobin content in plugs from HFD-fed mice was significantly reduced when compared with that of plugs from mice fed with normal chow (Fig. 4A, 4B). These results indicate that the function of BM-derived EPCs from HFD mice was reduced and was associated with defects of their ability to promote neoangiogenesis.

Next, similar experiments were performed in the presence of either GW7647 or MP^{PPAR α +/+}. As shown in Figure 4A, vascularization of the plugs was significantly stimulated when BM-derived cells were pretreated with GW7647 (1 μ M) or with MP^{PPAR α +/+}, but not with MP^{PPAR α -/-}, suggesting a key role of PPAR α in the effects evoked by MPs (Fig. 4A, 4B).

To confirm these results, we inhibited PPAR α harbored by MPs using MK886 for in vivo angiogenesis study. As observed in Figure 4A, the degree of vascularization was lower in the MP^{PPAR α +/+}-MK886 treated plugs from normal- and HFD-fed mice than in those treated with MP^{PPAR α +/+}. In addition, when

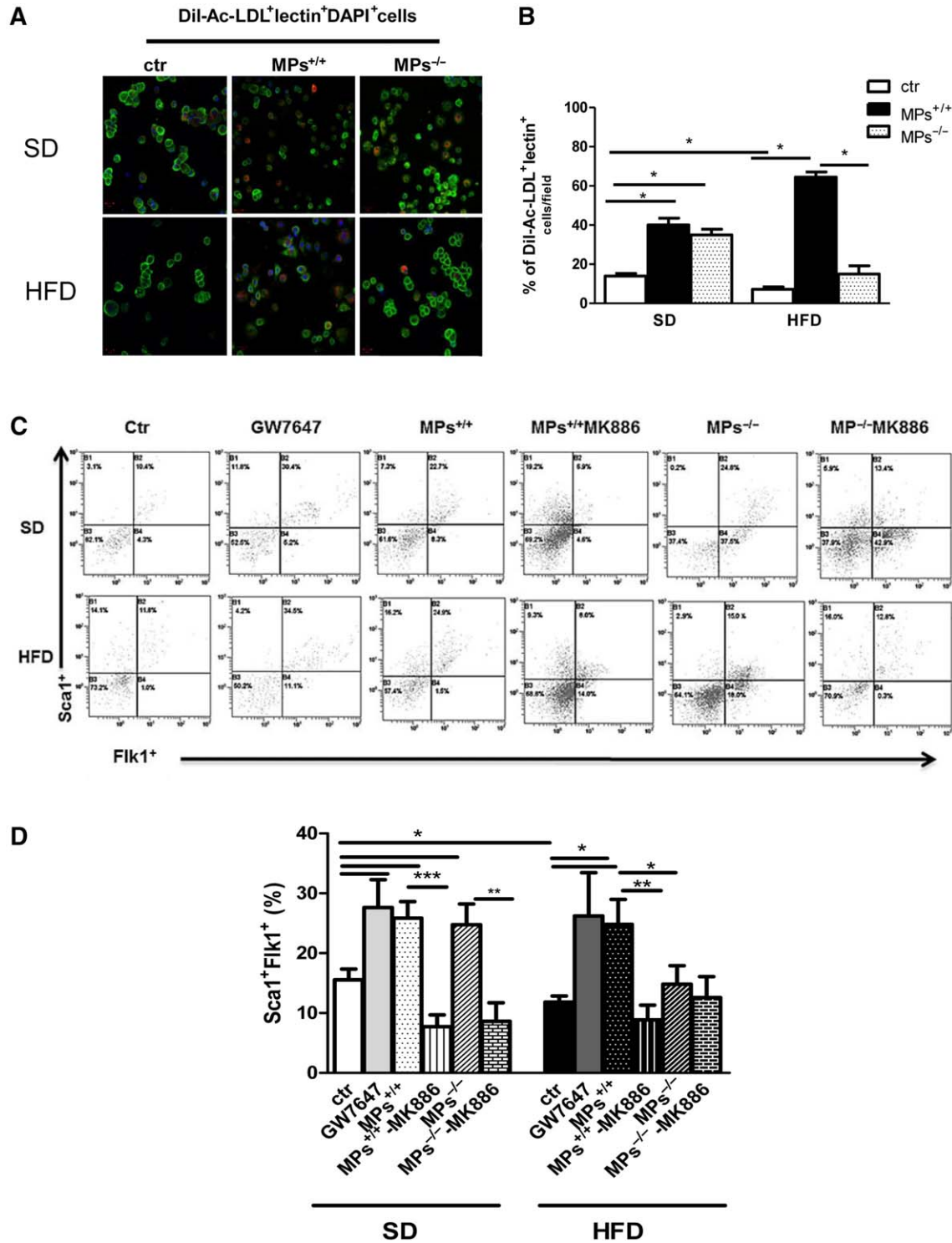


Figure 2. Peroxisome proliferator-activated receptor alpha (PPAR α) carried by MPs increases the number of bone marrow (BM)-derived progenitor cells undergoing in vitro differentiation in mice normal- and HFD-fed diet. **(A):** Confocal microscopy characterization of mice BM-derived endothelial progenitor cell (EPC) differentiation: cells were incubated in the absence or in the presence of either MPs^{PPAR α +/+} or MPs^{PPAR α -/-}. After 7 days in culture, cells with endothelial phenotype were characterized as adherent cells with double-positive staining for Dil-acetylated-LDL (red) and lectin (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). **(B):** Histograms showing the percentage of BM-derived cells labeled with Dil-Ac-LDL and lectin in the absence or in the presence of either MPs^{PPAR α +/+} or MPs^{PPAR α -/-}. **(C):** Representative flow cytometric plots for EPCs from normal- and HFD-fed mice after 7 days of treatment. **(D):** Percentage of BM-derived EPCs pre-treated with GW7647 (PPAR α agonist), MPs^{PPAR α +/+}, MPs^{PPAR α -/-}, in the absence or in the presence of MK886. Data are shown as mean values \pm SEM of at least 5 independent experiments for each condition. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: Dil-Ac-LDL, 19-diiodoacetyl-3,3,3,3-tetramethyl-indocarbocyanine-labeled acetylated-LDL; HFD, high-fat diet; MPs, microparticles; SD, standard diet.

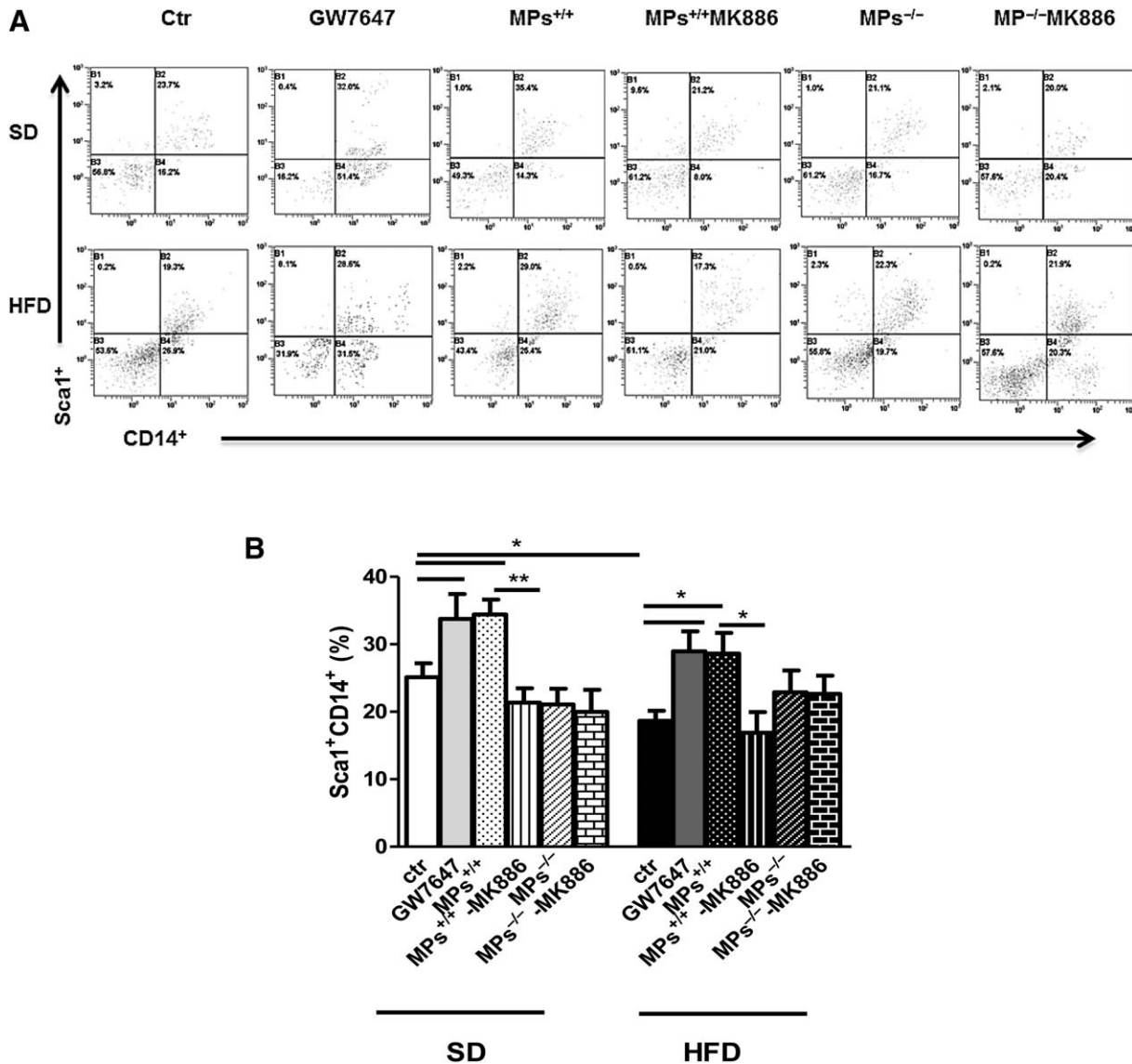


Figure 3. MP treatment increases the number of bone marrow (BM)-derived myeloid progenitors in normal- and HFD-fed diet mice. **(A, B):** Flow cytometer characterization of BM-derived monocyte differentiation: cells were incubated either in the absence or in the presence of GW7647 (PPAR α agonist), MPs^{PPAR α +/+} or MPs^{PPAR α -/-}, in the absence or in the presence of MK886. After 7 days in culture, cells with double-positive staining for Sca1⁺/CD14⁺ were identified as monocytes. Data are shown as mean values \pm SEM of at least 6 independent experiments for each condition. *, $p < .05$; **, $p < .01$. Abbreviations: HFD, high-fat diet; MPs, microparticles; Sca1, stem cell antigen 1; SD, standard diet.

cells were treated with MPs^{PPAR α -/-} alone or in presence of MK886, no significant changes on hemoglobin content was observed independently of the diet. Collectively, these data provide evidence that MPs carrying PPAR α participate in the regulation angiogenesis and restores the HFD-induced impairment of the angiogenic ability of BM-derived EPCs.

Effects of Circulating MPs^{PPAR α +/+} on the Activation of Mouse PPAR α

Here, the manifold effects of circulating MPs^{PPAR α +/+} suggest an underlying mechanism, similar to the activities of PPAR α agonists such as fibrates. Therefore, the effects of circulating MPs isolated from WT or PPAR α -deficient mice on the activation of mouse PPAR α were investigated using the previously described reporter assays. Upon binding to an agonist, the PPAR-GAL4 fusion protein activates a luciferase reporter. As shown in Figure 5B, a significant increase in PPAR nuclear signaling was observed with 1 μ M of

selective PPAR α agonist, GW7647, when compared with control. Remarkably, circulating MPs^{PPAR α +/+}, but not MPs^{PPAR α -/-}, increased the basal luciferase activity by 12.7-fold corresponding to \sim 281 nM of the specific and potent PPAR α activator GW590735, with an EC₅₀ of 124 nM (Fig. 5A, 5B). In contrast, this activity was inhibited by the preincubation of MPs^{PPAR α +/+} with MK886 (20 μ M). Furthermore, MPs^{PPAR α -/-} alone or MPs^{PPAR α -/-}-MK886 treatment did not modify the PPAR α signaling.

These findings indicate that MPs^{PPAR α +/+} have the ability to reinforce PPAR α activation as much as the specific and potent PPAR α agonist.

DISCUSSION

Herein, we demonstrate that metabolic dysfunctions altered BM-derived progenitor cells differentiation and functionality in mice.

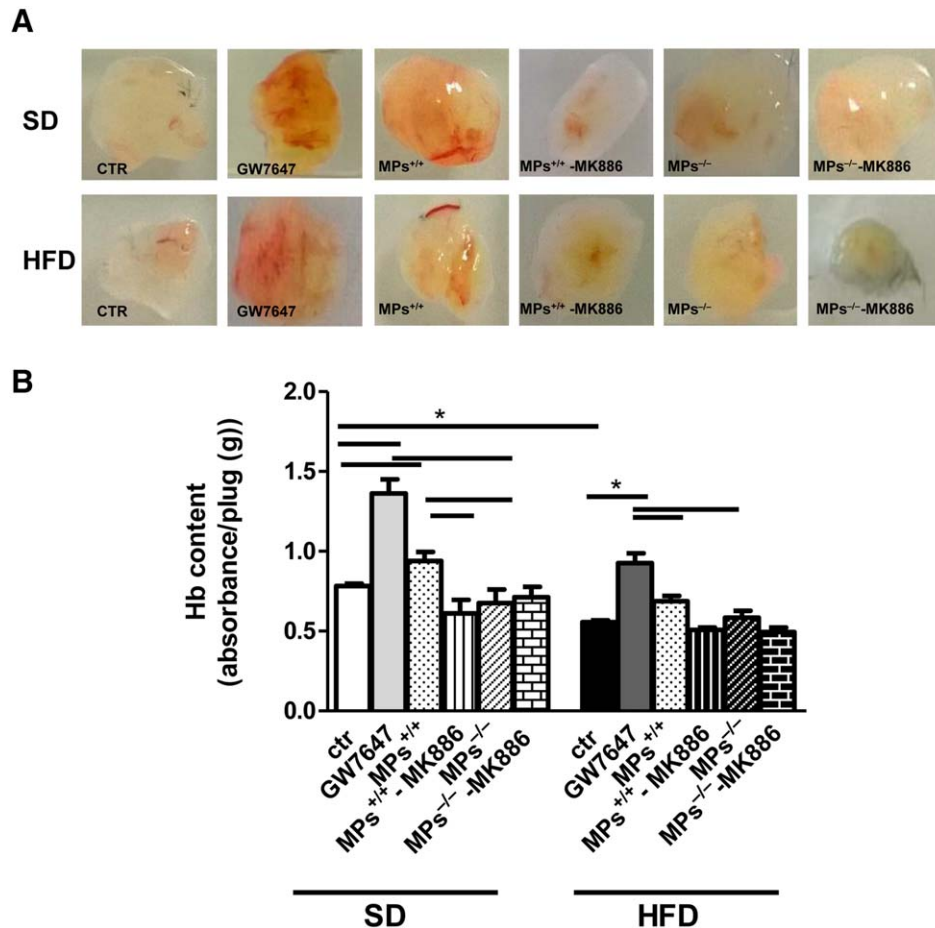


Figure 4. MPs harboring PPAR α promote in vivo bone marrow (BM)-derived EPC-mediated angiogenesis. **(A):** BM-derived cells from normal and HFD-fed mice were treated with GW7647 (1 μ M), MPs^{PPAR α +/+} or MPs^{PPAR α -/-} in the absence or in the presence of MK866. Then, cells were detached, mixed with Matrigel and basic fibroblast growth factor (bFGF) and injected subcutaneously into mice fed with normal or HFD. **(B):** Quantitative measurement of Hb was reported as absorbance/weight of plugs. Data are shown as mean values \pm SEM of at least 5 independent experiments for each condition. *, $p < .05$. Abbreviations: Hb, hemoglobin; HFD, high-fat diet; MPs, microparticles; SD, standard diet.

Most importantly, MPs carrying PPAR α are crucial in restoring both impaired functionality of BM-derived EPCs and failed angiogenesis induced by HFD.

The BM is a highly vascularized tissue and contributes to the transport of BM-derived cells to the systemic circulation. In the present study, the HFD-fed mouse model has been used as a robust model to study the development of impaired functions of BM induced by metabolic dysfunction [17, 18]. HFD feeding did not modify total body weight of mice after 12 weeks of treatment, but it significantly increased adiposity, total cholesterol and glucose levels and reduced the ratio HDL/LDL compared with mice fed with a normal chow diet. These results underscore an “early stage of diet-induced obesity.” This is in agreement with our previous report showing that, in the same model, HFD-induced metabolic alterations without affecting body weight [16].

Importantly, similar to human studies [19], we found that the EPC mobilization ability from BM to circulation is curtailed in HFD-fed animals. In agreement with the results obtained by flow cytometry analysis, HFD significantly reduced circulating MPs from progenitor cells. Consistent with this notion, it has been proposed that impaired phosphorylation of BM endothelial NO synthase leads to reduced mobilization of BM-derived EPCs into the circulation of diabetic mice [20]. We cannot rule out the possibility

that EPCs from HFD-fed mice display a reduced half-life in circulation. We showed both reduced numbers of these precursors in blood and impaired in vitro differentiation of BM-derived progenitor cells. Furthermore, after 7 days of culture, also BM-derived monocytes from HFD-fed mice are reduced, but unlike of EPCs, we observed an increase of circulating mature monocytes. One potential explication could be that blood sample analysis of circulating monocytes does not necessarily represent their mobilization from the BM but also from other niches such as liver or spleen. Hence, HFD appears to cause dysregulation to more primitive hematopoietic progenitor cells in BM. Our data are in agreement with those of Luo et al. [21] showing that obesity significantly alters hematopoietic and lymphopoietic function in BM, suggesting substantial changes in this primary tissue.

Metabolic diseases such as diabetes and obesity are associated with alterations in angiogenesis. Patients with these diseases display impairment of reendothelialization after vascular injury, associated not only with the reduction of number of EPCs but also with alterations of their functions [22, 23]. Several strategies are designed to improve EPC function in these patients, such as the use of statins [24] and PPAR γ agonists [25], and the ex vivo genetic modifications of EPCs [22, 26]. We have also shown that in vitro treatment with low concentrations of ethanol can improve

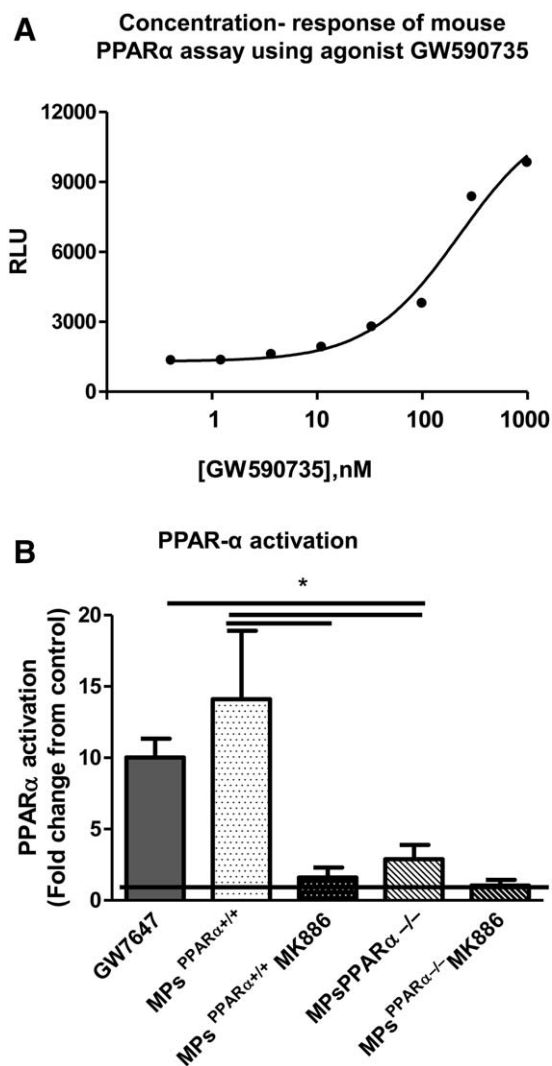


Figure 5. $MPs^{PPAR\alpha+/+}$ induce activation of PPAR α DNA-binding domains. **(A):** Concentration-response of mouse PPAR α Assay using reference agonist GW590735. **(B):** Reporter cells expressing mouse PPAR α were incubated either in the absence or in the presence of $MPs^{PPAR\alpha+/+}$, $MPs^{PPAR\alpha-/-}$ in the absence or in the presence of MK886 or the specific activator GW7647 for 24 hours. Luciferase activity was determined as described in Methods. Data are presented as the mean values \pm SEM of eight independent measurements. *, $p < .05$. Abbreviations: PPAR α , peroxisome proliferator-activated receptor α ; RLU, relative light units.

the functionality of EPCs from HFD-fed mice [16] suggesting that in vitro manipulation may be considered as a therapeutic tool regarding expansion and angiogenic properties of BM-derived cells. In this respect, it has been recently shown that MPs, small vesicles released by cells, can be potential therapeutic tools in pathologies associated with endothelium injury by inducing recruitment of EPCs in the sites of the vascular lesion. Indeed, treatments with endothelial- and platelet-derived MPs participate in the development of blood vessels or angiogenesis [8, 27]. For instance, the endothelial characteristics of “early EPCs” may also be acquired due to uptake of platelet MPs, as a simple transfer of antigens CD31 and vWF [28]. But, few studies have analyzed the effects of total circulating MPs on angiogenesis [7]. It has been reported that circulating MPs, regardless of their sources, participate in vessel remodeling following endothelial denudation [29].

In previous work, we have shown that circulating MPs harboring PPAR α seem to be essential for the proangiogenic reprogramming of EPCs. Since in the present study, differentiation of BM-derived EPCs and monocytes were significantly decreased by HFD, we studied the ability of MPs to restore them. We examined the behavior of these putative vascular progenitors because it is known that CD14 $^{+}$ cell populations may contain cells that can give rise to EPCs or be recruited to the site of neovascularization and directly contribute to angiogenesis [30, 31].

Here, we show that $MPs^{PPAR\alpha+/+}$ favor EPC and monocytic differentiation in BM-derived cells from mice normal- and HFD-fed mice. Interestingly, incubation of MPs with the PPAR α antagonist, MK886 prevented the effects induced by $MPs^{PPAR\alpha+/+}$, suggesting that the presence of PPAR α in MPs is necessary to re-establish EPC and CD14 $^{+}$ cell differentiation in HFD-fed mice.

Surprisingly, MK886 treatment appeared also to prevent EPC differentiation induced by $MPs^{PPAR\alpha-/-}$ in BM-derived cells from normal fed-mice, suggesting that its mechanism of action may extend beyond a specific nuclear receptor. Indeed, it is thought to prevent the conformational change in PPAR α required to dismiss corepressors and recruit coactivators for gene transcription [32]. Structural features of this shift are common to members of the nuclear receptor superfamily [33] and it is possible that MK886 blocks multiple nuclear receptors implicated in EPC differentiation. These data are in accordance with those reported by Kehrer et al. [32] showing that MK886 inhibits PPAR α and, though less effectively, either PPAR β or PPAR γ .

In contrast to the results of Benameur et al. [12], we observed that also $MPs^{PPAR\alpha-/-}$ affected EPC expansion in normal-fed mice. One possible explanation for this discrepancy might relate to the differences in mouse age (8 weeks old vs. 20 weeks in the present study). Indeed, mature BM-derived cells might respond differently to $MPs^{PPAR\alpha-/-}$ than progenitors from young mice.

According to the aforementioned data, the proangiogenic property of these MPs into Matrigel plugs was correlated, at least in part, with expression of EPC differentiation markers. Indeed, we demonstrate that isolated BM-derived cells from normal- and HFD-fed mice expanded by in vitro $MPs^{PPAR\alpha+/+}$ treatment are able to generate in vivo functional vessels. Conversely, in mice fed with normal chow, we found that $MPs^{PPAR\alpha-/-}$ treatment increased EPC differentiation, despite no change on the hemoglobin content in Matrigel plug. Several explanations can be advanced. Thus, it is possible that $MPs^{PPAR\alpha-/-}$ -treated EPCs are not functional or even they form nonfunctional vessels. This finding indicates that MPs carrying PPAR α play an essential role in neovascularization.

Despite the fact that all the above-described pleiotropic effects of MPs are specifically related to the activation of the PPAR α pathway, the molecular mechanism by which $MPs^{PPAR\alpha+/+}$ could affect BM niche and the development of progenitor cells is not yet clear. As previously shown by Benameur et al. [12], PPAR α expression is not transferred into BM-derived cells after $MPs^{PPAR\alpha+/+}$ treatment although it is expressed and functional in BM-derived cells. In this work, we demonstrate that $MPs^{PPAR\alpha+/+}$ activate PPAR α using a reporter cell line over expressing GAL4 fusion proteins to DBD of PPAR α , suggesting they serve as a natural ligand. Regardless of the exact nature of the interaction, it is possible that lipids, protein carried by $MPs^{PPAR\alpha+/+}$ may act as the endogenous ligands leading to modification of BM-derived cell phenotype. In this context, the presence of PPAR α within the MPs might induce through a nongenomic mechanism protein and pi tlbipid

modification or direct protein/protein binding with key signaling pathways. Indeed, it is possible that PPAR α , characterized as a “transcription factor,” has also nontranscriptional functions [34]. For example, it has been described that PPAR α may regulate neuronal nicotinic acetylcholine receptor function by a nongenomic stimulation of tyrosine kinases [35]. Furthermore, it was demonstrated that PPAR α and AP-1 or NF- κ B can functionally interact by interfering with signaling pathways (such as protein phosphorylation), and this modulates transcription factor activity [36]. Thus, further studies are needed for a better understanding of the mechanism evoked by MPs^{PPAR α +/+} on BM-derived progenitor cells in vitro, and on in vivo in order to elaborate an efficient strategy for their potential application to correct failed angiogenesis in obesity or diabetes.

A limitation of our study is the lack of MP characterization, a longer time course of analysis may be necessary to evaluate their components. For this reason, we cannot exactly assess how PPAR α carried by MPs is implicated in BM-derived cells differentiation pathway. Here, we demonstrated that this process is likely to be regulated by crosstalk between PPAR α and his surrounding microenvironment in MPs. For instance, a mechanism by which PPAR α exerts EPC differentiation might be through modulating lipid levels. Indeed, it is well known that lipids, and especially lipoproteins can act on the target cells via MPs inducing phenotypic and functional changes in the recipient cells [37]. In this connection, PPAR α deletion could influence the compositions of both external and internal membranes of MPs from PPAR α -null mice. Indeed, it was reported that basal fatty acid homeostasis in PPAR α -null mice is altered, that is, abnormal accumulation of lipid in the liver, lower serum triglycerides and altered constitutive expression of fatty acid-metabolizing enzymes [38]. These alterations might well be related.

CONCLUSION

Overall, these data support that HFD-related changes impair EPC mobilization from BM to circulation and alters the stem cell niche, independently of obesity development. In addition, in HFD-fed mice, MPs carrying PPAR α restore differentiation of hematopoietic cells and their recruitment into target sites for new blood vessel

formation without typical drug associated side effects. These insights will open new possibilities to impact BM-derived cells function by MPs carrying PPAR α . In this respect, MPs carrying PPAR α can represent a true therapeutic potential tool on pathophysiological conditions associated with failed EPC differentiation such as diabetes, although the mechanisms involved in these effects are not completely elucidated. However, the fact that circulating MPs are from diverse cell origins may lead to heterogeneous or multiple-directional effects of MPs. Further studies are needed to completely understand the effects of MPs carrying PPAR α on progenitor differentiation during HFD conditions in which BM microenvironment and, in consequence, BM niche function is altered.

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AUTHOR CONTRIBUTIONS

L.V.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; E.L. and R.S.: collection of data; R.A.: manuscript writing, final approval of manuscript; M.C.M.: conception and design, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

NOTE ADDED IN PROOF

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