International Journal of Obesity
https://doi.org/10.1038/s41366-018-0167-1

ARTICLE

Adipocyte and Cell Biology

Regulation of PKCβ levels and autophagy by PML is essential for high-glucose-dependent mesenchymal stem cell adipogenesis

Claudia Morganti[1] · Sonia Missiroli[1] · Magdalena Lebiedzinska-Arciszewska[1,2] · Letizia Ferroni[3] · Lucia Morganti[4] · Mariasole Perrone[1] · Daniela Ramaccini[1] · Savino Occhionorelli[4] · Barbara Zavan[3] · Mariusz R. Wieckowski[2] · Carlotta Giorgi[1]

Received: 21 February 2018 / Revised: 15 May 2018 / Accepted: 15 June 2018

Abstract

Background/Objectives Obesity is a complex disease characterized by the accumulation of excess body fat, which is caused by an increase in adipose cell size and number. The major source of adipocytes comes from mesenchymal stem cells (MSCs), although their roles in obesity remain unclear. An understanding of the mechanisms, regulation, and outcomes of adipogenesis is crucial for the development of new treatments for obesity-related diseases. Recently an unexpected role for the tumor suppressor promyelocytic leukemia protein (PML) in hematopoietic stem cell biology and metabolism regulation has come to light, but its role in MSC biology remains unknown. Here, we investigated the molecular pathway underlying the role of PML in the control of adipogenic MSC differentiation.

Subjects/Methods Muscle-derived stem cells (MDSCs) and adipose-derived stem cells (ADSCs) obtained from mice and voluntary patients (as a source of MSCs) were cultured in the presence of high glucose (HG) concentration, a nutrient stress condition known to promote MSCs differentiation into mature adipocytes and the adipogenic potential of PML was assessed.

Results PML is essential for a correct HG-dependent adipogenic differentiation, and the enhancement of PML levels is fundamental during adipogenesis. Increased PML expression enables the upregulation of protein kinase Cβ (PKCβ), which, in turn, by controlling autophagy levels permits an increase in peroxisome proliferator-activated receptor γ (PPARγ) that leads the adipogenic differentiation. Therefore, genetic and pharmacological depletion of PML prevents PKCβ expression, and by increasing autophagy levels, impairs the MSCs adipogenic differentiation. Human ADSCs isolated from overweight patients displayed increased PML and PKCβ levels compared to those found in normal weight individuals, indicating that the PML-PKCβ pathway is directly involved in the enhancement of adipogenesis and human metabolism.

Conclusions The new link found among PML, PKCβ, and autophagy opens new therapeutic avenues for diseases characterized by an imbalance in the MSCs differentiation process, such as metabolic syndromes and cancer.

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells, able to differentiate into a variety of cell lineages and having a high self-renewal potential [1]. Therefore, the biology of MSCs, their capacity to treat various diseases, and their potential role in managing the components of metabolic syndrome have been extensively studied [2, 3].

Hyperglycemia is a common nutrient stress condition that can occur in patients with type 2 diabetes, who are typically obese. Obesity is considered the main cause of metabolic syndrome due to the increased adipocyte size and number [4–6]. MSCs are a major source of adipocyte generation [7]; thus, the identification of new molecular adipogenesis regulators could provide innovative

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41366-018-0167-1) contains supplementary material, which is available to authorized users.

✉ Carlotta Giorgi
grgclt@unife.it

[1] Department of Morphology, Surgery and Experimental Medicine, Section of General Pathology and Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy

[2] Nencki Institute of Experimental Biology PAS, Warsaw, Poland

[3] Department of Biomedical Sciences, University of Padova, Padova, Italy

[4] Department of Morphology, Surgery and Experimental Medicine, Acute Care Surgery Service, University of Ferrara and S.Anna University Hospital of Ferrara, Ferrara, Italy

Published online: 06 August 2018

therapeutic strategies for metabolic-related and obesity-related disorders.

Several important regulatory pathways, particularly the Wnt signaling pathway, have been found to participate in the regulation of MSC proliferation and differentiation. The effects of Wnt signaling can induce different or even opposing biological functions [8–10]; indeed, the complex effect of Wnt signaling is closely related to its target genes. Interestingly, the promyelocytic leukemia protein (PML) gene can function as a target of the Wnt signaling pathway. Indeed, it was found that PML forms a complex with β-catenin, and the two proteins colocalize in the nucleus [11]. Thus, PML has newly emerged as a possible regulator of stem cell biology.

PML was first identified as a fusion partner of human retinoic acid receptor α (RARα) when a chromosomal translocation was discovered in acute promyelocytic leukemia (APL) [12–14], and its role in solid tumors and leukemia pathogenesis has been thoroughly investigated [15–18].

Recently, PML has been shown to be required for hematopoietic stem cell (HSC) maintenance and neural progenitor cell (NPC) differentiation. Indeed, PML knockout (KO) HSC exhausted due to increased cycling rate [19] and loss of PML leads to an increased number of proliferating NPC and a defective exit from cell cycle [20], but its role in MSC biology remains unknown. Moreover, different groups have reported that the expression of PML is relevant to the response to metabolic insults, nutritional disorders and obesity, but the results of these studies are contradictory [21–23]. Thus, in this work we investigated the molecular pathway involved in the process.

Materials and methods

Cell cultures

Primary MDSCs cultures were prepared from newborn C57BL/6 WT, PML KO, and protein kinase C β (PKCβ) KO mice. Five-day-old mice were sacrificed, and the skeletal muscles were isolated. After washes and bone removal, the muscles were minced and digested for 1 h with 0.2% collagenase A (103586, Roche, Basel, Switzerland) at 37 °C. Using a 75-μm cell strainer, the obtained cells were purified from the undigested tissue and plated in Dulbecco's modified Eagle's medium (DMEM) low glucose (LG) medium (5 mM glucose). After 2 h, the suspension containing MDSCs was transferred to a new dish, and this passage was repeated after 24 h. The cells able to attach after 24 h were considered MDSCs.

Human adipose-derived stem cells (hADSCs) were extracted from human subcutaneous adipose tissues from patients undergoing surgical procedures, after obtaining their informed consent. The adipose tissues were digested with 0.075% collagenase (type 1A; Sigma-Aldrich, Saint Louis, USA) in Hank's balanced salt solution for 3 h at room temperature. After inactivating the digestion process by adding DMEM LG containing 10% fetal bovine serum, the samples were centrifuged at 1200 rpm for 4 min, and the pellets containing hADSCs were washed and plated.

For the high-glucose-dependent adipogenic differentiation, DMEM LG was replaced with DMEM high glucose (HG) (25 mM glucose) for 7 days or the indicated time. For the classical adipogenic differentiation, the MDSCs were cultured in DMEM HG plus 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1 mM indomethacin, and 1 μM dexamethasone for 3, 7, and 21 days.

Classical tissue culture plates (Thermo Scientific, Waltham, USA) were used for all experimental condition, avoiding the use of specific plate's morphologies which have been demonstrated to conditionate the naïve commitment of MSCs in basic culture medium [24].

In vivo animal model

Four-week-old male C57BL/6 wild-type (WT) and PML KO mice were used in this study. All mice were individually housed in structures with stainless-steel grid lids, and wood shavings were scattered on the floor. The vivarium was maintained at 23 °C under a 12-h light/12-h dark cycle with lights off at 7 pm. The mice in the high-fat diet group had access to pelleted Teklad Rodent Diet with 60% of calories from fat (MV2 Envigo RMS S.R.L., San Pietro al Natisone, Italy). Water and food were available ad libitum.

Oil Red O staining

The Oil Red O (ORO) staining (O0625, Sigma-Aldrich, Saint Louis, USA) of the cytoplasmic drops of neutral lipids was performed according to the standard procedure (see in ref. [25]), and images were acquired using light microscopy. Briefly, after fixing with 4% Paraformaldehyde, the cells were permeabilized with 2-propanol for 5 min and then stained with ORO for 15 min.

Immunoblotting

For the immunoblotting, the following primary antibodies were used: mouse anti-PML [MAB3738] (1:3000) from Millipore, Burlington, USA; rabbit anti-peroxisome-proliferator-activated receptor γ (PPARγ) [2435] (1:1000) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [2118] (1:5000) from Cell Signaling, Danvers, USA; rabbit anti-GLUT4 [2213] (1:1000), mouse anti-β-actin [A1978] (1:10000), and rabbit anti-LC3B [L7543]

(1:1000) from Sigma-Aldrich, Saint Louis, USA; and PKCβ [ab32026] (1:1000) and rabbit anti-PML [ab72137] (1:1000) from Abcam, Cambridge, UK. Finally, the membranes were incubated with the appropriate horseradish peroxidase-labeled secondary antibodies (Thermo Fischer Scientific, Waltham, USA), followed by detection by chemiluminescence (Thermo Scientific, Waltham, USA) using Image Quant LAS4000 (GE Healthcare, Little Chalfont, UK).

Reagents and viral vectors

For the pharmacological deletion of PML, arsenic trioxide (Ato) (A1010, Sigma-Aldrich, Saint Louis, USA) (500 nM) in DMEM HG was used. Rapamycin (Rapa) (553210, Calbiochem, Burlington, USA) (100 nM) was used to induce autophagy in the WT MDSCs. LY-294002 (L9908, Sigma-Aldrich, Saint Louis, USA) (50 μM) was used to inhibit autophagy in the PML KO MSCs. As indicated, the cells were infected with green fluorescent protein-light chain 3 (GFP-LC3) adenovirus and PKCβ adenovirus.

Fluorescence microscopy and quantitative analysis of GFP-LC3 dots

The cells were cultured in 24-mm glass coverslips and infected at 50% confluence with the GFP-LC3 virus. After 36 h, images were obtained under a Nikon LiveScan Swept Field Confocal Microscope Eclipse Ti equipped with NIS-Elements microscope imaging software (Nikon Instruments, Tokyo, Japan). For each condition, the number of GFP-LC3 dots was counted in at least 20 independent visual fields.

Quantitative real-time PCR

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, USA). Real-time PCR was performed using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche, Basel, Switzerland), following the manufacturer's protocol. The values were normalized to the expression of GAPDH, which served as an internal reference.

Statistical analysis

All results are expressed as the mean ± SD obtained from at least three independent experimental days. The probability of significant differences among the experimental groups was determined by analysis of variance (ANOVA), and the results following treatments showing significant overall changes were subjected to post hoc Bonferroni tests. Student's *t* test was performed to determine the statistical significance between two groups. *P* values <0.05 were considered statistically significant. Different labels indicate $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$, and $****p < 0.00001$.

Results

PML is essential for HG-dependent adipogenic differentiation

To determine the involvement of PML in MSCs differentiation into adipocytes, MDSCs (Fig. 1a) and ADSCs (Fig. S1a) obtained from PML WT and KO mice were used.

A HG concentration, which is one of the most common conditions leading to an increase in adipose tissue in vivo [26], promotes MSCs differentiation into mature adipocytes in vitro [27]. The adipogenic potential of PML WT and KO MSCs was assessed after a 7-day HG treatment by immunoblotting and by measuring the levels of PPARγ, which is a key transcriptional factor for adipogenic commitment [28]. As shown in Fig. 1b, c and Fig. S1b, the PPARγ protein levels increased in the WT MSCs following the addition of HG; in contrast, in the PML KO MSCs, the PPARγ protein levels remained unaltered both in the LG and HG conditions. Accordingly, since PPARγ expression is under control of PPARδ [29], deletion of PML was linked to a decrease of PPARδ levels (Fig. S1c), as also reported by Ito et al. [30].

During adipogenesis, differentiating cells begin to accumulate lipids in their cytosol. The ORO staining, selective for the lipids depots, revealed a substantial adipose differentiation in WT MSCs grown in HG, whereas PML KO MSCs cultured with HG were unable to accumulate lipids and differentiate (Fig. 1d, e), indicating a less adipogenic conversion of MSCs in the absence of PML.

To confirm that PML is critical for adipogenic differentiation, the PML protein levels were pharmacologically decreased in the WT MSCs using arsenic trioxide (Ato), which is typically used in APL therapy to degrade PML-RARα [31]. The Ato treatment drastically reduced the PML protein levels in our model (Fig. 1b), and due to this reduction, the adipogenesis process was prevented. Indeed, the PPARγ protein levels did not increase after the addition of HG (Fig. 1b, c), and the number of ORO-positive cells was significantly reduced in comparison to the untreated cells (Fig. 1d, e).

We then investigated whether PML deletion can prevent or only delay adipogenic differentiation. The accumulation of lipid drops was monitored for 21 days and the ORO staining was analyzed. The percentage of ORO-positive WT MSCs in the HG medium constantly increased, while the PML KO MSCs were unable to differentiate (Fig. 1f), indicating a general block of the adipogenic process.

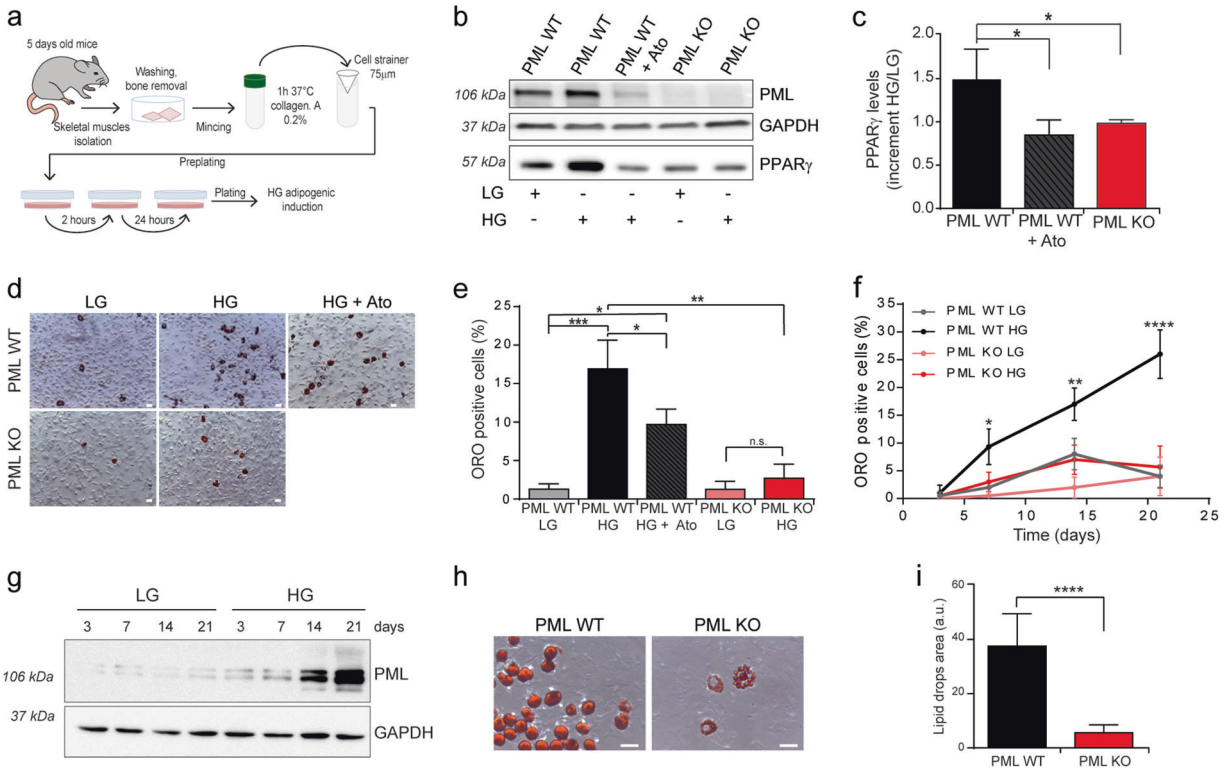

Fig. 1 PML is essential for high-glucose-dependent adipogenic differentiation. **a** Schematic representation of MDSCs isolation using the preplate technique. **b** Representative immunoblot of PML, GAPDH, and PPARγ in WT and PML KO MDSCs. Where indicated, the cells were cultured with 5 mM glucose (LG), 25 mM glucose (HG), and 25 mM glucose plus 500 nM arsenic trioxide (Ato) for 7 days. **c** Quantification of the increase in PPARγ levels (HG/LG). **d** Representative images and **e** quantification of lipid drops by ORO staining. Magnification ×10; scale bar 10 µm. **f** Quantification of lipid drops in WT and PML KO MDSCs cultured with LG or HG for 3, 7, 14, and 21 days. **g** Representative immunoblot of PML and GAPDH in WT MDSCs cultured with LG or HG for 3, 7, 14, and 21 days. **h** Representative images and **i** quantification of lipid drop area in WT and PML KO MDSCs cultured with HG for 21 days. Magnification ×40; scale bar 10 µm. Student's *t* test. All data represent the mean ± SD obtained from at least three independent experimental days. ANOVA (unless indicated otherwise); $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$, $****p < 0.00001$ and n.s. $p > 0.05$

Accordingly, we also observed increased PML levels over time in in vitro MSC cultures during HG-dependent adipogenic differentiation (Fig. 1g).

Furthermore, analyzing the morphology and area of the lipid drops it was clear that the alteration of adipogenesis observed in the PML KO MSCs was not only associated with the number of cells able to differentiate but also with a deficiency in the correct lipid drop formation. As shown in Fig. 1h, i, after 21 days of HG administration, in contrast to the WT MSCs, the few PML KO MSCs that were able to differentiate contained small lipid droplets that failed to fill the cytosol.

Altogether, these data support the hypothesis that PML is essential for correct HG-dependent adipogenic differentiation.

PML affects HG-induced adipogenic differentiation through autophagy modulation

Macroautophagy (termed autophagy in this manuscript) is a bulk degradation process in which proteins and organelles are sequestrated into double-membrane vesicles called autophagosomes and subsequently degraded through the fusion of autophagosomes with lysosomes [32]. By selectively degrading harmful protein aggregates or damaged organelles, autophagy maintains intracellular homeostasis and performs an essential quality control function within the cell [33].

Among its myriad of cellular and developmental functions, autophagy level regulation has emerged as a key regulator of lipid metabolism and adipogenesis [34–38]. Knowledge regarding the role of autophagy in MSCs biology relies on the observation that primary human bone marrow MSCs have high levels of constitutive autophagy that decrease as these cells differentiate into osteoblasts [39].

Similarly, through the analysis of the conversion of LC3-I to LC3-II via immunoblotting, we show that autophagic levels in WT MSCs decrease after HG-dependent adipogenic differentiation (Fig. 2a and Fig. S1b).

Moreover, since we previously published that PML is essential for repressing the autophagic process in primary

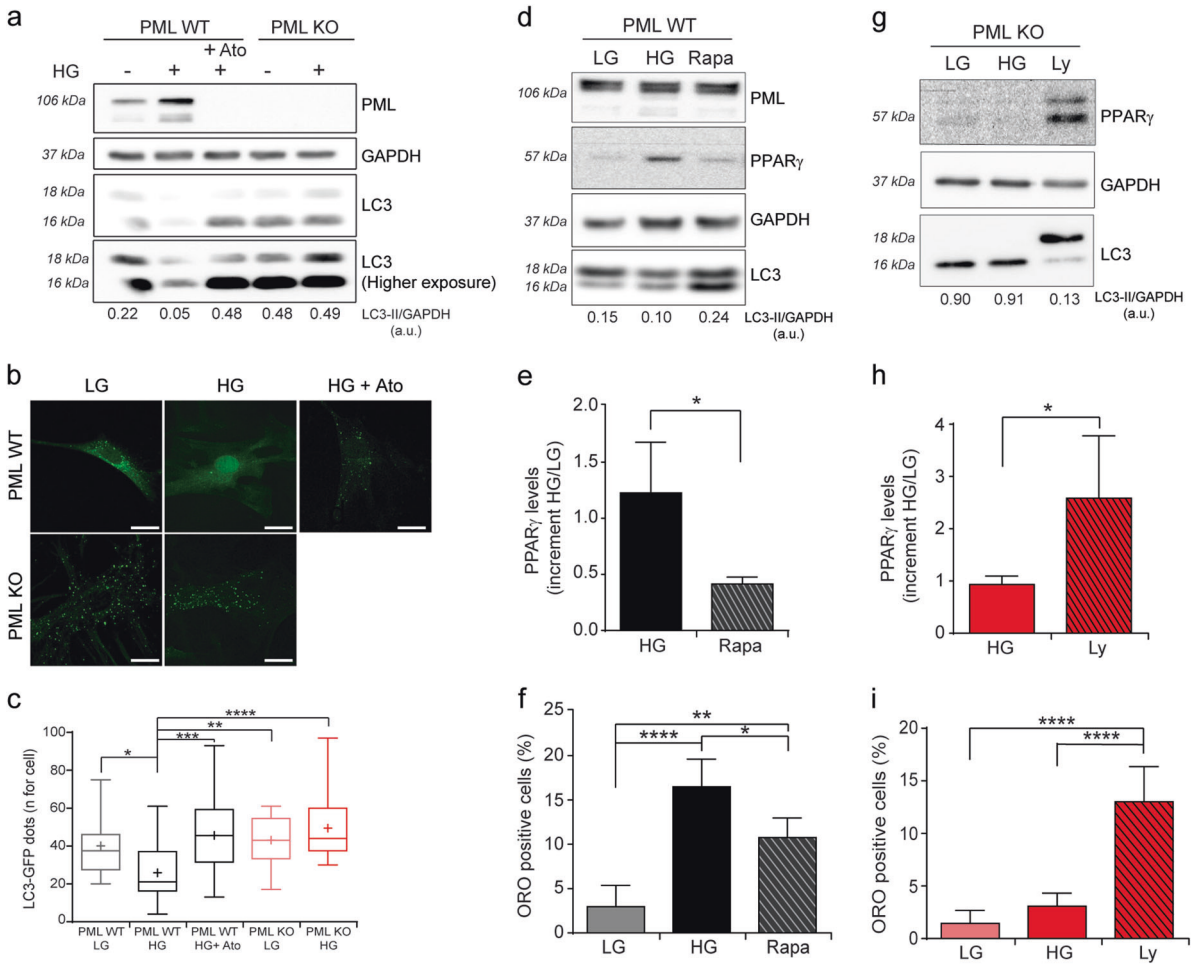

Fig. 2 Autophagic levels regulated by PML affect high-glucose-dependent adipogenic differentiation. WT and PML KO MDSCs were cultured, as indicated, in 5 mM glucose (LG), 25 mM glucose (HG), and HG plus 500 nM arsenic trioxide (Ato), or plus 100 nM rapamycin (Rapa) or plus 50 μM Ly-294002 (Ly) for 7 days. **a** Representative immunoblot of PML, GAPDH, and LC3. **b** Representative images and **c** quantification of GFP-LC3 clustering in MDSCs. Magnification: ×60; scale bar 10 μm. The data are shown as the median (+) plus the min to max values (box). **d**, **g** Representative immunoblot of PML, GAPDH, PPARγ, and LC3. Adipogenic differentiation is shown as (**e**, **h**) an increase in the ratio of the HG/LG PPARγ and (**f**, **i**) quantification of lipid drops with ORO staining. Data are shown as the % mean ± SD (unless indicated otherwise) obtained from at least three independent experimental days. ANOVA; $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$, and $****p < 0.00001$

mouse embryonic fibroblasts and mice [40], here we confirmed that PML plays the same role also in MSCs. Indeed, PML KO MDSCs or WT MDSCs treated with Ato display higher levels of LC3-II compared to WT MDSCs and prevent the decrease of LC3-II levels followed by HG adipogenic differentiation (Fig. 2a and Fig. S1b).

To measure the autophagic degradation activity, MSCs were treated with NH_4Cl, which abolishes the acidification of lysosomes. The accumulation of LC3-II after the NH_4Cl administration (Fig. S2) in both the WT and KO genotypes suggests that PML induces a real increase in the autophagic process under our experimental conditions.

We then confirmed the involvement of autophagy during the adipogenesis process in live imaging experiments analyzing autophagosomes as fluorescent cytoplasmic dots that contained LC3 fused to GFP. Autophagy was decreased after HG differentiation in WT MDSCs, while PML deletion caused an increase in autophagic levels which opposed to HG differentiation effect (Fig. 2b, c).

To verify the hypothesis that autophagy controls the adipogenic differentiation process, and in turn explains the differences in adipose differentiation efficiency observed in PML WT and KO MSCs, we modulated autophagy in our experimental conditions by using pharmacological agents suggested by recent guidelines [41], because of the strong difficulty of transfection in this primary cell type.

Thus, WT MSCs were treated with Rapa for 7 days to increase the autophagy level, which was detected by the LC3-II conversion (Fig. 2d). Following the Rapa treatment, the PPARγ protein levels did not increase (Fig. 2d, e), and consequently, the % of ORO-positive cells in the WT

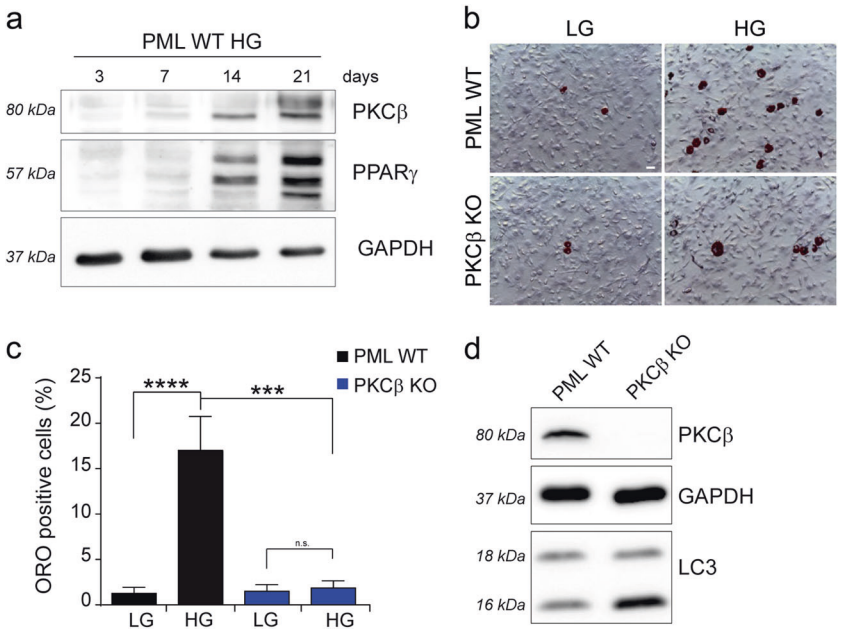

Fig. 3 PKCβ KO MSCs are unable to differentiate in adipocytes due to high levels of autophagy. **a** Representative immunoblot of PKCβ, PPARγ, and GAPDH in WT MDSCs cultured with HG for 3, 7, 14, and 21 days. **b** Representative images and **c** quantification of lipid drops by ORO staining in WT and PKCβ KO MDSCs cultured with LG or HG. The data are shown as the % of positive cells relative to the total number of cells. Magnification ×10; scale bar 10 µm. **d** Representative immunoblot of PKCβ, GAPDH, and LC3. Data represent the mean ± SD obtained from at least three independent experimental days. ANOVA; ***$p < 0.0001$, ****$p < 0.00001$ and n.s. $p > 0.05$

MDSCs significantly decreased (Fig. 2f) compared to that in the WT MDSCs cultured under the control HG conditions.

However, the Ly-294002 (Ly) treatment used to inhibit autophagy (Fig. 2g) restored the ability of PML KO MSCs to differentiate into adipocytes, which was demonstrated by an increase in the PPARγ protein levels (Fig. 2g, h) and accumulation of lipid drops (Fig. 2i).

Altogether, these results demonstrate that a tight control of autophagy is essential for correct adipocyte differentiation and that PML plays a key role in this regulation.

PKCβ level regulation by PML enables HG-dependent adipogenic differentiation

PKC family members play essential roles in a variety of physiological functions, including cell metabolism, proliferation, differentiation, migration, and apoptosis [42–44]. Moreover, PKC isoforms have been shown to be involved in the regulation of adipocyte differentiation [45–47], and in particular, the specific isoform PKCβ was reported to inhibit autophagy [48].

Thus, we sought to investigate the direct involvement of PKCβ in the signaling route of HG-dependent differentiation process regulated by PML through autophagy control in MSCs.

Accordingly with data showed in Fig. 1g, we detected increased PKCβ protein levels during the HG-dependent adipogenesis in WT MSCs (Fig. 3a).

Expectedly, the PKCβ KO MSCs were unable to accumulate lipid drops following the HG administration (Fig. 3b, c) since their autophagic levels are higher than those in the WT MSCs (Fig. 3d) but comparable to those in the PML KO MSCs (Fig. 2a).

We found out that the pharmacological and genetic deletion of PML was associated with a reduction in the PKCβ protein and mRNA levels (Fig. 4a–c). The introduction of PKCβ by an adenovirus carrying PKCβ-GFP rescued the adipogenic potential of the PML KO MSCs (Fig. 4d, e) by restoring the correct autophagy levels and enhanced adipogenesis in the WT MSCs (Fig. S3a, S3b).

hADSCs derived from overweight patients show increased PML levels

Although PML upregulation is required for the correct adipogenic differentiation, an excessive increase in PML could contribute to the promotion of obesity, which is a complex disease characterized by the accumulation of excess body fat caused by an increase in adipose cell size and number. Thus, we investigated the levels of PML in hADSCs isolated from subcutaneous adipose tissue of voluntary patients undergoing surgical procedures (Fig. 5a). The patients were divided into the following three groups (according to the World Health Organization definitions): normal weight (body mass index (BMI) <25 kg/m^2), overweight (25 < BMI < 30 kg/m^2), and obese (BMI >30 kg/m^2).

Interestingly, the levels of PML were significantly increased in the overweight hADSCs compared to those in the normal weight hADSCs (Fig. 5b, c), and the increase in PML was accompanied by increased PKCβ levels, supporting our hypothesis that the PML-PKCβ pathway is directly involved in the enhancement of adipogenesis.

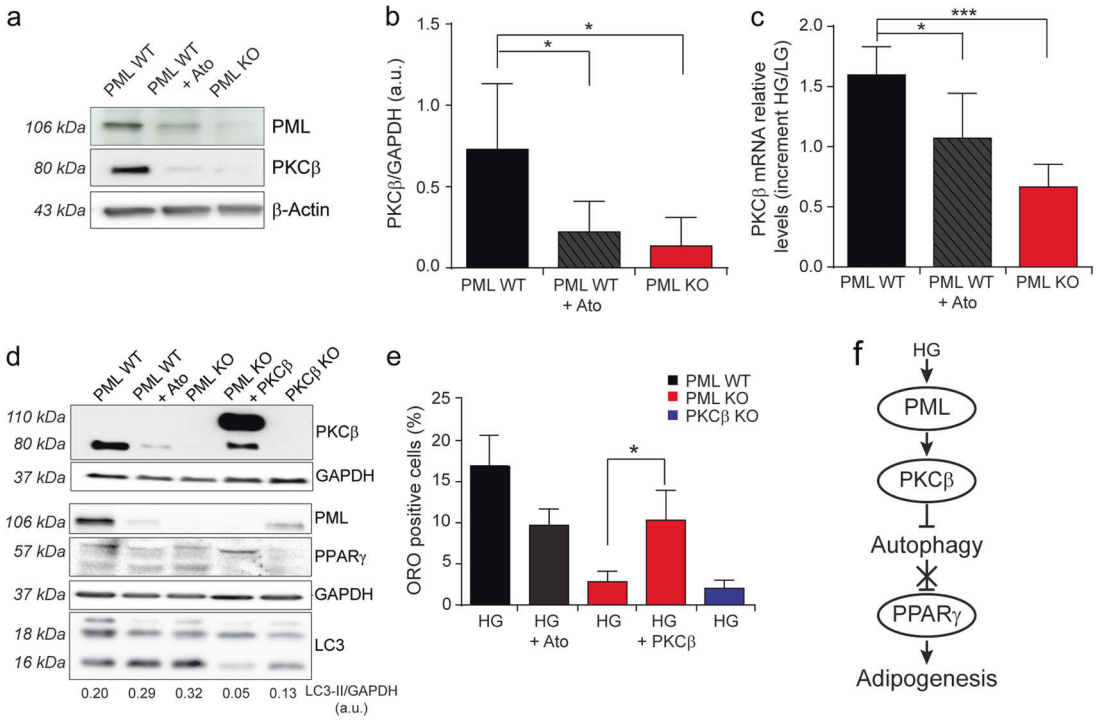

Fig. 4 Lack of PKCβ expression in PML KO prevents high-glucose-dependent adipogenic differentiation. **a** Representative immunoblot of PML, PKCβ, and β-actin, **b** quantification of relative PKCβ protein, and **c** mRNA levels in WT and PML KO MDSCs cultured with HG and, as indicated, HG plus 500 nM arsenic trioxide (Ato). **d** Representative immunoblot of PML, PKCβ, PPARγ, LC3, and GAPDH in WT, PML KO, and PKCβ KO MDSCs cultured with HG and, as indicated, HG plus 500 nM arsenic trioxide (Ato) or infected with PKCβ-encoded virus for 7 days. **e** Quantification of lipid drops with ORO staining in WT, PML KO, and PKCβ KO MDSCs cultured with HG and, as indicated, HG plus 500 nM arsenic trioxide (Ato) or infected with PKCβ-encoded virus for 7 days. **f** A schematic model representing HG-dependent adipogenesis regulated by PML. Data represent the mean ± SD obtained from at least three independent experimental days. ANOVA; $*p < 0.05$ and $***p < 0.0001$

Surprisingly, the PML levels in the obese hADSCs were decreased relative to those in the overweight hADSCs and were comparable to those in the normal weight hADSCs (Fig. 5b, c).

In order to investigate these unexpected data, we performed a single-cell immunofluorescence analysis for PML and PPARγ in WT MSCs population after 21 days of HG differentiation. We found out that those MSCs completely differentiated in adipocytes (with high PPARγ levels and visible lipid drops formed) showed decreased PML levels compared to the surrounding cells (Fig. S4a).

Furthermore, the PML levels in mature adipose tissues of mice feed with high-fat diet were markedly reduced compared with those in other tissues, such as the liver (Fig. S4c), while a substantial increment of PML is shown in livers of mice feed with high-fat diet (Fig. S4b). Therefore, PML increment seems only necessary during the first steps of the adipogenesis process to allow PKCβ expression and regulate autophagy. However, during the final phase, the PML levels must be down-regulated to avoid unnecessary lipid accumulation. Abnormal conditions that bypass the impact of PML on adipogenesis can drive an uncommitted and faster lipids accumulation (Fig. S4b) with progression of metabolic and obesity-related disorders. Indeed, PML WT MSCs cultured in classical adipogenic medium (DMEM HG plus 10 µg/mL insulin, 0.5 mM IBMX, 0.1 mM indomethacin, and 1 µM dexamethasone) differentiated more than those subjected to the HG-dependent adipogenic induction (Fig. S4e) without increases in PML levels, indicating that a higher % of MSCs completely matured into adipocytes bypassing the PML-dependent adipogenic pathway (Fig. S4c). Accordingly, also the PML KO MSCs were able to differentiate under adipogenic medium (Fig. S4e), suggesting a complete loss of control on HG-dependent adipogenic route regulated by PML.

Discussion

Obesity has become a major public health problem worldwide due to its increasing incidence and because it is a major risk factor for type 2 diabetes and cardiovascular disease [49], as well as for certain cancers [50]. Obesity is a complex disease characterized by the accumulation of excess body fat, which is caused by an increase in adipose

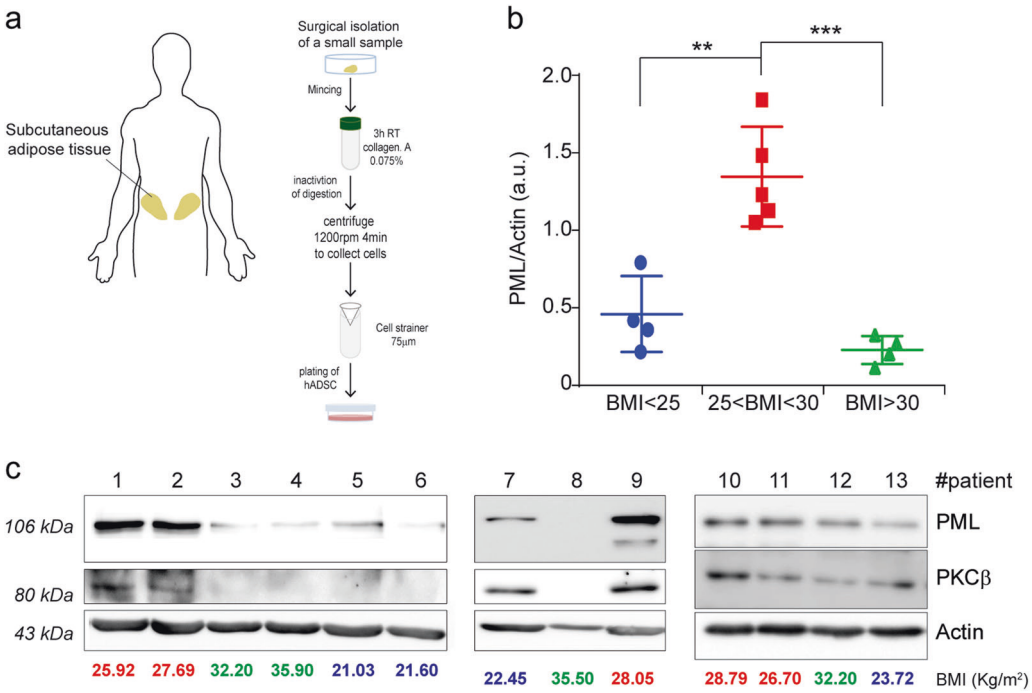

Fig. 5 PML levels increased in hADSCs from overweight patients. **a** Schematic representation of hADSCs isolation from human subcutaneous adipose tissue. hADSCs samples were divided into the following three groups according to the body mass index (BMI) of the patient: normal weight BMI <25 kg/m^2 (blue), overweight 25 < BMI < 30 kg/m^2 (red), and obese BMI >30 kg/m^2 (green). **b** Quantification of PML protein levels in hADSCs and **c** representative immunoblot of PML, PKCβ, and β-actin in hADSCs. The data are shown as the mean ± SD. ANOVA; $^{**}p < 0.01$ and $^{***}p < 0.001$

cell size and number. MSCs are a major source of adipocyte generation; indeed, MSCs are multipotent cells that can differentiate into a variety of cells of mesodermal lineage, including adipocytes [51]. However, the roles of MSCs in obesity remain unclear, and an understanding of the mechanisms, regulation, and outcomes of adipogenesis is crucial for the development of MSC-based treatments for obesity-related diseases.

The regulatory mechanisms of MSCs adipogenesis are complex, but most mechanisms involve the regulation of a range of transcription factors, such as PPARγ and several members of the CCAAT/enhancer-binding proteins.

In this study, we focused on new possible upstream regulators of the adipogenesis process, which finally merged in PPARγ upregulation. Previously, we have identified that HG exposure plays a primary role in adipogenic differentiation, providing a direct link between hyperglycemia and an increase in adiposity [27], which, in turn, may play a key role in the progression of metabolic dysfunction, such as an irreversible diabetic state.

Diet can be considered one of the major cause of obesity [52] due to both caloric intake and the synthetic additive increasingly used in industrial highly processed food [53]. In addition to hyperglycemia, patients's habits and systemic stress were reported to promote inflammation and oxidative stress impacting on adipocyte disorders [54–56].

Here, we identified PML as a critical player in the HG-dependent adipogenic process; indeed, the genetic and pharmacological deletion of PML in MSCs impaired PPARγ expression, lipid droplet accumulation, and thus adipogenic differentiation.

These data are coherent with previous findings that highlighted a critical role for PML in stem cell biology and in particular in HSCs, in which the absence of PML resulted in the loss of HSC maintenance, loss of asymmetric division, and, as a consequence, reduced HSC rate of differentiation [19].

More recently, PML has been shown to promote osteogenic differentiation in MSCs, which is associated with the upregulation of integrin-binding sialoprotein [57], confirming our hypothesis that PML is directly involved in the regulation of MSC differentiation process.

Interestingly, in 2015, an evaluation of PML transcript abundance in a cohort of human liver biopsies from lean or morbidly obese subjects was published. This study revealed a significant PML upregulation in obese individuals and demonstrated that PML accumulates in hepatocytes under obesity conditions [58]. Here, we showed that the PML levels also increase in livers of mice fed a high-fat diet compared with those fed a standard diet (Fig. S4b), indicating a possible correlation with liver steatosis, which is a condition of lipid accumulation in hepatocytes that is

frequently associated with obesity [59]. Surprisingly, we found very low PML levels in adipose tissues from adult mice (Fig. S4c) and in subcutaneous adipose tissue of patients (Fig. S4f) as well as in hADSCs derived from obese patients (Fig. 5).

Our hypothesis is that appropriate levels of PML are necessary for the proper regulation of MSC differentiation and metabolic homeostasis, and that PML is fundamental in the first step of adipogenesis, while at the end of differentiation, as in mature adipose tissues, PML must be downregulated to avoid excessive lipid accumulation. Abnormal conditions that bypass the impact of PML on adipogenesis can drive an uncommitted and faster lipids accumulation (Fig. S4d, e) followed by diseases development.

Overall, the identification of PML as a key regulator of MSC differentiation could provide a new target for the treatment of diseases in which an imbalance in MSCs differentiation is observed. For instance, in 2015, Cheng et al. [60] published a paper regarding the ability of Ato to inhibit the adipogenic process in bone marrow MSCs from aplastic anemia patients. The typical pathological feature of aplastic anemia is an increase in the number of fat cells and a reduction in the number of osteoblasts in the bone marrow. Both fat cells and osteoblasts in bone marrow are derived from MSCs. Generally, adipogenic and osteogenic differentiation is a dynamic and balanced process, and an imbalance in this process may participate in the occurrence and progression of many diseases. In this study, the authors reported that Ato inhibits adipogenic differentiation and promotes osteogenic differentiation in MSCs from aplastic anemia patients. The authors did not correlate the observed effects with the PML levels, but consistently with the data shown here and with the ability of Ato to downregulate PML expression [31], such a correlation could explain the efficiency of Ato therapy.

Another fundamental point that we addressed here is the molecular mechanism by which PML can affect adipogenesis. PML performs different functions due to its interaction with several proteins and regulation of cell processes, including autophagy [40].

A balance in autophagy appears to be a key feature of efficient MSCs differentiation and function [39, 61], and, accordingly, in this work we observed that the ability of PML to modulate the autophagic levels is fundamental in the adipogenesis process. Loss of PML in MSCs leads to higher autophagy levels which oppose to adipogenesis process, while modulation of autophagy levels (in WT MSCs by Rapa and in PML KO MSCs by Ly) restores the effects of PML.

Consistently, similar data showing that autophagy modulation with Rapa inhibited adipocyte formation, while autophagosome blockade with bafilomycin accelerated fat accumulation [62] support our hypothesis that PML regulates adipogenesis by modulating the autophagy levels.

Regarding the signals driving the differentiation of MSCs into adipocytes in a PML-dependent manner, our data also suggest a crucial role for PKCβ.

PKC is a member of the serine/threonine protein kinase family that plays important roles in the control of a variety of cellular functions. Interestingly, PKCβ shares common aspects with PML as follows: PKCβ is involved in the regulation of adipocyte differentiation [44] and inhibits autophagy [48]. In addition, PKCβ KO mice consumed more food than WT mice daily but gained less weight, suggesting that important alterations in energy expenditure and disposition were present [63].

Our results show that PKCβ upregulation is necessary for HG-dependent adipogenic differentiation (Fig. 3) and that PML deletion prevents PKCβ upregulation, resulting in adipogenesis impairment. The reintroduction of correct levels of PKCβ to PML KO MSCs rescued the adipogenic process restoring correct levels of autophagy (Fig. 4).

Altogether, our data indicate that, under HG conditions, increased PML expression enables the upregulation of PKCβ, which, in turn, by controlling autophagy levels permits an increase in PPARγ, that leads to adipogenic differentiation (Fig. 4f). In the absence of PML, and in turn without PKCβ expression, the increased autophagy levels favor a deregulation of adipocyte differentiation through a blockade of PPARγ activity. This signaling pathway appears to be governed upstream by PML since HG promotes PML upregulation also in PKCβ KO MSCs (Fig. S3c). Autophagy levels' correction in PKCβ KO MSCs is able to determine the incremement of PPARγ (Fig. S3f). Moreover, the direct stimulation of PPARγ by the addition of troglitazone [64] in PML KO MSCs promotes adipogenic differentiation, bypassing PKCβ upregulation (Fig. S3d, S3e).

In conclusion, our data demonstrate a critical role for PML in orchestrating the adipogenic process in MSCs, providing insight into the mechanisms underlying this process. We found that PML is fundamental for maintaining the correct autophagy level during HG-dependent adipogenesis in MSCs by allowing PKCβ expression, which enables the differentiation process. This new link among PML, PKCβ, and autophagy opens new therapeutic avenues for diseases characterized by an imbalance in the MSC differentiation process, such as metabolic syndrome.

Acknowledgements CG is supported by local funds from the University of Ferrara, the Italian Association for Cancer Research, the Italian Ministry of Health, and by Cariplo grant. MRW is supported by the FOIE GRAS and mtFOIE GRAS projects.These projects received funding from the European Union'sHorizon 2020 Research and Innovation programme under the MarieSkłodowska-Curie Grant

Agreement No. 722619 (FOIE GRAS) andGrant Agreement No. 734719 (mtFOIE GRAS).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–7.
2. Tolar J, Le Blanc K, Keating A, Blazar BR. Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells. 2010;28:1446–55.
3. Bernardo ME, Pagliara D, Locatelli F. Mesenchymal stromal cell therapy: a revolution in regenerative medicine? Bone Marrow Transplant. 2012;47:164–71.
4. Brook CG, Lloyd JK, Wolf OH. Relation between age of onset of obesity and size and number of adipose cells. BMJ. 1972;2:25–7.
5. Faust IM, Johnson PR, Stern JS, Hirsch J. Diet-induced adipocyte number increase in adult rats: a new model of obesity. Am J Physiol. 1978;235:E279–86.
6. Hirsch J, Batchelor B. Adipose tissue cellularity in human obesity. Clin Endocrinol Metab. 1976;5:299–311.
7. DeGaris RM, Pennefather JN. Prolonged supersensitivity to noradrenaline of smooth muscle of the epididymal half of the rat vas deferens denervated by vasectomy. J Auton Pharmacol. 1987;7:267–79.
8. Chen BY, Wang X, Chen LW, Luo ZJ. Molecular targeting regulation of proliferation and differentiation of the bone marrow-derived mesenchymal stem cells or mesenchymal stromal cells. Curr Drug Targets. 2012;13:561–71.
9. Ling L, Nurcombe V, Cool SM. Wnt signaling controls the fate of mesenchymal stem cells. Gene. 2009;433:1–7.
10. De Boer J, Wang HJ, Van Blitterswijk C. Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng. 2004;10:393–401.
11. Shtutman M, Zhurinsky J, Oren M, Levina E, Ben-Ze'ev A. PML is a target gene of beta-catenin and plakoglobin, and coactivates beta-catenin-mediated transcription. Cancer Res. 2002;62:5947–54.
12. de The H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell. 1991;66:675–84.
13. Rowley JD. Identification of the constant chromosome regions involved in human hematologic malignant disease. Science. 1982;216:749–51.
14. Kakizuka A, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR, Murty VV, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. Cell. 1991;66:663–74.
15. Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH, Pandolfi PP. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol. 2004;6:665–72.
16. Trotman LC, Alimonti A, Scaglioni PP, Koutcher JA, Cordon-Cardo C, Pandolfi PP. Identification of a tumour suppressor network opposing nuclear Akt function. Nature. 2006;441:523–7.
17. Bernardi R, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, et al. PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR. Nature. 2006;442:779–85.
18. Giorgi C, Ito K, Lin HK, Santangelo C, Wieckowski MR, Lebiedzinska M, et al. PML regulates apoptosis at endoplasmic reticulum by modulating calcium release. Science. 2010;330:1247–51.
19. Ito K, Bernardi R, Morotti A, Matsuoka S, Saglio G, Ikeda Y, et al. PML targeting eradicates quiescent leukaemia-initiating cells. Nature. 2008;453:1072–8.
20. Regad T, Bellodi C, Nicotera P, Salomoni P. The tumor suppressor Pml regulates cell fate in the developing neocortex. Nat Neurosci. 2009;12:132–40.
21. Carracedo A, Weiss D, Leliaert AK, Bhasin M, de Boer VC, Laurent G, et al. A metabolic prosurvival role for PML in breast cancer. J Clin Invest. 2012;122:3088–100.
22. Kim MK, Yang S, Lee KH, Um JH, Liu M, Kang H, et al. Promyelocytic leukemia inhibits adipogenesis, and loss of promyelocytic leukemia results in fat accumulation in mice. Am J Physiol Endocrinol Metab. 2011;301:E1130–42.
23. Cheng X, Guo S, Liu Y, Chu H, Hakimi P, Berger NA, et al. Ablation of promyelocytic leukemia protein (PML) re-patterns energy balance and protects mice from obesity induced by a Western diet. J Biol Chem. 2013;288:29746–59.
24. Marrelli M, Pujia A, Palmieri F, Gatto R, Falisi G, Gargari M, et al. Innovative approach for the in vitro research on biomedical scaffolds designed and customized with CAD-CAM technology. Int J Immunopathol Pharmacol. 2016;29:778–83.
25. Hope RG, McLauchlan J. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J Gen Virol. 2000;81(Part 8):1913–25.
26. Chuang CC, Yang RS, Tsai KS, Ho FM, Liu SH. Hyperglycemia enhances adipogenic induction of lipid accumulation: involvement of extracellular signal-regulated protein kinase 1/2, phosphoinositide 3-kinase/Akt, and peroxisome proliferator-activated receptor gamma signaling. Endocrinology. 2007;148:4267–75.
27. Aguiari P, Leo S, Zavan B, Vindigni V, Rimessi A, Bianchi K, et al. High glucose induces adipogenic differentiation of muscle-derived stem cells. Proc Natl Acad Sci USA. 2008; 105:1226–31.
28. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 1994;8:1224–34.
29. Bastie C, Holst D, Gaillard D, Jehl-Pietri C, Grimaldi PA. Expression of peroxisome proliferator-activated receptor PPARdelta promotes induction of PPARgamma and adipocyte differentiation in 3T3C2 fibroblasts. J Biol Chem. 1999;274:21920–5.
30. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med. 2012;18:1350–8.
31. Lallemand-Breitenbach V, Zhu J, Puvion F, Koken M, Honore N, Doubeikovsky A, et al. Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. J Exp Med. 2001;193:1361–71.
32. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol. 2007;9:1102–9.
33. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell . 2011;147:728–41.
34. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431:200–5.
35. Carnevalli LS, Masuda K, Frigerio F, Le Bacquer O, Um SH, Gandin V, et al. S6K1 plays a critical role in early adipocyte differentiation. Dev Cell. 2010;18:763–74.
36. Le Bacquer O, Petroulakis E, Paglialunga S, Poulin F, Richard D, Cianflone K, et al. Elevated sensitivity to diet-induced obesity and

insulin resistance in mice lacking 4E-BP1 and 4E-BP2. J Clin Invest. 2007;117:387–96.
37. Polak P, Cybulski N, Feige JN, Auwerx J, Ruegg MA, Hall MN. Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. Cell Metab. 2008;8:399–410.
38. Yoon MS, Zhang C, Sun Y, Schoenherr CJ, Chen J. Mechanistic target of rapamycin controls homeostasis of adipogenesis. J Lipid Res. 2013;54:2166–73.
39. Liu F, Fang F, Yuan H, Yang D, Chen Y, Williams L, et al. Suppression of autophagy by FIP200 deletion leads to osteopenia in mice through the inhibition of osteoblast terminal differentiation. J Bone Miner Res. 2013;28:2414–30.
40. Missiroli S, Bonora M, Patergnani S, Poletti F, Perrone M, Gafa R, et al. PML at mitochondria-associated membranes is critical for the repression of autophagy and cancer development. Cell Rep. 2016;16:2415–27.
41. Galluzzi L, Bravo-San Pedro JM, Levine B, Green DR, Kroemer G. Pharmacological modulation of autophagy: therapeutic potential and persisting obstacles. Nat Rev Drug Discov. 2017;16:487–511.
42. Clemens MJ, Trayner I, Menaya J. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci. 1992;103(Part 4):881–7.
43. Gallicano GI, Yousef MC, Capco DG. PKC—a pivotal regulator of early development. Bioessays. 1997;19:29–36.
44. Zhou Y, Wang D, Li F, Shi J, Song J. Different roles of protein kinase C-betaI and -delta in the regulation of adipocyte differentiation. Int J Biochem Cell Biol. 2006;38:2151–63.
45. Artemenko Y, Gagnon A, Aubin D, Sorisky A. Anti-adipogenic effect of PDGF is reversed by PKC inhibition. J Cell Physiol. 2005;204:646–53.
46. Fleming I, MacKenzie SJ, Vernon RG, Anderson NG, Houslay MD, Kilgour E. Protein kinase C isoforms play differential roles in the regulation of adipocyte differentiation. Biochem J. 1998;333(Part 3):719–27.
47. McGowan K, DeVente J, Carey JO, Ways DK, Pekala PH. Protein kinase C isoform expression during the differentiation of 3T3-L1 preadipocytes: loss of protein kinase C-alpha isoform correlates with loss of phorbol 12-myristate 13-acetate activation of nuclear factor kappaB and acquisition of the adipocyte phenotype. J Cell Physiol. 1996;167:113–20.
48. Patergnani S, Marchi S, Rimessi A, Bonora M, Giorgi C, Mehta KD, et al. PRKCB/protein kinase C, beta and the mitochondrial axis as key regulators of autophagy. Autophagy. 2013;9:1367–85.
49. Matsushita K, Dzau VJ. Mesenchymal stem cells in obesity: insights for translational applications. Lab Invest. 2017;97:1158–66.
50. De Pergola G, Silvestris F. Obesity as a major risk factor for cancer. J Obes. 2013;2013:291546.
51. Chen Q, Shou P, Zheng C, Jiang M, Cao G, Yang Q, et al. Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? Cell Death Differ. 2016;23:1128–39.
52. Caballero B. The global epidemic of obesity: an overview. Epidemiol Rev. 2007;29:1–5.
53. Tatullo M, Simone GM, Tarullo F, Irlandese G, Vito D, Marrelli M, et al. Antioxidant and antitumor activity of a bioactive polyphenolic fraction isolated from the brewing process. Sci Rep. 2016;6:36042.
54. Boyer F, Vidot JB, Dubourg AG, Rondeau P, Essop MF, Bourdon E. Oxidative stress and adipocyte biology: focus on the role of AGEs. Oxid Med Cell Longev. 2015;2015:534873.
55. Marrelli M, Gentile S, Palmieri F, Paduano F, Tatullo M. Correlation between Surgeon's experience, surgery complexity and the alteration of stress related physiological parameters. PLoS ONE. 2014;9:e112444.
56. Tatullo M, Gentile S, Paduano F, Santacroce L, Marrelli M. Crosstalk between oral and general health status in e-smokers. Medicine (Baltim). 2016;95:e5589.
57. Sun J, Fu S, Zhong W, Huang H. PML overexpression inhibits proliferation and promotes the osteogenic differentiation of human mesenchymal stem cells. Oncol Rep. 2013;30:2785–94.
58. Carracedo A, Rousseau D, Douris N, Fernandez-Ruiz S, Martin-Martin N, Weiss D, et al. The promyelocytic leukemia protein is upregulated in conditions of obesity and liver steatosis. Int J Biol Sci. 2015;11:629–32.
59. Fracanzani AL, Valenti L, Bugianesi E, Vanni E, Grieco A, Miele L, et al. Risk of nonalcoholic steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease and low visceral adiposity. J Hepatol. 2011;54:1244–9.
60. Cheng HC, Liu SW, Li W, Zhao XF, Zhao X, Cheng M, et al. Arsenic trioxide regulates adipogenic and osteogenic differentiation in bone marrow MSCs of aplastic anemia patients through BMP4 gene. Acta Biochim Biophys Sin (Shanghai). 2015;47:673–9.
61. Oliver L, Hue E, Priault M, Vallette FM. Basal autophagy decreased during the differentiation of human adult mesenchymal stem cells. Stem Cells Dev. 2012;21:2779–88.
62. Nuschke A, Rodrigues M, Stolz DB, Chu CT, Griffith L, Wells A. Human mesenchymal stem cells/multipotent stromal cells consume accumulated autophagosomes early in differentiation. Stem Cell Res Ther. 2014;5:140.
63. Bansode RR, Huang W, Roy SK, Mehta M, Mehta KD. Protein kinase C deficiency increases fatty acid oxidation and reduces fat storage. J Biol Chem. 2008;283:231–6.
64. Camp HS, Li O, Wise SC, Hong YH, Frankowski CL, Shen X, et al. Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. Diabetes. 2000;49:539–47.