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

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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 adipocyte 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 MSCs biology remains unknown.

Here, we investigated the molecular pathway underlying the role of PML in the control of adipogenic MSCs 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 PKCβ, which, in turn, by controlling autophagy levels permits an increase in 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 with the potential to differentiate into a variety of cell lineages and 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 therapeutic strategies for metabolic and obesity-related disorders.

Several important regulatory pathways, particularly the Wnt signaling pathway, have been found to participate in the regulation of MSCs 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 PML gene can function as a target of the Wnt signaling pathway (11). Thus, promyelocytic leukemia protein (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 alpha (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 maintenance and neural progenitor cell differentiation (19, 20), but its role in MSCs 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 MDSC cultures were prepared from newborn C57BL/6 WT, PML KO and 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 hour with 0.2% collagenase A (Roche 103586) at 37°C. Using a 75-µm cell strainer, the obtained cells were purified from the undigested tissue and plated in DMEM LG (5 mM glucose). After 2 hours, the suspension containing the

MDSCs was transferred to a new dish, and this passage was repeated after 24 hours. The cells able to attach after 24 hours were considered MDSCs.

Human adipose-derived stem cells (hADSCs) were extracted from human subcutaneous adipose tissues from patients undergoing surgical procedures. The adipose tissues were digested with 0.075% collagenase (type 1A; Sigma–Aldrich) in Hank's Balanced Salts solution for 3 hours at room temperature. After inactivating the digestion process by adding DMEM containing 10% FBS, 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 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 IBMX, 0.1 mM indomethacin, and 1 μM dexamethasone for 3, 7 and 21 days.

In vivo animal model

Four-week-old male C57BL/6J 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.). Deionized water and food were available ad libitum.

Oil Red O staining

The Oil red (Sigma-Aldrich O0625) staining of the cytoplasmic drops of neutral lipids was performed according to the standard procedure, and images were acquired using light microscopy. Briefly, after fixing with 4% PAF, 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; rabbit anti-PPARγ [2435] (1:1000) and rabbit anti-GAPDH [2118] (1:5000) from Cell Signaling; rabbit anti-GLUT4 [2213] (1:1000), mouse anti-β-Actin [A1978] (1:10000), and rabbit anti-LC3B [L7543] (1:1000) from Sigma-Aldrich; and PKCβ [ab32026] (1:1000) and rabbit anti-PML [ab72137] (1:1000) from Abcam. Finally, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibodies (Thermo

Fischer Scientific), followed by detection by chemiluminescence (Thermo Scientific) using Image Quant LAS4000 (GE Healthcare).

Reagents and viral vectors

For the pharmacological deletion of PML, arsenic trioxide (Ato) (Sigma-Aldrich A1010) (500 nM) in DMEM HG was used. Rapamycin (Calbiochem 553210) (100 nM) was used to induce autophagy in the WT MDSCs. LY-294002 (Sigma-Aldrich L9908) (50 μM) was used to inhibit autophagy in the PML KODSCs. As indicated, the cells were infected with GFP-LC3 adenovirus and PKCβ adenovirus.

Fluorescence microscopy and quantitative analysis of GFP-LC3 dots

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

Quantitative RT-PCR

Total RNA was extracted with the TRIzol Reagent (Invitrogen). Real-time PCR was performed using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche), 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. The probability of significant differences among the experimental groups was determined by 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

1. PML is essential for high-glucose-dependent adipogenic differentiation.

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

A high glucose (HG) concentration, which is one of the most common conditions leading to an increase in adipose tissue in vivo (24), promotes MSCs differentiation into mature adipocytes in vitro (25). The adipogenic potential of PML WT and KO MSCs was assessed by measuring in immunoblotting the protein levels of PPARγ, which is a key transcriptional factor for adipogenic commitment (26), after a 7-day HG treatment. As shown in Fig. 1b, 1c 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 from the low-glucose (LG) condition to the HG condition. Accordingly, since PPARγ expression is under control of Peroxisome-proliferator activated receptor delta (PPARδ) (27), deletion of PML was linked to a decrease of PPARδ levels (Fig. S1c), as also reported by Ito et al.(28).

During adipogenesis, differentiating cells begin to accumulate lipids in their cytosol. The oil red O (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 and 1e), 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 (29). 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 and 1c), and the number of ORO-positive cells was significantly reduced relative to that of the untreated cells (Fig. 1d and 1e).

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.

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

Furthermore, analyzing the morphology and area of the lipid drops, was clear that the adipogenic deficiency 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 and 1i, 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.

2. 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 (30). By selectively degrading harmful protein aggregates or damaged organelles, autophagy maintains intracellular homeostasis and performs an essential quality control function within the cell (31).

Among its myriad of cellular and developmental functions, autophagy level regulation has emerged as a key regulator of lipid metabolism and adipogenesis (32-36). 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 (37).

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 mouse embryonic fibroblasts and mice (38), 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 suggest that PML induce 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 and 2c).

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,

231 we modulated autophagy in our experimental conditions by using pharmacological agents suggested
232 by recent guidelines (39), because of the strong difficulty of transfection in this primary cell type.
233 Thus, WT MSCs were treated with rapamycin (Rapa) for 7 days to increase the autophagy level,
234 which was detected by the LC3-II conversion (Fig. 2d). Following the Rapa treatment, the PPARγ
235 protein levels did not increase (Fig. 2d and 2e), and consequently, the % of ORO-positive cells in
236 the WT MDSCs significantly decreased (Fig. 2f) compared to that in the WT MDSCs cultured
237 under the control HG conditions.
238 However, the Ly 294002 (Ly) treatment used to inhibit autophagy (Fig. 2g), restored the ability of
239 PML KO MSCs to differentiate into adipocytes, which was demonstrated by an increase in the
240 PPARγ protein levels (Fig. 2g and 2h) and accumulation of lipid drops (Fig. 2i).
241 Altogether these results demonstrate as a tight control of autophagy is essential for correct
242 adipocyte differentiation and PML plays a key role in this regulation.
243
244 *3. PKCβ level regulation by PML enables HG-dependent adipogenic differentiation.*
245 We then decided to get more insight into the mechanisms that drive autophagy-induced adipocytic
246 differentiation in HG conditions.
247 PKC family members play essential roles in a variety of physiological functions, including cell
248 metabolism, proliferation, differentiation, migration, and apoptosis (40-42). Moreover, PKC
249 isoforms have been shown to might be involved in the regulation of adipocyte differentiation (43-
250 45), and in particular, the specific isoform PKCβ was reported to inhibit autophagy (46).
251 Thus, we sought to investigate the direct involvement of PKCβ in the signaling route of HG-
252 dependent differentiation process regulated by PML through autophagy control in MSCs.
253 Accordingly with data showed in Fig. 1g, we detected increased PKCβ protein levels during the
254 HG-dependent adipogenesis in WT MSCs (Fig. 3a).
255 Expectedly, the PKCβ KO MSCs were unable to accumulate lipid drops following the HG
256 administration (Fig. 3b and 3c) since their autophagic levels are higher than those in the WT MSCs
257 (Fig. 3d) but comparable to those in the PML KO MSCs (Fig. 2a).
258 We found out that the pharmacological and genetic deletion of PML was associated with a
259 reduction in the PKCβ protein and mRNA levels (Fig. 4a-4c). The introduction of PKCβ by an
260 adenovirus carrying PKCβ-GFP rescued the adipogenic potential of the PML KO MSCs (Fig. 4d
261 and 4e) by restoring the correct autophagy levels and enhanced adipogenesis in the WT MSCs (Fig.
262 S3a and S3b).
263 Altogether our data indicate that, under HG conditions, increased PML expression enables the
264 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 (47) in PML KO MSCs promotes adipogenic differentiation, bypassing PKCβ upregulation (Fig. S3d and S3e).

4. hADSCs derived from overweight patients show increased PML levels.

Above, we described that the genetic and pharmacological deletion of PML is associated with a lack of adipogenic differentiation (Fig. 1b-f, 1h and 1i), which indeed requires PML upregulation (Fig. 1g). However, 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 human adipose-derived stem cells (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 (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 and 5c), 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.

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 and 5c).

In order to investigate this 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 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 (FigS4b). 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 control of PML on adipogenesis can drive an uncommitted and faster lipids accumulation (Fig. S4b) with degeneration in 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 and faster 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 by-passing the adipogenic pathway PML-dependent (Fig. S4c). Accordingly, also the PML KO MSCs were able to differentiate under adipogenic medium (Fig. S4e) suggesting a completely 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, cardiovascular disease (48), as well as for certain cancers (49). 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. Mesenchymal stem cells (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 (50). 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 MSCs-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 peroxisome proliferator-activated receptor-gamma (PPARγ) and several members of the CCAAT/enhancer-binding proteins (C/EBPs).

In this study, we focused on new possible upstream regulators of the adipogenesis process, which finally merged in PPARγ upregulation. We previous identified that HG exposure plays a primary role in adipogenic differentiation, providing a direct link between hyperglycemia and an increase in adiposity (25), which, in turn, may play a key role in the progression of metabolic dysfunction, such as an irreversible diabetic state.

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 hematopoietic stem cells (HSCs), in which the absence of PML resulted in the loss of HSC maintenance, loss of asymmetric division and as a consequence reduced HSCs rate of differentiation (20).

More recently, PML has been shown to promote osteogenic differentiation in MSCs, which is associated with the upregulation of integrin-binding sialoprotein (51), confirming our hypothesis that PML is directly involved in the MSCs differentiation process and regulation.

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 (52). Here, we showed that the PML levels also increase in livers from mice fed a high-fat diet compared with those in mice 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 (53). Surprisingly, we found very low PML levels in adipose tissues from adult mice (Fig. S4c) and in subcutaneous adipose tissue of human 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 MSCs 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 down-regulated to avoid excessive lipid accumulation. Abnormal conditions that bypass the control of PML on adipogenesis can drive an uncommitted and faster lipids accumulation (Fig S4d and S4e) 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. 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 (54). 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 (29), 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 (38).

A balance in autophagy appears to be a key feature of efficient MSC differentiation and function (37, 55), 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 rapamycin and in PML KO MSCs by Ly) restores the effects of PML.

Consistently, similar data showing that autophagy modulation with rapamycin inhibited adipocyte formation; while autophagosome blockade with bafilomycin accelerated fat accumulation (56) 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 suggest also a crucial role for PKCβ.

Protein kinase C (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 (42) and inhibits autophagy (46). 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 (57).

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).

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 syndromes.

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Conflict of Interest

No conflicts of interest to disclose.

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Figure Legends

Figure 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 3 independent experimental days. ANOVA (unless indicated otherwise); *$p<0.05$, ** $p<0.001$, *** $p<0.0001$, ****$p<0.00001$ and n.s. $p> 0.05$

Figure 2. Autophagic levels regulated by PML affect high glucose dependent adipogenic differentiation

WT and PML KO MDSCs were cultured, as indicated, in 5mM glucose (LG), 25 mM glucose (HG) and HG plus 500 nM arsenic trioxide (Ato), or plus 100 nM Rapamycin (Rapa) or plus 50 μM Ly294002 (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 3 independent experimental days. ANOVA; *$p<0.01$, ** $p<0.001$, *** $p<0.0001$, and ****$p<0.00001$.

Figure 3. Authopagy and adipogenic potential in PKCβ KO MDSC.

(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 3 independent experimental days. ANOVA; *** $p<0.0001$, ****$p<0.00001$ and n.s. $p>0.05$.

Figure 4. Lack of PKCβ expression in PML KO prevents high glucose dependent adipogenic differentiation.

(a) Representative immunoblot of PML, PKCβ and β-Actin (b) and 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 3 independent experimental days. ANOVA; *$p<0.05$ and *** $p<0.0001$.

Figure 5. PML levels increased in hADSCs from overweight patients

(a) Schematic representation of hADSC isolation from human subcutaneous adipose tissue. hADSC samples were divided into the following 3 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 *** $p<0.001$.

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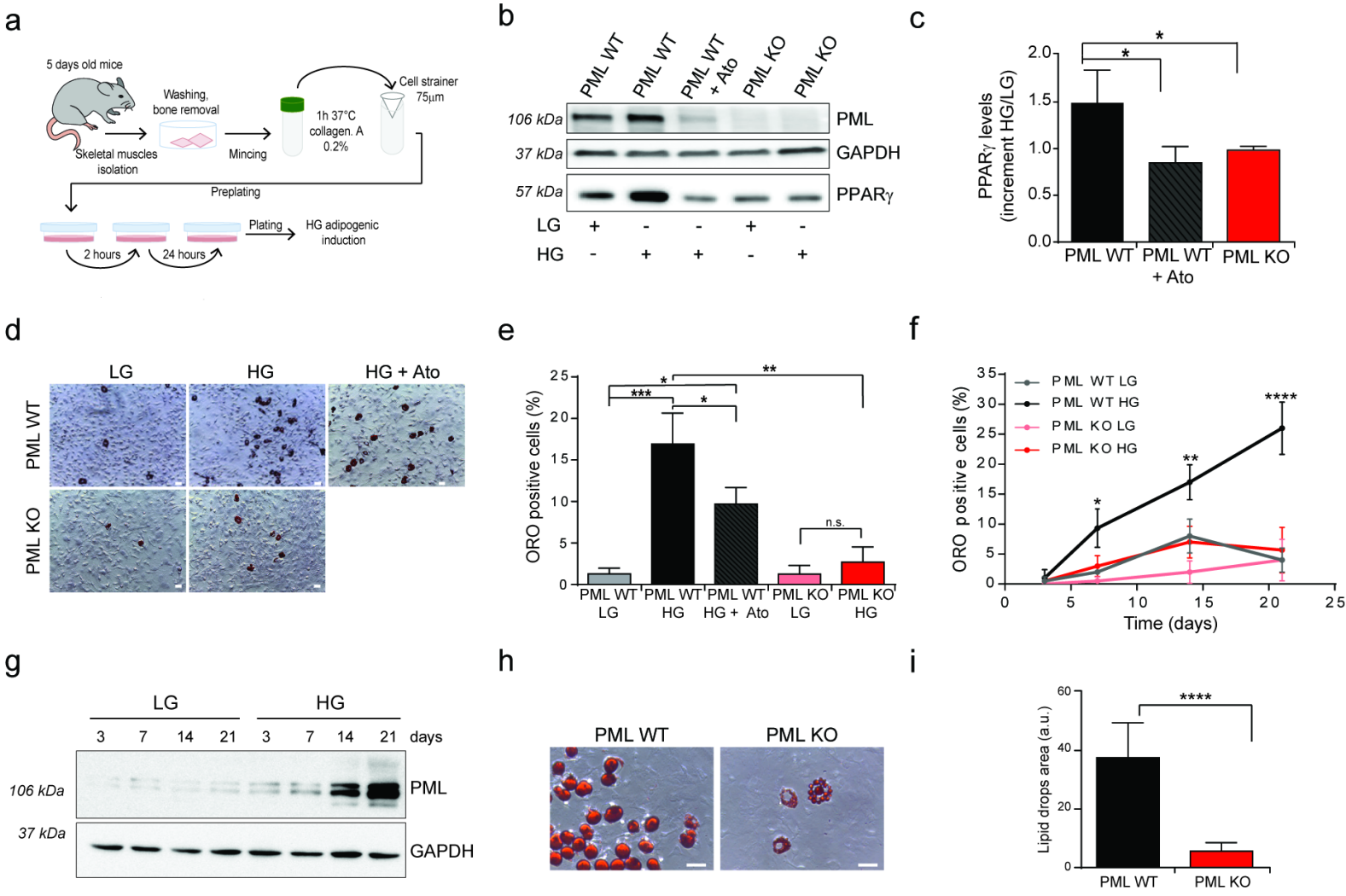
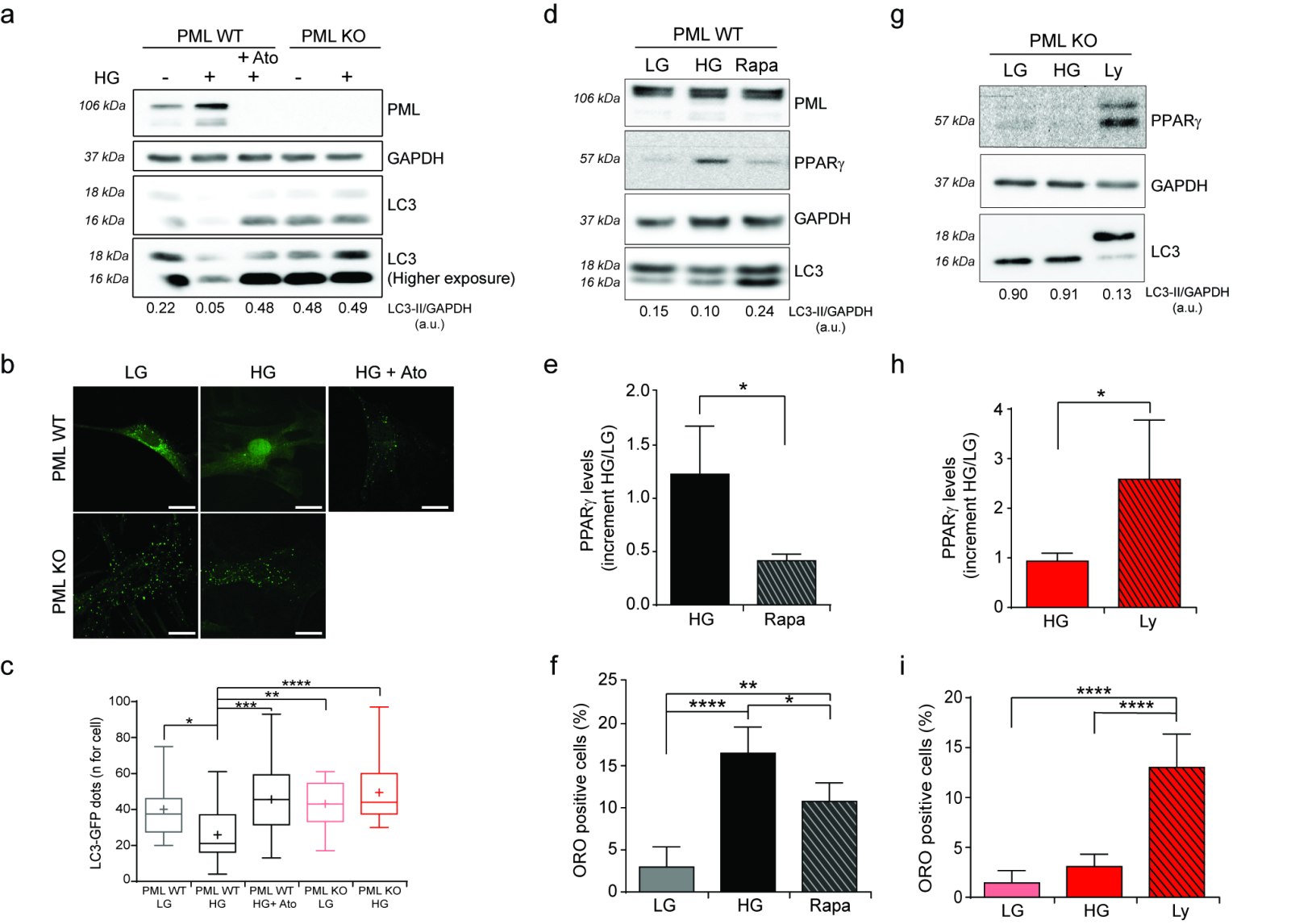


Figure 1_Morganti et al.



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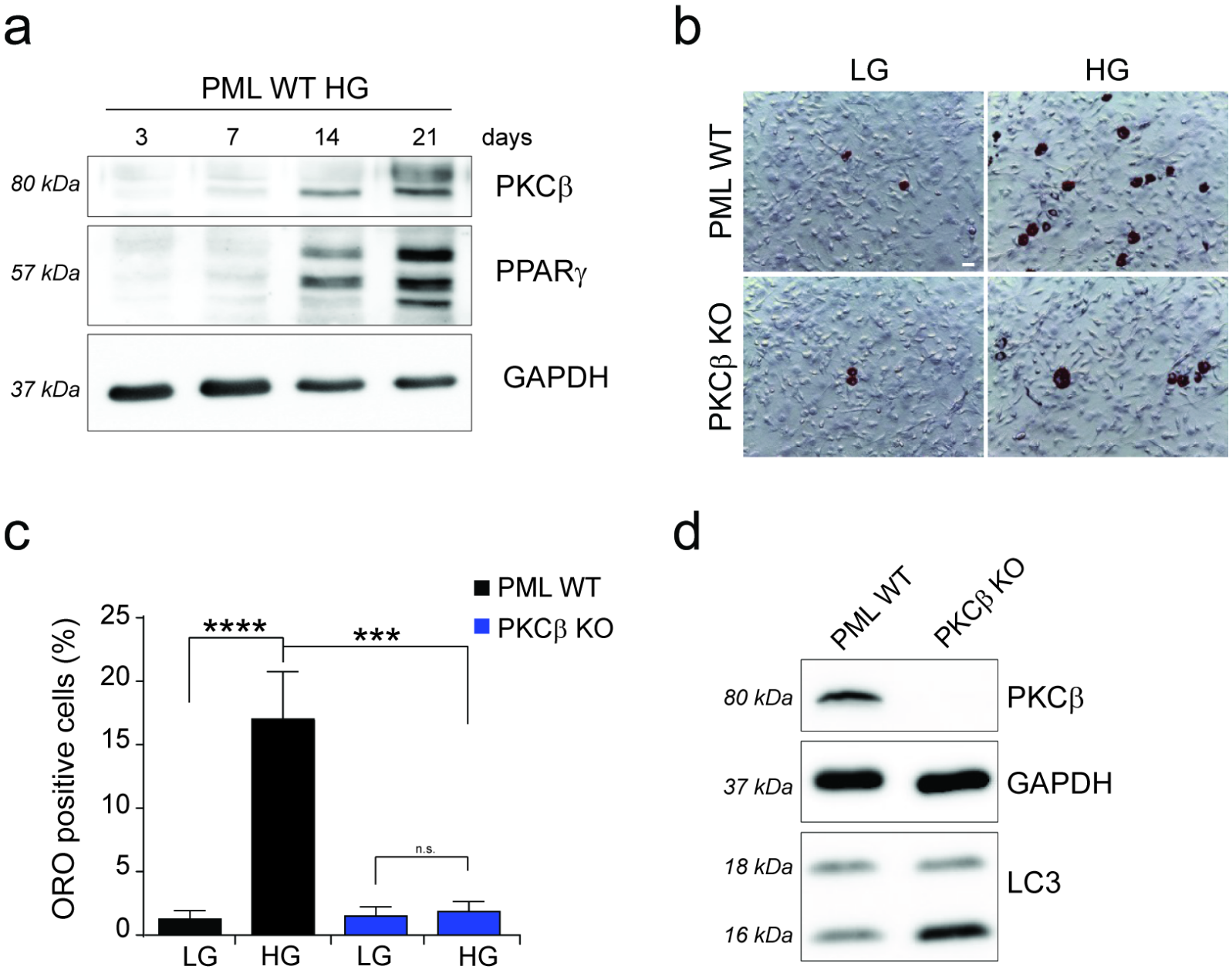

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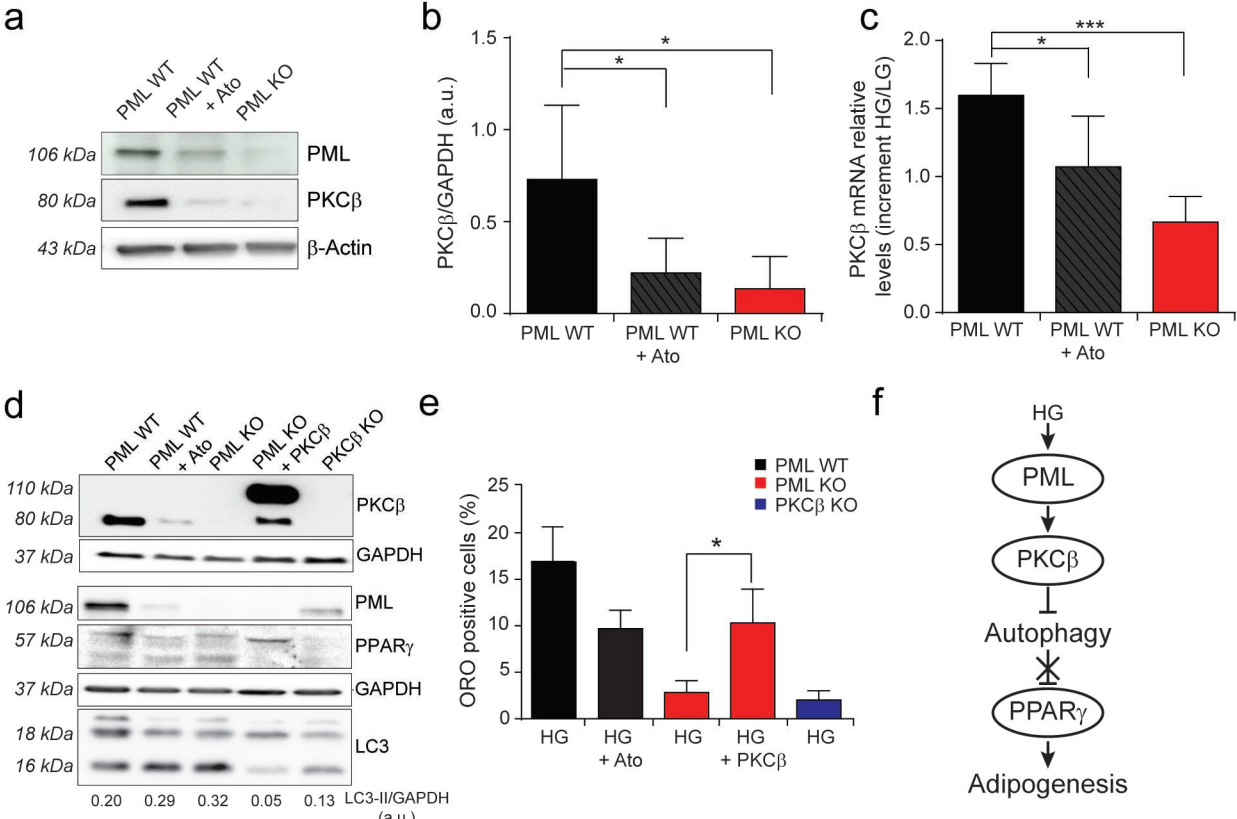

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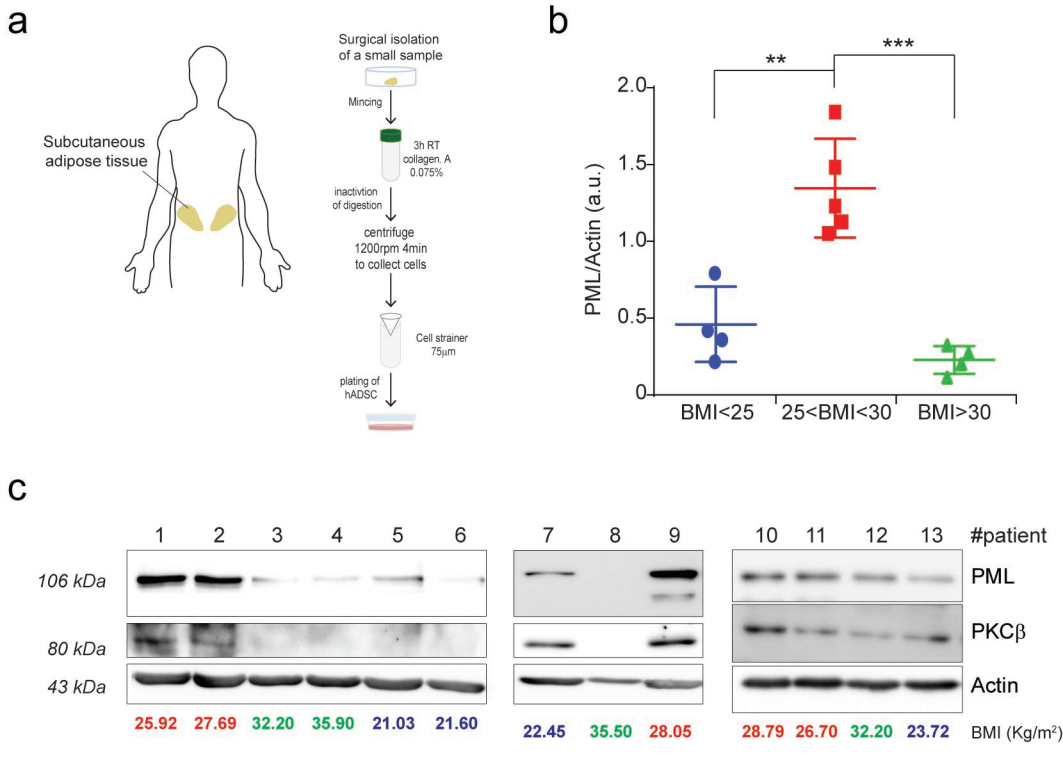

Figure 5_Morganti et al.