Evidence of mature adipocyte proliferation regulated by proliferin

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SUMMARY

Despite much research, whether mature adipocytes proliferate remains controversial. Here, we examined 5-bromo-2′-deoxyuridine (BrdU)-labelling of mature adipocytes. Although BrdU incorporation into subcutaneous adipocytes was less than that in visceral adipocytes, pioglitazone (Pio) treatment increased BrdU incorporation in subcutaneous, but not visceral, adipocytes in rats. Fully differentiated 3T3-L1 adipocytes exhibited an increase in cell number and BrdU incorporation with time, with this increase enhanced by Pio treatment. We therefore screened for genes that encode growth factors regulated by Pio, and selected proliferin (PLF). Both gene silencing of PLF by small interfering RNA and treatment with anti-PLF antibody suppressed proliferation in 3T3-L1 adipocytes. In adipocytes isolated from Pio-treated rats, the tissue-specific pattern of PLF expression was similar to that of BrdU incorporation. Administration of an anti-PLF antibody to mice reduced BrdU incorporation into adipocytes. Mature adipocytes thus have the ability to replicate, and this proliferation is positively regulated by PLF.

INTRODUCTION

There is growing evidence that adipocytes regulate whole-body energy homeostasis and insulin sensitivity. A "Western lifestyle" including a high-fat diet and lack of exercise causes excessive fat accumulation in adipocytes, which in turn leads to obesity, a major risk factor for type 2 diabetes, hypertension, dyslipidemia and atherosclerosis. Underlying insulin resistance plays a pivotal role in the pathogenesis of these disorders; however, the mechanisms that link obesity and impaired insulin signaling are not fully understood. Obesity is associated with a low-grade inflammation characterized by macrophage infiltration into adipose tissue[1, 2]. Monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes, leukocytes and other inflammatory cells, plays a crucial role in this process[3]. Insulin resistance and hepatic steatosis induced by a high-fat diet are reduced in MCP-1 KO mice[4]. It has been proposed that the capacity of adipose tissue to expand, rather than the absolute amount of adipose tissue, is critical for metabolic homeostasis. When this capacity is exceeded, lipid accumulates in skeletal muscle and liver, which, in turn, attenuates insulin action[5]. Therefore, understanding the mechanism by which new adipocytes are generated is important. The finding that approximately 10% of fat cells are renewed annually at all adult ages and levels of body mass index indicates that the ability to provide newborn adipocytes is limited[6]. It is

generally considered that the mature adipocyte is unable to proliferate[7] and that it is generated from a mesenchymal stem cell in the adipose tissue[8]. In the first phase, this multipotent stem cell turns into a preadipocyte, which cannot be distinguished morphologically from the precursor stem cells. In the second phase, terminal differentiation, the preadipocyte acquires the characteristics of the mature adipocyte, including lipid droplets, insulin sensitivity, and the ability to secrete adipokines. Terminal differentiation consists of a cascade of transcriptional events. The transient induction of the CCAAT-enhancer binding proteins C/EBPβ and C/EBPδ leads directly, or in concert with Krüppel-like factor 5 (KLF5), to the induction of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα, the central transcriptional regulator of adipocytes. They play a major role in the individual or synergistic induction of adipocyte-specific genes, including those encoding adipocyte fatty acid-binding protein (aP2), lipoprotein lipase (LPL), adiponectin and glucose transporter type 4 (Glut4).

Adipose tissue is classified into visceral and subcutaneous adipose tissue according to its anatomical location in the body. Multiple lines of evidence indicate that an increase in visceral adipose tissue is associated with insulin resistance, hyperlipidemia, type 2 diabetes and atherosclerosis[9, 10], whereas the finding that transplantation of subcutaneous adipose tissue improves insulin sensitivity[11] indicates a beneficial role for subcutaneous adipose tissue. Treatment with thiazolidinedione, which specifically targets PPARγ, increases subcutaneous adipose tissue and decreases visceral adipose tissue[12], a process associated with improved insulin sensitivity.

Thiazolidinedione treatment also decreases the cell size of adipocytes, with a concomitant increase in the number of cells[13]. These findings show that thiazolidinedione accelerates adipocyte hyperplasia, especially in subcutaneous adipose tissue; however, as its target, PPARγ, first appears during terminal differentiation, it is difficult to understand how this agent could have an effect on preadipocyte or mesenchymal stem cells. Accordingly, two possibilities arise: the first is that thiazolidinedione might stimulate mature adipocytes to secrete substances that promote the proliferation of precursor cells; the second is that thiazolidinedione might directly induce the proliferation of mature adipocytes.

In this study, we investigated the mechanism by which pioglitazone (Pio), a member of the thiazolidinedione class of drugs, increases the number of adipocytes. First, we ascertained that mature adipocytes incorporate 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog, which indicates that the mature adipocytes undergo DNA synthesis. Second, we found that Pio accelerated mature adipocyte proliferation *in vivo* and *in vitro*. We then screened for genes encoding proteins that could mediate the proliferative signals of PPARγ, and noted that a previous microarray study showed that proliferin (PLF) is upregulated by Pio, rosiglitazone and troglitazone[14]. We confirmed that Pio elevated the mRNA and protein level of PLF in 3T3-L1 adipocytes, and that this was associated with increased cell proliferation. Since PLF acts *via* a paracrine mechanism[15], we found that treatment with an anti-PLF antibody resulted in reduced adipocyte proliferation *in vivo* and *in vitro*.

Taken together, our results indicate that PLF regulates adipocyte proliferation.

RESULTS

Effect of Pio on cell proliferation in adipose tissue

To evaluate cell proliferation, we used immunohistochemistry to measure BrdU incorporation into adipose tissue and non-adipose tissue (*i.e.*, intestine). Because cell proliferation in adipose tissue is much less active than that in intestine (Fig. 1a), repeated intraperitoneal injections of BrdU were required. A single BrdU injection labels cells for 5–6 h[16], and so we expected that cells in S-phase would be detected for approximately 12–18 h by our method. Because the animals were sacrificed 24 h after the first injection, the possibility that labeled cells might transform from preadipocytes into mature adipocytes was excluded. In the epididymus, BrdU-labeled cells were detected in the perivascular area, which consists of the stromal vascular fraction (SVF; Fig. 1b). Furthermore, BrdU-labeled cells with the characteristics of mature adipocytes were observed (Fig. 1c). Double-color immunostaining for BrdU and the mature adipocyte-specific protein adiponectin confirmed that adiponectin was present in the cytoplasm of the BrdU-labeled cells (Fig. 1d). Taken together, these results suggest that mature adipocytes have the ability to proliferate. Furthermore, BrdU-labeled mature adipocytes in contiguous sections were concomitantly

stained with anti-proliferating cell nuclear antigen (PCNA) antibody (Fig. 1e,f), another marker of proliferating cells[17]. No apoptotic cells labeled with terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) were detected in the BrdU-positive mature adipocytes (Supplementary Fig. 1).

To confirm that mature adipocytes incorporate BrdU, the mature adipocytes were separated from the SVF by collagenase digestion, DNA was extracted, and BrdU incorporation was quantified. No significant difference was observed in BrdU incorporation between mature adipocytes and SVF cells (Fig. 2a). Therefore, we can exclude the possibility that the apparent BrdU incorporation in mature adipocytes was due to contamination with SVF cells. Visceral adipose tissues (*i.e*, epididymal, mesenteric and perirenal adipose tissues) were more active in incorporating BrdU than subcutaneous adipose tissue (epididymal vs subcutaneous; p= 0.032, mesenteric vs subcutaneous; p=0.044). Treatment with Pio for 14 d significantly (0.005% Pio vs control; P=0.048, 0.025% Pio vs control; P=0.026) increased BrdU incorporation in subcutaneous adipocytes, but not in visceral adipocytes, (Fig. 2b). These results suggest that, in mature subcutaneous adipocytes, activation of PPARγ by its agonist Pio leads to an increase in the rate of proliferation. The pattern of PCNA protein expression levels in isolated mature adipocytes from subcutaneous and visceral fat (Fig. 2c) was similar to that of BrdU incorporation (Fig. 2a,b). Moreover, the protein expression of PCNA was increased by Pio treatment in subcutaneous adipocytes (Fig. 2c), supporting the results for BrdU

incorporation. Although quantitative estimation was difficult, treatment with Pio did not increase the number of preadipocyte factor-1 (Pref-1)-labeled cells in adipose tissue including subcutaneous fat (Supplementary Fig. 2). Because Pref-1 is a marker of preadipocytes, this result implies that the Pio treatment did not influence preadipocyte proliferation.

Effect of Pio and 15-deoxy-Δ12,14-prostaglandin J2 on cell proliferation in cultured adipocytes

Fully differentiated 3T3-L1 adipocytes were re-seeded into 6-well plates to determine if they are capable of proliferation. Although adipocyte proliferation was less active at 10 d than at 5 d after differentiation, the cell number increased markedly between 0 h, 24 h and 48 h after the cell attachment to the plate (Fig. 3a). Both Pio and 15-deoxy-Δ12,14-prostaglandin2 **(**PGJ2) enhanced the proliferation rate (control vs Pio; P=0.002, control vs PGJ2; P=0.027: Fig. 3b). Adiponectin was detected in more than 90% of re-seeded adipocytes, whereas Pref-1 was not detected in these cells (Supplementary Fig. 3). Hence, we can rule out the possibility that the increases in cell number were due to contamination by preadipocytes. Furthermore, lipid droplets, a characteristic of mature adipocytes, were detected in more than 90% of the cells examined (Fig. 4a). Supporting the results in Fig. 3b, BrdU incorporation in the 3T3-L1 adipocytes was increased by treatment with Pio or PGJ2 (control vs Pio; P=0.0494, control vs PGJ2; P=0.017: Fig.

3c,d). Together, these results suggest that activation of PPARγ in fully differentiated 3T3-L1 adipocytes leads to cell proliferation. To further confirm whether these mature adipocytes are capable of proliferating, we performed cell cycle analyses using flow cytometry. Cells in G0/G1, S and G2/M phase were observed amongst the fully differentiated 3T3-L1 adipocytes (Fig. 3g). In addition, we examined cell proliferation in primary cultures of subcutaneous and mesenteric adipocytes. Since passage of primary cultured mature adipocytes is difficult, differentiation was induced at 70% confluence. The efficiency of differentiation in the primary cultured adipocytes was inferior to that of 3T3-L1 adipocytes; however, we were able to discriminate the differentiated cells by staining for adiponectin. We therefore carried out double staining for adiponectin and BrdU. More than 80% of the subcutaneous and mesenteric cells stained positive for adiponectin (Fig. 3e). The proportion of BrdU-labeled cells within the adiponectin-positive cell population was significantly smaller in subcutaneous adipocytes than in mesenteric adipocytes; however, treatment with 10 μM Pio significantly increased the proportion of BrdU-labeled cells within the adiponectin-positive cells (subcutaneous vs mesenterin; P=0.018, control vs Pio 10 μM in subcutaneous; P=0.036: Fig. 3f).

Incubation of fully differentiated 3T3-L1 adipocytes with 10 μM aphidicolin, an inhibitor of DNA polymerase α, δ and ε, for 48 h resulted in marked lipid droplet formation (Fig. 4a). Although treatment with Pio significantly decreased the PPARγ mRNA level, it significantly increased adiponectin and fatty acid synthase (FAS) mRNA levels (Fig. 4b–e).

Treatment with Pio significantly decreased the MCP-1 mRNA level (Fig. 4h). Our result supports the previous finding that Pio downregulates PPARγ mRNA expression in mature, but not partially differentiated adipocytes[18]. Treatment with aphidicolin significantly suppressed expression of PPARγ mRNA and its downstream genes, including the genes encoding aP2, LPL and adiponectin (Fig. 4b–d,f), and elevated the mRNA expression of genes encoding cytokines such as MCP-1 and interleukin-6 (IL-6; Fig. 4h,i). These results indicate that DNA polymerase influenced the morphology and gene expression of mature adipocytes and support our contention that these cells proliferate.

Role of PLF on adipocyte proliferation

Next, we explored the factors regulating mature adipocyte proliferation. Since we observed that proliferation was accelerated by Pio, we screened for genes regulated by the thiazolidinedione class of drugs. In published microarray data from 3T3-L1 adipocytes[14], we noted that the growth factor PLF was significantly upregulated by three thiazolidinediones (i.e., Pio, rosiglitazone and troglitazone). PLF, also termed mitogen-regulated protein (MRP), is a member of the prolactin/growth hormone family, and is involved in the angiogenesis of placenta[19, 20]. PLF, secreted as a paracrine agent, binds to the insulin-like growth factor II (IGF-II)/ cation-independent mannose 6-phosphate (M6P) receptor, the signal of which can be mediated with Gi protein[21, 22]. Here, we detected PLF mRNA at day 2 after the initiation of differentiation of 3T3-L1 adipocytes; the

mRNA level then remained stable at days 5 and 9 (Supplementary Fig. 4). Four PLF/MRP genes, *Plf1*, *Plf2*, *Mrp3* and *Mrp4*, have been cloned and shown to share 91% amino acid sequence identity[15]. To determine which subclass is expressed in 3T3-L1 adipocytes, PCR of PLF cDNA was performed as previously described[15]. Direct sequence analysis revealed that the PCR product completely coincided with PLF1 (Supplementary Fig. 5). Incubation with Pio increased PLF mRNA as well as protein levels in cell lysates and culture media (Fig. 5a,b). Gene silencing of PLF by siRNA significantly decreased both the cell number and BrdU incorporation in the fully differentiated adipocytes (P=0.001, P=0.007: Fig. 5c,d). Addition of goat anti-PLF antibody to the non transfected adipocyte culture medium significantly suppressed cell proliferation, when compared with the effect of addition of goat IgG (P=0.001, P=0.001: Fig. 5c,d). Incubation with IGF-II significantly increased cell proliferation, whereas pertussis toxin (PTx) treatment significantly decreased cell proliferation (control vs IGF2;P=0.001, P=0.014, control vs PTx;P=0.001, P=0.011: Fig. 5e,f). Both gene silencing and antibody treatment reduced the mRNA expression levels of PPARγ, LPL and adiponectin, but not MCP-1 in the 3T3-L1 adipocytes (Supplementary Fig. 6b,e).

We then examined the expression of the PLF protein in rat adipocytes. PLF was expressed abundantly in white adipose tissue and brown adipose tissue, whereas low PLF levels were detected in pancreas, liver and skeletal muscle (Supplementary Fig. 7). Interestingly, PLF protein expression in epididymal and mesenteric fat was significantly higher than

that in subcutaneous fat, and treatment with Pio significantly increased PLF expression in subcutaneous fat only (control vs Pio; P=0.001: Fig. 6a,b). These results were similar to the results for BrdU incorporation shown in Fig. 2b., supporting our speculation that PLF regulates mature adipocyte proliferation *in vivo*. To confirm this, we studied the effect of an anti-PLF antibody *in vivo*. Administration of the anti-PLF antibody to mice resulted in a decrease of BrdU incorporation in adiponectin-positive epididymal adipocytes (P=0.006: Fig. 6c).

DISCUSSION

Numerous researchers have clarified the origin of adipocyte progenitor cells and the mechanism(s) by which they differentiate into adipocytes, but there has been little debate regarding the possibility of mature adipocyte proliferation. A recent study concluded that adipocyte number increases in the early stage in man[6]. The authors claimed that adipocytes do not divide or express mitotic markers, although no evidence is cited. The notion that mature adipocytes are unable to replicate is poorly described in recent reviews[23, 24]. Rodeheffer *et al.* [25], who identified the undifferentiated adipocyte precursor subpopulation within adipose tissue stroma, stated the inability of mature adipocytes to replicate on the basis of a microscopic morphological study carried out by Simon[26] in 1965. During the period from 1970 through 1990, the possibility of mature adipocyte proliferation

was a matter of debate. Several *in vivo* studies were performed using labeled thymidine[27, 28] Sugihara *et al.*[29] provided evidence that "ceiling" cultures of mature white adipocytes exhibit morphologically dividing cells. Although there has been no conclusive evidence to support the assertation, terminally differentiated and lipid-filled adipocytes have been generally considered, since the 1990's, to be incapable of division. This is despite recent research demonstrating mature adipocyte proliferation by BrdU-labeling[30] or microscopic observation of organotypic culture system[31].

Here, we assessed mature adipocyte proliferation by measuring BrdU incorporation in adipose tissue. Our detection of BrdU-labeled mature adipocytes was consistent with the detection of BrdU-labeled white mature adipocytes after sympathetic denervation reported by Foster *et al.*[30]. We confirmed that the labeled cells were actually mature adipocytes by using double-color immunostaining for BrdU and adiponectin, a marker of mature adipocytes. Our finding that a proportion of BrdU-positive mature adipocytes were also positive for PCNA, a different marker of proliferation, further supports our contention of mature adipocyte proliferation. BrdU can integrate into apoptotic cells as a result of DNA damage and repair[32], and thiazolidinediones can induce apoptosis in adipocytes[33]. Therefore, BrdU incorporation in mature adipocytes, particularly those treated with thiazolidinediones, should be interpreted carefully. Other evidence suggests that the use of BrdU labeling criteria makes it highly unlikely that apoptotic cells would be mistaken for newborn cells *in vivo*[34]. Indeed, in our study,

no adipocytes that simultaneously stained for BrdU and TUNEL were detected, even in visceral adipose tissue. Since the animals were sacrificed 24 h after the first BrdU injection, the possibility that BrdU-labeled preadipocytes turned into mature adipocytes can be ruled out. Therefore, our results demonstrate that DNA synthesis, the early phase of cell division, occurs in mature adipocytes *in vivo*.

To quantify BrdU incorporation, adipocytes were separated from SVF by collagenase digestion. Surprisingly, BrdU incorporation in adipocytes was not inferior to that in SVF. Our experiments demonstrated that BrdU incorporation was higher in visceral than in subcutaneous adipocytes. Treatment with Pio increased BrdU incorporation in subcutaneous adipocytes in a dose-response manner, whereas visceral adipocytes did not respond to Pio. We suggest that a Pio-induced increase in the proliferation of subcutaneous adipocytes may be the mechanism behind the Pio-induced increase in subcutaneous fat mass in type 2 diabetes patients reported by Miyazaki *et al.*[12]. Conversely, treatment with Pio did not increase preadipocyte number. Hence, the effect of Pio on cell number cannot be explained by recruitment of progenitor cells.

To examine whether cultured mature adipocytes proliferate, we re-seeded fully differentiated 3T3-L1 adipocytes onto plates to remove contact inhibition. Both the number of adipocytes and the level of BrdU incorporation increased with time in culture. Furthermore, cells that had entered the cell cycle were detected by flow cytometry. Although the possibility that undifferentiated preadipocytes contributed to these results

cannot be excluded completely, our observations that more than 90% of cells stained with an anti-adiponectin antibody, but not anti-Pref-1 antibody, and that lipid droplets were present in these cells, imply that the vast majority of the cells examined were differentiated adipocytes. Our result that subcutaneous adipocytes have a lower proliferative activity than visceral adipoctyes, but only subcutaneous adipocytes respond to Pio, was confirmed in primary culture. Although we are unable to explain this difference between the behavior of these two cell types, it may be related to their distinct expression profiles of multiple genes involved in embryonic development and pattern specification, which are intrinsic and persist during *in vitro* culture and differentiation[35].

Our result that the PPARγ agonist Pio and the PPARγ ligand PGJ2 both accelerated 3T3-L1 adipocyte replication strongly suggests that activation of PPARγ promotes mature adipocytes proliferation. We therefore screened the published literature for growth factors regulated by PPARγ. Among numerous studies, most of which showed that thiazolidinediones inhibit cell proliferation in various cell types, including cancer cells[36], a microarray study performed by Sears *et al.*[14] indicated that thiazolidinediones upregulate PLF in 3T3-L1 adipocytes. PLF was identified by Linzer *et al.* in 1984 as a member of the prolactin/growth hormone family in mouse 3T3 cells[19]. Four PLF/MRP genes, *Plf1*, *Plf2*, *Mrp3* and *Mrp4*, have been cloned in the mouse and shown to share 91% amino acid sequence identity[15]. PLF genes are expressed markedly in uteroplacental tissue and are thus thought to be involved in angiogenesis of

the uterus and placenta[37]. *Mrp3* is induced in wound-edge keratinocytes during cutaneous wound healing, whereas *Mrp3* and *Mrp4* are found in the hair follicles[38]. PLFs are speculated to be involved in the growth of neuroblastoma cells[39] and hematopoietic stem cells[40]. Here, we showed that, among various organs in adult rats, PLF protein was most abundantly expressed in white adipose tissue. We found that Pio increased not only the expression level of PLF mRNA, but also the level of protein secreted into the medium in 3T3-L1 adipocytes. Accordingly, we postulated that PLF might regulate adipocyte proliferation. Our finding that 3T3-L1 adipocyte proliferation is inhibited when PLF action is attenuated by either anti-PLF antibody or PLF siRNA strongly supports this. The observation that inhibition of PLF action led to a decrease in the mRNA expression of PPARγ and its downstream genes, LPL and adiponectin, in a similar manner to treatment with the DNA polymerase inhibitor aphidicolin suggests that cell proliferation might influence adipocyte functions to regulate glucose and lipid metabolism. Since PLF action is mediated *via* the IGF-II/cation-independent M6P receptor, which is PTx sensitive [22], we evaluated the effects of IGF-II and PTx on cell proliferation in 3T3-L1 adipocytes. As expected, PTx abolished cell replication, whereas IGF-II stimulated cell replication. These results support the notion that PLF acts on the IGF-II/cation-independent M6P receptor in adipocytes; however, further study is necessary. Interestingly, Connor *et al.*[41] reported that PLF mRNA levels are increased by estradiol and reduced by dexamethasone in the BNL cell line. We observed that treatment with dexamethasone reduced

proliferation and PLF mRNA levels in 3T3-L1 adipocytes (data not shown). Considering the effect that glucocorticoids and estrogen have on the distribution of adipose tissue and insulin sensitivity, further research into the role of PLF in adipocyte proliferation can be expected to provide new insight in diabetic research. In addition, we found that the tissue-specific pattern of PLF protein expression was similar to that of BrdU incorporation in adipocytes isolated from subcutaneous adipose and various visceral adipose tissues following treatment with Pio. These results imply that PLF regulates the proliferation of mature adipocytes *in vivo*, and that the difference between subcutaneous and visceral adipocyte proliferation might be explained by their ability to secrete PLF. Moreover, our results indicating that treatment with anti-PLF antibody significantly decreased BrdU incorporation and elimination of PLF throughout the body resulted in a decrease of mature adipocytes proliferation.

In conclusion, our results strongly suggested that mature adipocytes have the ability to proliferate in adipose tissue, as well as in cultured adipocytes. PLF may contribute to the proliferation of these mature adipocytes.

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AUTHOR CONTRIBUTIONS

K. Kajita, experiment design and performance, manuscript preparation; I. Mori, and T. Hanamoto, cell culture including transfection of siRNA and immunohistochemistry; W. Zhiliang, real-time PCR; T. Ikeda, K. Fujioka, M Yamauchi and H. Okada, animal treatment; A. Minami and T. Kajita, dot blot, Western blot analysis and immunostaining; Y. Uno, and H. Morita, statistics; I. Nagano, real-time PCR; M. Takemura, flow cytometry, M. Seishima, experimental design; Y. Takahashi, real-time PCR and experimental design; T. Ishizuka, project development, coordination and manuscript preparation.

FIGURE LEGENDS

Fig. 1. Immunohistochemical staining of BrdU in the adipose tissue.
Male Wistar rats were intraperitoneally injected with 200 mg/kg of BrdU three times 24 h before sacrifice. The small intestinal tract (control) **(a)** and epididymal adipose tissue **(b–f)** were harvested, processed for paraffin sections, and immunohistochemically stained as described in the Methods. **(a)** Tissue from the small intestinal tract contained abundant BrdU-labeled cells. **(b)** BrdU-labeled cells were observed in the stromal vascular area in epididymal adipose tissue. **(c)** BrdU-labeled nuclei were detected in cells with the appearance of mature adipocytes. **(d)** Double-color

immunohistochemical staining for BrdU and adiponectin. BrdU-positive nuclei (3,3'-Diaminobenzidine, DAB: brown) were detected (black arrow) in adiponectin-positive cytosols (tetramethylbenzidine, TMB: blue). **(e, f)** Immunohistochemical staining for BrdU **(e)** and PCNA **(f)** in contiguous sections. The same nucleus was stained simultaneously.

Fig. 2. Analysis of BrdU incorporation in adipocytes and SVF isolated from subcutaneous and visceral adipose tissues, and the effect of Pio treatment

(**a**) To quantitatively evaluate BrdU incorporation, the adipocytes and SVF in each adipose tissue isolated from male Wistar rats were separated by collagenase digestion. DNA extracted from adipocytes and SVF cells was spotted onto a nitrocellulose membrane, and BrdU incorporation and total DNA levels were visualized with an anti-BrdU antibody (top panel) and anti-DNA antibody (middle panel), respectively. A histogram of BrdU incorporation into adipocytes (white bar) and SVF (black bars) in visceral adipose tissues relative to that in subcutaneous adipose tissue (100%) is shown; n = 5; *, $P<0.05$ *vs* subcutaneous adipose tissue (bottom panel). Sub, subcutaneous; Epi, epididymal; Mes, mesenteric; Ren, perirenal. **(b)** BrdU incorporation in adipocytes isolated from male Wistar rats housed with food containing various amounts of Pio. The top panel shows a typical result for the effect of food containing 0.005% Pio on BrdU incorporation into adipocytes. The bottom panel shows the effect of food containing 0% Pio (Control, white bar), 0.005% Pio (black bar) and 0.025% Pio (grey bar)

on BrdU incorporation in various visceral adipose tissues; all values are relative to the level of BrdU incorporation in the subcutaneous adipocyte isolated from the control (100%). n = 6; **, $P < 0.01$ *vs* control. **(c)** Expression of PCNA protein in subcutaneous, epididymal and mesenteric adipocytes isolated from untreated rats (Control) and rats housed with food containing 0.005% Pio. The typical results of Western blot in four separate experiments were shown. Each adipocyte sample was subjected to electrophoresis in a separate gel; however, the electrophoresis, transblot, incubation with antibody and enhanced chemiluminescence of the various samples were performed simultaneously.

Fig. 3. Cell proliferation in 3T3-L1 adipocytes and the effect of PPARγ agonists

(a) To evaluate cell proliferation, the 3T3-L1 adipocytes were induced to fully differentiate, and then 5 d (■) or 10 d (○) later, the cells were suspended by trypsin-EDTA treatment and re-seeded into 6-well plates at a density of 3×10^3 cells/cm^2. The medium was exchanged 24 h later, when the cells had attached to the plate, and the cell number was counted immediately (0 h), and 24 h and 48 h later. **(b)** The effect of Pio and PGJ2 on adipocyte number was examined. After the re-seeded 3T3-L1 adipocytes attached to the plate (0 h), the medium was exchanged and the cells were incubated for 24 h without treatment (Control, ■), or with 10 μM Pio (●) or 10 μM PGJ2 (▲). The cell number was counted at 0 h and 24 h. n = 6, *, $P < 0.05$ *vs* control. **(c**, **d)** To assess BrdU incorporation, 3T3-L1 adipocytes

were re-seeded into chamber slides at a density of 10^3 cells/cm^2. After the cells attached, they were incubated for 24 h without treatment (Control), or with 10 μM Pio or 10 μM PGJ2 (J2); the cells were treated with 10 μM BrdU for the last 4 h of this incubation period. Immunostaining for BrdU was performed, and the cells were counted. Representative images of the immunostaining **(c)** and histograms showing the proportion (%) of BrdU-labeled cells in each sample **(d)** are shown. Data are presented as the mean ± s.e.m of 6 independent experiments. *, $P < 0.05$ *vs* control. **(e, f)** Primary cultures of preadipocytes derived from subcutaneous and mesenteric adipose tissue were cultured in chamber slides. When the cells reached 70% confluence, differentiation was initiated. Five d after the differentiation, the cells were incubated for a further 24 h with or without various concentrations of Pio. Double-color immunostaining for BrdU (brown) and adiponectin (red) was performed. Typical results for subcutaneous and mesenteric adipocytes **(e)** and histograms showing the proportion (%) of BrdU-positive cells in sets of 5000 adiponectin-positive cells derived from subcutaneous adipocytes (black bars) or mesenteric adipocytes (white bars) **(f)** are shown. Data are presented as the mean ± s.e.m of 3 independent experiments. *, $P < 0.05$ *vs* subcutaneous control. **(G)** The cell cycle in fully differentiated 3T3-L1 adipocytes was analyzed by flow cytometry. A typical result from 5 independent experiments is shown.

Fig. 4. Effects of Pio and aphidicolin on cell morphology and gene

expression

(a) Differentiated 3T3-L1 adipocytes were suspended by trypsin-EDTA treatment, and re-seeded at 5×10^3 cells/cm^2 in chamber slides. After treatment with 10 μM Pio or 10 μM aphidicolin for 48 h, Papanicolau staining (upper panel) and Oil Red O staining (lower panel) was performed. **(b–i)** Passaged differentiated 3T3-L1 adipocytes were incubated without treatment (Control), or with 10 μM Pio, 10 μM aphidicolin or 10 μM Pio plus 10 μM aphidicolin for 48 h. Total RNA was extracted, and the mRNA levels of PPARγ **(b)**, aP2 **(c)**, adiponectin **(d)**, FAS **(e)**, LPL **(f)**, Glut4 **(g)**, MCP-1 **(h)** and IL-6 **(i)** were measured by real-time PCR, as described in Methods. Aphi, aphidicolin. n = 6, *, $P < 0.05$ *vs* control. **, $P < 0.01$ *vs* control.

Fig. 5. Expression of PLF in 3T3-L1 adipocytes

(a, b) Fully differentiated 3T3-L1 adipocytes, 7 d after induction of differentiation, were incubated without treatment (Control) or with 10 μM Pio for 24 h, and total RNA, cell lysate and medium were harvested for real-time PCR and Western blots. Treatment with Pio increased not only the PLF mRNA level **(a)**, but also the PLF protein level in cell lysates and media **(b)**. The values in panel **(a)** are the mean ± s.e.m. of 4 separate experiments. **, $P < 0.01$ *vs* control. Panel **(b)** represents a typical result of 4 independent experiments. **(c,d)** To assess the effect of PLF knockdown on adipocyte proliferation, PLF siRNA was transfected into fully differentiated 3T3-L1 adipocytes. One day later, cells were suspended by trypsin-EDTA

treatment. Cells were re-seeded at a density of 3×10^3 cells/cm^2 in 6-well plates and the cells were counted **(c)**, as described in Methods. The effect of treatment with goat anti-PLF antibody, or goat IgG, was concomitantly evaluated. ●, Control; ○, Goat IgG; ■, PLF siRNA; ▲, anti-PLF antibody. Cells were also re-seeded in chamber slides at a density of 10^3 cells/cm^2 and incubated with or without antibodies, as described above, and BrdU incorporation was measured **(d)**. n = 5; **, $P < 0.01$ *vs* control, ##, $P < 0.01$ *vs* Goat IgG. **(e, f)** In addition, the effects of treatment with IGF-II and PTx were studied to assess the involvement of IGF-II/ cation-independent mannose 6-phosphate receptor. The effects of IGF-II and PTx on adipocyte number **(e)** and BrdU incorporation **(f)** are shown. ●, Control; ■, IGF-II; ▲, PTx. n = 5; *, $P < 0.05$ *vs* control.

Fig. 6. Expression levels of PLF protein *in vivo*, and effect of anti-PLF antibody on *in vivo* BrdU incorporation in adipocytes.

(a, b) Expression of PLF in adipocytes isolated from subcutaneous (Sub), epididymal (Epi), mesenteric (Mes) and perirenal (Ren) adipose tissue by collagenase digestion, in male Wistar rats housed with food containing 0% Pio (Control, white bar), 0.005% Pio (black bar) or 0.025 Pio (grey bar). PLF protein levels were assayed by Western blot **(a)** and quantified **(b)**. Data are presented as the mean ± s.e.m of 5 independent experiments. *, $P < 0.05$ *vs* control. **(c)** C57BL-CR mice were intraperitoneally injected with 4 mg/kg goat anti-PLF antibody or goat IgG, and BrdU was administered 24 h later. BrdU-labeled adipocytes in epididymal adipose tissue were

detected with double-color immunohistochemical staining for BrdU and adiponectin. The values given are the proportion (%) of BrdU-labeled cells in the adiponectin-positive cells, expressed as the mean ± s.e.m. of 6 independent experiments. **, $P < 0.01$.

METHODS

Effect of Pio on cell proliferation in adipose tissue

Male Wistar rats weighing 250 g were treated with food containing either 0%, 0.005% or 0.025% Pio for 14 d. Then 200 mg/kg BrdU was intraperitoneally administered 3 times over a period of 12 h (*i.e.*, every 4 h). The rats were then sacrificed 12 h later, and subcutaneous, epididymal, mesenteric and perirenal fats were harvested. Animal care and experimental procedures were performed under the approval of the Animal Care Committees of Gifu University School of Medicine.

Immunohistochemical staining

BrdU antibody staining of paraffin sections of the fat tissues was carried out using a BrdU *In-Situ* Detection Kit (Becton Dickinson, Franklin Lakes, NJ, USA). As a positive control, tissue from the small intestinal tract of the same animal was also harvested and immediately stained, as described above. Other immunostaining was preformed using aint-adiponectin

antibody (R & D System, Minneapolis, MN, USA), anti-PCNA antibody (Santa Cruz, Santa Cruz, CA, USA), anti-Pref-1 antibody (Santa Cruz) and Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

Dot blot analysis

To confirm the hypothesis that mature adipocytes incorporate BrdU *in vivo*, isolated mature adipocytes and SVF were obtained by collagenase digestion of each adipose tissue. DNA extracted from adipocytes and SVF was spotted on a nitrocellulose membrane. Incorporated BrdU was visualized with an anti-BrdU antibody (Santa Cruz Biotechnology). The detailed method is described in the S**upplementary Methods.**

Measurement of cell number and BrdU uptake in 3T3-L1 adipocytes and primary cultured adipocytes

3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and differentiation induced with 3T3-L1 differentiation medium (DS Pharma Biomedical, Osaka, Japan). Fully differentiated 3T3-L1 adipocytes were suspended by trypsin-EDTA treatment and then seeded at a density of $3 \times 10^3/cm^2$ in 6-well dishes. Subsequently, cells were incubated without treatment (control), or with 10 μM Pio (Takeda, Osaka, Japan), 10 μM aphidicolin, or 10 μM PGJ2 in DMEM for 24 h. The cells were suspended again and counted using a hemocytometer. To measure BrdU uptake *in vitro*, isolated adipocytes were

seeded in chamber slides and incubated. Ten μM BrdU was added for a period of 4 h. The detailed method for assessment of the proliferation of primary cultured adipocytes is described in the S**upplementary Methods.**

Flow cytometry

Fully differentiated 3T3-L1 adipocytes were suspended by trypsin-EDTA treatment, and cell populations in the G0/G1, S and G2/M phases were separated using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Becton Dickinson, San Diego, CA, USA). The data were analyzed using Cell Quest software (Becton Dickinson). In each sample, 10 000 fluorescent cells were counted.

Real-time PCR analysis

To determine the mRNA expression levels of PPARγ, aP2, LPL, fatty acid synthase (FAS), adiponectin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), real-time PCR analysis was performed using a Thermal Cycler Dice (TAKARA, Ohtsu, Japan). Expression levels, calculated as copy numbers in each sample, were normalized to the expression level of GAPDH. The primer sequences are described in the S**upplementary Methods.**

Antibody administration *in vivo*

C57BL-CR mice were injected intraperitoneally with either 4 mg/kg goat anti-PLF antibody (Santa Cruz) or goat IgG, in parallel, and then

administered with BrdU 24 h later, as described above. BrdU-labeled adipocytes were detected with double-color immunostaining using an anti-BrdU antibody and anti-adiponectin antibody.

Statistics

Statistical comparisons were performed with Student's t-test (Figs. 5a and 6c) and two-factor ANOVA (Fig. 5c and 5d). Other data were analyzed with one-factor ANOVA (Dunnett test). All statistical tests were two-tailed. Data are given as mean ± s.e.m.. Values of $P < 0.05$ were considered statistically significant.

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SUPPLEMENTARY METHODS

Dot blot analysis

Isolated mature adipocytes and SVF were obtained by collagenase (type 1, Sigma-Aldrich, St. Louis, MO, USA) digestion of each adipose tissue dissociated from male Wistar rats. DNA was extracted with proteinase K from each cell. Equal amounts of DNA were spotted onto a nitrocellulose membrane (Protran® BA85, Whatman, London, UK) using an S&S Minifold® Slot Blot System (Whatman). The membrane was dried at 80°C for 2 h, and then heated to 95°C for 10 min in sodium citrate buffer (pH 6.0). After blocking non-specific binding with 7% (w/v) nonfat dry milk for 2 h, the membrane was incubated with an anti-BrdU antibody (Santa Cruz) in parallel with an anti–single stranded DNA antibody (Millipore, Billerica, MA, USA) overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz). The reaction was visualized by means of the ECL system (GE Healthcare, Buckinghamshire, UK).

BrdU incorporation into primary cultured adipocytes

Primary cultured subcutaneous and mesenteric adipocytes were harvested as described previously[1]. Cells were cultured in Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and seeded in a 6-well plate or chamber slide. When the cells reached 70% confluence, the medium was changed to differentiation medium (DS Pharma Biomedical). Two days later, the

medium was exchanged for DMEM, and differentiated adipocytes were maintained for 5 d. To assess the proliferation of primary cultured adipocytes, the primary adipocytes were incubated with or without 0-100 μM Pio for 24 h. Ten micromolar BrdU was added for 4 h prior to the end of incubation. BrdU-labeled nuclei and adiponectin-positive cytosols were visualized by immunostaining using BrdU *In-Situ* Detection Kit (Becton Dickinson), anti-adiponectin antibody and Vectastain ABC kit (Vector Laboratories). The proportion of BrdU-labelled cells in a total of 5000 adiponectin-positive cells was calculated.

Primer sequences

Quantitative real-time RT-PCR was performed for the validation of microarray results using the Thermal Cycler Dice Real Time System (TAKARA). Total RNA was isolated and purified as mentioned above. Reverse transcription was performed using a PrimeScript Reverse Transcriptase (TAKARA, Japan) according to the manufacturer's instructions. Optimal conditions for all investigated genes were established using SYBR Premix Ex Taq Kit (TAKARA) according to the manufacturer's instructions. Twenty μl of the reaction solution consisted of 2 μl of the template, 10 μl of SYBR Premix Ex Taq, 0.4 μl of 10 μM of each primer and 0.4 μl of ROX Reference Dye. PCR amplification was performed as follows: predenature for 1 cycle at 95℃ for 30s, and 40 cycles at 95℃for 5s, 60-62℃ for 30s. Oligonucleotide primers were

designed, based on sequences from the GenBank database.

PPARγ: 5′-CCCTTTGGTGACTTTATGGA-3′ (f) and

5′-CTGCCTGAGGTCTGTCATCT-3′ (r);

aP2: 5′-AAAGACAGCTCCTCCTCGAAGGTT-3′ (f) and

5′-TGACCAAATCCCCATTTACGC-3′ (r);

LPL: 5′-AGGATGCAACATTGGAGAAG-3′ (f) and

5′-TCTCTTGGCTCTGACCTTGT-3′ (r);

FAS: 5′-AGATCCTGGAACGAGAACACGAT-3′ (f) and

5′-GAGACGTGTCACTCCTGGACTTG-3′ (r);

adiponectin: 5′-AAGGACAAGGCCGTTCTCT-3′ (f) and

5′-TATGGGTAGTTGCAGTCAGTTGG-3′ (r);

IL-6: 5′-GACTTCCATCCAGTTGCCTTCT-3′ (f) and

5′-TTTTCTCTTTCCACGATTTCC-3′ (r);

Socs-1: 5′-GACACTCACTTCCGCACCTT-3′ (f) and

5′-GCAGTTCCGTTGGCGACT-3′ (r);

MCP-1: 5′-TCACCTGCTGCTACTCATTCAC-3′ (f) and

5′-CCATTCCTTCTTGGGGTCAG-3′ (r);

Glut4: 5′-AGTTGGAGAGAGAGCGTCCA-3′ (r) and

5′-ACCGAGACCAACGTGAAGAC-3′ (r);

proliferin (PLF): 5′-GGTCGTTGCTTTATGTCCTTTG-3′ (f) and

5′-GGCTTGTTCCTTGTTTTCTGG-3′ (r);

GAPDH: 5′-GGCATTGTGGAAGGGCTCAT-3′ (f) and

5′-GACACATTGGGGGTAGGAACAC-3′ (r).

To identify the PLF subtype expressed in mature 3T3-L1 adipocytes, PCR

for RT product was preformed using the following primers; plf downstream exon I, 5′-TAAGCCTGGGTAGGACTCTGC-3′ (45-65) and upstream exon V, 5′-CTCTGCAGAGATGCTCCCTTC-3′ (778-756). PCR amplification was performed as follows: predenature for 1 cycle at 95 ℃ for 2m, and 40 cycles at 95℃for 30s, 66℃ for 1m and 72℃ for 1.5m.

siRNA and antibody treatment

To examine the effect of elimination of PLF on mature 3T3-L1 adipocyte proliferation, PLF knockdown with siRNA was preformed. Fully differentiated 3T3-L1 adipocytes were re-seeded in 6-well dishes and cultured in DMEM. When the cells reached 80% confluence, commercial PLF siRNA or negative control (control) siRNA (Santa Cruz) was transfected using specific siRNA reagents (TransIT-TKO, Mirus, Madison, WI, USA) according to the manufacturer's instructions. The next day, the transfected cells were trypsinized again and seeded in a 6 well plate and chamber slide at a density of $3 \times 10^3/cm^2$. When the cells had attached to the plate, the cell number was counted immediately (0 h), and 24 h later. BrdU uptake in transfected 3T3-L1 adipocytes was measured as mentioned above. Furthermore, re-seeded 3T3-L1 adipocytes at a density of $3 \times 10^3/cm^2$ were incubated with 2 μg/ml goat anti-PLF antibody or goat IgG (Santa Cruz) for 24 h, followed by cell count and measurement of BrdU uptake. In addition, to evaluate whether IGF-II/cation-independent M6P receptor and Gi protein, the effects of treatment with 50 ng/ml IGF II and

100 ng/ml PTx for 24 h on cell count and BrdU incorporation were assessed.

SUPPLEMENTARY REFERENCE

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 1 Comparison between BrdU staining and TUNEL staining in individual cells in adipose tissues

(a, b) To examine the possibility that the BrdU staining in mature adipocytes could be attributed to apoptosis, BrdU and TUNEL staining were performed on contiguous sections of mesenteric adipose tissue isolated from male Wistar rats housed with food containing 0.025% Pio for 2 wk. The BrdU-labeled cells **(a)** were not simultaneously stained with TUNEL system **(b)**. The solid line arrows represent the BrdU-positive, TUNEL-negative cells. The broken line arrows indicate TUNEL-positive cells.

Supplementary Fig. 2 Immunohistochemical staining of Pref-1 in adipose tissue

(a, b) Subcutaneous adipose tissues isolated from rats housed with food containing no Pio **(a)** or 0.024% Pio **(b)** for 2 wk were immunohistochemically stained for Pref-1 (red) to determine whether preadipocyte growth is influenced by Pio administration.

Supplementary Fig. 3 Expression of Pref-1 and adiponectin in 3T3-L1 preadipocytes and adipocytes

To rule out the possibility that undifferentiated preadipocytes influenced the results for cell number, cell cycle and BrdU incorporation in 3T3-L1 adipocytes, we assessed the expression of Pref-1 and adiponectin in 3T3-L1 adipocytes. Immunostaining for Pref-1 in 3T3-L1 preadipocytes (left), re-seeded fully differentiated 3T3-L1 adipocytes (middle), and for adiponectin in re-seeded fully differentiated 3T3-L1 adipocytes (right) is shown.

Supplementary Fig. 4 Expression of PLF during differentiation in 3T3-L1 adipocytes

Expression of PLF mRNA (black bars) and PPARγ mRNA (white bars) increased during the differentiation of 3T3-L1 adipocytes. The values are mRNA copy numbers adjusted for GAPDH mRNA levels. Differentiation was initiated on Day 0. Data are presented as mean ± s.e.m of 3 independent experiments.

Supplementary Fig. 5 Sequence comparisons of the PCR product (cDNA) with other PLFs

Total RNA was isolated from 3T3-L1 adipocytes and then RT and PCR of PLF were carried out as mentioned in SUPPLEMENTARY METHODS. The sequencing of the PCR product was performed by Dragon GenomicsCtr, Takarabio Inc. (Mie, Japan) using Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems).

Supplementary Fig. 6 Effects of PLF knockdown and treatment with an anti-PLF antibody on gene expression in 3T3-L1 adipocytes

(a–e) Effects of siRNA-mediated PLF gene silencing and anti-PLF antibody on gene expression, including PLF **(a),** PPARγ **(b,)** LPL **(c),** adiponectin **(d),** and MCP-1 **(e)**, in 3T3-L1 adipocytes. n = 5, *, $P < 0.05$ *vs* control, **, $P < 0.01$ *vs* control, #, $P < 0.05$ *vs* Goat IgG.

Supplementary Fig. 7 Expression of PLF in various organs in rat

PLF protein levels were assayed by Western blot. The images presented are representative of 4 independent experiments. BAT, brown adipose tissue; WAT, white adipose tissue.

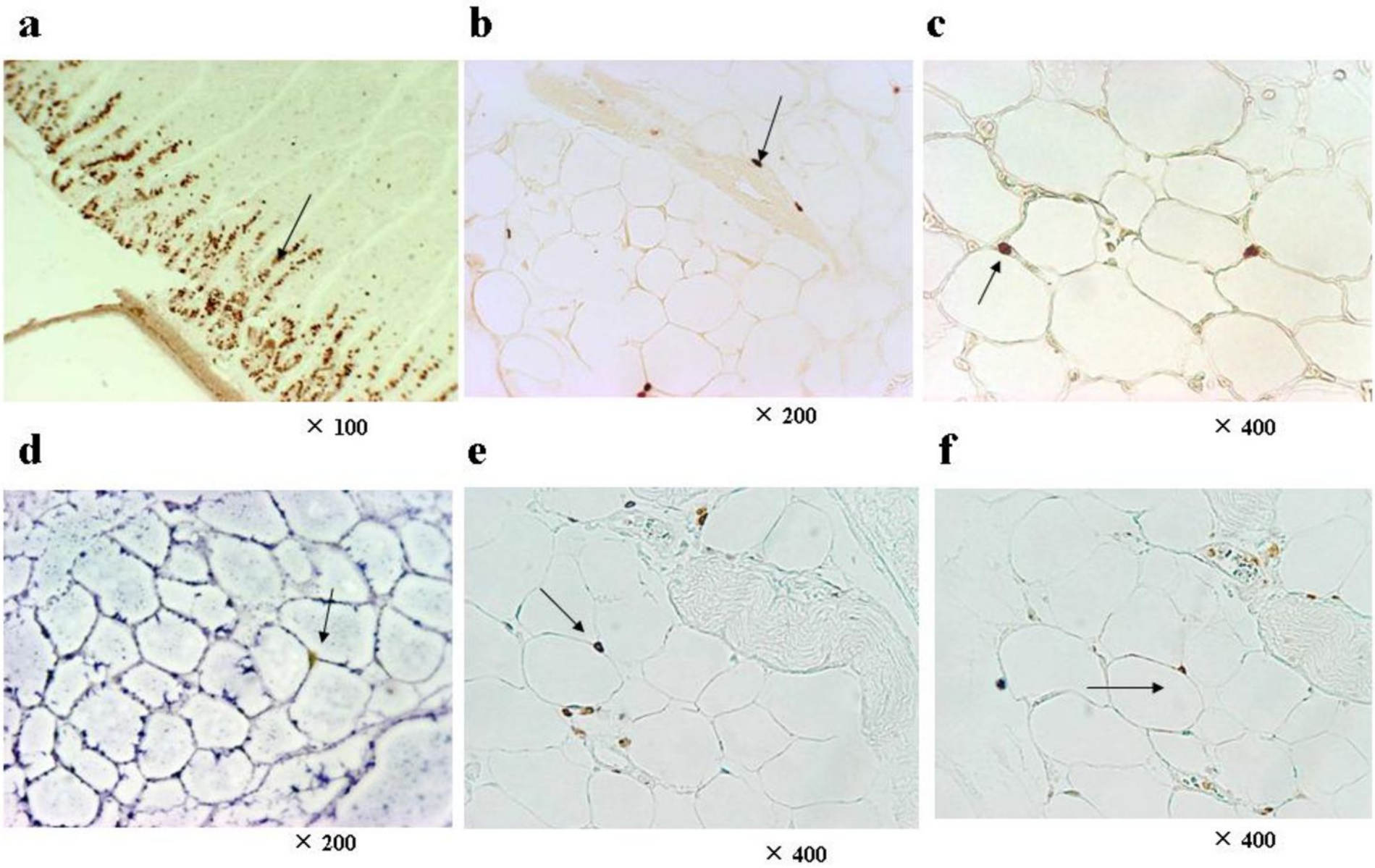


Fig. 1

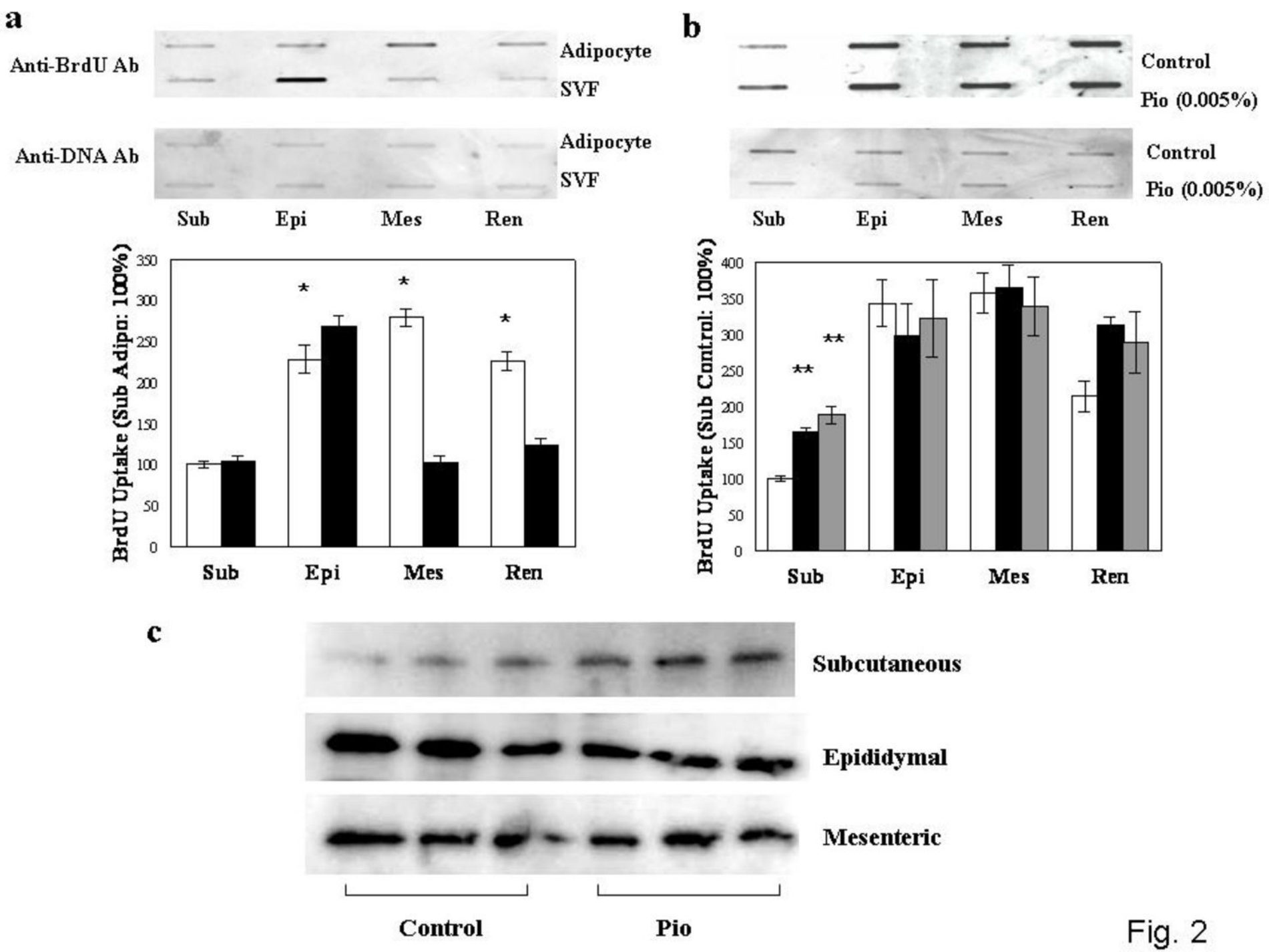
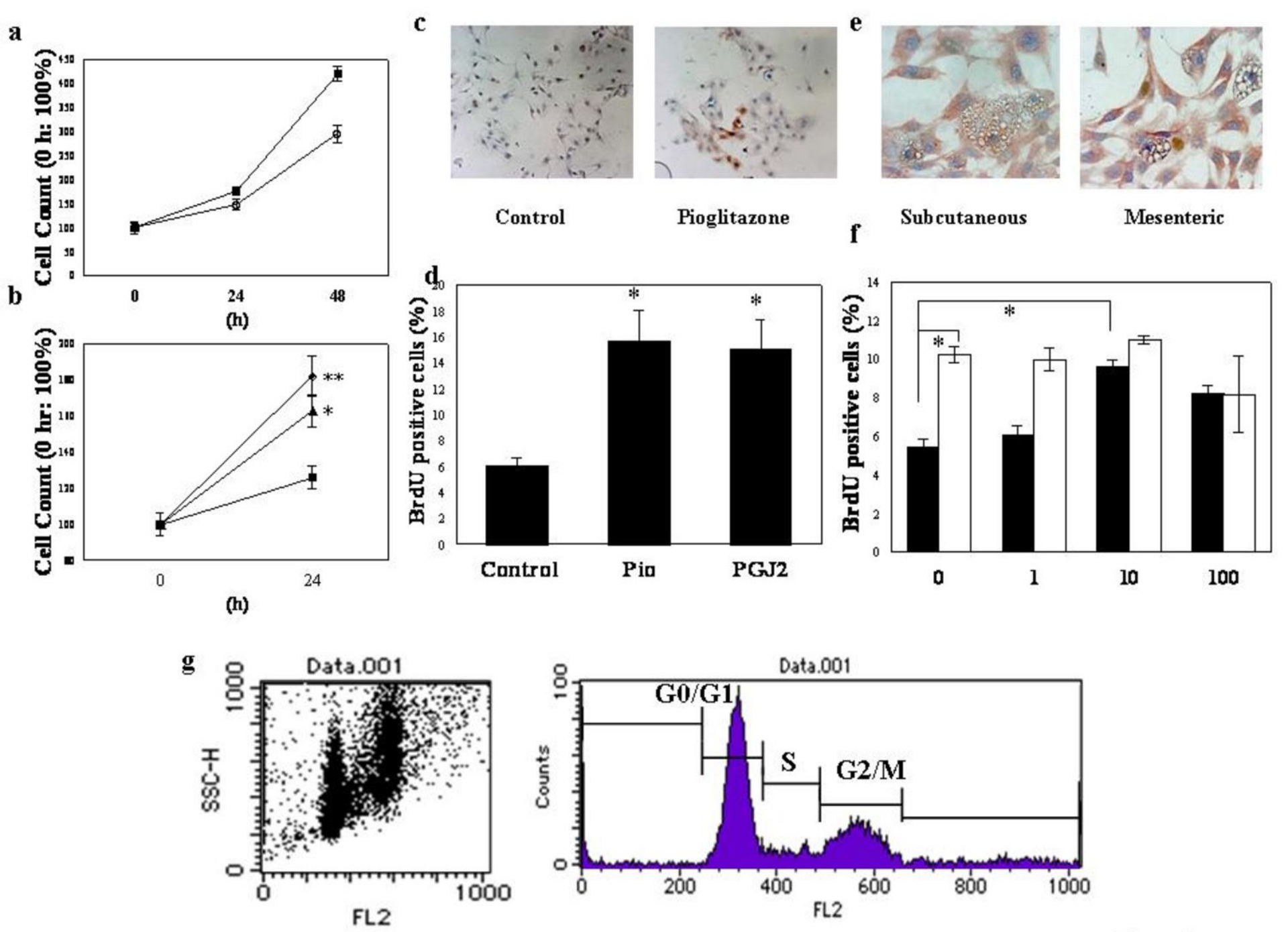

a
Anti-BrdU Ab
Adipocyte
SVF
Anti-DNA Ab
Adipocyte
SVF
Sub
Epi
Mes
Ren
b
Control
Pio (0.005%)
Control
Pio (0.005%)
Sub
Epi
Mes
Ren
BrdU Uptake (Sub Adipo: 100%)
350
300
250
200
150
100
50
0
*
*
*
Sub
Epi
Mes
Ren
BrdU Uptake (Sub Control: 100%)
400
350
300
250
200
150
100
50
0
**
**
Sub
Epi
Mes
Ren
c
Subcutaneous
Epididymal
Mesenteric
Control
Pio

Fig. 2


a
Cell Count (0 h: 100%)
0
24
48
(h)
b
Cell Count (0 hr: 100%)
**
*
0
24
(h)
c
Control
Pioglitazone
e
Subcutaneous
Mesenteric
d
BrdU positive cells (%)
*
*
Control
Pio
PGJ2
f
BrdU positive cells (%)
*
*
0
1
10
100
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Data.001
SSC-H
FL2
Data.001
G0/G1
S
G2/M
Counts
FL2

Fig. 3

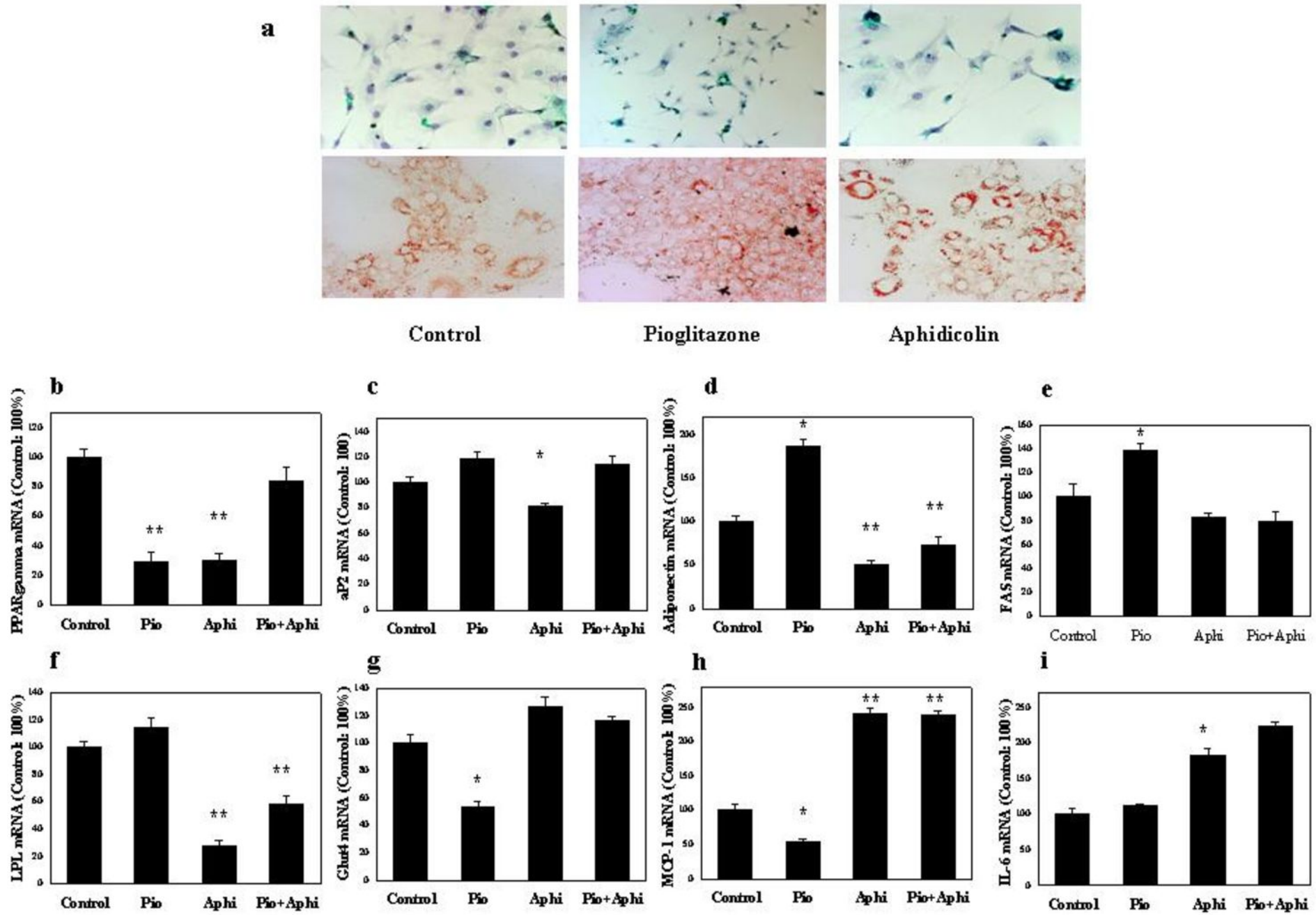

a
Control
Pioglitazone
Aphidicolin
b
PPARgamma mRNA (Control: 100%)
c
aP2 mRNA (Control: 100)
d
Adiponectin mRNA (Control: 100%)
e
FAS mRNA (Control: 100%)
f
LPL mRNA (Control: 100%)
g
Glut4 mRNA (Control: 100%)
h
MCP-1 mRNA (Control: 100%)
i
IL-6 mRNA (Control: 100%)
Control
Pio
Aphi
Pio+Aphi

Fig. 4

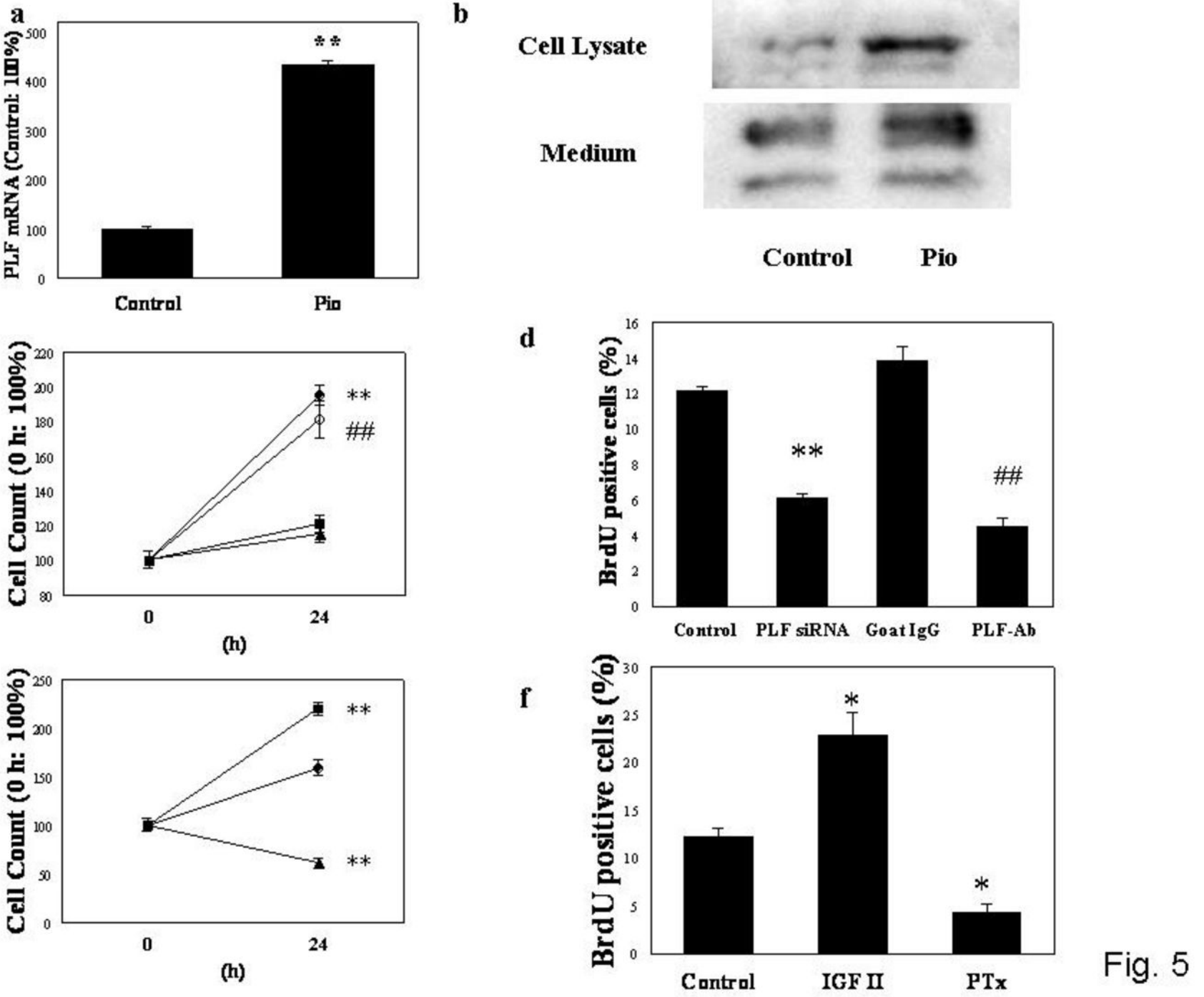
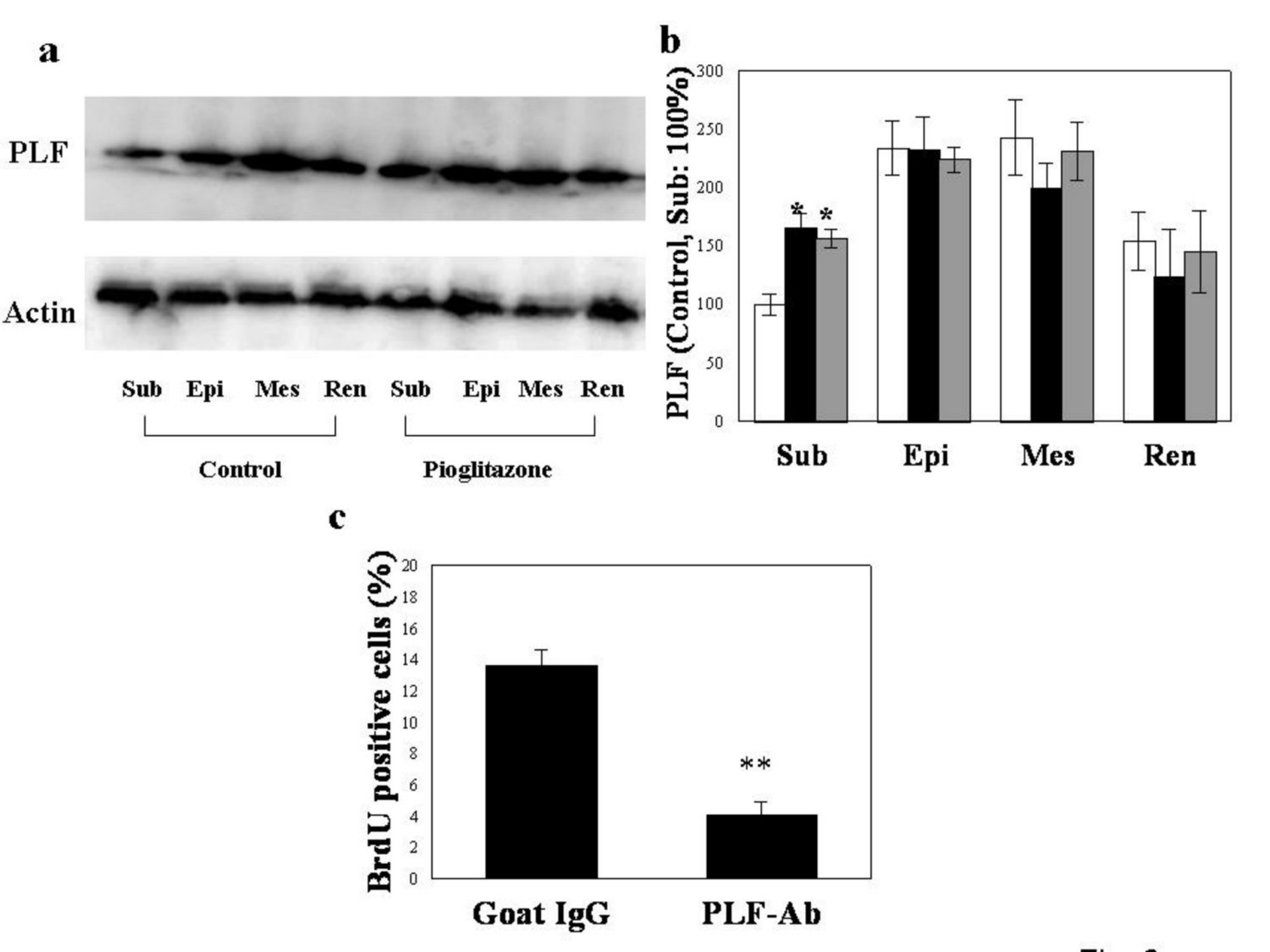

a
PLF mRNA (Control: 100%)
500
400
300
200
100
0
**
Control
Pio
b
Cell Lysate
Medium
Control
Pio
c
Cell Count (0 h: 100%)
220
200
180
160
140
120
100
80
**
##
0
24
(h)
d
BrdU positive cells (%)
16
14
12
10
8
6
4
2
0
**
##
Control
PLF siRNA
Goat IgG
PLF-Ab
e
Cell Count (0 h: 100%)
250
200
150
100
50
0
**
**
0
24
(h)
f
BrdU positive cells (%)
30
25
20
15
10
5
0
*
*
Control
IGF II
PTx

Fig. 5


a
PLF
Actin
Sub Epi Mes Ren Sub Epi Mes Ren
Control
Pioglitazone
b
PLF (Control, Sub: 100%)
300
250
200
150
100
50
0
*
*
Sub
Epi
Mes
Ren
c
BrdU positive cells (%)
20
18
16
14
12
10
8
6
4
2
0
**
Goat IgG
PLF-Ab