國立臺灣大學醫學院分子醫學研究所

碩士論文

Institute of Molecular Medicine

College of Medicine

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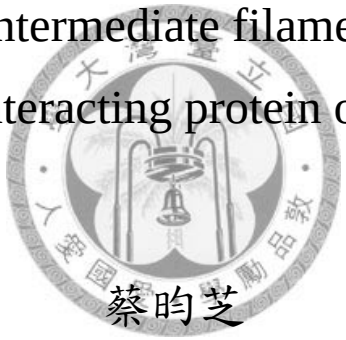
Master Thesis

中間絲蛋白 vimentin 為 PPARγ 的結合蛋白之鑑定與研究

Identification of intermediate filament protein vimentin as an interacting protein of PPARγ



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謝　誌

依稀記得當初口試時，是懷著怎樣忐忑的心情踏進偌大的會議室，也依稀記得最終接獲錄取通知時，是如何蜿蜒曲折的心情，那情那景，還彷若在眼前。

時光流轉，倏地，碩士生涯走到尾聲，即將要畢業了。

回顧這七百多個日子，我很感激也很慶幸自己進了莊醫師的實驗室，謝謝老師對我不吝惜的協助和指導，即便實驗結果不如預期，也總是以正面的態度溫言鼓勵我，從進度報告、口試到論文定稿引領我逐步完成；謝謝呂勝春老師在實驗設計上給予相當寶貴的建議和指導；也謝謝羅椀升老師在實驗及論文寫作上提供寶貴建議，使論文能更形完備。

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這一路上接受了許多幫助，謹於此致上由衷的感謝，謝謝你們，我做到了。最後，我想將這份論文獻給我的父母，感激你們無怨無悔的付出與包容。

昀芝 2008.8　于 台大醫院

摘要

過氧化體增殖劑活化受體γ (PPARgamma)是nuclear receptor家族中受配體(ligand)調控而活化的轉錄因子，它調控了參與分化、新陳代謝和免疫反應的基因表現。配體對PPARγ的結合使得轉錄輔抑制子脫離，並促使轉錄輔活化子返回與PPARγ結合進而活化基因轉錄的進行。PPARγ是信息傳遞的中樞因子，它的活性在不同的胞內運作機制下受到很完善的調控。致裂原(mitogen)的刺激會對PPARγ進行負向調控進而抑制其基因調控的活性，這樣的負調控主要是由胞外調節激酶（extracellular signal-regulated kinase ; ERK）/致裂原活化蛋白激酶(mitogen-activated protein kinase; MAPK)下游的信息梯瀑所主導。在致裂原與配體的刺激之下，MEKs和ERKs會快速轉移至細胞核中，ERKs進而在PPARγ上絲胺酸(Serine) $^{82/112}$的位置將其磷酸化，再由MEKs藉其上的NES(nuclear export signal)調控PPARγ被磷酸化後的核輸出。這樣大量的核輸出會降低PPARγ活化核目標基因的能力，進一步抑制其基因調控的功能。然而，PPARγ核輸出的實際運作機制及其送至細胞質後際而的命運仍有待後續研究。

運用 LC/MS/MS 的分析技術，我們從誘導分化的 3T3-L1 脂肪細胞萃取物中鑑定出一蛋白質 vimentin 會與 PPARγ 進行結合。隨著脂肪細胞的分化過程，vimentin 與 PPARγ 的蛋白質表現量會相對應增加。在 3T3-L1 脂肪細胞中，我們運用免疫沉澱-西方墨點法及免疫細胞化學染色分析進一步驗證了 vimentin 與 PPARγ 會在細胞質間進行結合。而在 PPARγ 的配體誘導之下，vimentin 會擇優地與磷酸化的 PPARγ 結合。

我們的實驗同時指出，在配體誘導之下 PPARγ 會被磷酸化，進而被送至細胞質與 vimentin 進行結合，這樣的一個運輸路徑是依賴 exportin-1/CRM1 來執行並受到 leptomycin B 所抑制。進一步詳細的實驗也說明了，vimentin 和 pPPARγ 的結合

除了如預期在細胞萃取物中不溶性的細胞骨架可以見到，同時也會在粒線體和內質網進行。

關鍵字：PPARγ, vimentin, 細胞核輸出, 細胞內區間, 非基因組信息傳遞

Abstract

Peroxisome proliferators-activated receptor-gamma (PPARγ) is a ligand-activated transcription factor of the nuclear receptor family that regulates genes involved in differentiation, metabolism and immunity. Upon ligand binding, PPARγ releases bound corepressors and recruits coactivators for transcriptional activation. As a central signaling component, the activity of PPARγ is well regulated under various cellular processes. Mitogenic stimulation exerts negative regulation that suppresses PPARγ's genomic activity. This downregulation is mediated largely by the extracellular signal-regulated kinase 1/2 (ERKs)/mitogen-activated protein kinases (MAPKs) signaling cascade. Upon mitogen and ligand stimulation, MEKs and ERKs rapidly translocate into the nucleus followed by ERK-mediated PPARγ phosphorylation on Serine $^{82/112}$. Upon binding onto the phosphorylated PPARγ, the NES in the MEKs then mediated the export of phosphorylated PPARγ out of the nucleus. This massive nuclear export reduces the ability of PPARγ to transactivate nuclear target genes and thereby inhibits its genomic function. However, the exact mechanism of nuclear export of PPARγ and the subsequent fate of cytoplasmic PPARγ remain further elucidated.

With advent of LC/MS/MS technique, we have identified a protein vimentin which was associated with PPARγ from the cell extracts of 3T3-L1 adipocytes upon

induction of differentiation. During adipocyte differentiation, the expression of vimentin was increased in parallel of the increases of PPARγ. We confirmed the association of vimentin and PPARγ in the cytoplasmic compartment of 3T3-L1 adipocytes with immunoprecipitation-western blot and immunocytochemistry studies. Interestingly, vimentin was preferentially associated with the phospho-PPARγ especially after treatment of PPARγ ligand.

Our data also suggest that phosphorylation of PPARγ appears after ligand treatment which leads to subsequent export of phosphorylated PPARγ to cytoplasm, at a leptomycin B-sensitive exportin-1/CRM1-dependent pathway, where interaction with vimentin occurs. Further detailed studies showed that the interaction of vimentin and pPPARγ may take place in mitochondria and ER in addition to the expected cytoskeleton in the insoluble portion of cell extracts.

Key words: PPARγ, vimentin, nuclear export, cellular compartment, nongenomic signaling

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Introduction

Peroxisome proliferator-activated receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) comprise an important subfamily of the nuclear hormone receptor (NHR) superfamily. The subfamily consists of three isoforms namely PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) that are encoded by different genes as shown in Figure I. PPARs play a central role in regulating the combustion and storage of dietary lipids, essentially by serving as sensors for fatty acids and their metabolic intermediates (Reddy & Rao, 2006). While PPARα is the master regulator of fatty acid oxidation in liver, PPARγ is expressed at a relatively high level in adipose tissue and controls energy conservation and adipogenesis (Desvergne & Wahli, 1999). PPARδ/β is expressed in most tissues and is also responsible for widespread energy burning mainly in the extrahepatic tissues (Desvergne & Wahli, 1999).

It has been shown that PPARγ participates in the regulation of a large number of cellular processes including differentiation, immune response, and metabolism (Rosen & Spiegelman, 2001). PPARγ has two isoforms, PPARγ1 and PPARγ2. The two protein isoforms are encoded by the same gene and generated by differential promoter usage and alternative splicing (Lazar, 2002; Zhu et al, 1995); both are induced during

adipogenesis. PPARγ2, harboring an additional 30 amino acids at its N-terminal end, is expressed specifically in adipocytes, whereas PPARγ1 is also relatively abundant in macrophages, colon epithelia and endothelium (Marx et al, 1999; Ricote et al, 1998). However, the relative roles of PPARγ1 and PPARγ2 in adipogenesis remain an open question.

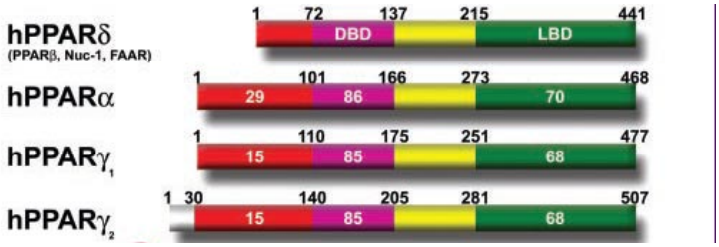


Figure I. Comparison of human PPARs.

1. Functional domain of PPARs

PPARs share a common structure of four main domains named A/B, C, D, and E/F (Desvergne & Wahli, 1999). Key functions have been assigned to each of these domains.

The N-terminal A/B domain harbors a ligand-independent transcriptional activating function (AF-1). The C domain, or DNA binding domain (DBD), is formed by two zinc finger-like motifs folded in a globular structure that can recognize a PPAR response element (PPRE) present on target genes. PPREs are specific DNA sequences formed by the repetition of a consensus hexanucleotide sequence (AGGTCA), separated

by one or two nucleotides (direct repeat 1 or 2, DR1 or DR2). Moreover, the 5'-AACT extension of this consensus sequence ensures a polarity for the heterodimer binding. The D domain is a hinge region that can modulate the DNA binding ability of the receptor and that is involved in cofactor interaction. The E/F domain, or ligand-binding domain (LBD), is multifunctional. In addition to the ligand binding, this domain exhibits a region involved in the dimerization with a partner nuclear receptor, the 9-cis retinoic acid receptor (RXR) and a strong ligand-dependent transcriptional activating function (AF-2).

Recent analysis of chimeric PPAR proteins suggests that the N-terminus of each receptor is the key determinant of isotype-selective gene expression and function, in part, to limit receptor activity (Hummasti & Tontonoz, 2006; Qi et al, 2000).

2. Activation of PPARγ

PPARγ is activated by low-affinity natural ligands, like hydroxyoctadecadienoic acid (HODE), 15-desoxy-D-12,14-prostaglandin J2 (15-dPGJ2) which was derived from long-chain fatty acids in nutrients or produced at inflammatory lesions. There are still another group of high-affinity synthetic PPARγ ligands named thiazolidinediones (TZDs), including rosiglitazone and pioglitazone, which were marketed as insulin sensitizer for treatment of type II diabetes mellitus (Knouff & Auwerx, 2004).

PPARγ forms heterodimer with a second member of the nuclear receptor family, retinoic X receptor (RXR) and bind to specific DNA sequence PPRE present in the promoter region of PPARγ target genes. In living cells, It has been experimentally demonstrated that PPARγ efficiently heterodimerizes with RXRα in a ligand-independent manner (Feige et al, 2005). Ligand binding leads to a reduction of receptor mobility in the nucleus implying that ligand-bound receptor exhibits enhanced interactions with cofactor complexes of very high molecular mass (Feige et al, 2005). In the absence of ligand, PPARγ has the potential to actively silence genes to which it is bound by the recruited transcriptional corepressor complexes containing nuclear receptor corepressor (N-CoR) or SMRT (silencing mediator of retinoid and thyroid receptors). Upon ligand binding, PPARγ undergo a conformation change that attracts transcriptional coactivators, including members of the steroid receptor coactivator (SRC) family (McKenna & O'Malley, 2002). The transcriptional coactivators and corepressors exist in multiprotein complexes including histone-modifying enzymes, such as histone acetyltransferases (notably CBP/p300) and histone deacetylases (notably HDAC3), respectively. The activity of these histone-modifying enzymes affects gene transcription by altering chromatin structure (Figure II, McKenna & O'Malley, 2002).

Posttranscriptional modification can also modulate the activity of PPARγ. It has been demonstrated that phosphorylation of serine residue 112 in the N terminus of

PPARγ2 reduces its transcriptional activity (Hu et al, 1996) and promotes sumoylation on lysine 107, which further lowers its ability to act as a transcriptional activator (Yamashita et al, 2004).

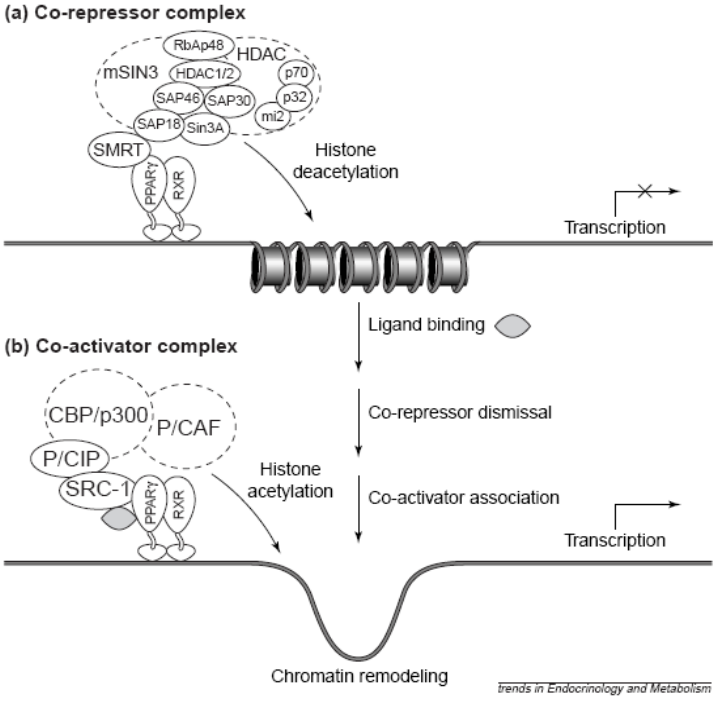


Figure II. Schematic diagram of the mechanism of action of PPARγ.

3. Selective PPAR modulator (SPPARM) model

It has been demonstrated that different ligands can directly bind to the ligand-binding domain of PPARγ. According to the selective PPAR modulator (SPPARM) model, each ligand–receptor complex assumes a different conformation that leads to unique and differential interactions with co-factors, histones, other transcription factors, etc. These distinct interactions lead to differential, but overlapping, sets of gene expression. For example, each ligand activates (or represses) a certain set of genes,

some of which are common to other ligands and some of which are not (McDonnell, 1999). The SPPARM model greatly expands the signaling repertoire of a specific nuclear receptor, PPARγ.

4. Phosphorylation of PPARγ

The activity of PPARγ is normally induced by binding of specific ligands that activate its genomic transcriptional activity and thus initiate the expression of several effector genes. In addition, as a central signaling component, the activity of PPARγ is well regulated and can be inhibited under various cellular conditions such as stimulation of cells with growth factors and protein kinase C activators. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) decrease the ligand-dependent transcriptional activity of PPARγ while increasing its phosphorylation through MAPK signaling in adipocyte cell lines (Camp & Tafuri, 1997; Hu et al, 1996).

A unique MAPK phosphorylation site, which can be used by both ERK- and JNK-MAPK (Camp et al, 1999), was mapped at serine 82 in the N-terminal domain in mouse PPARγ1, which corresponds to serine 112 of mouse PPARγ2 (Shao et al, 1998). Substitution of this serine by alanine leads to a loss of PDGF-mediated repression of PPARγ activity. Similar MAPK dependent phosphorylation and inhibition of PPARγ activity are observed in 3T3-L1 cells when treated with prostaglandin F2 alpha (PGF2α),

an arachidonic acid derivative that acts through a membrane receptor and has a potent inhibitory effect on adipogenesis (Reginato et al, 1998).

5. Nuclear-cytoplasmic shuttle of PPARγ

Although the nuclear receptor superfamily (NR) was named after its prominent nuclear localization and its genomic functions, evidence has been accumulated to demonstrate some extra-nuclear localization and non-genomic actions (Kampa & Castanas, 2006; Losel et al, 2003). Dynamic changes in subcellular localization of NRs upon stimulation with their respective ligands serve important mechanism for the NRs functions in different compartments. Most NRs contain a nuclear localization signal (NLS), interspersed in the DNA-binding (DBD) or ligand-binding (LBD) domains, which drive them to the nucleus immediately upon their translation or stimulation with ligand (Gervois et al, 1999; Hsieh et al, 1998). However, these proteins do not contain a classical leucine-rich nuclear export signal (NES) (Black et al, 2001; Saporita et al, 2003), and therefore, the molecular mechanisms that allow their cytoplasmic localization are a topic of recent investigation.

PPARγ, once known as a member of the orphan NRs, has been shown to function both in the nucleus and in the cytoplasm. However, so far neither an NES nor an NLS has been identified in PPARγ. Unlike most steroid hormone receptors, which are

complexed in the cytoplasm with chaperones such as the heat shock proteins (hsp70 or hsp90) and undergo ligand-dependent nuclear-cytoplasmic translocation, PPARγ has been previously reported (Berger et al, 2000; Gurnell et al, 2000) to reside mainly in the nucleus, similar to the vitamin D3 and thyroid hormone receptors. However, there is also evidence for a significant cytosolic localization upon ligand binding (Shibuya et al, 2002; Thuillier et al, 1998; Varley et al, 2004).

Therefore, although PPARγ functions primarily in the nucleus, it can redistribute to different subcellular compartments under distinct physiological conditions. It has been recently shown that during various cellular processes, PPARγ is exported out of the nucleus upon mitogen and ligand stimulations, and this shuttle is mediated by the NES in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2). Mitogen and ligand stimulation leads to a rapid nuclear translocation of MEKs and ERKs. ERKs phosphorylate PPARγ on Ser 82/112, thus facilitating ubiquitination and sumoylation of PPARγ that can then repress transactivation of PPREs and inhibit its genomic responses. Mitogen and ligand stimulation also leads to rapid nuclear export of PPARγ by MEK1 (Figure III). This massive nuclear export reduces the ability of PPARγ to transactivate nuclear target genes and thereby inhibits its genomic function. It further targets PPARγ to proteasomal degradation or allows PPARγ to interact with certain cytosolic and membrane proteins

(cytoskeleton, caveolae, lipid droplets, GPCRs) (Burgermeister et al, 2007). However, the cytosolic function of PPARγ and its interacting components in the cytosol still remain to be investigated.

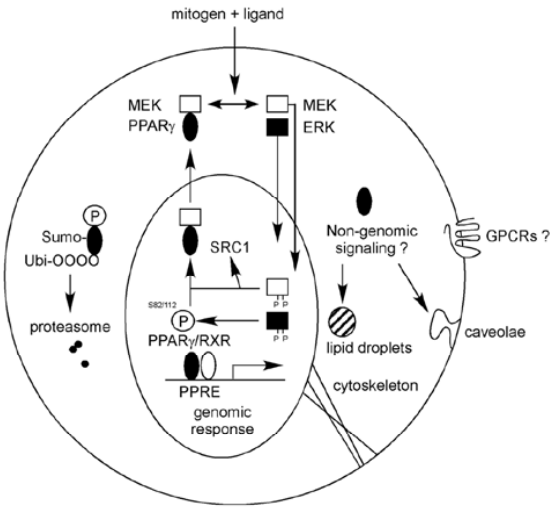


Figure III. Regulation of PPARγ by postranslational modification and compartmentalization by MEK1.

Intermediate filament: Vimentin

Among the large protein family of intermediate filaments (IFs), vimentin is one of the major members that are characteristic of many vertebrate cells of mesenchymal origin and frequently used as a developmental/differentiation marker of cells and tissues. Vimentin also shows a very high degree of sequence homology among different species, from fishes and Xenopus to humans (Herrmann et al, 1989; Schaffeld et al, 2001), suggesting some important and evolutionary conserved physiological functions of this IF protein. Furthur analyses of vimentin −/− mice have then revealed that loss of

vimentin leads to, for example, morphological changes in glia cells (Colucci-Guyon et al, 1999), impaired wound healing (Eckes et al, 2000) due to defects in the migratory capacity of fibroblasts (Eckes et al, 1998), decreased flow-induced dilation of resistance arteries reflecting a role in the mechanotransduction of shear stress (Henrion et al, 1997), disturbed homing of leukocytes to lymph nodes (Nieminen et al, 2006), and lack of integrity in vascular endothelium (Nieminen et al, 2006).

1. Vimentin and signal transductions

Recent studies have bringing insight to the roles of vimentin, as it emerges as an organizer of a number of critical proteins involved in adhesion, migration, structural support, and cell signaling. Members of 14-3-3 protein family are dimeric intracellular proteins that regulate cell cycle progression, apoptosis, and signal transduction (Bridges & Moorhead, 2005). Phosphorylation-dependent binding to 14-3-3 can obscure a specific region or induce a conformational change on a specific target protein or alter the subcellular localization of targets, thereby, modulating their functions (Bridges & Moorhead, 2005). However, it seems that vimentin interacts with 14-3-3 and instead limits its ability to interact with other target molecules such as Raf (Tzivion et al, 2000). Therefore, vimentin could participate in the modulation of signaling processes involving 14-3-3 and its interacting proteins.

It has been also suggested that vimentin would act as signaling platforms and scaffolds for docking of signaling molecules (Robidoux et al, 2006). Vimentin was recently identified as a β-adrenergic receptor (βAR)-interacting protein in modulating lipolysis in adipocytes (Kumar et al, 2007). The Erk1/2 pathway is one of the signaling pathways activated as a result of catecholamine stimulation of βARs, thereby initiating lipolysis (Kumar et al, 2007). Src kinase, directly recruited to the receptor, was demonstrated to act as a crucial mediator of the observed Erk activation (Robidoux et al, 2006). However, in the absence of a normal vimentin network, Erk activation was inhibited and the release of stored fatty acids was reduced (Kumar et al, 2007).

Vimentin was also demonstrated to enable the transport of phosphorylated and thereby activated pErk1 and pErk2 MAP kinases from the site of axonal lesion to nerve cell body, where they are needed to activate substrates, such as Elk1, critical for the repair of the lesion (Perlson et al, 2005). The activated pErks were linked to importin beta and, thereby, dynein-mediated retrograde transport via vimentin (Perlson et al, 2005).

2. The association of vimentin with cell organelles and DNA

Vimentin also involves in organelle positioning in the cell. The highly curved morphology of these organelles is maintained and stabilized by specific mechanisms,

such as the attachment to a rigid cellular structure, that is, the cytoskeleton. Vimentin has been reported to interact with several organelles such as the nucleus, Golgi apparatus, endosomes and lysosomes (Gao & Sztul, 2001; Hartig et al, 1998; Styers et al, 2004). It also has been recently reported that vimentin co-localized and interacted with mitochondria to a greater extent than other cytoskeletal components such as microtubules and actin filaments. This result suggested that vimentin could participate in the association of mitochondria with microtubules and is critical to maintain mitochondrial support (Tang et al, 2008).

On the other hand, vimentin has been demonstrated to function as a potential regulator of transcription, as it is able to interact and sequester transcriptional determinants such as p53 (Yang et al, 2005) and menin (Lopez-Egido et al, 2002). The latter of which co-operates with transcription factors AP1 and JunD. Some reports also have illustrated that the ability of vimentin to interact with various specialized DNA structures containing satellite DNA (Tolstonog et al, 2000) , telomere DNA (Tolstonog et al, 2001), retroposons (Tolstonog et al, 2001), and mitochondrial DNA (Tolstonog et al, 2001). These studies reveal a completely new aspect to the cellular functions of vimentin. Furthermore, the interaction between cytoplasmic vimentin and genomic DNA is supported by the fact that vimentin filaments actually seem to tightly associate with nuclei and mitochondria.

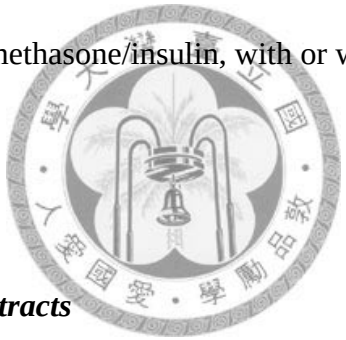
3. The reorganization of vimentin filament

Vimentin polymers are highly dynamic with an active subunit exchange between polymer and soluble subunits. Vimentin have been shown to undergo a dramatic and specific reorganization during the differentiation of preadipocytes into adipocytes (Franke et al, 1987). During this reorganization, vimentin filaments surround the nascent lipid droplets, forming a regularly spaced cage-like structure around them. Similar structures of vimentin filaments also have been reported in cholesterol-loaded macrophages (McGookey & Anderson, 1983) and the foam cells in the atherosclerotic lesions (Amanuma et al, 1986). It also has been reported that disruption of vimentin IFs during adipose differentiation of 3T3-L1 cells inhibits lipid droplet accumulation (Lieber & Evans, 1996).

Materials and Methods

Cell Culture

3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) plus 10% calf serum. Two days after confluence, differentiation was induced by the addition of DMEM containing 10% fetal bovine serum (FBS), 172 nM insulin, 1μM dexamethasone (Dex), and 0.5mM methylisobutylxanthine(Mix) for 4 days. The medium was then replaced with DMEM containing 10% FBS for full differentiation in 2–3 days. The effect of PPARγ ligand was assessed by inducing 3T3-L1 cells with either dexamethasone/insulin, with or without 0.5 μM BRL49653 or 10 μM 15-ketoPGE2.

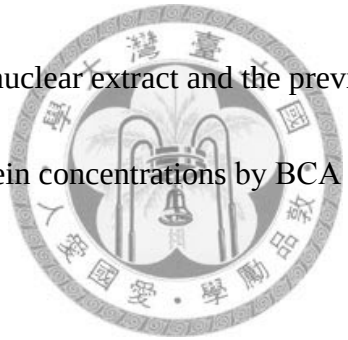


Preparation of Whole Cell Extracts

Cell monolayers were washed with phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM EGTA, 5 mM EDTA, 1% (vol/ vol) Triton X-100, 10% (vol/ vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1mM sodium pyrophos phate, 1 mM sodium orthovanadate, and proteinase inhibitor mixture (Roche, Basel, Switzerland). Samples were extracted on ice for 30 min prior to centrifugation at 12,000 rpm for 30 min at 4 °C. The resulting supernatants were analyzed for protein content by BCA analysis (Pierce) and stored at -80 °C until used.

Preparation of Nuclear/Cytosolic Extracts

Cell monolayers were washed with PBS, scraped, swelled in hypotonic cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl ,0.5% Nonidet P-40 and proteinase inhibitor mixture (Roche, Basel, Switzerland), and incubated on ice for 5 min followed by centrifugation at 12,000 rpm for 15 min to pellet the nuclei, and the supernatant was saved as cytosolic extract. The nuclear pellet was washed twice with PBS, resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) , and incubated on ice for 1 hr. After sonication, the sample was centrifuged at 12,000 rpm for 15 min at 4 °C. The resulting nuclear extract and the previously obtained cytosolic extract were analyzed for protein concentrations by BCA analysis (Pierce) and stored at -80 °C until used.



Preparation of Four Subcellular Fractions

Cell extracts were collected with Qproteome Cell Compartment kit (Qiagen). By sequential addition of different extraction buffers to a cell pellet, proteins in the different cellular compartments can be selectively isolated. 3T3-L1 cells in 6 cm dish were washed, trypsinized, and the cell suspension containing $5x10^6$ cells were transferred into a conical tube followed by centrifugation. Extraction Buffer CE1 was added to cell pellet and selectively disrupted the plasma membrane without solubilizing

it, resulting in the isolation of cytosolic proteins. The pellet from the first step was resuspended in Extraction Buffer CE2, which solubilized the plasma membrane as well as all organelle membranes except the nuclear membrane. After solubilization, the sample was centrifuged. The resulting supernatant contained membrane proteins and proteins from the lumen of organelles (e.g., the ER and mitochondria), while the pellet consisted of nuclei.

In the next step, nuclei were solubilized using Extraction Buffer CE3 in which all soluble and most membrane-bound nuclear proteins were extracted. Addition of Benzonase allowed release of the proteins which were tightly bound to nucleic acids (e.g., histones). After another centrifugation, Extraction Buffer CE4 was used to solubilize all residual, mainly cytoskeletal, proteins in the pellet. To further concentrate proteins, the extracts were then subjected to acetone precipitation to remove salts and lipid soluble contaminants. In brief, pre-cool the required volume of acetone to -20°C. Then, place protein sample in acetone-compatible tube and add four times the sample volume of cold (-20°C) acetone to the tube. Vortex tube, incubate for 30 minutes on ice and centrifuge 10 minutes at 12,000 rpm. Remove the supernatant and allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes to dry the pellet. Add Tris buffer (pH 8.0) and vortex thoroughly to dissolve protein pellet for western blot analysis or immunoprecipitation assay.

Immunoprecipitation

After 3T3-L1 cells were fully differentiated, whole cell lysates and nuclear/cytosolic/membrane/cytoskeletal extracts were collected as described above. For co-precipitation with PPARγ, 1 mg of protein was incubated with 2 μg of anti-PPARγ-agarose conjugate (Santa Cruz Biotechnology) and the samples were rotated overnight at 4 °C on a rotating platform. For immunoprecipitation with anti-vimentin and nonspecific mouse IgG, 2 μg of each antibody was first incubated with 40 μl protein G-agarose beads for 1 hr. Then, 1 mg of cellular extracts was added to the antibody-beads conjugates. After incubation for overnight at 4 °C on a rotating platform, the samples were centrifuged at 12,000 rpm for 1 minute at 4°C and supernatants were removed. The beads were washed four times in cold PBS, boiled in 2X Laemmli Sample Buffer (125 mM Tris, pH 6.8, 4% SDS, 0.02% bromophenol blue and 20% glycerol) before being resolved by SDS-PAGE, as outlined below.

Western blot

Cell extracts were resolved on 7.5, 10 or 15% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated at room temperature for 1 hr in a Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5 % skimmed milk. After being washed three times for 5 min each with TBS-T, the

membrane was incubated with primary antibodies in the TBS-T containing 3% bovine serum albumin (BSA) overnight at 4°C. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence using the ECL Detection Kit (Pierce).

Immunofluorescence microscopy

3T3-L1 cells were grown and differentiated on glass coverslips. Cells were fixed on coverslips in 4% (wt/vol) paraformaldehyde in PBS, followed by a 1-min permeabilization in 0.1% (vol/ vol) Triton X-100 in PBS at room temperature. After blocking with phosphate-buffered saline containing 10% normal goat serum, cells were incubated with antibodies directed against vimentin (Abcam Ltd.), pPPARγ (Chemicon Ltd.), and PPARγ (Santa Cruz Biotechnology, Inc.) for 1 hour at 37°C. Preparations were then incubated with a combination of tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma). Nuclei were visualized upon a 15-min incubation with 0.1 mg/ml 4,6-diamidino-2-phenylindole (DAPI). ER and mitochondria were stained with ER-Tracker™ Blue-White DPX (Invitrogen) and Mito-Tracker® Red (Invitrogen). Cells were observed, and images were acquired with an LSM510 confocal laser-scanning microscope using a Zeiss 63X oil immersion lens. At least two

independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment. For studying the effect of leptomycin B on intracellular localization of PPARγ, pPPARγ and vimentin, preadipocyte or differentiated adipocytes were pretreated with or without 20 nM leptomycin B (Sigma) for 8 h prior to the addition of BRL49653 for 1 h.

Peptide Identification by Mass Spectrometry and Bioinformatics Analysis

The gel pieces containing polypeptides of interest were first reduced and pyridylethylated as previously described (Tsay et al, 2000). Up to 0.2 μg of trypsin (Promega) was added to the dried gel to incubate overnight. The supernatant was removed and the gel was extracted with the adequate amount of 0.1% formic acid. After formic acid extraction, supernatant and extracts were combined together and dried in Speed-Vac. Electrospray mass spectrometry was performed using a Finnigan Met LCQ ion trap mass spectrometer interfaced with an ABI 140D HPLC (Perkin-Elemer). A 150 x 0.5 mm PE Brownlee C18 column (Perkin-Elemer) (5 mm particle diameter, 300 pore size) with mobile phases of A (0.1% formic acid in water) and B (0.085% formic acid in aceteonitrile) were used. The peptides were then eluted using the aceteonitrile gradient and analyzed by “triple-play” experiment as described (Tsay et al, 2000). Data interpretation and correlation between the spectra and amino acid sequences within a

human EST database was done by Finnigan Corporation software package, the SEQUEST Browser.

Results

Identification of PPARγ interacting proteins

Our previous study has demonstrated that the prostaglandin (PG) metabolite 15-keto-PGE2 is a newly identified ligand of PPARγ. Binding of 15-keto-PGE2 to PPARγ can increase coactivator recruitment, thus activating PPARγ-mediated transcription and enhancing adipogenesis of 3T3-L1 cells (Chou et al, 2007). To further investigate whether these two distinct ligand–receptor complexes exhibit differential interactions with any co-factors, histones, or other transcription factors, we treated 3T3-L1 cells with 15-keto-PGE2 and the canonical PPARγ ligand BRL. It has been reported that treating 3T3-L1 preadipocytes with insulin (I) and dexamethasone (Dex, D) is unable to induce adipocyte differentiation unless methylisobutylxanthine (Mix, X) is added together to stimulate the generation of endogenous PPARγ ligands via cAMP signaling pathways. This DI system allows us to evaluate the effect of supplementation of potential PPARγ ligands on promoting adipogenesis in the absence of Mix.

Cell lysates were then collected from the 3T3-L1 cells treated with DI only, DI and 15-keto PGE2 or DI and BRL to evaluate different ligand-induced effect. To identify the interacting proteins with PPARγ, we performed immunoprecipitation using anti-PPARγ antibody, followed by SDS-PAGE and the gel was subsequently stained with Coomassie blue. As shown in Figure 1, there was a 57-kd band which showed

differential activity in interaction with PPARγ under BRL treatment as compared to 15-keto-PGE2 treatment.

To characterize the nature and function of the interacting protein of PPARγ, we then isolated two protein bands, as indicated as number 1and 2, for LC/MS/MS analysis. The results revealed that these protein bands contain several cytoskeleton proteins (Table 1). Among them, the intermediate filament vimentin is the major cytoskeleton protein. We then focus to investigate its probable underlying role in regulating the metabolism of PPARγ in adipocyte.

Expression and interaction of vimentin with PPARγ during 3T3-L1 adipocyte differentiation

The expression level of vimentin was increased during the differentiation of 3T3-L1 cells at protein level (Figure 2), showing similar expression pattern to PPARγ and a typical PPARγ target gene aP2 (Figure 2). By use of immunoprecipitations with reciprocal antibodies, we confirmed the interaction between PPARγ and vimentin upon ligand treatment (Figure 3A, B). Notably, vimentin seemed to preferentially interact with PPARγ2 isoform (Figure 3B).

Subcellular compartment of interactions for vimentin and PPARγ during 3T3-L1 adipocyte differentiation

Vimentin is exclusively localized in the cytoplasm; however, it is well-studied that PPARγ is mainly localized in the nucleus (Berger et al, 2000; Gurnell et al, 2000). Intriguingly, how do these two proteins interact with each other? Previously, it has been shown that, under certain stimulations, downregulation of the PPARγ's genomic activity occurs via MEK/ ERK signaling cascade, which attenuates PPARγ's transactivation function either by an inhibitory phosphorylation of PPARγ (pPPARγ) or by modulating PPARγ's nuclear-cytoplasmic compartmentalization (Burgermeister et al, 2007). Therefore, we fractionated total cellular extracts into cytosolic and nuclear extracts to further analyze whether the interactions occurs in specific cellular compartments. As shown in Figure 4, the expression level of PPARγ and pPPARγ in different cellular compartments is very different during adipocyte differentiation. The cytosolic level of pPPARγ is quite stable at different time point of adipocyte differentiation, but not that of PPARγ (Figure 4A), and is slightly increased upon BRL treatment (Figure 4A, lane 2,4,6). On the contrary, the nuclear level of PPARγ could only be detected upon ligand treatment (Figure 4B, lane 2,4,6) , whereas the nuclear level of pPPARγ was hardly detected in the early stage of differentiation (Day 2 & 4; Figure 4B, lane 2 and 4) but was highly expressed in the terminally differentiated cells (Day 6; Figure 4B, lane 6).

There were low level of PPARγ detected in the nuclear extracts in early stage of differentiation (Day 2 and 4; Figure 4B, lane 1 and 3) when the cells was not treated with BRL49653.

To further study the regulation of phosphorylation and localization of PPARγ upon PPARγ agonist, we studied the effect of BRL49653 treatment for 1 hr in the adipocytes induced with regular DIM treatment. As shown in Figure 5, the expression level of both PPARγ1 and PPARγ2 isoforms were induced during differentiation (Figure 5A), and the localization of the PPARγ and pPPARγ was mostly in the nucleus, although pPPARγ protein could be readily detected in the cytosol (Figure 5A). The distribution of vimentin is more complex as it was more preferentially purified in the nuclear extracts at day 1 after induction of differentiation, but return to basal levels after day 3 (Fig. 5A). The presence of vimentin in the nuclear extracts are possibly due to incomplete separation of the cytoplasm and nucleus because the presence of vimentin in the perinuclear zone evidenced by immunocytochemistry (Fig. 5E). To illustrate compartments for the interactions of vimentin and PPARγ, we further performed immunoprecipition assays in nuclear and cytosolic extracts. As shown in Figure 5B, the interaction between vimentin and pPPARγ was clearly detected in the cytosol (Figure 5B, upper panel). However, these interactions were also observed in the nuclear extracts (Figure 5C, D, lower panel), probably due to incomplete separation of the cytoplasm

and nucleus (Fig. 5E).

To confirm the interaction between vimentin and PPARγ in selective subcellular compartment, we performed immunocytochemistry studies in pre-adipocytes and differentiated 3T3-L1 adipocytes to demonstrate if vimentin and PPARγ/pPPARγ colocalized in specific cellular compartments. In the pre-adipocytes, vimentin was localized in the cytoplasm (Figure 6A, E), whereas PPARγ (Figure 6B) and pPPARγ (Figure 6F) were localized exclusively in the nucleus. There is no colocalization of vimentin and PPARγ/pPPARγ in the 3T3-L1 pre-adipocytes, indicating there is no interaction between these molecules (Figure 6C and 6G). Following differentiation, we found that both vimentin and PPARγ/pPPARγ were gradually increased in the well-differentiated 3T3-L1 adipocytes. Notably, PPARγ was almost entirely localized in the nucleus (Figure 7B, F, J) whereas pPPARγ was found in both nucleus and cytosol. The cytosolic pPPARγ was only noted after ligand treatment in a time-dependent manner (Figure 7N, R, V). Interestingly, there was no obvious colocalization between vimentin and PPARγ (Figure 7G, K), however, pPPARγ showed clear colocalization with vimentin in the cytoplasm, esp. after PPARγ ligand treatment (Figure 7R, V), indicating the interaction of vimentin and pPPARγ occurs in cytoplasmic compartment following activation with PPARγ agonist.

Identification of nuclear-cytoplasmic translocation of pPPARγ upon ligand treatment

It has been reported that the export of some nuclear proteins is sensitive to the treatment of leptomycin B, an inhibitor that blocks the NES-receptor protein exportin-1/CRM-1 at the nuclear pore. Therefore, we treated cells with leptomycin B for indicated time to study whether the translocation of PPARγ/pPPARγ is dependent on this shuttle or not. As shown in figure 8, PPARγ was stably localized in the nucleus after PPARγ ligand treatment irrespective of presence of leptomycin B or vehicle (Figure 8B, F). By contrast, the cytoplasmic distribution of pPPARγ upon ligand treatment was remarkably inhibited by leptomycin B treatment (Figure 8J, 8N). The colocalization between vimentin and pPPARγ decreased dramatically when the nuclear export was blocked with leptomycin B (Figure 8O) compared to vehicle control (Figure 8K). Sqaured areas are enlarged as shown in Figure 8I', K', M', O', respectively.

Interaction of vimentin and PPARγ/pPPARγ in cytoplasmic compartments

To further demonstrate the compartments for the interactions of vimentin and pPPARγ, we fractionated cellluar extracts into four different fractions, i.e. cytosol, membranes, nucleus, and cytoskeleton portions under canonical DIM treatment. Immunoblots were performed using antibody against pPPARγ, vimetin, and proteins specific to each fraction as shown in Figure 9. Vimentin was preferentially present in

the cytoskeleton fraction (Figure 9A, lane 10, 11, 12), esp. at the day 0 and 7. The protein level of vimentin was decreased at day3 (Figure 9A, lane 11), but it could then be detected in the cytosol and nuclear fractions (Figure 9A, lane 2 and 8), suggesting that the intermediate filament vimentin may undergo marked reorganization during adipocyte differentiation. It is notable that both pPPARγ and vimentin could be also detected in the membrane fractions under long exposure (Figure 9A, lane 5 and 6). To establish the compartments where vimentin interacts with pPPARγ, we then performed immunoprecipitation assays in the four subcellular fractions of cellular extracts. The interaction of these two proteins was clearly detected in the cytoskeletal fraction as well as in the membrane fraction (Figure 9B).

The membrane fraction contains membrane and lumen proteins of the organelles, including endoplasmic reticulum (ER), mitochondria, and Golgi apparatus. To further distinguish where vimentin-pPPARγ interacting complex associates with, immunocytochemisrty studies were performed using makers of ER, mitochondria, and, Golgi, as shown in Figure10-12. PPARγ did not colocalize with vimentin (Figure 10C, D) as shown before, and also not colocalize with markers for ER (Figure 10F), mitochondrial (Figure 11G, H), or Golgi (Figure 12 G, H). However, vimentin and pPPARγ showed clear colocalization with markers for ER (Figure 10M, N, O, P) and mitochondria (Figure 11C, D, K, L), but not Golgi (Figure 12 C, D, K, L).

Discussion

Our present study shows for the first time that an interaction between vimentin and phospho-PPARγ occurs during differentiation of 3T3-L1 adipocytes. Our data also suggest that phosphorylation of PPARγ appears after ligand treatment which leads to subsequent export of pPPARγ to cytoplasm. Based on our immunocytochemistry data, the vimentin-pPPARγ interacting complexes were colocalized to certain organelles, such as mitochondria and ER, in addition to the expected site of cytoskeleton where vimentin locates.

Modulation of PPARγ activity via phosphorylation

As a central transcriptional regulator of metabolism, the activity of PPARγ is highly regulated by various mechanisms (Rochette-Egly, 2003). Mitogenic hormones, growth factors, stress and pro-inflammatory signals are all known to reduce the ability of PPARγ to respond to ligand stimulation. The mechanisms underlying this downregulation are complex and comprise a set of post-translational modifications including phosphorylation (Diradourian et al, 2005; Rochette-Egly, 2003), ubiquitination (Genini & Catapano, 2006), sumoylation (Yamashita et al, 2004), and cytoplasmic shuttling (Burgermeister et al, 2007). These regulatory modifications are in concordance

with the anti-proliferative and anti-inflammatory role of PPARγ.

Various MAPKs participate in a key downregulating machinery via serine/threonine phosphorylation of the substrates. It was shown that PPARγ can be phosphorylated by ERKs, JNKs and p38, resulting in inhibition of transactivating activity of PPARγ (Diradourian et al, 2005). Phosphorylation is directly against Ser^{82}/Ser^{112} ($Ser^{82/112}$) within a MAPK consensus motif (PXSPP) located in the AF1 domain of PPARγ1/PPARγ2 (Adams et al, 1997; Camp & Tafuri, 1997), and decreases basal and ligand-dependent transactivation through PPARγ.

Several factors inhibit PPARγ-mediated regulations by the induced phosphorylation. Thus the phosphorylation of PPARγ upon epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) treatment further inhibits adipogenic differentiation of murine fibroblasts (NIH-3T3) and preadipocytes (3T3-L1) (Adams et al, 1997; Aouadi et al, 2006; Camp & Tafuri, 1997; Hu et al, 1996). Moreover, It was proposed that upon $Ser^{82/112}$ phosphorylation evoked by IFNγ, PPARγ is subjected to the subsequent poly-ubiquitination and proteasomal degradation in adipocytes (Floyd & Stephens, 2002).

On the other hand, it was also demonstrated that the transcriptional activity of PPARγ and adipogenesis were increased upon $Ser^{82/112}$ phosphorylation mediated by

cyclin-dependent kinase 9 (CDK9) (Iankova et al, 2006) and CDK7 (Compe et al, 2005). Thus, phosphorylation of PPARγ at the same residue by different kinases may result in either activation or repression of its activity. The exact explanation of this discrepancy is still lacking.

The nuclear-cytoplasmic shuttle of PPARγ

According to a previously mentioned model (Burgermeister & Seger, 2007), upon mitogenic or PPARγ ligand stimulation, cytoplasmic MEKs and ERKs are released from scaffold proteins of the MAPK-module to rapidly translocate to the nucleus while ERKs phosphorylate PPARγ at $Ser^{82/112}$ leading to subsequent MEK1-dependent nuclear export of PPARγ which was demonstrated in an exportin-1/CRM1-dependent manner in our study (Burgermeister & Seger, 2007). This massive nuclear export then further reduces the genomic activity of PPARγ through its removal from target gene promoters involved in cell cycle control, differentiation or apoptosis. The cytoplasmic redistribution may facilitate the cytoplasmic effects of PPARγ including its association with caveolae and other cytoplasmic proteins. It was shown that PPARγ can also associate with caveolin-1 and this association upregulates the expression of caveolin-1 in human MCF-7 breast and HT-29 colon adenocarcinoma and leukemia cells

(Burgermeister et al, 2003; Chintharlapalli et al, 2004; Llaverias et al, 2004). However, no other interacting protein in the cytosol has been identified so far.

Our current study showed that upon ligand stimulation, PPARγ was subjected to serine phosphorylation and was subsequently transported to the cytoplasm. Upon exporting out of the nucleus, pPPARγ subsequently interacted with a cytoskeleton protein, vimentin. This is the first evidence linking the nuclear-cytoplasmic shuttling of PPARγ to a certain interacting partner in the cytoplasm. This mechanism was confirmed by our immunostaining data which was demonstrated that the level of cytoplasmic pPPARγ and also the colocalization with vimentin in the cytoplasm were decreased upon leptomycin B treatment, suggesting a exportin-1/CRM-1-dependent nuclear export was involved. Further detailed studies, we demonstrated that the interaction of vimentin and pPPARγ may take place in mitochondria and ER in addition to the expected cytoskeleton in the insoluble portion of cell extracts.

According to the newly established model, a question is raised here, what is the ultimate fate of PPARγ upon transporting to the cytoplasm where interaction with vimentin occurs in the ER and mitochondria? To our knowledge, there are several possibilities, e.g. PPARγ may be only sequestered by vimentin in the cytoplasm and would be further transported back to the nucleus or targeted to proteasomal degradation

under certain cellular circumstances. Thus, the phenomenon observed in this study might be just a temporal change of subcellular compartmentalization of PPARγ in order to modulate its activity. Nevertheless, this confinement of PPARγ to different subcellular compartments may probably provide a direct spatial and temporal control mechanism that separates nuclear from cytoplasmic responses and allows nongenomic function of PPARγ to take place. Further investigations will be required to elucidate the exact cytoplasmic function of PPARγ.

Vimentin was previously regarded as an exclusively intracellular cytoplasmic protein. However, in a recently published paper, vimentin was shown to be phosphorylated by protein kinase C (PKC) and secreted by activated human macrophages in a process that is regulated by pro- and anti-inflammatory cytokines (Mor-Vaknin et al, 2003). Regarding to the fact that there are many similarities between adipocyte and macrophage, the biological function of vimentin (together with its interacting partner PPARγ) in adipocyte remains to be further explored.

It was previously mentioned that vimentin can undergo a dramatic and specific reorganization during the differentiation of preadipocytes into adipocytes (Franke et al, 1987). Regarding to the various size of lipid droplets in certain metabolic active or inactive adipocytes, vimentin may play an essential role on regulating lipid droplet

formation. However, the underlying mechanism still remains to be determined.

To further illustrate the interaction of vimentin and PPARγ, we will construct GST-fusion proteins to determine which functional domain of PPARγ is responsible for its binding to vimentin. We will also perform receptor activity assays to demonstrate whether the activity of PPARγ is altered upon silencing of vimentin.

Due to the differential ligand-induced effects mediated by 15-keto PGE2 and BRL49653 treatment as shown in Figure 1, we will further continue our investigations on the differential physiological regulation induced by these two ligands in this newly established vimentin-PPARγ interacting model.

In summary, this study demonstrates that a cytoskeleton protein, vimentin, direct binds to pPPARγ in the cytoplasm and thus implicated that vimentin may involve in the cytoplasmic regulation of PPARγ. Further studies should provide greater insight into the exact interacting mechanism underlying these two proteins and, most importantly, the cytoplasmic function of PPARγ.

Figures

Figure 1.

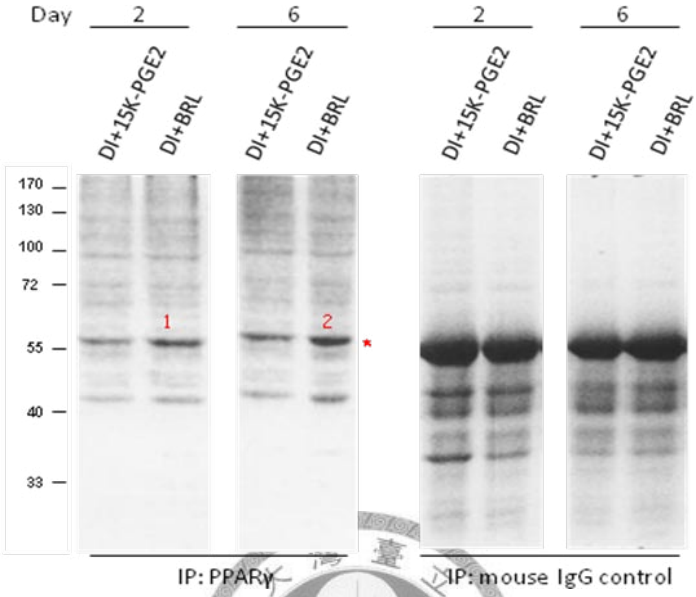


Figure 1. Identification of proteins showing differential activity in interaction with PPARγ under different ligand treatment.

To evaluate different ligand-induced effect, total cell lysates were collected from the 3T3-L1 cells under DI and 15-keto PGE2 or DI and BRL treatment at day2 and day6 during differentiation. Immunoprecipitation assay was performed using anti-PPARγ antibody (left panel) or anti-mouse IgG (right panel), followed by SDS-PAGE and the gel was subsequently stained with Coomassie blue. Distinct protein bands, as indicated as number 1 and 2 , were subjected to LC/MS/MS analysis. Days of differentiation indicate early differentiation (day2), and well-differentiated (day 6) cells.

Figure 2.

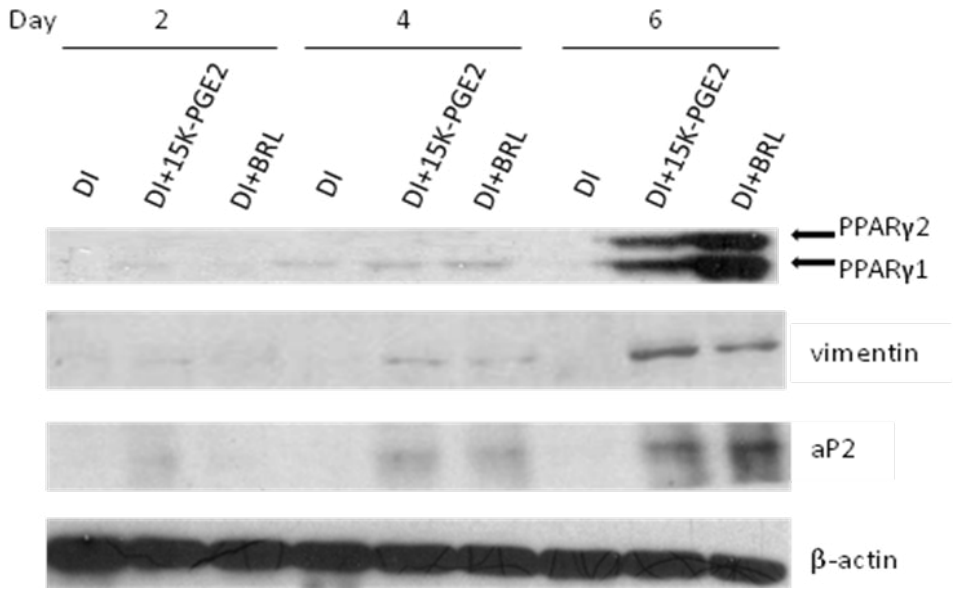


Figure 2. The expression level of vimentin during 3T3-L1 adipocyte differentiation at protein level.

Total cell lysates were collected from the 3T3-L1 cells under DI only, DI and 15-keto PGE2 or DI and BRL treatment at day 2, day 4, and day 6 during differentiation and were subjected to Western blot analyses with anti-PPARγ, anti-vimentin, and anti-aP2 antibodies, respectively.

Figure 3.

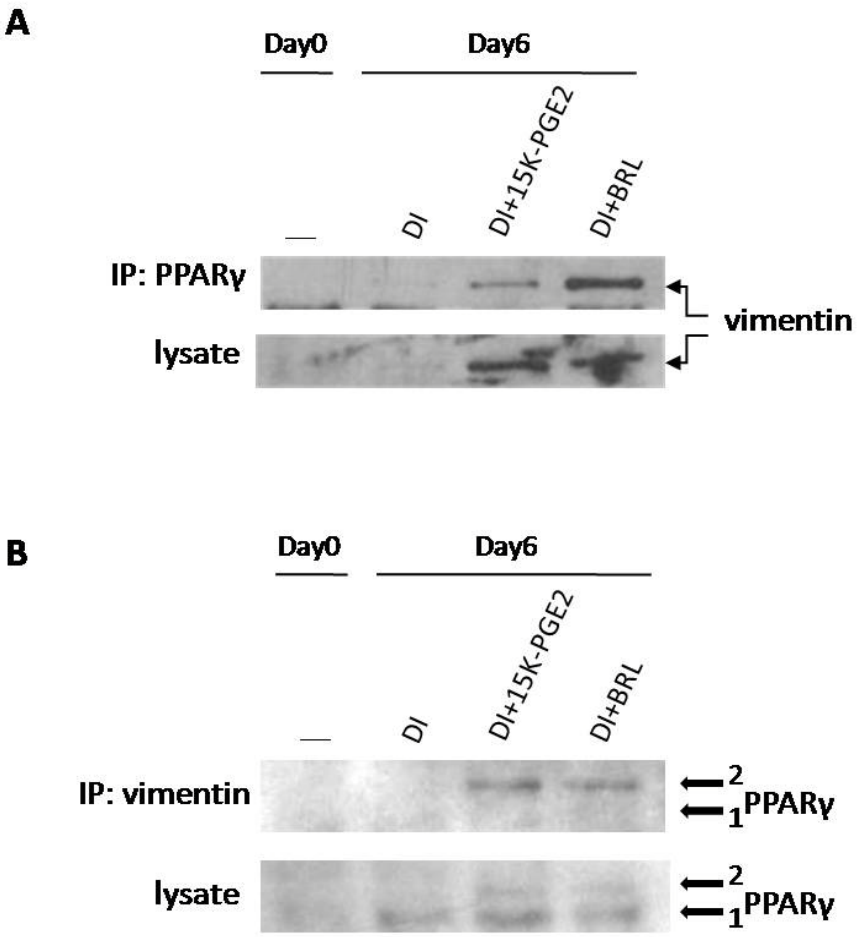


Figure 3. Interaction of vimentin with PPARγ during 3T3-L1 adipocyte differentiation.

Total lysates were collected under different treatments as described in figure 2 at day0 and day6 during differentiation. Day0 was served as control. For reciprocal immunoprecipitation assays, lysates were incubated with either PPARγ-agarose conjugate (Santa Cruz Biotechnology) or pre-incubated vimentin-protein agarose G beads complex. Samples were then resolved by SDS-PAGE.

Figure 4.

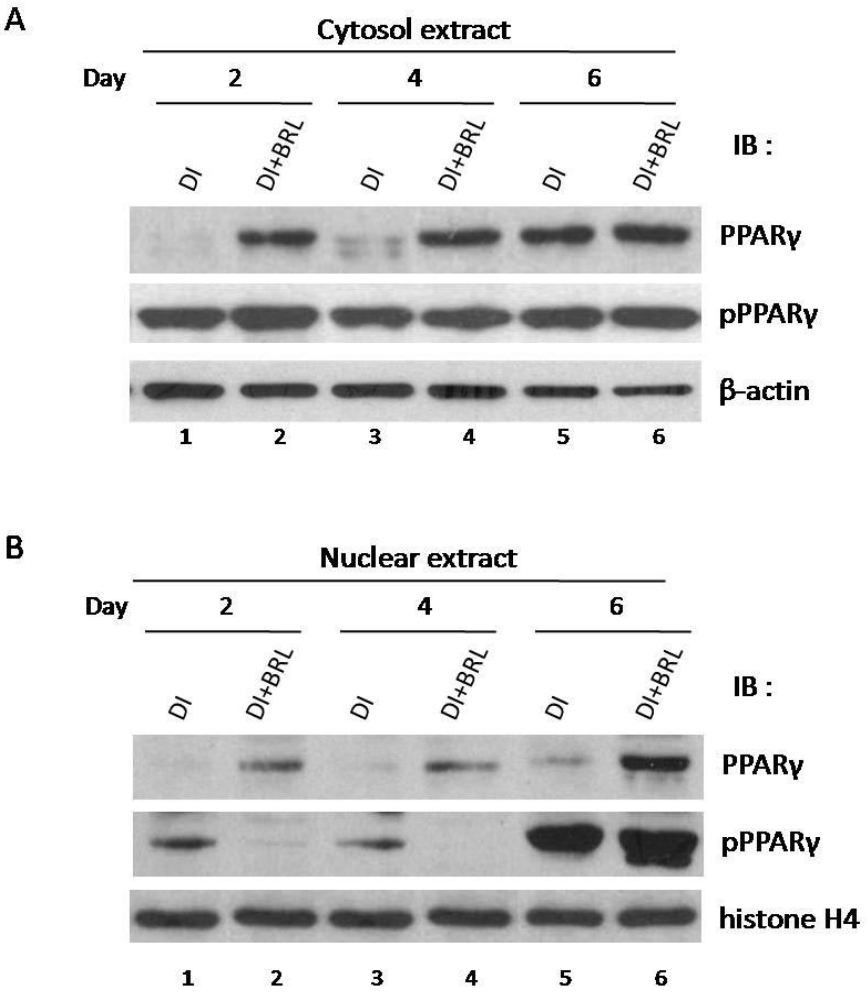


Figure 4. The expression level of PPARγ and phospho-PPARγ in different cellular compartments.

Total cellular extracts were fractionated into cytosolic (A) and nuclear extracts (B) from 3T3-L1 cells at indicated times during differentiation to determine the expression level of indicated proteins using antibody against PPARγ and phospho- PPARγ (pPPARγ), respectively.

Figure 5.

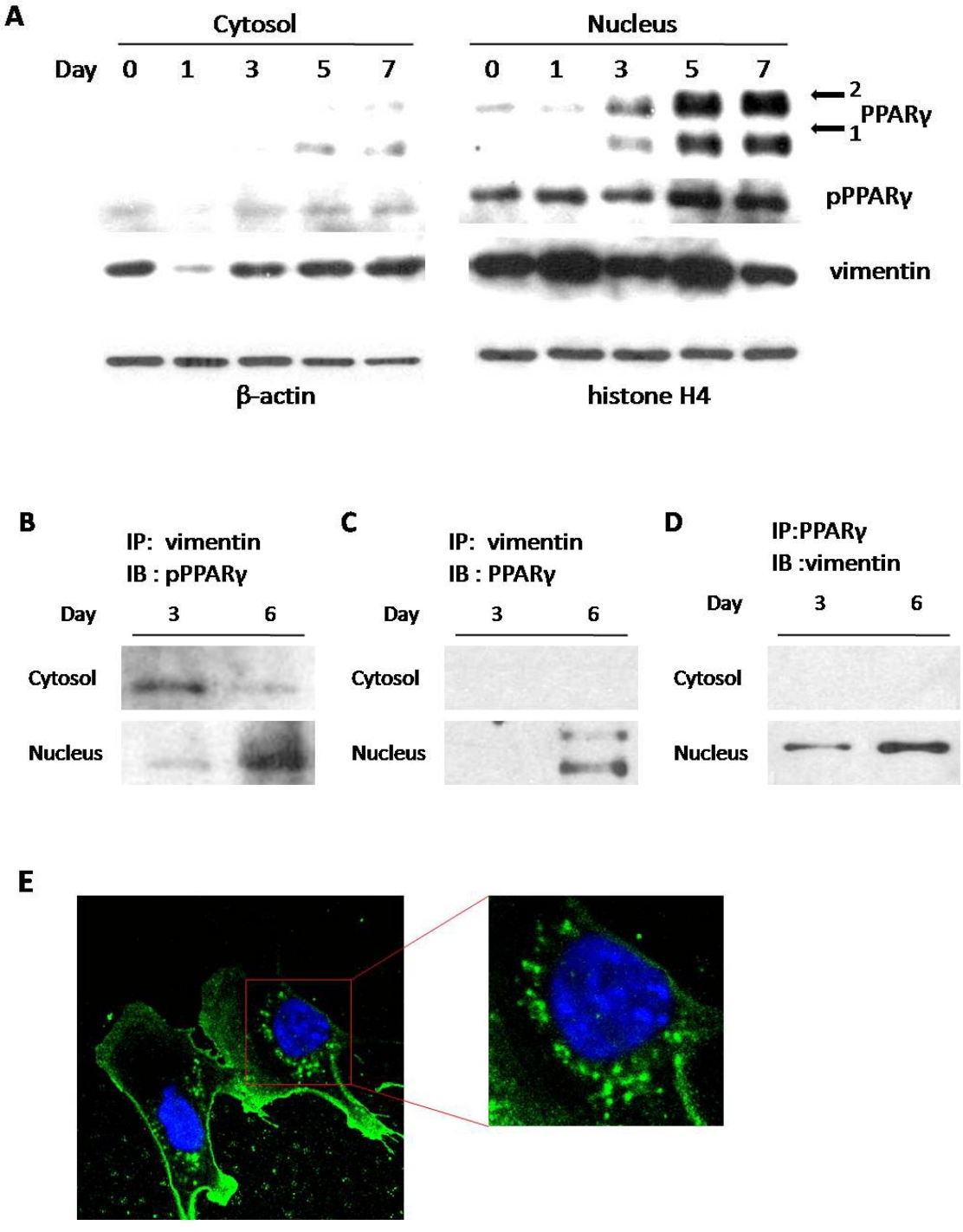

A
Cytosol
Nucleus
Day 0 1 3 5 7
0 1 3 5 7
2
PPARγ
1
pPPARγ
vimentin
β-actin
histone H4
B
IP: vimentin
IB : pPPARγ
Day 3 6
Cytosol
Nucleus
C
IP: vimentin
IB : PPARγ
Day 3 6
Cytosol
Nucleus
D
IP:PPARγ
IB :vimentin
Day 3 6
Cytosol
Nucleus
E

Figure 5. The distribution of vimentin in different cellular compartments.

A,B,C, 3T3-L1 cells induced under regular DIM treatment were treated with BRL49653 for 1 hr and the lysates were fractionated into cytosolic and nuclear extracts from the cells at indicated times during differentiation. Samples were then subjected to Western blot analyses with anti-PPARγ (A), anti-pPPARγ (B), and anti-vimentin (C) antibodies. D,E,F, The cell lysates were immunoprecipitated and blot with indicated antibodies. G, Confocal microscopy image of preadipocyte, with outlined squared areas enlarged at 5x magnification (right panel).

Figure 6.

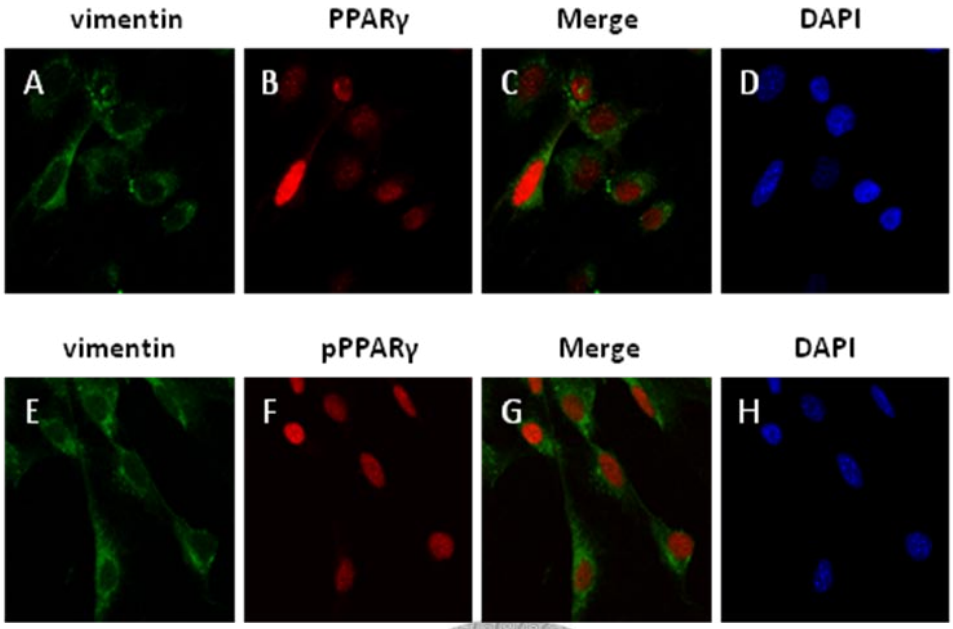


Figure 6. The interaction between vimentin and PPARγ/p-PARγ in selective subcellular compartment in preadipocytes.

Preadipocytes were grown on coverslips at day0 (confluence) during differentiation and were incubated with antibodies against vimentin, PPARγ, and pPPARγ, respectively. Nuclei were visualized upon DAPI staining (blue). Images were acquired with an LSM510 confocal laser-scanning microscope using a Zeiss 63X oil immersion lens. Three independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 90% of the cells had similar staining patterns.

Figure 7.

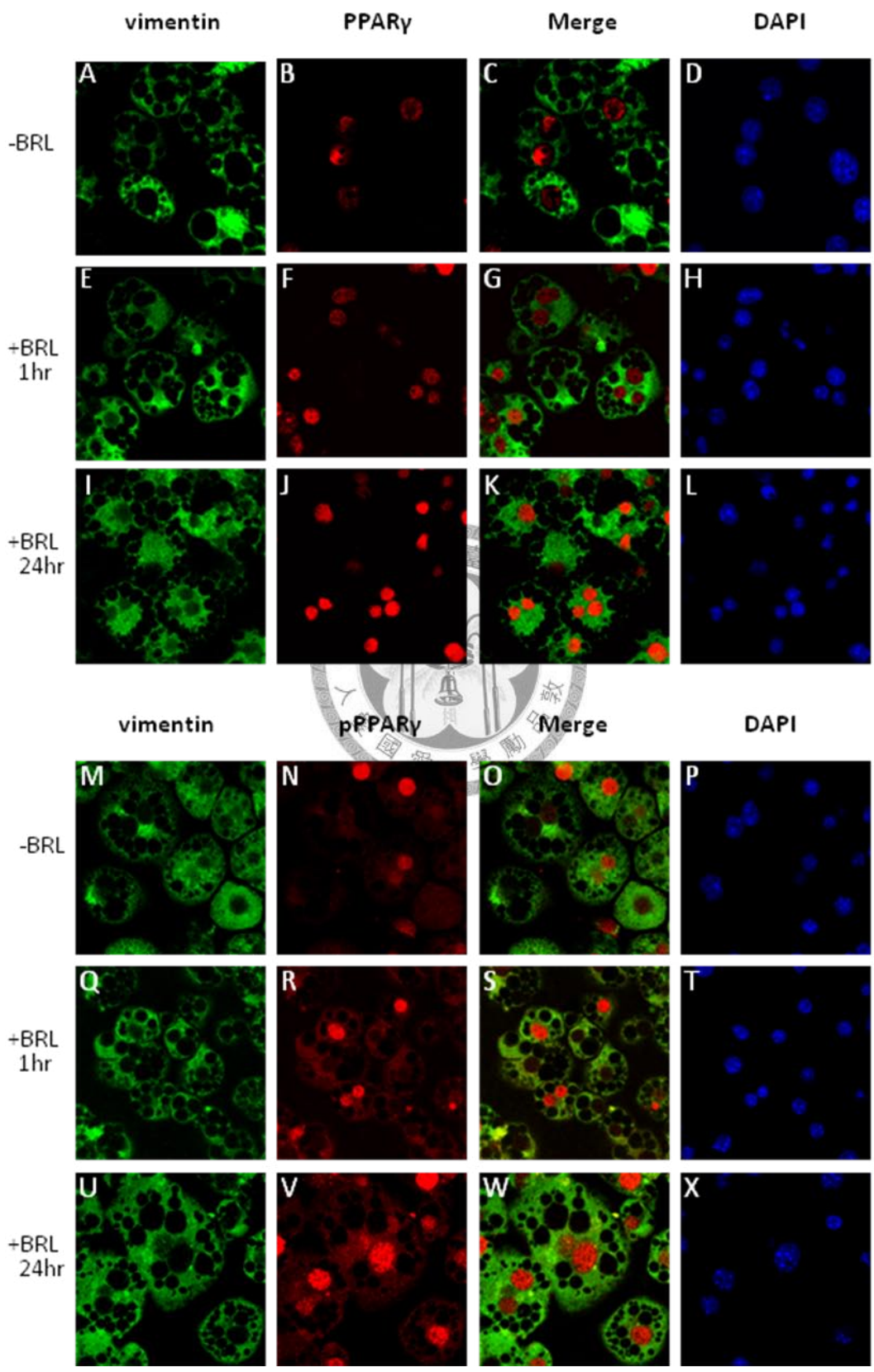

vimentin
PPARγ
Merge
DAPI
A
B
C
D
-BRL
E
F
G
H
+BRL
1hr
I
J
K
L
+BRL
24hr
vimentin
pPPARγ
Merge
DAPI
M
N
O
P
-BRL
Q
R
S
T
+BRL
1hr
U
V
W
X
+BRL
24hr

Figure 7. The colocalization of vimentin and pPPARγ in differentiated 3T3-L1 adipocytes under BRL49653 treatment.

Differentiated 3T3-L1 adipocytes (day 7) treated with BRL or not for indicated times were incubated with antibodies against vimentin, PPARγ, and pPPARγ, respectively. Nuclei were visualized by DAPI staining (blue). Five independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 80% of the cells had similar staining patterns.

Figure 8.

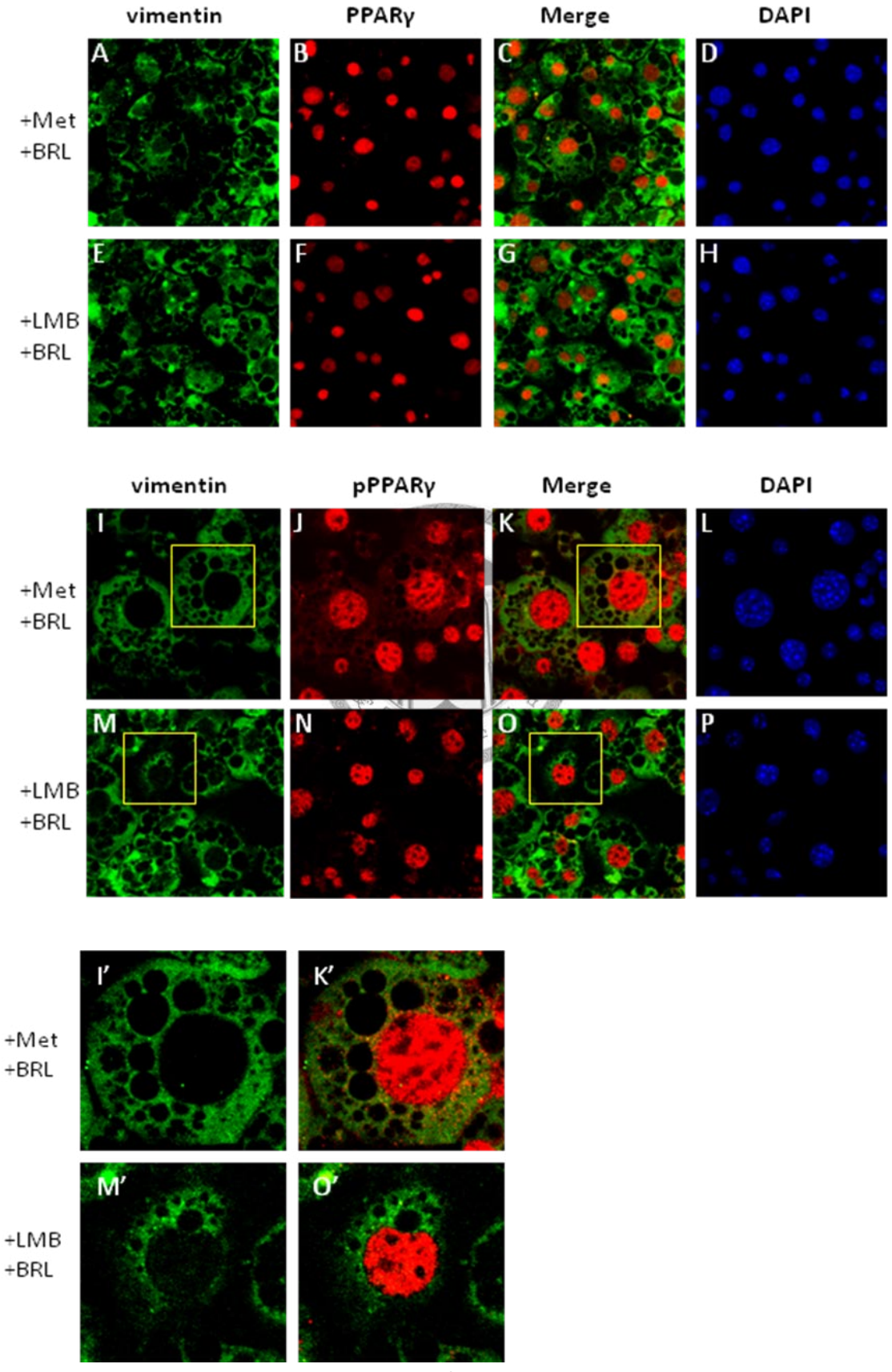

vimentin
PPARγ
Merge
DAPI
A
B
C
D
+Met
+BRL
E
F
G
H
+LMB
+BRL
vimentin
pPPARγ
Merge
DAPI
I
J
K
L
+Met
+BRL
M
N
O
P
+LMB
+BRL
I'
K'
+Met
+BRL
M'
O'
+LMB
+BRL

Figure 8. Identification of nuclear-cytoplasmic translocation of pPPARγ upon ligand treatment.

Differentiated 3T3-L1 adipocytes (day 7) were treated with vehicle control (methanol, +MET), or 20 ng/mL nuclear export inhibitor leptomycin B (+LMB) for 8 hr prior to the addition of BRL49653 for 1hr (+BRL). The cells were then fixed and incubated with antibodies against vimentin (A, E, I, M), PPARγ (B, F), and pPPARγ (J, N), respectively. Nuclei were visualized by DAPI staining (blue). Three independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 80% of the cells had similar staining patterns.

Figure 9.

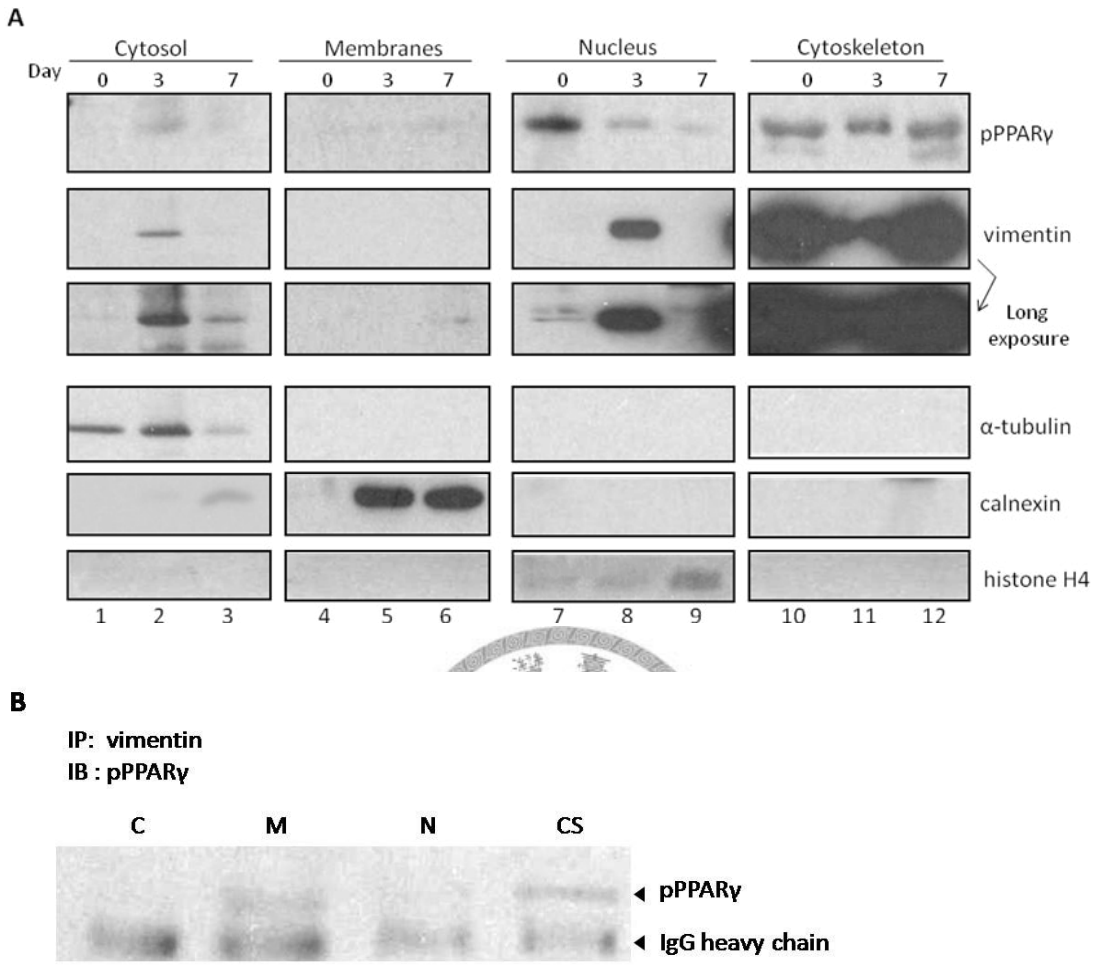


Figure 9. The interaction of vimentin and pPPARγ occured in the membrane and cytoskeleton fractions.

3T3-L1 cells were induced under regular DIM treatment and the lysates were fractionated into cytosolic and nuclear extracts from 3T3-L1 cells at indicated times during differentiation. A, Samples were then subjected to western blot analyses using antibodies against pPPARγ and vimentin. α-tubulin, calnexin, and histone H4 were served as markers of cytosol, membrane, and nucleus fractions. B, The cell compartment fractions were immunoprecipitated and blotted with indicated antibodies. (C, cytosol; M, membranes; N, nucleus; CS, cytoskeleton)

Figure 10.

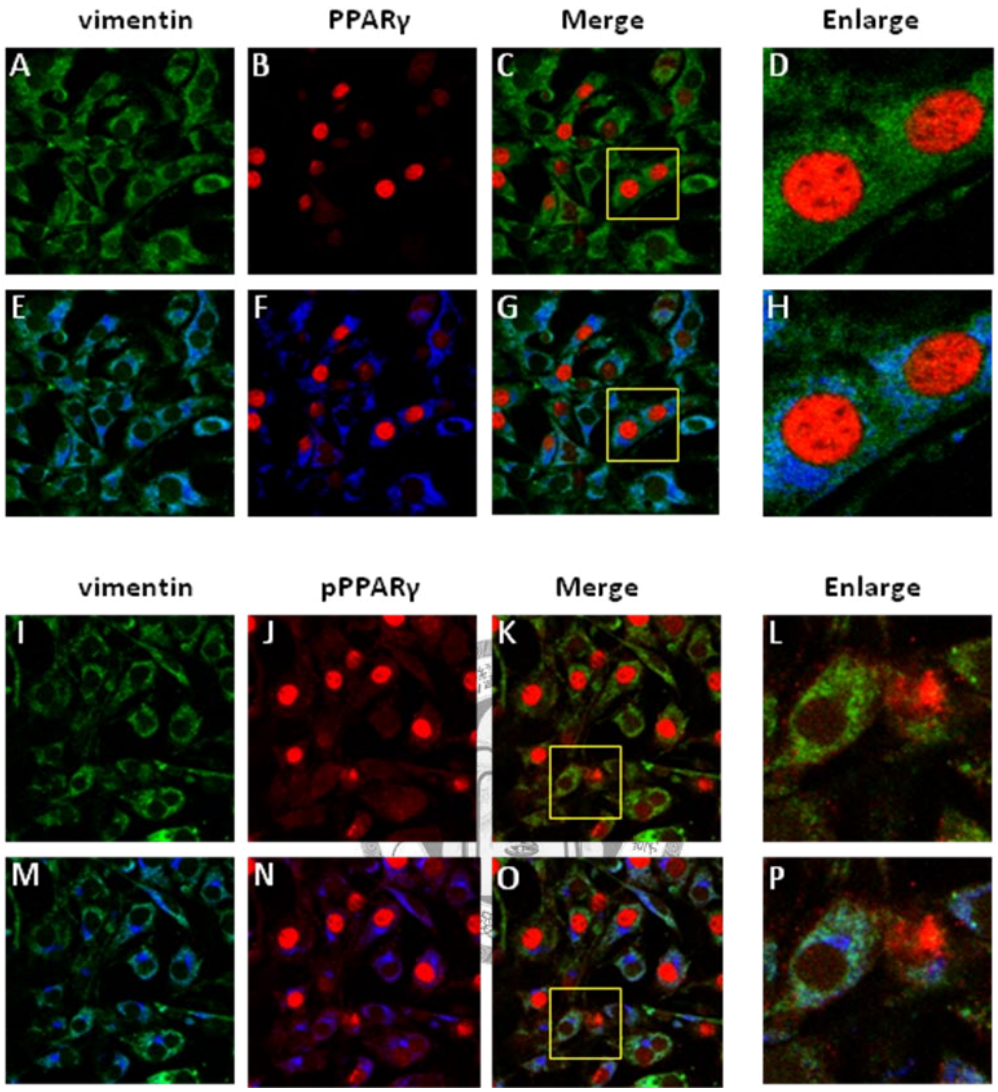


Figure 10. Colocalization of vimentin, pPPARγ and ER.

Early differentiated 3T3-L1 adipocytes (day 3) induced with DIM treatment were fixed and incubated with antibodies against vimentin (A, I), PPARγ (B), and pPPARγ (J). ER was stained in blue (E-H, M-P). Nuclei were visualized by DAPI staining (blue). Five independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 90% of the cells had similar staining patterns. Outlined squared areas are enlarged at 10x magnification (L, P).

Figure 11.

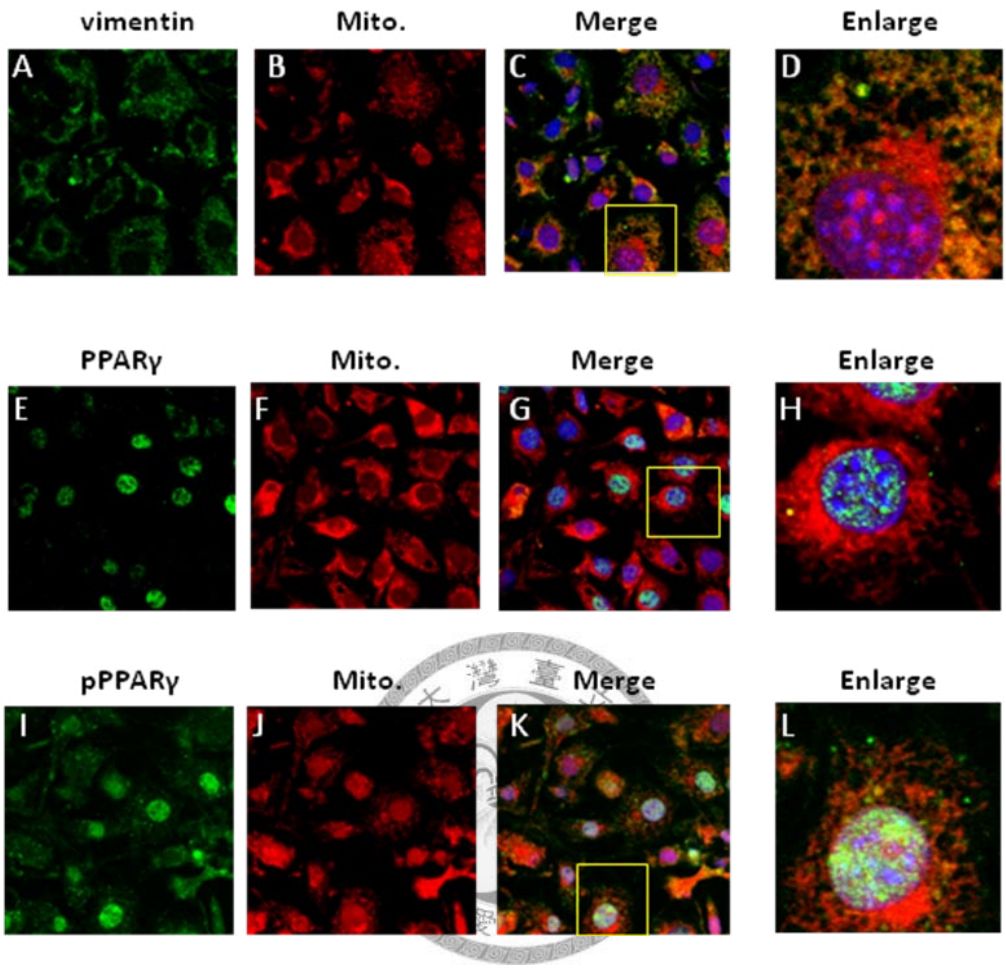


Figure 11. Colocalization of vimentin, pPPARγ and mitochondria.

Early differentiated 3T3-L1 adipocytes (day 3) induced with DIM treatment were fixed and incubated with antibodies against vimentin (A), PPARγ (E), and pPPARγ (I). Mitochondria were stained in red (B-D, F-H, J-L). Nuclei were visualized by DAPI staining (blue). Four independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 90% of the cells had similar staining patterns. Outlined squared areas are enlarged at 10x magnification (D, H, L).

Figure 12.

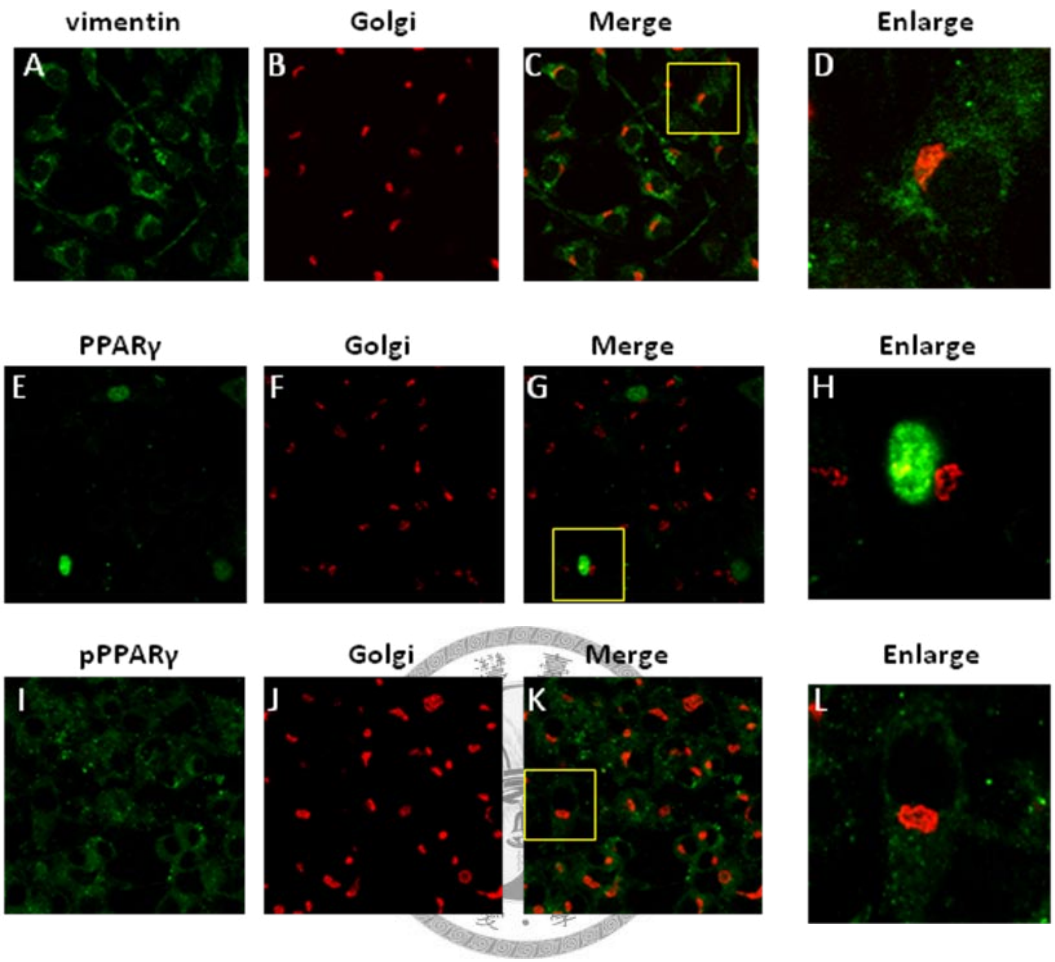


Figure 12. Colocalization of vimentin, pPPARγ and Golgi apparatus.

Early differentiated 3T3-L1 adipocytes (day 3) induced with DIM treatment were fixed and incubated with antibodies against vimentin (A), PPARγ (E), and pPPARγ (I). Golgi were stained in red (B-D, F-H, J-L). There independent experiments were performed, and 5 or more fields per sample were analyzed in each experiment and over 90% of the cells had similar staining patterns. Outlined squared areas are enlarged at 10x magnification (D, H, L).

Tables

Table 1. Results the proteins identified with LC/MS/MS in anti-PPARγ immunoprecipitates.

VIME_HUMAN	**Vimentin**.- Homo sapiens (Human).
K2C7_HUMAN	Keratin, type II cytoskeletal 7 (**Cytokeratin-7**) (CK-7) (Keratin-7) (K7) (Sarcolectin).- Homo sapiens (Human).
Q3SY84_HUMAN	**Keratin 6** irs.- Homo sapiens (Human).
DESM_HUMAN	Desmin.- Homo sapiens (Human).
AAA61281	HUMVIM10 NID: - Homo sapiens
AAA61282	HUMVIM3 NID: - Homo sapiens
Q65ZQ1_HUMAN	Anti-colorectal carcinoma heavy chain.- Homo sapiens (Human).
CAA67203	HSCYTOKE1 NID: - Homo sapiens
ABA00080	DQ065670 NID: - Homo sapiens
PL0122	Ig heavy chain V-III region (TD-Vq) - human (fragment)
BAC01495	Immunoglobulin heavy chain VHDJ region (Fragment).- Homo sapiens (Human).
BAA36314	Immunoglobulin heavy chain variable region (IgM) (Fragment).- Homo sapiens (Human).
Q9H552_HUMAN	OTTHUMP00000021786.- Homo sapiens (Human).
BAC02117	Immunoglobulin heavy chain VHDJ region (Fragment). - Homo sapiens (Human).
AAQ05536	AF471361 NID: - Homo sapiens

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