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SIRT1 mediates the actions of PPARδ on the oxLDL-triggered migration and proliferation of vascular smooth muscle cells

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Running title:

PPARδ inhibits VSMC activation via SIRT1.

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Abbreviations:

CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nLDL, native low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; siRNA, small interfering RNA; TGF, transforming growth factor; VSMC, vascular smooth muscle cell

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Abstract

Peroxisome proliferator-activated receptor (PPAR) δ has been implicated in the vascular pathophysiology. However, its functions in the atherogenic changes of the vascular wall are not fully elucidated. GW501516-activated PPARδ significantly inhibited oxidized low-density lipoprotein (oxLDL)-triggered migration and proliferation of vascular smooth muscle cells (VSMCs). These GW501516-mediated effects were significantly reversed by PPARδ-targeting siRNA, indicating that PPARδ is involved in the action of GW501516. Anti-proliferative effect of GW501516 was directly linked to cell cycle arrest at the G_0/G_1 to S phase transition, which was followed by the down-regulation of cyclin-dependent kinase 4 along with increased levels of p21 and p53. In VSMCs treated with GW501516, the expression of SIRT1 mRNA and protein was time-dependently increased. This GW501516-mediated upregulation of SIRT1 expression was also demonstrated even in the presence of oxLDL. In addition, GW501516-dependent inhibition of oxLDL-triggered migration and proliferation of VSMCs was almost completely abolished in the presence of SIRT1-targeting siRNA. These effects of GW501516 on oxLDL-triggered phenotypic changes of VSMCs were also demonstrated via activation or inhibition of SIRT1 activity by resveratrol or sirtinol, respectively. Finally, gain or loss of SIRT1 function imitated the action of PPARδ on oxLDL-triggered migration and proliferation of VSMCs. Taken together, these observations indicate that PPARδ-dependent upregulation of SIRT1 contributes to the anti-atherogenic activities of PPARδ by suppressing the migration and proliferation of VSMCs linked to vascular diseases such as restenosis and atherosclerosis.

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Introduction

Oxidized low-density lipoprotein (oxLDL) is a pro-atherogenic risk factor that promotes the migration and growth of vascular smooth muscle cells (VSMCs) and macrophages in atherosclerotic lesions (Chatterjee and Ghosh, 1996; Sakai et al., 1996; Mine et al., 2002; Liu et al., 2014). VSMC proliferation and migration triggered by oxLDL were recently demonstrated as an important phenotypic change in the multiple human vascular disorders such as atherosclerosis, and thus facilitate the formation and vulnerability of atherosclerotic plaque (Witztum and Steinberg, 2001). VSMCs play major roles in the maintenance of plaque stability as an important structural component of atherosclerotic plaque cap (Clarke and Bennett, 2006). Several studies demonstrate that intimal migration and excess growth of VSMCs is a critical risk factor leading to restenosis and atherosclerosis (Stintzing et al., 2009; Brown et al., 2010). In fact, these phenotypic changes of VSMCs are regulated by interactions and networking between local cells and humoral factors within the arterial wall (Jiang et al., 2013). Thus, fine-tuned modulation on the migration and proliferation of VSMCs may have therapeutic potential in atherosclerotic complications.

Peroxisome proliferator-activated receptor (PPAR) δ participates in the multiple physiological processes including cellular energy homeostasis, cell growth and differentiation, and inflammatory responses as a nuclear transcription factor activated by specific ligands (Giordano Attianese et al., 2015). This receptor heterodimerizes with retinoid X receptor in the consensus sequences named PPAR response elements (PPRE) to modulate gene expression of target genes (Tugwood et al., 1992). It was recently observed that specific ligand-mediated activation of PPARδ in vascular cells exerts anti-

atherosclerotic effects through antisenescent or antiinflammatory mechanisms (Takata et al., 2008; Jandeleit-Dahm et al., 2009; Lee et al., 2003). Activation of PPARδ also suppresses proliferation and inflammation of VSMCs by upregulating transforming growth factor (TGF)-β1 (Kim et al., 2008). We also demonstrated that ligand-activated PPARδ may stabilize atherosclerotic plaques by upregulating extracellular matrix proteins in VSMCs (Kim et al., 2009). Furthermore, activation of PPARδ counteracts oxLDL-triggered apoptosis of VSMCs through TGF-β and focal adhesion kinase signaling (Hwang et al., 2015). Therefore, it is especially important to examine whether PPARδ modulates the phenotype of VSMCs induced by oxLDL.

SIRT1 functions as a protein deacetylase to remove the acetyl groups of diverse proteins in a NAD^+-dependent manner (Michan and Sinclair, 2007). SIRT1 participates in the multiple cellular processes such as modulation of the cell cycle, metabolism, and aging through interaction with diverse substrates including forkhead transcription factor, histones, MyoD, PPARγ coactivator-1α, and p53 (Brunet et al., 2004; Langley et al., 2002; Vaziri et al., 2001). Inhibitory effects of SIRT1 on the migration and growth of VSMCs were reported in the multiple studies (Gorenne et al., 2013; Li et al., 2011; Miyazaki et al., 2008). Recently, a study reported that ligand-activated PPARδ enhances transcription of SIRT1 through Sp1, but not PPRE, in HuH7 cells (Okazaki et al., 2010). Therefore, we hypothesized that the adverse effects of PPARδ on VSMC migration and growth may be in part stemmed from the upregulation of SIRT1. The present findings indicate that PPARδ-induced upregulation of SIRT1 mediates the ability of this transcription factor to inhibit the phenotypic changes of VSMCs induced by oxLDL.

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Materials and methods

Materials. GW501516 and low-density lipoprotein were purchased from Enzo Life Science (Farmingdale, NY, USA) and Calbiochem (La Jolla, CA, USA), respectively. Anti-β-actin antibody, 4',6-diamidino-2-phenylindole (DAPI), mithramycin A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Sirtinol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Resveratrol and GSK0660 were obtained from Calbiochem and Tocris Bioscience (Bristol, UK), respectively. Polyclonal antibodies specific for horseradish peroxidase-conjugated IgG, c-Myc, PPARδ, and SIRT1 as well as monoclonal antibodies specific for cyclin D1, cyclin-dependent kinase (CDK) 2, p21, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A monoclonal anti-CDK4 antibody was purchased from Cell Signaling (Beverly, MA, USA).

Cell culture. Aortic VSMCs were isolated and incubated in DMEM containing 1% antibiotics and 20% fetal bovine serum (FBS) at 37°C under a condition of 5% CO_2 and 95% air, as essentially described (Hwang et al., 2015). Briefly, the aortic explants dissected from rats were longitudinally cut, and the endothelial cells were removed. The isolated membrane was chopped into small pieces and incubated for a few days in DMEM supplemented with 20% FBS. When VSMCs had fully migrated into the medium, the cells were maintained in the same culture medium.

Preparation of oxLDL. oxLDL was prepared as essentially described (Hwang et al., 2015). Briefly, native low-density lipoprotein (nLDL) was reacted with $CuSO_4$ (final 5

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μmol) at 37°C for 6 h, and then 1 mM EDTA was added to stop the reaction. After thorough dialysis in phosphate-buffered saline (PBS), the extent of LDL oxidation was determined by measuring thiobarbituric reactive substances (Siow et al., 1999).

Small interfering (si)RNA study. Gene-specific small interfering RNA (siRNA)-mediated gene silencing was performed as essentially described (Hwang et al., 2015). Briefly, VSMCs were transfected with nonspecific sequence-targeting siRNA (Ambion, Austin, TX, USA), SIRT1-targeting siRNA (Bioneer, Daejeon, Korea), PPARδ-targeting siRNA (Ambion) using Welfect-Q (WelGENE, Daegu, Korea) in serum-free medium. The effects of siRNA were analyzed after incubation for 24-48 h in fresh medium.

Assay of cell proliferation. VSMCs were synchronized to quiescence by incubating in DMEM containing 0.1% FBS for 24 h. The cells were then treated with the indicated reagent for the indicated time in fresh DMEM containing 5% FBS, and exposed to nLDL or oxLDL. Following incubation for 72 h, the cells were maintained for an additional 4 h in the culture medium containing MTT solution (final 0.1 mg/ml). Following removal of the culture medium, the resultant formazan crystals were dissolved in acidified isopropanol and the optical density was measured using a microplate reader at 570 nm as essentially described (Ham et al., 2013).

Cell cycle determination. VSMCs were synchronized to quiescence via incubation in 0.1% FBS-containing DMEM. Following an overnight incubation, VSMCs were pretreated with GW501516 for 24 h in fresh DMEM. Finally, the cells were incubated in DMEM containing oxLDL for an additional 72 h, and then centrifuged at 1,000 rpm for

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5 min to collect the cells. The cells were then fixed by incubating with 70% (v/v) ethanol at -70°C for 1 h. Following washing with PBS, a staining solution [10 mM Tris (pH 7.0), 0.1% NP-40, 1 mM NaCl, 0.7 μg/ml ribonuclease A, and 5 μg/ml propidium iodide] was added to the cells. The cellular DNA content was determined after incubation for 30 min in the dark, and cell cycle distributions were estimated with a FACSCalibur™ system (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and CellQuest Pro™ software.

Measurement of cell migration. A wound healing assay was adopted to measure VSMC migration as essentially described (Ham et al., 2014). To pause proliferation, VSMCs grown to confluency were incubated in culture medium containing mitomycin C (final 8 μg/ml). Following treatment with the indicated reagents for the indicated time periods, the VSMC monolayers were scratched with a sterile scraper, and then treated with nLDL or oxLDL for an additional 72 h in fresh DMEM containing 5% FBS. The cells were fixed by incubating with 70% (v/v) ethanol at -70°C for 1 h, and then stained in 0.2% crystal violet solution. Cell migration was determined by counting the cells migrated across the wound edge under a microscope.

Real-time PCR. Expression of SIRT1 mRNA was analyzed by real-time PCR as essentially described (Kim et al., 2013). Briefly, total RNA was isolated and reverse-transcribed into cDNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and TOPscript RT DryMIX kit (Enzynomics, Seoul, Republic of Korea), respectively. An aliquot of cDNA was amplified in a 10 μl reaction solution containing 10 pM primers and 2 × Real-Time PCR mix (Solgent, Daejeon, Korea), followed by 40 cycles of PCR

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amplification (10 s at 95°C, 10 s at 56°C, and 10 s at 72°C). The primer sequences used: *SIRT1*, 5'-AGA ACC ACC AAA GCG GAA A-3' and 5'-TCC CAC AGG AGA CAG AAA CC-3'; and *GAPDH*, 5'-CAT GGC CTT CCG TGT TCC TA-3' and 5'-CCT GCT TCA CCA CCT TCT TGA T-3'. The cDNA levels of target gene were determined as the fold ratio relative to *GAPDH* by the $\Delta\Delta C_T$ method (Hwang et al., 2014).

Western blot analysis. The levels of protein were analyzed by immunoblotting as essentially described (Hwang et al., 2015). In brief, whole cell lysates containing 50 μg protein per lane were fractionated on a 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P^+, 0.45 μm pore, Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% skim milk, the membranes were probed with specific antibodies (anti-PPARδ diluted to 1:500; anti-SIRT1 diluted to 1: 1000; anti-p53 diluted to 1:500; anti-p21 diluted to 1:500; anti-CDK4 diluted to 1:1000; anti-CDK2 diluted to 1:1000; anti-cyclin D diluted to 1:500; anti-β-actin diluted to 1:3000) overnight at 4°C. Following incubation with a peroxidase-conjugated secondary antibody, immunoreactive band was visualized by conjunction with West-ZOL Plus (iNtRON Biotechnology).

Vector constructs. Recombinant adenoviral vectors were constructed for *lacZ* and Myc-SIRT1 (GenBank: AF083106) expression. All cDNAs were ligated into the pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase II and Gateway system (Invitrogen). Recombinant adenoviral vector was linearized with *Pac*I and transferred into 293A cells, and then cultured for 2 weeks in complete DMEM. The pAd/CMV/V5-GW/*lacZ* vector (Invitrogen) as a control was also introduced to produce a *lacZ*-bearing

adenovirus. The KIST virus facility (Seoul, Korea) was used to produce adenoviruses. The mammalian expression vector pcDNA3.1-Flag-Sp1 was presented by Prof. Jae-Hwan Kim (CHA University, Korea).

Reporter gene assay. The $PPRE_3$-tk-luc luciferase reporter vector was a gift from Prof. Frank J. Gonzalez (National Cancer Institute, NIH, Bethesda, MD, USA). VSMCs transfected with 0.5 μg pcDNA3.1-PPARδ, 0.5 μg pTK-PPRE-luc, 0.5 μg pSV β-gal, and/or 0.5 μg pcDNA3.1-Myc-SIRT1 for 48 h were incubated with or without GW501516 and/or resveratrol for indicated times. The cells were then lysed and the reporter gene activity was measured as essentially described (Hwang et al., 2015).

Statistical analysis. One-way ANOVA followed by the Tukey-Kramer test was used to determine the statistical significance.

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Results

Activation of PPARδ inhibits oxLDL-induced proliferation of VSMCs. oxLDL increases the proliferation of cultured VSMCs (Chatterjee and Ghosh, 1996); therefore, we assessed whether activation of PPARδ by GW501516 affects the VSMC growth stimulated by oxLDL. A concentration-dependent increase in proliferation was demonstrated in VSMCs treated with oxLDL relative to nLDL-treated cells, as determined by the MTT assay (Fig. 1A). By contrast, the oxLDL-stimulated VSMC proliferation was dose-dependently inhibited in cells treated with GW501516, a specific ligand for PPARδ (Fig. 1B).

To investigate the involvement of PPARδ in the inhibition of VSMC proliferation triggered by oxLDL, VSMCs were treated with PPARδ-targeting siRNA or GSK0660, a PPARδ-specific antagonist (Shearer et al., 2008). The expression of PPARδ protein was reduced in VSMCs transfected with PPARδ-targeting siRNA, whereas non-specific control siRNA did not (Supplemental Fig. 1). The addition of PPARδ-targeting siRNA or GSK0660 significantly reversed the inhibitory effect of GW501516 on VSMC proliferation triggered by oxLDL (Fig. 1, C and D).

To further characterize the anti-proliferative action of PPARδ in oxLDL-treated VSMCs, cell cycle distribution was analyzed in VSMCs treated with or without oxLDL in the presence or absence of GW501516. The cells were synchronized by incubating in medium containing 0.1% FBS for 24 h, and then exposed to vehicle (DMSO) or GW501516, followed by stimulation with oxLDL. A significant decrease in the G_0/G_1 population was observed in VSMCs exposed to oxLDL for 72 h (Fig. 2, A and B). By contrast, the population of cells proceeding S phase was significantly increased.

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GW501516, however, significantly reversed the oxLDL-induced reduction in the cell populations of G_0/G_1 phase. Especially, the increased transition of oxLDL-stimulated cells into S phase was also significantly suppressed by GW501516. These findings suggest that activation of PPARδ by GW501516 causes the cell cycle arrest of VSMC treated with oxLDL.

Because a dynamic network of cell cycle-related molecules such as CDKs, cyclins, and CDK inhibitors is associated with the regulation of cell cycle (Pavletich, 1999), we examined the expression levels of key regulatory proteins in VSMCs treated with oxLDL in the presence or absence of GW501516. oxLDL markedly suppressed the levels of p21 and p53 (Fig. 2, C and D), key regulators of G1 to S phase transition checkpoint (Pavletich, 1999; Toyoshima and Hunter, 1994). This oxLDL-dependent decrease in both protein levels was significantly reversed in the presence of GW501516. In addition, upregulation of CDK4, but not CDK2, induced by oxLDL was also significantly downregulated by GW501516. These observations provided evidence that GW501516-activated PPARδ arrests oxLDL-induced cell cycle progression through expressional modulation of cell cycle-related proteins.

Activation of PPARδ inhibits oxLDL-stimulated migration of VSMCs. Next, we investigated the role of GW501516 on oxLDL-triggered migration of VSMCs by an *in vitro* wound healing assay. By the proliferation index as a reference basis described previously (Kim et al., 2010), we found that oxLDL significantly enhanced the migration of VSMCs, whereas this effect of oxLDL on cell migration was significantly suppressed by GW501516 treatment (Fig. 3, A and B). GW501516-mediated reduction in VSMC migration was not observed in cells transfected with PPARδ-targeting siRNA

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(Fig. 3, C and D). These observations suggested that the action of GW501516 is dependent on PPARδ in the suppression of VSMC migration triggered by oxLDL.

Activation of PPARδ induces SIRT1 expression in VSMCs. Because SIRT1 is implicated in the neointima formation through modulation of VSMC migration and proliferation (Li et al., 2011), the expression level of SIRT1 was determined in VSMCs treated with GW501516. The levels of both SIRT1 mRNA and protein in VSMCs were significantly increased by GW501516 treatment in a time-dependent manner (Fig. 4, A and B).

To confirm whether this effect of GW501516 on the upregulation of SIRT1 is PPARδ-dependent, the effects of PPARδ-targeting siRNA and GSK0660 were examined. PPARδ-targeting siRNA, but not non-specific control siRNA, markedly inhibited GW501516-induced upregulation of SIRT1 in VSMCs (Fig. 4C). In addition, the effect of GW501516 on SIRT1 expression was also suppressed by GSK0660 pretreatment (Fig. 4D), indicating the involvement of PPARδ in the upregulation of SIRT1.

Since Sp1 is implicated in the PPARδ-mediated SIRT1 expression (Okazaki et al., 2010), the effect of mithramycin A, a specific Sp1 inhibitor, was examined in the PPARδ-mediated SIRT1 expression. When VSMCs were incubated in the presence of mithramycin A, the GW501516-induced upregulation of SIRT1 was markedly inhibited (Fig. 4E). This effect of mithramycin A was also observed in the GW501516-mediated suppression of VSMC proliferation triggered by oxLDL (Supplemental Fig. 2A). In addition, ectopic expression of Sp1 potentiated the suppressive effect of GW501516 on the VSMC proliferation triggered by oxLDL (Supplemental Fig. 2B). These findings

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indicate that GW501516-regulated SIRT1 is an important factor in the modulation of VSMC proliferation stimulated by oxLDL. On the other hand, because SIRT1 was reported to inhibit the transcriptional activity of PPARγ (Picard et al., 2004), another member of PPAR nuclear family, we assessed the effects of SIRT1 overexpression and activation on the transcriptional activity of PPARδ. Although GW501516 significantly increased the transcriptional activity of PPARδ, the ectopic expression of SIRT1 or addition of resveratrol did not significantly affect to this GW501516-activated increase in the transcriptional activity of PPARδ, suggesting that SIRT1 did not directly influence to the transcriptional activity of PPARδ in VSMCs (Supplemental Fig. 3).

Downregulation of SIRT1 abrogates the inhibitory effect of PPARδ on VSMC migration and proliferation triggered by oxLDL. Because the expression of SIRT1 was upregulated in VSMCs by PPARδ activation, the role of SIRT1 in PPARδ-associated modulation of cell migration and proliferation was examined in VSMCs stimulated with oxLDL. Expression of SIRT1 was time-dependently decreased in the presence of oxLDL, whereas this oxLDL-triggered reduction of SIRT1 was dose-dependently reversed by GW501516 (Fig. 5, A and B).

To further examine whether this GW501516-mediated recovery of SIRT1 expression directly affects oxLDL-stimulated migration and proliferation of VSMCs, the effect of SIRT1-targeting siRNA was examined in VSMCs (Supplemental Fig. 4). Although GW501516 significantly inhibited oxLDL-stimulated migration and proliferation of VSMCs, siRNA-mediated knockdown of SIRT1 in VSMCs almost completely abolished the effect of GW501516 on oxLDL-triggered migration and proliferation (Fig. 5, C and D). These observations suggested that PPARδ inhibits

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oxLDL-triggered cell migration and proliferation through modulation of SIRT1 expression.

Ectopic overexpression of SIRT1 mimics the suppressive action of PPARδ in VSMC migration and proliferation triggered by oxLDL. To further characterize the critical role of SIRT1 in oxLDL-triggered cell migration and proliferation, the effects of SIRT1 overexpression were examined in VSMCs. The ectopic expression of SIRT1 increased the level of SIRT1 in VSMCs (Supplemental Fig. 5). In line with the levels of SIRT1 expression, oxLDL-triggered migration and proliferation of VSMCs were significantly attenuated, indicating that SIRT1 is an important protein in PPARδ-mediated inhibition of VSMC migration and proliferation triggered by oxLDL (Fig. 6).

SIRT1 is an important factor in PPARδ-mediated suppression of VSMC migration and proliferation triggered by oxLDL. To confirm the functional significance of PPARδ-mediated induction of SIRT1, the impact of SIRT1 activity on PPARδ-mediated inhibition of VSMC migration and proliferation was investigated in cells triggered by oxLDL. Blockade of SIRT1 activity by sirtinol, a specific SIRT1 inhibitor, reversed the inhibitory actions of PPARδ on the VSMC migration and proliferation triggered by oxLDL (Fig. 7; Supplemental Fig. 6). By contrast, a SIRT1 activator resveratrol significantly inhibited both oxLDL-stimulated cell migration and proliferation to a similar degree as GW501516 did in VSMCs (Fig. 7). This inhibitory effect of GW501516 was further enhanced in the presence of resveratrol, suggesting that the actions of PPARδ on the VSMC migration and proliferation are mediated through expressional regulation of SIRT1 (Fig. 7; Supplemental Fig. 6).

Discussion

Transcriptional activation of specific target genes by PPARδ elicits a broad range of cellular responses to maintain vascular homeostasis (Lee et al., 2003; Kim et al., 2008; Kim et al., 2009). Although diverse target genes participate in the vascular actions of PPARδ, the effector molecules associated with the vascular actions of PPARδ have not been fully elucidated. Here, we showed that a specific ligand GW501516-activated PPARδ inhibits oxLDL-stimulated migration and proliferation of VSMCs, which trigger formation of a fibrous capsule in the pathogenesis of atherosclerosis (Gorenne et al., 2013; Dzau et al., 2002). This PPARδ-mediated inhibition is dependent on SIRT1, a deacetylase that has a multitude of roles in the modulation of cardiovascular diseases (Ota et al., 2010). In VSMCs treated with GW501516, the mRNA and protein of SIRT1 were upregulated. Chemicals- or siRNA-mediated modulation of SIRT1 activity or expression abrogated the inhibitory actions of PPARδ on the migration and proliferation of VSMCs triggered by oxLDL. Furthermore, adenovirus-mediated ectopic expression of SIRT1 potentiated these effects of GW501516 on VSMCs.

The anti-atherogenic properties of PPARδ are, in part, associated with its activity to induce the intracellular multifunctional gene SIRT1 in VSMCs. The present observation is consistent with a previous finding demonstrating that activation of PPARδ suppresses premature senescence by upregulating SIRT1 in human endothelial cells exposed to angiotensin II (Kim et al., 2012). In addition, SIRT1 transgenic mice exhibited protection against atherosclerosis via inhibition of VSMC migration and proliferation, which underlie neointima formation (Li et al., 2011). By contrast, another

study showed that SIRT1 promoted proliferation by preventing senescence of primary endothelial cells cultured from porcine aorta (Zu et al., 2010). Although the action of SIRT1 in cell proliferation is debatable, this study demonstrates that activation of PPARδ inhibits oxLDL-stimulated VSMC migration and proliferation. In human atherosclerotic plaques, the reduced expression of SIRT1 has also been detected (Gorenne et al., 2013); therefore, it may be possible to control the stability of plaques through SIRT1 upregulation in a process mediated by PPARδ activation. Therefore, the current observations provide new clues in the role of PPARδ as a potential novel therapeutic target in cardiovascular diseases associated with VSMC migration and proliferation such as atherosclerosis and restenosis.

Reduced expression of SIRT1 was demonstrated in VSMCs isolated from plaques and human atherosclerotic plaques (Gorenne et al., 2013). Accordance with results in atherosclerotic plaques, this study demonstrate that the expression of SIRT1 in VSMCs was markedly suppressed by exposure to oxLDL, which is implicated in various aspects of cardiovascular diseases, in particularly atherosclerosis (Trpkovic et al., 2015). This oxLDL-induced suppression of SIRT1 expression was dose-dependently reversed by the activation of PPARδ in VSMCs. In fact, direct modulatory properties of SIRT1 in the migration and proliferation of VSMCs induced by oxLDL were demonstrated by siRNA- or adenovirus-mediated modulation of SIRT1 expression as well as chemical-mediated modulation of SIRT1 activity. Accordingly, oxLDL-induced downregulation of SIRT1 seems to be involved in the pathogenesis of vascular disorders triggered by oxLDL. Our finding shows that activation of PPARδ significantly enhances SIRT1 expression regardless of oxLDL in VSMCs. Although the roles of PPARδ in SIRT1 expression were not directly evaluated, Sp1 inhibitor mithramycin A is clearly inhibited

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the GW501516-induced expression of SIRT1. This finding was in line with reported results showing that ligand-activated PPARδ transcriptionally activates the *SIRT1* gene through Sp1 in VSMCs and hepatocyte-derived Huh7 (Kim et al., 2012; Okazaki et al., 2010). The present result indicates that Sp1 is a critical factor in the PPARδ-mediated induction of SIRT1 expression instead of PPRE. In addition, Sp1 also inhibited VSMC proliferation by transcriptionally increasing mitofusin-2 or growth inhibitory protein p27 (Sorianello et al., 2012; Andrés et al., 2001). In line with these previous reports, modulation of Sp1 by inhibitor or ectopic overexpression significantly affected to the actions of GW501516 on the VSMC proliferation triggered by oxLDL. SIRT1 also inhibited the vascular injury-induced cell proliferation in carotid artery-ligated mice (Li et al., 2011), stressing the importance of SIRT1 in restenosis. In fact, upregulation of SIRT1 by PPARδ inhibited platelet-derived growth factor- or interleukin-1β-induced migration and proliferation of VSMCs in a cell cycle-dependent manner (Lim et al., 2009; Kim et al., 2010). Furthermore, activation or inhibition of SIRT1 activity by resveratrol or sirtinol, respectively, modulated VSMC migration and proliferation triggered by oxLDL in the present experimental conditions. These observations indicate that PPARδ inhibits oxLDL-triggered migration and proliferation of VSMCs through a mechanism linked to SIRT1.

PPARδ-dependent upregulation of SIRT1 is a key event in the inhibition of VSMC migration and proliferation triggered by oxLDL. SIRT1, a NAD^+-dependent histone deacetylase, has a variety of cellular functions in the cell cycle regulation, organism longevity, and energy metabolism (Finkel et al., 2009). The present study showed that GW501516-activated PPARδ suppressed oxLDL-triggered migration and proliferation of VSMCs. This effect of PPARδ on VSMC migration and proliferation was linked to

the levels of SIRT1, indicating that SIRT1 is a critical factor in the modulation of atherosclerotic pathogenesis (Gorenne et al., 2013; Li et al., 2011). Thus, SIRT1 has emerged as an important modulator that promotes vascular functions (Li et al., 2011; Gorenne et al., 2013). Such findings provide a rationale for SIRT1 as a drug target in vascular disorders including restenosis and atherosclerosis. In fact, many other studies provided evidence that the level of SIRT1 expression is intimately linked to atherosclerotic vascular disorders (Gorenne et al., 2013; Kim et al., 2012; Li et al., 2011). Because downregulated level of SIRT1 was demonstrated in VSMCs of atherosclerotic plaque region (Gorenne et al., 2013), it may be possible to inhibit the pro-atherogenic effect of decreased SIRT1 in the vascular wall by inducing SIRT1 expression through activation of PPARδ.

In conclusion, SIRT1 was identified as a key molecular target of PPARδ-mediated anti-migration and anti-proliferation actions in VSMCs stimulated with oxLDL. Our these observations indicate that GW501516-activated PPARδ inhibits the migration and proliferation of VSMCs triggered by oxLDL by modulating SIRT1 expression, suggesting that reinforcement of SIRT1 expression by PPARδ could be a novel therapeutic strategy for vascular disorders including restenosis and atherosclerosis.

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Authorship Contributions

Participated in research design: JSH and HGS.

Conducted experiments: JSH, SAH, TY, WJL,

Performed data analysis: JSH, KSP, CHL and HGS.

Wrote or contributed to the writing of the manuscript: JSH, KSP and HGS.

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Footnotes

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Figure legends

Fig. 1. GW501516-activated PPARδ attenuates oxLDL-triggered proliferation of VSMCs. (A) VSMCs were stimulated with either nLDL or oxLDL for 72 h. (B) VSMCs treated with indicated concentration of GW501516 for 24 h were stimulated with either nLDL or oxLDL for 72 h. (C) GSK0660-pretreated cells were incubated for 30 min, and then exposed to GW501516. Following incubation for 24 h, the cells were stimulated with oxLDL for 72 h. (D) PPARδ siRNA-transfected cells were incubated for 24 h, and then pretreated with GW501516. Following incubation for 24 h, the transfectants were exposed to oxLDL for 72 h. MTT assay was performed to determine the cell proliferation. Results are expressed as the means ± SE (n=4 or 5). $^{**}P<0.01$ vs untreated cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs oxLDL-treated cells; $^{\dagger}P<0.05$ vs oxLDL plus GW501516-treated cells.

Fig. 2. GW501516-activated PPARδ regulates cell cycle progression of VSMCs triggered by oxLDL. (A and B) Cells synchronized to quiescence by incubating in DMEM containing 0.1% FBS were pretreated with GW501516 for 24 h. Following incubation for 72 h in DMEM containing 5% FBS and oxLDL, DNA content in cells stained with propidium iodide was analyzed by flow cytometry. Representative histograms (A) and the cell number of each cell cycle (B) are plotted as the means ± SE (n=3). (C and D) The expression of cell cycle-regulatory proteins was detected by Western blot analysis. Representative blots (C) and the fold changes in each proteins to β-actin ratio relative to untreated control (D) are shown as the means ± SE (n=4).

$^{*}P<0.05$, $^{**}P<0.01$ vs 5% serum-treated cells; $^{\#}P<0.01$, $^{\#\#}P<0.05$ vs oxLDL-treated cells.

Fig. 3. GW501516-activated PPARδ attenuates oxLDL-triggered migration of VSMCs. (A and B) Mitomycin C (8 μg/ml)-treated cell monolayers were incubated for 2 h, and then washed with PBS. Following pretreatment with or without GW501516 for 24 h, the cells were wounded by scraping with a razor blade, and then stimulated with either nLDL or oxLDL in fresh medium containing 5% FBS. Following incubation for 72 h, wound healing assay was performed to count the cells that migrated across the wound. (C and D) Cells transfected with PPARδ-targeting siRNA were treated as described above, and wound healing assay was performed as described above. Representative photographs (A and C) and migrated cell numbers (B and D) are shown as means ± SE (n=6). Bars, 100 μm. $^{*}P<0.05$, $^{**}P<0.01$ vs untreated cells; $^{\#}P<0.05$ vs oxLDL-treated cells; $^{\dagger}P<0.05$ vs oxLDL plus GW501516-treated cells.

Fig. 4. GW501516-activated PPARδ induces SIRT1 expression. (A and B) VSMCs were treated with GW501516 for the indicated time periods. Total RNA or cell lysate was analyzed by real-time PCR (A) or immunoblot (B), respectively, as described in the Materials and methods. The fold change in *SIRT1* to *GAPDH* cDNA ratio relative to the time 0 was determined and plotted as the means ± SE (n=4). $^{**}P<0.01$ vs untreated cells. (C) VSMCs transfected with PPARδ-targeting siRNA or non-specific siRNA for 24 h were stimulated with or without GW501516 for 24 h, and then harvested and subjected to immunoblot analysis. (D and E) VSMCs pretreated with GSK0660 (D) or mithramycin A (E) for 30 min were stimulated with or without GW501516 for 24 h and

then analyzed by Western blotting as described above.

Fig. 5. RNAi-mediated knockdown of SIRT1 abrogates the actions of GW501516 on the migration and proliferation of VSMCs triggered by oxLDL. (A) Cells were treated with oxLDL for the time period indicated. (B) GW501516-pretreated cells were incubated for 24 h, and then exposed to oxLDL for 72 h. Immunoblot analysis was performed to detect the expression of SIRT1 protein using specific antibodies for SIRT1 and β-actin. (C) SIRT1 siRNA-transfected cells were incubated for 24 h, and then pretreated with or without GW501516. Following incubation for 24 h, the transfectants were exposed to oxLDL for 72 h. MTT assay was performed to determine cell proliferation. (D) SIRT1 siRNA-transfected cell monolayers were incubated for 24 h, and then pretreated consecutively with or without mitomycin C (8 μg/ml) and/or GW501516 for 2 h and 24 h, respectively, prior to exposure to oxLDL for 72 h. A wound healing assay was performed to count the cells that migrated across the wound and expressed as the means ± SE (n=4). $^{*}P<0.05$, $^{**}P<0.01$ vs untreated cells; $^{\#}P<0.05$ vs oxLDL-treated cells; $^{\dagger}P<0.05$ vs oxLDL plus GW501516-treated cells.

Fig. 6. Adenovirus-mediated overexpression of SIRT1 potentiates the effects of PPARδ on the migration and proliferation of VSMCs triggered by oxLDL. (A) Ad-*LacZ*- or Ad-SIRT1-infected cells were incubated for 48 h, and then pretreated with or without GW501516 for 24 h. Following incubation for 72 h with or without oxLDL, MTT assay was performed to determine the cell proliferation. (B and C) Ad-*LacZ*- or Ad-SIRT1-infected cells monolayers were incubated for 48 h, and then treated with mitomycin C

(8 μg/ml) for 2 h. Following pretreatment with or without GW501516 for 24 h, the cells were stimulated with or without oxLDL for 72 h. Wound healing assay was performed (B) and the migrated cell numbers (C) are shown as the means ± SE (n=4). Bars, 100 μm. $^{*}P<0.05$.

Fig. 7. Activator- or inhibitor-mediated modulation of SIRT1 activity mimics the actions of PPARδ on the migration and proliferation of VSMCs triggered by oxLDL. (A) GW501516-pretreated cells were incubated for 24 h, and then exposed to sirtinol or resveratrol for 30 min. Following stimulation with oxLDL for 72 h, MTT assay was performed to analyze cell proliferation. (B) Mitomycin C (8 μg/ml)-treated cell monolayers were incubated for 2 h, and then pretreated with or without GW501516 for 24 h. Following exposure to sirtinol or resveratrol for 30 min, the cells were stimulated with oxLDL for 72 h. Wound healing assay was performed to analyze cell migration and expressed as the means ± SE (n=4). $^{**}P<0.01$ vs untreated cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs oxLDL-treated cells; $^{\dagger}P<0.05$ vs oxLDL plus GW501516-treated cells.

Figure 1

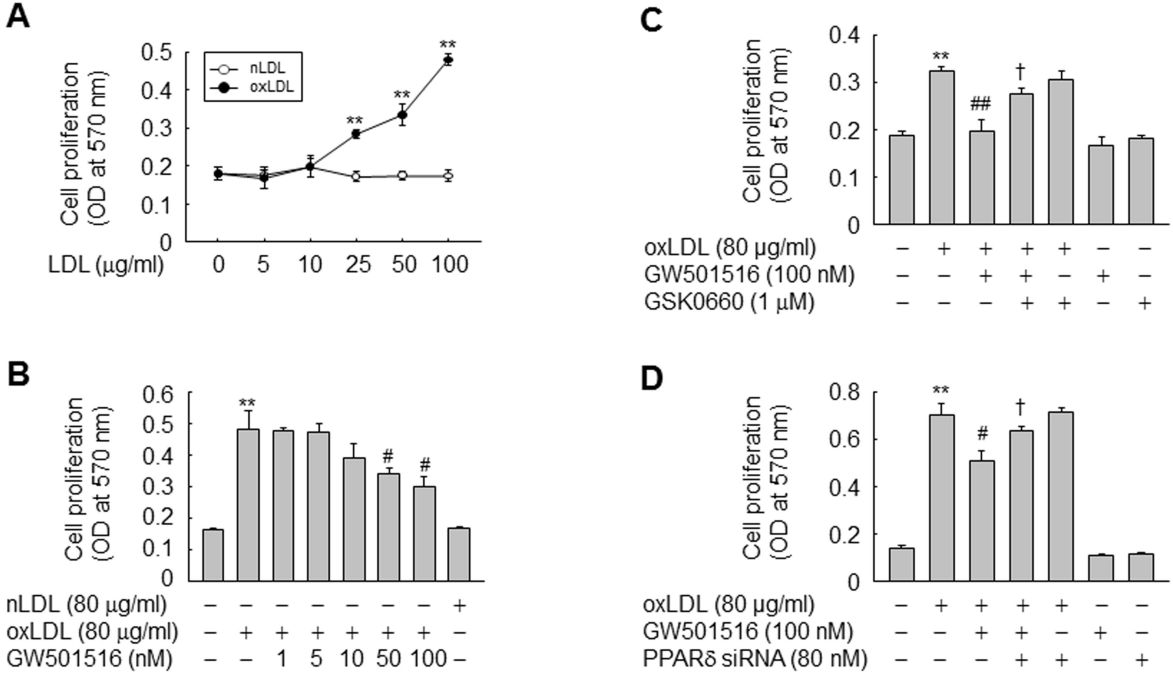

A
Cell proliferation (OD at 570 nm)
nLDL
oxLDL
LDL (μg/ml)
0 5 10 25 50 100
B
Cell proliferation (OD at 570 nm)
nLDL (80 μg/ml)
oxLDL (80 μg/ml)
GW501516 (nM)
C
Cell proliferation (OD at 570 nm)
oxLDL (80 μg/ml)
GW501516 (100 nM)
GSK0660 (1 μM)
D
Cell proliferation (OD at 570 nm)
oxLDL (80 μg/ml)
GW501516 (100 nM)
PPARδ siRNA (80 nM)

Figure 2

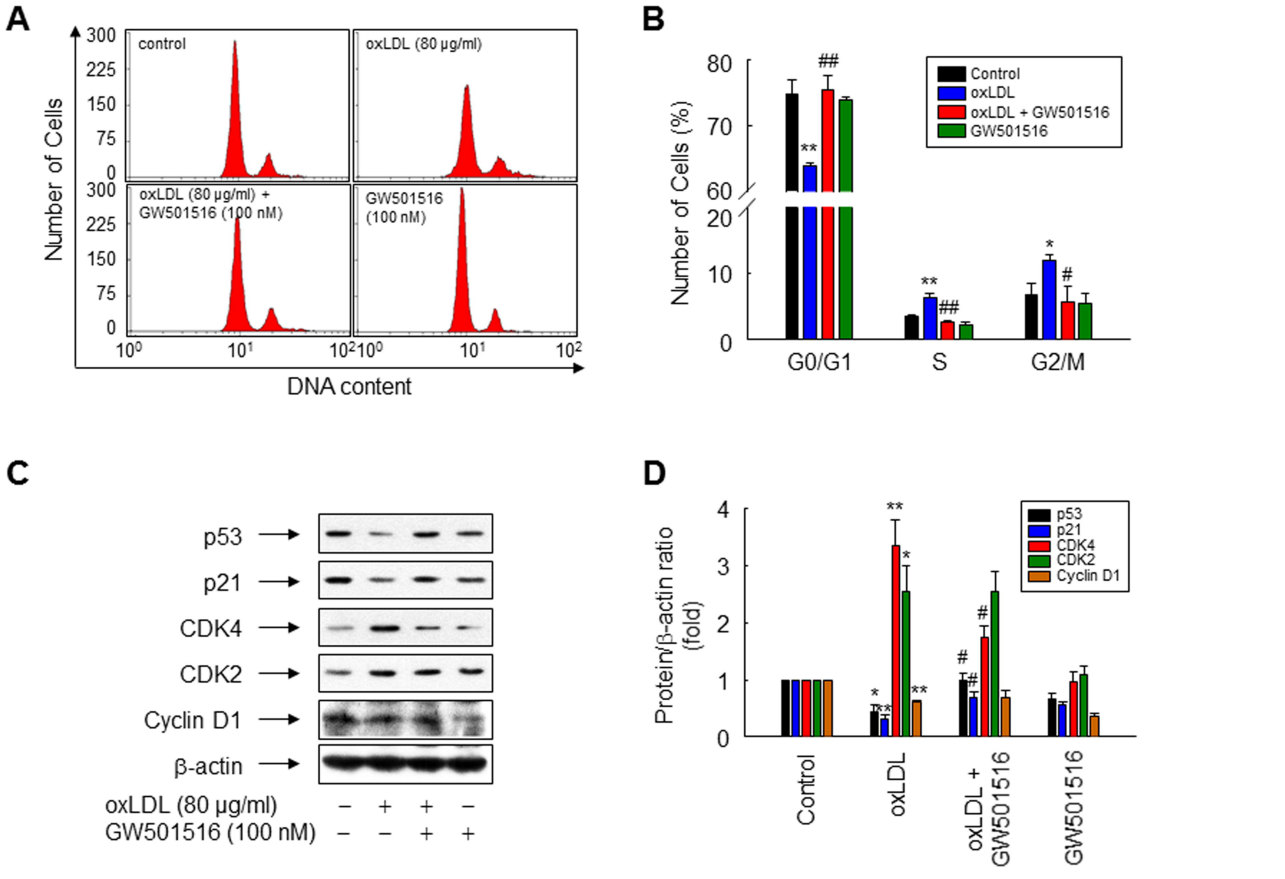
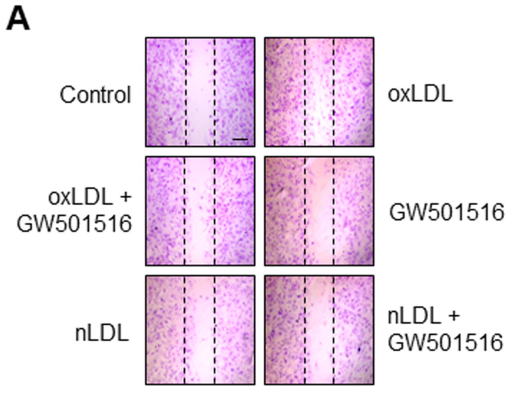
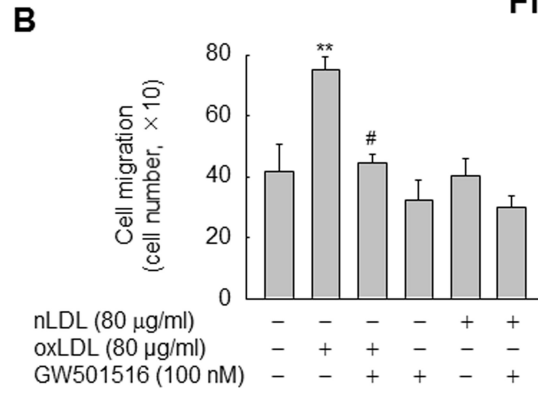
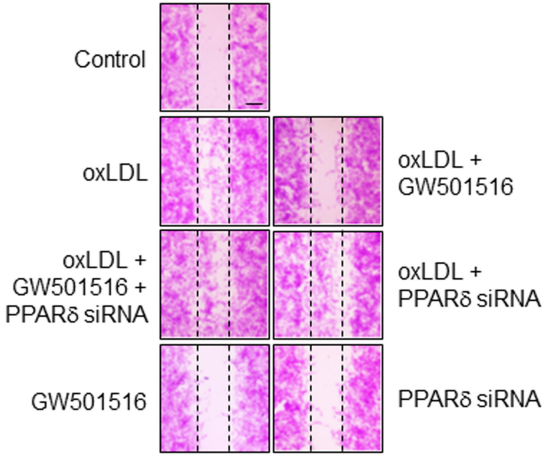

A
control
oxLDL (80 μg/ml)
oxLDL (80 μg/ml) + GW501516 (100 nM)
GW501516 (100 nM)
Number of Cells
DNA content
B
Control
oxLDL
oxLDL + GW501516
GW501516
Number of Cells (%)
G0/G1
S
G2/M
C
p53
p21
CDK4
CDK2
Cyclin D1
β-actin
oxLDL (80 μg/ml)
GW501516 (100 nM)
D
p53
p21
CDK4
CDK2
Cyclin D1
Protein/β-actin ratio (fold)
Control
oxLDL
oxLDL + GW501516
GW501516

Figure 3


A
Control
oxLDL
oxLDL +
GW501516
GW501516
nLDL
nLDL +
GW501516


B
Cell migration
(cell number, ×10)
80
60
40
20
0
**
#
nLDL (80 μg/ml) – – – – + +
oxLDL (80 μg/ml) – + + – – –
GW501516 (100 nM) – – + + – +


C
Control
oxLDL
oxLDL +
GW501516
oxLDL +
GW501516 +
PPARδ siRNA
oxLDL +
PPARδ siRNA
GW501516
PPARδ siRNA

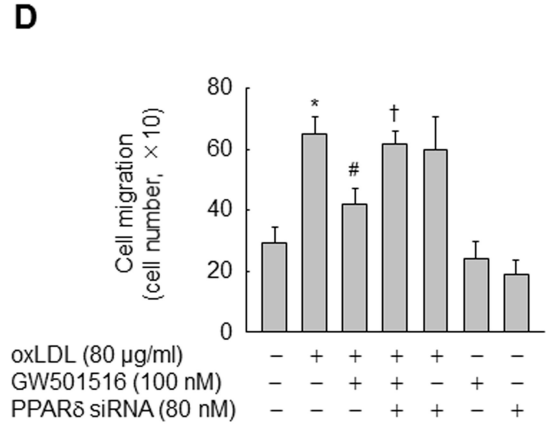

D
Cell migration
(cell number, ×10)
80
60
40
20
0
*
#
†
oxLDL (80 μg/ml) – + + + + – –
GW501516 (100 nM) – – + + – + –
PPARδ siRNA (80 nM) – – – + + – +

Figure 4

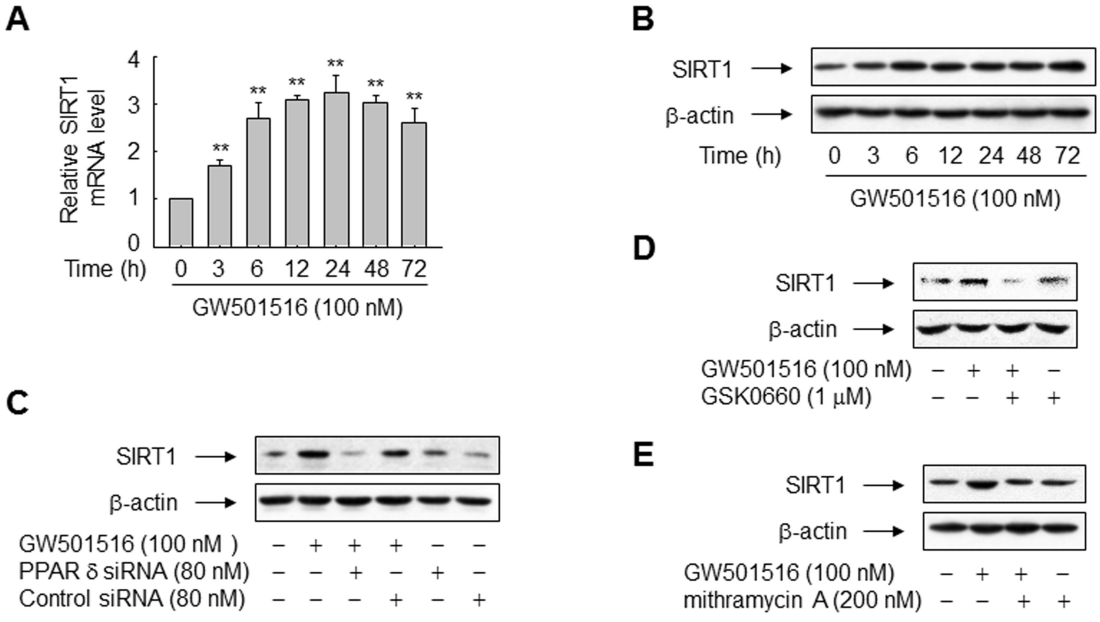

A
Relative SIRT1 mRNA level
4
3
2
1
0
**
Time (h)
0 3 6 12 24 48 72
GW501516 (100 nM)
B
SIRT1
β-actin
Time (h)
0 3 6 12 24 48 72
GW501516 (100 nM)
C
SIRT1
β-actin
GW501516 (100 nM) – + + + – –
PPAR δ siRNA (80 nM) – – + – + –
Control siRNA (80 nM) – – – + – +
D
SIRT1
β-actin
GW501516 (100 nM) – + + –
GSK0660 (1 μM) – – + +
E
SIRT1
β-actin
GW501516 (100 nM) – + + –
mithramycin A (200 nM) – – + +

Figure 5

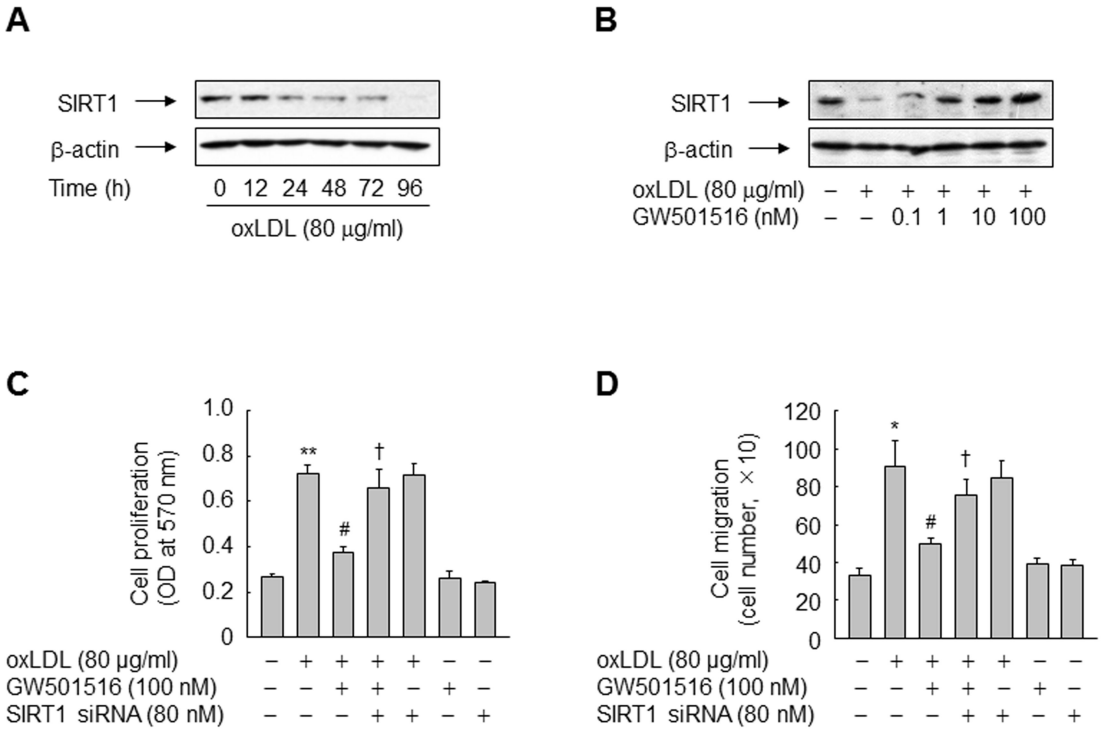

A
SIRT1
β-actin
Time (h)
0 12 24 48 72 96
oxLDL (80 μg/ml)
B
SIRT1
β-actin
oxLDL (80 μg/ml) – + + + + +
GW501516 (nM) – – 0.1 1 10 100
C
Cell proliferation (OD at 570 nm)
1.0
0.8
0.6
0.4
0.2
0
**
#
†
oxLDL (80 μg/ml) – + + + + – –
GW501516 (100 nM) – – + + – + –
SIRT1 siRNA (80 nM) – – – + + – +
D
Cell migration (cell number, × 10)
120
100
80
60
40
20
0
*
#
†
oxLDL (80 μg/ml) – + + + + – –
GW501516 (100 nM) – – + + – + –
SIRT1 siRNA (80 nM) – – – + + – +

Figure 6

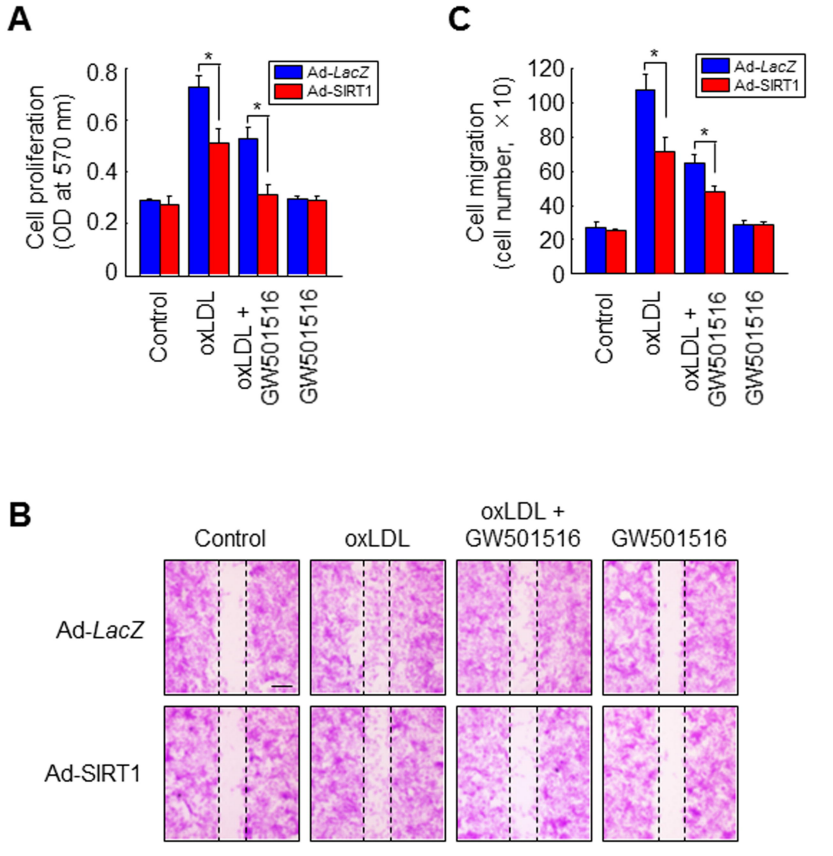
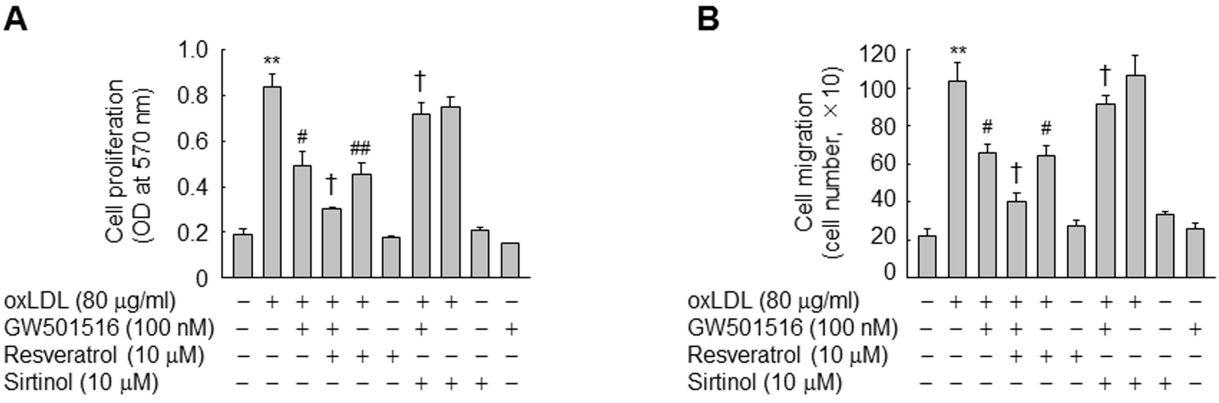

A
Cell proliferation (OD at 570 nm)
0.8
0.6
0.4
0.2
0
Ad-LacZ
Ad-SIRT1
*
*
Control
oxLDL
oxLDL + GW501516
GW501516
C
Cell migration (cell number, ×10)
120
100
80
60
40
20
0
Ad-LacZ
Ad-SIRT1
*
*
Control
oxLDL
oxLDL + GW501516
GW501516
B
Control
oxLDL
oxLDL + GW501516
GW501516
Ad-LacZ
Ad-SIRT1

Figure 7


A
Cell proliferation (OD at 570 nm)
1.0
0.8
0.6
0.4
0.2
0
**
#
†
##
†
oxLDL (80 μg/ml) – + + + + – + + – –
GW501516 (100 nM) – – + + – – + – – +
Resveratrol (10 μM) – – – + + + – – – –
Sirtinol (10 μM) – – – – – – + + + –
B
Cell migration (cell number, × 10)
120
100
80
60
40
20
0
**
#
†
#
†
oxLDL (80 μg/ml) – + + + + – + + – –
GW501516 (100 nM) – – + + – – + – – +
Resveratrol (10 μM) – – – + + + – – – –
Sirtinol (10 μM) – – – – – – + + + –