## SUPPLEMENTAL MATERIALS

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Experimental mice

Conventional PPARδ knock-out mice are difficult to generate due to frequent embryonic lethality [1, 2]. In this study, vascular smooth muscle cell (VSMC)-selective PPARδ knockout mice were generated by breeding our SM22α-Cre knock-in homozygous mice ($SM22\alpha^{Cre/+}$)[3] with PPARδ-floxed homozygous mice ($PPAR\delta^{flox/flox}$) [1]. Both $SM22\alpha^{Cre/+}$ and $PPAR\delta^{flox/flox}$ mice are derived from a C57BL/6J background. Offspring were genotyped by PCR as DNA was obtained from tail-snip biopsies using transgene-specific oligonucleotide primers for SM22α-Cre and PPARδ-floxP [1, 3]. Two groups of mice were selected for this study: 1) VSMC-selective PPARδ conditional knockout mice (SMPδ cKO) with a genotype of SM22α-Cre-knock-in heterozygous and PPARδ-floxed homozygous ($SM22\alpha^{Cre/+}/PPAR\delta^{flox/flox}$), and 2) littermate control mice (LC) with a genotype of SM22α wild-type and PPARδ-floxed homozygous ($SM22\alpha^{+/+}/PPAR\delta^{flox/flox}$, PPARδ-floxed control). Another line of LC could be mice with a genotype of SM22α-Cre heterozygous and PPARδ wild-type ($SM22\alpha^{Cre/+}/PPAR\delta^{+/+}$, SM22α-Cre control). We chose PPARδ-floxed mice as the experimental LC in this study since these two genotyped mice of LC show the same phenotype. Generally, VSMC-selective PPARδ conditional knockout mice showed normal development and fertility. All experiments were conducted in male, 8-10 week-old mice, and age- and weight-matched littermates were housed in a temperature-controlled animal facility with a 12-h:12-h light-dark cycle and free access to water and rodent chow. The animal study protocol was approved by the University of Michigan Animal Care and Use Committee.

Mouse model of transient focal cerebral ischemia

Focal cerebral ischemia was induced in mice by intraluminal middle cerebral artery occlusion (MCAO) using a nylon monofilament suture as described previously [4-6]. Briefly, mice were anesthetized with ketamine (100mg/kg) and xylazine (10 mg/kg). After a midline skin incision, the left common carotid artery was exposed and then its branches were electrocoagulated. A 2-cm length of 6-0 rounded tip nylon suture was gently advanced from the external carotid artery up to the internal carotid artery until regional cerebral blood flow (rCBF) was reduced to less than 16% of baseline. After 30 minutes of proximal MCA occlusion, blood flow was restored by removing the suture. Changes in cerebral blood flow (CBF) at the surface of the cortex were recorded using a laser-Doppler flowmetry monitor (BPM2 System, Vasamedic, St. Paul, MN). Sham control animals were subjected to similar operations to expose the carotid arteries without occlusion of the middle cerebral artery. After 30 minutes of MCAO, the mice were allowed to recover for 24 hours. Arterial blood gases, mean arterial pressure, and heart rate were also monitored in selected animals 30 min before, during, and 30 min after MCAO. The rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad (Harvard, Holliston, MA). After the ischemic insult, mice were kept in an air-ventilated incubator at 24.0 ± 0.5°C. The animals were sacrificed at 24 h of reperfusion, and the brains were quickly removed for biochemical assays as well as infarct determination.

Inhibition of MMP-9 levels in mouse brain by lentivirus-mediated infection

We employed a lentivirus-mediated RNA interference approach to achieve the inhibition of MMP-9 levels in the brain. In brief, mice were anesthetized and fixed in a stereotaxic apparatus. A burr hole was drilled in the pericranium 2.5 mm lateral to the sagittal suture and 0.94 mm posterior to the coronal suture. A 10-μl Hamilton syringe was stereotactically inserted into the lateral ventricle and caudate approximately 3.0 mm under the cortex. Four-microliter lentiviral

suspensions (Lenti-shMMP-9, Lenti-GFP, custom-made in Sigma, St Louis, MO) containing $5x10^{10}$ genome copies of each virus were injected into the left ventricle and caudate putamen at a rate of 0.2 μl per minute based on a prior publication [7]. The needle was withdrawn after 15 minutes of injection. Control animals received the same amount of PBS injection. The lentivirus-infected mice survived for 4 weeks before transient focal cerebral ischemia was induced in the left MCA territory for 30 min and all mice were sacrificed 24 h after MCA occlusion.

Mouse brains were removed and then subjected to 2% 2,3,5-*triphenyltetrazolium chloride* (TTC) staining for measurement of infarct volume, gelatin-based zymography for MMP activity, and quantitative analysis of Evans Blue extravasation for BBB permeability.

Measurement of infarct volume and neurological deficit

Infarct volume was measured using 2% TTC staining as described previously [5, 6, 8]. Mouse brains were removed at 24 h after MCAO, and sliced into 8 coronal sections (1 mm thick) by a mouse brain matrix. The slices were stained with 2% TTC for 15 min at 37°C, scanned and the infarct area was estimated by Metamorph software. The infarct volume was calculated using a derived formula [9] in which infarct volume as a percentage of the contralateral hemisphere was calculated as 100 (contralateral hemisphere volume – non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume.

Following cerebral ischemia, mice were also tested for neurological deficits and scored on a 5-point scale [5, 6, 8]: 0, no observable neurological deficits (normal); 1, failure to extend right forepaw (mild); 2, circling to the contralateral side (moderate); 3, falling to the right (severe); 4, mice could not walk spontaneously; depressed level of consciousness (very severe).

Quantitation of Evans Blue extravasation

For analysis of cerebrovascular permeability after MCAO, mice were injected with 100 µl of 4% Evans Blue (EB) (Sigma-Aldrich) 23 h after MCAO. One hour later, animals were perfused with PBS and the brains were removed and separated into hemispheres ipsilateral and contralateral to the MCAO. Each hemisphere was then homogenized in N,N-dimethylformamide (Sigma-Aldrich) and centrifuged for 45 min at 25,000 rcf. The supernatants were collected and quantitation of EB extravasation was performed as described [5, 10]. Briefly, EB levels in each hemisphere were determined from the formula: ($A_{620}$nm – (($A_{500}$nm + $A_{740}$nm) / 2)) / mg wet weight. Background EB levels in the non-ischemic hemisphere were subtracted from the ischemic hemisphere ipsilateral to the MCAO.

Isolation of cerebral vessels

Cerebral vessels were isolated using a previously described method with some modifications[11] . Briefly, mice were sacrificed by decapitation under anesthesia. The brains were immediately removed from the skull and immersed in ice-cold buffer A (103 mM NaCl, 4.7mM KC1, 2.5 mM $CaC1_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 15 mM Hepes, pH 7.4). The brain was homogenized in a 5-fold volume excess of buffer B (103 mM NaCl, 4.7mM KC1, 2.5 mM $CaC1_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 15 mM Hepes, 25 mM $HCO_3$, 10 mM glucose, 1mM sodium pyruvate, and 1 g/100 ml dextran pH 7.4) with a Teflon homogenizer. The homogenate was suspended in an equal volume of 25% BSA and was centrifuged at 5,800 x g at 4 $^oC$ for 30 min. The vessel pellet was recovered in buffer B and then spun at 500 x g for 5 min. The final vessel pellet was stored at -80 $^oC$ until various biochemical assays were performed.

Gelatin-substrate zymography:

Animals were perfused with saline, and then the cerebral cortex and hippocampus were removed and snap-frozen at –80˚C. Brain samples were then homogenized in 500 µl lysis buffer (50 mM Tris--HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% $NaN_3$) and the supernatant was collected after centrifugation. The brain supernatant or 1 ml of conditioned medium from VSMC culture after various treatments was incubated with 50 µl of gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) for 1 h with constant shaking. After centrifugation, the gelatin-sepharose pellet was rinsed with 500 µl lysis buffer and the solution was centrifuged again. The pellet was then incubated for 30 min with 50 µl of elution buffer consisting of lysis buffer plus 10% dimethylsulfoxide (DMSO). 25 µl of eluted sample from a specific mouse brain region was mixed with 25 µl 2x non-reducing sample buffer (0.125 M Tris-HCl, 20% glycerol, 4% SDS, 0.003% bromphenol blue, pH 6.8), loaded onto 7.5% SDS-PAGE containing 0.1% gelatin, and electrophoresed at 180 V. The gel was incubated for 20 h at 37˚C in buffer containing 21 mM Tris-HCl, 10 mM $CaCl_2$ and 0.04% $NaN_3$, pH 7.6 after washing twice in 2.5% Triton X-100 for 20 min. After incubation, the gel was stained for 1 h with 0.1% Coomassie blue (diluted with 40% methanol and 10% HAc) and destained until clear proteolytic bands appear on a contrasting blue background [12, 13].

Cell cultures

Aortic smooth muscle cells were prepared from wild-type mice as previously described [14, 15]. The cultured cells were maintained in DMEM/F-12 containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 200 mM L-glutamine. VSMCs (4-10$^{th}$ passage) uniformly positive for α–smooth muscle actin immunohistochemical staining (>95% vascular smooth muscle cell purity), were grown to 85-95% confluence before OGD exposure. In some experiments, a specific PPARδ agonist, GW 501516, at a dose of 1µM was added 12 h prior to OGD onset.

Oxygen-glucose deprivation (OGD)

To mimic ischemia-like conditions *in vitro*, mouse VSMC cultures were exposed to OGD for various times [5, 6, 16]. Briefly, confluent VSMCs were transferred into a temperature-controlled (37 ± 1°C) anaerobic chamber (Forma Scientific, Marietta, OH) containing a gas mixture composed of 5% $CO_2$, and 95% $N_2$. The culture medium was replaced with deoxygenated glucose-free Hanks' Balanced Salt Solution and cells were maintained in the hypoxic chamber for 0, 4 and 24 h. Control VSMC cultures were not exposed to OGD.

Real-time PCR

A quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was carried out with a Bio-Rad thermocycler and an SYBR green kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations [5, 6, 11]. Specific primers used for the reaction are as described in Supplemental Table I. The relative expression of target mRNAs was normalized by 18S RNA levels.

Western blot analysis

Total protein was isolated from the cerebral cortex, cerebral vessels, aortas or VSMC cultures as described previously [5, 6, 11, 16, 17]. Samples (20-40 μg of protein) were electrophoresed onto 10-15% SDS/polyacrylamide gels (SDS/PAGE) and transferred to PVDF membranes. The membranes were blocked in TBST buffer containing 20 mM Tris-HCl, 5% nonfat milk, 150 mM NaCl, and 0.05% Tween-20 (pH 7.5) for 1 h at room temperature. Thereafter, the blots were incubated respectively with a primary rabbit anti-PPARδ antibody (1:200; Santa Cruz, CA), or mouse anti-actin antiserum (1:500; Santa Cruz, CA) for 1-2 h at room temperature. The

membranes were washed with TBST 3 times at 10 min intervals, incubated with the secondary antibody (1:5000; anti-rabbit, or anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, WI) at RT for 1 h, then washed three times each at 10 min intervals with TBST and two times each for 10 min with TBS. The color reaction was developed by the Blot AP System (Promega).

Adenovirus-mediated gain-of-PPARδ function in VSMCs

We employed a previously described method that facilitates adenoviral vector construction [18]. Briefly, a ~1.4 kb segment of a mouse PPARδ gene coding sequence was amplified by RT-PCR from mouse total RNA and then cloned into pCMVTrack, an adenovirus shuttle vector. This plasmid was co-transformed with AdEasy, a backbone of the adenovirus, into E.coli. The clone from *in vivo* recombination was isolated, digested and used for transfection in the packaging cell line, HEK 293. Transfected cells were monitored for GFP expression and collected 7-10 days later by scraping and pelleting along with any floating cells in the culture. After amplification in HEK 293 cells, viruses were purified by a CsCl gradient; final yields were generally $10^{11}$ to $10^{12}$ plaque-forming units. The generated adenovirus was used to infect VSMCs for 48-72 h, and the expression levels of the transgene were determined by immunoblotting. Comparisons were made using control adenoviral vectors with empty inserts or green fluorescent protein (GFP) [5].

Retrovirus-mediated loss-of-PPARδ function in VSMCs

A retrovirus vector carrying shRNA targeting the mouse PPARδ gene (pRS-shPPARδ) or a non-effective 29-mer shGFP cassette (pRS-shGFP) was purchased directly from the Origene company (Origene, Rockville MD). For production of a high-titer retrovirus vector expressing PPARδ small hairpin RNA, the recombinant vector was transfected into EcoPack-293 cells and

incubated at 32°C for 2 d. The supernatant fraction containing the viral vector was collected to infect VSMCs for 3 h. The supernatant fraction was then removed and replaced with normal growth medium for 48-72 h. Infected populations exhibiting between 70-90% green fluorescent cells were used for further experimentation [11].

Chromatin Immunoprecipitation (ChIP) Assay

Mouse VSMCs were infected with an adenovirus carrying mouse PPARδ or GFP. After 48 h, cells were fixed and a ChIP assay was performed by using the EZ-ChIP assay kit from Upstate Biotechnology (Lake Placid, NY). The size of the sonicated DNA fragments subjected to immunoprecipitation was 0.5-1 kb as determined by ethidium bromide gel electrophoresis. Purified chromatin was immunoprecipitated using an anti-PPARδ antibody (Santa Cruz). Eluted DNA fragments were purified to serve as templates for PCR amplification. The input fraction corresponded to 2% of the chromatin solution before immunoprecipitation. By using bioinformatics software to analyze the mouse MMP-9 promoter, a potential PPARδ binding site (PPRE) was identified, which is located -944/-921 bp upstream of the transcription start site. Primers used to amplify the area containing this PPARδ binding site are: Forward primer 5′-aagcacatgaaggtctgggcacac-3′ and reverse primer 5′- tgacctggatggggcaaacacc-3′, resulting in a 236 bp fragment. To monitor the specificity of each assay, ChIP experiments with normal IgG immunoprecipitates served as background control [5]. To further confirm the specificity of the ChIP assay, a nonspecific region distal to the PPRE site (at nucleotides -3613 to -3282 of the MMP-9 promoter) was also amplified by PCR using the following primers: Forward primer 5′-aagtgttggtgcggctaagttgaa-3′ and reverse primer 5′-cctgggtgagtgggaagagaaga-3’.

Plasmid constructs and luciferase assays

A 1,309 bp segment from the promoter region (-1253/+55) of the mouse MMP-9 gene (MMP-9 PPRE WT) was amplified by PCR from mouse genomic DNA and then cloned into the *Kpn I/Xho I* site of the pGL 4.10 Luciferase vector (Promega). The following primer sets were used to generate specific fragments: Forward, 5'-cggggtaccaaggtggttgggaaatgacgaggttg-3'; reverse, 5'-ccgctcgagtgcagccgaaagccaggagagc-3'. According to bioinformatics analysis, a potential PPAR binding site (PPRE) at locations of -944/-921 bp was identified in this promoter fragment. Thus, we also generated a mutant MMP-9 promoter construct (MMP-9 PPRE mut.) with substitutions of 4 bp respectively from the PPRE site of perfect complementarity by using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The sequence of the mutant PPRE MMP-9 promoter segment contained 5'-ctggaggAttGGAattGTCCatg-3' (the four underlined nucleotides are mutated). Wild-type and mutant inserts were confirmed by sequencing [5].

MMP-9 promoter plasmids (MMP-9 PPRE WT or PPRE mut.) together with the *Renilla* luciferase control reporter vector (pRL-TK) were transfected into HEK 293 cells with lipofectamine 2000 (Invitrogen) for 4 h, then changed to normal growth medium for 48 h. HEK 293 cells were also co-infected with an adenovirus carrying mouse PPARδ or GFP for 48 h. Promoter activity was examined using the Dual-Luciferase Assay Kit (Promega) with a TD-20/20 luminometer (Turner Designs). MMP-9 promoter-firefly luciferase activity was normalized to the TK promoter–*Renilla* luciferase activity [5]. In parallel experiments, either the specific PPARδ agonist GW 501516 (1μM) or the specific PPARδ antagonist GSK 0660 (1μM) [19], was added 12 h prior to cell harvest for promoter activity assay.

Statistical analysis

Quantitative data are expressed as mean ± SD or SEM based on at least three independent experiments of triplicate samples. Differences among three or more groups were

statistically analyzed by one-way analysis of variance followed by Bonferroni's post hoc test. Comparisons between two experimental groups were based on a two-tailed t-test. A *p-value* less than 0.05 was considered significant.

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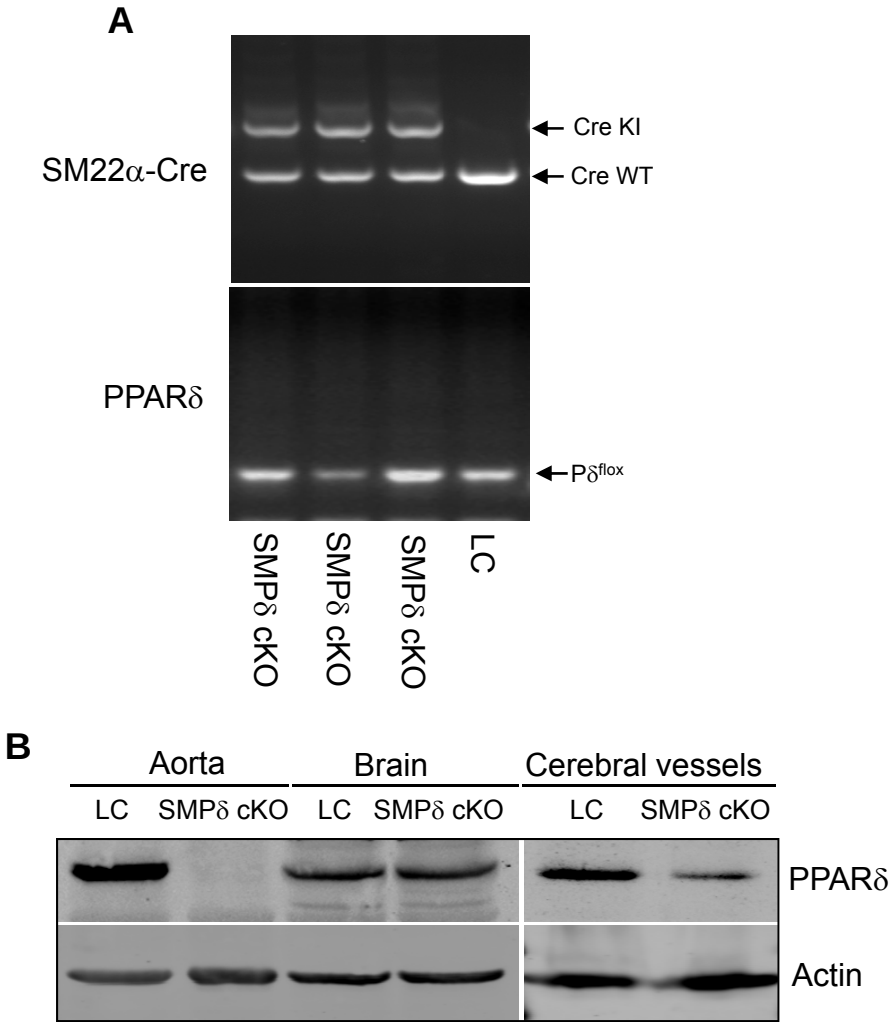
**SUPPLEMENTAL FIGURE LEGENDS**

**Supplementary Figure I: Identification of SMPδ cKO mice.** (A) Genomic PCR genotyping showed that VSMC-selective PPARδ knockout (SMPδ cKO) mice and littermate controls (LC) were generated by crossing SM22α-Cre knock-in mice with PPARδ$^{flox/flox}$ mice. (B) Western blot analysis showing PPARδ expression in lysates from aortas, brains, and cerebral vessels of SMPδ cKO and LC mice. Cre-mediated recombination in VSMCs led to a lack of PPARδ expression in the aortic SMC layer but not in the brains of SMPδ cKO mice. Also, a significant reduction of PPARδ levels in the cerebral vessels of SMPδ cKO mice is evident. Results shown are representative of three separate experiments with similar results.

**Supplementary Figure II: Expression of pro-inflammatory genes in brains of SMPδ cKO after focal cerebral ischemia.** Quantitative PCR data shows that the expression of indicated cytokines was significantly increased in VSMC-selective PPARδ knockout (SMPδ cKO) mice after transient MCAO and 24 h of reperfusion when compared with littermate control (LC) mice. * $P<0.05$ vs LC group. Data are expressed as mean $\pm$ SEM from three separate experiments in triplicate.

**Supplementary Figure III: Adenoviral-mediated PPARδ gene transfer in mouse VSMCs.** (A) Fluorescence microscopic observation indicates a successful infection of the adenovirus carrying PPARδ or GFP into mouse VSMCs. Scale bar, 50μm. (B) Upregulation of PPARδ in mouse VSMCs after adenoviral-mediated gene transfer is confirmed by Western blotting. (C) Knockdown of PPARδ in mouse VSMCs after retrovirus-mediated RNA interference is confirmed by Western blotting. Results shown are representative of three separate experiments with similar results.

**Supplementary Table I: The specific primers for real time PCR**




Supplemental Figure I

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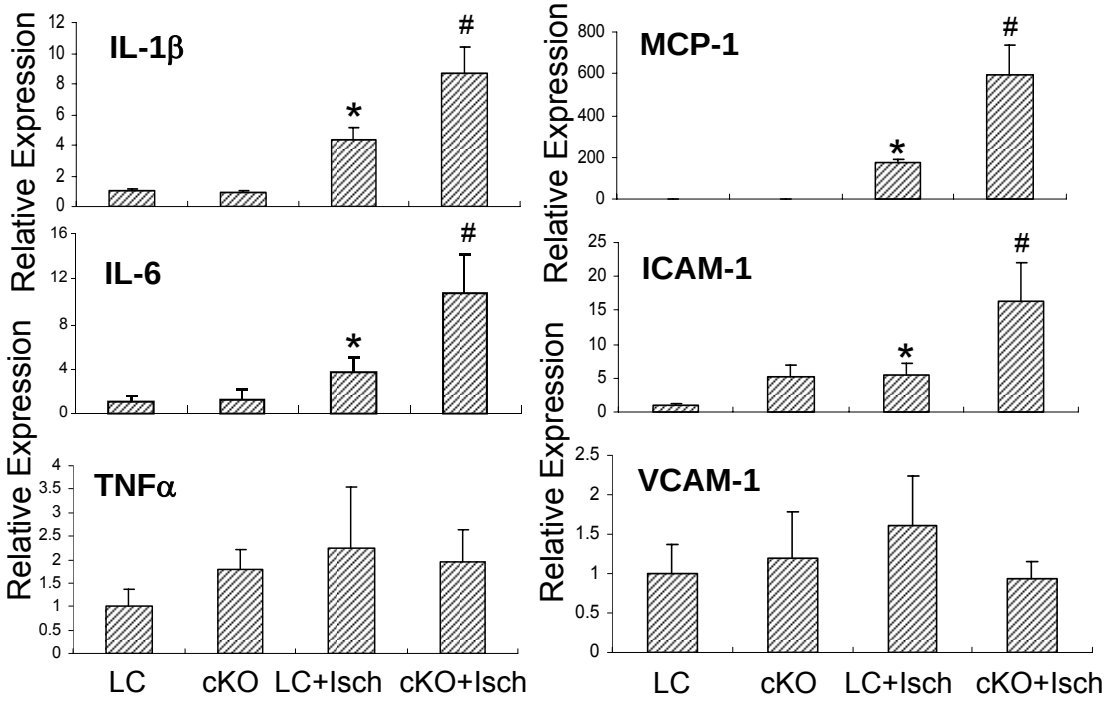


Supplemental Figure II

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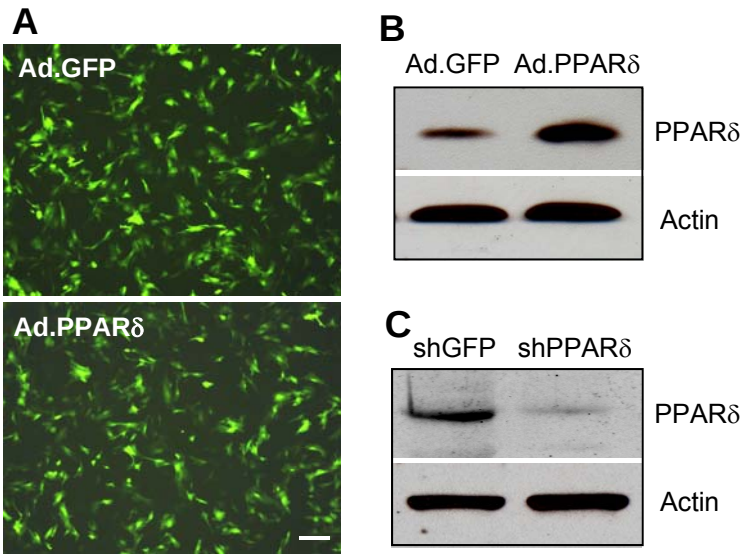


Supplemental Figure III

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## Table I: PCR primers

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| PPARδ | 5’-AGGCCCGGGAAGAGGAGAAAGAGG-3' | 5'-CGCGTGGACCCCGTAGTGGA-3' |
| IL-1β | 5’-AGGAGAACCAAGCAACGACAAAATAC-3’ | 5’-TGGGGAACTCTGCAGACTCAAACT-3’ |
| IL-6 | 5’-AGTTGCCTTCTTGGGACTGA-3’ | 5’-TCCACGATTTCCCAGAGAAC-3’ |
| TNFa | 5’-CTCCTCACCCACACCGTCAGC-3’ | 5’-AACACCCATTCCCTTCACAGAGCA-3’ |
| MCP-1 | 5’-GCACCAGCACCAGCCAACTCTCACT-3’ | 5’-CATTCCTTCTTGGGGTCAGCACAG-3’ |
| ICAM-1 | 5’-TTCACACTGAATGCCAGCTC-3’ | 5’-GTCTGCTGAGACCCCTCTTG-3’ |
| VCAM-1 | 5’-ATTTTCTGGGGCAGGAAGTT-3’ | 5’-ACGTCAGAACAACCGAATCC-3’ |
| MMP-2 | 5’-GTCGCCCCTAAAACAGACAA-3’ | 5’-GGTCTCGATGGTGTTCTGGT-3’ |
| MMP-9 | 5’-CGTCGTGATCCCCACTTACT-3’ | 5’-AACACACAGGGTTTGCCTTC-3’ |
| 18S RNA | 5’-CATTCGAACGTCTGCCCTATC-3’ | 5’-CCTGTGCCTTCCTTGGA-3’ |

Supplemental Table I

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