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PPAR-γ Promotes Endothelial Cell Migration By Inducing the Expression of Sema3g

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ABSTRACT

In addition to regulating lipid and glucose metabolism, the nuclear receptor PPAR-γ has emerged as a potentially relevant player in regulating endothelial cell function. Despite the identification of numerous PPAR-γ targets involved in vascular development, the targets downstream of PPAR-γ that directly affect endothelial cell function remain to be elucidated. In this report, we identify Sema3g as a novel PPAR-γ-regulated gene playing a substantial role in endothelial biology, particularly with respect to endothelial cell migration. Sema3g expression is induced by either overexpression of PPAR-γ or PPAR-γ ligands treatment in human umbilical vein endothelial cells (HUVECs). Chromatin immunoprecipitation (ChIP) and transient transfection assays revealed that PPAR-γ binds to the Sema3g promoter and activates transcription. Furthermore, we show that overexpression of Sema3g augments PPAR-γ-driven HUVECs migration, whereas silencing of Sema3g expression almost completely abrogates PPAR-γ or Sema3g-mediated cell migration. Accordingly, the anti-neuropilin-2 (Sema3g receptor) neutralizing antibody treatment markedly inhibits Sema3g-induced cell migration. Collectively, these results identify Sema3g as one of the downstream effectors of PPAR-γ, which is centrally involved in regulating endothelial cell migration. J. Cell. Biochem. 116: 514–523, 2015.

KEY WORDS: Semaphorin3g; PPAR-γ; TRANSACTIVATION; ENDOTHELIAL CELL; MIGRATION

Angiogenesis is a precise process between pro- and anti-angiogenic mediators, and results in the formation of blood vessel networks. Endothelial cell migration is an important process in angiogenesis that involves three major mechanisms, namely, chemotaxis, haptotaxis, and mechanotaxis [Lamalice et al., 2007]. Numerous cytokines are involved in the regulation of endothelial cell migration during angiogenesis. The three major promoters of this type of actin-based motility are VEGF, bFGF, and angiopoietins [Stratman et al., 2011]. Other contributing cytokines include: hepatocyte growth factor, platelet-derived growth factor, epidermal growth factor, and transforming growth factor. Published data suggest that axon guidance molecules such as Ephrins, Netrins, Semaphorins, and Slits are also involved in the formation of vascular networks.

Peroxisome Proliferator-Activated Receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. PPARs are divided into at least three distinct subtypes: PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-γ forms a heterodimer with another nuclear receptor, retinoid X receptor (RXR). The PPAR-γ/RXR heterodimers can be activated by either PPAR-γ or RXR ligands. These heterodimers bind to specific DNA sequences—termed PPAR response elements (PPREs)—in the

Abbreviations: Ad-GFP, adenovirus expressing the green fluorescence protein; Ad-PPAR-γ, adenovirus expressing PPAR-γ; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; HUVECs, human umbilical vein endothelial cells; mRNA, messenger RNA; Nrp, neuropilin; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator responsive element; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; Sema, semaphorin; TESS, transcription element search system.

All authors have read the manuscript and have no conflict of interest to declare.

Weiwei Liu and Jingjin Li contributed equally to this work.

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regulatory regions of target genes (McKenna and O'Malley, 2002). PPAR-γ is mainly expressed in adipose tissue and plays an important role in lipid metabolism, adipogenesis, and glucose homeostasis [Guan et al., 2005]. However, PPAR-γ is also expressed in vascular cells, including endothelial cells, and mediates migration of human umbilical vein endothelial cells (HUVECs) [Lehrke and Lazar, 2005]. PPAR-γ has been recently recognized as an important regulator in endothelial biology [Duan et al., 2008]. Thiazolidinediones, a class of oral antidiabetic agents, are selective agonists of PPAR-γ. In addition to their function in adipogenesis and increasing insulin sensitivity, thiazolidinediones also play an important role in regulating endothelial migration and angiogenesis [Duan et al., 2008].However, details of the molecular mechanisms responsible for thiazolidinediones-induced endothelial migration remain enigmatic.

Semaphorins are secreted, membrane-bound proteins and are potent neural axon guidance factors. More than 20 semaphorin genes have been identified in mammals. All semaphorin proteins have a conserved Sema domain of about 500 amino acids at their amino terminus and fall into eight semaphorin subclasses based on their structural features [Quinn et al., 1999]. Class-3 semaphorins are found in vertebrates only. They are composed of seven members ranging from Sema3a to Sema3g, a large and diverse group of proteins that function as potent repellents or attractants during neural development [Yazdani and Terman, 2006]. Recent studies have revealed that several semaphorins (Sema3a-g, 4d, and 6a) also significantly affect endothelial cell migration, proliferation, adhesion, and survival [Adams and Eichmann, 2010].

Expression of Class-3 semaphorins is controlled by different signaling pathways; cis-elements and trans-acting factors involved in the induced expression of Class-3 semaphorins are quite diverse. For example, Sema3b has been reported to be a direct transcriptional target of p53, suggesting that Sema3b may be involved in p53–dependent cell growth regulation [Ochi et al., 2002]. GATA6, a zinc finger transcription factor, has been found to directly regulate Sema3c expression in the cardiac neural crest, while Sema3c expression in these cells is essential for cardiovascular morphogenesis [Lepore et al., 2006]. A recent study has shown that Id2 (inhibitor of DNA binding/differentiation 2) represses Sema3f expression, which induces tumor cell migration and invasion [Coma et al., 2010]. Although Sema3g was found to be expressed in different cell types, no detailed study of transcriptional regulation of the Sema3g gene in human endothelial cells has yet been reported. To investigate transcriptional regulation of Sema3g, we analyzed the promoter region sequence of Human Sema3g using the Transcription Element Search System (TESS) and found a motif highly similar to canonical Peroxisome proliferator-gamma-responsive elements (PPRE) in the 5′ flanking region of the human Sema3g gene. Bioinformatics-based analysis indicated that Sema3g may be one of the PPAR-γ target genes.

This study was initiated to determine whether PPAR-γ promotes endothelial cell migration by inducing Sema3g expression. We demonstrate that PPAR-γ directly regulates the expression of Sema3g at the transcriptional level, which appears to be critical for the motility of endothelial cells in response to PPAR-γ activators.

Our results indicate that PPAR-γ activation promotes endothelial cell migration at least partially through the Nrp2–Sema3g signaling pathway.

METHODS

CELL LINES AND CELL CULTURE

Human umbilical vein endothelial cells (HUVECs) were cultured at 37°C in a humidified incubator with 5% CO_2 in Medium 199 (M199) supplemented with 20% fetal bovine serum, 20 mM HEPES, 1 ng/ml of recombinant human FGF-1, and 90 μg/ml of heparin and antibiotics. Bovine aortic endothelial cells (BAECs) were from bovine aorta and cultured in DMEM with 10% FBS. Cells were from national infrastructure of cell line resources of China. Human samples procedure conforms to the principles outlined in the Declaration of Helsinki, The procedures received approval from the ethics review board of Peking University Health Science Center.

REAL-TIME SEMI-QUANTITATIVE-PCR

Total cellular RNA was extracted from HUVECs with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed into cDNA using Superscript II reverse transcriptase and an oligo-dT primer (Invitrogen). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) using specific primers for Sema3g and GAPDH genes were performed on an Opticon continuous fluorescence detection system (MJ Research Inc, Waltham, MA) with SYBR green fluorescence (Molecular Probes, Eugene, OR). The primers (Table I) were used for the detection of Sema3g mRNA expression. GAPDH were used for internal amplification control. Gene expression was quantified by using the comparative CT method, normalized to GAPDH, and expressed as fold induction of control.

RNA INTERFERENCE

Short interfering RNAs specific for Human Sema3g gene silencing were synthesized (Gene Pharma, Shanghai, China) (Table I), annealed, and transfected into HUVECs using Lipofectamine 2,000 (Invitrogen) as previously described [Toyofuku et al., 2005].

ADENOVIRAL INFECTION

Cells were infected with adenoviruses together with Ad-tTA, which encodes a tetracycline-responsive transactivator. These adenoviruses construction and infect cells presented with or without tetracycline(0.1 μg/ml, a tet-off expression) as previously described [Wang et al., 2002]. The 78 amino acid VP16 transactivation domain fused to the gene. The cDNA fragment was subcloned into a shuttle plasmid pAdlox and recombined with an E1 and E3 deleted ψ5 viral DNA in CRE8 cells. The tetracycline-responsive transactivator (tTA) control the expression in a tet-off manner.

IMMUNOBLOTTING AND IMMUNOPRECIPITATION

Whole-cell extracts from HUVECs were prepared using a lysis buffer containing 20 mM Tris, 150 mM NaCl, 1% NP-40, 1% SDS, 5% glycerol, and the Complete-Mini protease inhibitor cocktail tablet

TABLE I. Sequences for Primers and Probes

Gene		Sequences
Primer for PCR		
Human semaphorin 3g	forward	5′-ACAGTACCATGGGTAGGGTGTG-3′
	reverse	5′-TAACCTATCAACGCCCTGAGAA-3′
Human GAPDH	forward	5′-ACCACAGTCCATGCCATCAC-3′
	reverse	5′-TCCACCACCCTGTTGCTGTA-3′
Primers for ChIP assay		
Semaphorin 3g PPRE-1		5′-CTCTCTCCCCACTTGTCACTTT-3′
		5′-GCTTCTCCTTCCACCTTGTTG-3′
Semaphorin 3g PPRE-2		5′-TTTGGAAGGTCTCTGCTGTG-3′
		5′-AGATGTCCACAAATGCAGGTC-3′
Semaphorin 3g PPRE-3		5′-GCTGGGTGTCTGGCTGAG-3′
		5′-GACTGCTTCTGTCTCTGTTTCC-3′
siRNA		
Semaphorin siRNA	sense	5′-GCGGGUGCUGGUGAACAAATT-3′
	anti-sense	5′-UUUGUUCACCAGCACCCGCTG-3′

(Roche, Nutley, NJ). Cells were lysed on ice for 60 min and insoluble material was pelleted by centrifugation at 16,000*g* for 10 min. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Rabbit anti-human polyclonal Sema3g antibody (Sigma) and mouse anti-human monoclonal β-actin antibody were used for immunoblotting. At the indicated time, supernatant from HUVECs were incubated with the anti-human Sema3g antibody. After an overnight incubation with protein A-coupled Sepharose beads, the immunoprecipitates were electrophoresed on an 8% SDS–polyacrylamide gel (SDS–PAGE) and transferred to a nitrocellulose membrane. Immunoprecipitated proteins were blotted with the anti-human Sema3g antibody, followed by detection using a horseradish peroxidase-labeled secondary antibody and an ECL chemiluminescence kit (Amersham, Piscataway, NJ).

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAYS

Chromatin Immunoprecipitation (ChIP) assay was performed according to the method recommended by the manufacture (CST, MA), with minor modifications. In brief, a plate of cells was fixed with 1% formaldehyde in isotonic phosphate buffer for 10 min at room temperature. The reaction was then stopped by adding glycine to a final concentration of 0.125 M. A soluble chromatin fraction containing fragmented DNA of 500–2,000 bp was obtained after cell lysis and sonication. The fraction was diluted ten times and pre-cleared with Protein A-agarose slurry. Samples were incubated with Rabbit anti-human PPAR-γ (Santa Cruz) or rabbit IgG (as a negative control), followed by immunoprecipitation. DNA was used as a template for PCR to amplify the region of the PPAR-γ binding sites in the Sema3g promoter. Specific primers were shown in Table I.

PLASMIDS CONSTRUCTION AND LUCIFERASE ACTIVITY ASSAY

The 5′ flanking region of Human Sema3g spanning +/− 2,000 bp was amplified by PCR from the genomic DNA with specific primers and subcloned into the pGL3 basic vector (Promega, Madison, WI) to generate Sema3g-Luc. The mut-Sema3g-Luc (negative control) was generated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as described [Zhang et al., 2008]. The putative PPRE sites (at −769 and −1234) were disrupted with the mutagenic primers respectively. HUVECs were placed in well plates 24 h before transfection. Reporter plasmids were transfected using the Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol pRSV-β-galactosidase (Promega) was cotransfected as an internal control. Twenty-four hours after transfection, the cells were treated with rosiglitazone, GW9662 (a PPAR-γ antagonist) or DMSO (vehicle control). The cells were lysed 24 h after drug treatment and subjected to a luciferase assay according to standard protocols. All experiments were performed at least three times. Luciferase activity was calculated as the activity of the reporter constructs compared to the β-galactosidase activity.

HUVECs MIGRATION AND INVASION ASSAYS

The migration of HUVECs was assessed by scratch-wound assay as described previously [Xu et al., 2012]. HUVECs invasion was analyzed using modified Boyden chambers (Kurabo, Osaka, Japan). Cell culture supernatants were placed in the bottom wells of the chambers. Polycarbonate filters with 8-mm pores were coated with 50 mg/ml collagen and placed between the bottom wells and the upper wells of the chambers. Cultured HUVECs were harvested by trypsinization, washed twice with M199, resuspended in M199 containing 1% BSA, plated at a density of 2.5×10^5 cells/well in the upper wells of the chambers and incubated for 5 h at 37°C under a humidified atmosphere of air containing 5% CO_2. After incubation, a cotton ball was used to remove the cells that did not migrate from the upper side of the filters. The filters were then fixed with 100% methanol for 10 min, mounted onto microscope slides, and stained with Giemsa solution. Cells that had migrated to the bottom side of the filters were visualized under a microscope (100× magnification) and counted. Ad-Sema3g- and Ad-GFP-infected (100:1 multiplicity of infection) HUVECs were used at 24 h post-infection. Meanwhile, supernatants were collected for use.

IMMUNOFLUORESCENCE

To assess actin polymerization (F-actin formation), HUVECs were stained with rhodamine-phalloidin. Briefly, the HUVECs were fixed with 3% buffered formaldehyde (pH 7.0) and then stained for 120 min at room temperature with a solution of 10 U/ml of rhodamine-phalloidin in PBS containing 0.1% Triton X-100 and 1% BSA. After washing with PBS, several drops of 90% glycerol/10% PBS containing 0.1 M N-propylgallate were added and the membrane was covered with a coverslip. The cells were examined using a confocal laser scanning microscope system (LSM 410, Zeiss, Oberkochen, Germany) coupled to a Axioverd 135 fluorescence microscope (Zeiss).

STATISTICAL ANALYSES

All values are expressed as mean ± SEM. Statistical differences were analyzed using Student *t*-test (paired groups) or one-way ANOVA (multi-group comparisons). A P-value < 0.05 was considered to indicate statistical significance.

RESULTS

PPAR-γ INDUCES EXPRESSION OF Sema3g mRNA IN HUVECs

To examine the effect of PPAR-γ on Sema3g mRNA expression, HUVECs were treated with rosiglitazone, a PPAR-γ agonist at different concentrations (1–20 μM) or different durations (20 μM

from 3 to 24 h) or were infected with Ad-PPAR-γ. Expression levels of Sema3g mRNA were measured by qRT-PCR. Of note, the mRNA levels of Sema3g were significantly increased (by approximately five-fold) after rosiglitazone treatment at 20 μM for 24 h (Fig. 1A,B). Pretreatment with GW9662, an antagonist of PPAR-γ, blocked rosiglitazone-induced Sema3g mRNA expression (Fig. 1C). Taken together, these results indicate that rosiglitazone upregulated Sema3g mRNA expression depends on PPAR-γ activation).

PPAR-γ PROMOTES Sema3g PROTEIN EXPRESSION IN HUVECs

To explore the effect of PPAR-γ on Sema3g protein expression, HUVECs were treated with rosiglitazone or infected with Ad-PPAR-γ. Total and secreted protein levels of Sema3g were assessed by western blot. Consistent with the above-mentioned mRNA expression, the total and secreted protein levels of Sema3g were significantly increased (by threefold) using incubation with rosiglitazone at 20 μM for 36 h (Fig. 2A). Pretreatment with GW9662 blocked rosiglitazone-induced Sema3g protein expression (Fig. 2B). These results demonstrate that PPAR-γ induces not only total but also secreted Sema3g protein expression in HUVECs.

PPAR-γ TRANSACTIVATES Sema3g

Since PPAR-γ activation increases Sema3g mRNA expression, we hypothesize that Sema3g may be a transcriptional target of PPAR-γ. Nucleotide-sequence analysis indicated that the promoter region of HUVECs Sema3g gene harbors three putative PPAR-γ response elements, named PPRE-1, -2 and -3 (Fig. 3A). To determine whether PPAR-γ binds to the Sema3g promoter in vivo, chromatin immunoprecipitation (ChIP) assays were performed. PCR primers were designed to flank these sites, and binding was assessed by enrichment of the PCR signal in the anti-Sema3g sample compared to the no-antibody and control IgG reaction mixtures. PCR amplification of a region that flanks another PPRE-1 site failed to show enrichment in binding signal in HUVECs. The two other consensus sites (PPRE-2 and PPRE-3) were also examined, and strong enrichment in binding signals was observed (Fig. 3B). These results indicate that PPAR-γ binds to the Sema3g promoter in vivo and that this binding is restricted to certain regions of the promoter. To assess the regions on the Sema3g promoter that are required for PPAR-γ-dependent activation, we conducted a series of luciferase-reporter assays. Luciferase constructs that carry wild-type and

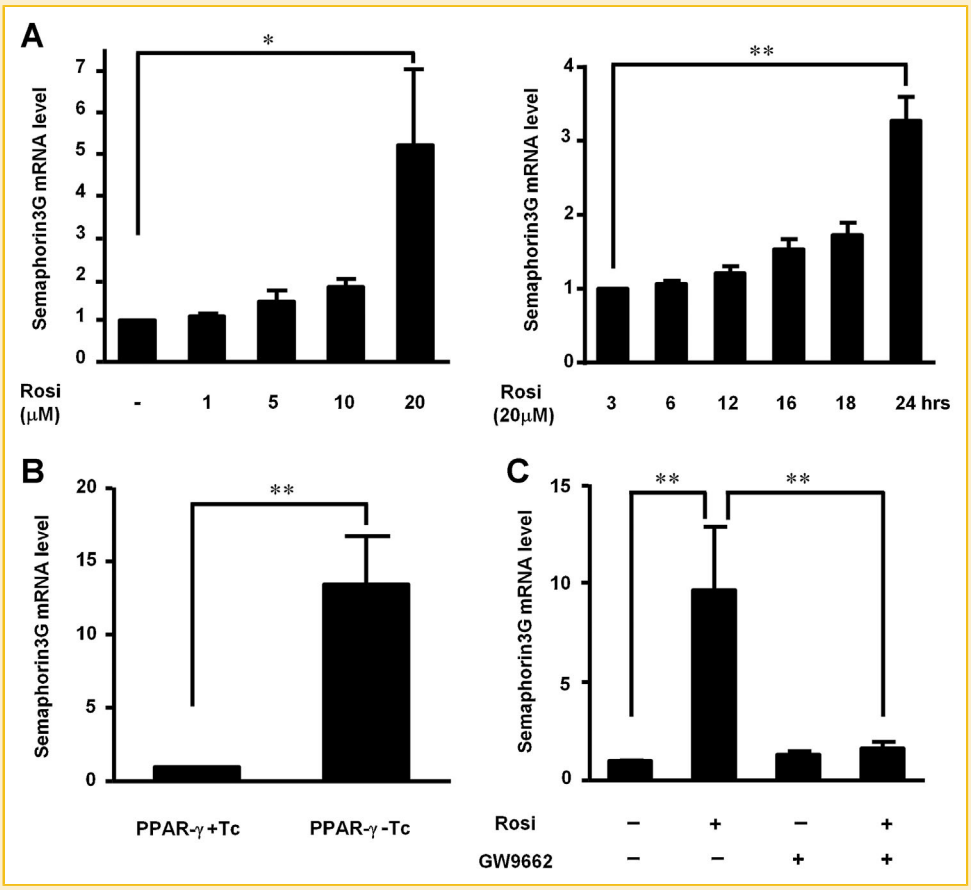

Fig. 1. PPAR-γ agonist (rosiglitazone) upregulated Sema3g mRNA expression is dependent on PPAR-γ activation. A: HUVECs were treated with rosiglitazone for the indicated time periods (20 μM for 0–24 h) or with the indicated doses (0–20 μM) for 24 h. B: HUVECs were infected with Ad-PPAR-γ and Ad-tTA (Tc:Tetracycline controlled transactivator). C: HUVECs were pretreated with GW9662 for 1 h and then treated with DMSO or rosiglitazone (20 μM). The relative mRNA level of the Sema3g gene was determined using qRT-PCR and, after normalization with GAPDH, expressed as the fold induction in relation to the control. Data represent mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control.

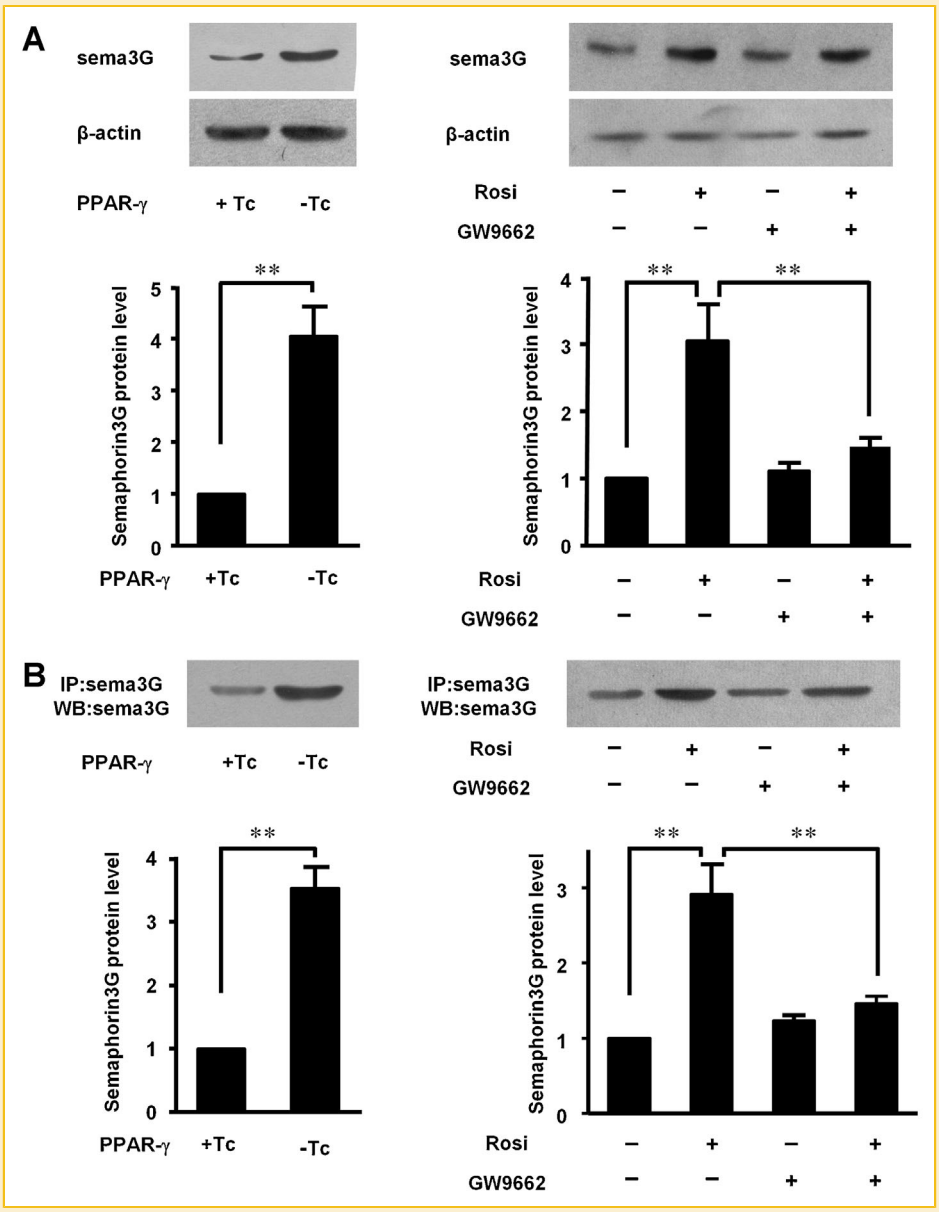

Fig. 2. PPAR-γ induces not only total Sema3g protein but also secreted Sema3g protein in HUVECs. A: HUVECs were infected with Ad-PPAR-γ and Ad-tTA, treated with GW9662 or not for 1 h, and exposed to DMSO or rosiglitazone (20 μM) for 24 h. Total protein levels of Sema3g were quantified by western blot and normalized to β-actin. B: HUVECs were treated the same as A. HUVEC supernatant underwent immunoprecipitation of Sema3g and secreted protein levels of Sema3g were quantified by western blot. Relative Sema3g protein was normalized to β-actin and expressed as fold of control. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

mutant segments of the Sema3g promoter were transiently transfected along with PPAR-γ or empty vector into HUVECs. We observed PPAR-γ-dependent activation of the wild-type luciferase-reporter construct: Ad-PPAR-γ transactivated the Sema3g promoter by 8-fold (Fig. 3C). Rosiglitazone treatment increased the Sema3g promoter activity by fivefold. To further examine the specificity of positive PPRE in the Sema3g promoter region, we conducted site-directed mutagenesis to construct mutant Sema3g promoters. As shown in Fig. 3D, the mutation of PPRE-2 (mPPRE-2) slightly reduced the PPAR response to fourfold (versus 5-fold) when the cells were co-transfected with mPPRE-2 or wild-type PPRE with PPAR-γ, respectively. PPAR-γ did not significantly activate the luciferase activity of mutation of PPRE-3 (mPPRE-3). These results indicate that PPAR-γ binds to PPRE-2 and PPRE-3 in the Sema3g promoter region. (Fig. 3D).

Sema3g PROMOTES ENDOTHELIAL CELL MIGRATION AND INVASION VIA Nrp2

To investigate the role of Sema3g in endothelial cell migration, scratch-wound assays and transwell assays were performed. HUVECs were infected with Ad-Sema3g or Ad-GFP at 100:1 multiplicities of infection. Ad-Sema3g-infected HUVECs

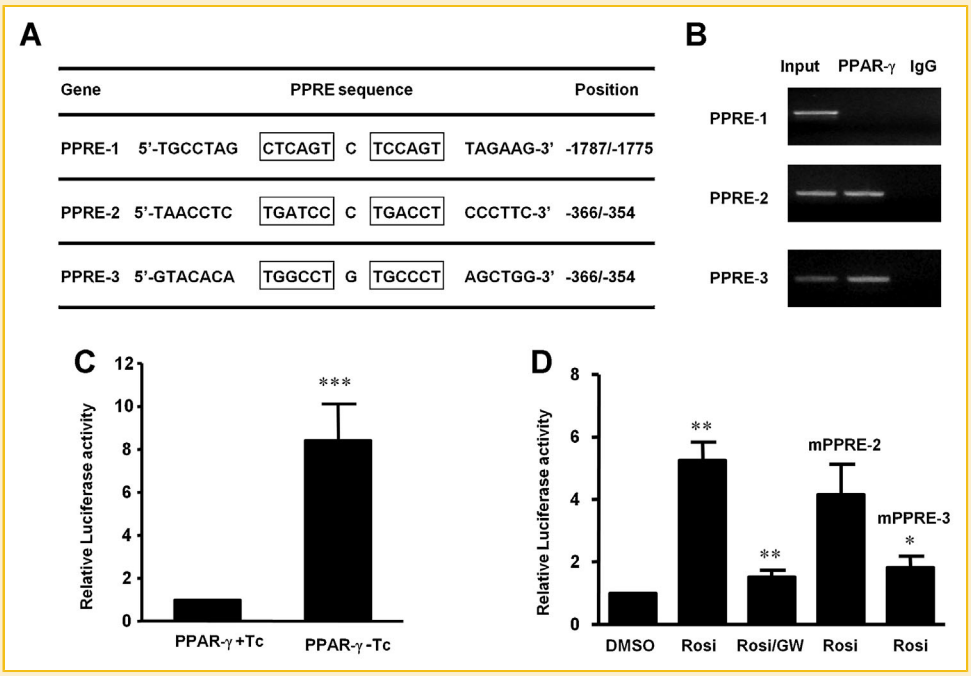

Fig. 3. ChIP assay and luciferase activity assay showed PPAR-γ bound to putative PPRE-2 and PPRE-3 in Sema3g promoter region. A: The diagram depicts the putative PPRE motifs located in the 5′ flanking region of the Human Sema3g gene. B: BAEC cells were coinfected with Ad-PPAR-γ and Ad-tTA for 24 h. ChIP assays were performed with the antibodies against PPAR-γ or IgG as control. The immunoprecipitates and the positive input control were amplified with the use of specific primer sets spanning the DNA segments containing the putative PPRE-1, PPRE-2, and PPRE-3. C: BAEC cells transfected with the Sema3g-PPRE-Luc, and the β-galactosidase plasmids were treated with Ad-PPAR-γ and Ad-tTA. Luciferase activity was normalized to b β-galactosidase activity and expressed as fold induction compared with control. D: BAEC cells transfected with the Sema3g-PPRE-Luc or Sema3g-mPPRE-Luc, and the β-galactosidase plasmids were treated with DMSO (control) or rosiglitazone (20 μM) with or without GW9662 (GW, 10 μM). Luciferase activity was normalized to β-galactosidase activity and expressed as fold induction compared with control. Data are mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. $\#P < 0.05$, $\#\#P < 0.01$ versus rosiglitazone.

significantly increased Sema3g mRNA and protein levels compared with Ad-GFP-infected HUVECs (Fig. 4A). Ad-Sema3g-infected HUVECs markedly enhanced endothelial cell migration in comparison with Ad-GFP-infected cells at 12 h in the scratch-wound assay (Fig. 4B). The number of cells that migrated to the lower chamber was significantly increased (by 1.4-fold) in the Ad-Sema3g-infected HUVECs compared to the Ad-GFP control (Fig. 4C). In contrast to Ad-GFP-infected cells, Ad-Sema3g-infected significantly enhanced HUVECs' migration. To examine if Nrp2 receptor would mediate Sema3g-induced cell migration, we used the Nrp2 neutralizing antibody. Treatment of HUVECs with anti-Nrp2 antibody significantly inhibited Ad-Sema3g-mediated cell migration (Fig. 4D). Collectively, these results strongly suggest that Sema3g can bind to Nrp2, which involves endothelial cell migration and invasion.

KNOCKDOWN OF Sema3g SUPPRESSES ENDOTHELIAL CELL MIGRATION

To examine whether knockdown of Sema3g affects endothelial cell migration and invasion, HUVECs were transfected with the Sema3g siRNA or scrambled siRNA. Sema3g siRNA transfection successfully inhibited Sema3g expression at both mRNA and protein level in HUVECs (Fig. 5A). Sema3g siRNA transfected HUVECs showed a significantly reduced endothelial migration compared with scrambled siRNA transfected HUVECs in the scratch-wound assay (Fig. 5B). Sema3g siRNA suppressed HUVECs migration by up to 50% (in cell numbers) compared to scrambled siRNA in a transwell assay (Fig. 5C). Scratch-wound assay demonstrated knockdown of Sema3g-attenuated, rosiglitazone-induced endothelial cell migration. These results reveal that PPAR-γ promotes endothelial cell migration, probably through Sema3g signaling (Fig. 5D).

Sema3g CAUSES LAMELLIPODIA FORMATION IN Rac1 DEPENDENT MANNER

Endothelial cell migration exhibits constant remodeling of the actin cytoskeleton, including the formation of filopodia, lamellipodia, and stress fibers. In order to explore cytoskeletal rearrangement in endothelial cells, we stained actin fibers with TRITC-labeled phalloidin. We found that FGF induced the formation of actin stress fibers and the protrusion of lamellipodia in HUVECs (supplementary Fig. S1B at arrowheads) and Ad-GFP-infected HUVECs displayed normal actin fibers, whereas Ad-Sema3g-infected HUVECs induced the formation of lamellipodia (supplementary Fig. S1D,E). To determine whether Rac1 is required for the cytoskeletal rearrangement mediated by FGF and Sema3g, we examined the effect of a Rac1 inhibitor on FGF and Ad-Sema3g-mediated morphological changes in HUVECs. The FGF and Ad-Sema3g-mediated protrusion of lamellipodia was suppressed by a Rac1 inhibitor (supplementary Fig. S1C, F). These results indicate that Sema3g can cause lamellipodia formation through Rac1 activation.

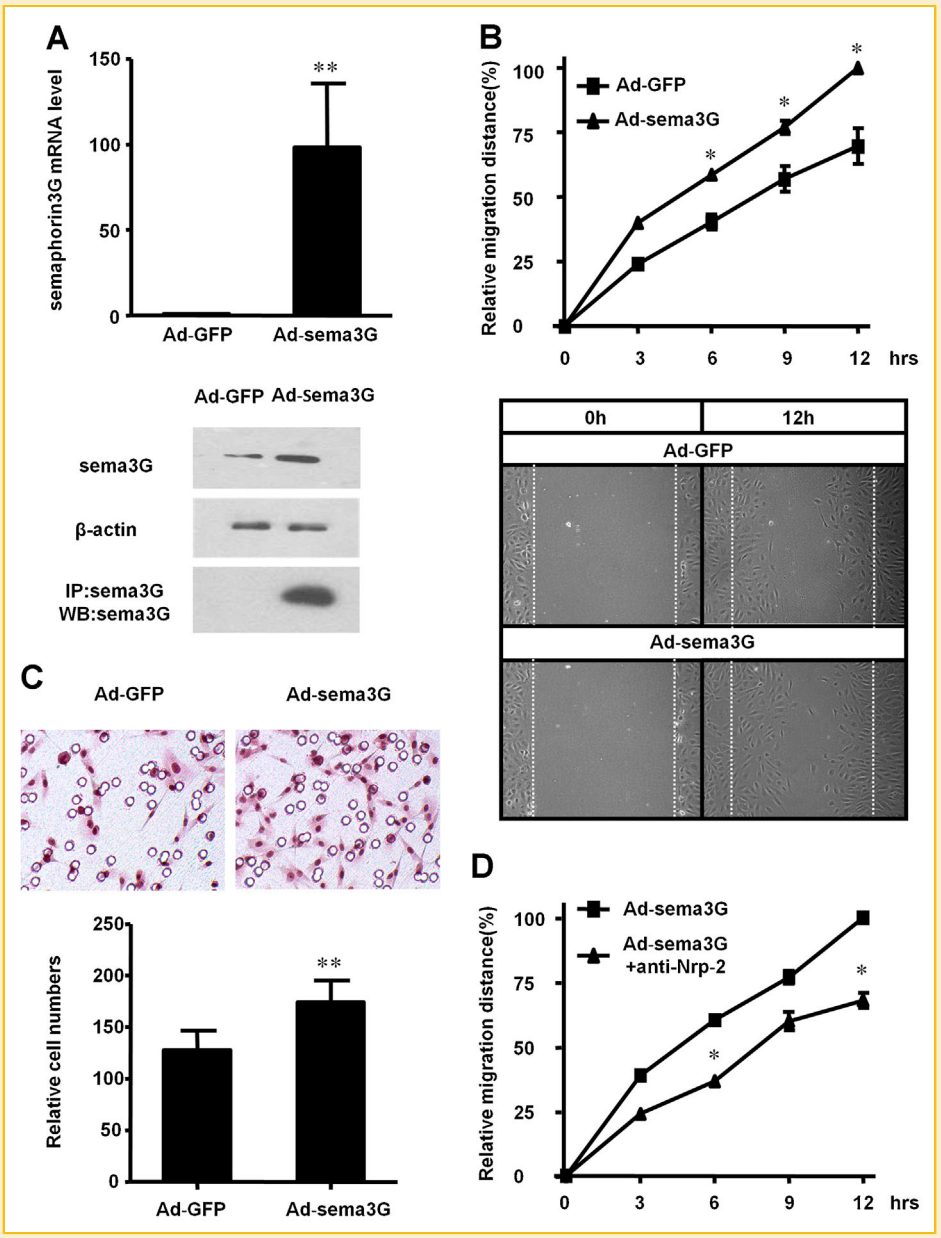

Fig. 4. Sema3g facilitates HUVECs migration in vitro. HUVECs were infected with Ad-Sema3g or Ad-GFP for 48 h. A: Relative mRNA or protein levels of Sema3g was determined by qRT-PCR or western blot. B: Migration assay. Confluent HUVECs were infected with Ad-Sema3g and Ad-GFP, then were recorded every 3 h from 0 to 12 h. C: Invasion assay. Confluent HUVECs monolayers infected with Ad-Sema3g and Ad-GFP were plated onto the upper chamber. HUVEC culture medium with 10% FBS was included in the lower chamber as a chemoattractant. Cells migrating across the filters at 6 h were stained. 100× magnification, $^*P < 0.05$. Representative results of three independent experiments are shown. Migrated cells were quantified by the average of four randomly chosen high-power fields (HPF) of three independent duplicate experiments. D: HUVECs infected with Ad-Sema3g or Ad-GFP were scratch-wounded 24 h after infection, then treated with Nrp2 neutralizing antibody (20 μg/ml) and normal rabbit immunoglobulin as a control. Cells were kept in culture for an additional 3, 6, 9, and 12 h before imaging. The mean distance migrated by HUVECs is quantified (as the average of four independent microscope fields for each of three independent experiments). $^*P < 0.05$.

DISCUSSION

PPAR-γ, which is adipose-enriched, controls the expression of genes involved in fatty acid storage and adipogenesis. PPAR-γ has also been increasingly recognized as a key player in the control of endothelial cell behavior, though the exact mechanism by which PPAR-γ regulates endothelial cell migration remains unclear. Here, we identify Sema3g as a novel physiologically relevant factor involved in the biological events associated with endothelial cell migration and invasion that acts downstream of PPAR-γ signaling.

The cellular signaling mechanism linking the PPAR-γ and Sema3g, Rac1 GTPase pathway remains unclear. Comprising a large family of conserved, secreted and membrane-associated proteins originally described as guidance cues for developing neurons,

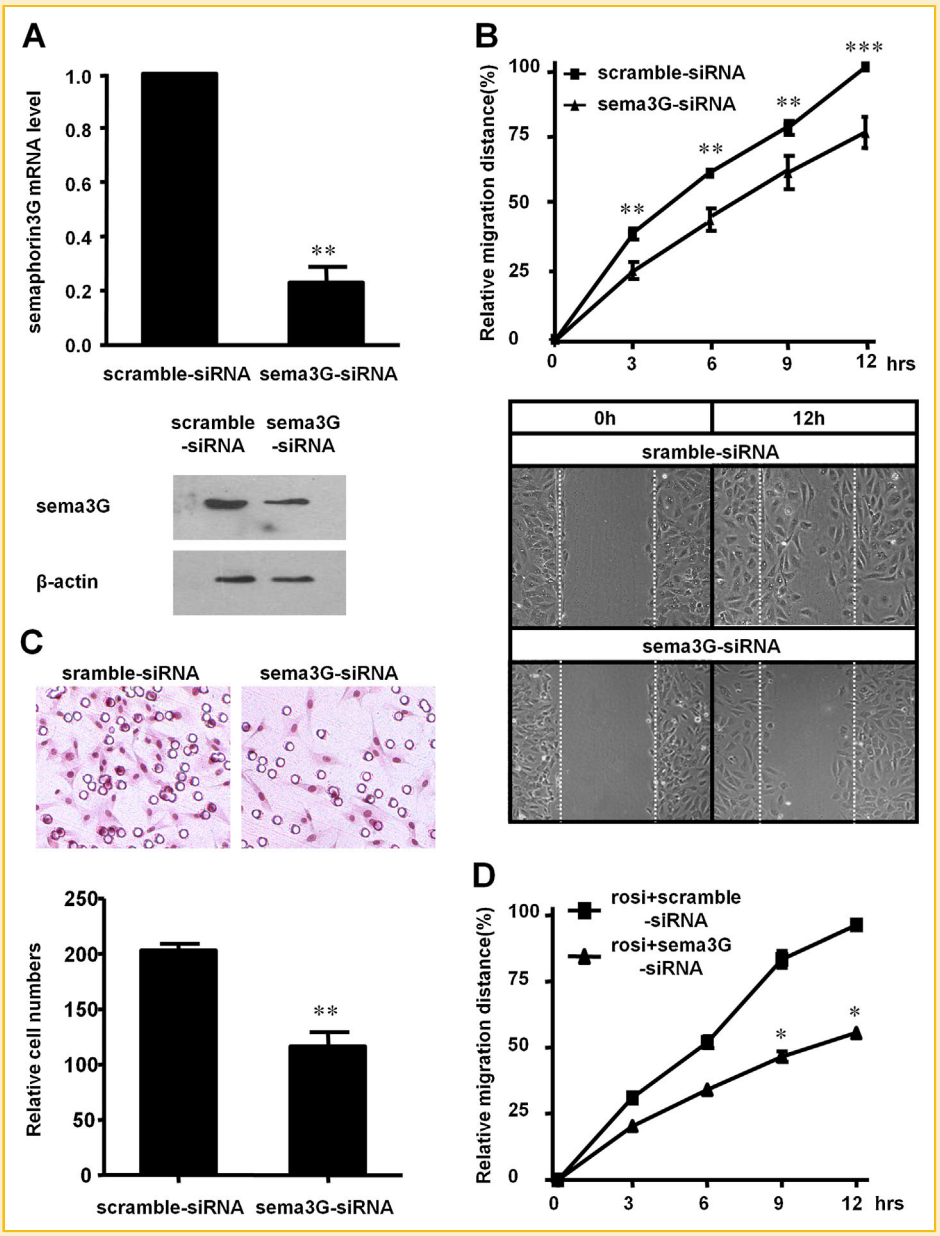

Fig. 5. Sema3g knockdown retards HUVECs migration in vitro. HUVECs were transfected with the Sema3g siRNA or scrambled siRNA and, after 24 or 48 h. A: relative mRNA and protein levels of Sema3g were determined using qRT-PCR or western blot. Compared to scrambled siRNA, Sema3g siRNA significantly reduces both the mRNA and protein level. $^{**}P < 0.01$ versus control. B: Migration assay. HUVECs transfected with Sema3g siRNA and scrambled siRNA were scratch-wounded, then recorded every 3 h from 0 to 12 h (the dotted line indicates wound edge). The mean distance migrated by HUVECs is quantified (as the average of four independent microscope fields for each of three independent experiments). Bottom, 100× magnification, $^{*}P < 0.05$. C: Invasion assay. Confluent HUVECs monolayers transfected with Sema3g siRNA and scrambled siRNA were plated onto the upper chamber. Huvec culture medium with 10% FBS was included in the lower chamber as a chemoattractant. Cells migrating across the filters at 6 h were stained. 100× magnification, $^{*}P < 0.05$. D: HUVECs scratch wounding healing transfected with Sema3g siRNA and scrambled siRNA and co-treated with Rosiglitazone, the Sema3g siRNA representative results of three independent experiments are shown. Migrated cells were quantified as the average of four randomly chosen high-power fields (HPF) of three independent duplicate experiments.

semaphorins have emerged as key regulators of vasculogenesis, angiogenesis, and tumorigenesis [Serini et al., 2003; Guttmann-Raviv et al., 2007]. A growing body of evidence has shown that Sema3a-regulated tumor cell migration [Raper, 2000]. Sema3g has significantly affected tumor cell growth and vessel density [Kigel et al., 2008]. Sema3c has increased migration of endothelial cells [Banu et al., 2006]. These publications strongly suggest that Class-3 semaphorins are closely related to endothelial cell function. Based on these findings, we can speculate that PPAR-γ-mediated endothelial cell migration occurs via a Sema3g-dependent pathway. Here, we therefore clarified the relationship between PPAR-γ activation, Sema3g expression and cell migration.

Our decision was to first investigate mechanisms involving PPAR-γ activation regulating Sema3g expression in HUVECs. Without

ruling out a role for post-transcriptional mechanisms, our data showed that PPAR-γ regulates Sema3g expression mainly at the transcriptional level. Indicating a transcriptional regulation of Sema3g by PPAR-γ agonists. PPAR-γ activation increased Sema3g mRNA expression by three to fivefold. Expectedly, the increased levels of Sema3g mRNA in response to the PPAR-γ agonist were detectable earlier than protein. The maximal increase in the level of Sema3g mRNA was observed at 24 h after PPAR-γ agonist treatment, but the maximal increase in Sema3g protein was seen only after 36 h, suggesting that increased protein levels most likely resulted from translation of increased numbers of Sema3g mRNA transcripts. Although its mechanism of control is unclear, PPAR-γ is known to bind to DNA in a sequence-specific manner and stimulate the transcription of genes downstream of the binding sites [Belanger et al., 2002; Goetze et al., 2002; Spiegelman, 1998]. DNA sequence analysis revealed that the 5′ flanking promoter region of the human Sema3g contains three putative PPAR-γ binding sites, which may allow PPAR-γ to bind to the Sema3g promoter region and enhance transcription of the gene. Our ChIP experiments confirmed that PPAR-γ is indeed associated with the Sema3g promoter. In vitro mutagenesis and a luciferase reporter gene analysis further demonstrated that PPAR-γ specifically transactivates Sema3g transcription, since even a single base change at the PPRE site of the Sema3g promoter resulted in a nearly 90% reduction in transcription. Collectively, these data demonstrate that Sema3g is a physiologically relevant target gene of PPAR-γ in HUVECs, and that PPAR-γ regulates Sema3g expression primarily at the transcriptional level.

Cell migration is a complex vascular response that involves numerous cytosolic and nuclear signaling events. Among the cytosolic signaling steps that contribute to the migration process, a recent study identified the protein kinase Akt as an important signaling molecule in PPAR-γ-mediated endothelial cell migration, indicating that PPAR-γ could have an antimigratory function via Akt signaling pathway inhibition [Goetze et al., 2002]. Sema3a, a family member of Class-3 semaphorins, affects tumor cell migration and invasion [Bachelder et al., 2003]. Moreover, experimental evidence has indicated that Sema4d can inhibit or promote cell migration, depending on the receptor complexes that are expressed by the tumor cells [Barberis et al., 2004; Swiercz et al., 2008]. These reports indicate that Sema3g may also control endothelial cell migration. Our experiments in cell lines both overexpressing Sema3g and silencing Sema3g expression confirmed that Sema3g promotes endothelial cell migration in a dose-dependent manner. Sema3g silencing by specific siRNA significantly inhibited PPAR-γ induced endothelial cell migration, indicating that Sema3g is a contributing factor to PPAR-γ mediated cell migration.

The mechanism by which Sema3g influences cell migration represents an interesting avenue for future research. Our work characterizing Sema3g function provides some clues as to how it might work. Sema3g has been shown to induce cell migration and its expression is upregulated in PPAR-γ activated cells, indicating that Sema3g is a downstream target of PPAR-γ. Data from recent studies indicate that Sema3g binds Nrp2 and induces the repulsion of sympathetic axons, suggesting that Sema3g utilizes Nrp2 as a receptor to repel specific types of axons [Taniguchi et al., 2005]. Thus, we believe that the Sema3g-initiated pathway involving cell migration also requires ligation of the Nrp2 receptor with Sema3g. Our data demonstrate that an Nrp2 receptor is indeed required for Sema3g-mediated cell migration because anti-Nrp2 antibody treatment can severely impair Sema3g-induced cell migration. The promigratory effect of Sema3g indicates a possible role in initiating the Rho GTPase Rac1 and Cdc42-mediated cell motility, since it is well established that the Rho GTPases (Rac1, Cdc42, and RhoA) are key effectors of actin cytoskeleton protrusion machinery [Van Aelst and D′Souza-Schorey, 1997]. Future studies on the influence of Sema3g on Rac1 signaling during cell migration would also be of interest.

It has been reported that Sema3g possesses anti-tumorigenic and anti-angiogenic properties and Sema3g overexpression can inhibit tumor cell invasion [Karayan-Tapon et al., 2008]. Thus, we should recall that Sema3g-induced cell migration and invasion probably occurs only in normal endothelial cells. Like Sema3g, Sema3e was also reported to have either promigratory or antimigratory activities in different types of endothelial cells [Christensen et al., 2005; Gu et al., 2005]. Therefore, these discrepancies might be explained by the involvement of different receptor complexes.

In conclusion, we provide compelling evidence showing for the first time that PPAR-γ promotes rather than inhibits endothelial cell migration by induction of Sema3g expression, thus identifying a novel downstream target for the promigratory actions of PPAR-γ.

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