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Objective—Circulating angiogenic cells (CACs) expansion is a multistage process requiring sequential activation of transcriptional factors, including STAT5. STAT5, in concert with peroxisome proliferator-activated receptors (PPARs), seems to induce discrete biological responses in different tissues. In the present study we investigated the role of STAT5 and PPARγ in regulating CAC expansion in normal and diabetic settings.

Methods and Results—Normal and diabetic CACs were used. siRNA technology, EMSA, and chromatin immunoprecipitation (ChIP) assay as well as site-directed mutagenesis of the STAT5 response element in the PPARγ promoter enabled us to demonstrate that STAT5 transcriptional activity controls PPARγ expression. Moreover, FACS analysis, coimmunoprecipitation experiments, and ChIP assay revealed that a STAT5/PPARγ transcriptional complex controls cyclin D1 expression and CAC progression into the cell-cycle. Conversely, PPARγ agonists, by preventing the expression of STAT5 and the formation of the STAT5/PPARγ heterodimeric complex failed to promote CAC expansion. Finally, we demonstrated that diabetic CAC functional capability can be recovered by molecules able to activate the STAT5/PPARγ transcriptional complex.

Conclusions—Our data identify the STAT5/PPARγ heterodimers as landmark of CAC expansion and provide evidences for a mechanism that partially rescues CAC bioavailability in diabetic setting. (***Arterioscler Thromb Vasc Biol.* 2009;29:114-120.**)

Key Words: STAT5 ■ PPARγ ■ diabetes ■ EPC ■ angiogenesis

Vascular complications, such as atherosclerosis, are a primary cause of mortality associated with diabetes and obesity. Thus, vascular protection is critical to decrease mortality and improve public health.[1] To accomplish this protection one member of the peroxisome proliferator-activated receptors (PPARs) has emerged.[2] Mammalian PPARs include 3 subtypes (α, β/δ, and γ), which are characterized by unique functions such as ligand specificities and tissue distributions.[3] PPARs are ligand-activated transcription factors that, by retinoid X receptors (RXRs) heterodimer formation and binding to specific DNA response elements (PPREs), modulate gene expression.[4,5] PPARγ is a key mediator in adipogenesis,[6] lipid metabolism,[7] and glucose homeostasis.[8] Moreover, compelling evidence suggests that PPARγ can influence target genes and processes that are of central relevance to endothelial biology.[9] In addition, PPARγ inhibits the expression of inflammatory genes and negatively interferes with proinflammatory transcription factor signaling pathways in inflammatory cells.[10–12] Recently, it has been reported that treatment of diabetic patients with the PPARγ ligands, possibly by modulating subclinical inflammatory activity or attenuating the detrimental effects of C reactive protein (CRP), increases the number and improves the functional capacity of endothelial progenitor cells (EPCs), thus providing evidence for PPARγ-mediated vascular protection.[13]

See accompanying article on page 10

From the initial report,[14] intense efforts have been focused on defining the role of circulating bone marrow–derived EPCs in the repair of damaged vascular endothelium and on translating this information into human clinical trials. To date, two types of EPCs have been described: true EPCs and circulating angiogenic cells (CACs).[15] Expansion of CACs is a multistep process that requires the activation of signaling pathways that are still under investigation. We recently demonstrated that the inflammatory cytokine interleukin (IL)-3, by activating STAT5, promotes CAC expansion.[16] STAT5 is a latent cytoplasmic transcription factor, ubiquitously expressed, that requires the JAK or the Src kinases to undergo activation.[16–18] In addition, STAT5, in concert with PPARs, has been reported to induce discrete biological responses in different tissues.[19–22]

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Herein we investigated the potential targets of STAT5 in regulating CAC expansion. In particular the relevance of the STAT5/PPARγ cross-talk in regulating this event was evaluated both in normal and in diabetic CACs.

Methods

Reagents and Antibodies

Reagents and antibodies used are listed in the supplement materials (available online at http://atvb.ahajournals.org).

Patients and Controls

Blood was recovered from 5 type 2 diabetic patients, arrived in our patient clinic (sex, M/F 2/3; HbA1c, 6.4±0.6%; age-years, 50.0±5; creatinine, 1±1 mg/dL; no retinopathy, no hypertension: blood pressure <140/90 mm Hg; Chol/apoB, 1.3±0.1). None of them was under insulin and all were treated only with diet. Ten blood donors were used as controls (sex, M/F 5/5; HbA1c, 5±01.0%; age-years, 50.0±1; creatinine, 0.7±0.4 mg/dL, no retinopathy, no hypertension: blood pressure ≤140/90 mm Hg, Chol/apoB, 1.6±0.1). The approval was obtained both from SIMT (Servizio Immunoematologia e Medicina Trasfusionale) and from the Institutional Review Board of S. Giovanni Battista Hospital, Turin, Italy. Informed consent was provided according to the Declaration of Helsinki. We also declare that for the present study, we had no direct contact with human subjects.

Cell Purification and Transfection

CACs, recovered from healthy subjects and diabetic patients, were isolated as described by Hill et al[23] and cultured as described in the supplement materials. In selected experiments CACs were cultured in the presence of troglitazone (10 μmol/L), 15dPGJ2 (5 μmol/L) or retinoic acid (RA; 10 μmol/L), or in EGM-2 standard medium. Experiments were also performed on cells transiently transfected with the activated form of STAT5 (STAT5 1*6)[24] or the empty vector pCNeo.

Isolation and Culture of BM-CACs From Transgenic Mice

Bone marrow (BM)-CACs from wild-type (WT), Tie2-ΔSTAT5A, and Tie2-ΔSTAT5B transgenic mice (Tie2-Δ5A and Tie2-Δ5B)[16] were isolated and cultured as described in the supplement materials.

Endogenous Depletion of STAT5 and PPARγ by Small Interfering RNAs

To obtain inactivation of endogenous STAT5 or PPARγ, IL-3-cultured CACs were processed as described in the supplement materials.

Western Blot Analysis and Coimmunoprecipitation Experiments

Cells were lysed and protein concentration was obtained as previously described.[25] For co-IP experiments cytosolic and nuclear extracts were obtained as previously described,[25] immunoprecipitated (IP) with the indicated antibodies, and processed.

Flow Cytometry

To analyze cell-cycle progression, FACS analysis was performed as previously described[25] and in the supplement materials.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

mRNA quantification from CACs, cultured with or without IL-3, as indicated, was performed by quantitative real-time polymerase chain reaction (Q-RT-PCR) as described in the supplement materials. The relative expression of PPARγ1 (defined as PPARγ throughout the study) and PPARγ2 were calculated by using comparative threshold cycle methods. The primer sequences are listed in the supplement materials.

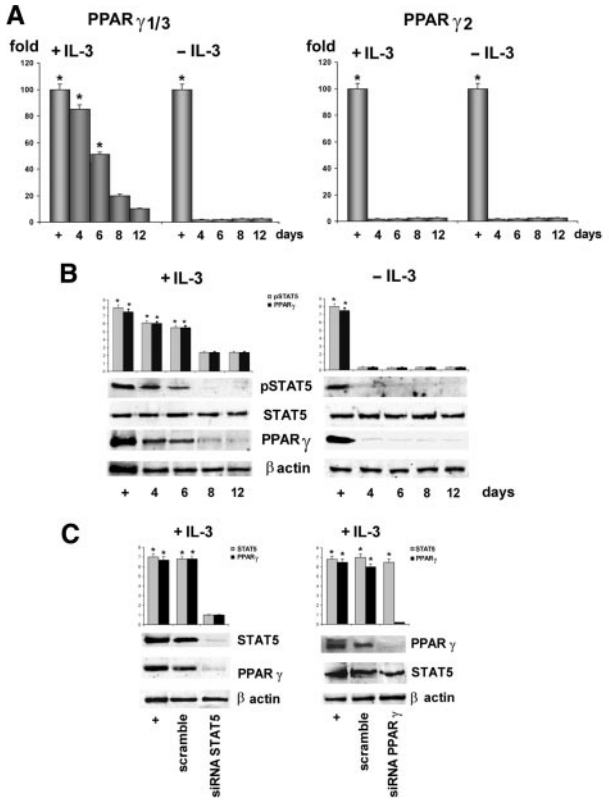


Figure 1. PPARγ expression temporally correlates with STAT5 activation. A, Q-RT-PCR of PPARγ1/3 and PPARγ2 on CACs (*$P<0.05$, 4 and 6 days of culture vs day 8 and 12). B, Western blot of untreated or IL-3-treated CACs. C, Western blot on STAT5- or PPARγ-depleted CACs.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts from CACs, cultured with or without IL-3, were prepared as described by Sadowski et al[26] and used for EMSA, as described in the supplement materials.

Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation (ChIP) assay was performed on CACs, recovered from healthy donors and diabetic patients and from WT, Tie2-Δ5A, and Tie2-Δ5B transgenic mice[16] using Magna ChIP A kit (Millipore), according to the vendor's instructions, as described in the supplement materials.

Luciferase-Report Assay

The luciferase reporter assay was performed as described in the supplement materials.

Statistical Analysis

Statistical analysis was performed as described in the supplement materials.

Results

STAT5 Transcriptional Activity Regulates PPARγ Expression

We recently demonstrated that the inflammatory cytokine IL-3, by activating the STAT5 signaling pathway, induces

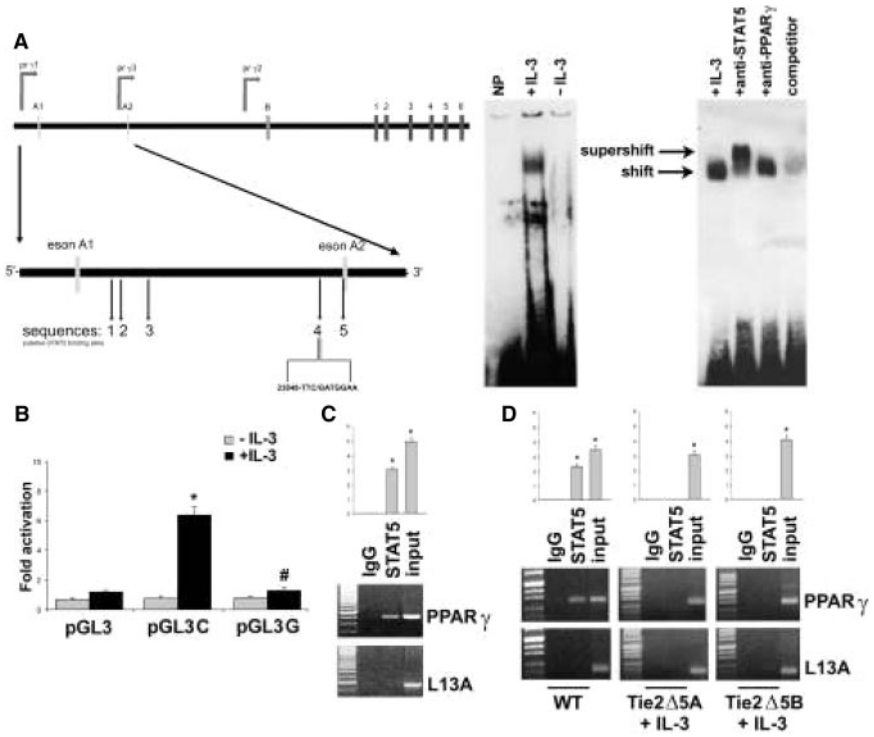


Figure 2. STAT5 transcriptional activity regulates PPARγ expression. A, Human PPARγ gene scheme. EMSA analysis on CACs. B, Luciferase activity on pGL3, pGL3/C, or pGL3/G transfected CACs (*$P<0.05$, pGL3/C vs pGL3; #$P<0.05$, pGL3/G vs pGL3/C). C) D, ChIP analysis on CACs from human (C) or transgenic mice (D).

CAC expansion.[16] STAT5 dimers bind to specific DNA sequences and regulate the expression of genes involved in different cell functions.[27] In addition, STAT5, in concert with PPARγ, modulates tissue specific signals.[19–22] Experimental and clinical evidence suggests a crucial role of PPARγ in regulating CAC functional activity in diabetic patients.[13] To investigate whether a modulatory effect of STAT5 over the PPARγ signaling pathway could account for the IL-3–mediated CAC expansion, quantitative real-time PCR and Western blot analysis on IL-3–cultured CACs was first performed. Kinetic analysis (Figure 1A and 1B) demonstrated that PPARγ expression, but not PPARγ2, temporally correlates with STAT5 activation. In addition, we found that PPARγ expression could be prevented by knocking down STAT5 (Figure 1C, left panel). Conversely (Figure 1C), STAT5 expression was not affected by the depletion of PPARγ. To verify the involvement of STAT5 in the control of PPARγ gene transcription, we selected 5 distinct putative STAT5 response elements in the PPARγ gene promoter (supplemental Table I), and used for EMSA. As shown in Figure 2A, reporting a representative consensus sequence (supplemental Table I, sequence 1), when nuclear extracts from IL-3–cultured CACs were assayed for their DNA-binding activity, the formation of PPARγ-binding complex was detected. Moreover, STAT5 binding to the DNA-binding complex was confirmed by the supershift assay using the anti-STAT5 antibody (Figure 2A). Similar results were obtained with all STAT5 consensus sequences tested (data not shown). To validate the above data, a C681G site-directed mutagenesis of the STAT5 response element in the PPARγ gene promoter[28] was performed. Two different constructs, containing the −681C (pGL3C) and the −681G (pGL3G) sequences, were used for the luciferase-reporter assay. As shown in Figure 2B, in CACs expressing the pGL3C PPARγ-luciferase-reporter vector, IL-3 was able to induce a high luciferase activity. Conversely, no promoter activity was detected when the pGL3G PPARγ-luciferase-reporter vector, containing the sequence corresponding to the mutated STAT5 response element, was used. To further confirm the transcriptional relevance of STAT5 in regulating PPARγ expression, ChIP assay was performed on CACs recovered from healthy subjects and from the recently described Tie2ΔSTAT5A and Tie2ΔSTAT5B transgenic mice.[16] The results, reported in Figure 2C and 2D, demonstrate the binding of STAT5 to the genomic DNA region encompassing the putative response elements on the PPARγ gene promoter in CACs recovered from humans or from wild-type mice. Conversely, no STAT5 binding could be detected when CACs recovered from Tie2ΔSTAT5A or Tie2ΔSTAT5B mice were used (Figure 2D).

Both STAT5-Dependent PPARγ Expression and Formation of an Heterodimeric STAT5/PPARγ Complex Are Required for CAC Expansion

To determine the role of PPARγ in regulating CAC cell-cycle progression, FACS analysis was performed on PPARγ silenced cells. As reported in Figure 3A, in these cells IL-3 failed to induce cyclin D1 expression and to promote CAC progression into the cell-cycle. This data indicates that both STAT5 and PPARγ are required for CAC expansion, raising the possibility that, by forming an heterodimeric transcriptional complex, STAT5 and PPARγ could control the expression of cell-cycle related genes, as cyclin D1. To validate this possibility, coIP experiments were first performed on cytosolic and nuclear extracts obtained from IL-3–cultured CACs. As reported in Figure 3B, although both cellular

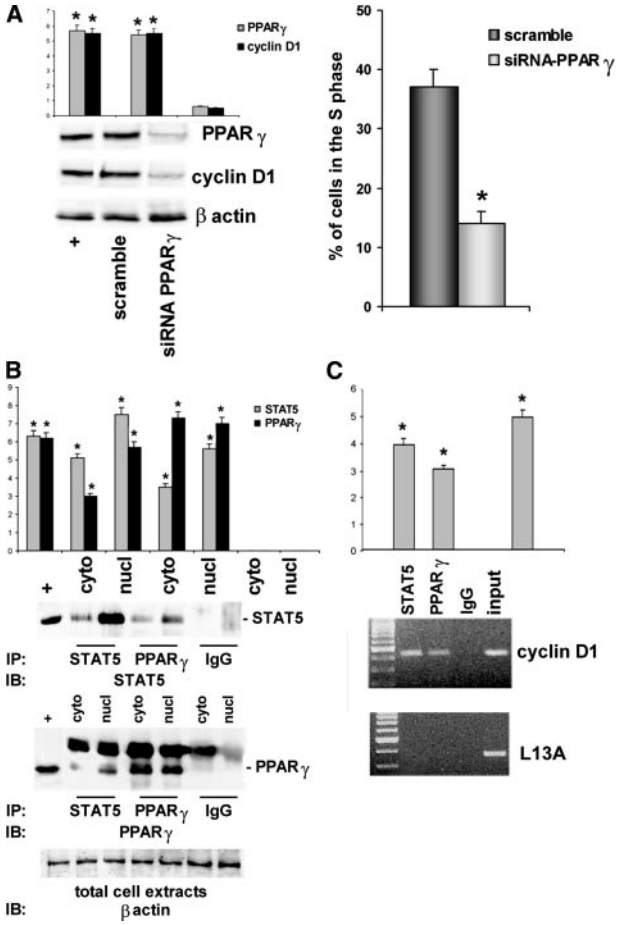


Figure 3. STAT5-dependent PPARγ expression and the STAT5/PPARγ complex are required for CAC expansion. A, Western blot and cell-cycle progression on PPARγ-silenced CACs (*$P<0.05$, experimental group vs scramble). B, Co-IP on cytosolic and nuclear extracts. C, ChIP analysis on human CACs.

compartments contained the STAT5/PPARγ molecular complex, the complex was mainly present in the nuclear fractions. To further investigate the transcriptional relevance of this heterodimeric complex, ChIP assay was performed. As shown in Figure 3C, either STAT5 and PPARγ were able to bind to the genomic DNA region encompassing the putative response elements on the cyclin D1 gene promoter. Conversely, no significant signal was detected using specific primers to the L13A gene (Figure 3C). Thus, these results identify PPARγ as a novel STAT5 heterodimeric partner involved in the control of cyclin D1 expression.

PPARγ Agonists Prevent CAC Expansion

The synthetic ligands thiazolidinediones (TZDs), besides ameliorating insulin sensitivity, also improve CAC functional activity in diabetic patients.[13] This prompted us to evaluate whether physiological and synthetic PPARγ agonists could, by themselves, promote in vitro expansion of CACs recovered from healthy subjects and diabetic patients. To this end, CACs cultured with troglitazone or with 15 deoxy--12,14-prostaglandin (PG) J2 (15 dPGJ2) were first assayed for PPARγ expression. Data reported in Figure 4A demonstrated that PPARγ ligands induced PPARγ expression both in normal and diabetic CACs, without affecting the promoter activity of the pGL3C PPARγ-luciferase-reporter vector (supplemental Figure I). Indeed, as reported in hemopoietic progenitor cells,[29] PPARγ ligands reduced the expression of STAT5 (Figure 4A) and failed to induce cyclin D1 expression and CAC cell-cycle progression (Figure 4A and 4B). Consistently, no STAT5 and PPARγ binding to the putative response elements of cyclin D1 could be detected (Figure 4C). The finding that RA also failed to induce cyclin D1 expression and CAC expansion (data not shown) further confirmed this data, suggesting that STAT5 expression and activation are required to promote CAC expansion. To validate this possibility, the constitutive active STAT5 (STAT5 1*6)[24] was used. As shown in Figure 4D and 4E, the constitutively activated STAT5 1*6 prevented TZD effects by rescuing both cyclin D1 expression and progression into the cell-cycle.

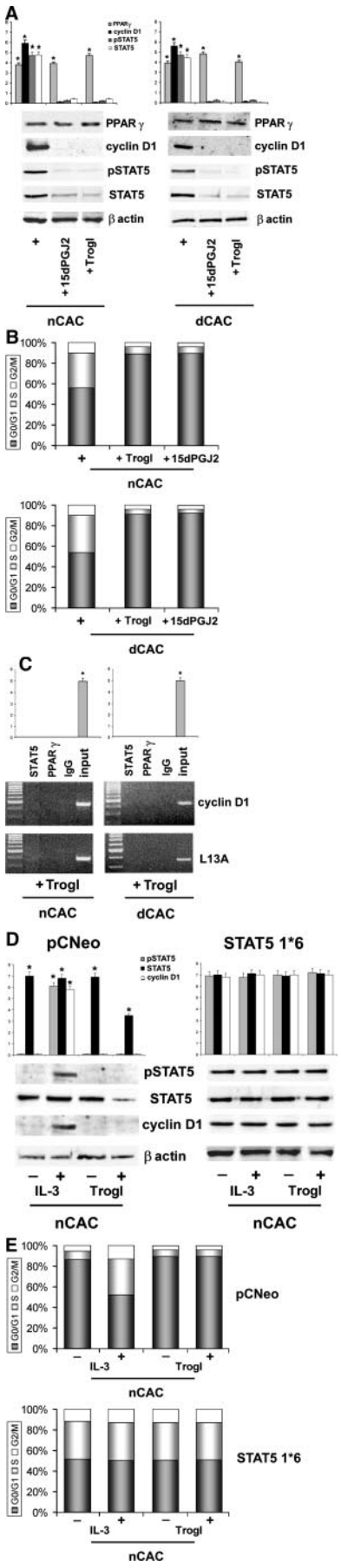
Diabetic CACs Retain the Ability to Activate the STAT5/PPARγ Complex and to Proliferate in a IL-3-Containing Microenvironment

To assess whether the activation of signals upstream to PPARγ could partially recover diabetic CAC functions, the cells were cultured in the presence of IL-3. As shown in Figure 5B, IL-3 was able to elicit STAT5 activation, PPARγ, and cyclin D1 expression. The finding that the number of cycling cells and of colonies was higher in healthy subjects than in diabetic patients depends on the different number of peripheral blood clonogenic cells (Figure 5A and 5C).[30] EGM-2 medium did not significantly affect neither normal nor diabetic CAC expansion, and the activation of signaling pathway leading to this event. To further investigate the role played by STAT5 and PPARγ in the control of cyclin D1 expression, ChIP assay was performed. The results, reported in Figure 5D, demonstrate that, similarly to non-diabetic, diabetic CACs, when cultured with IL-3, form a STAT5/PPARγ transcriptional complex that binds to the putative response elements and induces the expression of cyclin D1. These data identify IL-3 as a potential modulator of diabetic CACs ex vivo expansion.

Discussion

Data presented herein lead to the following conclusions: (1) STAT5 transcriptional activity regulates the expression of PPARγ; (2) both STAT5 and PPARγ are required for CAC expansion; (3) the STAT5/PPARγ transcriptional complex controls cyclin D1 expression; and (4) this complex can partially rescue diabetic CAC bioavailability.

As recently shown, CAC expansion at the site of vascular damage contributes to blood vessel formation.[14–16] However, the molecular mechanisms accounting for these events are still under investigation. We recently demonstrated that CACs exposed to IL-3 undergo proliferation, acquire vasculogenic property, and directly participate to neovessel formation by activating the STAT5 signaling pathway.[16] The aims of the present study were to characterize the molecular targets of STAT5 in mediating this event and to assess the relevance



of this signaling pathway in the control of CAC fate in diabetic setting. Although several lines of evidence indicate that PPARγ improves CAC functional activity in diabetic patients,[13] the mechanisms associated with this effect are still undefined. We herein demonstrate that PPARγ expression temporally correlates with STAT5 activation. In addition, we provide evidence that STAT5 transcriptional activity controls PPARγ expression in CACs exposed to an IL-3 containing microenvironment. In addition, by reproducing the −681C/G polymorphism, known to prevent STAT5 binding to the PPARγ gene promoter,[28] we strengthen the relevance of STAT5 transcriptional activity on PPARγ gene expression.

It is known that PPARγ mainly forms heterodimers with the nuclear retinoid X receptor (RXR)-α.[11,12] The PPARγ/RXR-α heteorodimers are permissive, in that they can be activated by either PPARγ or RXR-α ligands and they bind to specific PPAR response elements in the regulatory regions of target genes, mainly involved in the anti–inflammatory response and in cell differentiation.[2,3,10–12,31] Herein we identify STAT5 as a novel PPARγ transcriptional partner and we provide the first evidence that the STAT5/PPARγ transcriptional complex is required to control cyclin D1 expression and CAC cell-cycle progression.

PPARγ may also interact with other transcription factors, such as the activator protein (AP)-1 and NF-κB, without involving direct DNA binding to regulate gene transcription.[31,32] In particular, NF-κB is the major target of PPARγ to suppress inflammation, a crucial event in the development of vascular dysfunction. Very recently PPARγ agonists have been also shown to hamper the functionality of hemopoietic progenitors by inhibiting STAT5 gene expression.[29] Consistently, recent reports have mentioned unexplained hematopoietic abnormality in a large cohort of patients with type 2 diabetes participating in clinical trials with the PPARγ agonist pioglitazone.[33] Finally, Ricote et al[34] showed that PPARγ ligands can inhibit STAT activity in a PPARγ-dependent manner. Similarly, we found that physiological and pharmacological PPARγ agonists failed to induce CAC expansion possibly by affecting STAT5 expression, the formation of the STAT5/PPARγ transcriptional complex and its binding to the regulatory region of cyclin D1. Indeed, the observation that the expression of the activated variant of STAT5 prevents the inhibitory effect of trogitazone adds further insight into the mechanisms accounting for the results herein presented and for the above mentioned hemopoietic cell defects.

The reduced number and the impaired function of CACs in diabetes have been extensively documented;[23,35,36] however, the molecular mechanisms accounting for these events remain to be elucidated. Consistent with previous reports,[23,30] we found that the number of CACs recovered from diabetic patients was lower then that from sex and age matched normal subjects. However, diabetic CACs, when exposed to

Figure 4. PPARγ ligands fail to sustain normal and diabetic CAC expansion. Western blot (A), cell-cycle progression (B), and ChIP analysis (C) on normal (nCACs) or diabetic CACs (dCACs). Western blot (D) and cell-cycle progression (E) on nCACs transfected with the empty vector (pCNeo) or the STAT51*6 construct.

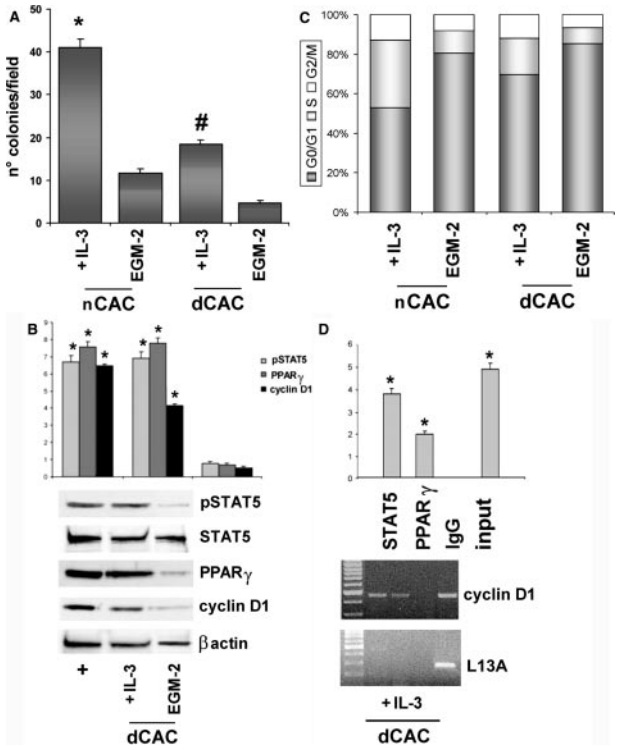


Figure 5. STAT5/PPARγ complex partially rescues diabetic CAC bioavailability. A, Colonies of IL-3– or EGM-2–cultured nCACs and dCACs (*$P<0.05$, nCACs+IL-3 vs nCACs EGM-2; #$P<0.05$, dCACs+IL-3 vs dCACs EGM-2). Western blot (B) and cell-cycle progression (C) on CACs, cultured as above. D, ChIP analysis on IL-3–cultured dCACs.

IL-3, acquire the ability to undergo cell cycle progression via STAT5 activation, the formation of the STAT5/PPARγ transcriptional complex, and cyclin D1 expression. Currently, impaired CAC functions are considered one mechanism by which risk factors worsen cardiovascular health. Herein, we provide evidence that a cytokine released in inflammatory environments can partially recover CAC bioavailability and possibly vascular regenerative capability in a diabetic setting.

Although human genetic studies and animal studies sustain the beneficial function of PPARγ in controlling susceptibility to vascular diseases,[10–12] recently reported clinical studies[37,38] raise some concern about cardiovascular adverse effects of PPARγ agonists. We provide evidence that agonist-independent PPARγ expression exerts a pivotal role in preventing vascular damage. Finally, our finding that PPARγ, by forming a different heterodimeric complex, can dictate discrete biological responses (supplemental Figure II), may make possible the generation of novel therapeutic strategies able to modulate vascular remodeling.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

METHODS

Reagents.

M199 medium, fetal bovine serum (FBS), fibronectin (FN), protein A Sepharose beads, proteinase K, propidium iodide, retinoic acid (RA) and troglitazone were from Sigma-Aldrich (St Louis, MO, USA). 15-deoxy-Δ 12, 14-prostaglandin J2 (15dPGJ2) was from Cayman Chemicals (Ann Arbor, MI, USA). EBM-2 medium supplemented with 10% of FBS and EGM-2 medium (10% FBS, hydrocortisone, human Fibroblast Growth Factor, Vascular Endothelial Growth Factor, Insulin Growth Factor 1, ascorbic acid, human Epidermal Growth Factor, gentamicin and amphotericin-B) were from Lonza (Walkersville, MD, USA). Trypsin was from Difco (Detroit, MI, USA). Nitrocellulose filters, HRP-conjugated anti-rabbit IgG and anti-mouse IgG, molecular weight markers, chemiluminescence reagent were from Amersham (Braunschweig, Germany). The presence of endotoxin contamination was tested by the Limulus amebocyte assay (concentration was <0.1 ng/ml). Human IL-3 was a gift from Sandoz Pharma Ltd (Basel, Switzerland).

Antibodies.

Anti-STAT5, anti-β actin, anti-cyclin D1, anti-mouse IgG and anti-PPARγ antibodies were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anti-p-STAT5 antibody was from Cell Signaling Technology (Beverly, MA, USA).

Cell purification and transfection.

Peripheral-blood mononuclear cells (PB-MNC), recovered from healthy subjects and diabetic patients, were isolated and cultured as described by Hill et al. [1]. Sorting of CD45+ cells from human PB was performed on MoFlo Cell Sorter (DakoCytomation Inc., Fort Collins, CO). CD45+ sorted cells, defined as CAC, were characterized by FACS for CD14, CD13, CD33 and CD11b expression, as previously described [2]. Sorted cells were cultured until 12 days on 20 µg/ml FN-coated dishes in EBM-2 with or without 10 ng/ml of IL-3. The major of the experiments were performed at day 4.

Isolation and culture of BM-CAC from transgenic mice. Bone marrow (BM)-MNC, isolated from murine BM flushed from the femurs of wild-type (WT), Tie2-ΔSTAT5A and Tie2-ΔSTAT5B transgenic mice (Tie2-Δ5A and Tie2-Δ5B) [2], were cultured on 20 μg/ml fibronectin-coated dishes in EBM-2, with or without IL-3 (10 ng/ml).

Endogenous depletion of STAT5 and PPARγ by Small Interfering RNAs (siRNAs).

To obtain inactivation of endogenous STAT5 or PPARγ, IL-3-cultured CAC were transiently transfected with siRNA for STAT5 or PPARγ and with duplex siRNAs purchased by Qiagen (Valencia, CA, USA), as scramble controls. Transfection was performed according to the vendor's instructions. 48 hours later whole cell extracts were prepared and processed for Western blot. Cell viability was evaluated at the end of the experiments.

Western blot analysis and co-immunoprecipitation experiments.

Cells were lysed and protein concentration was obtained as previously described [4]. For co-immunoprecipitation (co-IP) experiments cytosolic and nuclear extracts were obtained as previously described [4] and then immunoprecipitated (IP) with the indicated antibodies and processed.

Flow Cytometry.

To analyze cell-cycle progression, FACS analysis was performed as previously described [4]. Briefly, after treatment, the cells were fixed with 70% ethanol, DNA was stained with propidium iodide and analyzed with a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House, Inc., Topsham, ME).

RNA isolation and quantitative real-time PCR (Q-RT-PCR).

mRNA quantification from CAC, cultured with or without IL-3, for different times, as indicated, was performed by Q-RT-PCR using the ABI PRISM 7700 Sequence detection system and the SYBR Green Master Mix Kit (Applied Biosystem, Foxter Cyto CA). GAPDH gene was used as standard reference. The relative expression of PPARγ1/3, that will be defined as PPARγ throughout

the study, and PPARγ2 were calculated by using comparative threshold cycle methods. The primer sequences were as follows: PPARγ1/3, sense, 5'-CGTGGCCGCAGATTTGAA-3'; antisense, 5'-CTTCCATTACGGAGAGATCCAC-3': PPARγ2, sense, 5'-GGTGAAACTCTGGGAGATTCT-3'; antisense, 5'-CTCTGTGTCAACCATGGTCA-3'.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA).

Nuclear extracts from CAC, cultured in the presence or in the absence of IL-3, for 4 days, were prepared as described by Sadowski et al. [5]. The sequences corresponding to the putative STAT5 response element on the PPARγ promoter gene, selected by Gene bank analysis, reported in Table S1, were used for EMSA. EMSA was performed using LightShift Chemiluminescent EMSA kit (PIERCE, Rockford, IL, USA), according to the vendor's instructions.

Chromatin immunoprecipitation (ChIP) assay.

ChIP assay was performed using Magna ChIP A kit (Millipore, Temecula, CA, USA), according to the vendor's instructions. Briefly, CAC, recovered from healthy subjects and diabetic patients and from WT, Tie2-Δ5A and Tie2-Δ5B transgenic mice (generated as previously described) [2], were cross-linked with 1% formaldehyde and quenched before harvest and sonication. The sheared chromatin was immunoprecipitated with an anti-STAT5 or an anti-PPARγ antibody or control IgG on protein A Sepharose magnetic beads. The eluted IP were digested with proteinase K and DNA was extracted and underwent PCR with primers specific for cyclin D1 promoter region: sense, 5'-GATGCAGTCGCTGAGATTCTT-3'; antisense, 5'-TTGCCCCTGTAGTCCGGTTTT-3'; with primers specific for a PPARγ promoter region containing STAT5 response element (sequence 1): sense, 5'GATGTGACCATGACCCTGAATT3'; antisense, 5'AACGTATATTCCCCAGGAGCAA-3'; or with primers for part of the L13A gene: sense, 5'GCAAGCGGATGAACACCAACC-3'; antisense, 5'-TTGAGGGCAGCAGGAACCAC-3'. The non-immunoprecipitated genomic DNA was also analyzed using semi-quantitative real-time PCR and expressed as % of the input.

Luciferase-report assay.

The luciferase reporter assay was performed using a construct generated by subcloning the PCR products amplified from CAC genomic DNA in the KpnI/HindIII restriction sites of the luciferase reporter vector pGL3 (Promega, Madison, WI, USA). The PCR products were obtained from PPARγ1/3 promoter region, containing the original STAT5 response element, located at –681 bp from A2 exon of the human PPARγ1/3 promoter gene, as described by Meirhaeghe et al. [6] (pGL3/C), using the following primers: sense,5'-ATGGTCTACTACATTTATGCCATGTGT-3';antisense,5'-TGCATAGTCCAACTGACTGGAA-3': A site-direct mutagenesis on the same amplified PCR product was performed to obtain the mutated STAT5 response element, with –681 C/G mutation, (pGL3/G) [6]. The sequence was generated using the Quik-Change Site-Direct Mutagenesis kit (Stratagene, La Jolla, CA, USA). The oligonucleotide (TTTTGGCATTAGATGCTGTTTTGTCTT *G* ATGGAAAATACAGCTATTC) containing the desired mutation was designed according to the manufacturer's instructions (the mutated nucleotide is underlined and italicized). The insert identity was verified by sequencing. The pGL3, pGL3/C and pGL3/G reporter vectors were transiently co-transfected in CAC, cultured with IL-3 or PPARγ agonists, at 50:1 molar ratio with the pRL vector, coding for the *Renilla* luciferase, used as internal control of luciferase assay. Luciferase activities were analyzed by Dual-Luciferase Report Assay System (Promega), according to vendor's instructions, using a TD20/20 double injector luminometer (Turner Designs, Forlì, IT). The results are expressed as fold activation, calculated by normalizing the ratio of the firefly/renilla luminescences.

Statistical analysis.

The results are representative of at least three independent experiments, performed at least in triplicate. Densitometric analysis using a Bio-Rad GS 250 molecular imager was used to calculate the differences in the fold induction of protein activation or expression (* and # $p < 0.05$, statistically significant between experimental and control values). Significance of differences was calculated using analysis of variance with Newman-Keuls multicomparison test.

EXTENSIVE FIGURE LEGENDS

Figure 1. PPARγ expression temporally correlates with STAT5 activation. (A) Q-RT-PCR was performed on CAC, cultured with or without IL-3, for different times, to evaluate PPARγ1/3 and PPARγ2 expression. Expression levels are presented as fold increase (logarithmic scale) in comparison with baseline levels and normalized by using GAPDH as housekeeping gene. The mRNA isolated from adipose tissue samples was used as positive control (+) (* $p < 0.05$, 4 and 6 days of culture vs day 8 and 12). **(B)** Cell extracts from CAC, challenged with or without IL-3, were subjected to SDS-PAGE and the filters were immunoblotted (IB) with anti-PPARγ, anti-pSTAT5, anti-STAT5 or anti-β-actin antibodies. **(C)** STAT5- or PPARγ–depleted CAC, cultured with IL-3 for 4 days, were analyzed by WB. Scrambled sequences (scramble) were used as control. The filters were IB with anti-STAT5, anti- PPARγ or anti-β-actin antibodies. In B and C, EC extracts were used as positive control (+).

Figure 2. STAT5 transcriptional activity regulates PPARγ expression. (A) Schematic representation of the genomic structure of the human PPARγ gene. The gene is drawn to scale. The region encompassing the regulatory elements of PPARγ 1-3 is higher magnificated. The arrows indicate the location of the selected putative STAT5 binding sites **(**left panel). Nuclear extracts from CAC, cultured with or without IL-3, were analyzed by EMSA, in the presence or in the absence of an anti-STAT5 or an anti-PPARγ antibody. Arrows indicate the PPARγ-binding complex and the supershifted species (right panel). **(B)** CAC, treated as above, were transfected with luciferase reporter pGL3, pGL3/C or pGL3/G vectors. After 48 h, luciferase activity, expressed as fold activation, was evaluated (*$p < 0.05$, pGL3/C vs pGL3; # $p<0.05$, pGL3/G vs pGL3/C). **(C) (D)** ChIP assay was performed on chromatin, derived from IL-3-cultured human CAC (C) and WT, Tie2Δ5A or Tie2Δ5B transgenic mice (D), IP with anti-STAT5 antibody and anti-mouse IgG and

amplified with primers for PPARγ promoter or L13A. Control PCR was done with non-IP genomic DNA (input).

Figure 3. STAT5-dependent PPARγ expression and the STAT5/PPARγ complex are required for CAC expansion. (A) PPARγ-silenced CAC were lysed. The filters were IB with anti-PPARγ, anti-cyclin D1 or anti-β-actin antibodies (left panel). The percentage of cells in the S phase was evaluated by FACS analysis on PPARγ-depleted CAC (right panel). Scrambled sequence (scramble) was used as control. * $p < 0.05$, experimental group vs scramble. **(B)** Co-immunoprecipitation (co-IP) experiments were performed on cytosolic and nuclear extracts from IL-3-cultured CAC using anti-STAT5 and anti-PPARγ antibodies. The filter was normalized with an anti-β-actin antibody. In A and B, EC extracts were used as positive control (+). **(C)** ChIP assay was performed on IL-3-cultured CAC chromatin, IP with anti-STAT5, anti-PPARγ antibodies and anti-mouse IgG and amplified with primers for cyclin D1 promoter or L13A. Control PCR was done with non-immunoprecipitated genomic DNA (input).

Figure 4. PPARγ ligands fail to sustain normal and diabetic CAC expansion. (A) Cell extracts from normal (nCAC) or diabetic CAC (dCAC), cultured for 4 days with troglitazone or 15dPGJ2, were analyzed by WB. The filters were IB with anti-PPARγ, anti-cyclin D1, anti-pSTAT5, anti-STAT5 and anti-β-actin antibodies. **(B)** FACS analysis was performed to evaluate cell-cycle progression of nCAC and dCAC treated as above. **(C)** ChIP assay was performed on troglitazone-cultured nCAC and dCAC, as above described, and amplified with primers for cyclin D1 or L13A. Control PCR was done with non-immunoprecipitated genomic DNA (input). In A and B, IL-3-cultured nCAC were used as positive control (+). **(D)** nCAC, treated as indicated, were transfected with the empty vector (pCNeo) or with the STAT51*6 construct. After 48h, cells were lysed and analyzed by WB. The filters were IB with anti-cyclin D1, anti-pSTAT5, anti-STAT5 and anti-β-

actin antibodies. **(E)** FACS analysis was performed to evaluate cell-cycle progression of pCNeo- or STAT51*6-transfected nCAC, treated as above.

Figure 5. STAT5/PPARγ complex partially rescues diabetic CAC bioavailability. (A) The number of colonies obtained by IL-3- or standard medium (EGM-2)-cultured nCAC and dCAC, for 4 days, is reported. Data are the mean of 10 fields ± SD (* $p<0.05$, nCAC+IL-3 vs nCAC EGM-2 ; # $p<0.05$, dCAC+IL-3 vs dCAC EGM-2). **(B)** dCAC, cultured as above, were lysed. The filters were IB with anti-pSTAT5, anti-STAT5, anti-PPARγ, anti-cyclin D1 and anti-β-actin antibodies. IL-3-cultured EC were used as positive control (+). **(C)** FACS analysis was performed to evaluate cell-cycle progression of nCAC and dCAC, cultured as above. **(D)** ChIP assay was performed on chromatin from IL-3-cultured dCAC and amplified with primers for cyclin D1 or L13A. Control PCR was done with non-immunoprecipitated genomic DNA (input).

LEGEND TO SUPPLEMENTAL FIGURES

Figure I. STAT5 is not involved in PPARγ ligand-dependent PPARγ expression CAC, treated as indicated, were transfected with luciferase reporter pGL3, pGL3/C or pGL3/G vectors. After 48 h, luciferase activity, expressed as fold activation, was evaluated (*$p < 0.05$, pGL3/C vs pGL3).

Figure II. Model of dual role of PPARγ (A) Ligand-induced PPARγ expression leads to the formation of the canonical PPARγ/RXR transcriptional complex regulating the inflammatory response, and to the reduction of STAT5 expression that prevents cell-cycle progression. **(B)** Cytokine-mediated STAT5-dependent PPARγ expression leads to the formation of a novel PPARγ heterodimer, the PPARγ/STAT5, permissive for cyclin D1 expression and CAC expansion.

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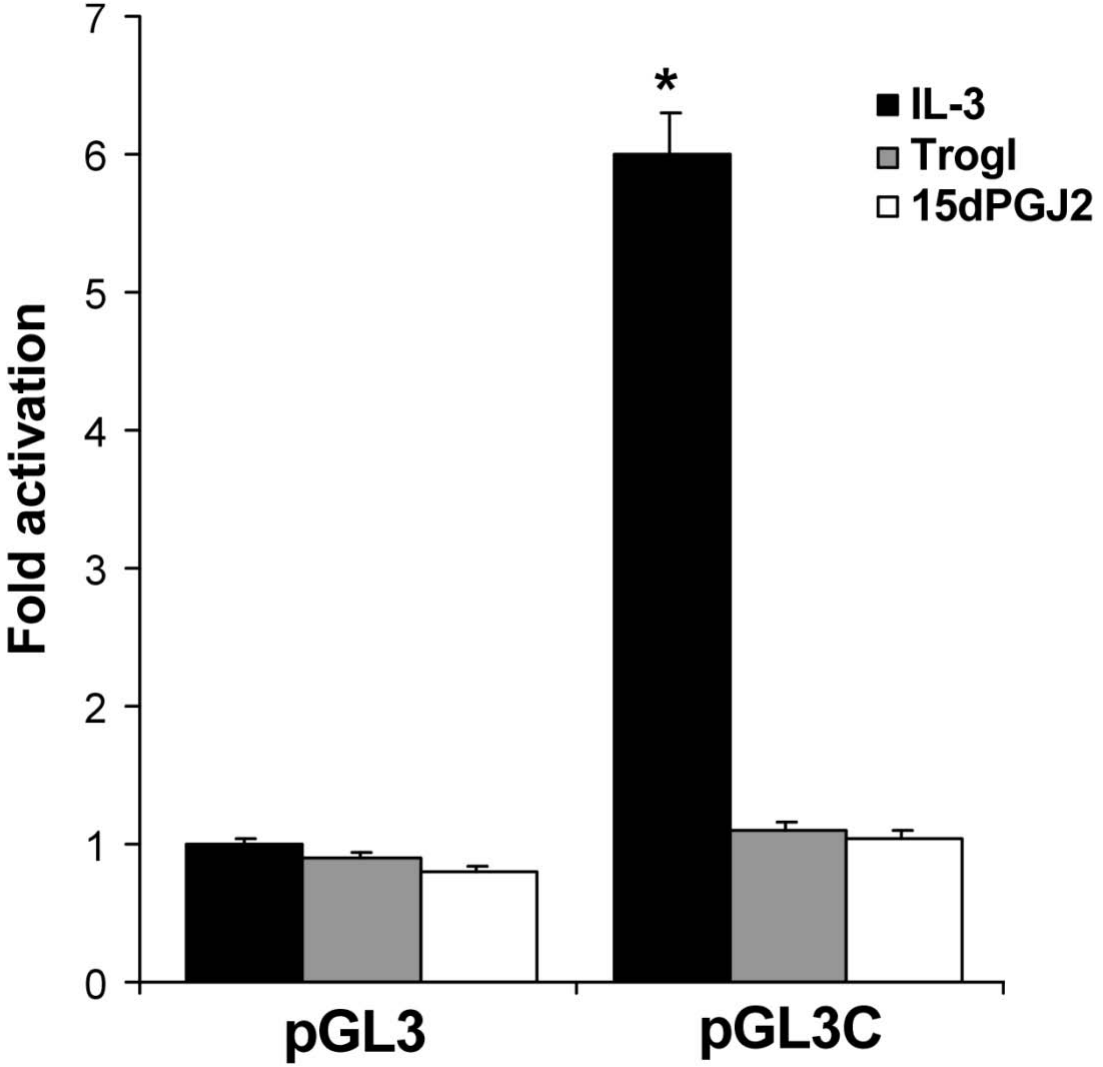
Table I. Putative STAT5 response elements in the PPARγ promoter gene.

Human PPARγ NC_000003.10|NC_000003:12304349-12450855 (gene ID: 5468):		
sequence	STAT5 response element	Binding activity
1	4291-ttctgggaa-4299	+
2	5581-ttctgagaa-5589	+
3	5783-ttctaagaa-5792	+
4	23848-ttcatggaa-23857	+
5	25097-ttcctggaa-25106	+


7
6
5
4
3
2
1
0
Fold activation
*
IL-3
Trogl
15dPGJ2
pGL3
pGL3C